CHARACTERISATION OF THE INVOLVEMENT OF RAB5 IN EARLY ENDOSOME FUSION

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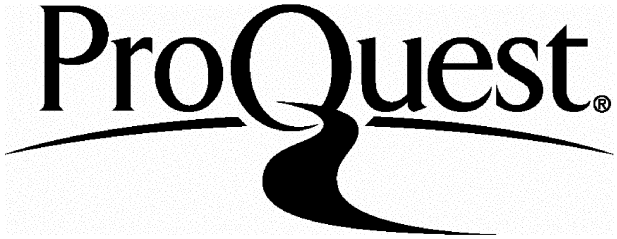

ABSTRACT

The rab5 protein is a member of the rab family of ras-related GTPases, which are known to be involved in the regulation of membrane transport in eukaryotic cells. Rab5 is localised to early endosomes, clathrin coated vesicles and the plasma membrane and has been shown to regulate an early step in the endocytic pathway both *in vivo* and *in vitro*. Although the exact function of the rab proteins is still unknown, their activity is believed to be dependent on their ability to cycle between GTP- and GDP-bound forms.

A cell-free assay has been used to characterise the role of rab5 in early endosome fusion. All three isoforms, rab5a, rab5b and rab5c, showed the same *in vitro* activity. The nucleotide requirement of rab5a was investigated using mutant proteins. The results of these experiments indicate that GTP-binding is required for rab5 activity, and that hydrolysis is required for inactivation and recycling of the protein. Data was also obtained indicating that the N-terminal domain of rab5 is required for its function.

Two phosphoproteins were detected which specifically co-immunoprecipitate with rab5. One of these was identified as the regulatory protein Rab-GDI. Although a small fraction of GDI was found on membranes, phosphorylated GDI was detected only in cytosol fractions. It is proposed that phosphorylation/dephosphorylation of GDI can regulate the specificity and directionality of the rab protein cycle.

Finally, the ability of the REP-1 protein to deliver rab proteins to membranes was investigated. Purified REP-1/rab5 complex significantly stimulated endosome fusion, confirming the hypothesis that REP-1 is an escort protein. This system should now enable us to study in detail the requirements for GDI and REP proteins in rab protein regulation.

Some of the work which is presented in this Thesis has been published or is in preparation for publication.

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ABBREVATIONS

The abbreviations and definitions used in this thesis are those recommended by the Editorial Board of the Biochemical Society (Biochem. J. 1994 **297**; 1-15)

In addition the following abbreviations have been adopted:

APS	Ammonium persulphate
ARF	ADP-ribosylation factor
BFA	Brefeldin A
BHK	Baby hamster kidney
b-insulin	Biotin-labelled insulin
DMEM	Dulbecco's modified Eagle medium
DOTAP	N-[1-(2,3-dioleoyloxy)propyl]-N,N,N trimethyl ammonium methylsulphate
GAP	GTPase activator
GDI	Guanine nucleotide dissociation inhibitor
GDS	Guanine nucleotide dissociation stimulator
GEF	Guanine nucleotide exchange factor
GM	Growth medium
GMEM	Glasgow's minimal essential medium
GRF	GDI release factor
HB	Homogenisation buffer
HRP	Horseradish Peroxidase
IEF	Isoelectric focusing
IM	Internalisation medium
KLH	Keyhole Limpet haemocyanin
LB	Lysis buffer
MCLM	Methionine/cysteine labelling medium
MLM	Methionine labelling medium
PLM	Phosphate labelling medium
PNS	Post-nuclear supernatant
RTB	Rosenbusch-Tenside buffer
TB	Transfection buffer
TBS	Tris buffered saline
TPA	12-O-tetradeconylphorbal-13-acetate
TPCK	N$^{\alpha}$-Tosyl-Phenylalanyl-Chloromethyl Ketone

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INTRODUCTION

OVERVIEW

The eukaryotic cell contains a vacuolar apparatus which is made up of a number of discrete membrane bound compartments, including the nucleus, endoplasmic reticulum (ER), Golgi, lysosomes, and endosomes, contained within the plasma membrane. These organelles are responsible for the biosynthesis, processing, targeting, secretion, internalisation and degradation of proteins and lipids. On the biosynthetic pathway, for example, secretory proteins synthesised on the cytoplasmic face of ER membranes are translocated co-translationally into the lumen of the ER. From there they progress sequentially through the Golgi stack and, after being sorted in the trans Golgi network (TGN), are finally released at the cell surface. Similarly proteins and other molecules internalised from the plasma membrane or the extracellular milieu into early endosomes, can either be transported to late endosomes and then lysosomes, or be recycled to the plasma membrane. In addition, there is considerable "cross-talk" between the endocytic and biosynthetic pathways. How then does the cell maintain the unique characteristics of each of its many compartments while actively transporting content and membrane from one to another?

Almost all, if not all, of these intra-organelle transport steps are carried out by a process known as vesicular transport (Palade, 1975). Transport vesicles bud from the membrane of a "donor" organelle and then fuse with the membrane of an "acceptor" organelle, thus delivering their fluid contents and membrane lipids and proteins to the acceptor organelle. The best characterised of these transport vesicles are the two forms of clathrin coated vesicles which mediate endocytosis and transport from the TGN to endocytic compartments (Pearse and Robinson, 1990). Clearly the fidelity of this system is essential; transport vesicles must be targeted to, and fuse with, the correct acceptor organelle.

In addition to vectorial vesicular transport between different organelles within the cell, many of these organelles, for example the early endosome, appear to be highly dynamic structures which are continually carrying out fission and fusion events (Gruenberg and Howell, 1989; Mellman and Simons, 1992). This process is seen at its most extreme during cell division when single copy organelles such as the Golgi apparatus and ER must be divided between two daughter cells (review: Warren, 1993b). In the

case of the Golgi apparatus the organelle is first broken into fragments and then vesicularised, following the completion of cell division it is then reassembled by the fusing together of the cognate parts in a "homotypic" fusion process (Thyberg and Moskalewski, 1985; Lucocq et al., 1989). Irrespective of whether these processes are occurring in interphase or mitotic cells, the salient point is that the fusion process requires the same fidelity as that required in vesicular transport. Therefore, these two systems could be expected to share common factors which are required for the maintenance of fidelity. In fact, as the mechanisms regulating membrane fusion begin to be unravelled it has become obvious that they are highly conserved, both between different species and for different fusion steps within the cell (review: Rothman and Warren, 1994).

Among the many proteins which have now been found to be involved in membrane transport events within the cell are the family of small GTP-binding proteins called the rab proteins. In this work the function and regulation of one member of this family, the rab5 protein which regulates an early membrane fusion event of the endocytic pathway (Gorvel et al., 1991; Bucci et al., 1992), has been investigated. This introduction is divided into two parts, the first being devoted to the rab proteins and the second to the endocytic pathway and the regulation of early endosome fusion *in vitro*.

RAB PROTEINS

In the last decade cell biologists have finally been able to begin to understand the mechanisms involved in eukaryotic membrane transport. The success in this field owes much to the development of three different, but complementary systems; genetic analysis of vesicular traffic mutants in the yeast *Saccharomyces cerevisiae*, isolation of genes by molecular biology and reconstitution of specific membrane transport steps in cell-free assays. One of the outstanding successes to have come from the use of these techniques was the identification of the Ypt1/Sec4/rab family of small GTP-binding proteins as regulators of vesicular traffic. The involvement of these GTPases in membrane transport was first indicated by the characterisation of two transport mutants in *S. cerevisiae*, *sec4* and *ypt1*, which showed that the products of both of these genes are small GTP-binding proteins, closely related to the proto-oncogene p21ras (Salminen and Novick, 1987; Goud et al., 1988; Segev et al., 1988; Schmitt et al., 1988). Shortly thereafter, intra-Golgi transport was shown to be inhibited by a non-hydrolysable GTP analog in a cell-free transport assay, suggesting a role for GTPases in mammalian membrane transport (Melançon et al., 1987). However, it is now known that

multiple GTP-binding proteins are involved in the regulation of transport and the GTPγS-sensitive GTPase is unlikely to be a member of the Ypt1/Sec4/rab family. Since that time the powerful tools now available in the field of molecular biology have allowed the identification of a large number of Ypt1/Sec4 related proteins, which have been classified into different subgroups according to their amino acid sequences (Valencia et al., 1991). The members of two of these subgroups, the ARF/Sar and Ypt1/Sec4/rab families, are now known to be exquisitely involved in the process of membrane transport, though their precise functions remain unclear (reviews: Pfeffer, 1992; Zerial and Stenmark, 1993; Simons and Zerial, 1993; Novick and Brennwald, 1993; Takizawa and Malhotra, 1993; Marsh and Cutler, 1993).

GTPases are required for membrane transport in eukaryotic cells

The reconstitution of distinct transport steps of both the endocytic and biosynthetic pathways using cell-free systems has been used extensively to study membrane transport in eukaryotic cells and yeast. These *in vitro* assays have been shown to be extremely sensitive to GTPγS, a non-hydrolyzable analog of GTP. On the biosynthetic pathway GTPγS inhibits protein transport between the ER and Golgi (Baker et al., 1988; Ruohola et al., 1988; Beckers and Balch, 1989) and between Golgi sub-compartments (Melançon et al., 1987), it also inhibits budding of transport vesicles from the TGN (Tooze et al., 1990) and recycling of the mannose-6-phosphate receptor from the late endosome to the TGN (Goda and Pfeffer, 1988).

On the endocytic pathway the effect of GTPγS is not so clear, as it appears to be able to either inhibit or stimulate homotypic fusion of early endosomes, depending on the cytosol conditions or the assay system (Mayorga et al., 1989a; Mayorga et al., 1989b; Tuomikoski et al., 1989; Bomsel et al., 1990). In fact GTPγS can also stimulate the formation of coated vesicles on the secretory pathway (Orci et al., 1993). The dual effects of GTPγS are now known to be due to the involvement of different kinds of GTP-binding protein (Colombo et al., 1992a; Lenhard et al., 1992; Orci et al., 1993; Palmer et al., 1993; Elazar et al., 1994), which will be discussed later.

Small GTP-binding proteins are required for vesicular transport in Saccharomyces cerevisiae

The first direct evidence for the involvement of small GTP-binding proteins in membrane transport came from genetic studies using the budding yeast *S. cerevisiae.* In the temperature sensitive *sec4* mutant, secretion is blocked and secretory vesicles accumulate at the restrictive temperature

(Salminen and Novick, 1987). The *SEC4* gene product (Sec4p) is a 23.5 kD protein, which is present on the cytoplasmic surface of post-Golgi secretory vesicles and the plasma membrane (Goud et al., 1988). When Sec4p was sequenced it was found to share approximately 30% sequence identity with the product of the proto-oncogenes H-ras and K-ras and subsequently it was shown to be able to bind and hydrolyse GTP (Kabçenell et al., 1990). Another mutant, *ypt1*, inhibits transport from the ER to the Golgi and causes proliferation of the ER (Schmitt et al., 1988; Segev et al., 1988; Baker et al., 1990). The *YPT1* gene product, Ypt1p, is a 23 kD GTP-binding protein with 30% amino acid identity with H-ras and 45% with Sec4p (Gallwitz et al., 1983).

Identification of Ypt1/Sec4 related GTP-binding proteins in eukaryotic cells

The results obtained from *S. cerevisiae* secretion mutants and the use of non-hydrolysable GTP analogs indicated that small ras-related GTP-binding proteins could play an integral role in membrane transport. On the basis of these observations Henry Bourne (Bourne, 1988) proposed that these proteins would regulate transport in a manner analogous to the regulation of protein synthesis by the elongation factor Tu. Although this model will be discussed in detail later in this introduction, it is important to already stress one major prediction of the model; namely that each step in membrane transport would require a different GTP-binding protein and thus that a large number of these proteins should be present in eukaryotic cells. This prediction was soon fulfilled as molecular biologists started to search intensively for related proteins. Although the first mammalian Ypt1/Sec4 related proteins were isolated from a rat brain cDNA library (Touchot et al., 1987), using an oligonucleotide corresponding to a motif (DTAGQE) conserved among ras and ras-related proteins, a large number of rab (from **ra**t **b**rain) proteins were rapidly discovered in yeast and mammalian cells (Chardin and Tavitian, 1986; Haubruck et al., 1987; Bucci et al., 1988; Matsui et al., 1988; Pizon et al., 1988; Sewell and Kahn, 1988; Didsbury et al., 1989; Polakis et al., 1989; Zahraoui et al., 1989). In 1990 Chavrier and colleagues (Chavrier et al., 1990a; Chavrier et al., 1990b) screened a kidney epithelial cell (MDCK) cDNA library using an oligonucleotide corresponding to an amino acid motif (WDTAGQE), which is highly conserved between members of the Ypt1/Sec4/rab family of proteins, with the long term goal of identifying proteins involved in membrane transport steps specific to polarised epithelial cells. Initially 11 cDNA sequences were isolated (Chavrier et al., 1990b), of which several were identical, or highly homologous with previously described

mammalian GTP-binding proteins (Haubruck et al., 1987; Touchot et al., 1987; Bucci et al., 1988; Haubruck et al., 1989; Zahraoui et al., 1989). Three of these proteins were then shown to be localised to specific exocytic and endocytic compartments (Chavrier et al., 1990a), thus further implicating rab proteins in membrane transport. Since that time a large number of rab proteins have been described and several of them have been characterised both *in vitro* and *in vivo*. It is clear from these studies that they play essential roles in the regulation of membrane transport in all eukaryotic cells (review: Simons and Zerial, 1993).

Rab proteins are members of the ras superfamily of small GTP-binding proteins

In addition to the rab proteins many other monomeric GTP-binding proteins, which show clear sequence homology with the p21ras proto-oncogene proteins, have been described in recent years (Hall, 1990a; Bourne et al., 1991; Grand and Owen, 1991; Valencia et al., 1991). This ras superfamily is made up of five subfamilies; ras, rho, ran, ARF and rab, of which the rab family is the largest (Fig. 1). The similarity in sequence which allows the grouping of these proteins into subfamilies is also reflected in the functions of the individual family members. The ras proteins are components of one of the signal-transduction pathways mediating cell growth and differentiation, and were first discovered on the basis of the oncogenic activity of viral ras proteins, they are now known to be present in all eukaryotes including yeast (Lowy and Willumsen, 1993). The rho proteins link growth factor receptors to the assembly of cytoskeletal elements and regulate the NADPH oxidase in macrophages (Hall, 1992; Zhang et al., 1993; Bokoch and Knaus, 1994; Bromberg et al., 1994). The ran/TC4 protein is required for protein import into the nucleus (Melchior et al., 1993; Moore and Blobel, 1993) or the regulation of nuclear structure and may play a role in the signaling pathways which mediate progression through the cell cycle (Kornbluth et al., 1994). The ARF family of proteins are, like the rab proteins, involved in the regulation membrane transport and have recently also been implicated in the regulation of phospholipase D activity (Brown et al., 1993; Orci et al., 1993; Ostermann et al., 1993; Teal et al., 1994).

With the exception of ran, the biological activity of all of these proteins is dependent on membrane association. Post-translational modifications, which in the case of the rab proteins is covalent attachment of a geranylgeranyl group to a C-terminal cysteine residue, are required for membrane association for all of these proteins except Sar1p (review: Newmann and Magee, 1993).

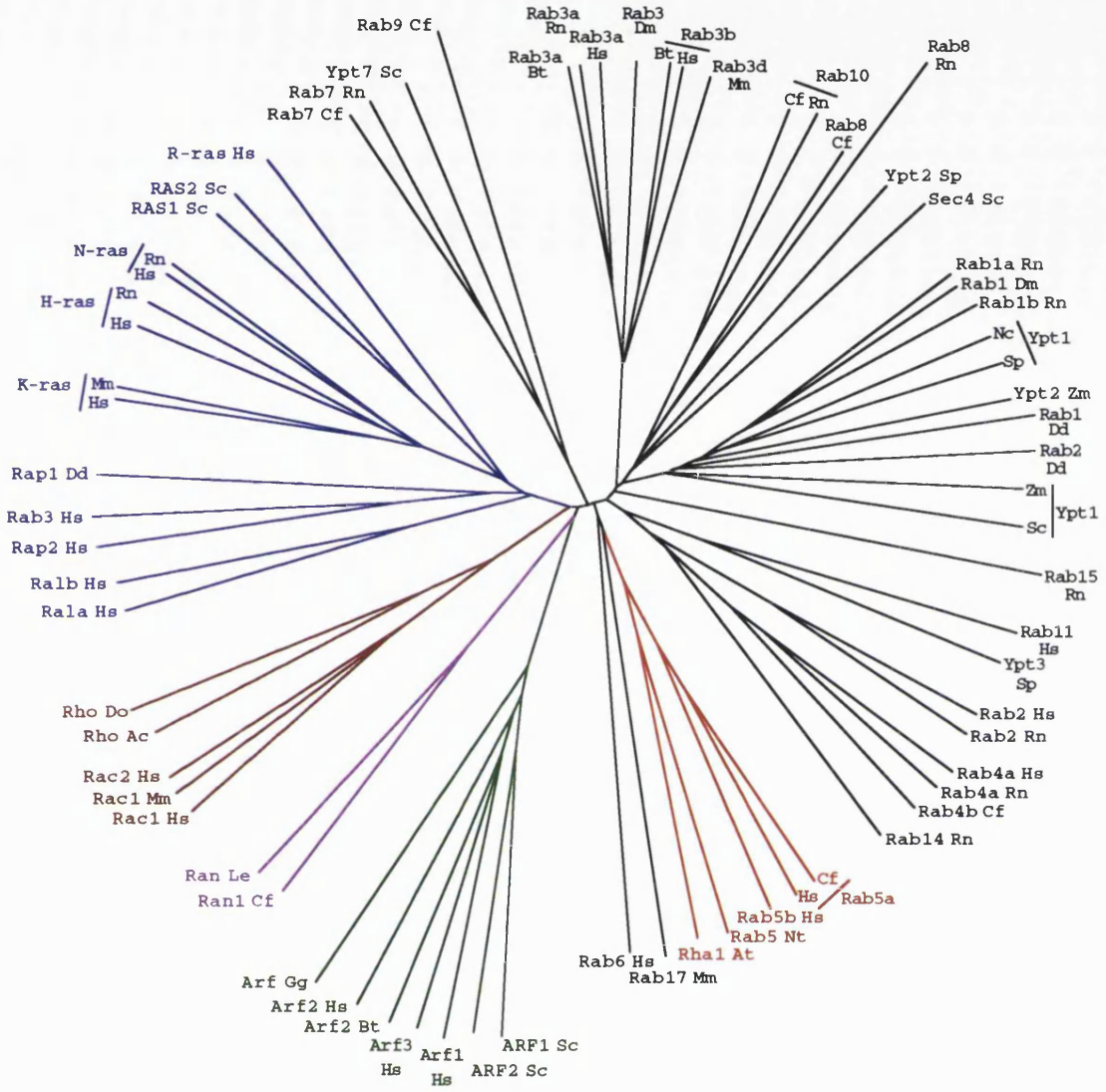


Figure 1 Evolutionary tree of the ras-related superfamily of small GTP-binding proteins. The five major families; ras (blue), rho (brown), ran (purple), arf (green) and rab (black) are indicated. The rab5 subfamily is highlighted in red. Alignments were carried out using Clustalw and the tree was plotted using Phylip3.5. The species abbreviations used are; Ac, Aplysia californica; At, Aribidopsis thaliana; Bt, Bovine; Ce, Caenorhabditis elegans; Cf, canine; Dd, Dictyostelium discoideum; Dm, Drosophila melanogastor; Do, Dicopyge ommata; Gg, chick; Hs, human; Le, tomato; Mm, mouse; Nc, Neurospora crassa; Nt, tobacco; Rn, rat; Sc, Saccharomyces cerevisiae; Sp, Shizosaccharomyces pombe; Zm, maize.

While the functions of the ras superfamily proteins are diverse, they all utilise their ability to bind and hydrolyse GTP in order to regulate reversible interactions with other effector molecules. The GTP-binding domains of these proteins are highly conserved, consisting of two phosphate/Mg^{2+}-binding loops with the consensus sequences of GxxxxGK{S,T} and DTAG in the N-terminal sub domain and two guanine base binding loops, NKxD and ExSAK, in the C-terminal sub domain (Fig. 2) and probably all have a similar tertiary structure (Valencia et al., 1991). Because of this high degree of conservation it has been possible to design mutants for many of these proteins which are based on well characterised ras substitution mutants which affect nucleotide binding, exchange or hydrolysis. Such mutants have proven invaluable in the study of rab proteins, both *in vivo* and *in vitro*.

The rab family is further subdivided into groups of highly related proteins

The rab family of GTP-binding proteins now includes more than 30 members, most of these proteins share amino acid identity of approximately 30%, however, some of them are >90% identical (reviews: Goud, 1992; Gruenberg and Clague, 1992; Pfeffer, 1992; Zerial and Stenmark, 1993). These highly homologous proteins have been put into subgroups and labelled alphabetically, for example; rab1a and rab1b, or rab3a, rab3b, rab3c and rab3d. The significance of these subgroups of rab proteins is not clear, although the high degree of homology seen in isoforms from different species indicates that there is a functional constraint (Fig. 2). However, the data so far accumulated on the activity of the different isoforms indicates that if there are functional differences between these proteins, they may be very subtle and difficult to assess. The existence of multiple isoforms is not limited to the rab proteins and seems to be a characteristic of the ras and ras-related GTPases, thus the reason for their evolution may only become clear as our understanding of the different families is increased.

The rab1a and rab1b proteins both regulate ER to Golgi transport indicating that they have similar, or identical, functions (Tisdale et al., 1992). However, this may not be true for all of the subgroups as indicated by the following observations. Firstly, antibodies raised against rab5a, which do not recognise rab5b or rab5c, inhibit early endosome fusion *in vitro* (Gorvel et al., 1991). Secondly, rab1a and rab4a can be phosphorylated by the $p34^{cdc2}$ kinase, whereas rab1b and rab4b, in common with other rab proteins, do not contain the consensus phosphorylation sites (Bailly et al., 1991; van der Sluijs et al., 1992b). Finally, expression of some members of the rab3 subgroup is

Figure 2 Multiple alignment of Rab5 proteins. Identical (red) and conserved (green) residues are indicated. The sequence of p21-ras is shown underneath. The four GTP-binding domains are boxed in yellow, the rab5 specific sequence in grey. Other structural domains of the proteins, which are refered to in the text, are indicated by black bars. Species abbreviations are as in Fig. 1. Alignment was carried out using the Clustalw program.

cell type specific and/or dependent on the differentiation state of cells. Rab3a appears to be only expressed in cells which carry out regulated secretion such as endocrine and exocrine cells and neurons (Ayala et al., 1989; Sano et al., 1989; Mizoguchi et al., 1990), rab3b is expressed preferentially in polarised epithelial cells (Weber et al., 1994) and rab3d protein expression is increased during differentiation of 3T3-L1 cells into adipocytes (Baldini et al., 1992).

Structure of the rab proteins

All of the ras and ras-related proteins, including the Ypt1/Sec4/rab family, share the same basic structure. Figure 2 shows a sequence alignment of the known rab5 proteins with H-ras, illustrating the four regions which make up the GTP-binding pocket and are the most highly conserved regions in all of the ras-related proteins (Valencia et al., 1991). The region labelled G3 includes the WDTAGQE motif, which was used to isolate many of the rab proteins (Chavrier et al., 1990b). The effector domain is another region that is highly conserved between the members of the Ypt1/Sec4/rab family; identified in the ras proteins this domain interacts with GAP (GTP-ase activating) proteins and its conformation changes according to whether ras is bound to GDP or GTP (Barbacid, 1987; McCormick, 1989; Bourne et al., 1991). Multiple sequence alignments identify the N- and C-terminal regions of the rab proteins as being the most divergent (Valencia et al., 1991) and the C-terminal domain can be divided into two parts, which are required for membrane association and localisation. At the extreme C-terminus a 2-5 amino acid motif, containing one or two cysteine residues, is absolutely required for the attachment of geranylgeranyl groups, and thus membrane attachment of the rab proteins (Chavrier et al., 1990a; Peter et al., 1992). Immediately upstream from this motif is the hypervariable region of 30-40 amino acids, which shows the most variability between the rab proteins (Valencia et al., 1991).

Localisation of rab proteins

Within the eukaryotic cell each rab protein is found in the cytosol and also attached to the cytoplasmic face of specific organelles. Almost all of the organelles within the cell have now been shown to have their own complement of rab proteins (Fig. 3). The localisation signal required for attachment to the correct membrane was shown by the construction of chimeric proteins to reside in a 35 amino acid stretch of the hypervariable carboxy-terminal region of the rab proteins (Chavrier et al., 1991; Brennwald and Novick, 1993; Stenmark et al., 1994a). This was initially demonstrated in

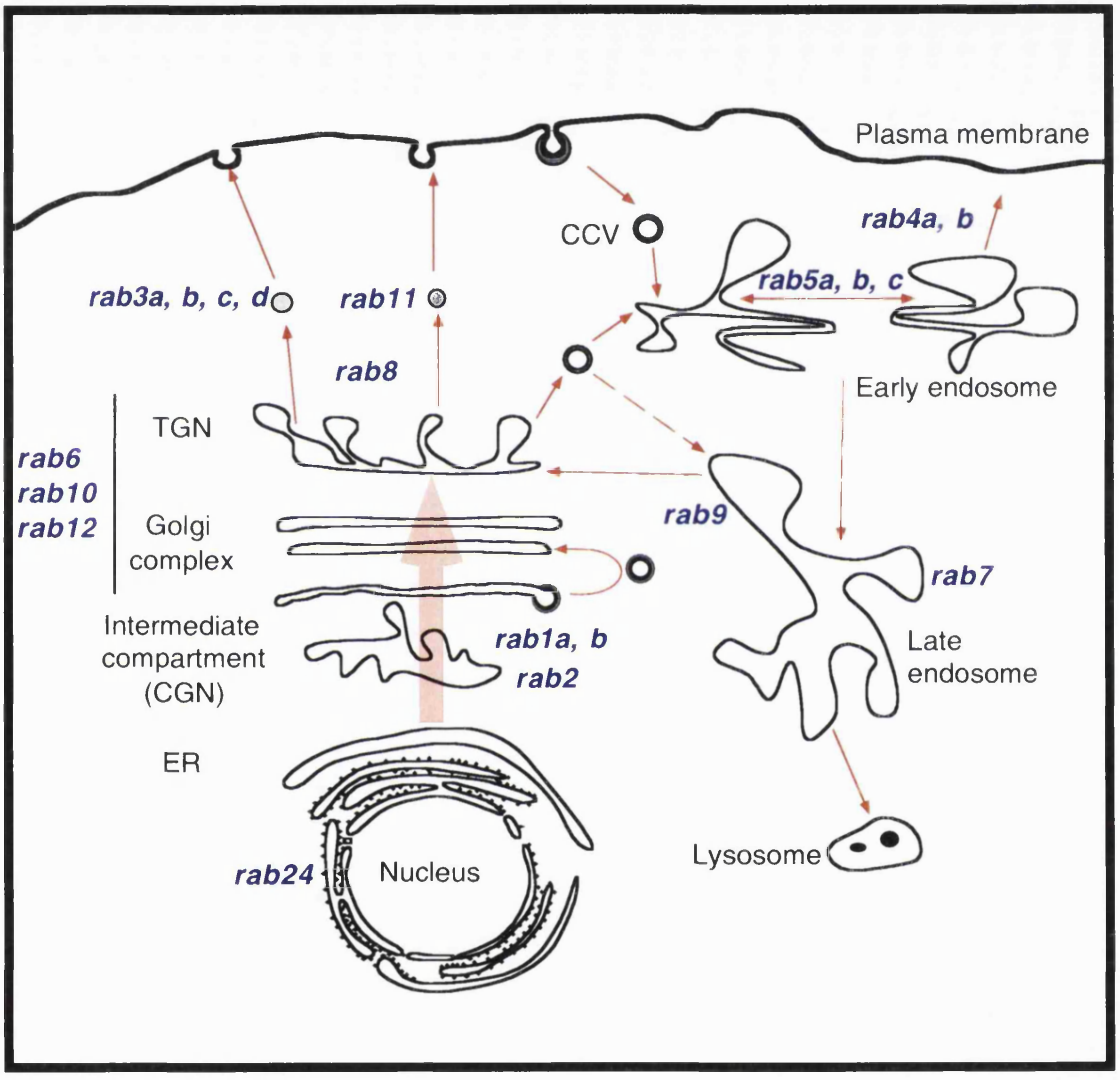


Figure 3 Localisation of rab proteins in mammalian cells.

"tail-swapping" experiments where the rab2 and rab7 proteins were retargeted, from the intermediate compartment or late endosome respectively, to the early endosome by replacing their C-terminals with that of rab5 (Chavrier et al., 1991). Interestingly the cysteine motif at the extreme C-terminus of the rab proteins, which is required for membrane attachment via lipid modification of cysteine residues, does not seem to affect localisation. When the CCSN motif of rab5 was replaced with the CSC sequence of rab7 the chimeric protein showed the same localisation as wild type rab5 (Chavrier et al., 1991).

The targeting of rab proteins via the localisation signal to distinct organelles indicates the existence of specific rab receptors on the membranes of these compartments, especially as overexpression of rab proteins does not lead to their mislocalisation but rather cytoplasmic accumulation (Chavrier et al., 1990a; Chavrier et al., 1991). However, overexpression also leads to a several-fold increase in the amount of protein associated with the specific membranes. Thus, if there is a saturatable receptor for each rab protein it must be present at levels much higher than those of the endogenous rab protein. So far it has not been possible to identify a receptor with these characteristics. It is possible that, rather than a receptor in the classical sense, the rab proteins may be recognised by a membrane associated protein that only transiently associates while inserting them into the membrane. This would agree with the observations that expression of rab proteins using recombinant vaccinia virus, which produces a slow accumulation of the overexpressed protein, leads to a greater increase in membrane associated rab protein than the T7 polymerase recombinant-vaccinia virus overexpression system, which produces large amounts of protein in a very short time and leads to very high cytosolic levels of rab proteins (Chavrier et al., 1990a; Gorvel et al., 1991; Bucci et al., 1992). Alternatively this observation may be explained by saturation of the machinary involved in C-terminal prenylation of the rab proteins.

Post-translational modification of rab proteins

Carboxy-terminal modification

The Ypt1/Sec4/rab family of proteins, like the ras proteins, are synthesised as soluble precursors, and membrane attachment is mediated by post-translational isoprenylation of cysteine residues at the C-terminus (review: Newmann and Magee, 1993). All known ras proteins terminate with a motif known as the CAAX box (C, cysteine; A, aliphatic; X, serine/methionine/alanine) which is subjected to three post-translational

modifications. Farnesylation of the cysteine residue is followed by proteolytic cleavage of the AAX sequence and finally carboxyl-methylation of the C-terminal α-carboxyl group (Gutierrez et al., 1989; Hancock et al., 1989; Hancock et al., 1991). Although most of the ras-related proteins appear to be prenylated, except for the ARF proteins which lack a cysteine motif at the C-terminus but are myristilated at the N-terminus (Kahn et al., 1988) and the ran/TC4 and SAR1 proteins which have no consensus prenylation site (Nakano and Muramatsu, 1989; Bischoff and Ponsteig, 1990a; Bischoff and Ponsteig, 1990b), farnesylation seems to be restricted to the true ras proteins. Most other members of the ras super family are modified by a C_{20} geranylgeranyl isoprenoid. This is true even for ras-related proteins which possess the CAAX motif, and may be determined by the hydrophobicity of the X residue (Newmann and Magee, 1993).

The rab proteins usually have C-terminal motifs of the type CC or CXC (where X is any amino acid) deletion of which prevents isoprenylation and membrane association (Chavrier et al., 1990a; Gorvel et al., 1991; Peter et al., 1992). All of the rab proteins seem to be geranylgeranylated, including rab8, rab11 and rab13 which have the classical CAAX motif seen in ras proteins (Joberty et al., 1993). At least one of these proteins, the rab3a (smg25A) protein, acquires geranylgeranyl groups on both of its C-terminal cysteines (Farnsworth et al., 1991). However, it is not clear if this is true for other members of the rab family.

The enzyme responsible for prenylation of rab proteins was recently purified from rat brain, it is a multisubunit enzyme consisting of two major components; a catalytic subunit and another component which is required to present the rab protein to the catalytic subunit (Seabra et al., 1992a; Seabra et al., 1992b). The catalytic subunit, previously referred to as component B but now usually called rab geranylgeranyl transferase (Rab GG transferase), is a tightly bound dimer of α and β subunits with molecular masses of 60 kD and 38 kD respectively (Seabra et al., 1992a; Seabra et al., 1992b; Armstrong et al., 1993b). These subunits show clear sequence homology with the α and β subunits of the ras farnesyltransferase (Armstrong et al., 1993b). Unlike the ras farnesyl transferase, component B of Rab GG transferase requires an accessory protein, the 95 kD component A (now called REP-1, for **r**ab **e**scort **p**rotein), which is required for geranylgeranyl transferase activity (Seabra et al., 1992a; Seabra et al., 1992b). Two closely related REP proteins, REP-1 and REP-2, have been identified in human and rat cells. While they both facilitate the prenylation of all rab proteins which have been tested, the efficiency of modification varies (Cremers et al., 1994).

Sequence analysis of the REP-1 and REP-2 proteins from rat revealed that they are the counterparts of the human choroideremia and choroideremia-like gene products respectively (Andres et al., 1993; Seabra et al., 1993). The choroideremia gene was identified as the site of mutation in patients suffering from an X-linked form of late-onset retinal degeneration and it has been shown that lymphoblasts isolated from patients with choroideremia do not contain functional REP-1 (Cremers et al., 1990; Merry et al., 1992; Seabra et al., 1993). Another interesting observation is that both REP-1 and REP-2 have several regions of homology with the rab regulatory protein GDI (Fig. 4), which will be discussed in detail later in this section (Cremers et al., 1994). The homology between GDI and the REP proteins, and the fact that, in contrast to other prenyltransferases, the rab geranylgeranyl transferase requires structural determinants other than, or in addition to, the cysteine motif (Moores et al., 1991; Khosravi-Far et al., 1992; Kinsella and Maltese, 1992; Wilson and Maltese, 1993) suggests that these two proteins may recognise the same conserved sequences in rab proteins (Seabra et al., 1992b).

In vitro REP-1 binds to unmodified rab proteins, and presents them to Rab GG transferase, and it has been suggested that, following geranylgeranyl transfer and the subsequent dissociation of the transferase, it escorts the modified rab protein to the specific membrane (Andres et al., 1993). However, *in vitro*, the modified rab protein is unable to dissociate from REP in the absence of detergent (Andres et al., 1993; Alexandrov et al., unpublished data) and it is possible that, *in vivo,* this process is mediated by interaction with another protein, perhaps GDI or a membrane associated "receptor" protein.

Whether the C-terminus of rab proteins is further processed by proteolytic cleavage and carboxyl-methylation as for ras proteins is still unclear. *In vivo* rab3a has been shown to be carboxy-methylated (Farnsworth et al., 1991) as is the *Schizosaccharomyces pombe* ypt5 protein (Newman et al., 1992). Interestingly both of these proteins have the CXC motif and examination of two other *S. pombe* proteins, ypt1p and ypt3p, and the rab2 protein, all of which have the CC motif, did not reveal carboxy-methylation (Newman et al., 1992; Wei et al., 1992), indicating that methylation may be directed by the CXC motif. The enzyme responsible for methylation has not yet been identified, although Newman and colleagues speculate that it is not the same enzyme that acts on the ras proteins. Palmitoylation has been reported for *S. pombe* ypt3p and *S. cerevisiae* Ypt1p (Molenaar et al., 1988; Newman et al., 1992), however, most of the rab proteins do not have suitably

Figure 4 Multiple sequence alignment of GDI and REP proteins. Positions of amino acid identity are indicated in red and those of similarity in green. The alignment was carried out using the Clustalw program.

positioned cysteine residues for this modification. The ras proteins appear to require either palmitoylation or a polybasic domain, in addition to farnesylation, for efficient membrane attachment (Hancock et al., 1989; Hancock et al., 1990; Hancock et al., 1991a) but it is possible that the greater hydrophobicity of the geranylgeranyl group relative to farnesyl negates the requirement for such a "secondary" modification in rab proteins.

Phosphorylation

Two rab proteins, rab1a and rab4a, have been shown to be phosphorylated by $p34^{cdc2}$ in mitotic cells (Bailly et al., 1991; van der Sluijs et al., 1992b). It is not clear what the significance of this observation is particularly as phosphorylation seems to have different effects on the two proteins; the rab4 protein dissociates from membranes whereas the rab1 protein shows reduced cytosolic concentrations in mitotic cells. Recently it has been reported that three other rab proteins, rab3b, rab6 and rab8, are phosphorylated in thrombin activated human platelets although neither the effect of phosphorylation nor the kinase activity responsible for phosphorylation were investigated (Karniguian et al., 1993).

Regulation of membrane transport by rab proteins

Since the discovery of the first rab proteins a large amount of data has been produced confirming the initial proposal that they may regulate membrane transport in eukaryotic cells (Bourne, 1988). As indicated by the specificity of their localisation, the activity of these GTPases is restricted to specific membrane transport steps (reviews: Gruenberg and Clague, 1992; Simons and Zerial, 1993; Zerial and Stenmark, 1993).

Rab proteins on the secretory pathway

On the exocytic pathway in animal cells the involvement of rab proteins has been shown for four distinct membrane transport steps; ER to Golgi, intra-Golgi, regulated fusion of exocytic vesicles with the plasma membrane and constitutive vesicle transport to basolateral/dendritic membranes (Fischer von Mollard et al., 1990; Plutner et al., 1991; Schwaninger et al., 1992; Tisdale et al., 1992; Huber et al., 1993a; Huber et al., 1993b; Elazar et al., 1994).

ER to Golgi vesicular transport appears to be regulated by at least three rab proteins; rab1a, rab1b and rab2 (Plutner et al., 1991; Tisdale et al., 1992). The rab1a and rab1b proteins, are 70-80% identical to *S. cerevisiae* Ypt1p (Haubruck et al., 1987; Valencia et al., 1991) and the rab1a protein can complement *S. cerevisiae* which lack the *YPT1* gene (Haubruck et al., 1989).

In mammalian cells both rab1a and rab1b proteins are found on the ER and Golgi compartments and have been shown to regulate transport between these compartments *in vitro* and *in vivo* (Plutner et al., 1991; Schwaninger et al., 1992; Tisdale et al., 1992). The third rab protein which is involved in ER to Golgi transport is the rab2 protein (Tisdale et al., 1992), which localises to the post-ER, pre-Golgi intermediate compartment (Chavrier et al., 1990a). The requirement for three rab proteins in ER to Golgi transport has still to be clarified. It is possible that the rab1a and rab1b proteins, which are 95% identical (Zahraoui et al., 1989), have the same function, but the rab2 protein shares less than 40% identity with the rab1 proteins (Valencia et al., 1991) and has a different subcellular localisation (Chavrier et al., 1990a; Plutner et al., 1991).

That the rab1 and rab2 proteins probably have different functions is also indicated by the activity of mutant proteins *in vivo* (Tisdale et al., 1992). Using the T7 polymerase recombinant vaccinia virus system to transiently express high levels of protein the effect of mutations in the GTP-binding domains of rab1a, rab1b and rab2 was studied. While overexpression of the wild type proteins had no detectable effect on the transport of the VSV G protein from the ER to the Golgi complex, overexpression of mutants equivalent to the ras N116I mutant (Der et al., 1988), resulted in inhibition of transport. The ras mutant has an increased GDP dissociation rate and is therefore predominantly in the GTP-bound form, but it appears that the equivalent rab mutants may also bind inefficiently to either GTP or GDP (Gorvel et al., 1990; Pind et al., 1994). However, mutations affecting the rate of nucleotide exchange or hydrolysis, equivalent to the ras S17N and Q61L mutants, had opposite effects in the rab1 and rab2 proteins. In ras the S17N mutation inactivates the protein, as the rate of nucleotide exchange is decreased and the mutant is primarily in the GDP-bound form, whereas the Q61L mutation impairs GTP hydrolysis and thus results in an increase in the amount of the active GTP-bound form of ras (Barbacid, 1987). The ras S17N equivalent rab1b mutant (rab1b S22N) was a potent inhibitor of ER to Golgi transport, but the rab2 mutant (rab2 S20N) had no effect. Thus GDP/GTP exchange is essential for the activity for the rab1 protein. In contrast, whereas the ras 61L equivalent rab1b mutant (rab1b Q67L) did not inhibit transport, the rab2 mutant (rab2 Q65L) significantly inhibited transport of the VSV G protein into the Golgi. These results strongly indicate that there is a difference in the activity of the rab1 (a and b) and rab2 proteins, although, the significance of the differences with respect to requirement for nucleotide binding and hydrolysis remain unclear.

The expression of members of the rab3 subgroup, which consists of at least 4 members (rab3a-d), is restricted to certain cell types and each of these proteins appear to regulate cell-type specific membrane transport events on the secretory pathway (Fischer von Mollard et al., 1994b). Two of these proteins, rab3a and rab3c, are found only in neurons where they are present on synaptic vesicles, but redistribute to the cytosol following nerve terminal stimulation (Fischer von Mollard et al., 1990; Fischer von Mollard et al., 1991; Matteoli et al., 1991; Fischer von Mollard et al., 1994a). Synthetic peptides of the rab3a effector domain also stimulate exocytosis (Oberhauser et al., 1992; Padfield et al., 1992; Senyshyn et al., 1992; Richmond and Haydon, 1993), and there is some indication that this activity is mediated by interaction of the peptides with G proteins (Law et al., 1993). Though whether this is a true reflection of the activity of the intact effector domain rather than a property of the isolated domain, which like the wasp venom peptide mastoparan is polybasic, remains unclear.

In pituitary cells rab3b appears to be the only rab3 protein which is expressed (Zahraoui et al., 1989; Baldini et al., 1992) and knock out of this protein using antisense oligonucleotides causes inhibition of calcium dependent exocytosis without affecting endocytosis (Lledo et al., 1993). Antisense oligonucleotides directed against rab3a had no effect in these cells (Lledo et al., 1993). In epithelial cells the localisation of rab3b is dependent on the polarisation state of the cells, and it has been suggested that this protein plays a role in apical and/or junctional traffic (Weber et al., 1994). The fourth rab3 protein, rab3d, is expressed predominantly in adipocytes, in which its expression is increased during differentiation, and it has been suggested that it may regulate the insulin-dependent exocytosis of glucose transporter-containing vesicles in these cells (Baldini et al., 1992).

A rab3-like protein which may be a fifth member of the rab3 family has been found in pancreatic acinar cells and localises primarily to the cytoplasmic surface of zymogen granules (Jena et al., 1994). Upon stimulation the zymogen granules fuse with the plasma membrane, but unlike rab3a (Matteoli et al., 1991) this rab3 isoform is not detected on the plasma membrane after stimulation. In contrast it seems to recycle directly to newly forming secretory granules at Golgi and the cytosolic concentration does not perceptibly increase (Jena et al., 1994).

Another rab protein which has been shown to regulate membrane traffic on the exocytic pathway in specialised cells is the rab8 protein. In cells in which the plasma membrane is divided into two domains the proteins destined for each domain are sorted in the TGN into separate populations of

vesicles which are then transported to the correct membrane. This system has been best studied in polarised epithelial cells, specifically in MDCK cells, where the plasma membrane is divided into apical and basolateral domains (Mostov et al., 1992). The rab8 protein localises to the Golgi, vesicular structures and the basolateral plasma membrane in these cells (Huber et al., 1993a). In vitro studies using perforated MDCK cells showed that a peptide derived from the C-terminal region of rab8 inhibited transport from the TGN to the basolateral plasma membrane, but did not affect the delivery of proteins to the apical surface (Huber et al., 1993a). In a parallel study it was demonstrated that rab8 fulfils a similar function in cultured neurons (Huber et al., 1993b). Like epithelial cells the plasma membrane of neurons can be divided into two domains with the sorting pathways to the axonal and somatodendritic domains being equivalent to those involved in sorting to the apical and basolateral domains, respectively (reviews: de Hoop and Dotti, 1993; Rodriguez-Boulan and Powell, 1992). In agreement with the idea that similar sorting mechanisms may be responsible for the generation and maintenance of the separate plasma membrane domains in these cells, rab8 antisense oligonucleotides inhibited delivery of plasma membrane proteins to the dendritic, but not to the axonal, plasma membrane in cultured hippocampal neurons (Huber et al., 1993b).

Rab proteins on the endocytic pathway

In addition to the rab4 and rab5 proteins, which will be discussed later, at least three other rab proteins, rab18, rab20 and rab22, are associated with early endosomes and two, rab7 and rab9, with late endosomes in mammalian cells (Chavrier et al., 1990a; van der Sluijs et al., 1992a; Lombardi et al., 1993; Olkkonen et al., 1993). While it is clear that rab5 and rab4 regulate an early step in endocytosis and recycling to the plasma membrane, respectively, there is no functional data for the other early endosome rab proteins (Gorvel et al., 1991; Bucci et al., 1992; van der Sluijs et al., 1992a). Presumably each of these proteins regulates a membrane traffic step within the early endocytic pathway, or between early endosomes and other organelles not directly associated with the endocytic pathway (Marsh and Cutler, 1993).

Two rab proteins, rab7 and rab9, have been localised to late endosomes in mammalian cells (Chavrier et al., 1990a; Lombardi et al., 1993). Although there is no functional data for rab7 the characterisation of homologs in *S. cerevisiae* (Ypt7p), Soybean (sRab7) and *Vigna aconitifolia* (vRab7) suggests that the protein has a role in the regulation of transport between endocytic compartments (Wichmann et al., 1992; Cheon et al.,

1993). While the endocytic pathway in yeast has not been well characterised, early and late endosomal compartments have been described and Ypt7p appears to be involved in the later part of the endocytic pathway (Singer and Riezman, 1990; Singer-Krüger et al., 1993). Ypt7p is 63% identical to rab7 and *Ypt7* null mutants display altered vacuole morphology, with several small vacuoles rather than a large single vacuole, and α-factor is degraded very slowly although its internalisation does not seem to be affected (Wichmann et al., 1992). In addition, the suppression of vRab7 (65% identity with rab7) expression in *V. aconitifolia* by the antisense oligonucleotide approach causes the accumulation of late endosome-like structures and multivesicular organelles (Cheon et al., 1993). From these observations it seems likely that rab7 is involved in the regulation of membrane transport at a step which involves late endosomes. Considering that canine rab5 can complement *YPT5* deletion mutants in *S. pombe* (Armstrong et al., 1993a) it will be interesting to see if rab7 can functionally replace Ypt7p in *S. cerevisiae*, especially as the identities between the yeast and mammalian proteins are the same (63%) in both cases. While the effector regions appear to be highly conserved, the C-terminal hypervariable regions, shown for the mammalian proteins to be required for correct localisation (Chavrier et al., 1992), show little homology (Armstrong et al., 1993a; Wichmann et al., 1992).

The rab9 protein, which localises to perinuclear mannose 6-phosphate receptor (MPR) positive structures and the TGN (Lombardi et al., 1993), appears to function in transport between late endosomes and the TGN. This has been demonstrated using a cell-free transport assay in which the transport of MPR from late endosomes to the TGN is reconstituted (Lombardi et al., 1993). Using the T7 polymerase-recombinant vaccinia virus system rab9 was transiently overexpressed at high levels in BHK cells, cytosol prepared from these cells was then used to complement the cell-free transport assay. Whereas cytosols containing rab4 or rab7 had no stimulatory effect on transport the rab9 cytosol stimulated transport by approximately 2.5-fold. Furthermore, recombinant rab9, purified after overexpression in *E. coli*, was also able to stimulate transport with maximum stimulation being achieved in the presence of geranylgeranyl diphosphate. As *E. coli* expressed rab proteins are not C-terminally prenylated this result suggested that C-terminal modification was occurring in the transport assay. This was confirmed by demonstrating that under the same conditions, in the presence of cytosol and ATP, *E. coli* recombinant rab9 could incorporate ^{3}H-labelled geranylgeranyl groups (Lombardi et al., 1993). The involvement of the C-terminal and requirement for membrane attachment was further illustrated by the lack of activity of a C-terminally truncated rab9 protein in the transport assay.

