

Inhibition of intra-Golgi transport in mitotic extracts

**presented for the degree of Doctor of Philosophy at
the University of London**

Deborah J. G. Mackay

October 1993

**Imperial Cancer Research Fund
44, Lincoln's Inn Fields,
London WC2A 3PX**

**University College, London
London WC1E 6BT**

ProQuest Number: 10045779

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



ProQuest 10045779

Published by ProQuest LLC(2016). Copyright of the Dissertation is held by the Author.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code.
Microform Edition © ProQuest LLC.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

Many stages of vesicle-mediated exocytic and endocytic membrane traffic are inhibited in mitotic mammalian cells. The fact that transport between the ER and the Golgi is inhibited in mitosis made it technically very difficult to monitor mitotic intra-Golgi protein transport *in vivo*. Therefore, a cell-free assay was supplemented with heterologous cytosols to study transport inhibition *in vitro*.

A high-speed supernatant ('cytosol') with high histone kinase activity was prepared from mitotic cells and markedly inhibited intra-Golgi transport. The inhibition was mimicked by treatment of interphase cytosol with the p34^{cdc2}-associated protein cyclin A, and was reversed by the kinase inhibitor staurosporine, strongly linking a mitotic kinase-phosphatase cycle with control of the assay. The histone kinase activity of the S-phase kinase p33^{cdk2} did not promote transport inhibition, and destruction of p34^{cdc2} in a temperature sensitive cell line prevented the cyclin effect. These results supported the hypothesis that the mitotic kinase p34^{cdc2} was responsible for transport inhibition, though probably not directly. Pharmacological and biochemical experiments suggested that the fusion of transport vesicles with their target was the site of the inhibition. The proteins involved are not known at present. These data support a model which links inhibition of vesicle fusion with the observed vesiculation of the Golgi during mitosis.

Table of Contents

	Abstract	2
	Table of Contents	3
	Table of Figures	6
	<i>Table of Abbreviations</i>	7
1	Introduction	8
	Overview	8
1.1	The exocytic pathway	9
1.1.1	The organelles of the exocytic pathway	9
1.1.2	The mechanism of intracellular transport	14
1.1.3	Protein localisation in the exocytic pathway	15
1.2	An in-vitro assay for intra-Golgi transport	19
1.2.1	The principle of the assay	19
1.2.2	Identification of proteins using the assay	20
1.3	Mitosis	28
1.3.1	Early work	30
1.3.2	p34 ^{cdc2} and the cell cycle	31
1.3.3	Proteins implicated in the control of p34 ^{cdc2}	34
1.3.4	The control of Start and S-phase	36
1.4	Intracellular organelles in mitosis	39
1.4.1	Organellar morphology in mitotic cells	39
1.4.2	Organellar function in mitosis	41
1.4.3	Structure and function of the mitotic Golgi apparatus	42
	Objective of the thesis	44
2	Materials and methods	45
2.1	Chemicals	45
2.2	Cell culture	46
2.3	Making and titrating Vesicular stomatitis virus	46
2.3.1	Virus preparation	46
2.3.2	P.f.u. assay	47
2.4	Making Golgi membrane fractions	50
2.4.1	Donor Golgi homogenate	50
2.4.2	Acceptor Golgi homogenate	50
2.4.3	Sucrose density gradient centrifugation for preparation of donor and acceptor Golgi membranes	51
2.4.4	Salt-washing Golgi membranes	51
2.5	Making interphase and mitotic cytosol	52
2.5.1	Cytosol preparation	52
2.5.2	Cell counting and evaluation of mitotic index	53
2.6	Intra-Golgi transport assay	54
2.7	Enzyme assays	57
2.7.1	Histone kinase assay of cytosol	57
2.7.2	β 1,4-Galactosyltransferase assay	58
2.7.3	N-acetylglucosaminyltransferase I assay	59
2.7.4	Protein assay	60
2.8	Expression of bacterial proteins	60
2.8.1	MBP-cyclin A	60
2.8.2	GST-cyclin B	61

3	Characteristics of the interphase and mitotic transport assay	63
3.1	Aims	63
3.2	The standard assay	65
3.2.1	The transport buffer	65
3.2.2	The cell-derived components	65
3.2.3	Antibody titrations	68
3.2.4	Time course of the assay	68
3.3	Titration of membranes in the assay	72
3.4	Interphase and mitotic cytosol	76
3.4.1	Histone kinase assay of cytosol	76
3.4.2	Durability of mitotic kinase activity under transport conditions	76
3.4.3	Interphase and mitotic cytosol and the transport assay	79
3.5	Salt-washing Golgi membranes	79
3.5.1	Salt-washed membranes and the transport assay	79
3.5.2	Time course of transport by K-Golgi	83
3.5.3	Variation in salt-washed membranes	83
3.5.4	Mitotic inhibition of K-Golgi transport	83
3.6	Summary	87
4	Control of the intra-Golgi transport assay by phosphorylation	88
4.1	Aims	88
4.2	Kinase inhibitors	89
4.2.1	Staurosporine	89
4.2.2	Optimal time of addition of staurosporine to the mitotic assay	91
4.2.3	Effect of dimethylaminopurine on mitotic transport	91
4.3	Cyclin A	94
4.3.1	Activation of cytosol by cyclin A	94
4.3.2	Effect of cyclin A on the transport assay	94
4.3.3	Optimal time of addition of cyclin A to the transport assay	97
4.3.4	Abrogation of the cyclin A effect by boiling or by simultaneous staurosporine treatment	99
4.4	Other cell cycle-related agents	101
4.4.1	Cyclin B	101
4.4.2	Phosphatase inhibitors	104
4.5	Summary	106
5	Further studies of the control of intra-Golgi transport by phosphorylation: the role of p34^{cdc2}.	107
5.1	Aims	107
5.2	The roles of protein kinases A and C in transport inhibition	108
5.2.1	Protein kinase A	108
5.2.2	Protein kinase C	108
5.3	The S-phase kinase, p33 ^{cdk2}	111

5.4	Mouse cell cytosols with temperature-sensitive p34 ^{cdc2}	114
5.4.1	Characterisation of FM3A and FT210 cytosols	114
5.4.2	Heat-treatment and the effect of cyclin A on FT210 cytosol	117
5.4.3	p33 ^{cdk2} and FT210 cytosol	123
5.4.4	Effect of microcystin on FT210 cytosol	125
5.5	Summary	127
6	The target of mitotic kinase in the intra-Golgi transport assay	128
6.1	Aims	128
6.2	Effects on GTP-binding proteins	129
6.2.1	Effect of GTP γ S	129
6.2.2	Effect of AlF _{3.5} and mastoparan	132
6.2.3	Effect of Brefeldin A	138
6.3	Rescue of arrested cytosol	140
6.4	Effect of purified transport proteins	142
6.5	Effect of cytosol on donors and acceptors in isolation	148
6.6	Summary	151
7	Discussion	152
7.1	Overview	152
7.2	The transport assay	152
7.3	Mitotic inhibition of transport	155
7.4	The role of p34 ^{cdc2}	157
7.5	The target of mitotic inhibition	159
7.6	Future directions	161
7.7	Conclusion	162
	Acknowledgements	163
	References	165

Table of Figures

1	Introduction	8
1.1	Overview of the exocytic pathway	10
1.2	Glycosylation in the Golgi apparatus	12
1.3	A model of Golgi structure based on oligomeric association of resident enzymes	18
1.4	A mode for coatomer and ARF involvement in intra-Golgi traffic	21
1.5	A model for the interaction of NSF, SNAPs and SNAREs in vesicle fusion	25
1.6	The mammalian cell cycle	29
1.7	p34 ^{cdc2} in interphase and mitosis	32
1.8	The control of p34 ^{cdc2}	35
1.9	Cyclins and cdk proteins throughout the cell cycle	38
1.10	The Golgi apparatus in interphase and mitosis	40
1.11	A model for transport inhibition and Golgi breakdown	43
2	Materials and methods	45
2.1	Pfu assay of vesicular stomatitis virus	49
3	Characteristics of the interphase and mitotic transport assay	63
3.1	The transport assay	64
3.2	The interphase transport assay	66
3.3	Optimisation of immunoprecipitation conditions	69
3.4	The time course of the intra-Golgi transport assay	71
Table 3.1	Characteristics of typical membrane preparations	73
3.5	Golgi membrane titrations	75
3.6	Capacity of the histone kinase assay	77
3.7	Time course of histone kinase activity under transport conditions	78
Table 3.2	Characteristics of typical cytosol preparations	80
3.8	Mitotic cytosol inhibition of the standard assay	81
3.9	Salt-washed Golgi and the transport assay	82
3.10	Time course of transport by salt-washed Golgi	84
3.11	Examples of salt-washed Golgi titrations	85
3.12	Comparison of two paired cytosols with the same membranes	86
4	Control of the intra-Golgi transport assay by phosphorylation.	88
4.1	Effect of staurosporine on the transport assay	90
4.2	Time course of the staurosporine effect	92
4.3	Effect of DMAP on mitotic cytosol	93
4.4	Histone kinase activation of interphase cytosol by cyclin A	94
4.5	The effect of cyclin A on the intra-Golgi transport assay	96
4.6	Time course of cyclin effect on transport	98
4.7	The effect of staurosporine and heat treatment on cyclin A with interphase cytosol	100

4.8	Effect of cyclins A and B on interphase cytosol	102
Table 4.1	Failure of recombinant cyclin B to activate sHeLa cytosol	103
4.9	The effect of microcystin on the transport assay	105
5	Further studies of the inhibition of transport by phosphorylation: the role of p34^{cdc2}	107
5.1	Forskolin effect on mitotic, interphase and cyclin-activated cytosol	109
Table 5.1	Protein kinase C activity in interphase and mitotic cytosol	110
5.2	Effect of cyclin A/p33 ^{cdk2} on the cyclin response of interphase cytosol	112
5.3	Correlated effects on transport inhibition and histone kinase activity of p33 ^{cdk2}	113
5.4	Titration of mouse cytosols and HeLa cytosol	115
5.5	Activation of mouse cytosols by cyclin A	116
5.6	Cytosol made from FT210 cells at 39°C remained cyclin A-sensitive	118
5.7	Heat treatment and the cyclin response of FT210 cytosol	119
5.8	Transport inhibition by cyclin A depends on p34 ^{cdc2} activity	120
Table 5.2	Addition of cyclin A to FT210 cytosol ameliorates the heat-lability of p34 ^{cdc2}	122
5.9	The effect of p33 ^{cdk2} and cyclin A on FT210 cytosol	124
5.10	Microcystin and FT210 cytosol	126
6	The target of mitotic kinase in the intra-Golgi assay	128
6.1	Effect of GTP γ S on transport	130
6.2	Effect of aluminium fluoride on the transport assay	133
6.3	Effect of mastoparan 1 on interphase and mitotic transport	134
6.4	Effect of cytotropic peptides on the transport assay	137
6.5	Effect of brefeldin A on interphase and mitotic Golgi transport	139
6.6	'Rescue' of mitotic transport by interphase cytosol	141
6.7	Effect of SNAPs and NSF on interphase and cyclin-treated cytosol	144
6.8	Effect of transport proteins on interphase cyclin-treated and arrested cytosol	146
6.9	Effect of transport proteins on interphase, mitotic and arrested cytosol	147
6.10	Effect of mitotic cytosol preincubations of separated membranes	149

Abbreviations

20S	complex of NSF, α - and γ -SNAP with size 20S based on sedimentation behaviour
ARF	ADP-ribosylation factor
BFA	brefeldin A
BHK	baby hamster kidney
cdc	cell division cycle
CGN	cis-Golgi network
CHO	Chinese hamster ovary
COP	coat protein/ coat protomer
EM	electron microscopy
GNTI	N-acetylglucosaminyltransferase I
GST	glutathione S-transferase
GT	galactosyltransferase
HeLa	see sHeLa
IPTG	isopropylthiogalactoside
LCRI	low cytosol-requiring intermediate
Mann II	mannosidase II
MBP	maltose binding protein
mc	microcystin
MPF	maturation promoting factor (p34 ^{cdc2} and cyclin B)
m/s	microcystin + staurosporine
NE	nuclear envelope
NEM	N-ethyl maleimide
NSF	NEM-sensitive factor
OA	okadaic acid
pCoA	palmitoyl coenzyme A
PKA	protein kinase A
PKC	protein kinase C
PP1/2A	protein phosphatases 1/2A
PTA	phosphotungstic acid
sHeLa	suspension HeLa (also referred to as HeLa occasionally in the text)
TGN	trans-Golgi network
SNAP	soluble NSF-associated protein
SNARE	SNAP receptor

Introduction

Overview

The Golgi apparatus lies at the heart of the exocytic pathway. It consists, in mammalian cells, of three or more flat, apposed cisternae lying close by the nucleus, and is flanked on one side by the endoplasmic reticulum and on the other by the trans-Golgi network. The Golgi stack contains, among other proteins, the enzymes responsible for N-linked glycosylation of newly-synthesised proteins. These enzymes are functionally polarised in the organelle.

Proteins traverse the Golgi and the whole exocytic pathway by means of nonselective transport vesicles, while the residents do not enter the vesicles, but are retained.

At mitosis in mammalian cells, intracellular traffic is inhibited at almost every stage so far studied. It is likely that transport inhibition is directed by the mitotic kinase, p34^{cdc2}, or subsidiary kinases. At the same time, the Golgi apparatus undergoes a gross morphological change, being vesiculated and dispersed throughout the mitotic cytoplasm. It has been proposed that it does so to facilitate partitioning between the daughter cells (Warren, 1985), and its breakdown may be at least partly due to inhibition of transport vesicle fusion.

This thesis contains experiments which study transport through the Golgi apparatus under interphase and mitotic conditions. The experiments employed a cell-free intra-Golgi transport assay originally characterised by Rothman and co-workers. This system was supplemented with cytosol derived from mitotic and interphase HeLa cells. The assay was used firstly to determine whether intra-Golgi transport was inhibited in the presence of mitotic cytosol.

When transport was found to be inhibited under mitotic conditions, experiments were performed to find out whether the inhibition was directed by kinase activity, particularly that of p34^{cdc2}, and also whether its target could be identified.

This introduction will first give a brief description of the organelles and mechanism of the exocytic pathway as it is currently understood. Since endocytic transport is not dealt with in this thesis, it will only be mentioned when it adds information to the picture of exocytosis. Next there will be a discussion of the intra-Golgi transport assay, explaining the principle and describing some of the proteins discovered with its aid. The last section will be a brief discussion of mitosis and its regulation, and a description of the structural and functional changes in intracellular organelles during mitosis. In general, the introduction will be confined to description of the situation in mammalian cells; other systems will be discussed where necessary.

1.1 The exocytic pathway

1.1.1 The organelles of the exocytic pathway

This section is a brief description of the structure and function of the exocytic organelles in the order of their action (Figure 1.1). The Golgi apparatus will be described at greater length since it is the subject of this thesis, but it is set in its functional context.

The endoplasmic reticulum

The endoplasmic reticulum (ER) is an extensive tubular reticulum which is contiguous with the nuclear envelope (NE) (see Palade, 1975). The ER comprises two subcompartments with no visible separation by means of EM. These are the smooth ER, which is a site of lipid manufacture, and the rough ER, which is decorated with ribosomes and forms the entry point of protein into the exocytic pathway. The ER colocalises with the microtubule network in cells by immunofluorescence (Lee and Chen, 1988), and can be caused to extend along microtubules in vitro (Dabora and Sheetz, 1988).

Admittance to the ER requires a signal sequence, which has no conserved residues, but a conserved pattern of hydrophobic and hydrophilic sequences (von Heijne, 1985). Other sequences within the protein direct its final orientation and number of passages through the ER membrane, and thus its final membrane conformation. The nascent polypeptide is inserted when the ribosome contacts the ER via the signal receptor particle and its receptor (reviewed in Walter and Lingappa, 1986).

In the ER, proteins are folded with the assistance of chaperone proteins (reviewed in Rothman, 1989), and do not leave the ER until they are correctly folded (reviewed in Rose and Doms, 1988). Many oligomerise, and this again is probably with chaperone intervention since incomplete hetero-oligomers do not leave the ER (Gething et al., 1986); reviewed in (Hurtley and Helenius, 1989), or at least do not cease to cycle between the ER and the CGN. Most importantly for the study of membrane traffic, in the ER nascent glycoproteins receive oligosaccharide units from a dolichol donor (reviewed in Kornfeld and Kornfeld, 1985). Proteins depart the ER in vesicles which bud from its transitional elements, tubules which resemble antennae extending from the ER (Saraste and Kuismanen, 1984).

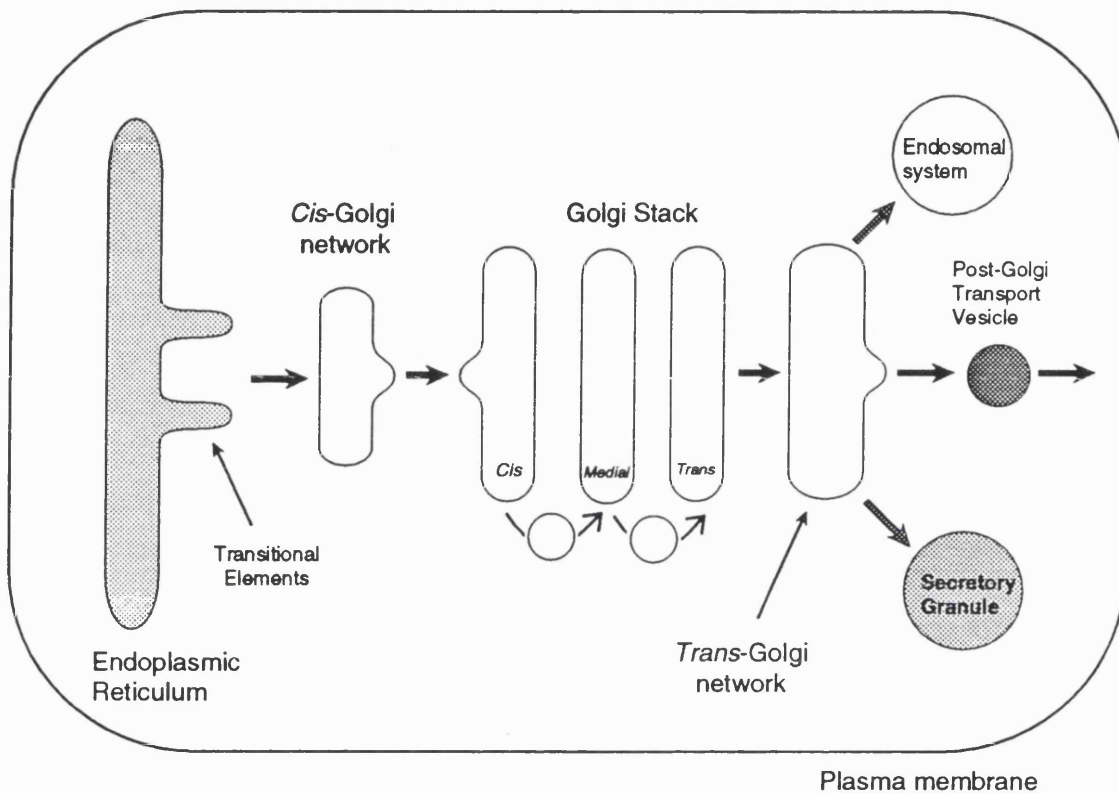


Figure 1.1: the exocytic pathway in mammalian cells

The arrows represent vesicle-mediated transport stages. Only a single vesicle-mediated step is thought to lie between the ER and the Golgi: it is not known whether or not the CGN is discrete from the ER.

The cis-Golgi network

At the cis face of the Golgi lies the cis-Golgi network or CGN. This resembles the cis-Golgi in being ramifying and fenestrated, but functionally is linked with the ER, and its structural relationship with these compartments is not entirely clear (see, for example, Beckers et al., 1990; Schwaninger et al., 1992; reviewed in Hauri and Schweizer, 1992). It is most easily studied when expanded by incubation of cells at 15°C (Saraste and Kuismanen, 1984).

Proteins localised to this compartment include proteins of 53 and 63kD, and rabs 1b and 2; also, COP-coated vesicles may bud off this compartment for the Golgi (reviewed in Hauri and Schweizer, 1992).

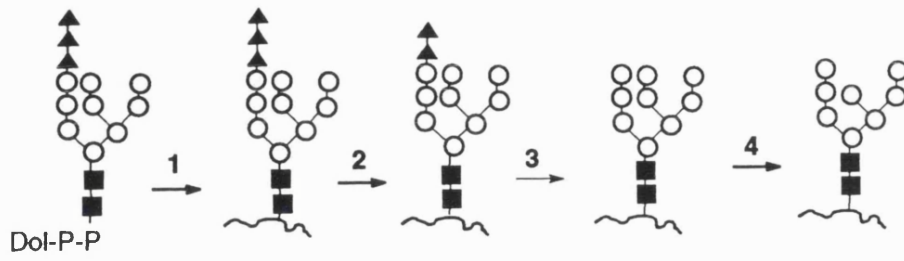
The CGN has been described as a salvage compartment (Warren, 1987). ER resident proteins and incompletely-folded proteins which escape the ER with the departing transients are selectively removed and returned to the ER from the CGN (reviewed in Pelham, 1989).

The Golgi apparatus

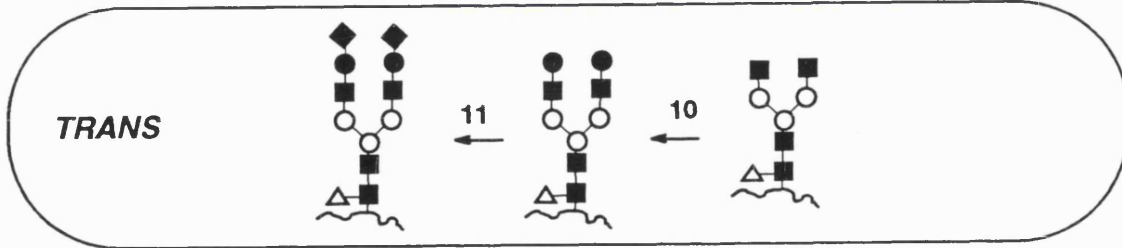
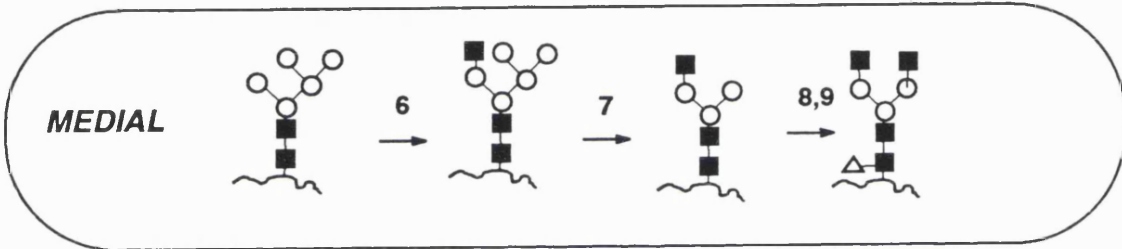
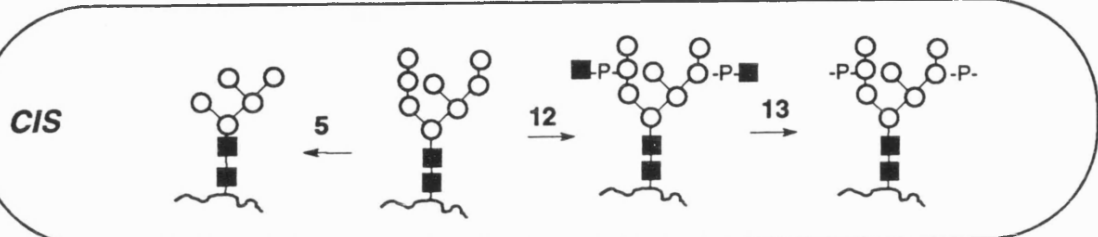
The Golgi apparatus has a distinctive structure and a well-established function (reviewed in Kornfeld and Kornfeld, 1985; Roth, 1987; Rambourg and Clermont, 1990). The organelle lies close to the cell nucleus and consists, in mammalian cells, of three or more flattened, apposed cisternae with fenestrated rims, the first cisterna being the most fenestrated. It is believed that the Golgi is a single-copy organelle, but it undulates so much that careful serial sectioning (eg Lucocq et al., 1989) or high-voltage EM (Rambourg and Clermont, 1990) is required to capture its full extent. Different glycosylation enzymes are enriched at the cis (early, towards the ER) or trans (late, towards the TGN) faces of the Golgi, or in the central, medial cisternae. Proteins passing through the Golgi apparatus undergo sequential modifications by the enzymes in the different cisternae.

The asymmetrical nature of the Golgi apparatus was initially revealed by EM in the presence of different stains; the cis face of the Golgi is stained with osmium tetroxide (Friend and Murray, 1965), whereas the trans face is stained with thiamine pyrophosphatase (Novikoff and Novikoff, 1977). Later, enzymes of oligosaccharide synthesis were found to reside in specific cisternae of the Golgi. Their distribution has been worked out by a combination of electron microscopy (eg Roth and Berger, 1982; Dunphy et al., 1985), lectin staining (Griffiths et al., 1982), studies of glycosylation mutants (reviewed in Stanley, 1984) and subcellular fractionation (eg Goldberg and Kornfeld, 1983; Dunphy and Rothman, 1983). Figure 1.2 shows the sequence and localisation of modification which has been deduced (reviewed in Kornfeld and Kornfeld, 1985).

ENDOPLASMIC RETICULUM



GOLGI



- | | | | |
|-----------|-----------------------|-------------|---------------|
| ○ mannose | ■ N-acetylglucosamine | ● galactose | ◆ sialic acid |
| △ fucose | | ▲ glucose | |
-
- | | |
|-------------------------------------|--|
| 1 oligosaccharyltransferase | 8 N-acetylglucosaminyltransferase II |
| 2 alpha-glucosidase I | 9 fucosyltransferase |
| 3 alpha-glucosidase II | 10 galactosyltransferase |
| 4 ER alpha 1,2-mannosidase | 11 sialyltransferase |
| 5 Golgi alpha-mannosidase I | 12 N-acetylglucosaminylphosphotransferase |
| 6 N-acetylglucosaminyltransferase I | 13 N-acetylglucosamine-1-phosphodiesterase |
| 7 Golgi alpha-mannosidase II | alpha-N-acetylglucosaminidase |

Figure 1.2: glycosylation in the Golgi apparatus

after Kornfeld and Kornfeld, 1985

note; some trans-Golgi enzymes also located in the TGN

The modifications outlined in the Figure represent only a few of those performed by the mammalian Golgi. The actual oligosaccharide patterns seen on a given protein depend on the organism, the organ of the body, the protein environment of each potential glycosylation site and microheterogeneity (ie a small range of related structures seen on each site when all other sources of variation are eliminated) which may reflect the stochastic nature of the enzyme-substrate reaction during transit through the Golgi. The sequential nature of the modifications has permitted the enzymes to be localised in the Golgi apparatus. At the same time, the glycosylation state of a protein may indicate its location in the exocytic pathway (as for example Endo H resistance, the condition of a glycoprotein which has been acted on by mannosidase II and GNTI in the medial Golgi, and is resistant to the clipping enzyme endoglycosidase H, whose effect is normally readily detectable on protein SDS-PAGE). However, the Golgi enzymes act in the same way with or without their spatial organisation. This has been seen in cells treated with the fungal metabolite Brefeldin A (BFA: Misumi et al., 1986). This drug causes the Golgi apparatus to redistribute to the ER (among other changes in the structure of the protein traffic organelles) so that ER residents receive Golgi modifications when they would not normally do so (Doms et al., 1989; Klausner et al., 1992). The modifications by these mislocalised Golgi enzymes are similar to those of the normal Golgi, though very careful oligosaccharide analysis would be required to detect small alterations in glycosylation.

Interestingly, the distributions of the Golgi enzymes established by single-label immuno-EM have recently been challenged by a double-label immuno-EM study of the Golgi in HeLa cells (Nilsson et al., 1993). It has been shown that GNT occupies both medial and trans cisternae, and GT both trans cisternae and TGN; and that the two enzymes have overlapping distributions (see also Velasco et al, 1993). This notable finding suggested a model of Golgi structure (Warren, 1993) in which like enzymes in different cisternae associate via a Golgi matrix (Cluett and Brown, 1992). Recognition of like enzymes would provide a conceptual basis for the compartmentation of the Golgi and its reassembly after mitosis, and also explain how expanded Golgi with multiple, replicated cisternae (eg plant Golgi; Driouich et al., 1993) may associate.

The mammalian single-copy Golgi is associated with microtubules (Ho et al., 1989; Kelly, 1990), to which it is anchored by cytoplasmic dynein (Corthésy-Theulaz et al., 1992); microtubule disruption in vivo by nocodazole, and dynein cleavage in vitro by UV light both result in release of Golgi anchorage. The Golgi functions normally in nocodazole-treated cells; indeed, 300 discrete Golgi stacks are seen immediately after mammalian mitosis. The yeast *S. pombe* contains multiple Golgi stacks, and these do not fragment at mitosis (K. Ayscough, PhD Thesis, University of London, 1993).

The trans-Golgi network

The trans-Golgi network, or TGN, is a tubulovesicular network which lies at the trans face of the Golgi. The best marker for the TGN at present is the Golgi enzyme sialyltransferase (Roth et al., 1985). This compartment is the site at which several pathways meet, including constitutive and regulated exocytic transport, transport to and from the early and late endocytic pathways (the TGN and endosomal network are closely associated in the cell), and diversion to the lysosomes. In the TGN of polarised cells, proteins destined for delivery to the different cell surfaces are separated, having traversed the ER and Golgi together (reviewed in Griffiths and Simons, 1986).

1.1.2: the mechanism of intracellular transport

Vesicular transport

It is a central tenet of the theory of exocytosis that its compartments do not move or change, but that the transiting proteins move from one to another by means of lipid vesicles. Membrane buds are probably formed continuously from all intracellular compartments with unconstricted boundaries (such as the fenestrated rims of Golgi cisternae). Such membrane buds possess equal potential to form sealed vesicles and extended tubules, either of which could fuse with a target membrane (see Cluett et al., 1993). To prevent extensive tubulation of membranes (which would cause fusion between compartments rather than selective transfer of their contents) vesicles going between non-mixing compartments are provided with coats. These could complete vesicle formation, and then maintain the fusion machinery in a cryptic state until the target had been reached.

A different mechanism has been proposed for traffic through the exocytic pathway: that of compartment maturation, by which complete compartments would move through the cell and gradually change their nature by movement of enzymes etc (eg Brown and Romanovicz, 1976). This would involve vesicular transport of compartment residents backwards to meet their substrates, rather than the substrates going forwards. Such a model is supported by the fact that Golgi enzymes are capable of retrograde movement under some circumstances, eg in BFA-treated cells (though the TGN mixes with the endosomal system under those conditions), but there is no evidence that they do so in vivo on the scale required by cisternal maturation.

On the other hand, the cell-fusion experiments by Rothman and co-workers (Rothman et al., 1984a, b) indicated strongly that glycoproteins could be transferred between non-contiguous Golgi membranes. Instead, when intra-Golgi transport is inhibited in vitro, Golgi-derived vesicles are detected which contain the transported proteins (Malhotra et al., 1989), and in a

yeast system, such vesicles are consumed on relief of the blockade (Rexach and Scheckman, 1991).

Bulk flow and default transport

It was mentioned at the beginning of this section that proteins require a signal sequence to enter the ER. It is thought that for plasma membrane and secreted proteins the signal sequence is the only 'passport' that must be displayed by a correctly-assembled protein in order to traverse the secretory pathway. This is the concept of default transport. It would be structurally complex and intellectually unsatisfactory if all exocytosed proteins had to display several signals, each one required to go one stage further through the exocytic pathway. Rather, exocytosed proteins only bear signals if they are to be diverted from, retrieved to or retained at a site proximal to the plasma membrane. A prediction of this theory was that proteins of diverse species might be exocytosed, since only one signal would be required for entry into the pathway. This was borne out by the finding that bacterial β -lactamase could be exocytosed from *Xenopus* oocytes (Wiedmann et al., 1984).

Another prediction was that proteins should have a maximum rate of transport. If they bore multiple signals, then proteins with several signals might go as fast or faster than proteins with none; if bulk flow was operating, then proteins with minimal structural information should be transported fastest of all. This has been harder to demonstrate unambiguously, but it appears that the transport rate of newly-synthesised proteins depends on the rate of folding in the ER rather than the rate of exit of the folded proteins (reviewed in Rose and Doms, 1988).

1.1.3 Protein localisation in the exocytic pathway

The ordered passage of proteins through the exocytic pathway requires that the proteins which act on the transients at each stage remain resident themselves (assuming a vesicular model of protein transport). Two concepts have arisen to account for compartmental residence; **retrieval** and **retention**. The retrieval idea is that proteins which leak from their own compartment are recognised and brought back; retention requires that resident proteins do not enter transport vesicles.

Retrieval was hypothesised to account for the properties of the immunoglobulin heavy-chain binding protein (BiP) a luminal protein of the ER. When the last four amino-acids of the protein (KDEL) were deleted, the protein was secreted slowly, with a $t_{1/2}$ of three hours. The same C-terminal amino-acids were present on another ER resident, protein disulphide isomerase, and were sufficient to confer ER localisation on a secreted protein (lysozyme) in the ER (Munro and Pelham, 1987). When attached to the lysosomal protein cathepsin, the

protein accumulated in the ER, but nevertheless received early Golgi modification, and this suggested that it was escaping the ER but being retrieved (reviewed in Pelham, 1989). The notion of retrograde transport through the exocytic pathway has since been given support by the identification of a HDEL receptor in the Golgi of *S. cerevisiae*, which was capable of retrieving HDEL-tagged chimaeric proteins (Semenza et al., 1990). Its specificity was demonstrated by an elegant experiment involving the ERD2 protein of the yeast *Kluyveromyces lactis*, which recognises the retrieval signal -DDEL. When ERD2 of *S. cerevisiae* was replaced by that of *K. lactis*, the specificity of retention was altered such that -DDEL-tagged chimaeras, and not -HDEL proteins were retrieved (Semenza et al., 1990; Lewis et al., 1990).

By homology cloning, the human ERD2 homologue has been identified, and this retrieves KDEL-bearing proteins of the ER (Lewis and Pelham, 1992). It remains to be determined how ERD2 is located in the Golgi, and how it binds ER proteins there to release them in the ER. However, the idea of proteins being 'localised' in a cycle between two compartments is exemplified by ERD2, and the idea of retrieving a protein which travels beyond its own residence originates with BiP.

Other organelle residents owe their localisation to retrieval. For example, some membrane-spanning proteins of the ER bear a cytoplasmic (C-terminal) double-lysine motif (Nilsson et al., 1989; Jackson et al., 1990). These proteins have been shown to receive O-glycosylation characteristic of the intermediate compartment, and to localise with p58 there; ie they remain in the ER by a retrieval mechanism (Jackson et al., 1993). The double lysine signal, when grafted onto secreted proteins, causes their retrieval to the ER (Nilsson et al., 1989).

The Golgi enzyme galactosyltransferase has a transmembrane sequence which causes its retention in the trans-Golgi (Nilsson et al., 1991). Chimaeric proteins were created between the cytoplasmic and transmembrane sequences of galactosyltransferase and the marker protein invariant chain, and these sequences retained the chimaera in the trans-Golgi. A sequence comprising the luminal half of the transmembrane sequence was also sufficient to cause retention of invariant chain. It was suggested that the retention required homodimerisation, and in support of this a truncated protein containing only the cytoplasmic and transmembrane regions of GT, and a few luminal residues, was expressed in bacteria and found to exist as a dimer.

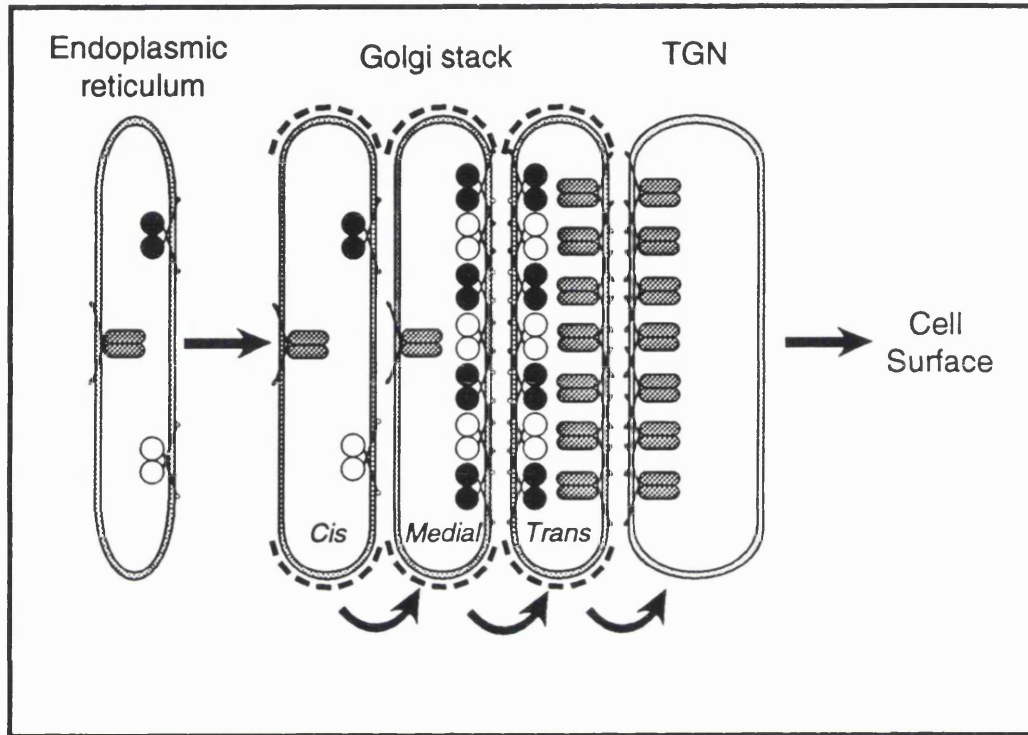
It is possible to generate a model for Golgi structure which requires little more than retention of its enzyme residents and some form of matrix (Figure 1.3; Warren, 1993). It has been suggested that the Golgi enzymes form oligomers by alternate dimerisation between luminal enzyme heads and transmembrane helices. Such oligomers might be too large to enter transport vesicles, and therefore be retained in the Golgi. If the enzymes were also capable of

selecting newly-synthesised or retrieved co-residents out from the transient population they could also contribute to the compartmental organisation of the complex. It has also been suggested that the enzymes are anchored to a proteinaceous Golgi matrix (P. Sluszarewicz, personal communication). It is possible that the structure of the Golgi apparatus is linked to the retention of its enzyme population, and thus to its function in glycosylation. This implies that the Golgi is a self-regulating organelle, ie that its form is dictated by its population of resident and transient protein and the metabolic requirements of the cell.

One might imagine that proteins which cycle between different sites would require both retention and retrieval signals. For example, the membrane protein TGN38 cycles between the TGN and the cell surface (Luzio et al., 1990). It bears a cytoplasmic tetrapeptide containing a tyrosine residue which can direct inclusion in a budding vesicle by interacting with its coat adaptor proteins (Bos et al., 1993). TGN38 appears to contain an additional, weak transmembrane motif for retention in the TGN (S. Ponnambalam, personal communication). Another example is BiP, which is secreted very slowly from cells when its -KDEL motif is removed (Munro and Pelham, 1987); this may well be due to a retention motif.

Proteins at a fixed intracellular location might require only retention signals - but in fact it is more likely that the cell has molecular contingency plans for the failure of retention signals in all compartmentalised proteins. Thus, Golgi enzymes which escape retention may be retrieved and relocalised. This suggestion is supported by work with BFA, which causes the redistribution of Golgi enzymes into the ER. In the presence of the drug, coated transport vesicles are not formed, and instead tubulovesicular processes travel along cytoplasmic microtubules to deposit the contents of the Golgi in the ER (Lippincott et al., 1990; Cooper et al., 1990). It has been proposed that this drug halts forward transport to reveal a retrograde transport pathway used for resorting compartmental contents (Lippincott-Schwartz, 1993). The hypothesis that the Golgi acts as a kind of 'distillation apparatus' to prevent the loss of ER proteins (Rothman, 1981) seems to contain a valuable concept of the cell continuously redefining its organelles by vetting and re-sorting their contents.

It is suggested that dimerisation between alternate transmembrane and luminal domains of Golgi residents leads to the formation of protein oligomers or rafts too large to be incorporated into transport vesicles. The proteins GNTI and Mann II appear to be retained in the medial Golgi in hetero-oligomers. The association of similar hetero-oligomers between cisternae may account for the observed polarity of the stack but, since the cytoplasmic tails of the enzymes could not span the intercisternal distance, it is necessary to postulate the existence of an enzyme-binding Golgi matrix such as has been isolated in this laboratory (Sluszarewicz et al, J. Cell Biol. 1994, in press).







 'Medial' enzymes 'Trans' enzyme

dashed regions at ends of Golgi cisternae represent active budding regions

Figure 1.3: a model for Golgi structure based on oligomeric recognition of resident enzymes

1.2 An in-vitro assay for intra-Golgi transport

1.2.1 *The principle of the assay*

The in-vitro assay reconstitutes the vesicle-mediated transport of the vesicular stomatitis virus glycoprotein (VSV-G) between successive cisternae of the Golgi apparatus (Balch et al., 1984a, b; Balch and Rothman., 1985: the rationale of the assay is shown in Figure 3.1). The G protein originates in a Golgi-enriched "donor" membrane fraction prepared from a VSV-infected mutant CHO cell line (clone 15B; Gottlieb et al., 1974) which lacks the medial Golgi enzyme GNT I. VSV infection of cultured cells results in the secretion of very large quantities of the G-protein, so that 'donor' membranes contain high levels of VSV-G. The donor membranes are incubated with an "acceptor" Golgi fraction prepared from uninfected wild-type cells. In its original form, the transport of G-protein was then quantified by determining the fraction of the protein which had become resistant to endoglycosidase H. This could only occur if VSV-G had been transferred from the donor membranes to the medial Golgi of the acceptor, and there encountered GNT I. Later, the assay was made far more convenient by use of tritiated UDP-GlcNAc, which is taken up by the Golgi sugar transporter, to label the transported G-protein to high efficiency. The process requires ATP and cytosolic factors normally supplied by CHO cytosol.

The extraction of the Golgi from the cellular milieu has allowed unparalleled experimental flexibility in determining its requirements. The proteins involved in the transport process have been identified by use of drugs as inhibitors of the assay, studies of transport kinetics, and biochemical complementation with heterologous proteins and cytosols. However, this isolation of a transport process has also been a problem for the assay since, as with all in-vitro systems, it must be proved that observations made on the assay reflect the situation in vivo.

Alongside the biochemical studies using the transport assay, understanding has been gained by the genetic analysis of the secretory pathway by Novick and Scheckman (Novick and Schekman, 1979; Novick et al., 1980). *S. cerevisiae* was mutagenised and genes with transport mutant phenotypes were identified and subsequently classified into ER-Golgi, intra-Golgi, Golgi-vacuole and Golgi-cell surface phenotypes.

Something over fifty SEC (secretory pathway) gene products have been studied after this method, and the work has been linked to mammalian-system studies in three ways. First, it has been possible to identify sequentially-acting or interacting components by classical genetic methods. Second, it has been possible to mutate or delete genes from *S. cerevisiae*, and then study the effects of a deletion or its rescue, long before similar conditions could be generated in mammalian systems. Last, on many occasions when transport proteins have been identified biochemically in a mammalian system, their *S. cerevisiae* homologues have

been recognised almost at once from the existing bank of SEC gene products. This can provide opportunities to rescue a mammalian phenotype with a yeast gene or vice versa, as described above for *S. pombe* p34^{cdc2}. The limitation of *S. cerevisiae* as a system of study is its relatively undeveloped Golgi apparatus and endocytic system. This means that its repertoire of compartment-specific proteins is probably different from, and not as great as that of mammalian cells.

1.3.2 Identification of transport proteins using the assay

Vesicles and vesicle budding

It was early established that transport was inhibited by GTP γ S, which caused the accumulation of uniformly-sized transport vesicles and indicated that GTP hydrolysis was required for vesicle fusion (Melancon et al., 1987; Orci et al., 1989). When transport vesicles were purified, a family of externally-disposed proteins was found in a stoichiometric ratio (Serafini et al., 1991; Waters et al., 1991), ranging in size from 170kD to 21kD. In addition, the 100kD coat protein (β -COP) has a homologue in the adaptin of clathrin-coated vesicles. As a group, they are referred to as coat protomers or COPs, and their immunodepletion prevented budding of vesicles from donor membranes (Orci et al., 1993).

Vesicles purified from exocytic systems fall into two categories on the basis of their coats. The first-discovered type carries vesicles at the very end of the pathway, from the trans-Golgi to the cell surface, and they also carry traffic in the endocytic pathway. These are coated externally with clathrin, and this surrounds clathrin adaptor proteins or adaptins, which can select the contents of the vesicle (reviewed in Pearse and Robinson, 1990). The clathrin is in the form of triskelions, whose rearrangement encloses the nascent vesicle (Brodsky et al., 1991).

The second type carries protein from the ER and through the Golgi, and is coated with clathrin- and adaptin- related coat proteins (COPs). Uncoated vesicles have not been purified biochemically. Since they would be late-stage, pre-fusion vesicles according to the terms stated above, this may simply reflect conditions inappropriate to detect them.

The smallest coat protein is ADP-ribosylation factor (ARF), which was originally identified as a cofactor for the ribosylation of the alpha-subunit of adenylate cyclase by cholera toxin (Kahn and Gilman, 1984). ARF associates with membranes only when both myristoylated and complexed with GTP (Serafini et al., 1991). The association of ARF with donor Golgi membranes is irreversible when ARF is bound to GTP γ S (Taylor et al., 1992). More recently, ARF has also been implicated in clathrin coat assembly (Stamnes and Rothman, 1993) (Figure 1.4).

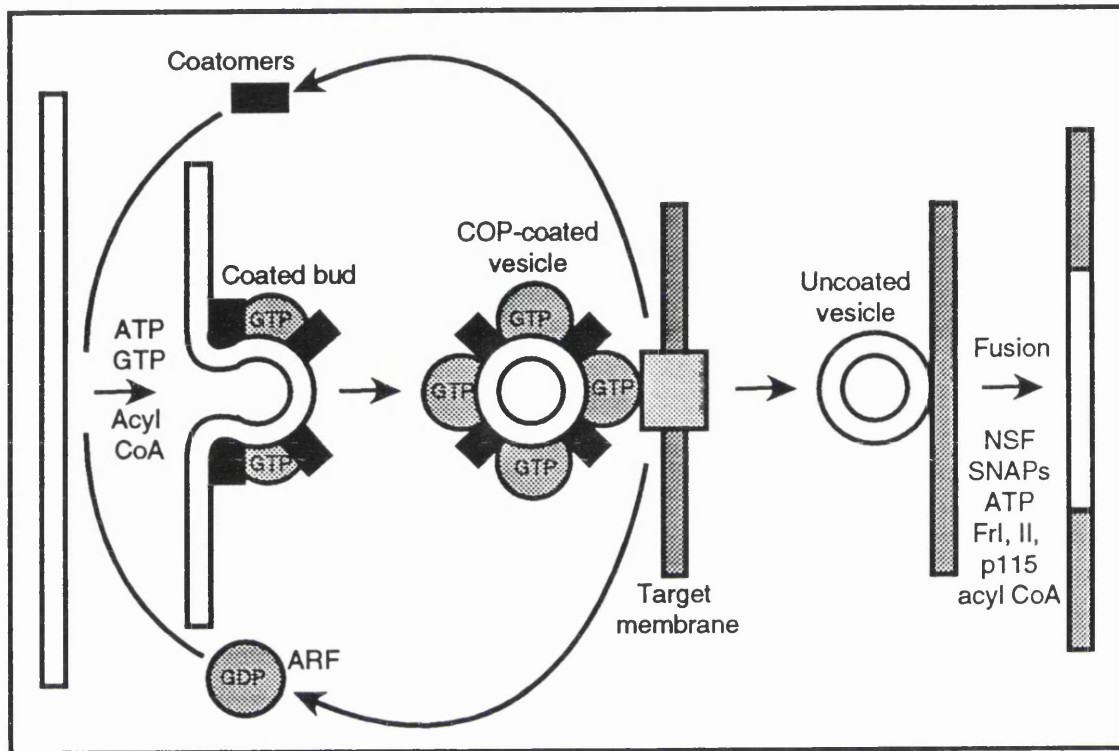


Figure 1.4: the involvement of ARF and coatamer in intra-Golgi transport

A number of ARF proteins have been identified by cloning, suggesting that the proteins are required for different events. ARF has indeed been found to be involved in a variety of budding events; in ER-Golgi transport (Balch et al., 1992; Schwaninger et al., 1992), of both clathrin-coated and Golgi coated exocytic vesicles (Serafini et al., 1991; Taylor et al., 1992; Stamnes and Rothman, 1993), and of clathrin-coated endocytic vesicles (Lenhard et al., 1992), and in almost all budding events examined in yeast (see d'Enfert et al., 1992). A number of ARF proteins have been cloned and shown to be structurally and functionally conserved (Kahn et al., 1991), suggesting that different ARF subtypes may be involved in different stages of transport.

Additional information about mammalian ARF-dependent budding has come from experiments with BFA which, as mentioned in section 1.1.3, causes the redistribution of the Golgi apparatus to the ER. The drug was found to cause the release of β -COP from the fenestrated rims of the Golgi, by inhibiting GDP-GTP exchange on ARF (Helms and Rothman, 1992; Donaldson et al., 1992a). As a result, the Golgi did not form coated vesicles, but rather coatless tubules which extended from the Golgi to the ER (Cooper et al., 1990). Also, BFA treatment circumvents the GTP γ S block to fusion, since GTP γ S only acts on vesicle uncoating, which is a later stage in the transport process (Helms and Rothman, 1992). The exception to the universal role of ARF is yeast ER-Golgi transport. There, a mutant deleted for ARF accumulated invertase transport intermediates in an early-Golgi state of glycosylation, and not with ER core-glycosylation, as would be expected if ER exit had been blocked. The gene responsible turned out to be SAR1, another small GTP-binding protein with 35% homology to ARF and which, like ARF, is incorporated into transport vesicles (Nakano and Maramatsu, 1989).

Unlike ARF, Sar1p lacks an N-terminal myristoylation site, but associates with ER membranes even at 4°C. It associates with Sec12p, a membrane-integral protein required for vesicle budding from the ER (d'Enfert et al., 1991), along with Sec 13, 16 and 23p (Kaiser and Schekman, 1990). It also possesses GTPase activity which is enhanced by interaction with Sec23p, one part of a heterooligomeric complex with GAP activity (Barlowe et al., 1993; Yoshihisa et al., 1993). The use of SAR1 for ER-Golgi transport is conserved in *S. pombe* and in at least one plant (d'Enfert et al., 1992).

Vesicle fusion

The first protein discovered with the transport assay was a factor required for membrane fusion. The assay is inhibited by mild treatment with the alkylating agent N-ethylmaleimide (NEM); a protein was purified from cytosol which rescued transport in the NEM-treated assay, and was termed NEM-sensitive factor or NSF (Block et al., 1988; Malhotra et al.,

1988). This protein, a homotetramer of 76kD subunits, was found to be essential for vesicle fusion with acceptor membranes. It was found that, in the presence of ATP, the NSF was released from the Golgi membranes, whereas it was membrane-bound in the absence of ATP. When NSF was cloned on the basis of its ability to reconstitute transport in an NEM-treated assay, it was found to bear significant homology with the yeast protein SEC18 (Wilson et al., 1989). NSF has been implicated in every stage of transport so far examined, including ER-Golgi (Beckers and Balch, 1989; Davidson and Balch, 1993), and endocytic vesicle fusion (Diaz et al., 1989).

The sequences and characteristics of SEC18 and NSF indicated that the proteins were probably soluble, and thus it was necessary to identify their mechanism of membrane attachment. For this reason, an assay was devised using membranes depleted of cytosolic attachments by treatment in 1M KCl (salt-washed or K-Golgi: Weidman et al., 1989). Whereas the normal assay can be supported by cytosol from *S. cerevisiae*, a K-Golgi assay could not; ie some of the components removed from the membranes by the salt-wash could not be supplied by the yeast cytosol. It was found that, in addition to NSF, two mammalian cytosol fractions were required for full reconstitution; these were termed Fraction I (average Mr 500kD) and Fraction II (average Mr 40kD). Using K-Golgi and several fractions of bovine brain cytosol in an assay the critical proteins were purified and found to be components of 35, 36 and 39kD (in bovine brain cytosol), and were termed α , β and γ SNAP (soluble NSF attachment protein: Clary et al., 1990; Clary and Rothman, 1990). β -SNAP was found to be a brain-specific isoform of α -SNAP, since γ -SNAP could form a complex with either of them in NSF binding. NSF could not bind Golgi membranes in the absence of SNAPs (Whiteheart et al., 1993).

It was then found that a complex of NSF, SNAPs and a membrane protein could be solubilised from rat liver Golgi membranes in the presence of ATP γ S, and dissociated in the presence of Mg⁺⁺-ATP. This complex migrated at 20S on a glycerol gradient, and it was demonstrated that membrane binding of SNAPs was a prerequisite for NSF-SNAP association (Wilson et al., 1992).

The membrane receptor for the 20S complex was identified by an elegant affinity-isolation procedure (Soellner et al., 1993) in which NSF-myc was immobilised on a column in the presence of ATP γ S and EDTA (to prevent any ATP hydrolysis by chelating Mg⁺⁺), and then α and γ SNAP and a detergent-solubilised membrane extract passed over the matrix. After washing in the presence of Mg-ATP γ S (to remove proteins bound to the matrix via ATP γ S or ATP) the remaining protein was eluted from the matrix by addition of Mg⁺⁺-ATP. Aside from a small number of non-specific bands, the only proteins eluted were found to reside in the range of 18-25kD. They were recognised by protein microsequencing as SNAP-25, synaptobrevin and syntaxin. These proteins were known to be involved in fusion of

neurosecretory vesicles (bearing synaptobrevin) with the presynaptic membrane (bearing syntaxin). Thus, the biochemical data obtained in the cell-free assay was linked with long-established observations in a different field (see Warren, 1993). It may be suggested that syntaxin and synaptobrevin (renamed t-SNARE and v-SNARE respectively, to indicate their locations) are general components of the vesicle-target fusion machine via their NSF-SNAP binding (Figure 1.5), and also that they are members of a multigene family with representatives in different organellar membranes and in different species. If this were so, their localisation to specific vesicle cycles between adjacent cisternae might be linked with the localisation of Golgi residents to similar cisternal pairs.

Recently, a new candidate has been presented for control of traffic through the Golgi apparatus, the trimeric G-protein. These proteins have classically been implicated in signal transduction from the cell surface (reviewed in Gilman, 1987), but some have been localised to Golgi membranes by immunofluorescence (Ercolani et al., 1990) and by specific functional tests, such as inhibition by aluminium fluoride (Kahn, 1991) or by mastoparan (Gil et al., 1991; Higashijima et al., 1990). Increasing the expression level of $G\alpha_{i-3}$ decreases the rate of constitutive transport through the cell surface (Stow et al., 1991). Trimeric G-protein function has also been implicated in control of endosome fusion (Colombo et al., 1992).

Vesicle targeting

What proteins are used to target the vesicles to their acceptor membranes? The most plausible answer has until recently been rab proteins. These are small GTP-binding proteins, whose archetypes YPT1 and SEC4 of yeast perform essential functions in ER-Golgi and Golgi-cell surface transport respectively (Segev and Botstein, 1987; Segev et al., 1988; Salminen and Novick, 1987). Homology screening identified a plethora of rabs in yeast and all other organisms studied; numbers sufficient for a 'one compartment-one rab' hypothesis of vesicle targeting. Indeed, several rabs were located to different stages of transport, by immuno-EM, or inhibition of stages with rab-derived peptides, or implication of rabs in specific transport events. For example, rab1 and 2 are implicated in ER-Golgi transport (Plutner et al., 1991; Chavrier et al., 1990); rab 6 is localised to the medial- and trans-Golgi (Goud et al., 1990); rab 4 to the early endosomes (van der Sluijs et al., 1991) and rab 5 and 7 to early and late endosomes respectively (Gorvel et al., 1991). The rab proteins are highly homologous in their GTP-binding regions, but their C-termini are more variable. At the C-terminus there is an acylation site, and these proteins require geranylgeranylation for association with their membranes, and transport activity (Farnsworth et al., 1991).

A model for Golgi vesicle targeting based on the synaptic vesicle fusion process. The synaptic vesicle protein VAMP and the synaptic membrane proteins syntaxin or SNAP-25 (also known as v- and t-SNAREs respectively) may direct specific vesicle targeting. Premature vesicle fusion could be blocked by a 'fusion clamp' such as synaptotagmin. α -SNAP and the 20S complex of α - and γ -SNAPs and NSF could displace synaptotagmin from the SNARE complex. ATP hydrolysis by NSF could be required for the release of the soluble complex, the dissociation of the SNARE pair and the fusion between the two membranes. Some of these studies have recently been published by the Rothman laboratory (Sollner et al, Cell 75, 409-418 (1993)).

