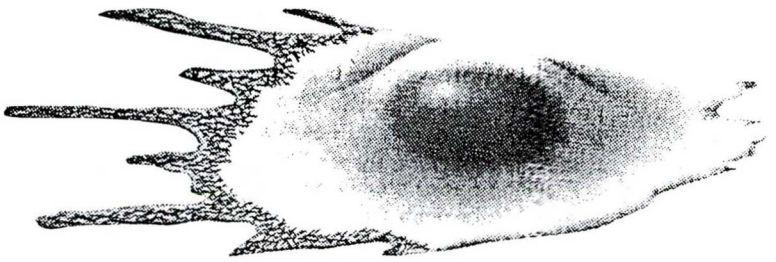
Direct Linkage between the Plasma Membrane and the Cytoskeleton: Importance of Actin-Binding Membrane Proteins



Margit Keresztes M.D.

Ph. D. Thesis

1999.

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Publications - related to the subject of the thesis

I. **Margit Keresztes**

Three actin-binding proteins are developmentally regulated in rat liver plasma membranes

FEBS Letters (1989) 249, 51-55.

II. **Margit Keresztes** and Zsuzsa Lajtos

Major laminin-binding and F-actin-linked glycoproteins of neutrophils

Cell Biology International (1997) 21, 543-550.

III. **Margit Keresztes**, Zsuzsa Lajtos, János Fischer and László Dux

Moesin becomes linked to the plasma membrane in attached neutrophil granulocytes

Biochemical and Biophysical Research Communications (1998) 252, 723-727.

1. Introduction

1.1. Actin and actin-binding proteins: a short overview

1. 1.1. Actin: a unique molecule

Actin is a highly conserved protein present in all eukaryotic organisms which is characterized by its intrinsic capability to polymerize into long filaments and to interact with multitude of actin-binding proteins. Actin is thought to rank among the five most abundant proteins on Earth: together with the associated proteins, it constitutes more than 25% of the total cell protein in non-muscle cells (in muscle, this value is over 60%) (Kreis and Vale, 1993). It is essential not only for muscle contraction and for many other forms of cellular motility, but for the structure and mechanical properties of the cytoplasmic cytoskeletal matrix as well (Pollard, 1986). Since the discovery of actin over 50 years ago by Brúnó Straub (1942, Institute of Medical Chemistry, University of Szeged), enormous amount of data have been obtained about the structure and biochemistry of both (monomeric) G-actin and (filamentous) F-actin and, in addition, vast number of papers have been published on the molecular features and actions of the different actin-binding proteins, the number of which is still continuously growing.

Comparing the primary protein structures composed of 374-375 amino acids, only minimal differences (<5%) were found in the sequence of muscle and cytoplasmic actins of animals and protozoa (except for some variable acidic residues at the N terminus); i.e. actin molecules in organisms as diverse as amoebae and humans have strikingly similar sequences (Pollard, 1986; Egelman and Orlova, 1995). (Plants diverging from animals 10^9 years ago evolved multiple actin genes /three classes/ that differ from animal actin genes and from each other by more than 10%.) All metazoan cytoplasmic actins (and the muscle actins of invertebrates) probable derived directly from the cytoplasmic actins found in protozoa and fungi, while the muscle actins of vertebrates of higher order form a distinguishable family which are phylogenetic derivatives of the ancient actin type (Pollard, 1986). Warm-blooded vertebrates express six actin forms in a tissue specific manner: two striated muscle actins (skeletal muscle and cardiac actin), two smooth muscle actins (vascular and visceral actin) and two non-muscle actins; the three groups have distinct isoelectric points and are designated as α-, β- and γ-actin, respectively (Kreis and Vale, 1993).

According to X-ray diffraction analysis, G-actin has two domains separated by a cleft containing the nucleotide- and the high-affinity divalent cation binding sites (Kabsch et al., 1990) (Fig. 1a). The large domain at the C terminal comprises subdomains 3 and 4, while the small, flexible domain at the N terminal contains subdomains 1 and 2. According to recent investigations, subdomains 3-4 and subdomain 1 form two rigid units, while the second subunit is a semi-rigid one; these three units can rotate independently, to a limited extent (Page et al., 1998). Surprisingly, some allosteric interactions are likely to exist already in G-actin, resulting in an opening of the ATP-binding pocket (Chik et al., 1996; Page et al., 1998). The small, so-called outer domain is a favourite target for numerous actin-binding proteins binding to "hot spot" sites (primarily some acidic residues) (Lorenz et al., 1993; Reisler, 1993; McGough, 1998) (Fig. 1b).

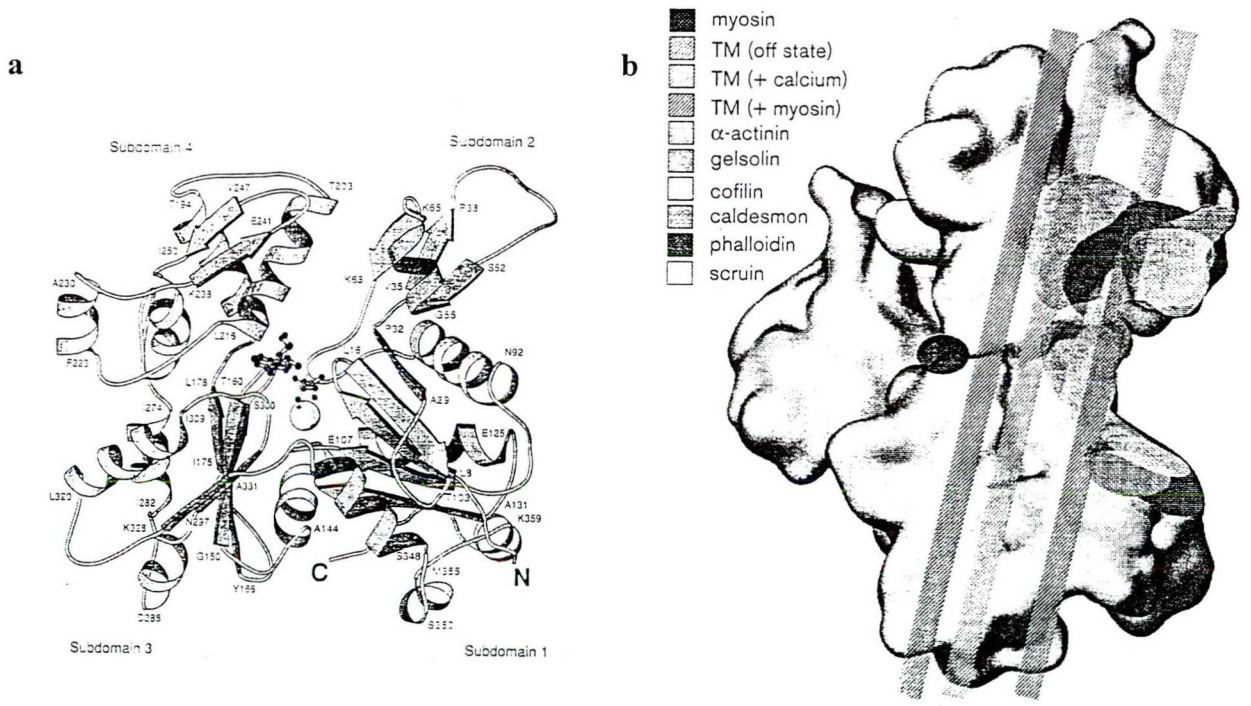


Fig. 1. **a** The secondary structural elements of G-actin (crystal structure). ADP and the metal cation are located in the cleft between the large and small domain (Lorenz et al., 1993).

b Molecular footprints of some F-actin-binding proteins. All the proteins examined so far have been shown to interact with at least two monomers, and on a monomer, mostly with subdomains 1 and 2. (TM: tropomyosin, phalloidin is a fungal toxin) (McGough, 1998)

Actin is a self-assembling protein, which - following the relatively slow formation of a trimer (nucleation) - can rapidly polymerize into double helical structures of 10 nm thickness, depending on the conditions (actin concentration, concentrations of cations - like K, Ca, Mg, the form of the bound nucleotide: ATP/ADP, presence of different actin-binding proteins and regulatory factors) (Pollard, 1986). The kinetics of polymerization differs at the two ends of growing filaments; the polarity of which can be detected by decoration with heavy meromyosin into arrowhead-like structures, thus defining barbed and pointed filament ends (Pollard, 1986; Kreis and Vale, 1993). The diffusion controlled monomer exchange at the barbed end is an order of magnitude faster than that at the pointed end (Pollard, 1986). Actin filaments - these non-covalent assemblies of monomeric subunits - exhibit both flow resistant and elastic properties. Individual filaments are capable of some torsional motions around their axis and can flex along their length (Pollard, 1986; Egelman and Orlova, 1995). Large-scale cooperativity within the actin filament was observed, where structural changes occuring in one monomer could affect the conformation of many surrounding subunits; e. g. when gelsolin is bound to F-actin, actin subunits thousands of Ångströms away from this actin-binding protein appeared to display a conformational change (Orlova et al., 1995). However, according to recent findings, the conformational changes elicited by polymerization and/or complex formation with actin-binding proteins are probably limited; they can be best described as a rotation of the two rigid units with some additional rotation of the semi-rigid subdomain facilitated by the changes in the hinge and shear regions between the rigid domains at/in the cleft (Page et al., 1998).

The ubiquiter expression, the unusually high degree of sequence conservation, and the lethal effect of gene disruption of the actin molecule apparently reflect that actin has a pivotal role and indispensible functions in the cell (Kreis and Vale, 1993).

1.1.2. Major classes of actin-binding proteins and their main properties

The multitude of actin-binding proteins can be grouped at least to five major classes according to the nature of the binding and the functions (Pollard, 1986; Ayscough, 1998). However, this parsimonius classification gives one only a faint idea about these proteins, since it is not able to cover or define the variations in actin linkage and in actions of the actin-binding protein families/superfamilies forming ~50 classes according to sequence homologies (Kreis and Vale, 1993). The striking diversity of the actin-binding proteins has evolved

probably not only to result in distinct interactions with actin but also with other proteins, since they can have additional, non-cytoskeletal activities, too (e.g. ABP-50 is elongation factor-1α) (Kreis and Vale, 1993). Not only actin-binding proteins interact with each other; actin itself is promiscuous in cells: i.e. it can interact with several partners at the same time. The complex array of interactions - more precisely, the balance in the actions of different actin-binding proteins controlled by numerous factors - will determine many of the morphological changes in sessile, migratory or dividing cells via remodelling of the actin cytoskeleton (Way and Weeds, 1990). Rearrangement of the actin system must go on a fast time scale to allow e.g. relatively quick cell migration and rapid cell shape changes (e.g. during platelet activation), that is based on the relatively low affinities of actin-binding proteins for actin (Kreis and Vale, 1993).

Most actin-binding proteins function by counteracting the intrinsic property of actin to polymerize into few, very long filaments - in order to create diverse and complex F-actin structures (Pollard, 1986; Stossel, 1993). When the filaments are crosslinked and form a three-dimensional network, they can manifest bulk elastic behaviour and resist stretching, twitching or bending; this "stiff gel" character is a prominent feature of the leading lamellae of migrating cells (Stossel, 1993). On the other hand, special F-actin bundles called stress fibers terminating at cell-substrate adhesion sites have a contractile property - similarly to the myofibers in muscles; this actin filament structure is characteristic primarily for cells adhering to substrate (Stossel, 1993). Almost all actin-binding proteins are present in the different species of the eukaryotic kingdom, vital functions of some of these proteins (e.g profilin, cofilin) was demonstrated also in higher vertebrates in gene disruption experiments (Ayscough, 1998).

The five major classes of actin-binding proteins are the following: actin monomer binding, capping, severing, F-actin side-binding proteins and myosins (Fig. 2). Members of the first class are thought to ensure a pool of G-actin for subsequent filament assembly by sequestering actin monomers (Pollard, 1986; Way and Weeds, 1990). A chief representative of the first class is profilin, some isoforms of which, surprisingly, might promote actin polymerization, according to recent in vivo studies (Ayscough, 1998). Capping proteins bind to the ends of actin filaments and inhibit further linkage of monomers there; most proteins block the barbed ends. Some exert no additional action on actin (Cap 32/34, Cap Z), while others have also severing activity (gelsolin, villin, severin, fragmin).

