Regulation of the activity of integrins during the cell cycle

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SUMMARY

I compared the expression and activity of integrins in HeLa cells between interphase and mitosis. Cultures enriched in mitotic cells (>90%) were obtained by thymidine and nocodazole blocks. Early synchronisation experiments suggested that serum improved the rate of exit from metaphase in nocodazole-blocked cells. This was confirmed by quantification of post-metaphase cells at different times after release of the nocodazole block.

Immunoprecipitation and immunoblotting showed that HeLa cells express at least three integrin subunits, probably α_5, α_v and β_1. However, I could not detect $\beta 3$ subunits. Flow cytometry showed that integrins in HeLa cells were not expressed differently in mitosis and interphase. However, I found that, in spreading assays, cells arrested in metaphase with the microtubule inhibitor nocodazole were unable to spread on fibronectin, whereas control interphase cells spread normally in the presence of nocodazole. I could just detect spreading of mitotic cells on fibronectin if 1mM Mn^{2+} was present in the medium, or on glass surfaces in growth medium if the metaphase block was continued for 3-4 hours. Immunofluorescence showed that integrins were redistributed in this limited spreading, as well as in the process of respreading of non blocked, mitotic cells.

Analysis of fibronectin receptor activity using affinity chromatography of detergent extracts on the cell binding fragment of fibronectin showed that the mitotic receptor partially loses its activity. No phosphorylation of either mitotic or interphase receptors could be detected by immunoprecipitation from ^{32}P-labelled cells. I further investigated integrin phosphorylation and activity in cellular rounding caused by the phosphatase inhibitor calyculin A (CL-A), as it offered a possible model for cellular rounding at mitosis. Although fully spread HeLa cells did not respond to CL-A at concentrations reported to specifically stimulate CDK activity (i.e. 1-2nM), cellular rounding was achieved at ~50nM CL-A. Fibronectin receptor activity was lost from CL-A-treated cells, to a much larger extent than from mitotic cells, but again no phosphorylation was detected. Interestingly, low concentrations of CL-A (0.5-2nM) caused small but significant improvement of cell spreading, both on haemoglobin and on fibronectin, at the early stages (i.e. less than 30 min.). However, this effect was reversed on the long term.

Analysis of the biosynthesis of integrins during the cell cycle was also carried out using immunoprecipitation from ^{35}S-labelled cells. Asynchronous

cultures were compared with cell populations enriched in G1+S phases. The evidence suggests that, although there is a basal level of synthesis throughout the cell cycle, there may be a peak of synthesis in G2, especially of α subunits. The results also show the presence of a large cytoplasmic pool of β subunit.

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LIST OF ABBREVIATIONS

AJ.	Adherens-type junction
ATP	Adenosine triphosphate
CAM	Cell adhesion molecule
cAMP	Cyclic adenosine monophosphate
CBF	(Fibronectin) cell-binding fragment
CL-A	Calyculin A
CDC	Cell division cycle
CDK	Cyclin-dependent kinase
cDNA	Complementary deoxy ribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
EHS	Englebreth-Holm-Swarn (murine tumor)
FA	Focal adhesion
FN	Fibronectin
GAG	Glycosaminoglycan
GBP	(Bacterial) galactose-binding protein
GTP	Guanosine triphosphate
HA	Hyaluronic acid
H NMR	Proton nuclear magnetic resonance
ICAM	Intercellular CAM
IF	Intermediate filament
LFA	Lymphocyte function-associated antigen
MAP	Mitogen-activated protein
MPF	Maturation-promoting factor
N-CAM	Neural CAM
Ng-CAM	Neural tissue CAM
OA	Okadaic acid
PDGF	Platelet-derived growth factor
PMA	Phorbol myristate acetate
PSA	Polysialic acid
PSP	Protein serine phosphatase
PTP	Protein tyrosine phosphatase
RPA	(DNA-binding) replication protein A
SH	Src homolgy domain
TGFβ	Transforming growth factor β
VLA	Very late after activation-antigen
VN	Vitronectin
VWF	von Willebrand factor

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A mis padres

CHAPTER 1

INTRODUCTION

1. EXTRACELLULAR MATRIX

The existence of an extracellular matrix (ECM), the ground substance secreted by cells, has been known for many years. Ultrastructural studies were able to identify well organised collagen fibrils, the only ECM component showing some degree of structural order. Little more advance was achieved until the discovery that there were several types of collagen (see below, 1.4. collagens), which suggested that there could be different, possibly cell-type specific, ECM components (Bernfield, 1989). The discovery of fibronectin and the characterization at the molecular level of its interactions (Bernfield, 1989; Ruoslahti, *et al.*, 1981; Ruoslahti, 1988) stimulated many investigators to search for more components and it soon became clear that the ECM is extremely complex. There are two main types of ECM: the basal lamina and the interstitial matrix, each containing collagens as the major components, together with proteoglycans and glycoproteins (Bernfield, 1989).

I will give a brief introduction to some of the most studied components of the ECM: fibronectin, vitronectin, laminin, collagens and proteoglycans, and also discuss briefly their interactions with cells. I will focus particularly on fibronectin and vitronectin, since the work described in this thesis deals with receptors for these glycoproteins.

1.1. Fibronectin

One of the most studied macromolecules in the ECM is fibronectin. Fibronectin is a glycoprotein that mediates both cell anchorage and dynamic processes such as migration and ECM remodelling in wound healing and embryonic development (Ruoslahti, 1988; Schwarzbauer, 1991; Yamada, 1989).

1.1.1. Fibronectin structure

The amino acid sequence of the fibronectin polypeptide has been determined for bovine, rat and human species (Hynes, 1990). They share a high degree of sequence similarity. The structure of fibronectin is shown in figure 1.1. The actual fibronectin molecule is composed of two subunits linked through a pair of disulfide bonds near the COOH-terminus, resulting in a dimer of ~550kDa. Each subunit consists of three types of homologous sequences termed I, II and III. These sequences constitute units of folded secondary structure, which may assemble to form domains with specific affinities. For example, four type I and two type II repeats near the N terminus constitute a domain with affinity for collagen (see figure 1.1).

Type I homologies consist of 45 amino acids containing two disulfide bonds. The two type II sequences present in fibronectin (see figure 1.1) contain two disulfide bonds, as do type I sequences, and are 60 residues long. Type III residues are around 90 amino acids long and lack disulfide bonds (Hynes, 1990).

The structure of a type 1 module has been elucidated using H NMR (Baron *et al.* 1990). The main feature is the existence of two anti-parallel β-sheets. Both sheets have a right-handed twist and are stacked on top of one another, enclosing a hydrophobic core containing three highly conserved aromatic residues and the two consensus disulphide bonds. The secondary structure of a type III module has also been elucidated using NMR (Baron *et al.* 1993). There are seven β-strands in two antiparallel β-sheets, with the RGD sequence located on a loop between two β-strands.

The molecular weight of fibronectin varies according to the source, since cell type specific alternative splicing occurs within the fibronectin gene (Tamkun, Schwarzbaure *et al.*, 1984). Thus, plasma fibronectin, synthesized mainly in the liver (Tamkun, Schwarzbaure *et al.*, 1983), has two subunits with different molecular weight and is smaller than fibrillar fibronectin produced by other cell types. Alternative splicing is also responsible for at least 20 variants of the human fibronectin family (Reviewed by K.M. Yamada, 1989).

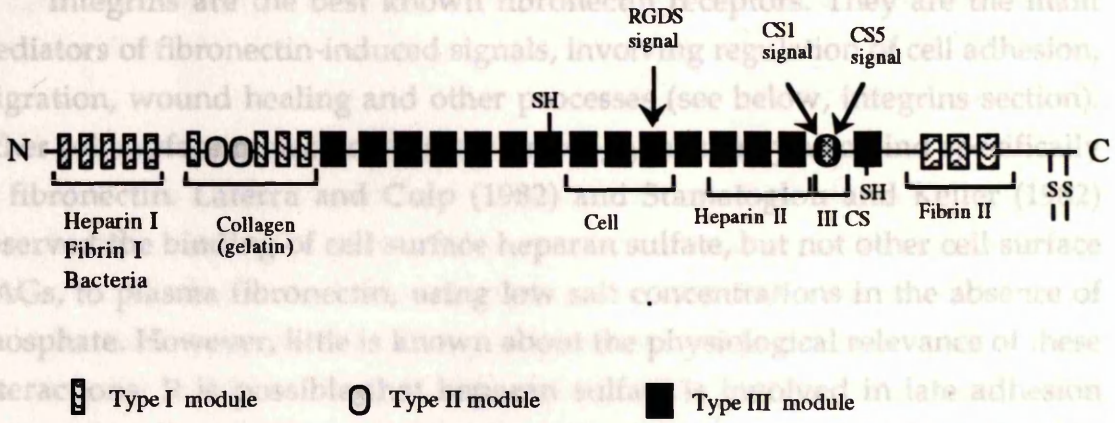


Figure 1.1.Structure of fibronectin. Modules I, II and III are assembled into domains with specific affinities. IIICS region is regulated by alternative splicing. CS1 signal consists of the octapeptide EILDVPST and CS5, which is active in binding to melanoma cells, contains REDV as the active sequence. See text for detail. Sekiguchi *et al.* 1991.

1.1.2. Cell-fibronectin interactions.

Fibronectin promotes cell adhesion and spreading by binding directly to its receptor(s) (see below, integrins section). Some molecular details about this interaction have been unveiled. Experiments involving controlled proteolysis of the fibronectin molecule and further sequence analysis (Pierschbacher and Ruoslahti, 1984; Pierschbacher *et al.*, 1981) demonstrated that the presence of a tetrapeptide (RGDS) is sufficient to promote cell adhesion significantly. This sequence is found in a type III repeat (III-10) in the fibronectin molecule, is conserved between species and is present also in other extracellular matrix components with cell binding capacity, like vitronectin, collagen, thrombin and fibrinogen. There are other sites for cell adhesion in the fibronectin molecule in the vicinity of III-7 or III-8, which act cooperatively with the RGDS sequence (Obara *et al.*, 1987; Obara *et al.*, 1988), and in the IIICS (also called V) region. This region, which has no homology to any other segment of fibronectin, is subject to alternative splicing and is less conserved among species. It has cell-attachment activity contained in the sequence REDV (melanoma cells) and in the segment CSI (with EILDVPST as the active sequence), in neural crest-derived cells and lymphocytes (Humphries *et al.*, 1986; Humphries *et al.*, 1987; Yamada and Kleinman, 1992). It has been reported that the heparin-binding site at the C-terminal side of III-10 also has cell-binding capacity, probably through the proteoglycans present in the cell surface (reviewed by Hynes, 1990).

Integrins are the best known fibronectin receptors. They are the main mediators of fibronectin-induced signals, involving regulation of cell adhesion, migration, wound healing and other processes (see below, integrins section). Other cell surface molecules, mainly proteoglycans, may also bind specifically to fibronectin. Laterra and Culp (1982) and Stamatoglou and Keller (1982) observed the binding of cell surface heparan sulfate, but not other cell surface GAGs, to plasma fibronectin, using low salt concentrations in the absence of phosphate. However, little is known about the physiological relevance of these interactions. It is possible that heparan sulfate is involved in late adhesion events or cell spreading, since treatment with heparinase does not affect attachment, although spreading on fibronectin is inhibited (Laterra *et al.*, 1983; McInnes *et al.*, 1987).

1.1.3. ECM-Fibronectin interactions

As mentioned above, fibronectins (both plasma and cellular forms) also bind to other extracellular matrix components. Their binding to collagen and gelatin has been extensively studied. Collagens I, II, III, IV and V all show some

degree of affinity for fibronectin, which is enhanced when heat-denatured forms of collagen (gelatin) are used (Engvall and Ruoslahti, 1977; Engvall, Ruoslahti *et al.*, 1978). Fibrin and fibrinogen also bind to plasma fibronectin in a non covalent way, although covalent cross-linking occurs in the presence of blood clotting factor XIIIa (Mosher, 1975; Mosher, 1976). K.M. Yamada et al (1980) showed that cellular chicken fibronectin binds reversibly to the glycosaminglycans (GAGs) heparin and hyaluronic acid (HA) but not to chondroitin sulfate.

The interactions of fibronectin with other ECM macromolecules to form fibronectin-rich matrices are very likely to play central roles in the regulation of its structure and function.

1.2. Vitronectin

Early experiments using gel filtration chromatography to assess the spreading activity of serum (Knox and Griffiths, 1980) identified two peaks, one corresponding to fibronectin and the other to an unrelated 70 kDa protein (serum spreading factor). (Knox and Griffiths, 1980). The same authors (Knox and Griffiths, 1980; Knox and Griffiths, 1982) showed that the 70K protein is the cell spreading factor under normal culture conditions, rather than fibronectin. Other authors, using different approaches , have identified a serum spreading factor, vitronectin, which apparently refers to the same glycoprotein, so called because of its affinity for glass beads (Hayman *et al.* 1983; Barnes and Silnutzer, 1983).

1.2.1 Vitronectin structure and function

The amino acid sequence of vitronectin has been elucidated from cDNA (Suzuki *et al.*, 1985). The study of the gene intron-exon organization (Jenne and Stanley, 1987) shows that there are eight exons and seven introns and there is no indication of alternative splicing. Vitronectins from different species are in the range of 56K-80K (Kitagaki-Ogawa*et al.*, 1990), and there is a substantial immunological cross-reactivity between porcine, human, horse and bovine vitronectins (Preissner, 1991). Unlike fibronectin, vitronectin has no series of repeating domains. However, there are several domains with strong homologies to other peptides. The most obvious example is the somatomedin B domain, at the N terminus (see figure 1.2), so called from its identical sequence to the peptide somatomedin B (Suzuki *et al.*, 1984). Another protein with strong homology is the human membrane glycoprotein PC-1 (Buckley *et al.*, 1990), which contains tandem repeats of the somatomedin B domain with eight

interlinked cysteines in identical positions. The physiological relevance of those domains as independent peptides or as part of other proteins, like vitronectin, remains obscure. It is possible that, in vitronectin, the somatomedin B domain is released after proteolysis between Thr-44 and Arg-45 or Arg-45 and Gly-46, interfering with the activity of the R45-G46-D47 sequence (Preissner, 1991), in which the main cell-binding activity of vitronectin resides. This sequence constitutes the first three residues of the connecting segment.

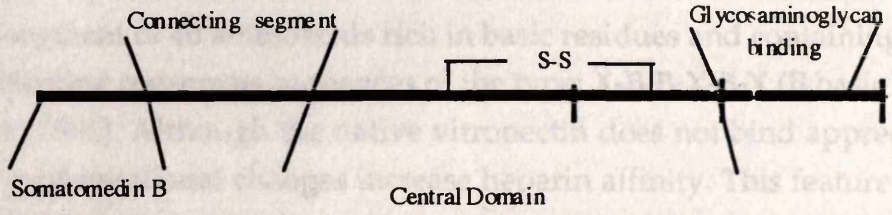


Figure 1.2 Vitronectin structure. The connecting segment starts with the sequence RGD, followed by a cysteine-free region from residues 48 to 130. The central domain contains six hemopexin-type repeats (see text for details); it also contains the protease-sensitive site between Arg379 and Ala380. Modified from Suzuki et al. (1984).

RGD is present in other ECM molecules (see above, fibronectin section) and is recognized by many integrins (D'Souza *et al.*, 1991; Humphries, 1990), which are the main vitronectin receptors identified so far (Humphries, 1990).

The connecting segment also contains a region free of cysteines, between residues 48 (just after RGD) and 130, with no homology to other known sequences, and responsible for the collagen-binding activity of vitronectin (Gebb *et al.*, 1986; Izumi *et al.*, 1988). As this segment also contains crosslinking sites at Gln 93 and two adjacent Gln, through which factor XIIIa/transglutaminase may catalyse the formation of vitronectin oligomers (Sane *et al.*, 1988), it is possible that vitronectin may bind irreversibly, via those sites, to collagen-rich matrices (Preissner, 1991).

Vitronectin also contains two domains homologous to hemopexin. Hemopexin is a serum glycoprotein that binds heme groups and transports them to the liver for salvage of iron. It contains two homologous domains, each made up of four homologous repeats and connected by a hinge region (Takahashi *et al.*, 1985). In the vitronectin molecule, six hemopexin-type repeats span from residues 132 to 459, with a putative hinge region around Pro-268, suggesting an incomplete four repeat-domain duplication during evolution (Preissner, 1991). As with hemopexin, binding of heme groups to vitronectin

may alter its conformation, although it is not known if heme-vitronectin interactions have physiological relevance (Preissner, 1991).

Although vitronectin is synthesized as a single chain glycoprotein, some plasma vitronectin may appear as two chains (65kDa and 10kDa) when run on SDS gels under reducing conditions. This is due to the presence of a protease sensitive site at Arg-379/Ala380 (see figure 1.2), near the C terminus (Preissner, 1991). The possible relevance of this proteolytic site is unknown.

The heparin-binding domain participates in cell binding. It binds to vessel-wall heparan sulfate and other cell surface GAGs. It consists of a C-terminal segment of 40 aminoacids rich in basic residues and containing several heparin-binding consensus sequences of the type: X-B-B-X-B-X (B:basic residue) (Preissner, 1991). Although the native vitronectin does not bind appreciably to heparin, conformational changes increase heparin affinity. This feature is likely to have physiological relevance: vitronectin seems to play a role in haemostasis via its heparin-binding activity (Preissner *et al.*, 1987b). Various GAGs (including heparin) act as cofactors enhancing the inhibition of thrombin and other coagulation enzymes (Damus *et al.*, 1973). The heparin-binding ability of vitronectin inhibits the heparin-accelerated interaction of thrombin or factor Xa with antithrombin, thus neutralizing the anticoagulant properties of heparin (Preissner, 1991; Preissner and Muller-Berghaus, 1987a; Preissner *et al.*, 1987b). The mechanism involves protecting thrombin from fast inactivation by the complex antithrombinIII-heparin. Vitronectin competes directly with the inhibitor for the binding site on heparin. Heparin-binding forms of vitronectin are present in plasma in quantities that suggest that the above mechanism may occur *in vivo* (Preissner, 1991).

Vitronectin may also form a ternary complex: vitronectin-thrombin-antithrombin, with properties still unknown (Preissner *et al.*, 1987b; Tomasini and Mosher, 1988). Its formation also involves a change in vitronectin conformation, exposing the heparin-binding domain. These ternary complexes are recognized by integrins at the vitronectin and possibly the thrombin RGD sites. As these complexes are likely to be concentrated in vessel walls, due to their affinity for endothelial cell GAGs, it is possible that they have a role in integrating integrin- and non integrin- mediated responses at sites of vessel wall injury (Preissner, 1991).

Vitronectin has also been shown to block lytic pore formation by the complement system by a mechanism dependent on the heparin-binding domain (Preissner, 1991).

It is clear from this information that vitronectin cannot be considered just as a substrate molecule on which cells adhere and spread. It is rather a versatile molecule that links cell adhesion to other processes, particularly those involved in immune defense, hemostasis and wound healing.

1.3. Laminin

Laminin is a glycoprotein present in basement membranes, the thin ECM surrounding epithelial tissues, nerves, fat cells and muscles. It was initially characterised in Englebreth-Holm-Swarm (EHS) murine tumor (Timpl *et al.* 1979). EHS laminin consists of three large polypeptide chains, A, B1 and B2. Their sizes are 300kDa (440 glycosylated), 205kDa (225 glycosylated) and 185kDa (205 glycosylated) respectively. These subunits are held together in a cruciform structure via many inter- and intra-chain disulfide bonds (Martin and Timpl, 1987). Studies with cDNA clones have shown that the A chain has three globular domains at the amino terminus separated by three EGF-like repeats (see figure 1.3.). The remainder forms part of the long arm and has a coiled-coil structure, finishing in a large globular domain. B1 and B2 chains are structurally similar, containing two globular domains and two EGF-like repeats (reviewed by Kleinman and Weeks, 1989).

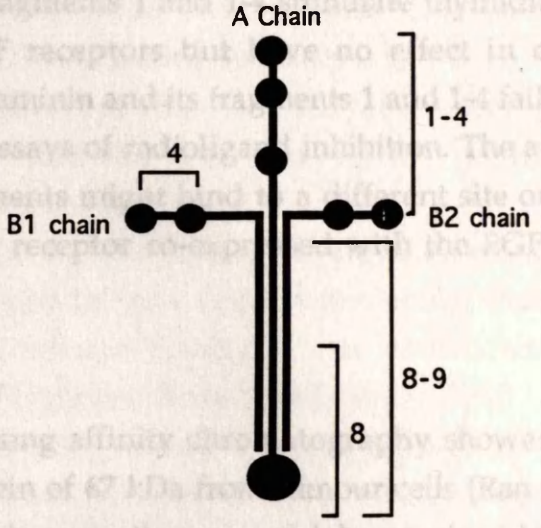


Figure 1.3. **Schematic model of laminin structure. Filled circles represent globular domains. Numbers indicate proteolytic fragments from elastase treatment. See text for details. Adapted from Mecham, 1991.**

Laminin exhibits several biological activities, including promotion of cell adhesion, migration and neurite outgrowth. Several data suggest that laminin is central in development. It is synthesised at the two-cell stage (Mecham, 1987),

it is able to cause differentiation of mesenchymal cells into epithelium in the kidney, and it is localised along the path of neural crest cell migration in developing tissues but not in the adult (reviewed by Kleinman and Weeks, 1989).

The active sites within the laminin molecule have been identified using controlled proteolysis (Rao *et al.* 1982: Edgar *et al.* 1984: Palm *et al.* 1985). Numbers in figure 1.3. indicate different fragments obtained by digestion with elastase. Fragment 1-4 (also E1-4), the three short arms, binds to collagen IV and promotes the attachment of MCF-7 epithelium-derived cells (Terranova *et al.* 1983). Fragment 1 (E1; not shown) contains the short arms, except the terminal globular domains. It retains cell attachment activity, but lacks the collagen-binding activity (Terranova *et al.* 1983). Fragment 8 (E8) spans the lower 35 nm of the long arm and stimulates neurite outgrowth (Edgar *et al.* 1984). Fragment 4 (E4) binds to collagen (Martin and Timpl. 1987). The large globular domain at the end of the long arm (C terminus) contains a heparin-binding site (Ott *et al.* 1982).

The rod-like segments between the globular domains in the short arms exhibit homology to EGF, and fragments 1 and 1-4, both containing EGF-like repeats, show growth-factor activity, possibly mediated through the EGF receptor. The evidence for this function comes from studies (Panayotou *et al.* 1989) showing that fragments 1 and 1-4 stimulate thymidine incorporation in cells expressing EGF receptors but have no effect in cells lacking those receptors. However, laminin and its fragments 1 and 1-4 failed to compete with the EGF receptor in assays of radioligand inhibition. The authors suggest that laminin and its fragments might bind to a different site on the receptor than EGF. Alternatively, a receptor co-expressed with the EGF receptor might be involved.

1.3.1. Laminin receptors

Early studies using affinity chromatography showed the presence of a laminin-binding protein of 67 kDa from tumour cells (Rao *et al.* 1983; Malinoff and Wicha, 1983). Subsequently, several laboratories identified the 67 kDa receptor in several other cell types (reviewed by Mecham, 1991). Later on this receptor was shown to be identical to the elastin receptor, a galactose-specific lectin (Mercurio, 1990). Other lectins have been reported as laminin-binding proteins *in vitro* (reviewed by Mecham, 1991). However, their physiological relevance as *in vivo* receptors for laminin is not yet understood.

The best characterised laminin receptors belong to the integrin family (see below, integrins section). $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$ and $\alpha_v\beta_3$ have been reported to bind to laminin *in vitro* (reviewed by Hynes, 1992). Although the laminin molecule contains an RGD sequence (see above, fibronectin section) in the central EGF-like repeat of the A chain, this sequence is inactive in integrin binding. Elastase-derived fragment 8 binds to $\alpha_3\beta_1$ and $\alpha_6\beta_1$, and fragment 1 binds to $\alpha_1\beta_1$.

Integrin $\alpha_6\beta_4$ adheres to the fragment 8 of laminin, at least in a colon adenocarcinoma-derived human cell line. However, the role of this integrin as a laminin receptor in other cell lines remains controversial (Lee *et al.* 1992).

1.4. Collagens

Collagens are the most abundant components of the extracellular matrix, forming the banded fibres seen ultrastucturally in the majority of vertebrate tissues (Burgeson, 1988). At present there are fourteen known types of collagen, all containing triple helical and globular domains. The triple helix is made up of three α chains, which are left-handed helices wound together to produce a right-handed helix. The overall structure is approximately 1.4 nm in diameter, with variable length (Burgeson, 1988). Figure 1.4 shows the structure of some collagens in a simplified form. Each α chain consists of more than 1000 aminoacids. The triple helix-forming sequence is $(gly\text{-}x\text{-}y)_n$, where x is usually a proline and y is a hydroxyproline. The alicyclic nature of both aminoacids prevents rotation around the C-N bond in the peptide backbone, thus providing rigidity to the α chain.

In some cases, the triple helices aggregate in an overlapping way to form collagen fibrils, stabilised by intra and intermolecular crosslinking. In further levels of organization, collagen fibrils can form fibres which, in turn, associate to form fibre bundles (Light and Bailey, 1981; Reid, 1991).

The role of collagens is mainly structural, being the main contributors to the determination and stabilization of adult tissue architecture. In development, collagen degradation is an important process, as tissues are continuously being remodeled. The enzymes responsible for collagen degradation fall into three categories: metalloproteinases, neutral proteinases and lysosomal cathepsins (reviewed by Reid, 1991). These enzymes are also active in wound healing and in some pathological processes involving destruction of connective tissue, such as lytic tumor invasion and rheumatoid arthritis (Cawston *et al.*, 1984; Maciewicz *et al.*, 1989).

Collagens have multiple receptors. The best characterized are β_1 and β_3 integrins (see below, integrins section). Syndecan, a proteoglycan, binds also to collagens I, III and V (see below, proteoglycans, section). In HeLa cells, four proteins of 102, 87, 45 and 38 KDa have been claimed to be integrin-unrelated collagen receptors by several criteria (Beacham and Jacobson, 1990; Lu *et al.*, 1989). In fibroblasts and chondrocytes, anchorin CII has also been characterized as a collagen receptor (Fernandez *et al.*, 1988). It is interesting that so many apparently unrelated proteins should act as collagen receptors. Obviously, further work will be needed to establish the possible relationships between them, and their role *in vivo*.

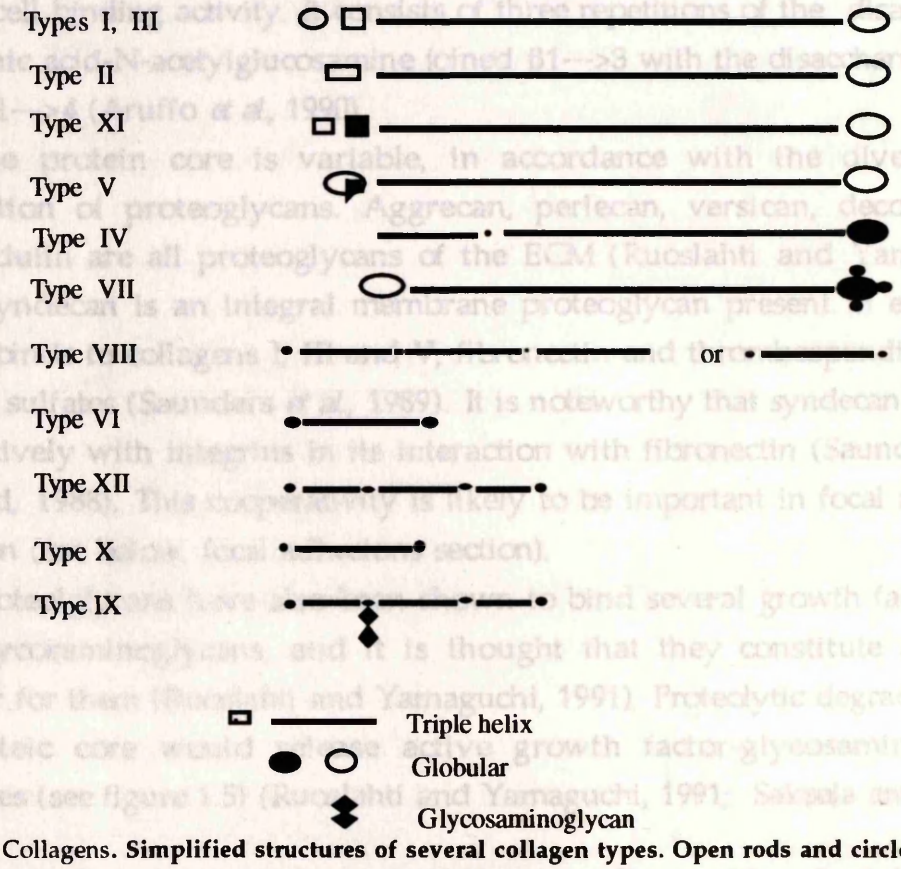


Figure 1.4 Collagens. **Simplified structures of several collagen types. Open rods and circles indicate portions removed prior to incorporation into insoluble matrices. The rest of the molecule is indicated in closed circles and rods. Modified from Burgesson, 1988.**

1.5. Proteoglycans

Proteoglycans are present at the cell surfaces and in the ECM in multiple forms. They consist of a protein core covalently bound to glycosaminoglycans,

which are large carbohydrates made up of disaccharide repeats. Glycosaminoglycans exist in four different forms: hyaluronic acid, keratan sulfate, chondroitin sulfate and dermatan sulfate, and heparan sulfate and heparin (Ruoslahti and Yamaguchi, 1991). They all have strong negative charge, especially the sulfated ones, which enable them to bind to a large number of components. However, binding specificity is provided by specific carbohydrate sequences, rather than by the charge itself. In fact, the potential of carbohydrate sequences to encode biologic information is remarkable. This potential becomes obvious if we realize that glucose may form up to 11 different disaccharides and 35,560 different tetrasaccharides, against 2 dipeptides and 24 tetrapeptides respectively for aminoacids, although a small subset of oligosacccharides actually exists.

There is a known hexasaccharide sequence in hyaluronic acid which confers cell binding activity. It consists of three repetitions of the disaccharide glucuronic acid-N-acetylglucosamine joined β1--->3 with the disaccharide units joined β1--->4 (Aruffo *et al.*, 1990)

The protein core is variable, in accordance with the diversity of distribution of proteoglycans. Aggrecan, perlecan, versican, decorin and fibromodulin are all proteoglycans of the ECM (Ruoslahti and Yamaguchi, 1991). Syndecan is an integral membrane proteoglycan present in epithelial cells. It binds to collagens I, III and V, fibronectin and thrombospondin via its heparan sulfates (Saunders *et al.*, 1989). It is noteworthy that syndecan may act cooperatively with integrins in its interaction with fibronectin (Saunders and Bernfield, 1988). This cooperativity is likely to be important in focal adhesion formation (see below, focal adhesions section).

Proteolglycans have also been shown to bind several growth factors via their glycosaminoglycans, and it is thought that they constitute an ECM reservoir for them (Ruoslahti and Yamaguchi, 1991). Proteolytic degradation of the proteic core would release active growth factor-glycosaminoglycan complexes (see figure 1.5) (Ruoslahti and Yamaguchi, 1991; Saksela and Rifkin, 1990).

Betaglycan, a cell membrane proteoglycan, and decorin, an ECM proteoglycan, bind to transforming growth factor β (TGF-β) through their proteic core. Interestingly, binding of decorin to TGF-β inactivates the growth factor and decorin synthesis is stimulated in several cell types by TGF-β (Ruoslahti and Yamaguchi, 1991).

The role of proteoglycans as growth factor reservoirs in the ECM and/or at the cell surfaces may be important in the control of their activity. It is

possible that immobilization of growth factors is important for keeping growth factor activity spatially controlled, helping in the process of formation and maintenance of different tissues (Ruoslahti and Yamaguchi, 1991).

From these data it is clear that proteoglycans are extremely interesting components of the ECM and cell surfaces, regulating cell adhesion and cell growth. A better knowledge of polysaccharide conformational complexity will help understand further the physiological roles of proteoglycans.

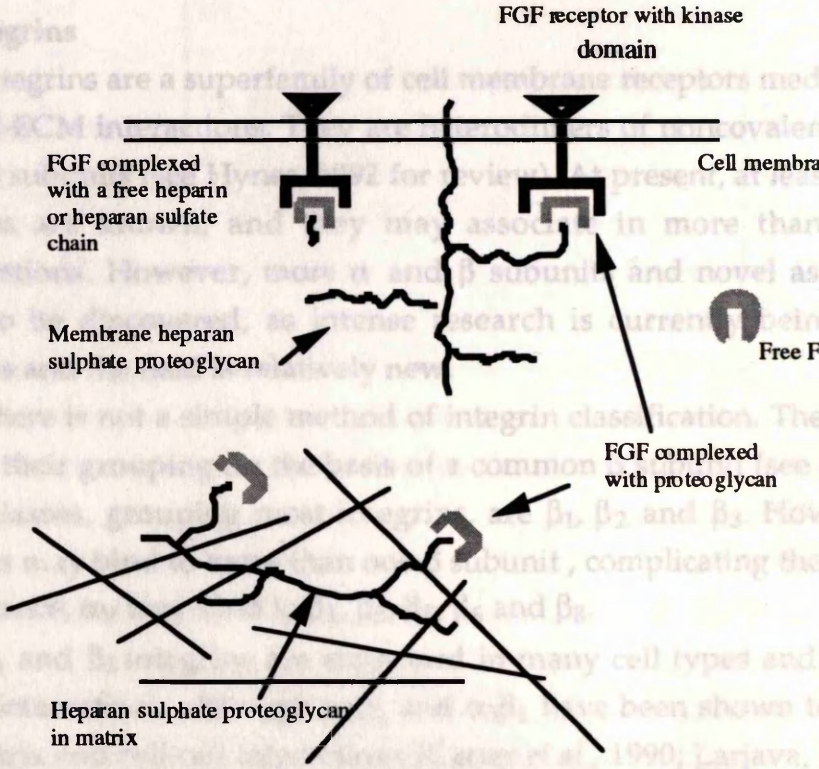


Figure 1.5. Proteoglycans. Schematic representation of membrane and ECM proteoglycans and their role as growth factors reservoirs. FGF: fibroblast growth factor. Adapted from Ruoslahti and Yamaguchi (1991).

2. CELL ADHESION MOLECULES

The evolution of mechanisms for cell adhesion was obviously a crucial step in the creation of multicellular organisms. During development, adhesion events play an important role in morphogenesis. These events involve interactions between cells and the ECM, and also between cells, and may be functionally divided into different steps: a first specific interaction between a cell membrane receptor and its ligand(s) is followed by specific responses, which may include cytoskeleton reorganization and generation of second

messengers, activation of kinase cascades and, ultimately, changes in gene expression. These responses may result in changes in cell differentiation, or specific immune responses, among others.

Next, I will describe the main adhesion molecules identified so far, including the integrin superfamily of cell-cell and cell-ECM adhesion molecules, the immunoglobulin superfamily of CAMs, cadherins and selectins. In section 5, I will focus in more detail on integrin regulation, since it is directly relevant to my own research.

2.1. Integrins

Integrins are a superfamily of cell membrane receptors mediating cell-cell and cell-ECM interactions. They are heterodimers of noncovalently associated α and β subunits (see Hynes, 1992 for review). At present, at least 8 β and 14 α subunits are known, and they may associate in more than 20 different combinations. However, more α and β subunits and novel associations are likely to be discovered, as intense research is currently being focused on integrins and the field is relatively new.

There is not a simple method of integrin classification. The most widely used is their grouping on the basis of a common β subunit (see table 1.1). The major classes, grouping most integrins, are β_1, β_2 and β_3. However, some α subunits may bind to more than one β subunit , complicating the classification. For instance, α_V may bind to β_1, β_3, β_5, β_6 and β_8.

β_1 and β_3 integrins are expressed in many cell types and mediate cell-matrix interactions, although $\alpha_2\beta_1$ and $\alpha_3\beta_1$ have been shown to support both cell-matrix and cell-cell interactions (Carter *et al.*, 1990; Larjava, *et al.*, 1990). β_3 are found in a vascular context. $\alpha_{IIb}\beta_3$ is essential in platelet activation (see below, integrin regulation section). β_2 integrins mediate cell-cell adhesion and are restricted to leukocytes.

The specificity of integrins for their ligands is not well defined. As seen in table 1.1, some integrins are able to bind to many ligands and, in the same way, some ligands may bind to several integrins. The reason why receptor-ligand specificities should be "degenerate" is not clear. One possibility is that integrins that share the same ligands serve different functions (Humphries, 1990). For instance, a fibronectin receptor that is expressed in a cell type at some stage of development might promote cell migration through a loose interaction with fibronectin whereas another fibronectin receptor with a stronger affinity, expressed at a different time, might promote cell attachment to the substrate. Obviously, this would imply the participation of integrins in signal

Table 1.1. Integrin subunits and their interactions

Subunits		Ligands	Binding sites
β1	α1	CO, LN	
	α2	CO, LN	DGEA*
	α3	CO, LN, FN	RGD?
	α4	FN , VCAM-1	EILDV
	α5	FN	RGD
	α6	LN	
	α7	LN	
	α8	?	
	αv	VN, FN?	RGD
β2	αL	ICAM-1, ICAM-2	
	αM	ICAM-1, FG, factor X, C3b(inact)	
	αX	FG, C3b(inact?)	GPRP
β3	αIIb	FG, FN, VWF, VN, TP	RGD, KQAGDV
	αv	FG, FN, VWF, VN, TP, OP, CO	RGD
β4	α6	LN ?	
β5	αv	VN	RGD
β6	αv	FN	RGD
β7	α4	FN, VCAM-1	EILDV
	αIEL	?	
β8	αv	?	