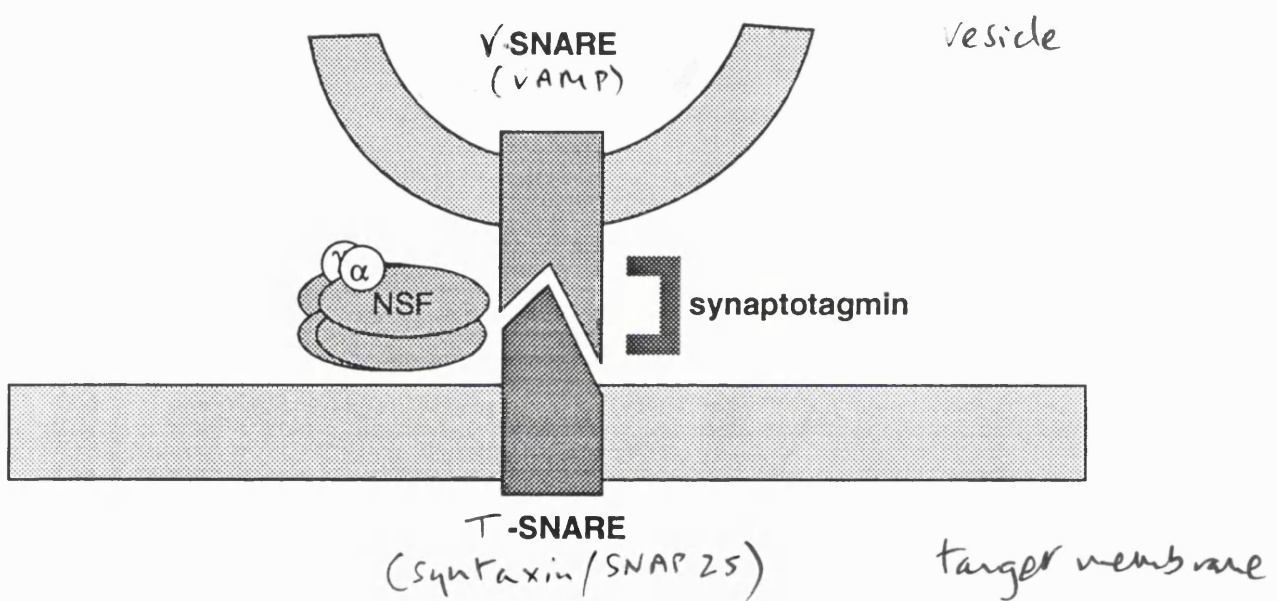


Figure 1.5: model of the vesicular fusion complex

it is believed that the NSF-SNAP complex causes the removal of the synaptotagmin clamp and breakdown of the SNARE complex, so that the vesicle may fuse with its target

More recently, however, some results have been adduced against rabs as the mediators of vesicle targeting. When chimaeras were made between YPT1 (a budding yeast rab active between the ER and the Golgi) and SEC4 (Golgi to cell surface), a nine amino-acid peptide in the YPT1 sequence, substituted into the SEC4 protein, generated a protein which could perform the functions of both (Dunn et al., 1993; Brennwald and Novick, 1993). The chimaera could direct the full glycosylation and secretion of invertase, and the lysosomal targeting of carboxypeptidase 1, without misdirection of either protein. This shows that neither protein can be solely responsible for accurate targeting to its designated compartment. It appears from recent data that the SNAREs themselves may be responsible for targeting. This conclusion comes from the study of certain SLY (Suppressor of Loss of YPT) genes (Dascher et al., 1991; Ossig et al., 1991), which were identified by their ability to permit YPT-independent ER-Golgi transport and growth. SLY2 and SLY12 are related to synaptobrevins or v-SNARE, and rescue *ypt1* mutants at high copy number. One may imagine that they are targeting molecules, which at high copy number enable the incoming vesicle to remain in contact with the target membrane for long enough to enter the fusion pathway without functional YPT1. In addition to this, the gene SED5, identified as a suppressor of ERD2 mutation, is a syntaxin (t-SNARE) homologue (Hardwick and Pelham, 1992). A chain of association can be built up between rab and SNARE, since the target of rab3A is a ~80kD protein designated rabphilin (Shirataki et al., 1993), with domains homologous to synaptotagmin, which is a synaptobrevin-associated protein that binds membrane phospholipid in a Ca⁺⁺-dependent manner. Thus, by a flight of fancy one arrives at the suggestion that SNARE interactions target the incoming vesicle, with rab proteins in a subsidiary role. The truth of the matter will not be known until the number and variety of SNAREs has been assessed, and the mechanism of vesicle recognition and fusion is better understood.

This leaves one wondering about the function of this army of rab proteins; they are still present in every organelle examined to date. It has been suggested that they fulfil a proof-reading role as 'molecular clocks'. This idea has arisen from the precedent of the GTP-binding protein at the heart of amino-acid selection at the ribosome. At the elongation site, there is constant sampling of amino-acid-charged tRNAs, which enter and leave the site with rapid turnover. However, the appropriate tRNA, by virtue of its association with its cognate mRNA codon, remains in contact with the active site. Now, the elongation factor Ef-Tu is a GTPase with a slow turnover. Turnover is only possible if a tRNA occupies the ribosome, but most do not remain for long enough to allow GTP hydrolysis; in fact, only the correct match remains for long enough. GTP hydrolysis then seals the fate of the tRNA and ensures (with a very low error rate) appropriate polypeptide elongation.

Since rabs and fusion complexes are thought to associate (by a tenuous chain of association) it is possible that the on-site rab protein proof-reads and confirms the information provided by the docking SNARE complex. The intrinsically low GTPase activity of the rabs could be increased by a GTPase activating protein (GAP) (see Strom, et al., 1993) which might interact with rab only once proofreading was complete. Afterwards, the rab must dissociate from the acceptor compartment, and this might be connected with GTP hydrolysis. This suggestion is borne out by the properties of rab3A, which associates with synaptic membranes and vesicles in its GTP-form. A rab GDI interacts with rab3A and removes it from membranes when in the GDP form, and nucleotide exchange (by a GDS : see Kawamura et al., 1991) is necessary to permit release of the rab from the GDI (Araki et al., 1990). The number of rab GDIs is not known at present, but it seems appropriate that the proofreading process requires GTP hydrolysis and must be completed by removal of the proofreader.

In the Golgi complex, the number of cisternae and other activities can vary markedly, yet there is only one known Golgi-localised rab protein. One explanation for this may be that transport between the cisternae of the Golgi is proof-read by a single rab protein. It is possible, moreover, that targeting itself within the Golgi is not rigidly defined. Ordered glycosylation does not absolutely require access to the enzymes to be sequential, and it has been shown that the enzymes overlap in the Golgi so that activities formerly connected with medial and trans cisternae now appear to colocalise (Nilsson et al., 1993). Thus, provided that vesicular entry into and exit from the Golgi domain was monitored, the vesicles could come and go relatively freely within the Golgi domain.

To summarise: in the presence of the ras-related protein ARF, coatamer mediates budding from the donor compartment and transport vesicles are formed. On arrival at a suitable acceptor membrane, the coat is removed, possibly signalled by interaction of v-SNARE and t-SNARE. The docking appears to involve dissociation of the interacting SNAREs by the NSF-SNAP complex, and is finalised by GTP and ATP hydrolysis so that the vesicle may fuse with the acceptor (reviewed in Rothman and Orci, 1992).

Clearly, this picture is incomplete. It has not been proven that transport vesicles can be formed *in vitro* or *in vivo*; it is strongly surmised from the observation of vesicles accumulated in the presence of GTP γ S (where uncoating cannot occur), the existence of coatamer, and the current model of the Golgi apparatus as a static structure. It remains unclear how (or indeed whether) a vesicle specifically locates its target, though rab proteins would be good candidates for acceptor markers. The rab proteins, having made their outward journey, would have to be released from the membrane into the cytoplasm and return to their donor cisterna.

So far, this chapter has described the structure and function of the mammalian exocytic pathway in interphase. In mitosis, intracellular traffic ceases at almost every point so far studied. The cytoarchitecture alters to prepare for the division of the cell and, most importantly for the present work, the Golgi apparatus fragments and is dispersed throughout the cell.

The next part of this chapter describes the present understanding of mitosis, its causation and its action on the cell.

1.3 Mitosis

The cell division cycle comprises DNA replication (S-phase) and mitosis (M-phase), and two gap phases in which those events are prepared, called G1 and G2 (Figure 1.6). Mitosis is that part of the cell cycle in which the DNA of the cell is divided into two, and in mammalian cells it is almost always connected with cytokinesis. The cell must divide at an appropriate time: to fail to do so could result in its death. For mammalian cells, entry into the cell cycle is normally prompted by reception of a proliferation signal, which is linked to commencement of the cell cycle by a pathway not yet fully understood. Mitosis commences when the DNA has been replicated. For unicellular eukaryotes, entry into the cell cycle normally implies selection of the mitotic instead of the conjugative proliferation pathway, and then mitosis waits on both DNA replication and achievement of adequate cell size. This implies that the mitotic machinery is capable of being restrained by factors which detect the metabolic state of the cell.

The mitotic mechanism must, once activated, direct the reorganisation of the cell such that the DNA and all other cellular contents are divided equably (cell divisions need not be equal, but the daughter cells must be able to perform their designated functions).

The mechanism of mitosis is a complex series of protein phosphorylations, in which the key kinase is the protein p34^{cdc2} (also known as p34^{cdk1}: Nurse, 1990; Norbury and Nurse, 1992; Cross et al., 1989). The control and activity of p34^{cdc2} has been elucidated by both biochemical study and yeast genetics.

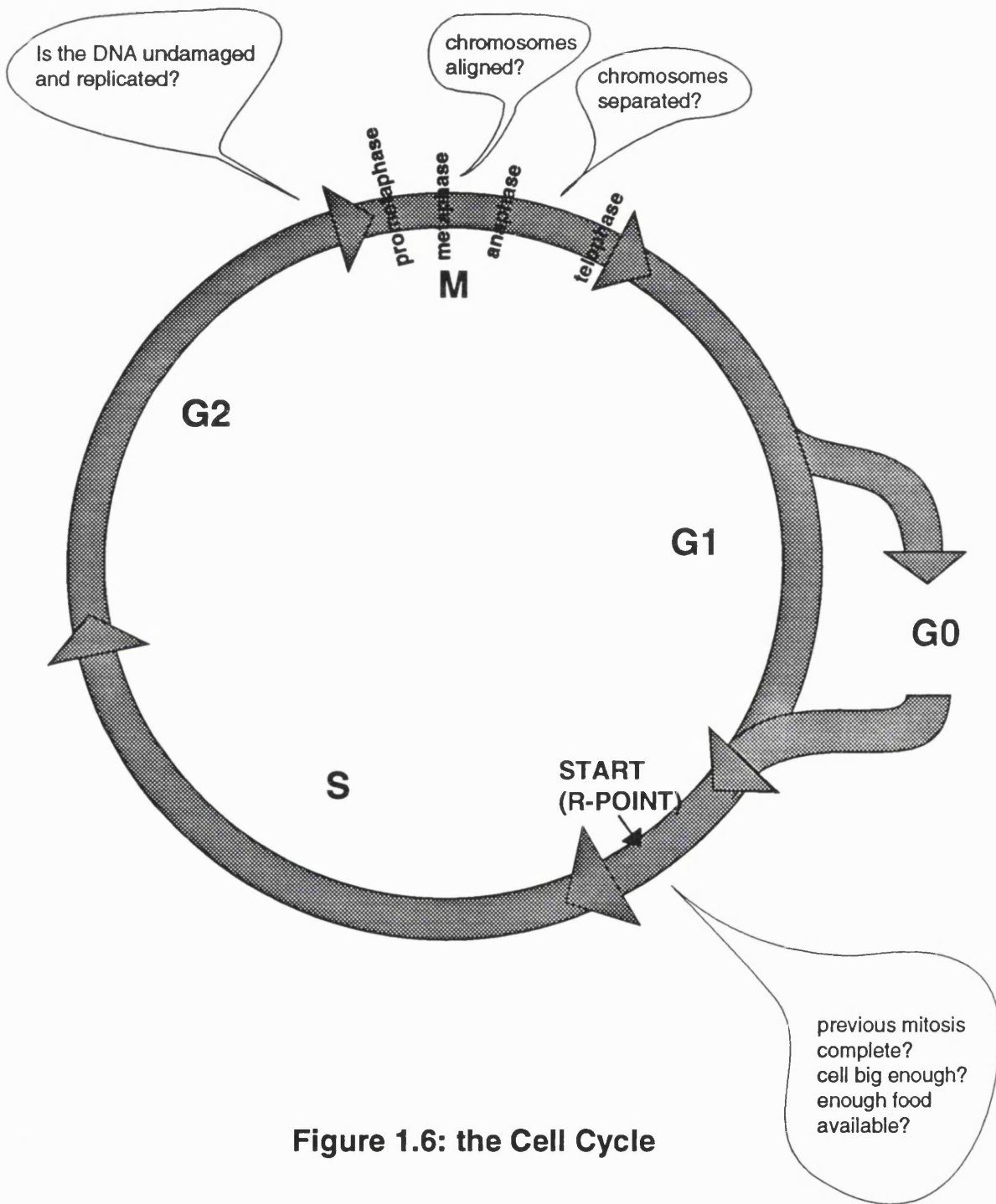


Figure 1.6: the Cell Cycle

1.3.1 Early work

The discovery of p34^{cdc2} had its basis in studies in three fields; mammalian cell biochemistry, *Xenopus* embryology and yeast genetics. In synchronised mammalian cultured cells, mitosis was shown to involve increased phosphorylation of cellular proteins on specific sites (Lake and Salzman, 1972; Lake, 1973; Maller et al., 1977). Also, protein kinases and kinase cascades have been known to cause catalytic activation of the pathways on which they operate (Smith and Ecker, 1965, 1971). The activating substance was identified in mammalian cells as a 90kD kinase (Schlepper and Knippers, 1975).

At about the same time it was found that *Xenopus* oocytes could be activated catalytically by very small quantities of extract from activated oocytes (Wasserman and Masui, 1976; Gerhart et al., 1984), which was called maturation-promoting factor or MPF. The process could be repeated with several eggs until the material from the first activated egg must have been diluted to insignificant levels. This showed that each egg was being activated in its turn by catalytic quantities of the material from the previous activated oocyte. It also showed that the maturation process required nothing not already present in the cell, and it was indeed found to be independent of protein synthesis (Maller, 1982). The activating substance of *Xenopus* was partially purified (Wu and Gerhart, 1980) and its kinase activity shown to rise and fall concurrently with mitosis (Dorée et al., 1983; Picard et al., 1987; reviewed in Maller, 1985). However, it was not fully purified for a long time due to its instability.

S. pombe geneticists studied conditional mutations in cell cycle control (Nurse, 1975; Nurse and Thuriaux, 1980), taking advantage of the small generation time of the organism, its genetic tractability and the resemblance of some facets of its cell biology to those of mammalian cells. Some defects gave rise to extended cell cycle length and hence abnormally long cells, and these were grouped into a class *cdc* (for cell division cycle). The mutation *cdc2* led to a block before mitosis (and also a block at G1), and this gene was found to encode a 34kD protein kinase which was essential for mitotic onset (Simanis and Nurse, 1986). *S. pombe cdc2* mutant cells could be rescued by transformation with a plasmid bearing the human *cdc2* gene (Lee and Nurse, 1987); this rescue demonstrated the conservation of function between the human and yeast genes, and underlined the fundamental nature of mitotic control for all organisms. Studies in *S. pombe* also revealed control of *cdc2* by other genes; negative control by genes *wee1* and *nim1*, which are also both protein kinases (Moreno et al., 1989), and positive control by *cdc25*, a serine/threonine and tyrosine phosphatase (Russell and Nurse, 1986; Gould and Nurse, 1989). The gene *cdc13* was also required for mitosis (Booher and Beach, 1988). When MPF was purified from *Xenopus* extract, it was found to consist of p34^{cdc2} and cyclin B (*cdc13*) (Lokha et al., 1988; Dunphy et al., 1988; Labbé et al., 1988), and to be recognised by antibodies to the yeast kinase (Gautier et al., 1988). Thus work in three fields converged on the *cdc2* gene product. The

mammalian cell line FT210, which contains a temperature-sensitive *cdc2* gene, was unable to enter mitosis at restrictive temperature (Th'ng et al., 1990) which further underlined the critical nature of this key mitotic kinase. Biochemical and genetic analysis have gone hand in hand to elucidate the nature of mitotic control. Some of the information relevant for the thesis will be outlined below.

1.3.2 p34^{cdc2} and the cell cycle

Although the cellular levels of p34^{cdc2} are approximately constant throughout the cell cycle, it is only active during mitosis (Moreno et al., 1989: Figure 1.7). Its activation is under both transcriptional and post-translational control (Solomon et al., 1990) and, by means which are still almost completely unknown, cell status is reflected in the control of p34^{cdc2}, by presence of cyclin and by phosphorylation of the kinase.

Cyclin provides the first level of control for p34^{cdc2}. It was described biochemically as a protein of clam eggs which was expressed in increasing quantity through the cell cycle, being most abundant at mitosis, and disappearing before its end (Evans et al., 1983). Cyclins A and B were described on the basis of slightly different size and properties, and MPF was found to comprise an equimolar ratio of p34^{cdc2} and cyclin B (Lokha et al., 1988: the cyclin B complex is the archetypal one and the main subject of consideration below). Cyclins have no intrinsic kinase activity, but are essential for the kinase activity of p34^{cdc2}, and thus limit its activity broadly to the times when they are available. However, the combined levels of cyclins A and B are lower than that of p34^{cdc2}, and in the cell cycle the protein requirement for MPF is achieved considerably before mitosis (Minshull et al., 1989). Thus, though cyclin is the only protein which must be synthesised de novo for entry into mitosis (Murray and Kirschner, 1989) it is clearly insufficient on its own to activate the mitotic kinase. MPF activity is regulated by multiple phosphorylations. These may conveniently be outlined by describing the state of p34^{cdc2} throughout the cell cycle; unless noted, the information describes the mitotic kinase of HeLa cells.

At the beginning of the cell cycle, p34^{cdc2} is unphosphorylated and little or no cyclin is present in the cell. Phosphorylation on Thr167 is essential for kinase activity in *S. pombe* p34^{cdc2} (Gould et al., 1991) and is thought to take place on association of the kinase with cyclin (Krek and Nigg, 1991). Cyclin B is first detectably expressed at the outset of S phase; it is thought to attain 25% of p34^{cdc2} levels by mitosis. It binds to p34^{cdc2} and p34^{cdc2} is phosphorylated on Thr 167 and immediately inactivated by phosphorylation on Thr 14 and Tyr 15 (Solomon et al., 1990; Norbury et al., 1991): mutation of either or both of these sites to unphosphorylatable ones increases the rate of progression into mitosis (Krek and Nigg, 1991). The phosphorylated p34^{cdc2}-cyclin complex accumulates through S phase and G2, and is mainly cytoplasmic in location (Pines and Hunter, 1991).

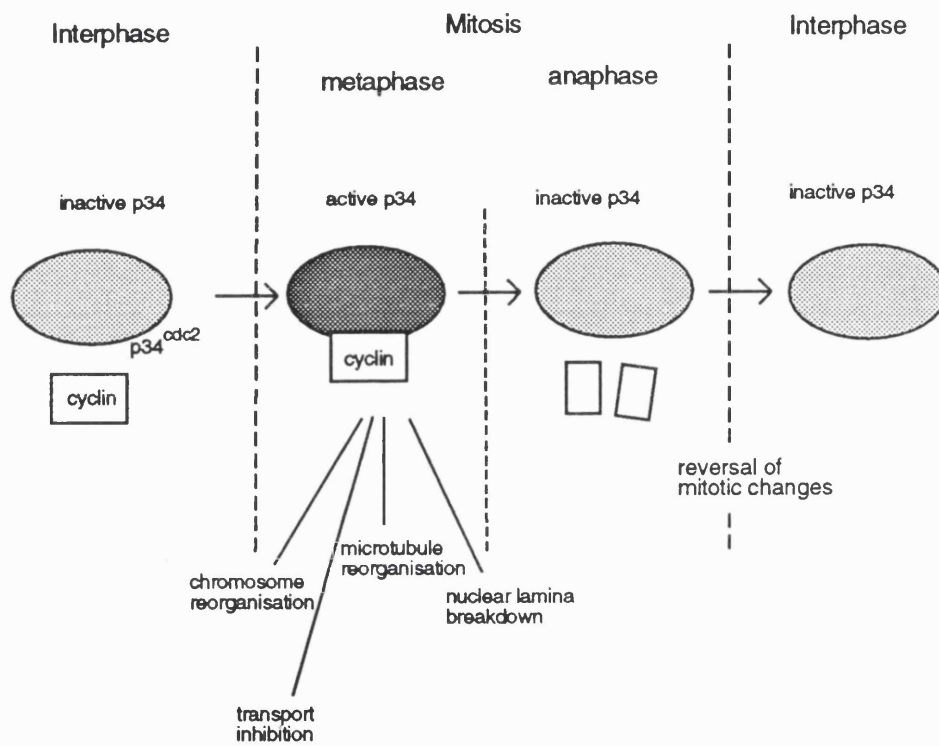


Figure 1.7: p34^{cdc2} in interphase and mitosis

The G₂-M transition is marked by rapid dephosphorylation of Thr 14 and Tyr 15 (Gould and Nurse, 1989) by the dual threonine-tyrosine phosphatase p80^{cdc25} (Millar et al., 1991) (Millar and Russell, 1992). In *S. pombe*, inactivity of CDC25 gives rise to the over-long cdc phenotype, whereas circumvention of p80^{cdc25} activity causes premature entry into mitosis; in particular in the presence of damaged or unreplicated DNA (Enoch and Nurse, 1991; Dasso and Newport, 1990). By immunofluorescence, as the p34^{cdc2}-cyclin B complex enters the nucleus, the NE breaks down (Pines and Hunter, 1991). The complex retains kinase activity until the end of metaphase, and then the cyclin is rapidly degraded, apparently by the ubiquitin pathway (Glotzer et al., 1991). The cyclin appears to contain a 'cyclin box'; an ubiquitination sequence, which is required for its destruction and hence for exit from mitosis (Luca et al., 1991). The uncomplexed p34^{cdc2} is then completely dephosphorylated (Lorca et al., 1992) and the cell exits mitosis. (Curiously, in *S. cerevisiae* cells, studies with mutants defective in anaphase and telophase functions have shown that histone kinase activity persists at least until mitotic telophase in this organism (Surana et al., 1993))

The synthesis of cyclin A commences near the G₁-S-phase transition, and the protein complexes with p34^{cdc2} like cyclin B. The p34^{cdc2}-cyclin A complex has been shown to undergo little or no regulatory phosphorylation (Clarke et al., 1992); therefore the kinase activity of the complex is proportional to the quantity of cyclin A present. The complex, according to immunofluorescence studies, localises to the nucleus (Pines and Hunter, 1991), and its activity remains high till metaphase, like that of the cyclin B complex.

Further light has been shed on the relative activity of cyclins A and B by some very elegant experiments using clam oocytes. Activated oocyte extracts undergo cyclic accumulation and destruction of cyclin, and careful analysis revealed that cyclin A destruction preceded that of cyclin B by some three minutes, and the metaphase-anaphase transition followed three minutes after that. If, however, the extract was treated with nocodazole, the destruction sequence was suspended between the time of the two cyclins; thus, cyclin A was destroyed, leaving cyclin B the only partner of p34^{cdc2} (Hunt et al., 1992). Similar experiments on HeLa cells treated with nocodazole showed that, over 24 hours, the cyclin A in the extract was totally degraded, while the kinase activity of the cyclin B-p34^{cdc2} complex remained high (Pines and Hunter, 1990). Nocodazole, a microtubule-disrupting drug, prevents assembly of the mitotic spindle (de Brabander et al., 1976). Thus, the cyclin B complex might be required to 'sense' completion of the mitotic spindle and its inactivation would signal spindle completion and permit onset of chromosome separation. The cyclin A complex could then perform other mitotic functions, and its inactivation could represent the completion of its mitotic tasks while the cyclin B complex remained to await spindle assembly. It is likely that the two cyclins direct the active kinase to different tasks at different locations in the cell (Pines and Hunter, 1991).

1.3.3 Proteins implicated in the control of p34^{cdc2}

p34^{cdc2} is controlled by a net of interacting phosphorylations, and its activity reflects the balance of activity of the kinases and phosphatases acting upon it (Figure 1.8). Some of these will be outlined below, to give an indication of the complexity of the situation and the number of pathways by which metabolic information can impinge on the mitotic kinase.

(i) cyclins; as mentioned above, mammalian cells have at least two cyclins in active complexes during mitosis, cyclins A and B (Minshull et al., 1990). These two cyclins may direct p34^{cdc2} to different sets of cellular substrates, or to different cellular locations (Pines and Hunter, 1991) or perhaps both. In *S. pombe*, the situation appears more complex since, in addition to *cdc13*, two other B-type cyclin genes have been cloned to date, designated *cig 1* and *2*. These proteins certainly have different functions in mitosis, since *cig 2* is required for commencement of mitosis, whereas *cdc13* is implicated in the return to interphase (Bueno and Russell, 1993).

In mammalian cells, there is some indication that the p34^{cdc2}-cyclin A complex, which does not undergo inhibitory phosphorylation, is used for catalytic activation of MPF. This information comes from study of frog oocyte extracts, some of which require kinase activity to 'jump-start' their MPF. It may do this by phosphorylating and activating *cdc25* (Hoffman et al., 1993; Galaktionov and Beach, 1991), or by phosphorylating and inactivating *wee1* (Devault et al., 1992).

(ii) kinases; the kinases *wee1* and *mik1* have been implicated in the inhibition of p34^{cdc2} during interphase in *S. pombe*. So far, only *wee1* has been an object of biochemical study. It is a 107kD kinase which has been shown to act both autocatalytically and on p34^{cdc2}, though only on Tyr15; it only recognises Thr14 *in vitro* and the true *in-vivo* kinase for that site is not known at present (McGowan and Russell, 1993). The human homologue of *wee1* has been cloned and indeed phosphorylates and inactivates p34^{cdc2} *in vitro*. It also appears to be a target for regulation, being inhibited by phosphorylation by the *Nim1* gene product (Russell and Nurse, 1987a, b; Lundgren et al., 1991; Parker et al., 1993). *Wee1* is activated by an okadaic acid-sensitive phosphatase (Devault et al., 1992). The kinases responsible for this are thought to be *wee1* and *nim1* (Russell and Nurse, 1987b).

The kinase which performs the Thr161 phosphorylation of cyclin-associated kinases has recently been identified as a protein closely related to the MO15 gene product (Solomon et al., 1993), which was itself identified in a screen for p34^{cdc2}-related proteins in *Xenopus* (Shuttleworth et al., 1990). In starfish (Fesquet et al., 1993) and *Xenopus* extracts the 37kD MO15 gene product is active in association with a 40kD protein, but when purified from bacteria it requires incubation with a cytosolic extract and ATP to be activated (Poon et al., 1993). This suggests that it is itself a cell cycle-controlled phosphoprotein.

At the beginning of the cell cycle, p34^{cdc2} is unphosphorylated and little or no cyclin is present in the cell. Phosphorylation on Thr 161 (Thr 167 in *S.pombe*) is essential for kinase activity and is thought to take place on association of the kinase with cyclin. Cyclin B is first detectably expressed at the outset of S phase. It binds to p34^{cdc2}, and p34^{cdc2} is phosphorylated on Thr 167 and then immediately inactivated by phosphorylation on Thr 14 and Tyr 15. The phosphorylated p34^{cdc2}-cyclin complex accumulates through S phase and G2. The G2-M transition is marked by rapid dephosphorylation of Thr 14 and Tyr 15. Cyclin A is first expressed a little later than cyclin B. Its binding to p34^{cdc2} also brings on phosphorylation on Thr 161, but this complex is not subject to inhibitory phosphorylation, so that its histone kinase activity increases in proportion with its level in the cell.

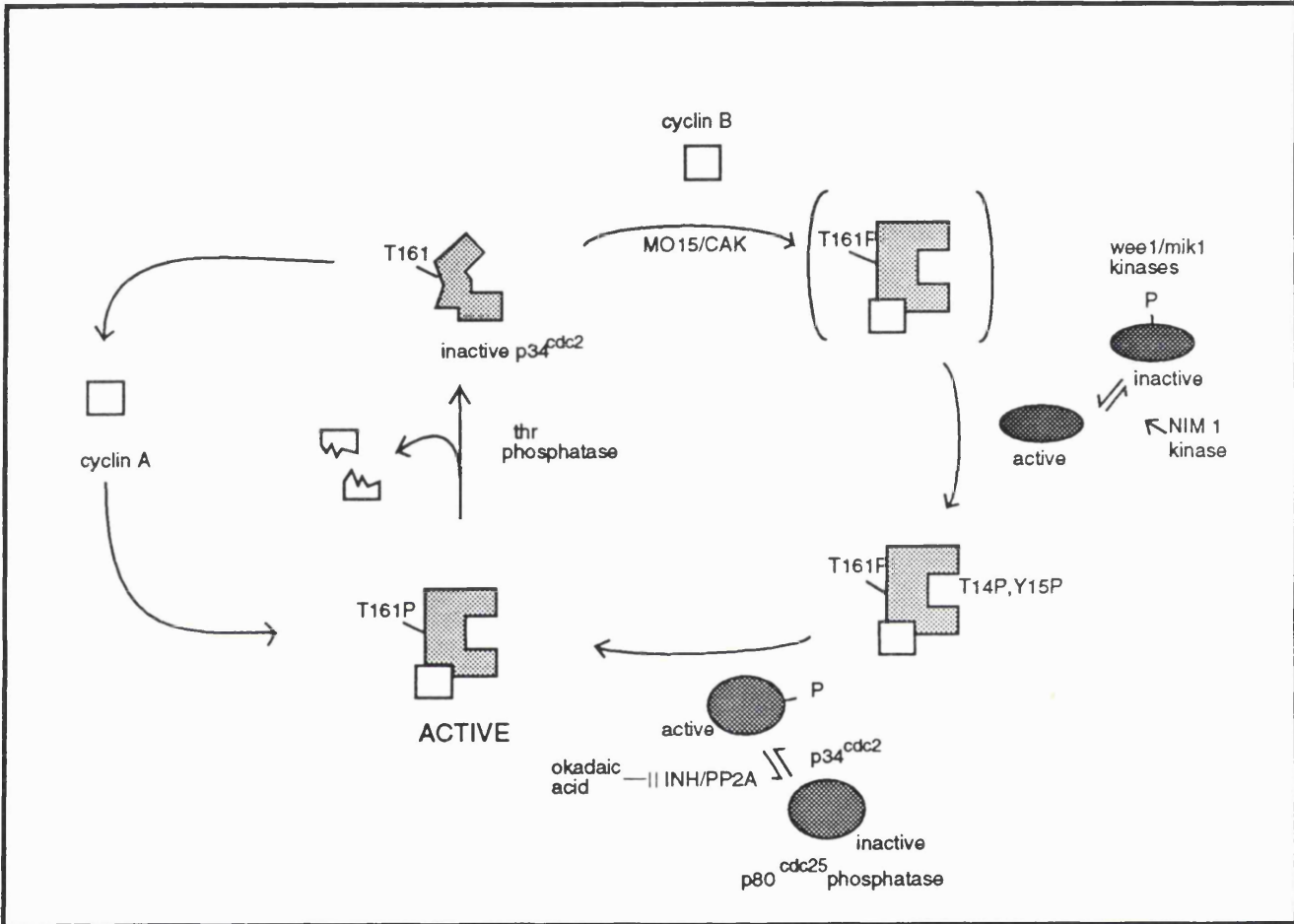


Figure 1.8: the control of p34^{cdc2}

It is interesting to note that constitutive activation of a small portion of the cell's p34^{cdc2} does not cause activation of the remainder except before mitosis. The Thr161-phosphorylated, cyclin-associated p34^{cdc2} is theoretically capable of catalysing full activation of the mitotic kinase pool, but it was not found to do so in a HeLa system (Norbury et al., 1991). The mechanism by which the kinase's activity is reserved for the appropriate time is unknown. (iii) phosphatases; the chief of these is the product of the *cdc25* gene in *S. pombe*, or its functional homologues of other organisms. As mentioned above, this unusual protein is both a threonine and tyrosine phosphatase. It too is affected by okadaic acid, through the inactivation of the phosphatase which inactivates it (Kumagai and Dunphy, 1992). Its activating kinase is not yet identified, but may be MPF itself (Hoffman et al., 1993). In addition, a factor designated INH was purified from oocyte extracts on the basis of MPF inhibition, and found to contain PP2A (Cyert and Kirschner, 1988; Lee et al., 1991). In *S. pombe*, p80^{cdc25} is not the only phosphatase capable of activating p34^{cdc2}. Deletion of both *wee1* and *cdc25* is not lethal because the *mik1* protein phosphorylates Thr14 of the mitotic kinase, while the product of the *pyp3* gene appears to dephosphorylate it (Millar et al., 1992b; reviewed in Labib and Nurse, 1993). The *pyp1* and *pyp2* phosphatases are (possibly overlapping) activators of the *wee1* kinase in *S. pombe*; deletion of both, but not either alone, is lethal since it permits unregulated advance into mitosis (Millar et al., 1992a). Okadaic acid (Takai et al., 1987) has been a useful tool for examining some cell cycle control proteins. Through its inhibitory action on type 1 and type 2A phosphatases, it is capable of inducing a mitotic or pseudomitotic phenotype in at least one cell type. It causes premature MPF activation in *Xenopus* oocyte extracts (Felix et al., 1990) and mitotic changes in BHK cells (Yamashita et al., 1990). In HeLa cells it causes mitosis-like changes to the cytoarchitecture, but does not cause elevation of histone kinase activity, suggesting that it is acting there without the mediation of p34^{cdc2} (Lucocq et al., 1991).

1.3.4 The control of Start and S-phase

Yeasts enter the mitotic cell cycle via a commitment not to undergo sexual replication, and mammalian cells do so by leaving G₀, the non-cycling state (reviewed in Reed, 1992). The first indication that a cell-cycle kinase was responsible for mitotic commitment came from the finding that p34^{cdc2} of *S. pombe* and p34^{cdc28} of *S. cerevisiae* are required for both entry into mitosis and commitment to S-phase (reviewed in Forsburg and Nurse, 1991). Mammalian cells do not use p34^{cdc2} for both events, but instead place S-phase entry under the control of a related kinase, p33^{cdk2}. This protein is a regulated, cyclin-dependent kinase like p34^{cdc2}, but its major cyclin partners are cyclins A and E, and these complexes are regulated by similar phosphorylations to the cyclin B-p34^{cdc2} complex (Gu et al., 1992). The p33^{cdk2}-cyclin A complex is required for DNA synthesis but not for mitosis (Dasso and

Newport, 1990; Fang and Newport, 1991). Mitosis cannot be undertaken until S-phase is completed if cyclin A is present (Walker and Maller, 1991). Under certain pharmacological conditions, for example treatment with caffeine, mitosis may be attempted before S-phase is over, or in the presence of unreplicated DNA; this results in cell death. Such cells experience early and inappropriate activation of p80^{cdc25} (Enoch and Nurse, 1991) and Wee 1 (Smythe and Newport, 1992).

p33^{cdk2} is interesting in having not only a cell-cycle role but an apparent link with cell-cycle dependent gene transcription. It associates with the transcription factor EF2 during S-phase (Pagano, et al., 1992) and the retinoblastoma-associated protein p107, in its hypophosphorylated state, in G₁ (Shirodkar et al., 1992). It is thought to phosphorylate p107 to block its growth-factor binding, thus permitting escape from G₁ (reviewed in Nevins, 1992).

Start itself may be dependent on a cascade of activation of a cell-cycle kinase. Cyclin regulation in budding yeast is an example of this. In that organism, Start requires the activity of start-type cyclins, designated CLN1-3 (reviewed in Hadwiger et al., 1989). CLN1 and CLN2 increase dramatically in expression just before commitment to Start, but CLN3p is expressed at constant low levels throughout the cell cycle, and the histone kinase activity associated with it is lower than with the other two cyclins. However, this baseline cyclin in association with CDC28 appears to be capable of directing the expression of the others by causing activation of the cell-cycle dependent transcription factors SWI4 and SWI6 (Dirick and Nasmyth, 1991). These TFs can then direct CLN1 and CLN2 expression, leading to further TF activation and so on; the result is positive feedback and an eruption of CLN activity (Cross and Tinkelenberg, 1991). The initial up-regulation of SWI4 and SWI6 is due not to CLN3 alone (whose histone kinase activation is insufficient to direct the process), but probably to CLN3 along with another cyclin, perhaps one of those identified more recently in budding yeast (Tyers et al., 1993).

In mammals, several novel cyclins (some with scant homology to cyclins A or B) have been shown to peak in abundance at different times in the cell cycle (reviewed in Sherr, 1993; Pines, 1993; Figure 1.9). Of these, cyclins A, C, D and E associate with p33^{cdk2}. Cyclin A and E complexes peak in activity just before S-phase, but cyclin C levels scarcely oscillate throughout the cell cycle; this situation is reminiscent of the yeast *cln* proteins (and indeed, some of these were isolated by rescue of *cln* mutants: see Lew et al., 1991). D-type cyclins are unstable even when complexed with a kinase, and expressed only in the presence of growth factors; this suggests that they may be more intimately connected in the release from G₀ (Matsushime et al., 1991). Other cyclin-like and cdk-like proteins are yet to find even putative functions (for example, Meyerson et al., 1992).

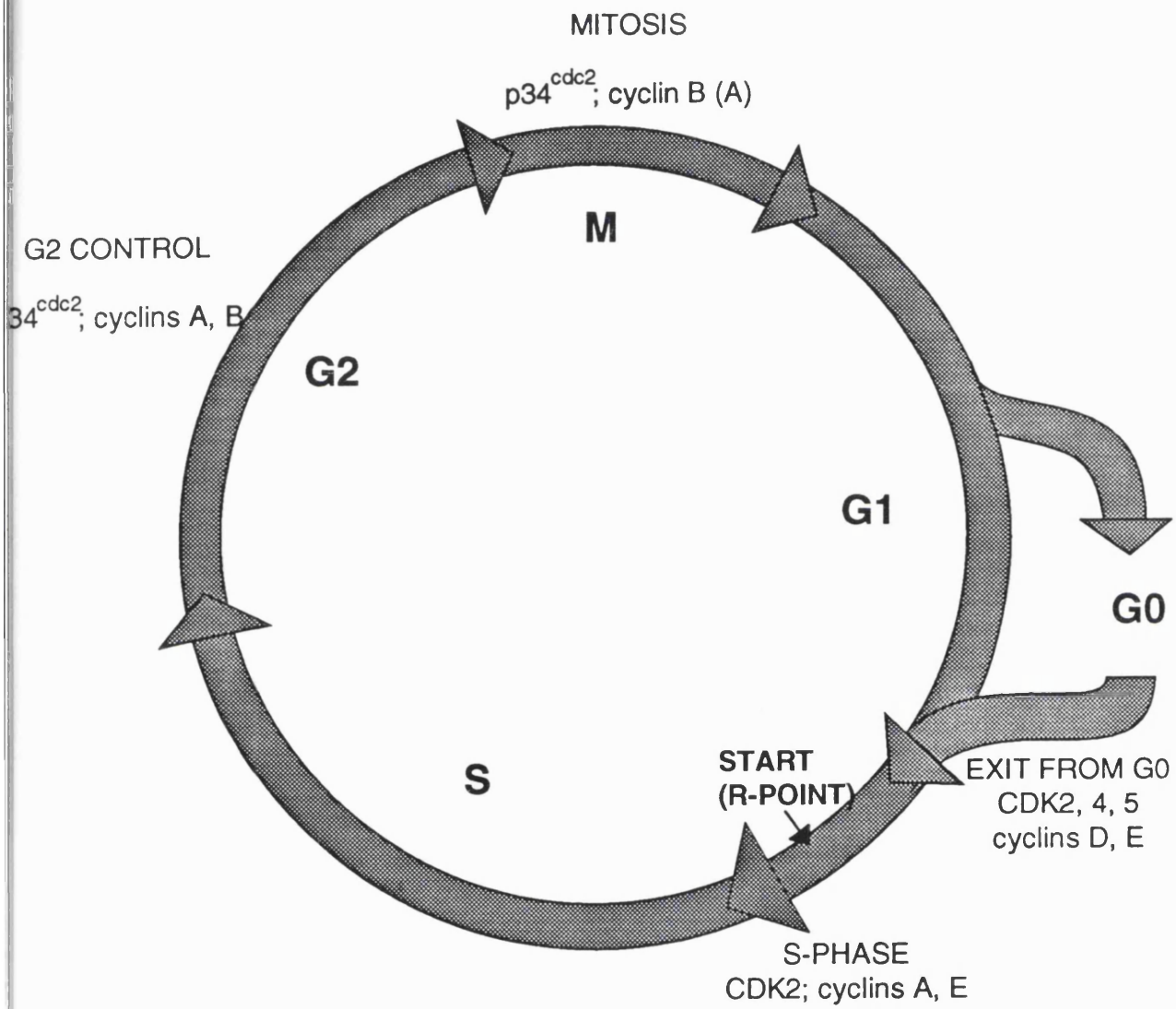


Figure 1.9: cyclins and cdks in the cell cycle

1.4 Intracellular organelles in mitosis

1.4.1 Organellar morphology in mitotic cells

In mitosis in mammalian cells, the ER fragments to a variable extent, the nuclear envelope and nuclear pores are fragmented, and the Golgi is completely broken down into thousands of vesicles scattered throughout the cytoplasm (see Zeligs and Wollman, 1979: reviewed in Warren, 1993).

Golgi breakdown at the onset of mitosis has not been observed by EM, and must be inferred from its reverse, reassembly at telophase, or by studies of the Golgi of oocytes arrested at meiotic prophase. This is because the synchronised cells leave prophase before harvesting of mitotic cells is complete. The best synchronisation method available is treatment with nocodazole. Nocodazole-treated cells accumulate in prometaphase, and complete mitosis synchronously on release from the block. There is presently no method of accumulating cells at the G2-M border which is quickly and totally reversible, although okadaic acid promises to make at least a pseudomitotic phenotype available for study (Lucocq, 1992). Thus, Golgi reassembly will be described (Lucocq et al., 1987, 1989).

The mitotic Golgi consists of thousands of vesicles, some single and some in 'mitotic clusters' which appear near small lengths of membrane probably derived from transitional elements of the ER. The clusters have been shown by specific Golgi stains like osmium tetroxide and TPPase, and also by immuno-EM of Golgi markers (Lucocq et al., 1989; Pypaert et al., 1993). As telophase proceeds, the vesicles fuse to form approximately 300 Golgi clusters which themselves fuse to form multiple Golgi stacks. These finally move towards the nucleus, and fuse to form the complete juxtannuclear interphase stack. From this description it is inferred that, at mitotic onset, the Golgi breaks down by shedding membrane and disintegrating more and more completely until only vesicles remain (Figure 1.10).

In mammalian cells the nuclear envelope fragments to facilitate the division of the chromosomes by the mitotic spindle (reviewed in Gerace and Burke, 1988). The nuclear pores disassemble and the membrane fragments relocate to the spindle poles and spindle axis. This is accompanied, or perhaps caused, by the breakdown of the nuclear lamina. It appears from work in a cell-free system (Allan and Vale, 1991) that movement of vesicles (such as endocytic vesicles) along microtubules is inhibited, while the microtubules themselves are severed (Vale, 1991); this would free the microtubules from interphase duties and redirect them to centrosome formation. The nucleation activity of centrosomes is stimulated by the cyclin A in *Xenopus* oocyte extracts (Buendia et al., 1992). However, microtubule shortening, an anaphase event, is stimulated mainly by the cyclin B complex (Verde et al., 1992).

Mitotic Golgi breakdown must be inferred from the study of model systems like cell-free assays or phosphatase inhibitor-treated cells. The interphase Golgi may break into multiple small stacks in prophase, and also lose membrane by vesicle shedding. This would lead to the accumulation of small vesicles and scattered cisternae. Golgi breakdown may also require the dissociation of any proteinaceous matrix present, and the breakdown of the putative rafts of resident enzymes.

The prometaphase Golgi consists of thousands of vesicles, some single and some in 'mitotic clusters' which appear near small lengths of membrane probably derived from transitional elements of the ER. As the cell passes metaphase, and telophase proceeds, the vesicles fuse to form approximately 300 Golgi clusters which themselves fuse to form multiple Golgi stacks. These finally move towards the nucleus, and fuse to form the complete juxtannuclear interphase stack at the end of telophase or the beginning of G1.

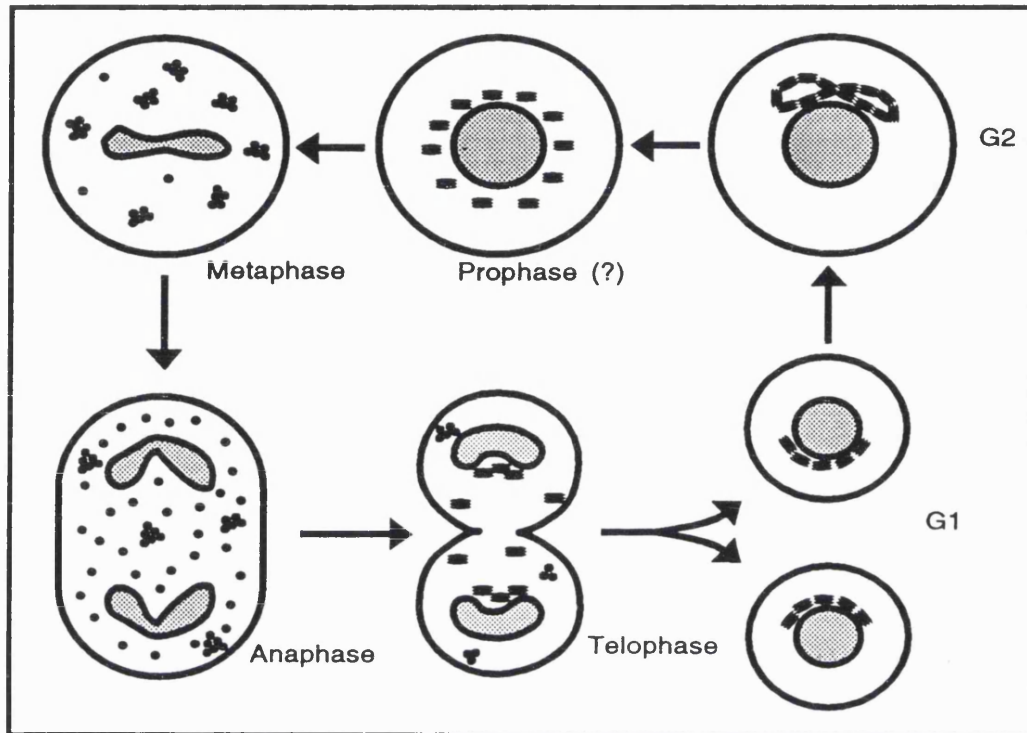


Figure 1.10: the Golgi apparatus in interphase and mitosis

The breakdown of the nuclear lamina is one of the few mitotic events shown to be directly due to MPF. The lamina is formed of lamins A, B and C, which have some homology with intermediate filament proteins, and which form a stable underlying structure for the NE (reviewed in Gerace and Burke, 1988). At mitosis, the soluble lamins A and C are released into the cytoplasm, whereas lamin B is acylated at the C-terminus (Farnsworth et al., 1989) and thereby remains associated with the membrane fragments (Krohne et al., 1989). In vivo and in vitro, the breakdown of the nuclear lamina is caused by phosphorylation of the lamins by p34^{cdc2} (Peter et al., 1990; Ward and Kirschner, 1990), and MPF causes dissociation of nuclear membrane vesicles from chromatin in an in-vitro system, apparently via action on a kinase-phosphatase system (Pfaller et al., 1991). It should be remembered, that, though much work has been done on the control of nuclear lamina breakdown, the disassembly and reassembly mechanisms of the nuclear membrane and pores are only just coming to be understood (see for example Foisner and Gerace, 1993; Chaudhary and Courvalin, 1993). Okadaic acid has been a useful tool for the study of cell morphology in mitosis (reviewed in Lucocq, 1992). As mentioned in section 1.4.3, it induces a mitosis-like state in BHK cells, with transient activation of histone kinase activity dependent on cellular cyclin B levels (Yamashita et al., 1990). Its action appears to be cell-type specific since, in HeLa cells, it causes Golgi breakdown and transport inhibition (Lucocq et al., 1991) but no change in nuclear envelope morphology or histone kinase activity and almost no chromatin condensation (Steinman et al., 1991)

1.4.2 Organellar function in mitosis

Aside from morphological change, a large number of cellular functions are altered in mitosis. Some systems have been studied in vivo, using synchronised cells, and in other cases transport events have been studied in vitro, to provide unimpeded experimental access to the compartment of interest.

Transcription is inhibited, as might be expected given the inaccessibility of genes in the packed mitotic chromosomes, but has also been shown to be inhibited by inactivation of a component of the transcription machinery, when the DNA substrate is available (Hartl et al., 1993). Protein translation is reduced, reflecting the reduction in cellular mRNA levels. In mammalian cells, both exocytosis (Warren et al., 1983) and endocytosis (Berlin and Oliver, 1980) are inhibited. Transport from the ER to the Golgi is inhibited both in vivo (Featherstone et al., 1985) and using phosphatase inhibitors is inhibited both in vivo and in vitro (Davidson et al., 1992). Transport of lipid through the Golgi apparatus is inhibited in vivo (Collins and Warren, 1992). Transport from the TGN to the cell surface was examined in vivo using exogenously-supplied xylosides to act as substrates for the formation of glycosaminoglycans. In mammalian cells, the process is not inhibited (Kreiner and Moore,

1990) though, curiously, in *Xenopus*, it is transport between the TGN and the cell surface which is inhibited (Leaf et al., 1990) and ER-medial Golgi transport which is not (Ceriotti and Colman, 1989). Regulated secretion, exemplified by the release of histamine, is inhibited in synchronised mitotic mast cells (Hesketh et al., 1984). Multiple stages of endocytosis are inhibited, including the formation of coated pits and their invagination (Pypaert et al., 1991), their fusion (Woodman et al., 1992; Thomas et al., 1992) and their recycling to the cell surface (Warren et al., 1984). Interestingly, in an in-vitro system containing *Xenopus* cytosol, inhibition of fusion was found to require p34^{cdc2} and cyclin B rather than cyclin A (Tuomikoski et al., 1989; Thomas et al., 1992); whereas in a mammalian cell-free system containing HeLa mitotic cytosol, fusion was inhibited preferentially by the cyclin A complex, and also mimicked by okadaic acid (Woodman et al., 1992, 1993). Rab4 was found to be phosphorylated in mitotic cells and also in vitro by p34^{cdc2}, and this reversible phosphorylation led to reversible redistribution into the cytoplasm without altering the C-terminal modification of the protein (van der Sluijs et al., 1992). Curiously, the authors were apparently unable to duplicate this phenomenon by incubating endosomes with purified p34^{cdc2} and cyclin B.

1.4.3 Structure and function in the Golgi apparatus

The Golgi apparatus has a remarkable structure-function relationship, in that its membrane turns over rapidly in vesicular traffic while its enzymes remain stably resident. The mitotic fragmentation of the Golgi has also been described, and the fact that exocytosis generally ceases at mitosis. From these observations, it has been suggested that the vesiculation of the Golgi at mitosis arises from a failure of exocytic transport (see Warren, 1985). If vesicles budded from mitotic Golgi, but failed to fuse, then the Golgi would shrink through loss of membrane. If that process were coupled either with loss of retention, so that resident enzymes were progressively incorporated into vesicles, or with loss of stacking and cohesion, by some method other than traffic-related budding, then a conceptually simple model of Golgi breakdown is the result. The model is represented in Figure 1.11.

It is not simple to show in vivo that transport through the Golgi apparatus is inhibited at mitosis. The main problem is that transport from the ER to the Golgi ceases, so that intra-Golgi transport may be reduced by lack of substrates without requiring a specific brake for the Golgi. One solution to the problem was adopted by Collins and Warren, who studied the transport of glycosphingolipids through the Golgi of mitotic cells, since the transport of these molecules from the ER to the Golgi is not subject to mitotic inhibition (Collins and Warren, 1992). However, in order to study protein traffic through the mitotic Golgi at a molecular level, it is necessary to employ an in-vitro system, such as the intra-Golgi transport assay described in section 1.2.

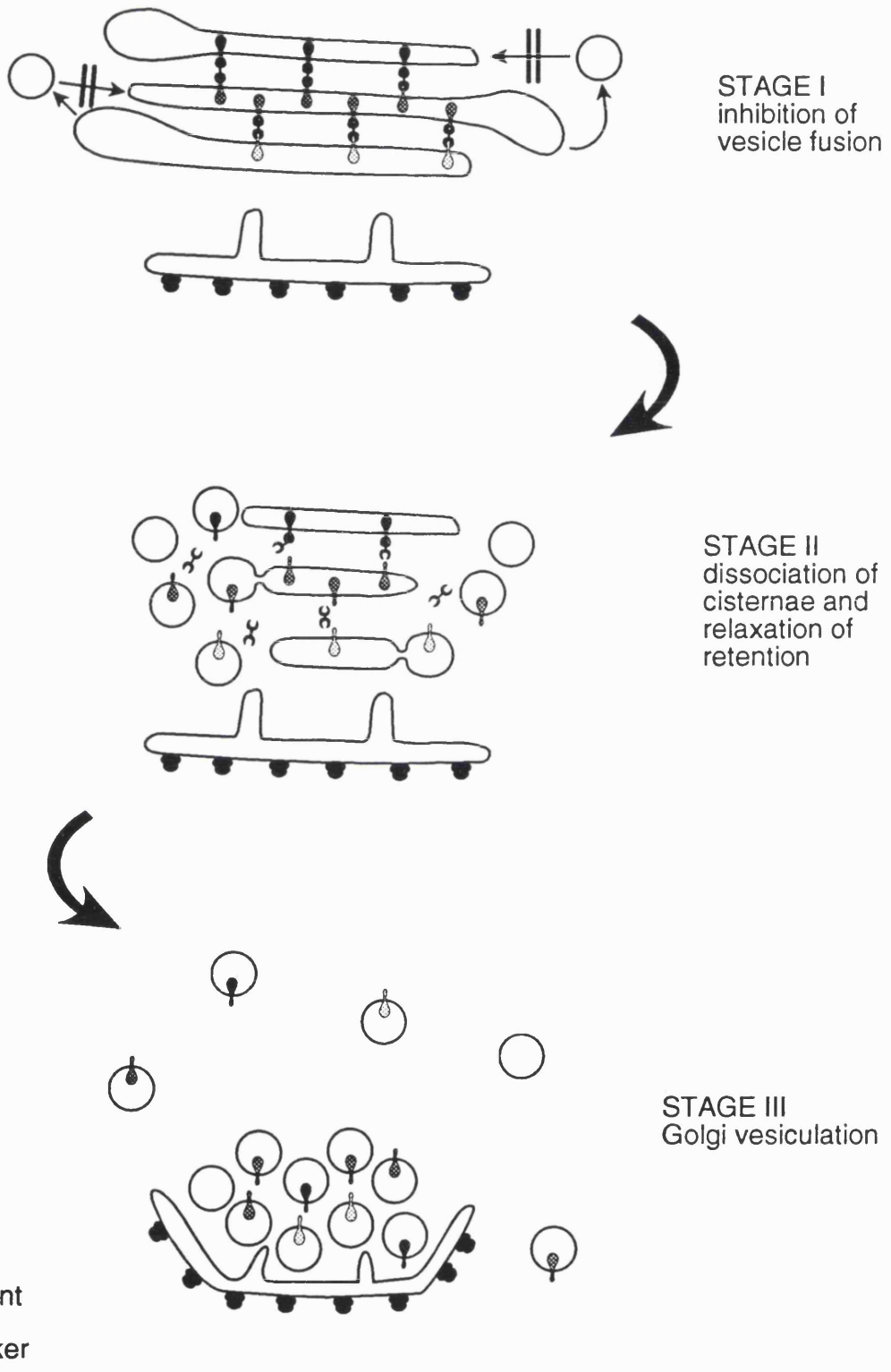


Figure 1.11: Golgi breakdown via inhibition of vesicular transport

Objective of the thesis

The thesis describes work performed using the Rothman transport assay. The assay was supplemented with a high-speed supernatant or 'cytosol' derived from HeLa cells, which could be either interphase or mitotic. The initial goal was to characterise the assay with HeLa cytosol, and to determine that the transport assay was inhibited by cytosol derived from mitotic cells. Then, experiments are described which link the control of the assay to the kinase activity of the cytosol. Others, using cyclin A, implicate a cell-cycle kinase specifically in mitotic control. Further experiments with cell-cycle proteins are used to try and find out whether transport was inhibited by the direct action of p34^{cdc2}.

The other goal of the thesis was to determine whether transport was actually inhibited via an effect on acceptor membrane function. Some drugs known to inhibit certain facets of transport, for example the actions of ARF and trimeric G proteins, are used to test this. Also, the effect of mitotic cytosol on donor and acceptor membranes is explored. Some studies are made of NSF and SNAPs, to try to find out whether any of their activities are altered in mitosis.

Chapter 2

Materials and Methods

2.1 Chemicals

All chemicals were obtained from Sigma unless specified. Chemicals were made up in Millipore-filtered water, or in analytical grade solvents.

Amylose-sepharose was purchased from New England Biolabs as a preswollen suspension in ethanol.

Bio-Gel P6 DG was purchased from Bio-Rad and swollen in water, then stored at 4°C for up to a month.

Borosilicate glass tubes were bought from Samco, via FSA.

Cell culture media were obtained from Gibco BRL, with the exception of RPMI medium and trypsin-versene, which were prepared in-house. Tissue culture sterile plastics were obtained from Falcon and Nunc.

Glass-fibre filters (934-AH) for use in the transport assay were obtained from Whatman, as were the P81 paper used for the histone kinase assay and the 3MM paper used in various methods.

Glutathione-Sepharose was purchased from Sigma.

His-bind was bought from Qiagen as a pre-activated suspension, or from Novagen as dry gel swollen and activated according to the manufacturers' before use.

IPTG was bought from Novabiochem and made up at 200mM in water.

Microcystin (Calbiochem) was prepared at 1mM in 10% methanol and kept at -20°C for up to a month. It was diluted into water or buffer shortly before use.

Okadaic acid (Moana Bioproducts, Inc) was made up at 1mM in water and stored at -20°C. It was stable to repeated freeze-thawing but was kept for only a month. It was diluted into buffer shortly before use.

Skimmed milk powder was purchased from Boots the Chemist.

Sodium fluoride was obtained from BDH and kept as a 1M aqueous solution at room temperature.

Staurosporine (Calbiochem) was made up at 1mM in DMSO and kept at -20°C for up to three months. These agents were all diluted into water or buffer shortly before use.

3H-UDP-GlcNAc and 3H-UDP-galactose were bought from New England Nuclear as ethanolic solutions at 5-25Ci/mmol, and kept at -20°C.

2.2 Cell culture

HeLa cells were grown in RPMI medium with 2% bicarbonate, supplemented with 10% foetal calf serum, 200mM glutamine, 1% penicillin/streptomycin and 1% non-essential amino acids. They were split 1:5 every 2-3 days for up to 30 passages. When grown in suspension, they were grown in the same medium and passaged by 1:5 split from a suspension of $2-6 \times 10^5$ cells/ml.

CHO and 15B cells were grown in Alpha-MEM supplemented with 10% foetal calf serum, 200mM glutamine, 1% penicillin/streptomycin and 1% non-essential amino acids. The cells were split 1:10 every 2-3 days for up to 25 passages.

BHK cells were grown in G-MEM supplemented with 10% foetal calf serum, 10% tryptose broth, 200mM glutamine, 1% penicillin/streptomycin and 1% non-essential amino acids. They were split 1:10 every 2-3 days for up to 35 passages.

FM3A and FT210 cells were maintained in suspension in RPMI medium with 2% bicarbonate, supplemented with 10% foetal calf serum, 200mM glutamine, 1% penicillin/streptomycin and 1% non-essential amino acids. They were grown at 30°C and split 1:4 or 1:5 every 2-3 days. They were maintained close to confluence to assist their growth.

The 8G5 hybridoma cell line was maintained in D-MEM supplemented with 20% foetal calf serum, 1% penicillin/streptomycin, 1% glutamine and 1% non-essential amino acids. The cells were split 1:5 every 2-3 days. The cells were loosely adherent.

2.3 Making and titrating Vesicular Stomatitis Virus

2.3.1 Virus preparation

Stocks

BHK infection medium: BHK growth medium (including 10% tryptose phosphate broth) in which the serum was replaced by 0.2% BSA and 10mM HEPES-KOH pH 7.4.