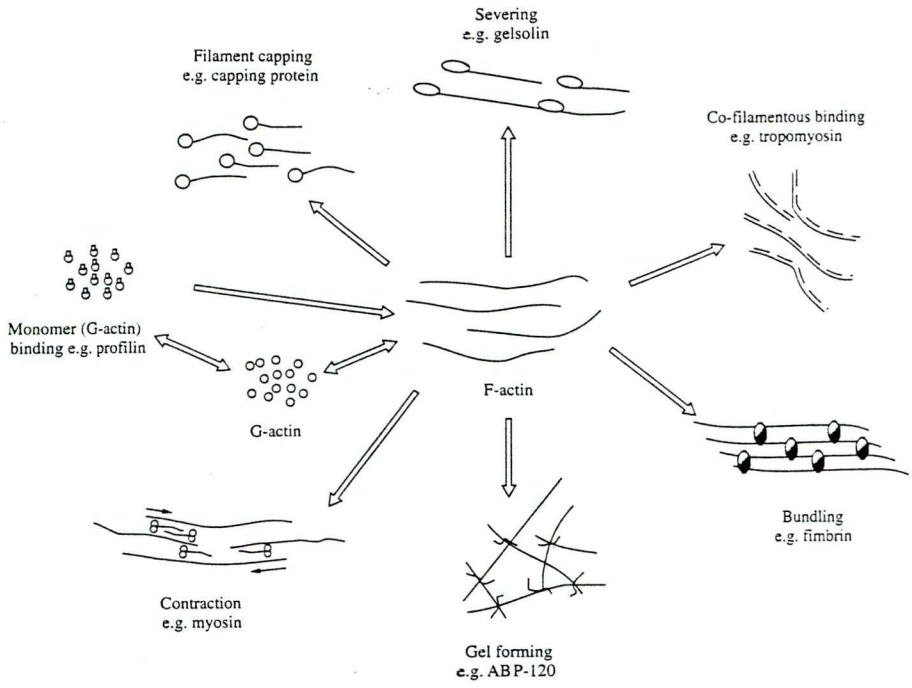


Fig. 2. Main functions of the actin-binding proteins with a representative of the class/subclass (Ayscough, 1998). (The low molecular mass severing proteins are not depicted.)

(Kreis and Vale, 1993). Villin contains an additional actin binding site responsible for filament bundling in the intestinal microvilli (Pollard, 1986; Weeds and Maciver, 1993). Gelsolin is thought to have roles in cell motility and, on the level of the organism, in stress responses like hemostasis, inflammation and wound healing (Ayscough, 1998); in addition, it was suggested to be involved in cell differentiation (Way and Weeds, 1990). The small class of severing proteins includes some low molecular mass actin-binding proteins as e.g. depactin (Pollard, 1986; Kreis and Vale, 1993). In contrast, the class of lateral F-actin-binding proteins comprises three subclasses: bundling, cross-linking and co-filamentous,"one-sided" lateral binding proteins. The first group of proteins connect actin filaments in the form of bundles (e.g. fascin, fimbrin), while the crosslinkers forming gels are able to link filaments into a three-dimensional network (e.g. filamin /ABP-280/, ABP-120, spectrin/fodrin) (Pollard, 1986; Ayscough, 1998). Co-filamentous proteins bind to actin filaments without connecting them; a representative example for this subclass is tropomyosin. Cofilin is somewhat special in the respect that it binds also to actin monomers and can stimulate depolymerization of actin filaments as well; it could be involved in the fast remodelling of the actin cytoskeleton e.g. during cell division, cytokinesis and phagocytosis (Ayscough, 1998). Myosins, some

microtubular proteins (e.g. Tau), some special actin-binding proteins (e.g. the phospholipid- and Ca-binding annexins) and the membrane-associated actin-binding proteins (see below) make up four more classes, although some of the classes/subclasses apparently overlap (Table 1 - classification is based on that of Pollard, 1986 and Kreis and Vale, 1993).

Table 1

Functional classification of actin-binding proteins

Class and subclass	*Protein examples*
Monomer binding proteins	profilin, thymosin, ABP-50
Capping proteins	
no severing activity	Cap Z, Cap 32/34
with severing activity	gelsolin, villin, severin, fragmin
Small severing proteins	depactin
Lateral F-actin-binding proteins	
bundling proteins	fascin, fimbrin, scruin, (villin)
crosslinkers (gel formers)	filamin, ABP-120, spectrin/fodrin, α-actinin
co-filamentous proteins	tropomyosin, caldesmon, cofilin
Myosins	myosin I, II
Microtubule-binding proteins	Tau, MAP-2
Special proteins	
phospholipid-binding proteins	annexins, MARCKS
Membrane-associated proteins	
binding to integrins	talin, α-actinin, filamin
binding to β-dystroglycan	dystrophin
binding to other receptors (e. g. CD44)	ezrin, radixin, moesin
binding to unknown proteins	supervillin, LSP1
binding to phospholipids	annexins, MARCKS
actin-binding transmembrane receptor	EGF receptor

1.2. F-actin binding anchor proteins and their role in cell-substrate adhesion and in cell migration

1.2.1. General considerations

Actin-binding proteins capable to associate with the plasma membrane are in a unique position: they can directly transmit signals from the membrane proteins (lipids) to the cytoskeleton and vica versa. This class of actin-binding proteins belong to the huge group of cytoskeletal anchor proteins on a functional basis: they play a central, pivotal role in cell adhesion and in cell migration as well as in the regulation of cell proliferation and cell differentiation - via the formation of various cytoskeletal networks at the cell cortex (membrane skeleton) and through the regulation of membrane structure and dynamics (Luna and Hitt, 1992; Kreis and Vale, 1993). Evidently, this group includes also indirect actin-binding cytoskeletal proteins like e.g. zyxin, paxillin, ankyrins etc. (Kreis and Vale, 1993; Stossel, 1993; Yamada and Geiger, 1997).

Only a few of the actin-binding anchor proteins were shown to bind directly to a transmembrane protein (Table 1); these are the ones binding to integrins: talin, α-actinin, filamin (ABP-280), (Stossel, 1993; Hitt and Luna, 1994; Yamada and Geiger, 1997); or to β-dystroglycan: dystrophin and related proteins (present primarily in muscle and nerve cells) (Bloom, 1995); or to other membrane receptors (e.g. CD44): members of the ezrin-radixin-moesin family (Tsukita et al., 1997a, b; Vaheri et al., 1997). In addition, supervillin (a member of the gelsolin/villin family) and LSP1 (lymphocyte-specific protein 1) were also found to be actin-binding proteins capable of membrane linkage (Pestonjamasp et al., 1997; Matsumoto et al., 1995). Aside from these, some special actin-binding proteins: annexins and MARCKS (myristoylated alanin-rich C-kinase substrate) can link to the phospholipids of the plasma membrane (Kreis and Vale, 1993).

Furthermore, direct interaction even between a transmembrane protein and actin could be also possible; however, it is well documented only for the EGF receptor in vertebrate cells (den Hartigh et al., 1992). Direct binding to actin was shown in vitro also for the α2β1 integrin receptor (Kieffer et al., 1995), and in the case of neutrophils, it was proposed for the f-Met-Leu-Phe chemoattractant receptor (Klotz and Jesaitis, 1994).

Clearly, F-actin-binding proteins in direct or indirect association with the plasma membrane play a key role in cell migration and cell-substrate adhesion by regulating the interactions between the membrane and the actin cytoskeleton, and between the actin

filaments (monomers) (Fig. 3.) Upon cell-substrate adhesion, cells become attached to the surrounding extracellular surface (usually the extracellular matrix) at contact areas of the plasma membrane (focal adhesions), which are linked to the cortical actin filament network and to growing long microfilaments. It is worth noting that adhesion to the extracellular substrate essentially determines the future fate of the cell: cell proliferation or apoptosis or further differentiation (Giancotti, 1997). It has been long known that normal cells need to adhere to extracellular matrix components (adhesion molecules like laminin, fibronectin,

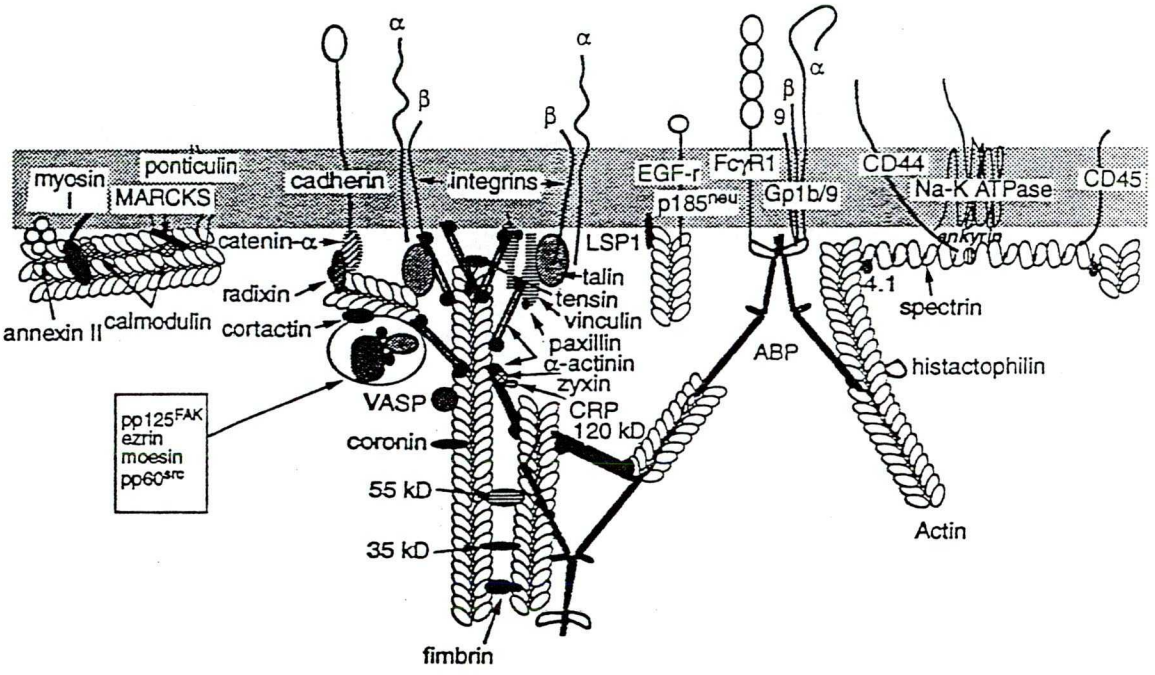


Fig. 3. The spectrum of possible actin - plasma membrane and actin - actin relations: a summary of proteins mediating connections between the plasma membrane and actin filaments and between actin filaments in motile cells (Stossel, 1993)

vitronectin etc.) in order to initiate cell proliferation (anchorage-dependent growth) and to promote the survival of the cell (Giancotti, 1997; Assoian and Zhu, 1997). Surprisingly, some cells undergo differentiation even in the presence of otherwise mitogenic concentrations of growth factors - if there is an interaction with specific extracellular matrix proteins (plus soluble differentiation factors are also present) (Giancotti, 1997). On the other hand, growth factors and extracellular matrix components may act in synergism in the stimulation of cell

proliferation (Giancotti, 1997; Assoian and Zhu, 1997). Transformed cells are characterized by anchorage-independent growth and by the absence of contact inhibition (leading to further cell divisions in high density cell populations). All these phenomena can be explained on the basis of interactions in cell adhesion networks which function also as huge signalling complexes: there is an intensive "talk" or "cross-talk" between the various members of the web (actin-linked proteins, extracellular substrate- and growth-factor activated molecules etc.) Furthermore, it is becoming more and more clear that the distinction between "structural proteins" (performing a mechanical role) and "signalling molecules" is rather artificial, and adhesion complexes are signalling centers as well (Yamada and Geiger, 1997).