CO: collagens; **LN**: laminin; **FN**: fibronectin; **C3b(inact)**: C3b component of complement (inactivated); **FG**: fibrinogen; **VWF**: von Willebrand factor; **TP**: thrombospondin; **OP**: osteopontin.
*This binding site has been defined for type I collagen only.

transduction pathways, not as mere anchorage sites for the cell. See below, integrin regulation section, for more details of participation of integrins in signal transduction pathways.

3.1.1. Integrin structure

Figure 1.6 shows schematically the overall structure of a typical integrin. Structurally, all α subunits are related. They all have short cytoplasmic

domains and three or four repeats homologous to the Ca^{2+}-binding loop found in the EF-hand structure of calmodulin.

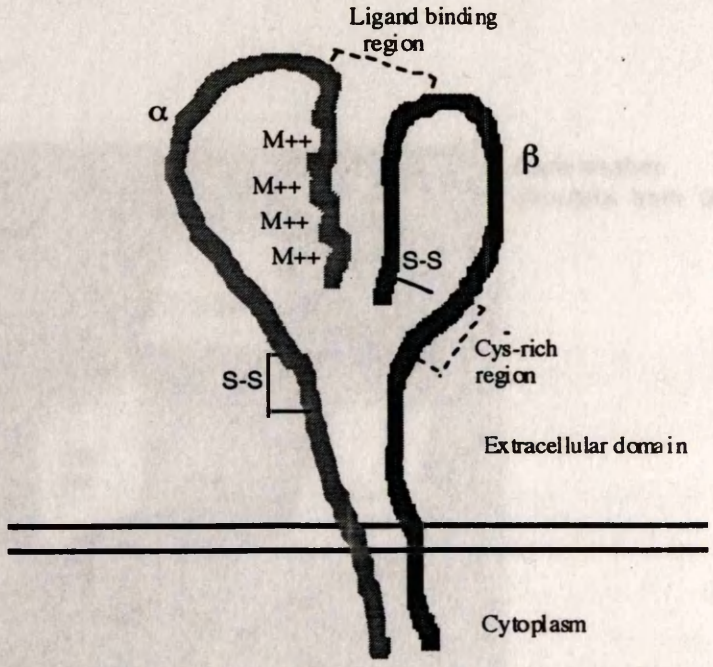


Figure 1.6 Integrin structure. All α subunits contain calcium-binding sites. The large loop in the β subunit is stabilized by intrachain disulfide binding (S-S). The β subunit contains 4 cysteine-rich repeats. Both subunits participate in the making of the ligand-binding region. The cytoplasmic domains of both subunits may be linked to cytoskeletal structures in focal adhesion structures. M++ sites make reference to metal binding sitesSee text for detail.

However, there is a fundamental difference between these structures and the homologous repeats of the integrins. Edwards *et al.* (1988) drew attention to the fact that the Ca^{2+}-binding sites present in α_v resembled more the sequence found in the so called "lock-washer" (Vyas *et al.*, 1987) structures of the bacterial galactose binding protein (GBP). Six carboxylate oxygens of three aspartates and a glutamate, a main chain carbonyl oxygen and an oxygen of a water molecule coordinate to each Ca^{2+} (see figure 1.7). The nine residues of the GBP loop are homologous to the first 9 of the loop of calmodulin and other proteins (see table 1.2).

However, co-ordinating position 12 of the lock-washer comes from the distant Glu205 residue. Two carboxylate oxygens from the Glu205 coordinate with Ca^{2+} in the same way as the Glu in position 12 of the EF-hand. This lack of coordinating sites at position 12 is shared with the integrin subunit α_v (see table 1.2). The missing carboxylate has to be supplied from elsewhere and this

feature is likely to be important in the regulation of integrin activity (see below, 3.1.2.integrin regulation).

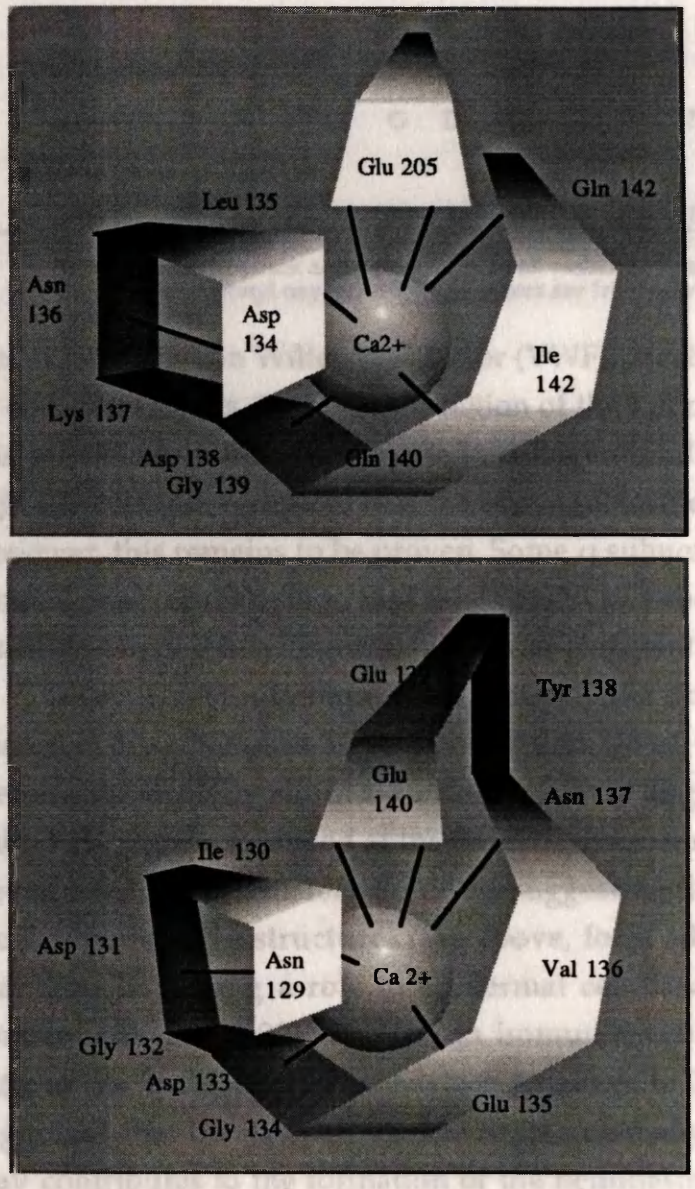


Figure 1.7. EF-hands and lock-washers. The nine-residue loop in the lock-washer has a similar conformation to that of the twelve-residue loop in the EF-hand. Also, the Glu205 from the lock-washer is equivalent to the invariant Glu residue at position 12 in the calmodulin loop. The sequence of the Ca^{2+}-binding sites in α_V is homologous to that of the lock-washer structure in GBP (see table 1.2), and it is likely to adopt a similar conformation (see text for details).

Some α subunits (α_1, α_2, α_L, α_X and α_M) contain an extra segment known as I-domain (I for inserted) of about 180 aminoacids, before the homologous repeats containing the cation-binding domains. It is homologous to the A-type

Table 2. Sequence alignment of Ca2+-binding sites in GBP and EF-hand loops

		*		*		*		*		*				
									142		204			
GBP	134	D	L	N	K	D	G	Q	I	Q	-	I	E	205
α_V		D	I	D	K	N	G	Y	P	D	L	-	-	
Calmodulin														
Loop IV	129	N	I	D	G	D	G	R	V	N	Y	E	E	140
Troponin C														
Loop IV	142	D	K	N	N	D	G	R	I	D	F	D	E	153

The sequences of the lock-washer structure of GBP (Vyas *et al.* 1987) is compared with two EF loops from troponin and calmodulin. In all cases, the ligand at position 7 is provided by the peptide carbonyl oxygen while the others are from side chain oxygens. Adapted from Vyas *et al.* 1987.

repeat found in von Willebrand factor (VWF), cartilage matrix protein and type VI collagen (Hynes, 1992). The function of the I domains is unknown, although it is possible that they participate in collagen-binding activity, since $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are collagen receptors and the above molecules may also bind to collagen. However, this remains to be proven. Some α subunits undergo post-translational proteolysis so that they consist of a short transmembrane segment disulfide-bonded to a larger, extracellular fragment.

Most β subunits have short cytoplasmic domains. The only exception known is β_4, which has a cytoplasmic domain of ~1000 aminoacids. Why this membrane protein should contain such a large cytoplasmic segment is unknown. $\alpha_6\beta_4$ is localized at the basal domain of most epithelial cells, in areas free of F-actin (Marchisio*et al.*, 1991), suggesting that it does not participate in focal adhesion-like structures (see above, focal adhesions section). It is likely that $\alpha_6\beta_4$ is playing a role in epidermal cell-basement membrane adhesion. Sonnenberg *et al.* (1991) showed in immunoelectron microscopy studies that $\alpha_6\beta_4$ at the basal epidermal surface localizes to hemidesmosomes, and they suggested that the β_4 subunit, due to its unusually large cytoplasmic domain, may contributes to the formation of the hemidesmosomal cytoplasmic plaque (see below, hemidesmosomes section).

Crosslinking experiments with β_3 integrins (vitronectin receptor and glycoprotein IIb-IIIa) show that RGD has discrete interactions near the lock-washer repeats in the α subunits (D'Souza *et al.*, 1988; D'Souza *et al.*, 1990; Smith and Cheresh, 1988; Smith and Cheresh, 1990a), although it also binds to a region on the β subunit, near its N-terminal, with homology (although more relaxed than in the α subunits) to an EF-hand repeat, suggesting that both subunits participate in the making of the ligand binding site.

All β subunits have four repeats rich in cysteine residues, believed to be internally disulfide-bonded, which cause them to run slower electrophoretically under reducing conditions.

2.2. Non-integrin cell adhesion receptors.

The main non-integrin cell adhesion molecules are involved in cell-cell interactions (CAMs) (Albelda and Buck, 1990). However, a few cell-substratum non-integrin receptors have also been identified. Cell surface proteoglycans are perhaps the best example. As mentioned above, they play important roles in cell physiology and may act cooperatively with integrins.

2.2.1. CAMs of the immunoglobulin superfamily

The CAMs of the immunoglobulin (Ig) superfamily mediate Ca^{2+}-independent cell-cell interactions (Williams and Barclay, 1988). N-CAM, Ng-CAM, and fasciclin II are examples of homophilic receptors (acting as both receptor and ligand), in neural tissues. N-CAM is present in many vertebrate species. It exists in three alternatively spliced forms, which differ in the length of the cytoplasmic region (C-terminus). They all contain polysialic acid (PSA), which varies during development and its amount correlates inversely with the binding strength of the receptor (Albelda and Buck, 1990). N-CAM is first detected in gastrulae. In chicken and frog the PSA content is low during early embryonic stages, increases during later embryonic development, and returns to a low content in the adult (Sunshine *et al.*, 1987). The authors suggest that the low content in PSA provides strength in cell-cell interactions during initial morphogenesis, and stabilization in adult tissues. A higher content of PSA would allow more flexibility in establishing cell-cell contacts, essential during histogenesis.

Many of the functions of the immune system also depend on CAMs from the Ig superfamily (Springer, 1990). CD2 (LFA-2) from T cells and LFA-3 in erythrocytes (also present in non hematopoietic cells) bind to each other and are examples of heterophilic receptors (Albelda and Buck, 1990). They are part of the Lymphocyte Function-related Antigens (LFA), together with LFA-1 ($\alpha_L\beta_2$), a member of the integrin family (see above). CD2/LFA-3 interaction is inhibited in resting T cells mainly by charge repulsion due to the presence of sialic acid on the cell membrane (Springer, 1990). These repulsive interactions

are reduced in activated T lymphocytes. Interestingly, the presence of polysialic acid in N-CAM also regulates its binding capacity (see above).

ICAM-1 and ICAM-2, also Ig members, are receptors for LFA-1. Expression of ICAM-1 is induced in inflammation in a wide variety of cells. However, ICAM-2 is not responsive to inflammatory mediators (Springer, 1990).

2.2.2. Cadherins and morphogenesis

Embryonic morphogenesis is brought about by a complex series of interconnected processes. Selective cell-cell and cell-ECM interactions may trigger cellular responses leading to differentiation, migration or cell aggregation. Cadherins have been shown to play important roles in some of those processes. They are calcium-dependent CAMs, mediating preferentially homophilic interactions (Geiger and Ayalon, 1992). There are three major groups: N-cadherins, found in adult neural tissues and muscle, E-cadherins, in adult epithelial cells, and P-cadherins, expressed transiently in development in several tissues, and present in placenta and epithelial cells (Albelda and Buck, 1990).

A classic paper by Townes and Holtfreter (1955) showed that dissociated cells tend to cluster according to their histogenetic origin and form characteristic tissue patterns. Recent work has shown that cadherins may play a role in those histogenetic segregation processes. When N-cadherin and E-cadherin expressing cells are mixed, a preference for homotypic interactions is found, although weaker, heterotypic contacts are also detectable (Volk *et al.*, 1987).

Cadherins are also involved in cell condensation, the transition of cell populations from a dispersed pattern to a compact one. Uvomorulin seems to play a role in cell compaction in the mouse morula (Hyafil *et al.*, 1980).

Cadherins are closely associated with transmembrane-cytoskeleton interactions, in the so called adherens-type junctions (AJs), which are structures typical of epithelia. In polar epithelial cells AJs are part of a junctional complex, with the more apical tight junctions and the more basal desmosomes (see figure 1.8). The transition from mesenchyme to epithelium is another morphogenetic process in which cadherins are involved. Many studies have shown that mesenchymal cells transfected with cadherin cDNA undergo epithelialization (reviewed by Geiger and Ayalon, 1992). Remarkably, transfection of fibroblasts with cadherin cDNA also induces epithelialization.

AJs have some similarities with focal adhesions (Geiger, 1989). For example, many of the cytoskeletal components of AJs are the same, and both contain proteins linking the actin filaments to the integral membrane components. However, there are proteins that associate selectively with FAs or AJs (i.e. talin *versus* plakoglobin; Geiger and Ayalon, 1992).

E-cadherin is required for the formation of tight junctions, AJs and desmosomes, as measured by the inhibitory effect of specific antibodies (Gumbiner *et al.*, 1988).

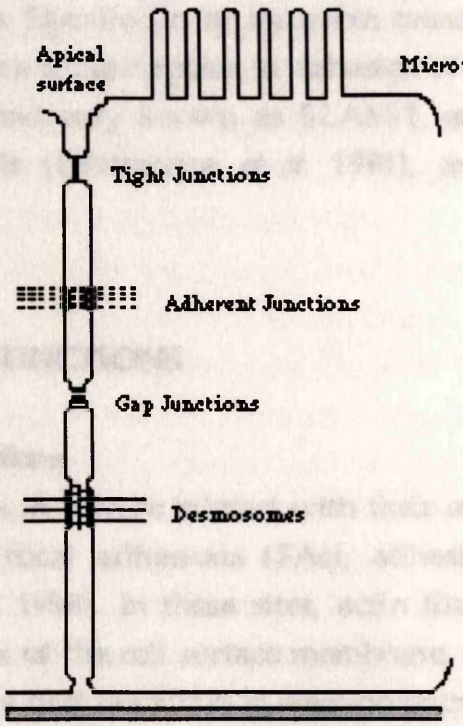


Fig 1.8.Cell-cell adhesion structures in polar epithelial cells. Adherent junctions contain cadherins as key components, linking cytoskeleton to the extracellular area (see text for details). Gap and tight junctions are sites of electrical conductanceand metabolic coupling between cells, and control of the extracellular space, respectively; in desmosomes, electron dense plaques are present in the intercellular space (25-35nm wide) and in the cytoplasm. These are attached to tonofilaments, usually of cytokeratin (Garrod, 1984). Figure modified from Garrod, 1984.

2.2.3. *Selectins*

Selectins are a class of integral membrane proteins involved in lymphocyte and neutrophil interaction with vascular endothelium (Springer, 1990).

They are expressed in leukocytes and endothelial cells. They contain an N-terminal lectin-like domain, followed by an EGF-like module and several repeats characteristic of complement-binding proteins. They contain a single transmembrane domain and a short cytoplasmic tail (Belvilacqua *et al.* 1991).

To date, three different selectins have been described. L-selectin was previously known as the lymph node homing receptor LEC.CAM-1, LECAM-1, LAM-1 or the MEL-14, Leu-8, TQ1 and DREG.56 antigens originally described on lymphocytes (Bevilacqua *et al.* 1991). It functions as a lymphocyte recirculation receptor and contributes to neutrophil emigration at inflammatory sites (Springer, 1990). P-selectin (or CD62), previously known as GMP-140 or PADGEM, is stored in α-granules of platelets and in Weibel-Palade bodies of endothelial cells. Stimulation by thrombin causes mobilization to the surface of these cells, where it participates in adhesion of neutrophils and monocytes. E-selectin was previously known as ELAM-1, and is also present in activated endothelial cells (Bevilacqua *et al.* 1991), and participates in neutrophil adhesion.

3. CELL-ECM JUNCTIONS

3.1. Focal adhesions

Many cells in culture interact with their substrate through well delimited regions called focal adhesions (FAs), adhesion plaques or focal contacts (Burridge *et al.*, 1988). In these sites, actin filament bundles are anchored to specialized areas of the cell surface membrane, and the cell-substrate contact is closest. FAs were first described in electron microscope studies (Abercrombie *et al.*, 1971) and were confirmed as focal contacts by interference reflexion microscopy (IRM), a method devised by Curtis (1964) (Izzard and Lochner, 1976; Abercrombie and Dunn, 1975).

FA formation can be divided in different stages: first, attachment of cells occurs on ECM components. Subsequent spreading is then accompanied by the formation of submembranous microfilament bundles, which associate with discrete areas of the membrane, in what can be considered as FA precursors. Finally, the microfilament bundles may enlarge to form fully developed stress fibers, firmly anchored to the membrane, possibly through other cytoplasmic components such as talin, vinculin and α-actinin, which concentrate at FAs concomitantly with actin (Burridge and Connell, 1983; Burridge and Feramisco, 1980; Woods *et al.*, 1986) .

3.1.1. FA components

Since the early descriptions of FAs, many of their components have been identified, mainly using immunofluorescence microscopy. These components have been extensively reviewed by Burridge *et al.* (1988). In the ECM, the first component identified was fibronectin (FN). However, FN is present only when serum-free medium is used (Grinnell, 1986). In the presence of serum, vitronectin (VN) is the detectable ECM component. Since cells are normally cultured in the presence of serum, it is possible that VN is the critical ECM component of FAs (Burridge *et al.*, 1988). Cell surface proteoglycans have also been shown to act cooperatively in FA formation in the presence of fibronectin (Culp *et al.*, 1986). However, integrins are the main cell surface components. They are the obvious candidates for the role of linking cytoskeletal structures with the ECM since, apart from their role as receptors for specific ECM components, their cytoplasmic domains also allow interaction with the cytoskeleton (see below, integrins section).

Talin and vinculin are cytoplasmic components of FAs (Burridge and Connell, 1983; Burridge and Feramisco, 1980). Although they may distribute differently in several cell types (Burridge *et al.*, 1988), they have been shown in smooth muscle to bind to each other with high affinity (Burridge and Mangeat, 1984). A vinculin-binding component has been identified, consisting of two bands of 150kDa and 200 kDa. This component was named tensin and is thought to cap the ends of actin filaments, although its particular role in FA formation is not yet clear (Burridge *et al.*, 1988). Fimbrin and α-actinin are the only actin-binding proteins so far identified in FAs. Fimbrin is present in *microvilli* and crosslinks actin filaments *in vitro*, whereas α-actinin is more widely distributed and its actin-crosslinking activity is lower (Bennett *et al.*, 1984; Bretscher, 1981).

Figure 1.9 summarizes the main FA components identified so far, and their *in vitro* interactions. It should be noted, however, that other components are likely to be involved, and that *in vitro* interactions might represent experimental artifacts rather than *in vivo* equivalents (Burridge *et al.*, 1988).

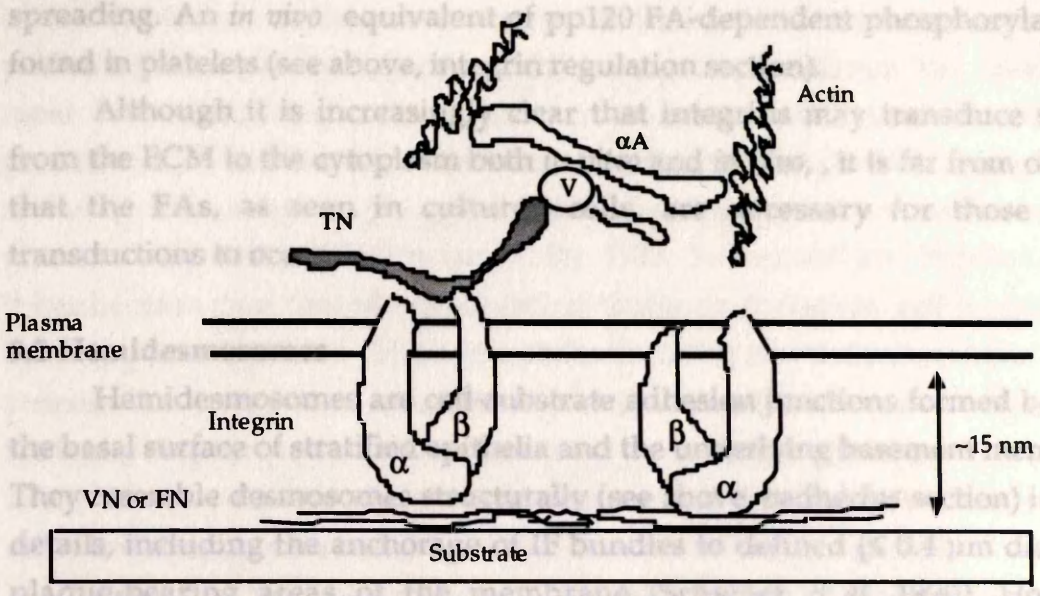


Figure 1.9. Focal adhesions. Schematic representation of the main FA components identified so far. α and β are integrin subunits. VN: vitronectin; FN: fibronectin; αA: α-actinin; TN: talin.

3.1.2 Physiological relevance of focal adhesions

The role of FAs *in vivo* is not well established. Although they are present in many cultured cells, FA-like structures within whole organisms have been reported in very few cases. The dense plaque of smooth muscle cells involves a link between ECM and cytoskeleton *via* integrins, and vinculin and talin are also present (Geiger *et al.*, 1981; Geiger *et al.*, 1985). α-actinin has also been reported, although other laboratories have failed to identify it, and its presence has been questioned (Small, 1985).

Similarly, the myotendinous junction of skeletal muscle represents an FA-like structure, with a link between the tendon and actin filaments *via* integrins. Vinculin and talin are present, although the presence of α-actinin, as with the dense plaque, remains controversial (Tidball, 1987). Other examples are the postsynaptic region of neuromuscular junctions and the *zonula adherens* junctions between epithelial cells, both with some components also present in FAs (reviewed by Burridge *et al.* 1988).

The similarities between these structures and FAs have been established mainly on the basis of their components, and physiological links are difficult to verify. Recently, it has been shown that focal adhesions may be involved in signal transduction pathways triggered by ECM-cell interactions. As mentioned above (see integrin regulation section), tyrosine phosphorylation of several components occurs upon interaction of ECM with integrins, the best characterized of which is a protein of ~125kDa. Interestingly, phosphorylation of this protein correlates with FA formation, rather than with cell adhesion or

spreading. An *in vivo* equivalent of pp120 FA-dependent phosphorylation is found in platelets (see above, integrin regulation section).

Although it is increasingly clear that integrins may transduce signals from the ECM to the cytoplasm both *in vitro* and *in vivo*, , it is far from obvious that the FAs, as seen in cultured cells, are necessary for those signal transductions to occur.

3.2 Hemidesmosomes

Hemidesmosomes are cell-substrate adhesion junctions formed between the basal surface of stratified epithelia and the underlying basement membrane. They resemble desmosomes structurally (see above, cadherins section) in a few details, including the anchorage of IF bundles to defined (≤ 0.4 μm diameter) plaque-bearing areas of the membrane (Schwarz *et al.* 1990). However, antibodies raised against desmosomal components do not react with hemidesmosomes (Jones *et al.* 1986).

Hemidesmosomes are resistant to Ca^{2+} depletion (Owaribe *et al.* 1990). However, extracellular Ca^{2+} and calmodulin are required for the formation of new hemidesmosomes (Trinkaus-Randall and Gipson 1984).

3.2.1. Hemidesmosome components

To date there is only one well characterised component of hemidesmosomes, a polypeptide localised at the plaque, of ~230kDa, recognised by autoantibodies from patients suffering from bullous pemphigoid and therefore called bullous pemphigoid antigen (Stanley *et al.* 1981). This component is not present in desmosomes or in plaques of cell-substrate junctions of single-layered epithelia, indicating that not all asymmetric plasma membrane plaques represent the same type of hemidesmosome (Schwarz *et al.* 1990).

The integrin $\alpha_6\beta_4$ (see above, integrins section) has also been shown to associate with hemidesmosomes (Stepp *et al.* 1990; Sonnenberg *et al.* 1991). It has been suggested (Sonnenberg *et al.* 1991) that the large β_4 cytoplasmic domain could play a role in assembling IFs to the hemidesmosomal plaque.

4. PROTEIN MODULATION BY PHOSPHORYLATION

Reversible covalent modifications of proteins are major regulatory mechanisms in a wide variety of processes (Uy and Wold, 1977). Reversible

phosphorylation, methylation, acetylation and S-S/SH interconversions are perhaps the best known examples. Of these, phosphorylation has proved the most interesting, since the regulation of many fundamentally different processes is based on this reversible modification. Since early experiments showing that enzyme phosphorylation was a regulatory mechanism in glycogen metabolism (Fischer and Krebs, 1955; Sutherland and Wosilait, 1955), it has become clear that phosphorylation is also important in cell division (see below), gene expression (Meisner and Czech, 1991) and indeed in almost every process involving outside-inside signal transduction (Taylor *et al.*, 1992). Protein phosphorylation is brought about by enzymes called protein kinases, which catalyze the transfer of a phosphate group from a nucleotide, typically ATP, to the target substrate (Krebs and Beavo, 1979).

There are two major classes of protein kinases: those whose specific substrates are Ser and/or Thr and those with specificity for Tyr. However, there is at least one kinase able to phosphorylate Ser/Thr and Tyr. wee1 (see below, cell cycle section) is one example. MAP protein kinase (MAP-KK) activators may also belong to the family of dual-specificity protein kinases. Growth factor treatment of cells results in the activating Tyr and Thr phosphorylation of MAP-PKs. Either the activator(s) are dual-specificity kinases or they induce autophosphorylation in Thr and Tyr by MAP-KK (Lindberg *et al.* 1992). However, evidence for this dual role in most cases comes from studies on autophosphorylation *in vitro*,, and physiological relevance of these results in all cases remains to be proven.

There is also a great diversity in terms of subcellular localization, size and mechanism of activation. For example, regulators of kinase activity include Ca^{2+}, cAMP, phorbol esters and diacylglycerol (Hunter, 1987).

Despite the great diversity of protein kinases, all of them share a catalytic core of approximately 260 residues (Taylor *et al.*, 1992; Taylor *et al.*, 1993). The conserved motifs are, starting from the N-terminus (see figure 1.10), a glycine-rich loop followed by Lys72, both involved in stabilizing the portion of MgATP not directly involved in phosphotransfer, and Asp184, which chelates the Mg^{2+} ion that bridges the β and γ phosphates (Taylor *et al.*, 1993).

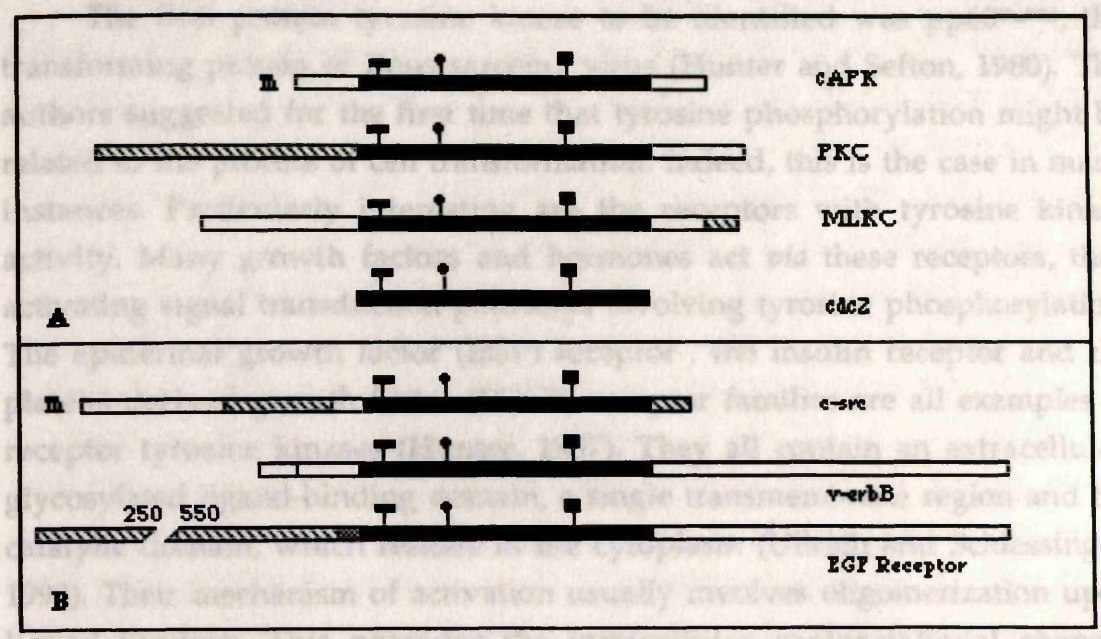


Figure 1.10. Protein kinases. The conserved catalytic core is shown in black. The catalytic subunit of cAPK contains 350 amino acids and the core extends from residue 40 to 300. Regions thought to be important for regulation are cross-hatched. The putative membrane-spanning segment in the EGF receptor is stippled. Small black rectangles indicate three conserved sequence motifs corresponding to the glycine-rich loop. Lys72 (filled circles) and Asp184 (filled squares) are also indicated. "m" refers to N-terminal myristoylation. A. Ser/Thr kinases. B. Tyr kinases.

Among the Ser/Thr protein kinases, one of the first to be fully characterized was the cAMP-dependent protein kinase (cAPK), and its structure can serve as a general template for the entire protein kinase family (Taylor *et al.*, 1993). It consists of two regulatory subunits (R, which is the primary cAMP receptor), and two catalytic (C) subunits. The activating mechanism is as follows: $R_2C_2 + 4cAMP \rightarrow R_2(cAMP)_4 + 2C$. The two catalytic subunits can now phosphorylate cytoplasmic proteins containing the consensus sequence RRXSY (X: any aminoacid; Y: hydrophobic aminoacid) (Taylor *et al.*, 1993). Protein kinase C (PKC) is a cytoplasmic kinase reversibly associated with membranes. It is activated by Ca^{2+}, diacylglycerol, phosphatidylserine and phorbol esters, all of them acting at major regulatory sites near the catalytic core (Nishizuka, 1988). Ca^{2+}/calmodulin-dependent protein kinases are normally specific for one substrate and present a great structural diversity. Thus, myosin light chain kinase (MLCK) is a monomer, and glycogen phosphorylase kinase is a hexadecamer (Taylor *et al.*, 1992). Cdc2 kinase is one of the smallest kinases (34kDa). It is essential for cell division and its activity is regulated by phosphorylation/dephosphorylation and association with other proteins whose expression is cell cycle regulated, cyclins (see below, cell cycle section).

The first protein tyrosine kinase to be identified was $pp60^{v\text{-}src}$, the transforming protein of Rous sarcoma virus (Hunter and Sefton, 1980). The authors suggested for the first time that tyrosine phosphorylation might be related to the process of cell transformation. Indeed, this is the case in many instances. Particularly interesting are the receptors with tyrosine kinase activity. Many growth factors and hormones act *via* these receptors, thus activating signal transduction pathways involving tyrosine phosphorylation. The epidermal growth factor (EGF) receptor , the insulin receptor and the platelet-derived growth factor (PDGF) receptor families are all examples of receptor tyrosine kinases (Hunter, 1987). They all contain an extracellular, glycosylated ligand-binding domain, a single transmembrane region and the catalytic domain, which resides in the cytoplasm (Ullrich and Schlessinger, 1990). Their mechanism of activation usually involves oligomerization upon ligand binding. This provides the intracellular conformational changes necessary to activate the catalytic domain, thus triggering the cellular responses. Recently, several of the components of the signal transduction pathway from receptor tyrosine kinases to Ras have been described. The first components in the cascade of events are proteins containing SH domains (Src Homology domains). One example is the protein Grb2, which contains SH2 and SH3 domains, which have been identified in several proteins involved in signal transduction. SH2 has affinity for phosphotyrosine phosphoproteins, and SH3 has affinity for proline-rich regions. Grb2 binds to the active phosphotyrosine-containing receptor via the SH2 domain, and uses its SH3 domains to bind to the next component in the pathway, which is a Ras activator, Sos, thus forming a complex: receptor-Grb2-Ras activator. Bound Ras activator converts inactive RasGDP to the active RasGTP. The sequence of event is then as follows: first, binding of the ligand causes oligomerization and autophosphorylation of the receptor. Newly formed phosphotyrosine-rich domains in the receptor bind to proteins containing SH2 domains, i.e. Grb2. The activated complex receptor-Grb2 binds to Sos which, in turn, binds to RasGDP, initiating a kinase cascade (McCormick, 1993).

Reversibility of protein phosphorylation implies the existence of enzymes able to remove the phosphate from kinase targets, although there are a few examples of direct reversal of protein kinase-catalyzed reactions (Krebs and Beavo, 1979). Those enzymes are called phosphatases and their characterization is obviously essential for an understanding of phosphorylation-dependent processes. As with kinases, phosphatases can be classified, according to their substrate specificity, as Ser/Thr (PSP) or Tyr (PTP) phosphatases (Tonks, 1990).

PSPs can be divided in four groups. Types 1, 2A, 2B and 2C (reviewed by Cohen, 1989). Of these, only 2B (calcineurin) responds directly to a second messenger, Ca^{2+} in this case, and is involved in signal transduction pathways. Types 1, 2A and 2B are structurally related, whereas 2C derives from a different gene family (Tonks, 1990). PSP1 and 2A are involved in cell cycle regulation, although their exact roles are still unclear. It is possible that PSP1 dephosphorylates phosphothreonine 161 in cdc2 kinase (see below, cell cycle section), allowing, in conjunction with cyclin degradation, exit from metaphase (Pallen *et al.*, 1992). Evidence from studies in *Xenopus* suggests that PSP1 is also involved in mitosis initiation, as addition of inhibitor-2, a specific PSP1 inhibitor, induces cdc2 activation, and addition of PSP1 causes mitosis retardation (Walker *et al.*, 1992). PSP-2A is required for entry into mitosis. Its exact role could be to maintain cdc25 in a dephosphorylated form (see below, cell cycle section), thus blocking thr14 and tyr15 dephosphorylation of cdc2, since treatment of interphase *Xenopus* extracts with okadaic acid, a PSP-2A inhibitor that accelerates entry into mitosis, causes cdc25 hyperphosphorylation, stimulating its cdc2 phosphatase activity (Kumagai and Dunphy, 1992).

PTPs can be divided into non-receptor and receptor-like phosphatases. All PTPs share a common segment of ~240 aminoacids, which contains the catalytic domain (Charbonneau and Tonks, 1992). The receptor-like PTPs have a highly conserved cytoplasmic domain where two tandem catalytic domains reside, a single transmembrane segment, and a variable extracellular region. CD45 is included in this group. It is a surface glycoprotein restricted to hematopoietic cells, with a role in signal transduction involving dephosphorylation of phosphotyrosyl residues. Its activity is necessary for the increased phosphatidylinositol turnover observed upon stimulation of the T-cell receptor (Koretzky *et al.*, 1990).

Non-receptor PTPs contain a single catalytic domain and unrelated regulatory sequences, whose main function is to direct the enzyme to specific subcellular compartments (Charbonneau and Tonks, 1992).

There is also a dual-specificity phosphatse, cdc25 (see below, cell cycle section). Although it is remarkable that cdc25 should have threonine and tyrosine as substrates, as PSPs and PTPs bear no sequence similarity, the experiments give overwhelming evidence of this double role for cdc25 (Kumagai and Dunphy, 1991; Millar and Russell, 1992). This is a clear difference from the dual-specificity kinases (see above), whose ability to act on

Ser/Thr and Tyr residues has been proved solely in autophosphorylation experiments.