Virus was prepared in BHK cells. The cells were grown to confluence in 8 150cm² flasks (which took 2-3 days for cells split at 1:10). The cells were washed twice with 10ml infection medium, then infected at 0.05pfu/cell (1.5×10^6 pfu per flask, diluted in 10ml infection medium per flask) for ~20 hours. Then the supernatant was removed and spun at 5000 rpm

30 minutes, 4°C in a Sorvall bench-top centrifuge. The resulting supernatant was snap-frozen in 5ml aliquots and stored at -80°C.

If the supernatant contained less than 5×10^9 pfu virus /ml, it was concentrated by sucrose gradient centrifugation. Gradients containing 2ml 55% (w/w) and 5ml 20%(w/w) sucrose (in 10mM Tris-HCl pH 7.2, 150mM NaCl, 0.5mM EDTA) were prepared in SW40 tubes. The supernatant was laid over these gradients, and after 1.5 hours at 25, 000 rpm 4°C in a Beckman ultracentrifuge, the 20-55% interface was collected. The bands were pooled, diluted in 10mM Tris-HCl pH 7.2, 150mM NaCl, 0.5mM EDTA, and centrifuged again on similar gradients. Then the pooled virus was snap-frozen in 5ml aliquots and stored at -80°C.

2.3.2 PFU assay

Stocks

Calcium and magnesium-free Dulbecco's PBS (CMF-PBS) and PBS with calcium chloride and magnesium chloride (CM-PBS): ICRF stock solutions.

CM-PBS + 3% formaldehyde: in a fume hood, 15g paraformaldehyde was added to 400ml CMF-PBS at 80°C. The solution was cooled to room temperature and Ca^{++} and Mg^{++} salts stocks added, then was filtered through Whatman No.1 paper. Stored at 4°C for a few weeks or at -20°C long-term.

50mM NH_4Cl in CMF-PBS: prepared fresh.

0.2% fish skin gelatin (FSG) in CMF-PBS: prepared fresh.

0.1% Triton X-100 (TX-100) in CMF-PBS: stored at 4°C

Hoechst 33258: prepare at 0.5mg/ml in water, stored at 4°C.

BHK cells were grown from 5% to 50% confluence on glass coverslips in 6-well plates, then transferred to fresh plates. The cells on one coverslip were released using trypsin-EDTA for counting. The remainder were washed twice with BHK infection medium and infected with VSV at 10^2 - 10^6 dilution in infection medium. 0.6ml of diluted VSV was added to each well for 1 hour at 37°C, and then was replaced by 2ml growth medium containing 0.1mM chloroquine (made fresh and diluted in growth medium; prevents reinfection by VSV) for 4 hours. Then the cells were prepared for immunofluorescence microscopy.

Immunofluorescence preparation of the cells was as follows:

- 1 The cells were washed in 2ml 3% formaldehyde in CM-PBS for 20 minutes (or overnight at 4°C). Then they were washed 3 times in CMF-PBS, and quenched for 10 minutes in 2ml 50mM NH_4Cl in CMF-PBS. The cells were then washed 3 times in CMF-PBS and blocked 3 times in CMF-PBS + 0.2% FSG over 5 minutes. They were washed 3 times in CMF-PBS.

2 The cells were incubated with anti-VSV for 30 minutes at room temperature. The antibody, the supernatant from the 8G5 cell line (supernatant from cells spun at 1000rpm for 5 minutes at 4°C in a Jouan benchtop centrifuge), was precleared in an Eppendorf centrifuge for 5 minutes at 14,000rpm. The incubation was performed in a wet box, made of a plastic box containing a layer of moistened Whatman 3MM covered with a strip of parafilm; 100µl of antibody was placed on the parafilm and the slide (after draining excess fluid by touching the side to 3MM) was inverted on top. Meanwhile the CMF-PBS in the wells was replaced by CMF-PBS/0.2% FSG. Then the slips were placed (cells uppermost) in wells, and washed 3 times in CMF-BS/0.2% FSG.

3 For secondary antibody, the cells were incubated with rhodamine-conjugated anti-mouse exactly as with anti-VSV; the antibody was diluted into CMF-PBS/0.2% FSG. Then the slips were returned to the wells and washed 3 times with CMF-PBS/0.2% FSG and 3 times with CMF-PBS.

4 The cell nuclei were Hoechst stained for DNA. The cells were washed in CMF-PBS/0.1% TX-100 for 4 minutes, then three times with CMF-PBS. Then CMF-PBS containing 2µg/ml Hoechst 33258 was added for 5 minutes. The cells were washed 3 times in CMF-BS. The slides were washed by immersion in 3 beakers containing distilled water and then dried by touching the edges with Whatman 3MM. Then each slide was mounted by being lowered onto 15µl of Moviol on a slide, and then the excess removed on Whatman 3MM; the Moviol was left to set over 1-2 hours at room temperature, then the slides were stored in the dark.

5 To estimate the number of infected cells, 5 fields of 100-300 cells were counted on the Hoechst channel for number of nuclei and on the rhodamine channel for infection level. The 50% infection point was established by plotting the percentage of infected cells vs VSV dilution. Then using the number of cells counted on one coverslip, the pfu of the virus was estimated. Virus was normally prepared at $1-10^{10}$ /ml as tissue culture supernatant.

An example of a preparation is shown in Figure 2.1. 50% of the cells on the coverslip (1.9×10^5) were infected by 0.6ml of virus at 3.7×10^4 dilution. Therefore the number of plaque-forming units per millilitre virus suspension (pfu/ml) was given by (cells on coverslip/1.2) x dilution factor = virus concentration (pfu/ml).

In this case the virus had a titre of 5.9×10^9 /ml.

All VSV preparation, and infection of 15B cells (below), was carried out by Ms R. Kieckbusch, who had permission to work with the virus.

Figure 2.1: Pfu assay of vesicular stomatitis virus

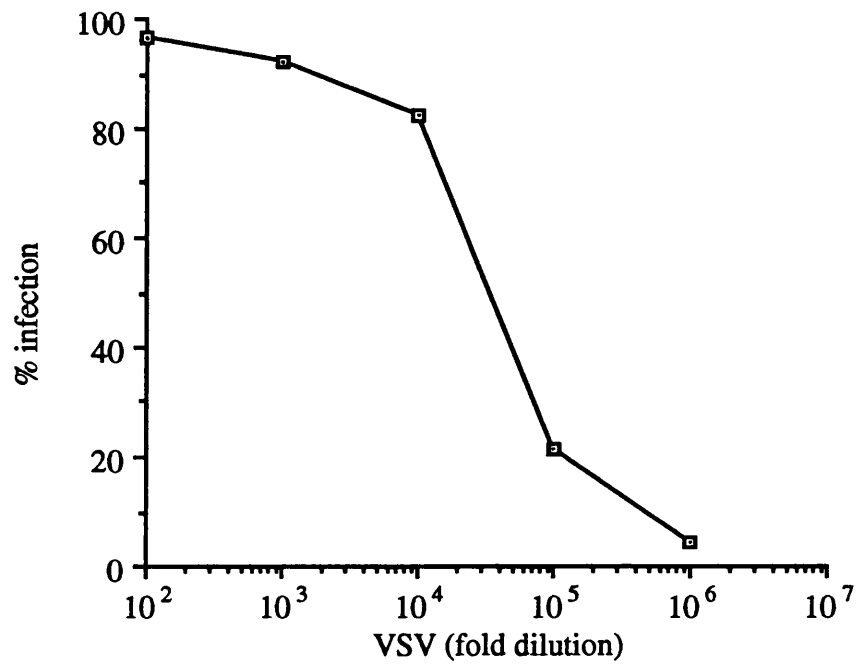


Figure 2.1: titration of infectivity of vesicular stomatitis virus. The virus was diluted into infection medium at the dilutions shown, and then 0.6ml aliquots used to infect BHK cells on coverslips. The percentage infection was assessed as the ratio of cells stained with anti-VSV-G antiserum to total cell nuclei.

2.4 Making Golgi membrane fractions

2.4.1 Donor Golgi homogenate

Stock solutions

0.25ST; 0.25M sucrose in 10mM Tris pH7.4, made freshly for each preparation, and used ice-cold.

- 1 For preparing donor membranes, 5 x 576cm² plates of 15B CHO cells were cultured to confluence. This was done by seeding with 3 x 150cm flasks of 15B cells and growing for 2-3 days. To infect, the growth medium of the cells was removed and saved, and washed with 10ml of infection medium (complete growth medium without foetal calf serum, with 10mM Hepes/KOH pH 7.4, 0.2% BSA), and then infected with 20ml of infection medium containing 50pfu/cell, ie 5.8x10⁹ pfu/plate, for 1 hour at 37°C. Then the medium was replaced by the 50ml growth medium, and incubated for a further 2.5 hours.
- 2 To harvest the cells, each plate in turn was drained of medium and washed twice in trypsin/EDTA, and then knocked to release the cells. The cells were then washed off into the saved growth medium, and thereafter kept on ice or at 4°C.
- 3 The cells were pelleted at 1500 rpm for 5 minutes at 4°C in a Jouan swinging-bucket rotor. The pellets were pooled in 2x 50ml Falcon tubes and made up to 100ml in 0.25ST, and then spun at 1500 rpm at 4°C for 5 minutes.
- 4 The supernatants were removed by aspiration, and the loose pellets were pooled in one 15ml Falcon tube and made up to 15ml in 0.25ST. The cells were pelleted at 2500 rpm for 5 minutes at 4°C.
- 5 The pellet volume was noted, and then the supernatant was removed by aspiration, and the pellet resuspended in an equal volume of 0.25ST using a 1ml Gilson pipette.
- 6 The cells were homogenised in a ballbearing homogeniser using a ballbearing of 0.253" diameter. Under standardised conditions, the cells were broken with 15-25 passages, the extent of cell breakage being assayed by microscopic examination in PBS/1µg/ml Hoechst 33258. Cell breakage ideally led to maximal cell disruption with minimal nuclear disruption. The volume of the homogenate was adjusted to 5ml with 0.25ST.

2.4.2 Acceptor Golgi homogenate

Stock solutions

Ice-cold 0.25M ST; 0.25M sucrose in 10mM Tris pH7.4, made freshly for each preparation.

- 1 The cells used in acceptor preparations were wild-type CHO cells. 8x576cm² plates were seeded by 5x150cm² flasks and grown to confluence over 2-3 days.

- 2 To harvest the cells, the plates were, four at a time, emptied of medium and washed in trypsin/EDTA, and then knocked to release the cells. The cells were washed clear of the plates and back into their medium. Thereafter all procedures were carried out at 4°C or on ice.
- 3 The cell suspensions were spun down at 1500 rpm for 5 minutes at 4°C, and then washed in one 50ml Falcon tube of 0.25ST.
- 4 The cells were resuspended in 1x15ml Falcon tube of 0.25ST, and spun at 2500 rpm for 5 minutes at 4°C. The volume of the pellet was noted, the supernatant aspirated, and the pellet resuspended in an equal volume of 0.25ST.
- 5 The cells were broken, under standardised conditions, by 20-30 passes of a ballbearing homogeniser, to yield a homogenate of maximum cell breakage and minimum nuclear breakage. This was monitored in the same way as for donor membranes. The homogenate was adjusted to 5ml with 0.25M ST.

2.4.3 Sucrose density gradient centrifugation for preparation of Donor and Acceptor Golgi membranes

Stock solutions

Sucrose solutions; at specified molarities in 10mM Tris-HCl at pH7.4.

EDTA; 100mM pH7.4

- 1 7-8ml of 2.3M sucrose was added to 5ml of CHO or 15B cell homogenate to produce a solution of 44-45% (w/w) sucrose.
- 2 EDTA was added to 1mM final from a 100mM stock (pH7.4), and the solution vortexed to yield an even suspension.
- 3 The homogenate was placed in the bottom of a SW28 tube, and then layered with 14ml 1.2ST and 8ml 0.8ST. A balance tube was prepared containing 5ml 0.25ST, 7ml 2.3ST, 14ml 1.2ST and 8ml 0.8ST
- 4 The gradient was spun at 25,000rpm for 2.5 hours in an SW28 rotor in a Beckman ultracentrifuge.
- 5 The membrane fraction was recovered by syringe puncture from the 0.8 - 1.2M interface in 2-3ml. It was either salt-washed immediately, or snap-frozen in 50µl aliquots and kept at -80°C. Membrane aliquots were thawed only once.

2.4.4 Salt-washing Golgi membranes

Stock solutions

1M sucrose, 0.72M sucrose and 1.5M KCl, all in 10mM Tris-HCl pH7.4 (1.0ST, 0.72ST and 1.5KT respectively).

- 1 Membranes were salt-washed immediately after recovery from float-up, with all steps being performed on ice or at 4°C. 2ml of membranes at 0.25-0.5mg/ml were salt-washed.
- 2 Gradients were prepared in thin-walled TL100.3 tubes, comprising 0.25ml 1.0ST and 0.75ml 0.72ST.
- 3 The Golgi was mixed with 4ml 1.5KT and a timer was set for ten minutes. During this time, the 6ml of salt-washing membrane was divided between the four tubes and placed in the swing-out rotor of a Beckman TL-100 tabletop ultracentrifuge.
- 4 At the end of the ten minutes the membranes were spun for 20 minutes at 50,000 rpm in a Beckman TL-100 benchtop ultracentrifuge, with acceleration and deceleration at setting five.
- 5 The membranes were then recovered from the 1.0-0.72MST interface and pooled. They were snap-frozen in 65µl aliquots and stored at -80°C. Their activity appeared to decline after about three months of storage. Salt-washed membranes were thawed only once and applied to transport reactions with alacrity, while being maintained on ice.

Cell homogenates and unwashed and salt-washed membrane fractions were characterised by protein and galactosyltransferase assay, and the yield and purification calculated. They were principally characterised by performance in the transport assay. Examples of purification tables and membrane titrations are shown in Table 3.1 and Figures 3.5, 3.9 and 3.11.

2.5 Making interphase and mitotic cytosol

2.5.1 Cytosol preparation

Stock solutions:

Nocodazole, 200µg/ml in DMSO

TEP - KCl 10mM triethanolamine, 150mM KCl pH 7.2. This buffer was made fresh for each preparation

EBS breaking buffer; 80mM β-glycerophosphate, 20mM EGTA, 15mM MgCl₂, 1mM DTT, 1mM PMSF, 2mM ATP; for mitotic cytosol 1µM okadaic acid or microcystin, pH 7.2. This buffer was made freshly for each preparation.

The preparation was normally used to generate cytosol from one litre each of interphase and mitotic suspension HeLa cells.

- 1 Ten 150cm³ flasks of adherent sHeLa cells were grown to confluence and then passaged into a 1l spinner culture. If used for interphase cytosol, the culture was harvested after 1 day, if for mitotics or a mixed prep it was treated after one day. The cell density required was 4-7x10⁵ cells/ml.

2 For a combined interphase and mitotic preparation, the culture was divided into two after one day's culture. One half was spun under sterile conditions at 2000 rpm for 2 minutes at 4°C in a J6 or equivalent centrifuge, and the supernatant was reserved under sterile conditions. The pellet was resuspended thoroughly in 10ml of medium containing 10µg/ml nocodazole, and then resuspended in 1l of medium to give a final nocodazole concentration of 100ng/ml. The remaining cells were made up to 1l in fresh medium to generate the interphase population. These two preparations were allowed to grow for a further 24 hours.

3 The interphase and mitotic cells were then spun down non-sterilely in 250ml bottles in a J6 or equivalent centrifuge for 10 minutes at 2000 rpm and 4°C, and thereafter kept at 4°C or on ice. The medium was discarded and the pellets resuspended in 50ml Falcon tubes in TEA-KCl, incubated on ice for ten minutes, and washed at 2000 rpm for 2 minutes.

4 The cells were washed in 10ml of EBS buffer in 15ml Falcon tubes, then the pellet volumes were noted, and the cells were resuspended in an equal volume of EBS buffer. For the mitotic cell pellet, either okadaic acid or microcystin was added to 1µM final from a 1mM stock. The agents had indistinguishable effects upon mitotic cytosol preparations.

5 The cells were broken in a ballbearing homogeniser with the number 30 ball, with the number of passes varying between preparations. For interphase cells, 30-50 passes were required, and for mitotic cells 15-30 passes.

6 The cell homogenates were placed in thick-walled TL100 tubes and spun for 30 minutes at 100,000 rpm.

7 The high-speed supernatant, or 'cytosol', was recovered from the tubes, avoiding the lipid layer and the cell pellet, and then snap-frozen in 50-100µl aliquots and stored at -80°C. Cytosol aliquots could be thawed twice, and could be kept on ice for up to 30 minutes either before or after desalting.

(NB: later cytosol preparations employed two spins to reduce the loss of cytosol due to lipid contamination. The homogenate was first spun for 10 minutes at 10,000 rpm in the TL-100, then the supernatant was transferred to a second tube and spun for 30 minutes at 100,000 rpm. The lipid was left behind after the first spin because it adhered to the tube walls.)

Cytosols were characterised by protein concentration, histone kinase activity and behaviour in the transport assay. Examples of cytosols are listed in Table 3.2.

2.5.2 Cell counting and evaluation of mitotic index

To calculate the cell density in each preparation, 1ml volumes were removed just before harvesting, pipetted several times to break up cell clumps, and then counted in a haemocytometer. Cell densities of 1×10^5 and 1×10^6 were required for healthy cells and reasonably-sized preparations.

λ
ml⁻¹

To establish the mitotic index, 1ml of the mitotic cells was removed into an Eppendorf tube and spun down for 10 seconds at maximum speed, and then resuspended in 50µl of Hoechst dye. This could be left on ice for up to 1 hour while the cytosol preparation was in progress. The cell suspension was placed on a slide, covered with a coverslip, and inspected under the fluorescence channel of the microscope using an oil-immersion lens. Several fields of cells (>300) were examined, and the mitotic (with tightly-aggregated, bright DNA) and interphase (with diffuse, dim DNA) cells were counted. A mitotic index of >94% was a prerequisite for mitotic cytosol to have a strong inhibitory effect on the transport assay.

2.6 Intra-Golgi transport assay

The transport assay measures transport of the VSV G-protein between the cis Golgi of the donor membranes and the medial Golgi of the acceptor membranes. This transfer requires cytosol and an ATP-regenerating system. The assay was almost exactly as described in Balch et al (1984) and Balch and Rothman (1985). The assay as described was used for studies of cell cycle-related control of transport.

Stock solutions

10x buffered salts (10xB/S): 250mM HEPES-KOH (pH 7.0), 250mM KCl, 25mM MgOAc: either prepared as 2ml aliquots and frozen at -20°C for up to three months, or kept at 4°C for up to two months.

Buffered salts stocks: 500mM HEPES-KOH pH 7.0, 1M KCl, 100mM MgOAc; these were all kept at -20°C for up to a year.

ATP (Sigma): 100mM in 250mM HEPES-KOH pH 7.0 and frozen at -20°C for up to a year; aliquots were not thawed above four times.

UTP (Sigma): 100mM in water and frozen at -20°C for up to a year; aliquots were not thawed above four times.

Creatine phosphate (CP) (Boehringer Mannheim): 200mM in water and frozen at -20°C for up to a year; aliquots were not thawed above four times.

Creatine phosphokinase (CPK) (Boehringer Mannheim) was made up at 2mg/ml in 50% glycerol and kept at -20°C for up to a month.

DTT (Boehringer Mannheim): prepared for each use at 100mM in water.

Uridine diphosphate-N-acetyl-D-glucosamine [glucosamine-6-³H(N)-] (UDP-³H-GlcNAc): purchased from NEN at 5-25Ci/mmol and kept at -20°C.

Anti-VSV-G monoclonal was prepared by affinity isolation and stored indefinitely at -20°C. Once opened, the aliquots were stored at 4°C

Rabbit anti-mouse IgG was purchased from Dakopatts and stored at 4°C.

Detergent stop buffer: 50mM Tris-HCl pH 7.5 (2M stock; room temperature), 250mM NaCl (2M stock; room temperature); 1mM Na₂EDTA pH 7.0 (500mM stock; room temperature)

1% Triton X-100, (20% w/v stock; 4°C);

1% Na cholate, (10% stock; -20°C): stored at room temperature

Washing buffer: 50mM Tris-HCl pH 7.5, (2M stock, room temperature): 250mM NaCl; 5mM Na₂EDTA pH 7.0 (500mM stock, room temperature); 1% Triton X-100; stored at room temperature

1 Desalting columns were prepared for cytosol. This was necessary to remove endogenous, unlabelled UDP-GlcNAc in the cytosol, which would otherwise compete with the labelled substance and reduce the transport signal below detectability. 100µl cytosol could be desalted over one column, and 20% more cytosol was desalted than was required, to allow for losses. Each column consisted of a capless Eppendorf with a hole punched in the bottom with a syringe needle, packed with glasswool and filled with preswollen Bio-Gel P6 DG, and supported in a Sarstedt 6ml polystyrene assay tube. The column was prepared during the preparation of the assay by washing through 5ml of 1xB/S with 1mM DTT and 1mM ATP. The column was centrifuged at 2000 rpm for 2 minutes at 4°C in a Jouan benchtop centrifuge. After the final spin, the collecting tube was changed and the newly-thawed cytosol loaded. The cytosol collected in the subsequent spin was exchanged into assay buffer, and could remain on ice for a few minutes. There was normally 80-90% volume recovery from such a spin-column.

2 The assay mixture for ten assays was prepared as follows (such a mixture might be used for nine samples, since there were small dispensing losses). 50µl ³H-UDP-GlcNAc (in ethanolic solution) was dispensed into a borosilicate glass tube and dried under a stream of nitrogen. To this was added 50µl 10xB/S, 12.5µl CP, 2.5µl CPK, 5µl ATP, 5µl UTP, 5µl DTT, 10µl pCoA and 260µl H₂O. This was mixed by brief vortexing, and then 35µl mix was placed in borosilicate glass tubes (the transport assay cannot be performed in Eppendorf tubes). Note that this reaction mix volume permits of no additions; on occasions up to 5µl of other reagents was added to some assays. Then the volume of water in the reaction premixture was reduced appropriately, and samples made up to 35µl with active agent or an appropriate control substance.

3 5µl desalted cytosol was added to the premixture (or sometimes less, according to the optimum performance established for each cytosol preparation). At this point, assays could be preincubated for some time, normally 20 minutes at 37°C, to allow effects upon the cytosol. At the end of any preincubation, the assays were returned to ice.

4 Membrane fractions were thawed and added to the assays. The volume of each added was normally 5 μ l, but could be less, particularly for non-salt-washed membranes. The membranes were thawed directly before addition to the assay, by holding in a 30°C or 37°C waterbath for a few seconds, until almost completely thawed, and then transferring to ice. The thawed membranes were added to the transport assays with speed. The assays were mixed by gentle flicking, and never by vortexing.

5 For the transport incubation, non-salt-washed assays were covered and transferred directly to a 37°C waterbath and incubated for 60 minutes. Salt-washed assays were warmed gently by standing at room temperature for 10 minutes, then covered, transferred to a 30°C waterbath and incubated for 120 minutes. The lower temperature and room temperature warming was partly a concession to the reduced robustness of the membranes, and partly to avoid the increased background counts which apparently result from higher-temperature incubation.

6 10 minutes before the end of the incubation time, the immunoprecipitating complex was prepared by mixing together anti-VSVG monoclonal and rabbit-anti-mouse IgG in appropriate ratio (in this work, normally 0.5 μ l anti-VSVG and 2 μ l rabbit anti-mouse per assay, with 10% more prepared than required, to account for dispensing losses). The antibodies were incubated at 37°C for at least 2 minutes, and complex formation was shown by cloudiness of the suspension. Then 50 μ l of stop solution per assay was mixed with the antibodies by inversion, and the suspension added to the assays. The stopped assay was mixed by vortexing, and allowed to immunoprecipitate at 37°C for at least 45 minutes or at 4°C overnight (which resulted in slightly elevated recovery of counts).

7 The immunoprecipitates were collected as follows. A 50-100ml solution of 2.5% skim milk powder in wash buffer was made and assay filters were soaked for at least 5 minutes. Meanwhile, the filter apparatus was set up with a mild vacuum line and a trap, and a heat lamp was positioned over a sheet of 3MM paper marked with the numbers of the (otherwise unmarked) filters. Wash buffer was placed in a beaker, and normally a multipipetter was used for washing.

For each assay, a filter circle was placed in the apparatus and washed with 3ml wash buffer, meanwhile 3ml more was added to the assay tube, and this was then poured over the drained filter. The tube was washed out with 3ml more buffer, then the filter was washed three times in 3ml wash buffer. The filter was then removed from the apparatus and placed on the 3MM paper. The filters were allowed to dry, and then loaded into 4ml scintillation vials, immersed in 4ml Beckman scintillation fluid, and counted.

Normally, duplicate assays or titrations were performed to control for slight inter-assay variation. In relatively simple assays, less than 10% variation was expected between

duplicates. As a control for antibody specificity, a 'minus-donor' control was normally appended to each assay, being complete but for donor membranes, and treated exactly as the other samples; it was expected to show less than 5-10% of the positive counts of a corresponding interphase assay. In some cases, particularly when comparing interphase and mitotic cytosol, counts were represented as a percentage of corresponding interphase counts.

2.7 Enzyme assays

2.7.1 Histone kinase assay of cytosol

The histone kinase assay was slightly adapted from Felix et al (1989). It was often used with a 2 μ l sample volume containing 0.2 μ g cytosol.

Stock solutions

100mM ATP; as for transport assay

KEHM; 50mM KCl, 10mM EGTA pH 7.2, 50mM Hepes/KOH pH 7.2, 1.92mM MgCl₂; stored at 4°C.

beta-glycerophosphate (BGP); 1M pH 7.2, stored at 4°C

NaF; 1M, stored at room temperature

histones; type III from calf thymus, Sigma H5505; prepare in KEHM, snap-frozen and stored at -20°C; thawed only once.

1 A reaction mixture for 10 assays contained 30 μ l 1xB/S (omitted for 5 μ l samples), 16 μ l 1M BGP, 10 μ l 1M NaF, 4 μ l 50mM MgCl₂, 2 μ l 1mM ATP, 2 μ l ³²P-ATP (10 μ Ci/ μ l), and 58 μ l of KEHM+/- histones.

(NB: the quantity of ³²P-ATP specified is more than actually required for a clear signal in the assay. If fresh ³²P-ATP was used, 1/2-1/3 of the specified quantity was found to be ample, and the volume specified gave clear results for ³²P-ATP up to four weeks old.

Generally, for single samples, for example in tests of mitotic cytosol, duplicate assays and controls were performed. In multiple assays involving the same cytosol, for example cyclin A titrations, it was found necessary to perform duplicate negative controls for each cytosol, but not for each treatment. This kept the number of samples to a minimum. In an assay with 6 treatments of a single cytosol, for example, a reaction mixture for 20 samples was prepared containing all chemicals but the KEHM/histones; ie 60 μ l B/S, 32 μ l BGP, 20 μ l NaF, 8 μ l MgCl₂, 4 μ l ATP and 4 μ l ³²P-ATP, total 128 μ l. 32 μ l would be withdrawn and mixed with 58 μ l KEHM, sufficient for 5 negative controls. The remainder would be mixed with 174 μ l histones in KEHM, sufficient for 15 samples.)

2 Each assay contained 18 μ l reaction mix in an Eppendorf tube in a bored aluminium block on ice. 2 μ l cytosol samples were added on ice, and the tubes were closed and incubated under convenient conditions; generally for 10 minutes at 37°C in a shielded waterbath, but in some experiments incubations were carried out for 15 or 20 minutes at 30°C.

3 During the reaction, Whatman P81 paper was ruled into 1.5cm squares, one for each assay and 4-6 positive controls. The samples and positives were numbered in pencil, cut into separate blocks and pinned separately to a board. Positives consisted of 2 μ l samples of the undiluted assay mixture, and were used to calculate ^{32}P cpm/pmol ATP.

4 At the end of the reaction, the tubes were returned to ice, and 12 μ l of each spotted onto the squares of P81 paper. This block of squares (NOT the positives) was unpinned and washed for 3x15 minutes in (20x no. of samples)ml of 150mM H₃PO₄, with occasional stirring. It was then rinsed in 95% ethanol, and placed on Whatman 3MM under a heat lamp to dry. Then the washed assays and unwashed positives were cut up and placed in 5ml scintillation tubes, immersed in 4ml scintillation fluid and counted.

The cpm of the negative controls was averaged, if necessary. Then the activity of the ATP was calculated from the cpms of the positives, as
average cpm / [(20/18)x2x1000] = cpm/pmol ATP.

The activity of each sample was calculated as
(sample cpm - bkd cpm)/cpm/pmol ATP = sample pmol ATP.

For sample specific activity,

Sample pmol ATP x (20/12) x (1/sample vol in μ l) x (1/assay time in minutes) x (1000/[cytosol]mg/ml) = specific activity in pmol ATP/minute/mg protein.

2.7.2 β 1,4-Galactosyltransferase assay

Stock solutions

sodium cacodylate; 0.4M pH6.6, stored at room temperature

ovomuroid, (trypsin inhibitor) 175mg/ml in water. Stored at -20°C in 500 μ l aliquots.

10mM UDP-galactose: in water, stored at -20°C

10% Triton X-100; stored at 4°C

200mM ATP; dissolve in water and adjust pH to 6-7 with 1M NaOH. Stored at -20°C in

200 μ l aliquots and thawed up to 4 times.

2M MnCl₂; in water, stored at 4°C

PTA-HCl: 0.5M, with 11ml c.HCl added per 500ml, stored at 4°C.

- 1 The assay mixture for 10 assays comprised: 100µl Na cacodylate pH6.6, 100µl ovomucoid, 3µl 14.3M β-mercaptoethanol, 20µl 10mM UDP-galactose, 20µl 10% (w/v) Triton X-100, 10µl 200mM ATP pH7.0, 20µl 2M MnCl₂ and 5µl 100mCi/ml ³H-UDP - galactose; H₂O 0.52ml; total, 0.8ml
- 2 80µl reaction mix was placed in screw-capped tubes in a bored aluminium block on ice, and 20µl samples (made up with water if necessary) were added; also a blank was prepared with 20µl water.
- 3 The reactions were incubated for 30 minutes at 37°C, and then removed to ice and cooled for 10 minutes.
- 4 Samples were washed three times in 1ml PTA-HCl, by vortexing to resuspend the protein precipitate in PTA-HCl, and then centrifuging the precipitate for 5 minutes at 2000 rpm in a microfuge and aspirating the supernatant.
- 5 The precipitate was washed once in 1ml ice-cold 95% ethanol, and then resuspended in 50µl 2M unbuffered Tris and 200µl 1% SDS. To the solubilised samples was added 1ml Beckman scintillation fluid. After vortexing, the samples were loaded into 4ml scintillation tubes and counted. Positives were prepared by mixing 10µl of the unadulterated assay mixture with 40µl Tris and 200µl SDS.

The activity of galactosyltransferase in the mixture was calculated as
 standard dpm x 80/1µmol = activity in µmol/ml, then sample activity was calculated as
 (sample - bkd) x 1/0.5h x 1000/20µl = activity in dpm/ml/h
 (activity/activity of GT) x [protein]= specific activity of GT in µmol/hr/mg protein.

2.7.3 *N-acetylglucosaminyltransferase I assay*

Stock solutions

0.5M Tris-maleate; 6.06g Tris, 5.81g maleic acid, pH to 6.9 with NaOH and make up to 100ml, stored at room temperature

ovalbumin; 200mg/ml in water. Stored at -20°C in 500µl aliquots.

1M KCl; stored at room temperature

50mM UDP-GlcNAc; in water, stored at -20°C

10% TX-100; stored at 4°C

2M MnCl₂; in water, stored at 4°C

2M MgCl₂; in water, stored at 4°C

PTA-HCl: 0.5M, with 11ml c.HCl added per 500ml, stored at 4°C.

- 1 The assay mixture for 10 samples comprised 100µl Tris/maleate buffer, 100µl ovalbumin, 10µl KCl, 20µl UDP-GlcNAc, 10µl Triton X-100, 2.5µl MgCl₂, 2.5µl MnCl₂, 10µl 100µCi/ml [³H]-UDP-GlcNAc, 0.55ml water; total volume 0.8ml.
- 2 80µl of reaction mixture was dispensed into screw-capped eppendorf tubes. 20µl of sample (made up with water if necessary) was incubated with the mixture for 2.5 hours at 37°C, and then removed to ice and cooled for 10 minutes.
- 3 Samples were washed three times in 1ml PTA-HCl, by vortexing to resuspend the protein precipitate in PTA-HCl, and then centrifuging the precipitate for 5 minutes at 2000rpm in a microfuge and aspirating the supernatant. The precipitate was then washed once in 1ml ice-cold 95% ethanol.
- 4 The precipitate was resuspended in 50µl 2M unbuffered Tris and 200µl 1% SDS. To the solubilised samples was added 1ml Beckman scintillation fluid. After vortexing, the samples were loaded into 4ml scintillation tubes and counted. Positives were prepared by mixing 10µl of the unadulterated assay mixture with 40µl Tris and 200µl SDS.

The activity of GlcNAc transferase in the mixture was calculated as
 standard dpm x 80/1µmol = activity in µmol/ml, then sample activity was calculated as
 (sample - bkd) x 1/2.5h x 1000/20µl = activity in dpm/ml/h
 and (activity/activity of GNT) x [protein]= specific activity of GNT in µmol/hr/mg protein.

2.7.4 Protein assay

Protein assays were carried out using either the Bio-Rad protein assay kit or the Pierce BCA assay kit, according to the manufacturers' instructions. The Bio-Rad assay was the more commonly used since it suffered less inaccuracy in the presence of cellular lipids than the Pierce assay.

2.8 Expression of bacterial proteins

2.8.1 MBP-cyclin A

Stock solutions

Bacterial L-broth; prepared in-house

Ampicillin; 50mg/ml in 50% ethanol, stored at -20°C

IPTG

Lysis buffer; 20mM Tris 7.4, 200mM NaCl, 1mM EDTA, 1mM DTT, 0.25µM PMSF; prepared freshly

Lysozyme

KEHM; 50mM KCl, 10mM EGTA pH 7.2, 50mM HEPES pH 7.2, 1.92mM MgCl₂; store at 4°C.

beta-glycerophosphate; 1M pH 7.2, store at 4°C

The fusion protein of maltose binding protein and cow cyclin A was constructed by Jorg Adamczewski from the Cell Cycle Control group at the ICRF Clare Hall laboratories, who kindly made the clone available for expression by workers in this laboratory. It was based on a protein A-cyclin A fusion protein developed there [Kobayashi, 1992 #158]. The protein was overexpressed in E.coli.

- 1 2 litres of E. coli cells were grown to an A₆₀₀ of 0.5 and then induced for 4 hours at 30°C or overnight at room temperature with 0.1mg/ml IPTG.
- 2 The cells were pelleted and washed twice in 0.9% NaCl, resuspended in 20ml lysis buffer.
- 3 Lysozyme was added to 0.1mg/ml and the suspension was incubated for 10 minutes on ice and then sonicated to break. Breakage was monitored by spinning down a 1ml sample of the bacterial homogenate in an eppendorf centrifuge for 1 minute, and checking the A₂₈₀ of a 200-fold dilution of the supernatant.
- 4 The cell suspension was clarified by centrifugation for 30 minutes at 4°C and 45,000 rpm in a Beckman SW55 rotor and then by passage through 0.45µm filters. The activity of the preparation could be checked at this stage by its effect on the histone kinase activity of interphase cytosol.
- 5 The supernatant was loaded onto a 5ml amylose-Sepharose column pre-equilibrated in lysis buffer, and the column was then washed in 8 volumes of the buffer (normally overnight).
- 6 The protein was eluted in lysis buffer plus 10mM maltose and desalted into KEHM; the yield was 4ml of protein at 3mg/ml.

2.8.2 GST-cyclin B

Stock solutions

hypertonic buffer; 2.3M sucrose, 50mM Tris pH7.5, 10mM EDTA, 1mM PMSF, made fresh and kept on ice (10ml)

lysis buffer; 50mM Tris pH7.5, 100mM KCl, 1mM EDTA, 1mM DTT, 1mM PMSF, made fresh and kept on ice (40ml)

wash buffer; 10mM Hepes, 1mM DTT, 1µg/ml pepstatin, leupeptin, chymostatin, made fresh and kept on ice (100ml)

wash buffer + 150mM NaCl (150ml)

wash buffer + 5mM glutathione pH8.0 (10ml)

4.1 litres LB-Amp

16ml 100mM IPTG

0.5ml 10mg/ml lysozyme (Boehringer Mannheim; stored at -20°C and made up fresh in buffer)

0.2ml 5mg/ml DNAase I (Boehringer Mannheim; stored at -20°C and made up fresh in buffer)

10% (w/v) deoxycholic acid (Sigma; stored at room temperature)

Cyclin B from *Xenopus* was engineered to incorporate a glutathione-S-transferase moiety at the N-terminus Guan and Dixon, (1991); Frorath et al, (1992). The engineered construct in *E.coli* was generously provided by Dr C. Smythe at the university of Dundee, and the protein was expressed according to a protocol he provided.

- 1 4 flasks of 1 litre LB-Amp were infected with 10ml of a 100ml overnight culture of the *E.coli* strain (*E.coli* pLys S containing pGST D13cyc). The bacteria were grown to an A_{600} of 0.4 (taking ~4 hours), and IPTG added to 25 μ M final.
- 2 The protein was expressed for a further 3 hours at 37°C.
- 3 The cells were then spun down for 5 minutes at 5000 rpm in a Sorvall rotor, resuspended in 200ml 0.9% NaCl and spun down as previously.
- 4 The cells were resuspended in 10ml of hypertonic buffer for 30 minutes on ice. Then they were diluted in lysis buffer containing 5ml lysozyme and incubated on ice for 1 hour to lyse the cells.
- 5 Sodium deoxycholate was added to 0.1% (w/w) and MgCl₂ to 10mM. 1mg DNAase I was added and the suspension left on ice till no longer viscous.
- 6 The homogenate was then centrifuged for 30 minutes at 25,000 rpm in an SW28 rotor, and the supernatant recovered (the preparation could be snap-frozen overnight at this stage).
- 7 It was added to 3ml glutathione-Sepharose prepared in washing buffer and then rotated for 45 minutes at 4°C in a 50ml Falcon tube.
- 8 The beads were then spun down briefly and the supernatant discarded.
- 9 The beads were washed three times in washing buffer supplemented with 150mM NaCl.
- 10 The cyclin was eluted by three 3ml washes of washing buffer supplemented with 5mM glutathione, pH8.0.
- 11 The protein was concentrated to 2ml in a Centricon, and exchanged into KEHM buffer over a column of Bio-Gel P6-DG, as described for cyclin A.

Chapter 3

Characteristics of the interphase and mitotic transport assay

3.1 Aims

As described in the Introduction and detailed in Materials and Methods, the intra-Golgi transport assay was the basic tool used in this thesis to study the activity of cytosols derived from interphase and mitotic cells.

The assay uses membranes from two sources. The first, the 'donor', is derived from the CHO mutant cell line 15B, which lacks the medial Golgi enzyme N-acetylglucosaminyltransferase I (GNTI), and is thus incapable of initiating the synthesis of hybrid and complex oligosaccharides. The 15B cells are infected with vesicular stomatitis virus (VSV) before isolation of the Golgi membranes. The second source, the 'acceptor', is uninfected CHO wild-type cells.

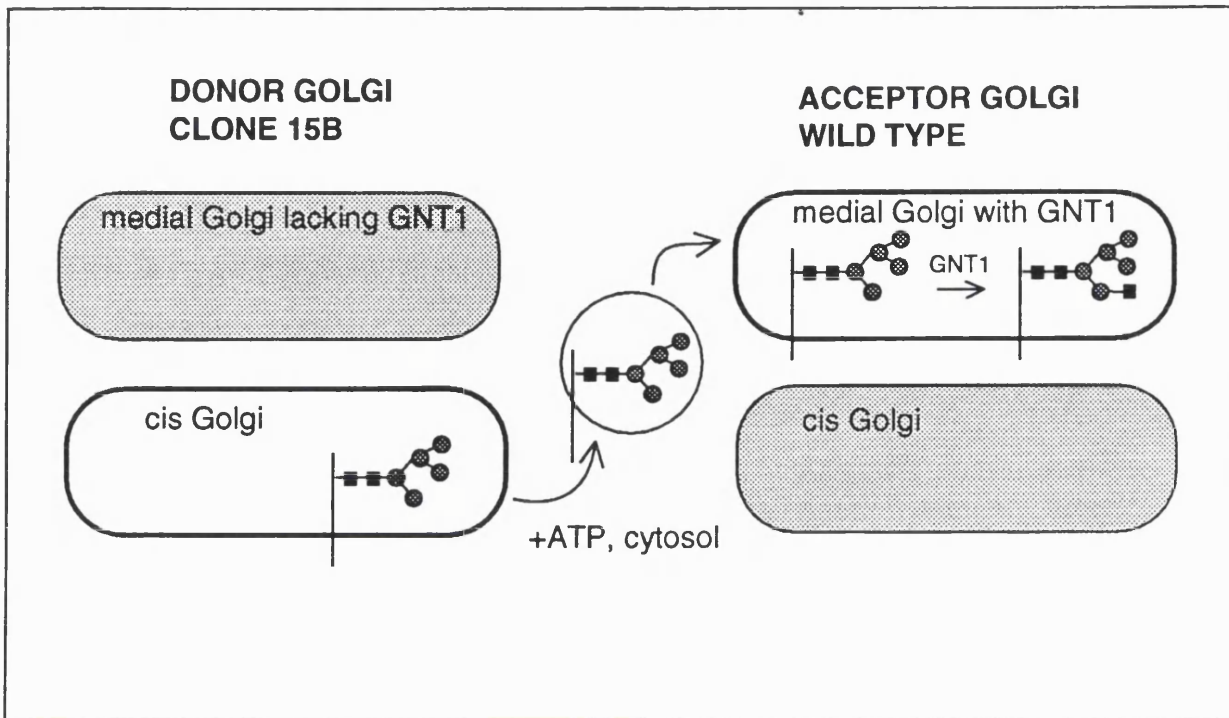
When these purified membranes are mixed with cytosol and supplied with energy, vesicular transport occurs. Vesicles containing VSV glycoprotein (VSV-G) derived from the cis-Golgi of the donor may fuse with the medial Golgi of the acceptor, and there the VSV-G is modified by GNTI. The extent of transport is monitored by incorporation of tritiated N-acetylglucosamine (GlcNAc) into the VSV-G immunoprecipitated at the end of the assay. Incorporation of ³H-GlcNAc into VSV-G at high levels requires removal of endogenous GlcNAc from the cytosol (by gel-filtration) to maximise the concentration of tritiated sugar available for incorporation into oligosaccharide.

The first part of the chapter describes the optimisation of the basic assay, using as cytosol a high-speed supernatant from sHeLa cells. Transport was supported by interphase cytosol, but less well by cytosol from mitotic cells. The later part of the chapter describes the maximisation of the difference between interphase and mitotic cytosol by a salt-treatment of the membranes.

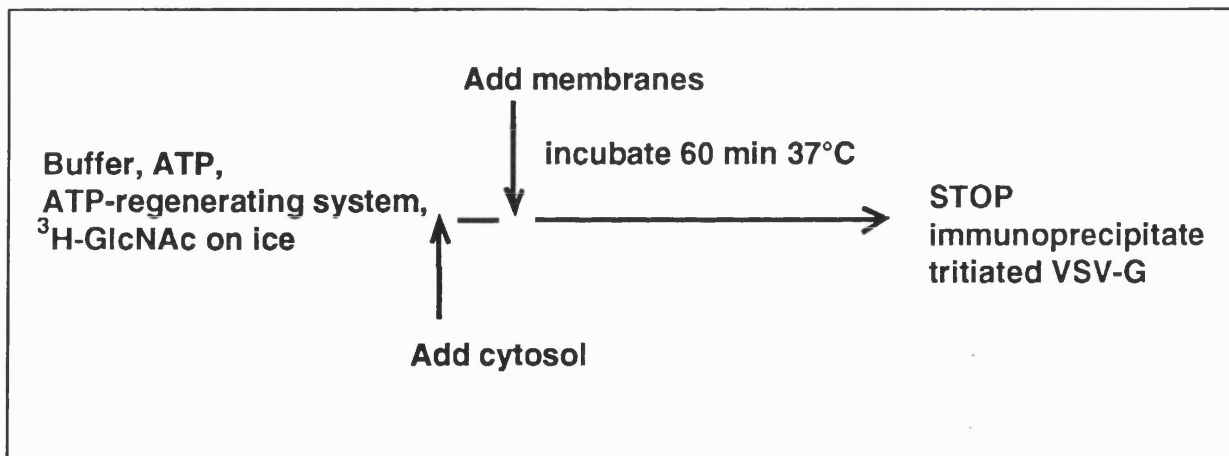
The sHeLa cytosol was the only component of the assay which differed significantly from that originally described by Rothman and co-workers. Because of the complexity of the assay, it seemed wise to characterise it in this laboratory, for two reasons: firstly, to determine any differences between this laboratory's methods and others'; secondly, to optimise it for work with sHeLa interphase and mitotic cytosols.

Figure 3.1: The transport assay

a: the principle of the assay



b: the experimental layout of the assay



3.2 The standard assay

The standard transport assay contained ³H-GlcNAc, buffer, ATP and an ATP regenerating system, donor, acceptor and cytosol (for details see Materials and Methods). The assay mixture was prepared first, in a tube in which labelled UDP-GlcNAc had been evaporated to dryness; containing buffer, ATP and an ATP-regenerating system and DTT. Then desalted cytosol was added, and finally membranes. The tubes were then incubated for 60 minutes at 37°C (except for a control maintained on ice) and then immunoprecipitated. The protocol for the assay is shown in Fig 3.1, and tests shown in Fig 3.2 show the effect of omission of assay components.

3.2.1 The transport buffer

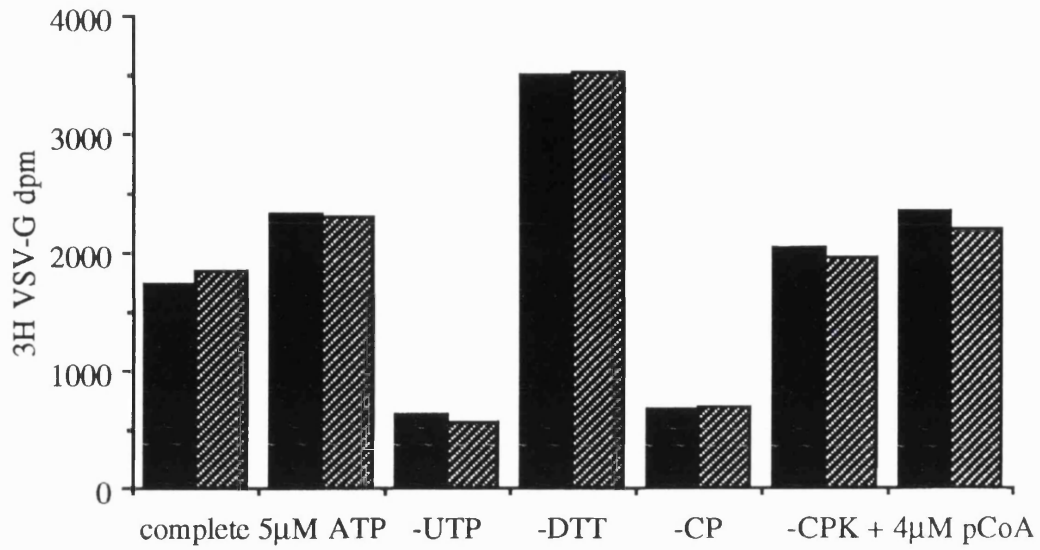
Figure 3.2a shows the importance of buffer components in the transport assay. Reduction in ATP concentration to 5µM had no ill effect on the transport assay, provided that the ATP-regenerating system was intact. The published K_m for ATP in the assay is 4µM (Balch et al, 1985), but higher levels were required to maintain the histone kinase activity of mitotic cytosols (Stuart et al, 1993). However, assays performed in the absence of CP or UTP supported almost no transport. CPK could be omitted from the assay since this protein is present in the cytosol and not removed by desalting. When DTT was absent from the transport reaction, the signal increased slightly; this was possibly due to decreased activity of DTT-requiring proteases. DTT was required in the assay to maintain the activity of mitotic cytosol (Stuart et al, 1993). Addition of 4µM palmitoyl CoA (pCoA) to the buffer caused 20% enhancement of the transport, and therefore it was routinely added to transport assay incubations.

3.2.2: The cell-derived transport components

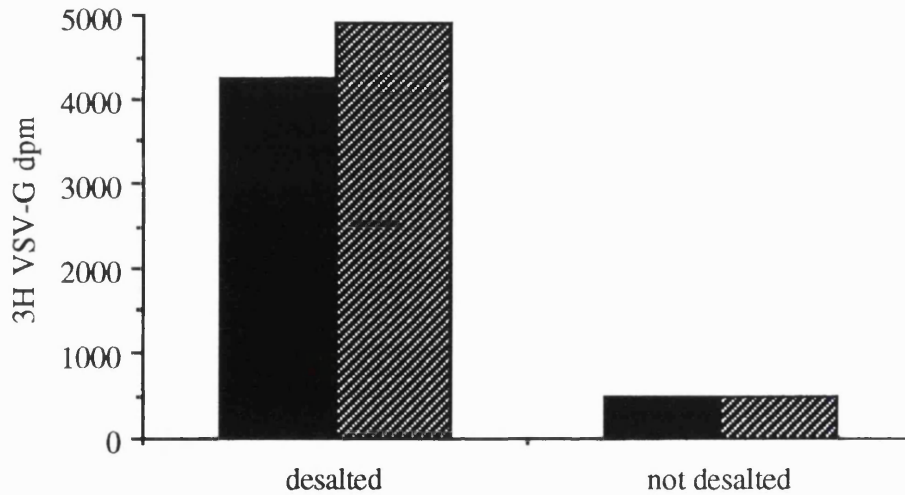
The cytosol was desalted into 1mM ATP and DTT in order to support the activity of the mitotic cytosol. All transport cytosols are desalted or dialysed into buffer in order to remove endogenous ³H-GlcNAc, which would otherwise dilute the glycosylation signal of tritiated ³H-GlcNAc. The fall in tritiated VSV-G levels in the absence of desalting is shown in Fig 3.2b. Figure 3.2c shows the effect of omitting any of the cell-derived fractions from the assay. In the absence of either donor or acceptor membranes, essentially no signal was obtained. In the absence of cytosol, however, a small signal was seen, presumably because of low levels of cytosolic proteins remaining specifically or non-specifically associated with the membranes during their purification.

Figure 3.2: the interphase transport assay

a: transport buffer components



b: the importance of desalting sHeLa cytosol



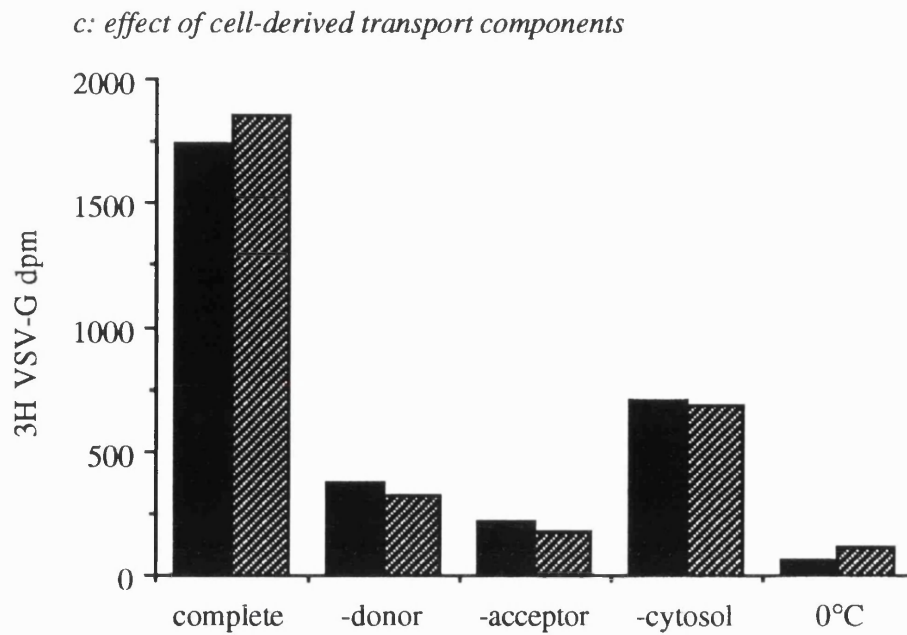


Figure 3.2: In these experiments, transport assays were prepared by addition of isolated components, rather than by the creation of an assay mixture, so as to be able to omit each component in turn. The duplicate assays contained 26 μ l H₂O, 5 μ l B/S and all but one of the following: 1 μ l 4 μ M pCoA, 0.5 μ l 100mM ATP, DTT, UTP; 1.25 μ l 200mM CP, 0.25 μ l (diluted in B/S) CPK, 5 μ l cytosol, 5 μ l donor, acceptor membranes. The complete assays were incubated at 37°C for 60 minutes, then immunoprecipitated with 0.5 μ l primary and 2 μ l secondary antibody. *The black and hatched bars show duplicate assays.*

~~purification~~. Essentially no transport was detected when the reaction was maintained on ice, despite the presence of all its components. This showed that the assay has a high minimum temperature due to the requirement for formation of transport vesicles; transport does not occur at all at 4°C.

3.2.3 Antibody titrations

Detection of tritiated VSV-G protein required efficient and rapid immunoprecipitation. This was performed by forming a pre-complex of anti-VSV-G monoclonal and rabbit anti-mouse monoclonal antibodies, then diluting this in assay stop buffer before addition to completed assays. Under these conditions, immunoprecipitation was close to maximal in 45 minutes at 37°C under optimal conditions. (Transport assays were also immunoprecipitated at 4°C overnight in some cases.)

To assess the importance of the antibodies in the assay, each was omitted from the immunoprecipitating buffer. The result of this is shown in Figure 3.3a. The signal was markedly reduced when either the primary or secondary antibody of the immunoprecipitation was omitted. The small signal detected probably reflected retention of VSV-G antibody aggregates on the filter. If the antibodies were not preincubated together before addition to the assay, a similarly low signal was obtained after 45 minutes of incubation (data not shown); this showed the effectiveness of the preincubation of the antibodies.

Different batches of monoclonal antibody were titrated to give the optimal recovery of VSV-G with minimal background. Such a titration is shown here (Fig 3.3b, c).

To establish the capacity of the antibody mixture, multiple reactions were prepared, and single assays' worth of antibody mixture was used to precipitate them (in stop buffer volumes increased in proportion to the assay mixture). Fig 3.3d shows that these levels of antibody were sufficient to precipitate VSV-G from up to five assay equivalents, and linear up to two assay equivalents.

3.2.4 Timecourse of the transport assay

In order to determine a suitable incubation time for the transport assay, a timecourse was performed by preparing identical transport reactions containing complete assay mixture, cytosol and membranes. The tubes were incubated at 37°C waterbath and, at various times, removed to ice to prevent further transport. After 60 minutes, all the assays were immunoprecipitated. The timecourse (Figure 3.4) shows that a 60 minute incubation was adequate for maximal signal with normal membranes. There was a lag of 10 minutes before signal was detected, and then a linear rise in signal which slowed after about 30 minutes.

Transport assays involving these membranes were therefore incubated for 60 minutes at 37°C.

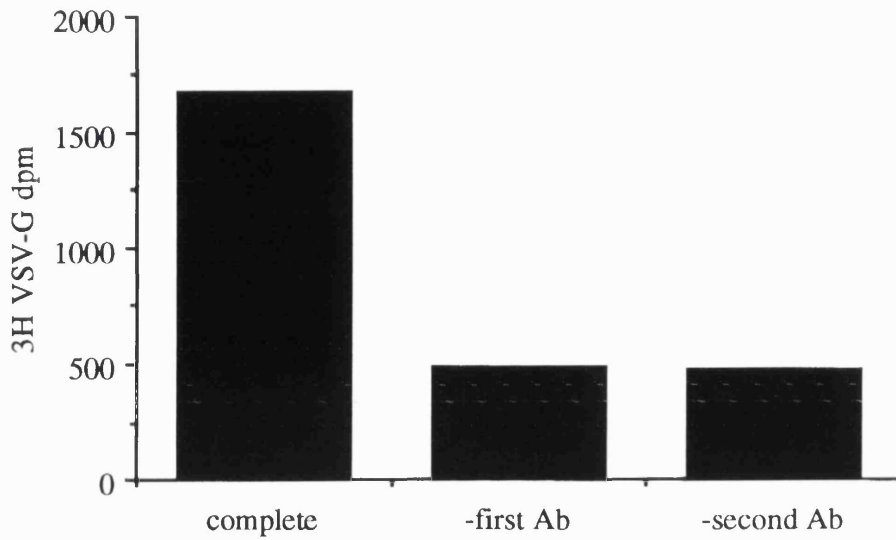
Figure 3.3: optimisation of immunoprecipitation conditions

In these experiments, assays were performed using standard buffer conditions, and 5µl each of HeLa cytosol, donor and acceptor membranes. After incubation for 60 minutes at 37°C, the assays were precipitated with the quantities of antibodies stated. In all cases, antibodies were preincubated in eppendorf tubes at 37°C for five minutes before the addition of stop buffer and mixing with the assays. In Figure 3.3d, the assays were incubated separately, and then combined and precipitated with 0.5µl primary and 2µl secondary antibody in stop buffer of equivalent volume to the transport assays.