Amoeboid cell movement implies rapid cell shape changes and reversible adhesion to extracellular surfaces, which require the formation of highly dynamic cytoskeletal structures, especially at the cell cortex. During the fast cell-substrate adhesion steps, cells seek contact with the help of steering microextensions followed by the appearance of relatively stable focal adhesion sites. Sensory cues on/of the extracellular matrix stimulate the formation and advance of the leading lamella in the form of filopodia (microextensions, spikes), small bubbles or by vertical thickening; backward bending can be also often observed (ruffling). All these phenomena are encompassed by the term "cortical flow", which stresses the reversibility of the plasma membrane - actin cytoskeleton connections during cell crawling at given focal contacts; anchoring complexes are continuously forming and disassembling at the capping adhesion receptors (Stossel, 1993).

1.2.2. Direct F-actin-binding membrane proteins of liver cells and neutrophils

Although data on actin-binding and anchor proteins are almost exponentially growing, relatively few reports were published on direct F-actin-binding proteins in association with the plasma membrane. However, these proteins probably play a crucial role in the fast information transfer between the cell surface and the interior of the cell - via the actin cytoskeleton. Surveying the literature from 1966, only three types were reported from these direct F-actin-binding proteins in liver cells: α-actinin, in bile canaliculus of normal human hepatocytes (Tsukada et al., 1995); a 50 kD integral membrane protein in rat hepatocytes (Barmann et al., 1986); annexin I (calpactin II) and annexin VI, in T51B rat liver epithelial cells and in bovine liver, respectively (Hosoya et al., 1992; Campos-Gonzales, 1990). Interestingly, annexin I was observed to undergo a rapid and reversible translocation (in 2-5 min) from the cytosol to

membrane ruffles upon EGF-dependent phosphorylation in T51B rat liver epithelial cells, probably concurrent with the induction of mitogenesis (Campos-Gonzales, 1990). No related developmental studies could be found.

In the case of neutrophils, in addition to α-actinin (Yürüker and Niggli, 1992), filamin (Sheterline and Hopkins, 1981), supervillin (Pestonjamasp et al., 1997) and ezrin plus moesin (Pestonjamasp et al., 1995) were detected in human (α-actinin), porcine (filamin) and in bovine (supervillin, ezrin and moesin) neutrophil granulocytes. Aside from these, the lymphocyte-specific protein 1 (LSP 1) was shown to be present also in human monocytes and in neutrophils (Li et al., 1995).

1.3. Neutrophil adhesion and migration: the importance of laminin receptors

Laminins are large glycoproteins found primarily in basement membranes underlying or covering endothelial, epithelial, fat and muscle cells. The laminin network provides the major structural frame for basement membranes in addition to the type IV collagen network (Mosher et al., 1992; Yamada and Kleinman, 1992). Several heterotrimer isoforms of laminin have been described (Tryggvason, 1993), of which the one isolated first from murine basement membrane-forming Engelbreth-Holm-Swarm (EHS) tumor is widely used for cell-substrate adhesion studies. Laminin forms have diverse biological activities including stimulation of cell adhesion, migration and differentiation of some cell types etc. (Yamada and Kleinman, 1992).

Laminin receptors include nine members of the (vertebrate) integrin family and some non-integrin cell surface proteins: e.g. ß-galactoside-binding lectins called galectins, galactosyl transferase; sulfated polysaccharides (heparin) and glycolipids (sulfatides) are also able to bind to laminins (Mecham, 1991; Mercurio, 1995). Galectins are reported to bind poly-N-acetyl-lactosamine chains on laminin (Barondes et al., 1994; Mercurio 1995).

The possession of laminin receptors is of fundamental importance for neutrophils during the early phase of acute inflammation, when they migrate through the intact endothelium (at tricellular corners poor in tight junctions) and across the basement membrane of venules towards the site of tissue injury or inflammation (Burns et al., 1997). A gradient of soluble intact and degraded laminin stimulates the extravasation of these leukocytes by a chemotactic effect, even at nanomolar concentrations (Bryant et al., 1987). While the selectin- and integrin-mediated interactions in the initial phases of rolling, arrest and firm adhesion

were characterized in detail, the final stage of transmigration have not been clarified yet (Hogg and Berlin, 1995). In respect of laminin, the α6β1 (Frieser et al., 1996), β2 (CD11/CD18) integrins (Bohnsack et al., 1990), and galectin-3 (Kuwabara and Liu, 1996) were shown to act as its receptors in neutrophils.

The aim of the present study was to investigate direct F-actin-binding proteins of the plasma membrane which could be involved in postnatal development of rat liver cells or could play a basic role during cell-substrate/laminin adhesion of porcine neutrophils.

The experimental objectives could be summarized in four points:

1. To identify direct F-actin-binding proteins in rat liver cells and in porcine neutrophil granulocytes.
2. To investigate if the appearance of some of these proteins in the plasma membrane of liver cells might undergo a change during postnatal development of rats - reflecting a possible developmental control.
3. To explore whether adhesion of neutrophils to an indifferent extracellular surface could affect the plasma membrane appearance of some direct F-actin-binding proteins and some major adhesion receptors (laminin receptors).
4. To develop a reliable, sensitive and user-friendly assay for the detection of direct F-actin-binding proteins in membrane samples.

2. Materials and methods

2.1. Preparation of membrane fractions

2.1.1. Liver cells

First, a crude plasma membrane-enriched fraction was prepared from livers of CFY rats according the method of Maeda et al. (1983). Shortly, the excised livers were rinsed in a buffer (10 mM tris, pH 7.3, 2 mM $MgCl_2$, 30 mM NaCl, 1 mM DTT, 100 U/l aprotinin) and cut to tiny pieces that was followed by mincing with a Polytron-type homogeniser in 250 mM sucrose-containing washing buffer. The tissue homogenate was layered over 41% of sucrose and centrifuged at 95 000 g for 1 h; the white band of the membrane sample was collected and pelleted by centrifugation at 95 000 g for 20 min. This membrane sample was purified on Percoll gradient prepared with sucrose (starting density: 1.05 g/ml; Pharmacia) by centrifugation at 20 000 g for 30 min. The final sample was sedimented by centrifugation at 100 000 g for 2 h. (All procedures were carried out at 4 °C.)

2.1.2. Neutrophil granulocytes

Isolation of porcine neutrophils was carried out essentially as described by Keller et al. (1980). Shortly, leukocytes in a modified Hank's buffer (138 mM NaCl, 5 mM KCl, 1.1 mM $MgCl_2$, 5 mM $NaHCO_3$, 0.64 mM Na_2HPO_4, 0.66 mM KH_2PO_4, 5.6 mM glucose, 20 mM tris, 1.5 mM $CaCl_2$, 0.5 mM PMSF, and 50 U/ml aprotinin, pH 7.3; after Southwick et al.; 1989) were separated in a Ficoll 400 - Uromiro gradient (4.94% w/v Ficoll 400 /Pharmacia/, and 15.55% v/v Uromiro 75% /Bracco, Italy/; density: 1.06-1.07 g/ml).

Preparation of membrane fractions was based on the method of Mollinedo (1986). First, neutrophil cytoplasts - lacking cell nuclei and most of their granules - were obtained by incubating the cells in a Percoll suspension (28% v/v, density: 1.04 g/ml; Pharmacia) containing 10 μg/ml cytochalasin B (in Hank's buffer) for 5 min at 37°C. Cytoplasts of different densities were separated in Percoll gradient (44% v/v, starting density: 1.06 g/ml) containing 1 μg/ml cytochalasin B (centrifugation: 20 000 g, 30 min, 40-44°C). Cytoplast fractions between the densities (g/ml) of 1.018-1.045 and 1.045-1.059 (well above the layer of cells) were collected. (Densities were assessed with the help of coloured density marker beads /Pharmacia/.) Membrane-enriched samples were gained after disruption of cytoplasts by freezing (-20°C for 30 min), thawing and homogenization (teflon/glass potter, 300 rpm, 4°C)

in a homogenizing buffer (100 mM KCl, 2 mM $MgCl_2$, 5 mM NaCl, 10 mM Hepes, 1 mM DTT, 100 U/ml aprotinin, 1 mM ATP, 0.1 mM PMSF, pH 7.4); finally, samples were sedimented by ultracentrifugation (70 000 g, 20 min).

2.2. Adhesion studies on neutrophils

Neutrophil granulocyte suspensions in pre-warmed Hank's buffer (37°C) were poured to Nunc (polystyrene) plates (600 cm^2, $2 \cdot 10^5$ cell/cm^2), and were incubated for 35 min at 37°C. Following rinsing with EDTA-Hank's buffer (containing 1 mM EDTA and 2.75 mM $MgCl_2$, free of calcium) to reduce cell aggregation and to eliminate floating cells, attached cells were detached with rubber policeman.

2.3. Enzyme assays

Na^+K^+-ATPase, acidic phosphatase and glucose-6-phosphatase were assayed according to the methods of Sulakhe et al., (1971), Fishman and Lerner (1953), and Hubscher and West (1965), respectively, at 37°C.

Assay of alkaline phosphatase activity was performed as described by Bessey et al. (1946) at 37°C using 4-nitrophenylphosphate (Serva) as substrate.

2.4. Electron microscopy

Membrane samples (protein conc: 1 mg/ml) on carbon-coated grids were examined following treatment with 1% uranyl acetate (0.5 min, 4°C).

2.5. F-actin affinity chromatography

Actin was extracted from rabbit skeletal muscle with the help of acetone by the traditional way described by Feuer et al. (1948). Actin was prepared from this aceton powder by polymerization and depolymerization according to Spudich and Watt (1971). The resulting sample was further purified by gel filtration on Sephadex G 150 column using a G buffer (2 mM tris pH 7.5, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM 2-mercaptoethanol). The purity of the actin-containing fractions was checked by SDS-polyacrylamide gel electrophoresis.

Coupling of actin to CNBr-activated Sepharose 4B (Pharmacia) was performed as suggested by the manufacturer. As a result, generally about 1.5 mg actin was linked to 1 ml packed column. Control columns were uncoupled ones. Prior to the affinity chromatography,

the 2-ml columns were washed with 20 vols of an F-actin stabilizing buffer containing detergent (100 mM tris, pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 0.2 mM DTT, 100 U/l aprotinin and 2% v/v Triton X-100). Thereafter, plasma membrane samples of 8-10 mg protein content were circulated through the columns for 12-14 h at 4° C followed by column washes with 40 vols of the detergent-containing buffer. Elution was carried out with 1.5 vols of 1% SDS as suggested by Luna et al. (1984).

2.6. Actin-cytoskeleton extraction

Cytoskeletal extracts were obtained from attached and non-attached neutrophils according to the method of van Bergen en Henegouwen et al. (1992). Briefly, cytoskeletons were isolated by incubating the cells for 5 min in a 0.15% Triton X-100 containing cytoskeleton-stabilizing buffer (10 mM Hepes, pH 6.8, 250 mM sucrose, 3 mM $MgCl_2$, 150 mM KCl, 1 mM EGTA, 1 mM PMSF) at room temperature. Following depolymerization plus removal of tubulin by cooling (10 min at 4° C) and centrifugation, the actin fraction was extracted with 0.6 M KI in the cytoskeleton-stabilizing buffer (20 min at 4° C).

2.7. SDS-PAGE analysis

Proteins were separated on 10% SDS-polyacrylamide gels (spectrin detection: on 8% gel); samples were applied without prior heating. Gels were stained with Coomassie blue G-250 or with silver (Blum et al., 1987). Some silver-stained gels were scanned using a computer-assisted laser densitometer (Biometra ScanPack). For statistical analysis, Mann-Whitney tests were carried out.

2.8. Western blotting

2.8.1. Protein blotting in general

Some gels were used for the electrotransfer of proteins onto nitrocellulose (Towbin et al., 1979). Protein transfer was carried out at 25-30 V (about 10 V/cm) for 1.5 h at 4° C. Generally, a part of the blots was stained with amido black to reveal the polypeptide patterns of the transferred samples and molecular mass markers; the rest was treated with reagents diluted in 1% Blotto/Tween (Harlow and Lane, 1988). Quenching was done usually with 5% Blotto/Tween overnight (at 4°C) (Harlow and Lane, 1988); in the early experiments, 10% normal chicken serum in PBS-Tween (0.05% Tween 20) was used. In case of

biotinylated primary reagents, blots were incubated thereafter with peroxidase-avidin (generally at 50 μg/ml; ExtrAvidin, Sigma) for 1 h. In the earlier experiments, visualization of labelled membrane proteins was achieved by treatment with diaminobenzidine solution (0.05% w/v 3,3'-diaminobenzidine-HCl, 0.03% w/v $NiCl_2$, and 0.05% v/v 30% H_2O_2 in TBS). In competitive blotting experiments and in the 80 kD-protein-related experiments, chemiluminescence reaction was performed (Renaissance reagents, NEN Life science). A 0.2% Tween-20, 0.9% NaCl, 50 mM tris, pH 7.5 buffer was used for all washes.