Regulation of rab proteins

It now appears that the ras and ras-related proteins are exquisitely controlled by interactions with three different classes of regulatory proteins (review: Boguski and McCormick, 1993) which can stimulate either GTPase activity (GTPase activating proteins) or nucleotide exchange (guanine-nucleotide exchange factors, also called guanine-nucleotide dissociation stimulators or guanine-nucleotide release factors), or inhibit dissociation of nucleotide (guanine-nucleotide dissociation inhibitors). Given the homology between the ras-related proteins it is not surprising that most of these regulatory proteins are specific for the particular subfamilies rather than for individual proteins, and also that there is considerable conservation of specific motifs between the different regulatory proteins. One major similarity between almost all of the regulatory proteins is the size difference between them and the proteins they regulate; the ras-related proteins being relatively simple with molecular masses of 20-30 kD whereas the regulatory proteins can be many-fold larger and contain several functional domains. The significance of this observation is that not all of the regulatory proteins interact only with the small GTPases; many of them associate themselves with a variety of other proteins thus integrating the ras-related proteins into complex intracellular pathways.

Guanine-nucleotide dissociation inhibitor (GDI)

Proteins which inhibit the release of bound-GDP have only been described for the rab and rho families of GTPases. They seem to be involved not only in the regulation of nucleotide exchange but also in membrane association (Araki et al., 1990; Matsui et al., 1990; Sasaki et al., 1990; Ueda et al., 1990; Hori et al., 1991; Hiraoka et al., 1992; Hancock and Hall, 1993; Ullrich et al., 1993).

Rab-GDI was first purified from bovine brain cytosol on the basis of its ability to inhibit dissociation of GDP from, and the binding of GTPγS to, rab3a (Sasaki et al., 1990). Further characterisation of rab-GDI showed that its expression is not limited to tissues where rab3a is expressed, indicating that it could interact with multiple rab proteins (Matsui et al., 1990). It is now known that rab-GDI interacts with many, if not all, of the rab proteins including Sec4p and that it binds preferentially to the GDP-bound form of these proteins (Araki et al., 1990; Sasaki et al., 1991; Regazzi et al., 1992; Soldati et al., 1993; Ullrich et al., 1993). A protein with high homology to rab-GDI was recently cloned and characterised from skeletal muscle, although it seems to be widely expressed (Shisheva et al., 1994). Because of the similarity between these two proteins, and as they both appear to interact with multiple

rab proteins they are referred to as GDI-1 and GDI-2. In addition to these two proteins another rab-GDI has been identified, however, it seems to be specific for the rab11 protein (Ueda et al., 1991).

GDI interacts only with geranylgeranylated rab proteins (Araki et al., 1991; Ullrich et al., 1993), although the lipid moiety can not be the only requirement for GDI recognition as is does not bind to other proteins with geranylgeranyl modifications and binds preferentially the GDP-bound form of rab proteins (Sasaki et al., 1990). It is thought that the region of homology shared between GDI and the REP-1 and REP-2 proteins, which can associate with unmodified rab proteins, is involved in recognition of a conserved motif in rab proteins (Andres et al., 1993). Recent studies have indicated that the cytosolic rab proteins, excepting the small fraction of newly synthesised unmodified protein, are most likely all associated with GDI (Regazzi et al., 1992). However, GDI does not merely associate with C-terminally modified rab proteins in the cytosol it can also both remove them from and insert them into the specific membranes (Regazzi et al., 1992; Ullrich et al., 1993). This is an extremely important fact as it is otherwise difficult to imagine how the rab proteins, with their attached geranylgeranyl moiety, could cycle between membranes and cytosol as proposed (Bourne, 1988; Magee and Newman, 1992; Pfeffer, 1992; Zerial and Stenmark, 1993).

The recent identification and characterisation of a GDI homolog in *S. cerevisiae* establishes the vital role played by GDI in regulation of membrane transport (Garrett et al., 1994). Gdi1p has 50% homology with GDI-1 and also with the *D. melanogaster* homolog dGDI (Zahner and Cheney, 1993). Deletion of the *GDI1* gene is lethal and depletion of the protein leads to a decrease in the soluble pool of Sec4p and causes inhibition of membrane transport along the secretory pathway (Garrett et al., 1994). Thus it appears that Gdi1p, like the mammalian GDI proteins, is essential for the recycling of proteins of the Ypt1/Sec4/rab family from target to donor membranes via the cytosol.

As a general regulator of rab proteins, which removes them from membranes and maintains them in the GDP-bound form, GDI itself is a likely target for regulation. An indication that GDI may be post-translationally modified comes from the identification of a *Drosophila melanogaster* homolog of GDI (Zahner and Cheney, 1993). In the developmental mutant *quartet* three abundant proteins undergo a shift in their isoelectric points. One of these proteins is the *D. melanogaster* homolog of rab-GDI, with 68% identity and 81% similarity to the mammalian protein. It seems likely that the product

of the wild type quartet gene is involved in the, as yet uncharacterised, post-translational modification of *D. melanogaster* GDI.

Guanine-nucleotide exchange factor (GEF)

A number of proteins which catalyse the exchange of bound GDP for free GTP have been described for the ras and ras-related GTPases (review: Boguski and McCormick, 1993). Many of these proteins share regions of homology and contain motifs often seen in proteins involved in signal transduction, including the SH3 and pleckstrin homology domains (Musacchio et al., 1993). Until recently, however, no GEF activity had been described for the Ypt1/Sec4/rab family of proteins.

The only rab-specific GEF activity that has been reported was detected by using rab3a, either purified from bovine brain or after expression in *E. coli*, to detect cytosolic factors capable of increasing the nucleotide exchange rate. A GEF activity with a calculated molecular mass of 295 kD was partially purified from rat brain cytosol, and shown to increase the GDP off-rate of rab3a by approximately 10-fold. It did not exhibit activity on either H-ras or rab2 proteins (Burstein and Macara, 1992). More recently, suppressor analysis in *S. cerevisiae* has been used to identify two related GEF proteins; the 17 kD Dss4 and a related 14 kD mammalian protein, Mss4 (Burton et al., 1993a; Moya et al., 1993). While both of these proteins were isolated on the basis of their ability to suppress the *sec4-8* mutant, they showed GEF activity, albeit considerably less than for Sec4p, when tested on Ypt1p. Mss4 also seems to have some GEF activity for the rab proteins when tested *in vitro* (Burton and De Camilli, 1993b; Burton et al., 1993a; Moya et al., 1993). Neither of these proteins show significant sequence homology with other known proteins, but they share two regions of high homology and have an overall identity of 27% and similarity of 51% (Burton et al., 1993a). In contrast to the cytosolic rab3 GEF (Burstein and Macara, 1992) and Mss4 protein, Dss4p is membrane associated, though it does not have a membrane spanning region (Burton et al., 1993a; Moya et al., 1993).

It is not yet clear if indeed Dss4/Mss4 type GEF proteins will be found which interact specifically with rab proteins. Particularly considering that the Dss4 and Mss4 proteins show no homology with, and are considerably smaller than, the GEF proteins which have been described for other ras-related GTPases.

GTPase activating protein (GAP)

The small GTP-binding proteins have low intrinsic GTPase activities and are usually converted from the GTP-bound "active" to the GDP-bound "inactive" form by interaction with GAP proteins. Oncogenic ras mutants are resistant to GAP and remain permanently in their active state (review: Hall, 1990b). Many GAP proteins have been described for the ras, rho and rac GTPases, and, like the GEF regulatory proteins, they contain conserved sequences and motifs associated with proteins involved in signal transduction (review: Boguski and McCormick, 1993).

Rab-GAP proteins have been found in both yeast and mammalian cells. Interestingly, in contrast to the GDI and GEF proteins, they seem to demonstrate specificity for particular rab proteins and those which have so far been described are specific for rab3a, Ypt1p/rab1 or Ypt6p (Burstein et al., 1991; Tan et al., 1991; Burstein and Macara, 1992; Strom et al., 1993). To date only the Ypt6p-GAP has been sequenced and it shows no homology with any known protein, including the ras, rap, rac and rho GAP proteins (Strom et al., 1993). Given the specificity displayed by the rab GAP proteins which have so far been described it is possible that individual membrane transport pathways, or indeed each rab protein, may have their own specific GAP proteins.

It is not clear how GAP proteins interact with rab proteins although it has been shown that C-terminal processing of the rab protein is not required (Burstein et al., 1991; Tan et al., 1991; Strom et al., 1993) and in common with ras-GAP proteins recognition is probably via the effector domain (Becker et al., 1991; Tan et al., 1991).

Function of rab proteins

The targeting model

Based on the identification of the Ypt1 and Sec4 proteins in yeast and the effects of GTPγS on *in vitro* vesicular transport within the Golgi apparatus, Henry Bourne (Bourne, 1988) proposed that these small GTP-binding proteins could mediate vesicular transport in a manner analogous to that of the elongation factor EF-Tu. The salient point of his hypothesis was that the small GTP-binding proteins would function not as signal amplifiers but as energy dependent switches which would "proof read" the binding of transport vesicles with their target membranes. Central to this model is the capacity of GTP-binding proteins to exist in different conformational states which are dictated by the bound nucleotide, thus the ability to function as

molecular switches which are turned "on" or "off" depending on whether they are bound to GTP or GDP. Bourne proposed that the cytosolic rab proteins, in the GDP or inactive state, would be inserted into the membrane of a specific donor organelle by interaction with an exchange protein. This would be followed by recognition and recruitment of the rab protein, now in the GTP-bound or active form, into a budding vesicle probably via a protein component (X) of the nascent vesicle. Binding of the budded transport vesicle to the correct acceptor membrane would then be mediated via an interaction between the rab-protein X complex and a docking protein (Y) on the acceptor protein. GTP-hydrolysis would then release the rab protein back into the cytosol, in the GDP-bound form, and the vesicle fusion process would proceed independently.

Although this model was proposed six years ago, and a great deal of effort has gone into studying the rab proteins, their function remains unclear. This is due in part to the lack of success in identifying components of the transport machinery which interact with the rab proteins and in part to the involvement of multiple GTP-binding proteins, which has complicated the identification of the GTPγS-sensitive factor. According to Bourne's model GTP hydrolysis is required for the release of the rab protein, from the docking machinery into the cytosol, and results in fusion of the transport vesicle with the acceptor membrane. Thus in the presence of GTPγS both membrane fusion and rab protein recycling would be inhibited, and both vesicles and rab proteins would be expected to accumulate on the acceptor membrane. It is now known, however, that GTPγS inhibition of vesicular transport is mediated by at least one other small GTP-binding protein. This is the ARF protein which is required for coat assembly on Golgi and plasma membrane derived transport vesicles. Disassembly of the coat, which is required for fusion, is dependent on GTP-hydrolysis which results in release of ARF and coatomer complexes into the cytosol (Orci et al., 1993; Ostermann et al., 1993; Palmer et al., 1993; Stamnes and Rothman, 1993; Elazar et al., 1994). In the presence of GTPγS uncoating is blocked and there is an accumulation of coated vesicles in the cytosol (Melançon et al., 1987). The identification of ARF as the GTPγS inhibitory factor in *in vitro* intra-Golgi transport exemplifies this point (Tanigawa et al., 1993). The involvement of heterotrimeric GTP-binding proteins further complicates the interpretation of results obtained with GTPγS, particularly as both stimulatory and inhibitory G proteins are now implicated in membrane transport (Barr et al., 1991; Stow et al., 1991; Colombo et al., 1992a).

Studies in yeast indicate that rab proteins may lack the specificity required for targeting

One of the predictions of the targeting model is that each rab protein directs a specific class of transport vesicles to their appropriate target membrane. In order to directly test this hypothesis two separate groups have investigated the activities of chimeric proteins in *S. cerevisiae*. As the rab family members share close structural similarity with one another it appears to be possible to interchange structural domains of different proteins without causing major changes in the tertiary structure of the proteins. This approach was used to perform the "tail-swapping" experiments which demonstrated that localisation of the rab proteins is determined by the hypervariable region at the C-terminus (Chavrier et al., 1991). More recently it has been used to study the function of specific domains within the Ypt1 and Sec4 proteins in *S. cerevisiae* (Brennwald and Novick, 1993; Dunn et al., 1993). Chimeric proteins were constructed in which different domains of the Ypt1 and Sec4 were combined and their ability to complement *ypt1* or *sec4* mutations or deletions was then tested. Substitution of two Ypt1 domains, corresponding to loop7 and the effector region by analogy with p21ras, into Sec4p transforms the protein into a functional Ypt1 protein without residual Sec4 activity (Dunn et al., 1993), although the converse chimera in which the two Sec4 domains are substituted into the Ypt1 protein is apparently inactive (Brennwald and Novick, 1993). Intriguingly another chimera in which the Ypt1 C-terminal and loop7 domains, but not the effector, were substituted into the Sec4 protein was shown to be able to function as either Sec4 or Ypt1 (Brennwald and Novick, 1993). This chimeric protein was able to efficiently complement deletion of either *SEC4* or *YPT1*, and even the simultaneous deletion of both genes, without inducing missorting of transported proteins.

As the Ypt1 and Sec4 proteins regulate ER-to-Golgi and Golgi-to-cell surface membrane transport respectively (Novick et al., 1980; Schmitt et al., 1988; Segev et al., 1988; Baker et al., 1990), the targeting model would predict that the dual-function Ypt1/Sec4 chimera would induce fusion of ER-derived transport vesicles with the cell surface. As no missorting is observed it appears that, while these small GTP-binding proteins are undoubtedly involved in the targeting of transport vesicles, they can not be the sole source of targeting information. On the basis of this data it has been proposed that the rab proteins may function in regulating interactions between other components of the recognition/fusion machinary, including those responsible for specific targeting (Brennwald and Novick, 1993; Rothman and Warren, 1994).

Rab proteins and the recognition/fusion machinery

The recent identification of many of the components of the recognition/fusion machinery involved in exocytosis in yeast and mammalian cells, indicates that common mechanisms and molecules may regulate different steps in membrane transport and that many of these molecules are highly conserved. The proteins which have so far been identified can be fitted roughly into three classes; 1) Proteins which are universal components of the recognition/fusion machinery, for example the NEM-sensitive factor (NSF), 2) Families of proteins of which the individual members are involved in specific transport steps, thus conferring specificity to the process, for example the rab or VAMP (v-SNARE) protein families, and 3) Proteins which are involved in specific transport steps which are subject to additional mechanisms of regulation an example of which is the synaptic vesicle-specific protein synaptotagmin.

The SNARE hypothesis (Söllner et al., 1993a), was formulated on the basis of the synaptic vesicle docking/fusion process, and was then proposed as a universal mechanism by which vesicle docking and fusion events could be mediated. According to this hypothesis each transport vesicle will posses a specific protein (v-SNARE) which will recognise its cognate receptor protein (t-SNARE) on the acceptor membrane. In the synaptic vesicle system the v-SNARE is VAMP/synaptobrevin and the t-SNARE is syntaxin, but the discovery of VAMP- and syntaxin-related proteins in both yeast and mammalian cells (Newman et al., 1990; Dascher et al., 1991; Ossig et al., 1991; Hardwick and Pelham, 1992; Newman et al., 1992a; Aalto et al., 1993; Bennett et al., 1993; Bennett and Scheller, 1993) supports the idea that each membrane transport step will have its own t- and v-SNARES. Following SNARE mediated docking of the transport vesicle it is proposed that the binding of soluble NSF attachment proteins (SNAPs) will mediate NSF binding, and hydrolysis of ATP by NSF will then dissociate the SNARE complex and enable membrane fusion.

While this hypothesis does not take the involvement of rab proteins into account, evidence suggesting that they interact with some of the proposed components of the recognition/fusion machinery comes from the biochemical characterisation of interactions between synaptic vesicle proteins and also from genetic studies in yeast (review: Bennett and Scheller, 1993). In synaptic vesicles rab3A has been shown to exist in a complex with the synaptic vesicle-specific proteins SV2 and synaptophysin. Both of these proteins have been isolated in complexes containing VAMP, synaptotagmin and the vacuolar proton pump (Bennett et al., 1992a). While this study does

not indicate direct interaction between rab3A and SNARE proteins, another study has shown that rab3A is present in a complex consisting of two t-SNARES (SNAP-25 and syntaxins) and one v-SNARE (VAMP) which can be isolated from bovine brain presynaptic terminals (Horikawa et al., 1993).

In yeast VAMP- and syntaxin-like proteins have been identified which interact genetically with Ypt1p and Sec4p. On the ER to Golgi pathway which is regulated by Ypt1p, two VAMP-like proteins, BET1/SLY12 and SEC22/SLY2, and one syntaxin homolog, SED5, are also required (Newman et al., 1990; Dascher et al., 1991; Ossig et al., 1991; Hardwick and Pelham, 1992; Newman et al., 1992a). Similarly SNC1 and SNC2, VAMP homologs and SSO1 and SSO2, syntaxin homologs, are required on the Sec4 regulated TGN to plasma membrane pathway (Gerst et al., 1992; Aalto et al., 1993; Protopopov et al., 1993). Another syntaxin-like protein, PEP12, has been identified on the Golgi to vacuole pathway (Pelham and Munro, 1993).

Thus at least three groups of proteins; the rab, syntaxin and VAMP families, appear to be involved in membrane transport, with individual members of each family being required at each specific transport step. How these proteins interact with one another or with other proteins which are implicated in membrane fusion, such as NSF (Block et al., 1988), SNAPs (Clary et al., 1990) or annexins (Gruenberg and Emans, 1993), remains unclear.

Rabphilin-3A

Despite the intensive search for membrane proteins interacting with rab proteins, only one putative target protein has been identified. Rabphilin-3a, which was first identified by incubating GTPγS-bound rab3a with bovine brain membranes in the presence of a cross linker, interacts preferentially with the GTP-bound rab3a protein (Shirataki et al., 1992). The function of rabphilin-3a remains obscure but it appears to maintain the rab3a protein in the GTP-bound state by inhibiting its GAP activity (Kishida et al., 1993). Interestingly the sequence of rabphilin-3a contains two internal repeats which are homologous to the C2 domains of protein kinase C and synaptotagmin (Kishida et al., 1993; Shirataki et al., 1993), both of which are known to bind to membrane phospholipid in a calcium dependent manner (Perin et al., 1990; Perin et al., 1991a; Perin et al., 1991b; Perin, 1994). In both rabphilin-3a and synaptotagmin the C2 domains are found in the C-terminal half of the protein and there is high sequence homology in these areas, in contrast the N-terminal of rabphilin-3a does not share homology with synaptotagmin. Although the significance of the C-terminal homology is

unclear, C2 domains have been suggested to be involved in calcium-dependent membrane interactions and, possibly, fusion (Perin et al., 1990). In addition, micro-injection of synaptotagmin C2 domain peptides inhibits regulated exocytosis in PC12 cells and squid giant presynaptic terminals (Bommert et al., 1993; Elferink et al., 1993).

THE ENDOCYTIC PATHWAY

In the most simple sense the endocytic pathway in eukaryotic cells can be described as a number of membrane bound compartments which are responsible primarily for the uptake, and subsequent sorting and transport, of molecules from the external melieu. However, it is becoming increasingly obvious that this description does not begin to do justice to the complexity, either morphologically or functionally, of this pathway. This is well illustrated by recent studies on the processing and presentation of foreign antigens via class II molecules. Several groups have now described an endocytic compartment which is implicated in this process (Amigorena et al., 1994; Qiu et al., 1994; Tulp et al., 1994; West et al., 1994). But it remains unclear whether this corresponds to a previously characterised endocytic compartment or a novel endocytic structure.

Structure of the endocytic pathway

As there is still considerable controversy over the biogenesis and structural integrity of the compartments making up the endocytic pathway it is necessary at this point to define the compartments as they will be discussed here. The four morphologically distinct compartments which can be labelled by internalised fluid phase markers will be referred to as; early endosomes, endosomal carrier vesicles (ECV), late endosomes and lysosomes. Early endosomes are defined as those organelles which are reached within five minutes of internalisation of a fluid phase marker, they are typically located at the cell periphery and usually have a complex tubular-vesicular appearance, although this varies considerably between different cell types (Griffiths et al., 1989; Gruenberg et al., 1989; Hopkins et al., 1990; Tooze and Hollinshead, 1991). Late endosomes are those organelles which are labelled after longer periods of internalisation, 10-30 min, in a microtubule dependent process (De Brabander et al., 1988; Gruenberg et al., 1989; Bomsel et al., 1990; Aniento et al., 1993b). In contrast to early endosomes, late endosomes are usually found in the perinuclear region of the cell and are generally large vesicular organelles, often containing many intracellular membranes, with which tubular-reticulate elements are in continuity (Griffiths et al., 1990a). In the

absence of microtubules, fluid phase markers do not reach the late endosome but accumulate in large (0.4-0.7 μm diameter) spherical endocytic carrier vesicles (ECVs), characterised by their multi-vesicular appearance (Hopkins, 1983a; Griffiths et al., 1988; Gruenberg et al., 1989). ECVs appear to be responsible for microtubule dependent transport from early to late endosomes in a variety of cell types (Bomsel et al., 1990; Parton et al., 1992; Aniento et al., 1993b). Morphologically the ECV resembles the multivesicular body described by many groups (Marsh et al., 1983; Neutra et al., 1985; Dunn et al., 1986; Mueller and Hubbard, 1986; Park et al., 1991), and may be equivalent to the late endosomes described by Schmid and co-workers (Schmid et al., 1988). The final organelle on the endocytic pathway to be accessed by internalised markers is the lysosome. Usually spherical the lysosome is characterised by high concentrations of lysosomal glycoproteins (lgp) and acid hydrolases (Griffiths et al., 1990b).

Markers of endocytic organelles

That certain membrane proteins are highly enriched within specific organelles on the endocytic pathway has been well documented in morphological studies, it should be noted, however, that most of these proteins are receptor proteins which are continually cycling between compartments and their distributions vary considerably in different types of cells. The large mannose 6-phosphate receptor (MPR), which has been well characterised and is often used as a marker for late endosomes, is a good example. In NRK and MDCK cells the bulk of this receptor localises to late endosomes with very little in the TGN and early endosomes (Parton et al., 1989; Griffiths et al., 1990b), but in other cell types, notably rat liver and HS4 cells, the bulk of the receptor appears to be found in the TGN with small amounts present in endocytic organelles (Geuze et al., 1984; Geuze et al., 1988).

Although a considerable amount of effort has been put into the identification of organelle specific proteins in the endocytic pathway, with the long term goal of understanding endocytic membrane transport, success has been limited. Subcellular fractionation combined with two-dimensional gel electrophoresis has been used to show that there are significant differences in the protein composition of endocytic organelles (Schmid et al., 1988; Beaumelle et al., 1990; Aniento et al., 1993b; Emans et al., 1993) but it was not until recently that individual proteins could be identified. Using subcellular fractionation to separate early endosomes from later stages of the pathway and then raising monoclonal antibodies against the membrane proteins of these fractions, Pitt and Schwartz (Pitt and Schwartz, 1991a), identified a 195

kD membrane protein which localises specifically to early endosomes. It is not known if this protein plays a role in membrane transport. The discovery of the ras-related family of rab GTP-binding proteins dramatically affected the field of membrane transport, particularly once the specificity of their localisation became clear. Each organelle within the eukaryotic cell seems to have its own complement of rab proteins so that now it is becoming possible to identify organelles on the basis of their rab protein content (review: Goud, 1992). Significantly the sequences and localisation patterns of rab proteins, which have been identified in cells from organisms as diverse as yeast, mammals and plants, are highly conserved; allowing not only comparison of organelles in different cells of one organism but also from different organisms. Thus the identification of rab5 and rab7 homologs in *S. cerevisiae* and *S. pombe* has been a major advance in the study of endocytosis in these organisms, which up till now had been severely restricted by the lack of endocytic markers (Wichmann et al., 1992; Armstrong et al., 1993a; Singer-Krüger et al., 1994).

Recently another family of proteins, the annexins, have become prominent as potential mediators of membrane fusion and the indications are that they also may show a organelle specific localisation (review: Gruenberg and Emans, 1993; Klaus Fiedler and Kai Simons, personal communication). Antibodies raised against a conserved sequence which recognise all annexins have been used to show that annexin proteins are associated with the membranes of all of the organelles involved in the biosynthetic and endocytic pathways (Gruenberg and Emans, 1993). On the endocytic pathway, the annexins I, II and VI, have been implicated in membrane transport. Annexin II, which mediates fusion of early endosomes *in vitro*, localises to the plasma membrane, clathrin coated pits and vesicles and the early endosome, it is not found on organelles involved in later stages of the pathway (Emans et al., 1993). Annexin VI is localised to the plasma membrane and has been implicated in clathrin coated vesicle formation (Lin et al., 1992). However a recent study has shown that this protein does not affect internalization and recycling of transferrin in A431 cells, which do not express annexin VI (Smythe et al, 1994). Annexin I is associated with the plasma membrane and ECVs, and its phosphorylation by the epidermal growth factor receptor kinase appears to mediate the formation of ECVs (Sawyer and Cohen, 1985; Lin et al., 1992; Futter et al., 1993).

Receptor-mediated endocytosis

Eukaryotic cells appear to have at least three routes by which molecules can be internalised from the external milieu, which are mediated by

clathrin coated vesicles, phagocytic vacuoles or caveolae. It is also possible that fluid-phase endocytosis and internalisation of small ligands such as ricin, may occur via a pathway which is different from the above (reviews: Sandvig and van Deurs, 1991; Watts and Marsh, 1992; Anderson, 1993b). Although multiple pathways are clearly involved in internalisation it is not clear if all of them interact with the endosomal apparatus (Hansen et al., 1994; Hewlett et al., 1994) and only clathrin-coated vesicle mediated endocytosis will be discussed here.

Much of our understanding of internalisation comes from studies on receptor-mediated endocytosis of ligands from the external environment (reviews: Hubbard, 1989; Morris et al., 1989; Courtoy, 1991; Schmid, 1992; Watts and Marsh, 1992; Trowbridge et al., 1993). Essentially receptor internalisation consists of four steps; 1) Receptors on the cell surface cluster into clathrin coated pits, 2) The coated pits pinch off to form clathrin-coated vesicles, 3) The vesicle is uncoated in the cytosol, and 4) The uncoated vesicle fuses with the early endosome. In this way receptor-ligand complexes, membrane proteins, membrane lipids and fluid phase molecules are delivered to the early endosome.

The initial step in receptor-mediated endocytosis begins with clustering of cell surface receptors in coated pits which for many receptors is mediated by internalisation signals in their cytoplasmic tails. Internalisation signals are specified by short stretches of amino acids, the best characterised being the tyrosine containing tight-turn signals (Mostov et al., 1986; Davis et al., 1987; Rothenberger et al., 1987; Lazarovitz and Roth, 1988; Lobel et al., 1989a; Lobel et al., 1989b; Miettinen et al., 1989; Collawn et al., 1990; Jing et al., 1990; Bansal and Gierasch, 1991; Eberle et al., 1991; Lehman et al., 1992). It is now believed that the internalisation sequences of the receptors interact with the plasma membrane specific assembly protein complex (AP-2) found in the interior of the clathrin lattice making up the coated pit (Chin et al., 1989; Glickman et al., 1989). Other receptors, primarily those involved in hormone and growth factor binding, are not clustered into pits and are internalised only upon ligand binding. Localisation signals have not been identified in the cytoplasmic domains of these proteins, although it has been shown that at least one, the epidermal growth factor receptor, can interact with AP-2 (Sorkin and Carpenter, 1993).

The formation of clathrin coated pits and vesicles, has been extensively studied, both *in vitro* and *in vivo*. However, although it seems that most of the major components have been identified, there is still some uncertainty as to the actual mechanics of vesicle formation and its regulation

(reviews: Pearse and Robinson, 1990; Robinson, 1992; Anderson, 1993a; Schmid, 1993). The initial step in clathrin-coated vesicle formation, formation of planar clathrin lattices, requires the association of clathrin triskelions and AP complex on the membrane. The AP complexes undoubtedly play a major role in this process as, in addition to interacting with the cytoplasmic tails of receptors, they also bind clathrin with high affinity and mediate its membrane association (Hanspal et al., 1984; Virshup and Bennett, 1988; Mahaffey et al., 1989). Recently it has been shown that purified AP-2 can stimulate coated pit formation *in vitro* (Smythe et al., 1992) and that AP self-aggregates can form nucleation sites for clathrin lattices (Chang et al., 1993; Hansen et al., 1993), though receptor-AP interactions may contribute to this process (Chang et al., 1993). This may explain why overexpression of receptors containing internalisation signals, such as the transferrin receptor, increases the area of planar lattices (Miller et al., 1991). Potassium depletion, which inhibits receptor-mediated endocytosis, causes lattices and coated pits to disassemble and the disappearance of coated vesicles from the cytosol but it also increases the amount of AP-2 associated with the EGF receptor, indicating that receptor-adaptin complexes form before lattice assembly (Hansen et al., 1993; Sorkin and Carpenter, 1993). Interestingly, signal-transduction is implicated in lattice assembly as self-aggregation of APs is affected by physiological concentrations of inositol phosphate (Chang et al., 1993).

Initial formation of a flat clathrin lattice is followed by spontaneous curvature which produces a nascent vesicle still attached to the membrane, a step which *in vitro* is sensitive to pH (Heuser and Keen, 1988) and inhibited by the addition of mitotic cytosol (Pypaert et al., 1991). Other *in vitro* studies have shown that vesicle fission from the plasma membrane requires cytosol and ATP (Lin et al., 1991; Schmid and Smythe, 1991), although the requirement for ATP at earlier steps seems to be dependent on the assay system (Moore et al., 1987; Mahaffey et al., 1989; Smythe et al., 1989; Schmid and Carter, 1990; Schmid and Smythe, 1991). In vitro assays have also produced conflicting results in respect to the involvement of calcium and the calcium and phospholipid-binding protein annexin VI in vesicle fission (Lin et al., 1992; Schmid, 1993). Budding reconstituted in a system using isolated plasma membranes requires calcium and is stimulated by annexin VI (Lin et al., 1992). But purified annexin VI has no effect on coated vesicle formation in perforated A431 cells which do not express this protein (Smythe et al., 1994).

The effects of GTP and GTP-analogs on vesicle formation *in vitro* indicate that GTP-binding proteins are involved at more than one of the

stages of vesicle formation (Carter et al., 1993). Coat assembly and budding are both inhibited by GTPγS, whereas invagination is stimulated by GTP but inhibited by GDPβS. Although it is unclear exactly which GTP-binding proteins are involved, both members of the ras superfamily of small GTP-binding proteins and trimeric G proteins are implicated. The effects of the amphipathic peptide mastoparan and aluminium fluoride (AlF_4^-), both of which appear to activate Gα (Chabre, 1990; Mousli et al., 1990), suggest a role for the trimeric G proteins in regulation of clathrin-coated vesicle formation. Both mastoparan and AlF_4^- inhibit coated-pit formation and coated-vesicle budding, although they do not seem to have an effect on invagination (Carter et al., 1993).

Following budding of clathrin-coated vesicles the coat is rapidly shed and the uncoated vesicle fuses with the early endosome. In vitro it has been shown that the heat shock protein hsc70 is the "uncoating ATPase" which is responsible for catalysing the ATP dependent release of clathrin from vesicles (Rothman and Schmid, 1986), but the precise role of hsc70 *in vivo* remains unclear. How the uncoating ATPase differentiates between coated pits, from which it does not remove clathrin (Heuser and Steer, 1989), and vesicles, is not known, but conformational changes in clathrin light chains may be involved (Schmid et al., 1984; DeLucca-Flaherty et al., 1990). In vitro, clathrin is removed from vesicles in a stoichiometric complex with the uncoating ATPase, but in this form it is unable to reassemble clathrin cages suggesting that other cellular factors are required for its recycling to the membrane (Rothman and Schmid, 1986). The AP complexes are not released from vesicles during uncoating and recent work suggests that AP-2 may be involved in fusion of uncoated vesicles either with one another or with the endosome (Beck et al., 1992).

The recycling pathway

Although 30-70% of fluid phase markers are directed to the degradative pathway, eventually reaching the lysosome, almost 100% of internalised receptors are recycled from the early endosome to the plasma membrane (Steinman et al., 1976; Besterman et al., 1981; Adams et al., 1982; Greenspan and St Claire, 1984; Goldstein et al., 1985; Salzman and Maxfield, 1988; Griffiths et al., 1989). The transferrin receptor cycle has been well studied and is a good example of a rapidly recycling receptor. Like the LDL receptor it is constitutively internalised via clathrin coated vesicles, irrespective of ligand binding. Other receptors such as the EGF receptor are internalised efficiently only when associated with their ligand (Schlessinger et al., 1978; Dunn et al., 1994). Cellular uptake of iron begins with binding of

diferric transferrin to the transferrin receptor at the plasma membrane, and the complex is then internalised via clathrin coated vesicles (Pearse, 1982; Bliel and Bretscher, 1982; Hopkins and Trowbridge, 1983). The low pH of the early endosome induces release of the two iron molecules and conversely increases the affinity of transferrin receptor for apotransferrin (Morgan, 1981; Rao et al., 1983; Klausner et al., 1984). The apotransferrin-receptor complex then returns to the plasma membrane where the neutral pH facilitates release of apotransferrin and binding of diferric transferrin, thus allowing the cycle to begin again (Dautry-Varsat et al., 1983; Harford et al., 1983; Klausner et al., 1983). Fluorescent studies in intact cells have shown that transferrin remains at neutral pH for 2-3 min, the pH then drops rapidly to about 6.0 before rising again to about 6.3-6.5 prior to appearance on the cell surface (Yamashiro and Maxfield, 1984; Sipe and Murphy, 1987; Yamashiro and Maxfield, 1987). The heterogeneity of the early endosome is also seen morphologically and the pH 6.0, or sorting, endosome is typically vacuolar whereas the less acidic recycling endosome tends to be tubular (Willingham et al., 1984; Marsh et al., 1986; Murphy, 1991). The pathway of molecules destined for degradation diverges from that of transferrin immediately after the pH 6.0 compartment, as they are transported to the more acidic late endosomes (Tycko et al., 1983; Willingham et al., 1984; Sipe and Murphy, 1987).

Transport to late endosomes and lysosomes

Morphological studies have clearly shown that internalised fluid phase markers are detectable in early endosomes within approximately 5 min, but require at least 15 to 20 min to reach late endosomes and longer periods of time to reach the lysosome (Griffiths et al., 1989; Gruenberg et al., 1989; Ludwig et al., 1991; Pieters et al., 1991; Killisch et al., 1992), however, the mechanism of transport from early to late endosomes, and then to the lysosomes, is still unclear. Although many models have been proposed they can be divided roughly into two classes, based on whether the endocytic pathway is made up of; 1) a series of transient compartments which undergo gradual remodelling to give rise finally to lysosomes (review: Murphy, 1991) or 2) stable compartments connected by vesicular transport (review: Griffiths and Gruenberg, 1991). It should be pointed out that one model of endocytic transport exists that does not fall into either of these two categories. Hopkins and co-workers (Hopkins et al., 1990) internalised fluorescent markers into epidermoid carcinoma (Hep-2) cells and studied the fate of the marker using video microscopy. An extensive endosomal reticulum was observed along which swellings containing the fluorescent markers were seen moving towards the perinuclear region. Electron microscopy identified these swellings

as multivesicular bodies, or ECVs, which instead of being discrete cytoplasmic organelles where retained within the membranes of the endosomal reticulum.

As pointed out by Murphy (1991), not all of the maturation models which have been proposed are compatible and they differ significantly in some aspects, though they are all in agreement on one point: the organelles which make up the endocytic pathway are in a constant state of flux and development. At its most basic the maturation model proposes that early endosomes are formed by the fusion of uncoated endocytic vesicles, that these early endosomes then become inaccessible to incoming markers and mature into late endosomes, which undergo further development into lysosomes. In answer to the argument that intermediate forms of these organelles should then be detectable, the proponents of maturation propose that the formation of the intermediate forms is rate limiting and that they may be very short lived (Murphy, 1991). In order to demonstrate maturation of endosomes several groups have studied the kinetics of internalisation of both fluid phase markers and membrane bound receptors (Salzman and Maxfield, 1988; Stoorvogel et al., 1991; Dunn and Maxfield, 1992). The maturation of early endosomes, usually called sorting endosomes in these studies, into late endosomes requires fission events which will allow the recycling membrane and receptors to return to the plasma membrane. In fact when the localisation of co-internalised ligands destined for the degradation pathway and recycling ligands, was studied it was shown that, although they initially co-localise, the recycling ligand relocalises to tubular recycling endosomes while the ligand destined for degradation remains in the sorting endosome (Yamashiro and Maxfield, 1984; Dunn et al., 1989). Thus it is proposed that fission of the tubular/recycling endosome from the vesicular sorting endosome would be the first step in the maturation of the latter into a late endosome. Using digital image analysis Dunn and Maxfield (1992) studied the trafficking of low density lipoprotein (LDL), a ligand which is directed to the lysosome whereas its receptor is recycled back to the plasma membrane (Goldstein et al., 1979), to show that sorting endosomes containing labelled LDL lose the ability to fuse with incoming primary endocytic vesicles in a time dependent manner. In addition, they showed that sorting endosomes, labelled with a 2 min pulse of LDL, retained the ligand for up to 18 min. The conclusions drawn from these experiments are that early endosomes mature into late endosomes, as if the early endosome was a stable compartment it would not be expected to retain lysosomally directed ligand and should still be able to fuse with incoming vesicles.

The stable compartment model proposes that the three main compartments of the endocytic pathway; early endosomes, late endosomes and lysosomes, are stable organelles between which membrane transport is carried out by vesicular intermediates (Griffiths and Gruenberg, 1991). Although the transport step from late endosomes to lysosomes has not been well studied, early to late endosome transport is proposed to occur via the ECV. Several lines of evidence support this theory, firstly when microtubules are depolymerized by treatment with nocodazole, fluid phase markers are not transported from early to late endosomes, but rather accumulate in ECVs the number of which increases significantly (Gruenberg et al., 1989; Bomsel et al., 1990). Secondly, in neurons ECV-like vesicles have been described which move from the axonally positioned early endosomes to the late endosomes in the cell body (Parton et al., 1992; Hollenbeck, 1993). Thirdly, in polarised epithelial cells the basolateral and apical endocytic pathways converge at late endosomes, and it has been shown *in vitro* that early endosomes from the two domains do not fuse (Parton et al., 1989; Bomsel et al., 1990). Therefore, if maturation was occurring the maturing endosomes would have to gain the ability to fuse with one another. In contrast, the ECVs of the stable compartment model could, independently of whether they originate from apical or basolateral endosomes, be targeted to a common late endosome (Griffiths and Gruenberg, 1991). Finally, *in vitro* studies (see below) have shown that ECVs fuse with late endosomes, but not with early endosomes, and that this process is microtubule dependent (Aniento et al., 1993b).

It is possible that a combination of vesicular transport and maturation are involved in endocytic transport. Certainly the budding of the large ECV from the early endosome, or fission of tubular recycling endosomal elements from a vacuolar sorting endosome, could be considered strikingly similar processes. Perhaps the crux of the issue is whether or not there are proteins, for example t-SNARES, which are restricted to certain stages of the endocytic pathway and do not undergo cycles of retrieval and introduction. On this point the two models are clearly not compatible and the matter will not be resolved until the molecular composition of endosomes is defined. According to the stable compartment model each of the endocytic organelles will contain resident proteins, but, as discussed above, none of the endosome marker proteins which have been well studied are confined to one organelle. Since the discovery of the rab proteins it has been suggested that they will be the best markers of intracellular organelles (review: Goud, 1992), however, as already mentioned each of these proteins is usually found on more than one compartment. The intriguing question at the moment is whether or not membrane proteins which interact with specific rab proteins, for example in

mediating membrane attachment, will be found which are truly organelle specific.

The rab5a and rab4a proteins regulate receptor-mediated endocytosis and recycling in vivo

At least two rab proteins, rab4a and rab5a, are involved in receptor-mediated endocytosis, although their exact roles are as yet unknown (review: Marsh and Cutler, 1993). Rab5a is found on clathrin coated vesicles as well as on the cytosolic surface of early endosomes and plasma membrane (Chavrier et al., 1990a; Bucci et al., 1992) and *in vivo* it has been shown to regulate an early step of the endocytic pathway (Bucci et al., 1992). Using the T7 polymerase recombinant vaccinia virus system (Fuerst et al., 1986) to overexpress the rab5 protein in BHK cells it was shown that overexpression of wild type rab5 protein caused an increase in the size of early endosomes and increased the rate of internalisation of endocytic markers. In contrast, overexpression of the rab5 N133I mutant which, like the corresponding ras N116I mutant, has a decreased affinity for nucleotide, caused a drastic change in the morphology of early endosomes, inducing accumulation of tubular structures and small vesicles, and inhibited the uptake of endocytic markers (Bucci et al., 1992). As rab5 N133I has also been shown to inhibit lateral fusion of early endosomes *in vitro* (Gorvel et al., 1991; and see below) this accumulation of tubular and small vesicular structures *in vivo* is most easily explained by envisaging the early endosome as a highly dynamic organelle undergoing multiple fission and fusion events, as has been previously suggested (Gruenberg and Howell, 1989). Thus expression of the rab5 N133I mutant, which inhibits fusion, would result in the fragmentation of the early endosome. The decrease in the rate of transferrin internalisation caused by overexpression of this mutant could be either an indirect result of membrane fragmentation, resulting in missorting or retention of the transferrin receptor or of a component required for one of the steps of internalisation. Alternatively, the rab5 protein may be required both in the lateral fusion event and for fusion of uncoated endocytic vesicles with the early endosome. In this case the rab5 N133I mutant would prevent fusion of uncoated clathrin-coated vesicles with endosomes, thus causing an accumulation of intermediate structures.

The rab4a protein, which localises like rab5a to the early endosome but has not been detected on the plasma membrane (van der Sluijs et al., 1991), is implicated in the regulation of the recycling pathway (van der Sluijs et al., 1992a). The effect of either wild type rab4 or mutant rab4 proteins on endocytosis was studied using CHO cell lines which

overexpressed these proteins. In cells overexpressing the wild type protein, the most dramatic effect was seen in the distribution of transferrin receptor, of which 75-80% was found on the cell surface compared to 20-25% in control cells (van der Sluijs et al., 1992a). A GTP-binding deficient mutant, rab4 N121I, which is equivalent to the rab5 N133I mutant (Gorvel et al., 1991; Bucci et al., 1992), had a similar though less remarkable effect with approximately 50% of the transferrin receptor being found on the plasma membrane. This may not be a true reflection of the relative activities of the two proteins as the mutant was expressed at much lower levels than the wild type (van der Sluijs et al., 1992a). As neither the initial rates of receptor internalisation nor the rates of fluid phase endocytosis were affected in cells overexpressing rab4, the change in distribution can not be attributed to a decrease in the rate of endocytosis. Furthermore, transferrin containing organelles prepared from cells overexpressing rab4 exhibited markedly reduced ATP-dependent acidification *in vitro*. This was not due to a general defect in endosome acidification as organelles containing FITC-dextran, which had been internalised for 1 hour, had identical acidification properties as those prepared from control cells. These data, together with the finding that iron is very inefficiently transported into rab4 overexpressing cells, indicates that rab4 overexpression prevents transferrin from accumulating in acidic endosomes. It is, however, unlikely that rab4 overexpression prevents the delivery of transferrin receptor to acidic early endosomes since receptor-mediated endocytosis of Semliki Forest virus (SFV), which is transported from early to late endosomes, is not affected. Therefore, perhaps the most straightforward explanation is that the residence time for transferrin receptor in the acidic early endosome is reduced, possibly as the result of an increase in the rate of transport to the recycling, or neutral pH, endosome (Trowbridge et al., 1993; Yamashiro and Maxfield, 1984).

While the functions of the rab4 and rab5 proteins are not clear, they obviously have very different roles in the regulation of endocytosis. In the light of this information it is tempting to postulate that the three other rab proteins which have been shown to localise to early endosomes also regulate specific membrane traffic events involving this organelle (Olkkonen, et al., 1993). In addition only the rab4a and rab5a proteins have been investigated, and the significance of the rab4b or the rab5b and rab5c (Bucci et al., 1994; Chavrier et al., 1990b) isoforms remains unclear.

Other GTP-binding proteins in endocytosis

It is becoming increasingly evident that several GTP-binding proteins are involved in the early stages of receptor-mediated endocytosis,

although it is not clear what roles they play. One of these proteins has now been shown to be the mechano-chemical GTP-binding protein dynamin (Shpetner and Vallee, 1989), which was first implicated in endocytosis when the gene product of the *Shibire* mutant in *D. melanogaster* was found to be a dynamin homolog (Chen et al., 1991; van der Bliek and Meyerowitz, 1991). Temperature sensitive paralysis in adult flies carrying the *Shibire* mutation (Grigliatti et al., 1973) is believed to be caused by a block in endocytosis which prevents membrane recycling and thus depletes synaptic vesicles from nerve terminals (Kosaka and Ikeda, 1983; Koenig and Ikeda, 1989). That dynamin is required in receptor-mediated endocytosis has recently been demonstrated in two separate studies (Herskovits et al., 1993; van der Bliek et al., 1993), where it was shown that expression of GTP-binding domain mutants in mammalian cells inhibited transferrin uptake and invagination of coated pits but did not affect clathrin lattice formation or AP-2 localisation. Thus dynamin appears to be involved in a process which occurs between lattice formation and budding of clathrin coated vesicles.

IN VITRO ANALYSIS OF EARLY ENDOSOME FUSION

Many of the steps involved in membrane transport along the biosynthetic and endocytic pathways have been reconstituted *in vitro* . And the use of these cell-free assays has clearly demonstrated that the mechanisms involved in transport are highly conserved both between different organisms and along the different pathways. However, there are still many important questions which remain to be answered one of the most notable being that of specificity. What is the mechanism responsible for maintaining membrane fusion fidelity within the cell? In order to answer this question cell-free transport assays and genetic studies in yeast are being used to identify and characterise the proteins involved in the regulation of targeting and recognition.

Early endosome fusion in vitro

Cell-free assays in which the fusion of early endosomes can be measured have been developed by several groups (Gruenberg and Howell, 1989; Steele-Mortimer et al., 1993). These assays are usually based on the principal that fusion of endosomes will result in mixing of their contents. Thus in most cases two markers, which upon mixing will interact with one another in a quantifiable manner, are internalised into separate populations of cells. In order to label the early endosome markers are internalised for 5 minutes at 37°C, longer internalisation times decrease the amount of measurable fusion

as markers become distributed throughout the endocytic pathway (Gruenberg et al., 1989). After homogenising the cells, endosome fusion can be measured directly by mixing post-nuclear supernatants or the endosomes can be separated from cytosol and other organelles before the fusion assay. Cytosol-free endosomes can be prepared by centrifugation, either by pelleting the membranes or separating them on flotation gradients. The later technique has the advantage that early endosomes have a characteristic density and can thus be efficiently separated from most other organelles including other endocytic organelles. Early endosomes can be further purified by the technique of immuno-isolation, with the benefit that the endosomes will then be associated with a solid matrix and can easily be retrieved (Gruenberg and Howell, 1988).

While most other cell-free fusion assays measure vectorial transport from one compartment to another, early endosome fusion is a lateral event occurring between identical acceptor and donor fractions. The propensity of early endosomes to undergo lateral fusion *in vitro* suggests that, *in vivo*, the early endosome is a highly dynamic organelle, consisting of a network of elements connected by fusion and fission events (Gruenberg and Howell, 1989). Morphological studies of fusogenic immuno-isolated early endosomes show that they maintain much of the complexity seen *in vivo*, with tubular and vesicular elements in continuity with one another. It is not known which of these structures are fusogenic, or whether *in vitro* fusion reflects events occurring between like or unlike elements. It is clear, however, that this lateral fusion event is extremely specific, since early endosomes do not fuse with late endosomes or other distal stages of the endocytic pathway (Gruenberg et al., 1989; Gorvel et al., 1991; Aniento et al., 1993b). Furthermore, this specificity extends to sub-populations of early endosomes as illustrated by endosomes prepared from MDCK cells. These polarised cells have two populations of early endosomes, apical and basolateral, which are topologically and functionally distinct *in vivo* (Bomsel et al., 1989; Parton et al., 1989). *In vitro*, fusion only occurs between endosomes of the same population, and apical and basolateral endosomes do not fuse with one another (Bomsel et al., 1990).