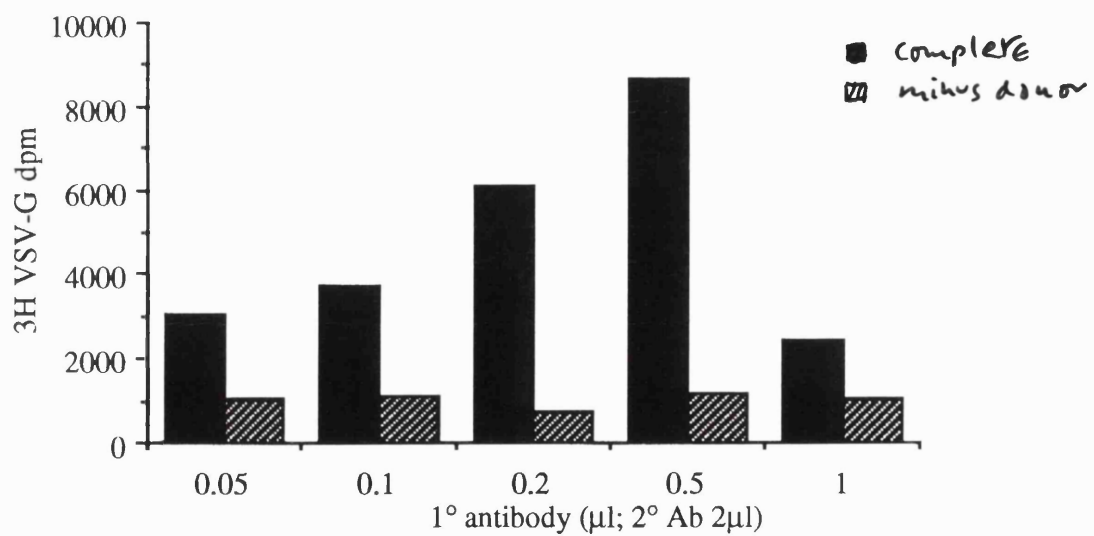
Assays containing donor and acceptor membranes (black bars) were compared with control assays lacking donor membranes (hatched bars)

Figure 3.3: optimisation of immunoprecipitation conditions

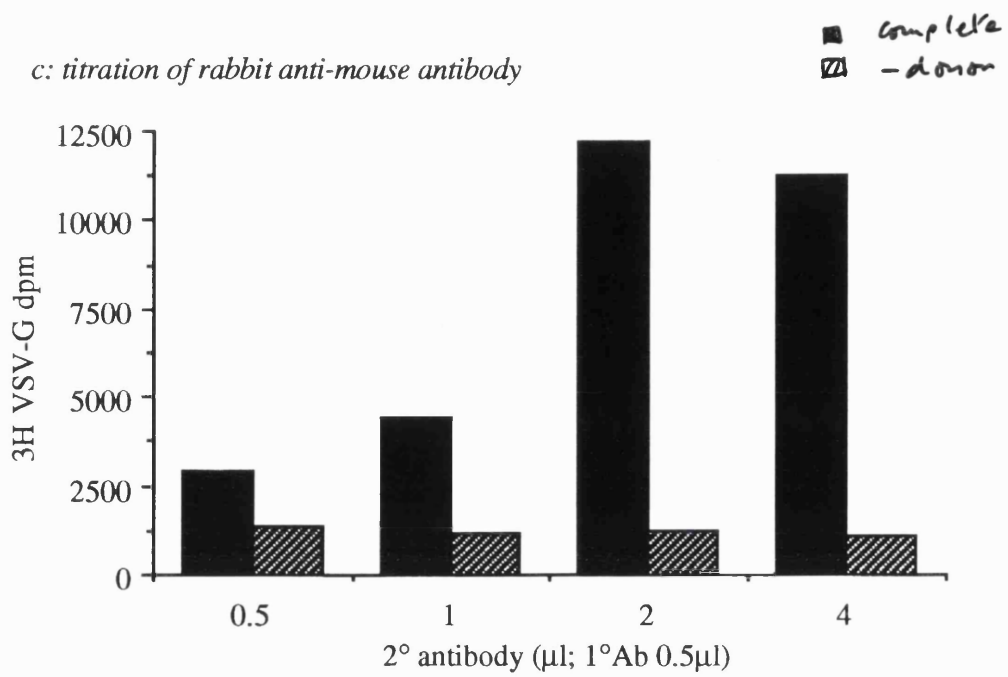
a: effect of omission of antibodies



b: titration of anti-VSV-G (primary antibody)



c: titration of rabbit anti-mouse antibody



d: capacity of transport assay immunoprecipitation

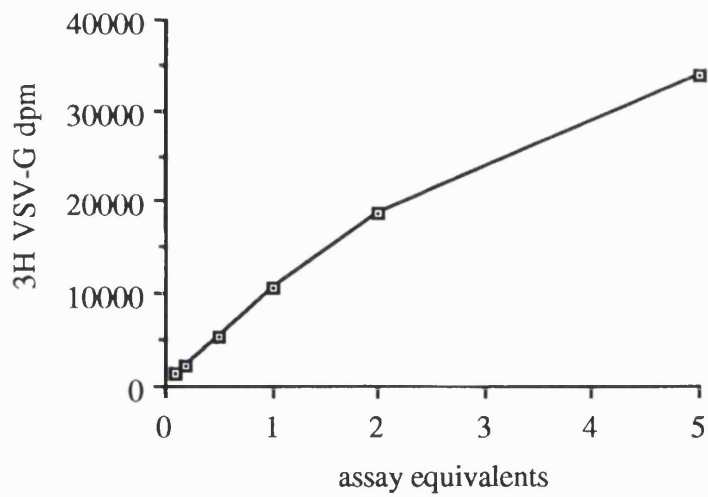


Fig 3.4: the time course of the intra-Golgi transport assay

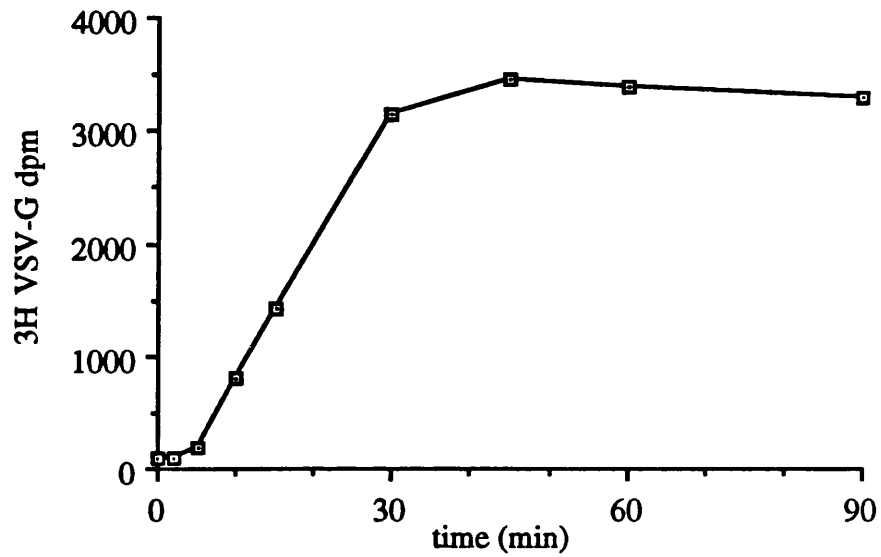


Figure 3.4: time course of intra-Golgi transport. In this experiment, identical reactions were prepared containing 5 μ l of cytosol, donor and acceptor membranes. The reactions were incubated at 37°C for various times and then removed to ice.

3.3 Titration of membranes in the assay.

To determine the optimal levels of each component, new batches were titrated alongside old ones with previously-tested components.

The membranes were the most delicate component of the whole transport assay. The occasional acceptor preparation was entirely inactive in transport, though entirely normal by such criteria as galactosyltransferase and protein assays. It was eventually realised that these preparations were derived from CHO cells which had been passaged more than about 25-30 times. Moreover, the transport activity of preparations did vary substantially: table 3.1 lists some examples of membrane batches and their transport activity. The variation could arise from a number of sources, including (i) passage age of cells, (ii) homogenisation behaviour (which was monitored separately for each preparation) and particularly in salt-washing (see section 3.5) and (iii) the assay components and immunoprecipitation (though these were kept as close to standard as possible).

For these reasons, it was almost never possible to compare transport assays directly in terms of counts obtained. Often, transport activity is presented as a percentage of interphase (or other appropriate standard) for that assay, where the interphase activity of the assay was comparable with the normal behaviour of the batches being used. Assays were generally performed several times, often with different membranes and cytosols to avoid anomalous results due to particular batches of components.

All forms of assay except large timecourses comprised duplicate samples, in order to avoid the small variations between incubations. These differences appeared to be due mainly to membrane variation. This impression was gained in experiments where duplicate histone kinase assays were performed on samples withdrawn from duplicate transport assays. In such cases, the histone kinase assays gave better duplicates than the transport assays, suggesting that the cytosol and other reaction components were less variable than the added membranes. Fig 3.5 shows some examples of inter-assay variation. The figure shows tests performed on some batches of acceptor membranes using the same donor membranes (28/9/92) and a single cytosol (29/9/92). While there was good correlation between the acceptors 10.8.92, 6.10.92 and 25.10.92, that of 19.10.92 was a 'dead' preparation made from high-passage CHO cells. The figure also shows how different preparations gave optimal transport signals at different concentrations, despite the fact that their protein concentrations were quite similar. Generally, the only guide to the activity of a new batch of membranes was not protein concentration or galactosyltransferase activity, but observed performance in transport.

Table 3.1

Some purification tables of membranes used in this study

Purification Table: donor membranes

Date: 10.8.92

Sample	Volume (ml)	[protein] (mg/ml)	total protein (mg)	GalT activity (nmol/hr/ml)	total GalT activity (nmol/h)	GalT sp.act (nmol/mg/hour)	Yield (%)	purification (fold over homogenate)	interphase transport (dpm)	mitotic transport (dpm)
Homogenate	12	7.6	91.2	117.5	1410	15.5	100%	1x	-	-
Golgi	2.7	0.45	1.22	291.9	788	685	56	44	6622	2909
K-Golgi*	2	0.2	0.4	82.2	164	411	12	27	3555	1038

Purification Table: acceptor membranes

Date: 10.8.92

Sample	Volume (ml)	[protein] (mg/ml)	total protein (mg)	GalT activity (nmol/h/ml)	total GalT activity (nmol/h)	GalT sp.act (nmol/mg/hour)	Yield (%)	purification (fold over homogenate)	interphase transport (dpm)	mitotic transport (dpm)
Homogenate	13	6.6	85.8	145	1885	21.9	100%	1x	-	-
Golgi	2.65	0.5	1.33	249	695	497	35	23	5455	5253
K-Golgi*	2	0.2	0.4	93.6	187	468	10	21	2702	879

* Golgi membranes washed in 1M KCl

Purification Table: donor membranes

Date: 17.7.92

Sample	Volume (ml)	[protein] (mg/ml)	total protein (mg)	GalT activity (nmol/hr/ml)	total GalT activity (nmol/h)	GalT sp.act. (nmol/mg/hour)	Yield (%)	purification (fold over homogenate)	interphase transport (dpm)	mitotic transport (dpm)
Homogenate	2	5.6	67.2	94.64	11135	16.9	100%	1x	-	-
Golgi	3.2	0.325	1.04	155.4	497.4	478.3	44	28	11776	10123
K-Golgi*	2	0.1	0.2	68.6	137.1	685.7	12	41	4339	1322

Purification Table: acceptor membranes

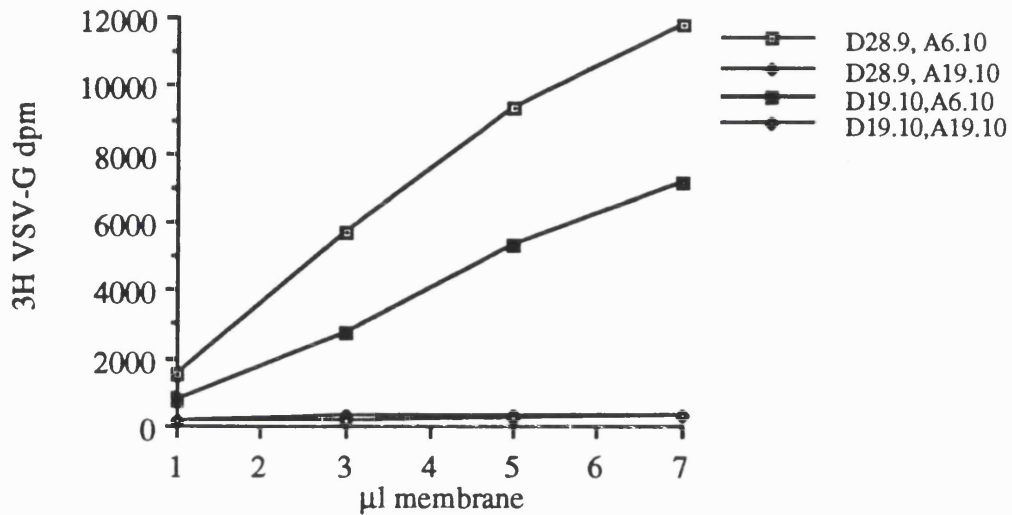
Date: 22.6.92

Sample	Volume (ml)	[protein] (mg/ml)	total protein (mg)	GalT activity (nmol/hr/ml)	total GalT activity (nmol/h)	GalT sp.act. (nmol/mg/hour)	Yield (%)	purification (fold over homogenate)	interphase transport (dpm)	mitotic transport (dpm)
Homogenate	3	12.9	167.7	49.45	643	3.9	100%	1x	-	-
Golgi	3	1.6	4.8	89.28	268	55.8	42	14.6	11338	6633
K-Golgi*	2	0.625	1.25	33.94	67.9	54.3	11	14.2	4169	1279

* Golgi membranes washed in 1M KCl

Figure 3.5: Golgi membrane titrations

a: comparisons of D28.9 with D19.10, and A6.10 with A19.10



b: comparison of A6.10 with A25.10 using D28.9

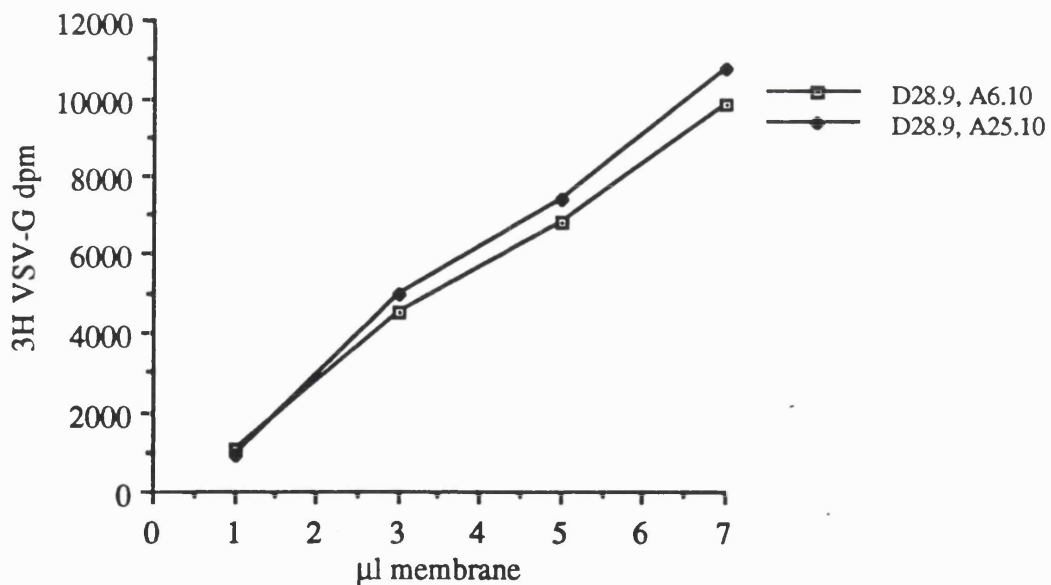


Figure 3.5: membrane titrations. In these titrations, standard assays were prepared containing 5µl cytosol (I 7.8, at 10.2mg/ml) and 5µl of the invariant membrane, but the indicated volume of the variable membranes, and then assayed for 60 minutes at 37°C.

3.4 Interphase and mitotic cytosol.

As described in Chapter 2, HeLa cells were used as the source of the cytosol used in almost all these experiments. Suspension HeLa (sHeLa) cells were made mitotic by being pelleted and thoroughly resuspended in nocodazole-containing medium, and then incubated in nocodazole for 24 hours. This caused them to accumulate in prometaphase because of the nocodazole inhibition of mitotic spindle formation. In almost all cases, a single flask of sHeLa cells was divided to generate interphase and mitotic cell populations and parallel (frequently referred to as 'cognate') interphase and mitotic cytosol preparations (though the 24h nocodazole block meant that about twice as much interphase as mitotic cytosol was isolated).

The mitotic index of the mitotic population was noted for every preparation, and was a good guide to the success of the batch in mitotic transport inhibition. A mitotic index of at least 94% was prerequisite for active mitotic cytosol. The other guide to cytosol activity was a histone kinase assay very similar to that used by researchers studying regulation of mitosis in *Xenopus* embryos.

3.4.1 histone kinase assay of cytosol

The histone kinase assay was performed by incubating cytosol samples in the presence of the histone substrate, EGTA, to inhibit PKA and Ca⁺⁺-dependent PK (Moreno et al, 1989), and phosphatase inhibitors, and $\gamma^{32}\text{P}$ -ATP. To determine the capacity of the assay, tests were performed on a single mitotic cytosol which was serially diluted and then assayed in the presence of varying quantities of histone substrate (Figure 3.6).

The assay was linear with respect to the cytosol up to 0.5 μg for histones at 2mg/ml, their normal concentration in the assay. Since HeLa cytosols were normally approx. 10mg/ml, this showed that about 0.5 μl of undiluted cytosol or 5 μl of transport assay containing cytosol could be tested in the linear range of the histone kinase assay. In fact approx. 2 μg of cytosol withdrawn from a transport assay was normally used for histone kinase assay, which was economical on samples withdrawn from transport assays. The figure also shows that histones were present in reasonable excess at 2mg/ml.

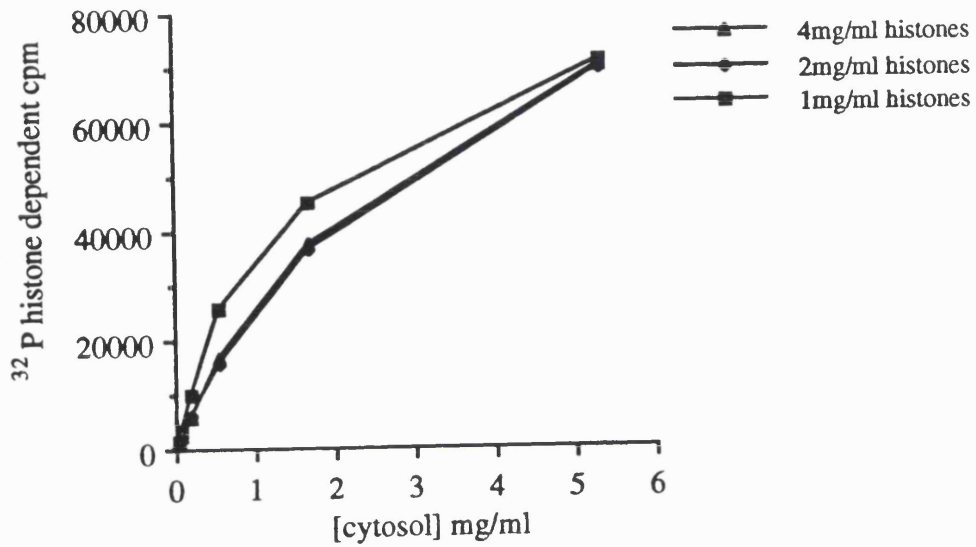
3.4.2 Durability of mitotic kinase activity under transport conditions

Fig 3.7 shows the activities of mitotic and interphase cytosol with incubation time under mock-transport assay conditions. For this experiment, scaled-up mitotic and interphase assays were prepared and incubated, and samples withdrawn and snap-frozen at the times indicated; then all the samples were assayed together at the end of the incubation. This pattern of histone kinase activity was also seen during normal transport assays.

Figure 3.6: capacity of the histone kinase assay. In this experiment, histone kinase assay mixtures were prepared as described in Chapter 2, except that histones were added to the concentrations shown. Then mitotic cytosol was added at the levels shown in 5µl volume. The assays were incubated for 15 minutes at 37°C.

Fig 3.6: capacity of the histone kinase assay

a. ^{32}P incorporation as a function of cytosol and histone concentration



b: variation in calculated specific activity

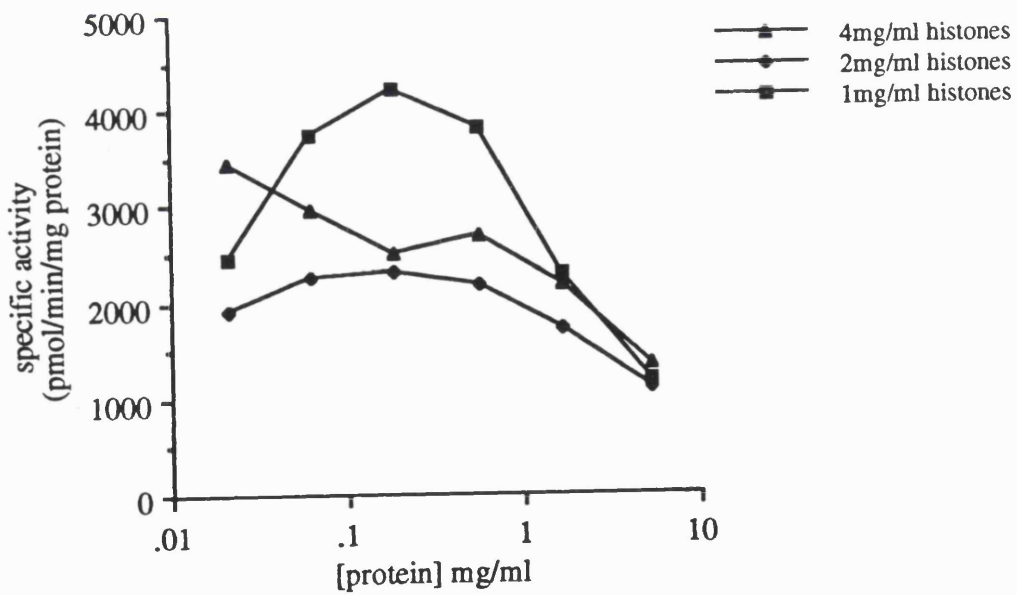


Fig 3.7: timecourse of histone kinase activity under transport conditions

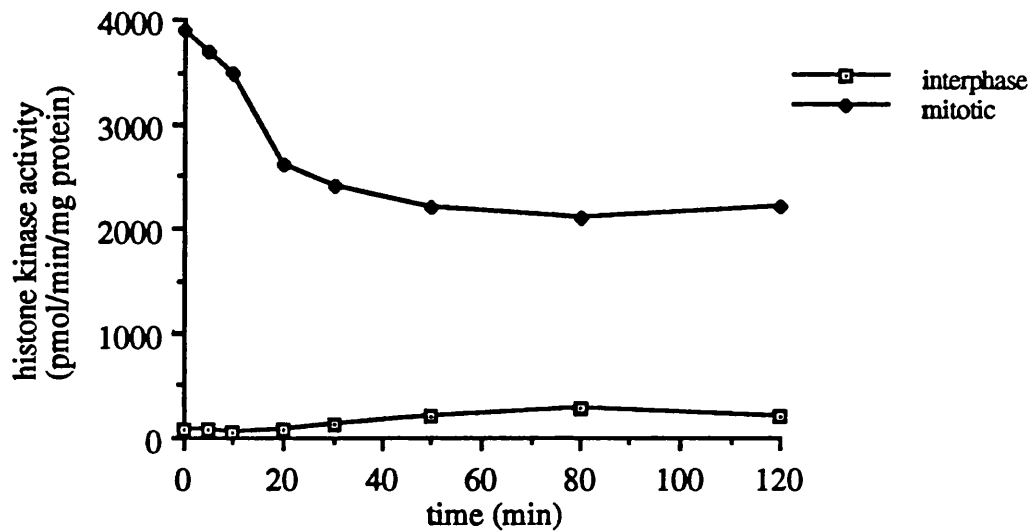


Figure 3.7: durability of histone kinase activity under transport conditions. Mock transport assays were prepared containing transport buffer, 5 μ l cytosol but no membranes (volume corrected with 1M ST). The mock assays were incubated at 37°C and at various times, samples were withdrawn and snap-frozen until all assayed together at the end of the time-course.

The histone kinase activity of the mitotic cytosol was highest at the beginning of the assay (approx 20-fold higher than interphase), declined over the first twenty minutes, and then remained stable at approximately 10-fold higher than interphase. Since mitotic cytosol had stable activity after 20 minutes at 37°C (as did cytosol incubated with cyclin A or cdk2; as seen in chapter 5) whenever histone kinase assays were performed in parallel with transport assays, the histone kinase samples were taken 20 minutes into the incubation or shortly thereafter (this does not apply to timecourses or mouse cytosols).

There was significant variation in the histone kinase (and transport) activities of different mitotic cytosol preparations. Table 3.2 lists the activities of some of the cytosols used in this study, along with some details of the preparations.

3.4.3 Interphase and mitotic cytosol and the transport assay

When a standard transport assay was performed with mitotic cytosol, transport was inhibited by 40-75% depending on the batches of membranes and cytosol used. Transport was compared between duplicate assays performed at the same time on single aliquots of membranes, and with cognate interphase and mitotic cytosols. All this reduced the chances that apparent inhibition was actually due to artefacts in the preparation of the cytosols or in the assays. Examples of good and poor transport inhibition are shown in Figure 3.8.

This variable inhibition was perhaps due to the preparation of the membranes. The float-up centrifugation used might cause the retention of CHO cytosol contaminants on the membranes. If some of these were transport components, then added mitotic cytosol would probably have little effect on the transport assay.

If this were true, the removal of any cytosolic proteins associated with the membranes could render them dependent on added cytosol, and thus increase the effect of the mitotic cytosol. To this end, a salt-wash was introduced into the membrane preparations.

3.5 Salt-washing Golgi membranes

3.5.1 Salt-washed membranes and the transport assay

When donor or acceptor membranes were removed from their first centrifugation step, they were incubated for 10 minutes on ice with 1M KCl, and then purified from the KCl back into sucrose by another centrifugation step. This generated 'K-Golgi' which, according to research from the laboratory of Prof. Rothman, is fully depleted of NSF and almost entirely depleted of coatamer and SNAPs, and therefore is dependent on the addition of these components (in cytosol) for transport activity. Salt-washing caused a drop in protein concentration and GT activity, ie very little change in overall purification of Golgi membranes (see Table 3.1). The transport activity of the membranes also fell.

Table 3.2

These tables list examples of the cytosols used in this study, giving characteristics of the preparations and their behaviour in the transport system.

cytosol	protein conc (mg/ml)	mitotic index (%)	histone kinase p/m/m	typical transport 3H dpm
I 20.12.91	12.6		381	5727
M20.12.91	12.7	95	3790	780
I26.2.92	12.9		166	9633
M26.2.92	9.1	94	2874	3570
I20.3.92	8		145	5565
M20.3	8.4	95	3600	1491
I10.4.92	10.7		109	2124
M10.4.92	11.5	96.5	2375	672
I7.5.92	13		190	2702
M7.5.92	17.4	95	8767	946
I5.6.92	6		189	2722
M5.6.92	8	97	3877	697
I15.7.92	4.7		213	4532
M15.7.92	5.7	95	4285	1527
I7.8.	10.2		104	3555
M7.8	11.2	94	2958	1038

H1 kinase activities are shown for cytosols before desalting in pmol/min/mg protein. Transport activities are examples from assays, to show typical mitotic inhibition of transport for salt-washed Golgi incubations.

Fig 3.8: mitotic cytosol and normal transport

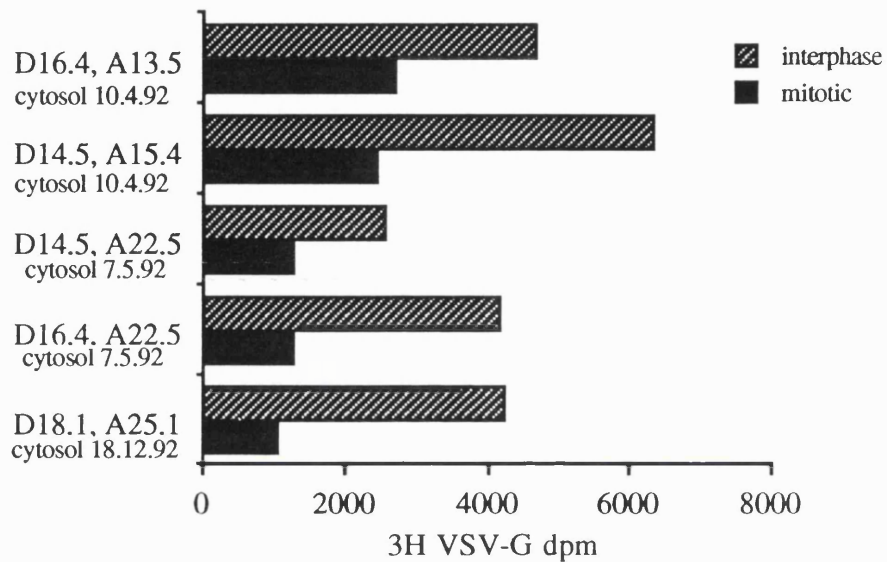


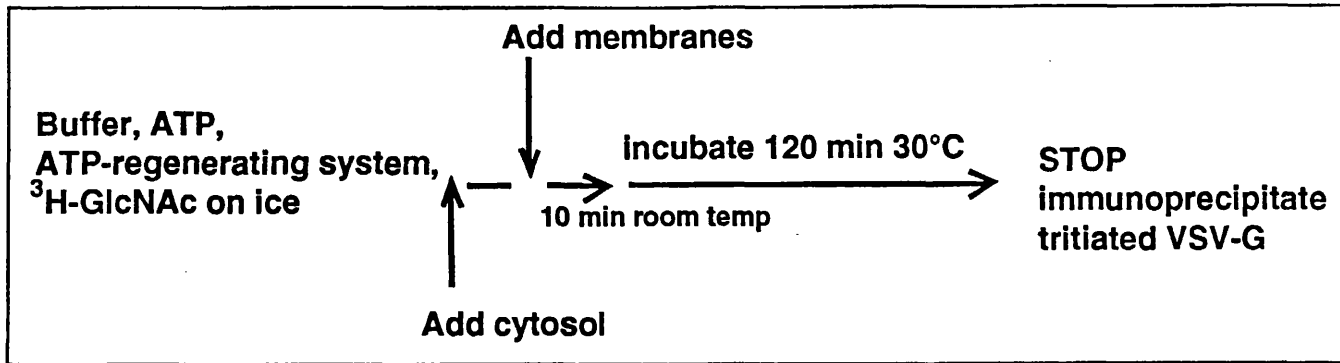
Figure 3.8: mitotic cytosol and transport by unwashed membranes. This figure represents typical examples of the behaviour of various cytosols and membrane preparations, chosen to show the variation in results due to preparations. All assays contained similar quantities of cytosol and membranes, and were incubated under standard conditions.

Figure 3.9: salt-washed Golgi and the transport assay. In these experiments, assays were prepared containing equal volumes (typically 5 μ l) of cytosol, but the volumes stated of either salt-washed or unwashed Golgi. Unwashed Golgi were assayed for 60 minutes at 37°C; salt-washed assays for 120 minutes at 30°C, after a 10 minute preincubation at room temperature.

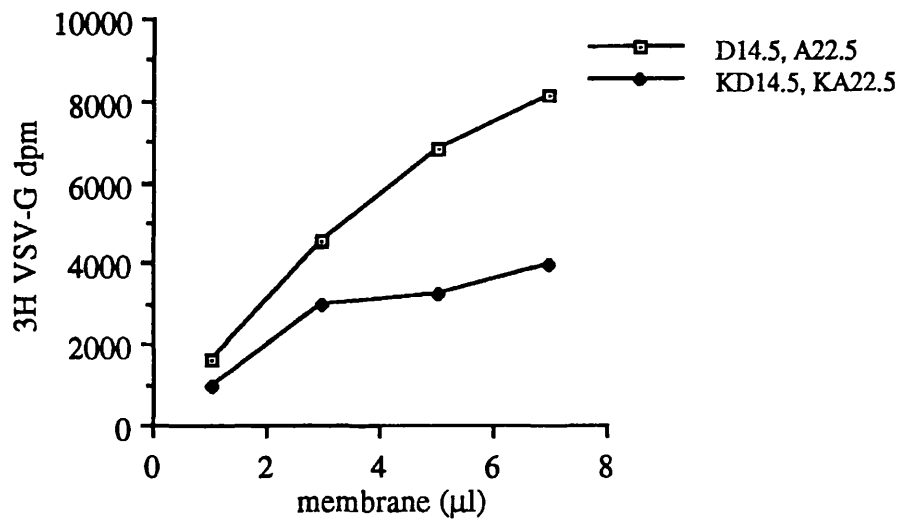
KD + KA represent salt-washed donor and acceptor membranes, respectively; batches made on dates indicated.

Figure 3.9: salt-washed Golgi and the transport assay

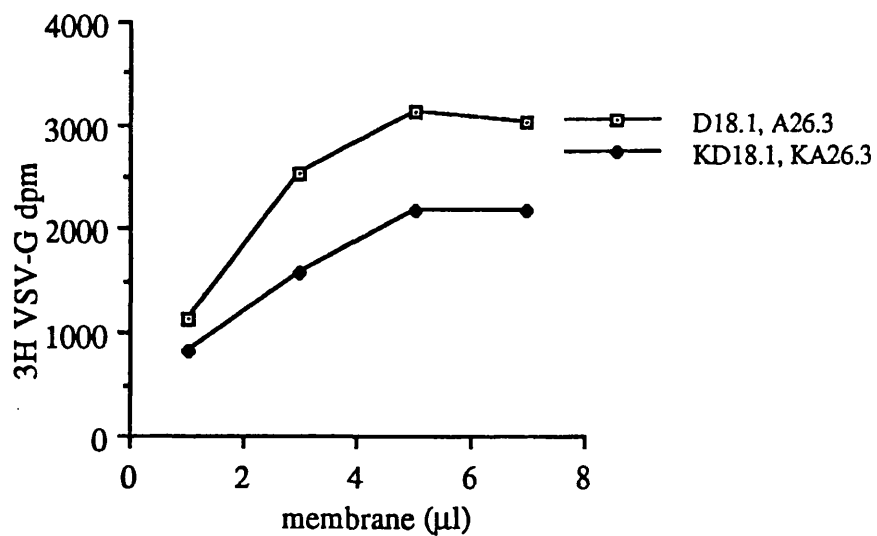
a: outline



b: salt-washing of D14.5.92 and A22.5.92



c: salt-washing of D18.1.93 and A26.3.93



The method of the assay using salt-washed Golgi is outlined in Figure 3.9a, and two examples are shown in Fig 3.9b, c. Transport by K-Golgi ranged from 25% to 75% of that of the parent non-salt-washed membranes despite great efforts to keep the preparations invariable, and the reason for the variation is not understood.

3.5.2 Timecourse of transport by K-Golgi

It has been suggested that K-Golgi transport at 37°C suffers a high non-specific labelling background (S.Whiteheart, personal communication), and also that salt-washed membranes are relatively fragile. For this reason, when K-Golgi was added to transport assays, they were removed from ice and allowed to stand at room temperature for 10 minutes, and then incubated at 30°C. The time course of transport (Fig 3.10) was investigated at this temperature by the same means as for non-K Golgi (Fig. 3.4). It was found that transport had not reached a plateau in 60 minutes, and therefore the assay incubation was extended to 120 minutes. Transport with mitotic cytosol followed a timecourse of similar proportions to the interphase assay, so it seemed reasonable to sample both after 120 minutes.

3.5.3 Variation in salt-washed membranes

The activity of salt-washed membranes in the transport assay was as variable as that of their unwashed progenitors, because of slight variations between preparations. The examples shown in Fig 3.11. illustrate this; the preparations shown had different maximum activities and different titration curves from one another, and the same membranes assayed at different times gave different levels of transport. The level of transport from K-Golgi might have been correlated with its inhibition by mitotic cytosol if, for example, any batch of membranes which was ineffectually salt-washed had high transport activity but could not be inhibited by mitotic cytosol. In fact no strong correlation was observed, but salt-washed membranes with high transport activity were often inhibited in mitotic assays, whereas unwashed membranes were often inhibited by less than 40%.

3.5.4 Mitotic inhibition of K-Golgi transport

Mitotic cytosol inhibited transport with salt-washed Golgi membranes by 60-85%, depending on both the membranes and the cytosol. Mitotic and interphase cytosol were titrated against salt-washed membranes to obtain the maximum signal-to-noise ratio for each batch. Fig 3.12 shows two paired batches of cytosol with somewhat different characteristics.

The cytosols in this figure did not plateau in efficiency, but many did at approximately 50-60µg protein/assay; the cytosol performance was influenced by that of the membranes. The level of cytosol used was fixed by the optimum interphase: mitotic ratio of transport, optimum transport performance, and optimum response to cyclin treatment (see Chapter 4).

Figure 3.10: time course of transport by salt-washed Golgi

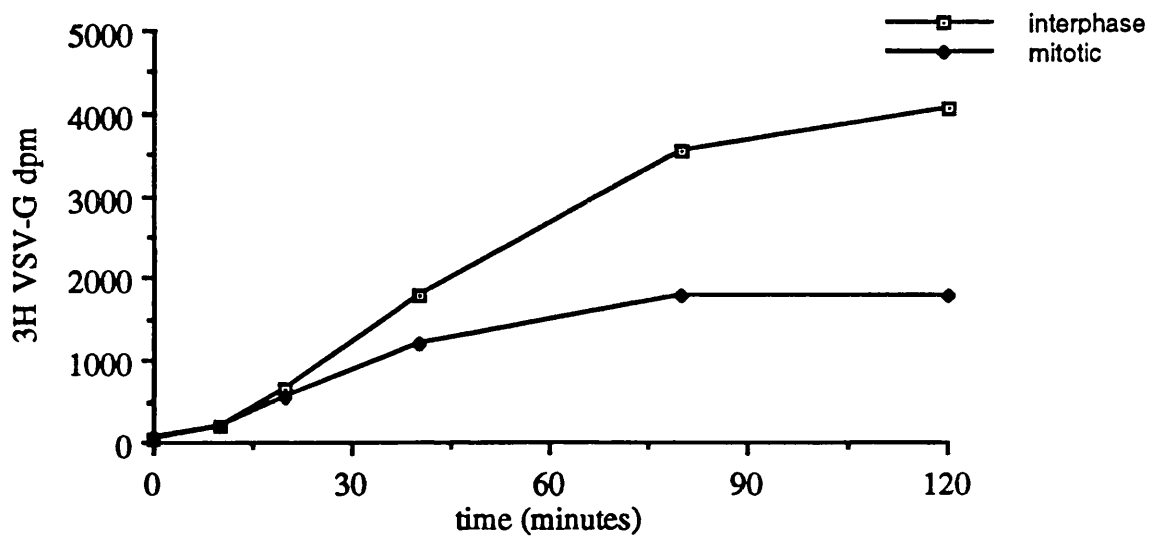
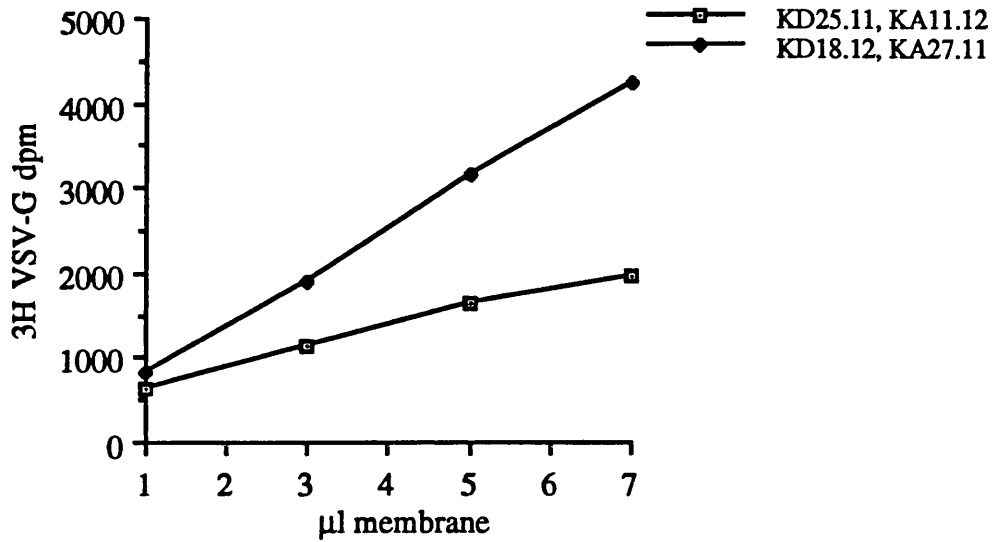


Figure 3.10: time course of transport by salt-washed Golgi. In this experiment, identical assays were prepared containing 5 μ l of cytosol, K-donor and K-acceptor. The zero time-point was never removed from ice. The remainder were incubated at room temperature for 10 minutes, and then at 30°C for the times indicated, before being returned to ice.

Fig 3.11: examples of salt-washed Golgi titrations

a: KD25.11 and 18.12 vs KA11.12 and 27.11



b: KD19.10 vs KA25.10 and 16.11

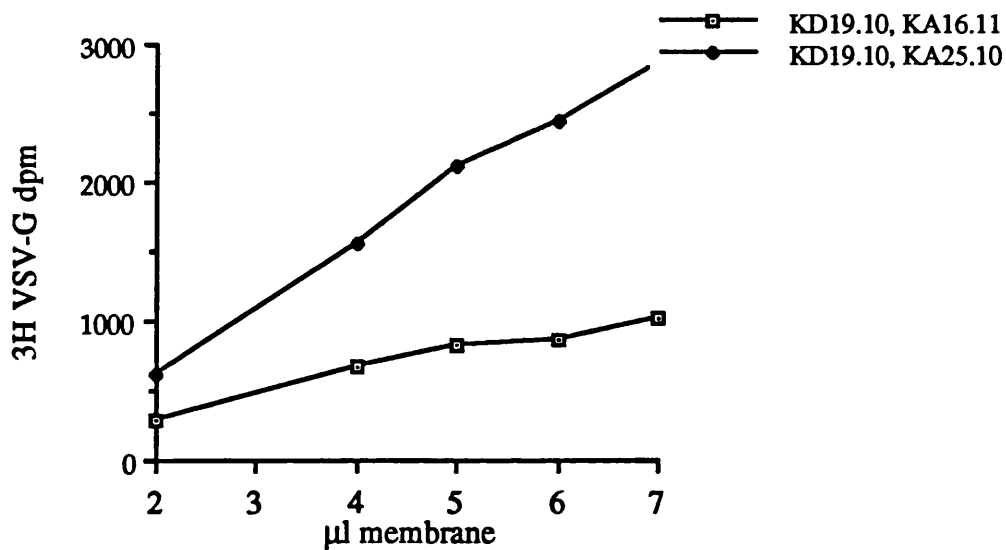
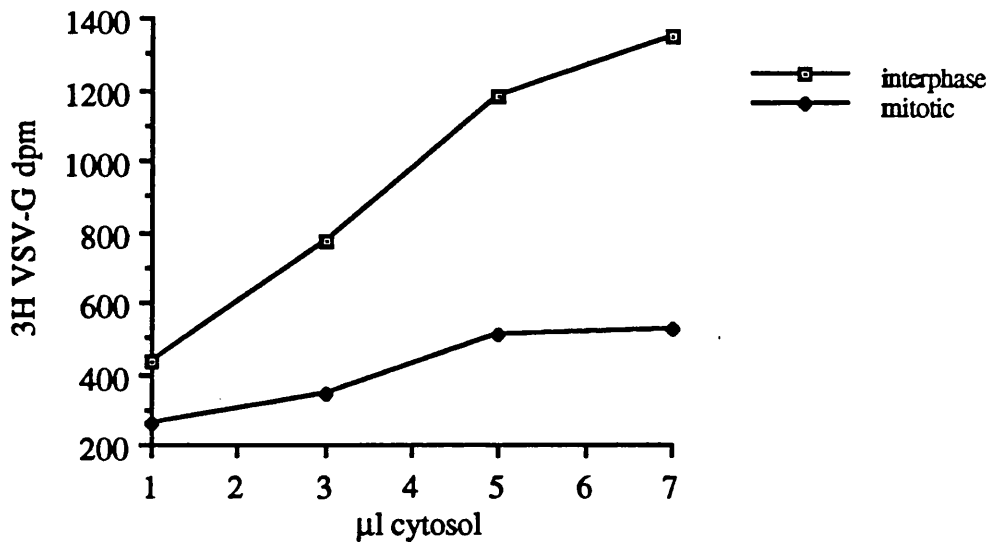


Figure 3.11: titrations of salt-washed Golgi. In these experiments, standard transport assays were prepared containing 5µl of cytosol and 5µl of the invariant membrane, but the quantity shown of the variable membrane and 1.0 ST to make up volume. Transport was assayed for 120 minutes at 30°C.

Figure 3.12: comparison of two paired cytosols with the same membranes (KD25.11, KA 27.11)

a: cytosols 9.12



b: cytosols 18.12.

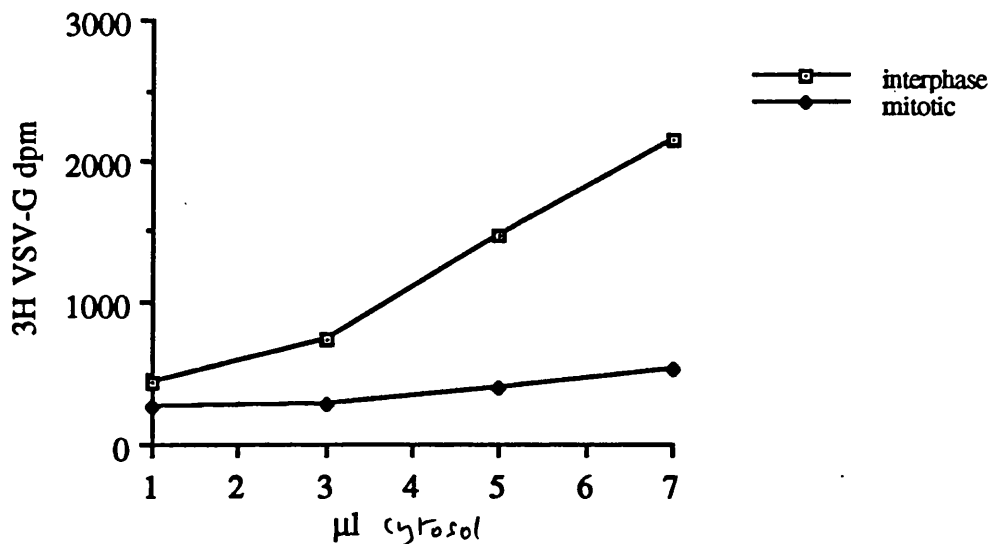


Figure 3.12: comparison of transport by two paired cytosols using the same salt-washed membranes. In these two experiments, transport assays were prepared containing 5µl each of salt-washed membranes but the specified volumes of the various cytosols. Transport was assayed for 120 minutes at 30°C.

3.6 Summary

The results presented in this chapter show the basic requirements of interphase transport, and then show how transport was reduced in the presence of mitotic cytosol. The inhibition of transport was more consistent and marked when the membranes were salt-washed, and salt-washed membranes were used in almost every experiment described in the following chapters.

It has been suggested that these membranes are incompetent for vesicle-mediated transport due to the depletion of coat proteins. It has been shown that Golgi membrane fusion can occur in the absence of cytosol, requiring only the components of the fusion complex; but it is suggested that this fusion occurs without completion of vesicle budding, ie it is promiscuous. There was a possibility that the salt-washed Golgi would undergo promiscuous fusion in this assay even in the presence of complete cytosol. The transport assay does not admit a direct approach to this question, since it measures nothing more than glycosylation of VSV-G which comes into contact with GNTI, regardless of the mechanism of its arrival. Some drugs influence different events of vesicular transport, and it appeared that mitotic cytosol did so also, and in that sense, experiments could be designed to touch on the question of vesicle-mediated transport by salt-washed Golgi. Such experiments are described in Chapter 6. Even if it were shown that K-Golgi was incapable of vesicular transport, the effect of mitotic cytosol would still be important, since it would unambiguously identify membrane fusion as the target of inhibition.

The fact that K-Golgi transport was inhibited by mitotic cytosol suggested that the inhibition acted on either the cytosol or an active complex of cytosolic and membrane proteins. It was suggested above that unwashed Golgi was only erratically inhibited by mitotic cytosol because it bore cytosol-derived transport proteins which enabled it to evade the transport block. The more potent inhibition of salt-washed Golgi would be due to its absolute requirement for transport proteins from the transport-incompetent mitotic cytosol. It remained a possibility, however, that transport was reduced by an irreversible change or decay of the cytosol under the conditions of mitotic block. Therefore, it was necessary to identify conditions under which the transport block was reversed in mitotic cytosol, or mimicked by interphase cytosol, and experiments to this end are described in Chapter 4.

Chapter 4

Control of the intra-Golgi transport assay by phosphorylation.

4.1 Aims

Chapter 3 described the basic working of the transport assay using interphase HeLa cytosol, and then showed how transport was reduced in the presence of mitotic cytosol. The difference between interphase and mitotic transport was more marked with salt-washed than with unwashed membranes, and this suggested that the K-Golgi, which was stripped of associated cytosolic protein, became acutely responsive to some component of the mitotic cytosol. However, the apparent inhibition of transport by mitotic cytosol might have been nothing more than an artefact of mitotic cytosol preparation which caused the irreversible degeneration of its transport capacity. To eliminate this possibility it was necessary to find a way to reverse the effect of mitotic cytosol, and also if possible to mimic its action. If mitotic cytosol could not be reverted, even if its activity was bona fide, it would be practically useless for studying its mechanism of action.

This chapter first describes how mitotic cytosol was reverted to interphase activity by kinase inhibitors. This established the reversibility of the mitotic effect, and also showed how the histone kinase activity of the cytosol was related to its ability to inhibit transport. Next, bacterially-expressed cyclin A is described as an agent capable of mimicking the action of mitotic cytosol. Its inhibition of transport was linked to its histone kinase activity, and established a strong link between a cell cycle kinase and transport inhibition. As shown in Chapter 3, transport inhibition was most marked when the assay was performed using salt-washed Golgi. For this reason, all of the data shown here will be taken from experiments using K-Golgi. Almost all of them were performed at some stage with unwashed membranes, and the effects, though far less profound, were still seen.

4.2 Kinase inhibitors

4.2.1. staurosporine

As described in the Introduction, protein phosphorylation increases during mitosis, and falls again at the return to interphase. Since a significant characteristic of the mitotic HeLa cytosols was high histone kinase activity, it was possible that mitotic cytosol exerted its effect on transport via phosphorylation. Therefore, it was possible that kinase inhibitors could reduce the histone kinase activity of mitotic cytosol and relieve its inhibition of the transport assay. If mitotic cytosol could be 'reverted', then it would be clear that it was capable of supporting transport, and not simply irreversibly inactivated during its preparation. To test this, experiments were performed using the general kinase inhibitor staurosporine, which is known to inhibit cell division kinases (Gadbois et al, 1992).

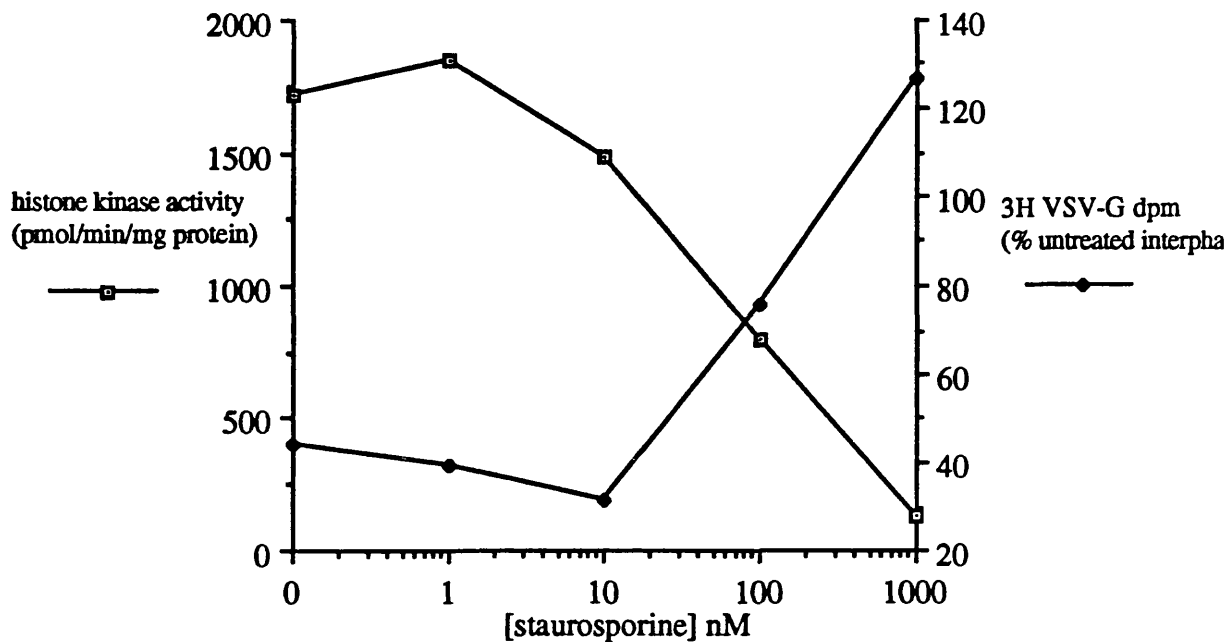
The general kinase inhibitor staurosporine was diluted from a 1mM stock in DMSO into water and added to the transport assay mixture at concentrations between 1nM and 1 μ M. Mitotic or interphase cytosol and salt-washed membranes were then added and incubated in a standard assay. 2 μ l aliquots of these assays were also tested for histone kinase activity.

As seen in Figure 4.1, staurosporine caused a titratable fall in histone kinase activity of mitotic cytosol, which reached interphase levels at a drug concentration of 1 μ M. Transport activity recovered to interphase levels in a titratable manner equivalent to the fall in kinase activity; in fact, it was reproducibly observed that staurosporine-treated mitotic cytosol supported 120-140% of the transport of its staurosporine-treated interphase cognate. This result showed that the low transport activity of mitotic cytosol was not due to an irreversible inactivation of some transport protein, but was fully reversible. Moreover, the effect was titratable, in that increasing inhibition of kinases by staurosporine caused increasing reversion of transport, and proportionate reduction in histone kinase activity. By contrast, staurosporine had no effect upon the interphase assay, though it slightly and reproducibly lowered its already-small histone kinase activity in line with the effect on mitotic cytosol.

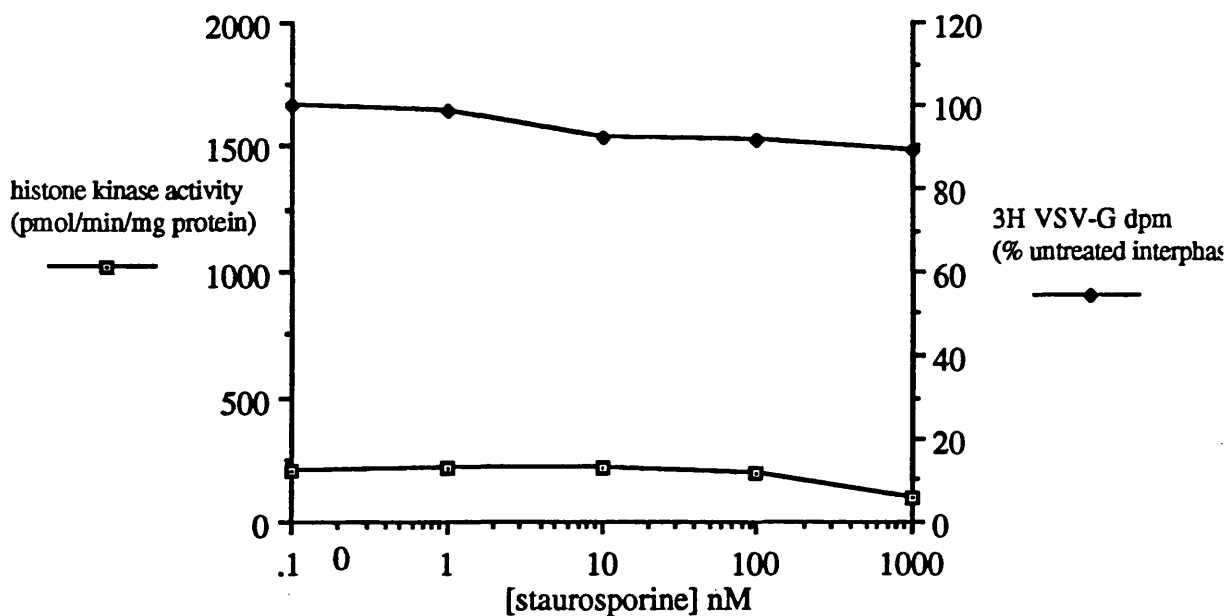
The effect of staurosporine on histone kinase activity was instantaneous by experimental measurement. Staurosporine was added to mitotic cytosol to a final concentration of 1 μ M, on ice, and the histone kinase activity of the cytosol was measured immediately, and found to be at interphase levels (data not shown).

Figure 4.1: effect of staurosporine on mitotic cytosol. In this experiment, transport assays were prepared containing staurosporine at the concentrations shown, diluted from a 1mM stock in DMSO. Equal quantities of interphase and mitotic cytosol were added to each, and then salt-washed membranes. Transport was assayed, after a 10 minute preincubation, for 120 minutes at 30°C. Histone kinase assays were performed on 2 μ l samples withdrawn from the transport incubations.

Figure 4.1: effect of staurosporine on the transport assay
a: mitotic cytosol



b: interphase cytosol



4.2.2 optimal time of addition of staurosporine to the assay

The effect of staurosporine on mitotic cytosol prompted an experiment to find out when the drug was most effective in the course of a transport assay. A series of mitotic transport incubations was set up with salt-washed membranes (and supplemented with 0.5 μ g cyclin extract for maximal histone kinase activity; see below). At various points in the 120 minute assay, staurosporine was added to a final concentration of 1 μ M. The result of this assay is shown in Figure 4.2. The effect of the drug declined to a minimum during the assay, with a half-time of approximately 60 minutes. This result shows that, whatever transport event the drug inhibited, it was still occurring up to 80 minutes into the assay, since kinase activity could be blocked at that time and still relieve transport inhibition to some extent.

4.2.3 effect of dimethylaminopurine on the assay

The effect of a second kinase inhibitor, dimethylaminopurine (DMAP) was also tested, to check that the result obtained with staurosporine was due to kinase inhibition rather than a nonspecific effect on transport. DMAP is an ATP analogue and competitive general kinase inhibitor, and because of the use of 1mM ATP in the assay, it was difficult to add sufficient DMAP to compete out the ATP effectively (The assay was performed in 1mM ATP to support the mitotic activity of cytosol; see Chapter 3). The inhibitor was added to transport buffer from a 50mM aqueous stock to 5mM final, just before the addition of mitotic or interphase cytosol. Then membranes were added and transport was assayed for 120 minutes at 30°C. Histone kinase assay of the cytosol was also performed. Fig 4.3 shows the partial reversion of transport obtained with 5mM DMAP.

These experiments show that both staurosporine and DMAP were capable of restoring transport activity to mitotic cytosol concurrent with reduction in its histone kinase activity. This indicated that the inhibition of transport by mitotic cytosol was connected with its kinase activity. Thus, it appeared that mitotic cytosol was not irreversibly faulty, but reversibly inhibited with regard to transport.