5. REGULATION OF INTEGRINS

5.1. Role of lock-washer structures in integrin ligand-binding activity

Ca^{2+} and/or Mg^{2+} binding is a prerequisite for integrin ligand-binding activity. The divalent metal cation-binding sites present in the α subunits are thus essential structures on the integrin molecule.

Remaining unexplained is the ability of Mn^{2+} to induce spreading of cells even in the absence of adhesive ligands. As it is very likely that Mn^{2+} binds also to the lock-washer domains of integrins (Gailit and Ruoslathi, 1988), the possibility remains that cation binding to the receptor is the key step for its activation, rather than the interaction with the adhesive ligand (Edwards *et al.*, 1987), i.e., the role of RGD and other adhesion motifs would be to fold the putative metal-binding sequences in order to generate complete co-ordinating sites for Ca^{2+} or Mg^{2+}, whose binding would then, in some sense, switch on the receptor, involving a series of conformational changes. The fact that much higher concentrations of Mn^{2+} are necessary for activation of cell spreading in the absence of adhesive ligands (Edwards *et al.*, 1987; Rabinovitch and DeStefano, 1973) may be explained if the role of RGD and other adhesion motifs were to fold the integrin metal binding sites in a cooperative way (Edwards *et al.*, 1988).

The lack of co-ordinating sites at position 12 of the metal-binding sequences (see above, integrin structure) present in α subunits offers a possible mechanism for RGD-like activity. Edwards *et al.* (1988) proposed that the receptor binding to its ligand could alter its conformation thus drawing a distant Glu to the position 12. In RGD dependent integrin activity, it would also be possible that the missing carboxylate is supplied by the Asp in the RGD sequence itself.

It is remarkable that other sequences from ECM glycoproteins that have been shown to be active in integrin binding share the presence of an Asp residue. The best characterized of these are REDV, LDV and HHLGGAKQAGDV. This Asp might provide the missing coordinating group in α_v (Humphries, 1990).

A potential role in integrin cation dependence has also been proposed for β subunits. Integrins $\alpha_v\beta_1$ and $\alpha_v\beta_3$ respond in different ways to Ca^{2+} in the

presence of Mg^{2+} and use different cations for binding to their ligands. Since they share the same α subunit, it is possible that the β subunit somehow modulates the cation binding activity (Kirchhofer *et al.*, 1991). The authors suggest that the missing Glu at position 12 from the metal-binding sequence might be provided by the β subunit so that different subunits might account for different cation affinities.

As mentioned above, sequences with homology to those forming lock-washer structures are present in all α subunits, and the presence or absence of the appropriate cations will determine an active or inactive conformation for the receptor. However, the definition of active or inactive is not simple. Integrins may have a wide range of affinities for their ligands according to different environments, and regulation of cation seems unlikely to account for those different affinities *in vivo*. Moreover, different integrins present in the same cell could not be differently regulated by the same cation environment. So we could say that the presence of cations is indispensable for integrin activity, but additional mechanisms are needed for a fine regulation of that activity. In fact, there are several known mechanisms which may modulate integrin affinities, as part of important transduction pathways relevant to cell growth, immune response, cell migration, wound healing or development.

5.2. Role of integrins in focal adhesion formation

Integrins are important components of focal adhesions (see above, focal adhesions section). It was recently reported that the phosphorylation of a 120 kDa protein (pp120) is induced by the interaction between fibronectin and β_1 integrin receptor(s) in NIH3T3 cells (Guan *et al.*, 1991). The β1 cytoplasmic domain was necessary for phosphorylation of pp120. This phosphorylation was independent of soluble growth factors, was immediately prior to cell spreading, and cell rounding reversed the effect. A homologous protein from rat testis cells was later shown to be a protein tyrosine kinase, FadK (Hanks *et al.*, 1992). The authors suggested that aggregation of integrins at focal adhesions would induce autophosphorylation of FadK (pp120), which would become active, phosphorylating other substrates in a transduction system leading to cell spreading. Schaller *et al.* (1992) reported a similar protein (pp125FAK) in chicken embryo cells.

Thus, a model is possible in which interaction between fibronectin and its integrin receptor(s) may change integrin conformation, possibly via oligomerization, at the cytoplasmic region, leaving the cytoplasmic domain of the β subunit with increased affinity for pp120. The bound pp120 would

phosphorylate itself and subsequently other substrates. When fibronectin detaches from its receptor(s) (cell rounding), integrin conformation would reverse, releasing pp120, which would become dephosphorylated and inactive, interrupting any further signal into the cytoplasm. Alternatively, a cytoplasmic signal could reverse the transduction pathway, the final effect being the inactivation of fibronectin-receptor interaction. However, these considerations are merely speculative.

It is interesting that RGD-containing peptides cannot trigger pp120 phosphorylation (Guan *et al.*, 1991) and that central regions of fibronectin, including the cell-binding domain, induce only intermediate levels of phosphorylation. Only when both the cell-binding domain and the heparin-binding domain were present, high levels of pp120 phosphorylation were detected. This requirement is also necessary for the formation of focal adhesions, which are not seen when the cell-binding domain of fibronectin alone is present as substrate (Woods and Couchman, 1992), even though cells seem to spread well.

This correlation between pp120 phosphorylation and focal adhesion formation is likely to be relevant for the role of pp120. It is also possible that, if focal adhesions are simply artifacts in these systems (see above, FAs section), the phosphorylation of pp120 may also be part of those artifacts. On the other hand, focal adhesions are likely to have some equivalent *in vivo*, in circumstances probably involving strong cell attachment, when structures similar to focal adhesion are likely to develop . However, a role for the action of β_1 integrins on pp120 *in vivo* remains to be discovered.

5.3. Role of integrins in platelets and the immune system

In spite of the current controversy about focal adhesion relevance *in vivo*, integrins have been shown to be physically and functionally the link between insoluble ECM and the cytoskeleton in many systems, independently of their possible involvement in focal adhesions. Also, in other instances, they seem to transduce signals in pathways involving soluble second messengers, functioning similarly to growth factor receptors (see below, this section).

An excellent example of integrin participation in relevant physiological processes is given by the immune system. Interactions of cells from the immune system with other cells and with the ECM are essential for a rapid response to infections. Integrins, selectins and immunoglobulins are the three families of receptors regulating those interactions (Springer, 1990).

Integrins are important in the dynamic regulation of adhesion and migration in the immune system (Springer, 1990). Their activity can be modulated, mainly through induced changes of conformation. Integrins of the immune system are the β_2 integrins ($\alpha_L\beta_2$, $\alpha_M\beta_2$ and $\alpha_x\beta_2$), whose expression is restricted to leukocytes. They may bind to a variety of ligands in activated leukocytes (see table 1.1). Changes of conformation are also involved in different states of integrin activation. There are several mechanisms by which β_2 integrins may be activated. One example relatively well understood is that of $\alpha_L\beta_2$ in lymphocytes. Naive T lymphocytes may be induced by specific antigens to become memory lymphocytes. The process is characterized by the increased and persistent expression of several cell surface molecules, including the integrin $\alpha_L\beta_2$, also known as LFA-1 (lymphocyte function-related antigen-1). In T cell activation, T cell receptor/CD3 in the T lymphocyte recognizes the antigen presented by the major histocompatibility molecules in the antigen-presenting cell. This specific interaction activates $\alpha_L\beta_2$ on the T cell, which will bind avidly to ICAM-1 in the antigen-presenting cell, providing a stronger adhesion between both cells. How $\alpha_L\beta_2$ is activated is unclear, although some evidence points to a change of conformation induced by a protein kinase C (PKC)-dependent mechanism (van Kooyk *et al.*, 1989). Deletions in the cytoplasmic domain of β_2 inactivate the receptor (Hibbs *et al.*, 1991), suggesting a regulatory role for this domain. Mechanisms for signalling from the cytoplasmic regions of $\alpha_L\beta_2$ may include phosphorylation or interaction with soluble cytoplasmic messengers or with the cytoskeleton, similarly to the case of the $\alpha_{IIb}\beta_3$ integrin (see below, this section). In fact, Buyon *et al.* (1990) reported the phosphorylation of β2 in neutrophils and monocytes upon activation by PMA.

Clearly, further research will be needed to elucidate the mechanisms of activation of β_2 integrins.

β_1 integrins are expressed in many cell types. On lymphocytes, they were called the VLA (very late antigen) family of integrins, because some are expressed in lymphocytes 2-4 weeks after antigen stimulation *in vitro* (Springer, 1990). However, they are constitutively expressed in many other cell types.

In the immune system, they mediate adhesion between the ECM and lymphocytes and are important for migration of activated lymphocytes in tissues during immune responses (Springer, 1990). As with other immune system integrins, β_1 integrins may acquire different states of activity, brought about by conformational changes. Anti-β_1 antibodies strongly enhance binding of U-937 myelomonocytic cells to fibronectin (Arroyo *et al.*, 1992). They showed

that VLA-4 and VLA-5 are the mediator integrins in this response. Furthermore, the same antibody induced the ability of VLA-4 to bind to VCAM-1 and fibronectin. PMA had a comparable effect on these interactions, although with different morphological effects, suggesting the existence of more than one transduction signal for U-937 adhesion. As with previous examples, evidence points to a conformational change, directed through the β_1 cytoplasmic domain, to explain VLA activation, and this change might also involve the α subunit, since specific antibodies against VLA α subunits abrogated the effects mediated by the anti-β antibody. It is likely that connection with cytoskeleton is needed, as its disruption cancels this effect. Interestingly, an active Na^+/H^+ antiporter was also required, suggesting that alkalinization of the cytoplasm is necessary, as with $\alpha_{IIb}\beta_3$. This pH rise is likely to have implications in the modulation of other integrins activities (see below).

The platelet integrin $\alpha_{IIb}\beta_3$ is important in platelet activation. Although fibrinogen and VWF are present in blood at concentrations that would saturate an active $\alpha_{IIb}\beta_3$, this integrin in circulating platelets is inactive. When platelets are activated, through an agonist-induced pathway, $\alpha_{IIb}\beta_3$ undergoes a conformational change and binds to its soluble ligands. There is also an agonist-independent activation. Platelets exposed to the subendothelial matrix, after vessel damage, will bind to immobilized fibrinogen and VWF, and these interactions will induce $\alpha_{IIb}\beta_3$ binding to soluble adhesive ligands.

The mechanisms by which $\alpha_{IIb}\beta_3$ conformation is changed in platelet activation is unknown. Deletion of the cytoplasmic region of α_{IIb} constitutively activates the receptor (O'Toole *et al.*, 1991), suggesting that this domain is central in the regulation of the receptor affinity for its ligands, possibly through modulated interactions with the cytoskeleton or soluble messengers in the cytoplasm. Platelet activation involves G protein activation, rises in intracellular pH and Ca^{2+} and activation of protein kinases (Shattil and Brugge, 1991). One possibility is that cytoplasmic domains of $\alpha_{IIb}\beta_3$ are phosphorylated. Indeed, β_3 is phosphorylated in Ser/Thr, although with such a low stoichiometry (less than 3%) that it is likely to have no physiological relevance.

The cytoplasmic tail of β_3 has a Tyr in position 747 with homology to tyrosine phosphorylation sites in EGF and insulin receptors, and $pp60^{src}$ phosphorylates this residue *in vitro*. However, tyrosine phosphorylation has never been detected in $\alpha_{IIb}\beta_3$ in intact cells.

On the other hand, phosphorylation may have an important role in transducing a signal to the cytoplasm through $\alpha_{IIb}\beta_3$, since its activation and binding to fibrinogen, together with platelet aggregation, are necessary for

tyrosine phosphorylation of several proteins upon platelet activation by thrombin, including pp125FAK (Brugge and Shattil, 1990; Burridge *et al.*, 1992).

Other data are available about platelet and $\alpha_{IIb}\beta_3$ activation (Shattil and Brugge, 1991), although the overall picture is far from clear. Obviously, further investigation will be needed to understand how conformational changes in $\alpha_{IIb}\beta_3$ are brought about after platelet activation, and where they fit in the platelet activation process itself.

5.4. Changes in gene expression induced by integrins

Other studies involving occupancy of receptors with antibodies or synthetic peptides have provided some insight into the the role of integrins in other systems For instance, Werb *et al.* (1989) have reported that binding of a monoclonal antibody to $\alpha_5\beta_1$ blocks initial adhesion of fibroblasts to fibronectin and induces gene expression of the ECM-degrading metalloproteinases collagenase and stromelysin. However, unlike with phorbol esters, cytochalasins and growth factors, which also may induce metalloproteinase expression, the addition of the anti-$\alpha_5\beta_1$ antibody in solution to cells spread on collagen or serum adhesion proteins does not cause changes in morphology or actin organization. It is interesting that fibronectin cannot trigger the same effects as this antibody, but RGD peptides derived from the sequence of fibronectin itself can.

These data suggest that $\alpha_5\beta_1$ can mediate signal transduction from outside the cell to the nucleus. It is not difficult to imagine a situation in development in which interaction of integrins with ECM components triggers the expression of some proteases which will change ECM composition, and this in turn might modulate the pattern of integrin expression, giving place to a new stage in development.

Menko and Boettiger (1987) have also reported interesting results on the role of integrins in development, using the monoclonal antibody CSAT, which recognizes β_1. Chicken embryo myoblasts treated with CSAT fail to fuse and do not produce muscle-specific myosin. The removal of the antibody reverses this effect. Thus, occupation of β_1 integrins seems to be responsible for the withdrawal from the cell cycle, changes in gene expression and production of multinucleate myotubes typical of myogenesis. The authors suggest that β_1 integrins function in this case analogously to hormone or growth factor receptors and that integrin-ECM interactions permit signal transduction to the nucleus. However, it is possible that this transduction is downstream of cell adhesion, and not necessarily an effect of antibody occupancy of the integrin.

The use of RGD-containing peptides prevents gastrulation in Drosophila embryos. Naidet *et al.* (1987) reported that Drosophila embryos injected with RGD peptides at the syncytial blastoderm stage have a phenotype characterised by dorso-ventral simmetry and, later on, there will be no muscular movement or organogenesis, presumably due to the RGD inhibition of ventral furrow formation.

Another example was provided by Mackrell *et al.* (1988) when they reported that a β_1 integrin subunit was involved in the lethal (1) myospheroid mutation [l(1)mys] in *Drosophila melanogaster*. Embryos carrying this mutation appear normal until the first muscular contractions occur. Then the dorsal suture breaks and muscles detach from the ECM and become spheroidal. Besides these apparently mechanical effects on the mutant embryos, experiments using cells in culture show that myoblasts explanted from l(1)mys embryos fail to fuse (Donady and Seecof, 1972), as occurs in the experiments by Menko and Boettiger (1987). This similarity suggests a common mechanism in both cases and also that integrins are central in signal transduction pathways modulating development.

Adams and Watt (1990) showed that terminal differentiation of keratinocytes *in vitro* is accompanied by loss of adherence to several ECM molecules, including fibronectin, and precedes, by several hours, the loss of $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_5\beta_1$ integrins from the cell surface. They also showed that inactivation of $\alpha_5\beta_1$ occurs prior to its removal from the cell surface. Their results also suggest that $\alpha_2\beta_1$ and $\alpha_3\beta_1$ might equally be inactivated. It is tempting to speculate that these inactivations might cause loss of cell adherence to the basal layer, facilitating migration out of it and causing changes in integrin expression and other molecules relevant to terminal differentiation.

5.5. Role of integrins in cytoplasmic pH modifications

Other investigations have tried to understand integrin participation in transduction systems using a different approach. It has been known for some time that cytoplasm alkalinization is necessary for growth in a variety of cell types (Moolenaar, 1986). A similar pH modification has been reported by Schwartz *et al.* (1989) for a different process. They showed that cell spreading induced cytoplasmic alkalinization via the Na^+/H^+ antiporter. Integrins are likely to be involved since modification of spreading using anti-integrin antibodies and RGD peptides correlates with modifications in cytoplasmic pH. It is interesting that the constitutive action of *ras* and *src* oncoproteins (which localize in the plasma membrane) bypasses the necessity of cell spreading for

cytoplasmic alkalinization, suggesting that integrin-dependent pH rise in this system could be transduced via *src* and *ras* cellular homologues.

Complementary studies using capillary endothelial cells and defined media (Ingber *et al.*, 1990) showed clearly that fibronectin can induce DNA synthesis and cytoplasmic alkalinization independently of the presence of growth factors. More importantly, they showed that activation of the Na^+/H^+ antiporter, rather than alkalinization itself, is the key step for inducing DNA synthesis.

Further work on the role of integrins in cell alkalinization (Schwartz *et al.*, 1991) revealed that clustering of $\alpha_5\beta_1$ by insoluble fibronectin activated the Na^+/H^+ antiporter. However, fibronectin failed in this case to induce DNA synthesis, even at high doses. The same authors (Schwartz and Lechene, 1992) reported that platelet-derived growth factor (PDGF) activates the Na^+/H^+ antiporter through a protein kinase C dependent pathway, only after cells have spread, although the participation of integrins in this case was not assessed.

These results are important since they establish a link between cell spreading (probably integrin dependent) and a single molecular event, namely cytoplasm alkalinization. As this event is also found in transduction pathways led by growth factors, it seems reasonable to speculate about the existence of integrin-driven transduction pathways involving second messangers, analogous to those led by the "classical" growth factors.

The work described in this thesis shows that fibronectin receptor activity is modulated between mitosis and interphase (see below, Results and Discussion chapters). I will return to the subject of integrin regulation at interphase → mitosis transition in the Cell Cycle section in this chapter, and in the Discussion chapter.

6. CELL CYCLE

The cell cycle is the sequence of events leading to the duplication of cells. This sequence may be divided in four different stages: G_1, S (DNA synthesis phase), G_2 and M (Mitosis). Classically, G_1 and G_2 have been considered as mere gaps between the two easily identifiable mitotic and DNA synthesis phases (see figure 1.11). Biochemical and genetic research in the last decade has proved that cell cycle phases are intimately interconnected, and structural and functional details of several cell cycle-dependent processes, such as DNA replication, organization of the mitotic spindle, nuclear envelope breakdown

and others have been unveiled (Hartwell and Weinert, 1989). I will discuss in this section the most recent advances in our understanding of how the cell cycle is regulated.

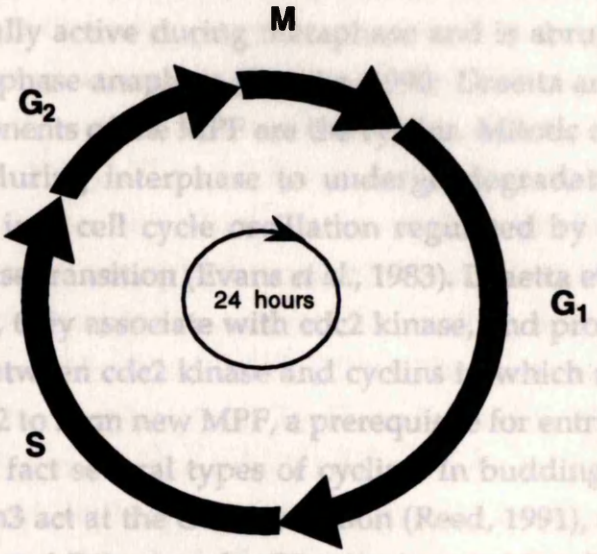


Figure 1.11. Phases of the cell cycle. A typical animal tissue cell in culture commited to the cell cycle takes ~24 hours to complete its division in two daughter cells. Quiescent cells are usually arrested in G_1, in a stage often called G_0 (Pardee, 1989).

6.1. Regulation of the cell cycle

Understanding the molecular events of the cell cycle proved difficult over many years. Geneticists isolated numerous cell division cycle (cdc) mutants from the fission (*Schizosaccharomyces pombe*; Hayles and Nurse, 1986)and budding (*Saccharomyces cerevisiae*) yeast (Hartwell and Weinert, 1989). One of those genes, $cdc2^+$, in *Schizosaccharomyces pombe*, was found to be of particular interest due first to its activity both at the onset of DNA synthesis and again at the transition G2--->Mitosis, and second because some alleles resulted in premature mitosis (wee phenotype; Nurse and Bissett, 1981). A gene in *Sacharomyces cerevisiae*, CDC28, was found to be homolog of $cdc2^+$. In fact, CDC28 rescues $cdc2^+$ mutations, and *vice versa* (Beach *et al.*, 1982). Both genes encode Ser/Thr protein kinases of ~34KDa (p34/cdc2 kinase), themselves susceptible to phosphorylation (Simanis and Nurse, 1986).

Subsequently, CDC28 homologues were found in clam, Xenopus, mouse and human (Draetta and Beach, 1988; Draetta *et al.*, 1989; Gautier *et al.*, 1988; Lee and Nurse, 1987) and, in fact, in all species so far studied, suggesting that this kinase forms part of a very well conserved mechanism by which G1 cells are driven into S phase and then into mitosis. Indeed, it soon became clear that

the cdc2 kinase was the catalytic subunit of a protein complex, called maturation-promoting factor (MPF), first described in amphibian oocytes and responsible for the regulation of G2/Mitosis transition in eukaryotic cell cycle (Draetta, *et al.*, 1989; Gautier *et al.*, 1988; Kishimoto and Kanatani, 1976). The enzyme is maximally active during metaphase and is abruptly inactivated at the transition metaphase-anaphase (Draetta, 1990; Draetta and Beach, 1988).

Other components of the MPF are the cyclins. Mitotic cyclins are proteins that accumulate during interphase to undergo degradation at the end of mitosis, resulting in a cell cycle oscillation regulated by proteolysis at the metaphase-anaphase transition (Evans *et al.*, 1983). Draetta et al. (1988) showed that, in HeLa cells, they associate with cdc2 kinase, and proposed a model for the relationship between cdc2 kinase and cyclins in which newly synthesized cyclin binds to cdc2 to form new MPF, a prerequisite for entry into mitosis.

There are in fact several types of cyclins. In budding yeast, G1 cyclins Cln1, Cln2 and Cln3 act at the G1-S transition (Reed, 1991), and mitotic B-type cyclins (B1, B2, B3 and B4) act at the G2-mitosis transition (Surana *et al.*, 1991). In higher organisms the picture is more complex. Cyclins A, B1 and B2 are active at the G2-mitosis transition. Cyclin A is also active at G1-S, binding not only to cdc2, but also to transcription factors E2F and DRTF1 and to the retinoblastoma protein (Elledge *et al.*, 1992). The situation is further complicated, since different cyclin-dependent protein kinases may play the role of cdc2 kinase at the G1-S transition. In *Xenopus*, a cdc2-like protein of ~32KDa is responsible for this transition and binds to two cyclin-like proteins of ~54KDa and to cyclin A (Fang and Newport, 1991). In humans, the kinase CDK2 (cyclin-dependent kinase 2) binds also to cyclin A and there is some evidence that this complex has some role in S phase (Elledge *et al.*, 1992). Both kinases have a high degree of homology with cdc2 and it is proposed that they are its functional homologues in G1/S. Indeed, there seems to be a family of cdc2-like kinases, called cyclin-dependent-kinases (CDK)* of which cdc2 is the prototypic member (Elledge *et al.*, 1992; Nigg, 1993).

Cyclin B is also known to be phosphorylated by mitotic cdc2 *in vitro* (Draetta and Beach, 1988; Morla *et al.*, 1989), and cyclin A is phosphorylated on Tyr in mouse cells (Hall and Vulliet, 1991). However, the physiological relevance of these phosphorylations is presently unknown.

In spite of the wealth of data about the regulation of cyclins during the cell cycle, why cells need several types of cyclins remains unclear. It seems

* I will use the terms CDK and cdc2 , as described by the different authors, although it is now clear that the cdc2 described in many papers does not correspond to p34/cdc2, and the term CDK activity seems more appropiate.

possible that different cyclins may provide different specificities, so that certain combinations of cyclin/CDK would phosphorylate only certain subsets of substrates. Further work will be needed before the full role for cyclins becomes apparent.

Cyclins are not the only proteins to regulate CDK activity. As mentioned above, cdc2 itself may undergo phosphorylation. Although Ser, Thr and Tyr residues have been shown to become phosphorylated (Draetta and Beach, 1988; Gould and Nurse, 1989), Thr and Tyr phosphorylation/dephosphorylation cycles seem to be the key elements in cdc2 activation. In particular, dephosphorylation of phosphothreonine14 and phosphotyrosine15, well conserved throughout evolution, activate the enzyme at the G2/M transition (Gould and Nurse, 1989; Morla *et al.*, 1989). The enzymes involved are the wee1 kinase, which phosphorylates Tyr15 and possibly Thr14, holding cdc2 inactive, and cdc25 phosphatase, which is responsible for both phosphotyrosyl and phosphothreonyl dephosphorylation (Kumagai and Dunphy, 1991; Millar and Russell, 1992). wee1 and cdc25 genes have been shown to determine the timing of mitosis in fission yeast, wee1 as an inhibitor and cdc25 as an activator (North, 1989). It has also been reported that cyclins B1 and B2 enhance cdc25 phosphatase activity 5-fold *in vitro*, suggesting that cdc25 may act on specific cdc2/cyclin complexes (Galaktionov and Beach, 1991). Further work in *Xenopus* (Kumagai and Dunphy, 1992) has established that cdc25 is itself a substrate for phosphorylation by cdc2 (see figure 1.12), being maximally active when phosphorylated in its N-terminus (corresponding with mitosis), thus establishing a positive feedback. cdc25 is weakly active when unphosphorylated (interphase), at which time the wee1 tyrosine kinase capacity on cdc2 is superior, resulting in a nett phosphorylated state for Tyr15. As for wee1 regulation, recent work (Coleman *et al.*, 1993) has shown that a protein kinase, nim1, also known as cdr1, directly phosphorylates wee1 at its C-terminus, causing its inactivation as a cdc2 tyrosine kinase. However, since the nim1 protein is not essential for viability (Russell and Nurse, 1987), there must be other components involved in the regulation of cdc2 tyrosine phosphorylation.

In summary, a clearer picture emerges from all these data, although there is obviously much still to be learned:

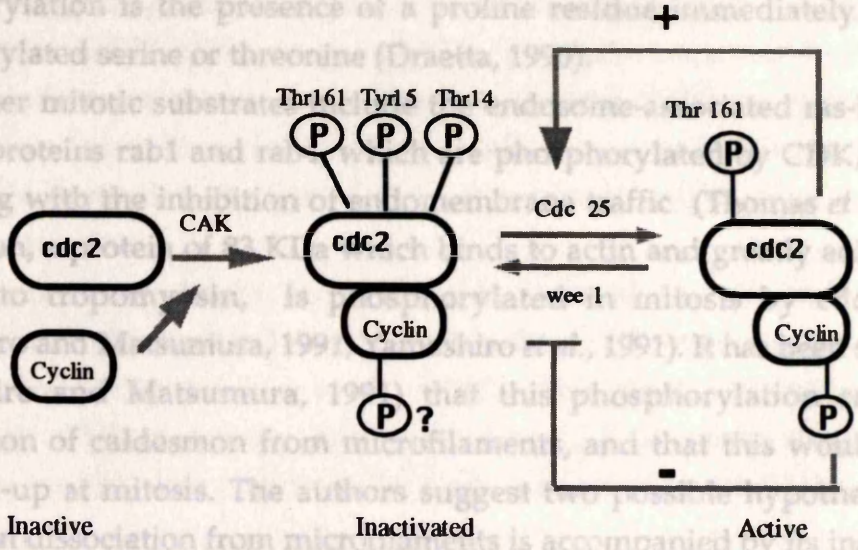


Figure 1.12. Regulation of cdc2 kinase. Binding of cyclin induces phosphorylation of Thr161, Tyr15 and Thr14. A novel kinase, CAK, is responsible for Thr161 phosphorylation (Solomon, 1993). wee1 accounts for Tyr15 and Thr14 phosphorylations. Full activation of cdc2 requires participation of cdc25, itself activated by phosphorylation by cdc2, thus establishing a positive feedback.

6.1.1. Substrates for mitotic CDKs

Much of the regulation of CDK activities has already been elucidated (see figure 1.12). Further work will eventually lead to the primary signal(s), internal and environmental, triggering mitosis. However, much less is known about the targets of such kinase activities. It is widely accepted that the structural reorganization of eukaryotic cells that takes place at the G2-Mitosis transition is brought about by specific CDK activities. This reorganization includes chromosome condensation, disassembly of nuclear *laminae*, rearrangement of cytoskeletal components and arrest of endomembrane traffic, among other events. Also, most mitotic cells in culture show a typical rounded morphology that will remain so until early anaphase, when daughter cells will respread (see Results and Discusion chapters). Much work has been focussed on the possible role of CDK activity in each of these processes. Early work showed the phosphorylation of mitotic histone H1, coinciding in time with chromosome condensation (Gurley *et al.*, 1974). More recently, the mitotic histone H1 kinase was shown to be identical to cdc2/cyclin (Arion *et al.*, 1988). However, a causal relationship between histone H1 phosphorylation and chromosome condensation remains unproven. The recognition sequence for cdc2 in histone H1 was shown to be S/TPXK. Since then, the study of candidate CDK substrates has shown that the requirement for CDK-dependent

phosphorylation is the presence of a proline residue immediately after the phosphorylated serine or threonine (Draetta, 1990).

Other mitotic substrates include the endosome-associated ras-like GTP-binding proteins rab1 and rab4, which are phosphorylated by CDK/cyclin B, coinciding with the inhibition of endomembrane traffic (Thomas *et al.*, 1992). Caldesmon, a protein of 83 KDa which binds to actin and greatly enhances its binding to tropomyosin, is phosphorylated in mitosis by cdc2 kinase (Yamashiro and Matsumura, 1991; Yamashiro *et al.*, 1991). It has been suggested (Yamashiro and Matsumura, 1991) that this phosphorylation causes the dissociation of caldesmon from microfilaments, and that this would lead to rounding-up at mitosis. The authors suggest two possible hypothesis. First, caldesmon dissociation from microfilaments is accompanied by its inactivation as an actomyosin ATPase inhibitor, resulting in the contraction of the actin-myosin system and eventual rounding. Alternatively, as tropomyosin-bound caldesmon inhibits the severing activity of actin by gelsolin, it is possible that its dissociation restores gelsolin activity, leading to the disassembly of stress fibres which, the authors claim, would induce cellular rounding. However, as disassembly of stress fibers is not necessarily followed by cell rounding, the possible role of caldesmon on stress fibers is unlikely to be relevant in mitotic rounding-up.

The mechanisms by which cells round up at mitosis are particularly relevant to the work described in this thesis, where one of the questions addressed is the possibility that integrins participate in mitotic rounding-up (see Results and Discussion chapters). To date, there is no published evidence that integrin activity is modulated during the cell cycle. However, it has been reported that a serine from the cytoplasmic domain of β_1, in $\alpha_5\beta_1$, becomes phosphorylated in mitosis, and that mitotic $\alpha_5\beta_1$ does not bind to fibronectin (unpublished data by Grandori and Hynes, quoted in Hynes (1992); see also Discussion chapter).

It is unlikely that $\alpha_5\beta_1$ is phosphorylated directly by cdc2, as it does not contain the consensus sequence P-S/T in either of its subunits (Chan *et al.* 1992; Marcantonio *et al.* 1990; but see Discussion chapter).

$pp60^{c\text{-}src}$ is another substrate for cdc2 that may be relevant for mitotic rounding-up. Two steps are required for $pp60^{c\text{-}src}$ activation. Firstly, residues Thr34 and Ser72, both conserved in frog, chicken, mouse and human, become phosphorylated by cdc2. Then, dephosphorylation of phosphotyrosine527 is further required for activation. When cdc2-induced phosphorylation of Thr34 and Ser72 is blocked by site-directed mutagenesis, $pp60^{c\text{-}src}$ is partially

inhibited, suggesting that cdc2 somehow participates in dephosphorylation of phosphotyrosine527. (Shenoy *et al.*, 1992). Downstream pp60$^{c\text{-}src}$ substrates might include components controlling cytoskeletal architecture and/or cell shape, known to be altered in transformed cells, as well as in mitosis (Shenoy *et al.*, 1992). In particular, changes in those components might cause the mitotic rounding-up.

Myosin-II regulatory light chain is claimed to be a substrate for CDK/Cyclin B, although it does not contain the S/TP consensus present in all other substrates (Satterwhite *et al.*, 1992). This phosphorylation inhibits actomyosin ATPase activity and, accordingly, it is postulated that this inhibition would delay contractile ring formation until CDK/Cyclin B inactivation.

As for the mitotic microtubule reorganization, microtubule dynamics and centrosome-nucleating activity can be modulated by addition of CDKs to cell-free systems, although the actual substrates have not yet been identified (Nigg, 1993). Nevertheless, some microtubule-associated proteins become phosphorylated in mitosis, and localize to the spindle fibers, in HeLa cells (Vandre *et al.*, 1991), and it is possible that they are CDK substrates.

Vimentin, an intermediate filament (IF) protein, is phosphorylated by cdc2 in mitosis (Chou *et al.*, 1991; Chou *et al.*, 1990). Also, phosphorylation of polymerized vimentin IFs *in vitro* by cdc2 causes their disassembly.

Another important event at the G2-Mitosis transition is nuclear envelope breakdown, which requires nuclear *laminae* A,B and C disassembly, brought about by CDK/Cyclin B phosphorylation (Heald and McKeon, 1990; Luscher *et al.* 1991). However, lamina phosphorylations are not sufficient for envelope breakdown, which probably requires additional CDK/cyclin B-directed action (Nigg, 1993).

The proto-oncogene c-myb is also a substrate for cdc2/cyclin B (Luscher and Eisenman, 1992). Myb is a DNA binding protein involved in transcriptional regulation. Its phosphorylation correlates with reduced DNA-binding ability. It has been suggested that its release from DNA might be necessary for an efficient chromosome condensation (Luscher and Eisenman, 1992).

6.1.2. Substrates for G1 and S CDKs

As mentioned above, CDK/cyclin activities also control the G1/S transition. Candidate substrates include tumor supressor gene products. In particular, the retinoblastoma protein (pRb) contains multiple CDK phosphorylation sites. pRb is kept inactive by hyperphosphorylation by

CDK/cyclin D2 on those sites in late G1 (Nigg, 1993), allowing progression through the cell cycle. p53 (Hartwell, 1992), is phosphorylated by CDK/p60 and CDK/cyclin B at Ser315 (Bischoff and Friedman, 1990), close to the onset of DNA replication. p53 is a tumour-supressor. When DNA is damaged, p53 acumulates and switches off replication, to allow for time for repair. If the repair fails, p53 triggers apoptosis (Lane, 1992).

CDK/Cyclin A and CDK/Cyclin B also participate in DNA synthesis control (Girard *et al.*, 1991). The only substrate identified so far is the 32-34 KDa subunit of the single-stranded DNA-binding replication protein A (RPA), a protein required for the unwinding of origin DNA. Both complexes phosphorylate RPA during S and G2, and CDK/cyclin B stimulates origin unwinding and DNA replication in G1 cell extracts (Dutta and Stillman, 1992).

Table 1.3 contains the known substrates for CDK/cyclin complexes, at least *in vitro*,, at different stages of the cell cycle. Further investigations will clarify their exact relevance *in vivo*, and new substrates will no doubt be identified.

G1 substrates
p53
pRb
G2 substrates
cyclin B
cdc25 phosphatase
S substrates
SV40 T
DNA polymerase α (?)
Mitotic substrates
Nuclear lamins
Vimentin
Myosin regulatory LC
Caldesmon
Neurofilament H
Histone H1
p60src
p150abl
Casein kinase II (α/β)
SW15
c-myb
oct-1 (?)
c-fos/jun (?)
Rab1/Rab4
EF-1γ
RNA polymerase α (?)

Table 1.3. **Substrates for CDK/cyclin complexes**

CHAPTER 2

MATERIALS & METHODS

1. MATERIALS

1.1. Media for cell culture

Analar grade reagents were used.

Hepes saline (HS)

NaCl	140mM
KCl	5mM
D-glucose	5mM
Hepes	10mM
0.5% phenol red	0.003mM
pH 7.5	

Hanks' Hepes (HH)

NaCl	140mM
KCl	5mM
$CaCl_2$ ($2H_2O$)	1mM
$MgCl_2$($6H_2O$)	1mM
D-Glucose	5mM
Hepes	10mM
0.5% phenol red	0.003mM
pH 7.5	

These buffers are dispensed in 100 ml aliquots, autoclaved and stored at 4°C.

Hepes is N-hydroxethylpiperazine-N'-2-ethane sulphonic acid.

Versene

NaCl	140mM
KCl	2.5mM
Na_2HPO_4	8mM
KH_2PO_4	1.5mM
EDTA	0.5mM
0.5%phenol red	0.003mM

EDTA is ethylenediaminetetra-acetic acid.

This salt solution is dispensed in 20ml aliquots, autoclaved and stored at 4°C.

Trypsin

2.5% sterile trypsin (Gibco) was dispensed in 2ml aliquots and stored at -20°C. 0.5ml of this solution were added to 20ml versene (trypsin/versene solution) for the suspension of cell monolayers.