2.8.2. Detection of F-actin binding proteins with the help of actin-peroxidase reagents

Actin-peroxidase conjugates were prepared according to the conjugation procedure described by Wilson and Nakane (1978) for antibodies. First, the horseradish peroxidase enzyme (Calbiochem) was oxidized by 0.1 M $NaIO_4$ (20 min incubation); the peroxidase-aldehyde solution was dialysed in 1 mM Na acetate (pH 4.4) overnight at 4° C. Thereafter, the pH of the solution was shifted to alkaline one between 9-9.5 with 0.2 M Na_2CO_3. Purified actin was also dialysed overnight in 0.01 M Na_2CO_3 (pH 9.5). Conjugation was carried out at room temperature (2 hours incubation). (Generally, 2 mg proxidase was coupled to about 2 mg actin.) The reaction was stopped by the addition of $NaBH_4$ (4 mg/ml; 2 hours incubation at 4° C). The proteins were collected by precipitation with 100% ammonium sulfate. The precipitate was washed in 50% ammonium sulfate, solved in distilled water and dialysed in TBS (overnight, at 4° C). For control experiments, ovalbumin-peroxidase conjugate was also prepared. The conjugates were stored in 50% glycerol at -20° C.

To detect F-actin-binding proteins, the blot slices were incubated for 40 min with the reagent in a F buffer (50 mM KCl, 2 mM $MgCl_2$, and 20 mM tris, pH 7) containing 1% Blotto-Tween or 5% normal chicken serum in TBS-Tween (early experiments), usually either in the presence of 1 mM Ca^{2+} or 1 mM EGTA. G-actin-binding was tested in a G buffer (0.2 mM $CaCl_2$, 1mM ATP, 0.5 mM 2-mercaptoethanol, 2mM tris, pH 7.6) in 1% Blotto-Tween.

2.8.3. Labelling glycoproteins

Blot strips were incubated with biotinylated Concanavalin A (Con A), wheat germ agglutinin (WGA), (Sigma), elderberry bark (EBL) (Vector) or Ulex europaeus I (UEA I) (Sigma) lectin (15-30 μg/ml) for 1 hour.

2.8.4. Detection of spectrin

Blot slices were reacted with a monoclonal anti-spectrin antibody recognizing primarily the β-subunit (mouse anti-human, Sigma; 1:20; incubation: overnight at 4°C or 1 h at room temperature), and subsequently, with peroxidase-conjugated secondary antibody (rabbit anti-mouse Ig, DAKO, 1:100; incubation: 1 h).

2.8.5. Labelling laminin-binding proteins

In overlay blotting experiments, blot strips were incubated with EHS laminin (Boehringer Mannheim; 2.5-20 μg/ml, 45-60 min), then with anti-laminin (rabbit anti-mouse laminin, Sigma; 1:100; incubation: 1 h), and finally, with peroxidase-conjugated anti-rabbit antibody (Human, Hungary or DAKO; 1:100; incubation: 1 h). For direct labelling studies, biotinylated EHS-laminin was prepared; biotinylation was performed with N-hydroxysuccinimide biotin (Bachem) according to Harlow and Lane (1989). (The coupling is done through free amino groups /normally lysyl residues/ of the protein generated by sodium borate treatment; conjugation was stopped by addition of NH_4Cl.) During competitive blotting, blot slices were preincubated with 0.2 M lactose (in TBS-Tween) for 1 hour prior to labelling with biotinylated laminin.

2.8.6. Identification of some membrane-associated proteins (CD14, moesin)

Monoclonal anti-human anti-CD14 antibody was a generous gift from Dr István Andó (Institute of Genetics, Biological Research Center of Hungarian Academy of Sciences; ref: Andó et al., 1989) and was used in 1:20, 1:50 and 1:100 dilutions (incubation: 1 h). Biotinylated sheep anti-mouse Ig antibody (Amersham; 1:100, 40 min) was employed as a secondary reagent.

A monoclonal anti-moesin antibody (mAb 38/87) and an affinity-purified rabbit polyclonal anti-moesin (90-7s) generated against calf moesin were tested (1:1000) (Dr Heinz Furthmayr, Department of Pathology, Stanford University) (Pestonjamasp et al., 1995; Amieva and Furthmayr, 1995). Peroxidase-labelled anti-mouse or anti-rabbit Ig antibodies (Sigma; 1:4000) were used as secondary reagents.

3. Results

3.1. Characterization of membrane fractions

3.1.1. Liver plasma membrane sample from rat

The simple protocol of Maeda et al. (1983) supplemented with additional purification steps on Percoll gradient proved to result in relatively contaminant-free membrane preparations. No contaminations were detectable by electron microscopy in the negatively stained samples (not shown). Enzyme activities characteristic for microsomes (glucose-6-phophatase) or for lysosomes did not rise considerably during the preparation, while the activity of Na^+K^+-ATPase - a characteristic marker of the plasma membrane - was found to be about 12-fold higher in the final sample than in the homogenate (Table 2). According to these results, the preparation can be regarded as a plasma membrane-enriched sample.

Table 2

Enzyme marker activities in the liver samples from adult rats

Enzyme activity (*μmol product/mg protein/h*)	***Liver homogenate***	***Membrane sample***
Na^+K^+-ATPase	1.44 ± 0.60 (5)	17.53 ± 3.88 (5)
Glucose-6-phosphatase	5.16 ± 0.90 (5)	5.90 ± 1.22 (5)
Acidic phosphatase	2.65 ± 0.63 (3)	3.86 ± 0.97 (3)

(Figures in parentheses indicate the number of assays performed.)

To reveal the polypeptide pattern of the membrane sample, SDS-gel electrophoresis was performed. About 40 polypeptide bands of different molecular masses could be distinguished in the Coomassie blue-stained tracks (Figs. 7 and 9; and Table 4).

3.1.2. Neutrophil membrane fractions from porcine blood

In the total cell sample, neutrophil granulocytes constituted about 92% of the cell suspension (staining: May-Grünwald and Giemsa, not shown). The two cytoplast fractions (d [g/ml]: 1.018-1.045 and 1.045-1.059) appeared to be clearly separated from the dense layer of the cell suspension (d: 1.081 g/ml) in the Percoll gradient. Checking and comparing the ultrastructure of the final membrane samples, we found that both the light and the dense membrane fractions had a similar, homogeneous appearance, and they seemed to be composed of tiny vesicles of about 0.01 μm (Fig. 4).

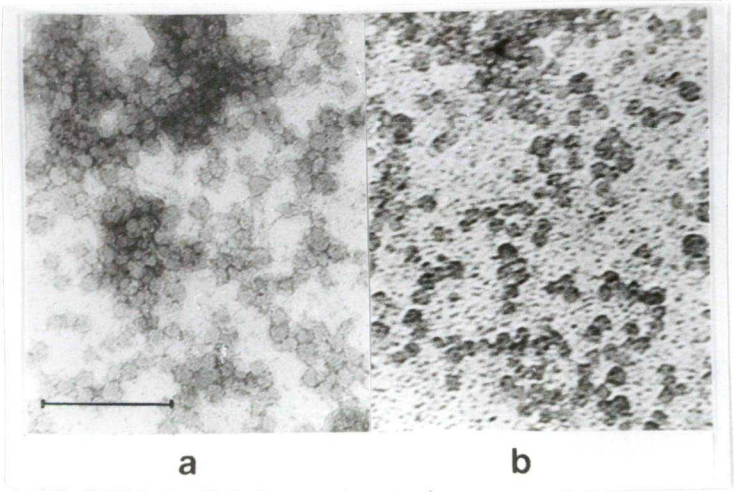


Fig. 4. Similar ultrastructure of the light (a) and the dense (b) membrane fraction (negative staining). Magnification: 150, 000x, bar is 0.1 μ.

Since almost all plasma membrane proteins are glycosylated (Gahmberg and Tolvanen, 1996), it seemed reasonable to characterize and compare our membrane fractions by their glycosylation patterns as well. Four lectins were used in blots for the detection of glycoproteins: Concanavalin A (Con A, specific for α-mannose, α-glucose and N-acetyl-α-glucosamine), wheat germ agglutinin (WGA, specific for terminal N-acetyl-ß-glucosamine of 3 or 2 units and sialic acid), Ulex europaeus agglutinin I (UEA I, specific for α-fucose), and elderberry bark lectin (EBL, specific for terminal sialic acid attached to galactose in α-2,6 or -2,3 linkage). Con A is a general glycoprotein marker, since a wide variety of glycoproteins have a core oligosaccharide structure including α-mannose residues. Terminal fucose and sialic acid residues are known to be in abundance in plasma membrane proteins, thus UEA I,

EBL (and WGA) bind preferentially to the surface membrane proteins. Checking our samples from this respect, we found that several glycoproteins were specifically labelled by UEA I and EBL, supporting the surface origin of these preparations. Interestingly, WGA recognized almost exclusively an 50 kD protein band, while Con A detected many glycoproteins above 45 kD. Comparing the patterns of the labelled glycoproteins, no significant differences could be demonstrated between the light and the dense fractions (with few exceptions e.g in the Concanavalin A lane in the range above 100 kD) (Fig. 5).

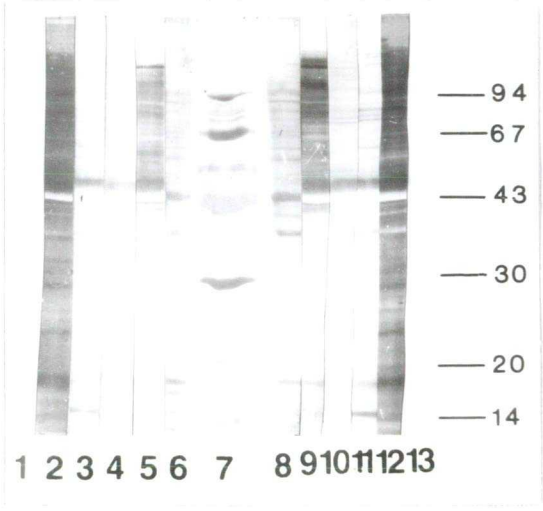


Fig. 5. Glycoprotein profiles of the studied membrane fractions derived from non-attached cells (lanes 1-5, dense fraction; lanes 9-13, light fraction). Blot slices were treated with concanavalin A (5, 9), with wheat germ agglutinin (4, 10), with Ulex europaeus I lectin (3, 11) or with elderberry bark lectin (2, 12); lanes 1 and 13, controls not treated with lectins; lanes 6 and 8, polypeptides of the samples; lane 7, molecular mass markers.

However, in the early series of experiments, the 220 kD β-subunit of spectrin (a cytoskeletal protein linked tightly to the plasma membrane via ankyrin) was detectable only in the low density fraction in immunoblot, while in the second series of investigations, this spectrin subunit was found to be enriched in both fractions (Fig. 6). (This difference is probably due to slight changes in the preparation procedure, and to some extent, also to a difference in antibody quality.) In addition, alkaline phosphatase activity, which is a common plasma membrane marker of neutrophils (Jesaitis et al. 1988), was significantly higher in the light membrane fraction than in the dense one (Table 3).

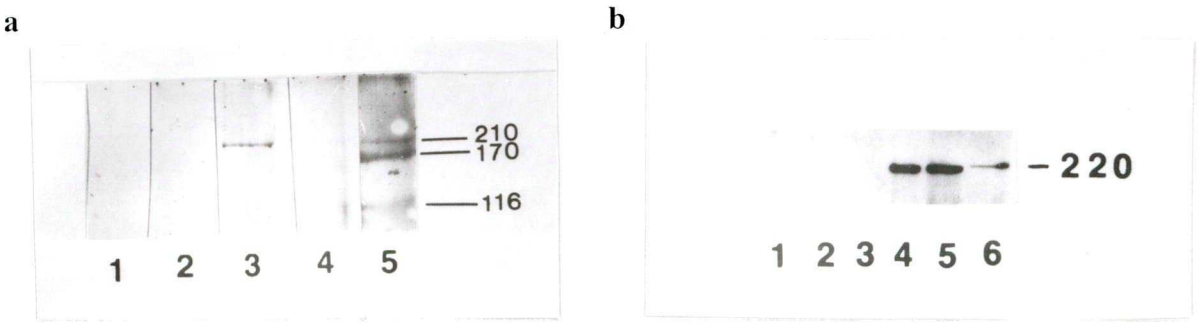


Fig. 6. Detection of spectrin in the membrane fraction(s) by immunoblotting.