Figure 4.2: time course of staurosporine effect

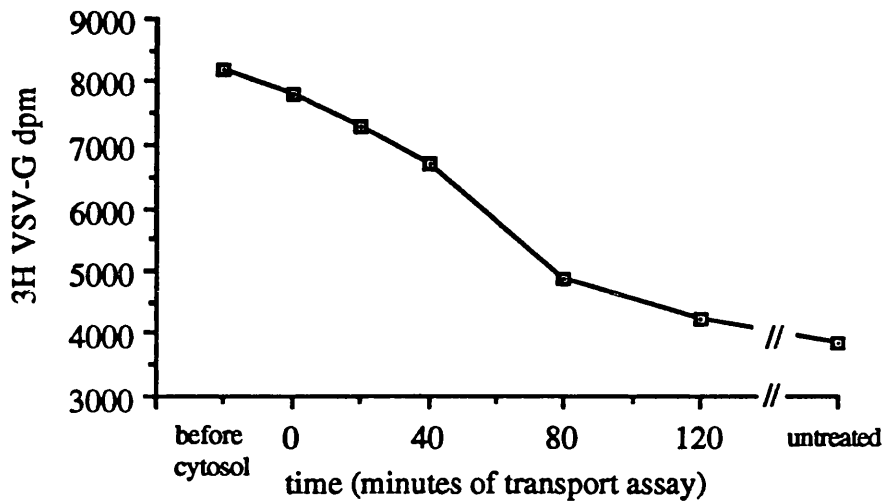
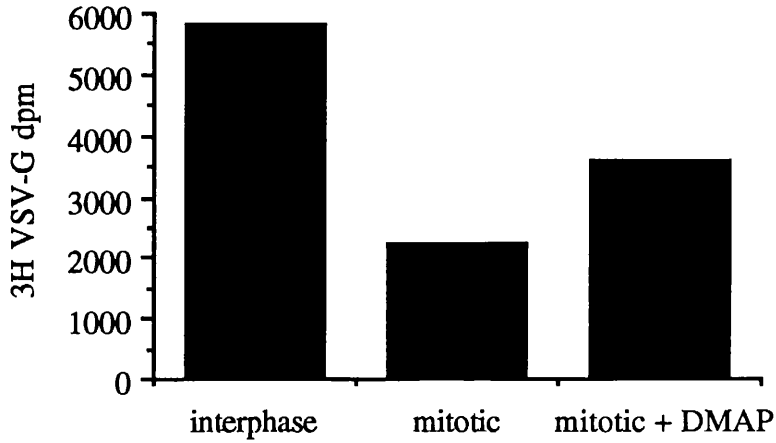


Figure 4.2: time course of the effect of staurosporine. Identical transport reactions were prepared, containing mitotic cytosol and salt-washed Golgi membranes. At various times, either before the cytosol or after the start of the full transport assay, staurosporine was added to a final concentration of $1\mu\text{M}$. Transport was assayed for 120 minutes at 30°C .

Fig 4.3: effect of DMAP on mitotic cytosol

a: transport activity



b: histone kinase activity

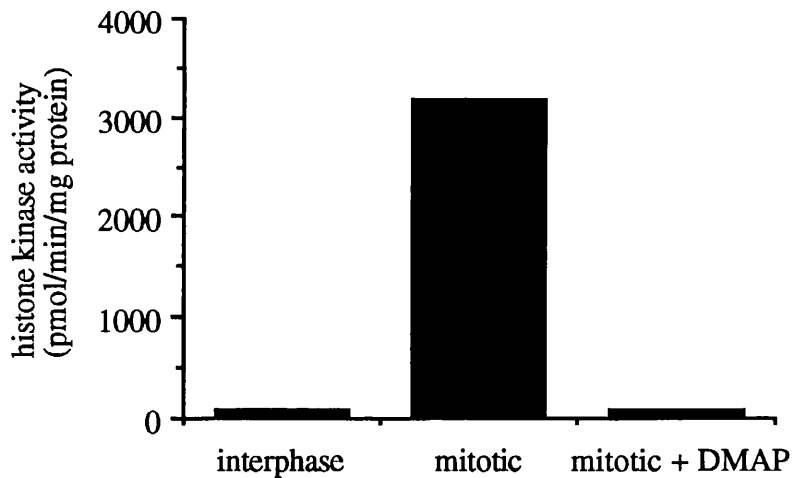


Figure 4.3: effect of dimethylaminopurine on mitotic transport. Transport assays were prepared containing interphase and mitotic cytosol, and DMAP was added to some at a final concentration of 5mM, before the addition of cytosol and membranes. Transport was assayed for 120 minutes at 30°C.

4.3 Cyclin A

Since it was shown that the mitotic cytosol was not irreversibly inactivated, but reversibly modified by phosphorylation, it seemed very likely that the kinase controlling this phosphorylation was the key mitotic kinase p34^{cdc2}. In order to test this hypothesis, interphase cytosol was treated with cyclin A, to see whether this protein would interact with mitotic kinase present in interphase cytosol and activate it. As stated in the Introduction, it has been shown that cyclin A associated with p34^{cdc2} does not undergo the complex regulatory phosphorylation associated with cyclin B/p34^{cdc2}; thus it seemed reasonable to expect that kinase activation would occur.

Human cyclin A protein was expressed in *E. coli* as a fusion protein with maltose binding protein (ie MBP-cyclin A, referred to henceforward as cyclin A) and was purified by amylose affinity-purification according to standard methods (Materials and Methods). The construct was made by Jorg Adamczewski in the laboratory of Tim Hunt at ICRF's Clare Hall laboratories, who also expressed the recombinant protein and supplied it to this laboratory in the first instance.

4.3.1 Activation of interphase cytosol by cyclin A

It was necessary to find out if, and how quickly, the cyclin A construct affected interphase cytosol. The protein was added to a mock transport assay mixture (to which 1.0 ST was added in place of membranes) with cytosol, and then incubated at 37°C, with samples being withdrawn for histone kinase assay at various times. Fig 4.4 shows the timecourse of acquisition of histone kinase activity by such incubations. From these timecourses it was clear that maximal cytosolic histone kinase activity was obtained after 20 minutes at 37°C. Longer preincubations resulted in cyclin-independent reduction in the transport signal, presumably due to deterioration of the cytosol (not shown). The cyclin A-dependent histone kinase activity remained high for up to 2 hours after it reached maximum levels.

4.3.2 Effect of cyclin A on the transport assay

A titration was performed to determine whether cyclin A could cause inhibition of transport. For this, increasing quantities of cyclin A were added to interphase cytosol in transport buffer, and preincubated for 20 minutes at 37°C. Afterwards salt-washed Golgi membranes were added and transport was assayed. During the transport assay, histone kinase assays were performed on 2µl samples withdrawn from the completed assay ten minutes into the incubation. The results are shown in Fig 4.5.

Fig 4.4: Histone kinase activation of interphase cytosol by cyclin A

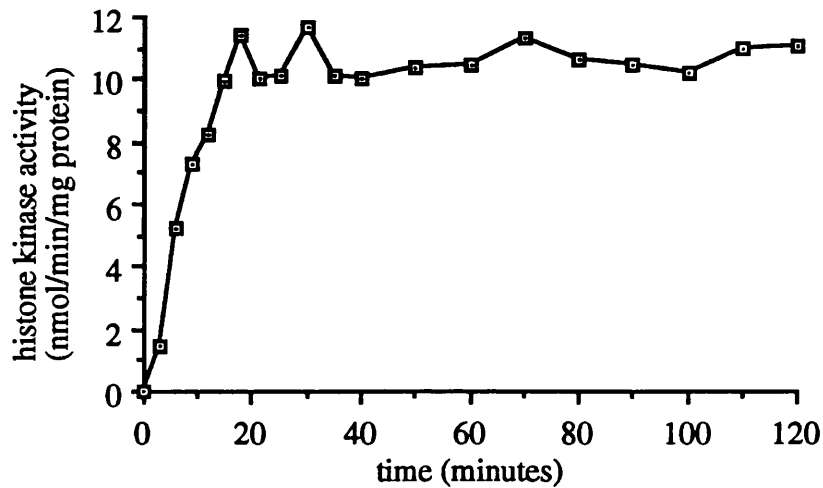
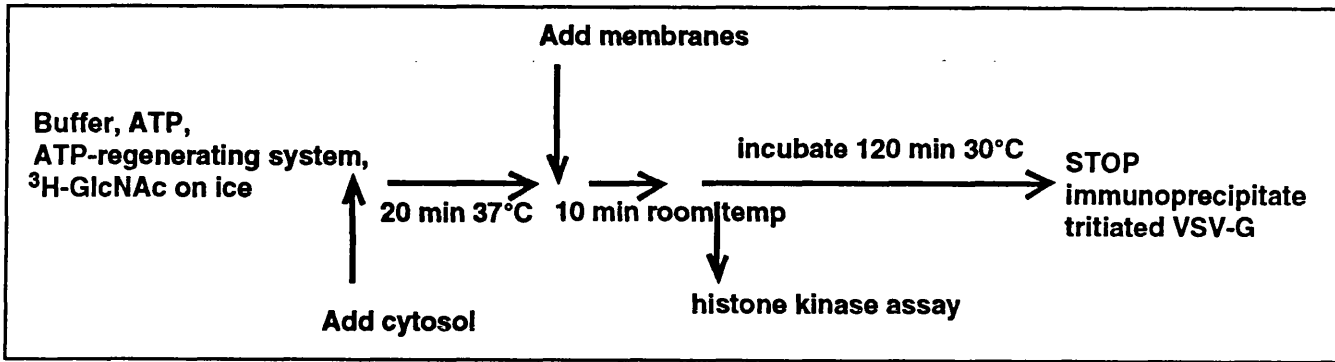


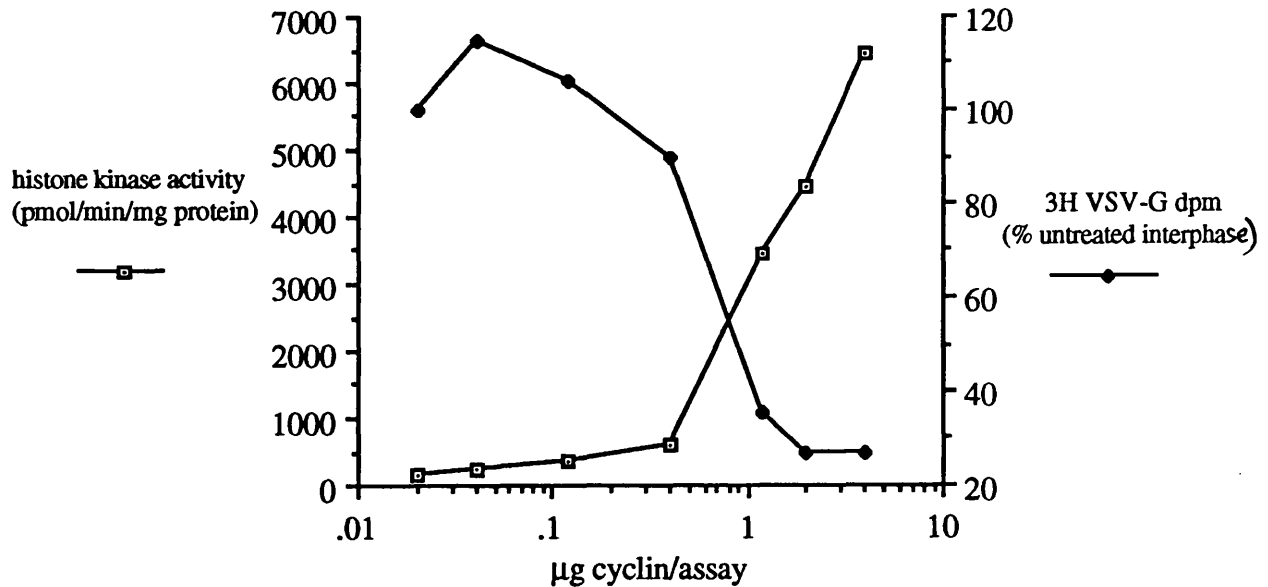
Figure 4.4: timecourse of cytosolic histone kinase activation by recombinant cyclin A. Mock transport assays were prepared containing no tritiated label, no membranes (but 1.0 ST to make up volume) and interphase cytosol. 2.5 μ g of cyclin preparation was added and the reaction incubated at 37°C. Samples were removed and snap-frozen until the end of the incubation, when they were assayed for histone kinase activity.

Figure 4.5: effect of cyclin A on the transport assay. Identical transport assays were prepared containing 5 μ l each of interphase cytosol and salt-washed donor and acceptor membranes. Initially, cyclin was added at the levels indicated. Cytosol was then added and the mixture incubated for 20 minutes at 37°C. Membranes were added on ice, and transport was assayed for 120 minutes at 30°C. Histone kinase assays were performed on 2 μ l samples from the assays 10 minutes into the transport reaction.

Figure 4.5: the effect of cyclin A on the intra-Golgi transport assay
a: layout of assay



b: effect of cyclin A on the assay



Transport was progressively inhibited by increasing quantities of cyclin A. The maximum inhibition seen was about 80%, by approx. 2.5 μ g of cyclin A-containing bacterial extract. At the same rate as transport declined, the histone kinase activity of the cytosol increased, until at maximal inhibition, the histone kinase activity was up to 1.5 times higher than that of mitotic cytosol, and 20-30 fold greater than interphase. The correlation of transport inhibition with histone kinase activation for cyclin A suggests strongly that the two effects are linked.

4.3.3 Optimal time of addition of cyclin A to the transport assay

Cyclin A added to interphase cytosol took 20 minutes at 37°C to cause full activation of histone kinase activity. This made it seem sensible to add the cyclin to the cytosol for activation before adding membranes. A time course was performed to find out whether cyclin had any effect when added during the transport assay itself.

To do this, several identical reactions were set up containing interphase cytosol and salt-washed donor and acceptor membranes in transport buffer. At various times, from the cytosol preincubation to the end of the assay, cyclin A was added. At the end of the timecourse, all the reactions were immunoprecipitated, and the results are shown in Figure 4.6.

The figure shows that, surprisingly, the best results were not obtained when cyclin was permitted to activate the cytosol before the membranes were added. The optimal time of addition was simultaneously with the membranes, and in fact, cyclin could be added up to 20-30 minutes into the transport assay and give similar transport inhibition to cyclin preincubated with cytosol before addition of membranes. This suggests that the membranes did not simply respond to the phosphorylation level of cytosolic transport components. Possibly the true target of the inhibition was not simply a cytosolic protein, but a cytosolic protein in complex with a membrane protein; ie cyclin A required both membranes and cytosol to be present in order to inhibit transport effectively. Also, the fact that cyclin still had a notable effect when added 20 minutes into the transport assay (even though it required 20 minutes further to activate cytosolic histone kinase) suggested that it was acting upon a relatively late event in the transport process. Note that this result was not used in a number of other experiments with cyclin, because it was necessary to obtain and manipulate cytosol of elevated histone kinase activity before adding membranes.

Figure 4.6: time course of cyclin effect on transport

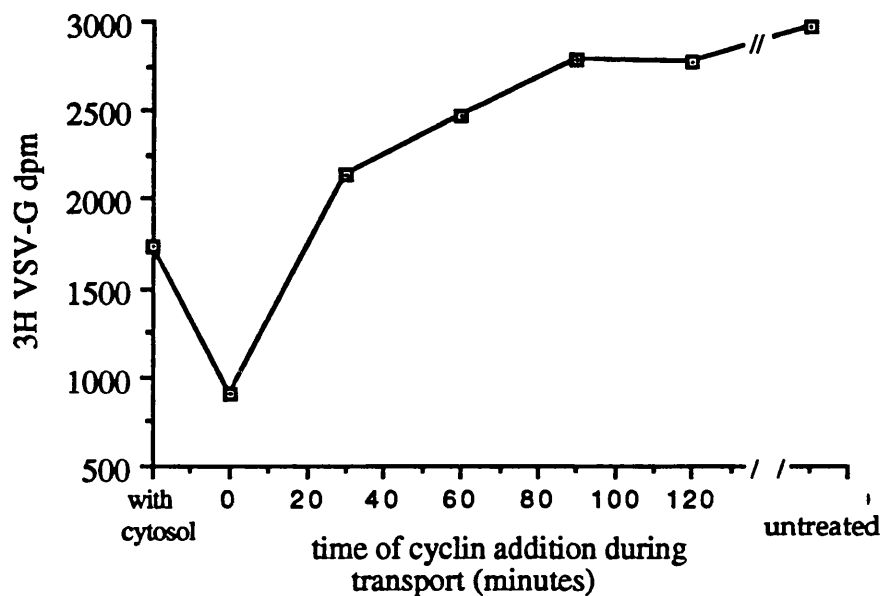


Figure 4.6: time course of the cyclin effect on transport: Identical transport assays were prepared, each containing 5 μ l interphase cytosol and K-donor and acceptor. At various times, either before addition of cytosol (for a 20 minute preincubation at 37°C) or during the transport assay proper, 2 μ g of cyclin protein was added. Transport was assayed for 120 minutes at 30°C.

4.3.4 Abrogation of the cyclin effect by boiling or by simultaneous staurosporine treatment

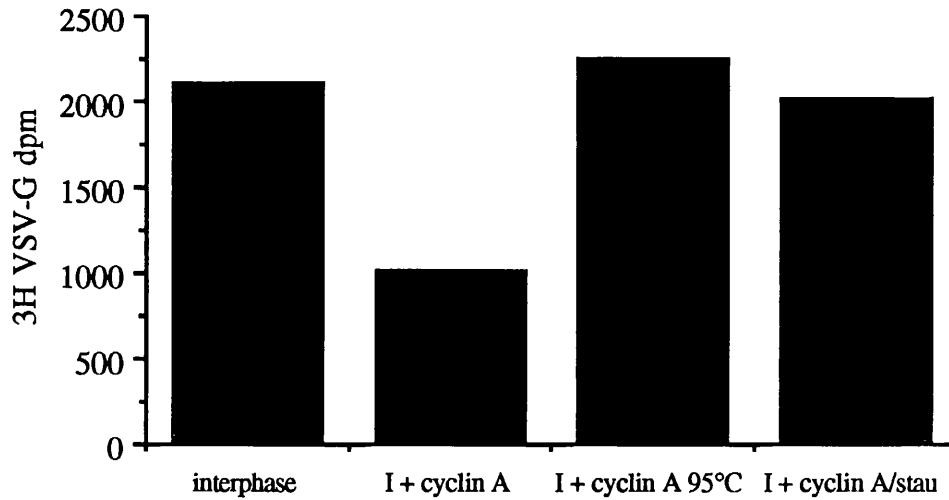
It was important to show that the effect of cyclin A was linked with its activation of mitotic kinase, rather than being due to some non-specific effect on transport. This was particularly significant, since a recombinant cyclin used an early stage in this study was a construct with protein A, designed for purification over an IgG affinity column. After a few months, some inactive point mutants were also made available for use as negative controls, and it was noted that these proteins inhibited transport in a titratable manner without having any effect on histone kinase activity, and even inhibited transport when added at the very end of the transport assay. It is likely that their effect was due to the protein A-tag interacting with the immunoprecipitating antibodies, and this was borne out by showing that protein A inhibited transport in a titratable manner at similar concentrations to these constructs (data not shown).

To show that the effect of cyclin was via its histone kinase activity, assays were performed in the presence of staurosporine. The drug was added at 1 μ M along with the cyclin and before preincubation with cytosol for 20 minutes at 37°C and addition of membranes. Figure 4.7 shows that 1 μ M staurosporine abrogated the effect of cyclin on the transport assay. In addition to this, when the cyclin A was heated to 95°C for 4 minutes before addition to the assay, its effects on histone kinase activity and transport were abolished. The experiments with cyclin A showed that the protein, not a kinase in its own right, was capable of evincing histone kinase activity from interphase cytosol. It seemed extremely likely that it did so by complexing with and activating a kinase of the cdk family. The activation of histone kinase was titratable and linked with a gradual reduction in transport capacity of the cytosol.

When cyclin A was added to mitotic cytosol (at approximately 10% of the levels added to interphase cytosol), it was frequently observed to increase its histone kinase activity to the maximum obtained with cyclin-treated interphase cytosol (data not shown).

Figure 4.7: the effect of staurosporine and heat treatment on cyclin A with interphase cytosol

a: transport activity



b: histone kinase activity

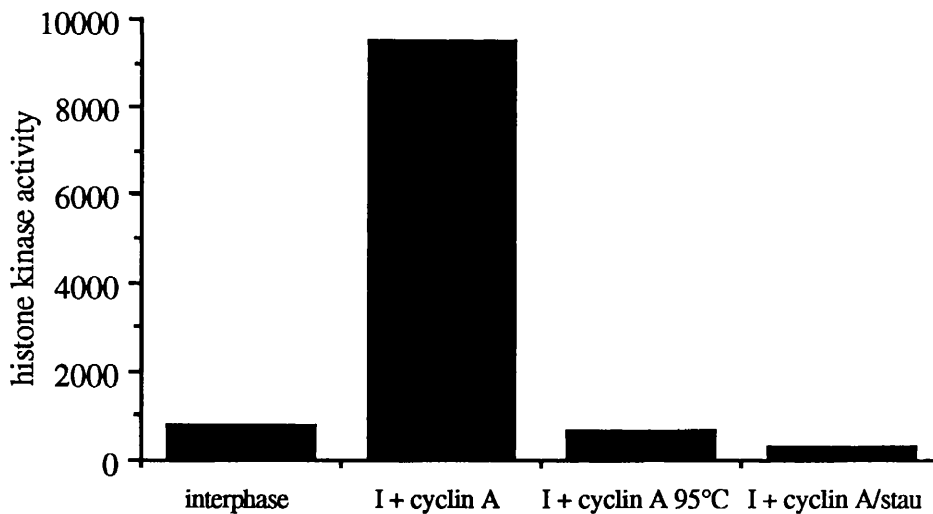


Figure 4.7: for these experiments, transport assays were prepared with 5 μ l of cytosol, K-donor and K-acceptor. To these was added 2 μ g of cyclin, in one case heated to 95°C, and in one case staurosporine (stau) at 1 μ M. The cytosols were incubated for 20 minutes at 37°C, and then assayed for transport for 120 minutes at 30°C. Histone kinase assays were performed on 2 μ l samples during the transport assay, and are expressed in picomoles phosphate per minute per mg cytosol.

4.4 Other cell cycle-related agents

4.4.1: Cyclin B

The experiments described above show how an inverse correlation appeared between the histone kinase activity of a cytosol and its ability to support transport, for mitotic cytosol and interphase cytosol activated by cyclin A. The behaviour of cyclin A in the transport system supported the contention that mitotic cytosol inhibited the transport assay by virtue of its histone kinase activity. However, mitotic cytosol made by nocodazole block of HeLa cells has been shown to contain only cyclin B (Pines and Hunter, 1990). Therefore, it was likely that the mitotic and cyclin A-activated cytosols represented cases of cytosol activation by cyclins B and A respectively. It seemed reasonable to attempt to activate cytosol using cyclin B, and cyclin B was obtained as a bacterially-expressed fusion protein with glutathione S-transferase (ie GST-cyclin B).

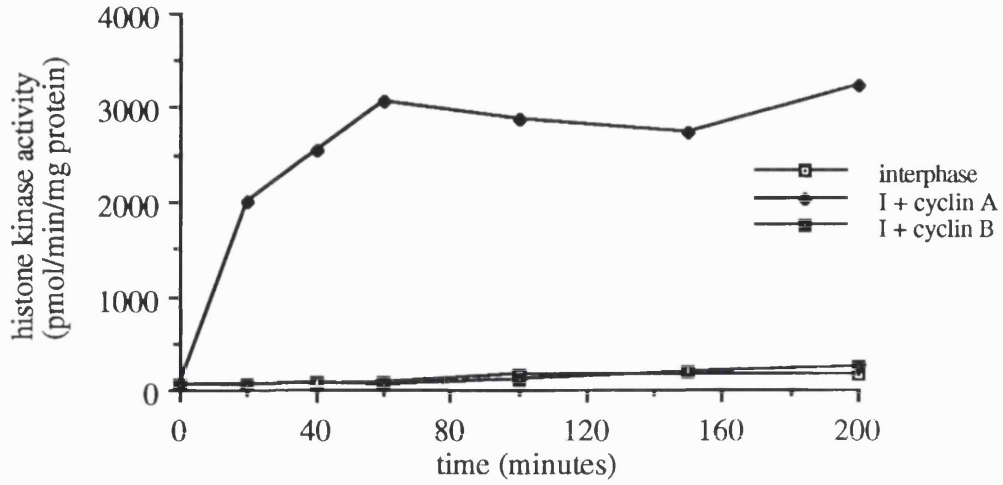
When this protein was added to interphase cytosol, there was no activation of histone kinase activity over an extended timecourse (fig 4.8a) and the protein had no significant effect upon a transport assay (fig 4.8b). Some efforts were made to generate a mammalian cytosol capable of activation by cyclin B, as listed in Table 4.2, but these were unsuccessful. It is possible that the recombinant cyclin B was only partially active, perhaps due to substantial misfolding in the bacterial system, or perhaps due to species specificity rendering it unsuitable for activation of the sHeLa cell kinase. The cyclin B possessed some activity since, when added to a *Xenopus* extract, it did stimulate histone kinase activity, though not to the same extent as an equivalent quantity of MBP-cyclin A. In addition, a mixture of HeLa cytosol and *Xenopus* extract could be activated by cyclin B, which suggested that HeLa cytosol lacked some activity necessary for response to cyclin B, rather than possessing some inhibitory factor.

It is not known why the protein evinced some kinase activity from *Xenopus* extract but none from HeLa cytosol, while both cytosols were activated by cyclin A. The difference may be due to the different preparation conditions of the two cytosols (in particular the high speed spin used for making the HeLa extract) or their buffer composition. It might also be connected with the extensive post-translational modification of the cyclin B-p34^{cdc2} complex.

Figure 4.8: effect of cyclins A and B on interphase cytosol. a, effect on undiluted cytosol; for this, 250µg of interphase cytosol was supplemented with an ATP regenerating system comprising 1mM DTT, UTP and ATP, 2mM CP and 10µg/ml CPK. To this was added 2µg of cyclin A or B, and the whole was incubated at 37°C and assayed at intervals. b, effect on the transport assay; for this, transport assays were prepared with 5µl of interphase cytosol, K-donor and K-acceptor, and 2µg of cyclin A or B was added and assayed for transport and histone kinase activities after a 20 minute preincubation at 37°C.

fig 4.8: effect of cyclins A and B on interphase cytosol

a: activation of histone kinase activity in undiluted cytosol



b: effect upon the transport assay

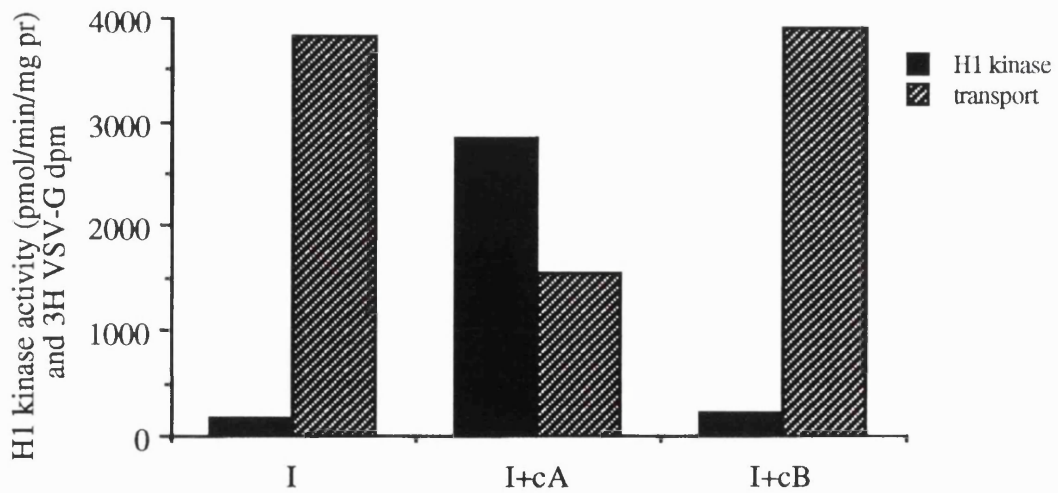


Table 4.1: Failure of recombinant cyclin B to activate *sHeLa* cytosol

Method	H1 kinase activity of cytosol (p/m/m)
Cyclin B incubated with reduced speed cytosol (100,000g 10 minutes; to avoid sedimentation of a fraction required for cyclin B-dependent cytosol activation)	interphase
Cyclin B incubated with interphase cytosol and recombinant cdc25 (to assist dephosphorylation of mitotic kinase)	interphase
Cyclin B incubated with post-nuclear supernatant (660g 10 minutes)	interphase
Cyclin B incubated with <i>Xenopus laevis</i> cytosol 60 minutes at room temp	5x over interphase
Cyclin B incubated with mixed interphase and <i>Xenopus</i> cytosol 60 minutes at room temp	4x over interphase
Cyclin B incubated with interphase cytosol and 1/10 volume mitotic cytosol 120 minutes (to provide some activated kinase to act catalytically)	interphase
Cyclin B incubated with interphase cytosol and 1/20 volume cyclin A 120 minutes (to provide catalytic kinase)	interphase
Cyclin B incubated with interphase cytosol and 1 μ M microcystin 120 minutes (to block activity of inhibitory phosphatases)	interphase
Cyclin B incubated with cytosol from thymidine-accumulated G2 cells (to provide cytosolic conditions resembling those shortly before mitosis)	interphase

In all cases the cytosols were incubated without desalting or dilution, at an appropriate temperature, with an ATP regenerating system added in 1/20 volume. Histone kinase determinations were taken at various times by removing small aliquots of cytosol and diluting into histone kinase assay mixture. Interphase controls were performed, and the fold-elevations of activity listed here are fold-increases over appropriate controls. The cyclin B determinations were performed on several occasions, using 2 batches of cyclin B purified in this laboratory, and also purified *Xenopus* cyclin B. The author is much obliged to Dr C Smythe of Dundee University for purified *Xenopus* cyclin B and *Xenopus* oocyte extract, and to J. Adamczewski for *Xenopus* extract. The rationales for these tests are indicated in the Table and are also described in the Introduction, with the exception of the idea of some particulate material being required for cyclin B activation of cytosol (Felix et al, 1989)

until

An extract containing considerable cyclin B may possess no histone kinase activity & the kinase complexes are activated by removal of the inhibitory phosphorylation. Cyclin A complexed with p34^{cdc2} does not experience these modifications and thus the histone kinase activity of an extract containing cyclin A is proportional to the quantity of cyclin A it contains.

4.4.2 Phosphatase inhibitors

A considerable amount of work has been performed on the drugs okadaic acid and microcystin. These drugs are both inhibitors of protein phosphatases 1 and 2A (OA has a K_i of 10nM for PP1 and 0.1nM for PP2A; those of microcystin are 0.06nM and 10pM, respectively: see Cohen et al, 1989 and MacKintosh et al, 1990) When HeLa cells were treated with okadaic acid, they rounded up and exhibited a pseudomitotic phenotype, and the Golgi apparatus in these cells fragmented, though there was no detectable elevation of histone kinase activity (Lucocq et al, 1991).

It was of interest to see whether phosphatase inhibitors affected the in-vitro transport system. The drug was added to transport assays at 1 μ M, and its effect on K-Golgi transport and cytosolic histone kinase activity was measured. Microcystin had no effect on the histone kinase activity of interphase cytosol and, rather than inhibiting transport, enhanced it slightly and reproducibly (Figure 4.9). The lack of effect on histone kinase activity is as expected from the published work, but the lack of effect of phosphatases on transport was unexpected, given the otherwise close correspondence between the transport system and observed effects of mitotic perturbation on other systems. It is possible that the buffers or the methods of purification in the system caused loss of phosphatase sensitivity, or the loss of some component required for a transport response to phosphatase inhibition, without interfering with the transport response to kinases.

Figure 4.9: the effect of microcystin on the transport assay

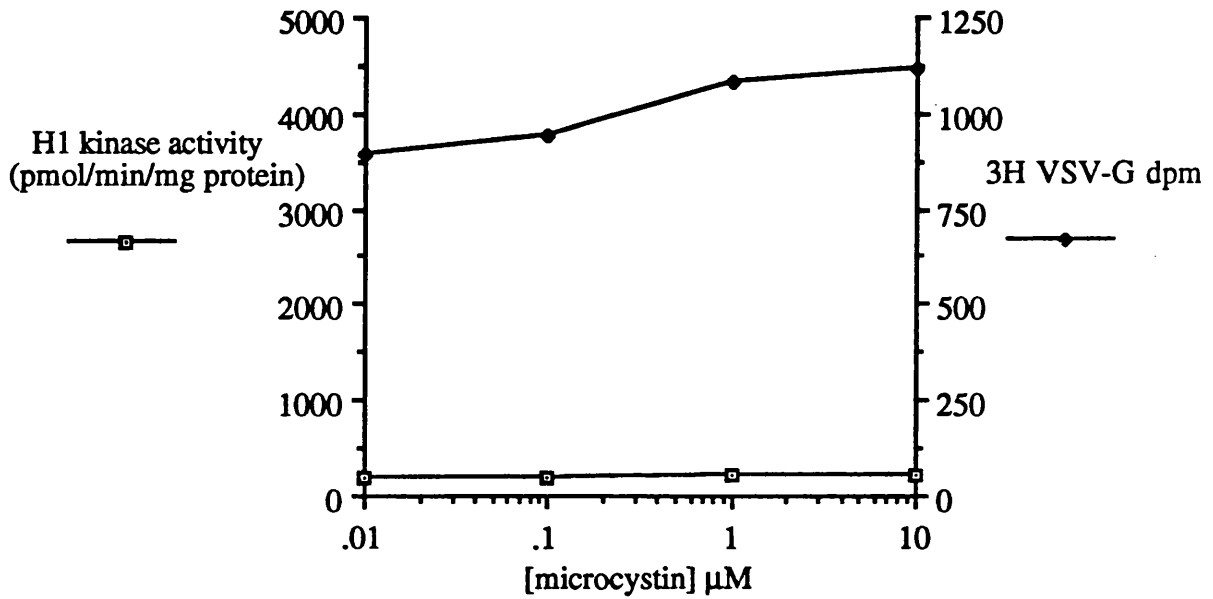


Figure 4.9: effect of microcystin on the transport assay: microcystin was added at the concentrations specified to transport buffer, preincubated with interphase cytosol for 20 minutes at 37°C , and then assayed for transport for 120 minutes at 30°C . Histone kinase activity of $2\mu\text{l}$ samples was determined during the transport assay.

4.5 Summary

The results in this chapter showed that mitotic cytosol inhibited transport by virtue of a phosphorylation event which could be reversed in a titratable manner by staurosporine. This phosphorylation was probably due to mitotic kinase activity since it could be mimicked by treatment of cytosol with cyclin A. The effect of cyclin A on transport depended on its activation of kinase activity, since staurosporine could block both kinase activation and transport inhibition by cyclin A. However, transport inhibition did not require elevated histone kinase activity throughout the assay; it was greatest when the cyclin was added at the same time as the membranes. Transport could not be inhibited by phosphatase inhibitors, and cytosol could not be activated from interphase by addition of cyclin B, for reasons which were not established. Overall, it appeared that the transport activity of a cytosol was inversely proportional to its histone kinase activity.

These results provided strong circumstantial evidence that transport inhibition in the *in vitro* system was due to mitotic kinase when activated beyond certain levels in a number of ways. But it was not clear that the transport effect was due to p34^{cdc2}, rather than one of the p34^{cdc2}-related kinases known to be present in cells, and it was not clear whether the kinase was directly or indirectly responsible for the effect. Therefore experiments were performed to establish whether the p34^{cdc2}-related kinase p33^{cdk2} could influence transport, and also whether inactivation of p34^{cdc2} could affect inhibition of transport. These experiments are described in Chapter 5.

Chapter 5

Further studies of the control of intra-Golgi transport by phosphorylation: the role of p34^{cdc2}.

5.1 Aims

In Chapter 4, a number of observations indicated that the transport activity of a cytosol was closely linked to its phosphorylation, and it was suggested that the active kinase of mitotic cytosol was the key p34^{cdc2} kinase, since its effects could be mimicked by addition of the p34-associated protein, cyclin A. This chapter describes some experiments which went further towards pinpointing p34^{cdc2} as the kinase active in mitotic or cyclin-activated interphase cytosol. The first portion of the data indicates that protein kinases A and C are irrelevant to the activity of mitotic cytosol on transport. The second section describes experiments using the S-phase kinase p33^{cdk2} which, like p34^{cdc2}, binds to cyclin A and acts as a cyclin-dependent histone kinase. The experiments were to determine whether p33^{cdk2} could mimic the phosphorylation-dependent transport inhibition due to mitotic cytosol. The third part of the chapter describes the use of a mouse cell line with a temperature-sensitive defect in p34^{cdc2} to find out the effect that removal of p34^{cdc2} had on transport inhibition by cyclin A.

Under some of these conditions, there was not a tight correlation between histone kinase activity and transport. The results suggest that while p34^{cdc2} is responsible for transport inhibition, it is probably not directly so. This will be described later in the chapter.

5.2 The roles of protein kinases A and C in transport inhibition

Since mitotic transport inhibition was apparently caused by mitotic kinase, and could be reversed by the kinase inhibitor staurosporine, it was of interest to determine whether other kinases were implicated in the process. Neither DMAP nor staurosporine is specific and in fact some kinases, for example protein kinase C, have no commercially-available specific inhibitors.

5.2.1 Protein kinase A

It was reported that inhibition of cAMP-dependent protein kinase (PKA) by a specific inhibitor peptide was synergistic with p34^{cdc2} activity in induction of mitotic effects in whole cells (Lamb et al, 1991). It was therefore decided to test whether activation of PKA could influence the cell-cycle response of the transport assay. The PKA activator forskolin was added at 10 μ M and 100 μ M to assay mixtures before preincubation with interphase, cyclin A-treated interphase and mitotic cytosol. The effects were examined by standard transport assay using salt-washed Golgi and by histone kinase assay (figure 5.1). It was found that forskolin at up to 100 μ M had no significant effect on interphase or mitotic transport and, more significantly, almost no effect on the transition from interphase to mitotic conditions as shown by cyclin A treatment. The small decline in the cyclin A-induced histone kinase activity in the presence of forskolin was not matched by an effect on transport inhibition. This strongly suggests that PKA is not a kinase downstream of p34^{cdc2} in the control of transport in vitro. In addition, the PKA activities of interphase and mitotic cytosol were assayed and found to be essentially the same (Rosemary Stuart; personal communication).

5.2.2 Protein kinase C

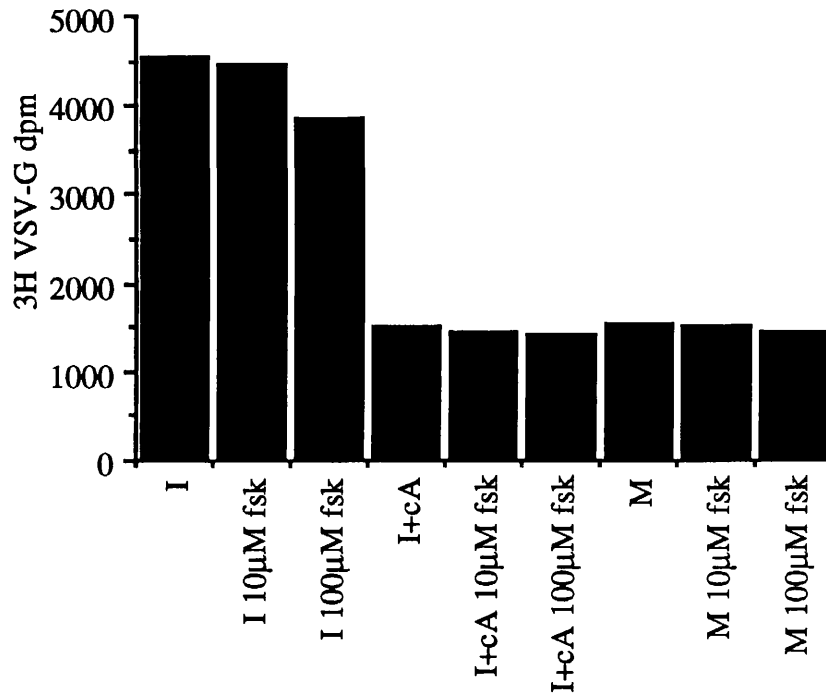
Since there is no commercially-available PKC inhibitor, the PKC activities of interphase and mitotic cytosol were assayed; if they were not markedly different, then PKC would be unlikely to be involved in mitotic transport inhibition. The PKC activity of interphase and mitotic cytosol were tested by Lodewyk Dekker from the laboratory of Dr Peter Parker at the ICRF. They were assayed by measuring the cofactor-dependent histone kinase activity of the cytosols. The cofactor-dependent histone kinase activities of mitotic and interphase cytosol, ie their PKC activities, were not significantly above the cofactor-independent activities (Table 5.1). The storage conditions of cytosol were not those normally used to preserve PKC activity (ie glycerol-containing solution at -20°C), so it is possible that fresh mitotic cytosol does have higher PKC activity than fresh interphase cytosol, but all the cytosols used in transport experiments had been stored at -80°C in buffer containing 0.2M sucrose, and thus had similar PKC activities.

Thus, when the cytosolic activities of PKC and PKA were studied, no evidence suggested that they play an important role in the control of the intra-Golgi assay.

Figure 5.1: the effect of forskolin on interphase, mitotic and cyclin-treated interphase cytosol. Transport assays were prepared containing forskolin (fsk) at 10 or 100 μ M. Interphase and mitotic cytosol, and interphase cytosol with 2 μ g cyclin A (cA) was preincubated for 20 minutes at 37 $^{\circ}$ C, then returned to ice. Then membranes were added, and transport assayed for 120 minutes at 30 $^{\circ}$ C; histone kinase assays were also performed on 2 μ l samples withdrawn from those assays 10 minutes into the incubation.

Figure 5.1: forskolin effect on mitotic, interphase and cyclin A-treated cytosol.

a: transport activity



b: histone kinase activity

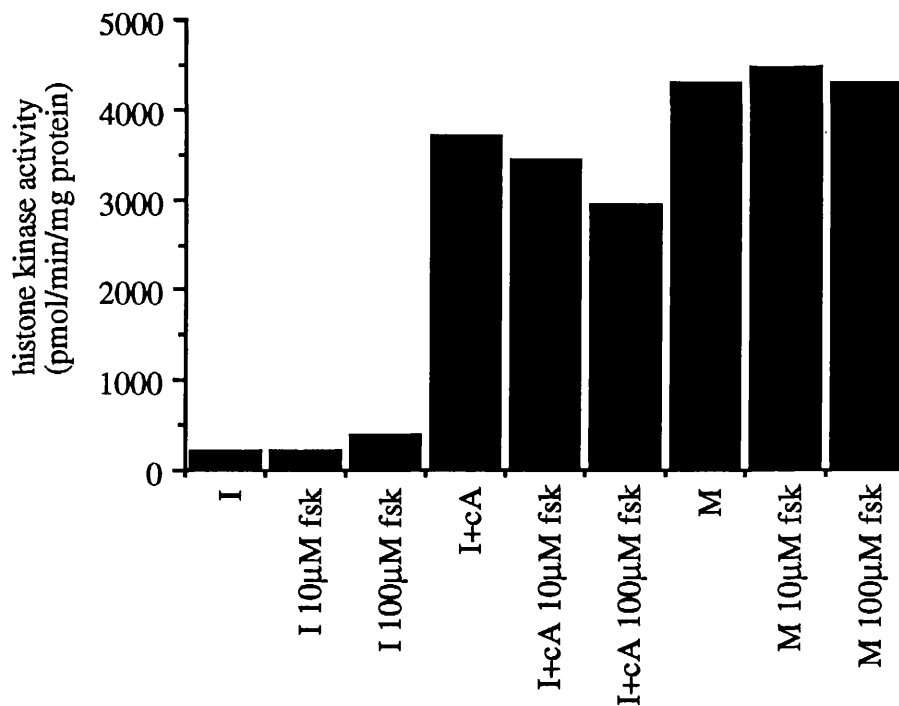


Table 5.1: Protein kinase C activity in interphase and mitotic cytosols

PKC activity	interphase	mitotic
cofactor-dependent	1647	20532
cofactor-independent	1896	18759

These results represent averaged triplicate histone kinase assays of interphase and mitotic cytosols in the absence or presence of PKC cofactors, ie Ca^{++} , phosphatidylserine and tetradecanoyl phorbol acetate; the method is a standard one for assay of PKC activity. The interphase samples contained no histone kinase activity. The mitotic cytosol contained histone kinase activity, but the level of cofactor-dependent histone kinase activity was not significant, according to Lodewyk Dekker, who performed this assay.

5.3 The S-phase kinase: p33^{cdk2}

The protein p33^{cdk2}, as described in the Introduction, is a kinase closely related to p34^{cdc2} and instrumental in the onset of S-phase in mammalian cells. It requires cyclins A or E for activity and, with cyclin A, is subjected to regulatory phosphorylation much like that of p34^{cdc2} with cyclin B.

This protein was made available as a bacterially-expressed construct with glutathione S-transferase. (The construct was made, expressed and generously given by Jorg Adamczewski at the ICRF laboratories at Clare Hall.)

Since transport was inhibited by cyclin A treatment, concurrent with a rise in histone kinase activity, it was of interest to determine whether the kinase involved was a cell-cycle-related kinase which interacted with cyclin A. This could be done by adding cyclin A to transport assays with or without p33^{cdk2}. If the kinase involved was p33^{cdk2}, then it was reasonable to expect that exogenous p33^{cdk2} and excess cyclin would result in greater transport inhibition than excess cyclin alone. If it were p34^{cdc2}, then perhaps p33^{cdk2} would compete with p34^{cdc2} for the available cyclin A, and thus ameliorate transport inhibition by limiting levels of cyclin A.

To test these predictions, cytosol preincubations were prepared containing increasing levels of cyclin A, in the presence or absence of p33^{cdk2}. These were preincubated with interphase cytosol and then assayed for transport in the normal way; also, 2 μ l aliquots of transport assay were removed for histone kinase assay.

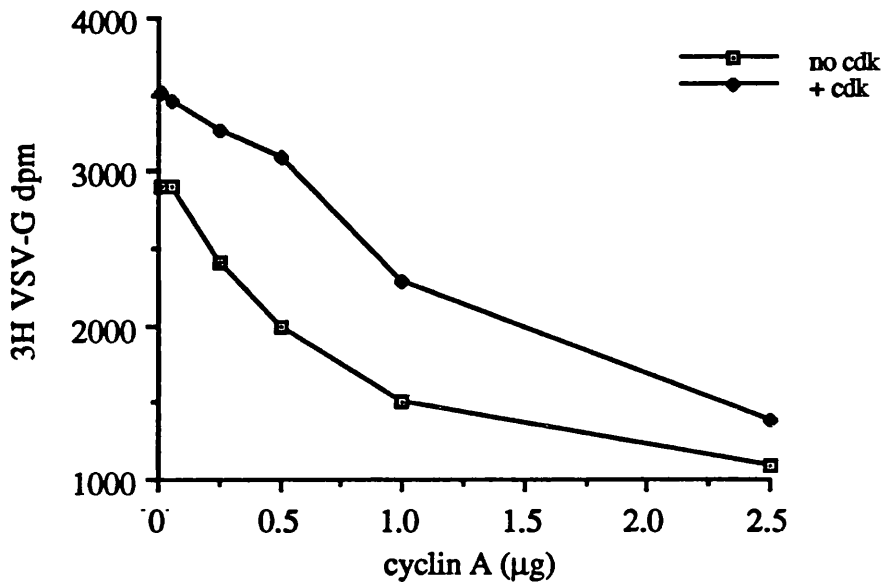
The results (Fig 5.2) show that low levels of p33^{cdk2} approximately doubled the histone kinase activity due to added cyclin A. This elevated histone kinase activity did not lead to increased transport inhibition; instead, for submaximal levels of cyclin A, transport inhibition was reduced. This indicated that the added p33^{cdk2} was competing for the cyclin A with another protein, very strongly suggesting that the inhibitory protein was p34^{cdc2}. At higher levels of cyclin A (when cyclin A was in excess) there was similar transport inhibition in the presence or absence of p33^{cdk2}. This gave some indication of the level of cyclin A required to complex with both the added p33^{cdk2} and the p34^{cdc2}.

The cyclin A level required to cause 50% transport inhibition increased as the quantity of competing p33^{cdk2} was increased. Figure 5.3 shows a summary of several experiments showing that the more p33^{cdk2} was added to an interphase cytosol, the greater the level of cyclin A required to inhibit transport, and the greater the level of cyclin-dependent histone kinase activation.

Figure 5.2: effect of cyclin A/p33^{cdk2} on the transport assay. These assays contained cyclin A at the levels shown, in either the presence or the absence of 0.2 μ g p33^{cdk2}. These were preincubated for 20 minutes at 37°C, and then membranes were added and transport assayed for 120 minutes at 30°C. Histone kinase assays were performed on 2 μ l samples withdrawn from the transport assay mixture 10 minutes into the incubation.

Figure 5.2: effect of cyclin A/p33^{cdk2} on the cyclin response of interphase cytosol

a: transport activity



b: histone kinase activity

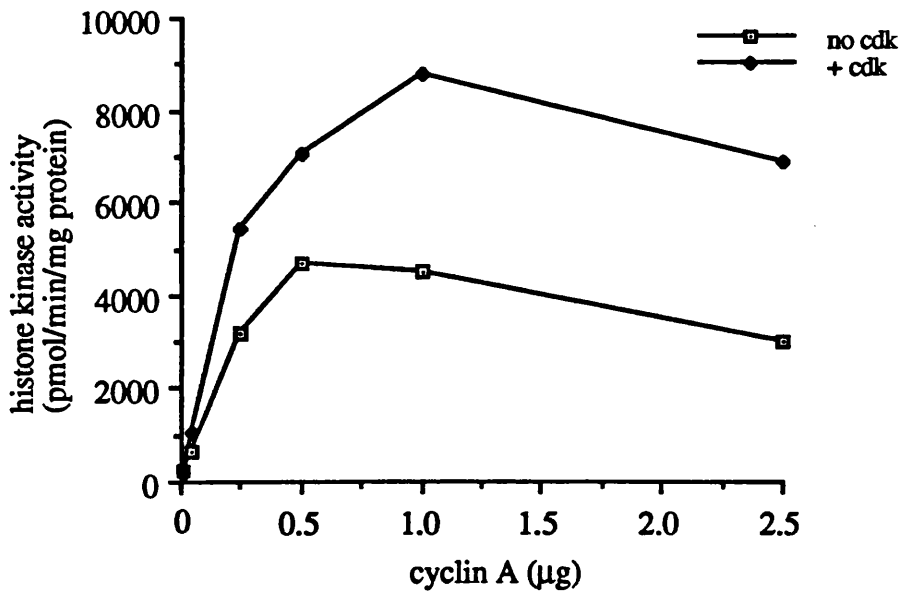


Figure 5.3: correlated effects on transport inhibition and histone kinase activity by p33^{cdk2}

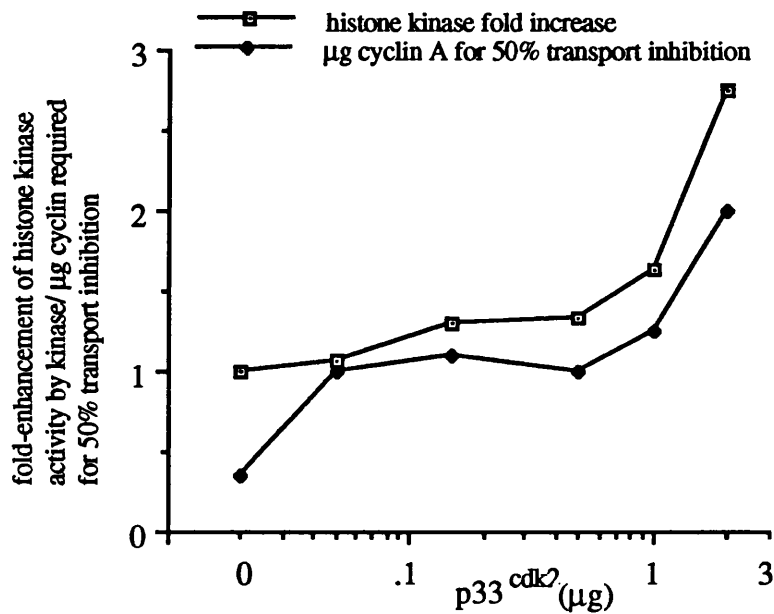


Figure 5.3: a correlation between cdk levels and amelioration of cyclin A effect. This graph represents a summary of several experiments, all performed in the same manner as that described in Fig 5.2, with different levels of the cdk2 protein and the same cytosol and membranes. Fold-increase of specific activity is the ratio of histone kinase activities with and without p33^{cdk2} at 1µg cyclin A. The transport inhibition level was selected as the quantity of cyclin present where transport was 50% of its maximal inhibition without p33^{cdk2}.

p33^{cdk2} had no effect on either transport or mitotic kinase activity when added to interphase cytosol in the absence of exogenous cyclin A. This indicated either that the interphase cytosol contained no appreciable levels of cyclin A, or that cyclin A was not available to interact with the added p33^{cdk2}. The latter is more likely to be true, since the interphase cytosol is taken from unsynchronised cells and therefore certainly contains low levels of endogenous cyclin A. Since it has been shown that the cyclin A-p33^{cdk2} complex does undergo regulatory phosphorylation, it is curious that the added kinase and cyclin A was not inhibited by the cytosol. It may be that the endogenous cyclin is all complexed and inactive, and added kinases are either incapable of being similarly regulated due to their bacterial origin, or are added in quantities too great to be inhibited in the timescale of the assay.

5.4 Mouse cell cytosols with temperature-sensitive p34^{cdc2}

5.4.1 Characterisation of FM3A and FT210 cytosols

As an alternative method for establishing the role of p34^{cdc2} in control of in-vitro transport inhibition, cytosol was prepared from a cell line with a temperature-sensitive defect in p34^{cdc2}. The line is a derivative of the FM3A mouse mammary carcinoma line established and generously provided by Mineo and co-workers (Mineo et al, 1986; Th'ng et al, 1990; Hamaguchi et al, 1992). The mutant is designated FT210. It was found that, when the FT210 cells were grown at 32°C, they had the same doubling time as the parent line. If, however, they were grown at 39°C, the FT210 cells arrested in G2 and eventually died. The kinase p34^{cdc2} was found to be denatured and degraded rapidly at 39°C, and despite elevated transcription of the gene, insufficient native protein was present to permit cell cycling. Degradation of p34^{cdc2} was significant 1-4 hours after temperature elevation.

The mutant and parent lines were obtained and maintained as recommended by the authors in spinner culture at 32°C. In order to generate cytosol lacking p34^{cdc2}, cultures of FM3A and FT210 cells were divided, and half the cells were transferred to 39°C for 7 hours before beginning cytosol preparation (this was to permit warming of the spinner medium to 39°C, which took up to four hours, and then to allow time for degradation of the kinase). Therefore, both FM3A and FT210 cytosols were prepared from cells submitted to high-temperature incubation, but only FT210 cytosol should be affected by it. (see Th'ng et al, 1990)

Figure 5.4: titration of mouse cytosols and HeLa cytosol

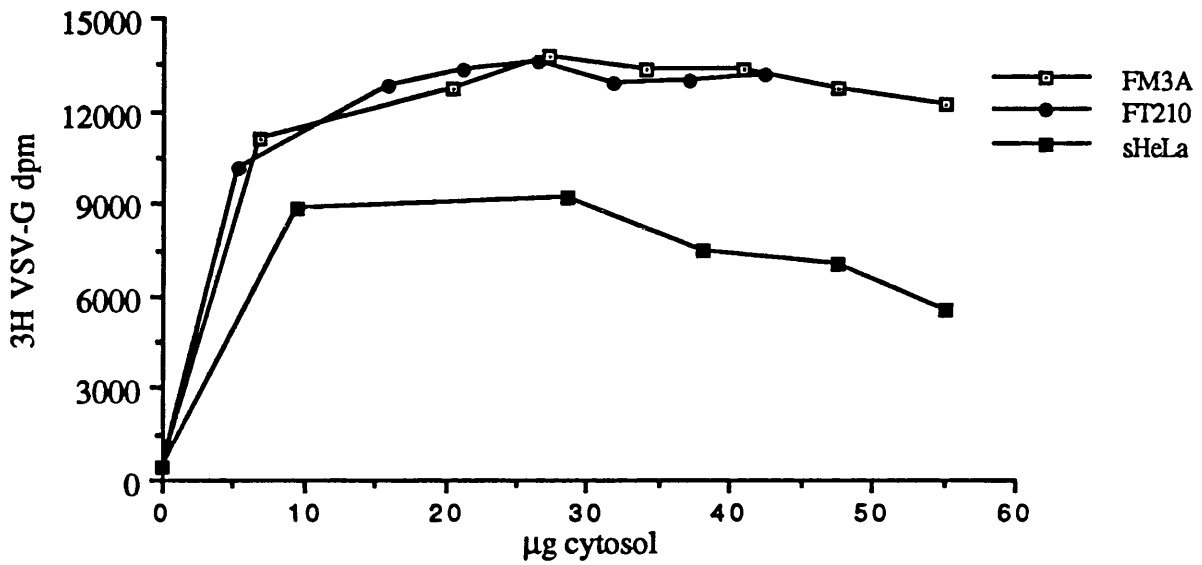


Figure 5.4: titration of mouse and HeLa cytosols. In these experiments, 50µl transport assays were prepared containing cytosol at the concentrations shown (with volumes adjusted with buffered salts) and equal levels of membranes. Transport was assayed for 120 minutes at 30°C. The FM3A cytosols were derived from cells grown at 32°C. Cytosol concentrations were: FM3A 6.8mg/ml, FT210 5.3mg/ml, sHeLa 9.5mg/ml.

Figure 5.5: activation of mouse cytosols by cyclin A

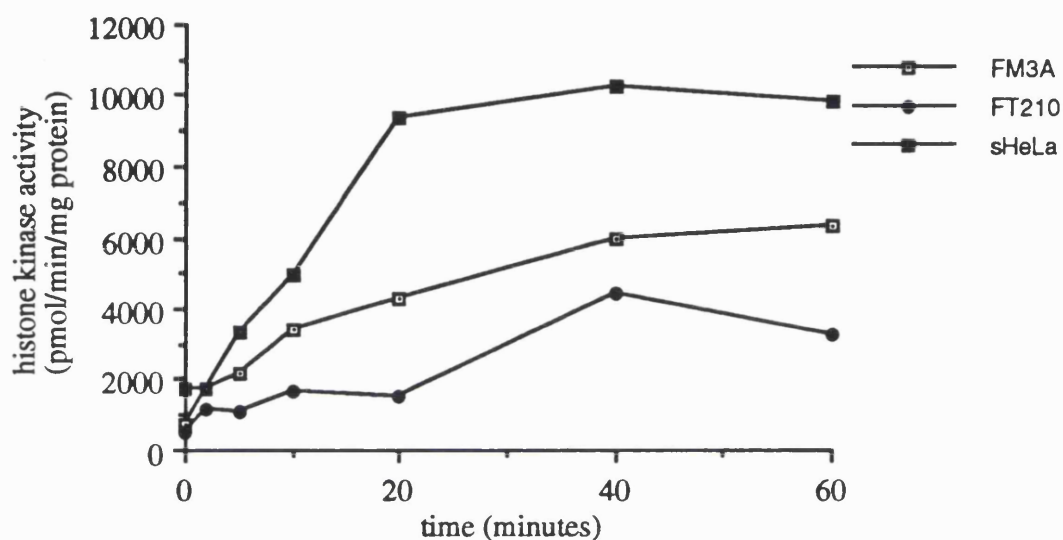


Figure 5.5: activation of mouse cytosols by cyclin A: In this experiment both FT210 and FM3A cytosols were derived from cells grown at 32°C. Mock transport assays were prepared as described in Figure 4.4*. Histone kinase assays were performed on 5µl samples of the assay, at 32°C for 20 minutes, in order to increase the ³²P incorporation into histones for the less active cytosols, and this method was used for all experiments with the mouse cytosols. The sHeLa cytosol was treated in the same way as the mouse cytosols.