Ham's F10 medium

Hepes water	180ml, 120mM
X10 HamsF10 (Gibco)	20ml
$NaHCO_3$	1ml
GPSA	5ml
TPB	20ml
Foetal calf serum	20ml

Tryptose Phosphate Broth (TPB)

29.5g/l TPB (Gibco) in double deionised water was dispensed in 20ml aliquots, autoclaved and stored at 4°C.
pH 7.4

GPSA

Glutamine	114mM
Penicillin	1905U/ml
Streptomycin	1905U/ml
Fungizone (Amphoterisin B)	11.9 µg/ml

Bicarbonate

$NaHCO_3$	7% (w/v)

Sterilised by Millipore ultra filtration and dispensed in 20ml aliquots.

Phosphate Buffered Saline (PBS)

NaCl	170mM
KCl	3.4mM
Na_2HPO_4	10mM
KH_2PO_4	1.8mM
$CaCl_2$	1mM

$MgCl_2$ 1mM
pH 7.4

1.2. Tissue culture vessels

Plastic tissue culture flasks and Petri dishes were from Greiner (Germany) or Sterilin (U.K.).

1.3. Solutions and reagents used for synchronization

Nocodazole

Methyl (5-(2-thienylcarbonyl)-1H-benzimidazol-2yl) carbamate (nocodazole) was from Sigma. It was used from a stock solution of 1.5mg in 1ml DMSO, kept at 4°C.

Thymidine

Thymidine (cell culture tested) was from Sigma. It was used from a filter-sterilized stock solution 200mM in HH and kept at -20°C.

1.4. Reagents used for Polyacrylamide Gel Electrophoresis

Materials and methods were as described by Laemmli (1970). Electrophoresis purity reagents were used. N,N,N',N' -tetramethylenediamine (TEMED) and Sodium Dodecyl Sulphate (SDS) were from Sigma. All other reagents were from BDH.

1.5. Antibodies

Antibodies were used for immunofluorescence, flow cytometry, immunoprecipitation and immunoblotting as described below. Lyophilized rabbit anti-human integrin $\alpha_5\beta_1$ and rabbit anti-human integrin $\alpha_V\beta_3$ polyclonal antibodies were from Telios (U.S.A.), and were redissolved in physiological buffer as indicated by the manufacturers. They were dispensed in 10 µl aliquots and kept at -70°C. Rabbit anti-

human integrin β_3 subunit polyclonal antiserum was from Chemicon International, Inc., USA. It was dispensed in 10 µl aliquots and kept at -70°C. Mouse anti-phosphotyrosine (PY20) and TRITC-conjugated anti-rabbit IgG were from Sigma; FITC-conjugated anti-rabbit IgG and preimmune rabbit serum were from the Scottish Antibody Production Unit (SAPU), U.K.

1.6. Solutions and reagents used for Western blotting

Electroblotting buffer

Tris base	250mM
Glycine	2M
Methanol	20%

Milk block buffer

Tris base	10mM
NaCl	150mM
Dry milk	4%
pH 8	

Tween buffer

Tris base	10mM
NaCl	150mM
Tween	0.2%

Tween is polyoxyethylenesorbitan.

1.7 Solutions and reagents used for immunoprecipitation

lysis buffer

Tris base	50mM
NaCl	150mM
$MgCl_2$ ($6H_20$)	3mM
NH_4VO_3	1mM
NaN_3	0.02%
PMSF (added just before use)	100µg/ml
Leupeptin (added just before use)	1µg/ml

Triton X-100	1%
NaF	5mM

Adjust pH to 8

PMSF (phenylmethylsulphonyl fluoride)

Used fresh for each experiment. The stock solution was made by dissolving 20mg of PMSF in 1ml DMSO (dimethyl sulfoxide), dispensed in 50 µl aliquots and .stored at -20°C.

Leupeptin

Used fresh for each experiment. The stock solution was made by dissolving 2mg of leupeptin in 1ml HH and aliquoted and stored as with PMSF.

Iodogen

1mg of 1,3,4,6-Tetrachloro-3a, 6a,-diphenylglycoluril (iodo-gen™) was placed in a glass universal. 1ml chloroform was then added and left overnight to evaporate.

Iodine-substituted HH

NaI	140mM
KCl	5mM
$CaCl_2(2H_2O)$	1mM
$MgCl_2(6H_2O)$	1mM
Hepes	5mM
NaN_3	0.01%
pH 8	

Protein A-agarose

Protein A was from Sigma, insolubilized on cross-linked 4% beaded agarose, in a suspension 0.5M NaCl containing 0.02% thimerosal. 1ml of this suspension had a binding capacity of 20-25 mg human IgG.

1.8. Solutions and reagents used for affinity chromatography

Urea

urea	8M
Tris	50mM
pH 7.5	

Citrate buffer

Na_2HPO_4	10mM
KH_2PO_4	1.5mM
NaCl	0.15M
Sodium Citrate	10mM
pH 7.2	

Digestion buffer

Tris	10mM
NaCl	50mM
EDTA	0.5mM
pH 7.6	

Chymotrypsin

Chymotrypsin was from Sigma. The stock solution was made to 1mg/ml in digestion buffer and stored at -70°C in 200µl aliquots.

$DPCCl_2$

Diphenylcarbamyl chloride was from Sigma. The stock solution was made to 1M in ddH_2O and stored at -70°C in 200µl aliquots.

Lysis buffer

$MnCl_2$	1mM
ßOG	100mM
NaF	5mM
NH_4VO_3	1mM
NaCl	150mM
Tris	50mM
PMSF	3mM
pH 7.3	

PMSF was added just before the cell extraction.

ßOG is n-octyl-ß-glucopyrannoside.

Washing buffer

Similar to lysis buffer, with ßOG 25mM instead of 100mM.

Eluting buffer

Similar to washing buffer, without divalent cations and with 20mM EDTA.

NaF and NH_4VO_3 were used as phosphatase inhibitors and in some experiments were omitted.

1.9. Materials and reagents used for spreading assays

Square glass coverslips were from Chance Propper Ltd. Coomassie blue was from Sigma and was used as a 1% solution in methanol:distilled water:glacial acetic acid 50:50:7. All reagents used in cell suspensions were of Analar grade. Lyophilized, sterile colchicine was from Sigma and was dissolved aseptically in HH to a concentration of 100 µg/ml and kept at 4°C. Hemoglobin was from Sigma and fibronectin was isolated as described below (see Methods section).

1.10 Radioactive materials

^{125}I was supplied by Amersham International plc. U.K., as NaI in dilute sodium hydroxide solution, ~17Ci/mg, pH 7-11. It was diluted in HH to a radioactive concentration of 4mCi/ml.

Tran^{35}s-label™ was from ICN Biomedicals Ltd. U.K., and contained ~70% L-methionine (^{35}S) and ~15% L-Cysteine (^{35}S). It was diluted in methionine and cysteine-deficient medium to a concentration of 0.5 mCi/ml.

^{32}P was supplied by Amersham International plc. U.K., as orthophosphate in acid free solution, 8500-9120Ci/mmol. It was diluted in HH to 5mCi/ml.

1.11 Calyculin A

Calyculin A, a phosphatase inhibitor, was the kind gift of Dr. L. Gasmi. A stock solution of 1 µg/ml in ethanol was kept at -70°C.

2. METHODS

2.1. Cell culture and growth conditions

HeLa cells were grown as monolayers on plastic, in Ham's F10 medium containing 7.5% foetal bovine serum and 10% tryptose phosphate broth, and checked periodically for mycoplasma. Cells were subcultured when almost confluent, usually every 3-4 days, depending on the initial number seeded. To obtain cells in suspension, cell monolayers were washed twice with HS and trypsin/versene suspension was added for 1 minute. Excess fluid was poured off and cells left at 37°C until rounded. Ham's medium was then added to stop trypsin activity. Cell suspensions were centrifuged for 5 min. at 1,000 r.p.m., resuspended in HH and aspirated in a Pasteur pipette to achieve single cell suspensions.

Stocks of frozen cells were kept at -70°C and prepared as follows: cell suspensions were centrifuged for 5 min. at 1,000 r.p.m., resuspended at a concentration of $5x10^6$ cells per ml in foetal calf serum containing 10% DMSO, aliquoted in cryotubes and kept at -70°C. For recovery, cells (1 ml) were thawed in a 37°C bath, added to 10ml of Ham's F10 medium, and centrifuged as indicated above. Cells were then resuspended in fresh medium, seeded in a $25cm^2$ culture flask and kept at 37°C.

2.2. Fluorochrome staining for mycoplasma

10^5 cells per well were grown overnight on 13 mm coverslips in a 5x5 wells sectored box. Next day cells were fixed for 2 minutes in glacial acetic acid:methanol 1:3. The fixative was removed and more fixative was added for a further 10 minutes. Cells were then rinsed twice with distilled water. Water was removed and fluorochrome bis benzimide "Hoechst 33258" (50µg/ml) was added and incubated for 30 minutes at 37°C. Coverslips were then rinsed thoroughly with distilled water and mounted on slides with PBS/glycerol 1:1 and examined using a Vickers Photoplan fluorescence microscope, using filters as for fluorescein.

2.3. Cell synchronization

Populations enriched in mitotic cells were obtained basically as described (Zieve *et al.* 1980). Briefly, aliquots of $4x10^6$ cells were seeded in 175 cm^2 culture flasks and incubated for 16-18 hours with 2.5 mM thymidine. Cells were then washed twice with HH, incubated for seven hours with normal medium and finally 0.05 µg/ml nocodazole were added for a further 8 hours. This treatment gave a population highly enriched in mitotic cells, which could be easily detached from the substrate by shaking off. Each flask yielded ~10^6 cells with a mitotic index of >95%, as measured by flow cytometry. For immunofluorescence experiments, the same treatment was applied using 2.5 cm tissue culture dishes instead of culture flasks.

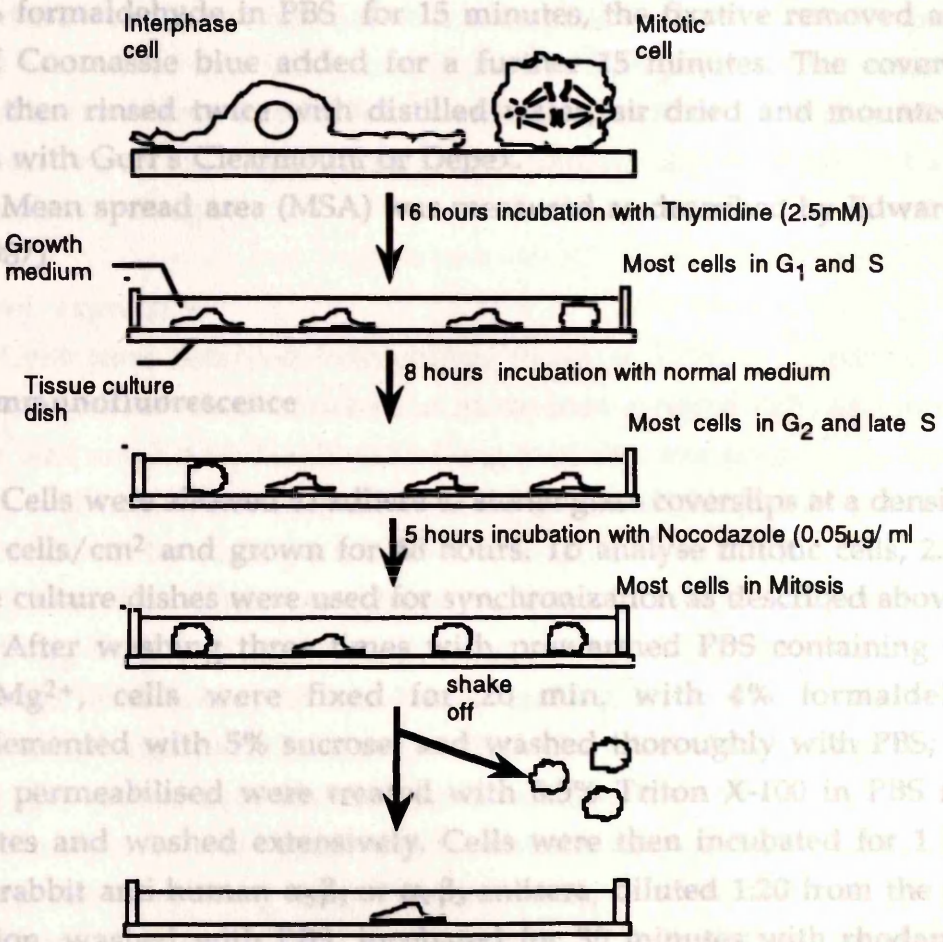


Figure 2.1. Synchronization of HeLa cells by thymidine and nocodazole blocks. See text for details.

2.4. Spreading assays

2 ml cells at $25x10^3$/ml in HH, harvested as indicated above, were normally used for all spreading experiments. 22 mm square coverslips were cleaned in an acid bath (sulphuric acid, nitric acid 1:1) for 30 minutes, rinsed thoroughly with tap water, then rinsed twice with ddH_2O, placed in 2.5 cm Petri dishes and precoated with fibronectin (40µg/ml in HH), calf serum (1% in HH) or haemoglobin (0.5mg/ml in HH). These solutions were left for at least 30 minutes at room temperature and the dishes were washed twice with HH before plating the cells.

Various additions to the cell suspensions were made depending on the experiment: Mn^{2+}, nocodazole, colchicine, vanadate, calyculin A and serum. Cells were then incubated at 37°C. To stop incubation, duplicates of samples were fixed at the indicated time intervals by addition of 1 ml of 4% formaldehyde in PBS for 15 minutes, the fixative removed and 2 ml of Coomassie blue added for a further 15 minutes. The coverslips were then rinsed twice with distilled water, air dried and mounted on slides with Gurr's Clearmount or Depex.

Mean spread area (MSA) was measured as described by Edwards *et al.* (1987).

2.5. Immunofluorescence

Cells were allowed to adhere to sterile glass coverslips at a density of $5x10^4$ cells/cm^2 and grown for 48 hours. To analyse mitotic cells, 2.5 cm tissue culture dishes were used for synchronization as described above.

After washing three times with prewarmed PBS containing Ca^{2+} and Mg^{2+}, cells were fixed for 20 min. with 4% formaldehyde supplemented with 5% sucrose, and washed thoroughly with PBS; cells to be permeabilised were treated with 0.5% Triton X-100 in PBS for 3 minutes and washed extensively. Cells were then incubated for 1 hour with rabbit anti-human $\alpha_5\beta_1$ or $\alpha_v\beta_3$ antisera, diluted 1:20 from the stock solution, washed with PBS, incubated for 30 minutes with rhodamine-conjugated anti-rabbit IgG, diluted 1:100 from the stock solution, and washed three times with PBS. Coverslips were then mounted in

undiluted glycerol, to which 5% n-propylgallate was added to inhibit photobleaching, and sealed with clear nail polish.

Representative fields of cells were photographed using a Vickers Photoplan microscope equipped for epifluorescence and Kodak T-Max 400 film.

2.6. Flow cytometry

Flow cytometry was used to monitor cell synchronization and to analyze integrin expression.

Cell synchronization

~10^6 cells treated for synchronization with thymidine and nocodazole as described above were centrifuged (5 min. 1000rpm), and resuspended for 5 min. in 70% ethanol at -20°C. Ethanol was then removed by centrifugation and cells resuspended in 1ml. PBS. Propidium iodide was added to a final concentration of 50 µg/ml and cells analyzed immediately using a FACScan machine (Beckton Dickinson & Co. Oxnard, Ca), in the Department of Immunology and Bacteriology at the University of Glasgow.

Integrin expression

Cells were detached from culture flasks as follows: for mitotic cells, a 175 cm^2 culture flask enriched in metaphase-arrested cells as described above was used: nocodazole-containing medium was poured off, HS was added and cells were then collected by gentle shaking. Interphase cells were detached by adding 1 mM EDTA for 20 min. at 37°C, after washing twice with HS. Both samples were then washed twice with PBS containing Ca^{2+} and Mg^{2+}, and aliquots containing $5x10^5$ cells were incubated for 1 hour with anti-human $\alpha_5\beta_1$ or $\alpha_v\beta_3$ antisera. Cells were then washed again and incubated for 30 min. with FITC-conjugated anti-rabbit IgG diluted 1:100 from the stock solution.

Finally cells were washed three more times, resuspended in 0.5 ml PBS and analysed using a FACScan machine.

Propidium iodide to a concentration of 2 µg/ml was used in the final incubation from a stock solution of 100 µg/ml in PBS, to gate out dead cells.

2.7. Immunoprecipitation of cell surface integrins

Immunoprecipitation of integrins and SDS-PAGE analysis was carried out as follows:

Interphase cells (~10^7) were washed twice with warm HS. Prewarmed versene was then added and changed for fresh versene after 5 min. Cells were incubated at 37°C until rounded. After pouring off the versene carefully, 10 ml HH were added. The resulting cell suspension was transferred to a plastic universal and cell clumps removed by pipetting. After centrifugation for 5min at 1,000 r.p.m., cells were resuspended in 10ml HH, centrifuged again and finally resuspended in 2ml HH + 5mM PMSF, 1µg/ml leupeptin.

Immediately, cells were transferred to an iodogen-coated glass universal and 1mCi ^{125}I was added, in a well ventilated fume hood. After iodination (20 min., 20°C, occasional agitation), cells were transferred to a glass conical bottomed tube containing ~20ml I-HH. Cells were then centrifuged, 5min., 1,000 r.p.m., supernatant discarded down the sink with plenty of water (tap was left on for a few minutes) and resuspended with another 20ml of I-HH. Centrifugation and discarding of supernatant was repeated again.

All subsequent steps were carried out in a cold room, at ~4°C:
1 ml per 10^7 cells lysis buffer was added to the cell pellet and left for 20 min. with occasional agitation. To remove nuclei and cytoskeletal structures, cells were transferred to an Eppendorf tube and centrifuged at 13,000 r.p.m. for 18 min. The supernatant (detergent-soluble fraction) was recovered and 0.05 volumes of preimmune rabbit serum +25µl proteinA-agarose beads were added for preclearing. This solution was left on a rotary mixer (Emscope) for 30min. Agarose beads were then centrifuged for 1 min. at 13,000 r.p.m. and supernatant split into several aliquots in Eppendorf tubes. Appropriate antibodies (specific or preimmune serum, 10µl) were then added and incubated for 2 hours.

After incubation with antibodies, 30µl of proteinA-agarose were added to each aliquot and incubated for a further hour. The suspension was centrifuged for 1 min.at 13,000 r.p.m. and supernatant carefully removed with a disposable pipette tip.

For washing, 0.4ml. of lysis buffer were added to each aliquot and the agarose beads were resuspended by vortexing. After a few minutes,

the operation was repeated. Finally, a wash with buffer without detergent (same composition as lysis buffer, but detergent-free) was carried out, taking care to remove all traces of buffer from walls of the Eppendorf tube. Samples were then analysed by SDS-PAGE, under reducing or non reducing conditions. Gels were dried under vacuum and radioactive proteins detected by autoradiography, using X-Omat film (Kodak) and intensifying screens (DuPont). Films were exposed at -70°C for 1-2 weeks, depending on the experiment.

2.8. Measurement of radioactivity by TCA precipitation

Radioactivity from radioiodinated samples was determined by TCA precipitation as follows: one ml of 0.1% calf serum in HH to act as a carrier was mixed with one volume of 10% trichloroacetic acid (TCA) in a test tube and 5µl of radioactive sample added. This mixture was left for a minimum of 15 min. at room temperature. Radioactive proteins were then collected on a Millipore filter (0.22 µm) and radioactivity counted on a γ-counter (for ^{125}I) or a scintillation counter (for ^{35}S).

2.9. Protein measurement from ^{32}P-labeled cells

Bradford's reagent was used to measure protein concentration in samples to be compared in immunoprecipitation experiments.

20 µl of different concentrations of BSA in lysis buffer (0.25µg/ml to 0.5mg/ml) were added to 10 ml Bradford's reagent and absorption readings at λ=595nm were obtained, in order to make calibration curves. 20µl from the samples to be compared were also added to 10ml Bradford's reagent and the readings at 595nm used to calculate protein concentration with the calibration curve.

2.10. Immunoblotting

Preparation of samples

For interphase samples, cells were grown in 10cm Petri dishes until near confluent, and mitotic cells removed by washing three times with

HH. Cold lysis buffer (0.5ml per plate, the same as described for immunoprecipitation, including 100µg/ml PMSF, 1mM NaF and 5mM NH_4VO_3) was then added and left at 4°C for 20 min. Cells were then scraped off, transferred to an Eppendorf tube and insoluble material pelleted by centrifugation 18 min. at 13,000 r.p.m. The supernatant was transferred to another Eppendorf and 0.25 volumes of boiling mix were added.

When the effect of calyculin A was to be studied, samples were prepared as follows: cells were grown and washed with HH as with interphase cells and 2ng/ml of calyculin A in 2ml of HH were finally added until cells rounded up, usually less than 30 min. This solution was then carefully removed with a pipette and the cells lysed and prepared for electrophoresis as with interphase cells.

Mitotic cells were obtained as described above for all other purposes, washed twice with HH by centrifugation (1,000 r.p.m. for 5min.) and the pellet lysed (1ml lysis buffer per 10^7 cells) for 20 min. at 4°C. Samples were then cleared of insoluble material and prepared for electrophoresis as with interphase cells.

SDS-PAGE and protein analysis

Western blotting was carried out as follows, in the CLIC laboratory, Department of Pathology and Microbiology, at the University of Bristol: samples were boiled for 3min. before loading on a 10% polyacrylamide minigel (protein from ~10^5 cells per well). Electrophoresis were run for 40min at 200V using rainbow molecular weight markers (Amersham International). Proteins were then transferred from the gel to nitrocellulose paper (Hybond-ECL, Amersham International) with a transblot apparatus (Bio-Rad). The electroblotting was left for 1 hour at 100V. After dismantling the blotting sandwich, blocking of non specific sites was carried out using milk blocking buffer overnight. Filters were then placed in polythene bags and incubation with first antibodies (anti $\alpha_5\beta_1$ at 1:1000 or anti phosphotyrosine at 1:500) took place for a minimum of 3 hours on a rocking platform, followed by a series of washes: twice with milk blocking buffer (10 min each), twice with Tween buffer (10 min each) and finally once with milk block buffer (10 min). These washes were performed in a sandwich box with continuous agitation.

Filters were then placed in new polythene bags and incubated with second antibodies (goat anti-rabbit IgG conjugated to horse radish

peroxidase (HRP) at 1:2000 or goat anti-mouse IgG-HRP at 1:2000) for 1 hour at room temperature on a rocking platform. Subsequent washes were performed in a sandwich box as follows: twice with milk block buffer (20 min each), four times with Tween buffer (10 min each) and a last rinse with distilled water.

Protein detection was performed using enhanced chemiluminiscence (ECL) according to the manufacturer's protocol (Amersham International). Horse radish peroxidase catalyses the oxidation of luminol in the presence of hydrogen peroxide. Immediately after oxidation, luminol is in an excited state, returning to the basal state by the emission of light, which is detected on a film (hyperfilmECL; Amersham).

2.11. Isolation of fibronectin

Fibronectin was isolated from calf serum (Gibco) on a gelatin-Sepharose column, as described by Engvall and Ruoslahti (1977). Briefly, the gelatine-Sepharose column (20 ml, prepared according to the manufacturer instructions, Pharmacia) was washed with three volumes of citrate buffer, and 500 ml of calf serum (Gibco) were added at a flow rate of 40 ml/h, followed by citrate buffer until the ultraviolet absorbance (280 nm) of the eluent reached that of the citrate buffer. Fibronectin was then eluted with 8M urea and stored at 4°C at a concentration of ~1 mg/ml until used. Fibronectin purity was assessed by SDS-PAGE.

2.12. Affinity chromatography

Isolation of the cell-binding fragment from fibronectin

Fibronectin, isolated as described above, was dialysed extensively from 8M urea against digestion buffer, and proteolysed by incubating with 2 µg/ml chymotrypsin at 37°C for 30 min. Normally, ~100ml of fibronectin, at 1.6-1.7 mg/ml were used. The digestion was stopped by adding $DPCCl_2$ and PMSF to a final concentration of 1mM each.

The digested fibronectin solution was fractionated on a gelatin-Sepharose column, and material that did not bind to the column was directly used as the cell-binding fraction (CBF). Usually, ~50 mg of protein

were recovered in this fraction, and were concentrated to 1mg/ml through vaccuum dialysis for coupling to Sepharose 4B. The digestion was monitored by SDS-PAGE under reducing conditions.

Proteolytic fragments that did not bind to gelatin were coupled to cyanogen-bromide-activated Sepharose 4B according to the manufacturer's instructions. This resulted in the coupling of an average of 4.2 mg of protein per millilitre of gel.

Isolation of the fibronectin receptor from CBF-Sepharose columns

The procedure was based on that described by Pytela *et al.* 1985. For each cell sample (i.e. interphase, mitotic or calyculin-treated cells), one milliliter columns of CBF-Sepharose were equilibrated in washing buffer, with or without phosphatase inhibitors (NaF 5mM, NH_4VO_3 1mM). Radioiodinated cell lysates from ~$2x10^7$ cells per sample, prepared exactly as for immunoprecipitation (except that β-octylglucoside was used as detergent instead of Triton X-100), were passed through the columns at the lowest rate possible, using a peristaltic pump (2132 Microperpex®, LKB). The columns were then washed with 10ml of washing buffer containing 1mM $MnCl_2$ (Gailit and Ruoslahti, 1988) and bound material eluted with 20mM EDTA (eluting buffer).

When phosphatase inhibitors were used, care was taken to include them in every buffer, from cell lysis to elution of the fibronectin receptor from the CBF-Sepharose column.

Material eluted with EDTA was directly analysed by SDS-PAGE, loading 50 µl per well, and autoradiographed, or previously immunoprecipitated with polyclonal anti-$\alpha_5\beta_1$ antibody.

Radioactivity, determined by TCA precipitation as described above, was measured for every fraction from the application of the cell lysate to the column, in order to monitor the presence of washed and eluted material.

2.13. Analysis of integrin phosphorylation

Immunoprecipitation of integrins from cells radiolabelled with ^{32}P was carried out in order to find out possible differences in phosphorylation between mitotic and interphase integrins. Integrins from calyculin-treated cells were also analysed using ^{32}P. Differences in

phosphotyrosine protein levels were analysed using the monoclonal PY20 antibody against phosphotyrosine, in all samples (i.e. mitotic, interphase and calyculin-treated cells). The procedure was as described for immunoprecipitation using radioiodinated cells, with the following modifications:

Mitotic cells (~$3x10^6$) were harvested exactly as described above, placed in a plastic universal and washed twice with phosphate-free medium by centrifugation at 1,000 r.p.m. for 5min. Cells were then resuspended in 1ml of phosphate-free medium and 1mCi of ^{32}P was added. This cell suspension was kept for 3 hours at 37°C, with occasional agitation. After incubation, cells were transferred to a cold room, at 4°C, washed twice by centrifugation (1000 rpm, 5min), using cold Tris buffer, which was the same as lysis buffer, but without detergent, and the final pellet was lysed for 20 min. with 1ml of lysis buffer. All subsequent steps were exactly as described for immunoprecipitation from radioiodinated cells.

For interphase cells, 10cm Petri dishes were used. Usually, ~1-1.2$x10^6$ cells per dish were seeded and grown at 37°C for 36 hours. Cells were then washed twice with Tris buffer in order to remove mitotic cells, and twice with phosphate-free medium. Finally, 1ml of this medium and 1mCi of ^{32}P were added and cells placed in a 37°C incubator for 3 hours, with occasional agitation, in order to keep cells from drying. After incubation, cells were transferred to a cold room, at 4°C, washed twice with Tris buffer, and lysed for 20 min. with 1 ml of lysis buffer. Lysed cells were scraped off using a rubber spatula and transferred to an Eppendorf tube for preclearing. All subsequent steps were exactly as described for immunoprecipitation from radioiodinated cells. Equal amounts of proteins were used for each sample. Protein concentration was determined in detergent-soluble fractions using Bradford's reagent. Radioactive proteins were detected by autoradiography, after 48 hours of exposure.

2.14. Biosynthesis of the fibronectin receptor during the cell cycle

Immunoprecipitation of integrins from cells metabolically labelled with ^{35}S was carried out exactly as described for ^{32}P labelled cells. 50 µCi of Tran^{35}Slabel, diluted in 100 µl, were used for each sample, and washes

were done using methionine-free and cysteine-free medium. All samples were pre-incubated for 15 min. in this medium prior to the addition of Tran^{35}Slabel. Mitotic cells were collected as described for ^{32}P labelled cells. Asynchronous cells were seeded and treated as described for interphase ^{32}P labelled cells, with more gentle washes, in order to keep the mitotic population. Cells in G1+S were obtained from 10 cm Petri dishes by seeding 1-1.2x10^6 cells and growing them in the presence of 2.5mM thymidine for ~20 hours. The medium was then removed, and fresh growth medium without thymidine added for a further 2 hours, after which cells were treated for immunoprecipitation as with asynchronous cells. Synchronization was monitored by flow cytometry (see fig. 3.17, Results chapter).

AIMS OF MY RESEARCH

The aim of my project was to gain some insight into the role of integrins in the transition from G2 to mitosis in HeLa cells, and thus perhaps to contribute to understanding of how the activity of integrins is regulated.

During this transition, most cells in culture undergo dramatic morphological changes, which eventually lead to cellular rounding-up. It is well known that cytoskeletal components participate in such changes (see Cell Cycle section, in Introduction). However, nothing seemed to be known about the state of integrins during mitosis. It was important to know if integrins continued to be expressed at the cell surface throughout mitosis. Two polyclonal antisera raised against human integrins were available to identify integrins in Hela cells (anti-$\alpha_5\beta_1$ and anti-$\alpha_v\beta_3$), which could be used in flow cytometry and immunoprecipitation to answer this question. Assays of the spreading activity of mitotic and interphase cells could then be used to give an indirect measure of integrin activity at the level of intact cells, and affinity isolation of integrins from detergent extracts could measure such activities in cell-free preparations. Since phosphorylation of integrins is a known mechanism for the regulation of their acitivity (see Integrins section, in Introduction) I planned to analyse the phosphorylation state of integrins in HeLa cells in mitosis and interphase by immunoprecipitation from ^{32}P-labelled cells.

Immunofluorescence was intended to localise integrins either at the cell surface on fixed cells, or intracellularly in permeabilised cells. I also considered of interest to study whether synthesis of integrins was regulated during the cell cycle or whether it was constitutive. Therefore, I intended to immunoprecipitate integrins from ^{35}S-labeled cells at different stages of the cell cycle. While optimising synchronisation procedures using the microtubule inhibitor nocodazole, I realised that serum seemed to have a positive effect on the transition from metaphase to anaphase after release of nocodazole block. Therefore, I planned to quantify the rate of exit from metaphase at different times after release of the nocodazole block.

During the course of the work, the phosphatase inhibitor calyculin A became available, offering a possible model in asynchronous cells for changes taking place at mitosis.

CHAPTER 3

RESULTS

1. ISOLATION OF CELL-BINDING FRAGMENT FROM FIBRONECTIN

Bovine fibronectin isolated from serum was used as a substrate for cells in spreading assays and as a source for the isolation of the cell-attachment site on the fibronectin molecule. Figure 3.1 shows the protein recovered in each step of fibronectin and cell-binding fragment isolations. The fraction recovered from the gelatin-Sepharose column with 8M urea contained mainly fibronectin, showing as two bands of ~200kDa under reducing conditions (lanes 5 and 6). Fibronectin digested with 2μg/ml chymotrypsin was then passed again through the gelatin-Sepharose column. The material immediately eluted, showed in lanes 3 and 4, constituted the cell-binding fraction (CBF). It consisted of a main band of ~130kDa, the cell-binding fragment, together with minor contaminants. The digested fragments bound to gelatin are shown in lanes 1 and 2.

2. SPREADING EXPERIMENTS

2.1. Interphase cells versus mitotic cells

Fig.3.2 shows the ability of mitotic and interphase cells to spread on glass surfaces pre-coated with fibronectin. Interphase cells responded readily to fibronectin and fully spread within one hour, unlike mitotic cells, which remained rounded and unable to spread. Nocodazole added to interphase cells during spreading had a negligible effect on spreading. Further experiments using different concentrations of Mn^{2+}, and haemoglobin and fibronectin as substrata, were then carried out. As fig.3.3 shows, interphase cells need concentrations of Mn^{2+} higher than 10^{-6}M to spread readily on fibronectin, and higher than 10^{-5}M to do so on haemoglobin, although there is a weak response to lower concentrations. Mn^{2+} at 10^{-4}M induces higher spreading of interphase cells on fibronectin than Ca^{2+} and Mg^{2+} both at 10^{-3}M. As for mitotic cells, although there is a significant increase in their MSA on fibronectin in the presence of >10^{-5}M Mn^{2+}, as compared with the same cells in the absence of Mn^{2+} (nearly 50% increase), this effect is negligible when compared with the area of fully spread interphase cells. Further experiments using several approaches (see below, section 2) proved that, given the right conditions, mitotic cells could be induced to spread to a limited extent, these results being compatible with the spreading results shown above. The apparently better spreading of mitotic cells on

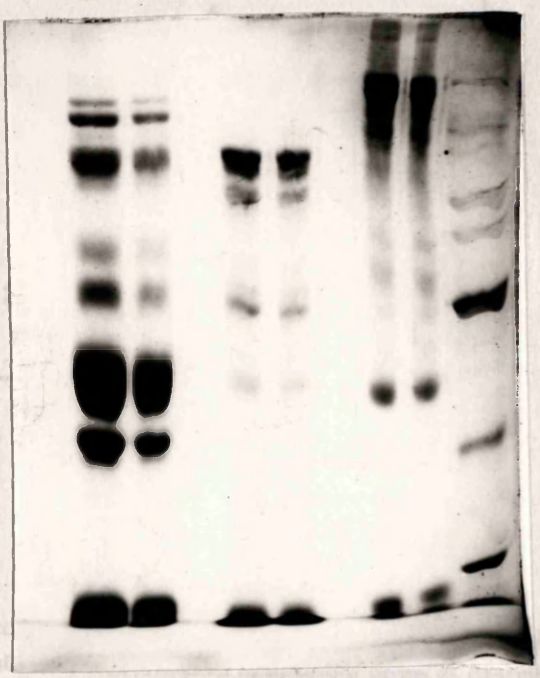


Figure 3.1. Isolation of the cell-binding fragment by affinity chromatography. Lanes 1 and 2, gelatin-bound fraction from fibronectin digest. Lanes 3 and 4, fraction of the fibronectin digest directly eluted from the gelatin column. It contains the cell-binding fragment as the main component. Molecular weight markers (lane 7) are 205, 116, 97.4, 66 , 48 and 29kDa.

haemoglobin at low concentrations of Mn^{2+} (observed in all experiments) is probably accounted for by the bigger size of mitotic cells.

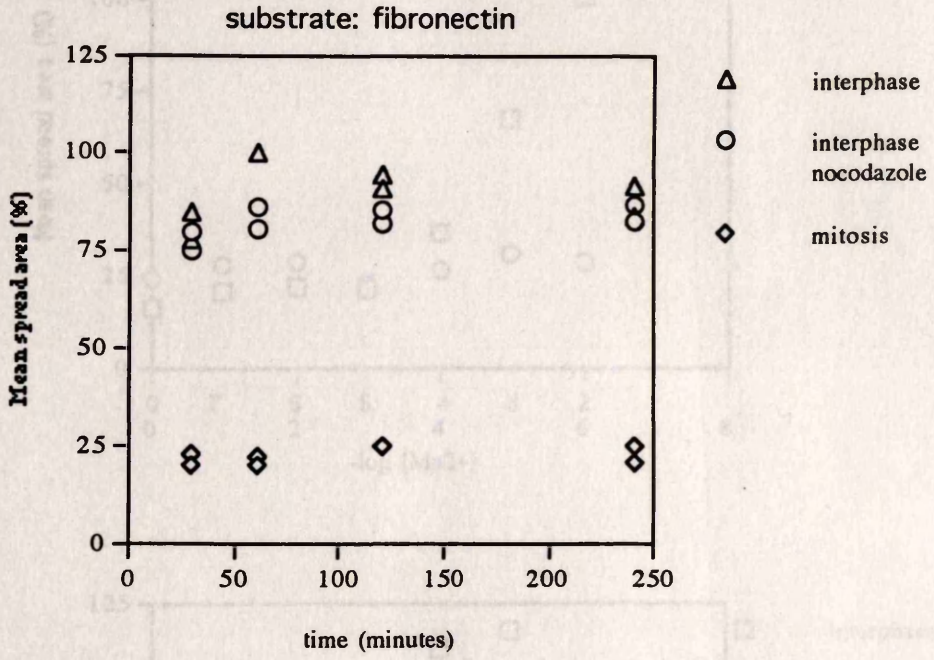


Figure 3.2. Time course of cell spreading on fibronectin (25 µg/ml). Cells were diluted in HH, incubated at 37°C, and analysed at different times as described in Materials and Methods. Results are expressed as mean spread area relative to interphase cells after 60 minutes incubation (value 100). Data from two different experiments

2.2. Effect of serum on metaphase→anaphase transition

Cells were blocked in metaphase as described in Materials and Methods, washed twice and plated on fibronectin in the presence or absence of nocodazole and serum. After different incubation times, cells were fixed and stained as for spreading essays. As seen in figure 3.4, cells which were incubated with nocodazole did not go through metaphase, as expected. Nocodazole-released cells, however, progressed faster in the presence of serum. Post-metaphase cells were defined as those showing a clear anaphase morphology (see figure 3.8b).