Part a (early experiments), lanes: 1, control not treated with primary antibody; 2, dense fraction; 3, light fraction; 4, cell suspension; 5, molecular mass markers.

Part b (recent experiments), lanes: 1-3, controls of the membrane samples and the total cell sample not treated with the primary antibody; 4, low density membrane fraction; 5, high density membrane fraction; 6, total cell sample.

Table 3

Alkaline phosphatase activities of the membrane fractions derived from non-attached neutrophils

	Light fraction	*Dense fraction*
Enzyme activity *(μmol product/mg protein/h)*	12.55 ± 2.40	5.15 ± 1.88

(Data represent the mean ± SEM of duplicate-triplicate determinations of 6 assays.)

According to these observations, the low density membrane fraction was regarded as a cell surface membrane sample, while the intracellular vesicular origin of the dense fraction (e.g. in the form of mobilizeable plasma membrane-like intracellular vesicles) could not be excluded (especially in the early investigations).

3.2. F-actin-binding proteins of the liver plasma membrane sample and the developmental change in their pattern

To show potential microfilament-associated proteins in the plasma membrane sample prepared from livers of mature rats, F-actin affinity chromatography and protein blotting with labelled actin in F-buffer were performed. 17 polypeptides could be discerned in the silver-stained gel pattern of the sample eluted from the affinity column (Fig. 7). (Some of these polypeptides appeared also in the eluate of the control column that is presumably due to aspecific binding to the column.) As the actin linked to column particles remains in bound form after elution, the eluted protein with an apparent molecular mass of 43 kD was associated probably with the cell membrane.

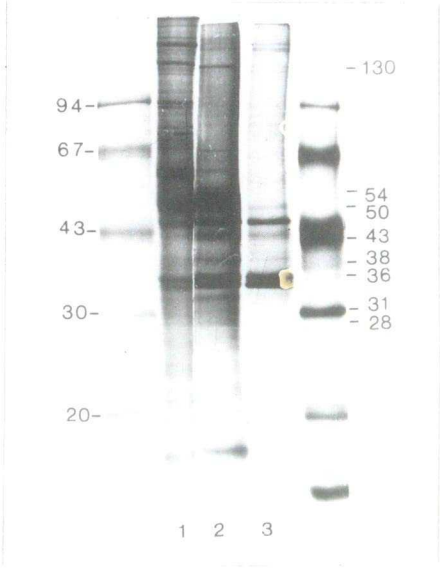


Fig. 7. Detection of F-actin-binding proteins by F-actin affinity chromatography. Lanes: 1, gel profile of the plasma membrane sample prepared from mature rats (Coomassie blue staining); 2, gel pattern of the sample eluted from the F-actin affinity column; 3, and from the ligand-free control column (silver staining). Numbers to the right of the figure designate the apparent molecular masses (in kD) of the polypeptides revealed also by the blot assay.

In the blot assay, transferred membrane proteins on nitrocellulose filter were treated with F-actin-peroxidase; ovalbumin-peroxidase was used in control experiments. 13 specific bands were shown on the blot incubated with the actin-peroxidase reagent. From these, 8 polypeptides with apparent molecular masses of 130 kD, 54 kD, 43 kD, 38 kD, 36 kD, 31 kD and 28 kD were found also in the eluate of the F-actin affinity column (Figs. 7 and 8).

Therefore, these 8 polypeptides from the 40 membrane polypeptides discerned are supposed to be direct F-actin-binding proteins (Table 4).

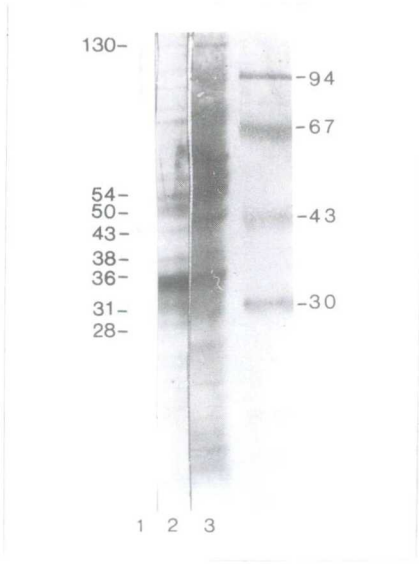


Fig. 8. Detection of F-actin-binding proteins by blot assay. Lanes: 1, control blot strip treated with ovalbumin-peroxidase; 2, blot strip probed with F-actin-peroxidase; 3, polypeptide profile of the transferred plasma membrane sample prepared from mature rats. Numbers to the left of the figure refer to the apparent molecular masses (in kD) of the polypeptides shown also by affinity chromatography.

Being interested whether any changes occur in the pattern of the F-actin-binding proteins during postnatal development, plasma membrane samples were prepared also from newborn and from 3-week-old rats. Comparing the polypeptide patterns, several differences could be observed among the three age groups in the relative abundance of some membrane proteins (Fig. 9). Three main differences were related to F-actin-binding proteins. The relative levels of the 130 kD, 50 kD and 36 kD polypeptides appeared to be significantly lower in newborn rats than in mature ones. In the case of the 3-week-old rat, the membrane sample was relatively enriched in the 130 kD and 36 kD proteins - similarly to the mature rats, while in respect of the relative abundance of the 50 kD protein, the young sample showed similarity with the neonatal one: they both contained relatively low amount of it. Taken together, these observations suggest a relative increase in the abundance of the 130 kD, 50 kD and 36 kD F-actin-binding proteins in the examined plasma membrane during postnatal development.

Table 4

Polypeptide pattern of the liver plasma membrane sample prepared from adult rat

Apparent molecular mass (kD)	***Actin-binding polypeptides***	*Apparent molecular mass (kD)*	***Actin-binding polypeptides***
400		70	
300		(65)	
260		(64)	
200		61	
170		60	
(155)		58	
(140)		55	
(135)		*54*	*
130	* +	52	
115		***50***	* +
100		(48)	
98		45	
95		*(43)*	*
94		*(38)*	*
(88)		***36***	* +
(84)		*(31)*	*
(80)		*(28)*	*
78		19	
75		17	
72		14	

Figures in parentheses denote minor polypeptides, * marks F-actin-binding proteins (molecular mass values in italic), + indicates a postnatal increase in the relative abundance in the plasma membrane sample (molecular mass values in boldface italic)

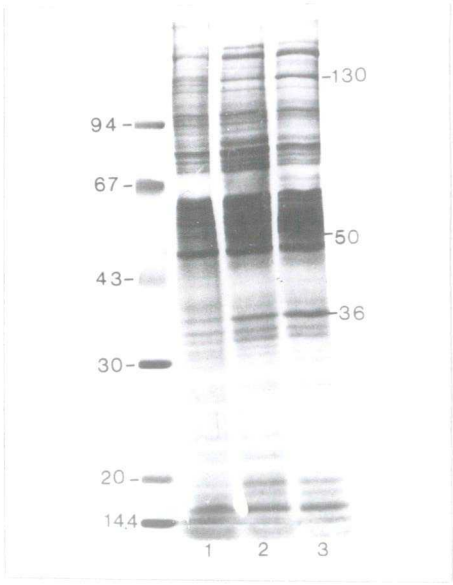


Fig. 9. Enrichment of three F-actin-binding polypeptides in the plasma membrane of the liver during postnatal development (SDS-PAGE analysis). Samples were prepared from newborn (lane 1), from 3-week-old (lane 2) and from mature (lane 3) rats. Molecular mass values to the right of the figure mark F-actin-binding polypeptides of different abundance in the three samples.

3.3. Major F-actin- and EHS-laminin-binding membrane-associated proteins of neutrophils

To detect direct F-actin-binding proteins in association with the plasma membrane, the polypeptides of the membrane fractions derived from attached and non-attached neutrophils were electroblotted and tested with F-actin-peroxidase conjugates - generally in the presence of Ca^{2+} (1 mM) in the labelling medium. Among the major plasma membrane proteins of the attached neutrophils, the ones with apparent molecular masses of about 80 kD, 65 kD, 50 kD, and 18 kD stained strikingly intensively with the F-actin reagent (dense adhesion membrane fraction; traditional developing system containing diaminobenzidine, $NiCl_2$, H_2O_2) (Fig. 10). It is important to note that labelling was specific and dependent on the assembly form of actin, since these bands were (almost) undetectable when probed with monomeric (G) actin reagent. Staining of these polypeptides was considerably reduced in the presence of EGTA (1 mM). The above mentioned proteins were heavily labelled also in the case of the control plasma membrane fraction of non-attached neutrophils (light fraction), with the exception of the 80 kD polypeptide, which was barely detectable in this membrane sample (Fig. 11).

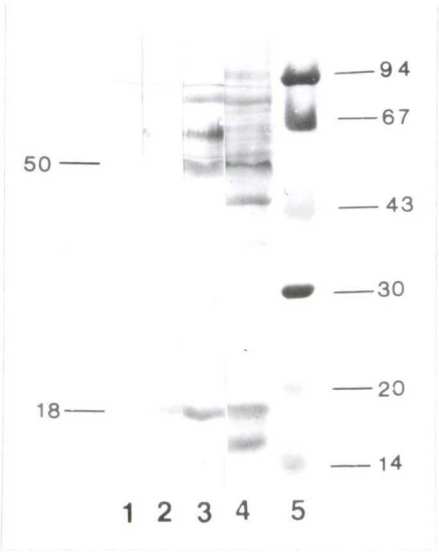


Fig. 10. Major F-actin-binding proteins in the "heavy" membrane fraction of attached neutrophils (A2). Blot slices were treated with with either peroxidase-labelled F-actin in the presence of 1 mM Ca^{2+} (3) or 1 mM EGTA (2); or with depolymerized (G-actin) conjugate /control/ (1). Lanes 4, sample polypeptides; 5, molecular mass markers.

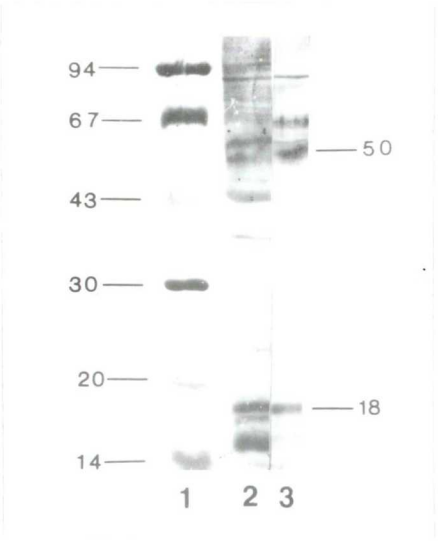


Fig. 11. Major F-actin-binding proteins in the light plasma membrane fraction of control neutrophils in suspension (C1). Lanes: 1, molecular mass markers; 2, polypeptide pattern of the sample; 3, blot slice tested with peroxidase-labelled F-actin in the presence of 1 mM Ca^{2+}.

Since laminin-binding plasma membrane proteins play a fundamental role during neutrophil migration, an overlay blot assay was performed with EHS laminin to detect the major laminin-binding proteins in our membrane samples. At 20 μg/ml and at 10 μg/ml

laminin concentration, the three major polypeptide bands discerned in the low-density membrane fraction of neutrophils in suspension (C1) had apparent molecular masses of 50 kD, 44 kD and 18 kD. From these, the 44 kD protein stained faintly at 10 μg/ml concentration (Fig. 12). While at these concentrations the 50 kD protein appeared to be labelled most intensively, at 5 μg/ml and at 2.5 μg/ml concentration staining of the 18 kD band was predominant - reflecting presumably its higher affinity for laminin.