Requirements for early endosome fusion in vitro

The requirements for early endosome fusion have been well documented and are strikingly similar to those of other *in vitro* membrane fusion events. Fusion is time and temperature dependent, with maximum fusion usually occurring within 30-45 minutes at 37°C (Braell, 1987; Woodman and Warren, 1988; Gruenberg et al., 1989; Wessling-Resnick and

Braell, 1990b; Colombo et al., 1992b). Cytosol is required and fusion efficiency decreases with dilution of either cytosol or endosome membranes (Woodman and Warren, 1988; Tuomikoski et al., 1989; Colombo et al., 1992b). The cytosolic factors required for fusion seem to be well conserved as fusion can be supported by cytosol prepared from many different sources. As an example endosomes prepared from BHK cells fuse efficiently using cytosol prepared from BHK cells, Xenopus oocytes or rat liver (Tuomikoski et al., 1989; Gorvel et al., 1991; Aniento et al., 1993b). Early endosome fusion is an energy dependent process requiring ATP hydrolysis; being inhibited by ADP, non-hydrolyzable analogs of ATP or ATP-depletion (Gruenberg and Howell, 1989; Gorvel et al., 1991; Colombo et al., 1992b). Finally fusion requires a salt concentration of 50-75 mM (Diaz et al., 1988; Colombo et al., 1992b; Jean Gruenberg, personal communication).

Regulation of early endosome fusion in vitro

Fusion is sensitive to the sulfhydryl alkylating reagent N-ethylmaleimide (NEM) and this inhibition can be partially reversed by the addition of purified NEM-sensitive factor (NSF) (Braell, 1987; Diaz et al., 1988; Woodman and Warren, 1988; Wessling-Resnick and Braell, 1990b; Gorvel et al., 1991), which was identified on the basis of its ability to restore fusion activity to NEM-treated Golgi membranes (Block et al., 1988). Indeed it has been proposed that NSF, a cytosolic protein which attaches to membranes in an ATP-dependent manner, is one of a soluble pool of proteins able to participate in fusion events at multiple intracellular locations (Wilson et al., 1989). The identification of an NSF homolog in *S. cerevisiae*, which is required for ER to Golgi transport *in vivo* and can functionally replace NSF in mammalian cell-free vesicular transport, supports the idea that the fusion machinery, or parts of it, will be found to be highly conserved (Wilson et al., 1989). The current view is that, while NSF and its soluble attachment proteins, the SNAPS, are universal components of the fusion machinery, the membrane receptors for SNAPS (SNARES) will provide specificity. Four SNARES, all restricted to the synapse, have recently been identified and seem to be members of a family of proteins showing specific intracellular localisations in both yeast and mammalian cells (Söllner et al., 1993a; Söllner et al., 1993b). One of these SNARES is the synaptic vesicle specific membrane protein synaptobrevin or VAMP and the discovery of a closely related protein, cellubrevin, which localises to early endosomes and clathrin coated vesicles (McMahon et al., 1993) suggested that cellubrevin may be an endosome-specific SNARE. It has now been shown that this potential SNARE

is not required for early endosome fusion *in vitro* (Link et al., 1993) but does seem to be involved in transferrin receptor recycling *in vivo* (Galli et al., 1994).

The regulation of *in vitro* early endosome fusion by phosphorylation and dephosphorylation events is now well documented (Tuomikoski et al., 1989; Thomas et al., 1992; Woodman et al., 1992; Woodman et al., 1993). Studies using mitotic and interphase cytosols prepared from Xenopus oocytes have shown that only the interphase cytosol can support *in vitro* endosome fusion and that inhibition in the mitotic cytosol is mediated by the $p34^{cdc2}$ kinase when it is complexed with cyclin B (Tuomikoski et al., 1989; Thomas et al., 1992). However, the addition of recombinant cyclin A to interphase cytosol prepared from mammalian cells also causes $p34^{cdc2}$ mediated inhibition of endosome fusion when high kinase activities are elicited (Thomas et al., 1992; Woodman et al., 1993). The targets of $p34^{cdc2}$ kinase responsible for inhibition have not been identified and it is unclear whether $p34^{cdc2}$ directly inactivates a component of the fusion machinery or if inhibition is mediated by a downstream kinase. Regulation of endosome fusion by $p34^{cdc2}$-independent phosphorylation in interphase cytosol has also been demonstrated, indicating that at least two kinases regulate fusion of early endosomes (Woodman et al., 1992).

Rab5 regulates fusion in vitro

The involvement of the rab5 protein in endocytosis was initially demonstrated by Gorvel and co-workers (Gorvel et al., 1991) who utilised an *in vitro* assay system in which early endosomes are first separated from other organelles and cytosol on a flotation gradient, so that fusion is carried out in the presence of exogenously added cytosol. This system allowed them to take advantage of the fact that cytosol containing high levels of rab protein can be prepared from cells in which the T7 polymerase-recombinant vaccinia virus system has been used to overexpress the specific protein. Thus they compared the effects of cytosol containing high levels of wild type rab5a protein, wild type rab2 protein or mutant rab5a proteins. While wild type rab5a protein stimulated fusion, the rab2 protein, which is localised on the biosynthetic pathway, had no effect. Furthermore, the rab5 N133I mutant protein, which has reduced nucleotide binding capacity, inhibited fusion, and a C-terminal deletion mutant, which is unable to associate with membranes but retains GTP-binding activity, had no effect. The significance of these *in vitro* results was demonstrated when the activities of the wild type rab5 and rab5 N133I mutant proteins were studied *in vivo* (Bucci et al., 1992). As discussed previously in this introduction the wild type and mutant rab5 proteins had opposite effects on the kinetics of internalisation and on the

morphology of early endosomes. Taken together these results demonstrate that rab5 regulates endosome fusion in a manner that is dependent on nucleotide binding and the ability to associate with membranes.

Regulation of in vitro endosome fusion by other GTP-binding proteins

Heterotrimeric GTP-binding proteins

The heterotrimeric GTP-binding proteins (G proteins) are best known for their classical role in the transduction of extracellular signals to intracellular effectors via coupling with transmembrane receptors (reviews: Spiegel, 1992; Clapham and Neer, 1993). G proteins consist of the α subunit which binds guanine nucleotides and the β and γ subunits which bind tightly to the inactive GDP-bound α subunit. Activation of the α subunit occurs upon exchange of GDP for GTP, a process which is stimulated by interaction with a ligand-bound receptor, and is accompanied by release of the β and γ subunits. The GTP-bound α subunit is then free to interact with, and activate, a downstream effector before its intrinsic GTPase activity returns it to the inactive state by GTP hydrolysis. The specificity of G proteins appears to be provided by the α subunits, of which there are about twenty, whereas the β and γ subunits show less diversity and are thought to act as general regulators of the α subunits, although recent studies have shown that they may have a more complex function (review: Lefkowitz, 1993).

G proteins were first implicated in membrane transport when it was shown that AlF_4^- could inhibit various transport steps in the secretory and endocytic pathways (Melançon et al., 1987; Mayorga et al., 1989a; Stow et al., 1991; Lefkowitz, 1993). AlF_4^- activates G proteins, by mimicking the γ phosphate of GTP when bound to the GDP form of Gα, without activating the ras-related proteins (Kahn, 1991). However, as it also affects other enzymes, including protein phosphatases and ATPases, it can not be used alone as proof of G protein involvement. More conclusive evidence for G protein involvement in membrane transport has come from the use of the amphiphilic peptide mastoparan, the βγ subunits of G proteins and the pertussis and cholera toxins (reviews: Balch, 1992; Barr et al., 1992; Bomsel and Mostov, 1992; Melançon, 1993).

The involvement of at least one G protein in endosome fusion has been demonstrated using a cell-free system (Colombo et al., 1992a). This study was based on the previously reported ability of GTPγS to either stimulate or inhibit fusion depending on the cytosol concentration (Mayorga et al., 1989b). At cytosolic concentrations of less than 0.5 mg/ml GTPγS is required for fusion whereas at cytosol concentrations of 1-2 mg/ml it inhibits

fusion. In contrast AlF_4^- inhibits fusion at high cytosol concentration but does not stimulate at low cytosol concentrations. It should be pointed out that even the high cytosol concentrations used in these studies are significantly lower than the *in vivo* cytosolic protein concentration, and in other cell-free systems higher concentrations of cytosol are required for maximum fusion (Braell, 1987; Gorvel et al., 1991). At low cytosol concentration the GTPγS induced stimulation was overcome by the addition of either mastoparan or purified βγ subunits. At high cytosol concentration the GTPγS induced inhibition was also reversed by mastoparan. As G proteins are usually activated by mastoparan, which stimulates the release of nucleotide by G_i and G_o, and inactivated by βγ subunits, which complex with the α subunits forming the inactive Gαβγ complex, the authors suggest that two G proteins, one stimulatory and one inhibitory, are involved (Colombo et al., 1992a). However, it has also been proposed that the βγ subunits could interact directly with a downstream effector and thus the involvement of only one G protein could produce these apparently diametric results (Bomsel and Mostov, 1992).

ARF proteins

The ARF proteins are small ras-related GTPases originally identified as a cofactor for the cholera toxin catalysed ADP-ribosylation of the G protein G_s (Kahn and Gilman, 1986). Myristilation of the N-terminal of ARF and GTP binding are required for membrane association, but the majority of the protein is cytosolic and bound to GDP (Kahn et al., 1988; Regazzi et al., 1991). There is now considerable evidence for the involvement of ARF in secretion and it has been shown to be required for the binding of coat components to both clathrin and non-clathrin-coated vesicles on the secretory pathway (Orci et al., 1993; Palmer et al., 1993; Stammes and Rothman, 1993; Traub et al., 1993). Activation of ARF is achieved by interaction with a membrane bound exchange factor and results in the association of GTP-bound ARF with the membrane followed by binding of coat components (Orci et al., 1993; Palmer et al., 1993; Stamnes and Rothman, 1993). Uncoating is then coupled with hydrolysis of GTP and the subsequent release of GDP-bound ARF into the cytosol (Helms et al., 1993; Teal et al., 1994; Zhang et al., 1994). Thus GTPγS or a mutant ARF protein, which hydrolyses GTP inefficiently, cause accumulation of coated pits and vesicles and inhibition of transport (Donaldson et al., 1991a; Donaldson et al., 1991b; Serafini et al., 1991; Palmer et al., 1993; Teal et al., 1994; Zhang et al., 1994). In contrast, the fungal metabolite Brefeldin A inhibits transport by inactivating the membrane bound ARF exchange factor thus preventing activation and membrane association of ARF (Orci et al., 1991; Donaldson et al., 1992b).

Although six ARF proteins have been identified in mammalian cells (Kahn et al., 1991; Tsuchiya et al., 1991) the role of specific ARF proteins has proven difficult to investigate. This is due to the high degree of homology between these proteins, which makes difficult both the production of specific antibodies and the preparation of purified fractions which do not contain multiple ARF proteins. Thus the localisation of membrane bound ARF proteins has not been well characterised and it is still unclear whether or not particular family members are involved in specific membrane transport steps. In *S. cerevisiae* there are two arf proteins, Arf1 and Arf2, deletion of both *ARF* genes is lethal but the single deletion mutants are viable. That the Arf proteins are interchangable is also demonstrated by the fact that the lethal *ARF1*$^-$ *ARF2*$^-$ deletion in yeast can be rescued by expression of either human ARF1 or ARF4 proteins (Kahn et al., 1991). These two proteins are 80% identical with each other and approximately 75% identical with each of the yeast proteins (Tsuchiya et al., 1991). Thus it appears that the individual ARF proteins share a conserved function.

Stahl and co-workers have studied the role of ARF in endocytosis by testing the activity of synthetic peptides and recombinant ARF1 in the same cell-free fusion assay that was used to demonstrate the involvement of G proteins in endosome fusion (Lenhard et al., 1992). The effects of two peptides corresponding to the sixteen N-terminal amino acids of human ARF1 and ARF4 were similar to those of mastoparan, in that they inhibited GTPγS dependent fusion at low cytosol concentration and antagonised GTPγS inhibition of fusion at high cytosol concentration (Colombo et al., 1992a; Lenhard et al., 1992). In the absence of GTPγS both peptides were slightly stimulatory at all cytosol concentrations. In contrast recombinant myristilated human ARF1 inhibited fusion at all cytosol concentrations in the presence, but not absence, of GTPγS and this inhibition could be reversed by the addition of the N-terminal ARF1 peptide. In addition pre-incubation experiments indicated that ARF is involved in the formation of a pre-fusion complex, as *in vitro* endosome fusion aquires resistance to ARF1 and GTPγS at a step preceding fusion.

Thus these results either indicate a, hitherto undetected, requirement for coat formation in endosome fusion or that the function of ARF in endosome fusion is different to its function on the biosynthetic pathway.

SCOPE OF THESIS

The involvement of the rab5a protein in an early step of endocytosis has been clearly demonstrated both *in vitro* and *in vivo* (Bucci et al., 1992; Gorvel et al., 1991), however, the function of this protein like that of the other rab proteins remains obscure. Henry Bourne proposed that the ability of these molecules to exist in two conformations, which are dependent on bound nucleotide, allows them to act as molecular switches which are used to confirm specificity on membrane fusion events within the cell, however, he also stressed the fact that these conformational states could be used in other ways (Bourne, 1988). Thus far it has only been possible to show that nucleotide binding is required for rab protein activity, as mutant proteins which do not bind nucleotide are non-functional. These experiments did not show whether, as predicted, GTP rather than GDP is required or what the requirement for GTP-hydrolysis is. Because of the involvement of other GTP-binding proteins in the regulation of membrane transport it is not easy to define these requirements. Inhibition of transport by GTPγS, for example, seems to be mediated by the ARF proteins, although this does not exclude the possibility that it could also exert an effect on the rab proteins.

Another question which has been raised is that of recycling of the rab proteins. Since their discovery it has been clear that the rab proteins exist both in membrane associated and cytosolic forms and it is now known that membrane association requires the attachment of geranylgeranyl groups to one or two C-terminal cysteine residues. The enzyme responsible for this modification, rab geranylgeranyl transferase, has now been characterised and modification can be achieved *in vitro* in the presence of the rab escort protein REP-1 (Andres et al., 1993; Armstrong et al., 1993b). It is predicted that the rab protein is then delivered to the specific membrane by REP-1, though this has not yet been demonstrated.

Once the rab protein is inserted into the membrane the question is how it is removed and recycled via the cytosol, since the presence of the highly hydrophobic lipid moiety will prevent it from readily dissociating from the membrane. Hence the requirement for the cytosolic protein GDI, which can remove GDP-bound rab proteins from membranes and, by binding to the lipid moiety, maintain them in a cytosolic form. Here though is another puzzle: GDI has been shown to not only remove rab proteins from membranes but also to insert them, presumably into the membrane of the correct organelle (Soldati et al., 1993; Ullrich et al., 1993; Ullrich et al., 1994). How is the directionality of this process maintained? Particularly as it has been shown

that, when purified, at least one rab protein will non-specifically associate with membranes (Araki et al., 1990).

Finally, although it has been possible, by analogy with p21 ras, to define the regions of the rab proteins which are involved in nucleotide binding and the C-terminal domain has been shown to be required for membrane association and localisation, nothing is known about the other regions of these proteins (Chavrier et al., 1991; Valencia et al., 1991). In particular the N-terminal domains show considerable heterogeneity between the various family members, but the significance of this variation remains unclear (Valencia et al., 1991).

In this work the rab5 protein has been studied using two main approaches. Firstly a cell-free assay, which was previously used to demonstrate the regulation by rab5 of fusion between early endosomes (Gorvel et al., 1991), has been utilised to further dissect the involvement of rab5 in this membrane transport step. Using this technique four major questions were approached; 1) Are all three isoforms of rab5 involved in the regulation of early endosome fusion?, 2) What are the requirements for nucleotide binding and hydrolysis for rab5 activity *in vitro*?, 3) Which domain of rab5 is involved in trypsin-mediated inhibition of early endosome fusion?, and 4) Can REP-1 be used to deliver rab5 to the early endosome membranes *in vitro*? In addition, the possible interaction of rab5 with phosphoproteins has been studied with the aim of demonstrating a convergence between two known regulators of the endocytic pathway; the rab5 protein and phosphorylation/dephosphorylation events.

MATERIALS AND METHODS

MATERIALS

General Reagents

Unless otherwise indicated in the text all chemicals, solid and liquid, were obtained from Merck (Germany) and were of the highest quality available.

Antibodies

Affinity purified polyclonal rab4 antibodies were a gift from Ira Mellman and Peter van der Sluijs (Yale, New Haven, USA). The polyclonal antibodies against C-terminal peptides of rab2 and GDI were kind gifts of Oliver Ullrich and Marino Zerial (EMBL, Heidelberg). The polyclonal antibodies raised against C-terminal peptides of rab5b (KQNLGGAAGRS-RGVDLHEQS) and rab5c (KQNAAGAPSRNRGVDLQENS) were a gift from Marino Zerial and Cecilia Bucci (EMBL, Heidelberg). The polyclonal antibody against GDI was a gift of Yoshimi Takai (Kobe university School of medicine, Japan). Polyclonal antibodies against avidin were a gift of Jean Gruenberg (EMBL, Heidelberg).

The production of polyclonal antibodies raised against a rab5a C-terminal peptide is described later in this section.

Peptides

All of the peptides used in this study were synthesised in the EMBL peptide synthesis group using an Applied Biosystems peptide synthesiser (ABI 431) utilising Fmoc chemistry, and purified by reverse phase HPLC.

The following peptides were used; rab5 C-terminal (PKNEPQNPGANSARGR), rab5 N-terminal (MANRGATRPNGPNTGNK), rab2 C-terminal (YEKIQEGVFDINNEANGIK).

Plasmid DNA

All of the plasmids used in this study were kind gifts of Philippe Chavrier, Harald Stenmark and Marino Zerial (EMBL, Heidelberg). The rab protein-encoding DNA sequences were inserted into the pGEM-1 plasmid (Stratagene) in front of the bacteriophage T7 promoter (Chavrier et al., 1990a; Gorvel et al., 1991; Stenmark et al., 1994a).

Purified recombinant proteins

Purified histidine tagged canine rab1a and human rab5a which had been expressed in *E. coli* (Andres et al., 1993) were gifts from Miguel Seabra (Southwestern Medical center, Dallas, Texas) and Marino Zerial (EMBL, Heidelberg), respectively. Purified REP-1 and rab geranylgeranyl transferase (Andres et al., 1993; Armstrong et al., 1993; Seabra et al., 1993) were a gift of Miguel Seabra (Southwestern Medical center, Dallas, Texas).

Vaccinia virus

The T7 RNA polymerase-recombinant vaccinia virus (Fuerst et al., 1986) used in this study was a gift of Marino Zerial (EMBL, Heidelberg).

Radioactive compounds

Guanosine (α-^{32}P) triphosphate, ^{32}P-orthophosphate and ^{35}S-methionine were obtained from Amersham-Buchler, Germany. ^{35}S-methionine/cysteine (EXPRE^{35}S^{35}S™) was obtained from Du Pont/NEN Research Products, Boston, MA.

Cell culture materials

All media and chemicals used for cell culture were obtained from Gibco-BRL (Scotland), unless otherwise indicated. Plastic flasks and dishes for cell culture were from Nunc (Denmark) or Flow (USA).

Biotinylated HRP

Biotinylated HRP (bHRP) was prepared by dissolving 20 mg of HRP (Sigma, St Louis, USA) in 9.5 ml of 0.1M $NaHCO_3/Na_2CO_3$, pH 9.0. The HRP was then mixed gently with 11.4 mg of biotin-X-NHS (biotinyl-e-aminocaproic acid N-hydroxysuccinimide ester; Calbiochem, USA) which had been solubilised in 0.5 ml dimethylformamide in a glass tube. After incubation at room temperature for 2 h with gentle stirring, the unreacted active groups were quenched by the addition of 1 ml of 0.2M glycine, pH 8.0, and incubated for a further 30 min. The bHRP was then dialysed extensively against internalisation medium (IM; MEM, 10mM HEPES, 5mM D-glucose, pH 7.4), then filter sterilised and stored at -20°C until use.

CELL CULTURE

Cells

Baby hamster kidney (BHK) cells, clone 21 (from Dr J. Gruenberg, EMBL), were grown in Glasgow's minimum essential medium (GMEM) supplemented with 5% foetal calf serum, 10% tryptose phosphate broth, 2mM glutamine and 100 units/l penicillin and streptomycin (Growth medium: GM), in humidified incubators (Heraeus) at 37°C and 5% CO2. Stock cells, which were prepared from the 3rd passage after cloning, were kept in liquid nitrogen in 50% FCS, 40% GM and 10% DMSO. For experiments cells were used which had been passaged not more than 20 times after cloning.

Maintenance cell culture

To set up new maintenance cells, an ampoule of stock BHK cells, containing approximately 15 x 10^6 cells, was rapidly thawed in a 37°C water bath and the cells immediately resuspended in 10 ml of GM. The cells were then pelleted by centrifugation at 1,000 rpm, for 5 min, at 37°C in a table top centrifuge (Heraeus). The pellet was resuspended gently, using a 5 ml pipette, in 5 ml of GM and the cells divided equally into two 75 cm^2 Falcon flasks containing 17.5 ml of GM. One of these flasks was passaged after 24 hr and the second at 48 hr, in both cases cells were seeded at a density of 1:10 (surface area:surface area) onto 10 cm diameter plastic dishes in 10 ml of GM. Once these cells reached confluency (2-3 days) they were passaged at a density of 1:20 (surface area:surface area) onto 10 cm diameter dishes and then routinely passaged in this manner every 3-4 days.

Passaging BHK cells

Confluent monolayers of cells on 10 cm diameter dishes were washed once with 10 ml PBS (137mM NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 6.5mM Na_2HPO_4, pH 7.4) and 5 ml of trypsin/EDTA solution (0.05% trypsin w/v and 0.02% EDTA w/v in Puck's saline solution) was added. After one minute at room temperature, 4 ml of the trypsin/EDTA solution was removed and the cells then left for a further 4-8 min, or until the monolayer began to break up into small patches of cells. The dishes were then swirled gently, in order to detach all of the cells from the surface of the dish, and the cells were carefully resuspended in 2.5 ml of GM, using a sterile 5 ml pipette. When a single cell suspension was obtained, the cells were diluted appropriately in GM and then aliquoted onto 10 cm dishes.

Preparation of cells for experiments

BHK cells required for subcellular fractionation were routinely prepared 14-18 hr before use. Three day old dishes of confluent cells were passaged at a dilution of 1:4 (surface area:surface area) so that the cells were just confluent (approximately 1.3 X 10^7 cells, or 2.5-3.0 mg total protein, per 10 cm dish) when experimental manipulation started.

CYTOSOL

BHK cytosol

For BHK cytosol cells were passaged onto 22.5 x 22.5 cm plastic dishes at a dilution of 1:4 (surface area:surface area) so that they were approximately 95% confluent (1.0 x 10^5 cells/cm^2) after 16 h. All the following steps were carried out on ice or at 4°C. The cells were first washed twice with PBS and then 10 ml of PBS added to each dish. One side of the dish was then raised so that, using a cell scraper (EMBL workshop), the monolayer could be scraped carefully into the PBS which collected at the bottom edge. The cells were then pelleted in a table top centrifuge (Sorvall RT6000 refrigerated centrifuge) at 1,000 rpm for 5 min, resuspended gently in 2 ml homogenisation buffer (HB; 250mM sucrose, 3mM imidazole, pH 7.4) and pelleted at 3,000 rpm for 10 min. The cell pellet was then gently resuspended in 0.5 ml HB and the cells homogenised by passing them through a 0.22g needle 4-7 times. The appearance of the cells was carefully monitored using a phase contrast microscope (Zeiss, Germany), so that as soon as 75% of the nuclei were free of cellular material homogenisation was stopped. Using this technique less than 5% of the nuclei were broken during homogenisation. The nuclei were then pelleted by centrifugation at 3,000 rpm for 15 min and the post nuclear supernatant (PNS) collected. The PNS was then centrifuged in a TL100 table top centrifuge (Beckman) using a TLA100.2 fixed angle rotor for 15 min at 22,000 rpm, the supernatant was collected and centrifuged for a further 30 min at 65,000 rpm. The supernatant (cytosol), from this second centrifugation was collected after the lipids, which collected near the surface, were removed by dipping a cold metal spatula into the top of the supernatant. After measurement of the protein concentration, typically it was 10-15 mg/ml, 100 µl aliquots of cytosol were flash frozen and then stored in liquid nitrogen.

Rab5 cytosol

BHK cells which had been infected with the T7 polymerase recombinant-vaccinia virus and transfected with rab5 DNA were washed

twice with PBS and cytosol was then prepared as described above and stored in liquid nitrogen in 50 µl aliquots. The protein concentration of cytosol prepared from cells overexpressing rab5 was routinely 6-8 mg/ml.

Rat liver cytosol

Rat liver cytosol was prepared from fresh livers obtained from female Wistar rats (Aniento et al., 1993a). The tissue was first minced in HB (4 ml per gram of tissue), and then homogenised by 6-8 passes in a Potter-Elvejehem homogeniser set at a speed of 3,000 rpm. The remaining tissue debris was removed by filtration through sterile surgical muslin and the filtrate centrifuged at 5,000 rpm (3,000 x g), for 10 min in a Sorvall SS34 rotor. The supernatant was collected and the pellet then resuspended in the same volume of HB as the liver was homogenised in and centrifuged for a second time. The supernatant of this centrifugation was pooled with that of the first centrifugation and the pooled material centrifuged at 14,500 rpm (25,000 x g) for 10 min. The supernatant was collected and centrifuged at 35,000 rpm (220,000 x g) for 1 h using an SW40 rotor and the supernatant (cytosol), harvested as for BHK cytosol. The protein concentration was then measured, typically it was 20-30 mg/ml, and the cytosol flash frozen and stored in liquid nitrogen in 0.5 and 1.0 ml aliquots.

PREPARATION OF EARLY ENDOSOME FRACTIONS

Internalisation of fluid phase markers

The cells, grown on 10 cm diameter dishes as described, were placed onto wet metal plates inserted into ice buckets on mechanical rockers, in this way they were maintained at 4°C during all manipulations, unless otherwise indicated. After two brief washes with 10 ml of ice cold PBS, the dishes were transferred onto wetted flat metal plates in a water bath at 37°C and allowed to equilibrate for 30 s, 3 ml of Internalisation medium (IM; MEM, 10mM HEPES, 5mM D-glucose, pH 7.4) containing either 3.3 mg/ml avidin or 1.8 mg/ml bHRP was then added to each dish. After 5 min the medium was removed, the cells were immediately returned onto the cold metal plates and 10 ml of ice cold PBS added. The monolayers of BHK cells were washed twice with PBS for 5 min each, once with PBS-BSA (5 mg/ml bovine serum albumin in PBS) for 20 min and then twice more with PBS. The cells were now ready for homogenisation.

Homogenisation and preparation of PNS

After washing the cells as described 2.5 ml of PBS was added to each dish and a cell scraper (EMBL workshop), was then used to carefully scrape the monolayer into the PBS. The cells from each 4 dishes were put into 15 ml plastic tubes (Falcon) and pelleted in a table top centrifuge (Sorvall RT6000 refrigerated centrifuge) at 1,000 rpm for 5 min. The resultant cell pellet was then resuspended gently, using a 3 ml disposable pasteur pipette, in 2 ml HB and repelleted at 3,000 rpm for 10 min. These cell pellets were then gently resuspended in HB (550 µl for 6 dishes of cells), using a 1 ml Eppendorf pipette tip, and homogenised by passing through a 0.22g needle 4-7 times. The appearance of the cells was carefully monitored using a phase contrast microscope (Zeiss, Germany), so that as soon as 75% of the nuclei were free of cellular material homogenisation was stopped. Using this technique less than 5% of the nuclei were broken during homogenisation. The nuclei were then pelleted by centrifugation at 3,000 rpm for 15 min and the PNS collected. Routinely approximately 500 µl (6 mg protein) of PNS was obtained from 6 dishes.

Preparation of early endosome fractions

Early endosome fractions were prepared using a flotation gradient which was previously developed in the laboratory (Gorvel et al., 1991). For each gradient 500 µl of PNS, prepared from six 10 cm dishes of BHK cells, was adjusted to 40.6% sucrose as measured on a densitometer, by addition of 62% (% weight) sucrose in 3mM Imidazole, pH 7.4. The PNS was then loaded into the bottom of a SW60 centrifuge tube (Beckman) and used to wet one side of the inner wall of the tube. A step gradient was then formed by sequentially adding; 1.5 ml of 35% (% weight) sucrose in 3mM Imidazole, pH 7.4, 1.0 ml of 25% sucrose (% weight) in 3mM Imidazole, pH 7.4 and finally 0.5 ml HB. In order to avoid mixing of the layers the solutions were poured down the wetted side of the tube, using a 2 ml syringe and wide gauge (18 1/2g) needle, so that drops did not form. The gradient was centrifuged at 35,000 rpm, for 60 min at 4°C, in a Beckman L5-65 ultracentrifuge using a SW60 rotor. The early endosome fraction (250-300 µl) was then collected at the 35%/25% sucrose interface using a 50 µl glass capillary tube fitted to a peristaltic pump (Pharmacia-LKB, Freiburg, Germany). The protein concentration of the fractions was measured and was routinely 0.45-0.55 mg/ml. These fractions were either used immediately or were flash frozen and stored in liquid nitrogen in 100 µl aliquots.

CELL-FREE ASSAY OF EARLY ENDOSOME FUSION

Early-endosome fusion was measured using the cell-free assay previously established in the group (Bomsel et al., 1990; Gorvel et al., 1991; Gruenberg and Howell, 1988). All of the steps were carried out on ice unless otherwise stated. Fusion was carried out in the presence of endogenous levels of nucleotides, except for ATP added exogenously as described.

Cell-free assay using early endosome fractions

For each assay 50 µl aliquots each of avidin (Molecular Probes Inc., Eugene, USA) and bHRP labelled early endosome fractions (containing approximately 25 µg protein) were mixed with 50 µl of cytosol and adjusted to 12.5mM HEPES (pH 7.4), 1.5mM MgOAc, 3mM imidazole, 1mM DTT (Saxon Biochemicals GmbH, Hannover, Germany), 75mM KOAc. In order to quench any avidin that might be released, 8 µg of biotin-insulin (Sigma, St Louis, USA) was added. An ATP-regenerating system, consisting of a 1:1:1 mixture of 800mM creatine phosphate (Boehringer Mannheim GmbH, Germany), 4 mg/ml creatine kinase (Boehringer Mannheim GmbH, Germany) and 100mM ATP (Sigma, St Louis, USA), mixed on ice immediately before use, was then added. In experiments where rat liver cytosol was used 4 µl of the ATP-regenerating system was added, when BHK cytosol was used 8 µl was added. For ATP depletion, 15 µl of hexokinase $(NH_4)_2SO_4$ precipitate suspension (Boehringer Mannheim GmbH, Germany) was pelleted by centrifugation for 1 min at full speed in an Eppendorf centrifuge, resuspended in 10 µl of 0.5mM D-glucose and added directly to the appropriate samples. All mixing steps were performed either by gentle tapping of the Eppendorf tube or with a cut off 1 ml pipette tip, in order to minimise damage to the endosome membranes. The samples were then incubated in a water bath at 37°C for 45 min, and then returned immediately onto ice. Before solubilising the membranes another 5 µg of biotin-insulin was added, to quench any remaining avidin binding sites. After addition of 5 µl of 20% Triton X-100, the samples were vortexed and incubated on ice for 30 min. Then 500 µl of PBS-BSA was added and any insoluble material removed by centrifugation at 9,000 rpm in an Eppendorf centrifuge for 1 min. The supernatant was then transferred into fresh Eppendorf tubes containing affinity purified anti-avidin antibodies (5 µg), which had been prebound to 50 µl (bed volume) of Protein A sepharose beads (Protein A Sepharose CL-4B, Pharmacia-LKB, Freiburg, Germany). Immunoprecipitation was allowed to proceed overnight at 4°C, with continuous rotation of the samples. The beads were then washed twice in PBS-BSA, containing 0.5% Triton X-100, and once in PBS.

In order to measure the total amount of avidin-bHRP complex (Total) which could be formed in the assay if all of the avidin and bHRP were allowed to mix, the avidin and bHRP labelled endosomes were mixed in the absence of biotin-insulin, and solubilization of the membranes, immunoprecipitation and HRP detection were carried out as for the other samples.

Testing the activity of rab5 and mutant rab5 proteins in the cell-free assay

In order to test the activity of cytosolic rab5 wild type and mutant proteins in early endosome fusion, the assay was carried out as described above. With the exception that 25 µl of rat liver cytosol was used and the assay was then complemented with 25 µl of BHK cytosol, prepared from cells overexpressing rab5, diluted in HB to the appropriate protein concentration.

Quantification of fusion

In order to quantify the amount of avidin-bHRP complex which had been immunoprecipitated the enzymatic activity of HRP was measured. This was done by adding 1 ml of HRP developing mixture consisting of 0.01% w/v o-dianisidine hydrochloride (Sigma, St Louis, USA) in 0.003% H_2O_2, 0.1% TX-100, 50mM $NaPO_4$, pH 5.0, and incubating in the dark at room temperature for 3 h. The OD455 was then measured using an Ultraspec II 4050 spectrophotometer (Pharmacia-LKB).

Fusion was calculated as the percentage of the value obtained when no b-insulin was added in the presence (TAv) or absence (TAv+bHRP) of excess bHRP. Thus, TAv is the value obtained when excess bHRP is added so that 100% of the avidin which is present binds to bHRP, and TAv+bHRP is the value obtained when all of the avidin and bHRP present in the endosomes are allowed to mix.

Measurement of early endosome latency

A 20 µl aliquot of PNS or early endosome fraction containing internalised bHRP, was loaded into an airfuge tube (Beckman) which was then filled with HB. The membranes were then pelleted by centrifugation in an airfuge for 20 min under 20 psi at 4°C. The supernatant and pellet were then collected and the amount of marker present in each was quantified using the HRP assay described above.

Latency is expressed as the percentage of total marker (supernatant and pellet) which is present in the pellet (intravesicular).

PROTEASE TREATMENT OF MEMBRANES AND CYTOSOL

Trypsin treatment of early endosome membranes

In order to quantify the effect of limited trypsinisation on the fusion activity of early endosomal membranes, early endosome membrane fractions (≈ 20 μg protein in 50 μl) labelled with either avidin or bHRP were separately treated with TPCK-treated trypsin (Worthington Biochemical Corp., New Jersey, USA) at concentrations of 0.1 to 1.0 μg/ml (corresponding to 0.25-2.5 ng trypsin/μg protein) for 30 min at 4°C. Trypsin activity was then stopped by adding 1 μl of HB containing 5 μg of soybean trypsin inhibitor (SBTI, Worthington Biochemical Corp., New Jersey, USA) and the mixture was further incubated for 30 min at 4°C. As a control, trypsin and SBTI were added simultaneously to the fractions and the mixture incubated for 60 min at 4°C. These fractions were then tested in the fusion assay (see above).

Trypsin treatment of cytosol

BHK cytosol was treated with 2.5 ng trypsin/μg cytosolic protein for 30 min on ice, the enzyme was then inactivated by the addition of 5.0 ng SBTI and the mixture incubated for a further 15 min. In control experiments, trypsin and SBTI were added simultaneously and the samples incubated for 45 min.

OVEREXPRESSION OF RAB5 PROTEINS IN BHK CELLS

In order to overexpress rab5 and mutant rab5 proteins the T7 RNA polymerase-recombinant vaccinia virus system was used (Fuerst et al., 1986). Cells were first infected with T7 RNA polymerase-recombinant vaccinia virus and then transfected with the appropriate DNA. This method has been shown to produce high levels of cytosolic rab proteins (Chavrier et al., 1990a; Gorvel et al., 1991).

Preparation of stock vaccinia virus

For each preparation of stock vaccinia virus twenty 10 cm dishes of 90% confluent BHK cells were used. The GM was removed and the monolayers were then rinsed with PBS. The virus (1 x 10^7 p.f.u. per 20 dishes) was diluted in 40 ml of PBS containing 20 μg/ml SBTI (Sigma), and 2 ml was used to infect each dish of cells. Infection was carried out at room temperature for 60 min, with occasional rocking of the dishes to ensure that the monolayer was evenly covered with the virus innoculum and to prevent drying out of the cells. The virus innoculum was then removed and 10 ml of

GM, containing only 2% foetal calf serum, was added onto each dish. The cells were then incubated for 48 h, at 37°C and 5% CO_2 in a humidified incubator. The following steps were all carried out at 4°C or on ice. All of the cells were scraped off the dish into the GM and then pelleted by centrifugation at 3,000 rpm for 10 min in a Sorvall RT6000 refrigerated centrifuge. The cell pellet was then resuspended in 10 ml of 10 mM Tris, pH 9.0, and homogenisation carried out using a Dounce homogeniser. The nuclei were then removed by centrifugation at 3,000 rpm for 15 min in a Sorvall RT6000 refrigerated centrifuge. The supernatant (PNS) was collected and layered on top of an equal volume of 10mM Tris, pH 9.0, 36% sucrose in an SW27 centrifuge tube (Beckman). The virus was then pelleted by centrifugation at 27,000 rpm, for 30 min in a Beckman L5-65 ultracentrifuge using a SW27 rotor. The supernatant was discarded and the pellet resuspended in 1-2 ml 10mM Tris, pH 9.0. Before freezing, the virus was trypsin treated by the addition of 100 µl of 2 mg/ml TPCK treated trypsin (Sigma) to 1 ml of virus stock, and incubated for 30 min with gentle vortexing every 10 min. Finally the virus stock was divided into 500 µl aliquots and stored at -80°C.

Infection with vaccinia virus

BHK cells were passaged 16 h before use, so that they were approximately 90% confluent at the time of infection. Stock vaccinia virus was thawed and diluted in GMEM, containing 20mM HEPES, pH 7.2, and 20 µg/ml SBTI (Sigma), to a concentration of approximately 3.4 p.f.u./ml. The monolayers were rinsed once with GMEM and then 3.5 ml of virus was added to each 10 cm dish of cells, in this way approximately 1 p.f.u. of virus was added per cell. The cells were then incubated at room temperature for 30 min, with occasional rocking of the dishes to ensure that the monolayer was evenly covered with the virus innoculum and to prevent drying out of the cells.

Transfection of virus infected cells

The plasmid DNA (2 µg per 10^5 cells) was first diluted in transfection buffer (TB; 20mM HEPES, 150mM NaCl, pH 7.4) to a final volume of 350 µl per 10 cm dish of cells. The transfection reagent N-[1-(2,3-dioleoyloxy) propyl]-N, N, N-trimethyl-ammonium-methylsulphate (DOTAP; Boehringer Mannheim) was then diluted (5 µl per 10^5 cells) in TB to a final volume of 340 µl per dish. The DNA and DOTAP solutions were then mixed and left at room temperature for 10 min. The virus innoculum media was then aspirated from the cells and replaced with 5 ml of GMEM supplemented with 10mM hydroxyurea, the DNA/DOTAP mixture was then added to the media

on each dish of cells, and the cells then incubated at 37°C. The incubation time varied between 6-7 h, so that the maximum levels of expression could be obtained whilst minimising the cytopathic effect of the virus.

PURIFICATION OF RAB5 PROTEIN AFTER EXPRESSION IN E. COLI

Canine rab5 protein was expressed in *E. coli* using the pET-vector expression system (Zahraoui et al., 1989; Wandinger-Ness and Zerial, in preparation). Transformed cells were grown on L-agar plates containing 100 µg/ml ampicillin at 37°C for 16 h. Twenty colonies were then picked and transferred into 100 ml of Superbroth + 100 µg/ml ampicillin in a 250 ml conical flask, and incubated for 2 h at 37°C with rotary shaking (200 rpm). The bacterial suspension was then divided into 10 2 l conical flasks, each containing 200 ml of Lurai Burtani medium, and incubated at 37°C with rotary shaking (200 rpm). The cells were grown until the OD600 reached 0.5, approximately 1.5 h. Induction was achieved by adding 0.5 ml of 50mM IPTG into each 200 ml culture, and incubating for a further 3 h at 37°C. The cells were then pelleted by centrifugation for 10 min at 4,000 rpm at 15°C using a Sorvall GS-3 rotor in a Sorvall GSA centrifuge. The supernatant was removed and the pelleted cells were frozen at -20°C.

In order to purify the overexpressed rab5 protein the frozen cell pellets were first resuspended in 15 ml of bacterial lysis buffer (BLB; 64.4mM Tris pH 8.5, 10mM $MgCl_2$, 0.5mM DTT, 10µM PMSF, 10µM Benzamidine, 1mM NaN_3). A French press, set at medium, was then used to break the cells. From this point on all steps were carried out at 4°C. Protamine sulphate powder was added directly to the stirred lysate to a final concentration of 2 mg/ml. The lysate was then stirred for a further 20 min. Bacterial debris was removed by centrifugation at 16,000 rpm for 15 min using a SW27 rotor in a Beckman L5-65 ultracentrifuge. The supernatant was then loaded onto a Q-Sepharose column (Pharmacia, 1 cm x 12 cm), which had been pre-equilibrated with BLB, washed through with BLB and the flow-through collected. The protein was further purified on a Superdex-75 gel filtration column (Pharmacia, 1 cm x 12 cm). Fractions of 0.5 ml were collected and run on a 12% mini-gel to estimate protein concentration and purity. Peak fractions were pooled and buffer exchanged for 50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM GDP, on a PD-10 column. The protein was divided into 200 µl aliquots and stored at -20°C.

METABOLIC LABELLING

^{35}S-methionine labelling

Confluent BHK cells were passaged at a dilution of 1:3 (surface area:surface area) and incubated for 3 hr so that they had attached to the surface of the dish and started to divide. The cells were then washed twice with PBS and 6 ml of methionine labelling medium (MLM; methionine free minimal essential medium supplemented with, 10% GM, 2.2g/l $NaHCO_3$ and 1.5 mg/l L-methionine, 1% BSA, and 1% HEPES, pH 7.5) containing 0.5mCi of carrier-free ^{35}S-methionine was added to each 10 cm diameter dish. The cells were incubated for 16 hr at 37°C in a 5% CO_2 atmosphere.

^{35}S-methionine/cysteine labelling

Confluent BHK cells were passaged at a dilution of 1:3 (surface area:surface area) and incubated for 3 hr so that they had attached to the surface of the dish and started to divide. The cells were then washed twice with PBS and 6 ml of methionine/cysteine labelling medium (MCLM; methionine and cysteine free Dulbeccos minimum essential medium [Sigma] supplemented with 10% GM) containing 1mCi of EXPRE^{35}S^{35}S™ was added to each 10 cm diameter dish. The cells were incubated for 16 hr at 37°C in a 5% CO_2 atmosphere.

^{32}P-orthophosphate labelling

Confluent BHK cells were passaged at a dilution of 1:5 (surface area:surface area) and incubated for 16 hr so that they were approximately 90% confluent. The GM was then removed and the cells washed twice with Tris-buffered saline (TBS; 50mM Tris-HCl, 150mM NaCl, pH 7.5) and 5 ml of phosphate labelling medium (PLM; phosphate free Dulbeccos minimum essential medium [Sigma] supplemented with 2% foetal calf serum which had been dialysed against TBS to remove organic phosphate) containing 0.5mCi ^{32}P-orthophosphate was added to each 10 cm diameter dish. The cells were then incubated for 3 hr at 37°C in a 5% CO_2 atmosphere.

IMMUNOPRECIPITATION

Unless otherwise indicated all steps were carried out at 4°C and all centrifugation steps were carried out in an Eppendorf centrifuge at 13,000 rpm for 2 min.

Preparation of protein A sepharose

The solid support used for all immunoprecipitations was Protein A sepharose CL-4B beads (Pharmacia LKB Biotechnology), which were prepared by swelling in distilled water according to the manufacturers instructions. The beads were always washed twice in the appropriate buffer and a 50% suspension was then prepared in the same buffer immediately before use. Routinely the immunoglobulin was pre-bound to the beads. This step was carried out by incubating the appropriate antiserum, diluted 100 fold in 1% NP40/PBS-BSA, with beads (3 µl of serum/15 µl of 50% Protein A sepharose suspension) for 60 min at 4°C on a rotating wheel, the beads were then washed twice with 1% NP-40/PBS-BSA, and twice with the appropriate immunoprecipitation buffer. In experiments where pre-clearing of the samples is indicated this step was carried out using beads which had been incubated with pre-immune sera instead of specific antisera.

Immunoprecipitation of ^{35}S-labelled proteins

Immunoprecipitation from early endosome fractions

BHK cells were labelled with ^{35}S-methionine/cysteine and early endosome fractions prepared as described elsewhere in this section. Aliquots of 200 µl (approximately 7 x 10^7 cpm) were flash frozen and stored in liquid nitrogen until use.

Membrane solubilisation and immunoprecipitation were carried by adding either 10% N-octylpolyoxyethlene (Octyl-POE/Rosenbusch-Tenside; Bachem-Biochemica GmbH, Heidelberg, Germany) or 10% NP-40 in PBS, to give a final detergent concentration of 1%, and incubating the samples for 30 min at 4°C. Then 700 µl of PBS-BSA was added and insoluble material removed by centrifugation. The supernatants were harvested and precleared with Protein A sepharose beads (30 µl), which had been incubated as described with pre-immune serum, for 30 min. The preclearing-beads were removed by centrifugation and the supernatants then incubated with specific antiserum or pre-immune serum, prebound to protein A sepharose beads (30 µl), overnight on a rotating wheel. These beads were then washed three times with either 1% Octyl-POE or 1% NP40 in 50 mM Tris, pH 7.4, and once with 50 mM Tris, pH 7.4. The bound protein was recovered from the beads in SDS-sample buffer (2% SDS, 10% glycerol, 100 mM DTT, 60 mM Tris-HCl, pH 6.8 and 0.001% bromophenol blue) and analysed by SDS-PAGE.

Immunoprecipitation from PNS, cytosol or membranes

When immunoprecipitated ^{35}S-labelled proteins and ^{32}P-labelled proteins were directly compared, the ^{35}S-labelled proteins were immunoprecipitated according to the protocol described for ^{32}P-labelled proteins.

Immunoprecipitation of ^{32}P- labelled proteins .

Immunoprecipitation from PNS

Monolayers of BHK cells, which had been labelled with ^{32}P-orthophosphate were washed twice with TBS and scraped into 2.5 ml of TBS. A PNS was prepared from the cells as described for cytosol preparation, except that the cells obtained from two 10 cm dishes were homogenised in 500 µl of HB. The PNS was then diluted with an equal volume of 2x lysis buffer (LB; 50mM Tris-HCl, pH 7.4, 20mM $MgCl_2$, 150mM NaCl, 0.5mM Na_3VO_4, 25mM NaF and 1% NP40), or 2x Rosenbusch-Tenside buffer (RTB; 12.5mM HEPES, 1.0mM DTT, 5mM EGTA, 100mM KOAc, 1mM $MgCl_2$, 1% Octyl-POE, pH 7.4) and incubated on ice for 30 min. Insoluble material was removed by centrifugation at full speed in an Eppendorf centrifuge for 2 min. The supernatant was divided into 10 equal aliquots which were then precleared by incubating them with 15 µl of protein A sepharose beads, which had been pre-incubated with pre-immune serum. After incubation for 15 min, the preclearing-beads were removed by centrifugation. The resulting supernatant then received 15 µl of protein A beads, which had been pre-incubated with specific antisera, and was incubated for 45 min on a rotating wheel. These beads were then washed four times with LB or RTB, and twice with 50mM Tris, pH 7.5. The immunoprecipitated protein was then recovered by boiling in SDS-sample buffer, containing 1mM GTP and 1mM GDP, and analysed by SDS-PAGE.

Immunoprecipitation from cytosol and membranes

The protocol used for immunoprecipitating proteins from cytosol and membranes of ^{32}P-orthophosphate labelled cells was the same as that described for immunoprecipitation from PNS except that an additional centrifugation step was added. The PNS was centrifuged for 15 min at 22,000 rpm (150,000 x g) in a TL100.2 rotor, using a Beckman TL100 tabletop centrifuge. The supernatant (cytosol) was then harvested and the pellet (membrane fraction) was resuspended in HB so that the final volume was the same as the volume of the cytosol. Solubilization and immunoprecipitation was then carried out as described above.