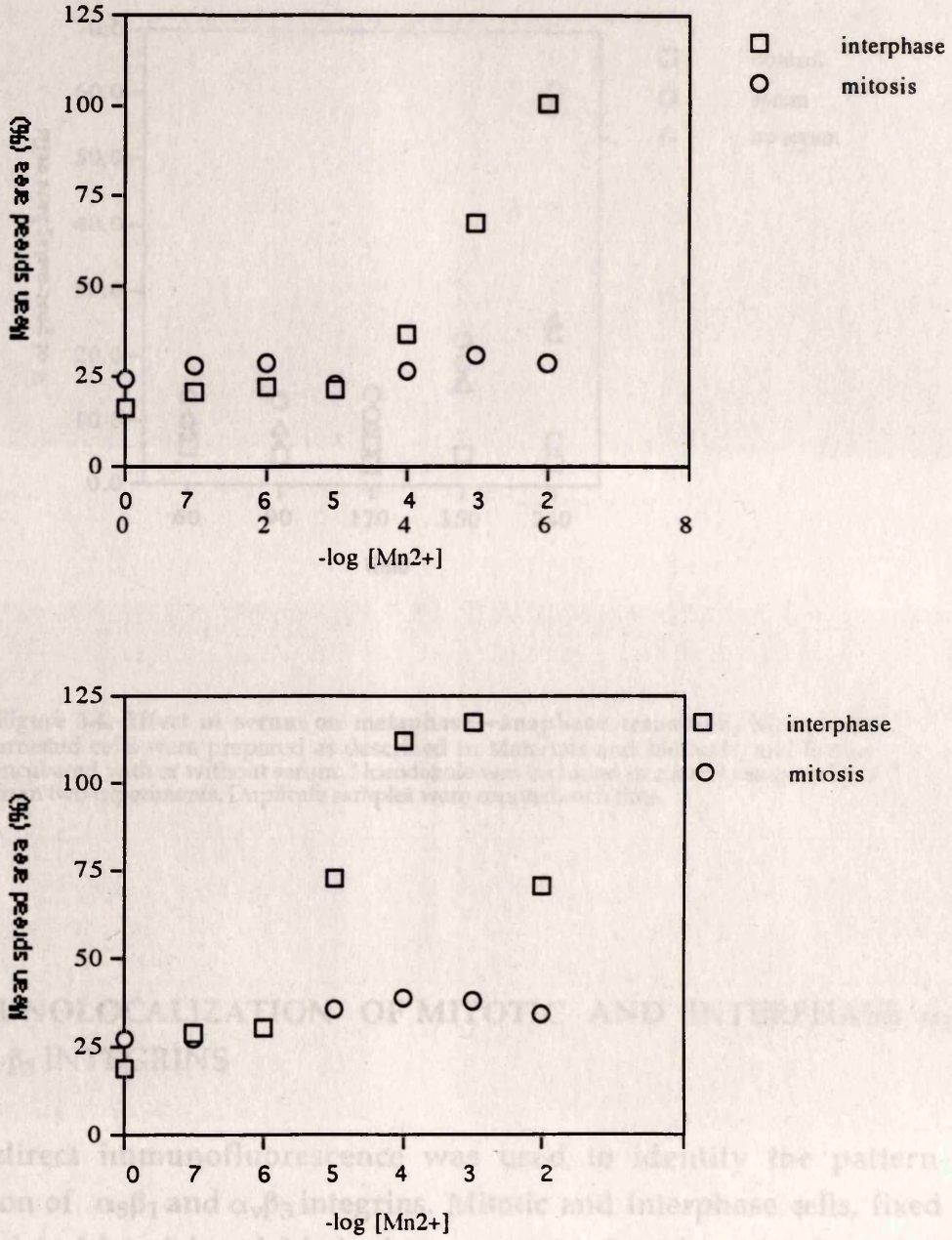


Figure 3.3. Spreading of mitotic and interphase cells in response to various concentrations of Mn^{2+}, using hemoglobin (top figure) and fibronectin as substrates. Cells were allowed to spread for 2 hours at 37°C in HS. Glass surfaces were pre-coated with fibronectin, from solution at 25 µg/ml, or haemoglobin, at 500 µg/ml. Data are expressed as mean spread area relative to spread interphase cells on fibronectin in 10^{-3}M Ca^{2+}, 10^{-3}M Mg^{2+}, without Mn^{2+} (value 100). Results are representative of 3 experiments.

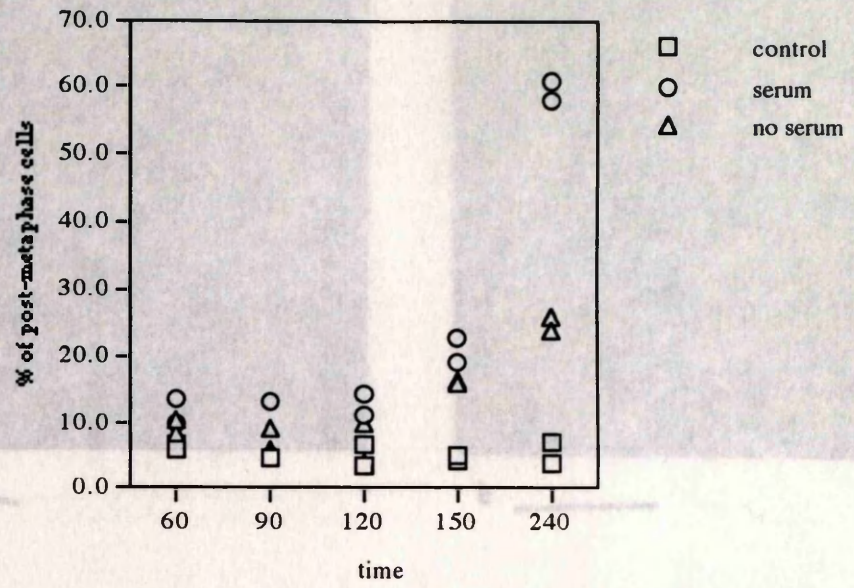


Figure 3.4. Effect of serum on metaphase→anaphase transition. Metaphase-arrested cells were prepared as described in Materials and Methods, and further incubated with or without serum. Nocodazole was included in control samples. Data from two experiments. Duplicate samples were counted each time.

3. IMMUNOLOCALIZATION OF MITOTIC AND INTERPHASE $\alpha_5\beta_1$ AND $\alpha_V\beta_3$ INTEGRINS

Indirect immunofluorescence was used to identify the pattern of expression of $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins. Mitotic and interphase cells, fixed as described in Material and Methods, were stained with anti-$\alpha_5\beta_1$ and anti-$\alpha_V\beta_3$ antibodies. Some samples were also permeabilised.

Non permeabilized interphase cells (fig.3.5a,b) show clear staining only at the edge of the cells. Mitotic cells showed positive staining for both antisera, in the form of a double ring pattern. (fig.3.5c,d). One possible explanation for the mitotic pattern of staining is that, as cells undergo a pre-treatment of several hours in nocodazole before being fixed for immunofluorescence, there is a percentage of the cell population which stays blocked in metaphase long enough to initiate spreading although, as

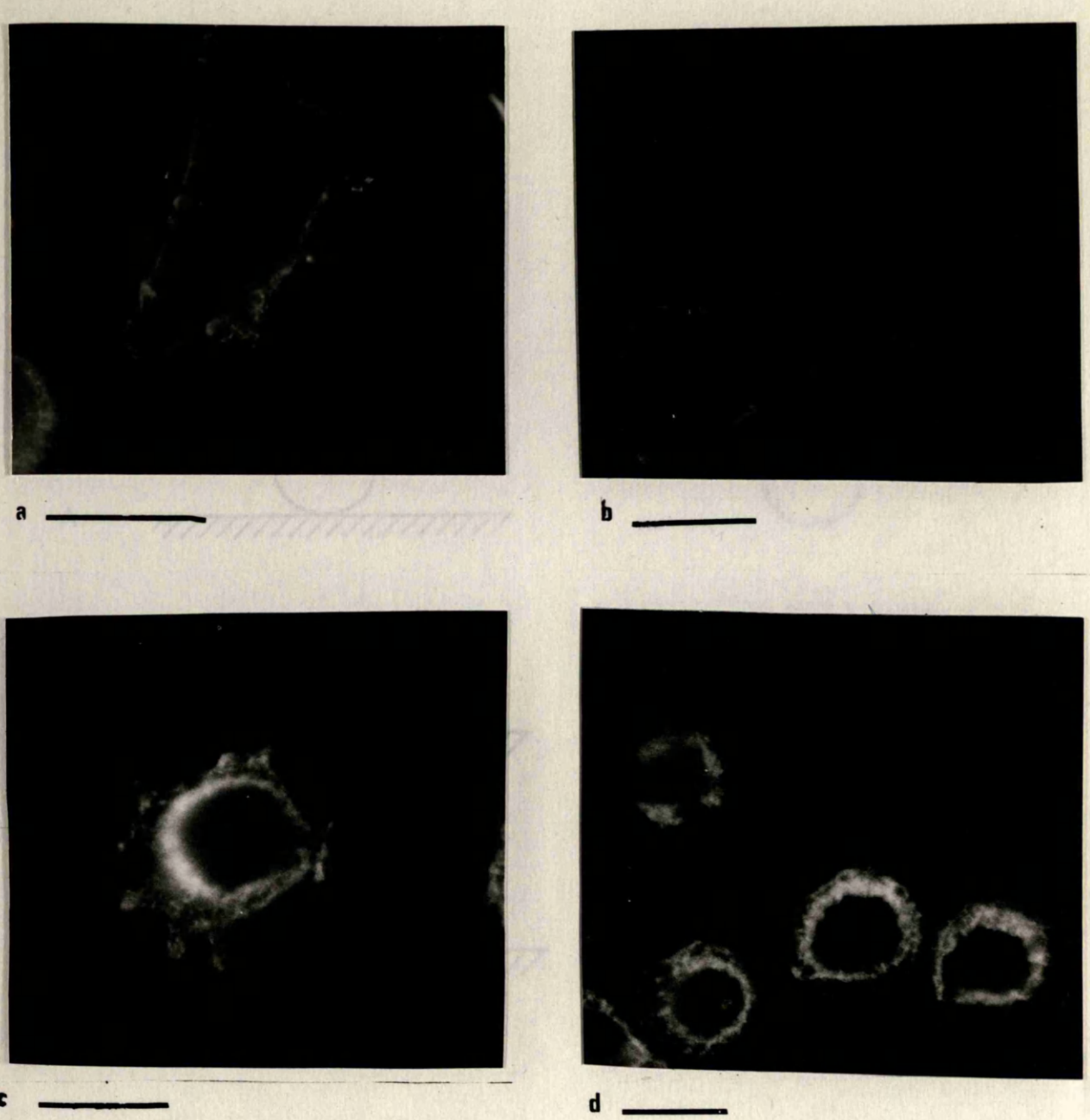


Figure 3.5. Immunolocalization of $\alpha_5\beta_1$ and $\alpha_V\beta_3$. Non permeabilised cells. a. Interphase cells stained with rabbit anti-human $\alpha_5\beta_1$. **b.** Interphase cells stained with rabbit anti-human $\alpha_V\beta_3$. **c.** Mitotic cells. Staining as in a. **d.** Mitotic cells. Staining as in c. Bar, 10μm.

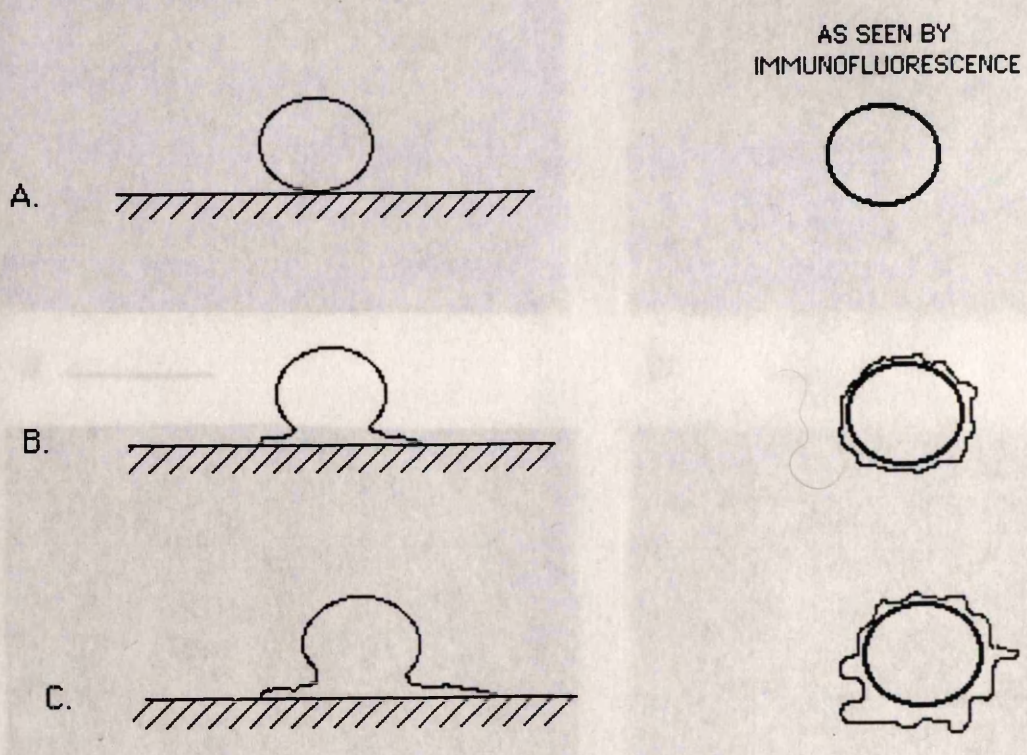


Figure 3.6. Limited spreading of mitotic cells, as seen by immunofluorescence. a. The cell rounds up at the onset of prophase and is blocked with nocodazole as it enters metaphase. **b**. After a few hours the cell starts spreading. **c**. Spreading stops at its early steps.

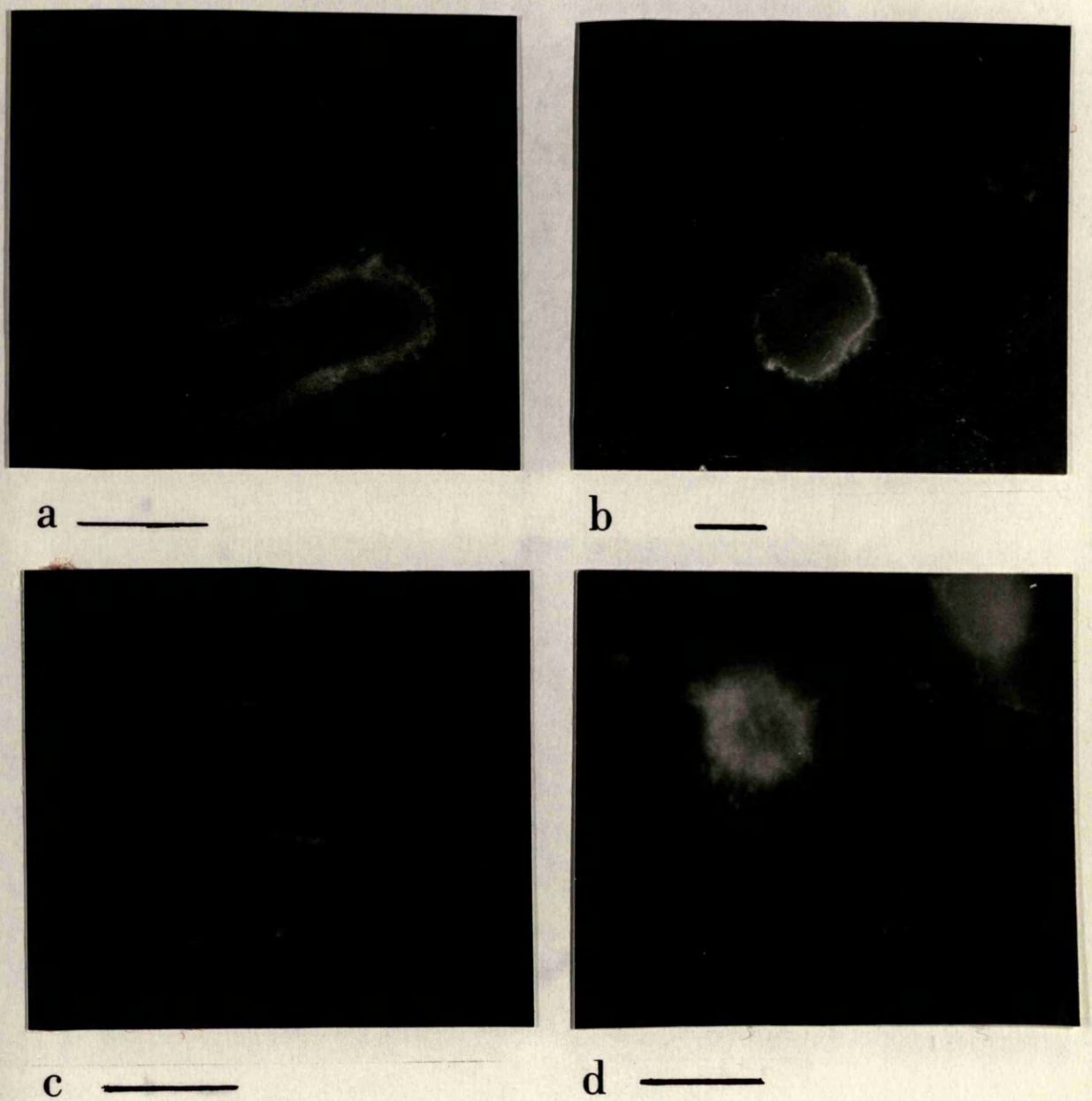


Figure 3.7. Immunolocalization of $\alpha_5\beta_1$ and $\alpha_V\beta_3$ from nonblocked, mitotic cells. a. Late telophase cell stained with anti-$\alpha_5\beta_1$. **b.** Anaphase/early telophase cell stained with anti-$\alpha_V\beta_3$. **c.** Daughter cells after parental cell has undergone cytokinesis. Staining as in a. **d** Cell in early prophase, in the process of rounding up. Staining as in a. Bar, 10μm.

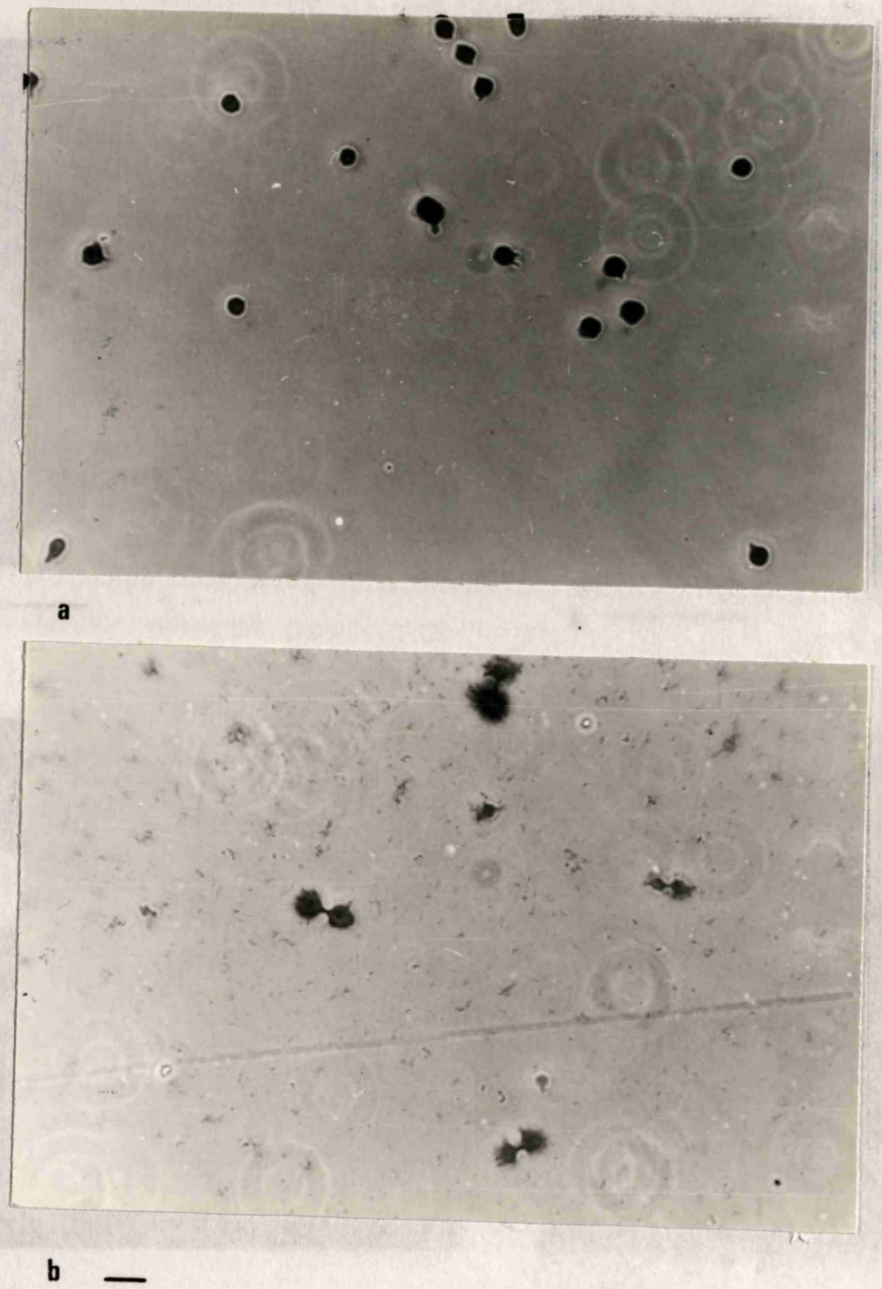


Figure 3.8. a. Mitotic cells harvested as described in Materials and Methods were washed twice with full medium and incubated on glass coverslips at 37°C for 4 hours in the presence of 0.05ug/ml nocodazole. Cells were fixed and stained as for spreading assays. **b**. Cells underwent same treatment, except that final incubation with nocodazole was omitted. Bar 20μm.

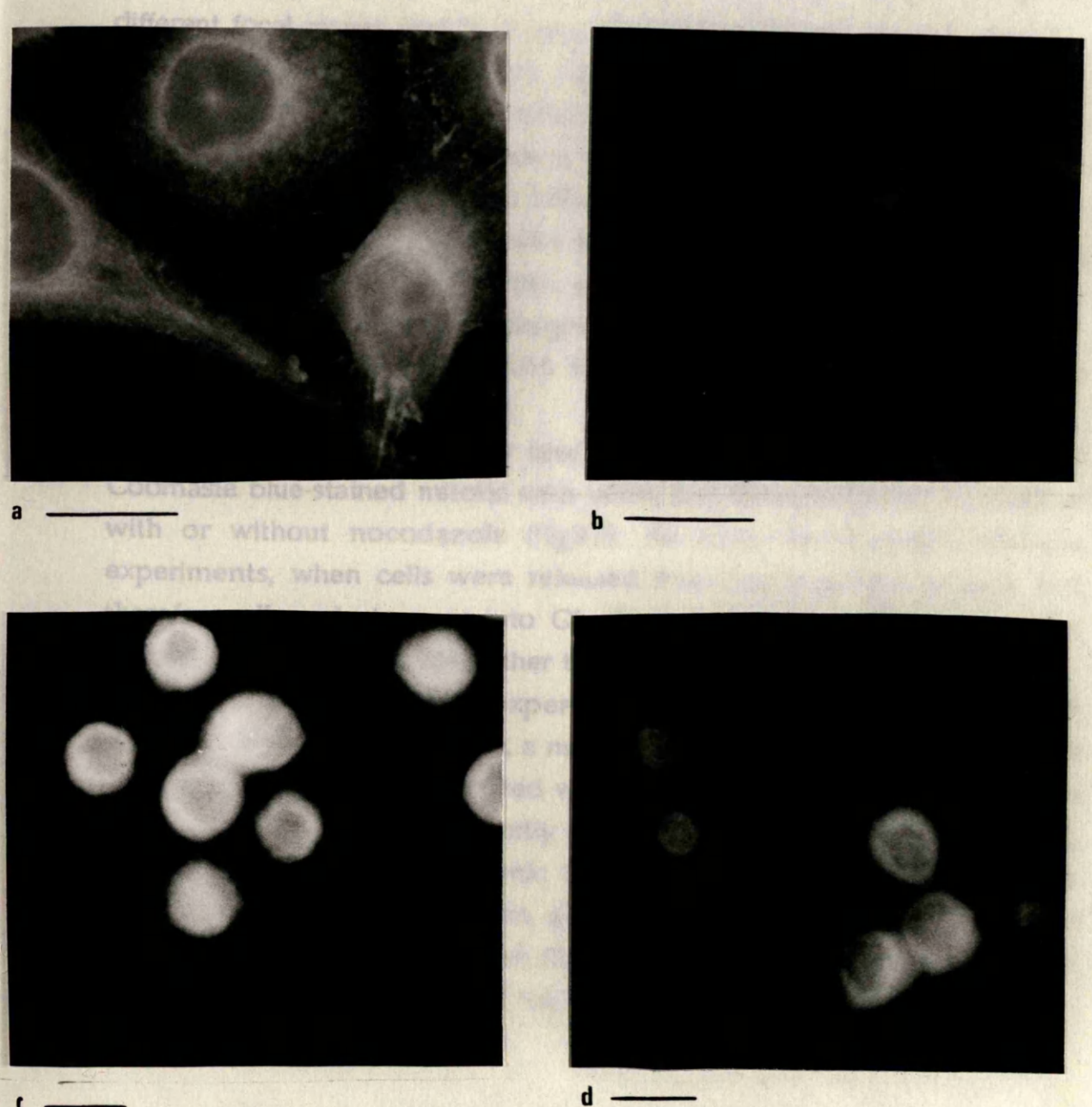


Figure 3.9. Immunolocalization of $\alpha_5\beta_1$ and $\alpha_v\beta_3$. a. Interphase cells stained with anti-$\alpha_5\beta_1$. **b.** Interphase cells stained with anti-$\alpha_v\beta_3$. **c.** Mitotic cells. Staining as in a. $\alpha_5\beta_1$. Mitotic cells. Staining as in b. Bars, 10μm.

immunofluorescence, there is a percentage of the cell population which stays blocked in metaphase long enough to initiate spreading although, as shown in spreading experiments using optimal conditions (fig. 3.3), this spreading is very limited. Figure 3.6 shows the sequence of steps involved in the process. The fact that the two rings in the same cell are situated at different focal planes (fig.3.5c,d) supports this hypothesis.

Further experiments were made to test this hypothesis. Cells were thymidine-blocked as in previous experiments, but blocking with nocodazole was omitted. Ten hours after the release of the thymidine block, when the percentage of mitotic cells is higher than usual, cells were treated for immunofluorescence. As seen in figure 3.7, this time no double ring pattern could be found in mitotic cells. Instead, a single ring pattern was identified in cells actually undergoing anaphase (fig.3.7a,b) or even after cytokinesis (fig.3.7c). Figure 3.7d shows a cell in early prophase in the process of rounding up.

These results were further confirmed by observing the morphology of Coomasie blue-stained mitotic cells which had been incubated in medium with or without nocodazole (fig.3.8). As with immunofluorescence experiments, when cells were released from the nocodazole block and therefore allowed to pass into G1 phase, only cells undergoing mitosis could be identified (fig.3.8b) rather than cells trying to spread (fig.3.8a).

Immunofluorescence experiments were also carried out with permeabilised cells. In this case, a neat cytoplasmic, Golgi-like, pattern could be seen in interphase cells treated with anti-$\alpha_5\beta_1$ antiserum, the staining at the cellular edges being apparently weaker than in non permeabilised cells, possibly due to the cytoplasmic brightness (fig. 3.9a). When cells were treated with anti-$\alpha_v\beta_3$ antiserum, a more diffuse and weaker cytoplasmic pattern of staining could be seen (fig.3.9b). Mitotic permeabilised cells were fully stained with both antisera (fig.3.9c,d).

4. EXPRESSION OF MITOTIC AND INTERPHASE INTEGRINS

Two different approaches were used to detect and compare relative amounts of integrins at both stages of the cell cycle: flow cytometry and immunoprecipitation of cell surface integrins. Immunoblotting was also carried out in interphase cells in order to test the presence of β_3 integrins in HeLa cells.

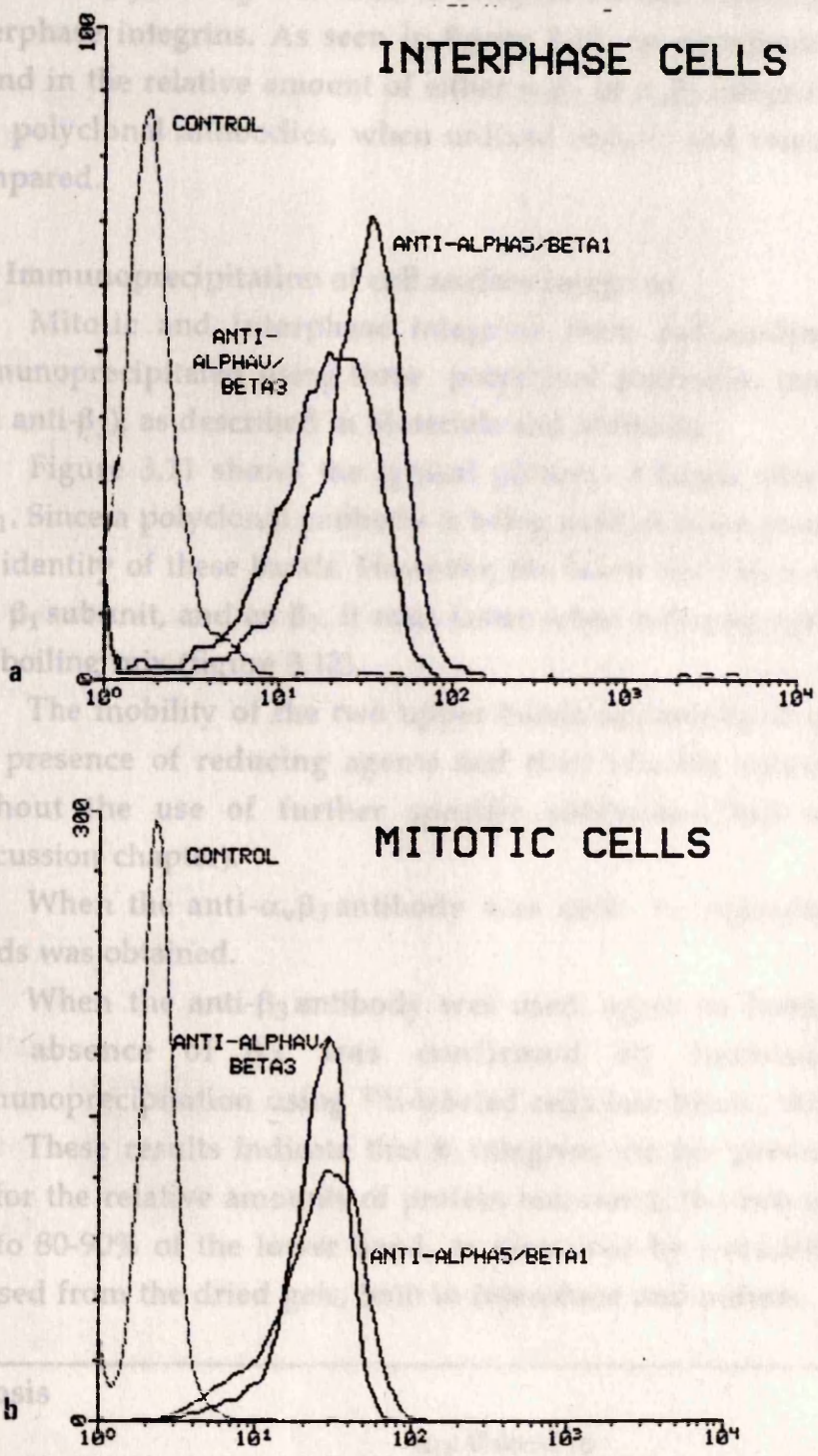


Figure 3.10. Analysis by flow cytometry of cell surface expression of integrins. a. Interphase cells. **b.** Mitotic cells. Control samples were incubated with preimmune serum as first antibody.

4.1. Flow cytometry

Flow cytometry was used to compare surface expression of mitotic and interphase integrins. As seen in figure 3.10, no significant difference was found in the relative amount of either $\alpha_5\beta_1$ or $\alpha_v\beta_3$ integrins, as detected by our polyclonal antibodies, when unfixed mitotic and interphase cells were compared.

4.2. Immunoprecipitation of cell surface integrins

Mitotic and interphase integrins from radioiodinated cells were immunoprecipitated using three polyclonal antibodies (anti-$\alpha_5\beta_1$, anti-$\alpha_v\beta_3$ and anti-β_3), as described in Materials and Methods.

Figure 3.11 shows the typical pattern of bands obtained using anti-$\alpha_5\beta_1$. Since a polyclonal antibody is being used, it is not possible to be sure of the identity of these bands. However, the lower one has a mobility expected of a β_1 subunit, and as β_1, it runs faster when reducing agents are added to the boiling mix (figure 3.12).

The mobility of the two upper bands apparently did not depend on the presence of reducing agents and their identity cannot be established without the use of further specific antibodies (but see section 3, in Discussion chapter).

When the anti-$\alpha_v\beta_3$ antibody was used, no reproducible pattern of bands was obtained.

When the anti-β_3 antibody was used, again no bands were detected. The absence of β_3 was confirmed by immunoblotting and immunoprecipitation using ^{35}S-labeled cells (see below, this section).

These results indicate that β_3 integrins are not present in HeLa cells. As for the relative amounts of protein recovered, the two upper bands sum up to 80-90% of the lower band, as measured by γ-counting of the bands excised from the dried gels, both in interphase and mitosis.

mitosis		
β:1	α_U: 0.48±0.16	α_L: 0.40±23
interphase		
β:1	α_U: 0.57±0.29	α_L: 0.29±0.21

Table 3.1. Relative amounts of integrin bands in interphase and mitosis. α_L: lower α subunit; α_u: upper α subunit. Data from four immunoprecipitations, means ± S.D.

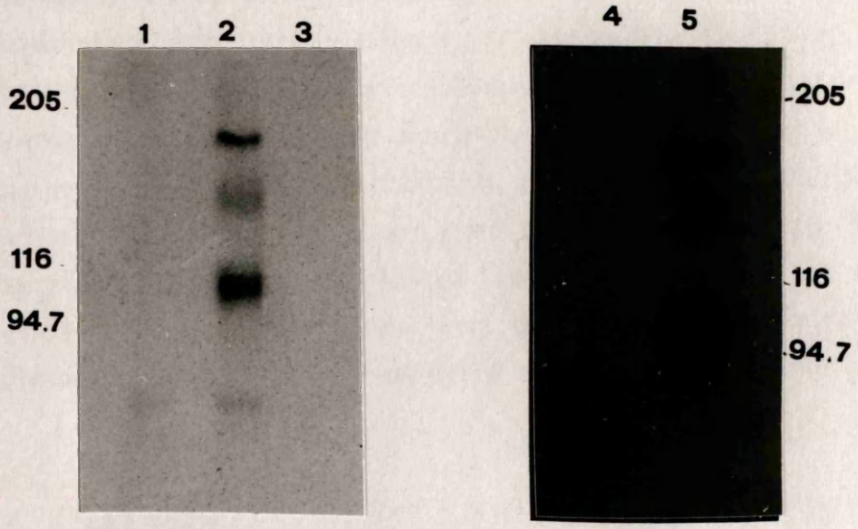


Figure 3.11. Analysis of cell surface expression of integrins by immunoprecipitation. Lane 1, preimmune rabbit serum. Interphase cells. Lane 2, anti-$\alpha 5\beta 1$. Interphase cells. Lane 3, anti-$\beta 3$. Interphase cells. Lane 4, preimmune rabbit serum. Mitotic cells. Lane 5. anti-$\alpha 5\beta 1$. Mitosis

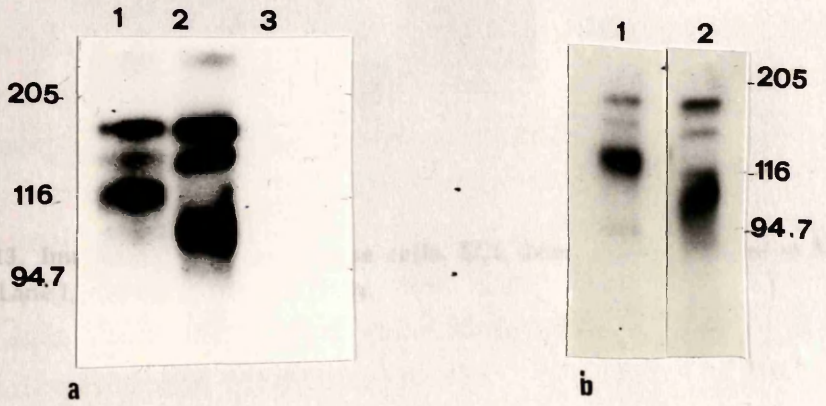


Figure 3.12. Immunoprecipitation as in 3.11. **a**. Interphase cells. Lane 1, anti-$\alpha 5\beta 1$, reducing conditions. Lane 2, as lane 1, nonreducing conditions. Lane 3, preimmune serum. **b**. Mitotic cells. Lanes 1 and 2, as in a.