* containing 2.5 µg recombinant cyclin A fusion protein

Cytosol was prepared from the parent and mutant cells both from 32°C and 39°C cultures, by the same methods as used for sHeLa cytosol. The cytosols had slightly lower protein concentration than sHeLa preparations (5.3mg/ml for FT210 and 6.8mg/ml for FM3A), but when titrated into transport assays, their ability to support transport, per µg cytosol, was greater than that of the HeLa cells (figure 5.4).

Mouse and human cytosols were compared for their response to cyclin A, to indicate the level of active mitotic kinase for assay. 2.5µg cyclin A was added to the cytosols in transport buffer, and they were incubated at 32°C. At various times aliquots were removed and histone kinase assays performed for 20 minutes at 32°C (to avoid compromising p34^{cdc2} activity in the FT210 line by incubation at 37°C). The results are shown in figure 5.5. The maximal histone kinase activity attained by the FM3A cytosol was approximately 1.5-fold lower than by the sHeLa cytosol incubated under identical conditions, and the activity of the FT210 cytosol was lower still. This is in accord with the original findings of Th'ng et al (1990). Unfortunately, the FT210 cytosol prepared at 39°C responded to cyclin A (Fig.5.6). Its cyclin-dependent histone kinase activity was greater than that of its sibling cytosol prepared at 32°C, though from a higher baseline so that the ratio of activation was in fact slightly lower. This suggested that the 39°C preparation conditions had not depleted p34^{cdc2} in the mutant cell line sufficiently to abrogate activation by cyclin A. However, an alternative explanation was that the p34^{cdc2} in the FT210 cytosol had been heat-inactivated, and the cyclin A was actually acting on a different kinase. To test whether response to cyclin A could be destroyed in FT210 cytosol, conditions were explored for heat-treatment of the isolated cytosol, and preparation of cytosol from heat-treated FT210 cells was subsequently abandoned.

5.4.2 Heat treatment and the effect of cyclin A on FT210 cytosol

In cytosol-heating experiments, the incubated cytosol had to be tested for its susceptibility to both cyclin A and elevated temperature (ie checking that incubation at high temperature did not lead to diminution of transport activity).

The FT210 cytosol (derived from cells grown at 32°C) was incubated at 38°C (which was found to cause less loss of transport activity than 39°C incubation). After various lengths of time from 5 to 20 minutes, it was removed to ice and cyclin A added, and then incubated for 30 minutes at 32°C (which was found to have no deleterious effect on FT210 cytosol).

Control incubations were incubated either without cyclin A, or with cyclin A but without heat-inactivation. After the preincubation, salt-washed membranes were added and a standard transport assay was performed. 5µl samples of the transport incubation were withdrawn for histone kinase assay for 20 minutes at 32°C. The layout of this experiment and its results are shown in Fig 5.7.

Figure 5.6: cytosol made from FT210 cells at 39°C remained cyclin A-sensitive

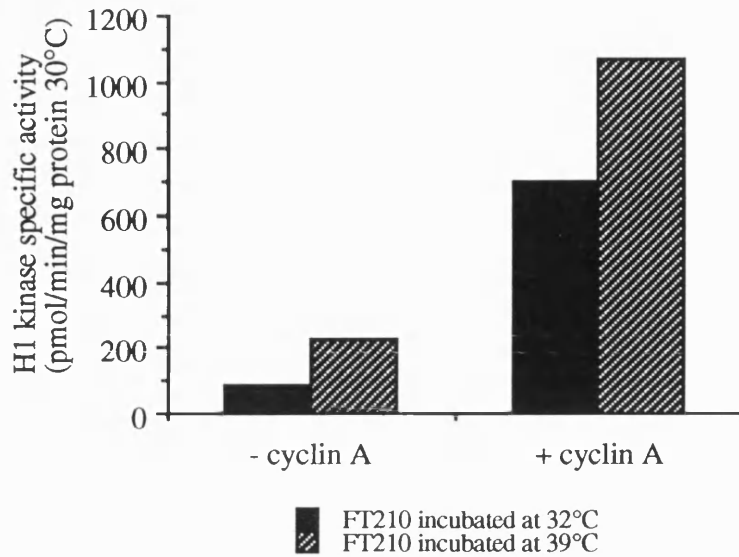
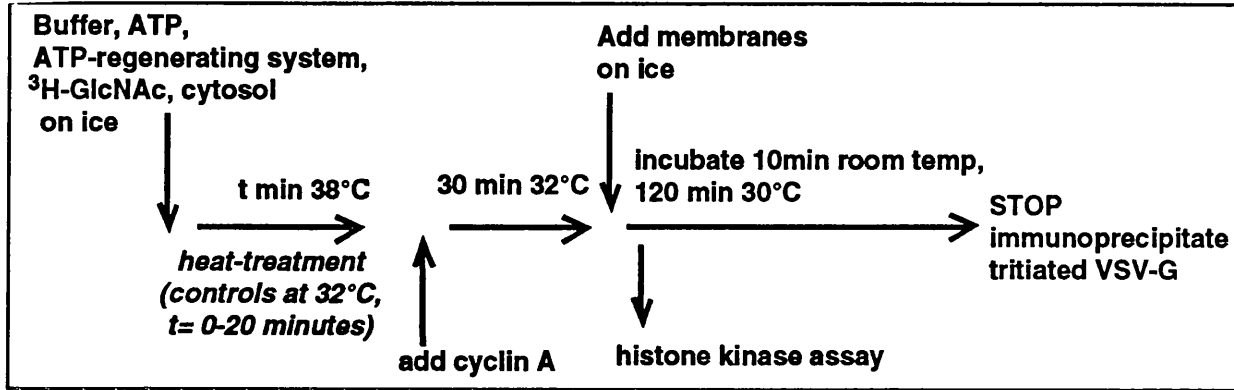


Figure 5.6: cytosol made from FT210 cells at 39°C remained cyclin A-sensitive. In this experiment, mock transport assays were set up as described for Figures 4.4 and 5.5. Equal quantities of cytosol were added to each, made from FT210 cells incubated at either 32°C or 39°C before harvesting. To this was added 2µg of cyclin A, then the samples were incubated for 30 minutes at 32°C. The histone kinase activity of the samples was then assayed as described in the legend to Figure 5.5.

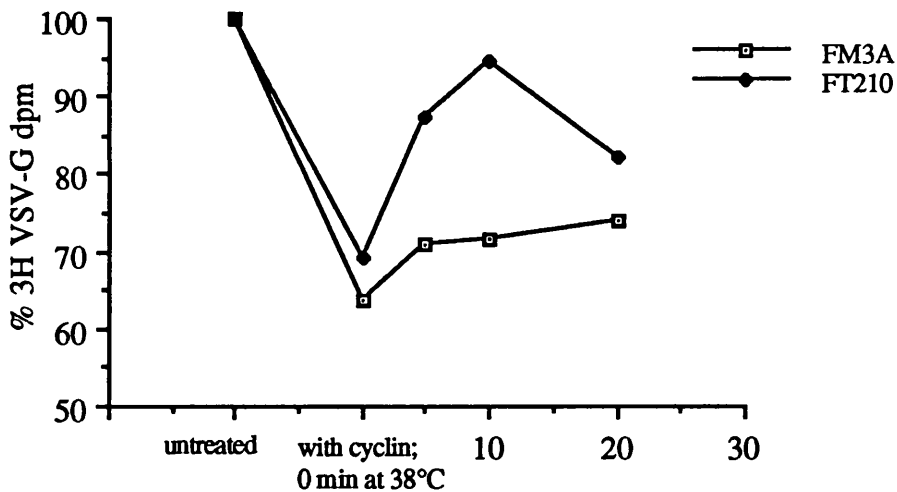
Figure 5.7: heat treatment and the cyclin response of FT210 cells. In these experiments, transport assays were prepared with equal levels of FT210 and FM3A cytosols. Some were preincubated for various times at 38°C, and then cyclin was added to them and they were transferred to 32°C for 30 minutes. Samples which were not preincubated at 38°C were incubated for 30 minutes at 32°C. After the preincubation, membranes were added and transport assayed for 120 minutes at 30°C, and histone kinase assays were also performed at 32°C.

Figure 5.7: heat treatment and the cyclin response of FT210 cytosol

a: experimental outline



b: transport activity



c: histone kinase activity

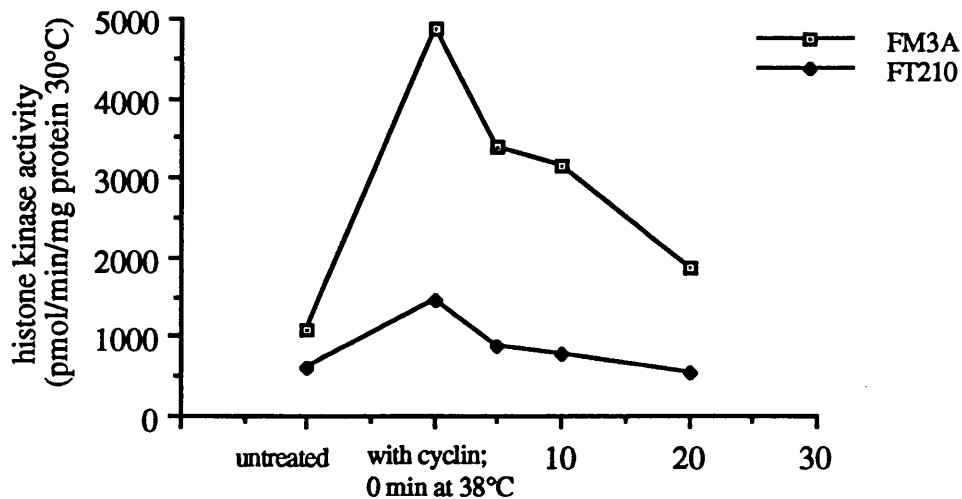
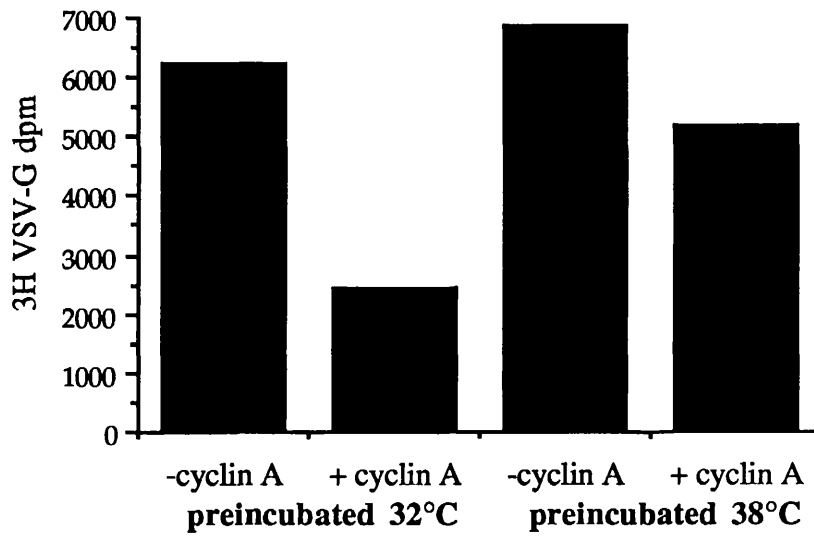


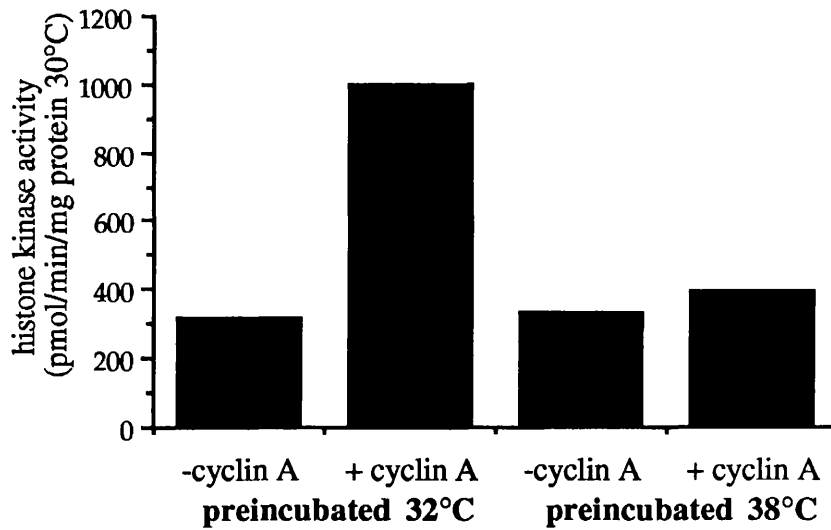
Figure 5.8: transport inhibition by cyclin A depends on activity of p34^{cdc2}. In this experiment, transport assay mixtures were prepared and FT210 cytosol added to them. The reactions were either preincubated with cyclin A for 30 minutes at 32°C (marked 32°C), or incubated at 38°C for 10 minutes before this incubation (marked 38°C). Control assays were incubated under similar conditions without cyclin A. Then membranes were added and transport assayed for 120 minutes at 30°C; also, histone kinase assays were performed at 32°C.

Figure 5.8: transport inhibition by cyclin A depends on p34cdc2 activity

a: transport activity



b: histone kinase activity



The results show that after 10 minutes' pretreatment at 38°C, there was almost total loss of histone kinase activity in response to cyclin A, and a concomitant loss of transport inhibition, compared with incubations not subjected to heat-treatment. The maximum transport inhibition obtained in this experiment was only 40%. This may have been due to the extended preincubation time in this experiment, or to an unfavourable combination of transport components. The FM3A and sHeLa cytosols also suffered a small loss of cyclin A response when incubated at 38°C for more than 15 minutes, and this probably reflected cytosol deterioration at high temperature (data not shown).

The effect of heat-treatment was compared between FT210 and FM3A cytosols. Samples were either treated at 38°C for 10 minutes, or maintained at 32°C. Then cyclin A was added to the treated and untreated cytosols, or omitted from control samples, and all samples were incubated for 20 minutes at 32°C. Then transport and histone kinase assays were performed as described above.

Figure 5.8 shows that the effect of cyclin A on FT210 cytosol was greatly impaired by heat-treatment. FT210 cytosol pretreated at 32°C was ^{approx.} 3-fold activated by cyclin A, but after heat-treatment was not significantly activated. For FT210 cytosol at 32°C, cyclin caused 60% inhibition of transport, but after heat-treatment no transport inhibition was observed. This experiment demonstrated that active p34^{cdc2} was required for transport inhibition, since the FT210 cell line, with temperature-sensitive p34^{cdc2}, could not respond to cyclin A after heat-treatment. The parent line, differing only in respect of p34^{cdc2}, showed constant cyclin-dependent transport inhibition (data not shown).

Interestingly, when FT210 cytosol was activated with cyclin, and then treated at 38°C, the histone kinase activity of the preparation was almost as high as controls cyclin-treated at 30°C, and transport inhibition was also almost at the low-temperature value (summarised in Table 5.2). This suggests that cyclin binding stabilised the kinase sufficiently to withstand brief treatment at 38°C.

Table 5.2: Cyclin A treatment of FT210 cytosol ameliorates the heat-lability of p34cdc2

	untreated FT210 cytosol	FT210 + cyc A	FT210 with 38°C treatment	cycA then 38°C treatment
histone kinase activity (pmol/min/mg protein)	460	1169	411	1011
3H VSV-G dpm	5779	3174	4741	3417

The FT210 cytosol was placed in transport buffer with or without optimal levels of cyclin A. Then it was preincubated either for 30 minutes at 32°C, or for 20 minutes at 32°C and then 10 minutes at 38°C. After this, salt-washed membranes were added and transport was assayed; also, histone kinase activity was tested as described in section 5.3.2.

5.4.3: *p33^{cdk2}* and FT210 cytosol

It was notable that the mouse cytosols inhibited transport at far lower histone kinase activities than HeLa cytosols; it was shown in Chapter 4 that HeLa cytosol with histone kinase activity of ~1000pmol/min/mg protein (whether due to cyclin activation of interphase cytosol or staurosporine treatment of mitotic cytosol) showed scarcely any inhibition of transport (approx. 20% in each case; see chapter 4). It is not clear why cyclin resulted in such small elevation of histone kinase activity with these cytosols. It is possible that cyclin was sequestered in some complex with another kinase, say, an inactivated complex with *p33^{cdk2}*. To gain some information on the activity of other kinases in the mouse cytosol, the *cdc2*-like kinase *p33^{cdk2}* was tested in FT210 cytosol. The cytosol was incubated with a small amount (0.25µg enriched bacterial extract) of *p33^{cdk2}* with or without cyclin A (1µg; this submaximal level was chosen so that if *p33^{cdk2}* inhibited transport in the presence of cyclin A, its effect could be seen) for 30 minutes at 30°C and then assayed for transport and histone kinase activity. The results are shown in Fig 5.9.

Addition of *p33^{cdk2}* alone to FT210 cytosol caused no elevation of histone kinase activity and no inhibition of transport; in fact, transport was markedly enhanced. The reason for this is not known, but HeLa cytosol incubated with *p33^{cdk2}* also showed transport elevation, though not so strongly. When *p33^{cdk2}* and cyclin A were preincubated together with FT210 cytosol, its histone kinase activity was enhanced by 20-fold, akin to the activation achieved by HeLa cytosols with cyclin A. At the same time, transport was inhibited more than with cyclin A alone. The fact that the percentage inhibition of transport was higher with the kinase than without (in the presence of cyclin) may be due simply to the fact that transport was elevated in the presence of the kinase alone. The actual counts achieved when cyclin was present were similar with or without kinase intervention.

The fact that high histone kinase activity could be attained by the mouse cytosols suggested that the kinases present in the cells were simply not very active in histone kinase assay, for an unknown reason - for example, if the cytosol contained very high levels of cell cycle-active phosphatases, then mitotic activity sufficient to inhibit the intra-Golgi transport assay might not be sufficient to cause high histone phosphorylation at equilibrium. Perhaps the exogenous *p33^{cdk2}* was structurally incapable of activation in that system, or perhaps not inactivable in the time course of the assay. Overall, however, it was clear that high histone kinase activity was not a prerequisite for transport inhibition for this cytosol, which was in this respect like HeLa cytosol treated with *p33^{cdk2}*.

Figure 5.9: the effect of p33^{cdk2} and cyclin A on FT210 cytosol

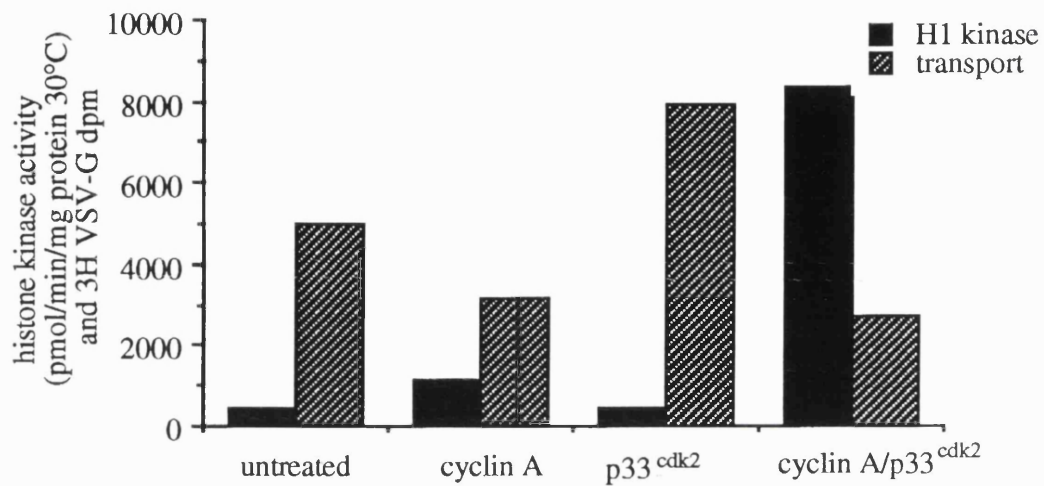


Figure 5.9: the effect of p33^{cdk2} and cyclin A on FT210 cytosol. In this experiment, FT210 cytosol was preincubated for 30 minutes at 32°C with combinations of cyclin A (1µg) and cdk2 protein (0.25µg). After this, membranes were added, transport assayed for 120 minutes on ice and histone kinase activity for 20 minutes at 32°C.

5.4.4: effect of microcystin on FT210 cytosol

The low histone kinase levels associated with transport inhibition suggested one further experiment. As mentioned in Chapter 4, HeLa cytosol treated with the phosphatase inhibitor microcystin showed no evidence of a pseudomitotic phenotype. This phenotype can arise when some cells or cell-free systems are treated with phosphatase inhibitors, due to a shift in the cytosolic equilibrium of phosphorylation. Since the mouse cytosols suffered strong transport inhibition at relatively low histone kinase activity, it was possible that they would be affected by microcystin treatment. Therefore, the effect of microcystin was tested on FT210 cytosols either replete with or depleted of p34^{cdc2} kinase activity.

Figure 5.10 shows that FT210 cytosol treated with 1 μ M microcystin showed strong inhibition of transport. This inhibition was observed at as little as 0.3 μ M of the drug, and was not accompanied by any elevation of histone kinase activity (data not shown). Notably, the inhibition did not require active p34^{cdc2}, since it was equally marked after 32°C and 38°C pretreatment.

This strongly suggests that transport is under control of a kinase-phosphatase cycle downstream of p34^{cdc2}, since phosphatase inhibition caused transport inhibition in the absence of cyclin-stimulated kinase activity. It is interesting that microcystin inhibited transport in mouse cytosol but not HeLa cytosol. Perhaps phosphatase levels in these cells are higher than in HeLa cells, so that phosphatase inhibitors can have a dramatic effect on the phosphorylation-sensitive transport mechanism. In the absence of more specific assays for the phosphorylation state of the cytosol, and particularly its transport components, this question cannot be resolved.

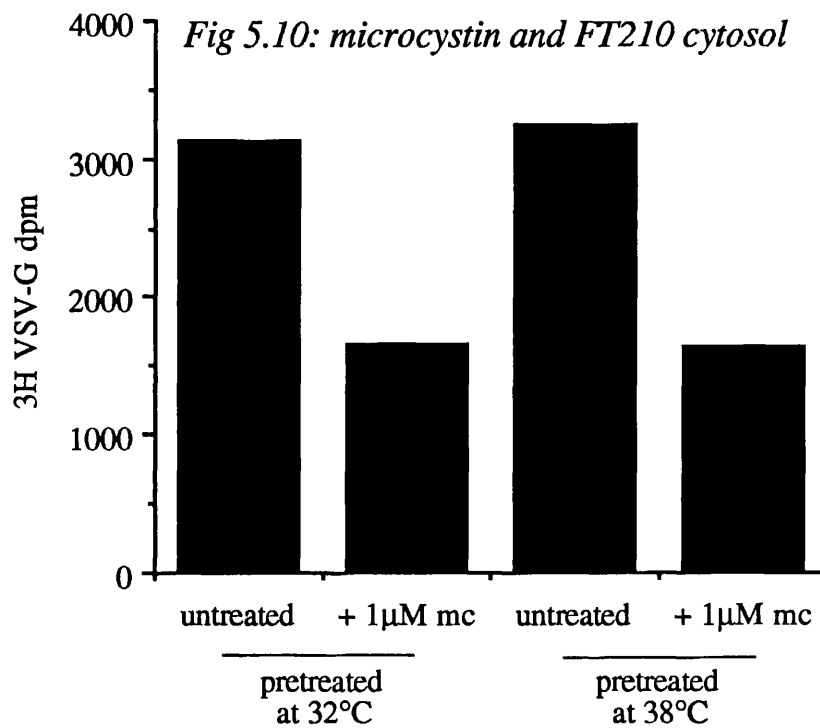


Figure 5.10: microcystin and FT210 cytosol. In this experiment, FT210 cytosol was added to reaction mixtures and incubated at either 32°C or 38°C for 10 minutes. Then all the preincubations were placed at 32°C, and microcystin was added to 1µM. After a further 30 minutes at 32°C, the reactions were moved to ice, K-Golgi added and standard transport assays performed.

5.5 Summary

The experiments in this chapter provided strong evidence that p34^{cdc2} kinase caused transport inhibition. Firstly, cyclin A added to cytosol was competed out by exogenous p33^{cdk2}; this showed that p33^{cdk2} was not the kinase on which the cyclin acted and, by elimination, indicated p34^{cdc2}. It also confirmed that the requirement for kinase activity was specific for p34^{cdc2}, and not for activation of any kinase in the cell. Secondly, the temperature-sensitive cell line FT210 was used to study the effect of ablation of mitotic kinase on the cyclin response of the cytosol. Heat-treatment of the cytosol abrogated its ability to respond to cyclin A, presumably because of inactivation of mitotic kinase, and this demonstrated that the inhibitory kinase was indeed p34^{cdc2}. The characterisation of the cytosol showed, however, that transport was inhibited at lower histone kinase activity than it was in a parallel HeLa cytosol, though exogenous S-phase kinase could cause strong elevation of histone kinase activity.

Experiments with the phosphatase inhibitor microcystin indicated that p34^{cdc2} was not directly responsible for transport inhibition. The evidence for this was that it was possible to inhibit transport with the phosphatase inhibitor microcystin in cytosol depleted of mitotic kinase activity.

Chapter 6

The target of mitotic kinase in the intra-Golgi transport assay.

6.1 Aims

In Chapter 3 it was shown that mitotic cytosol inhibited the intra-Golgi transport assay, particularly when the membranes used were salt-washed and thus dependent on cytosolic proteins for their activity. In Chapter 4 it was shown that the effect of mitotic cytosol was due to a mitotic phosphorylation event which could be mimicked by cyclin A-treated interphase cytosol or reversed by the kinase inhibitor staurosporine. In Chapter 5 data was presented which indicated that the mitotic kinase p34^{cdc2} was responsible for the transport effect, but probably not directly so. It was of great interest to identify the mechanism of mitotic transport inhibition. The data already available suggested that the effect was either due to a phosphorylation event involving a cytosolic transport protein, or due to an extremely rapid effect on the Golgi membranes.

It has been observed that many critical events in the transport assay take place within its first 30 minutes. For example, the early papers show that labelling of VSV-G proceeded with a lag of 7-10 minutes (Balch et al, 1984a), and indicate that a GTP γ S block could not be rescued by GTP after 10 minutes, indicating that ν ^{GTP γ S} had been incorporated into ν ^{GTP-binding}-proteins by that time (Melançon et al, 1987). Also, studies using the budding inhibitor primaquine, and observations of the LCRI (which is a post-fusion kinetic stage of the assay) indicate that budding and fusion have half-times of 10 and 25 minutes respectively (Hiebsch et al, 1991; Wattenberg et al, 1986). Kinetic studies have also suggested that NSF and SNAP are incorporated into transport vesicles (Wattenberg et al, 1992). All this taken together suggests that, if mitotic cytosol were to have a direct effect on membranes, it would have to act within 10 minutes to affect vesicle budding, and within 30 minutes to affect vesicle fusion (assuming transport at 37°C). Since mitotic cytosol exerted its strongest effect on membranes washed free of cytosolic contaminants, it seemed that if a kinase was being activated to generate a late effect on Golgi membranes, it would have to be on an integral membrane protein normally obscured by peripheral membrane proteins. It was felt that the most likely target of the mitotic kinase was a cytosolic transport protein, possibly one that cycled between the membranes and cytosol. Additionally, since it was hypothesised in the Introduction that Golgi transport is inhibited by inhibition of vesicle fusion (thus leading to Golgi breakdown), the target might serve a function connected with uncoating or docking of vesicles.

This Chapter lists various types of experiment designed to indicate the mechanism of transport and the target of the mitotic effect. Some are based on the assumption that the inhibition of transport requires only the activity of mitotic kinase on a cytosolic transport component, without the presence of membranes. An example of this is the 'rescue' experiment, where the kinase activity of mitotic cytosol is blocked, and then agents added to it in an attempt to restore transport activity. Others are based on the assumption that inhibition must act at some site on the membranes, and these use various methods both physical and pharmacological to separate the activities of donor and acceptor membranes. Some of these experiments also gave information on the mechanism of transport by the unwashed and salt-washed membranes. No attempt has been made to separate this information from that about mitosis, since the two strands of thought cross later in the chapter.

6.2 Effects on GTP-binding proteins

6.2.1 Effect of GTP γ S

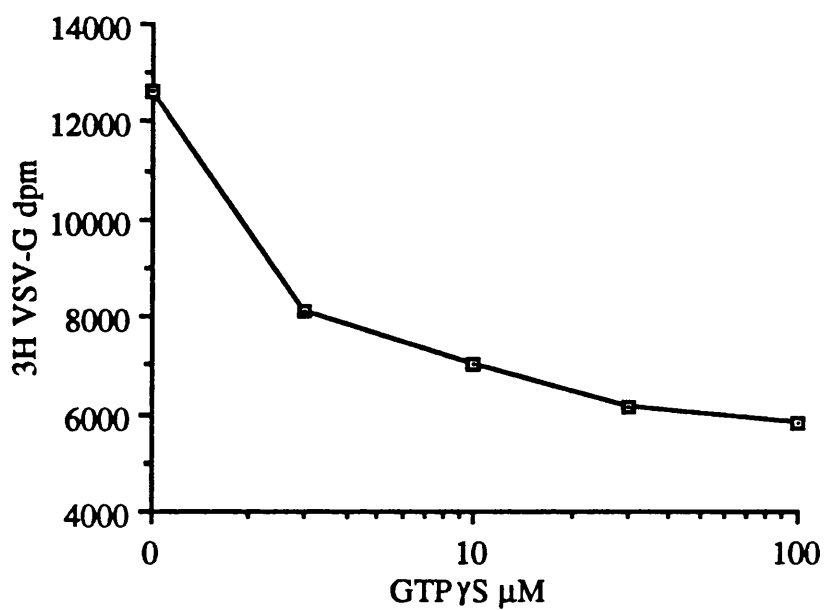
ARF, which is a 21kD GTP-binding protein, is believed to participate in the cycle of vesicle budding and fusion. It is essential for assembly of the vesicle coat and, in the GTP-bound state, forms a part of the coat complex. When the vesicle is uncoated before fusion with its target membrane, the coatomer dissociates from it in a process requiring hydrolysis of ARF-bound GTP.

The GTP analogue GTP γ S cannot be hydrolysed, and it irreversibly inhibits ARF from dissociation from membranes (Donaldson et al, 1992; Helms and Rothman, 1992). This results in accumulation of COP-coated transport vesicles in the transport assay. To test the effect of GTP γ S on normal and salt-washed Golgi, a titration of GTP γ S was prepared in transport assays containing each kind of membrane, and incubated for 60 minutes at 37°C for non-salt washed Golgi, and for 120 minutes at 30°C for salt-washed Golgi (Figure 6.1). When the two assays were compared, it was found that both kinds of assay were inhibited by GTP γ S. The inhibition is not extreme (perhaps because of low levels of GTP remaining in the cytosol after desalting). Inhibition was maximal at 30-40 μ M of the drug, but the salt-washed Golgi assay was marginally more inhibited than the 'normal' assay. This suggests that both forms of transport proceed via coatomer- and ARF-associated stages, but does not prove it.

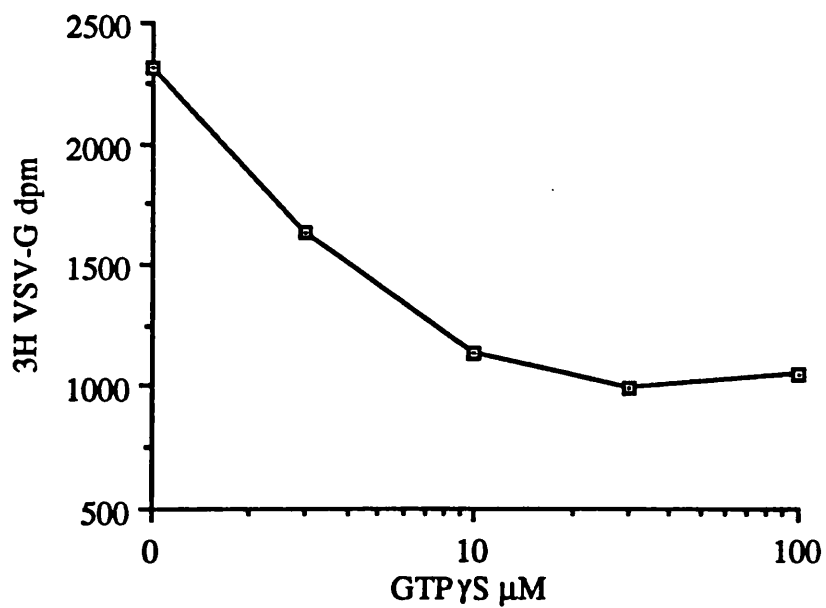
Figure 6.1c shows that both mitotic and interphase transport were inhibited by GTP γ S. In an interphase reaction, the drug had little effect on transport when added 20 minutes into the assay. A mitotic assay was still inhibited at that stage. This suggests that in the mitotic system, the formation of coated vesicles proceeded more slowly than in interphase, where the budding stage of the reaction was apparently substantially complete after 20 minutes.

Figure 6.1: effect of GTP γ S on transport

a: unwashed Golgi



b: salt-washed Golgi



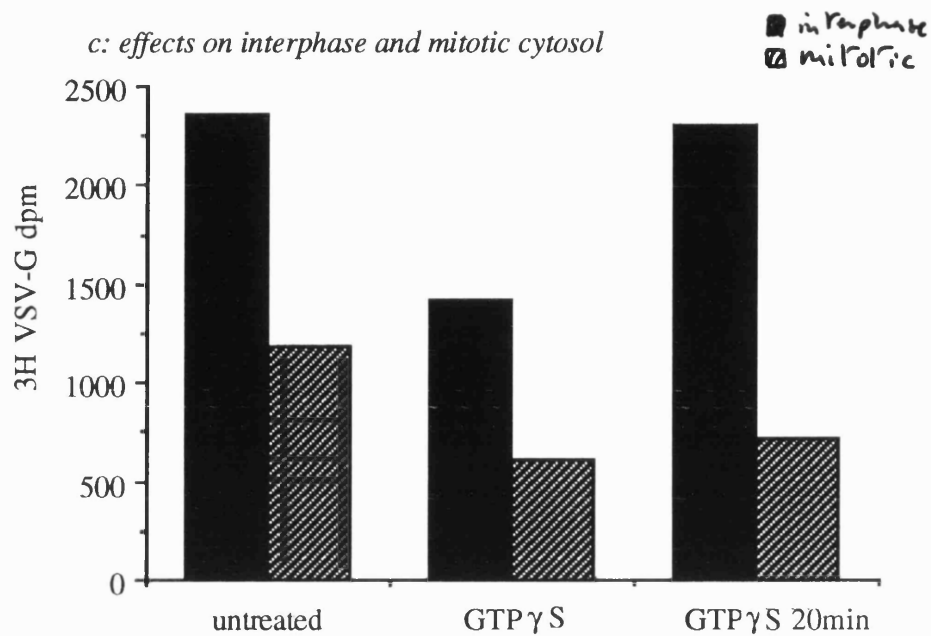


Figure 6.1: effect of GTP γ S on transport. The compound was made up at 100mM in 10mM Hepes/KOH, 1mM DTT and either used fresh or stored for up to a few weeks at -80°C. It was diluted into transport buffer just before addition of cytosol and membranes or, in (c), added just before the start of the transport assay or 20 minutes into it. The tubes were not removed to ice for late addition of the GTP γ S.

6.2.2 Effect of *AlF_{3.5}* and mastoparan

As described in the Introduction, it has lately been shown that exocytic transport may be controlled by trimeric G-proteins. Aluminium fluoride irreversibly inhibits trimeric G proteins (Kahn, 1991), and inhibits the intra-Golgi transport assay (Melançon et al, 1987) and cell-free transport between the ER and the Golgi apparatus (Schwaninger et al, 1992).

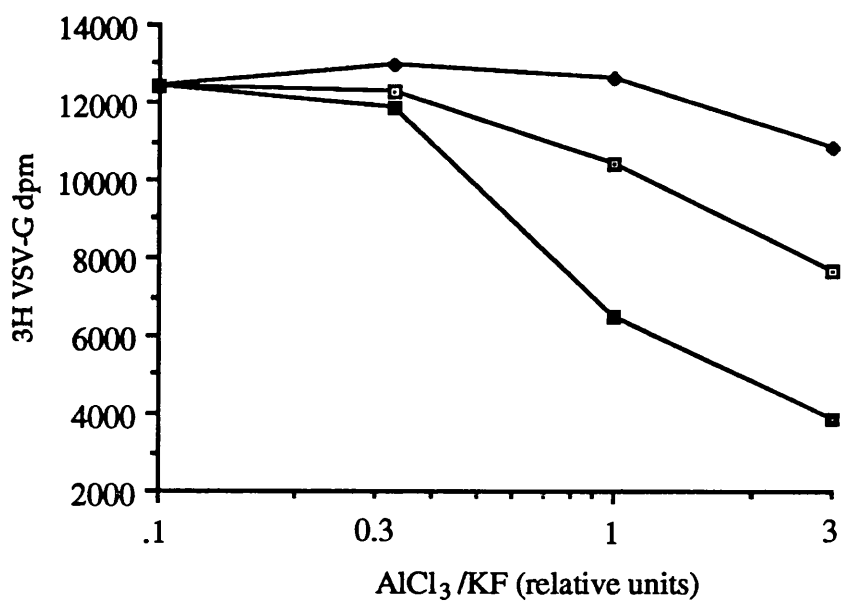
Mastoparan is an amphiphilic peptide (Hirai et al, 1979) which is believed to associate with and activate G α G-protein subunits in the same way as G-protein-coupled receptors (Higashijima et al, 1990). It was shown to promote association of β -COP with Golgi membranes and thereby antagonise the effect of BFA on cells, while cells pretreated with pertussis toxin lost the protection of mastoparan (Ktistakis et al, 1992).

The trimeric G-protein G α_{i-3} was shown to colocalise with Golgi mannosidase II of epithelial cells by immunofluorescence, and its overexpression found to reduce the rate of constitutive transport of heparan sulphate glycoprotein, in a pertussis toxin-sensitive manner (Stow et al, 1991). These findings suggested that high activity of trimeric G proteins may reduce transport through the Golgi apparatus.

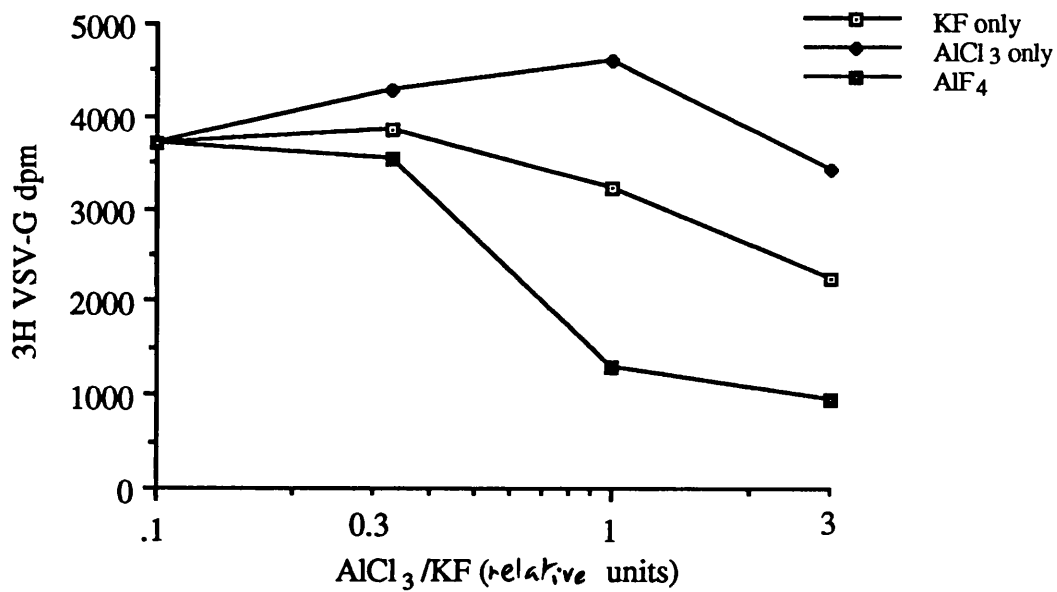
Aluminium fluoride was titrated into transport assays containing either salt-washed or unwashed Golgi membranes, and interphase cytosol. As seen in Figures 6.2a and 6.2b, the compound had very similar effects on both types of assay. Aluminium chloride at equivalent concentrations was almost without effect, whereas potassium fluoride slightly inhibited transport, most likely because of traces of aluminium salts leached from glassware into the transport buffer. Aluminium fluoride inhibited transport to the same proportional extent in both assays, and this indicated that trimeric G-proteins are required for transport in both normal and salt-washed Golgi transport assays.

Figure 6.2c shows the result of an assay of aluminium fluoride in relation to some cytosol conditions. The compound was added at 50 μ M to interphase or cyclin-treated interphase cytosol, or to mitotic or staurosporine-treated mitotic cytosol. It did not affect the histone kinase activities of any of these cytosols (data not shown). It inhibited transport in all conditions of cytosol to a very similar proportional effect, and this indicated that the role of the *AlF_{3.5}*-sensitive protein in transport was not influenced by the phosphorylation state of the cytosol, and hence was probably not a target for mitotic inhibition. In contrast to the situation with GTP γ S, in both interphase and mitotic assays, there was still substantial transport inhibition after 20 minutes (data not shown), which suggested that the requirement for the *AlF_{3.5}*-sensitive factor was not restricted to the early part of the transport process.

Figure 6.2: the effect of aluminium fluoride on the transport assay
 a: unwashed Golgi



b: salt-washed Golgi



c: effect of AlF_4 on different cytosols

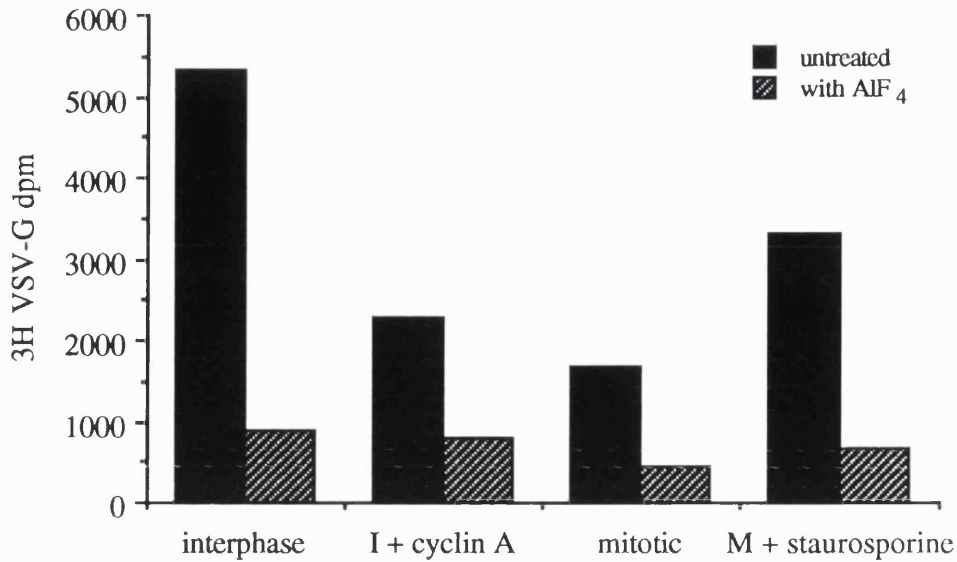


Figure 6.2: the effect of aluminium fluoride on transport. The compound was prepared by mixing $AlCl_3$ (100mM stock) and KF (500mM stock) at the required concentration. In a. and b., the standard concentration (arbitrarily fixed at 1) was $50\mu M$ $AlCl_3$ or 3mM KF, or both. This was added to transport assay mixtures before interphase cytosol and membranes, and transport was assayed as appropriate. In c., the agent was added at a final concentration of $50mM$ $AlCl_3$, 3mM KF to transport mixtures with cytosol (cyclin A being maximal, and staurosporine at $1\mu M$). The mixtures were incubated for 10 minutes at $37^\circ C$ and then salt-washed membranes were added and transport assayed. Histone kinase assays were performed on samples withdrawn 20 minutes into the transport assay, and activities were similar in the presence or absence of the compound (data not shown).

Fig 6.3: effect of mastoparan 1 on interphase and mitotic transport

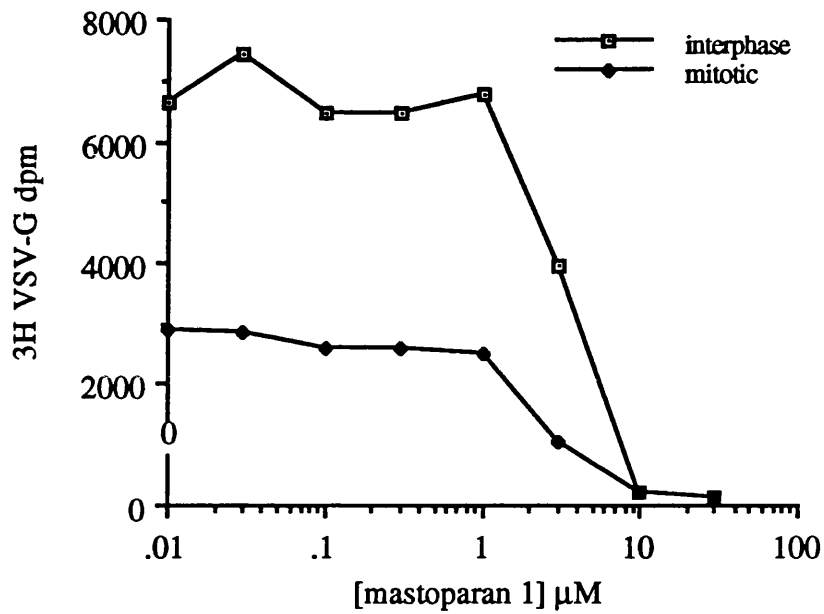


Figure 6.3: effect of mastoparan on interphase and mitotic transport. The peptides were kept as a 2mM stock at -80°C and thawed shortly before use. They were diluted into transport buffer before addition of cytosol (without preincubation) and membranes.

To test the effect of mastoparan 1 on the intra-Golgi transport assay, the peptide was diluted from a 2mM aqueous stock into transport buffer, to concentrations of 30nM to 30μM. Interphase or mitotic cytosol was added, and finally Golgi membranes, and standard transport assays were performed. The effect of mastoparan on non-salt-washed Golgi is shown in Fig 6.3 (the effect was similar on K-Golgi; see Figure 6.4).

The effect on unwashed and salt-washed, interphase and mitotic samples was the same: at 10μM mastoparan 1, transport was inhibited more severely than by any other agent tested; the signal was reduced to less than normal background levels (ie tests lacking donor membranes). The proportional inhibition of interphase and mitotic samples by low levels of mastoparan was identical.

The total and ineluctable loss of transport due to mastoparan, far greater than with AlF₃₋₅, might be attributable to a detergent effect of the peptide upon the Golgi membranes. To test this, other peptides were added to transport assays. Figure 6.4a shows that mellitin, HR1 and HR2 (which affect cellular functions-mellitin as bee venom and HR1/2 as mast cell degranulation peptides: reviewed in Mousli et al, 1990) all inhibited transport at micromolar concentrations. However, mastoparan 17, a weakly-active analogue of mastoparan 1, showed less devastating effects on transport than the parent compound (Fig 6.4b). This might indicate specificity of the effect of mastoparan 1, but might simply reflect a weaker detergent effect (see Mousli et al 1990), considering the range of other peptides which also acted on the assay. The only agents capable of inhibiting transport this far were organic solvents or detergents at 1% w/v in the assay. Having said that, experiments by Ktistakis et al (1992) did show that mastoparan 1 was much more effective than GTPγS in antagonising the effect of BFA on β-COP binding to membranes.

The experiments with mastoparan were abandoned at this stage. The transport assay seemed ineffective to study what appeared to be a detergent effect which disabled the assay, without any detectable difference between washed and unwashed Golgi, or interphase and mitotic cytosol. In order to proceed further, it would probably have been necessary to prepare both membranes and cytosol in the presence of pertussis toxin and study intra-Golgi transport under those conditions, involving a good deal of calibration for possibly little result.

Figure 6.4: effects of cytotropic peptides on transport.

a) effects of mellitin, HR1 and HR2. Melittin (Mel) at 10mg/ml (3.5mM); HR1 and HR2 at 3mg/ml (2mM), mastoparan 1 (Mas) at 20mM; all at -80°C. The peptides were added to the assay mixtures before cytosol and membranes.

b) comparison of the potency of mastoparans 1 and 17: the peptides were diluted from 20mM and 10mM stocks respectively into assay mixtures just before the addition of cytosol and membranes.

Figure 6.4a: effect of cytotropic peptides on the transport assay

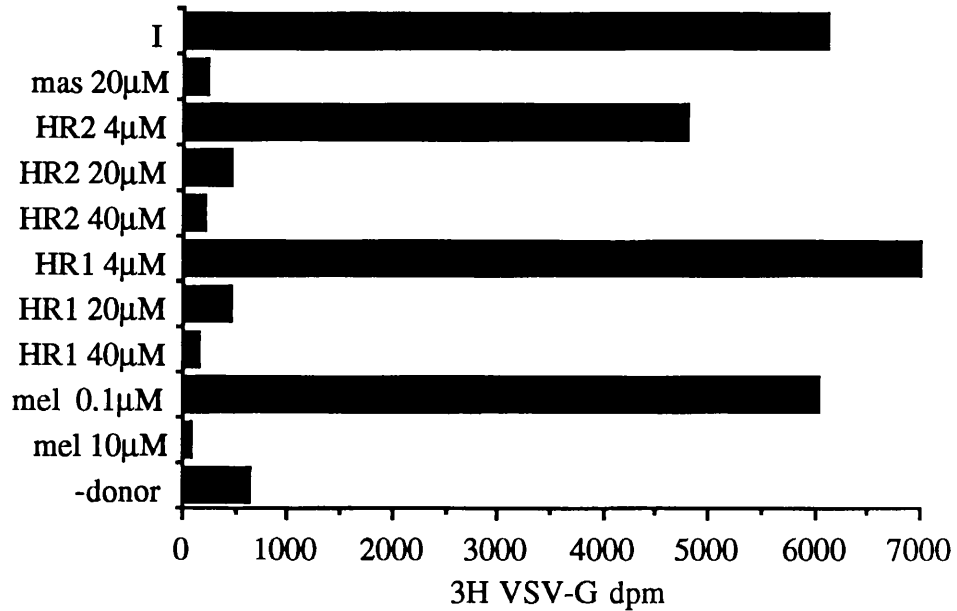
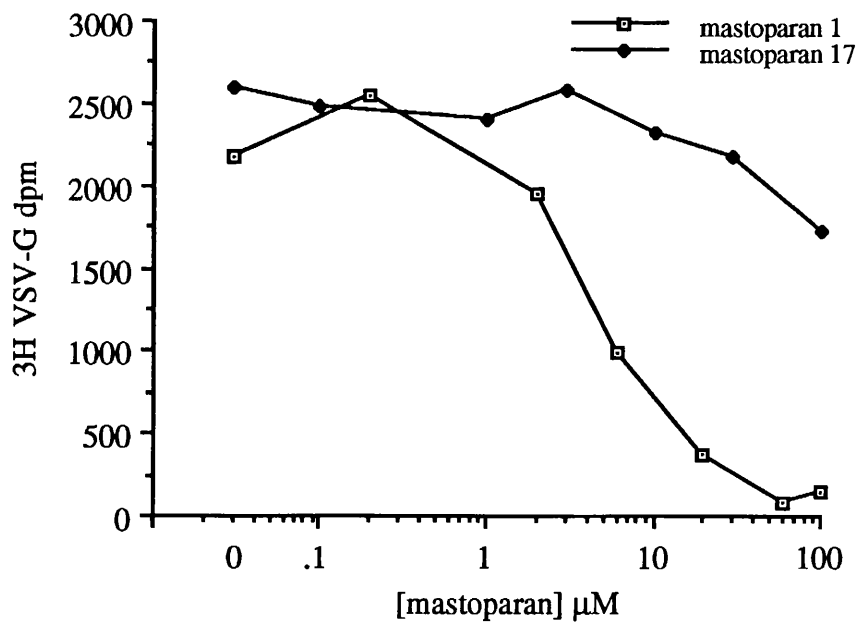


Figure 6.4b: comparison of mastoparan 1 and 17 effects on the transport assay



6.2.3 Effect of Brefeldin A

This fungal metabolite was found to cause the disappearance of the Golgi apparatus and the cessation of transport to the cell surface in cultured cells (Misumi et al, 1986) . Its action is inhibited by prior treatment with GTP γ S, which causes irreversible attachment of coatomer to the Golgi vesicle buds. In terms of the transport assay, one would expect that BFA would cause increased transport signal, because the Golgi compartments might fuse. Treatment with GTP γ S and then BFA would give rise to reduced signal, because uncoating and fusion of vesicles would be inhibited, and the action of BFA prevented.

The case for salt-washed Golgi is not so clear, since it has been suggested (see sections 3.6 and 6.1.3) that K-Golgi does not obtain enough coatomer from the cytosol to prevent non-specific fusion anyway. If that were true, BFA might have little effect on transport, but GTP γ S would also have little effect on transport, since there would be few coated vesicles whose uncoating would be inhibited. To examine the situation with the K-Golgi from this laboratory, transport assays were performed in the presence of interphase or mitotic cytosol, K-Golgi, and GTP γ S or BFA.

In this assay, interphase and mitotic incubations were treated with BFA or GTP γ S . These agents were added singly, or simultaneously, or one before the membranes and the other 15 minutes into transport. (by which time fusion events are apparently occurring in the salt-washed assay; see section 3.5.2). The results are shown in Fig 6.5.

Firstly, BFA caused slightly increased transport under interphase conditions, and this increase was blocked by simultaneous addition of GTP γ S. This is consistent with the data in section 6.2.3 in indicating that vesicles account for most of the transport in the K-Golgi in vitro assay. When a mitotic inhibition was treated with BFA, no increase in transport was observed. This can be interpreted to show that mitotic cytosol inhibited transport at a fusion stage, since if BFA could cause fusion of membranes, only a direct and final block on fusion could prevent its activity being seen. However, it may also be that mitotic cytosol acted very early, such that no structures of any kind became available for fusion, either with or without BFA. Thus, the experiment with BFA gave indications that the mechanism of this K-Golgi transport was vesicular, but could not give unambiguous results about the site of mitotic transport inhibition.

Figure 6.5: effect of brefeldin A on interphase and mitotic Golgi transport

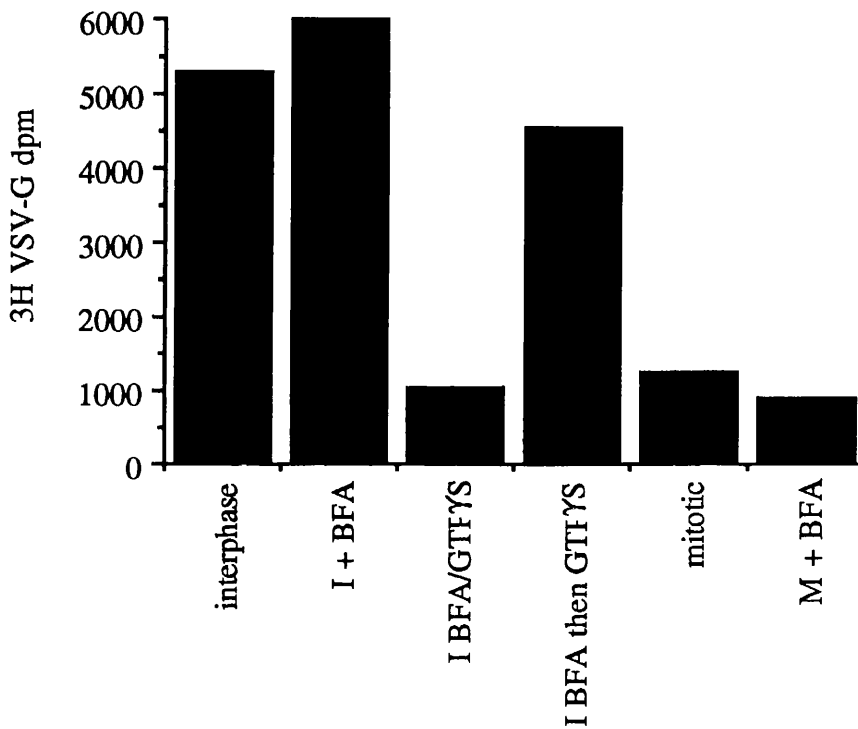


Figure 6.5: effect of Brefeldin A on interphase and mitotic transport. Salt-washed Golgi transport was performed with interphase or mitotic cytosol, some in the presence of BFA at 60μM and GTPγS at 50μM. Column 2 shows BFA added at the start of the assay, and Column 3 shows BFA and GTPγS added at the start of the assay. Column 4 shows BFA added at the start of the assay, and GTPγS added 15 minutes later. Column 6 shows a mitotic assay and column 7 one with BFA added at time zero.

6.3 Rescue of arrested cytosol

The purpose of the rescue experiments was to find out whether mitotic kinase caused the inhibition of a specific transport component. The rationale was to supply a transport-incompetent mitotic cytosol with interphase cytosol, to see whether the transport signal would then improve.

Normally, it would be relatively uninformative to incubate mitotic and interphase cytosol together, since the two have different kinase and phosphatase activities. In such mixtures, the mitotic and interphase cytosols tend to yield transport activity in proportion to their ratio, depending on the activities of different preparations (data not shown). In order to maintain the phosphorylation states of the cytosols, the mitotic cytosol could ^{be} treated with both staurosporine and microcystin, to inhibit the kinases and most of the phosphatases present. Then interphase cytosol could be added without either cytosol being able to affect the other by phosphorylation. Transport would be 'rescued' if a mitotic reaction could be supplied with transport activity by interphase cytosol and not by mitotic cytosol.

The assay was set up as follows. Interphase or mitotic cytosols were preincubated for 20 minutes at 37°C, then staurosporine and microcystin were added to 1µM. Then another assay mixture was added which had been preincubated at the same time as the first, containing either interphase or mitotic cytosol, or a 'blank' mixture containing neither cytosol nor membranes, but equivalent buffer. Then membranes were added to the mixture, at final volume 100µl, and transport was assayed.