ANTIBODY PREPARATION

Preparation of polyclonal anti-rab5 antisera

Rab5 polyclonal antibodies were raised against the C-terminal peptide (PKNEPQNPGANSARGR) described by Chavrier et al., (Chavrier et al., 1990a) using a lymph node injection protocol (Louvard et al., 1982).

Preparation of peptides for injection

The peptide was conjugated to Keyhole Limpet haemocyanin (KLH) by mixing 2.5 mg of KLH with 2.5 mg of peptide dissolved in 1 ml PBS, adding 2.5 µl of 8% gluteraldehyde (EM grade) and leaving for 5 min at room temperature. The addition of glutaraldehyde was repeated four times, giving a final concentration of 10mM. The solution was then left at room temperature for a further 60 min and then 1M glycine was added to give a final concentration of 200mM. The peptide preparation was then divided into 2 aliquots containing 150 µg and the rest containing 50 µg each. The volume of the aliquots was made up to 500 µl with PBS and they were stored at -20°C until required. The first injection was prepared by mixing an aliquot of peptide mixture (150 µg) with 500 µl of Freund's complete adjuvant; in order to obtain a good emulsion the mixture was passed several times between two 1 ml syringes joined with a Leuer lock. The second injection was prepared in the same way, except that Freund's incomplete adjuvant was used.

Immunisation Procedure for rabbits

Week 0:	first injection, 150 µg in Freund's complete adjuvant. As much as possible (100-200 µl) into the popliteal lymph nodes and the remainder intradermally on the back. At the same time 10 ml of blood was taken from the rabbits for the preparation of pre-immune serum.
Week 3:	second injection, 150 µg in Freund's incomplete adjuvant. Half of the volume injected subscapularly, and the rest intradermally in the neck region.
Week 4:	first bleed.
Week 5:	1st day; third injection, 50 µg in PBS, intramuscular. 2nd day; fourth injection, 50 µg in PBS, intravenous.
Week 7:	1st day; second bleed. 2nd day; fifth injection, 50 µg in PBS, intramuscular.
Week 8:	third bleed.

Week 9:	fourth bleed.
Week 10:	sixth injection, 50 μg in PBS, intramuscular.
Week 11:	fifth bleed.
Week 12:	sixth bleed.
Week 13:	seventh injection, 50 μg in PBS, intramuscular.
Week 14:	seventh bleed.
Week 15:	eighth injection, 50 μg in PBS, intramuscular.
Week 16:	eighth bleed.
Week 17:	ninth injection, 50 μg in PBS, intramuscular.
Week 18:	ninth bleed.
Week 19:	final bleed, obtained by cardiac puncture.

Preparation of antisera

Rabbits were bled from the ears, approximately 50 ml being obtained each time. The blood was put into 50 ml tubes (Falcon) and a 9 cm long wooden stick inserted into the blood, the tube was then sealed, incubated at 37°C for 1 hr and then left at 4°C overnight. The stick, and the blood clot which formed around it, was carefully removed and the remaining serum spun at 4,000 rpm for 20 min at 4°C in a bench top centrifuge (Heraeus), in order to remove the remaining cells and debris. Serum was aliquoted into 2.5 ml serum tubes and stored at -70°C.

Affinity purification of anti-rab5 antibodies

Preparation of affinity purification column

All steps were carried out at 4°C unless otherwise indicated. The peptide against which the antisera was raised was coupled to Affi-gel 10 (BioRad, Munich, Germany) by dissolving 20 mg of peptide in 50mM HEPES, pH 7.4, and mixing this with 1 ml (bed volume) of Affi-gel 10 matrix. Coupling was allowed to occur for 4 hr, with constant rotation. In order to block the remaining ester groups on the matrix, 100 μl of 1M triethanolamine-HCL, pH 8.0, was added and the mixture incubated for 1 hr. The matrix was then packed into a 1.5 ml Mobicol M1002 mini-column (Mobitec, Göttingen, Germany) and the column was washed with 10 bed volumes of each of the following; 50mM HEPES, pH 7.4; 10mM Tris, pH 7.5; 100mM glycine, pH 2.5; 10mM Tris, pH 7.5; 100mM triethanolamine, pH 11.5; 10mM Tris, pH 7.5. The

pH was measured after each of the Tris washes to ensure that it returned to 7.5.

Loading of serum and elution of antibodies

Antiserum was diluted with an equal volume of 10mM Tris, pH 7.5 and 2 ml of this mixture was loaded onto the column using a peristaltic pump so that the serum was passed several times over the column at a rate of 2 ml/hr. The column was then washed with 20 bed volumes of 10mM Tris, pH 7.5 followed by 20 bed volumes of 500mM NaCl, 10mM Tris, pH 7.5. Acid elution was carried out in 10 ml of glycine, pH 2.5, and all 10 ml of the eluate was collected in a tube containing 1 ml of 10mM Tris, pH 8. The column was then washed with 10 bed volumes of Tris, pH 8.8, so that the pH rose to 8.8. Basic elution was carried out in 10 ml of 100mM triethanolamine, pH 11.5, and all 10 ml of the eluate was collected in a tube containing 1 ml of 10mM Tris, pH 8. The column was then washed with 10 bed volumes of Tris, pH 7.5, so that the pH rose to 7.5, and was stored in this buffer supplemented with 0.1% sodium azide. The acid and basic eluates were pooled and then concentrated under pressure using a 10 ml volume Amicon concentrator until the volume was approximately 2 ml, 10 ml of 10 mM Tris, pH 7.5 was then added and the volume again reduced to approximately 1 ml. The protein concentration was then measured, typically it was 0.2-0.4 mg/ml, and the antibody solution mixed with an equal volume of glycerol and stored at -20°C in 250 µl aliquots.

Affinity purification of anti-avidin antibodies

Preparation of affinity purification column

Avidin (Molecular probes) was coupled to Affi-gel 10 using the same technique described for the rab5 affinity purification column, except for the following differences. The avidin (500 mg) was dissolved in 10 ml of 50mM HEPES, pH 7.4 and coupled to 12 ml (bed volume) of Affi-gel 10. The remaining ester groups were blocked by the addition of 1 ml 1M triethanolamine-HCL, pH 8.0. A glass column with a diameter of 1.5 cm and a length of 15 cm was used.

Loading of serum and elution of antibodies

The column was first washed with 20 bed volumes PBS. Binding of antibody to the column was carried out using the batch technique, 14 ml of a 1:1 mixture of anti-avidin antisera and PBS was added and the matrix resuspended in this mixture. After overnight incubation on a mechanical

rocker, the column was fixed in an upright position and the matrix allowed to settle. The column was then washed with 20 bed volumes of 50mM Tris, pH 8.0 followed by 20 bed volumes of 250mM NaCl, 50mM Tris, pH 7.5. Acid elution was carried out in 50 ml of glycine, pH 2.5, and 1 ml fraction were collected in Eppendorf tubes containing 50 µl of 1.5M Tris, pH 8.0. The OD280 of each fraction was measured and peak fractions were pooled, the antibody was then dialysed extensively using 1 l of PBS/ml of antibody, with at least 2 changes of PBS, for 24 h.

ANION-EXCHANGE CHROMATOGRAPHY

Early endosome fractions were prepared from BHK cells as described, they were flash frozen in 500 µl aliquots and stored in liquid nitrogen until required. Approximately 200 µg of protein was used for each experiment. The membranes were first diluted in a three-fold volume of 50mM Tris-HCl, pH 7.4, and then pelleted in a TL100 centrifuge (Beckman), using a TLA 100.2 rotor at 55,000 rpm, for 25 min at 4°C. The pellet was resuspended in 50mM Tris-HCl, pH 7.4, containing protease inhibitors (10µM leupeptin, 10 µg/ml aprotinin and 1µM pepstatin) and 1% NP40 in a total volume of 100 µl and incubated for 30 min on ice. The lysates were cleared by centrifugation as above and the supernatants loaded onto a Mono-Q column (SMART™ system, Pharmacia-LKB Biotechnology Inc., column dimensions; 0.10 ml, 1.6 x 50 mm) which had been equilibrated in 20mM ethanolamine, 0.2% NP40. The unbound material was washed out with the same buffer and the bound material eluted at 100 µl/min, with a 2.3 ml linear NaCl gradient (0.0-1.0M NaCl). Fractions of 100 µl were collected. For analysis of the total protein profile with ^{35}S-labelled proteins, 10 µl of each fraction recovered from the column was solubilised in SDS sample buffer before being subjected to SDS-PAGE and fluorography. To investigate the distribution of rab5 protein in the fractions, unlabelled early endosome membranes were used and the fractions were pooled to yield 15 x 200 µl samples, the proteins were then acetone precipitated by adding 1 ml of acetone at -20°C and 5 µg of haemoglobin, and incubated overnight at -20°C. The precipitated proteins were pelleted by centrifugation in a Biofuge A tabletop centrifuge at 15,000 rpm, for 15 min at 4°C, and were then resuspended in SDS sample buffer before being subjected to SDS-PAGE and Western blotting.

PREPARATION AND PURIFICATION OF REP-1/RAB COMPLEXES

In order to prepare the REP-1/rab protein complexes, the previously described *in vitro* reaction was used (Armstrong et al., 1993; Seabra et al., 1993). Firstly, 10 μM of the appropriate histidine tagged rab protein was mixed with 1 μM of rab geranylgeranyl transferase, 3 μM of REP-1 and 60 μM of geranylgeranyl pyrophosphate (American Radiolabeled Co) in buffer A (78 mM KCl, 50 mM HEPES-KOH pH 7.0, 7 mM $MgCl_2$, 10 mM EGTA, 8.37 mM $CaCl_2$, 1mM DTT, 1 μM GDP and 0.005% Triton-X 100) in a final volume of 550 μl. Following incubation at 30°C for 30 min the reaction mixture was loaded onto a Superose 12 10/30 column (Pharmacia-LKB) which had been pre-equilibrated in buffer A and the column was eluted using buffer A at a flow rate of 0.4 ml/min. Fractions (0.4 ml) were collected and the protein content analysed by SDS-PAGE. Fractions containing the highest concentration of REP-1/rab protein complex were pooled and stored in 100 μl aliquots at -80°C.

POLYACRYLAMIDE GEL ELECTROPHORESIS

One-dimensional gel electrophoresis

SDS-PAGE was carried out under reducing conditions on 12% acrylamide gels (Laemmli, 1970), using the Bio-Rad mini-gel system. The amounts given below are enough for two 1.5 mm thick gels.

Recipe for 12% gel

H_2O	5.0 ml
30% acrylamide mix	6.0 ml
1.5M Tris, pH 8.8	3.8 ml
10% SDS	0.15 ml
10% APS	0.15 ml
TEMED	0.006 ml

Recipe for stacking gel

H_2O	2.7 ml
30% acrylamide mix	0.67 ml
1.0M Tris, pH 6.8	0.5 ml

10% SDS	0.04 ml
10% APS	0.04 ml
TEMED	0.004 ml

Samples which had been resuspended in sample buffer were heated for 3 min using a 95°C heating block, were then briefly (30 s) centrifuged at 10,000 rpm in an Eppendorf centrifuge and immediately loaded into the wells of the stacking gel using 0.5-10 µl gel loading tips (Eppendorf, Hamburg, Germany). The sample volume loaded onto these gels was 20-50 µl. Gels were run at 100 V for the first 15 min and then at 200 V for approximately 30 min, or until the dye front reached the bottom of the gel.

Two-dimensional gel electrophoresis

A combination of isoelectric focusing (IEF) and SDS-polyacrylamide gel electrophoresis was used to separate proteins in two dimensions (Bravo, 1984; Celis et al., 1990). All chemicals and reagents used were of the highest grade available. Acrylamide for the 2nd dimension gels was obtained from BDH, Germany. Acrylamide for IEF gels, ammonium persulphate, sodium dodecylsulphate (SDS) and N, N'-methylenebisacrylamide (TEMED) were from BioRad. EEO research grade agarose was from Serva, Germany. Ampholines were obtained from either Pharmacia-LKB, or Serva, Germany as indicated. Urea was from Bethesda Research Labs, USA. DTT was from Saxon Biochemicals, Hannover, Germany. Nonidet P-40 was from Fluka, Germany.

Stock solutions for the first dimension

Lysis buffer

Urea	9.8M
Ampholines, pH 7-9 (LKB)	2.0% (v/v)
NP-40	4.0% (w/v)
DTT	0.1M

Lysis buffer was stored at -20°C in 500 µl aliquots.

Overlay buffer

Urea	8.0M
Ampholines, pH 7-9 (LKB)	1.0% v/v
NP-40	5.0% (w/v)

DTT	0.01M

Stored at -20°C in 500 µl aliquots.

Equilibration buffer

Tris-HCl, pH 6.8	60.0mM
SDS	2.0% (w/v)
DTT	50.0mM
Glycerol	10.0% (v/v)

Stored at room temperature.

IEF acrylamide solution

Acrylamide	28.38% (w/v)
Bis-acrylamide	1.62% (w/v)

Filtered (0.45 µm filter) and stored at 4°C in for up to two weeks.

IEF gel recipe for 15 capillaries

Urea	12.0 g
IEF acrylamide solution	2.79 ml
dH_2O	3.67 ml
Ampholines, pH 5-7 (Serva)	0.578 ml
Ampholines, pH 5-7 (LKB)	0.578 ml
Ampholines, pH 3.5-10 (LKB)	0.420 ml
10% (v/v) NP-40	4.2 ml
TEMED	0.0147 ml
10% (w/v) APS	0.021 ml
Total volume	24.2717 ml

This volume is sufficient for 15 tube gels.

Preparation of first dimension isoelectrical focusing tube gels

To prepare IEF gels the ingredients, without TEMED and APS, were first mixed and warmed to dissolve the urea, the solution was then degassed and the TEMED and APS added immediately before pouring. Using a 10 ml syringe with a 30 mm needle, the gel mixture was then loaded into glass tubes (diameter 2.3 mm, length 25 mm, outside diameter 4 mm; Preciver Activites, France), the bottoms of which were sealed with Parafilm™,

and allowed to polymerise for at least 2 hours. The cathode (lower) chamber of the IEF apparatus was filled with 20mM H_3PO_4 and the gels were then fitted into the chamber. The anode (upper) reservoir was filled with degassed 20mM NaOH and the gels overlaid with 10 µl of overlay buffer. The gels were pre-run at 0.33 mA/gel until the voltage reached 1,200 V (1-2 hr).

Loading and running first dimension isoelectrical focusing tube gels

The sample was solubilised in an equal volume of lysis buffer (usually 25-100 µl containing a maximum of 200 µg cellular protein) at 37°C for 30 min and then loaded, under the overlay buffer, onto the tube gel. The sample was further overlaid with 10 µl overlay buffer and the gels run for 16-20 hr at 1,200 V. The gels were then extruded from the glass tubes by water pressure using a syringe, incubated for 10 min in equilibration buffer, frozen on dry ice and stored at -20°C.

Stock solutions for the second dimension (SDS-PAGE)

Acrylamide solution for resolution gels

30% (w/v) Acrylamide, 0.15% (w/v) bis-acrylamide

Acrylamide	75.0 g
Bis-acrylamide	0.375 g
dd H_2O	to 250.0 ml

Filtered (0.45 µm filter) and stored at 4°C in for up to two weeks.

Resolution gel buffer

1.5 M Tris-HCL, pH 8.8

Tris base	291.2 g
Tris-HCL	94.8 g
dd H_2O	to 2.0 l

Stored at 4°C.

Acrylamide solution for stacking gels

10% (w/v) acrylamide, 0.5% (w/v) bis-acrylamide

Acrylamide	4.0 g
Bis-acrylamide	0.2 g
dd H_2O	to 40.0 ml

Filtered (0.45 µm filter) and stored at 4°C in for up to two weeks.

Stacking gel buffer

0.24M Tris-HCL, pH 6.8

Tris base	29.0 g
dd H_2O	to 1.0 l

Stored at 4°C.

Agarose sealing solution

Resolution gel buffer	31.25 ml
10% SDS	2.50 ml (0.9%)
87% Glycerol	28.70 ml (9.0%)
Agarose	2.75 g (1.0%)
Bromophenol blue	27.50 mg (0.01%)
dd H_2O	to 250.0 ml

5x Running buffer

Tris base	152.5 g
Glycine	720.0 g
dd H_2O	to 5.0 l

When diluting to 1x, SDS was added to a final concentration of 0.1%.

Stored at room temperature.

Recipes for the second dimension (SDS-PAGE)

All amounts are sufficient for 6 gels (gel size: 22 x 23 cm) with 1.22 mm spacers.

15% resolving gel recipe

Acrylamide solution (density = 1.0305 g/ml)	219.00 g
Resolution gel buffer (density = 1.0494 g/ml)	111.50 g
dd H_2O	100.00 g
10% SDS (0.1%)	4.25 ml
TEMED	0.075 ml
10% (w/v) APS	1.88 ml

5% stacking gel recipe

Acrylamide solution	30.0 ml
Stacking gel buffer	30.0 ml
10% SDS (0.1%)	0.6 ml
TEMED	0.022 ml
10% (w/v) APS	0.45 ml

Preparing second dimension (SDS-PAGE) gels

The gel system used for second dimension was made in the EMBL mechanical workshop, the dimensions of the glass plates were 22 x 23 cm and 1.2 mm spacers were used.

To prepare the resolving gels the ingredients, without TEMED and APS, were first mixed and degassed, TEMED and APS were then added and the gels were poured to approximately 2.5 cm from the top of the lower plate, and then overlaid with water-saturated isobutanol. Once the resolving gels had polymerised, approximately 2 hr, they were rinsed extensively with water, and a piece of filter paper was then used to remove the remaining water. The stacking gel ingredients, omitting the TEMED and APS, were mixed and degassed, and the TEMED and APS were then added immediately before pouring. The stacking gels were poured on top of the resolving gels, to within 2 mm of the top of the lower gel plates, and a single-slot comb was inserted at the left hand side of the stacking gel to allow loading of molecular weight standards.

Loading and running second dimension (SDS-PAGE) gels

The agarose solution was heated to 50°C in a water bath and then DTT added to a final concentration of 50mM. The IEF gel was laid onto the top of the stacking gel and sealed onto it with 2 ml of the molten agarose sealing solution. Once the agarose had set, molecular weight standards were loaded and the buffer tanks filled with running buffer (0.25M Tris-base, 1.9M Glycine). The gels were run for at 15 mA for 12-16 hr.

WESTERN BLOTTING

For Western blotting, proteins resolved by SDS-PAGE were electrophoretically transferred to nitro-cellulose (Burnette, 1981). Transferred antigens were detected using either an alkaline phosphatase colour development reaction or a chemilumescence reaction.

Transfer of proteins to nitrocellulose

Before starting, 6 sheets of Whatman 3MM filter paper were cut to the size of the gel and soaked in transfer buffer (TB: 20mM Tris-base, 150mM glycine, 20% v/v methanol) and a piece of nitrocellulose paper (Shleicher and Schuell, Dassel, Germany; 0.2 μm pore size) was cut and soaked in distilled water. The SDS-PAGE gel, and two support pads were then immersed in the TB and the transfer sandwich was assembled in the following order; the gel was placed onto 3 sheets of filter paper, and the nitrocellulose membrane was laid carefully onto the gel, another 3 sheets of filter paper were placed over the nitrocellulose and air bubbles were removed using a 10 cm rubber roller. The sandwich was then placed between the two support pads, and clamped into the blotting tank (EMBL Workshop), which was then filled with TB. Transfer was carried out at 50 mA for 14 hours, at 4°C.

Detection of transferred proteins and blocking of nitrocellulose

After transfer the nitrocellulose membrane was immersed in Ponceau red stain for 5 min, then briefly washed in dH_2O until the stained proteins became apparent, to confirm efficient transfer of the proteins. The nitrocellulose was then immersed in blocking buffer, consisting of 5% low fat milk powder (Natuaflor, Töpfer GmbH, Dietmannsried, Germany) in PBS, and rocked for 2 hr at room temp, or overnight at 4°C.

Binding of primary and secondary antibodies

The primary antibody, diluted in blocking buffer (1:50-1:400), was then incubated with the blot for 1.5 hr at room temperature. Blots were then rinsed briefly three times with blocking buffer, then washed once for 10 min in blocking buffer, once for 10 min in blocking buffer containing 0.1% TX-100 and once more with blocking buffer for 10 min. The secondary antibody was then diluted in blocking buffer, the dilutions used were as follows; affinity purified goat anti-rabbit, or anti-mouse, IgG alkaline phosphatase conjugate (Bio-Rad) was diluted 1:500, high activity affinity purified goat anti-rabbit, or mouse, IgG peroxidase conjugate (Sigma) was diluted 1:5,000. Incubation was for 60 min at room temperature. The blots were then washed using the same protocol that was used after binding of the primary antibody.

Detection of bound antigen using the alkaline phosphatase colour development reaction

The nitrocellulose was first rinsed briefly in carbonate buffer (0.1M $NaHC0_3$, 1.0mM $MgCl_2$, pH 9.8). Then 200 μl of a 30 mg/ml p-nitro blue

tetrazolium chloride (Bio-Rad) stock and 200 µl of a 15 mg/ml 5-bromo-4-chloro-3-indoyl phosphate p-toluidine (Bio-Rad) stock were added to 20 ml of carbonate buffer and the blot was immediately immersed in this solution. The blot was then incubated at room temperature until the required intensity of signal was obtained, usually 1-3 h. In the event that the signal was too weak to be clearly observed after two hours, the blot was left in the developing solution overnight at 4°C.

Detection of bound antigen using the chemilumescence method

The chemilumescence system used in this work was the ECL system (Amersham). According to the manufacturers instructions a 1:1 mixture was made of the solutions A and B, the nitrocellulose was then totally immersed in this mixture for exactly 1 min. Excess liquid was then removed from the nitrocellulose by holding it up and touching the bottom corner to a piece of filter paper. The nitrocellulose was then inserted into a clear plastic envelope and exposed directly to Kodak AR-X film in a light-proof film cassette, routinely several exposures of 1-10 min were made.

GTP-OVERLAY

In order to detect small GTP-binding proteins, the protein sample was first subjected to electrophoresis and the proteins then renatured and transferred to nitrocellulose. The nitrocellulose was then incubated with [α-^{32}P]GTP and the bound isotope detected by auto radiography.

Renaturation and transfer to nitrocellulose

Immediately after electrophoresis gels were immersed in renaturation buffer (20% glycerol, 50mM Tris, pH 7.5) for a total of 50 min, during which the buffer was changed three times. The proteins were then transferred to nitrocellulose using the technique described for Western blotting except that the transfer buffer was substituted by a carbonate transfer buffer (10mM $NaHCO_3$, 3mM Na_2CO_3, pH 9.9).

Binding of [α-^{32}P]GTP to small GTP-binding proteins on nitrocellulose

Immediately after electrophoretic transfer the nitrocellulose was immersed in binding buffer (10mM $MgCl_2$, 2mM DTT, 0.3% Tween 20, 4µM ATP, 50mM NaH_2PO_4, pH 7.5) and soaked for 20 min, with the buffer being changed after 10 min. The nitrocellulose was then incubated for 2 h in binding buffer containing 1-2 µCi/ml [α-^{32}P]GTP. After binding the blot was washed at least 7 times for a total of 60 min in binding buffer. The nitrocellulose was

then air-dried, inserted into a clear plastic envelope and exposed to Kodak AR-X film at -80°C. The exposure times were 2-5 days for immunoprecipitated proteins, or 15-90 min for overexpressed cytosolic rab proteins.

AUTO RADIOGRAPHY AND FLUOROGRAPHY

After electrophoresis, gels with ^{35}S-labelled samples were immersed for 20 (mini-gels) or 45 (large gels) min in solution A of the Entensify Kit (Dupont, NEN research products, USA) and then for the same length of time in solution B. The gels were then laid onto a double layer of filter paper (Whatman no. 3) and a piece of Parafilm™ spread over each gel. The gels were dried under vacuum at 60°C using a gel dryer manufactured in the EMBL mechanical workshop. After drying the gels were inserted into light-proof film cassettes and exposed to Kodak AR-X film at -80°C. Exposure times were between 2 days to 3 weeks.

Gels with ^{32}P-labelled samples were dried immediately after electrophoresis and were then exposed to Kodak AR-X film at -80°C in the presence of an intensifying screen. Exposure times were between 2 h to 4 days.

Kodak AR-X film was developed using an automated developer (Eastman Kodak).

PROTEIN DETERMINATION

Protein concentration was measured a commercially-available assay (BioRad) based on the change in absorbance which occurs when Coomassie Brilliant Blue binds to protein in an acidic solution (Bradford, 1976). The protein-containing sample (2-10 µg) was diluted in PBS to a total volume of 0.8 ml and this then mixed with 0.2 ml of the BioRad reagent. The samples were then left in the dark at room temperature for 5-10 min, after which the OD595 was read. Two blanks consisting of 0.8 ml PBS mixed with 0.2 ml of reagent were always prepared and read at the same time as the experimental samples. In order to prepare a standard curve, IgG (BioRad) was diluted in the range 1-10 µg/0.8 ml and prepared in parallel with the samples.

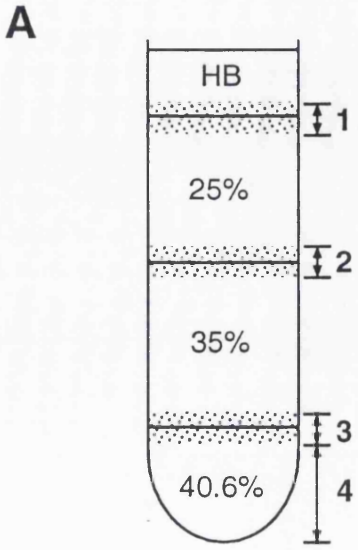
RESULTS

PREPARATION OF ENDOSOME FRACTIONS AND FUSOGENICITY OF EARLY ENDOSOMES IN VITRO

Separation of early and late endosomes on the flotation gradient

Previous studies have shown that fluid phase markers internalised into cells appear in early endosomes within 5 minutes and reach later stages of the endocytic pathway after 10 to 30 minutes (Griffiths et al., 1989; Gruenberg et al., 1989). In this way early endosomes can be labelled by incubating cells in the presence of fluid phase marker for 5 minutes at 37°C and late endosomes can be labelled by incubating the cells for five minutes in the presence of marker followed by a 30 minute incubation in the absence of marker (Gorvel et al., 1991). Early and late endosomes can then be separated from one another, and from other cellular organelles, including the plasma membrane, on the basis of their buoyant densities using a flotation gradient (Gorvel et al., 1991). After internalisation of fluid phase markers, cells were homogenised, using gentle conditions so that damage to organelles was minimised, and a postnuclear supernatant (PNS) produced. The PNS was then brought to 40.6% sucrose and loaded at the bottom of a step gradient consisting of 35% and 25% sucrose cushions overlaid with homogenisation buffer (HB; 250 mM sucrose). After centrifugation of these gradients, bands were clearly visible at each of the three interfaces (Fig. 5A).

When HRP was used as a general marker of endosomes, the amount of HRP present in each band was measured using the enzymatic assay described in the materials and methods section. Analysis of the three bands in the gradient showed that marker which had been internalised for five minutes was concentrated at the 35%/25% interface whereas marker internalised for 5 minutes followed by a 30 minute chase was concentrated at the 25%/HB interface (Fig. 5B). In both cases the HRP/protein ratio was enriched at least 10-fold compared to the PNS (Fig. 5B). The endosome fractions prepared in this way were also efficiently separated from free HRP but the enrichment was not simply due to the separation of membrane-enclosed HRP from free HRP present in the PNS, as approximately 80% of the HRP in the PNS could be recovered in the membrane pellet following high speed centrifugation (Fig. 6).



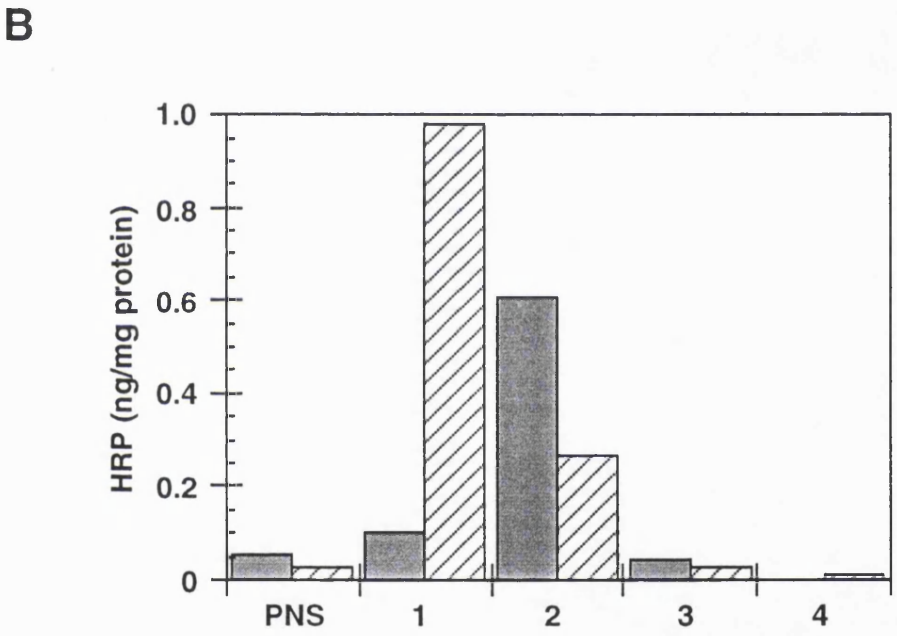


Figure 5 Separation of early and late endosomes using the flotation gradient. A) Schematic diagram of the sucrose step gradient used to separate endosomes. The sucrose concentrations of each of the steps are indicated. The fractions which were harvested for measurement of HRP activity are indicated on the right. B) HRP activity of fractions isolated from the sucrose step gradient. The numbers correspond to those indicated in Fig. 5A. Hatched bars represent the activities obtained when HRP was internalized into cells for 5 min at 37°C. Gray bars represent the activities obtained when the 5 min internalization was followed by a 30 min incubation in the absence of HRP. The data are taken from one experiment. Similar data were obtained in five separate experiments.

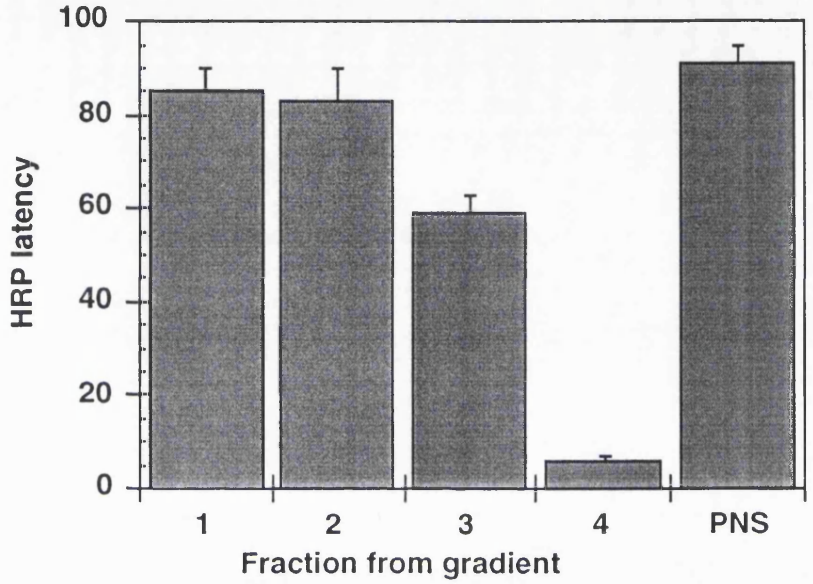


Figure 6 Latency of HRP in the postnuclear supernatant and in the flotation gradient fractions. 100 µl aliquotes were taken from the PNS or from the gradient and the latency was measured as described in the materials and methods section. For simplicity the total amount of HRP in each aliquot is expressed as 100% and HRP latency is expressed as the percentage of the total which is found in the membrane pellet after high speed centrifugation. The fraction numbers are as in Figure 5. The data are expressed as the mean ±SEM of duplicate samples from two experiments.

These results show that early endosomes were enriched at the 35%/25% interface of the flotation gradient and were separated from later stages of the endocytic pathway which were present at the 25%/HB interface.

Cell-free fusion of early endosomes

The cell-free assay of endosome fusion which has been used in this study is based on the concept that fusion of endosomes will result in mixing of their contents. Thus if two markers, which will form a stable complex upon coming into contact with one another, are internalised into separate populations of endosomes, fusion between endosomes from each population can be estimated by measuring the amount of complex formation. For these experiments the two fluid phase markers avidin and biotinylated-HRP (bHRP), were internalised for 5 minutes into separate populations of BHK cells and early endosome fractions prepared using the flotation gradient. In the cell-free assay fusion of avidin- and bHRP-labelled early endosomes, and the resultant formation of avidin-bHRP complex, could then be estimated by measuring the enzymatic activity of HRP immunoprecipitated with anti-avidin antibodies.

In all experiments the total amount of HRP which was present in the bHRP-labelled early endosomes used in each assay was less than that required to saturate the enzymatic assay. Figure 7 shows that, in the HRP detection assay which was used here, colour development was linear for amounts of HRP of up to at least 8 ng. The amount of bHRP which was precipitated from a single fusion assay did not exceed 6 ng (compare the TAv+bHRP signal in Fig. 8 with Fig. 7), and was therefore well within this linear range.

Fusion was carried out by mixing the avidin- and bHRP-labelled endosomes in the presence of cytosol and an ATP regenerating system and incubating at 37°C for 45 minutes. Excess biotinylated insulin was present at all steps in order to quench any avidin released upon endosome damage. Figure 8 shows that fusion is both ATP and cytosol dependent in agreement with previous studies (Diaz et al., 1988; Gruenberg and Howell, 1988; Woodman and Warren, 1988; Mayorga et al., 1989a; Mayorga et al., 1989b; Tuomikoski et al., 1989). In addition, it is shown that fusion of early endosomes prepared from BHK cells is efficiently supported by rat liver cytosol in an ATP-dependent manner (Fig. 8). In direct comparison with BHK cytosol, rat liver cytosol supports fusion of early endosomes to a similar extent when used at the same concentration. However, the fusion signal can be increased over that obtained with BHK cytosol by using higher

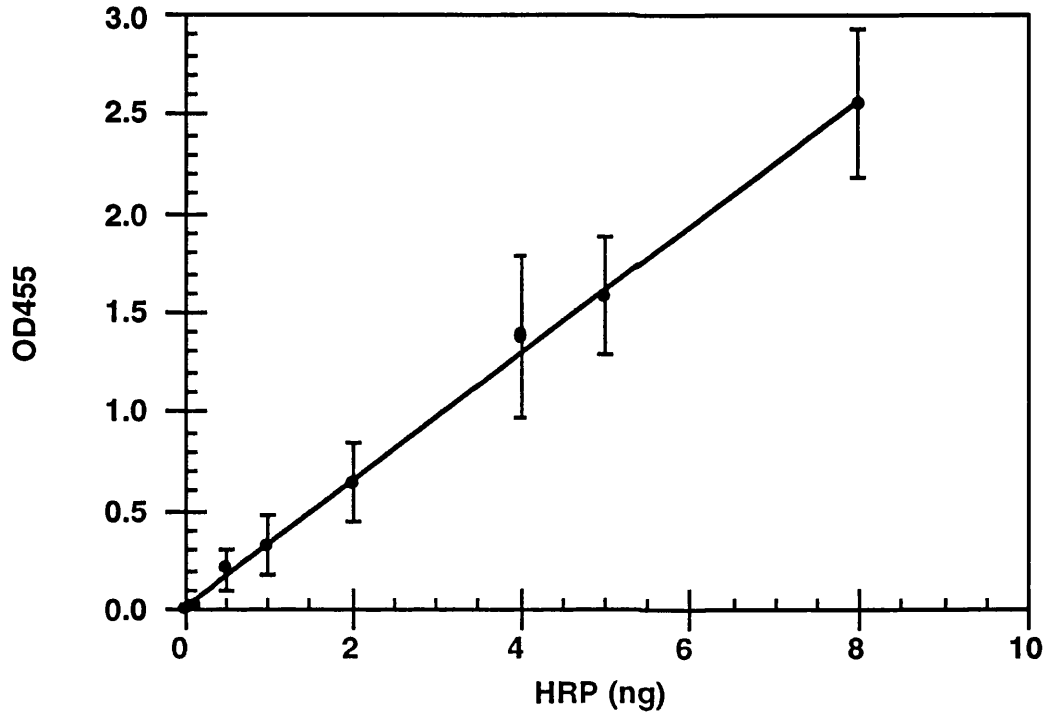


Figure 7 The HRP detection assay is linear within the range used in the fusion assay experiments. 100 µl aliquotes of HRP were prepared in PBS and mixed with 900 µl of the HRP detection reagent as described in the Materials and Methods section. The reactions were then developed in the dark, at room temperature for 3 h and the OD455 was read. Values are the average ±SEM from three separate experiments, in which duplicate samples were measured.

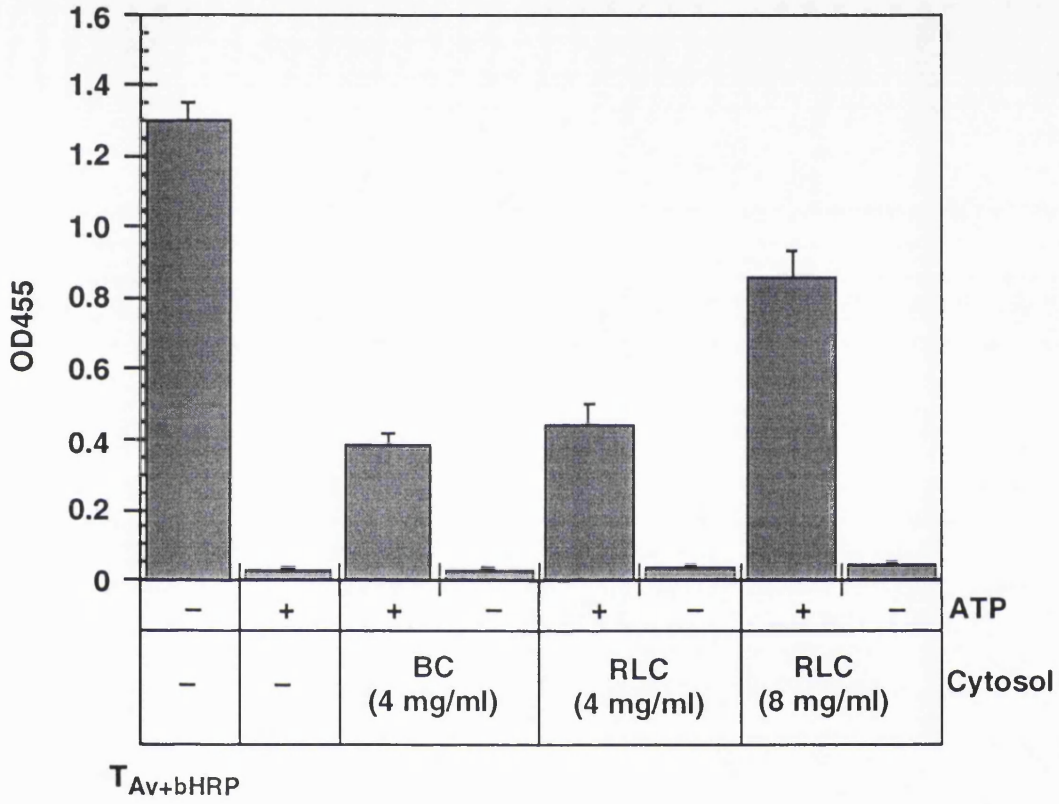


Figure 8 BHK and rat liver cytosol support ATP-dependent fusion of BHK cell early endosomes. The fusion assay was carried out for 45 min at 37°C, as described in Materials and Methods, in the presence of the indicated final concentrations of either BHK cytosol (BC) or rat liver cytosol (RLC). ATP regenerating (+) or depleting (-) systems were added as indicated. Values are the average ±SEM from two separate experiments, in which duplicate samples were measured. The signal obtained when the avidin and bHRP endosomes are solubilised in the absence of b-insulin (TAv+bHRP) is shown to illustrate the maximum signal obtainable in the system.

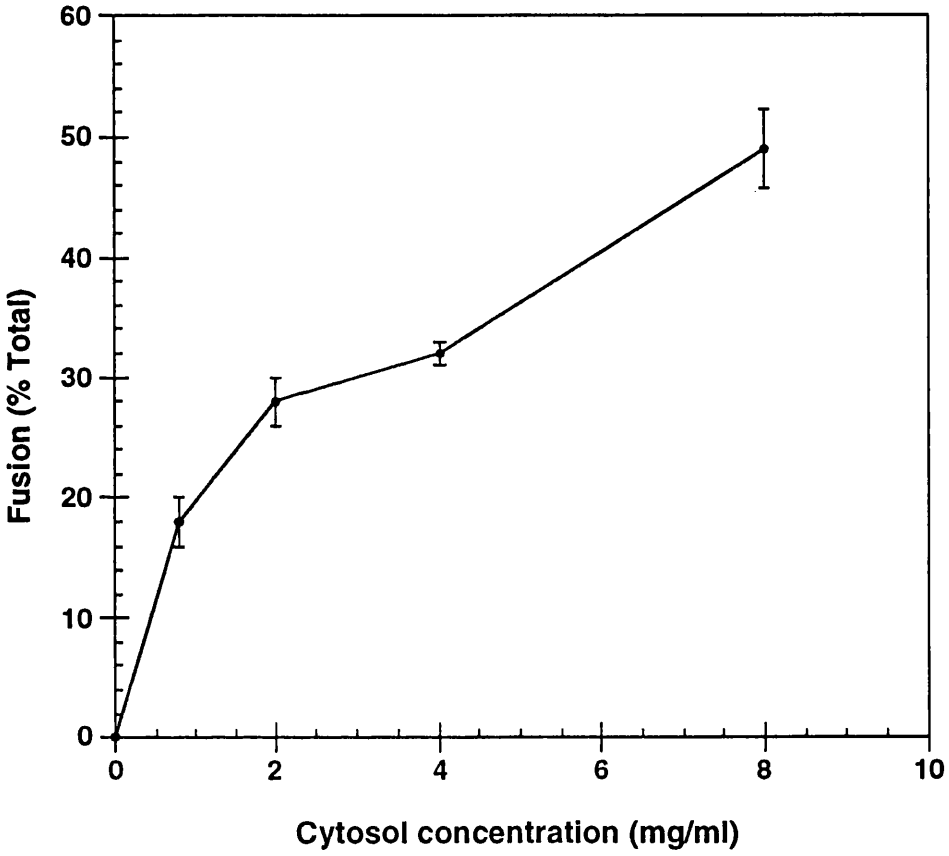


Figure 9 Fusion of early endosomes prepared from BHK cells is supported by rat liver cytosol in a concentration dependent manner. Fusion is expressed as the percentage of total signal which was obtained when the fractions were solubilised in the absence of b-insulin. The data are expressed as the mean ±SEM of duplicate samples from two separate experiments.

concentrations of rat liver cytosol (Fig. 8). Figure 9 shows the results of another experiment in which fusion efficiency is shown to be dependent on the concentration of rat liver cytosol.

As rat liver cytosol can readily be made at high protein concentrations (20-30 mg/ml) compared to BHK cytosol (8-12 mg/ml), it was routinely used in this study. A second advantage of the rat liver cytosol is that it can be produced in large volumes, typically one rat liver yielded approximately 20 ml of cytosol, which is sufficient for at least four hundred fusion assays, whereas thirty 10 cm plastic dishes of BHK cells yielded only 1 ml of cytosol. Thus by using the rat liver cytosol one source of variability between experiments was considerably reduced.

REGULATION OF IN VITRO EARLY ENDOSOME FUSION BY RAB5

Previous work in this laboratory demonstrated the involvement of the rab5 protein in *in vitro* endosome fusion (Gorvel et al., 1991). It was shown that antibodies raised against the C-terminal of rab5 inhibited fusion and also that cytosol containing overexpressed rab5 protein could stimulate fusion. In contrast, antibodies raised against the rab2 or rab7 proteins, or cytosol containing overexpressed rab2, had no effect on the efficiency of fusion. It was also shown that a mutant rab5 protein (rab5 N133I), which is defective in nucleotide binding, inhibited fusion and that deletion of the nine carboxyl-terminal amino acids, required for membrane association, rendered the protein inactive. In the following experiments the involvement of the rab5 protein in the cell-free fusion assay has been further characterised.

All three isoforms of rab5 regulate early endosome fusion

While many rab proteins exist in several isoforms the significance of this fact remains unclear and it is still not known to what extent these isoforms, which are more than 90% identical to one another (Zerial and Stenmark, 1993), can perform the same function. So far three isoforms of rab5 (rab5a, b and c) have been isolated from mammalian cells, all of which localise to early endosomes, although only the activity of the rab5a protein has been studied. Here the cell-free fusion assay has been used to compare the activity of the different wild-type isoforms and also of the N133I mutant proteins.

As described previously, cytosol containing high levels of rab5 can be prepared from cells in which the rab5 protein has been transiently

expressed using the T7 polymerase-recombinant vaccinia virus-mediated system (Chavrier et al., 1990a; Gorvel et al., 1991). Briefly, cells were infected with the T7 polymerase-recombinant vaccinia virus and then transfected with the pGEM rab5a, b or c vectors. Seven hours after transfection the cells were harvested and cytosol prepared. In order to detect the overexpressed protein in these cytosols antibodies specific for each isoform were used for Western blot analysis. All three antibodies were raised against C-terminal peptides of the rab5 isoforms and, by Western blotting, each one recognizes only the isoform against which it was raised (Cecilia Bucci and Marino Zerial, personal communication). In Figure 10A it is shown that similar levels of overexpressed protein was detectable in each case. Because of the possibility that the specific antibodies could have different affinities for the rab5 isoforms and therefore give an inaccurate estimation of the relative expression levels, the expression levels of the wild type proteins were also compared using the GTP-overlay technique. Using this method all three wild type proteins were detected in approximately equal levels (Fig. 10B) and as expected the N133I mutant proteins, which were expressed at similar levels according to the Western blot, did not bind GTP. Due to the fact that the different isoforms may have different affinities for GTP there may still be some undetectable variations in the level of expression, but the use of the two detection systems makes this less likely.

These cytosols were then used to complement the endosome fusion assay. In contrast to the previous study where the activity of rab5a was studied (Gorvel et al., 1991) and in which fusion was dependent on the rab5 overexpressing cytosol, these experiments have been carried out in the presence of rat liver cytosol. Rat liver cytosol was added at a final concentration of 4-7 mg/ml, under which conditions fusion occurs at a submaximal level, so that either stimulation or inhibition of fusion could be detected (Figures 8 and 9). The rab5 overexpressing cytosol was then added to a final concentration of ≈1 mg/ml. In this way only the overexpressed rab proteins should be supplied in significant quantities by the BHK cytosol and other cytosolic factors will be provided by the rat liver cytosol thus giving a greater level of reproducibility.