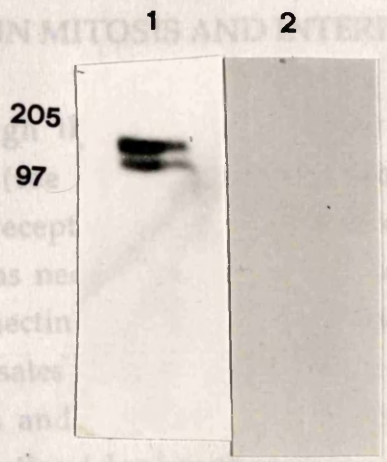


Figure 3.13. Immunoblots of interphase cells. ECL detection as described in Materials and Methods was used. Lane 1, anti-$\alpha 5\beta 1$. Lane 2, anti-$\beta 3$.

4.3. Immunoblotting

Integrin subunits were identified by immunoblotting using ECL detection as described in Materials and Methods. Anti-$\alpha_5\beta_1$ and anti-β_3 antibodies were used. As shown in fig. 3.13, with anti-$\alpha_5\beta_1$, a reproducible pattern of two bands was obtained, instead of the usual 3 from the immunoprecipitations (see also below, fig. 3.21). This is an expected pattern, since incubation with antibodies takes place on the nitrocellulose filter, where integrin subunits have previously been separated by SDS-PAGE. The apparent molecular weights of the two bands are ~130 kDa and 125 kDa under reducing conditions, and they represent α_5 (upper band) and β_1. However, no bands were detected using the anti-β_3 antiserum.

5. ANALYSIS OF THE CELL-FREE ACTIVITY OF THE FIBRONECTIN RECEPTOR IN MITOSIS AND INTERPHASE

Although the spreading ability of mitotic and interphase cells on fibronectin (see above, section 2) may be an indirect measure of the fibronectin receptor activity on those cells, it is obvious that a more direct approach was needed to assess such activity. This consisted of the isolation of the fibronectin receptor *in vitro* , using affinity columns.

Cell lysates were passed through CBF-Sepharose columns as described in Materials and Methods. The effect of a possible cell cycle-dependent phosphorylation/dephosphorylation on the activity of the fibronectin receptor (see below, Discussion chapter) was taken into account. Thus, cell lysates obtained and eluted from the columns using buffers containing phosphatase inhibitors were compared with similar samples where phosphatase inhibitors had been omitted. Figure 3.14 shows the radioactivity recovered from each fraction, after EDTA was applied, for interphase and mitotic samples.

It is clear that the presence of phosphatase inhibitors does not influence the recovery of protein with EDTA. However, there is a difference in recovery between interphase and mitosis. Table 3.2 shows the counts recovered from postEDTA fractions as a percentage of the total counts applied to each column.

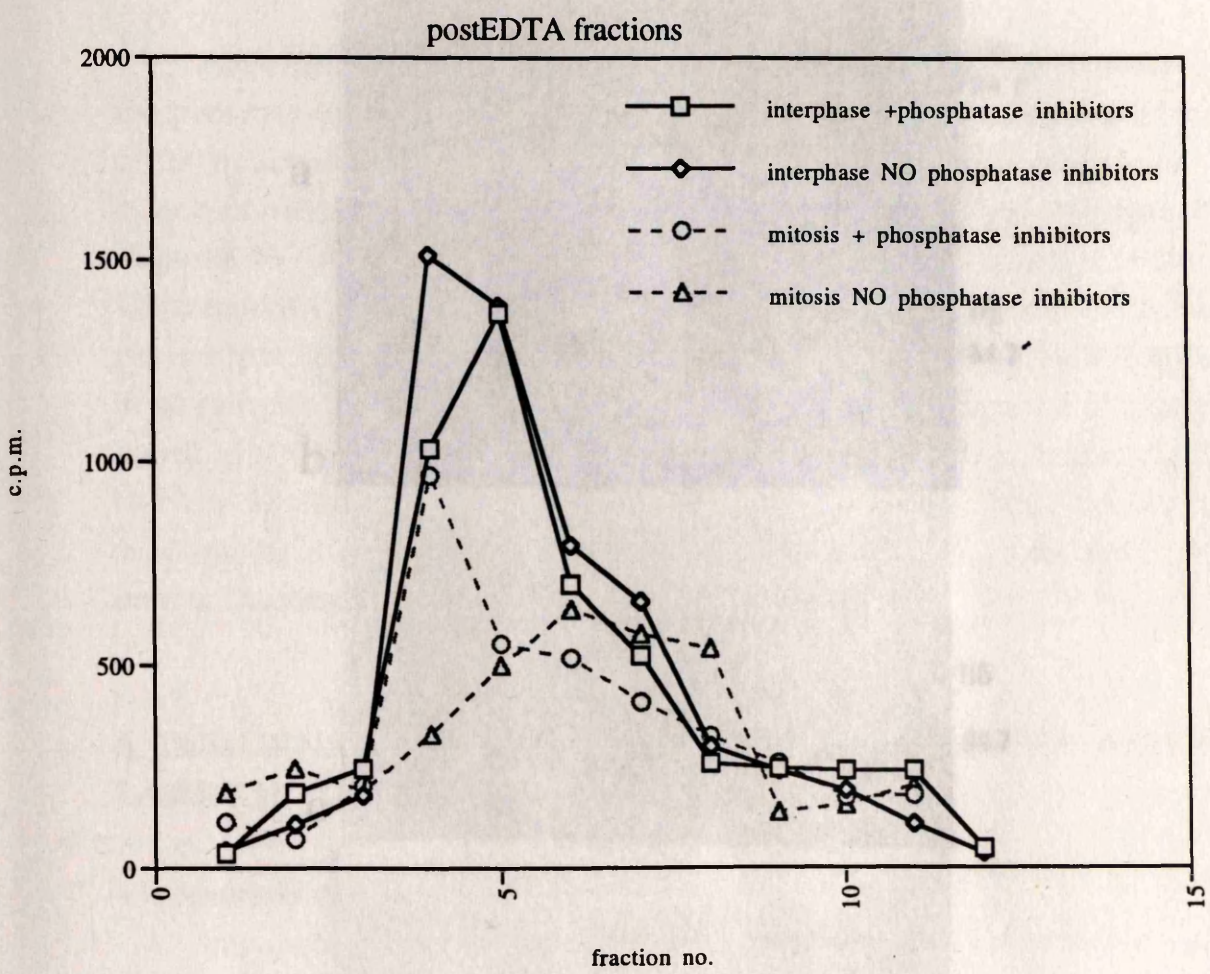


Figure 3.14. Affinity isolation of the fibronectin receptor. Mitotic and interphase cells were lysed with or without phosphatase inhibitors as described in Materials and Methods. c.p.m. are indicated for each postEDTA fraction.

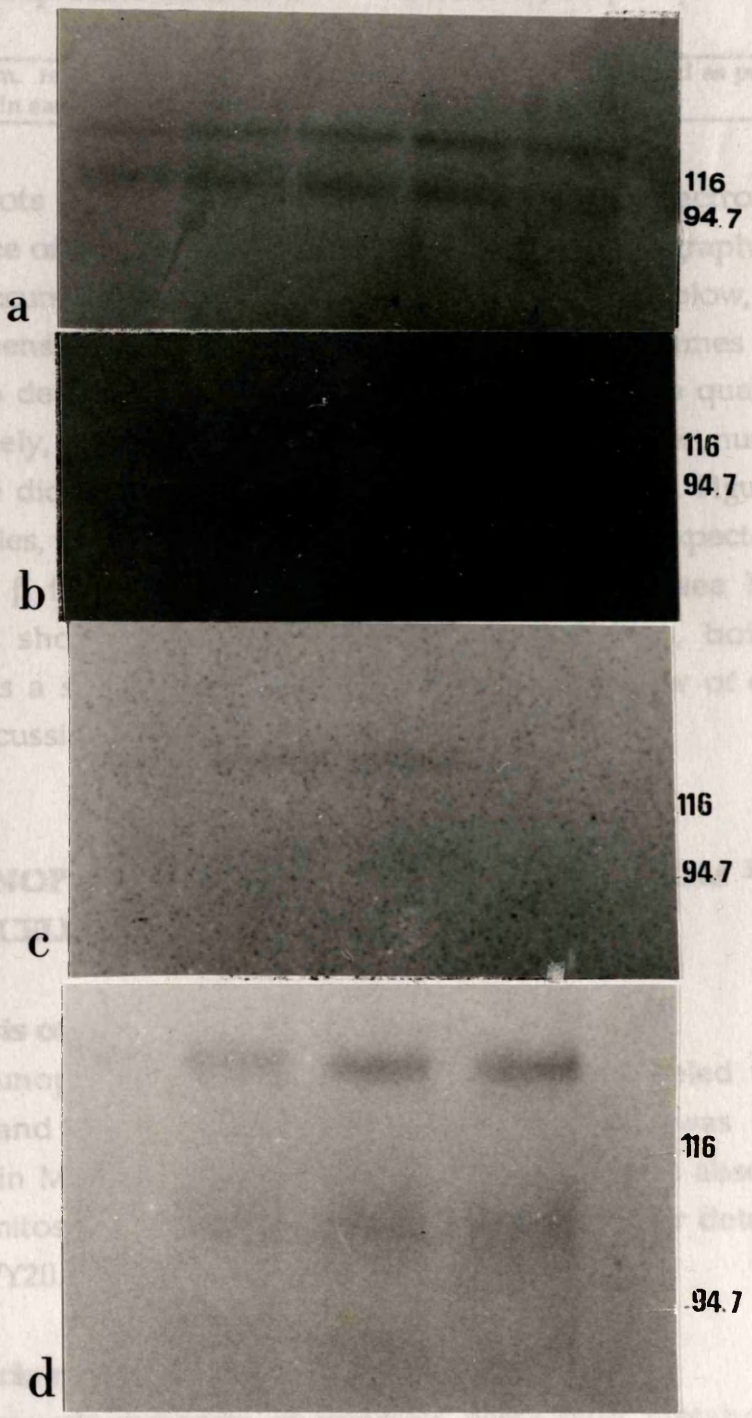


Figure 3.15. Affinity isolation of the fibronectin receptor. a. interphase cells, phosphatase inhibitors used. **b.** interphase cells, no phosphatase inhibitors. **c.** mitotic cells, phosphatase inhibitors. **d.** mitotic cells, no phosphatase inhibitors. Films were exposed for 28 days.

Interphase; phosphatase inhibitors 0.09%	**Interphase;** no phosphatase inhibitors 0.108%
Mitosis; phosphatase inhibitors 0.058%	**Mitosis;** no phosphatase inhibitors 0.054%

Table 3.2. c.p.m. recovered from CBF columns with EDTA. Data expressed as percentage of the total counts applied in each case

Aliquots (50µl) from postEDTA fractions were electrophoresed and the presence of radioactive bands detected by autoradiography. A persistent problem occurring in this type of experiments (see also below, section 6) was a lack of sensitivity, which translated into very long times of exposition required to detect recognizable bands, thus lowering the quality of the film. Unfortunately, the use of more radioactivity and higher numbers of cells per sample did not improve the final result (not shown). Figure 3.15 shows, in all samples, the recovery of two bands of a mobility expected of α (upper band) and β integrin subunits. Further experiments (see below, fig.3.23, section 6) showed that, under reducing conditions, both bands run similarly, as a single band, which is a typical behaviour of α_5 and β_1 (see below, Discussion chapter).

6. IMMUNOPRECIPITATION OF INTEGRINS FROM ^{32}P- AND ^{35}S-LABELED CELLS

6.1. Analysis of integrin phosphorylation

Immunoprecipitation of integrins from cells labeled with ^{32}P, using anti-$\alpha_5\beta_1$ and anti-phosphotyrosine (PY20) antisera was carried out as described in Materials and Methods. Fig. 3.16 shows the absence of integrin bands in mitosis and interphase . No bands were either detected using the antibody PY20.

6.2. Cell cycle-related biosynthesis of integrins

Immunoprecipitation of integrins using cells metabolicaly labeled with ^{35}S was carried out as described in Materials and Methods. Cells were partially synchronised in G1+S phase by one thymidine block, as described in Materials and Methods, followed by 2 hours incubation in normal medium (treatment T2). At this stage cells have recovered from the thymidine block and a significant percentage have entered S phase. Attempts to synchronise cells in G2 phase were unsuccessful, possibly

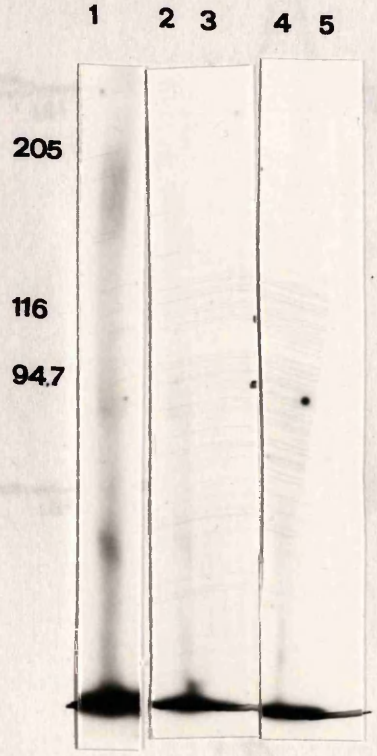


Figure 3.16. Immunoprecipitation of integrins and phosphotyrosine-containing proteins from cells labeled with ^{32}P. Lane 1, preimmune rabbit serum. Lane2, mitosis, anti-anti-$\alpha5\beta1$. Lane3, mitosis, PY20. Lane 4, interphase, anti-anti-$\alpha5\beta1$. Lane 5, interphase, PY20.

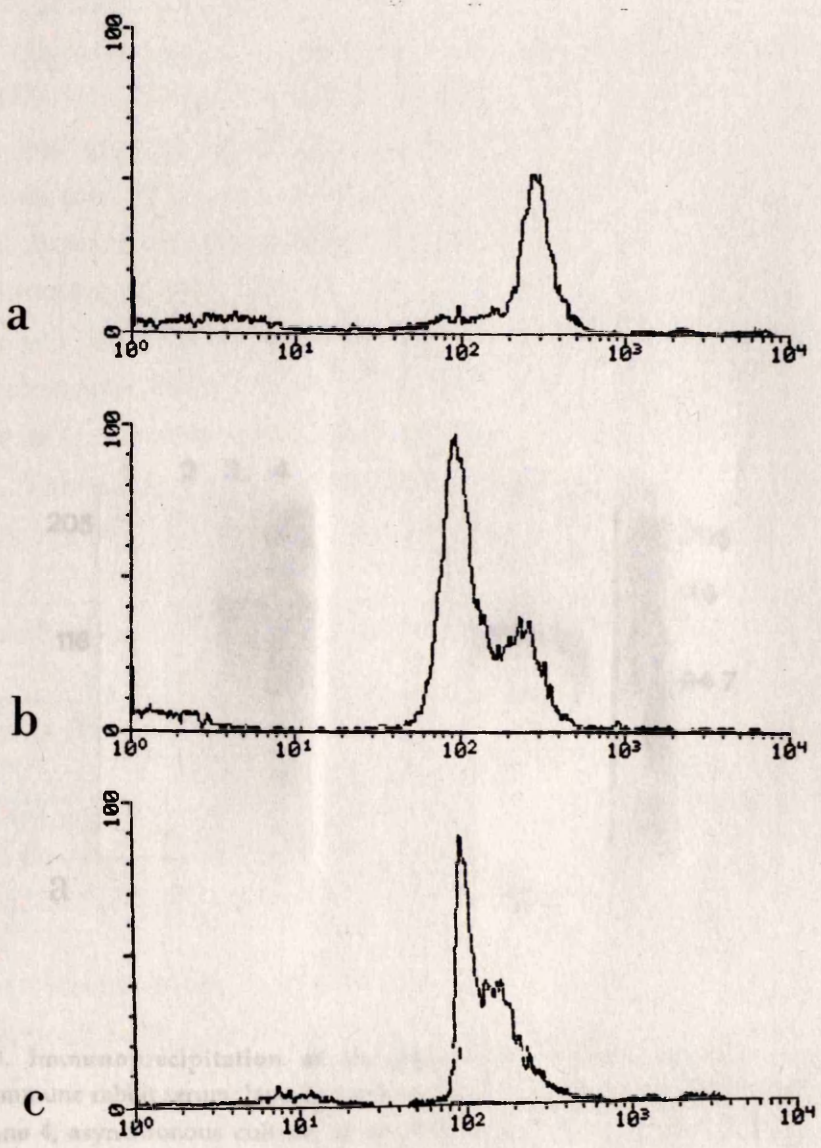


Figure 3.17. DNA content curves from synchronised samples. a. Mitotic cells. **b.** Asynchronous cells. **c.** T2 cells.

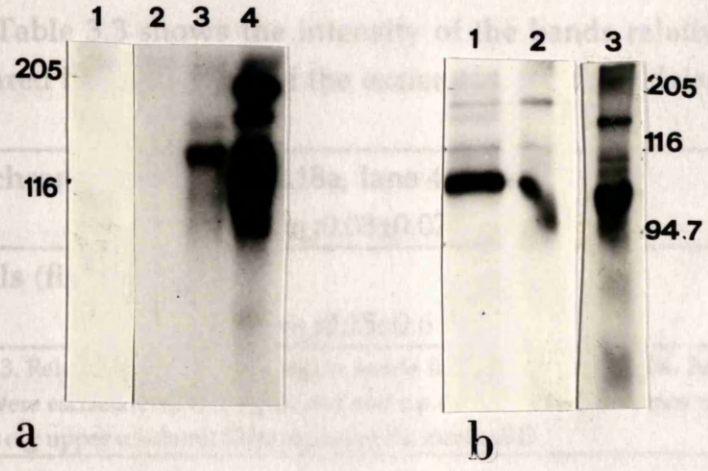


Figure 3.18. Immunoprecipitation of integrins from ^{35}S-labeled cells. a. Lane 1, asynchronous culture, preimmune rabbit serum; lane 2, asynchronous culture, anti-β_3 antibody; lane 3, T2 cells, anti-$\alpha_5\beta_1$ antibody; lane 4, asynchronous culture, as in lane 3. **b.** lane 1, T2 cells, anti-$\alpha_5\beta_1$ antibody, reducing conditions. lane 2, as in lane 1, nonreducing conditions; lane 3, asynchronous culture, anti-$\alpha_5\beta_1$ antibody, nonreducing conditions

because the length of G2 is very short compared with the partial de-synchronisation that occurs in the 6-7 hours required to pass through S phase, after the thymidine block. Synchronisation was monitored by flow cytometry using propidium iodide, as described in Materials and Methods. Figure 3.17 shows the typical curves obtained from the different synchronised samples.

Asynchronous and partially synchronised cells were incubated with Tran^{35}S-label™ for 3 hours, and integrins immunoprecipitated using anti-$\alpha_5\beta_1$ and anti-β_3 antibodies. Equal amounts of radioactivity were used for each aliquot. Figure 3.18 shows the pattern obtained for the two samples. Asynchronous cultures have a stronger β_1 band, and α subunits are not detected in T2 cells (fig. 3.18a). However, a further experiment (fig.3.18b) showed that both subunits are also present in T2 cells, although asynchronous cells still show stronger bands. These bands are probably the same as those obtained from radioiodinated cells (see above, figure 3.12).

Table 3.3 shows the intensity of the bands relative to the β subunit, as measured by γ-counting of the excised bands from dried gels.

Asynchronous cells (fig.3.18a, lane 4)		
β:1	α_L:0.08±0.025	α_U:0.16±0.02
T2 cells (fig.3.18b, lane 1)		
β:1	α_L:0.15±0.6	α_U:0.2±0.65

Table 3.3. Relative amounts of integrin bands from ^{35}S-labeled cells. Band intensities relative to β. Bands were escised from dried gels and and c.p.m. obtained three times from a γ-counter. α_L: lower α subunit; α_U: upper α subunit. Data represent the means±S.D.

Stronger bands were detected in asynchronous cultures. The presence of a much wider β_1-like subunit in this sample corresponds probably to the widely reported β_1 precursor, further supporting the idea that the lower band is indeed β_1. The differences in intensity compared with radioiodinated samples (table 3.3) could be due to the higher content in Cys and Met residues in the β_1 subunit (see above, Integrins section, in Introduction chapter) and to the presence of the β_1 precursor. The β_1 subunit contains 58 Cys and 15 Met, whereas α_5 contains 15 Cys and 8 Met residues (Argraves *et al.* 1987). Tran^{35}Slabel (see radioactive materials section, in Materials and Methods) contains 70% L-methionine (^{35}S) and 20% L-Cysteine (^{35}S). Assuming that the middle α subunit is α_5 and that there is no significant difference in Met and Cys content between α_5 and the

upper α, the expected relative intensities of α and the sum of both α subunits are as follows:

58Cys x 0.2 + 15Met x 0.7= 22.1 (β subunit)
15Cys x 0.2 + 8Met x 0.7= 8.6 (α subunit)

As two α subunits are present, the ratio is $2\alpha/\beta$= 17.2/22.1= 0.78. These calculations give an approximate estimation of the expected intensities of integrin bands as detected by autoradiography. However, the actual ratio encountered is 0.25-0.35 (see table 3.3). The presence of a large pool of cytoplasmic β subunit could then account for the difference.

No β_3 bands were detected, in accordance with the results shown in immunoblots and immunoprecipitation from radioiodinated cells (see above, section 4).

7. EFFECTS OF CALYCULIN A ON HeLa CELLS

The effects of calyculin A (CL-A) on the spreading ability and integrin activity of HeLa cells were studied.

7.1. Spreading of CL-A treated cells

Cells were prepared for spreading assays as described in Materials and Methods. Different concentrations of CL-A were used and cells were allowed to spread on fibronectin (from solution at 25 μg/ml) or hemoglobin (0.5mg/ml). CL-A was added at time zero.

The results are shown in figures 3.19a to 3.19d. As expected, cells spread best on fibronectin, in the presence of 1mM Mn^{2+}. On both fibronectin and hemoglobin, CL-A it appears to have a negative effect on spreading after one hour. However, spreading at its early stages (at 30 minutes after plating) is not affected in the presence of CL-A. The apparent difference between controls and CL-A treated cells was not statistically significant, as measured by Student's t test ($P<0.001$). However, morphology of control and CL-A treated cells was different. Control cells spread uniformly since the early stages until fully spread. However, CL-A treated cells adopted a stellate shape in all cases, after 30 minutes of spreading (see

fig. 3.19e). At longer times (1h and 2h), they had clearly retracted and had an appearance similar to that showed in figure 3.20.

Experiments with fully spread cells (see below, this section) showed that at least 50nM CL-A was needed to induce changes in cell shape. At that concentration, cells undergo a dramatic change of shape and become rounded after ~30 minutes (figure 3.20). Other concentrations had no detectable effect (1nM and 5nM) after 2 hours, or the effects were very slow (cell retraction and eventual rounding-up required longer than 1 hour with 25nM CL-A).

7.2. Effect of calyculin A on the integrin $\alpha_5\beta_1$

As mentioned above, fully spread cells treated with 50nM CL-A become rounded after ~30 minutes. In order to study the possible role of integrins in the CL-A-induced cellular rounding up, several approaches were used.

Integrin $\alpha_5\beta_1$ was detected by immunoblotting. Figure 3.21 shows the presence of two bands whose apparent molecular weights are 130kDa and 125kDa, under reducing conditions (see also fig. 3.13).

The activity of the fibronectin receptor from interphase, fully spread cells, and from cells treated with CL-A (2nM) was measured by affinity chromatography on the cell-binding fragment of fibronectin. Radioiodinated cell lysates from normal cultures (interphase) were prepared as described in Materials and Methods. Lysates from CL-A-treated cells were prepared after cell rounding had taken place. Figure 3.22 shows the radioactivity recovered from post-EDTA fractions. Higher counts are present in interphase fractions. The total c.p.m. recovered amount to 0.095% of the total c.p.m. applied to the column, comparable to other experiments (see table 3.2), whereas counts from CL-A-treated cells were negligible at this scale.

Aliquots (50µl) from post-EDTA fractions were electrophoresed and radioactive bands detected by autoradiography on dried gels, as described in Materials and Methods. Figure 3.23 shows the result of an autoradiograph from interphase post-EDTA fractions. Only fractions 5 (shown under reducing and nonreducing conditions) and 6 showed bands after 3 weeks of film incubation at -70°C. No bands appeared from post-EDTA fractions of CL-A-treated cells (not shown).

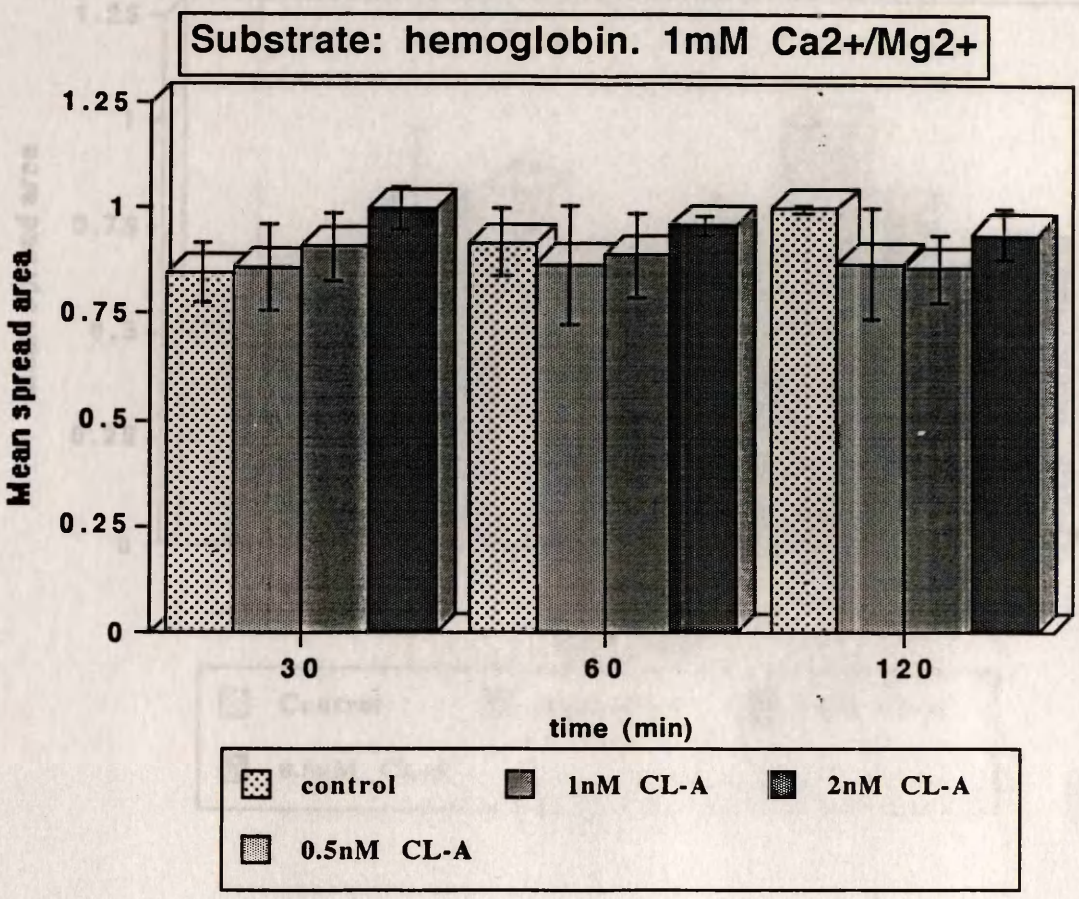


Figure 3.19a Spreading of CL-A treated cells on hemoglobin in the presence of 1mM Ca2+ / Mg2+. Cells were processed for cell spreading as described in Materials and Methods. Bars represent means ± S.D.

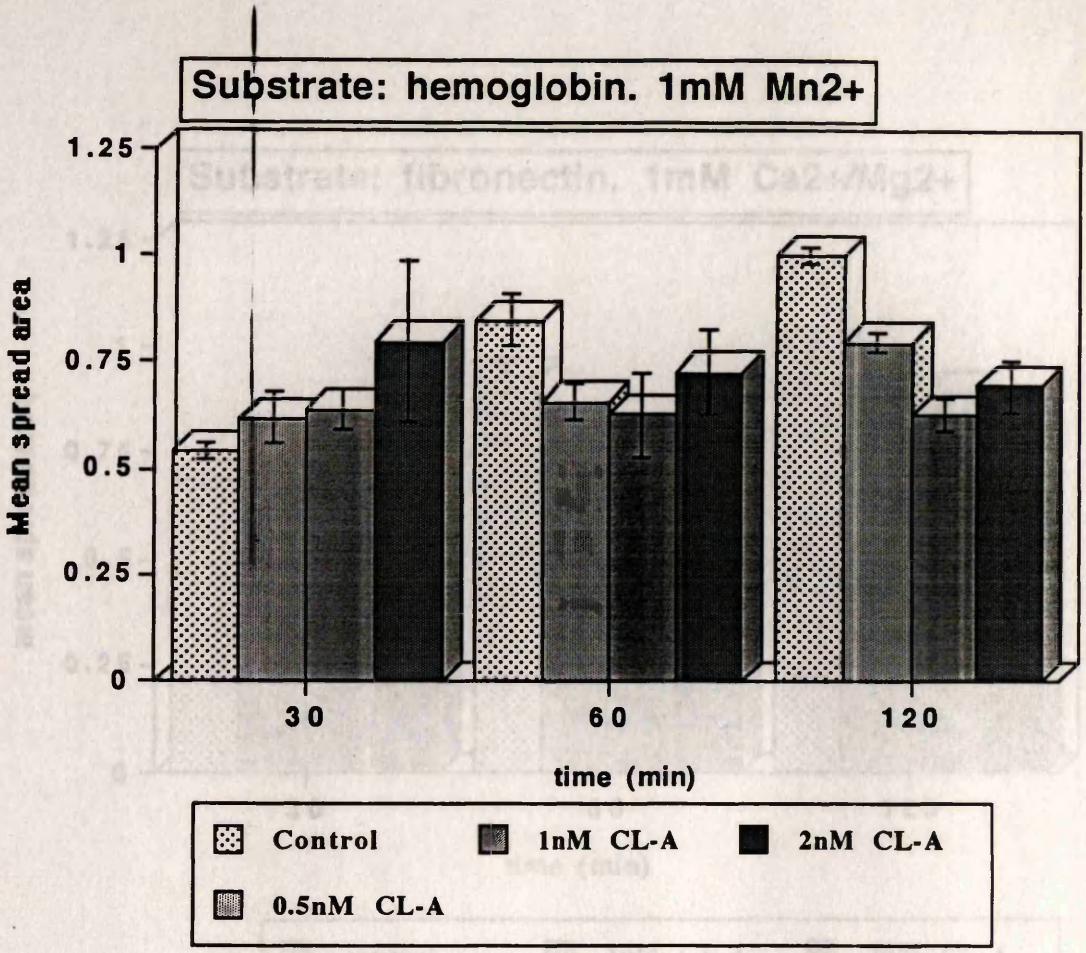


Figure 3.19a Spreading of CL-A treated cells on hemoglobin in the presence of 1mM Mn2+. Cells were processed for cell spreading as described in Materials and Methods. Bars represent means ± S.D.

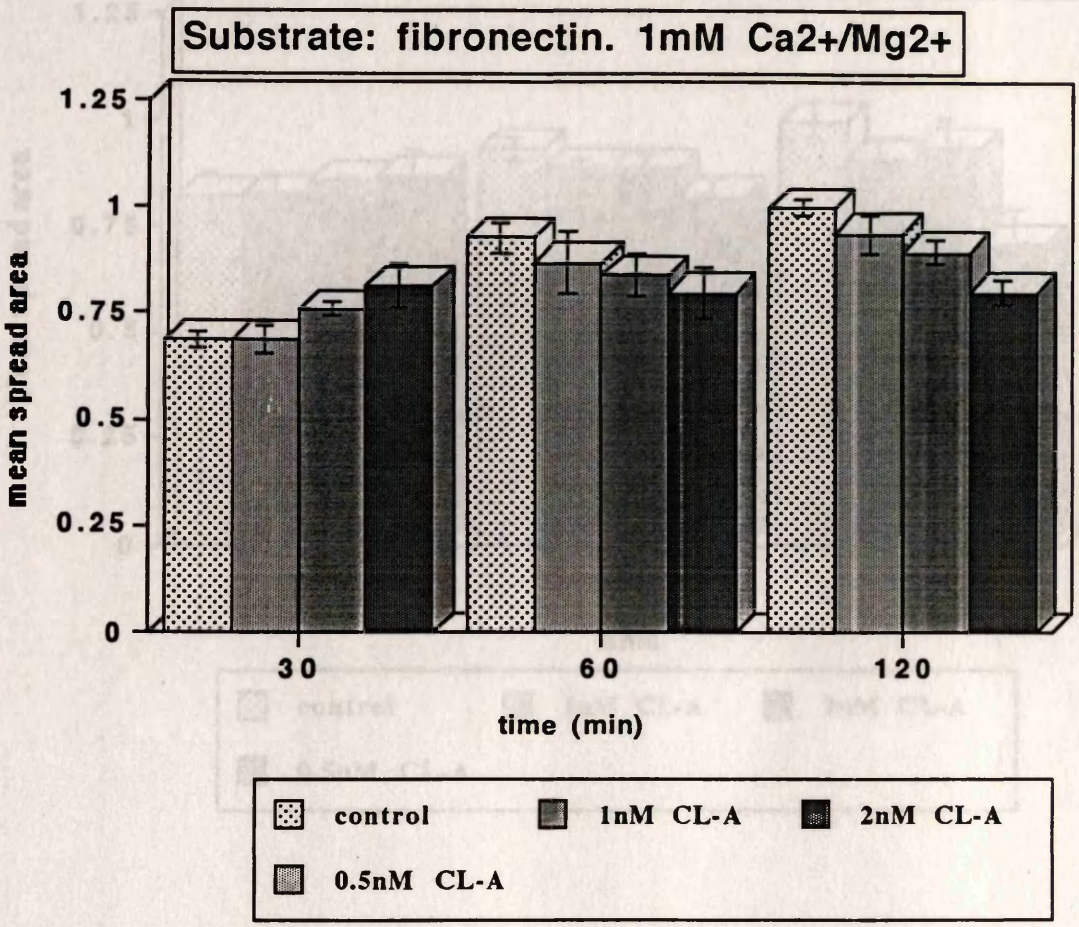


Figure 3.19c. Spreading of CL-A treated cells on fibronectin in the presence of 1mM Mg2+/Ca2+. Cells were processed for cell spreading as described in Materials and Methods. Bars represent means ± S.D.

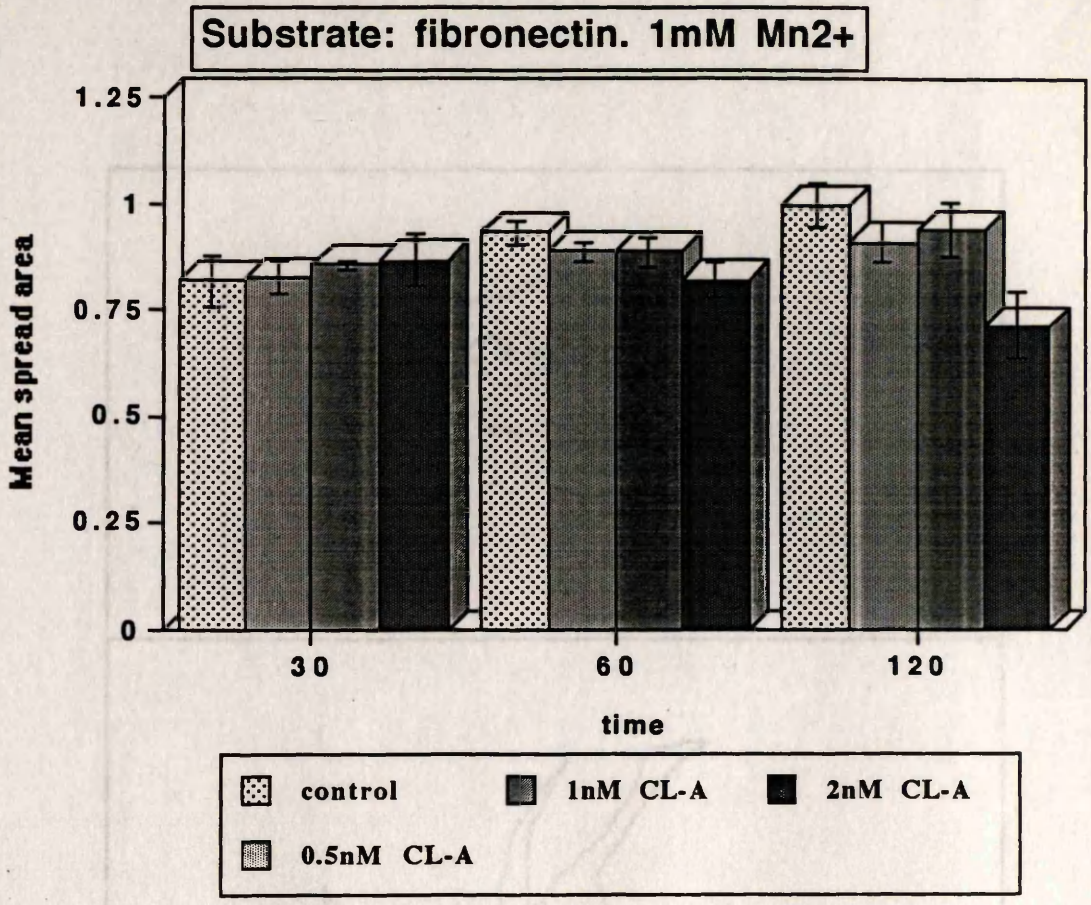


Figure 3.19d. Spreading of CL-A treated cells on fibronectin in the presence of 1mM Mn2+. Cells were processed for cell spreading as described in Materials and Methods. Bars represent means ± S.D.