This assay permitted study of the effect of interphase cytosolic components on a mitotic assay, without the accompanying problem of the two types of component interacting. Any observed effects were compared with those due to two mitotic cytosol equivalents or two interphase cytosol equivalents. The assay volume was doubled so that the volume of cytosol in an assay could be doubled without showing inhibition due to 'cytosol overdose' (the reduction in relative transport efficiency when cytosol forms more than approx. 10% of the assay volume: see Figure 5.4). The results are shown in Figure 6.6.

The first point to note is that mitotic cytosol treated with microcystin and staurosporine lost all histone kinase activity (data not shown), as did any cytosol incubated with 1µM staurosporine. Its transport activity, however, remained at a low mitotic level, showing that inhibition of phosphorylation led to the 'arrest' of the cytosol in a transport-incompetent state. In fact, in some assays, the transport of arrested mitotic cytosol did not remain low, but drifted up to 50% of interphase, an increase of some 10-15% in different assays, depending on the cytosol used (note that the Figure depicts an experiment where overall transport inhibition was not as great as in other cases, 55% as opposed to 75-80%).

Figure 6.6: 'rescue' of mitotic transport by interphase cytosol

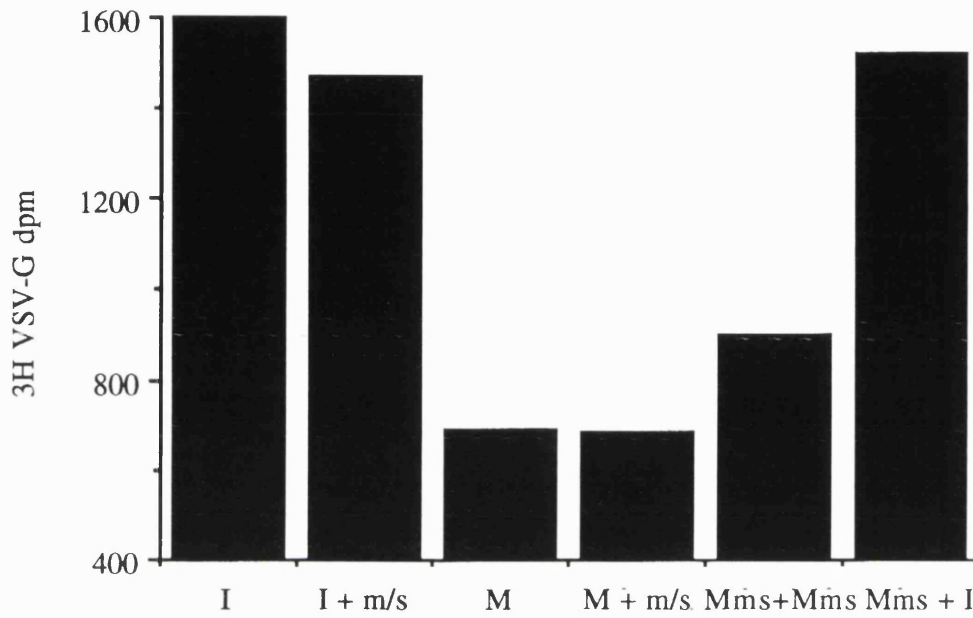


Figure 6.6: rescue of mitotic transport by interphase cytosol. Assay mixtures were prepared containing interphase or mitotic cytosol and preincubated for 20 minutes at 37°C. At the end of this time, the assays were returned to ice. Staurosporine and microcystin were added to 1µM final, from a 50µM mixture in water prepared shortly beforehand (designated arrested cytosol or Mms). Then another assay mixture was added which had been preincubated at the same time as the first, containing either interphase or mitotic cytosol, and 1M ST to make up volume for membranes (or a 'blank' mixture containing neither cytosol nor membranes, but equivalent buffer). Then membranes were added to the mixture, at final volume 100µl, and the whole assay was warmed to room temperature over 10 minutes and then incubated at 30°C for 2 hours. Each assay was immunoprecipitated in a volume of stop buffer sufficient for a doubled assay volume. I = *S. pombe* interphase cytosol; I+m/s = *S. pombe* interphase cytosol with just m/s before membrane addition; M = *S. pombe* mitotic cytosol; M+m/s = *S. pombe* mitotic cytosol + just m/s before membrane addition; Mms + Mms = 2 mitotic incubations containing just m/s; I+Mms = 1 interphase + 1 mitotic Mms-incubation. All assays contained 20µl membranes.

This loss of full mitotic nature might be due to incomplete inhibition of phosphatases. Alternatively, it may be that any cytosol with severely impaired phosphorylation control experiences loss of control of some phosphorylation-related pathways; this question was not addressed further.

When an arrested mitotic cytosol was supplemented with mitotic cytosol, its transport activity was scarcely elevated. However, addition of interphase cytosol caused almost complete recovery of transport.

This result strongly suggested either that all the components required for transport or transport inhibition reside in the cytosol, or that the mitotic cytosolic components interact with the membranes very rapidly in the timescale of the assay. Apparently, some transport component in mitotic cytosol was affected by phosphorylation, and could not be reactivated in arrested cytosol. However, the same component from interphase cytosol could 'rescue' the arrested mitotic cytosol.

6.4 Effect of purified transport components.

In the Introduction, it was described how a complex of NSF and SNAPs was implicated in intra-Golgi vesicle fusion, and quite likely in other vesicle fusion events. When the sequences of these proteins were examined, it was found that γ -SNAP and NSF bear weak and moderate cdc2 kinase consensus sequences, respectively (Dr. S. W. Whiteheart, personal communication). It seemed possible that one of these fusion proteins was the target of the mitotic inhibition, since that target appeared, from the 'rescue' experiments, to be a cytosolic transport component. NSF and SNAP proteins have been cloned and expressed in bacteria, and they were generously supplied to this laboratory by Dr S.W. Whiteheart in Prof. Rothman's laboratory. Experiments were designed to detect any possible recovery of mitotic transport by any of the proteins.

First, each of the components was titrated to find out whether they caused enhancement of transport. Each of the agents has an effect on the transport assay, but most particularly α -SNAP. Levels were suggested at which a SNAP- and NSF-dependent assay (Clary and Rothman, 1990) was supplied with the proteins sufficiently for interphase transport. NSF was required at 10ng/assay, and SNAPs at 50ng/assay; the different levels might reflect the difference between a 'catalytic' effect of NSF and a 'stoichiometric' effects of the SNAPs (S. W. Whiteheart, personal communication). Each agent was titrated separately into interphase and cyclin-treated interphase assays at levels spanning the concentration recommended, and the results are shown in Figure 6.7.

Each of the agents gave some enhancement to transport in both interphase and cyclin-treated interphase assays. The titrations showed that the recommended levels were not saturating for transport activation, so these were adhered to in further incubations (though the enhancement

of the assays did not plateau at the protein concentrations tested). However, it was also clear that there was little difference between the proportional activations of interphase and pseudomitotic transport. The whole complex, therefore, appeared to be very much limiting for the assay. NSF and α - and γ -SNAPs together could activate pseudomitotic transport to interphase levels, at concentrations lower than any of the proteins individually (Figure 6.7d). This result suggested that there was no difference (in terms of the fusion proteins) between interphase and cyclin-activated interphase cytosols; therefore, the pseudomitotic one must be inhibited in transport at another site. (It should be noted that these experiments had to be performed with great attention to detail, using matched preparations of membranes and cytosol, and yet experiments performed on different occasions could rarely be compared. This suggested that the different preparations contained different endogenous levels of the proteins.)

Next, assays were carried out to test whether arrested mitotic or pseudomitotic cytosol could be specifically activated by one or more of the fusion proteins. The protocol adopted was essentially that of a rescue experiment as described in section 6.3. In these experiments, the proteins were added (in a minimal volume) after any preincubations of cytosol, and after any inhibitors added, and before addition of membranes. The protein levels were fixed arbitrarily at those recommended by Dr. Whiteheart. Figure 6.8 shows the effect of the proteins on cyclin-activated (pseudomitotic) cytosol, with or without microcystin and staurosporine, while figure 6.9 shows the same data for interphase and mitotic cytosol. For convenience, the data are expressed both as recovered counts and as proportionate enhancement of transport relative to untreated samples.

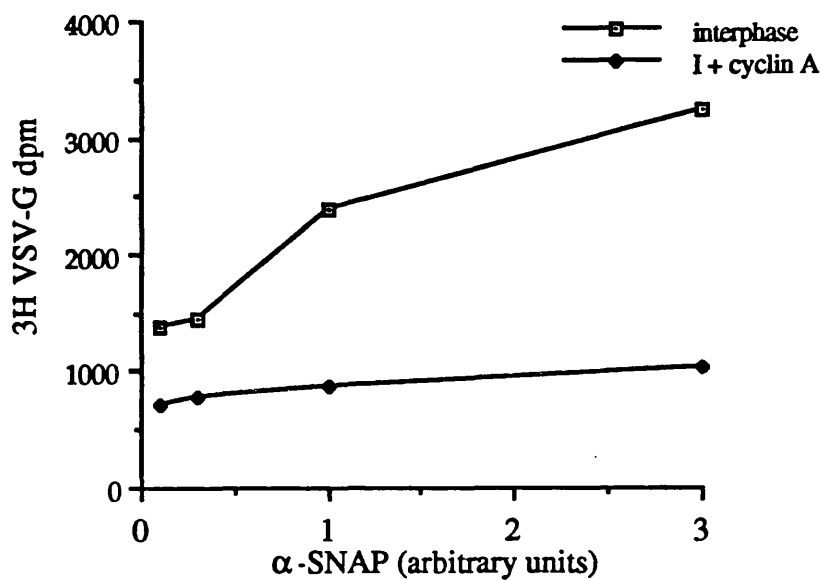
Figure 6.8 shows that the presence of cyclin in the cytosol made no difference to the proportional effects of the fusion proteins on transport, ie none of them either singly or together could rescue an arrested pseudomitotic cytosol. Figure 6.9 shows that use of mitotic versus interphase cytosol did affect the activity of the exogenous proteins in the transport reaction. While the proteins (particularly α -SNAP) enhanced interphase transport, they enhanced mitotic or arrested-mitotic transport by approximately 20% of the interphase level. As for the pseudomitotic assays, no conditions were found where transport was specifically enhanced under 'arrested' conditions; such assays were uniformly enhanced by addition of SNAPs and NSF despite their high histone kinase activity.

These results did not indicate any of the fusion proteins as the component of interphase cytosol which rescued an arrested mitotic cytosol, and they did not indicate whether the site of mitotic transport inhibition was before or after their point of action. However, the difference between mitotic and cyclin-treated interphase cytosol suggested that the two inhibited transport at different sites.

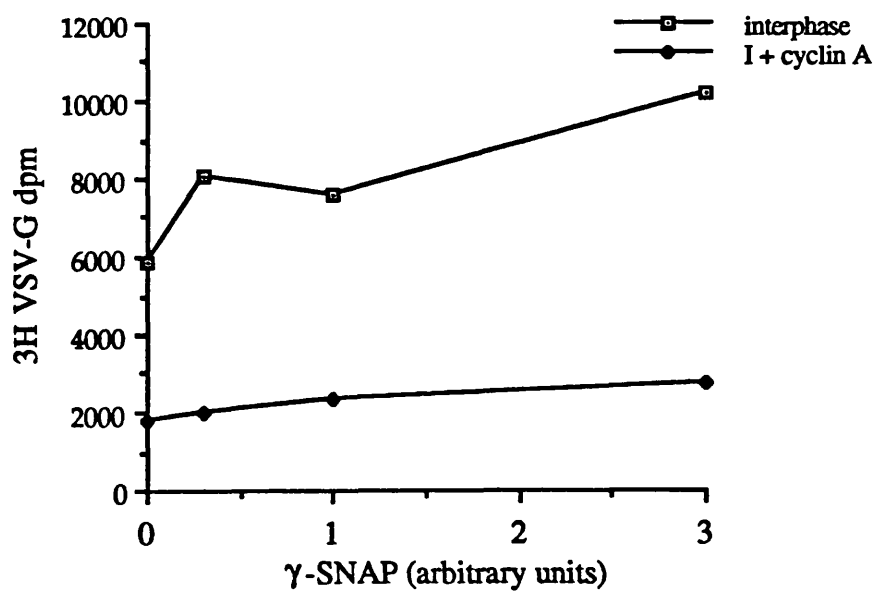
Figure 6.7: effect of SNAPs and NSF on interphase and cyclin-treated cytosol. Transport assays were prepared containing interphase cytosol with or without cyclin, and preincubated for 20 minutes at 37°C. Then, on ice, the proteins were added from stocks held at -80°C in KTDM (50mM KCl, 10mM Tris, 1mM DTT, 5mM MgCl₂). They were diluted as necessary into BS containing 0.1mg/ml soybean trypsin inhibitor as a carrier protein. After addition of proteins, transport was assayed for 120 minutes at 30°C. The recombinant proteins were supplied at the following concentrations: NSF, 1.3mg/ml; α-SNAP, 0.5mg/ml; γ-SNAP, 0.65mg/ml . They were added to the assay at: NSF - 10 ng = 1 arbitrary unit; SNAP - 50 ng = 1 arbitrary unit.

Figure 6.7: effect of SNAPs and NSF on interphase and cyclin-treated cytosol

a: *alpha*-SNAP



b: *gamma*-SNAP



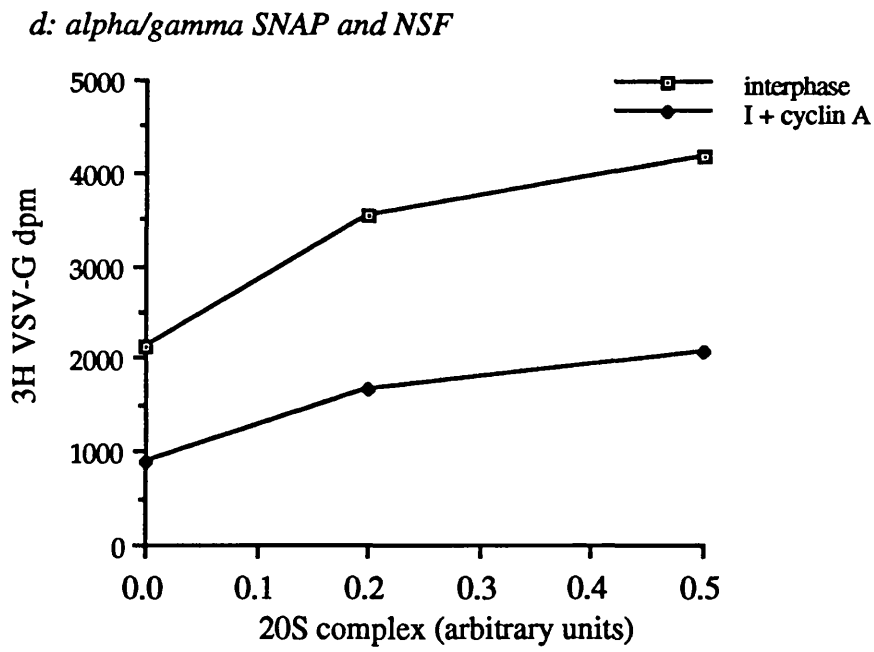
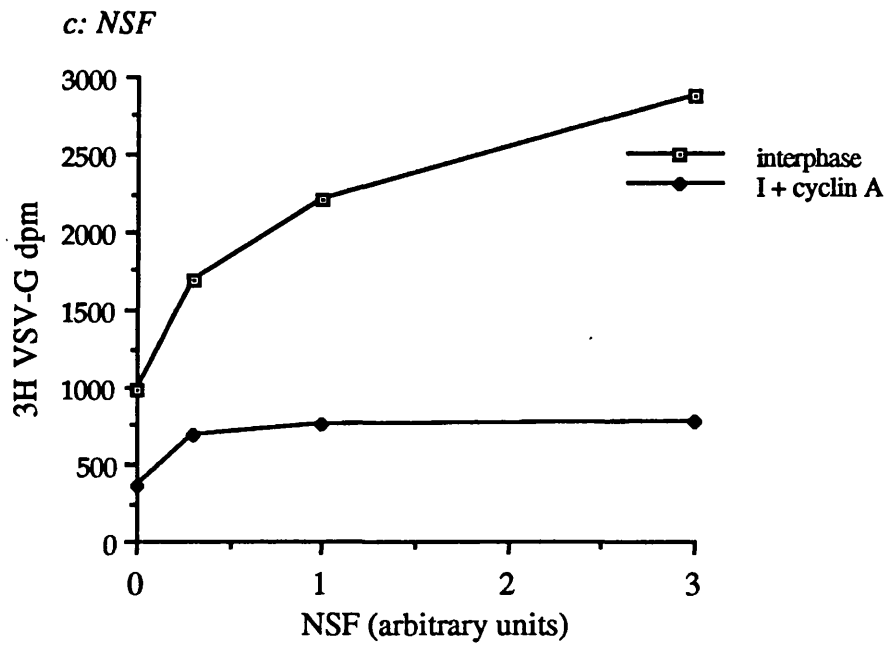
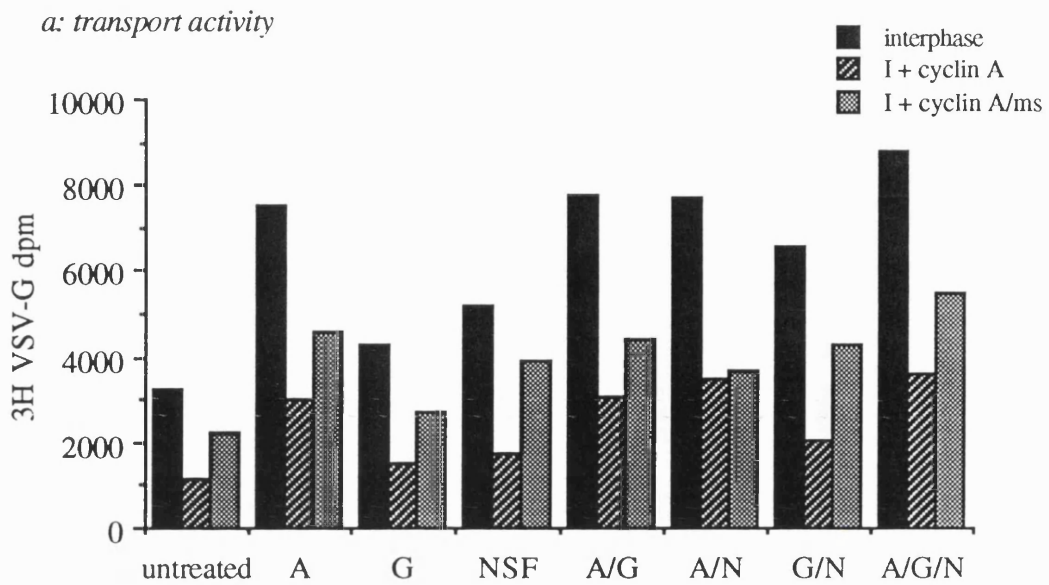


Figure 6.8: effect of transport proteins on interphase and cyclin-treated cytosol (with microcystin and staurosporine). Assays were prepared containing cytosol (30 μ g) with or without cyclin, and preincubated for 20 minutes at 37°C. Then, on ice, microcystin and staurosporine were added from a premixture to a final concentration of 1 μ M, also containing 1mM EGTA. Then proteins were added from dilutions in BS with soybean trypsin inhibitor. Membranes were added and transport assayed for 120 minutes at 30°C. Abbreviations: alpha-SNAP, A; gamma-SNAP, G; NSF, N.

ns, 1 μ M microcystin + staurosporine

Figure 6.8: effect of transport proteins on interphase, cyclin-treated and arrested cytosol



b: transport enhancement

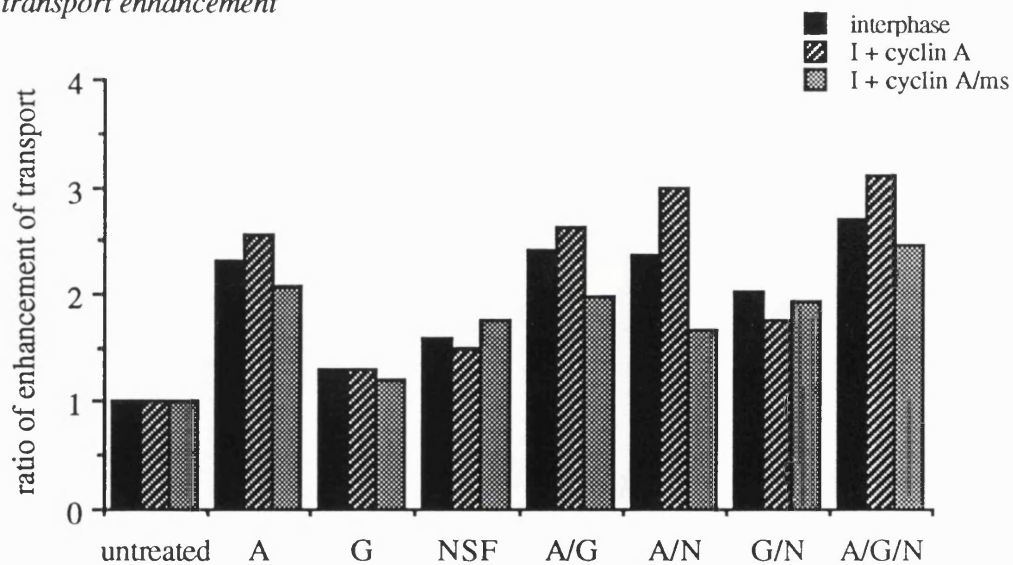
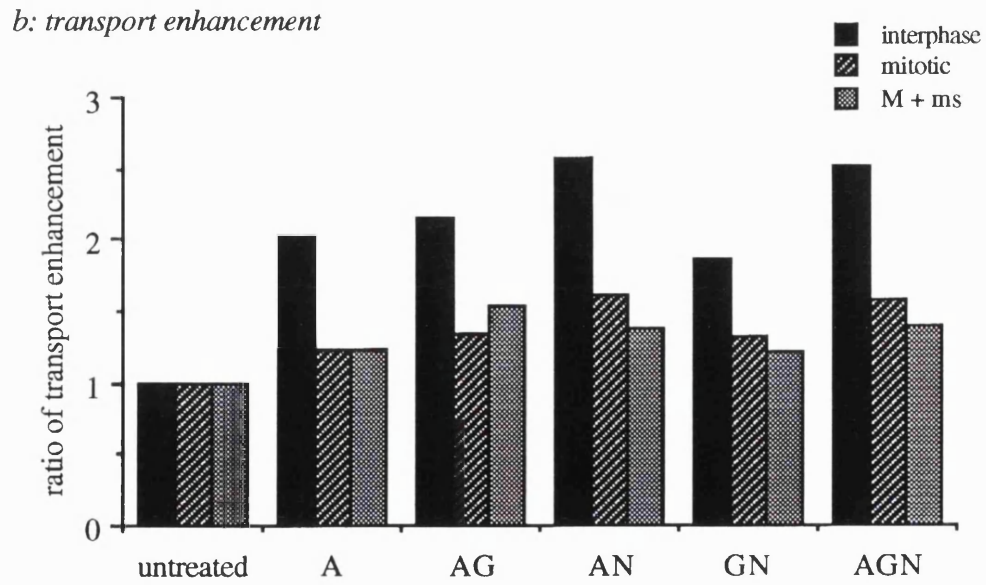
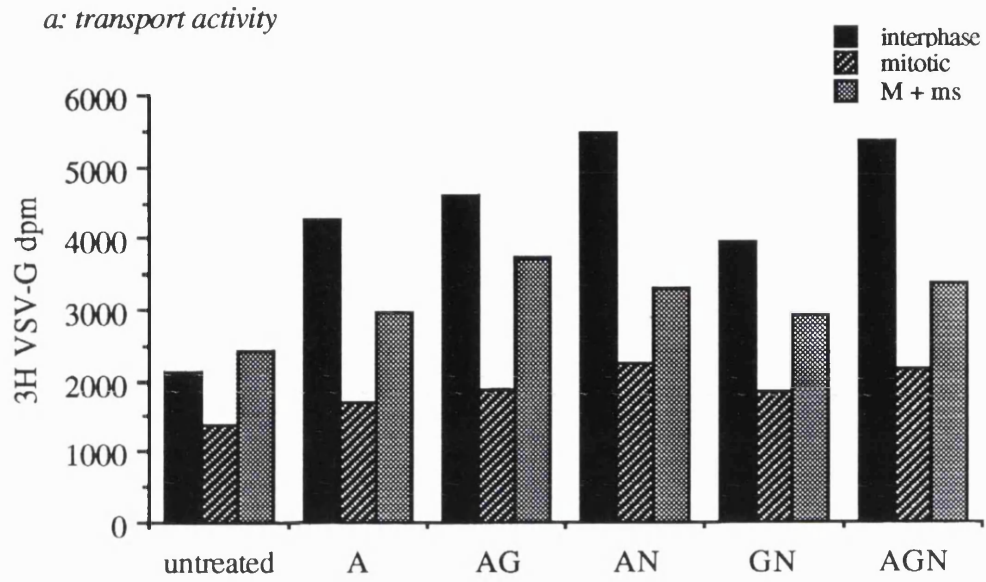


Figure 6.9: effect of transport proteins on interphase and mitotic cytosol (with microcystin and staurosporine). Assays containing cytosol were prepared and preincubated for 10 minutes at 37°C. Then, on ice, microcystin and staurosporine were added from a premixture to a final concentration of 1µM, also containing 1mM EGTA. Then proteins were added from dilutions in BS with soybean trypsin inhibitor. Membranes were added and transport assayed for 120 minutes at 30°C.

Abbreviations: alpha-SNAP, A; gamma-SNAP, G; NSF, N.

ms, 1µM microcystin + staurosporine

Figure 6.9: effect of transport proteins on interphase, mitotic and arrested cytosol



6.5 Tests of effect of mitotic cytosol on each membrane.

A relatively naïve interpretation of the effect of mitotic cytosol on transport is that it altered the function of the donor or the acceptor compartment. This suggestion would assume that the cytosol did contain a component which interacted with one membrane or other; for example, a cdc2-activated kinase which acted directly on a budding or fusion protein. In order to detect such an effect, it would be necessary to treat the membranes separately with mitotic cytosol, and then assess the effect on transport with the other component interphase-treated. To prevent the disruption of any effect generated, the completed assay would have to be treated with microcystin and staurosporine as described for the 'rescue' experiments in section 6.3. The experiment was designed as follows.

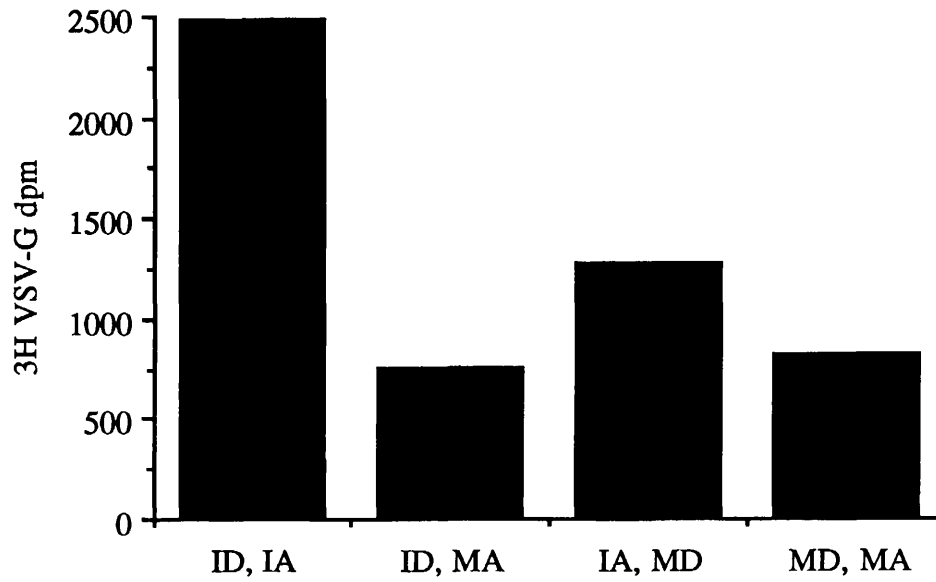
Reaction mixtures were prepared containing interphase or mitotic cytosol. To these were added 10 μ l of one or other membrane, or 5 μ l of each in control samples. These 'half-reactions' and the controls were incubated at 30°C for a short time, then removed to room temperature, and microcystin and staurosporine were added to 1 μ M final from a 50mM mixed stock. Then whole reactions were made by mixing 25 μ l portions of the halved ones. Fig 6.10 shows the result of such an experiment. The first point to note is that reactions that were divided for a short interval gave less than 50% the transport of the whole controls. The reason for this is unknown, but it is likely that after even 15 minutes of transport, a proportion of the transport vesicles were already committed to 'silent' events within their own membrane type, and thus were unavailable for detectable transport events. The time course of the transport assay showed that glycosylation of transported G protein was detectable after as little as 20 minutes, so it is not unreasonable to suggest that some transport vesicles were 'committed' before this time. The longer the donors and acceptors were separated, the more extreme this condition. Fig 6.10b shows the effect of separating donor and acceptor membranes for 30 minutes of the transport reaction. The second point to note is that, when acceptor membranes only were incubated with mitotic cytosol, the transport was slightly lower than when donor membranes were incubated in mitotic cytosol. This effect was small but reproducible, and 6.10b shows that the effect became more pronounced with time, though at the expense of the transport signal.

Figure 6.10: effect of mitotic cytosol preincubations on separated membranes. Here, standard reaction mixtures were prepared containing interphase or mitotic cytosol. To these were added 10 μ l of one or other membrane, and the assays were incubated for the times specified at 30°C. Then, without chilling the reactions, microcystin and staurosporine were added to them at 1 μ M. Then, 25 μ l lots of donor- and acceptor-containing reactions were mixed, and the control reactions were also pipetted, so that all samples contained equal volumes of membrane and were treated equally. The incubation proceeded for a total of 120 minutes.

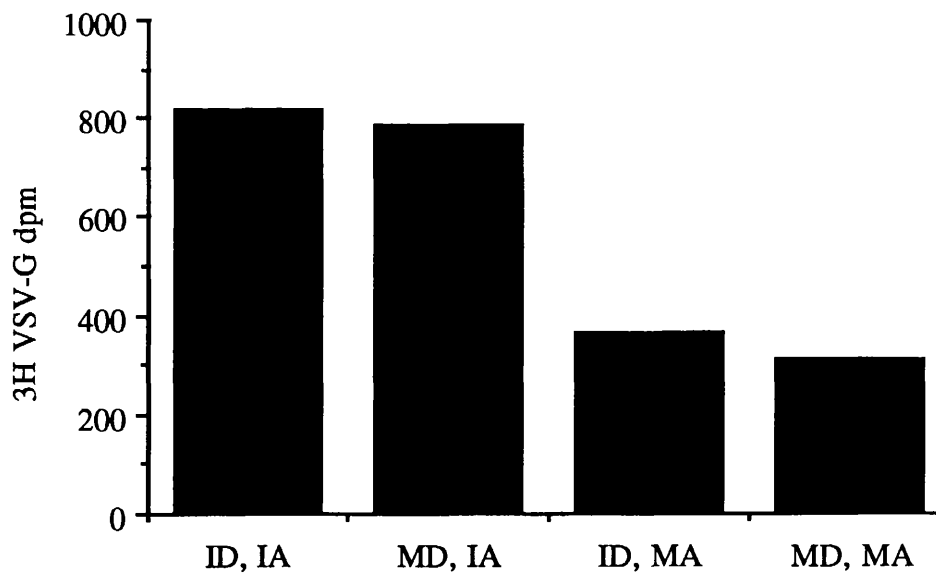
I = interphase-treated
M = mitotic-treated
A = acceptor membranes
D = donor membranes

Figure 6.10: effect of mitotic cytosol preincubations of separated membranes

a: 15 minute preincubation



b: 30 minute preincubation



This result can be interpreted in two opposite senses. It is likely that incubating acceptor membranes with mitotic cytosol renders them inimical to incoming vesicles. However, it is also possible that donor membranes incubated with mitotic cytosol do not generate vesicles, but when they come into contact with interphase cytosol they are enabled to generate those vesicles, so that the resultant transport signal is elevated. Given that vesicle formation is known to occur in mitotic incubations (T. Misteli, unpublished data) the first interpretation is the more likely, but this experiment alone offers no strong interpretations.

The third point about this experiment is that it worked in the same way with salt-washed and unwashed Golgi membranes (data not shown). It therefore supports the suggestion that transport uses the same mechanism (probably vesicular) in unwashed and salt-washed Golgi. Alternatively, if K-Golgi really undergoes unregulated budding and tubulation of membranes, then the fusion of those membranes is regulated in a similar way to 'normal' fusion in the transport assay. Also, it shows that if K-Golgi does undergo non-vesicular transport, the number of such fusion events is limited in the same way that the 'normal' transport assay is known to be limited to a single round of transport. If the K-Golgi assay were totally unregulated, then it might well show no fall in transport due to donor-acceptor separation, but in fact it does so.

Summary

The experiments in this chapter asked questions about the mechanism of transport in the interphase and mitotic transport assays. First, it appeared from experiments with GTP γ S that the mechanism of transport was similar for salt-washed and unwashed Golgi membranes, in that both types of assay were equally inhibited by that compound. This information suggested but did not prove that small GTP-binding proteins such as ARF were required for transport. Aluminium fluoride, which does not act on small GTP-binding proteins, also inhibited the assay. This implicated a GTP-binding protein, potentially a trimeric G-protein, in control of the assay, though not in its response to the phosphorylation state of the cytosol.

An experiment with brefeldin A and GTP γ S showed that in mitosis, transport is unresponsive to BFA. Since this drug prevents vesicle coat formation and causes promiscuous membrane fusion, this result suggested that membrane fusion functions might be disrupted in mitotic cytosol, though it remained possible that vesicle budding was inhibited. However, experiments in which donor or acceptor membranes were preincubated in mitotic cytosol also gave an indication that acceptor rather than donor function was challenged in mitosis.

To indicate the target of mitotic cytosol, a 'rescue' experiment was designed to see whether interphase cytosol could supply the transport deficiency of arrested mitotic cytosol, and it did so. Three fusion-active proteins, α - and γ -SNAP and NSF, were added to assays containing cytosol in different states. None of them appeared to 'rescue' arrested cytosol. Indeed, they enhanced transport in equal proportion in interphase and cyclin-treated assays, which clearly indicated that such cytosol did not control transport through their inactivation. On the other hand, mitotic incubations were relatively unresponsive to added transport proteins, which suggests that it is capable of blocking the formation or consumption of vesicle fusion complexes.

Chapter 7: Discussion

7.1: Overview

This thesis set out to answer some questions about the Golgi apparatus. The main question was whether protein transport through the Golgi apparatus is inhibited in mitosis, like virtually all other stages of membrane traffic studied to date. Subsidiary to that was the question of whether transport was inhibited directly or indirectly by the kinase activity of p34^{cdc2}. Also, given that the Golgi dissociates into vesicles in mitotic cells, it was of interest to see whether transport inhibition was connected with the observed breakdown of the organelle. The suggested link between inhibition of transport vesicle fusion and Golgi vesiculation gave rise to the possibility that a fusion protein would be found to be the target of mitotic inhibition.

The questions were tackled using the intra-Golgi transport assay. The reasons for this were twofold. Firstly, it is known that transport between the ER and the Golgi is inhibited in mitosis, and therefore *in vivo* studies might not distinguish between genuine intra-Golgi transport inhibition and a knock-on protein starvation from the ER. An *in-vitro* assay could be used to study Golgi transport events in isolation. Secondly, the well-characterised Rothman transport assay provided an experimental framework for studying the molecules involved in transport inhibition. Purified away from full cellular control, it was possible that the mechanism of the inhibition would be simpler to manipulate and dissect.

The results in Chapters 3-6 provide partial answers to the questions about Golgi activity in mitotic extracts. The transport assay was found to be inhibited in mitotic cytosol, in a manner apparently dependent on phosphorylation and p34^{cdc2}. Also, there were several results consistent with membrane fusion being the target of the effect.

7.2: The transport assay

The intra-Golgi transport assay, which generated almost all of the data in this thesis, is a well-established tool for studying many aspects of transport. As shown in the first part of Chapter 3, the properties of the assay were much as established by the Rothman laboratory. The assay has been refined to a high sensitivity and reproducibility, and that fact meant that the assay could be used without an extensive preamble of characterisation. The other luxury of the assay was that the effects of various drugs and active proteins had previously been studied, so that it was relatively simple to extrapolate the experiments to the mitotic system. In the section below, some of the problems encountered with interpreting the assay will be described. The problems frequently led to broader understanding of the system as a whole.

(i) the nature of the assay

The assay required isolated Golgi membranes, and a high-speed cell supernatant termed cytosol. The membranes are somewhat purified by enzymic criteria, but under the electron microscope they lack the elegance of the unmolested organelle. The donor cells are highly infected with VSV so that the Golgi contains high levels of the G protein. Experiments on semi-intact cells indicate that transport is a little slower *in vitro* than *in vivo*, and its overall efficiency is also reduced (Beckers et al, 1987).

The assay may also lack some level of cellular control. As mentioned in the introduction, Golgi transport is controlled by some or all of the following: rab proteins, trimeric G proteins, ARFs, cytoskeletal organisation, relative levels of resident proteins and cell cycle state. Membranes removed from this and placed in a dilute Tris solution may be easier to study than in the cell, but on the other hand, they may lose vital components of control mechanisms important for the understanding of the whole system. In addition, the cytosol added to the assay is a dilute version of the intracellular medium, and is gel-filtered to remove small molecules. The isolated cytosol presumably contains contaminants from disrupted organelles but lacks other sedimentable components which might be objects of fruitful study. The cytosol also presumably contains mitotic kinase, S-phase kinase, cyclins and a variety of other components of cell cycle control. It is curious that the cytosol as isolated contains almost no histone kinase activity, but is capable of strong activation by exogenous protein; it may be that these added proteins are not susceptible to control by the cytosolic machinery, by virtue of their bacterial origin, or are not susceptible to modulation in the time course of the experiment.

In general, the apparent tractability of the assay described here does not show that the assay also reflects the activity of the cytosol and the Golgi apparatus *in vivo*.

(ii) kinetics of the assay

The transport assay measures nothing more than glycosylation of the VSV-G protein. It provides little information about how the VSV-G protein gained access to the GNTI, nor about the rates of the steps involved. A simple timecourse of the assay (as in Chapter 3) shows that glycosylation of VSV-G commences after about 10-20 minutes, but kinetic experiments have suggested that fusion events are well advanced before glycosylation is marked. As mentioned in Chapter 6, the half-time of appearance of the LCRI (the 'low cytosol-requiring intermediate': actually vesicles fused with acceptor membranes) is 25 minutes, whereas glycosylation continues for over 30 minutes after that (Wattenberg et al, 1986). Within the framework of the transport assay it was clearly a problem being unable to dissociate budding and fusion events kinetically, since the only way of monitoring them was later than either.

The greatest kinetic limitation of the assay was that it was impossible to obtain information by incubating membranes for any length of time separately, or together in the absence of cytosol, or together in the presence of cytosol. In the absence of cytosol, the membranes lost transport capacity in a few minutes on ice, let alone at transport temperatures. In isolation from one another, the membranes probably conducted considerable transport, but in a manner unavailable for detection. If incubated together with cytosol, transport events commenced almost at once; it was technically very difficult to examine the effect of any treatment on the membranes without the presence or participation of the cytosol. Some experiments were performed where the membranes were preincubated and then centrifuged and transferred into different conditions, but the results of these were equivocal because of the sensitivity of the salt-washed membranes to centrifugation. Since it was likely that full transport inhibition required a combination of cytosolic and membrane-bound factors (as indicated by results like the sensitivity of the complete assay to cyclin A), this was a serious limitation to the assay's potential, and experiments with the membranes only partially circumvented these problems.

(iii) the nature of the fusion event for salt-washed Golgi

Research in the Rothman laboratory has led to the assumption that salt-washed Golgi, because it is highly limited in ARF, is incapable of supporting vesicular transport, and rather supports tubulation and uncontrolled fusion events. The data in this thesis suggested that the K-Golgi used here was capable of supporting transport in the same way as non-K-Golgi. The evidence for this is listed below.

a) both unwashed and washed Golgi could be inhibited by mitotic cytosol, though not to the same extent (Chapter 3); this suggests that they share at least one control mechanism.

b) both kinds of membranes showed equal transport inhibition in the presence of GTP γ S and aluminium fluoride (Chapter 6); GTP γ S inhibits vesicle uncoating and therefore suggests that vesicular transport is occurring, at least proportionately with the level of transport inhibition by the drug. The fraction of transport which is not inhibited by these agents may reflect the extent of non-vesicular transport.

c) both kinds of membranes suffered equally from separation early in the course of the assay (Chapter 6); if there were extensive unregulated fusion, the transport signal might not be greatly reduced by this treatment.

These results imply that vesicular fusion events were occurring, rather than the unregulated fusion one might expect from non-vesiculating membranes. In addition to this, experiments with primaquine (not shown) indicated that only a single wave of virus leaves the donor membranes at the outset of the assay.

Having said all this, these data are gleaned only from the transport assay, and the possibility remains that the K-Golgi do not perform transport like the unwashed membranes. If they do actually tubulate and fuse promiscuously rather than perform vesicular transport, the fact that their activity in the assay is reduced by mitotic cytosol is still of interest, since it strongly indicates membrane fusion as the site of the inhibition.

7.3: Mitotic inhibition of transport

Intra-Golgi transport was inhibited by mitotic cytosol in this cell-free system. This finding was the first direct proof that intra-Golgi protein transport, like other membrane traffic events, was cell-cycle sensitive.

Transport was most effectively inhibited with salt-washed Golgi, and reached 75-80% in favourable combinations of membranes and cytosol. It should be noted that the cytosol was potentially 75-80% inhibitory, but was derived from >95% mitotic cells. This indicates that the transport observed was not due to a residual percentage of interphase cytosolic activity, but was related to some other cytosolic characteristic. Considering that maximal inhibition by such agents as cyclin, or aluminium fluoride was also 70-80%, it is possible that the mitotic baseline represents inhibition of all vesicular or regulated transport, and leaves untouched only unregulated or non-vesicular transport (though it may instead represent a 'leakage' of interphase vesicular traffic through the mitotic block).

It was possible that the observed inhibition of transport was actually an inhibition of some process like sugar uptake of glycosylation which would lead to the same perceived effect, but this appeared unlikely. It has been shown previously that Golgi enzymes remain active during mitosis, so it was unlikely that apparent transport inhibition was caused by the cessation of glycosylation (Collins and Warren, 1992) Also, there was no detectable reduction in the association of UDP-GlcNAc with Golgi membranes incubated in mitotic cytosol (data not shown). Overall, it appeared that the acceptor membranes were capable of modifying VSV-G, but that VSV-G was not being exposed to GNT1 in the mitotic incubations.

The inhibition required cytosol with high histone kinase activity. To generate this, sHeLa cells were made mitotic by incubation in nocodazole for 24 hours. As described in Section 1.3.2, this treatment results in the degradation of all detectable cyclin A, and the histone kinase activity of the p34^{cdc2} is maintained by cyclin B alone (Pines and Hunter, 1990); D. Mundy, unpublished data).

The action of mitotic cytosol could be reversed by treatment with staurosporine, a nonspecific kinase inhibitor, and this led to the assumption that the kinase activity of the cytosol was the factor required for transport inhibition. The inhibition of transport by cyclin A treatment of interphase cytosol supported this suggestion, and also indicated that the kinase

involved was p34^{cdc2}, since that kinase alone is known to be activated by either cyclin A or cyclin B. The case for the involvement of phosphorylation in inhibition was strengthened by the staurosporine inhibition of cyclin A action.

Phosphorylation and transport - a link

The experiments with staurosporine and cyclin A indicated that the transport inhibition phenotype was not an 'all-or-nothing' event, but a titratable and reversible alteration. Also, it appeared that there was a tight correlation between the histone kinase activity of cytosol and its transport capacity. Mitotic cytosol, cyclin A-treated cytosol and staurosporine-treated cytosol showed similar transport inhibition at similar histone kinase activities. This observation suggests that mitotic cytosol and cyclin A-activated cytosol contain similar levels of a kinase which is acted on by staurosporine and capable of inhibiting the transport assay. That kinase, being present in mitotic cytosol and activated by cyclin A, may well be p34^{cdc2}. The critical importance of p34^{cdc2} was substantiated by showing that cytosol depleted of p34^{cdc2} did not show histone kinase activation or transport inhibition in the presence of cyclin A.

The idea of a reversible inhibition of transport by phosphorylation suggested that a vital transport event was under the control of a regulated kinase, similar to the control by p34^{cdc2} of the gamut of mitotic events. This comparison indicated that p34^{cdc2} might control mitotic Golgi vesiculation and transport inhibition directly, just as it has been shown to control nuclear structure and endocytic function directly. However, the fact that transport activity was inversely proportional to histone kinase activity does not prove that the histone kinase is directly responsible for transport inhibition. So far, the only proteins whose function is known to be altered by phosphorylation by p34^{cdc2} in vitro and in vivo are the nuclear lamins (Peter et al, 1990; Enoch et al, 1991), microtubules (reviewed in Karsenti, 1993) and rab4 (van der Sluijs et al, 1992). It was of great interest to try and find out whether there was a secondary kinase-phosphatase cycle dependent on p34^{cdc2}.

It should be noted that the lack of effect of staurosporine on interphase transport suggests that a kinase-phosphatase cycle is not required for normal turnover of transport components. Alternatively, it is possible that a kinase is required for transport but not inhibited by staurosporine; another possibility is that transport components are present in excess so that staurosporine could not inhibit overall transport by inhibiting their cycling (the fact that transport is limited for NSF and SNAPs argues against this, however).

Transport and phosphorylation: a missing link?

Very little information was obtained on a putative phosphorylation-dependent transport control mechanism. The clearest result was that mouse cytosol treated with a phosphatase

inhibitor showed inhibited transport without elevation of histone kinase activity. This is the in-vitro equivalent of the observation that cells treated with phosphatase inhibitors undergo several mitosis-like changes without histone kinase activation (reviewed in Lucocq, 1992). It is possible that microcystin permits activation of a kinase normally under the control of p34^{cdc2}. It is also possible, however, that the phosphatase inhibitor acts on a kinase-phosphatase cycle completely unconnected with cell cycle transport control, but rather with transport control.

This suggestion was substantiated by the difference in histone kinase activities of the mouse and human cytosols. Transport could be strongly inhibited in FT210 cytosol with a histone kinase activity which would have almost no effect on a HeLa cytosol incubation (though at similar levels of added cyclin A). This suggests that the kinase-phosphatase equilibria in these two cytosols are different, or that the mouse cytosol contains phosphorylation activity which the HeLa cytosol does not; for example, susceptibility to phosphatase inhibitors. Phosphatases impinge on p34^{cdc2}, and on many other cellular processes, at many sites, so it would be difficult to pinpoint a critical target of phosphatase inhibitors on transport. This kind of study was beyond the relatively crude tests available in this work, but identification of a cell-cycle linked transport control pathway would be of great interest.

7.4: The role of p34^{cdc2}

The inhibition of transport by cytosol from mitotic cells is a very clear argument for the role of p34^{cdc2} as a prime mover in mitotic events. This is reinforced by the effect of cyclin A on interphase cytosol. However, one of the puzzles about cyclin A was the apparently large quantity required to inhibit transport. The preparation was 30-50% pure, and the levels of active protein in that are not known, but almost microgram levels of the protein were added to achieve the pseudomitotic phenotype. These levels of protein were not deleterious to the assay in their own right, being entirely inactivated by boiling and entirely inhibited by staurosporine. p34^{cdc2} is approximately 800 nanomolar in *Xenopus* extracts (Nebreda and Hunt, 1993), and so it may be estimated that the highest levels of the cyclin A preparation used in this study were some 10-20 fold in excess of kinase.

Confirmation of the role of p34^{cdc2} was obtained by experiments with the S-phase kinase p33^{cdk2}. These two proteins are not active simultaneously in the cell cycle, but both are capable of activation when bound to cyclin A. Also, there is absolutely no indication that any intracellular transport event is modulated in S-phase. When added to the transport assay, p33^{cdk2} had no effect on transport or cytosolic histone kinase activity. This indicated either that the cytosol was devoid of cyclins A or E, or that those cyclins were sequestered in a form inaccessible to the added kinase, at least in the time of the assay. When cyclin and p33^{cdk2} were added to the assay together, there was strong activation of histone kinase activity. At

maximal (saturating) cyclin A levels, activation of histone kinase activity was doubled in the presence of 0.2 μ g of the purified p33^{cdk2} protein. This indicates that the cyclin A added to the reaction was in excess, but also that the levels of p34^{cdc2} and p33^{cdk2} present at that point were approximately equal in terms of histone kinase activity.

However, the p33^{cdk2} was not capable of inhibiting transport; in fact, it reproducibly enhanced the transport of both HeLa and FT210 cytosols, and in the presence of cyclin A, the p33^{cdk2} reduced the cyclin A-mediated inhibition of transport. The degree of amelioration depended on how much p33^{cdk2} was added to the cytosol. This result strongly suggested that cyclin A was incapable of inhibiting transport through the medium of the S-phase kinase, but required a kinase which competed with p33^{cdk2} for cyclin A. This was likely to be p34^{cdc2}. The other data on mitotic kinase came from the FT210 cell line, whose p34^{cdc2} was unstable to incubation at 38°C. If FT210, FM3A or HeLa cytosol were incubated at 38°C for 10 minutes, and then incubated with cyclin A and assayed for transport and histone kinase activity, only the FT210 cytosol had lost its capacity for transport inhibition. Since the FT210 cytosol was not reported to be deficient in any respect other than p34^{cdc2}, this was the best available indirect proof that mitotic kinase activity was required for transport inhibition. It would have been interesting to find out whether cyclin A or B was specifically implicated in transport inhibition. Thomas et al (1992) showed that p34^{cdc2} was directly responsible for inhibition of endocytic fusion in an in-vitro system based on *Xenopus* cytosol. They found that only cyclin B was suitable as the p34^{cdc2}-associated cyclin; a cyclin A-p34^{cdc2} complex could not direct fusion inhibition. While this particular finding was based on *Xenopus* cytosol, which does behave differently from mammalian cytosol in some respects, it is true that cyclin A and B direct p34^{cdc2} to different intracellular sites (Pines and Hunter, 1991). As stated above, mitotic cytosol (containing cyclin B) and interphase cytosol supplemented with cyclin A were both capable of inhibiting transport. This suggests that there is no cyclin preference in transport inhibition. However, it is possible that the interphase cytosol contained some quiescent cyclin B, which was activated on addition of cyclin A. To test this suggestion, it would be possible to make a cytosol lacking any cyclin, by synchronising cells in mitosis and then allowing them to escape into interphase (at which point the cyclins are degraded, and their resynthesis has not begun). Such a cytosol could then be tested for response to cyclin A in the absence of cyclin B. These experiments were attempted at the time of writing. The G1 cytosol had relatively low transport activity, and therefore transport reduction was not as noticeable as it would be in a highly-active cytosol. The experiment showed that transport was not significantly inhibited except at high levels of cyclin A, though histone kinase activity was elevated, but this result is not clear due to the poor transport capability of the G1 cytosol.

Unfortunately, no conditions were found under which purified cyclin B protein activated sHeLa cytosol, and this made it impossible to assess its effect on transport. Several methods were attempted; among them addition of proteins or agents known to assist activation of cyclin B-p34^{cdc2} in other systems, and isolation of cytosol from G2-phase cells. The fact that none of them worked, except marginally in the presence of *Xenopus* extracts, indicated that the sHeLa cytosol lacked some factor, or was in the wrong phosphorylation state for cyclin B activation of p34^{cdc2}, or that the cyclin preparation was incompatible with sHeLa cytosol. This problem was not overcome.

7.5: The target of mitotic inhibition

The ultimate aim of this work was to pinpoint a transport event or protein which was affected by mitosis. Initially, that was done by seeing whether membranes or cytosol were more affected. It was not feasible to isolate mitotic membranes, so they were always supplied to the assay in an 'interphase' state. The fact that mitotic or cyclin A-activated cytosols inhibited K-Golgi membranes more strongly than unwashed membranes suggested that a cytosol-derived factor was the primary target of the mitotic cytosol. K-Golgi is known to be stripped of a number of proteins which normally cycle on and off the membranes (Weidman et al, 1989), and it was an obvious suggestion that interphase proteins were removed by salt-washing to make way for the 'inactivated' mitotic ones.

This indication was supported by an experiment in which 'arrested' mitotic cytosol could be supplied with transport proteins by interphase cytosol. The simplest interpretation of that result was that transport components of cytosol had been inactivated by mitotic phosphorylation, thus immobilising an otherwise complete transport system. Then, addition of interphase cytosol resupplied the immobilised components, and transport could proceed. Given the attractive model in which inhibition of transport vesicle fusion leads to Golgi vesiculation, it would be satisfying if acceptor function were inhibited by mitotic kinase activity, leading to accumulation of vesicles in mitotic cytosol. It is also possible, however, that Golgi breakdown is conducted by an entirely separate process, and inhibition of vesicle fusion simply ensures against inappropriate vesicle mixing at any point in the entry to or exit from mitosis.

In one approach to this problem, donors or acceptors were preincubated in mitotic cytosol, and then arrested and added to interphase assays with the interphase-treated membranes. In this system, it was found that acceptors incubated in mitotic cytosol were less active in a complete assay than mitotic-treated donors. The assay was handicapped by the marked loss of counts in any form of assay where donors and acceptors were incubated separately under transport conditions.

Transport inhibition could also be studied by using inhibitors of various stages of transport. The agents mastoparan and aluminum fluoride both act on trimeric G-proteins, which have been localised to the Golgi and implicated in control of constitutive transport. Both of these had equal effects on interphase and mitotic assays, though in the case of mastoparan the effect was so extreme that it seemed to be due to a detergent action rather than influence on transport. Brefeldin A, which prevents assembly of COP-coated vesicles and causes promiscuous membrane fusion, increased apparent transport in interphase assays, but not mitotic ones. This suggested that fusion was inhibited in the mitotic assay at a post-uncoating stage, but it could not be ruled out that budding was profoundly inhibited.

GTP γ S inhibited mitotic and interphase transport assays to the same extent. Bearing in mind that salt-washed and unwashed transport were also equally affected by the drug, this result strongly suggested that coated vesicles were both formed and uncoated in interphase and mitotic cytosol, ie that the stage inhibited in mitosis was after the uncoating of the transport vesicles. Taken together with the effect of brefeldin, this experiment places the site of inhibition at a point after vesicle uncoating but before vesicle fusion. However, in interphase assays the effect of GTP γ S was almost nil after 20 minutes, whereas in mitotic cytosol the drug still inhibited transport at that time. This suggests that a stage before vesicle fusion was also affected by mitotic conditions. Overall, the results from experiments with transport inhibiting drugs did not unambiguously indicate a site of mitotic transport inhibition.

Another way of examining the mechanism of transport inhibition was to guess that the target might be one of the fusion proteins described by the Rothman group, and test whether any of those could be implicated in inhibition or recovery from inhibition. SNAPs and NSF were added to the transport assay either singly or in combination. The assay was notably stimulated by α -SNAP and less so by γ -SNAP or NSF, and this is in line with results of Rothman and co-workers. NSF is thought to act 'catalytically' in the assay, and therefore is stimulatory at a much lower level than the two SNAPs. α -SNAP appeared to be limiting in sHeLa cytosol at the levels normally used in transport, but γ -SNAP was not apparently limiting. Its role in fusion is not yet clear.

When these proteins were added to interphase or cyclin-treated cytosol, the assay was stimulated to just the same extent, whether or not it contained active phosphatases and kinases. Thus, under those conditions, none of the proteins were apparently targets for cyclin-mediated inactivation. This is consistent with the result that cyclin treatment of cytosol is less effective than simultaneous treatment of membranes and cytosol; if cytosol treatment alone were very inhibitory, then it might be expected to act on proteins like SNAPs and NSF. It also suggested that these proteins were after the rate-limiting stage of transport in the cyclin-treated assay.

In contrast to cyclin-treated cytosol, mitotic cytosol did not support significant transport enhancement by SNAPs or NSF, in the presence or absence of kinase/phosphatase inhibitors. This is interesting since the proteins were added to the cytosol before the membranes but after any arrest of cytosol, ie the proteins were functional targets of mitotic inhibition, or downstream of it, and kinase activity on them or the membranes was not required.

The finding that SNAPs and NSF had different effects on cyclin A-treated interphase cytosol and mitotic cytosol was unexpected, considering how alike the two had been in previous experiments. It suggested that the two conditions inhibited transport in different ways. This difference between cyclin and mitotic cytosol was the only indication in this work that cyclins A and B regulated transport differently, but the difference could not be explored further using the transport assay alone due to the failure of cyclin B to activate sHeLa cytosol. It appeared that the assay could be inhibited in more than one way. It was also plain that recombinant SNAP and NSF could not rescue transport in arrested mitotic cytosol. The simple idea that phosphorylation of a cycling fusion protein like NSF or SNAP inhibited the assay had to be abandoned.

Electron micrographs of mitotic and interphase transport assay mixtures (containing unwashed Golgi) did not show up any clear differences between them (T. Misteli, personal communication); perhaps this was due to the relatively disreputable appearance of CHO-derived Golgi membranes, or due to weak inhibition of transport in the reactions selected. However, rat liver Golgi (which can rapidly be purified over 200-fold) incubated with mitotic cytosol in EBS buffer (in which cytosol is made) sheds vesicles at a rate comparable with the published calculated rates of vesicle production by in-vitro systems (T. Misteli, manuscript in preparation). It will be extremely interesting to see whether the mechanism, rate and extent of vesicle shedding are comparable to those seen in transport.

7.6: Future directions

The transport assay used in this thesis has been used to yield much information about transport between the cisternae of the mammalian Golgi. Much of the information has been found applicable to other systems, such as ER-Golgi transport and endocytosis, and the transport assay has in turn benefited from research in other areas, most recently the study of synaptic vesicle proteins implicated in vesicle targeting. It appears that there is striking conservation of basic mechanisms between the various pathways of intracellular traffic. If a target could be identified for mitotic transport inhibition in the Golgi, then it might well be conserved in other organelles. This would be easily imaginable for a putative target such as NSF or another component of the identified fusion machinery.

It would also be useful to find out whether p34^{cdc2} was immediately responsible for inhibitory phosphorylation (and if so, with what cyclin) and if not, what was the nature of the

control intermediate. Very little is known at present about the mechanisms involved in mitotic alterations in cell architecture and function. If the transport assay could be used to identify and characterise a control mechanism regulated by p34^{cdc2}, it would be a useful tool and paradigm for other intracellular functions altered in mitosis.

The immediate aim is to discover the Golgi target of mitotic cytosol, by continuing experiments with isolated transport proteins. As well as transport assays, phosphorylation experiments are being used to label proteins altered in mitotic extracts, both in the Golgi and in the cytosol (N.Goldring, unpublished data). In addition, experiments are underway which study the binding of Golgi to fusion proteins under interphase and mitotic conditions (T.Levine, unpublished data). It is hoped that a phosphorylation target may be studied by a combination of these methods.