When the wild type rab5 isoforms were compared in this way all of them stimulated fusion (Fig. 10C). The rab5a isoform seemed more efficient since it caused a 100% stimulation when compared with the control, although the rab5b isoform appeared to be expressed at the highest levels. However, given the difficulty in accurately estimating the expression levels of each isoform, it is possible that these variations still reflect differences in the

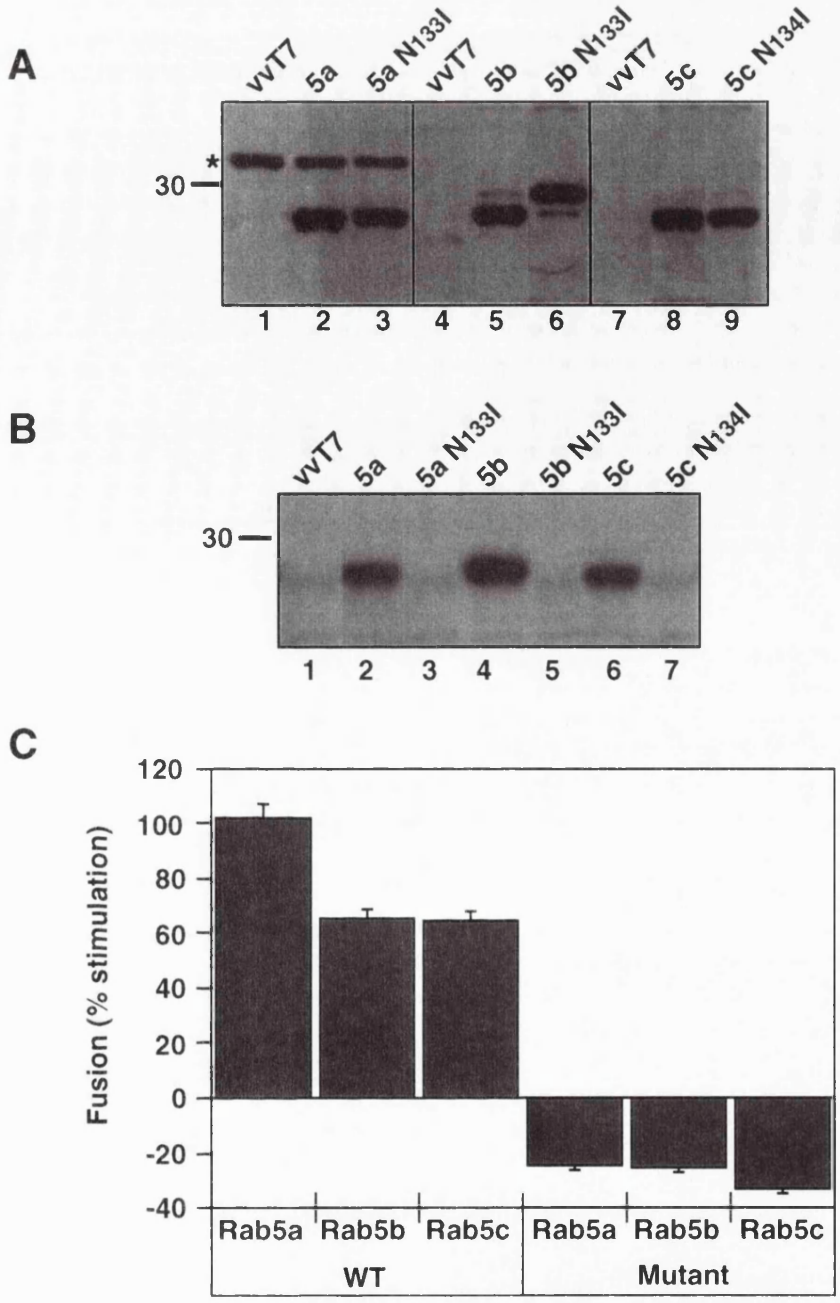


Figure 10 All three isoforms of rab5 regulate fusion of early endosomes *in vitro*. A) Western blot to show expression of all of the rab5 proteins in overexpressing cytosol. Antibodies specific for rab5a (lanes 1-3), rab5b (lanes4-6) or rab5c lanes (7-9) were used to detect the respective isoforms. The polypeptide indicated with an asterix on the left is a vaccinia virus protein recognized by the crude rab5a antiserum. B) GTP-overlay blot to show levels of expression of the rab5 wild type proteins in overexpressing cytosol. C) The fusion assay was carried out in the presence of 4 mg/ml rat liver cytosol as described. Each assay was complemented with 35 µg of BHK cytosol prepared from cells overexpressing the rab5 wild type proteins or mutant proteins as indicated. The control was complemented with cytosol prepared from vaccinia infected, mock transfected cells. Fusion is expressed as percentage of the control. The results shown are average±SEM of duplicate samples from two experiments.

amount of rab5 proteins present or in the amount of modified rab5. In contrast to the wild type proteins, the N133I mutants all inhibited fusion by approximately 25-30% compared to the control. Thus it appears that all three of the mammalian isoforms of rab5 are involved in the regulation of early endosome fusion and that nucleotide binding is required for function in each case. These results agree with *in vivo* data where it has been shown that overexpression of the wild type proteins increases the rate of endocytosis, whereas overexpression of the N133I mutant proteins causes a decrease in the rate of endocytosis (Bucci et al., 1994).

Although these experiments indicate that all three rab5 isoforms regulate fusion of early endosomes it is still unclear whether or not they have distinct functions or are redundant proteins which regulate one step in the recognition/fusion process. This issue could possibly be resolved if it were possible to perform mixing experiments to examine whether or not the effects of the different isoforms were additive. Unfortunately such experiments are technically difficult to perform using the fusion assay as described here. In particular it would be of interest to see if, after achieving the maximal amount of fusion possible with one isoform, a second isoform could then be used to further stimulate fusion. However, the volume of cytosol which can be added to the assay, without decreasing the efficiency of fusion, is limited (Woodman and Warren, 1988; Tuomikoski et al., 1989; Colombo et al., 1992b; Jean Gruenberg, personal communication). Thus such an experiment would not be possible unless the concentration of overexpressed rab5 in the BHK cytosol was much higher. Alternatively the functional rab5 isoforms would have to be produced using an alternative method so that highly concentrated protein could be added to the assay.

Nucleotide binding but not hydrolysis is required for rab5 function

One of the objectives of the present study was to investigate more thoroughly the requirement for nucleotide binding and hydrolysis by the rab5 protein in its role as a regulator of early endosome fusion. Since the involvement of other GTP binding proteins in endosome fusion (Colombo et al., 1992a; Lenhard et al., 1992) precluded the use of nonhydrolyzable analogues such as GTPγS or GDPβS, experiments were carried out using mutant rab5 proteins in which either nucleotide binding or hydrolysis were affected. As shown earlier, and also in the previous experiment, GTP-binding is required for rab5 activity, as the N133I mutant is inhibitory both *in vitro* and *in vivo* (Gorvel et al., 1991; Bucci et al., 1992). However, the requirement for GTP-hydrolysis or the nucleotide state (GTP or GDP) had not yet been investigated. For these reasons the activity of two rab5 mutants, Q79L and

S34N, based on the p21-ras mutations Q61L and S17N respectively, were tested in the cell-free assay. The Q79L mutant has been shown *in vitro to* hydrolyse GTP at a very low rate, corresponding to less than one hundredth of that of the wild type protein. In vivo studies have also indicated that the GTP-bound form of the mutant accumulates when compared to the wild type protein (Stenmark et al., 1994b). The S34N mutant in contrast, like the corresponding p21-ras and rac mutants, has a reduced affinity for GTP and is found predominantly in the GDP-bound form (Stenmark et al., 1994b).

Cytosol was prepared using the T7 polymerase-recombinant vaccinia system to obtain high levels of overexpressed cytosolic protein. As detected by Western blotting the levels of overexpressed proteins in these cytosols were 5- to 10-fold higher than that of the endogenous protein (Fig. 11A). In these Western blots two bands were often visible (Fig. 11A), this has been seen before (Chavrier et al., 1992) and the lower band appears to be unmodified protein. These cytosols were then used to complement the fusion assay, in the presence of 4mg/ml of rat liver cytosol, so that the activities of the S34N and Q79L mutant proteins could be compared to those of the wild-type and the N133I mutant rab5 proteins. These experiments were carried out in the presence of endogenous levels of GTP and GDP. As shown in Figure 11B, the S34N mutant inhibited fusion, even to a slightly greater extent than the N133I mutant. In contrast, the Q79L mutant significantly stimulated the level of fusion over the control, albeit to a lesser extent than the wild type protein. Although, there was consistently a lower amount of the Q79L mutant in cytosol preparations compared to the wild type protein or either of the other two mutants, this was apparently not due to decreased expression levels but rather a decrease in the amount of cytosolic versus membrane bound protein (Stenmark et al., 1994b). Thus it seems that the Q79L mutant is able to support fusion in a manner comparable to the wild type protein, whereas the S34N mutant is undoubtedly inhibitory.

Although these experiments indicate that, *in vitro*, GTP-binding, but not hydrolysis, is required for the activity of rab5 quantitative comparison of the activities of the wild type and mutant rab5 proteins was not possible. In order to do this the activity of the proteins at different concentrations would have to be compared. Such experiments are precluded by a number of technical restrictions. Firstly, it is at present not possible to accurately measure the concentration of overexpressed protein in the cytosol preparations which is available for insertion into the endosomal membranes. Secondly the volume of overexpressed cytosolic protein which can be added to the fusion assay is limited as fusion efficiency is decreased by diluting

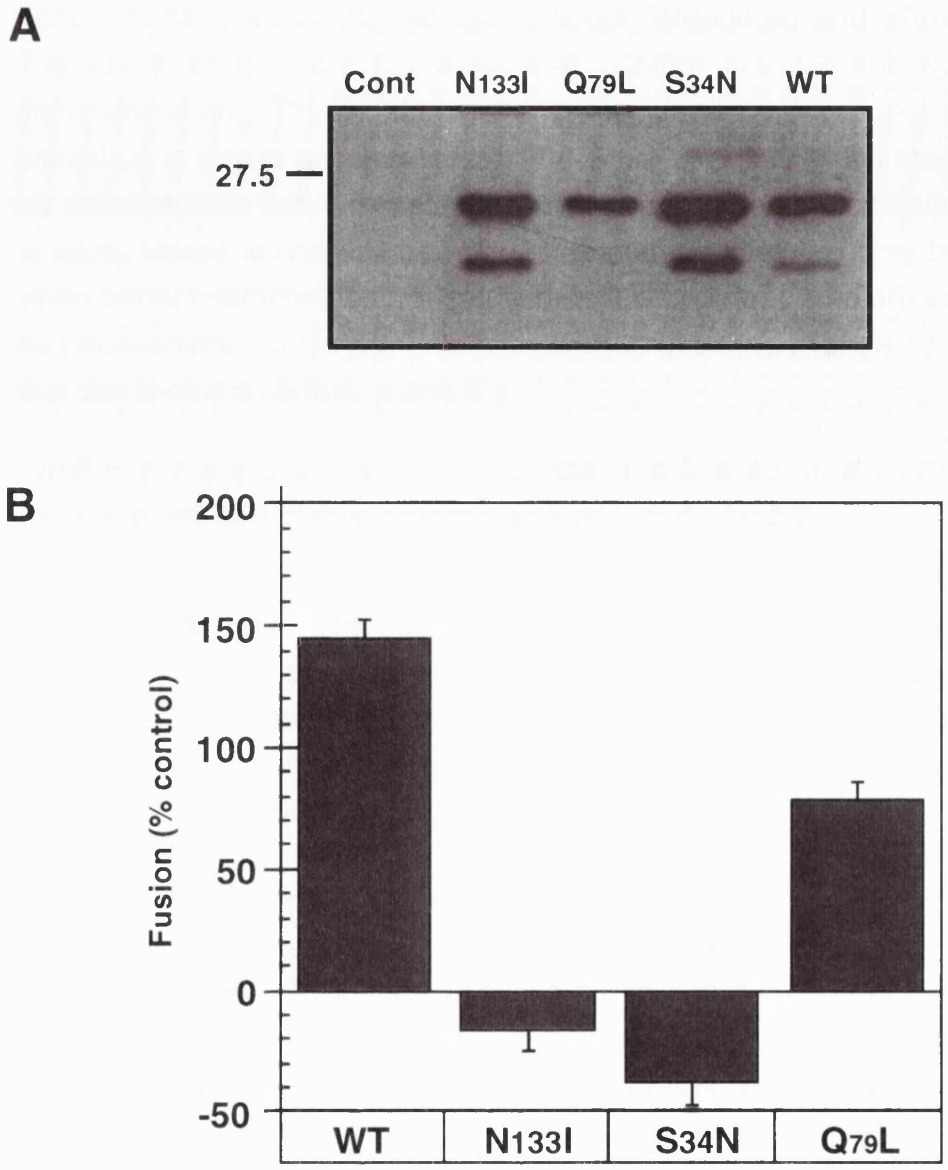


Figure 11 Activity of mutant rab5a proteins in the cell-free fusion assay. A) Western blot to show levels expression of the rab5 proteins in overexpressing BHK cytosol. As a control for endogenous levels of GTP-binding proteins the cytosol prepared from mock transfected cells was used (cont). B) Fusion assay. The experiment was carried out as described in Fig 6, using 4 mg/ml rat liver cytosol and complementing with 30 µg of BHK cytosol. The BHK cytosol was prepared from cells overexpressing wild type rab5a (WT), rab5a N133I (N133I), rab5a S34N (S34N) or rab5a Q79L (Q89L) as indicated. The control was complemented with cytosol prepared from vaccinia infected, mock transfected cells. Fusion is expressed as percentage of the control. The the data are expressed as the mean ±SEM of duplicates samples from four experiments.

either membranes or the rat liver cytosol (Woodman and Warren, 1988; Tuomikoski et al., 1989; Colombo et al., 1992b; Jean Gruenberg, personal communication). Thirdly, the fusion assay used here can not readily be scaled up in size to accommodate the number of points which are necessary for concentration curve experiments, particularily when the component which is being tested is not well defined. Thus such studies will only be possible when better characterised and purified rab5 protein fractions are available. In fact experiments which are described later in this thesis (page 138), indicate that this is now a realistic possibility.

Limited proteolysis of early endosome membranes renders them fusion incompetent and cleaves membrane associated rab5

In an attempt to identify proteins of the fusion/recognition machinery involved in early endosome fusion the assay has been used to titrate the effect of trypsin treatment of early endosome membranes. The rational for this approach being that it would allow the identification of a single membrane associated trypsin-sensitive polypeptide that is required for endosome fusion. Early endosome fractions were incubated with trypsin and the trypsin then inactivated by the addition of soybean trypsin inhibitor, the endosomes were then mixed with cytosol and the other components of the fusion assay and the assay carried out as usual. Figure 12A shows that treatment of endosome membranes with low amounts of trypsin, approximately 1:1,000 (w/w) trypsin to membrane protein, resulted in almost complete inhibition of fusion. The inhibitory effect was the same whether one or both sets of membranes in the assay were treated. Measurement of the latency of the early endosomes after trypsin treatment showed that, even at the highest concentration of trypsin which was tested, release of the fluid phase markers did not occur, thus fusion inhibition can not be explained by loss of membrane integrity (Fig. 12A).

In order to identify the trypsin sensitive factor responsible for the loss of fusion activity 2-D gel analysis was used to compare the polypeptide patterns of ^{35}S-methionine labelled early endosome membranes (Steele-Mortimer et al., 1994). These gels showed that the intensity of only one definable polypeptide spot appeared to be decreased when the membranes had been treated at the minimum concentration of trypsin required for inhibition of fusion. Surprisingly, this polypeptide had the migration characteristics of the rab5 protein. Furthermore, the intensity of another polypeptide, which was marginally more acidic and migrated slightly faster in the second dimension, was increased in the trypsin treated samples and both of these polypeptides could bind GTP on nitro-cellulose blots (Steele-

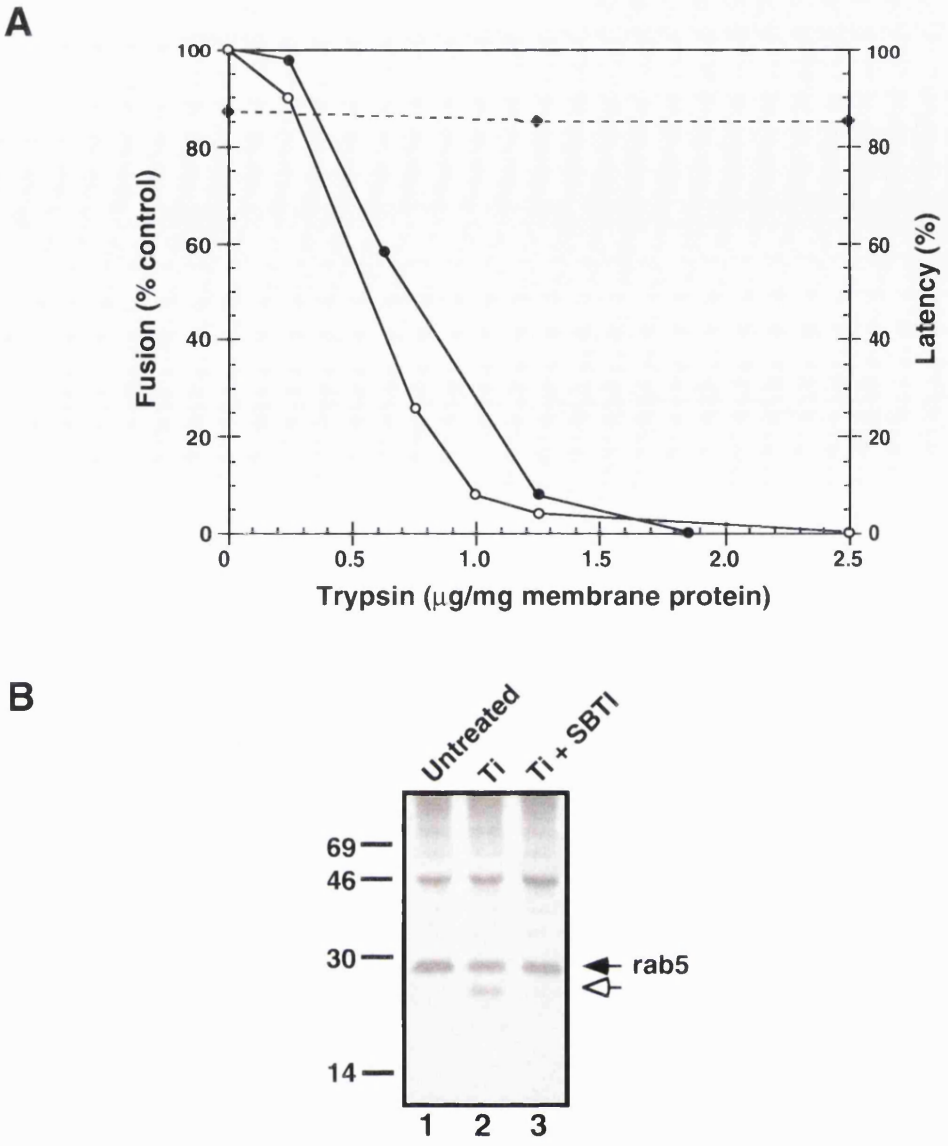


Figure 12 A) Trypsin-sensitivity of endosome fusion *in vitro*. Both endosome fractions (avidin and bHRP-labelled) were incubated separately for 30 min on ice with TPCK-treated trypsin (open circles). Alternatively only the bHRP-labelled fractions were treated with trypsin (closed circles). Trypsin activity was arrested by the addition of SBTI and the fractions were then used in the cell-free assay. Fusion is expressed as percentage of the control (membranes incubated simultaneously with trypsin and SBTI). Latency of the endosomes was measured as described and is expressed as the percentage of total HRP in the treated fractions which can be pelleted with the membranes (dashed line). B) Immunoprecipitation of rab5 from metabolically labelled early endosome membranes which had been treated with trypsin (1 μg/mg membrane protein) as described above. Rab5 was immunoprecipitated with the C-terminal specific antibody. Lane 1, untreated; Lane 2, trypsin treated; Lane 3, control (trypsin and SBTI). The rab5 protein (black arrow) and the faster migrating polypeptide which appears after trypsin treatment (open arrow) are indicated on the right. Molecular weight markers are indicated on the left.

Mortimer et al., 1994). These experiments indicated that one or more of the rab5 isoforms were the trypsin sensitive factor(s) on early endosome membranes.

In order to verify these results immunoprecipitation was carried out from ^{35}S-labelled membranes using a polyclonal antibody raised against the hypervariable C-terminal region of rab5a. Figure 12B shows the results of SDS-PAGE analysis of such immunoprecipitates. While only one protein band in the 25-30 kD region was detectable after immunoprecipitation from untreated membranes, immunoprecipitation from trypsin treated membranes resulted in the appearance of a second polypeptide with a slightly lower (~1kD) apparent molecular weight. It was apparent, both from this experiment and from experiments in which endosomes prepared from cells overexpressing the rab5a protein were trypsin treated and the rab5 protein then detected by GTP-overlay following 2-D gel electrophoresis (Steele-Mortimer et al., 1994), that only a fraction of the membrane associated rab5 was cleaved by trypsin. These experiments indicated that either the cleavage of a certain proportion of the rab5 protein is sufficient to inhibit fusion, or that inhibition is mediated by a different trypsin sensitive factor. Indeed membranes which had been trypsin treated remained fusion incompetent even in the presence of cytosol containing overexpressed rab5, suggesting that a factor other than rab5 has been inactivated by trypsin cleavage (Jean-Pierre Gorvel and Jean Gruenberg, personal communication). However, these results do not exclude the possibility that rab5 is the sole trypsin sensitive factor responsible for the observed inhibition of fusion. This would still be possible if the cleaved protein, which as shown remains associated with the membrane (Fig. 12B, becomes irreversibly associated with a component (X) of the fusion machinary, thus blocking the fusion process directly and preventing the association of uncleaved rab5 protein into the machinary. Likewise it is possible that only the fraction of membrane associated rab5 which is associated with component X is sensitive to trypsin under the conditions used here, perhaps because of a conformation change induced by the interaction.

It was not possible to determine whether the rab5b and rab5c isoforms are also cleaved under these conditions, as all three proteins have extremely similar migration characteristics by 2-D gel electrophoresis. Also the rab5b and rab5c specific antibodies were not available at this time.

Trypsin treatment of cytosol containing overexpressed rab5 inactivates the rab5 protein

It could still be argued that the cleavage of rab5 by trypsin, which evidently only removes a small fragment and does not affect all of the membrane associated rab5 protein, may not inactivate it and does not mediate fusion inhibition, although 2-D gel analysis did not reveal any other trypsin sensitive polypeptides (Steele-Mortimer et al., 1994). Therefore, in order to demonstrate the inactivation of rab5 protein the fusion assay was used to assay the effect of trypsinisation on the cytosolic protein. For these experiments the early endosome membranes were prepared as usual and fusion was carried out in the presence of rat liver cytosol as described in the materials and methods section. The assay was then complemented with BHK cytosol prepared from cells overexpressing wild type rab5 protein, which was treated under the same conditions and with the same concentrations of trypsin as used for the above experiments (1:1,000 [w/w] trypsin to cytosol protein). Figure 13 shows that the mock treated rab5 cytosol, to which trypsin and trypsin inhibitor were added simultaneously, stimulated fusion as expected. The trypsin treated rab5 cytosol, however, did not significantly affect the level of fusion when compared to that obtained in the presence of control cytosol. That this effect is not the result of inhibition by a trypsin sensitive factor other than the rab5 protein is demonstrated by the result that trypsin treatment of the control BHK cytosol had no effect on the amount of fusion compared to untreated cytosol (Fig. 13).

The N-terminal domain of rab5 is required for its activity

Given that the cleaved rab5 protein migrates only slightly faster than the uncleaved form as measured by SDS-PAGE, it seemed likely that only a few amino acids were being removed from the protein. Several observations indicated that the cleavage was occurring at the N-terminal of the protein. Firstly, the cleaved protein remained tightly associated with endosomal membranes. As the rab5 protein is associated to membranes via geranylgeranyl modification of one or both of the cysteine residues in the CCSN sequence at the extreme C-terminal of the protein, removal of this sequence would be expected to result in dissociation from the membrane. Alternatively, the protein could remain on membranes after cleavage of the C-terminal domain, if it associated tightly with another component of the endosomal membrane. However, no evidence has been found for such an association. Secondly, the cleaved product was still recognised by an antibody which was raised against a sequence which starts 14 amino acids upstream of the CCSN motif. Thirdly, treatment of purified recombinant *E. coli*

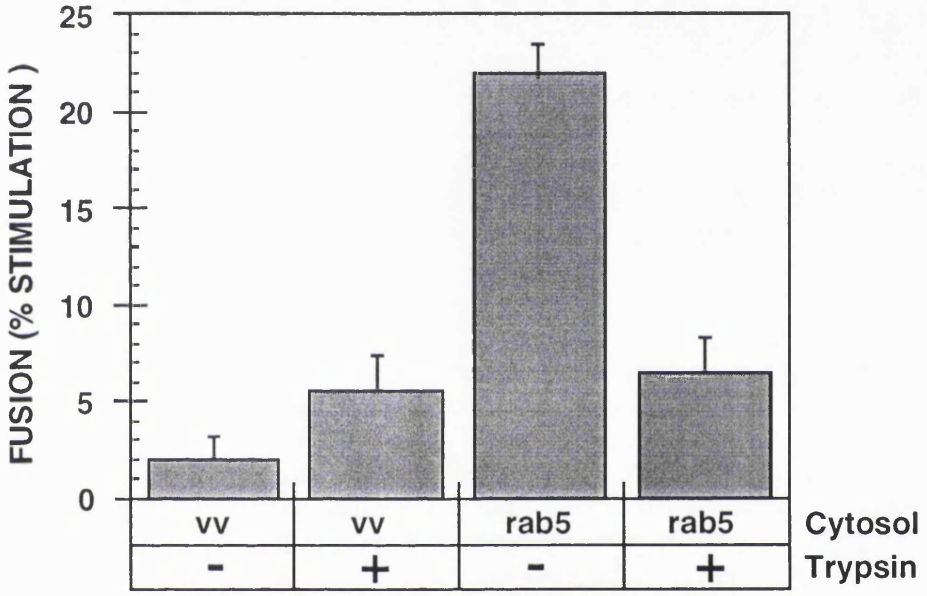


Figure 13 The cytosolic form of rab5 is inactivated by trypsin. BHK cytosol prepared from cells overexpressing wild type rab5 (rab5) or mock transfected cells infected with the T7 polymerase-recombinant vaccinia virus (vv) was treated with trypsin (1μg/mg of cytosol) which was then inactivated by the addition of SBTI (+), and added to the assay as described. Alternatively, cytosols were incubated with trypsin and SBTI simultaneously (-). The results are expressed as the percentage of fusion obtained in the absence of BHK cytosol. Values are the averages ±SEM of duplicate samples from twoe experiments.

expressed rab5 protein with trypsin produced a cleaved polypeptide which was approximately 1 kD smaller than the uncleaved protein, as measured by SDS-PAGE, and N-terminal amino acid analysis of the smaller polypeptide showed that four amino acids had been removed (Steele-Mortimer et al., 1994). Finally, a peptide consisting of the 17 extreme N-terminal amino acids of rab5 inhibited in vitro early endosome fusion whereas a similar peptide corresponding to the N-terminal of the rab3a protein had no significant effect at the same concentrations (Steele-Mortimer et al., 1994).

PROTEINS INTERACTING WITH RAB5

Detection of phosphorylated proteins associated with rab5

As endosome fusion is known to be regulated by phosphorylation and also by the rab5 protein, immunoprecipitation experiments were carried out to investigate whether it was possible to demonstrate interactions between rab5 and phosphorylated proteins. Similar experiments have been used to show that p21-ras is associated with phosphoproteins *in vivo* (Kaplan and Bar-Sagi, 1991).

BHK cells were labelled with ^{32}P-orthophosphate and a post-nuclear supernatant prepared, immunoprecipitation was then performed using three different rab5-specific antisera. While no phosphoprotein with a molecular weight corresponding to that of rab5 was detected, a phosphoprotein with an apparent molecular mass of approximately 55 kD was co-precipitated using antisera raised against either the C-terminal or N-terminal peptides of rab5. That this co-precipitation is specific is demonstrated by the fact that it is competed by the peptides against which the antibodies were raised (Fig. 14). In this figure the N-terminal peptide competition does not look as efficient as that of the C-terminal peptide, however, this is due to the fact that in this experiment more of the 55 kD protein is immunoprecipitated by the N-terminal antibody than by the C-terminal one. In fact the efficiency of the competition was within the same range. Another phosphoprotein with an apparent molecular mass of approximately 20 kD was co-precipitated with one of the C-terminal antisera (101), but not with the other (112) or with the N-terminal antiserum (218). Although both of the C-terminal antisera were raised against the same peptide they appear to recognise different epitopes on the rab5 protein. When these antisera were used to detect purified recombinant *E. coli* expressed rab5 protein which had been transferred to nitro-cellulose both of them recognised the major form of the rab5 protein (Fig. 15A). Antisera 112 also

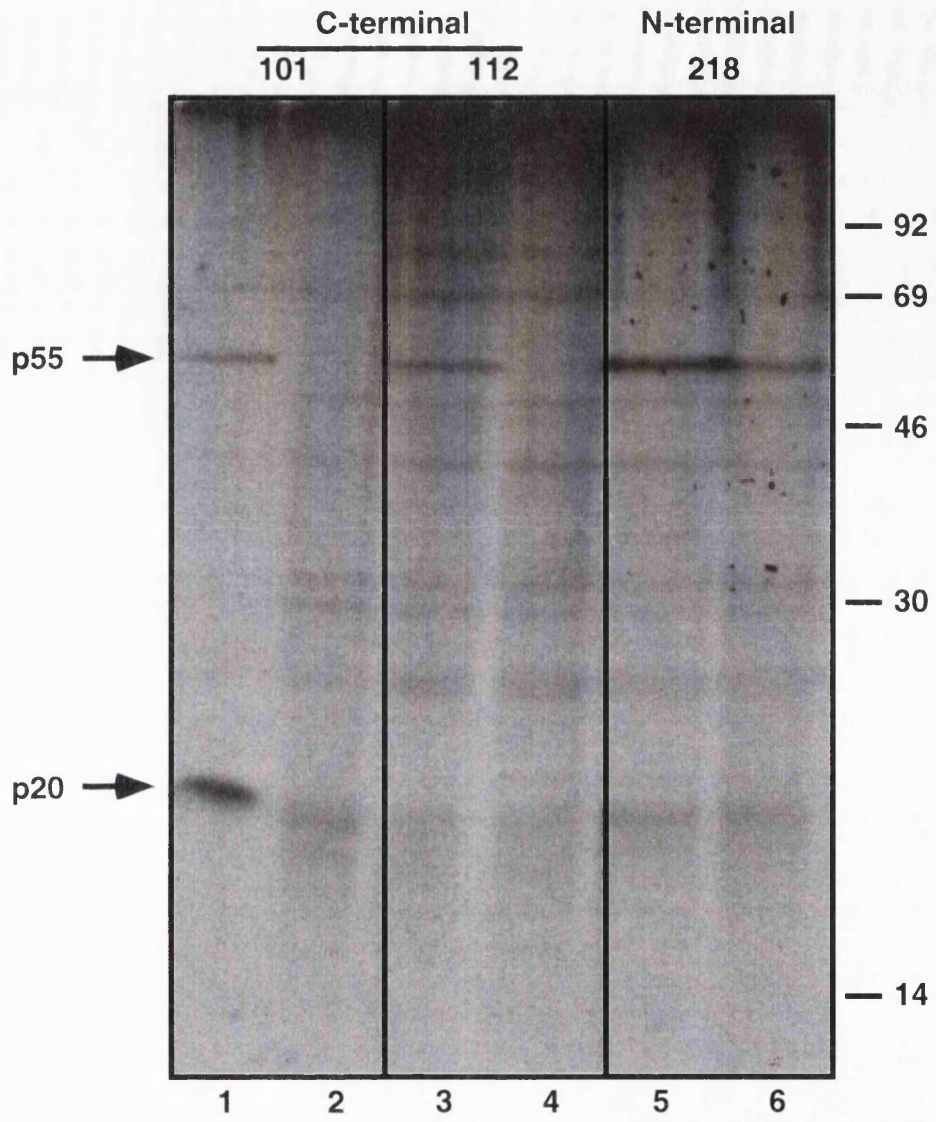


Figure 14 Two phosphoproteins specifically co-immunoprecipitate with rab5a. ^{32}P-labelling of BHK cells was carried out as described. Postnuclear supernatants were then prepared and immunoprecipitation carried out using rab5 antibodies which were raised against the C-terminal (lanes1-4) or N-terminal (lanes 5 and 6) peptides. For controls the antibody was pre-incubated with the peptide against which it was raised (lanes 2,4,6). The positions of the 55 kD and 20 kD phosphoproteins are indicated on the left. Molecular weight markers are indicated on the right.

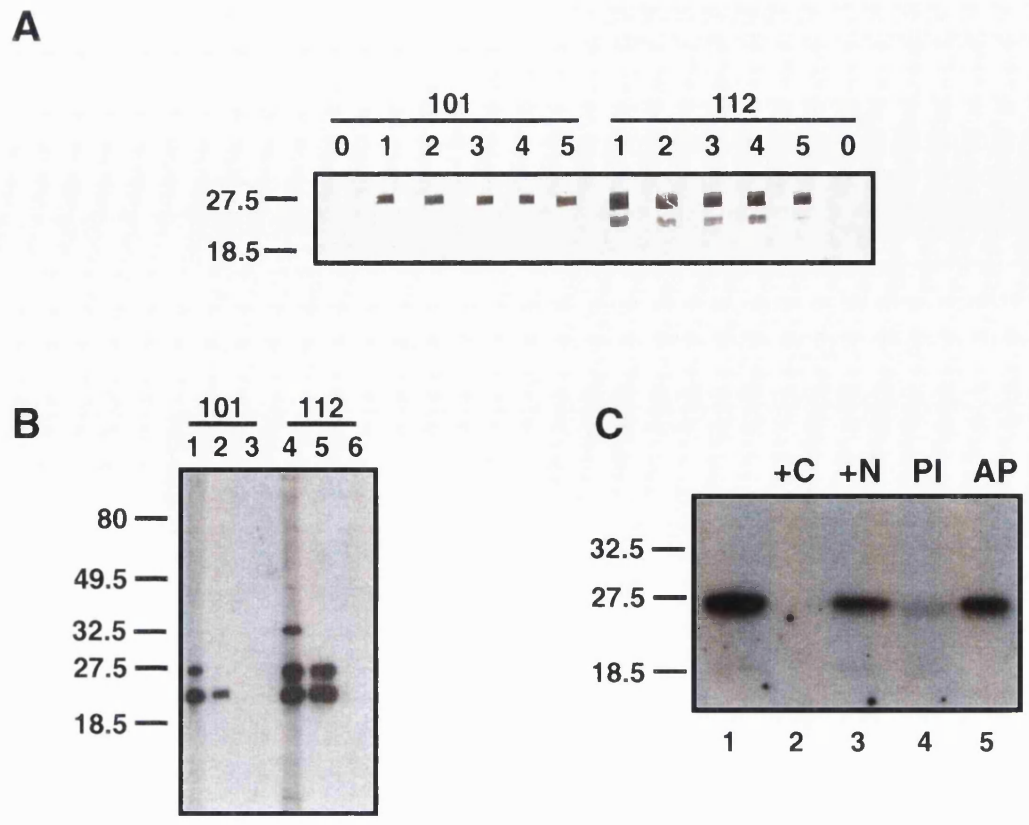


Figure 15 The two antibodies raised against the rab5 C-terminal peptide recognize different epitopes. A) Western blot of purified *E. coli* expressed rab5a protein. The purified rab5 protein was transfered onto nitrocellulose after separation by SDS-PAGE and identical nitrocellulose strips were then incubated with 1:500 dilution of the indicated C-terminal antisera (101 and 112). For each antisera the first 5 bleeds were tested (lanes 1-5) and also the pre-immune serum (lane 0). The bound antibody was detected using the alkaline phosphatase method. B) Western blot of overexpressed wt rab5a protein in cytosol fractions. Cytosol overexpressing rab5 was prepared as described and subjected to SDS-PAGE and the proteins were then transfered to nitrocellulose. The blots were incubated with 1:500 dilution of the indicated anitisera (101 and 112). Lanes 1 and 4, crude antisera; lanes 2 and 5, affinity purified antibody; lanes 3 and 6; affinity purifed antibody preincubated with the C-terminal peptide against which it was raised. The bound antibody was detected using the chemilumescence method. C) Immunoprecipitation of a GTP-binding protein by the rab5 C-terminal antisera 101. Lane1, crude antiserum. Lane 2, crude antiserum preincubated with the C-terminal peptide. Lane 3, crude antiserum preincubated with the N-terminal peptide. Lane 4, pre-immune seum. Lane 5, affinity purified antibodies (101). Immunoprecipitation was performed from BHK cell membranes. The immunoprecipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose. GTP-binding proteins were detected using the GTP-overlay technique.

recognised a lower molecular weight cleaved form of rab5 which was not recognised by the antisera 101, thus the epitope recognised by the latter is not present in the cleaved rab5 protein. In addition, Western blot analysis of cytosol prepared from BHK cells overexpressing rab5 protein demonstrates a difference in the activity of the antisera (Fig. 15B). The crude antisera both recognised two bands in the 20-27 kD region, an observation which had been made before (Chavrier et al., 1990a). When the affinity purified antibodies were used the 112 antibodies still recognised both forms of rab5 but the 101 antibodies recognised only one form of the protein. As the 101 C-terminal antibody was used extensively for the following experiments we further characterised its specificity by immunoprecipitation followed by GTP-overlay detection of small GTP-binding proteins. As shown in Figure 15C only one band was detected when immunoprecipitation was carried out from cells expressing endogenous levels of rab5, this protein was not recognised by the pre-immune sera and was efficiently competed by the C-terminal peptide, against which the antibody, but inefficiently competed by the N-terminal peptide.

The 55 kD phosphoprotein is associated with other rab proteins and is immunoprecipitated by antibodies raised against rab-GDI

In order to investigate whether the phosphoproteins which co-precipitated with rab5 were also associated with other members of the rab family of GTPases, immunoprecipitation experiments were carried out using antibodies raised against C-terminal peptides of rab2 and rab4. Both of these antibodies have previously been characterised (Chavrier et al., 1990a; van der Sluijs et al., 1991). Analysis of the immunoprecipitates showed that the 55 kD phosphoprotein was co-precipitated in both cases (Fig. 16A). The specificity of co-precipitation with rab2 and rab4 could not be assayed by peptide competition as the peptides against which these antibodies were raised were not available. The 20 kD phosphoprotein did not co-precipitate with rab2 or rab4 and in fact it was only ever found in immunoprecipitates using the C-terminal rab5 antisera 101.

By virtue of its mobility in 1-D gels and the finding that it interacts with multiple rab proteins, it seemed possible that the 55 kD phosphoprotein could be the regulatory protein rab-GDI. To test this hypothesis, immunoprecipitations were carried out using two different GDI-specific antisera. The 55 kD phosphoprotein was not immunoprecipitated by an antisera raised against recombinant *E. coli* expressed GDI but was efficiently immunoprecipitated by an antibody raised against a C-terminal peptide of GDI (Fig. 16B). It was not possible to demonstrate by Western blotting that

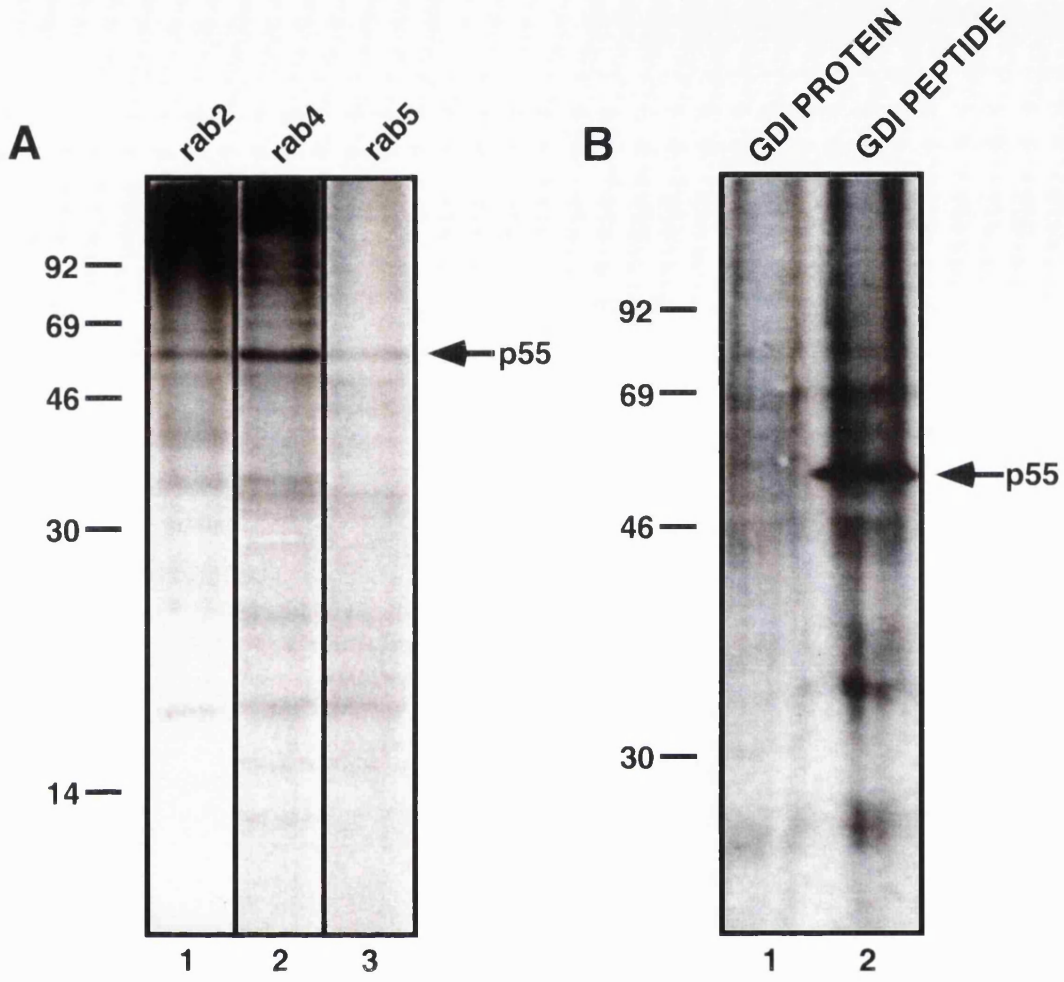


Figure 16 The 55 kD phosphoprotein also co-precipitates with the rab2 and rab4 proteins and can be immunoprecipitated with an antibody raised against rab-GDI. A) Immunoprecipitations were carried out from PNS prepared from ^{32}P-labelled BHK cells using antibodies raised against C-terminal peptides of rab2 (lane 1), rab4 (lane 2) or rab5 (lane 3). B) Immunoprecipitations were carried out as above, using antibodies raised against either recombinant GDI (lane 1) or a GDI C-terminal peptide (lane 2). Molecular weight markers are indicated on the left. The position of the 55 kD phosphoprotein is indicated on the right.

rab5, or other rab proteins, were co-precipitating with phosphorylated GDI in this experiment, as the rab proteins migrate in the same position as IgG light chains which are present in large quantities following immunoprecipitation.

Comparison of p55 and GDI by high resolution 2-D gel separation of ^{32}P-labelled proteins

The above results strongly indicated that the 55 kD phosphoprotein and GDI were the same protein, in order to confirm this identity high resolution 2-D gel electrophoresis was then used to compare the physical characteristics of these proteins. Cytosol was prepared from ^{32}P-labelled cells and immunoprecipitations carried out using the antibodies raised against the C-terminal peptides of either rab5 or GDI. Autoradiographs of the two immunoprecipitates were then compared after separation of the polypeptides by isoelectric focusing and SDS-PAGE (Fig. 17). Significantly, the major phosphoproteins detected in either case exhibited identical isoelectric points and molecular weights (Fig. 17A and B, insets). Both proteins migrated somewhat slower in the second dimension than was observed in 1D SDS-PAGE, demonstrating an apparent molecular weight of approximately 69 kD rather than 55 kD.

Comparison of p55 and GDI by high resolution 2-D gel separation of ^{35}S-labelled proteins

When the GDI-specific antibodies were used to immunoprecipitate GDI from cytosol prepared from ^{35}S-methionine/cysteine metabolically-labelled cells one major polypeptide spot, with an apparent molecular weight of 69 kD, was detected on 2-D gels (Fig. 18A). Upon longer exposure of this gel, however, several polypeptide spots could be detected which had a very similar distribution pattern to that of the 55 kD phosphoprotein (Fig. 18B). In comparison, the anti-rab5 and anti-rab4 antibodies almost exclusively co-immunoprecipitated the more acidic forms of GDI (Fig. 18C-F). When these ^{35}S-labelled immunoprecipitates were treated with alkaline phosphatase immediately prior to electrophoresis, these more acidic forms were shifted back to the position of unphosphorylated GDI confirming that they are the phosphorylated GDI (Fig. 19). These experiments establish that rab5, and other rab proteins, interact predominantly with the phosphorylated, rather than non-phosphorylated, forms of GDI in the cytosol (see Fig. 26 for a summary of the distribution of the different forms of GDI and rab proteins in cytosol and membranes).

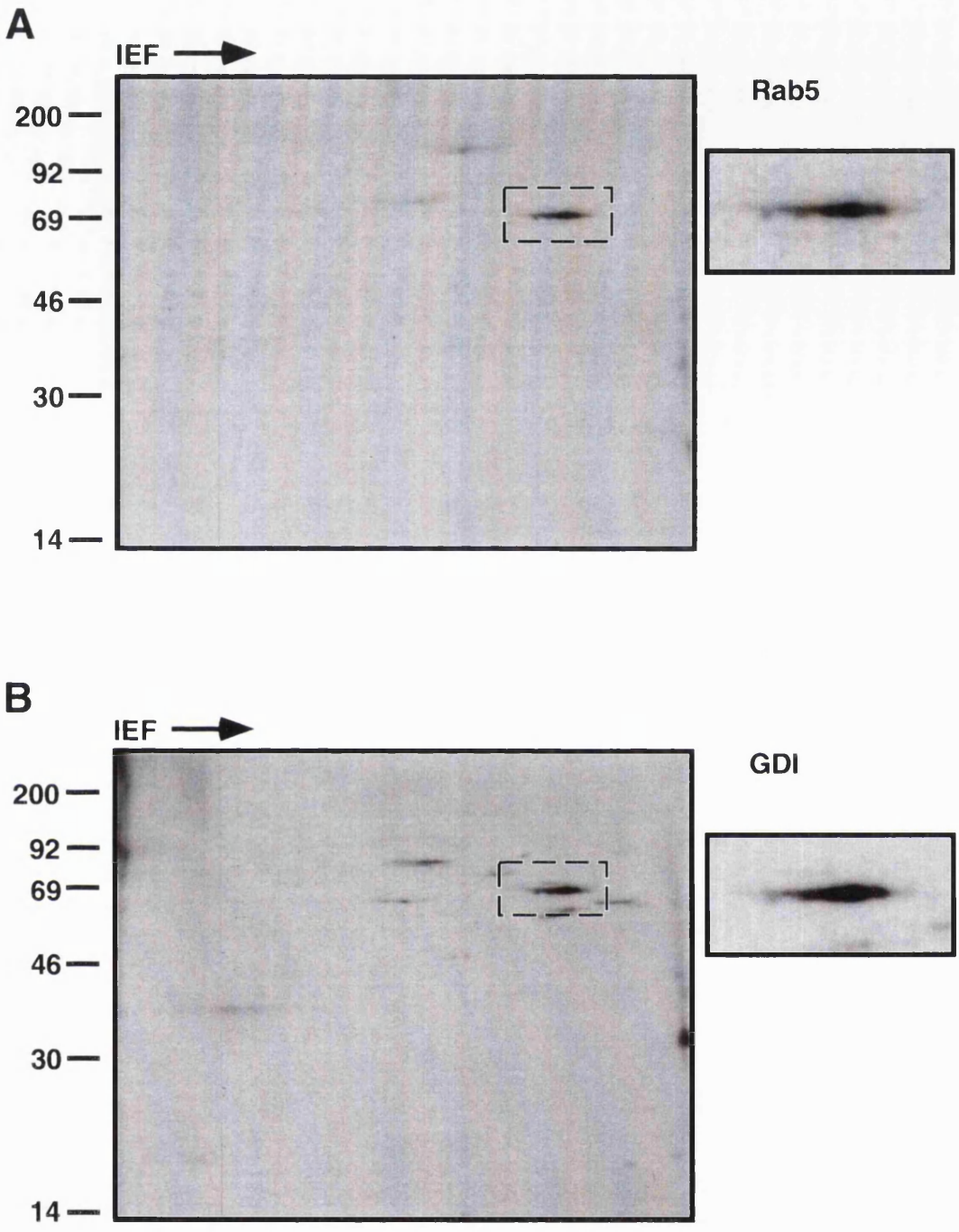


Figure 17 High resolution 2-D gel analysis of immunoprecipitates from cytosol of ^{32}P-labelled BHK cells. A) Immunoprecipitation with antibodies against rab5. B) Immunoprecipitation with antibodies against GDI. The boxed areas, which contain the 55 kD phosphoprotein (it migrates with a higher apparent molecular weight on 2-D gels), are shown enlarged in the panels on the right. Molecular weight markers are indicated on the left. The exposure time for the autoradiograph in B was twice that of A.