Using biotinylated EHS laminin in blots, specific labelling of the 50 kD and the 18 kD protein could be well demonstrated also in the dense membrane fraction (C2); some minor bands - mostly between 30 kD and 18 kD - were also observed (Fig. 13). According to these results, the 50 kD and the 18 kD proteins were considered as major EHS laminin-binding proteins in neutrophils.

Competitive inhibition experiments were performed to investigate whether these proteins could belong to the galectin class of laminin-binding proteins. Indeed, binding of biotinylated laminin to both of these membrane proteins - especially to the 18 kD one - could be considerably reduced by preincubation of the blot with 0.2 M lactose (Fig. 13).

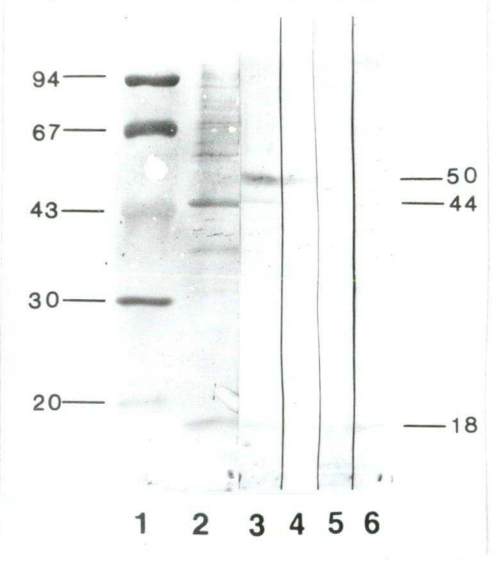


Fig. 12. Laminin-binding proteins of the neutrophil plasma membrane (light fraction of cells in suspension) shown by blot overlay. Lanes: 1, molecular mass markers; 2, protein pattern of the plasma membrane (C1); 3-6, blot overlays with 20 μg/ml (3), 10 μg/ml (4), 5 μg/ml (5) and 2.5 μg/ml (6) EHS laminin.

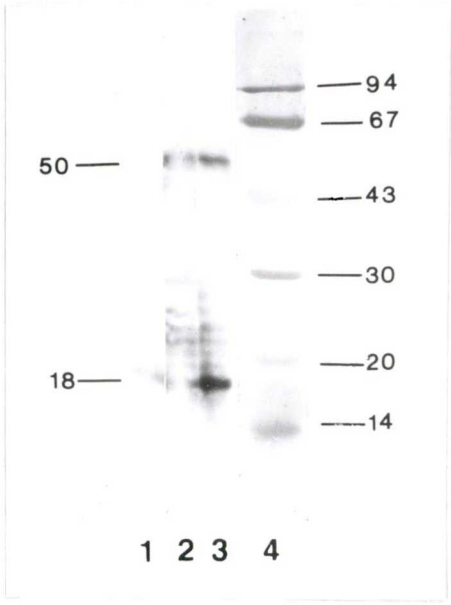


Fig. 13. Galectin-like laminin-binding proteins of neutrophils (dense fraction of control cells in suspension, C2) detected by biotinylated laminin (lane 3). Competitive blot analysis: preincubation with 0.2 M lactose (lane 2). Lane 1, control blot slice not treated with labelled laminin ; lane 4, molecular mass markers.

3.4. Characteristic changes in the pattern of the 50 kD, the 18 kD and the 80 kD polypeptides of the membrane fractions upon attachment of neutrophils

In order to estimate the quantitative differences between the control and the adhesion membrane fractions - focusing first on the appearance of these laminin- and F-actin-binding proteins - the SDS-polyacrylamide gel lanes of control and adhesion fractions were compared (Fig. 14). Two kinds of reproducible differences could be observed in our experiments: 1, the relative abundance of the 18 kD protein seemed to increase considerably in the membrane fractions of the attached cells; 2, the 50 kD protein, a major component of the neutrophil cell membrane, appeared to accumulate in the dense fraction following adhesion. In addition, the 80 kD protein could be observed primarily in the fractions of the attached cells (see below).

For quantitative analysis, the relative abundances of these laminin- and F-actin-binding proteins were calculated from the values of the gel densitometry (as percentages of the total protein, vs percentage of a 44 kD "stabile component" of the plasma membrane - to counterbalance increase of degradative products in the adhesion membrane fractions). A substantial alteration could be observed when the relative abundance of the 18 kD protein was considered: about 3-fold elevation following adhesion (Fig. 15). Control and adhesion data of

the 18 kD protein are averages of the light and the dense fractions (averages of C1,C2 vs A1, A2). No significant difference could be detected between the low and high density membrane fractions. In contrast, the 50 kD protein was shown to accumulate preferentially in the dense membrane fraction of the attached neutrophils, while no significant difference in its distribution could be detected between the control fractions (Fig. 16).

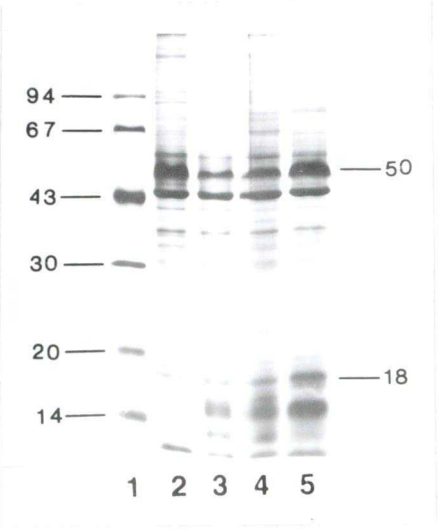


Fig. 14. Effect of adhesion of neutrophils on the appearance and/or on the preferential localization of the 50 kD and the 18 kD proteins in the light and in the dense membrane fractions. Lanes: 1, molecular mass markers; 2-3, membrane fractions prepared from neutrophils in suspension; 4-5, membrane fractions derived from attached neutrophils; 2 and 4, low density fractions (C1, A1); 3 and 5, high density fractions (C2, A2).

Focusing now on the surprisingly enhanced appearance of the 80 kD protein in the membrane fractions of the attached neutrophils, this phenomenon was reexamined. We were interested, whether this protein is present in the whole cell sample. A gel electrophoresis was performed (Fig. 17a), and thereafter, the blotted samples were probed with F-actin-peroxidase using the chemiluminescence developing system (Fig. 17b). As observed previously, the 80 kD band appeared preferentially and characteristically in the fractions of the attached cells, where it constituted a well-detectable component of the polypeptide profile. In contrast to the 50 kD protein accumulating in the dense membrane fraction of attached cells, no significant difference could be demonstrated between the light and dense adhesion membrane fractions in the relative abundance of this polypeptide band. It is important to note, that this 80 kD band

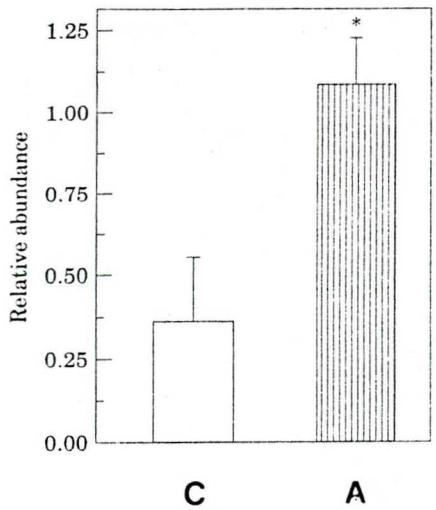


Fig. 15. Enhanced plasma membrane appearance of the 18 kD protein in neutrophils following adhesion. Values were calculated from the percentage data of gel scans-densitograms. Results shown are means (±SE) from 4/4 control/adhesion experiments (taking the average of light and dense fractions). C: contol, non-attached cells; A: attached cells.
*P<0.01 for attached vs control neutrophils (Mann-Whitney test).

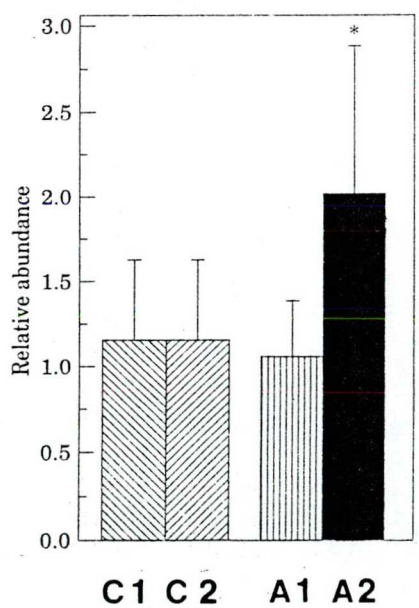


Fig. 16. Preferential accumulation of the 50 kD protein in the dense membrane fraction (A2) of attached neutrophils. Gel scan data (reflecting relative abundance) are means (±SE) from 5/5 control/adhesion experiments.
*P<0.02 for dense (A2) vs light fraction (A1) of attached neutrophils
*P<0.1 for "heavy" membrane fraction of attached neutrophils (A2) vs control "heavy" membrane fraction of cells in suspension (C2) (Mann-Whitney-test)

was well visible in the total cell sample, while it could be hardly seen in the membrane fractions derived from cells in suspension. In accordance with the findings described above, this protein turned out to be a major F-actin-binding one in the membrane fractions of the attached neutrophils, and it was well detectable with the F-actin probe in the total cell sample, too (Fig. 17b). Thus, the 80 kD protein appeared to be characteristic for the membrane fractions derived from attached cells and for the whole cell sample, where it binds specifically F-actin.

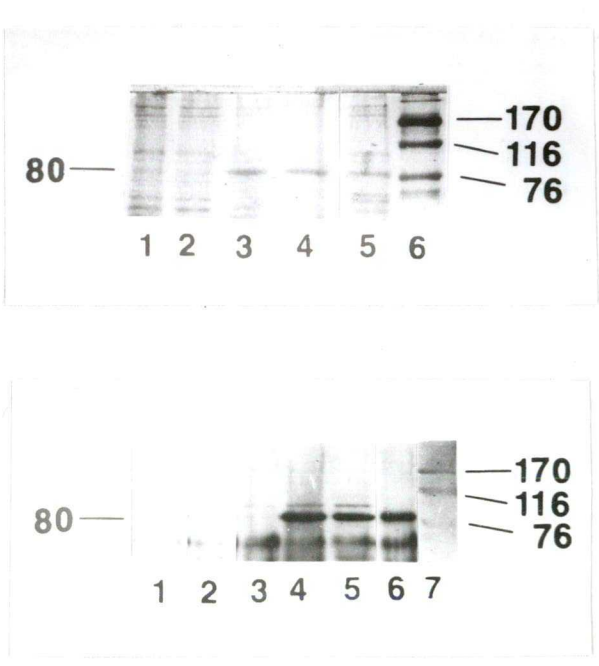


Fig. 17. **a** Characteristic presence of the 80 kD protein in the SDS-polyacrylamide gel patterns of the membrane fractions derived from attached neutrophils and in the gel lane of the total cell sample. Lanes: 1-2, membrane fractions of neutrophils in suspension; 3-4, membrane fractions of attached neutrophils; 1, 3, light fractions; 2, 4, dense fractions; 5, cell suspension; 6, molecular mass markers.

b Actin-binding proteins in the various fractions and in the cell sample examined by actin-peroxidase in blot. Lanes: 1, dense "adherent" membrane sample treated with monomeric (G-) actin; 2-6, blot strips probed with polymerized (F-) actin: light (2) and dense (3) membrane fractions of non-attached cells, light (4) and dense (5) membrane fractions of attached cells, total cell sample (6); 7, molecular mass markers.

3.4. Major components of the actin-cytoskeletal fractions of neutrophils

We further extended our investigations on the F-actin-binding of the studied proteins by cytoskeletal extraction. Actin-cytoskeletal samples were prepared from attached and non-attached neutrophils and their gel profiles were analyzed. In both samples, the 80 kD polypeptide band was found to be a major constituent of the protein pattern in the high molecular mass range (Fig. 18). In addition, a 43 kD protein (probably actin) and small molecular mass polypeptides of about 18 kD and 15 kD (and about 30-35 kD) could be observed. (The absence of the 50 kD protein band may be due to its relatively fast degradation in the sample.)