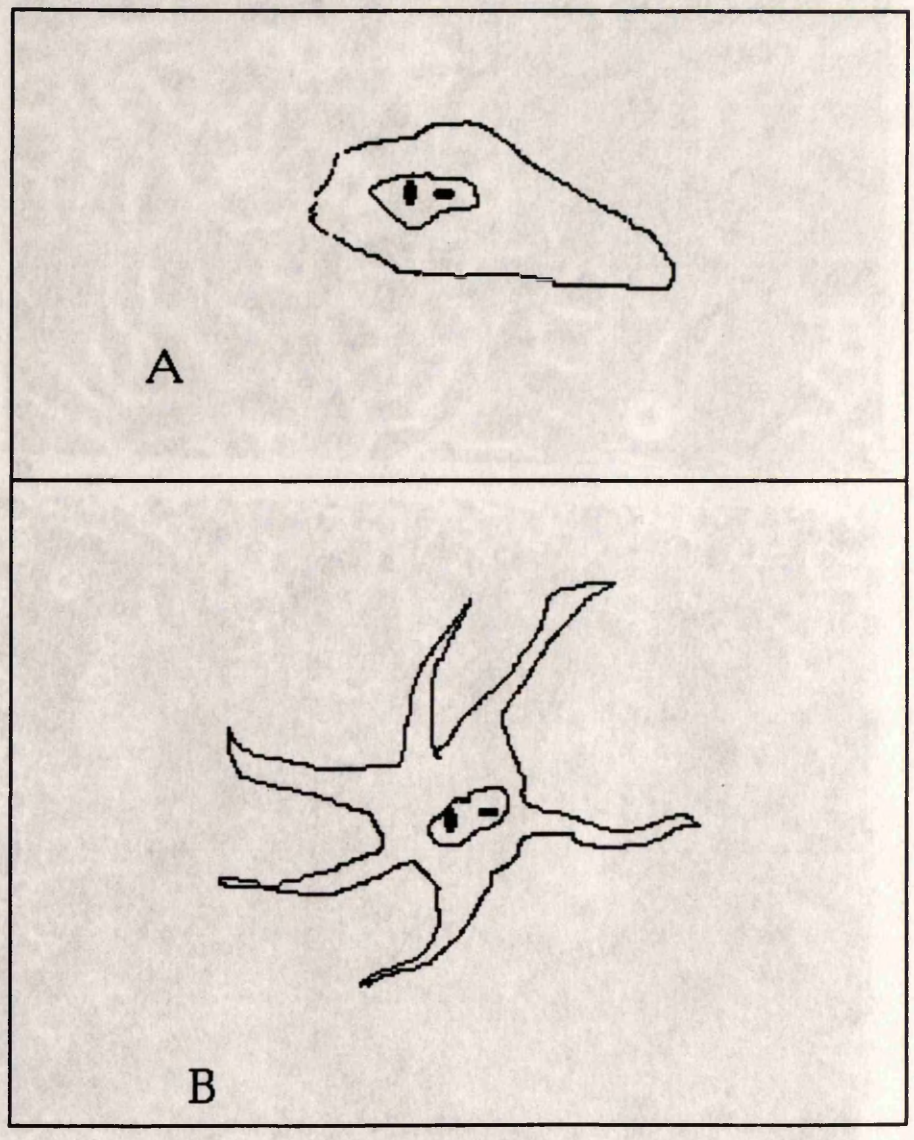


Figure 3.19e. A. Spreading of HeLa cells. Incubation of cells with Ca^{2+} / Mg^{2+} on fibronectin or with Mn^{2+} on a nonadhesive substrate (see fig. 3.3) causes cells to spread uniformly from the early stages. **B.** When cells are incubated with nM concentrations of CL-A (see fig. 3.19a-d) they adopt a more stellate morphology after 30 minutes. Longer times of incubation cause cellular rounding (see fig. 3.20b). See p. 98 for discussion,

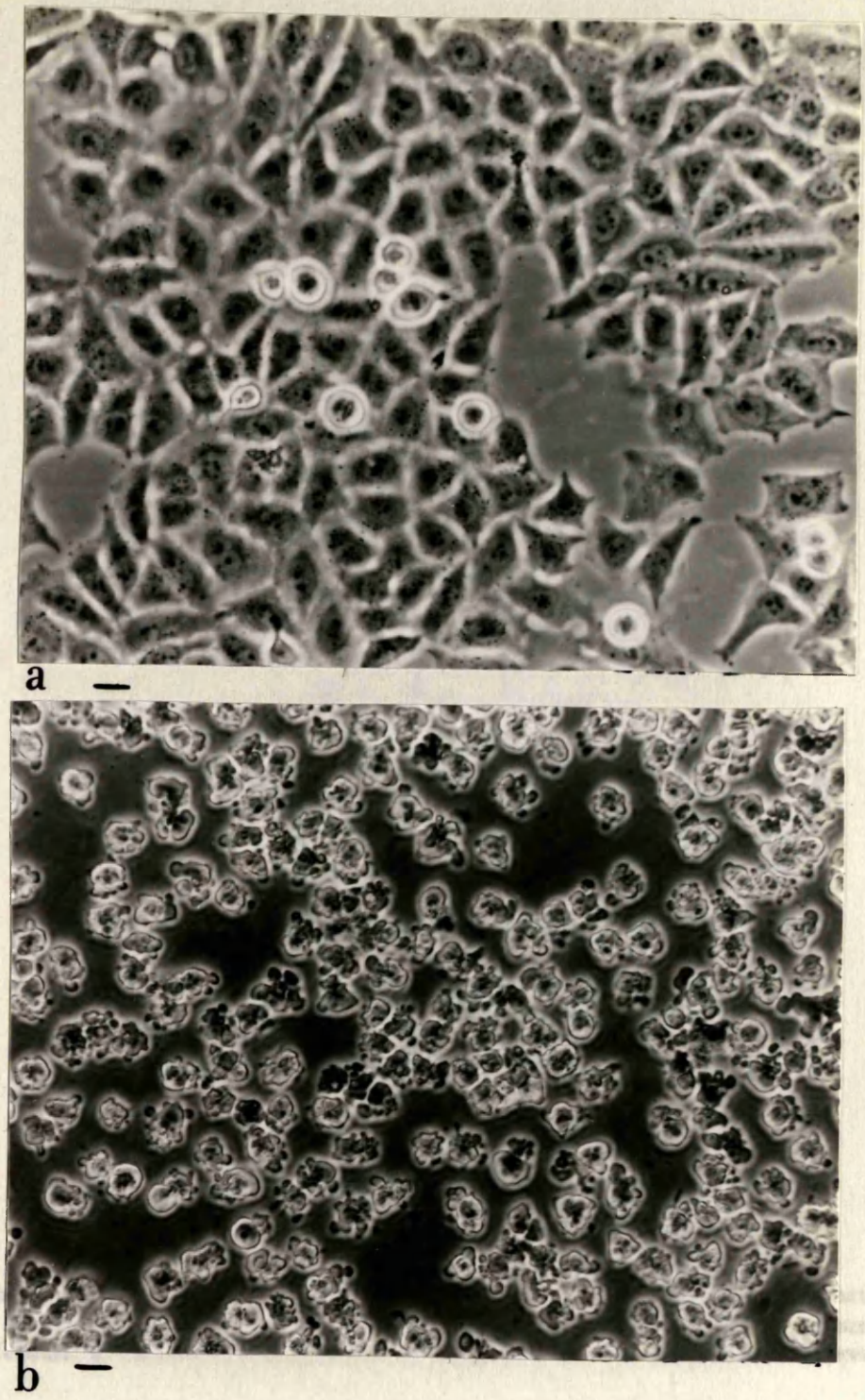


Figure 3.20. Effect of CL-A on the shape of HeLa cells. a. HeLa cells under physiological conditions. b. Same culture 30 minutes after the addition of 50nM CL-A. Bars, 10μm

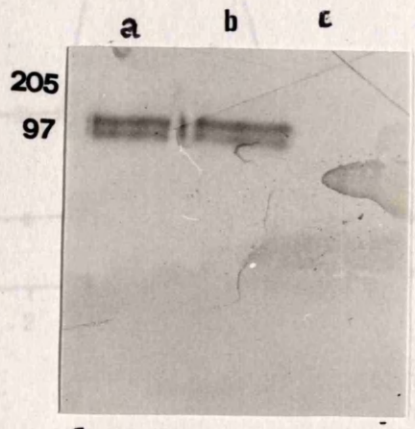


Figure 3.21. Immunoblotting using a polyclonal anti-$\alpha 5\beta 1$ antibody. Cell extracts were electrophoresed, transferred to nitrocellulose filters and integrins detected by ECL as described in Materials and Methods. **a**. Calyculin A treated cells. **b**. Interphase cells **c**. Interphase cells, preimmune serum

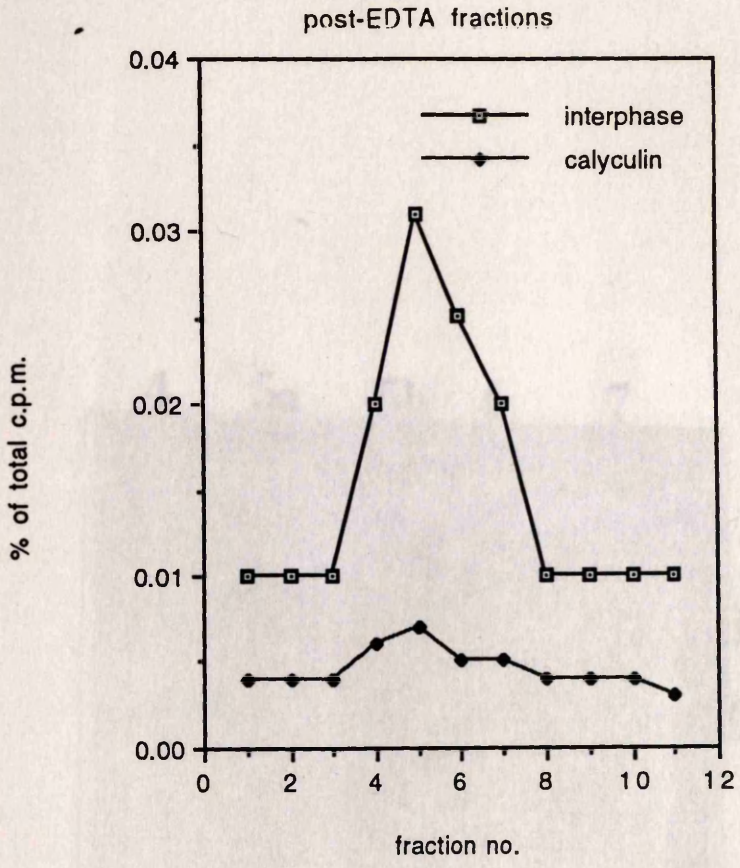


Figure 3.22. Affinity chromatography isolation of the fibronectin receptor. An aliquot (10ul) from eluted fraction, starting from cell extract application, was taken and its radioactivity measured as described in Materials and Methods. Only post-EDTA fractions are shown. c.p.m. are expressed relative to the peak fraction of each sample.

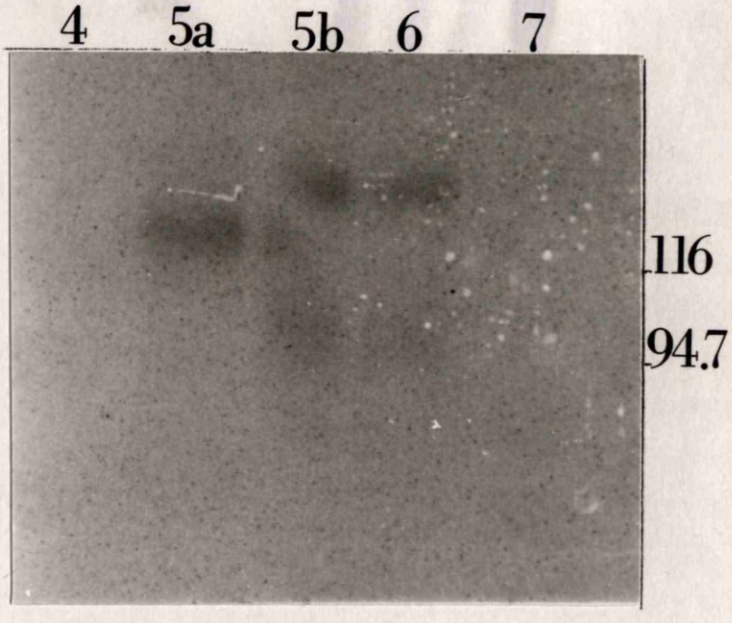


Figure 3.23. Detection of integrin bands from affinity chromatography isolated fractions. Lanes shown are from interphase cells. Numbers of tracks coincide with numbers of fractions from figure 3.22. **5a**. Fraction 5 under reducing conditions. **5b**. Fraction 5 under nonreducing conditions

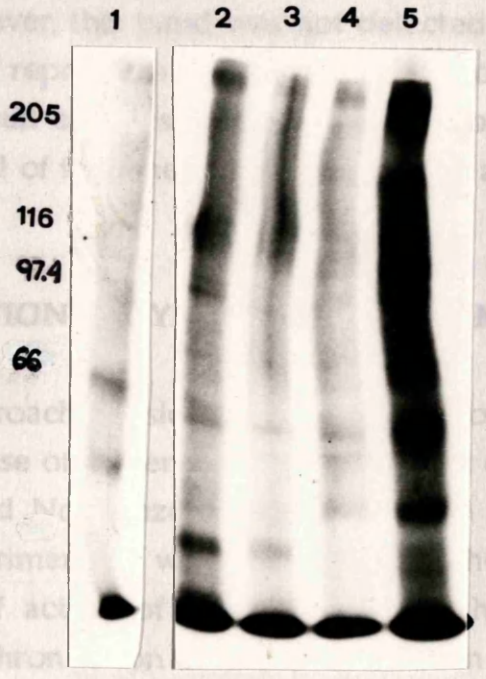


Figure 3.24. Analysis of integrin phosphorylation from interphase and CL-A-treated cells. Samples were analysed by SDS-PAGE under nonreducing conditions, using 7% running gel. **Lane1**. Preimmune rabbit serum. **Lane 2**. Interphase cells. Anti-$\alpha5\beta1$. **Lane 3.** Interphase cells. PY20. **Lane 4**. CL-A-treated cells. Anti-$\alpha5\beta1$. **Lane 5**. CL-A-treated cells. PY2O. Films were exposed for 48 hours.

Changes in integrin phosphorylation were also studied by immunoprecipitation from ^{32}P-labeled cells, using anti-$\alpha_5\beta_1$ and anti-phosphotyrosine (PY20) antibodies. Immunoprecipitation was carried out as described in Materials and Methods. CL-A (50nM) was added to cells 30 minutes before incubation time with ^{32}P was due to finish (i.e. 2.5 hours after addition of ^{32}P). The same amount of protein, as measured with Bradfords' reagent, was applied for each fraction. Figure 3.24 shows the absence of integrin bands in both samples. The interphase track shows a possible band in the range of 97 and 116kDa, compatible with being a β_1 subunit. However, this band was not detected in three further experiments and probably represents an artefact (see also above, figure 3.16). A more interesting result appears in the anti-phosphotyrosine tracks. CL-A clearly raises the level of tyrosine phosphorylation in a wide range of proteins.

8. OPTIMISATION OF SYNCHRONISATION METHODS

The approach considered to generate populations enriched in mitotic cells was the use of reagents that block cells at certain stages of the cell cycle (thymidine and Nocodazole), combined with serum-starvation of the cells in some experiments. I will discuss briefly the current knowledge on the mechanism of action of Nocodazole and thymidine, and why they are useful in synchronisation methods for cells in culture. Then I will describe the steps involved in the optimisation of a method suitable for synchronisation of HeLa cells.

8.1. Nocodazole and thymidine blocks

Nocodazole is an alkaloid similar to colchicine, which interferes reversibly with the polymerization of microtubules (MTs). Since the segregation of replicated chromosomes depends on the formation of a bipolar spindle composed primarily of MTs, Nocodazole can be used to inhibit formation of the mitotic spindle, therefore arresting cells at the onset of mitosis. Furthermore, concentrations of Nocodazole effective on mitotic MT polymerization have no effect on the MTs from interphase cells. Thus, treated cells will proceed normally through the cell cycle until mitotic spindle formation, which cannot be achieved. Removal of the drug allows the cells to continue through mitosis (Vandre *et al.* 1992).

Reversible inhibition of DNA synthesis allows the synchronisation of a high percentage of cells at the boundary G1/S. This can be accomplished by the use of high concentrations of thymidine, which will interfere with the synthesis of the necessary nucleotides. Uptaken thymidine is rapidly converted into dTTP, which is an allosteric inhibitor of ribonucleotide reductase. This enzyme is responsible for reducing all ribonucleotide diphosphates to the corresponding deoxy form (from NDP to dNDP). It is believed that high concentrations of dTTP reduce the supply of dCTP from CDP, which eventually will cause DNA polymerase to stop. In fact, addition of dCTP bypasses the thymidine block (reviewed by Adams, 1980).

A way to arrest cells before S phase is to stop their growth by removing serum from the medium. Serum contains growth factors and possibly other components which are essential for cell growth. Therefore, deprivation of serum causes most cells to withdraw from the cell cycle and remain at Go. Readdition of high concentrations of serum causes a high percentage of cells to reenter the cell cycle in G1.

8.2. Synchronisation methods

My first synchronisation experiments involved the use of 0.05 µg/ml Nocodazole on exponentially growing cultures, during 15-20 hours, and then isolation of the mitotic pool by shaking off. Although it has been reported that reversibility of Nocodazole effect is gradually lost after incubation of the drug for more than 8 hours (Zieve *et al.* 1980), treatment with Nocodazole for much longer times has been widely reported as a successful approach to block reversibly several cell types in mitosis. This suggests that Nocodazole reversibility could be to some extent cell type dependent.

In my hands, a single Nocodazole treatment of 0.05 µg/ml for 18 hours allowed the collection of 30-40% of all cells, with a mitotic index of >85% as measured by flow cytometry (fig. 3.25). However, when cells were extensively washed to remove Nocodazole, and plated with fresh medium, a high majority did not divide, and cells were clearly unhealthy after 24 hours (fig. 3.26). This result is in accordance with Zieve *et al* (1980). Therefore, a milder treatment was needed in order to obtain viable mitotic cells. This consisted of the use of serum-starvation of cells to obtain populations in Go which, after restimulation, would progress in a highly synchronised way towards mitosis. This would allow the use of a short block with Nocodazole, fully reversible. The actual protocol was as follows:

1. Plating of cells in fresh medium containing 0.1% fetal calf serum
2. After 24 h, restimulation with 20% fetal calf serum
3. After 20 h, addition of 0.05 µg/ml Nocodazole
4. After 8h, collect mitotic cells

This procedure did not yield significant amounts of mitotic cells. Modification of the time between restimulation and addition of Nocodazole (from 10 h to 24 h), or lengthening of serum starvation period up to 48 h did not improve the result (not shown). The most likely explanation at this stage was that cells were not being arrested at Go and, therefore, treatment with Nocodazole for 8 h could not yield high amounts of mitotic cells. Comparison of DNA profiles between serum-starved cells and control cells (i.e. incubated with serum-containing medium) showed no significant difference (see fig. 3.27) indicating that cells were not arresting at Go, but rather proceeding through the cell cycle. It is likely that, as HeLa cells are a transformed cell line (cervix carcinoma, containing several copies of the human papillomavirus genome), high growth is a default situation, and requirement of growth factors and/or other components from serum is bypassed by constitutively active signal transduction pathways involved in cell growth.

Therefore, I considered the use of a thymidine block in order to obtain most of the cell population in G1/S, followed by a block with Nocodazole several hours after removal of thymidine, when most cells are at the end of S phase or in G2. Thus, a Nocodazole block of no more than 8 hours will suffice to arrest reversibly a high proportion of cells in mitosis.

The actual protocol was as follows:

1. Addition of 4mM thymidine to an exponentially growing culture
2. After 20 h, change medium for fresh medium without thymidine
3. After 7 h, add 0.05µg/ml Nocodazole
4. After 8 h, collect mitotic cells

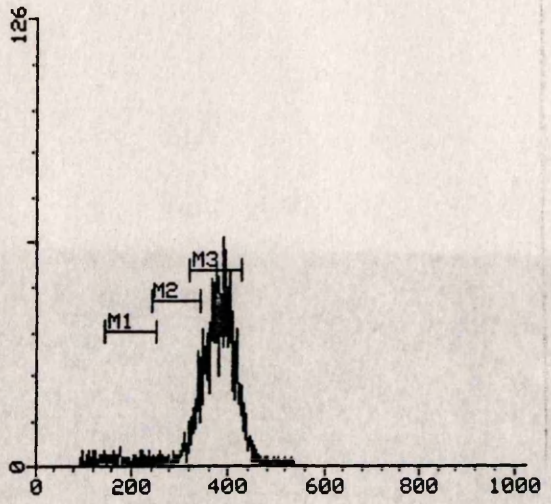
Initially, results were similar to those obtained with serum-starvation. It was possible that cells needed more than 7 hours to recover from the thymidine block. Therefore, I compared mitotic recovery from samples differing in the length of time between removal of thymidine and addition of Nocodazole. None of the samples yielded significant amounts of

mitotic cells (not shown), suggesting that the thymidine block was somehow cytotoxic. Indeed, the use of 2.5mM thymidine instead of 4mM, following the above protocol, yielded high amounts of mitotic cells at the end of the treatment (see fig. 3.28). This concentration was the lowest at which cells became blocked at G1/S and could recover fully. Cell viability was analysed by flow cytometry and by replating mitosis-arrested cells in Nocodazole-free medium. Cells started to divide within a few hours. This is shown if figure 3.29, which represents the DNA profile of a mitotic population 5 hours after Nocodazole release. Note the high percentage of cells that have already entered in G1. Full recovery was obvious 48 hours after plating (fig. 3.30).

Figure 3.31 shows the appearance of a culture after 20 hours of treatment with 2.5mM thymidine. Note the complete absence of mitotic figures.

Figure 3.32 shows the actual duration of mitosis for an untreated cell. About 2 hours are required between cell retraction (G2/M boundary) and complete separation of the two daughter cells. The period from metaphase to cell separation is ~1.5 hours.

My attempts to synchronise cells at the G2 stage for analysis of integrin biosynthesis were unsuccessful. The approach used involved a block with thymidine to arrest most cells at the G1/S boundary, and collection of cells at different times. Flow cytometry was then used to monitor exit from S phase. DNA profiles showed that desynchronisation occurs very early after release of the thymidine block. In fact, although relatively synchronised cells could be obtained in G1+S (see fig. 3.17), DNA profiles typical of an asynchronous culture were obtained after more than 5 hours (not shown). Using a double thymidine block to obtain more cells at the G1/S boundary gave similar results.



M	Left,Right	Events	%	Peak	PkChl	Mean	Median	SD	CV ?
0	0, 1023	3976	100.00	64	390.00	368.17	380.00	61.66	16.7
1	147, 253	149	3.75	5	176.00	194.73	190.00	30.54	15.6
2	244, 341	487	12.25	32	338.00	317.63	326.00	24.17	7.6
3	322, 428	3298	82.95	64	390.00	379.99	382.00	25.65	6.7

Figure 3.25. DNA profile of cells treated with 0.5µg/ml Nocodazole for 18 hours. Statistics at the right show percentage of cells in G1 (3.75%, defined as M1), S (12.25%, defined as M2) and G2+M (82.95, defined as M3). Note that, although this method does not allow separation of G2 from Mitosis, the method of collection (shaking off of loosely attached cells) makes it unlikely that the population defined as M3 (82.95%) contains any significant amount of cells in G2.

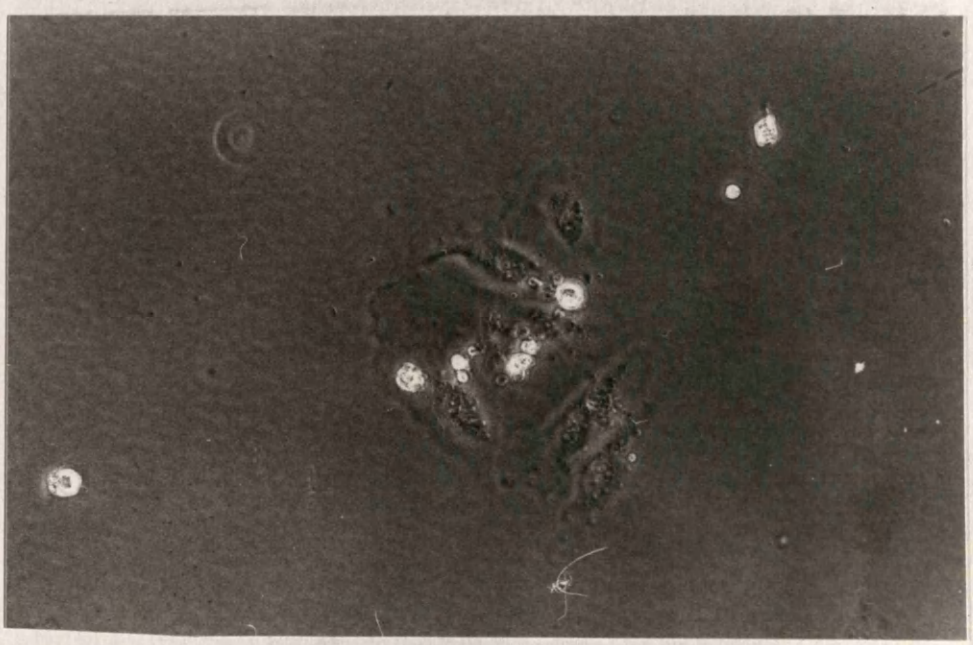
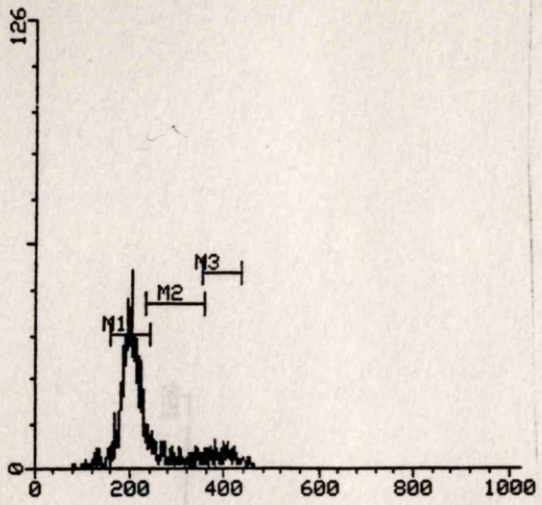


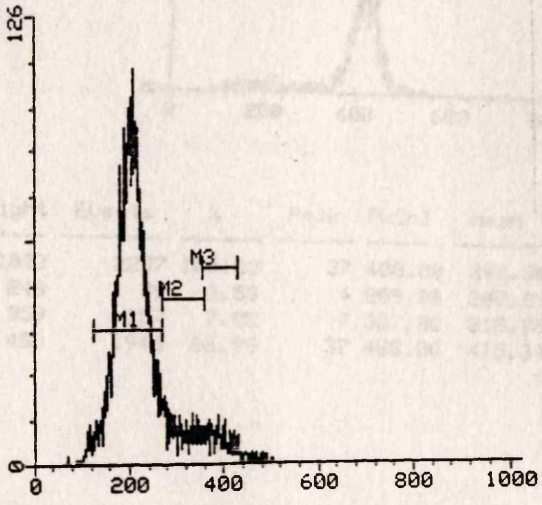
Figure 3.26. Cells treated with Nocodazole for 18 hours as described in the text were washed extensively with medium without Nocodazole and replated. Photograph was taken 24 hours after plating. Magnification x20

a)



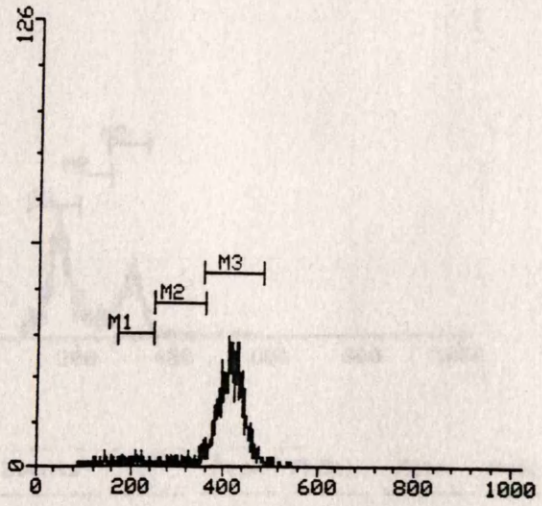
M	Left,Right	Events	%	Peak	PkChl	Mean	Median	SD	CV %
0	0, 1023	2469	100.00	55	204.00	235.37	210.00	71.30	30.29
1	161, 244	1753	71.00	55	204.00	203.46	203.00	17.35	8.53
2	235, 359	436	17.66	12	235.00	282.36	271.00	38.55	13.65
3	354, 437	251	10.17	8	378.00	394.57	395.00	21.87	5.54

b)



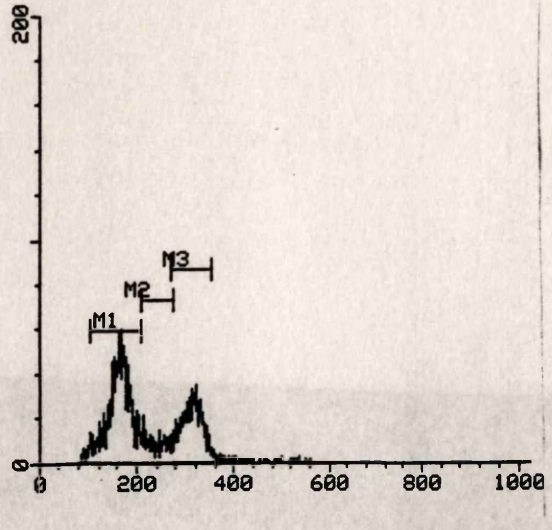
M	Left,Right	Events	%	Peak	PkChl	Mean	Median	SD	CV %
0	0, 1023	8146	100.00	111	208.00	228.60	213.00	65.40	28.61
1	129, 271	6610	81.14	111	208.00	205.42	206.00	28.99	14.11
2	271, 364	894	10.97	19	275.00	313.11	310.00	28.46	9.09
3	359, 433	497	6.10	13	388.00	391.67	389.00	21.15	5.40

Figure 3.27. DNA profile of a) asynchronous, untreated cells and b) cells serum-starved for 48 hours. Note that, although the serum-starved sample has a higher percentage of cells in G1 (defined as section M1 in the graph), there is still a significant proportion of cells in S and G2+Mitosis (17%, defined as section M2 and M3 in the graph), suggesting that cells are able to divide, even in the absence of serum.



M	Left,Right	Events	%	Peak	PkChl	Mean	Median	SD	CV %
0	0, 1023	2237	100.00	37	408.00	396.38	411.00	66.13	16.68
1	170, 248	79	3.53	4	209.00	207.29	209.00	20.54	9.91
2	248, 359	157	7.02	7	357.00	318.08	322.00	32.77	10.30
3	354, 483	1946	86.99	37	408.00	415.34	415.00	25.55	6.15

Figure 3.28. DNA profile of mitotic cells. Cells were arrested at pro-metaphase using 2.5mM thymidine for 20 h, incubation with fresh medium for 7-8 hours and a final incubation with 0.05 µg/ml Nocodazole for 7-8 hours. Cells were collected by shaking off and immediately processed for flow cytometry as described in Materials and Methods.



M	Left,Right	Events	%	Peak	PkChl	Mean	Median	SD	CV %
0	0, 1023	4692	100.00	60	168.00	222.84	193.00	76.42	34.29
1	105, 211	2533	53.99	60	168.00	164.42	166.00	22.91	13.93
2	211, 276	555	11.83	21	214.00	242.67	243.00	20.43	8.42
3	271, 354	1505	32.08	35	318.00	311.76	313.00	20.02	6.42

Figure 3.29. Mitotic cells were extensively washed with full medium to remove Nocodazole and allow to proceed through metaphase in suspension. The DNA profile of this population after 5 hours of Nocodazole release is shown. Cells were processed for flow cytometry as described in Materials and Methods.

Figure 3.30 Mitotic cells were washed extensively to remove Nocodazole and replated. Cells were photographed after 48 hours. Magnification x20.

Figure 3.31. HeLa cells after treatment for 20 h with 2.5mM thymidine. Note the absence of mitotic figures, indicating that most cells have been arrested after mitosis in G1/S, or during S phase. Magnification x20.

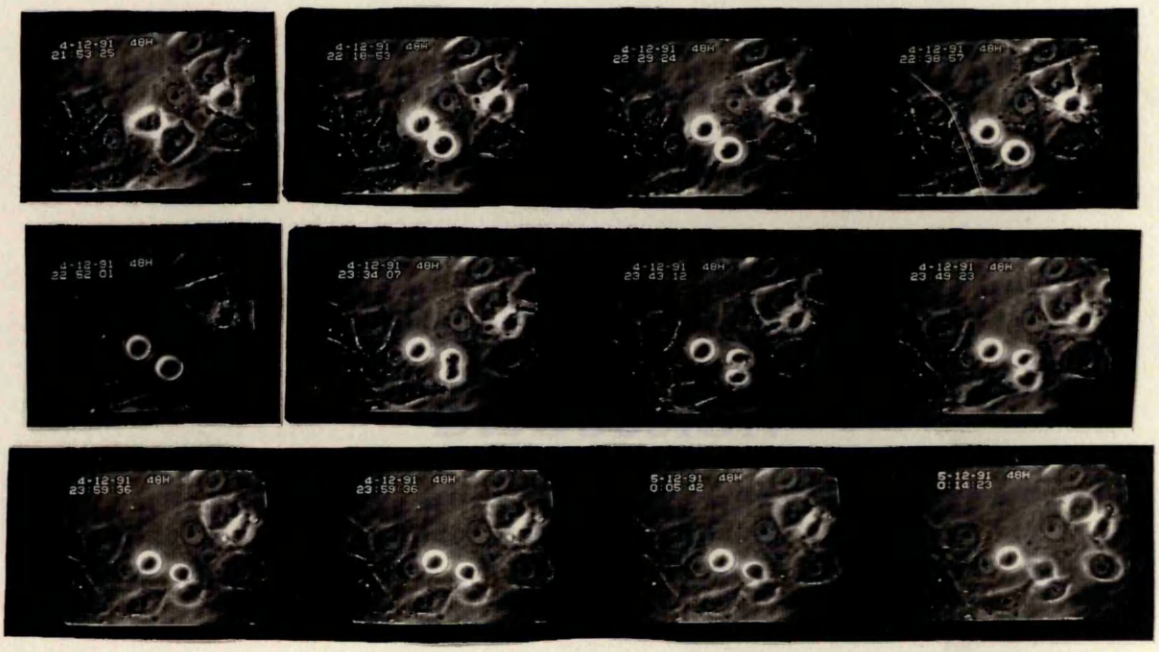


Figure 3.32 Time-lapse video recording of the mitotic process in HeLa cells. Asynchronous cultures were treated with 2.5mM thymidine for 20 hours to obtain a high percentage of cells at phases G1/S and S. Cultures were then allowed to grow in thymidine-free medium for a further 15 hours. At this stage, cells were placed on a 37°C chamber attached to a TMS Nikon microscope and video-recorded.