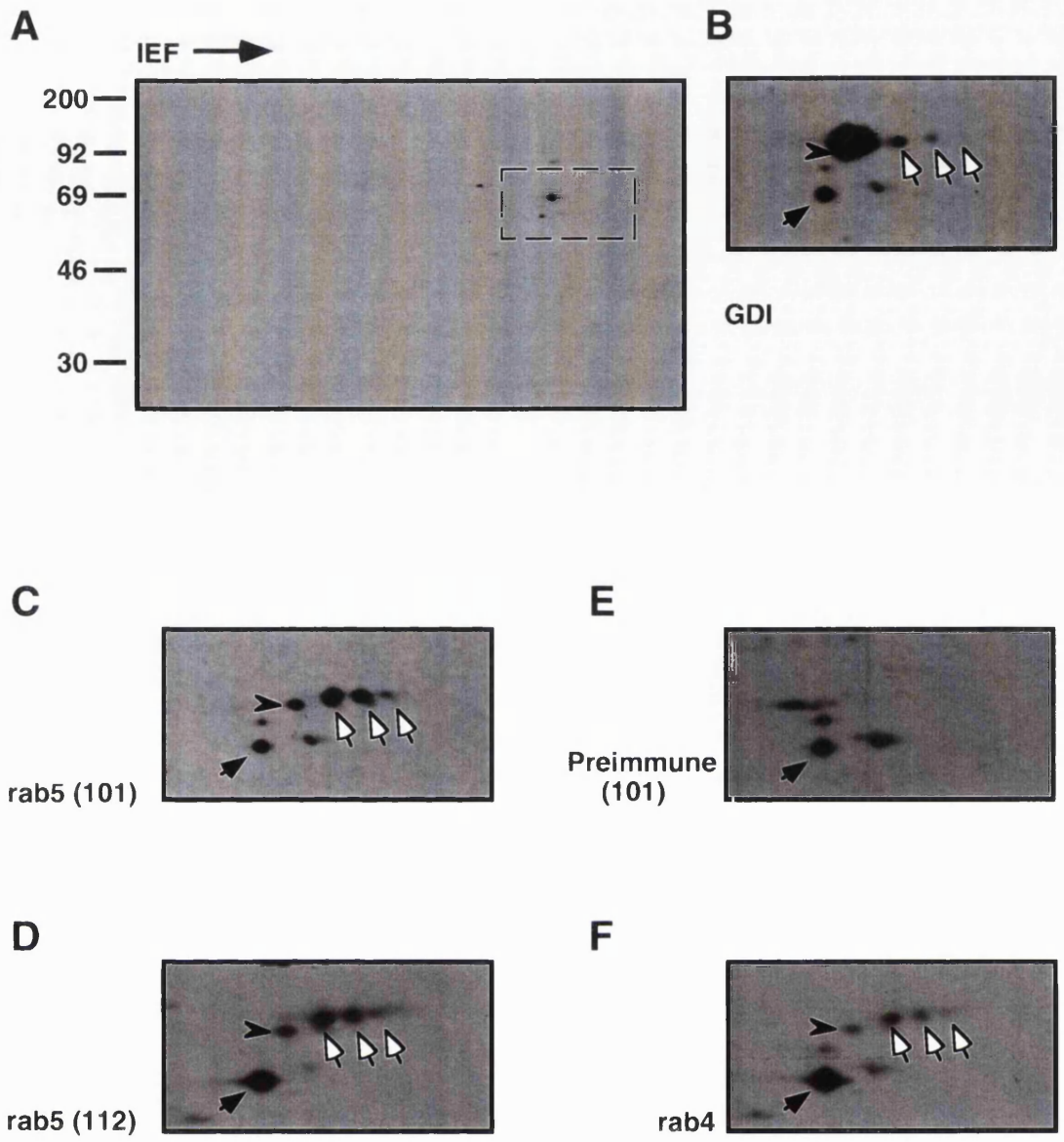


Figure 18 High resolution 2-D gel analysis of immunoprecipitates from cytosol of ^{35}S-labelled BHK cells. A) Immunoprecipitation with antibodies against GDI. The boxed area, which contains the major polypeptide recognized by the anti-GDI antibodies, is shown enlarged after longer exposure in panel B. The major polypeptide is indicated with an arrow head. Molecular weight markers are indicated on the left. C-F) Comparison of the major polypeptide pattern after immunoprecipitation with rab5 C-terminal antisera (C and D), pre-immune sera (E) or rab4 affinity purified antisera (F). Only the equivalents of the boxed area indicated in (A) are shown. The three polypeptide spots which migrate with the same characteristics as the phosphoproteins shown in Fig 13, are indicated by the open arrows. A background spot which was seen in all immunoprecipitates (black arrow) indicates the relative exposure times as it was present in all immunoprecipitates at the same level.

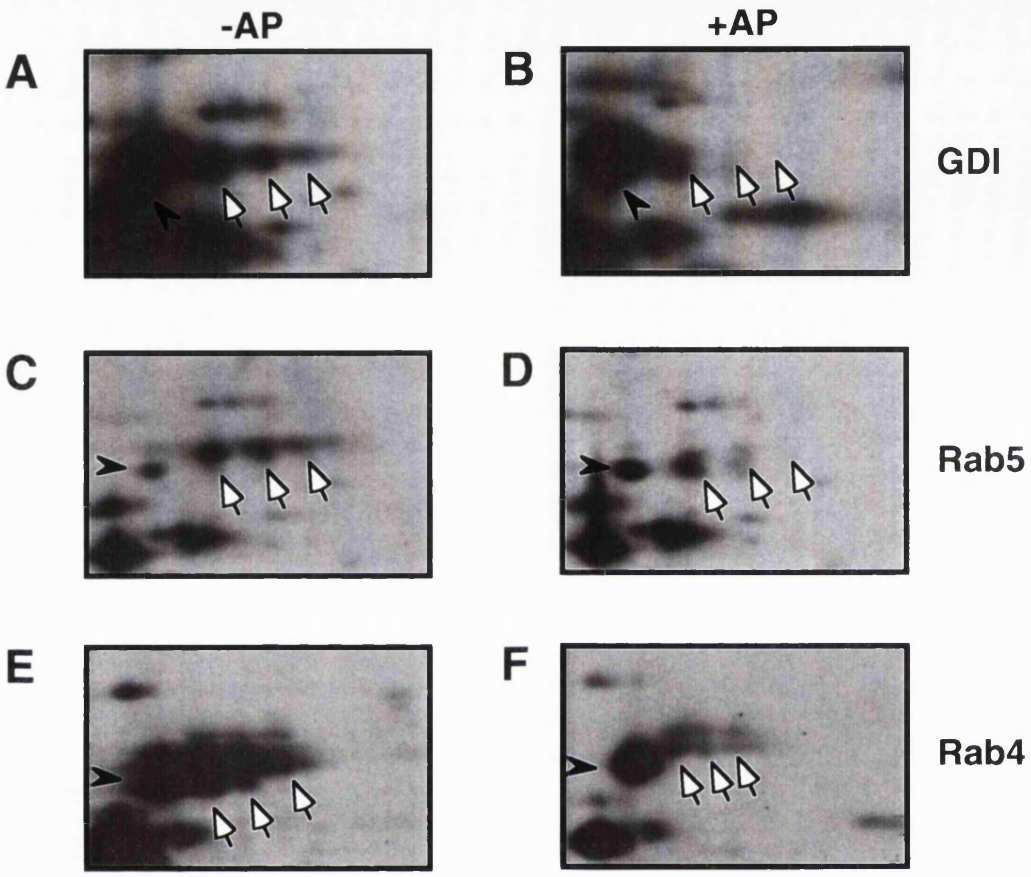


Figure 19 Comparison of the pattern of immunoprecipitated ^{35}S-labelled proteins before and after alkaline phosphatase treatment of the immunoprecipitates. For clarity, only the area of the 2-D gels in which GDI migrates is shown. Immunoprecipitations of cytosolic proteins were carried out using anti-GDI antiserum (A and B), anti-rab5 antiserum (C and D) or affinity purified anti-rab4 anitbodies (E and F). A, C, E; no phosphatase treatment (-AP). B, D, F; phosphatase treated (+AP). The three polypeptide spots which migrate with the same characteristics as the phosphoproteins shown in Fig 17, are indicated by the open arrows. The position of the major isoform recognized by the anti-GDI antibody is indicated by the black arrowheads.

Comparison of ^{35}S- and ^{32}P-labelled proteins in total cytosol

One surprising result of the above experiments was the observation that phosphorylated forms of GDI were more effectively precipitated by anti-rab protein antibodies than by the anti-GDI antibody (Fig. 17 and 18). Presumably the C-terminal epitope recognised by the anti-peptide GDI antibody may be masked by phosphorylation or by association with rab proteins. That the phosphorylated forms of GDI predominate in the cytosol is shown by two dimensional gel analysis of complete cytosol. When the patterns of phosphoproteins and ^{35}S-labelled proteins in total cytosol are compared it is possible to identify the characteristic pattern of phosphorylated GDI (Fig. 20A and B), conversely the major spot which was seen in the GDI immunoprecipitates was not visible in the ^{35}S-labelled cytosol (Fig. 20B). Thus it appears that the unphosphorylated isoform of GDI, which is immunoprecipitated very efficiently by the GDI antibody, is present in the cytosol at much lower concentrations than the phosphorylated form.

Phosphorylated GDI is not found on membranes

When immunoprecipitation experiments were carried out using membrane fractions in place of cytosol, no phosphorylated GDI was detected, in contrast the 20 kD phosphoprotein was precipitated from both cytosol and membranes (Fig. 21). In this experiment the phosphorylated proteins precipitated using the rab5 and GDI specific antibodies were compared to those precipitated using an antibody raised against p62. The p62 protein has been shown to be associated with the rab6 protein both in cytosol and on membranes and is phosphorylated in the cytosol (Jones et al., 1993). Although the sequence of p62 is not known it has several of the characteristics of GDI, however, in this experiment immunoprecipitation with the p62-specific antibody did not result in the same phosphoprotein pattern as that seen using the GDI antibody (Fig. 21A). This result is in agreement with Western blotting experiments and some sequencing data which indicate that GDI and p62 are not identical (K. Howell and S. Jones, personal communication).

Having demonstrated that phosphorylated GDI was not present on membranes, experiments were then carried out to investigate whether unphosphorylated GDI is present on membranes, and if so whether this GDI is also associated with rab proteins. Immunoprecipitation of ^{35}S-labelled GDI indicates that, while most of the GDI is cytosolic, a small fraction of the total protein is associated with membranes (Fig. 21B and also summary in Fig. 26). In addition, GTP-overlay analysis of immunoprecipitates from unlabelled

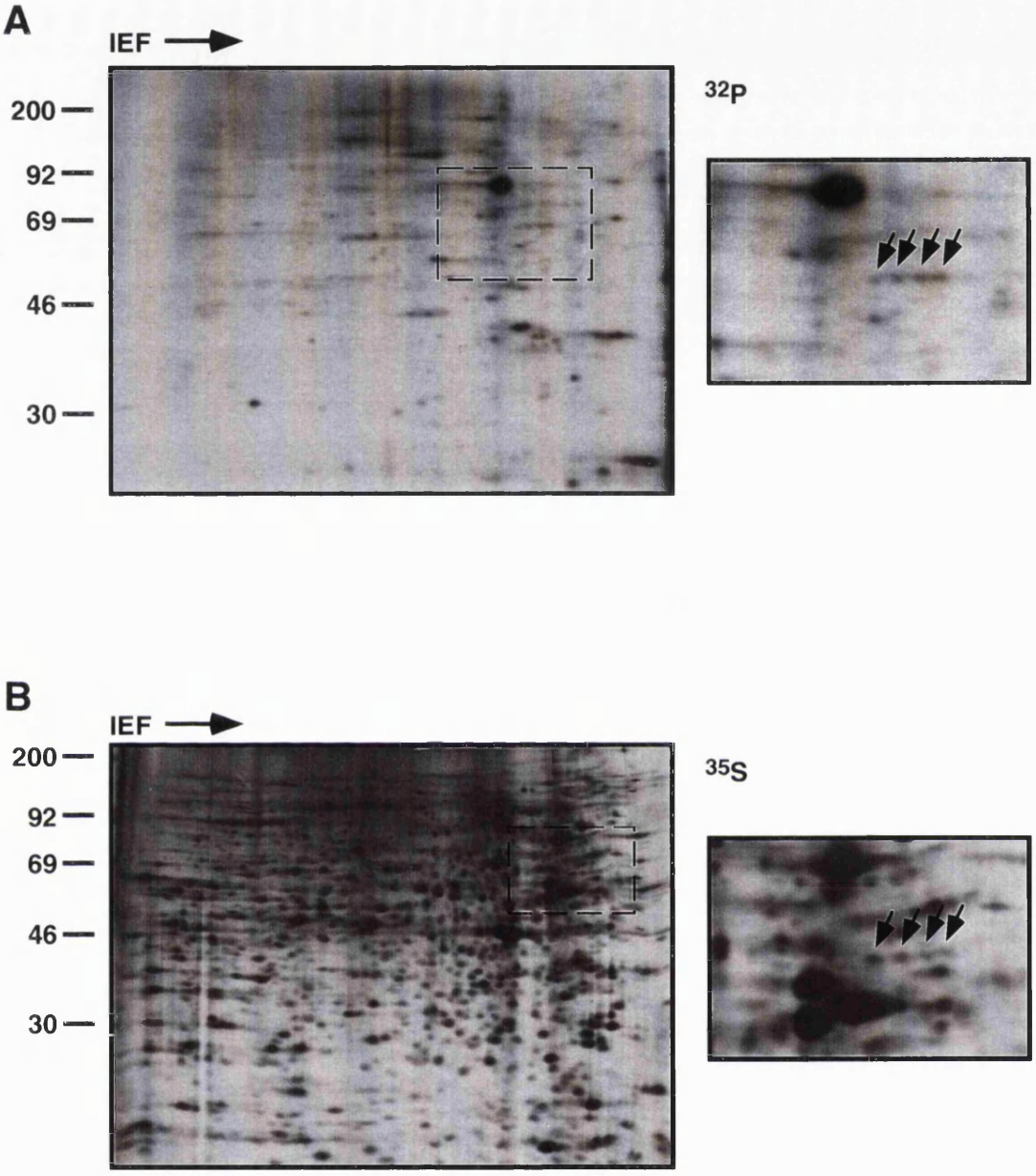


Figure 20 Comparison of total cytosolic proteins from ^{32}P- and ^{35}S-labelled cells. Cells were metabolically labelled and cytosol prepared as described. A) ^{32}P-labelled proteins. B) ^{35}S-labelled proteins. The boxed areas are shown enlarged on the left, with the characteristic polypeptide spots which migrate in the same position as those seen in the immunoprecipitation experiments indicated (arrows). Molecular weight markers are shown on the left.

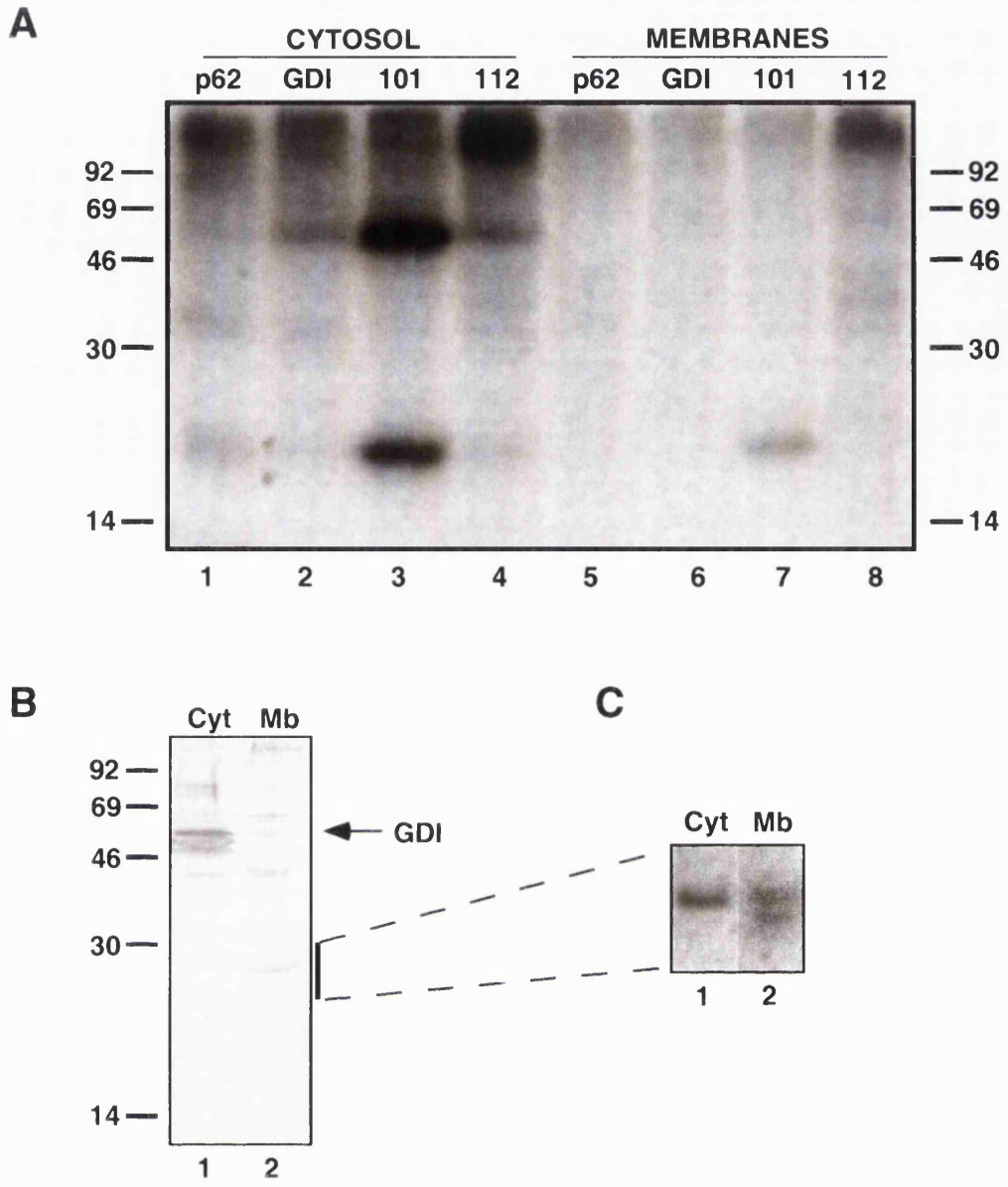


Figure 21 A) Comparison of ^{32}P-labelled proteins immunoprecipitated from cytosol and membrane fractions. Immunoprecipitations were carried out using cytosol (lanes 1-4) or membrane fractions (lanes 5-8) as described. Lanes 1 and 5, anti-p62 antibodies; lanes 2 and 6, anti-GDI peptide antiserum; lanes 3 and 7, anti-rab5 antiserum (101); lanes 4 and 8, anti-rab5 antiserum (112). Molecular weight markers are indicated on the right. The positions of the p55 and p20 phosphoproteins are indicated. B) and C) GDI can be immunoprecipitated from both cytosol and membrane fractions and is associated with small-GTP binding proteins in both fractions. B) Immunoprecipitation of ^{35}S-labelled proteins with the anti-GDI peptide antiserum from cytosol (lane 1) and membranes (lane 2). The position of GDI is indicated. Molecular weight markers are shown on the left. C) Immunoprecipitation was carried out from unlabelled cytosol (lane 1) and membranes (lane 2) using the anti-GDI peptide antiserum. The small GTP-binding proteins were detected using the GTP-overlay method as described. Only the portion of the blot corresponding to the area indicated in (B) is shown.

cells showed that both the cytosolic and membrane associated GDI fractions are associated with small GTP-binding proteins (Fig. 21C).

Anion exchange chromatography of early endosome proteins indicates that membrane-associated rab5 is partially associated to GDI

To investigate the possible association of rab5 with GDI on membranes, early endosome fractions were prepared and the membranes then solubilized in 1% NP40 and applied to a Mono Q anion exchange column. The proteins were eluted with a linear 0.0-1.0 M NaCl gradient and collected in 100 µl aliquots (Fig. 22A). Comparison of the elution profile with SDS-PAGE analysis of the ^{35}S-labelled proteins shows that two major protein peaks were obtained, the first peak which consisted of unbound material was collected in fractions 5 and 6 and the second peak was collected in fractions 16 and 17 (Fig. 22A and B). Western blot analysis of unlabelled proteins prepared in the same way showed that while approximately 50% of the rab5 protein was present in the unbound material (fractions 5 and 6) the remainder of the protein was detected in fractions 17 to 20 (Fig. 22C). In addition, Western blot analysis using the GDI-specific antibody showed that, while GDI was not detected in the unbound material (fractions 4 and 5), it was detected in fractions 17 and 18 and thus partially co-fractionates with rab5 (Fig. 22C). These results together with the immunoprecipitation studies (Fig. 21), strongly suggest that non-phosphorylated GDI is associated with rab proteins on membranes.

Treatment of cells with okadaic acid or TPA does not affect the amount of phosphorylated GDI which co-immunoprecipitates with rab5

The affects of the okadaic acid and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) on the interaction of phosphoproteins with rab5 were tested in co-immunoprecipitation experiments. Okadaic acid is a potent inhibitor of protein phosphatase-1 (PP1) and -2a (PP2A) and is known to inhibit fusion of endocytic vesicles *in vitro* (Woodman et al., 1992) and transport along the exocytic pathway both *in vivo* and *in vitro* (Lucocq et al., 1991; Davidson et al., 1992). In contrast, the phorbol ester TPA is an activator of protein kinase C, and has been shown to stimulate fluid phase and/or non-clathrin-mediated endocytosis in some cell types (Haigler et al., 1979b; Sandvig and van Deurs, 1990). Intact BHK cells were pre-treated with either 2 µM okadaic acid or 2 µM TPA and homogenisation and immunoprecipitation carried out as described. When the rab5 antibody (101) was used, no difference was detected in the phosphoproteins co-precipitating with rab5 (Fig. 23). Nor were the levels of the 55 or 20 kD phosphoproteins affected.

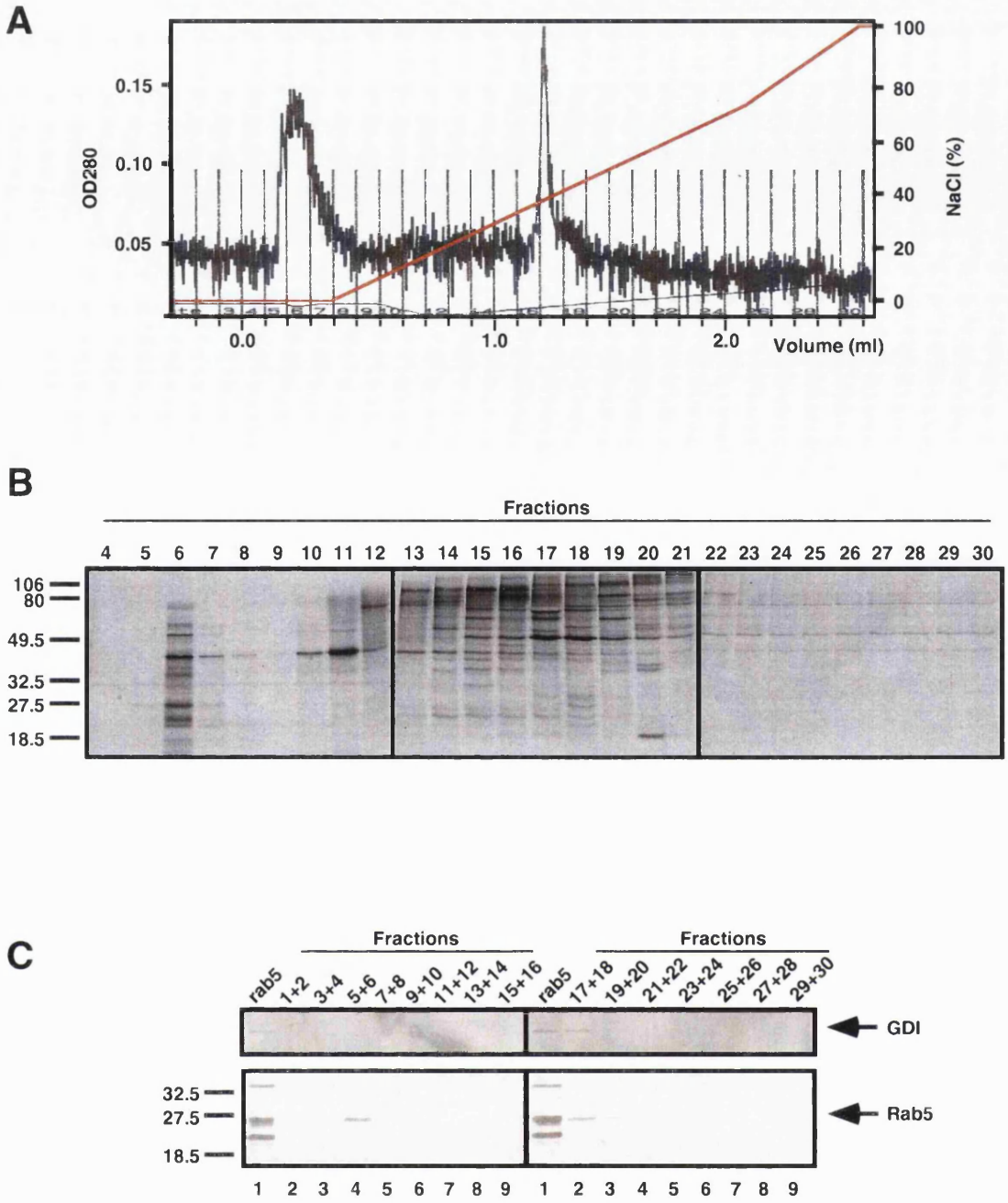


Figure 22 Rab5 and GDI are associated with one another on early endosome membranes. A) Elution profile of early endosome proteins from the Mono Q column. The NaCl gradient is shown in red. B) SDS-PAGE analysis of ^{35}S-labelled protein fractions. C) Western blot detection of GDI (top panel) and rab5 (lower panel) in fractions (lanes 2-9). Cytosol containing overexpressed rab5 was included on each gel as a control (lane 1). The GDI blot was carried out using the anti-GDI peptide antiserum and bound antibodies were revealed using the chemilumescence method. The rab5 blot was carried out using the rab5 anti-peptide antiserum (112) and was revealed by the alkaline phosphatase method. Molecular weight markers are indicated on the left.

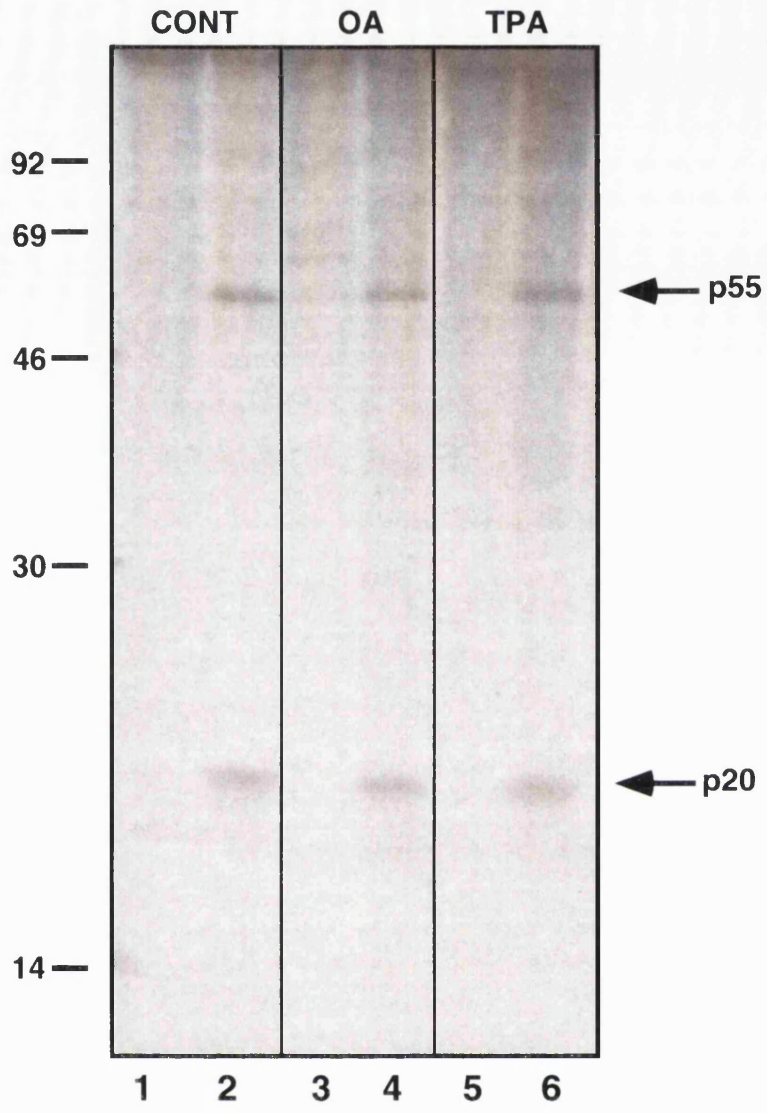


Figure 23 Pretreatment of cells with okadaic acid or TPA does not affect the amounts of the 55 kD and 20 kD phosphoproteins which co-precipitate with rab5. Cells were metabolically labelled with ^{32}P-orthophosphate and then incubated with 2 μM okadaic acid for 15 min (lanes 3 and 4) or 2 μM TPA for 10 min (lanes 5 and 6). Control cells received no treatment (lanes 1 and 2). A cytosol fraction was then immediately prepared and immunoprecipitation carred out using the rab5 anti-peptide antiserum 101 (lanes 2, 4 and 6) or the pre-immune serum (lanes 1, 3, and 5). Molecular weight markers are indicated on the left.

Stimulation of early endosome fusion by the REP-1/rab5 complex in vitro

Geranylgeranyl groups are transferred onto C-terminal cysteines of rab proteins by a rab-specific geranylgeranyltransferase (Rab-GG transferase). The Rab-GG transferase, previously called component B, consists of two tightly associated α and β subunits which make up the catalytic unit of the enzyme (Seabra et al., 1993). The activity of the transferase is, however, dependent on the presence of an additional component, the 95 kD REP-1 protein, previously called component A (Andres et al., 1993). Recent in vitro studies have shown that REP-1 acts as a rab escort protein which binds unprenylated rab proteins and presents them to the catalytic GG transferase. It has been suggested that it then transfers the modified rab protein (GG-rab) to the specific membrane, presumably via interaction with another protein factor, perhaps Rab-GDI (Andres et al., 1993). In order to test this model purified REP-1/GG-rab5 complex was prepared and used to complement the cell-free endosome fusion assay. Equimolar REP-1/GG-rab5 complex was obtained by first prenylating purified *E. coli* expressed rab5 *in vitro*, in the absence of detergent, using recombinant REP-1 and GG transferase in the presence of geranylgeranyl pyrophosphate as described (Alexandrov et al., 1994; Cremers et al., 1994). As the REP-1, but not GG transferase, remains associated with the prenylated rab protein under these conditions the REP-1/GG-rab5 complex could then be purified by gel filtration chromatography (Andres et al., 1993).

When added to the endosome fusion assay in the presence of limiting concentrations of rat liver cytosol the REP-1/GG-rab5 complex stimulated fusion in a concentration dependent manner (Fig. 24A). As shown in Figure 24B the purified REP-1/GG-rab5 complex did not contain detectable amounts of GG transferase and thus stimulation is due to the REP-1/GG-rab5 complex alone.

REP-1/GG-rab5 stimulation of early endosome fusion in vitro is specific

In order to demonstrate the specificity of stimulation of early endosome fusion by the REP-1/GG-rab5 complex its activity was compared to that of REP-1/GG-rab1 complex and to either purified recombinant rab5 or REP-1. In agreement with the previous experiment, addition of the REP-1/rab5 complex stimulated early endosome fusion by over 100% compared to the control, in contrast the REP-1/rab1 complex had no significant effect (Fig. 25). In addition, neither purified REP-1 nor unmodified rab5 had any effect on the amount of fusion (Fig. 25). This result indicates that in the presence of

cytosol and membranes, but absence of detergent, the modified rab protein is inserted into the early endosome membrane. These results are in agreement with studies showing that incubation of perforated cells with the REP-1/rab5 complex results in the formation of large endosomal structures similar to those seen when rab5 is overexpressed *in vivo* (Bucci et al., 1992; Alexandrov et al., 1994). The specificity of the membrane insertion of rab5 into the correct membrane has not been characterised here, however, in the perforated cell system the rab5 protein does appear to be inserted specifically into the membranes of a transferrin receptor-positive compartment (Alexandrov et al., 1994).

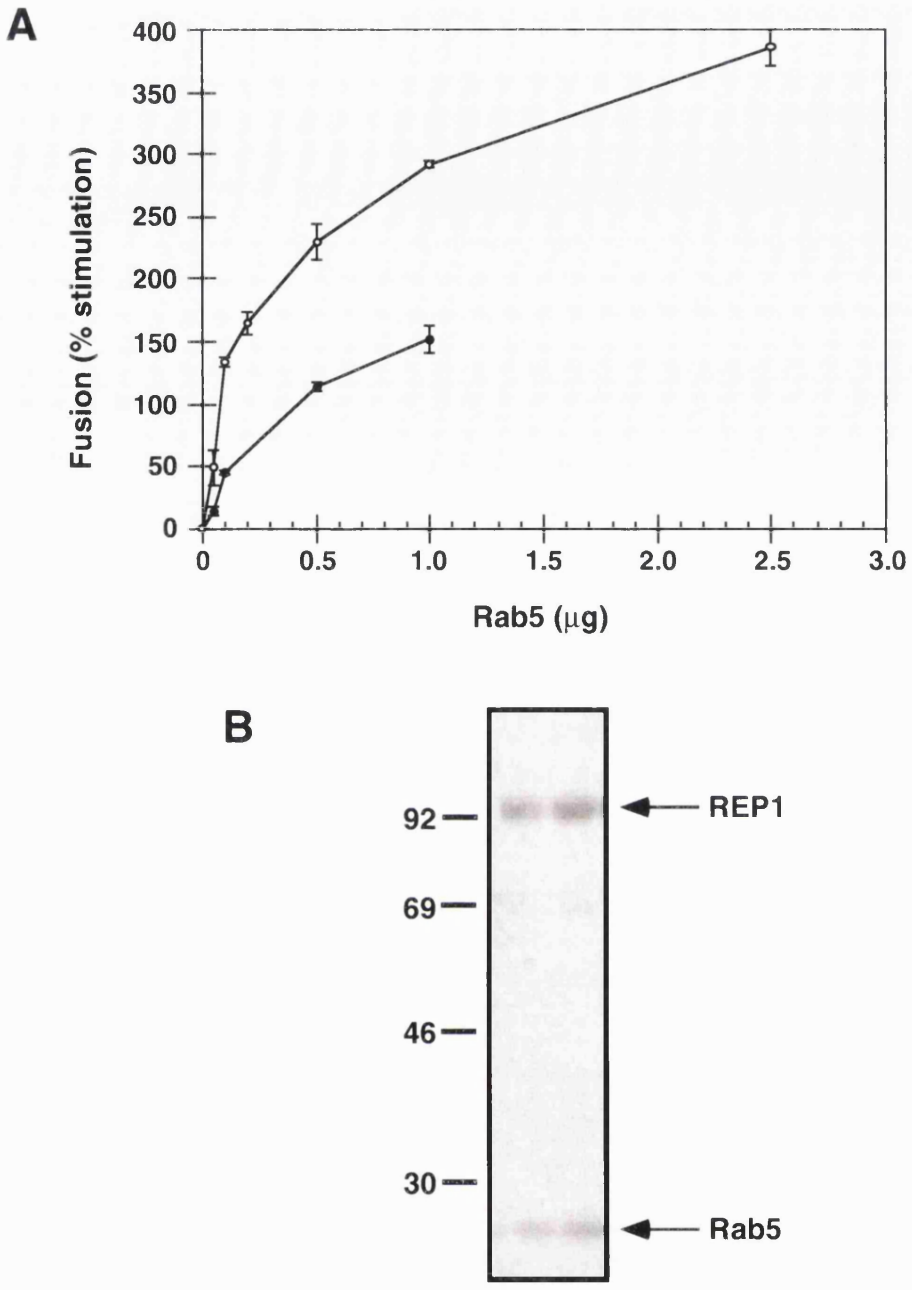


Figure 24 Addition of REP-1/rab5 complex to the cell-free endosome fusion assay stimulates fusion in a concentration dependent manner. A) Fusion was carried out in the presence of 4 mg/ml rat liver cytosol. The REP-1/rab5 complex was added to duplicate samples immediately before raising the temperature to 37°C. Fusion is expressed percentage stimulation compared to the control which recieved no REP-1/rab5 complex. Data are expressed as the average of two points ± boundry level. The results of two separate experiments are shown. B) SDS-PAGE analysis of the REP-1/rab5 complex which was used in the fusion assay. The proteins were detected by coomasie blue staining. Molecular weight markers are indicated on the left.

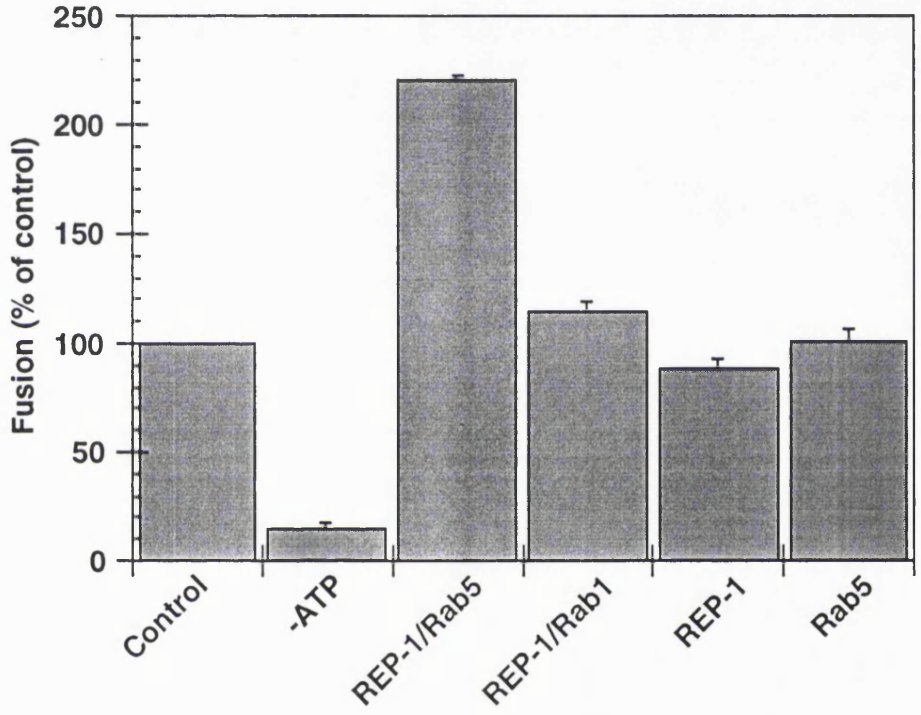


Figure 25 Stimulation of *in vitro* early endosome fusion by REP-1/rab5 is specific. The fusion assay was carried out as described in Fig. 24. Immediately before raising the temperature to 37°C, duplicate points were complemented with REP-1/rab5 complex, REP-1/rab1 complex, recombinant REP-1 or recombinant rab5, to a final concentration of 260 nM (1 μg of rab5). The control assay was carried out in the absence of exogenous REP-1 or rab proteins. The data are shown as the averages ± boundry levels of duplicate points from one experiment.

DISCUSSION

Since the discovery of the rab proteins it has become obvious that they are crucial to the process of membrane transport. However, it has proven extremely difficult to gain an insight into their function. In this study an in vitro system has been used to investigate the activity of rab5 in the fusion of early endosomes and a biochemical approach has been used to study the interaction of rab5 with other proteins.

Characterisation of the early endosome fusion assay

The *in vitro* assay of early endosome fusion which has been used in this study has been well characterised and described, although some minor modifications have been made (Tuomikoski et al., 1989; Gorvel et al., 1991; Thomas et al., 1992; Emans et al., 1993). As in the previous studies BHK cells have been used as they are easily handled and are readily homogenised using a combination of osmotic shock and mechanical shearing (Jean Gruenberg, personal communication). This is important as the complex morphology and large size of early endosomes makes them extremely sensitive to structural damage and any breakage of the endosomes would result in loss of fluid phase markers and a subsequent decrease in the sensitivity of the fusion assay. Here it is shown that less than 10% of an internalised marker is released during homogenisation, indicating that the endosomal membranes maintain their structural integrity during preparation of the fractions (Fig. 6).

Using the sucrose flotation gradient early endosomes were efficiently separated from later stages of the endocytic pathway (Fig. 5). Contamination of the early endosome fractions with late endosomes or endosomal carrier vesicles should not affect the fusion efficiency, as early endosomes do not fuse with these organelles (Bomsel et al., 1990; Aniento et al., 1993b). Also, because the two fluid phase markers, avidin and bHRP, are internalised into cells for only five minutes they are restricted to the early endosomes and avidin-bHRP complex formation is thus dependent on direct fusion between early endosomes. However, as the long-term goal of the study was to dissect the role of a rab protein in a specific membrane transport step, it was considered important to use the purified early endosome fractions.

Early endosome membranes prepared in this way were highly fusogenic, and fusion was dependent on the presence of cytosol and ATP

(Fig. 8) as previously described (Bomsel et al., 1990; Gorvel et al., 1991; Emans et al., 1993). Fusion was supported by either BHK or rat liver cytosol, but better efficiency was obtained with rat liver cytosol as it could be prepared at higher protein concentrations (Fig. 8 and 9). This ability of cytosol prepared from different species to support endosome fusion in vitro is well documented and suggests that the cytosolic factors involved are highly conserved (Tuomikoski et al., 1989; Gorvel et al., 1991; Aniento et al., 1993b).

Rab5a, rab5b and rab5c all regulate fusion of early endosomes in vitro

Previous work has shown that rab5 regulates the fusion of early endosomes *in vitro* and that it is a rate limiting factor at an early step in endocytosis *in vivo* (Bucci et al., 1992; Gorvel et al., 1991). These studies showed that cytosolic rab5 was functional, and that functionality was dependent on the ability to associate with membranes and bind nucleotide. However there were many more questions that remained to be answered, one of these being the question of redundancy. The rab5 protein exists as three isoforms, which appear to be expressed in all cell types (Bucci et al., 1994). In common with other ras-related GTPases the isoforms are extremely highly conserved, so that there is greater homology between the rab5a proteins from two different species, than there is between the rab5a and rab5b isoforms from one species (Fig. 2). This conservation indicates that there is strong selective pressure on the sequences of the different isotypes suggesting that they may have distinct functions. On the other hand there are also indications suggesting that the functions of these isoforms, if not identical, are very similar. The activity of these proteins in the cell-free assay of early endosome fusion also indicates that the rab5 isoforms regulate a common step in this membrane fusion process, as all the wild type proteins stimulated fusion and all of the N133I mutants inhibited fusion (Fig. 10). In agreement with these results, the rab5 isoforms also appear to behave identically *in vivo* (Bucci et al., 1994). All three of the rab5 proteins localise to the early endosome and plasma membrane and overexpression of the rab5b and rab5c wild type or N133I mutant proteins produced the same morphological changes as the rab5a proteins (Bucci et al., 1994).

These findings do not rule out the possibility that the isoforms may interact with the components of the recognition/fusion machinery with different affinities or that they may be responsible for different "fine tuning" events on the same pathway. In fact, recent results indicate that in *S. cerevisiae*, the three rab5 homologs, Ypt51p, Ypt52p and Ypt53p, are not

functionally identical (Singer-Krüger et al., 1994). Although deletion of the *YPT5* genes did not affect α-factor internalisation, all of them appear to be involved on the endocytic pathway and at an earlier step than the Ypt7 protein (Schimmoller and Riezman, 1993). Deletion of the *YPT51* gene produced the most severe defects in yeast, indicating that this protein plays a more essential role than either of the other two Ypt5 proteins, in contrast deletion of the *YPT53* gene had no detectable effect. In fact, the involvement of Ypt53p only became apparent in double or triple deletion mutants. While these results indicate that these proteins may play slightly different roles in the yeast endocytic pathway the relative abundancies of the proteins may also be significant, as it was estimated that Ypt52p and Ypt51p are expressed at higher levels than Ypt53p (Singer-Krüger et al., 1994).

While several of the other rab proteins also seem to exist as multiple isoforms only the rab1a and rab1b, rab3a and rab3c and rab3a and rab3b isoforms have been compared. So far no evidence has been obtained for any difference in the activity of the rab1 isoforms (Tisdale et al., 1992). The rab3 subfamily, however, appears to be unique in that the expression of the isoforms is cell-type specific. Rab3a is been localised to regulated secretory vesicles in neurons and neuroendocrine cells, and has been shown to relocalise into the cytosol following stimulation of secretion in neurons (Touchot et al., 1987; Zahraoui et al., 1989; Darchen et al., 1990; Fischer von Mollard et al., 1990; Mizoguchi et al., 1990; Fischer von Mollard et al., 1991; Matteoli et al., 1991; Johannes et al., 1994). Rab3b is expressed primarily in epithelial cells, but has also been shown to be essential for calcium-dependent exocytosis in pituitary cells (Lledo et al., 1993; Weber et al., 1994). Rab3c co-localises with rab3a on synaptic vesicles in neurons and both isoforms appear to have similar, if not identical activities (Fischer von Mollard et al., 1994a). Rab3d is predominantly found in adipocytes, although the subcellular localisation has not been studied, where it's expression level is apparently dependent on the differentiation state of the cells (Baldini et al., 1992). Stimulation of adipocytes with insulin results in the fusion of a population of vesicles containing a glucose transporter (GLUT4) with the plasma membrane, and is has been suggested that rab3d may regulate this process (Baldini et al., 1992; Fischer von Mollard et al., 1994b). Recently a rab3-like protein, which is recognised by a monoclonal antibody which cross-reacts with all of the known rab3 isoforms, has been localised in pancreatic acinar cells. This protein appears to be highly concentrated on zymogen granules in resting cells, but upon stimulation, when approximately 90% of the zymogen granules fuse with the plasma membrane, it redistributes to the TGN (Jena et al., 1994). This data

suggests that the rab3 proteins are required for regulated exocytosis in a variety of systems, and that, perhaps, the isoforms have evolved to interact with components specific to each of these systems.

As the existence of multiple isoforms is a common characteristic of the ras and ras-related proteins (Valencia et al., 1991), the reasons for this heterogeneity may also be related. Given the significant role played by these proteins in many different aspects of cellular regulation, the existence of the different isoforms may be a safety precaution. This apparent redundancy would then minimise the risk to the cell of mutation or inactivation of a single gene.

Nucleotide binding but not hydrolysis is required for rab5 function in vitro

The role of GTPase activity in rab protein function, although a critical point in the search for rab protein function, has been difficult to address because of the involvement of multiple GTP-binding proteins at each step of membrane transport (Barr et al., 1991; Oka et al., 1991; Stow et al., 1991; Colombo et al., 1992a; Lenhard et al., 1992; Herskovits et al., 1993; Palmer et al., 1993; van der Bliek et al., 1993). Currently there are two different views as to the function of GTP-hydrolysis. According to the model proposed by Henry Bourne (1988), GTP-hydrolysis is required immediately before membrane fusion and, by analogy to the function of EF-Tu in protein chain elongation, it may act as a proof-reading mechanism, ensuring that membrane fusion would only take place if the pre-fusion membrane-membrane interactions were longer lived than the GTP-bound form of rab protein. The second view is that GTP-hydrolysis is required to "inactivate" the rab protein by switching it into the GDP-bound "inactive form" (Takai et al., 1992; Novick and Brennwald, 1993). Here the activity of two rab5 mutants, in which the nucleotide exchange and GTPase activities are affected, has been examined in the *in vitro* fusion assay. While the rab5 S34N mutant, which is predominantly in the GDP-bound form, inhibited fusion, the rab5 Q79L mutant, which is predominantly in the GTP-bound form stimulated fusion (Stenmark et al., 1994b). This is in agreement with the results obtained from *in vivo* experiments in which the mutants were either expressed transiently or in stable cell lines (Li and Stahl, 1993; Stenmark et al., 1994b). Overexpression of the S34N mutant induced the accumulation of small endocytic vesicles, similar to those seen in cells overexpressing the N133I mutant (Bucci et al., 1992), and decreased the rate of internalisation of HRP and transferrin. Similarly, the corresponding mutant in rab1a inhibits ER to Golgi, and intra-Golgi, transport (Nuoffer et al.,

1994). In contrast, overexpression of the Q79L mutant resulted in the formation of extremely large endocytic structures, markedly larger than those seen with overexpression of the wild type protein, and increased the rate of internalisation of both HRP and transferrin to approximately the same level as wild type rab5 (Li and Stahl, 1993; Stenmark et al., 1994b).