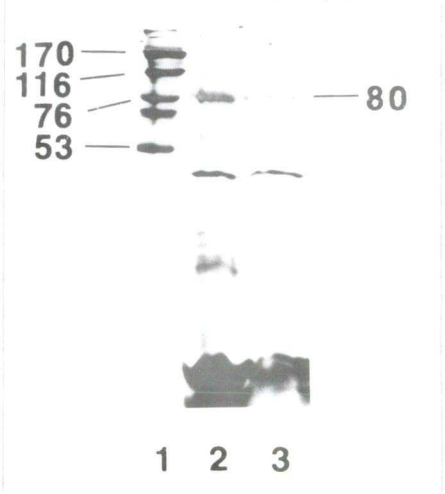


Fig. 18. Major components of the actin-cytoskeletal fractions prepared from non-attached (lane 2) and from attached (lane 3) neutrophils revealed in silver-stained gels. (Lane 1, molecular mass markers.)

When these cytoskeletal samples were checked with labelled F-actin following blotting, the 80 kD protein band appeared as an intensively stained one reflecting its functional capability to bind F-actin (Fig. 19). (The polypeptide of about 75 kD in the "non-adhesion" cytoskeletal sample is presumably a degradation product.) In addition, all the above mentioned polypeptides were detectable in the blot, including the 18 kD one. Labelling was specific for actin (no background was detectable using ovalbumin-peroxidase conjugate), and it was predominantly seen with actin in the polymerized state (F-actin versus G-actin staining), as observed before.

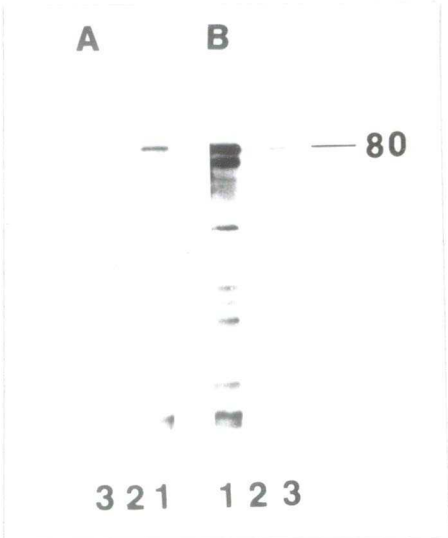


Fig. 19. Blotted actin-cytoskeletal samples from attached (A) and non-attached neutrophils (B) probed with actin-peroxidase to check functional F-actin binding. Lanes: 1, treatment with F-actin reagent; 3, treatment with G-actin reagent; 2, control slices tested with ovalbumin-peroxidase.

3.5. Characterization and identification of the studied membrane-associated proteins of neutrophils

Both the 50 kD and the 18 kD proteins turned out to be glycoproteins in several experiments judged by labelling by ConA lectin. As characteristic plasma membrane proteins, both proteins were detected by UEA I and EBL lectins which are specific either for fucose (UEA I) or for terminal sialic acid (EBL) (Fig. 5). In addition, the 50 kD glycoprotein appeared to bind WGA lectin almost exclusively among the membrane proteins.

Aiming at a more precise identification, several antibodies generated against different cell surface proteins of neutrophils (anti-CD antibodies) were tested in immunoblots. At last, we succeeded to find one, which bound to one of the studied proteins: the 50 kD protein band was recognized by a monoclonal anti-CD14 antibody (Fig. 20). As far as the identity of the 18 kD protein is concerned, it may be related to galectin 5 or 10 of 17-18 kD (which are characteristic for erythrocytes and eosinophils, respectively), or to a 17.5 kD galectin described in adult rat kidney (Jung and Fujimoto, 1994).

Trying further, we tested if our 80 kD polypeptide might be related to ezrin or to moesin (ERM proteins of 81 kD and 78 kD), since these proteins were reported to be

characteristic F-actin-binding proteins in association with bovine neutrophil plasma membrane (Pestonjamasp et al., 1995). As the immunblots of the "adhesion" plasma membrane fraction and the whole cell sample revealed, the 80 kD polypeptide band was recognized by mono- and polyclonal anti-moesin antibodies (Fig. 21). (The 65 kD polypeptide stained in the membrane fraction could be a related polypeptide.) The molecular mass of the studied protein turned out to be indeed 78 kD, when determined precisely (Fig. 17). No staining could be detected when applying anti-radixin antibody to the blot (not shown).

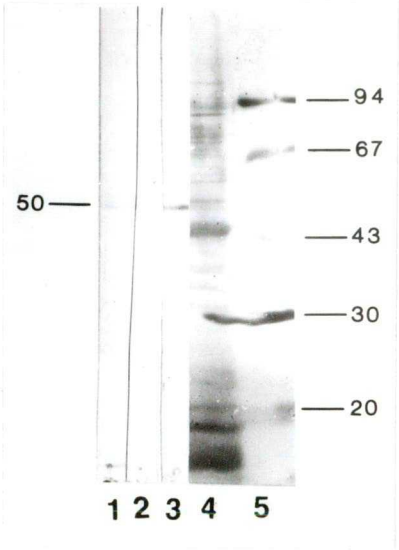


Fig. 20. Identification of CD14 in the 50 kD glycoprotein band in immunoblot. Lanes: 1-3, treatments with anti-CD14 monoclonal antibody of different concentration (dilution factors: 100x, 50x and 20x, respectively), 4, light membrane fraction, 5, molecular mass markers.

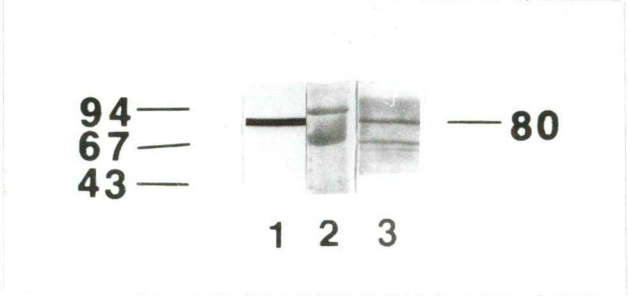


Fig. 21. Detection of moesin in the 80 kD protein band in immunoblot. Lanes: 1, total cell sample tested with a monoclonal anti-moesin antibody (mAb 38/87); 2, molecular mass markers; 3, light membrane fraction of attached neutrophils tested with an affinity-purified polyclonal anti-moesin antibody (90-7s).

4. Discussion

4.1. Evaluation of the methods used for the separation and labelling of F-actin-binding proteins

In the course of our study, two different experimental approaches were employed for the general identification of F-actin-binding proteins in the membrane samples. A traditional way to separate these proteins is by F-actin-cosedimentation or by actin affinity blotting using labelled proteins and actin immobilized on filter disks (e.g. Brown et al., 1983). A somewhat similar method is F-actin-chromatography, introduced by Luna et al. (1982, 1984) for a similar purpose as ours; it was applied also by Miller and Alberts (1989). (In contrast to these protocols, we did not use labelled F-actin, labelled membrane preparates and/or phalloidin.) The affinity chromatography method is considered to be a reliable and specific method for the separation of direct and indirect F-actin-binding proteins, although membrane lipids may disturb the specific binding of membrane proteins in tight association with phospholipids, as lipids were reported to bind readily to column particles (Luna et al., 1984). This can be the reason for the retention of the 36 kD protein - most probably a phospholipid-binding protein - by the ligand-free Sepharose column. The other problem with this method is, that it requires a relatively large amount of the membrane sample (mg quantities) - in the absence of specific sample labelling.

Gel or blot overlay assays with labelled F-actin turned out to be equally reliable, and at the same time, more user-friendly methods for the identification of actin-linked proteins. In an early report, actin blot overlay was carried out using actin plus actin-antibodies and secondary antibodies (Walker et al., 1984). Almost all other overlay procedures employ ^{125}I-labelled actin in a one-step assay for the detection of these proteins (e. g. Snabes et al., 1981; Barmann et al., 1986; Amrein-Gloor and Gazzotti, 1987; Pestonjamasp et al., 1995, 1997). Since our goal was to find a specific, simple blot overlay method for the detection of direct F-actin-binding proteins - without using potentially harmful substances, we introduced the application of peroxidase-labelled actin conjugates. In the early study, F-actin-chromatography and ovalbumin-peroxidase treatment were used as control experiments, while recently, a more proper control reagent: G-actin-peroxidase was used. Our assay solution did not contain phalloidin and gelsolin, in contrast to the one of Pestonjamasp et al. (1995, 1997). Similarly to the assays using ^{125}I- labelled actin, our procedure proved to be efficient, reliable and fast in

the detection of direct F-actin-binding proteins (evidently, without revealing indirect actin-binding proteins, as it might occur in the case of actin-chromatography). In addition, this method required small amounts of samples (about 10 μg/blot lane); furthermore, it is cheap, because no antibodies are required - since all F-actin-linked proteins are labelled at the same time, with the same reagent. On the other hand, the actin-peroxidase conjugates may be used even for years, if prepared and stored properly.

4.2. Development-dependent accumulation of the 130 kD, 50 kD and the 36 kD F-actin-binding proteins in the rat liver plasma membrane sample

Investigations on the cell membrane fraction of rat livers demonstrated that it contains at least 8 F-actin-binding proteins, as from the 40 polypeptides discerned on SDS-polyacrylamide gel, 8 were shown to bind F-actin both by actin-chromatography and by the blot assay. In contrast, Amrein-Gloor and Gazzotti (1987) detected only three such proteins, with molecular masses of 240 kD, 145 kD and 80 kD in rat liver plasma membranes (performing a ^{125}I-actin blot test). The low number of the revealed F-actin-linked proteins is apparently due to the "overpurity" of their plasma membrane preparate which is presumably devoid of several peripheral membrane proteins. On the other hand, the failure to detect the 240 kD protein in the present blot assay most probably resulted from the lower efficiency of the blotting procedure to transfer proteins with higher molecular masses, since a polypeptide of 260 kD was shown by the actin affinity chromatography. The 145 kD polypeptide might be the same as the 130 kD one detected by us.

The most interesting finding of this study is that the appearance of some F-actin-binding proteins at the plasma membrane seems to be developmentally regulated. According to our observations, the relative abundance of the 36 kD and 130 kD polypeptides in/at the plasma membrane is increased primarily during the first three weeks of postnatal liver development, while the cell membrane/cortical accumulation of the 50 kD polypeptide appears to be initiated only after this time.

Although these F-actin-binding polypeptides were not immunologically identified (caused by the lack of commercially available, proper antibodies), it is tempting to speculate - on the basis of the apparent molecular masses - that they could be identical or similar to proteins described in literature. The 50 kD polypeptide may be the same as the 50 kD actin-binding plasma membrane protein demonstrated in rat hepatocytes, which was found to

accumulate in the plasma membrane following induction of liver tumours by N-nitroso-morpholine and phenobarbital (Barmann et al., 1986). The 130 kD polypeptide may something to do with vinculin - a characteristic component of focal adhesion complexes - however, the membrane-association of this protein has not been proven yet (Yamada and Geiger, 1997).

A more precise characterization was possible in the case of the 36 kD polypeptide: it is thought to be a member of the annexin (calpactin/lipocortin family) according to its degradation products and its retention by the ligand-free Sepharose column. Annexins are characterized by their Ca^{2+}-dependent binding to acidic phospholipids, and they can have several other activities like e.g. inhibition of phospholipase A_2 (Brugge, 1986; Kreis and Vale, 1993.) From the several types of this protein family, types I and II (previously known as lipocortin I/calpactin II and lipocortin II/calpactin I, respectively) were shown to bind F-actin also in a Ca^{2+} -dependent manner (Glenney, 1986). Via membrane phospholipids, these proteins can be associated with plasma membranes; and especially annexin II was reported to participate in direct F-actin - plasma membrane linkage (Stossel, 1993). The 36 kD protein described in this study is presumably identical to annexin II (calpactin I/lipocortin II) heavy chain, since its instability during storage results in the degradation derivatives of 34.5-34 kD and 33 kD observed also by Huang et al. (1986). In addition, the unspecific binding of this protein to the ligand-free column can be explained by its close association with some phospholipids in the membrane sample, as lipids readily bind to the Sepharose column particles (Luna et al., 1984).