CHAPTER 4

DISCUSSION

1. SPREADING EXPERIMENTS

1.1. Spreading of mitotic cells

The spreading ability of mitotic and interphase cells under different conditions was assessed. It seemed reasonable to think that, if integrins (the fibronectin receptor(s) in this case) play a role in the cellular rounding at mitosis, optimum conditions might be found under which spreading of mitotic cells could be promoted. For example, the use of fibronectin as substrate and the optimum concentration of Mn^{2+} in the incubation medium might trigger mitotic cell spreading; Mn^{2+} is known to support spreading on fibronectin, probably via integrins, of variant BHK cells otherwise unresponsive to that surface (Edwards *et al.* 1987; Edwards *et al* 1988; section 4, in Final Conclusions chapter). Furthermore, Rabinovitch and DeStefano (1973) showed that sarcoma ascites cells, unable to spread in Ca^{2+}/Mg^{2+}-containing media, were induced to spread by the addition of Mn^{2+}.

A problem affecting the results of spreading assays is the presence of nocodazole in metaphase-arrested cells. This presence is unavoidable, as release from nocodazole starts metaphase—>anaphase transition, accompanied by morphology changes, thus distorting the results. The objective was to assess the ability of rounded mitotic cells to spread under various conditions, and metaphase block with nocodazole was appropriate, as its effects, at the concentrations used, are fully reversible for the length of time of my experiments, and there are no cytotoxic effects (Zieve *et al.* 1980). Interphase cells incubated with nocodazole were included as appropriate control.

Figure 3.2 shows that mitotic cells are unable to spread significantly on fibronectin. Although this could be due to nocodazole, this is unlikely, as its effects on interphase cells are negligible (see also below, section 4.2).

When Mn^{2+} is added to the medium (figure 3.3), mitotic spreading improves significantly in relation to the same cells without Mn^{2+}. However, the absolute values of spreading remain in the same range. Thus, neither the presence of an adhesive substrate nor Mn^{2+} in the medium can induce full spreading of mitotic cells. It is likely that cytoskeletal components essential for spreading are committed to the process of mitosis, therefore preventing full spreading. However, the machinery involved in the initial steps does not seem to be committed to the cell cycle, as mitotic cells, under the right

conditions, are able to spread to a limited, but significant, extent. Furthermore, if cells are blocked in metaphase for a few hours in normal medium, spreading is also detectable (see also figs. 3.7 and 3.10).

1.2. Effects of serum on mitotic cells

While analysing the reversibility of nocodazole effects on metaphase-blocked cells, I noticed that cells seemed to advance faster through anaphase if foetal serum was present in the medium (F-10 Hams'). Therefore I decided to quantify the rate of exit from metaphase in cells with and without serum. As shown in figure 3.4, when serum is present, the majority of cells have clearly divided and daughter cells are in the process of spreading. This is not the case in the absence of serum. It is noteworthy that even in the presence of serum, progression through anaphase is still slow (~4 hours). This is probably due to the time required by the cells to recover from nocodazole (Rieder, 1992).

The serum requirement for mitosis exit shown by HeLa cells is rather surprising, since serum-starvation of cells is a well known method to arrest cells in G1 phase (Morla *et al.* 1989), and there is no published evidence, to my knowledge, of mitotic arrest by serum-starvation. However, an important difference between G1-arrested cultures and my mitotic samples is that I was using highly synchronised cells, which could account for the different behaviour. For instance, in cultures deprived of serum, factors (normally present in serum) secreted from interphase cells, could allow progression through anaphase for the small percentage of mitotic cells existing at all times, until all cells are stopped at G1. However, if mitotic cells represent more than 90% of the total, as in my experiments, these factors might not be present, thus keeping cells in metaphase. Nevertheless, whatever the mechanism, it is not sufficient to arrest all cells in mitosis, since a small percentage of cells by-passes the necessity for serum and progresses through anaphase. Obviously, all possibilities must be considered speculative, since no more data are available.

These are potentially important observations, and further investigation will be necessary in order to confirm a role for serum in the G2/mitosis transition.

2. IMMUNOLOCALIZATION OF $\alpha_5\beta_1$ AND $\alpha_v\beta_3$ INTEGRINS

Indirect immunofluorescence was carried out in order to confirm the presence of $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins in HeLa cells. $\alpha_5\beta_1$ is a fibronectin receptor which binds to fibronectin through the central cell binding fragment, which contains the sequence RGD (Humphries, 1990). As one of the objectives of this thesis was the isolation of the fibronectin receptor by affinity chromatography on the central cell binding fragment, the use of the polyclonal anti-$\alpha_5\beta_1$ antibody was a reasonable choice.

The predominant adhesive glycoprotein in culture conditions, present in serum, is vitronectin. The presence of $\alpha_v\beta_3$, a strong vitronectin receptor (Hynes, 1992), was also screened with a polyclonal anti-$\alpha_v\beta_3$ antibody. The actual bands recognised by these antibodies are discussed below, in section 3.

Figure 3.5 shows a pattern of staining along the edge of interphase cells (figures 3.5a,b). The widely reported punctuate pattern, typical of fibroblasts, indicating the presence of focal contacts, was not detected, although it is possible that access of antibodies to the ventral surface of the cell is especially restricted. However, actin staining of HeLa cells reveals the absence of stress fibres and the presence of a strong staining along the cellular edge (not shown), suggesting that focal adhesions are not present in these cells. The edge pattern could be due simply to the presence of more membrane, in vertical section, at the cellular edge. Something similar would occur in the mitotic samples (Fig.3.5.c,d). The bright inner ring would represent the circumference of the rounded cell (fig.3.7.b,c), whereas the outer edge pattern would be analogous to that of interphase. It is interesting that the spread fringe of mitotic cells seems brighter than in interphase. It is possible that this brightness represents the transit of integrins from the rounded area to sites of spreading. In a fully spread cell (i.e., interphase cells), the receptor density at the dorsal surface is probably too low to detect (fig.3.5.a,b).

However, the pattern of spreading of nonblocked, mitotic cells may be different from that of metaphase-arrested cells, at least as identified with our polyclonal antibodies. As figure 3.7 shows, both antisera stain the cellular edges, but no inner ring is present. If we assume that the transit of integrins to the edge of the anaphase cell is relatively fast, then a bright staining at the cellular edge is to be expected. The difference with blocked cells is that, in the latter, only a small amount of integrins is able to function at the edge in the

process of spreading, as this process stops at a very early stage. Thus, a discontinuity between the main pool of cell surface integrins and the integrins at the spreading sites can be detected.

Figure 3.9 shows the pattern of staining from permeabilised cells. Interphase cells show a perinuclear, Golgi-like staining, revealing the presence of a cytoplasmic pool of the integrins recognised by the anti-$\alpha_5\beta_1$ antiserum (fig. 3.9a). However, the anti-$\alpha_v\beta_3$ antiserum reveals a much weaker and more diffuse pattern (fig.3.9b). Although a Golgi-like pattern is present, the nucleus is also slightly stained.

The significance of the differences shown between both antisera in permeabilised cells in unclear. It is possible that there is a quicker turnover of the integrins recognised by the anti-$\alpha_5\beta_1$ antiserum, thus showing a larger cytoplasmic pool of proteins being continuously exported to the plasma membrane. On the contrary, if the turnover were relatively slow, only a small cytoplasmic pool would be required, as seems to be the case with cells stained with anti-$\alpha_v\beta_3$ antiserum.

Alternatively, it is possible that the process of permeabilisation may account for the differences in cytoplasmic staining. For instance, the pattern along the edge of cells is not present at all in permeabilised cells stained with anti-$\alpha_v\beta_3$ antiserum (fig.3.9b), whereas it appears in unpermeabilised cells (fig. 3.5b). Note also that, when flow cytometry is used to quantify the amount of antigen per cell in the plasma membrane (fig. 3.10), no significant differences are found between the two antisera, further supporting this idea.

3. EXPRESSION OF INTEGRINS.

Immunofluorescence experiments permit the cellular localisation of the integrins recognised by the polyclonal anti-$\alpha_v\beta_3$ and anti-$\alpha_5\beta_1$ antisera. Information about the identity of those integrins can be obtained with immunoprecipitation experiments using radioiodinated cells. Cell surface proteins are ^{125}I-labeled, and integrins recognised by the antisera are visualised by autoradiography.

Figure 3.11 shows the bands immunoprecipitated with anti-$\alpha_5\beta_1$ antiserum. At least three subunits were recognised in all experiments, the lower one of an intensity in the range of the sum of the other two (see table

3.1). Several data suggest that the lower band is β_1. First, the antiserum is directed against the β_1 subunit, although other β subunits associating with α_5 would also appear. Second, its molecular weights under reducing/nonreducing conditions coincide with those published for β_1 (~125kDa/~110kDa). And finally, when the antiserum is used with ^{35}S-labeled cells, a wider β band appears (Fig.3.18), which may be indicative of the presence of a precursor, also reported for the β_1 band (Akiyama *et al.* 1990).

However, the identity of the two upper bands (~150kDa and ~135kDa) is less clear. Their mobility is not apparently affected by the presence of reducing agents. With no more data available, there are several possibilities. One could expect the presence of α_5 together with β_1, as the antiserum should also recognise α_5 epitopes. Immunoblotting experiments were carried out to confirm this possibility. Figure 3.13 shows the presence of two bands recognised by the anti-$\alpha_5\beta_1$ antiserum. Since the antigen-antibody reaction takes place after the integrin subunits have separated by SDS-PAGE, these bands are bound to be α_5 and β_1. Furthermore, the fibronectin receptor affinity-isolated on the cell binding fragment of fibronectin (to which $\alpha_5\beta_1$ binds; see fig. 3.23) consists of a β_1-like subunit associated to an α subunit which runs faster under reducing conditions, as expected from α_5.

On the other hand, figure 3.12 shows that the central band (the one with a molecular weight similar to α_5: ~135kDa) moves identically under reducing and nonreducing conditions. A possible explanation is that there are several subunits associated with the β_1 subunit. At least two of them would be present in the central band (e.g. α_2, α_3 and α_5 have similar mobilities, and all associate with β_1; Hemler *et al*, 1987) and, under reducing conditions, α_5 could move faster and run as a single band with β_1. Note that, although relative intensities of bands under reducing conditions were not compared, the intensity of the central band in relation to the rest, if anything, diminishes with respect to nonreduced subunits, thus supporting this hypothesis.

Although the presence of the α_5 and β_1 subunits is reasonably clear, the identity of the accompanying α bands cannot be firmly established without the use of antibodies against specific α subunits. Unfortunately, such antibodies were not available, and I continued my work on the assumption that my polyclonal antiserum recognised several β_1 integrins, one of them probably $\alpha_5\beta_1$.

Immunoprecipitation with the anti-$\alpha_v\beta_3$ antiserum proved unsuccessful. A polyclonal antiserum raised against the β_3 subunit did not recognise any band either. This antiserum was also used in immunoprecipitation experiments with ^{35}S-labeled cells and in immunoblottings (fig. 3.13), always with the same negative results. With these data, it is reasonable to conclude that there are no β_3 integrins in HeLa cells. However, it is obvious that, although the idea that no β_3 integrins are present in HeLa cells may be correct, the anti-$\alpha_v\beta_3$ antiserum seems to recognise other integrins, since immunofluorescence and flow cytometry experiments give a positive result. However, the affinity for these integrins is probably weak, as the best results are achieved with unfixed, living cells (flow cytometry), and with cells mildly fixed with 5% sucrose in 4% formaldehyde (see Materials and Methods section). Negative results, undistinguishable from the controls, were obtained if sucrose was omitted during the fixation. It is also clear that any integrin recognised by this antiserum should contain the α_v subunit, as β_3 subunits are not present. Note also that the upper α band identified using the anti-$\alpha_5\beta_1$ antiserum has a mobility expected of α_v, suggesting that the integrin $\alpha_v\beta_1$ is also present in HeLa cells.

In conclusion, then, it is reasonable to assert that no β_3 integrins are present in HeLa cells, and that there are several β_1 integrins, $\alpha_5\beta_1$ amongst them. We can also conclude that there is no apparent difference in β_1 integrin expression between mitosis and interphase as measured by flow cytometry and immunoprecipitation.

4. ACTIVITY AND PHOSPHORYLATION STATES OF MITOTIC AND INTERPHASE INTEGRINS

Great advance has been made in our understanding of how integrins achieve different states of activity (see integrin regulation section, in Introduction chapter). Phosphorylation is a mechanism known to affect such activity in cultured cells. Tapley *et al.* (1989) reported that phosphorylation of the β_1 subunit by pp60^{src} in Tyr diminished binding of β_1 integrins to fibronectin and talin. Possibly, this inactivation by phosphorylation helps to maintain the rounded phenotype typical of cells transformed with Rous sarcoma virus. In fact, transformation with a mutant pp60^{src} unable to induce

cell rounding and loss of fibronectin, is not accompanied by β1 phosphorylation (Horvath *et al.* 1990). Hirst *et al.* (1986) showed that transformation of cells with oncogenes encoding tyrosine kinases correlated with phosphorylation of the fibronectin receptor and rounded morphology, although receptor activity was not assessed.

The above data relate phosphorylation of integrins with rounded phenotypes in transformed cells. An obvious connection is the cell cycle. Cells in mitosis are also rounded, and it is reasonable to think that the mechanism of cell rounding in transformed cells may be related to that of mitosis. Thus, it is possible that, as phosphorylation of integrins is very likely to play a role in cell rounding in transformation, the same may apply for cell rounding in mitosis. In fact, protein phosphorylation is central to cell division. As already mentioned in the Introduction of this thesis (section 6), CDK kinase activities form part of a conserved mechanism by which cells are driven into cell division. Theses activities are responsible for the structural reorganisation taking place at the G2—>Mitosis transition, which includes disassembly of nuclear laminae, rearrangement of cytoskeletal components and arrest of endomembrane traffic, and coincides in time with cell rounding.

There are several substrates for CDK activity participating in these changes, of obvious interest for the work described in this thesis (see 6.1.2. in Introduction). $pp60^{c\text{-}src}$ is one of them (Shenoy *et al.* 1992). Its downstream candidates might well include cytoskeletal components, or even integrins. Caldesmon is another substrate for CDK. Yamashiro and Matsumura (1991) have suggested that phosphorylation of caldesmon would cause its disassociation from microfilaments, and that this would help in the process of rounding up at mitosis. Vimentin, an intermediate filament (IF) protein, is also phosphorylated by CDK, and this is accompanied by vimentin IF diassembly (Chou *et al.* 1991). Interestingly, microinjection of CDK into cells at any time of the cell cycle except in S phase caused cell rounding and reorganisation of microfilaments and microtubules (Lamb *et al.* 1990). Thus, correlation of cytoskeletal rearrangements and cellular rounding is apparent. However, it is also imaginable that a regulated control of rounding at G2/prophase and respreading at anaphase should involve not only cytoskeleton re-organisation, but also inactivation of the interactions between the ECM and their receptors at G2/prophase, followed by reactivation after

metaphase. Therefore, one of the objectives of my work was to find out whether integrins played a role in the cellular rounding up at mitosis, and a reasonable hypothesis was that integrins might be involved in the phosphorylation cascades triggered by CDK. For example, as mentioned above, β_1 integrins may be phosphorylated by $pp60^{src}$, which in turn is a substrate for CDK.

It is also noteworthy that to date there are no published data on the role of any ECM receptor in the cellular rounding up at mitosis. Nevertheless, Hynes (1992) makes reference to unpublished results from his group (C.Grandori and R. Hynes, unpublished data), mentioning that a Ser in the β_1 subunit of the fibronectin receptor $\alpha_5\beta_1$ is phosphorylated in mitosis and the receptor no longer binds to fibronectin. However, these results remain to be confirmed. There is some work published about cell-cell interactions at mitosis, in epithelial cells. Baker and Garrod (1993) reported that confluent epithelial cell layers retained cell-cell junctions (see 3.2., in Introduction) at mitosis. From their work it seems clear that these junctions remain functional during cell division and are not involved in the processes triggered by CDK. The authors also report that hemidesmosomes are retained during mitosis, in basal epidermal keratinocytes. Thus, it appears that retention of cell-basal lamina contacts through hemidesmosomes may be important in retaining dividing cells in the basal layer.

In conclusion, the above data present sufficient evidence in the direction of some role for integrins in mitosis. An obvious approach to test this idea was the use of affinity columns to analyse cell cycle-related differences in the binding ability of the fibronectin receptor $\alpha_5\beta_1$, together with integrin immunoprecipitation from ^{32}P-labeled cells, using anti-$\alpha_5\beta_1$ antiserum. This would allow detection of changes in the phosphorylation state of integrins which might correlate with different states of activity.

4.1 isolation of the fibronectin receptor using affinity chromatography

The use of the fibronectin cell-binding fragment to isolate the fibronectin receptor(s) was first reported by Pytela *et al.* 1985. The material coupled to the isolation column was a highly purified fragment obtained from digested fibronectin (Pierschbacher *et al* 1981), which contains the RGDS sequence (see fibronectin section, in Introduction chapter).

I followed basically the same procedure. As described in Materials and Methods, the fibronectin digest fraction directly eluted from the gelatin column (unbound fraction) was used as the coupling material for receptor isolation. Two differences from the published procedure were the use of Mn^{2+} in all buffers up to receptor elution, as Mn^{2+} improves receptor binding (Gailit and Ruoslahti, 1988), and the use of the cell-binding fragment as obtained from the gelatin column, instead of further purified from heparin and gel filtration columns (Pierschbacher *et al* 1981). In my experiments, the cell-binding fragment eluted from the gelatin column constituted practically the only major component (fig.3.1). Furthermore, this fraction has already been succesfully used to isolate the fibronectin receptor from BHK cells in our lab (Edwards and Campbell, unpublished results).

A major problem found using this approach was a very low sensitivity in the detection of the fibronectin receptor eluted with EDTA, which needed several weeks of film incubation. Although technical problems may account for this low sensitivity, it is also possible that very low amounts of the receptor are present in HeLa cells. In fact, although relatively high amounts of β_1 integrins are detected by immunoprecipitation using ^{125}I from as little as ~10^7 cells (figs. 3.11 and 3.12), the percentage of $\alpha_5\beta_1$ seems in fact much smaller (see section 3, this chapter). This low percentage may have also been the cause of the negative results achieved with immunoprecipitation of the eluted receptor using anti-$\alpha_5\beta_1$ antiserum (not shown).

Nevertheless, the main objective sought with this approach, namely the comparison of the activity of the fibronectin receptor between mitotic and interphase cells, was successfully carried out (see also below, section 6, for discussion on the activity of the fibronectin receptor in CL-A-treated cells).

Figure 3.14 shows the c.p.m. eluted from the cclumns with EDTA. Amounts in the same range are recovered from both interphase samples, and from both mitotic samples. Table 3.2 shows the total post-EDTA c.p.m. recovered from each sample as a percentage of the total c.p.m. applied to each column. c.p.m. recovered from mitotic cells represent ~50-60% of those from interphase, regardless of the presence of phosphatase inhibitors.

Although the high and uneven background present in the autoradiographs (fig.3.15) prevents a clear comparison between samples, the point that must be stressed is that integrin bands are present in all samples. Therefore, full inactivation of the mitotic fibronectin receptor does not occur.

If we compare the 50-60% of mitotic recovery (table 3.2) with the poor spreading (≤10% of that of interphase cells, and only using optimum conditions) shown by mitotic cells, it becomes apparent that the loss fibronectin receptor activity alone, as detected in solution, cannot account for mitotic rounding.

4.2. Changes in phosphorylation

Figure 3.16 shows clearly the absence of phosphorylated integrins in interphase and mitosis. No bands were either immunoprecipitated with the antibody PY20 (see also fig. 3.24).

Although the partial inactivation of $\alpha_5\beta_1$ cannot account for the poor spreading shown by mitotic cells, it is clearly significant. Phosphorylation of either subunit is a possible mechanism to account for such changes in activity. However, my results indicate that β_1 integrins are not phosphorylated. Also, they are in contradiction with the results mentioned by Hynes (1992), where $\alpha_5\beta_1$ is inactivated and β_1 becomes phosphorylated in mitosis, although in this latter case the results remain to be confirmed.

Therefore, a mechanism for the partial inactivation of the mitotic fibronectin receptor remains to be identified. On the other hand, it is quite possible that the poorer recovery from mitotic samples could be due solely to the differences between samples in cell harvesting for radioiodination. Integrins bind to the ECM and also to components in the cystoskeleton to achieve fully active conformations (section 5, Introduction chapter; Hynes, 1992). In mitotic cell rounding, G2 cells re-organise their cytoskeleton as they enter mitosis, which is likely to be accompanied by inactivation of components interacting with integrins, which in turn may affect integrin activity. The final result is cellular rounding. Therefore there is an inactivation of cell-ECM interaction initiated from inside the cell. On the other hand, interphase cells are detached from the substrate by removing divalent cations from the medium, so that cytoskeletal structures linked to integrins may not be essentially modified, as they are in mitosis.

Thus, it is possible that interactions of cytoskeletal components (e.g. talin or vinculin) with interphase integrins are not disrupted in the solubilisation procedure with nonionic detergent. However, such interactions have been already disrupted from inside in mitotic cells. The hypothesis is that this difference causes interphase and mitotic integrins to keep different

conformations when solubilised with β-octyl-glucoside, which could account for the different recovery from the fibronectin fragment columns. Note also that, although these considerations are speculative, they explain the irrelevance of using phosphatase inhibitors, while keeping the importance of the phosphorylation events triggered by CDK activity. In interphase, as no phosphorylation is involved, the use of phosphatase inhibitors is irrelevant. In mitosis, as the cytoskeleton is already rearranged when cells have rounded up, lysis is extremely unlikely to restore integrin-cytoskeleton interactions that are already disrupted, regardless of the effect of the absence or presence of phosphatase inhibitors.

In conclusion, then, it is possible that, in HeLa cells, $\alpha_5\beta_1$ is indeed less active in mitosis, due not to post-translational changes, but to modification of its interaction with the cytoskeleton. This would also explain the spreading results obtained with mitotic cells. Since much of the spreading machinery is committed to cell division, very limited spreading is possible in mitosis.

It is also possible that the use of Mn^{2+} in the affinity isolation accounts for the results obtained. It could be that the mitotic receptor has lost its activity, and that the presence of Mn^{2+} stimulates its activity to the extent of being detectable by affinity chromatography (see Integrin regulation section, in Introduction chapter). However, this possibility does not explain satisfactorily the irrelevance of the use of phosphatase inhibitors.

Future work should test the validity of these hypothesis. A possible experiment could be the use of electrophoresis under nondenaturing conditions, so that any changes in integrin mobility due to the presence of bound proteins are detected. These proteins could then be identified by immunoblotting using antibodies against vinculin, talin, α-actinin, etc.

5. BIOSYNTHESIS OF β_1 INTEGRINS

The results from figure 3.18 show metabolic labelling of the putative β1 subunit, together with its precursor and two α subunits, and the absence of β_3 integrins. This pattern of three bands is probably the same of that obtained from radioiodinated cells. However, the relative intensity of β is much higher from ^{35}S-labeled cells. This difference cannot be explained by the different contents of Cys and Met residues between α and β subunits (see

Biosynthesis of Integrins, in Results chapter). It is likely then that there is a higher turn-over of β subunit. There is also a difference between asynchronous and G1+S (T2) cells. Asynchronous cultures have a stronger β_1 band (fig. 3.18a), and α subunits are not present in T2 cells. A further experiment (fig.3.18b) showed that, although asynchronous cells still show stronger bands, α subunits were detectable in T2 cells. The main difference between asynchronous and T2 samples is the presence of a higher proportion of G2 cells in asynchronous cultures. Taking these results at face value, it would suggest that there is a peak of synthesis of integrins at G2, especially of α subunits. However, further work is clearly needed in order to understand the synthesis of integrins during the cell cycle. Analysis of mRNA at different stages of the cell cycle should clarify whether there is a regulated synthesis of integrins in G2 or such synthesis is constitutive during the cell cycle.

6. EFFECTS OF CALYCULIN A ON CELL SPREADING AND ON β_1 INTEGRINS

Two potent inhibitors of phosphatases PP1 and PP2A (see section 4, in Introduction), namely okadaic acid (OA) and calyculin A (CL-A) have recently been discovered (see Cohen *et al.* 1990; Ishihara *et al.* 1989 for reviews). ID_{50} values for OA are ~1nM for the catalytic subunit of type 2A, 100nM for the multisubunit 2A1 and 0.5μM for the catalytic subunit of type 1. Values for CL-A are similar, with the exception of type 1 phosphatase. ID_{50} values with CL-A in this case are 1-2nM (Chartier *et al.* 1991). Treatment of cells with these inhibitors enhances the overall state of phosphorylation due to phosphatase inhibition, and is a useful approach to investigate processes involving phosphorylation-dephosphorylation events.

These inhibitors have been shown to induce morphological changes similar to those in mitosis. For example, Kipreos and Wang (1990) showed that μM OA activates cdc2 kinase and induces cell rounding. Furthermore, c-abl protooncoprotein becomes phosphorylated at the same site as in mitosis. Yamashita *et al.* (1990) reported that treatment of cells synchronised in S phase with 0.5μM OA caused cellular rounding, nuclear envelope breakdown and chromosome condensation. In fact, cdc2 kinase may be activated by OA at

concentrations that inhibit PP2A but no PP1 (Cohen *et al.* 1990; see also section 4, in Introduction).

CL-A also induces shape changes in 3T3 cells at 0.1µM, accompanied by reorganisation of IFs, microfilaments and microtubules. These effects were fully reversible (Chartier *et al* 1991).

The effect of both OA and CL-A on cellular shape, and their possible link to the cell cycle make these inhibitors ideal reagents to study the activity and phosphorylation state of integrins in a model that closely mimicks the changes taking place in mitosis. As a small quantity of CL-A was available in our lab, I undertook such studies, using approaches similar to those used to compare mitotic and interphase integrins.

Figure 3.20 shows the typical morphology of normal HeLa cells (3.20a) and of cells treated with 50nM CL-A. Removal of CL-A restored the normal morphology (not shown). Spreading experiments (figS. 3.19a-d) showed that CL-A-treated cells spread to a limited extent, but retract after a short time. The negative effect of CL-A is clear only after 1 hour of incubation. There is no significant difference in spread area between control and CL-A treated cells after 30 min. However, the morphology of all CL-A treated cells after 30 min was different from that of control cells (fig. 3.19e). It showed a more irregular, stellate shape. With no more data available, it is not possible to know whether CL-A treated cells spread uniformly at the very early stages of spreading, and then retract in a way that would cause the stellate shapes, or their spreading involves initially the adoption of such morphology. Obviously, analysis of spreading at the very early stages (less than 30 min) will be necessary in order to understand better the effect of CL-A on the spreading of HeLa cells.

Integrin subunits, presumably α_5 and β_1, are shown in figure 3.21 (see also fig. 3.13). The activity of $\alpha_5\beta_1$ was analysed by affinity chromatography on the cell binding fragment of fibronectin. Figure 3.22 shows the difference between spread, interphase cells and cells treated with CL-A. Very little counts are present from CL-A-treated cells, and these counts do not translate into bands, even after long times of film incubation (>4 weeks). It seems then that the fibronectin receptor does not bind to fibronectin after CL-A has been added to the cells. However, as in mitotic cells, no phosphorylation of integrins could be detected in CL-A-treated cells, although a general increase

in tyrosine phosphorylation appears in proteins larger than ~60kDa (smaller bands are also present in other lanes and are probably nonspecific).

Therefore, it appears that there are at least two basic differences in the behaviour of the mitotic and CL-A-treated cells concerning the fibronectin receptor and the pattern of tyrosine phosphorylation. The mitotic fibronectin receptor still binds to fibronectin, although with a reduced affinity. On the contrary, the receptor from CL-A-treated cells apparently loses all its activity. Also, the level of tyrosine phosphorylation in mitosis does not change appreciably (fig. 3.16) whereas a clear rise in tyrosine phosphorylation is detected in CL-A-treated cells. A similar increase in overall phosphorylation has been reported by Chartier *et al* (1991) using 0.1μM CL-A.

It might be expected that mitosis and addition of CL-A would have a similar effect on the fibronectin receptor. The effect of CL-A at 1-2nM is thought to be the inactivation of PP2A, which would cause cdc25 hyperphosphorylation, increasing its cdc2 phosphatase activity. At the end, a mechanism similar to mitosis is triggered. However, activation of cdc2 also requires the apropriate level of cyclin A and/or B (see section 5 in Introduction). If that level is not met, CL-A-treated cells will pass normally through all phases of the cell cycle until they are blocked in metaphase. This is indeed the case with LLC-PK cells (Vandre and Wills, 1992). BHK cells, on the other hand, accumulate cyclin B from early S phase (Yamashita *et al* 1990), and treatment with OA induces premature mitosis in many of them. Accumulation of cyclin B in HeLa cells occurs at G2 (Pines and Hunter, 1989). Therefore, a result similar to that with LLC-PK cells is to be expected. My results are in accordance with these data. ~1-5nM and indeed much higher concentrations of CL-A have no effect on spread HeLa cells. In other words, no premature mitosis is induced with concentrations that would inactivate PP2A. In consequence, it must be assumed that the rounding induced by CL-A in my experiments is due to more general, cell cycle-unrelated phosphorylation processes, most likely taking place in the cytoskeleton, and involving many phosphatases. It is not unreasonable to think that the differences encountered in the activity of the fibronectin receptor between interphase, mitotic and CL-A-treated cells may depend on the way cells have rounded prior to cell labeling with ^{125}I. In interphase, removal of cations from the medium causes cell rounding but may leave some cytoskeleton/integrin interactions untouched. Mitosis may inactivate such

interactions more completely from inside but, obviously, is a highly regulated process, lasting only from early prophase until metaphase→anaphase transition. Finally, in CL-A-treated cells, the concentrations of CL-A used may disrupt a wider number of interactions in the cytoskeleton, achieving total inactivation of the receptor, as measured by affinity chromatography.

Also, as shown by immunoprecipitation of integrins from ^{32}P-labeled cells, I could find no evidence that integrin phosphorylation is involved in modulating the different degrees of activity (fig. 3.24). However, it is likely that phosphorylation of other components from the cytoskeleton must participate in integrin inactivation, as both treatment with CL-A and mitosis involve changes in the phosphorylation state of cytoskeletal components (see above, this section).

7. FINAL CONCLUSIONS

Expression of integrin subunits in HeLa cells

The use of the anti-$\alpha_5\beta_1$ and anti-$\beta 3$ antisera in immunoprecipitation experiments show the presence of β_1, but not β_3 integrins. The presence of $\alpha_5\beta_1$ is reasonably clear, and there is evidence to suggest that $\alpha_v\beta_1$ is also present. First, positive results are obtained with anti-$\alpha_v\beta_3$ antiserum in flow cytometry and immunofluorescence experiments, although no $\beta 3$ integrins are detectable by immunoblotting and immunoprecipitation, suggesting the presence of α_v associated with a β subunit different from β_3. Second, when anti-$\alpha_5\beta_1$ antiserum is used, at least three bands are detected, one of which corresponds to an α subunit of a mobility expected of α_v, suggesting the presence of the heterodimer $\alpha_v\beta_1$. Further work using antibodies against specific α subunits will be needed to confirm the presence of $\alpha_v\beta_1$ and $\alpha_5\beta_1$ in HeLa cells.

Expression of integrins: interphase versus mitosis

Flow cytometry experiments showed that integrins recognised by anti-$\alpha_5\beta_1$ and anti-$\alpha_v\beta_3$ antisera are not expressed differently in mitosis and interphase. This result is also suggested by immunoprecipitation experiments.

Activity of integrins: interphase versus mitosis

Spreading assays have revealed that rounded, metaphase-arrested cells do not spread on fibronectin. Only a very limited spreading ability is achieved when Mn^{2+} is present in the medium. Also, when arrested in metaphase for 2-3 hours in normal medium, cells are able to spread to a very limited extent (less than 10% of interphase cells), and immunofluorescence experiments reveal that integrins are involved in such process, and also in the process of respreading in mitotic, nonblocked cells. These experiments also showed the absence of focal contacts, although it cannot be discerned whether such absence is due to restricted acces of antibodies to the ventral surface or such structures are really absent. However, the absence of stress fibers, as detected with actin staining, suggests that focal contacts are not present.

Affinity chromatography showed that the fibronectin receptor $\alpha_5\beta_1$ partially loses its ability to bind to the cell binding fragment of fibronectin in

mitosis. However, the remaining activity is higher than expected if fibronectin receptor inactivation alone were responsible for mitotic rounding-up. Also, as spreading experiments have shown that mitotic spreading is very limited, even under optimum conditions for integrin activity, it is likely that the cytoskeletal spreading machinery in mitosis is mainly committed to the cell cycle. However, when such limited mitotic spreading is detected by immunofluorescence, it is apparent that integrins are present at the cellular edge, in the spread fringes, suggesting that, although integrin activity is modified at mitosis, it may be restored under optimum conditions. If this were the case, full spreading would not be achieved only because the cytoskeletal spreading machinery is committed to the cell cycle.

Mechanism of regulation of cell cycle-related activity of integrins

Integrin phosphorylation could not be detected in immunoprecipitation experiments. However, it is likely that phosphorylation events are involved in the mitotic partial inactivation of the fibronectin receptor, due to the nature of the mitotic events. They are triggered by CDK activities, and affect several cytoskeletal components (see introduction, phosphorylation section). It is possible that interactions of some cytoskeletal components with integrins are disrupted in mitosis via cytoskeletal phosphorylation, which in turn may modify integrin conformation and activity. Thus, the conditions of the experimental procedure may account for the different recovery between interphase and mitosis. Radioiodination of mitotic samples takes place on rounded cells, with a reorganised cytoskeleton. It is likely that components interacting with integrins are inactivated, which in turn may affect integrin conformation and activity. Thus, there would be an inactivation of cell-ECM interaction initiated from inside the cell. On the other hand, radioiodination of interphase samples takes place on cells previously detached from the substrate by removing divalent cations from the medium (outside—>inside inactivation of integrins), so that cytoskeletal structures linked to integrins (e.g. talin or vinculin) may not be disrupted in the solubilisation procedure with β-octylglucoside. In conclusion, this difference could cause interphase and mitotic integrins to keep different conformations when solubilised, which could account for the different recovery from the fibronectin fragment columns. It also would explain that the use of phosphatase inhibitors does not affect the recovery of fibronectin receptor, as phosphorylation would not

be involved in interphase cells, and in mitosis, as the cytoskeleton is already rearranged, lysis is extremely unlikely to restore integrin-cytoskeleton interactions that are already disrupted by cytoskeletal phosphorylation, even in the presence of phosphatases.

Alternatively, it is possible that the use of Mn^{2+} in all buffers up to receptor elution may explain the results obtained. It could be that inactivation of the mitotic receptor is complete, and that Mn^{2+} stimulates its activity to the extent of being detectable by affinity chromatography (see above, integrin section, Introduction chapter). Future work will be needed to assess the validity of these hypothesis.

Effect of calyculin A on integrin activity

Treatment of cells with calyculin A was a reasonable approach to create a model mimicking the conditions of mitotic cell rounding. However, HeLa cells do not respond to concentrations (1-2nM) at which this may be the case, and therefore cell rounding caused by larger concentrations cannot be compared with that of mitosis, as effects become unspecific. The use of calyculin A has also confirmed that phosphorylation events affect cell shape. The fibronectin receptor is fully inactivated when cells are treated with calyculin A and, similarly to mitosis, receptor phosphorylation could not be detected. Complete disruption of cytoskeletal/integrin interactions may account for these results, in a model similar to that proposed for mitotic cells. In CL-A-treated cells, such disruptions would achieve total inactivation of the receptor, as measured by affinity chromatography. The presence of Mn^{2+} does not cause an active receptor conformation, as might be the case for mitosis. Interestingly, spreading results showed that CL-A-treated cells respond faster than normal cells when spreading is at its early steps (less than 30 min.). Further work using calyculin A or okadaic acid in short-term spreading essays should give further information about a role for these phosphatase inhibitors in cell spreading.

Biosynthesis of integrins during the cell cycle

Integrin synthesis is detected in asynchronous cells and in cultures enriched in G1+S phases. A putative β_1 subunit is detected, together with its precursor. Two α subunits are also present, in a pattern similar to that obtained with radioiodinated samples. The evidence shows that there may be

a peak of synthesis at G2, especially of α subunits, although a basal level of synthesis seems to be present throughout the cell cycle. Further work using Northern blots and protein synthesis inhibitors should clarify whether there is G2-specific integrin synthesis or such synthesis is not cell cycle-dependent.

Effect of serum in metaphase—>anaphase transition

It has been shown that serum improves the recovery of metaphase-arrested cells after release of the nocodazole block. It is well known that serum-starved cells will arrest at G1 phase in the cell cycle. However, my results show that there is a clear improvement in the rate of recovery from metaphase to anaphase when serum is present in the medium. This discrepancy may be explained by the fact that I am using highly synchronised cells. In cells synchronised in mitosis, a component from serum may be needed for exit from metaphase. This component may be synthesised by non mitotic cells in asynchronous cultures, thus bypassing the necessity of serum for the small population in metaphase. However, these considerations are merely speculative, and further work will be required to assess a possible role for serum in mitosis.

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