The inhibitory effect of the S34N mutant, both *in vitro* and *in vivo*, suggests that GTP-binding is absolutely required for the activity of rab5. In the cell-free assay the residual fusion obtained in the presence of this mutant is presumably due to the presence of endogenous wild type rab5 present on the early endosome membranes. After the initial round of fusion, this rab5 is expected to become cytosolic and depleted from the membranes. The subsequent recruitment of this cytosolic form onto the membrane may then be inhibited in the presence of an excess of mutated rab5. The simplest mechanism to achieve this would be by titration of an exchange factor required for conversion of GDP-bound to GTP-bound rab5 on the membranes. In fact, data obtained from the equivalent ras and rab3a mutants indicate that this interpretation is likely to be realistic. The ras mutant inhibits ras-GDS (Medema et al., 1993) and the rab3a mutant has approximately 10-fold greater affinity for rab3a GRF compared to wild type rab3a (Burstein et al., 1992).

While the inhibitory activity of the S34N mutant indicates a requirement for GTP-binding by rab5 in a pre-fusion step, the stimulatory effect of the Q79L mutant demonstrates that the GTPase activity of rab5 is not required for membrane fusion. In contrast to the corresponding rab3a mutant (Brondyk et al., 1993), the rab5 Q79L mutant is apparently poorly sensitive to GAP activity, so that *in vivo* 60-70% of the mutant is found in the GTP-bound form compared to only approximately 20% of the overexpressed wild type rab5 (Stenmark et al., 1994b). Furthermore, the membrane bound/cytosolic ratio for the Q79L mutant is considerably higher than that of the wild type protein. However, previous studies in *S. cerevisiae*, showed that, although the Sec4 Q79L mutant could rescue Sec4 deletion mutants, the sec4 Q79L mutant cells were cold sensitive; with reduced TGN to plasma membrane transport and accumulation of vesicular transport intermediates (Walworth et al., 1989; Walworth et al., 1992). However, closer examination of their results shows that these effects are only noticeable after longer periods of time at the restrictive temperature. Growth rates of wild type and sec4 Q79L cells were identical for the first 8 hours at 14°C, and invertase secretion by the mutant cells was increased, compared to wild type cells, during the first 10 min at the restrictive temperature

(Walworth et al., 1992). Thus it may be possible that the initial effect of the mutant is stimulatory, but, due to a decrease in the amount of GDP-bound Sec4 Q79L which is available for recycling to the TGN, the long term effect could be inhibitory. Alternatively, it is possible that the Sec4 Q79L mutant is not stable at the restrictive temperature. Overexpression of the rab5 Q79L mutant does significantly inhibit transferrin recycling in mammalian cells, although the rate of transferrin uptake is similar to that seen in cells overexpressing the wild type protein (Stenmark et al., 1994b). As the inhibitory effects of the GTPase mutants in either Sec4 or rab5 were only seen when the proteins were expressed at high levels, or in the absence of endogenous wild type protein, it is possible that these effects are due to the titration of a factor required for membrane transport. It is also perhaps significant that the fusion assay used here measures a lateral fusion process rather than a vectorial process, so that the effect of reduced recycling of the rab protein would be an increase in the amount of rab5 on the endosome (fusogenic) membranes. Altogether, these results strongly indicate that GTP-hydrolysis by rab5 is not required for endosome fusion, but that the GTPase activity of the rab proteins may indeed be required to convert them from the "active" GTP-bound form to the "inactive" GDP-bound form and for recyling to the donor membranes (Takai et al., 1992; Novick and Brennwald, 1993).

The N-terminal domain of rab5 is required for its activity

The sensitivity of the early endosome membranes to trypsin treatment, which rendered them fusion incompetent, indicated that a factor essential for the recognition/fusion machinery was being cleaved by trypsin. That the rab5 protein was sensitive to trypsin under these conditions is demonstrated by the immunoprecipitation with rab5 specific antibodies of an additional polypeptide which migrated slightly faster on SDS-gels than the uncleaved protein. Significantly the immunoprecipitation was carried out from a membrane fraction recovered after trypsin treatment, indicating that the faster migrating form of rab5 remains membrane associated. Furthermore, other work in this laboratory has shown that no other polypeptides are detectably cleaved under these conditions (Steele-Mortimer et al., 1994). The inactivation of rab5 is also demonstrated by showing that when cytosol containing overexpressed rab5 is trypsin-treated it loses the ability to stimulate fusion in the cell-free assay. As the cleaved form of rab5 which was detected here by immunoprecipitation, migrates only slightly faster on SDS-gels, most likely only a few amino acids were cleaved from either the N- or C-terminal of the protein. Because the rab proteins are

attached to membranes via the geranylgeranyl groups on C-terminal cysteine residues, and in the case of rab5 there are only two residues between the cysteine motif and the C-terminal, it seemed unlikely that the rab5 protein would remain membrane associated if these cysteine residues were removed. For this reason the N-terminal domain was considered more likely to contain the trypsin cleavage site. This has been confirmed by N-terminal micro-sequencing of *E. coli* expressed rab5 protein, which showed that trypsin cleaved four amino acids from the extreme N-terminus of the protein (Steele-Mortimer et al., 1994). These experiments indicated for the first time that the N-terminus of a rab protein could be required for its function.

Subsequently several other experiments have provided further evidence for the role of the N-terminal in rab5 function. Firstly, it has been shown that a peptide consisting of the seventeen amino acids at the extreme N-terminal of rab5 can efficiently inhibit fusion of early endosomes in vitro. In contrast, a rab3 N-terminal peptide, which like the rab5 protein has an extended N-terminal domain compared to most other rab proteins, had no effect on endosome fusion (Steele-Mortimer et al., 1994). Secondly, a domain replacement approach has been used to show which of the rab5 domains are required for its activity (Stenmark et al., 1994a). Chimeric proteins were constructed from the rab5 and rab6 proteins and the activity of these proteins was then measured *in vivo*. Whereas the overexpression of wild type rab5 in BHK cells stimulated internalisation of fluid phase marker, a chimeric protein, in which the N-terminal (20 amino acids) of rab5 was replaced with that of rab6 (13 amino acids), could not stimulate internalisation of fluid phase marker. Furthermore, the authors showed that replacement of four specific rab6 domains with the equivalent domains from rab5 produced a protein which demonstrated the functional specificity of rab5. These four domains were; the two "switch" regions α2/loop5 and α3/loop7, which by analogy with ras should exhibit nucleotide-dependent conformational changes, the C-terminal domain, required for correct localisation, and the N-terminal domain. When only the C-terminal domains were switched, the rab6 protein was mislocalised to the early endosome but this hybrid had no effect on the internalisation of fluid phase marker into cells. The α2/loop5 region in rab5 is highly conserved, both in the different isoforms and between species suggesting that it is likely to play a role in rab5 specificity (Fig. 2). However, switching of this domain, with the C-terminal domain of rab5, into rab6 resulted in a protein which appeared to be unable to associate with membranes although it was geranyl-geranylated. The additional substitution of the α3/loop7, however, created a

protein which associated efficiently with membranes but inhibited internalisation. Intriguingly subsequent substitution of the N-terminal domain of rab5 into this dominant negative mutant resulted in a protein with rab5 activity.

The activities of the rab5/rab6 chimeric proteins suggest that the α2/loop5 and α3/loop7 domains are involved in interaction with other proteins and that in the absence of the correct N-terminal one or more of these interactions may become irreversible thus inhibiting endocytosis. All three of these regions may therefore be involved in interactions between the rab5 protein and regulatory or target molecules. Similar studies domain switching studies have previously been used to show that the α3/loop7, effector/loop2 and C-terminal domains are involved in the specificity of the Ypt1 and Sec4 proteins in *S. cerevisiae* (Brennwald and Novick, 1993; Dunn et al., 1993). But these studies did not identify a requirement for the α2/loop5 or N-terminal domains in the specificity of these two rab family members. This disparity may well reflect the diversity of interactions between individual rab proteins and other proteins, and it would be unwise to assume that all the members of this large family would have identical requirements for all of the structural domains.

Given the above data it is perhaps somewhat surprising that the rab5 homolog in *S. pombe*, Ypt5, can be functionally replaced by the canine rab5a protein as the N-terminals of these two proteins do not show high homology (Armstrong et al., 1993). However, although the Ypt5 deletion mutants were non-viable on minimal media some growth was observed when the mutants were grown on some rich media, suggesting that a minimal amount of rab5 function could be sufficient for rescue of the ypt5-disrupted cells. Unfortunately, the activities of the two proteins could not be directly compared as there is no assay for endocytosis in *S. pombe*, so it is possible that very low rab5 activity was required for rescue. In addition, it is possible that only certain conserved residues within the N-terminus are required for the function of this domain.

In spite of the accumulated evidence for the involvement of the N-terminal of rab5 in its function, it is difficult to reconcile the effects reported here, of cleaving the four N-terminal amino acids from rab5, with a recent report in which an N-terminal rab5 deletion mutant was shown to be active *in vivo* (Li and Stahl, 1993). Using a Sindbis virus vector to overexpress wild type rab5 in BHK cells these authors obtained a 2-3 fold stimulation in fluid phase marker uptake. Similar levels of stimulation were obtained when a rab5 mutant containing a deletion of the four N-terminal amino acids was

tested, however, deletion of nineteen amino acids from the N-terminus did reduce the activity of the rab5 protein. Although Li and Stahl (1993) used a human rab5 sequence and the other studies were performed using the canine rab5 sequence (Steele-Mortimer et al., 1994; Stenmark et al., 1994a), there are only four amino acids that are not identical in these two sequences, and only one of these substitutions is in the N-terminal region, therefore, it seems unlikely that the differences in results could be due to a significant difference in the activities of the proteins. In fact, the N-terminal homology between the human and canine rab5a proteins, and the human and murine rab5b proteins (100% identity), suggests functional restriction on mutation of these sequences and is thus a further, if indirect, evidence for function. It is, however, possible that the method used by Li and Stahl (1993) to study the activity of rab5 mutant proteins could in certain instances give misleading results. This is possible because these authors have only used one parameter to measure the activity of rab5, i.e. the amount of fluid phase marker (HRP) which is internalised into cells within one hour. They did not measure either the rate of internalisation or the rate of recycling. Thus either an increase in the rate of internalisation or a decrease in the rate of recycling could increase the total amount of HRP detectable within the cells. If the N-terminal mutants acted by inhibiting recycling, but not internalisation, perhaps by preventing fusion of incoming endocytic vesicles with the early endosome, an increase in the amount of internalised HRP would be measured. Without measuring the rates of both internalisation and recycling it is, therefore, not possible to exclude such events. It has been clearly shown that other rab5 mutants have remarkable effects on the morphology of early endosomes, with dominant negative mutants causing the disintegration of the normally complex endosome into a homogeneous collection of small tubules and stimulatory mutants causing the formation of large vacuolar endosomes (Bucci et al., 1992; Stenmark et al., 1994b). Therefore, morphological analysis of the early endosomes and rab5 positive structures in cells overexpressing the N-terminal mutants would probably give a better indication of the activity of these mutants.

In conclusion, while the role of the N-terminal in rab5 function remains to be determined, the bulk of evidence which has so far been obtained does indicate that this domain is required for rab5 activity. The N-terminal regions of the ras-related proteins show considerable variation in length and sequence, particularly between the different subgroups of proteins (Valencia et al., 1991). Indeed, the N-terminal domain of rab5 and certain other rab proteins, for example rab3 and sec4, is considerably longer than that of most members of this family, and it has been suggested

that these extended N-terminals may form additional sub domains, which could be involved in specific interactions with other proteins (Valencia et al., 1991). The significance of these observations should become clearer with the identification of regulatory factors which interact with the rab proteins.

GDI-phosphorylation and regulation of rab proteins

That endosome-endosome fusion is regulated by phosphorylation events, both in mitosis and interphase, is now well documented (Tuomikoski et al., 1989; Pitt and Schwartz, 1991b; Thomas et al., 1992; Woodman et al., 1992; Woodman et al., 1993). Up to the present time, although unique membrane phosphoproteins have been localised to endosomes (Rindress et al., 1993), the phosphoproteins involved in mediation of fusion events remain unidentified. There is now also a considerable amount of data showing that ras and ras-related proteins are regulated, either directly or indirectly, by phosphorylation (Ellis et al., 1990; Bailly et al., 1991; Kaplan and Bar-Sagi, 1991; Moran et al., 1991; Cichowski et al., 1992; Gross et al., 1992; Molloy et al., 1992; Polakis et al., 1992; van der Sluijs et al., 1992b; Albright et al., 1993; Altschuler and Lapetina, 1993; DeClue et al., 1993; Gale et al., 1993; Gulbins et al., 1993; Pronk et al., 1993; Scherle et al., 1993; Cherniack et al., 1994). Given this information it was considered possible that the rab5 protein, as a known regulator of endosome fusion, could itself be regulated by phosphorylation/dephosphorylation events. However, unlike the rab1a and rab4b proteins, rab5 does not contain a consensus site for p34cdc2 kinase and there is no evidence that it is phosphorylated (Bailly et al., 1991). Therefore it was considered that proteins interacting with, and regulating, rab5 would be possible targets for regulation by phosphorylation. Experiments carried out to test this hypothesis confirmed that the rab5 protein itself does not seem to be phosphorylated, and showed that it does interact specifically with phosphoproteins of 20 and 50 kD. While the 20 kD phosphoprotein has not been identified, the 55 kD phosphoprotein shows many of the characteristics of the regulatory protein Rab-GDI.

The integral role played by GDI in rab protein regulation is becoming increasingly clear. First isolated on the basis of its ability to inhibit dissociation of GDP from rab3a (Matsui et al., 1990; Sasaki et al., 1990), it is now known to interact with many, if not all, of the rab proteins. The current view is that GDI is responsible for both the removal and the insertion of GDP-bound rab proteins from and into membranes (Araki et al., 1990; Sasaki et al., 1991; Ueda et al., 1991; Regazzi et al., 1992; Soldati et al., 1993; Ullrich et al., 1993; Soldati et al., 1994; Ullrich et al., 1994). In this way

GDI is thought to be responsible for the cycling of rab proteins between membranes and cytosol. However, we are now faced with the conundrum that the two functions of GDI, membrane insertion and removal of rab proteins, must occur in a specific and directional manner. In other words a rab protein must first be inserted into the correct "donor" membrane and should be removed only after it has performed its function, presumably from the "acceptor" membrane. In addition, given that GDI appears to be a general regulator of rab proteins, and thus a universal regulator of membrane transport, it itself would seem a likely target of regulation. However, in spite of some indirect evidence which has suggested that this speculation may be correct, a mechanism for this regulation remains obscure (Sasaki et al., 1991; Zahner and Cheney, 1993).

Previous studies have indicated that cytosolic rab proteins are associated with GDI (Regazzi et al., 1992; Ullrich et al., 1993) and, in addition to confirming these data, the results described here show that this cytosolic rab-associated GDI is phosphorylated. In contrast, the minor portion of GDI which is found on membranes, although also associated with rab proteins, is not phosphorylated (the distribution of GDI and rab5 is summarized in Figure 26). These data suggests that the phosphorylation of GDI may regulate its association with rab proteins and, by inference, the membrane cycling of these small GTPases.

In Figure 27 a simple scheme by which this mechanism may regulate the association of rab proteins with membranes is depicted. This model was designed based on the phosphorylation data described here together with other data which were available at the time. It is proposed that the specific association of rab proteins with membranes is initiated by interaction of the cytosolic, phosphorylated GDI-rab complex with a membrane associated protein, presumably a nucleotide exchange factor. Exchange of GDP for GTP would promote dissociation of the GDI-rab complex, due to the vastly reduced affinity of GDI for the GTP bound form of the rab protein (Araki et al., 1990), thus freeing the lipid moiety of the rab protein to insert into the adjacent membrane. In this way the rab protein would be delivered to the membrane in its active conformation. Following GTP hydrolysis, the GDP-bound rab protein, by associating with free GDI, would be extracted from the membrane. Phosphorylation of GDI would prevent reinsertion of the rab protein into the membrane, except by interaction with the specific exchange factor.

Although the idea that guanine nucleotide exchange is coupled to insertion of the rab proteins into membranes is not new (Bourne, 1988),

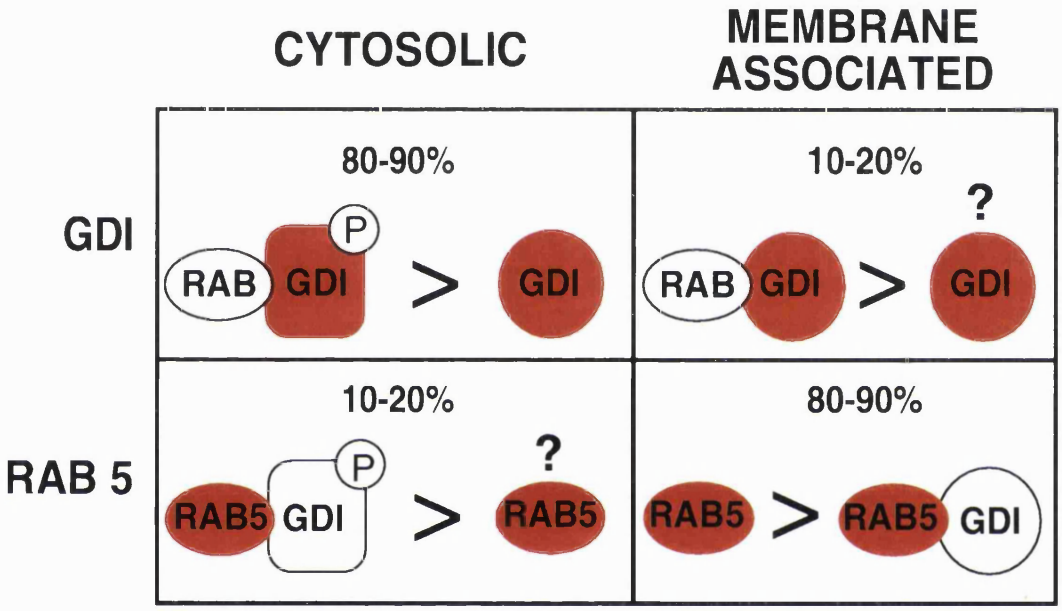


Figure 26 Summary of the distribution of GDI and rab5 complexes in membranes and cytosol. The distributions of rab5 and GDI between cytosol and membranes are indicated. For clarity the protein complexes are indicated in diagramatic form. Question marks (?) indicate forms of the proteins which may be associated with other unidentified factors. In each case the major form of the protein is shown on the left. GDI, guanine nucleotide dissociation inhibitor; P, phosphate group; RAB, all rab proteins.

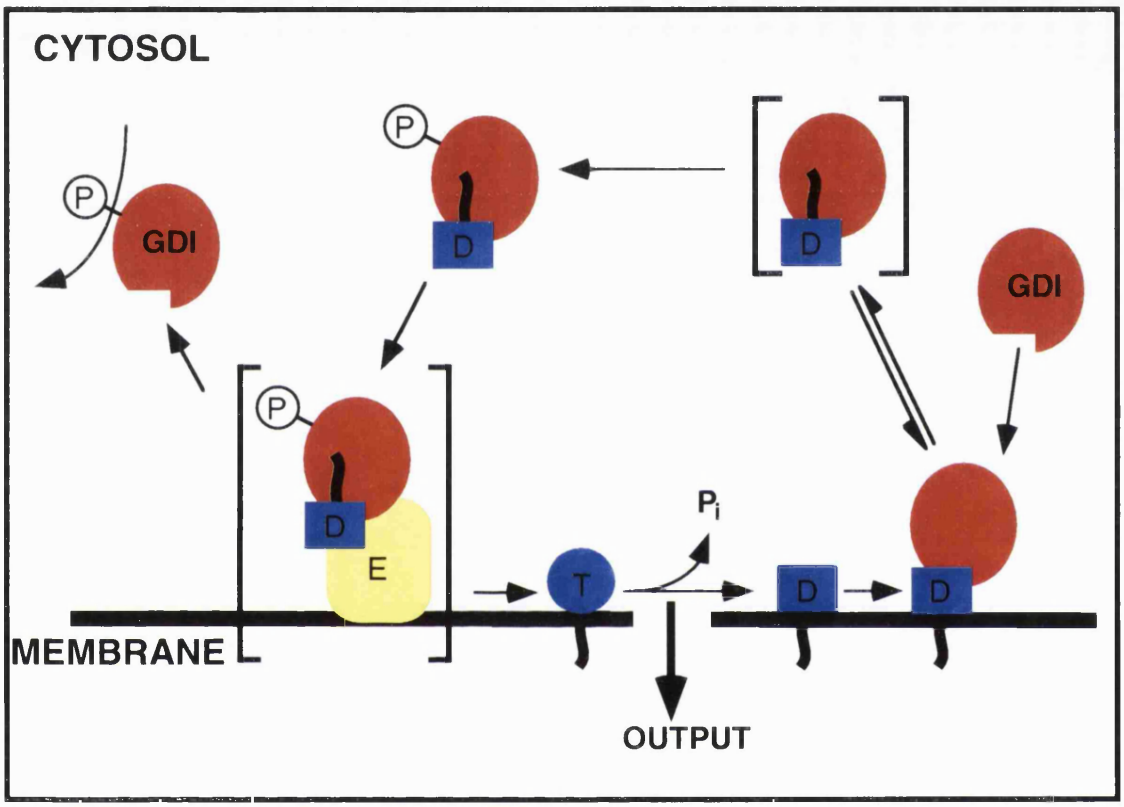


Figure 27 Model for the regulation of rab protein association with membranes (see text for explanation). Complexes of GDI with rab protein, which have not been detected and correspond to the putative intermediates, are shown in parenthesis.

D, GDP-bound rab protein; E, exchange protein; GDI, guanine nucleotide dissociation inhibitor; P, phosphate; P_i, inorganic phosphate; T, GTP-bound rab protein

evidence in support of this has only recently been obtained (Soldati et al., 1994; Ullrich et al., 1994). These two studies investigated the specific targeting of rab5 and rab9 to membranes using purified GDI-rab complexes which were either reconstituted using recombinant prenylated rab proteins and recombinant GDI or purified from cytosol. In agreement with the model shown here, both of these groups clearly demonstrate that GDI presents the rab protein to the specific membrane, and that membrane insertion is associated with nucleotide exchange. Both groups observed a slight lag between membrane association of the rab protein and nucleotide exchange suggesting that two separate activities could be involved in this process. Initially a GDI dissociation factor (GDF) would promote dissociation of GDI and insertion of the rab protein into the membrane and following this an exchange activity would promote exchange of GDP for GTP on the rab protein.The model is also consistent with properties of the unphosphorylated form of GDI reported by other groups; namely, inhibition of GDP dissociation and promotion of rab dissociation from the membrane (Araki et al., 1990; Ullrich et al., 1993). Firstly, it proposes that rab proteins are presented to the appropriate membrane in a complex with GDI, this makes comprehensible an earlier study (Araki et al., 1990) which showed that purified rab3a will non-specifically associate with membranes e.g. mitochondria and erythrocytes. A cycle of membrane association and dissociation is implicit in rab protein involvement in vectorial membrane transport (Bourne, 1988; Goud and McCaffrey, 1991; Magee and Newman, 1992; Pfeffer, 1992; Zerial and Stenmark, 1993), and this model predicts that GDI will be found on both donor and acceptor organelles but not on membranes of vesicular intermediates. Although the kinase and phosphatase activities have not so far been localised, they are depicted here as being cytosolic; in this configuration the homeostatic potential of GDI is most simply realised.

It has previously been shown that phosphorylation of the rab1a and rab4a proteins by the $p34^{cdc2}$ kinase determines their localisations (Bailly et al., 1991; van der Sluijs et al., 1992b). However, whereas rab4a becomes translocated into the cytosol upon phosphorylation, rab1a becomes predominantly membrane associated. How is it possible to explain the apparently diametric effects of phosphorylation on these two rab proteins? In the case of rab1a, phosphorylation may simply prevent its recognition by GDI and thus inhibit its removal from membranes. In contrast, it is possible that phosphorylation of rab4a, without affecting its interaction with GDI, could prevent the interaction of the GDI-rab4 complex with the nucleotide exchange factor thus inhibiting insertion of the rab4 protein into

the membrane. Thus phosphorylation appears to regulate the rab proteins at two levels; via GDI a general level of regulation affecting all of the rab proteins is achieved, whereas phosphorylation of individual rab proteins may be required for a finer or more specific level of regulation. Whether the phosphorylation state of GDI is altered in mitotic cells remains to be seen, although inhibition of rab protein activity via a common regulatory protein, of which GDI is as yet the only example, would be an efficient mechanism of effecting the well documented inhibition of membrane transport (Warren, 1989; Warren, 1993b).

In conclusion, the data suggest that GDI serves as universal regulator of membrane transport, perhaps controlling both the specificity and the directionality of the rab protein cycling pathway.

REP-1 functions as a rab escort protein in vitro

It has been suggested that the REP-1 protein performs the functions of an escort protein; firstly presenting the unprenylated rab protein to the geranylgeranyltransferase and then transferring the prenylated rab protein to the specific membrane (Andres et al., 1993). However, in the absence of membranes REP-1 remains associated with the modified protein, unless detergent is present at greater than the critical micellar concentration (Andres et al., 1993). In order to investigate the ability of REP-1 to insert rab proteins into membranes under more physiological conditions the activity of REP-1/rab complexes was studied using the *in vitro* assay of endosome fusion. Addition of the REP-1/rab5 complex to the fusion assay resulted in a concentration-dependent stimulation of fusion of up to almost four-fold. The amount of stimulation obtained seemed to be dependent on the endosome fractions used in the assay, as the same cytosol and REP-1/rab5 complex preparations were used in all experiments. This may be due to the slight differences observed in the amount of peripheral membrane proteins associated to endosomal membranes in different preparations (Jean Gruenberg, unpublished observations), or to other variables such as the state of the cells at the time of homogenisation. However, the specificity of the stimulation is clearly demonstrated by the fact that REP-1/rab1 was incapable of stimulating fusion, and neither purified REP-1 nor recombinant rab5 were active when added individually. Although the REP-1/rab5 complex was used at concentrations of up to 520 nM, saturation was not reached, indicating that at these concentrations rab5 is still a limiting cytosolic factor in endosome fusion. However, it is also possible that the complex is not 100% active and that the real saturation level is lower than determined.

The significance of these experiments are twofold. Firstly this system can now be used to further dissect the roles played by REP-1 and GDI in rab5 membrane association. And secondly it should now be possible to investigate more thoroughly the requirement for nucleotide binding and hydrolysis in rab5 function. In the first case *in vitro* studies indicate that REP-1 can prevent nucleotide dissociation from rab proteins and, in permeabilized cells, it can perform other functions similar to those of GDI, being able to insert and remove rab proteins either into, or from, their specific membranes (Alexandrov et al., 1994). It is not clear whether REP-1 would perform these functions *in vivo*, but given the activity of the REP-1/rab5 complex in the *in vitro* fusion assay it should be possible to investigate the relative activities of these two regulatory proteins.

The requirement by the rab proteins for GTP-hydrolysis and exchange has been difficult to study and, although it now seems clear that GTP-binding but not hydrolysis is essential for activity, the nucleotide requirement at specific steps is still undefined. *In vitro* studies suggest that it is possible to load the rab protein in the REP-1/prenylated rab protein complex with either GDP or GTP (Alexandrov and Zerial, unpublished data). In this case it should also be able to load the rab protein with non-hydrolyzable analogues of GTP, such as GTPγS, in order to investigate the requirement for GTP hydrolysis or exchange at different steps in the rab cycle.

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This Thesis is dedicated to my parents.

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Phosphorylation of GDI and membrane cycling of rab proteins

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Membrane transport is known to be regulated by protein phosphorylation and by small GTPases of the rab family. Using specific antibodies, we have identified a 55 kDa phosphorylated protein which co-immunoprecipitated with the cytosolic forms of rab5 and other rab proteins. We demonstrate, on the basis of its mobility in two-dimensional electrophoresis gels and its immunological properties, that this protein is rab GDI (p55/GDI). We also found that, a minor fraction of p55/GDI is membrane associated, but, whilst also complexed with rab proteins, it is not phosphorylated. On the basis of these data we suggest that the cycling of rab proteins between membranes and cytosol is regulated by phosphorylation of p55/GDI.

Small GTP-binding protein; Rab5; Rab GDI; Membrane traffic

1. INTRODUCTION

Intracellular membrane traffic events are known to be regulated by small GTPases of the SEC4/YPT1/rab family and also by protein phosphorylation [1]. In this study we have investigated whether there is a point of convergence between these two regulatory mechanisms, by looking for interactions between phosphorylated proteins and rab proteins.

More specifically we have studied the small GTP-binding protein rab5, which localizes to early endosomes and the plasma membrane [2], and has been shown to regulate both fusion between early endosomes in vitro [3] and early endocytic events in vivo [4]. Since early endosome fusion is also regulated by phosphorylation/dephosphorylation events, in both mitosis and interphase [5–7], we investigated the association of phosphorylated proteins with the rab5 protein. For these experiments we have used antibodies raised against the hypervariable carboxyl-terminal domain of the rab5 protein; antibodies raised against this domain are highly specific and have been extensively used in localisation studies [2,8,9].

By analogy with the related p21 ras proto-oncogene and by fluorescence spectroscopic measurements (M.J. Clague, unpublished observations), rab proteins undergo a conformational change upon hydrolysis of GTP. This molecular switching mechanism can be used to impart directionality, and specificity, to the process that they control [10]. Small GTP-binding proteins are regulated by interactions with other proteins which control the rate of this switching; proteins which inhibit GDP dissociation (GDI), facilitate nucleotide exchange (exchange factor), or enhance GTPase activity (GAP) of rab proteins have all been identified [11–14].

In this study we show that rab5, and other rab proteins, are associated with GDI both in cytosol and on membranes, and that in vivo the cytosolic GDI protein is phosphorylated when complexed to rabs. Previously GDI has been shown to associate with an array of rab proteins and to promote their dissociation from membranes [15–17]. However, it was not clear how this association could be regulated. Our results, together with an analysis of the membrane/cytosol distribution of the different forms, suggest that the cycling of rab proteins on and off membranes is regulated by GDI. We propose a simple model incorporating a universal role for GDI in specifying the directionality of this cycle.

2. MATERIALS AND METHODS

2.1. *Cells and reagents*

BHK cells were maintained and seeded for experiments as described previously [18]. The polyclonal antibody against rab5 was raised against the C-terminal peptide according to the method described by Louvard et al. [19]. The polyclonal antibodies raised against C-terminal peptides of GDI and rab2 were kind gifts from Oliver Ullrich and Marino Zerial (EMBL, Heidelberg). The affinity purified antibodies against the C-terminal of rab4 were a kind gift from Peter van der Sluijs and Ira Mellman (Yale University School of Medicine, New Haven). The rab5 C-terminal (PKNEPQNPGANSARGR, [2]) and N-terminal (MANRGATRPNGPNTGNK) peptides were synthesized by Dominique Nalis (EMBL, Heidelberg). Guanosine [α-^{32}P]triphosphate and [^{32}P]orthophosphate were obtained from Amersham-Buchler, Germany. [^{35}S]methionine/cysteine (EXPRE^{35}S^{35}S) was obtained from DuPont/NEN Research Products, Boston, MA. *N*-Octylpolyoxyethylene (Octyl-POE/Rosenbusch-Tenside) was obtained from Bachem-Biochemica GmbH, Heidelberg.

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Abbreviations: GDI, guanine nucleotide dissociation inhibitor; PNS, post-nuclear supernatant.

2.2. *Metabolic labeling*

For metabolic labeling of phosphorylated proteins, 90% confluent cells (16 h after seeding) were taken and incubated for 4–6 h with 0.5 mCi/ml of [^{32}P]orthophosphate in phosphate-free Dulbeccos modified Eagles medium (DME), supplemented with 1% fetal calf serum (Gibco BRL, Germany) which had been dialyzed for 15 h against Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris-HCl, pH 7.4). For [^{35}S]methionine/cysteine labeling, 40% confluent cells (4 h after seeding) were taken and incubated for 16 h in methionine/cysteine free DME supplemented with 1% fetal calf serum and 0.2 mCi/ml EXPRE^{35}S^{35}S.

2.3. *Immunoprecipitation*

Post-nuclear supernatant (PNS) was prepared from BHK cells as described previously [18], except that TBS was used in place of phosphate-buffered saline. Membrane and cytosol fractions were prepared by centrifuging PNS at 150,000 × *g* for 15 min in a Beckman TL100 ultracentrifuge, and taking the pellet or supernatant, respectively. Membrane pellets were resuspended in homogenization buffer (HB; 3 mM imidazole, 250 mM sucrose, pH 7.4) before solubilisation, so that the protein concentration was equal to that of the cytosol. Samples were then mixed with a 4-fold excess of Rosenbusch-Tenside buffer (RTB; 12.5 mM HEPES, 1.0 mM DTT, 0.5 mM EGTA, 100 mM KOAc, 1 mM $MgCl_2$, 1% *n*-octylpolyoxyethylene, pH 7.4) for 30 min at 4°C. Insoluble material was removed by centrifuging at 150,000 × *g*. The supernatant (250 mg) was mixed with antiserum (3 ml) for 1 h at 4°C. In peptide competition experiments, peptide (720 mg) was mixed with antiserum for 30 min at 4°C and the mixture then added to the supernatant. Protein A-Sepharose beads (5 ml/ml of antiserum), which were preblocked with cold solubilized PNS when immunoprecipitating metabolically labeled proteins, were added and mixed for 30 min at 4°C. The immune complexes were washed three times with RTB and three times with TBS. Finally the immunoprecipitated proteins were solubilized in sample buffer (2% SDS, 100 mM DTT, 60 mM Tris, pH 6.8, 0.001% Bromophenol blue, 15% glycerol) and SDS-PAGE carried out.

For immunoprecipitation of phosphorylated proteins all buffers were supplemented with 0.1 mM VO_3^- and 10 mM KF, and the final concentration of $MgCl_2$ was brought to 10 mM immediately after homogenization. Solubilized samples (60 mg protein) were added to antibodies (2 ml antiserum), which had been pre-coupled to Protein A-Sepharose beads (10 ml), and mixed at 4°C for one hour. When immunoprecipiating phosphorylated proteins from PNS a 10:1 volume ratio of immunoprecipitation buffer (IB; 50 mM Tris, 150 mM KCl, 1% NP40, 10 mM $MgCl_2$, 0.1 mM VO_3^-, 10 mM KF, pH 7.4) was added directly to the PNS and mixed for 30 min at 4°C, IB was then also used, instead of RTB, for washing immunoprecipitates. Insoluble material was removed from ^{32}P-labeled samples by centrifuging at high speed in a microfuge for 2 min.

2.4. *Electrophoresis and [^{32}P]GTP-overlay*

SDS-PAGE was carried out according to the system of Laemmli [20], using 12.5% gels. Labeled proteins were visualised by fluorography; gels with ^{35}S-labeled proteins were treated with Enhance (NEN) and intensifying screens were used for ^{32}P-labeled proteins. Gels were exposed for 24 h to 1 week. For two-dimensional gel electrophoresis, samples were run on IEF tube gels, with a linear pH gradient between pH 4.5 and 7.4, and then on 15% acrylamide second dimension resolving gels as previously described [21].

GTP-overlay was as previously described [4]. Briefly, after SDS-PAGE, the gels were equilibrated in 20% glycerol, 50 mM Tris-HCl, pH 7.5 and the proteins then transfered to nitrocellulose at 50 mA for 16 h (Transfer buffer; 3 mM Na_2CO_3, 10 mM $NaHCO_3$, pH 9.9). The nitrocellulose was then rinsed in binding buffer (BB; 50 mM NaH_2PO_4, 10 mM $MgCl_2$, 2 mM DTT, 0.3% Tween 20, 4 mM ATP, pH 7.5) and incubated for 2 h with [α-^{32}P]GTP (2 mCi/ml) in BB. The nitrocellulose was then washed extensively with BB, dried and exposed to film with an intensifying screen.

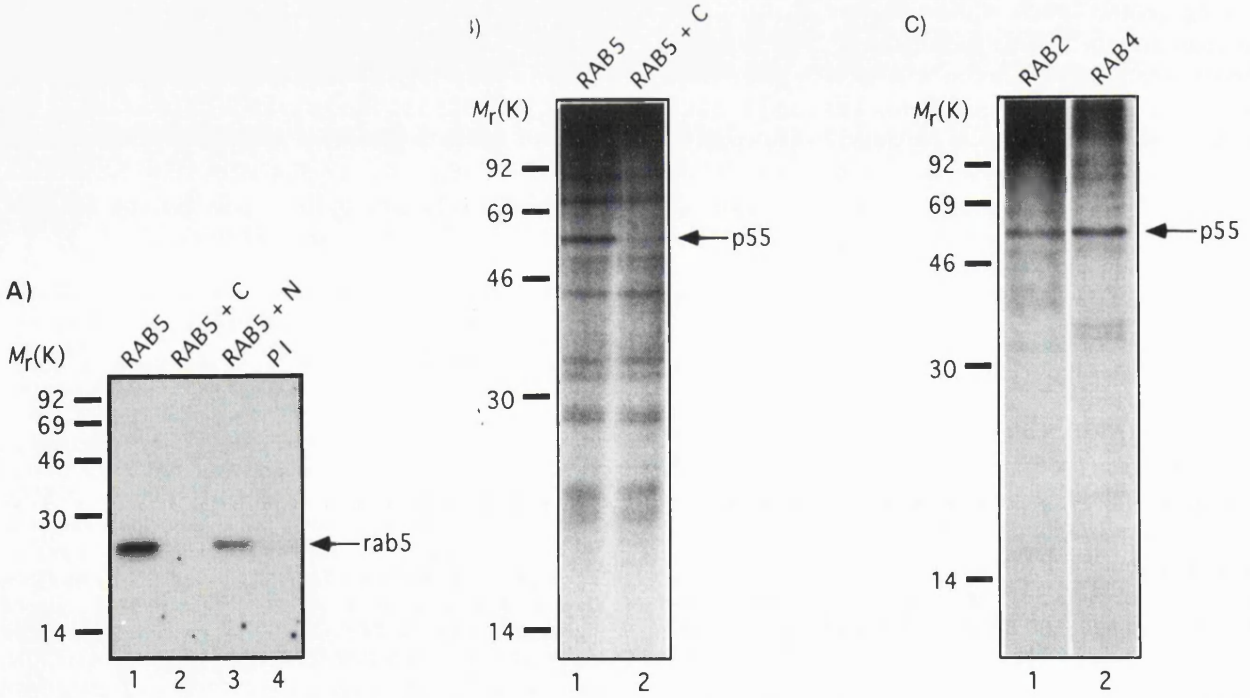


Fig. 1. Rab proteins are complexed with a 55 kDa phosphorylated protein. (A) The Rab5 C-terminal antiserum is specific, as shown by [^{32}P]GTP overlay after immunoprecipitation from membranes. Lane 1, rab5 antiserum; lane 2, rab5 antiserum with C-terminal rab5 peptide; lane 3, rab5 antiserum with N-terminal rab5 peptide; lane 4, preimmune sera. (B) and (C) Immunoprecipitation from post-nuclear supernatants (PNS), prepared from [^{32}P]orthophosphate labeled cells, with anti-rab antibodies. (B) lane 1, rab5 antiserum; lane 2, rab5 antiserum with C-terminal rab5 peptide. (C) lane 1, rab2 antiserum; lane 2, affinity purified rab4 antibodies. Molecular weight markers are indicated.

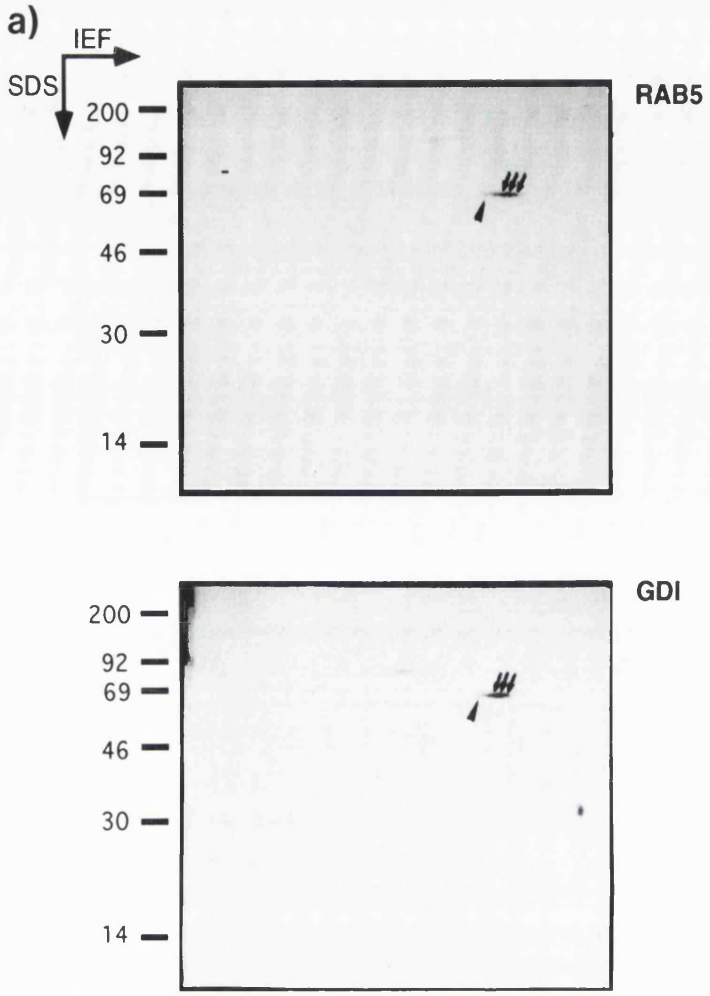
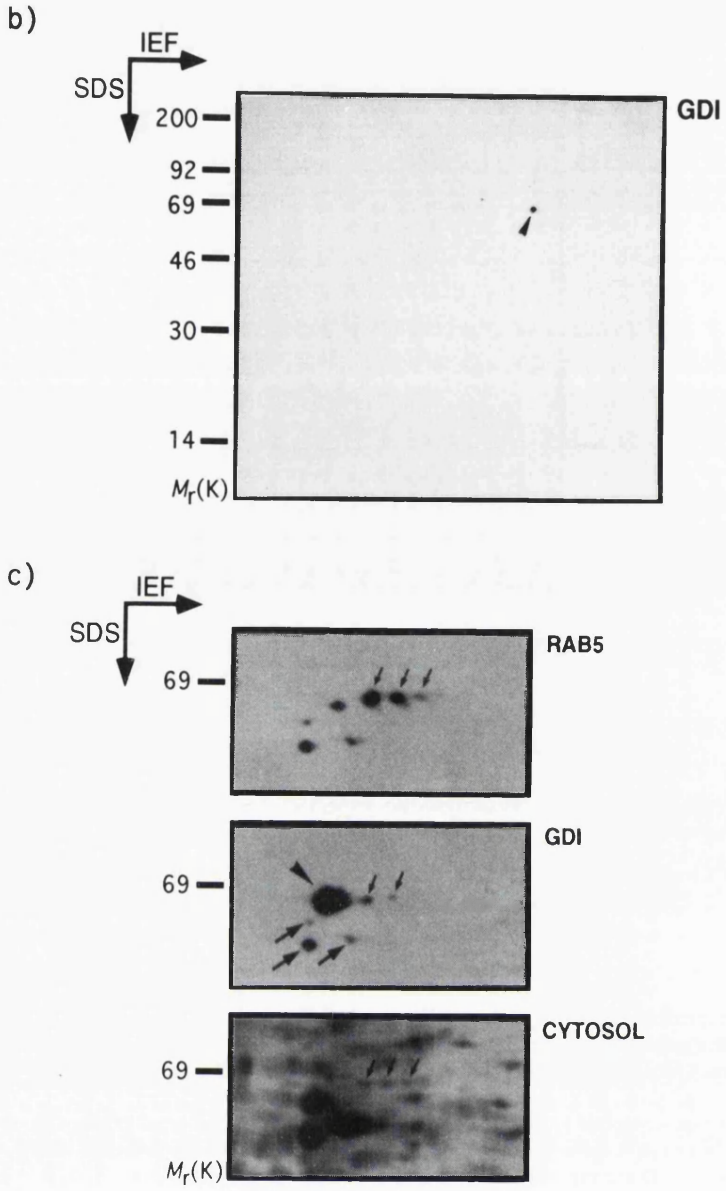


Fig. 2. 2D gel analysis of immunoprecipitates from cytosol of [^{32}P]orthophosphate (a) and [^{35}S]methionine (b and c) labeled BHK cells. (a) Immunoprecipitation with antibodies against rab5 (upper panel) or GDI (lower panel). (b) Immunoprecipitation with antibodies against GDI. (c) Sections of 2D gels, showing the area where GDI and its isoforms migrate, obtained after immunoprecipitation with antibodies against rab5 (upper panel) or after longer exposure of the gel shown in (b, middle panel). The lower panel shows the pattern of total cytosolic proteins seen in the same area of our 2D gels. The major isoform recognized by anti-GDI antibodies is indicated with an arrowhead and the other isoforms indicated with small arrows. The large arrows indicate major contaminants seen in all of the gels. The apparent molecular weight of p55/GDI is higher on our 2D gels (approx. 69 kDa) than on our 1D gels (approx. 55 kDa), we refer to it throughout this paper by its later apparent molecular weight.

3. RESULTS AND DISCUSSION

Fig. 1A shows that rab5 is specifically immunoprecipitated from BHK extracts by our C-terminal antiserum, and that immune complex formation is competed by the peptide against which the serum was raised. We then show that, when the same antiserum was used to immunoprecipitate from PNS prepared from ^{32}P-labelled cells, a phosphorylated protein of 55 kDa (p55) was specifically co-immunoprecipitated, although rab5 itself was not detectably phosphorylated (Fig. 1B). By virtue of its mobility in 1D gels, we suspected that p55 may be GDI. Since it has been proposed that essentially all cytosolic rab proteins are complexed to this protein [15,17], we tested antibodies specific for other rab proteins. Antibodies against rab2 and rab4 both immunoprecipitated phosphorylated p55 (Fig. 1B). Although rab4 co-localises with rab5 on endosomes [8], it is significant that rab2 specifically localises to organelles of the early biosynthetic pathway [2,22].

Next we used high resolution 2D gel electrophoresis to compare the physical characteristics of p55 with those of GDI. Cytosol was prepared from ^{32}P-labeled cells and immunoprecipitations carried out using either the anti-rab5 antibodies or an antibody raised against a peptide from the C-terminal region of GDI. Autoradiographs of the two immunoprecipitates were then compared (Fig. 2a). The isoelectric focusing of p55 indicates the existance of several phosphorylation states (indicated by arrowheads in Fig. 2a and 2b), and, significantly, the anti-GDI antibody recognizes a protein with the same physical characteristics. Phosphoamino acid analysis, after acid hydrolysis of p55, showed that serine residues were phosphorylated (not shown).



In order to investigate whether non-phosphorylated p55/GDI is also complexed with rab5 in cytosol, we repeated the immunoprecipitation experiments using cytosol prepared from cells metabolically-labeled with [^{35}S]methionine/cysteine. After immunoprecipitation with the anti-GDI antibody, a single polypeptide was revealed in 2D gels, which must be an unphosphorylated form of GDI, as its mobility is slightly more alkaline than any of the phosphorylated forms immunoprecipitated by the same antibody (compare with Fig. 2a). Upon longer exposure of this gel the phosphorylated forms of GDI become evident (Fig. 2c, middle panel). In contrast the anti-rab5 and anti-rab4 antibodies almost exclusively co-immunoprecipitated these phosphorylated forms of GDI (shown for rab5, Fig. 2c, upper panel). When we treated the ^{35}S-labelled anti-rab5 immunoprecipitate with alkaline phosphatase prior to electrophoresis, these more acidic forms were shifted back to the position of unphosphorylated GDI (not shown). These experiments establish that cytosolic rab5, and other rab proteins, predominantly interact with the phosphorylated forms of cytosolic GDI.