Another interesting application of transport assay technology may be the generation of Golgi transport vesicles. To date, transport vesicles have not been generated in quantity without the intervention of the fusion inhibitor GTP γ S, though recent studies in the Rothman laboratory have made much progress with this problem. It may be possible to generate vesicles in mitotic cytosol which could then be rescued to undergo normal transport, and they could be used to confirm the studies made of GTP γ S-generated vesicles.

7.7: Conclusion

This thesis has demonstrated that intra-Golgi protein transport is inhibited under mitotic conditions in an in-vitro system. This brings Golgi transport alongside almost all other membrane transactions studied to date. The assay permitted considerable manipulation of the phosphorylation state of the transport cytosol. It was shown that activity of p34^{cdc2} is essential for transport inhibition, either directly or via a kinase under its control. A variety of biochemical, kinetic and pharmacological experiments suggested that the cytosol contained transport factors inhibited by mitotic kinase, and there were some indications that their site of action was the acceptor compartment.

Acknowledgements

I would like to thank the following, for their parts in making me the scientist that I am now, and many others who are not named below. In chronological order:

First, to **GOD**, without whom none of this would have been possible.

Second, to my mother, who gave me everything, and to all my family. Then to Cath, my first and only co-worker; Sue and Chris, who showed me much good in science, and made me promise not to give up, and my friend Michael, who taught me to persevere faithfully.

Next, to Ginny, whose extreme skill and dedication made these experiments possible, and who has been a good friend to me as well. Also to Tommy, who provided me with a wide variety of learning situations. Also to Kathryn, a wise friend and most valued colleague. Also to Tom, for so many discussions and ideas, and to Marky for teaching me my stuff. My thanks to everyone else in the Warren and Armstrong labs, whose varied skills and talents made my time there so interesting; and of course, thanks to Graham, who formed me as a scientist. I wish also to thank Philip Woodman for many interesting discussions and experiments, Jorg Adamczewski for his knowledge, skill and recombinant proteins, and Tim Hunt for being so extremely inspiring.

Next, to all the musicians who allowed me to remain a singing biochemist: especially Jeremy and the Oxford Camerata, the McMullins, the Easter Day Opera players and the Romsey Singers.

Last, and most importantly, to Andy, whose love sustained me.

Thank you, my friends.

Afterword

Ἐὰν τοίνυν ἄλλων μετὰ ταῦτα ἐγκύμων ἐπιχειρήσῃ γίνεσθαι,
ὧ Θεαίτητε, εἴαντε γίγνη, βελτιόνων ἔσει πλήρης διὰ τὴν νῦν
ἐξέτασιν, εἴαντε κενὸς ᾗς, ᾗττον ἔσει βαρὺς τοῖς συνοῦσι
καὶ ἡμερῶ τερος, σωφρόνως οὐκ ὀϊόμενος εἰδέναι ἅ μὴ
οἶσθα

(And so, Theaetetus, you have finished this research. I hope that, if you succeed in your next project, your thinking will have improved after your experiences in this one. Or else, if you never finish a piece of research again, I hope that this has taught you to respect the work of others, and never to claim to know what you do not know.)

References

- Allan, V.J. and Vale, R.D. (1991). Cell cycle control of microtubule-based membrane transport and tubule formation in vitro. *J Cell Biol* 113, 347-59.
- Araki, S., Kikuchi, A., Hata, Y., Isomura, M. and Takai, Y. (1990). Regulation of reversible binding of smg p25A, a ras p21-like GTP-binding protein, to synaptic plasma membranes and vesicles by its specific regulatory protein, GDP dissociation inhibitor. *J. Biol. Chem.* 265, 13007-13015.
- Balch, W. E. and Rothman, J. E. (1985). Characterisation of protein transport between successive compartments of the Golgi apparatus: Asymmetric properties of donor and acceptor activities in a cell-free system. *Arch. Biochem. Biophys.* 240, 413-25.
- Balch, W.E., Dunphy, W.G., Braell, W.A. and Rothman, J.E. (1984a). Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of N-acetylglucosamine. *Cell* 39, 405-16.
- Balch, W.E., Glick, B.S. and Rothman, J.E. (1984b). Sequential intermediates in the pathway of intercompartmental transport in a cell-free system. *Cell* 39, 525-36.
- Balch, W.E., Kahn, R.A. and Schwaninger, R. (1992). ADP-ribosylation factor is required for vesicular trafficking between the endoplasmic reticulum and the cis-Golgi compartment. *J. Biol. Chem.* 267, 13053-13061.
- Barlowe, C., d'Enfert, C. and Schekman, R. (1993). Purification and characterisation of SAR1p, a small GTP-binding protein required for transport vesicle formation from the endoplasmic reticulum. *J. Biol. Chem.* 268, 873-879.
- Beckers, C.J. and Balch, W.E. (1989). Calcium and GTP: essential components in vesicular trafficking between the endoplasmic reticulum and Golgi apparatus. *J Cell Biol* 108, 1245-56.
- Beckers, C.J., Keller, D.S. and Balch, W.E. (1987). Semi-intact cells permeable to macromolecules: use in reconstitution of protein transport from the endoplasmic reticulum to the Golgi complex. *Cell* 50, 523-34.

Beckers, C.J., Plutner, H., Davidson, H.W. and Balch, W.E. (1990). Sequential intermediates in the transport of protein between the endoplasmic reticulum and the Golgi. *J Biol Chem* 265, 18298-310.

Berlin, R.D. and Oliver, J.M. (1980). Surface functions during mitosis II. Quantitation of pinocytosis and kinetic characterisation of the mitotic cycle with a new fluorescence technique. *J. Cell Biol.* 85, 660-671.

Block, M.R., Glick, B.S., Wilcox, C.A., Wieland, F.T. and Rothman, J.E. (1988). Purification of an N-ethylmaleimide-sensitive protein catalyzing vesicular transport. *Proc Natl Acad Sci U S A* 85, 7852-6.

Booher, R. and Beach, D. (1988). Involvement of *cdc13+* in mitotic control in *Schizosaccharomyces pombe*: possible interaction of the gene product with microtubules. *EMBO J* 7, 2321-7.

Bos, K., Wraight, C. and Stanley, K.K. (1993). TGN38 is maintained in the trans-Golgi network by a tyrosine-containing motif in the cytoplasmic domain. *EMBO J.* 12, 2219-2228.

Brennwald, P. and Novick, P. (1993). Interactions of three domains distinguishing the ras-related GTP-binding proteins Ypt1 and Sec4. *Nature* 362, 560-563.

Brodsky, F.M., Hill, B.L., Acton, S.L., Nathke, I., Wong, D.H., Ponnambalam, S. and Parham, P. (1991). Clathrin light chains: arrays of protein motifs that regulate coated-vesicle dynamics. *Trends Biochem. Sci.* 16, 208-213.

Brown, R.M. and Romanovicz, D.K. (1976). Biogenesis and structure of Golgi-derived cellulosic scales in *Pleurochrysis*. I. Role of the endomembrane system in scale assembly and exocytosis. *Appl. Polymer Symp.* 28, 537-585.

Buendia, B., Draetta, G. and Karsenti, E. (1992). Regulation of the microtubule nucleating activity of centrosomes in *Xenopus* egg extracts: role of cyclin A-associated protein kinase. *J Cell Biol* 116, 1431-42.

Bueno, A. and Russell, P. (1993). Two fission yeast B-type cyclins, Cig2 and Cdc13, have different functions in mitosis. *Mol. Cell. Biol.* 13, 2286-2297.

- Cerioti, A. and Colman, A. (1989). Protein transport from the endoplasmic reticulum to the Golgi complex can occur during meiotic metaphase in *Xenopus* oocytes. *J. Cell Biol.* 109, 1439-1444.
- Chaudhary, N. and Courvalin, J.-C. (1993). Stepwise reassembly of the nuclear envelope at the end of mitosis. *J. Cell Biol.* 122, 295-306.
- Chavrier, P., Vingron, M., Hauri, H.-P., Simons, K. and Zerial, M. (1990). Localisation of low molecular weight GTP-binding proteins to exocytic and endocytic compartments. *Cell* 62, 317-329.
- Clarke, P.R., Leiss, D., Pagano, M. and Karsenti, E. (1992). Cyclin A- and cyclin B-dependent protein kinases are regulated by different mechanisms in *Xenopus* egg extracts. *EMBO J* 11, 1751-61.
- Clary, D.O., Griff, I.C. and Rothman, J.E. (1990). SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast. *Cell* 61, 709-21.
- Clary, D.O. and Rothman, J.E. (1990). Purification of three related peripheral membrane proteins needed for vesicular transport. *J. Biol Chem.* 265, 10109-10117.
- Cluett, E.B. and Brown, W.J. (1992). Adhesion of Golgi cisternae by proteinaceous interactions: intercisternal bridges as putative adhesive structures. *J. Cell Sci.* 103, 773-784.
- Cluett, E.B., Wood, S.A., Banta, M. and Brown, W.J. (1993). Tubulation of Golgi membranes in vivo and in vitro in the absence of Brefeldin A. *J. Cell Biol* 120, 15-24.
- Cohen, P., Klumpp, S. and Schelling, D. (1989). An improved procedure for identifying and quantitating protein phosphatases in mammalian tissues. *FEBS Lett.* 250, 596-600.
- Collins, R.N. and Warren, G.B. (1992). Sphingolipid transport in mitotic cells. *J. Biol. Chem.* 267, 24906-24911.
- Colombo, M.I., Mayorga, L.S., Casey, P.J. and Stahl, P.D. (1992). Evidence of a role for heterotrimeric GTP-binding proteins in endosome fusion. *Science* 255, 1695-7.

- Cooper, M.S., Comell, B.A., Chernjavsky, A., Dani, J.W. and Smith, S.J. (1990). Tubulovesicular processes emerge from trans-Golgi cisternae, extend along microtubules, and interlink adjacent trans-Golgi elements into a reticulum. *Cell* 61, 135-45.
- Corthésy-Theulaz, I., Pauloin, A. and Pfeffer, S.R. (1992). Cytoplasmic dynein participates in the centrosomal localization of the Golgi complex. *J. Cell Biol.* 118, 1333-1345.
- Cross, F., Roberts, J. and Weintraub, H. (1989). Simple and complex cell cycles. *Annu Rev Cell Biol* 5, 341-96.
- Cross, F.R. and Tinkelenberg, A.H. (1991). A potential positive feedback loop controlling CLN1 and CLN2 gene expression at the start of the yeast cell cycle. *Cell* 65, 875-83.
- Cyert, M.S. and Kirschner, M.W. (1988). Regulation of MPF activity in vitro. *Cell* 53, 185-95.
- d'Enfert, C., Gensse, M. and Gaillardin, C. (1992). Fission yeast and a plant have functional homologues of the Sar1 and Sec12 proteins involved in ER to Golgi traffic in budding yeast. *EMBO J.* 11, 4205-4211.
- d'Enfert, C., Wuestehube, L.J., Lila, T. and Schekman, R. (1991). Sec12-dependent membrane binding of the small GTP-binding protein Sar1p promotes formation of transport vesicles from the ER. *J. Cell Biol.* 114, 663-670.
- Dabora, S.L. and Sheetz, M.P. (1988). The microtubule-dependent formation of a tubulovesicular network with characteristics of the ER from cultured cell extracts. *Cell* 54, 27-35.
- Dascher, C., Ossig, R., Gallwitz, D. and Schmitt, H.D. (1991). Identification and structure of four yeast genes (SLY) that are able to suppress the functional loss of YPT1, a member of the RAS superfamily. *Mol. Cell. Biol.* 11, 872-885.
- Dasso, M. and Newport, J.W. (1990). Completion of DNA replication is monitored by a feedback system that controls the initiation of mitosis in vitro: studies in *Xenopus*. *Cell* 61, 811-23.

- Davidson, H.W. and Balch, W.E. (1993). Differential Inhibition of multiple vesicular transport steps between the endoplasmic reticulum and the trans Golgi network. *J. Biol. Chem.* 268, 4216-4226.
- Davidson, H.W., McGowan, C.H. and Balch, W.E. (1992). Evidence for the regulation of exocytic transport by protein phosphorylation. *J Cell Biol* 116, 1343-55.
- de Brabander, M.J., Veire, R.M.L., Aerts, F.F.M., Borgers, M. and Janssen, P.A.J. (1976). The effects of methyl [5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl] carbamate, (R17934; NSO 238159), a new synthetic antitumoral drug interfering with microtubules, on mammalian cells cultured in vitro. *Cancer Res.* 36, 905-916.
- Devault, A., Fesquet, D., Cavadore, J.C., Garrigues, A.M., Labbe, J.C., Lorca, T., Picard, A., Philippe, M. and Dorée, M. (1992). Cyclin A potentiates maturation-promoting factor activation in the early *Xenopus* embryo via inhibition of the tyrosine kinase that phosphorylates cdc2. *J Cell Biol* 118, 1109-20.
- Diaz, R., Mayorga, L.S., Weidman, P.J., Rothman, J.E. and Stahl, P.D. (1989). Vesicle fusion following receptor-mediated endocytosis requires a protein active in Golgi transport. *Nature* 339, 398-400.
- Dirick, L. and Nasmyth, K. (1991). Positive feedback in the activation of G cyclins in yeast. *Nature* 351, 754-757.
- Doms, R.W., Russ, G. and Yewdell, J.W. (1989). Brefeldin A redistributes resident and itinerant Golgi proteins to the endoplasmic reticulum. *J Cell Biol* 109, 61-72.
- Donaldson, J.G., Cassel, D., Kahn, R.A. and Klausner, R.D. (1992a). ADP-ribosylation factor, a small GTP-binding protein, is required for binding of the coatamer protein beta-COP to Golgi membranes. *Proc Natl Acad Sci U S A* 89, 6408-12.
- Donaldson, J.G., Finazzi, D. and Klausner, R.D. (1992b). Brefeldin A inhibits Golgi membrane-catalysed exchange of guanine nucleotide onto ARF protein. *Nature* 360, 350-352.
- Dorée, M., Peaucellier, G. and Picard, A. (1983). Activity of the maturation-promoting factor and the extent of protein phosphorylation oscillate simultaneously during meiotic maturation of starfish oocytes. *Dev. Biol.* 99, 489-501.

- Driouich, A., Faye, L. and Staehelin, A. (1993). The plant Golgi apparatus: a factory for complex polysaccharides and glycoproteins. *Trends Biochem. Sci.* 18, 210-214.
- Dunn, B., Stearns, T. and Botstein, D. (1993). Specificity domains distinguish the Ras-related GTPases Ypt1 and Sec4. *Nature* 362, 563-565.
- Dunphy, W.G., Brands, R. and Rothman, J.E. (1985). Attachment of terminal N-acetylglucosamine to asparagine-linked oligosaccharides occurs in central cisternae of the Golgi stack. *Cell* 40, 463-72.
- Dunphy, W.G., Brizuela, L., Beach, D. and Newport, J. (1988). The *Xenopus* cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis. *Cell* 54, 423-431.
- Dunphy, W.G. and Rothman, J.E. (1983). Compartmentation of asparagine-linked glycosylation in the Golgi apparatus. *J. Cell Biol.* 97, 270-275.
- Enoch, T. and Nurse, P. (1991). Coupling M phase and S phase: controls maintaining the dependence of mitosis on chromosome replication. *Cell* 65, 921-3.
- Enoch, T., Peter, M., Nurse, P. and Nigg, E.A. (1991). p34^{cdc2} acts as a lamin kinase in fission yeast. *J Cell Biol* 112, 797-807.
- Ercolani, L., Stow, J.L., Boyle, J.F., Holtzman, E.J., Lin, H., Grove, J.R. and Ausiello, D.A. (1990). Membrane localization of the pertussis toxin-sensitive G-protein subunits α_{i-2} and α_{i-3} and expression of a metallothionein- α_{i-2} fusion gene in LLC-PK1 cells [published erratum appears in *Proc Natl Acad Sci U S A* 1990 Sep;87(18):7344]. *Proc Natl Acad Sci U S A* 87, 4635-9.
- Evans, T., Rosenthal, E.T., Youngblom, J., Distel, D. and Hunt, T. (1983). Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell* 33, 389-396
- Fang, F. and Newport, J.W. (1991). Evidence that the G1-S and G2-M transitions are controlled by different cdc2 proteins in higher eukaryotes. *Cell* 66, 731-42.

Farnsworth, C.C., Kawata, M., Yoshida, Y., Takai, Y., Gelb, M.H. and Glomset, J.A. (1991). C-terminus of the small GTP-binding protein smg p25a contains two geranylgeranylated cysteine residues and a methyl ester. *Proc. Natl. Acad. Sci.* 88, 6196-6200.

Farnsworth, C.C., Wolda, S.L., Gelb, M.H. and Glomset, J.A. (1989). Human lamin B contains a farnesylated cysteine residue. *J Biol Chem* 264, 20422-9.

Featherstone, C., Griffiths, G. and Warren, G. (1985). Newly synthesized G protein of vesicular stomatitis virus is not transported to the Golgi complex in mitotic cells. *J Cell Biol* 101, 2036-46.

Felix, M.A., Pines, J., Hunt, T. and Karsenti, E. (1989). Temporal regulation of cdc2 mitotic kinase activity and cyclin degradation in cell-free extracts of *Xenopus* eggs. *J Cell Sci Suppl* 12, 99-116.

Felix, M.A., Labbé, J.C., Dorée, M., Hunt, T. and Karsenti, E. (1990). Triggering of cyclin degradation in interphase extracts of amphibian eggs by cdc2 kinase. *Nature* 346, 379-82.

Felix, M.A., Pines, J., Hunt, T. and Karsenti, E. (1989). A post-ribosomal supernatant from activated *Xenopus* eggs that displays post-translationally regulated oscillation of its cdc2+ mitotic kinase activity. *EMBO J* 8, 3059-69.

Fesquet, D., Labbé, J.-C., Derancourt, J., Capony, J.-P., Galas, S., Girard, F., Lorca, T., Shuttleworth, J., Dorée, M. and Cavadore, J.-C. (1993). The MO15 gene encodes the catalytic subunit of a protein kinase that activates cdc2 and other cyclin-dependent kinases (CDKs) through phosphorylation of Thr161 and its homologues. *EMBO J* 12, 3111-3121.

Foisner, R. and Gerace, L. (1993). Integral membrane proteins of the nuclear envelope interact with lamins and chromosomes, and binding is modulated by mitotic phosphorylation. *Cell* 73, 1267-1279.

Forsburg, S.L. and Nurse, P. (1991). Cell cycle regulation in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. *Annu Rev Cell Biol* 7, 227-56.

Friend, D.S. and Murray, M.J. (1965). Osmium impregnation of the Golgi apparatus. *Am. J. Anat.* 117, 135-150.

- Frorath, B., Abney, C.C., Berthold, H., Scanarini, M. and Northemann, W. (1992). Production of recombinant rat Interleukin-6 in *Escherichia coli* using a novel highly efficient expression vector pGEX-3T. *BioTechniques* 12, 558-563.
- Gadbois, D.M., Hamaguchi, J.R., Swank, R.A. and Bradbury, E.M. (1992). Staurosporine is a potent inhibitor of p34^{cdc2} and p34^{cdc2}-like kinases. *Biochem Biophys Res Commun* 184, 80-5.
- Galaktionov, K. and Beach, D. (1991). Specific activation of cdc25 tyrosine phosphatases by B-type cyclins: evidence for multiple roles of mitotic cyclins. *Cell* 67, 1181-94.
- Gautier, J., Norbury, C., Lohka, M., Nurse, P. and Maller, J. (1988). Purified maturation-promoting factor contains the product of a *Xenopus* homolog of the fission yeast cell cycle control gene *cdc2+*. *Cell* 54, 433-9.
- Gerace, L. and Burke, B. (1988). Functional organization of the nuclear envelope. *Annu Rev Cell Biol* 4, 335-74.
- Gerhart, J., Wu, M. and Kirschner, M. (1984). Cell cycle dynamics of an M-phase-specific cytoplasmic factor in *Xenopus laevis* oocytes and eggs. *J Cell Biol* 98, 1247-55.
- Gething, M.J., McCammon, K. and Sambrook, J. (1986). Expression of wild-type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. *Cell* 46, 939-50.
- Gil, J., Higgins, T. and Rozengurt, E. (1991). Mastoparan, a novel mitogen for Swiss 3T3 cells, stimulates pertussis toxin-sensitive arachidonic acid release without inositol phosphate accumulation. *J Cell Biol* 113, 943-50.
- Gilman, A.G. (1987). G proteins: transducers of receptor-generated signals. *Ann. Rev. Biochem.* 56, 615-649.
- Glotzer, M., Murray, A.W. and Kirschner, M.W. (1991). Cyclin is degraded by the ubiquitin pathway. *Nature* 349, 132-8.

Goldberg, D.E. and Kornfeld, S. (1983). Evidence for extensive subcellular organization of asparagine-linked oligosaccharide processing and lysosomal enzyme phosphorylation. *J Biol Chem* 258, 3159-65.

Gorvel, J.-P., Chavrier, P., Zerial, M. and Gruenberg, J. (1991). Rab5 controls early endosome fusion in vitro. *Cell* 64, 915-925.

Gottlieb, C., Skinner, A.M. and Kornfeld, S. (1974). Isolation of a clone of Chinese hamster ovary cells deficient in plant lectin-binding sites. *Proc Natl Acad Sci U S A* 71, 1078-82.

Goud, B., Zahraoui, A., Tavitian, A. and Saraste, J. (1990). Small GTP-binding protein associated with Golgi cisternae. *Nature* 345, 553-6.

Gould, K.L., Moreno, S., Owen, D.J., Sazer, S. and Nurse, P. (1991). Phosphorylation at Thr167 is required for *Schizosaccharomyces pombe* p34^{cdc2} function. *EMBO J* 10, 3297-309.

Gould, K.L. and Nurse, P. (1989). Tyrosine phosphorylation of the fission yeast cdc2+ protein kinase regulates entry into mitosis. *Nature* 342, 39-45.

Griffiths, G. and Simons, K. (1986). The trans Golgi network: sorting at the exit site of the Golgi complex. *Science* 234, 438-43.

Griffiths, G.R., Brands, B., Burke, B., Louvard, D. and Warren, G. (1982). Viral membranes acquire galactose in trans Golgi cisternae during intracellular transport. *J. Cell Biol.* 95, 781-792.

Gu, Y., Rosenblatt, J. and Morgan, D.O. (1992). Cell cycle regulation of CDK2 activity by phosphorylation of Thr160 and Tyr15. *EMBO Journal* 11, 3995-4005.

Guan, K.L. and Dixon, J.E. (1991). Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal Biochem* 192, 262-7.

Hadwiger, J.A., Wittenberg, C., Richardson, H.E., de Barros Lopes, M. and Reed, S. (1989). A family of cyclin homologs that control the G1 phase in yeast. *Proc. Natl. Acad. Sci.* 86, 6255-6270.

Hamaguchi, J.R., Tobey, R.A., Pines, J., Crissman, H.A., Hunter, T. and Bradbury, E.M. (1992). Requirement for p34^{cdc2} kinase is restricted to mitosis in the mammalian cdc2 mutant FT210. *J Cell Biol* 117, 1041-53.

Hardwick, K.G. and Pelham, H.R.B. (1992). SED-5 encodes a 39kD integral membrane protein required for vesicular transport between the ER and the Golgi complex. *J. Cell Biol.* 119, 513-521.

Hartl, P., Gottesfeld, J. and Forbes, D.J. (1993). Mitotic repression of transcription in vitro. *J. Cell Biol.* 120, 613-624.

Hauri, H.-P. and Schweizer, A. (1992). The endoplasmic reticulum-Golgi intermediate compartment. *Curr. Opin. Cell Biol.* 4, 600-608

Helms, J.B. and Rothman, J.E. (1992). Inhibition by brefeldin A of a Golgi membrane enzyme that catalyses exchange of guanine nucleotide bound to ARF. *Nature* 360, 352-354.

Hesketh, T.R., Beaven, M.A., Rogers, J., Burke, B. and Warren, G.B. (1984). Stimulated release of histamine by a rat mast cell line is inhibited during mitosis. *J Cell Biol* 98, 2250-4.

Hiebsch, R.R., Raub, T.J. and Wattenberg, B.W. (1991). Primaquine blocks transport by inhibiting the formation of functional transport vesicles. Studies in a cell-free assay of protein transport through the Golgi apparatus. *J Biol Chem* 266, 20323-8.

Higashijima, T., Burnier, J. and Ross, E.M. (1990). Regulation of G_i and G_o by mastoparan, related amphiphilic peptides and hydrophobic amines: mechanism and structural determinants of activity. *J. Biol. Chem.* 265, 14176-14186.

Hirai, Y., Yasuhara, T.H., Nakajima, M., Fujino, M. and Kitada, C. (1979). A new mast cell degranulating peptide, "mastoparan" in the venom of *Vespula lewisii*. *Chem. Pharm. Bull.* (Tokyo) 27, 1942-1944.

Ho, W.C., Allan, V.J., van Meer, G., Berger, E.G. and Kreis, T.E. (1989). Reclustering of scattered Golgi elements occurs along microtubules. *Eur. J. Cell Biol.* 48, 250-263.

- Hoffman, I., Clarke, P.R., Marcote, M.J., Karsenti, E. and Draetta, G. (1993). Phosphorylation and activation of human cdc25-C by cdc2-cyclin B and its involvement in the self-amplification of MPF at mitosis.
- Hunt, T., Luca, F.C. and Ruderman, J.V. (1992). The requirements for protein synthesis and degradation, and the control of destruction of cyclins A and B in the meiotic and mitotic cell cycles of the clam embryo. *J Cell Biol* 116, 707-24.
- Hurtley, S.M. and Helenius, A. (1989). Protein oligomerization in the endoplasmic reticulum. *Annu Rev Cell Biol* 5, 277-307.
- Jackson, M.R., Nilsson, T. and Peterson, P.A. (1993). Retrieval of transmembrane proteins to the endoplasmic reticulum. *J. Cell Biol.* 121, 317-333.
- Jackson, M.R., Nilsson, T.J. and Peterson, P.A. (1990). Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. *EMBO J.* 9, 3153-3162.
- Kahn, R.A. (1991). Fluoride is not an activator of the smaller (20-25kDa) GTP-binding proteins. *J. Biol. Chem.* 266, 15595-15597.
- Kahn, R.A. and Gilman, A.G. (1984). Purification of a protein cofactor required for ADP-ribosylation of the stimulatory regulatory component of adenylate cyclase by cholera toxin. *J Biol Chem* 259, 6228-34.
- Kahn, R.A., Kern, F.G., Clark, J., Gelmann, E.P. and Rulka, C. (1991). Human ADP-ribosylation factors. A functionally conserved family of GTP-binding proteins. *J Biol Chem* 266, 2606-14.
- Kaiser, C. and Schekman, R. (1990). Distinct sets of sec genes govern transport vesicle formation and fusion early in the secretory pathway. *Cell* 61, 723-733.
- Karsenti, E. (1993). Severing microtubules in mitosis. *Current Biology* 3, 208-210.
- Kawamura, S., Kaibuchi, K., Hiroyoshi, M., Hata, Y. and Takai, Y. (1991). Stoichiometric interaction of smg p21 with its GDP/GTP exchange protein and its novel action to regulate

the translocation of smg p21 between membrane and cytoplasm. *Biochem. Biophys. Res. Commun.* 174, 1095-1102.

Kelly, R.B. (1990). Microtubules, membrane traffic and cell organisation. *Cell* 61, 5-7.

Klausner, R.D., Donaldson, J.G. and Lippincott-Schwartz, J. (1992). Brefeldin A: insights into the control of membrane traffic and organelle structure. *J. Cell Biol.* 116, 1071-1080.

Kobayashi, H., Stewart, E., Poon, R., Adamczewski, J., Gannon, J. and Hunt, T. (1992). Identification of the domains in cyclin A required for binding to, and activation of, p34^{cdc2} and p32^{cdk2} protein kinase subunits. *Molecular Biology of the Cell* 3, 1279-1294.

Kornfeld, R. and Kornfeld, S. (1985). Assembly of asparagine-linked oligosaccharides. *Annu Rev Biochem* 54, 631-64.

Kreiner, T. and Moore, H.P. (1990). Membrane traffic between secretory compartments is differentially affected during mitosis. *Cell Regul* 1, 415-24.

Krek, W. and Nigg, E.A. (1991). Mutations of p34^{cdc2} phosphorylation sites induce premature mitotic events in HeLa cells: evidence for a double block to p34^{cdc2} kinase activation in vertebrates. *EMBO J* 10, 3331-41.

Krohne, G., Waizenegger, I. and Hoger, T.H. (1989). The conserved carboxy-terminal cysteine of nuclear lamins is essential for lamin association with the nuclear envelope. *J Cell Biol* 109, 2003-11.

Ktistakis, N.T., Linder, M.E. and Roth, M.G. (1992). Action of brefeldin A blocked by activation of a pertussis-toxin-sensitive G protein. *Nature* 356, 344-6.

Kumagai, A. and Dunphy, W.G. (1992). Regulation of the cdc25 protein during the cell cycle in *Xenopus* extracts. *Cell* 70, 139-51.

Labbé, J.-C., Picard, A., Karsenti, E. and Dorée, M. (1988). An M-phase-specific kinase of *Xenopus* oocytes: partial purification and possible mechanism of its periodic activation. *Dev. Biol.* 127, 157-169.

Labib, K. and Nurse, P. (1993). Bring on the phosphatases. *Current Biology* 3, 164-166.

Lake, R.S. (1973). F1-histone phosphorylation in metaphase chromosomes of cultured Chinese hamster cells. *Nature New Biol* 242, 145-6.

Lake, R.S. and Salzman, N.P. (1972). Occurrence and properties of a chromatin-associated F1-histone phosphokinase in mitotic Chinese hamster cells. *Biochemistry* 11, 4817-26.

Lamb, N.J., Cavadore, J.C., Labbé, J.C., Maurer, R.A. and Fernandez, A. (1991). Inhibition of cAMP-dependent protein kinase plays a key role in the induction of mitosis and nuclear envelope breakdown in mammalian cells. *EMBO J* 10, 1523-33.

Leaf, D.S., Roberts, S.J., Gerhart, J.C. and Moore, H.P. (1990). The secretory pathway is blocked between the trans-Golgi and the plasma membrane during meiotic maturation in *Xenopus* oocytes. *Dev Biol* 141, 1-12.

Lee, C. and Chen, L.B. (1988). Dynamic behavior of endoplasmic reticulum in living cells. *Cell* 54, 37-46.

Lee, M.G. and Nurse, P. (1987). Complementation used to clone a human homologue of the fission yeast cell cycle control gene *cdc2*. *Nature* 327, 31-5.

Lee, T.H., Solomon, M.J., Mumby, M.C. and Kirschner, M.W. (1991). INH, a negative regulator of MPF, is a form of protein phosphatase 2A. *Cell* 64, 415-23.

Lenhard, J.M., Kahn, R.A. and Stahl, P.D. (1992). Evidence for ADP-ribosylation factor (ARF) as a regulator of in vitro endosome-endosome fusion. *J Biol Chem* 267, 13047-52.

Lew, J., Dulic, V. and Reed, S.I. (1991). Isolation of three novel cyclins by rescue of G1 cyclin (*cln*) function in yeast. *Cell* 66, 1197-1206.

Lewis, M.J. and Pelham, H.R.B. (1992). Ligand-induced redistribution of a human KDEL receptor from the Golgi complex to the endoplasmic reticulum. *Cell* 68, 353-364.

Lewis, M.J., Sweet, D.J. and Pelham, H.R. (1990). The ERD2 gene determines the specificity of the luminal ER protein retention system. *Cell* 61, 1359-63.

Lippincott-Schwartz, J., Donaldson, J.G., Schweizer, A., Berger, E.G., Hauri, H.-P., Yuan, L.C. and Klausner, R.D. (1990). Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway. *Cell* 60, 821-36.

Lippincott-Schwartz (1993). Bidirectional traffic between the endoplasmic reticulum and the Golgi apparatus. *Trends Cell Biol.* 3, 81-88.

Lokha, M.J., Hayes, M.K. and Maller, J.L. (1988). Purification of maturation promoting factor, an intracellular regulator of early mitotic events. *Proc. Natl. Acad. Sci.* 85, 3009-3014.

Lorca, T., Labbé, J.C., Devault, A., Fesquet, D., Capony, J.P., Cavadore, J.C., Le Bouffant, F. and Dorée, M. (1992). Dephosphorylation of cdc2 on threonine 161 is required for cdc2 kinase inactivation and normal anaphase. *EMBO J* 11, 2381-90.

Luca, F.C., Shibuya, E.K., Dohrmann, C.E. and Ruderman, J.V. (1991). Both cyclin A $\Delta 60$ and B $\Delta 97$ are stable and arrest cells in M-phase, but only cyclin B $\Delta 97$ turns on cyclin destruction. *EMBO J* 10, 4311-20.

Lucocq, J. (1992). Mimicking mitotic Golgi disassembly using okadaic acid. *J. Cell Sci* 103, 875-880.

Lucocq, J., Warren, G. and Pryde, J. (1991). Okadaic acid induces Golgi apparatus fragmentation and arrest of intracellular transport. *J Cell Sci*

Lucocq, J.M., Berger, E.G. and Warren, G. (1989). Mitotic Golgi fragments in HeLa cells and their role in the reassembly pathway. *J Cell Biol* 109, 463-74.

Lucocq, J.M., Pryde, J.G., Berger, E.G. and Warren, G. (1987). A mitotic form of the Golgi apparatus in HeLa cells. *J Cell Biol* 104, 865-74.

Lundgren, K., Walworth, N., Booher, R., Dembski, M., Kirschner, M. and Beach, D. (1991). mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2. *Cell* 64, 1111-1122.

Luzio, J.P., Burke, B., Banting, G., Howell, K.E., Braghetta, P. and Stanley, K.K. (1990). Identification, sequencing and expression of an integral membrane protein of the trans Golgi network. *Biochem. J.* 270, 97-102.

MacKintosh, C., Beattie, K.A., Klumpp, S., Cohen, P. and Codd, G.A. (1990). Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. *FEBS Lett.* 264, 187-192.

Malhotra, V., Orci, L., Glick, B.S., Block, M.R. and Rothman, J.E. (1988). Role of an N-ethylmaleimide-sensitive transport component in promoting fusion of transport vesicles with cisternae of the Golgi stack. *Cell* 54, 221-7.

Malhotra, V., Serafini, T., Orci, L., Shepherd, J.C. and Rothman, J.E. (1989). Purification of a novel class of coated vesicles mediating biosynthetic protein transport through the Golgi stack. *Cell* 58, 329-36.

Maller, J., Wu, M. and Gerhart, J.C. (1977). Changes in protein phosphorylation accompanying maturation of *Xenopus laevis* oocytes. *Dev. Biol.* 58, 295-312.

Maller, J.L. (1982). Interaction of steroids and growth factors with the plasma membrane in the induction of oocyte maturation in *Xenopus laevis*. *Prog Clin Biol Res* 91, 157-69.

Maller, J.L. (1985). Regulation of amphibian oocyte maturation. *Cell Differentiation* 16, 211-221.

Matsushime, H., Roussel, M.F., Ashmun, R.A. and Sherr, C.J. (1991). Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. *Cell* 65, 701-713.

McGowan, C.H. and Russell, P. (1993). Human wee1 kinase inhibits cell division by phosphorylating p34^{cdc2} exclusively on Tyr15. *EMBO J.* 12, 75-85.

Melançon, P., Glick, B.S., Malhotra, V., Weidman, P.J., Serafini, T., Gleason, M.L., Orci, L. and Rothman, J.E. (1987). Involvement of GTP-binding "G" proteins in transport through the Golgi stack. *Cell* 51, 1053-1062.

Meyerson, M., Enders, G.H., Wu, C.L., Su, L.K., Gorka, C., Nelson, C., Harlow, E. and Tsai, L.H. (1992). A family of human cdc2-related protein kinases. *EMBO J* 11, 2909-17.

Millar, J.B., McGowan, C.H., Lenaers, G., Jones, R. and Russell, P. (1991). p80^{cdc25} mitotic inducer is the tyrosine phosphatase that activates p34^{cdc2} kinase in fission yeast. *EMBO J* 10, 4301-9.

Millar, J.B. and Russell, P. (1992). The cdc25 M-phase inducer: an unconventional protein phosphatase. *Cell* 68, 407-10.

Millar, J.B., Russell, P., Dixon, J.E. and Guan, K.L. (1992a). Negative regulation of mitosis by two functionally overlapping PTPases in fission yeast. *EMBO J* 11, 4943-4952.

Millar, J.B.A., Lenaers, G. and Russell, P. (1992b). Pyp3 PTPase acts as a mitotic inducer in fission yeast. *EMBO J.* 11, 4933-2941.

Mineo, C., Murakami, Y., Ishimi, Y., Hanaoka, F. and Yamada, M. (1986). Isolation and analysis of a mammalian temperature-sensitive mutant defective in G2 functions. *Exp. Cell Res.* 167, 53-62.

Minshull, J., Blow, J.J. and Hunt, T. (1989). Translation of cyclin mRNA is necessary for extracts of activated *Xenopus* eggs to enter mitosis. *Cell* 56, 947-956

Minshull, J., Golsteyn, R., Hill, C.S. and Hunt, T. (1990). The A- and B-type cyclin associated cdc2 kinases in *Xenopus* turn on and off at different times in the cell cycle. *EMBO J* 9, 2865-75.

Misumi, Y., Misumi, Y., Miki, K., Takatsuki, A., Tamura, G. and Ikehara, Y. (1986). Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. *J Biol Chem* 261, 11398-403.

Moreno, S., Hayles, J. and Nurse, P. (1989). Regulation of p34^{cdc2} during mitosis. *Cell* 58, 361-372.

Munro, S. and Pelham, H.R. (1987). A C-terminal signal prevents secretion of luminal ER proteins. *Cell* 48, 899-907.

Murray, A.W. and Kirschner, M.W. (1989). Cyclin synthesis drives the early embryonic cell cycle. *Nature* 339, 275-280

Nakano, A. and Maramatsu, M. (1989). A novel GTP-binding protein, Sar1p, is involved in transport from the endoplasmic reticulum to the Golgi apparatus. *J. Cell Biol.* 109, 2677-2691.

Nebreda, A.R. and Hunt, T. (1993). The c-mos proto-oncogene protein kinase turns on and maintains the activity of MAP kinase, but not MPF, in cell-free extracts of *Xenopus* oocytes and eggs. *EMBO J.* 12, 1979-1986.

Nevins, J.R. (1992). E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. *Science* 259, 1908-1912.

Nilsson, T., Jackson, M. and Peterson, P.A. (1989). Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reticulum. *Cell* 58, 707-18.

Nilsson, T., Lucocq, J.M., Mackay, D. and Warren, G. (1991). The membrane spanning domain of beta-1,4-galactosyltransferase specifies trans Golgi localization. *EMBO J* 10, 3567-75.

Nilsson, T., Pypaert, M., Hoe, M.H., Slusarewicz, P., Berger, E. and Warren, G. (1993). Overlapping distribution of two glycosyltransferases in the Golgi apparatus of HeLa cells. *J. Cell. Biol.* 120, 5-13.

Norbury, C., Blow, J. and Nurse, P. (1991). Regulatory phosphorylation of the p34^{cdc2} protein kinase in vertebrates. *EMBO J* 10, 3321-9.

Norbury, C. and Nurse, P. (1992). Animal cell cycles and their control. *Annu Rev Biochem* 61, 441-70.

Novick, P., Field, C. and Schekman, R. (1980). Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell* 21, 205-15.

Novick, P. and Schekman, R. (1979). Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* 76, 1858-1862.

- Novikoff, A.B. and Novikoff, P.M. (1977). Cytochemical contributions to differentiating GERL from the Golgi apparatus *Histochem. J.* 9, 525-551.
- Nurse, P. (1975). Genetic control of cell size at cell division in yeast. *Nature* 256, 457-51.
- Nurse, P. (1990). Universal control mechanism regulating onset of M-phase. *Nature* 344, 503-8.
- Nurse, P. and Thuriaux, P. (1980). Regulatory genes controlling mitosis in the fission yeast *Schizosaccharomyces pombe*. *Genetics* 96, 627-37.
- Orci, L., Malhotra, V., Amherdt, M., Serafini, T. and Rothman, J.E. (1989). Dissection of a single round of vesicular transport: sequential intermediates for intercisternal movement in the Golgi stack. *Cell* 56, 357-68.
- Orci, L., Palmer, D.P., Ravazzola, M., Amherdt, M. and Rothman, J.E. (1993). Budding from Golgi membranes requires coatamer, a complex of non-clathrin coat proteins. *Nature* 362, 648-652.
- Ossig, R., Dascher, C., Trepte, H.H., Schmitt, H.D. and Gallwitz, D. (1991). The yeast SLY gene products, suppressors of defects in the essential GTP-binding YPT1 protein, may act in endoplasmic reticulum-to-Golgi transport. *Mol. Cell. Biol.* 11, 2980-2993.
- Pagano, M., Draetta, G. and Jansen-Dürr, P. (1992). Association of cdk2 kinase with the transcription factor E2F during S phase. *Science* 255, 1144-1147.
- Palade, G. (1975). Intracellular aspects of the process of protein synthesis. *Science* 189, 347-58.
- Parker, L.L., Walter, S.A., Young, P.G. and Piwnicka-Worms, H. (1993). Phosphorylation and inactivation of the mitotic inhibitor Wee1 by the nim1/cdr1 kinase. *Nature* 363, 736-8
- Pearse, B.M.F. and Robinson, M.S. (1990). Clathrin, adaptors, and sorting. *Ann. Rev. Cell Biol.* 6,
- Pelham, H.R.B. (1989). Control of protein exit from the endoplasmic reticulum. *Ann. Rev. Cell Biol.* 5, 1-23

Peter, M., Nakagawa, J., Dorée, M., Labbé, J.C. and Nigg, E.A. (1990). In vitro disassembly of the nuclear lamina and M phase-specific phosphorylation of lamins by cdc2 kinase. *Cell* 61, 591-602.

Pfaller, R., Smythe, C. and Newport, J.W. (1991). Assembly/disassembly of the nuclear envelope membrane: cell cycle-dependent binding of nuclear membrane vesicles to chromatin in vitro. *Cell* 65, 209-17.

Picard, A., Labbé, J.-C., Peaucellier, G., le Bouffant, F., le Peuch, C. and Dorée, M. (1987). Changes in the activity of the maturation-promoting factor are correlated with those of a major cyclic AMP and calcium-independent protein kinase during the first mitotic cell cycles in the early starfish embryo. *Develop., Growth and Differ.* 29, 93-103.

Pines, J. (1993). Cyclins and cyclin-dependent kinases: take your partners. *Trends Biochem. Sci.* 18, 195-197.

Pines, J. and Hunter, T. (1990). Human cyclin A is adenovirus E1A-associated protein p60 and behaves differently from cyclin B. *Nature* 346, 760-3.

Pines, J. and Hunter, T. (1991). Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport. *J Cell Biol* 115, 1-17.

Plutner, H., Cox, A.D., Pind, S., Khosravi, F.R., Bourne, J.R., Schwaninger, R., Der, C.J. and Balch, W.E. (1991). Rab1b regulates vesicular transport between the endoplasmic reticulum and successive Golgi compartments. *J Cell Biol* 115, 31-43.

Poon, R.Y.C., Yamashita, K., Adamczewski, J.P., Hunt, T. and Shuttleworth, J. (1993). The cdc2-related protein p40^{MO15} is the catalytic subunit of a protein kinase that can activate p33^{cdk2} and p34^{cdc2}. *EMBO J.* 12, 3123-3132

Pypaert, M., Mundy, D., Souter, E., Labbé, J.-C. and Warren, G. (1991). Mitotic cytosol inhibits invagination of coated pits in broken mitotic cells. *J. Cell Biol.* 114, 1159-1166.

Pypaert, M., Nilsson, T., Berger, E.G. and Warren, G. (1993). Golgi clusters are not tubular endosomes. *J. Cell Sci.* 104, 811-818.

Rambourg, A. and Clermont, Y. (1990). Three dimensional electron microscopy: structure of the Golgi apparatus. *Eur. J. Cell Biol.* 51, 189-200.

Reed, S.I. (1992). The role of p34 kinases in the G1 to S-phase transition. *Ann. Rev. Cell Biol.* 8, 529-561.

Rexach, M.F. and Scheckman, R.W. (1991). Distinct biochemical requirements for the budding, targeting and fusion of ER-derived transport vesicles. *J. Cell Biol.* 114, 219-229.

Rose, J.K. and Doms, R.W. (1988). Regulation of protein export from the endoplasmic reticulum. *Ann. Rev. Cell Biol.* 4, 257-88.

Roth, J. (1987). Subcellular organization of glycosylation in mammalian cells. *Biochem. Biophys. Acta* 906, 405-436.

Roth, J. and Berger, E.G. (1982). Immunocytochemical localization of galactosyltransferase in HeLa cells: codistribution with thiamine pyrophosphatase in trans-Golgi cisternae. *J Cell Biol* 93, 223-9.

Roth, J., Taatjes, D.J., Lucocq, J.M., Weinstein, J. and Paulson, J.C. (1985). Demonstration of an extensive trans-tubular network continuous with the Golgi apparatus stack that may function in glycosylation. *Cell* 43, 287-295.

Rothman, J.E. (1981). The Golgi apparatus: two organelles in tandem. *Science* 213, 1212-1219.

Rothman, J.E. (1989). Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. *Cell* 59, 591-601.

Rothman, J.E., Miller, R.L. and Urbani, L.J. (1984). Intercompartmental transport in the Golgi is a dissociative process: facile transport of membrane protein between two Golgi populations. *J. Cell Biol.* 99, 260-271.

Rothman, J.E. and Orci, L. (1992). Molecular dissection of the secretory pathway. *Nature* 355, 409-416.

Rothman, J.E., Urbani, L.J. and Brands, R. (1984). Transport of protein between cytoplasmic membranes of fused cells: correspondence to processes reconstituted in a cell-free system. *J. Cell Biol.* 99, 248-259.

Russell, P. and Nurse, P. (1986). *cdc25+* functions as an inducer in the mitotic control of fission yeast. *Cell* 45, 145-53.

Russell, P. and Nurse, P. (1987a). The mitotic inducer *nim1+* functions in a regulatory network of protein kinase homologs controlling the initiation of mitosis. *Cell* 49, 569-76.

Russell, P. and Nurse, P. (1987b). Negative regulation of mitosis by *wee1+*, a gene encoding a protein kinase homolog. *Cell* 49, 559-67.

Salminen, A. and Novick, P.J. (1987). A ras-like protein is required for a post-Golgi event in yeast secretion. *Cell* 49, 527-38.

Saraste, J. and Kuismanen, E. (1984). Pre- and post-Golgi vacuoles operate in the transport of Semliki Forest virus membrane glycoproteins to the cell surface. *Cell* 38, 535-49.

Schlepper, J. and Knippers, R. (1975). Nuclear protein kinases from murine cells. *Eur J Biochem* 60, 209-20.

Schwaninger, R., Plutner, H., Bokoch, G.M. and Balch, W.E. (1992). Multiple GTP-binding proteins regulate vesicular transport from the ER to Golgi membranes. *Journal of Cell Biology* 119, 1077-1096.

Segev, N. and Botstein, D. (1987). The ras-like yeast YPT1 gene is itself essential for growth, sporulation and starvation response. *Mol. Cell. Biol.* 7, 2367-2377.

Segev, N., Mulholland, J. and Botstein, D. (1988). The yeast GTP-binding YPT1 protein and a mammalian counterpart are associated with the secretion machinery. *Cell* 52, 915-924.

Semenza, J.C., Hardwick, K.G., Dean, N. and Pelham, H.R. (1990). ERD2, a yeast gene required for the receptor-mediated retrieval of luminal ER proteins from the secretory pathway. *Cell* 61, 1349-57.

Serafini, T., Orci, L., Amherdt, M., Brunner, M., Kahn, R.A. and Rothman, J.E. (1991). ADP-ribosylation factor is a subunit of the coat of Golgi-derived COP-coated vesicles: a novel role for a GTP-binding protein. *Cell* 67, 239-53.

Sherr, C.J. (1993). Mammalian G1 cyclins. *Cell* 73, 1059-1065.

Shirataki, H., Kaibuchi, K., Sakoda, T., Kishida, S., Yamaguchi, T., Wada, K., Miyazaki, M. and Takai, Y. (1993). Rabphilin-3A, a putative target for smg-p25A/rab3A p25 small GTP-binding protein related to synaptotagmin. *Mol. Cell. Biol.* 13, 2061-2068.

Shirodkar, S., Ewen, M., DeCaprio, J.A., Morgan, J., Livingston, D.M. and Chittenden, T. (1992). The transcription factor E2F interacts with the retinoblastome product and a p107-cyclin A complex in a cell cycle-regulated manner. *Cell* 68, 157-166.

Shuttleworth, J., Gdfrey, R. and Coleman, A. (1990). p40^{MO15}, a cdc2-related protein kinase involved in negative regulation of meiotic maturation of *Xenopus* oocytes. *EMBO J.* 9, 3233-3240

Simanis, V. and Nurse, P. (1986). The cell cycle control gene *cdc2+* of fission yeast encodes a protein kinase potentially regulated by phosphorylation. *Cell* 45, 261-8.

Smith, L.D. and Ecker, R.E. (1965). Protein synthesis in enucleated eggs of *Rana pipiens*. *Science* 150, 777-9.

Smith, L.D. and Ecker, R.E. (1971). The interaction of steroids with *Rana pipiens* Oocytes in the induction of maturation. *Dev Biol* 25, 232-47.

Smythe, C. and Newport, J.W. (1992). Coupling of mitosis to the completion of S phase in *Xenopus* occurs via modulation of the tyrosine kinase that phosphorylates p34^{cdc2}. *Cell* 68, 787-97.

Soellner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P. and Rothman, J.E. (1993). SNAP receptors implicated in vesicle targeting and fusion. *Nature* 362, 318-324

Solomon, M.J., Glotzer, M., Lee, T.H., Philippe, M. and Kirschner, M.W. (1990). Cyclin activation of p34^{cdc2}. *Cell* 63, 1013-24.

Solomon, M.J., Harper, J.W. and Shuttleworth, J. (1993). CAK, the p34^{cdc2} activating kinase, contains a protein identical or closely related to p40^{MO15}. *EMBO J.* 12, 3133-3142.

Stamnes, M.A. and Rothman, J.E. (1993). The binding of AP-1 clathrin adaptor particles to Golgi membranes requires ADP-ribosylation factor, a small GTP-binding protein. *Cell* 73, 999-1005.

Stanley, P. (1984). Glycosylation mutants of animal cells. *Annu Rev Genet* 18, 525-52.

Steinman, K.E., Belinsky, G.S., Lee, D. and Schlegel, R. (1991). Chemically-induced premature mitosis: differential response in rodent and human cells and the relationship to cyclin B synthesis and p34 cdc2-cyclin B complex formation. *Proc. Natl. Acad. Sci.* 88, 6843-6847.

Stow, J.L., de, A.J., Narula, N., Holtzman, E.J., Ercolani, L. and Ausiello, D.A. (1991). A heterotrimeric G protein, G α_{i-3} , on Golgi membranes regulates the secretion of a heparan sulfate proteoglycan in LLC-PK1 epithelial cells. *J Cell Biol* 114, 1113-24.

Strom, M., Vollmer, P., Tan, T.J. and Gallwitz, D. (1993). A yeast GTPase activating protein that interacts specifically with a member of the Ypt/rab family. *Nature* 361, 736-739.

Surana, U., Amon, A., Dowzer, C., McGrew, J., Byers, B. and K., N. (1993). Destruction of the CDC28/CLB mitotic kinase is not required for the metaphase to anaphase transition in budding yeast.

Takai, A., Bialojan, C., Toschka, M. and Ruegg, C.J. (1987). Smooth muscle myosin phosphatase inhibition and force enhancement by black sponge toxin. *FEBS Lett* 217, 81-84.

Taylor, T.C., Kahn, R.A. and Melancon, P. (1992). Two distinct members of the ADP-ribosylation factor family of GTP-binding proteins regulate cell-free intra-Golgi transport. *Cell* 70, 69-79.

Th'ng, J.P., Wright, P.S., Hamaguchi, J., Lee, M.G., Norbury, C.J., Nurse, P. and Bradbury, E.M. (1990). The FT210 cell line is a mouse G2 phase mutant with a temperature-sensitive CDC2 gene product. *Cell* 63, 313-24.

- Thomas, L., Clarke, P.R., Pagano, M. and Gruenberg, J. (1992). Inhibition of membrane fusion in vitro via cyclin B but not cyclin A. *J Biol Chem* 267, 6183-7.
- Tuomikoski, T., Felix, M.A., Dorée, M. and Gruenberg, J. (1989). Inhibition of endocytic vesicle fusion in vitro by the cell-cycle control protein kinase cdc2. *Nature* 342, 942-5.
- Tyers, M., Tokiwa, G. and Futcher, B. (1993). Comparison of the *Saccharomyces cerevisiae* G1 cyclins: Cln3 may be an upstream activator of Cln1, Cln2 and other cyclins. *EMBO J.* 12, 1955-1968.
- Vale, R.D. (1991). Severing of stable microtubules by a mitotically-activated protein in *Xenopus* egg extracts. *Cell* 64, 827-840.
- van der Sluijs, P., Hull, M., Huber, L.A., Male, P., Goud, B. and Mellman, I. (1992). Reversible phosphorylation-dephosphorylation determines the localization of rab4 during the cell cycle. *EMBO Journal* 11, 4379-4389.
- van der Sluijs, P., Hull, M., Zahraoui, A., Tavitian, A., Goud, B. and Mellman, I. (1991). The small GTP-binding protein rab4 is associated with early endosomes. *Proc. Natl. Acad. Sci.* 88, 6313-6317.
- Velasco, A., Hendricks, L., Moremen, K.W., Tulsiani, D.R.P., Touster, O. and Farquhar, M.G. (1993). Cell type-dependent variations in the subcellular distribution of α -mannosidase I and II. *J. Cell Biol.* 122, 39-51.
- Verde, F., Dogterom, M., Stelzer, E., Karsenti, E. and Leibler, S. (1992). Control of microtubule dynamics and length by cyclin A- and cyclin B-dependent kinases in *Xenopus* egg extracts. *J. Cell Biol.* 118, 1097-1108.
- von Heijne, G. (1985). Signal Sequences: the limits of variation. *J Mol Biol* 184, 99-105.
- Walter, P. and Lingappa, V.R. (1986). Mechanism of protein translocation across the endoplasmic reticulum membrane. *Ann. Rev. Cell Biol.* 2, 499-516.
- Ward, G.E. and Kirschner, M.W. (1990). Identification of cell cycle-regulated phosphorylation sites on nuclear lamin C. *Cell* 61, 561-77.

Warren, G. (1985). Membrane traffic and organelle division. *Trends Biochem. Sci.* 439-443.

Warren, G. (1987). Signals and salvage sequences. *Nature* 327, 17-18.

Warren, G. (1993). Membrane partitioning during cell division. *Ann. Rev. Biochem.* in press

Warren, G., Davoust, J. and Cockcroft, A. (1984). Recycling of transferrin receptors in A431 cells is inhibited during mitosis. *EMBO J.* 3, 2217-2225.

Warren, G., Featherstone, C., Griffiths, G. and Burke, B. (1983). Newly synthesized G protein of vesicular stomatitis virus is not transported to the cell surface during mitosis. *J Cell Biol* 97, 1623-8.

Warren, G.B. (1993). Bridging the gap. *Nature* 362, 297-298.

Wasserman, W.J. and Masui, Y. (1976). A cytoplasmic factor promoting oocyte maturation: its extraction and preliminary characterization. *Science* 191, 1266-8.

Waters, M.G., Serafini, T. and Rothman, J.E. (1991). 'Coatomer': a cytosolic protein complex containign subunits of non-clathrin-coated Golgi transport vesicles. *Nature* 349, 248-251.

Wattenberg, B.W., Balch, W.E. and Rothman, J.E. (1986). A novel prefusion complex formed during protein transport between Golgi cisternae in a cell-free system. *J. Biol. Chem.* 261, 2202-2207.

Wattenberg, B.W., Raub, T.J., Hiebsch, R.R. and Weidman, P.J. (1992). The activity of Golgi transport vesicles depends on the presence of the N-ethylmaleimide sensitive factor (NSF) and a soluble NSF-attachment protein (SNAP) during vesicle formation. *J. Cell Biology* 118, 1321-1332.

Weidman, P.J., Melancon, P., Block, M.R. and Rothman, J.E. (1989). Binding of an N-ethylmaleimide-sensitive fusion protein to Golgi membranes requires both a soluble protein(s) and an integral membrane receptor. *J Cell Biol* 108, 1589-96.

Whiteheart, S.W., Griff, I.C., Brunner, M., Clary, D.O., Mayer, T., Buhrow, S.A. and Rothman, J.E. (1993). SNAP family of NSF attachment proteins includes a brain-specific isoform. *Nature* 362, 353-355.

Wiedmann, M., Huth, A. and Rapoport, T.A. (1984). *Xenopus* oocytes can secrete bacterial β -lactamase. *Nature* 309, 637-639.

Wilson, D.W., Whiteheart, S.W., Wiedmann, M., Brunner, M. and Rothman, J.E. (1992). A multisubunit particle implicated in membrane fusion. *J Cell Biol* 117, 531-8.

Wilson, D.W., Wilcox, C.A., Flynn, G.C., Chen, E., Kuang, W.J., Henzel, W.J., Block, M.R., Ullrich, A. and Rothman, J.E. (1989). A fusion protein required for vesicle-mediated transport in both mammalian cells and yeast. *Nature* 339, 355-9.

Woodman, P.G., Adamczewski, J., Hunt, T. and Warren, G.B. (1993). In vitro fusion of endocytic vesicles is inhibited by cyclin A-cdc2 kinase. *Mol. Biol. Cell* 4, 541-553.

Woodman, P.G., Mundy, D.I., Cohen, P. and Warren, G. (1992). Cell-free fusion of endocytic vesicles is regulated by phosphorylation. *J Cell Biol* 116, 331-8.

Wu, M. and Gerhart, J.C. (1980). Partial purification and characterisation of the maturation-promoting factor from the eggs of *Xenopus laevis*. *Dev. Biol.* 79, 465-477.

Yamashita, K., Yasuda, H., Pines, J., Yasumoto, K., Nishitani, H., Ohtsubo, M., Hunter, T., Sugimura, T. and Nishimoto, T. (1990). Okadaic acid, a potent inhibitor of type 1 and type 2A protein phosphatases, activates cdc2/H1 kinase and transiently induces a premature mitosis-like state in BHK21 cells. *EMBO J* 9, 4331-8.

Yoshihisa, T., Barlowe, C. and Schekman, R. (1993). Requirement for a GTPase-activating protein in vesicle budding from the endoplasmic reticulum. *Science* 259, 1466-1468.

Zeligs, J.D. and Wollman, S.H. (1979). Mitosis in rat thyroid epithelial cells in vivo
I. Ultrastructural changes in cytoplasmic organelles during the mitotic cycle. *J Ultrastruct Res* 66, 53-77.