One surprising result of the above experiments was the observation that phosphorylated forms of p55/GDI were more effectively precipitated by anti-rab protein antibodies than by the anti-GDI antibody. In fact two-dimensional gel analysis of the complete cytosol (Fig. 2c, bottom panel), shows that the phosphorylated forms of GDI are much more abundant than the unphosphorylated form. We presume that the GDI antibody does not efficiently immunoprecipitate the phosphorylated GDI because the C-terminal epitope recognized by the antibody is masked by phosphorylation or by association with rab proteins.

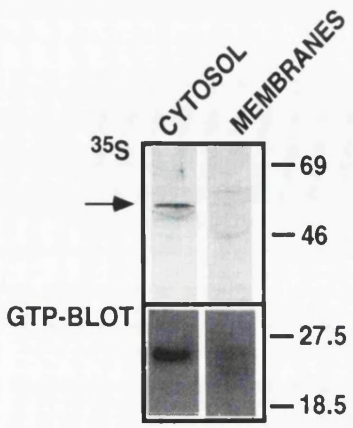


Fig. 3. GDI is associated with GTP-binding proteins in cytosol and on membranes. (a) Immunoprecipitation of ^{35}S-labeled proteins from cytosol or membranes by GDI antibodies. The position of GDI is indicated. (b) Visualization of small GTP-binding proteins by [^{32}P]GTP overlay following immunoprecipitation with GDI antibodies from membranes and cytosol of unlabeled cells. Only the 18–30 kDa range is shown.

When we repeated these immunoprecipitation experiments using membrane fractions in place of cytosol, we were unable to detect any phosphorylated p55/GDI (data not shown), although ≈ 10% of the total immunoprecipitable [^{35}S]methionine/cysteine-labeled GDI is membrane associated (Fig. 3). In contrast, approximately 80% of the rab5 protein is membrane associated [3]. We found that a significant fraction of the membrane associated form of GDI is complexed to rab proteins, as evidenced by [^{32}P]GTP overlay of small GTP-binding proteins after co-immunoprecipitation with anti-GDI antibodies (Fig. 3). Altogether, our data confirm that the cytosol contains a small proportion of rab proteins but the bulk of p55/GDI [3,17], and shows that these cytosolic rab proteins interact with a phosphorylated form of GDI. In contrast the fraction of membrane associated rab proteins which are complexed with p55/GDI interact with its unphosphorylated form (Fig. 4a).

Since its discovery it has been apparent that GDI function must be regulated. The involvement of post-translational modifications has been postulated, but remained obscure [16,23]. Phosphorylation/ dephosphorylation as reported here represents a strong candidate for fulfilling this role and, in Fig. 4, we depict a simple scheme by which this mechanism may regulate the association of rab proteins with membranes. We propose that the specific association of rab proteins with membranes is initiated by interaction of the cytosolic, phosphorylated GDI–rab complex with a membrane associated protein, presumably a nucleotide exchange factor. Exchange of GDP for GTP would promote dissociation of the GDI–rab complex, due to the vastly reduced affinity of GDI for the GTP bound form of the rab protein [24], thus the lipoyl moiety of the rab protein would be freed to insert into the adjacent membrane. In this way the rab protein would be delivered to the membrane in its active conformation. Following GTP hydrolysis, the GDP-bound rab protein, by associating with free GDI, would be extracted from the membrane. Phosphorylation of GDI would prevent reinsertion of the rab protein into the membrane, except by interaction with the specific exchange factor.

This model is consistent with properties of the unphosphorylated form of GDI reported by other groups; namely, inhibition of GDP dissociation and promotion of rab dissociation from the membrane [17,25]. Our model proposes that rab proteins are presented to the appropriate membrane in a complex with GDI, this makes comprehensible a study by Araki et al. [24], who have shown that purified rab3a will non-specifically associate with membranes (e.g. mitochondria, erythrocytes). A cycle of membrane association and dissociation is implicit in rab protein involvement in vectorial membrane transport [10], and we predict that GDI will not be associated with membranes of vesicular intermediates of membrane transport, but will be found on both donor and acceptor organelles. Although we have not so far localised the kinase and the phosphatase activities, we have supposed them to be cytosolic; in this configuration the homeostatic potential of GDI is most

a)

	CYTOSOL	MEMBRANES
GDI	80-90% RAB-GDI[P]> GDI	10-20% RAB-GDI > (GDI)
RAB 5	10-20% RAB5-GDI[P]> RAB5	80-90% RAB5 > RAB5-GDI

b)

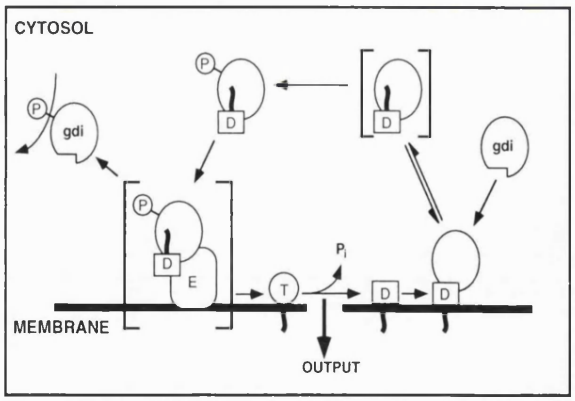


Fig. 4. (a) Summary of the distribution of GDI and rab5 complexes in membranes and cytosol (see text for refs.). (b) Model for the regulation of rab protein association with membranes (see text for explanation). Complexes of GDI with rab protein which have not been detected, and correspond to the putative intermediates, are shown in parenthesis. D, GDP-bound rab protein; E, exchange protein; GDI, guanine nucleotide dissociation inhibitor; P, phosphate; P_i, inorganic phosphate; T, GTP-bound rab protein.

simply realised. In conclusion, our studies suggest that GDI serves as universal regulator of membrane transport, perhaps controlling both the specificity and the directionality of the rab protein cycling pathway.

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The N-terminal domain of a rab protein is involved in membrane-membrane recognition and/or fusion

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Proteins of the YPT1/SEC4/rab family are well documented to be involved in the regulation of membrane transport. We have previously reported that rab5 regulates endosome-endosome recognition and/or fusion *in vitro*. Here, we show that this process depends on the rab5 N-terminal domain. Treatment of early endosomal membranes at a low trypsin concentration essentially abolished fusion and cleaved rab5 to a 1 kDa smaller polypeptide. Two-dimensional gel analysis suggested that rab5 is one of the few, if not the only, polypeptides cleaved by trypsin under these conditions. Whereas endosome fusion could be stimulated by cytosol prepared from cells overexpressing rab5 (and thus containing high amounts of the protein), this stimulation was abolished by trypsin-treatment of the cytosol. Trypsin-treated cytosol prepared from mock-transfected cells, which contains very low amounts of rab5, showed no inhibitory activity indicating that rab5 is the target of trypsin in these experiments. Purified rab5 prepared after expression in *Escherichia coli* was treated with trypsin, which cleaved the protein at the N-terminus. A synthetic peptide of rab5 N-terminal domain inhibited endosome fusion in our cell-free assay. A version of the same peptide truncated at the N-terminus or a peptide of rab3 N-terminal domain were without effects. Altogether, these observations suggest that the N-terminal domain of rab5 is involved in the process of early endosome recognition and/or fusion, presumably because it interacts with another component of the transport machinery.

Key words: endosome recognition and fusion/membrane transport/N-terminal/rab5

Introduction

The involvement of monomeric GTP-binding proteins, homologous to the protein encoded by the proto-oncogene *ras*, in the regulation of membrane transport is now well established (for reviews, see Goud and McCaffrey, 1991; Balch, 1992; Gruenberg and Clague, 1992; Pfeffer, 1992; Zerial and Stenmark, 1993). At least two families of these proteins, Sar/ARF and Ypt1/Sec4/rab, have been implicated in transport. Amongst proteins of the former family, Sar1p is required for vesicle formation in yeast (Rexach and Schekman, 1991; Oka *et al.*, 1991), and an ARF protein is a component of non-clathrin-coated vesicles in mammalian cells (Serafini *et al.*, 1991). More is known about the role of proteins of the YPT1/SEC4/rab family, which we will refer to as rab proteins. Several rab proteins have been shown to be required at specific steps of membrane transport, both in yeast (Goud *et al.*, 1988; Segev *et al.*, 1988) and in mammalian cells (Gorvel *et al.*, 1991; Plutner *et al.*, 1991; Bucci *et al.*, 1992; Lombardi *et al.*, 1993). Moreover, every member of that family that has been localized exhibits a specific subcellular distribution (Goud and McCaffrey, 1991; Gruenberg and Clague, 1992). Since rab proteins, by analogy with other GTP-binding proteins, are believed to undergo a conformational change upon GTP hydrolysis and thereby to act as a molecular 'switch', it has been proposed that they mediate membrane targeting (Bourne, 1988; Bourne *et al.*, 1990). This proposal is consistent with studies of SEC4 (Goud *et al.*, 1988) and YPT1 in yeast (Rexach and Schekman, 1991; Oka *et al.*, 1991; Segev, 1991). However, the precise function of rab proteins in membrane transport remains unclear.

Information on the structural organization of rab proteins has been obtained by comparision with the structure of ras (deVos *et al.*, 1988; Pai *et al.*, 1989; Tong *et al.*, 1989), and by mutagenesis. In their hypervariable C-terminal region (Valencia *et al.*, 1991), rab proteins contain a signal which is both necessary and sufficient for their association with the correct intracellular membrane (Chavrier *et al.*, 1991). Membrane association itself requires the prenylation of one or more C-terminal cysteine residues present in a motif functionally analogous to the ras CAAX box (Evans *et al.*, 1991). Other identified regions include the highly conserved GTP-binding motifs and the so-called 'effector' domain (Valencia *et al.*, 1991). By analogy with ras, the effector domain is believed to interact with a GAP protein, thereby stimulating the low endogenous GTPase activity of rab proteins. Effector domain peptides have, in fact, been shown to inhibit transport in the biosynthetic pathway (Plutner *et al.*, 1990). Proteins have been identified that can escort rab proteins in the cytosol (GDI), stimulate GTP hydrolysis or facilitate GDP/GTP exchange (Huang *et al.*, 1990; Sasaki *et al.*, 1990; West *et al.*, 1990; Burstein *et al.*, 1991; Tan *et al.*, 1991; Burstein and Macara, 1992). Other proteins, which include putative components of the transport machinery, are expected to interact with rab proteins. In fact, a protein interacting with rab3a/smg-25a has been identified (Shirataki *et al.*, 1992). However, it is not clear which regions of rab proteins would be involved in these interactions and except for the GTP-binding domain, the effector domain and the C-terminus, nothing is known about the role played by other regions of rab proteins.

Using an established cell-free assay, we have previously shown that rab5 is required for the fusion of early endosomes

in vitro (Gorvel *et al.*, 1991). In the present paper, we report that the N-terminal domain of rab5 is necessary for rab5 function in endosome–endosome recognition and/or fusion, presumably because this domain interacts with another component of the transport machinery.

Results and discussion

Early endosomes exhibit a striking tendency to undergo lateral (homotypic) fusion with each other *in vitro* (Davey *et al.*, 1985; Gruenberg and Howell, 1986, 1987, 1989; Braell, 1987; Diaz *et al.*, 1988; Woodman and Warren, 1988). This process is highly specific (Gruenberg *et al.*, 1989; Bomsel *et al.*, 1990; Aniento *et al.*, in press) and is regulated by NSF (Diaz *et al.*, 1989), heterotrimeric G-proteins (Colombo *et al.*, 1992), rab5 (Gorvel *et al.*, 1991), phosphorylation–dephosphorylation events (Tuomikoski *et al.*, 1989; Thomas *et al.*, 1992; Woodman *et al.*, 1992) and, possibly, annexin II (Emans *et al.*, 1992). The fusion process can be inhibited after trypsin treatment of the membranes (Diaz *et al.*, 1988; Woodman and Warren, 1988), and a high molecular weight trypsin-sensitive protein was shown to be required for the fusion of macrophage endosomes (Colombo *et al.*, 1991). Our initial goal was to use the fusion assay we have established (Gruenberg and Howell, 1986, 1987; Gruenberg *et al.*, 1989) to identify components of the early endosomal recognition/fusion machinery that may be sensitive to trypsin. Briefly, fusion is reconstituted in the assay by mixing two early endosomal fractions, one containing internalized avidin and the other internalized biotinylated horseradish peroxidase (bHRP). The avidin–bHRP complex formed upon fusion is then extracted in detergent and immunoprecipitated with antibodies against avidin, and the enzymatic activity of HRP quantified.

Trypsin sensitivity of early endosome fusion in vitro

To titrate the amount of trypsin required for inhition of fusion, early endosomal fractions (avidin- and bHRP-labeled) were pre-incubated separately, for 30 min at 4°C, in the presence of TPCK-treated trypsin. The reaction was arrested by the addition of excess soybean trypsin inhibitor. Control experiments were treated identically, except that trypsin and the inhibitor were added simultaneously. Treated fractions were then used in the fusion assay. As shown in Figure 1, early endosome fusion was essentially abolished by trypsin at a low concentration (~0.5 μg/ml), corresponding to a ratio of ~1:1000 (w/w) trypsin to the total amount of endosomal membrane protein present. Decreased fusion activity could not be explained by leakage of the fusion markers resulting from damage to endosomal membranes, since endosomes remained ≥85% latent at the end of the reaction. When only the bHRP-labeled fraction was treated with trypsin (instead of both the avidin- and the bHRP-labeled fractions), fusion was also inhibited at the same trypsin concentration (Figure 1).

Analysis of trypsin-sensitive polypeptides

In an attempt to identify the trypsin-sensitive polypeptide(s) responsible for inhibition of the fusion process, we analyzed the polypeptide composition of early endosomal membranes by two-dimensional gel electrophoresis. Cells were metabolically labeled for 16 h with [^{35}S]methionine, and then early endosomal fractions were prepared using a flotation gradient followed by immunoisolation (see

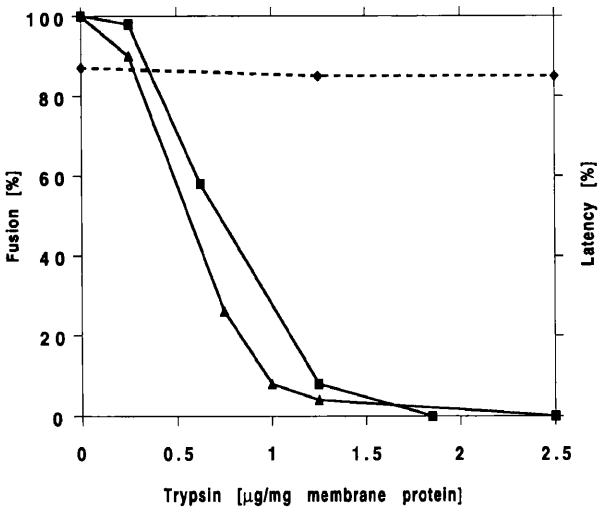

Fig. 1. Trypsin-sensitivity of endosome fusion *in vitro*. Both fractions (avidin and bHRP-labeled) were separately incubated for 30 min on ice with TPCK-treated trypsin (solid squares). Alternatively, only the bHRP-labeled fractions were treated with trypsin (solid triangles). Trypsin activity was arrested by adding soybean inhibitor (0.1 mg inhibitor/mg membrane proteins in the assay) and the fractions were used in the cell-free fusion assay. All values are expressed as a percentage of the control fusion (membranes incubated simultaneously with the inhibitor and 2.5 μg trypsin/mg membrane protein). Under all conditions, the fluid phase marker used to measure fusion (bHRP) retained its latency (~85% of the total present in the fraction) during the course of the experiment (solid diamonds). The amounts of TPCK-treated trypsin are expressed in μg per mg membrane protein present in the corresponding fractions.

Gruenberg and Gorvel, 1992, and references therein). Early endosomes were prepared with a 75-fold enrichment over the homogenate, and the *in vitro* fusion activity of these fractions was very high, corresponding to ~60% mixing of the markers following fusion in the assay (Thomas *et al.*, 1992; Emans *et al.*, 1993).

The immunoisolated fractions were treated with TPCK-trypsin as above, at the lowest concentration required to inhibit fusion [1:1000 (w/w) trypsin:endosomal protein], and the reaction was arrested with soybean trypsin inhibitor. The fractions were then analyzed using high resolution two-dimensional gels (Thomas *et al.*, 1992; Emans *et al.*, 1993) and autoradiography. Comparision of autoradiograms from several experiments showed that the polypeptide patterns of the trypsin-treated samples were essentially identical to those of the untreated controls (even after longer exposure times). However, the intensity of two low molecular weight polypeptides was changed by the trypsin treatment. In Figure 2, the enlarged lower half of a typical autoradiogram shows that the intensity of one labeled polypeptide decreased after treatment, whereas the intensity of a faster migrating (~1 kDa smaller), slightly more acidic polypeptide, which remained membrane-associated, increased.

Rab5 associated with early endosomal membranes is trypsin-sensitive

The only polypeptide sensitive to trypsin (Figure 2) under conditions inhibiting fusion (Figure 1) exhibited a molecular weight similar to that of the low molecular weight GTP-binding protein rab5, which is known to be required for early endosome fusion (Gorvel *et al.*, 1991). Therefore, we investigated the trypsin sensitivity of rab5 associated with early endosomal membranes.

Early endosomal fractions were prepared using the

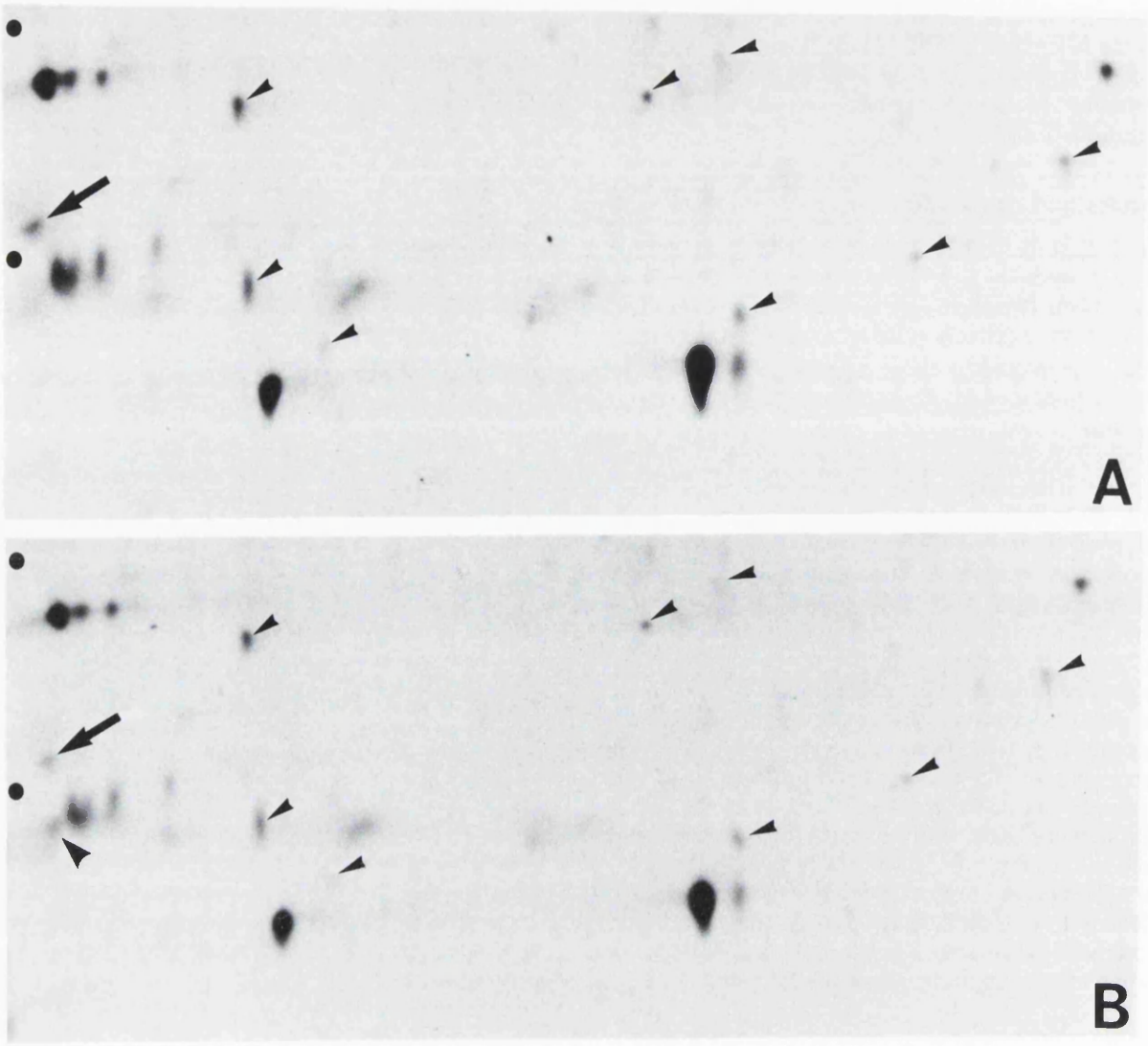

Fig. 2. Two-dimensional gels of trypsin-treated endosomal fractions. Immunoisolated early endosomal fractions were prepared from cells metabolically labeled with [^{35}S]methionone and treated with trypsin under conditions which inhibit fusion [corresponding to 1:1000 (w/w) trypsin:membrane protein] as in Figure 1. (**A**) Control: membranes were incubated simultaneously with trypsin and the inhibitor; (**B**) trypsin and inhibitor were added sequentially. The samples were analyzed by two-dimensional gel electrophoresis followed by autoradiography. Isoelectric focusing was from left (alkaline pH) to right (acidic pH) and the second dimension from top (high molecular weight) to bottom (low molecular weight). Only the lower halves of the gels are shown (molecular weight markers of 30 and 46 kDa are indicated). The arrow indicates the only polypeptide that exhibited a detectable decrease in intensity after the treatment (presumably rab5), and the large arrowhead the only spot that exhibited a detectable increase in intensity (presumably a cleaved form of rab5). Small arrowheads indicate examples of polypeptides not affected by the treatment.

flotation gradient, treated with trypsin as above [1:1000 (w/w) trypsin:endosomal protein], recovered by centrifugation and analyzed by Western blotting using an antibody raised against a C-terminal epitope of rab5 (see Chavrier *et al.*, 1990a). As shown in Figure 3A, the membrane-associated form of rab5 was cleaved by trypsin. The cleavage product, which was still recognized by the antibody after Western blotting (Figure 3) or immunoprecipitation (not shown), was ~1 kDa smaller, corresponding to a shift in mobility similar to that observed in two-dimensional gels (Figure 2). In addition, rab5 (Figure 3A) and the metabolically labeled trypsin-sensitive polypeptide (indicated by an arrow in Figure 2) exhibited a very similar behavior; in both cases, although a minor amount of cleaved protein was already detected in the control (presumably reflecting cleavage occurring during preparation of the membranes), only 50% of the total amount of protein was cleaved by trypsin and the cleavage product remained membrane-associated (Figure 2B and Figure 3A, lane 2). These experiments show that rab5 can be cleaved by trypsin and suggest that the target of trypsin in our assay is the rab5 protein.

The rab5 protein may be the target of trypsin

As rab5 was a good candidate for the trypsin-sensitive polypeptide, we determined its mobility in our two-dimensional gels. To establish its position unambigously, rab5 was overexpressed using the T7 RNA polymerase recombinant vaccinia virus system (Chavrier *et al.*, 1990a; Gorvel *et al.*, 1991) and early endosomal membranes were prepared using the flotation gradient. These fractions were then treated with trypsin as above [1:1000 (w/w) trypsin:endosomal protein], recovered by centrifugation and analyzed by two-dimensional gel electrophoresis. The polypeptides were then transferred to nitrocellulose and overlaid with [α-^{32}P]GTP (Lapetina and Reep, 1987; Bucci *et al.*, 1992; Huber *et al.*, 1993), in order to reveal the position of small GTP-binding proteins.

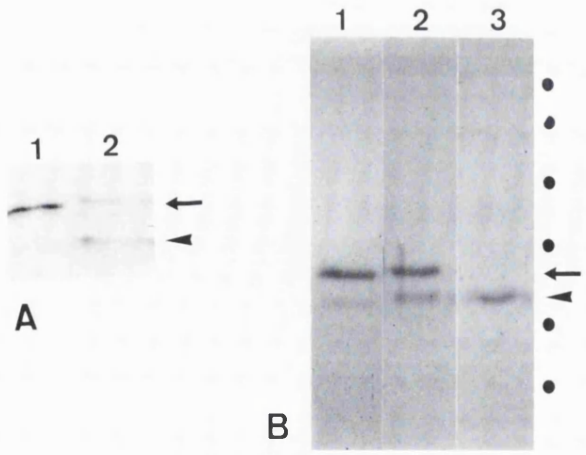

Fig. 3. Western blotting of endogenous rab5 and Coomassie-staining of rab5 produced in *E.coli*. (**A**) An immunoisolated endosomal fraction was treated without (lane 1) or with trypsin (lane 2) as in Figures 1 and 2, and then analyzed by electrophoresis in 12.5% acrylamide gels and Western blotting using the anti-rab5 antibody. (**B**) The rab5 protein produced in *E.coli* was purified and then treated as in Figures 1–3, using 0 (lane 1), 0.13 (lane 2) or 1.3 (lane 3) μg trypsin/mg rab5 protein. The molecular weight markers are indicated (14, 30, 46, 69, 92 and 200 kDa). In both panels, arrows indicate the position of intact rab5 and arrowheads the cleaved form of rab5. The cleaved form of rab5 exhibited the same apparent mobility (~1 kDa smaller than rab5) in panels A and B.

Figure 4 shows the enlarged lower half of a typical blot, comparable to the autoradiogram shown in Figure 2. In the absence of trypsin, several GTP-binding proteins could be detected in the fraction. As expected after overexpression, the rab5 spot, which migrates at the same position in the absence of overexpression (not shown), is most heavily labeled with [α-^{32}P]GTP. A comparision between autoradiograms showed that the mobility of overexpressed rab5 was identical to that of the trypsin-sensitive polypeptide observed after metabolic labeling in Figure 2. The other GTP-binding proteins present in the fractions have not yet been identified, and their pattern differs significantly from those obtained with other subcellular fractions (Huber *et al.*, 1993). They presumably include rab proteins involved in other steps of membrane transport connected to early endosomes (van der Sluijs *et al.*, 1991). After trypsin-treatment, overexpressed rab5 was cleaved to a slightly more acidic and ~1 kDa smaller polypeptide, while other GTP-binding proteins present in the fraction were not affected. The overexpressed rab5 appeared more sensitive to trypsin than endogenous rab5 (Figure 3A), although both are clearly membrane-associated, suggesting that the latter is partially protected, possibly via interactions with other proteins. The trypsin-treated form of rab5, which retained the capacity to bind GTP on blots, migrated at the same position as the metabolically labeled product of trypsin cleavage identified

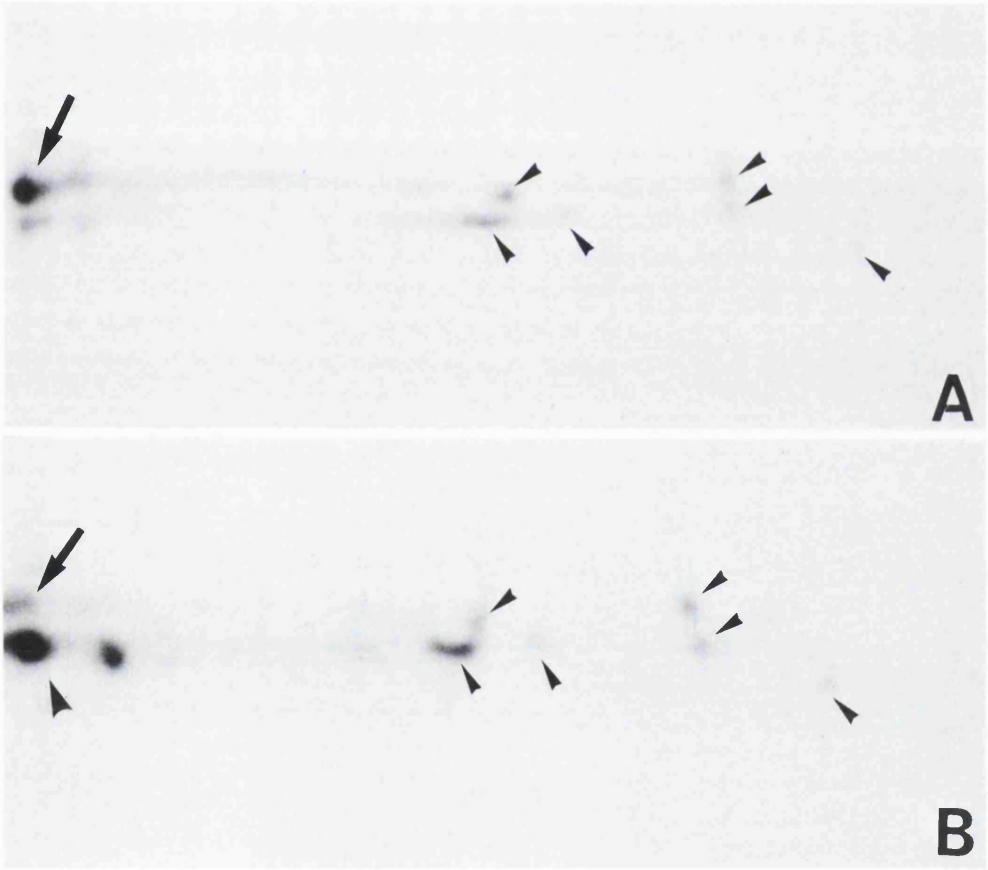

Fig. 4. GTP overlay after rab5 overexpression. Rab5 was overexpressed using the vaccinia/T7 RNA polymerase system and early endosomal fractions were prepared using the flotation gradient (Gorvel *et al.*, 1991). The fractions were then treated as in Figures 1–3. Two-dimensional gels are as in Figure 2. (**A**) Trypsin and inhibitor were added simultaneously; (**B**) trypsin and inhibitor were added sequentially. After treatment the fractions were analyzed on two-dimensional gels, transferred to nitrocellulose and overlaid with [α-^{32}P]GTP to reveal small GTP-binding proteins. The arrow indicates the position of intact overexpressed rab5 and the large arrowhead the cleavage product after trypsin treatment. As shown in panel B, the latter form still binds GTP. In this as in other experiments, we have observed that a fraction of the overexpressed rab5 protein migrates at a slightly more acidic position than the major spot indicated by the arrow (panel A). A similar spot is observed for the cleaved form of rab5 in panel B. Small arrowheads show the position of other small GTP-binding proteins present in the fraction, which were unaffected by trypsin.

in Figure 2. Altogether, these experiments demonstrate that rab5 is a target of trypsin in our assay.

Cytosolic rab5 is inactivated by trypsin

As a next step, we took advantage of our previous finding that cytosol prepared from cells overexpressing rab5 (and thus containing high amounts of the protein) stimulates endosome fusion *in vitro* (Gorvel *et al.*, 1991). In Figure 5, we show that this stimulation of fusion was abolished after pre-treatment of the cytosol with trypsin under the same conditions as used for the membranes [1:1000 (w/w) trypsin:cytosol protein]. As its membrane-associated form (Figures 3 and 4), rab5 was then cleaved but still recognized by our C-terminal antibodies (not shown). Addition of soybean trypsin inhibitor during the pre-treatment step blocked the effects of trypsin on fusion (Figure 5), and stimulation by cytosolic rab5 was then as effective as previously observed (Gorvel *et al.*, 1991). The inhibitory effect of trypsin on fusion activity depended on the rab5 protein alone, since trypsin treatment of cytosol prepared from mock-transfected cells, which contains very low amounts of rab5 (Gorvel *et al.*, 1991; Steele-Mortimer *et al.*, 1993), did not inhibit fusion.

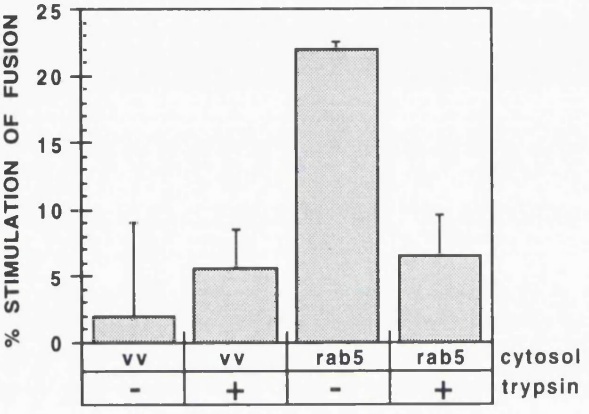

Fig. 5. The cytosolic form of rab5 is inactivated by trypsin. BHK cytosol prepared from cells overexpressing rab5 (rab5) or mock-transfected cells infected with vaccinia virus (vv) was pre-treated with trypsin (+), which was then inactivated with trypsin inhibitor, and then added to the fusion assay as described. In control experiments (−), cytosols were incubated with trypsin and trypsin inhibitor simultaneously. The stimulation of fusion is expressed as a percentage of the value obtained with rat liver cytosol only.

The N-terminus of rab5 is involved in endosome fusion

Cleavage of rab5 must have occurred near the C- or the N-terminus of the protein, since the cleavage product migrated only sightly faster in gels (~1 kDa). In addition, the cleaved form of rab5 remained membrane-associated, being recovered on endosomal membranes after flotation (Figures 3 and 4). Rab5, like other rab proteins, is associated with membranes via geranyl geranylation of a C-terminal cysteine (Kinsella and Maltese, 1991). Therefore, if trypsin cleaved the protein at a C-terminal site between the epitope of the anti-rab5 antibody (Figure 3A) and the geranyl geranylated cysteine, the cleavage product would have to bind to another membrane component in order to remain membrane-associated. This component would have to be relatively abundant, when compared with endogenous rab5, since the cleavage product remained membrane-associated even after ~5-fold overexpression of rab5 (Figure 4). Until now, we have not detected any such component after immunoprecipitation from endosomal membranes using anti-rab5 antibodies (with or without trypsin treatment), except for low amounts of the cytosolic escort protein GDI (Steele-Mortimer *et al.*, 1993). Alternatively the cleavage may have occurred at the N-terminus of the protein.

In order to characterize further the site of trypsin cleavage, rab5 was purified after expression in *Escherichia coli*. As with the endogenous and overexpressed rab5, a shift of ~1 kDa could be observed following trypsin treatment (Figure 3B). N-terminal sequencing of the cleavage product revealed that four amino acids had been removed by trypsin (see Figure 6). In none of our experiments did we detect additional cleavage products, and neither were other intermediates detected after cleavage of the endogenous

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rab3a   M A S A T D S R Y G Q K E S S D Q N F D Y M F K I L I I G N
rab3b   M A S V T D G K H G V K D A S D Q N F D Y M F K L L I I G N
rab4    - - - - - - - - - - - - - - - - M S E T Y D F L F K F L V I G N
rab6    - - - - - - - - - M S T G G D F G N P L R K F K L V F L G E
rab1    - - - - - - - - - - - M S S M N P E Y D Y L F K L L L I G D
rab2    - - - - - - - - - - - - - - - - M A Y A Y L F K Y I I I G D
rab5    - - M A N R↓G A T R P N G P N T G N K I C Q F K L V L L G E
rab8    - - - - - - - - - - - - - - - - M A K T Y D Y L F K L L L I G D
rab7    - - - - - - - - - - - - - - - - M T S R K K V L L K V I I L G D
rab9    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
rab10   - - - - - - - - - - - - - - - - M A K K T Y D L L F K L L L I G D
rab11   - - - - - - - - - - - M G T R D D E Y D Y L F K V V L I G D
rab4b   - - - - - - - - - - - - - - - - M A E T Y D F L F K F L V I G S
YPT1    - - - - - - - - - - - - - - - - M N S E Y D Y L F K L L L I G N
YPT3    - - - - - - - - - - - - - - M C Q E D E Y D Y L F K T V L I G D
SEC4    - - M S G L R T V S A S S G N G K S Y D S I M K I L L I G D
H-ras   - - - - - - - - - - - - - - - - - - - - - - M T E Y K L V V V G A
```

Fig. 6. N-terminal sequence of rab proteins. The N-terminal domains of different rab proteins were aligned according to Chavrier *et al.* (1990b). The sequences of the rab3b and rab5 N-terminal domain peptides are underlined. A small arrow indicates the trypsin cleavage site of *E.coli* rab5, as determined by N-terminal sequencing of the band recovered from the gel shown in Figure 3, lane 3.

protein present on early endosomal membranes (Figures 2–4), suggesting that cleavage had occurred at a single site.

These experiments suggest that the rab5 N-terminal domain might be involved in the process of endosome recognition and/or fusion. In order to test this hypothesis, we synthesized a peptide of 17 amino acids corresponding to the N-terminal domain of rab5, and tested this peptide in our endosome fusion assay. As a control, we used a peptide corresponding to the N-terminal domain of rab3b, a small GTP-binding protein associated with synaptic vesicles (Fischer von Mollard *et al.*, 1991) and possibly involved in secretion in acinar cells (Padfield *et al.*, 1992). This peptide was selected as a control, because, when compared with most other monomeric GTP-binding proteins (Chavrier *et al.*, 1990b; Valencia *et al.*, 1991), both rab3 and rab5 contain relatively long N-terminal extensions forming an additional domain (see Figure 6). As shown in Figure 7, fusion was inhibited in our assay by relatively low concentrations of the rab5 N-terminal peptide under conditons where the rab3b N-terminal peptide or a rab2 C-terminal peptide were without effect. Moreover, a truncated version of the rab5 N-terminal peptide, lacking the four N-terminal residues removed by trypsin (see Figure 6), had no effect on endosome fusion (not shown). Several peptides corresponding to other regions of rab5 have also been shown to be ineffective (Lenhard *et al.*, 1992). Our findings agree well with recent studies of rab5/rab6 chimeras showing that the N-terminal domain of rab5 is absolutely required for stimulation of endocytosis *in vivo* (Stenmark *et al.*, 1994). Until now, it has been difficult to demonstrate that a structural domain of rab proteins [other than the GTP-binding motifs and the effector domain in some cases, see Plutner *et al.* (1990)] is directly involved in the regulation of membrane transport. In fact, canine rab5 can rescue ypt5-disrupted *Schizosaccharomyces pombe* cells, despite little apparent sequence homology in the variable regions of the protein (Armstrong *et al.*, 1993). Our data strongly indicate that the rab5 N-terminus is necessary for rab5 function in mammalian cells, presumably because it is required for interactions with other components of the machinery controlling early endosome recognition/fusion.

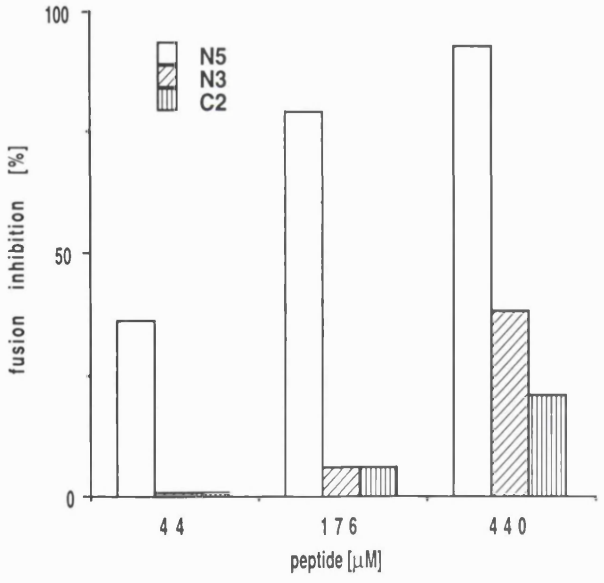

Fig. 7. Peptide inhibition of early endosome fusion. *In vitro* fusion of early endosomes was carried out as described in the legend to Figure 1. Before the assay, the mixture was preincubated on ice in the presence of either the rab5 (N5) or the rab3b (N3) N-terminal domain peptide (see Figure 6) at the indicated concentrations, and the peptides remained present throughout the assay. As an additional control, a peptide of the C-terminal domain of rab2 (C2) was used (Chavrier *et al.*, 1990a).

Materials and methods

Cells and reagents

Baby hamster kidney (BHK) cells were grown, maintained and metabolically labeled with [^{35}S]methionine for 16 h as previously described (Gruenberg *et al.*, 1989). The monoclonal antibody against a C-terminal peptide of rab5 was a kind gift of Angela Wandinger-Ness (Northwestern University, Evanson, IL) and David Vaux (Sir William Dunn School of Pathology, Oxford University). The monoclonal antibody against a C-terminal peptide of the spike glycoprotein G of vesicular stomatitis virus was a kind gift of Thomas Kreis (University of Geneva; Kreis, 1986). The peptides of canine rab2 C-terminal domain, human rab3 N-terminal domain and canine rab5 N-terminal domain were synthesized by Dominique Nalis (EMBL, Heidelberg); all were >98% pure, as measured by HPLC analysis.

Cytosol preparation

Cytosol was prepared as previously described (Gorvel *et al.*, 1991; Aniento *et al.*, 1993) at protein concentrations of 24 mg/ml for rat liver cytosol, 10–15 mg/ml for BHK cytosol and 5–8 mg/ml for mock-transfected or transfected cells. Transfection with rab5 cDNA was carried out using the vaccinia/T7 polymerase system; cells were infected with virus for 30 min and then transfected for 8 h using DOTAP (Boehringer, Mannheim) as described by Bucci *et al.* (1992).

Fusion assay

The cell-free assay we have established (Gruenberg and Howell, 1986; Gruenberg *et al.*, 1989; Tuomikoski *et al.*, 1989; Bomsel *et al.*, 1990; Gorvel *et al.*, 1991; Thomas *et al.*, 1992; Emans *et al.*, 1993; Aniento *et al.*, in press) was used to measure the fusion between early endosomes. Briefly, avidin and bHRP were internalized separately into two cell populations, by fluid phase endocytosis for 5 min at 37°C. The cells were homogenized and early endosomal fractions prepared using a flotation gradient (Gorvel *et al.*, 1991; Thomas *et al.*, 1992; Emans *et al.*, 1993). In the assay, the avidin- and bHRP-labeled endosome fractions were then combined at 4°C in the presence of 5 mg/ml BHK cytosol, ATP and salts. When indicated, peptides were added and the mixture pre-incubated for 60 min at 4°C. In some experiments, we used 25 μl (0.6 mg) rat liver cytosol (Aniento *et al.*, 1993) complemented with 25 μl (125 μg) BHK cytosol prepared from cells overexpressing rab5 or from mock-transfected, vaccinia-infected cells. In all cases, fusion was then allowed to proceed for 45 min at 37°C. At the end of the reaction, the avidin–bHRP complex formed upon fusion was immunoprecipitated with an anti-avidin antibody in the presence of detergents. The extent of fusion was quantified by measuring the enzymatic activity of the bound bHRP.

Trypsin-treatment of early endosomal membranes or cytosol

The cell-free assay was used to quantify the fusion activity of trypsin-treated early endosomal membranes. Each fraction (~20 μg protein in 50 μl) labeled with either avidin or bHRP was separately treated with TPCK-treated trypsin at concentrations varying between 0.1 and 1.0 μg/ml (corresponding to 0.25–2.5 ng trypsin/μg protein) for 30 min at 4°C. Trypsin activity was then stopped by adding 1 μl of homogenization buffer (3 mM imidazole pH 7.4, 250 mM sucrose) containing 5 μg of soybean trypsin inhibitor and the mixture was further incubated for 30 min at 4°C. As a control, TPCK-trypsin and the inhibitor were added simultaneously to the fractions and the mixture incubated for 60 min at 4°C. These fractions were then tested in the fusion assay (see above). The latency of trypsin-treated early endosomes containing avidin or bHRP was measured as described (Gorvel *et al.*, 1991). BHK cytosol was treated with 2.5 ng trypsin/μg cytosolic protein for 30 min on ice; the enzyme was then inhibited with 5.0 ng soybean trypsin inhibitor and the mixture was incubated for a further 15 min. In control experiments, trypsin and soybean trypsin inhibitor were added simultaneously and the samples were incubated for 45 min.

Analysis of early endosomal polypeptides

In order to analyze the polypeptide composition of early endosomes after trypsin treatment, fractions were prepared using a combination of two previously established protocols, a flotation gradient and immunoisolation. Briefly, cells were metabolically labeled for 16 h with [^{35}S]methionine and the spike glycoprotein G of vesicular stomatitis virus was then implanted

into the plasma membrane by low pH-mediated fusion of the virus envelope with the plasma membrane (White *et al.*, 1980; Gruenberg and Howell, 1985, 1986). The G-protein was internalized into early endosomes for 5 min at 37°C (Gruenberg and Howell, 1987; Gruenberg *et al.*, 1989; Thomas *et al.*, 1992; Emans *et al.*, 1993) and the cells were homogenized. Early endosomes were first separated from the plasma membrane and late endosomes by flotation on a sucrose/D_2O step gradient (Gorvel *et al.*, 1991; Emans *et al.*, 1993), and then immunoisolated (Gruenberg and Howell, 1986, 1987; Gruenberg *et al.*, 1989; Thomas *et al.*, 1992; Emans *et al.*, 1993) using a solid support coated with an antibody against the cytoplasmic domain of the G-protein (Kreis, 1986). The immunoisolated fraction was then washed in PBS to remove unbound vesicles, and treated sequentially with TPCK-trypsin and soybean trypsin inhibitor under the same conditions as described above. The fractions were analyzed by high resolution two-dimensional gel electrophoresis and autoradiography.

Transfer to nitrocellulose blots and [α-^{32}P]GTP overlay

Small GTP-binding proteins separated by two-dimensional gel electrophoresis were transferred to nitrocellulose and detected by GTP overlay. The protocol for transfer and GTP overlay (Bucci *et al.*, 1992; Huber *et al.*, 1993) was modified from the method of Lapetina and Reep (1987). Briefly, the two-dimensional gels were washed twice for 15 min each in 50 mM Tris–HCl pH 7.5 containing 20% glycerol and electrophoretically transferred to nitrocellulose paper in 10 mM $NaHCO_3$/3 mM Na_2CO_3 pH 9.8. The nitrocellulose was (i) rinsed for 30 min in binding buffer (50 mM NaH_2PO_4 pH 7.5, 10 μM $MgCl_2$, 2 mM DTT, with 4 μM ATP as competing substrate), (ii) incubated for 120 min with [α-^{32}P]GTP (1 μCi/ml, specific activity 2903 Ci/mmol, 1 Ci = 37 GBq) and (iii) rinsed for 60 min with several changes of binding buffer. The nitrocellulose was then air-dried and [α-^{32}P]GTP-binding was visualized by autoradiography (24 h, −80°C) using Kodak X-Omat AR film with an intensifying screen. To determine the molecular masses, prestained SDS–PAGE molecular weight standards (Bio-Rad) were co-electrophoresed in the second dimension and transferred to nitrocellulose.

Purification of rab5 produced in E.coli

Canine rab5 protein was expressed in *E.coli* using the pET-vector expression system (Zaharaoui *et al.*, 1989; A.Wandinger-Ness and M.Zerial, in preparation). The cells were then lysed and rab5 was purified using a Q-Sepharose column, as described by Tucker *et al.* (1986). The protein was further purified by running a 0–0.5 M NaCl gradient through an S-Sepharose column equilibrated with 25 mM HEPES pH 6.8, 10 mM $MgCl_2$, 100 mM GDP. Peak fractions were pooled and buffer replaced with 50 mM Tris pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM GDP, on a PD-10 column.

Analytical techniques

We used the high resolution two-dimensional gel electrophoresis system established by Celis and his collaborators (Celis *et al.*, 1990), as in our previous studies (Thomas *et al.*, 1992; Emans *et al.*, 1993). Protein determination was according to Bradford (1976).

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