4.3. Adhesion-dependent accumulation of the 50 kD, 18 kD proteins and moesin (80 kD) in the plasma membrane fractions of bovine neutrophils

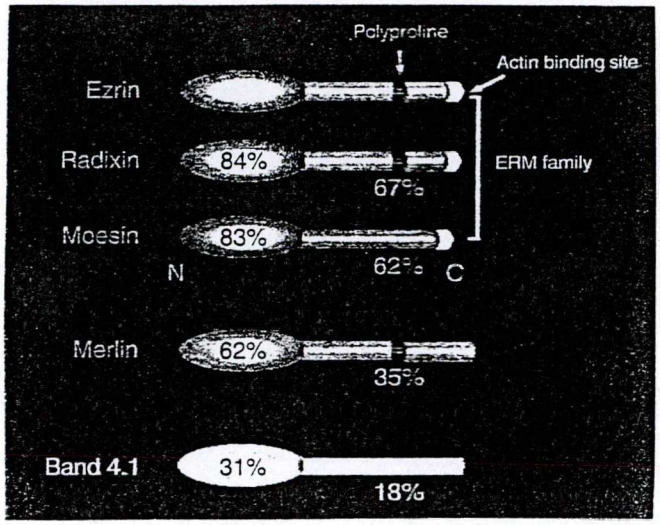
Our results propose that among the four major F-actin-binding proteins shown in the present study on the cell membrane of attached neutrophils, there are two galectin-like laminin-binding proteins with M_r of 50 kD and 18 kD. Following adhesion to plastic, the cell surface appearance of the 18 kD glycoprotein increased considerably in the attached cells; the 50 kD one accumulated characteristically in the dense membrane fraction, which presumably represents an intracellular, plasma membrane-like fraction.

As far as the identities of these proteins is concerned, the immunological assays led to a positive result only in the case of the 50 kD band: it contains most probably a CD14-like

protein (specific reaction with an anti-CD14 monoclonal antibody). Cell surface CD14 is known as a 55 kD glycosyl-phosphatidylinositol-anchored membrane protein characteristic for mature myeloid cells (monocytes, macrophages and granulocytes), which serves as a receptor for lipopolysaccaride (LPS) present in the outer membranes of Gram-negative bacteria (Read et al., 1993). Internalization of LPS in complex with CD14 was shown to occur (in 20 minutes) following its interaction with CD14 on the cell surface (Detmers et al., 1996). These data are consistent with our results suggesting that an adhesion receptor function and intracellular localization of the 50 kD glycoprotein may be possible. Moreover, a galectin: galectin-3 was demonstrated to bind LPS from Klebsiella pneumoniae (primarily) via the ß-galactosides in the polysaccharide chains; this binding was characteristically inhibited by lactose (Mey et al., 1996). Therefore, CD14 - a LPS-receptor - may function as a receptor for laminin (rich in ß-galactosides) as well; and conversely, a galectin (like the 50 kD protein) may act as a LPS-receptor. It is worth noting, that galectin-3 - expressed mainly in activated macrophages, basophils and mast cells - binds also laminin (Barondes et al., 1994). Recently, Dewitt et al. (1998) concluded that CD14 behaves as a multifunctional receptor of the phagocytes which is able to bind to different lipids and saccharides of e.g. the apoptotic cell and, regarding the interactions with saccharide-containing molecules, it can presumably function as a lectin. Considering the capability of the 50 kD membrane protein of direct F-actin-binding, it resembles the LSP1 membrane/cytosolic protein of similar molecular mass which is involved in the regulation of the microfilamentous cytoskeleton structure in leukocytes and in other hematopoetic cells by stabilizing F-actin bundles (Matsumoto et al., 1995; Li et al., 1995).

The 80 kD protein - identified as moesin - was shown to appear characteristically in the membrane fractions of attached neutrophils, the results presented here support some earlier observations which suggest that moesin is capable of direct binding to microfilaments and that it takes part in cell adhesion (reviews: Tsukita et al., 1997a; Vaheri et al.,1997). Moesin (*m*embrane-*o*rganizing *e*xtension *s*pike prote*in*) is a member of the ezrin-moesin-radixin (ERM) family belonging to the band 4.1 superfamily (Fig. 22a). The members of this superfamily show homology of the amino-terminal domain with the band 4.1. protein of erythrocytes, which connect the actin-spectrin network to the membrane protein glycophorin C. The highly conserved globular domain at the amino terminal was reported to bind to plasma membrane proteins (e.g. CD 44, ICAM-1, -2) also in case of ERM proteins (Fig. 22b)

a



b

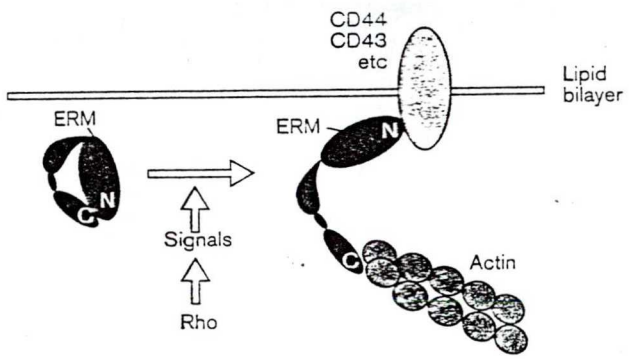


Fig. 22. **a** ERM family members (ezrin, radixin, moesin), merlin/schwannomin and band 4.1 protein. The extent of homology of the amino- and carboxyl-terminal halves is related to those of ezrin (Tsukita et al., 1997b).

b Activation of ERM proteins as membrane - actin filament crosslinkers by Rho via some unidentified downstream signals.The carboxy terminal of an ERM protein binds F-actin, whereas the amino terminal is linked to plasma membrane receptors like CD44, CD43 etc. (Tsukita et al., 1997a).

(Tsukita et al., 1997a; Vaheri et al., 1997; Bretscher et al., 1997). The basic carboxy-terminal domain is probably involved in F-actin-binding, it is highly conserved within the ERM family and shows sequence similarities to actin-binding sites in other proteins (Tsukita et al., 1997a; Pestonjamasp et al., 1995). These domains are connected by an extended α-helical domain

containing a polyproline region in ezrin and radixin (Fig. 22a). ERM proteins are supposed to function by linking microfilaments to some plasma membrane proteins (Fig. 22b) (Tsukita et al., 1997a; Pestonjamasp et al., 1995; Bretscher et al., 1997). They are thought to have an essential role in cell adhesion, since addition of ERM antisense oligonucleotides completely destroyed the preformed cell-cell and cell-substrate adhesion in mouse mammary tumour and in thymoma cells; moesin antisense oligonucleotides appeared to affect primarily microvilli causing their partial diappearance (Takeuchi et al., 1994). Moesin is expressed in a wide variety of cells (epithelial, vascular endothelial, fibroblastic-chondroblastic, neuronal, glial cells and different blood cells); it was seen to be concentrated in microextensions (filopodia, microvilli, microspikes and retraction fibres) (Amieva and Furthmayr, 1995).

In the F-actin blot overlay assay of bovine neutrophil plasma membrane fraction performed with ^{125}I -labelled actin by Pestonjamasp et al. (1995), polypeptides with M_r of 205 kD, 95 kD, ~80 kD, 69 kD, 67 kD were detected in the 250-30 kD polypeptide range. We failed to recognize high molecular mass polypeptides in our experimental system, (partially because of transfer problems); in contrast, our blotting system worked efficiently in specific labelling of polypeptides in the 15-100 kD range. The 95 kD and the 67 kD polypeptides may correspond to our 92 kD and 65 kD ones, respectively. The ~80 kD protein band was reported to be composed of ezrin, and primarily moesin. In accordance with these findings, we showed the presence of moesin in the 80 kD protein band in the "adhesion" membrane fractions; according to preliminary results, ezrin might be also present. (Radixin was not detectable neither by Pestonjamasp et al., nor by us.) Thus, the employment of these two F-actin labelling methods led to comparable results when examinig the 100-60 kD polypeptide range of mammalian neutrophil membrane fractions.

It is important to note, however, that we could show the 80 kD protein predominantly in the membrane fractions of attached cells with a very low detectability in those of non-attached cells; in contrast, moesin was present in the whole cell sample of non-attached neutrophils. This presumably reflects adhesion-triggered association of moesin with the plasma membrane in agreement with the widely accepted idea on the regulated translocation of ERM proteins to the plasma membrane; it was shown that ERM proteins bind to the cytoplasmic part of some membrane proteins only after the activation of some signalization pathways (e.g. growth factor receptor activation and/or activation of Rho) (Tsukita et al., 1997a; Vaheri et al., 1997; Bretscher et al., 1997). Immunofluorescence microscopic

examinations of Pestonjamasp et al. (1995) reveal moesin at the inner surface of the plasma membrane on neutrophils attached to glass. The detailed immunofluorescence-immunoelectron microscopic study of Amieva and Furthmayr (1995) on the subcellular localization of moesin in fibroblastic and macrophage cell lines demonstrated that there is a time-dependent change in the subcellular localization of moesin after attachment (to glass or to Permanox chambered slides). Moesin appeared immediately after cell contact at the plasma membrane in short surface protrusions; ten to thirty minutes later, it was detected in filopodia. Interestingly, moesin could not be observed in lamellipodia of fully spread cells (a day later), it was present only in apical microspikes, filopodia and in retraction fibers at the cellular edges. This latter finding supports the idea that ERM proteins are involved primarily in the initial formation of cadherin- and integrin-based attachment sites (Tsukita et al., 1997b).

In contrast to the model of signal-triggered activation of ERM to bind actin filaments (Tsukita et al., 1997a; Bretscher et al., 1997), we detected moesin in association with actin-cytoskeleton not only in attached cells, but also in cells in suspension. Some other experiments using detergent extraction also found a considerable portion of ezrin in the cytoskeletal fraction (Berryman et al., 1995), and Bretscher (1997) speculated that ezrin may be involved in the stabilization of microfilaments under certain circumstances.

5. Summary

1. Taken together, our study demonstrates that in rat liver cells and in bovine neutrophils, several plasma membrane-associated proteins can be directly bound to F-actin, thereby constituting a direct link between the plasma membrane and the actin-cytoskeleton.

2. Surprisingly, the appearance of some of the F-actin-binding polypeptides with apparent molecular mass of 130 kD, 50 kD and 36 kD (probably annexin II) in the liver plasma membrane were observed to increase relatively following birth and/or three weeks post partum in rats. This may reflect a developmental control: an increased targeting and/or enhanced synthesis during postnatal development of the liver cells.

3. In bovine neutrophils, two major membrane glycoproteins with apparent molecular mass of 50 kD and 18 kD are suggested to be involved in the direct linkage of laminin to actin filaments; these proteins appeared to act as galectin-like proteins. Considering their accumulation in the membrane fractions following 35 min cell-substrate adhesion, it seems

plausible to suppose that cell attachment could induce increased cell surface targeting of these adhesion receptors. In parallel with this idea, Singer et al. (1989) described some leukocyte specific granules as adhesomes because of their abundance in extracellular matrix receptors (e.g. fibronectin, vitronectin and 67 kD laminin receptors). Leukocyte activation e.g. by LPS was observed to induce fusion of specific granules with the plasma membrane resulting in the translocation of the stored adhesion receptors to the cell surface (immunelectron microscopic studies of Singer et al., 1989). It is intriguing to speculate that some adhesion receptors - like presumably the 50 kD glycoprotein (a CD14-like protein) - might undergo an internalization process as well following cell attachment.

According to our results on the 80 kD F-actin-binding protein identified as moesin, in parallel with previous reports, it appears to participate in cell-substrate adhesion. In contrast to the general idea, moesin might be bound to F-actin prior to its translocation to the plasma membrane that is presumably initiated by some extracellular adhesion signals.

4. In addition, it is worth noting, that a sensitive, simple and harmless blot assay procedure was developed for the detection of direct F-actin-binding proteins in the plasma membrane-enriched fractions of rat livers and bovine neutrophils.

All in all, F-actin-binding proteins in association with the plasma membrane probably have a fundamental role in cell adhesion, cell motion and related signalling events by anchoring microfilaments to the intracellular surface of the cell membrane. As a consequence, these proteins are certainly involved in many developmental (and regenerative) processes; they could be essential e.g. for embryonic organogenesis, similarly to other vital actin-binding proteins like profilin, cofilin /Ayscough, 1998/.) Our developmental study appears to stress the importance of these cytoskeletal proteins even during postnatal liver development in rats.

Early inflammatory processes involving the migratory neutrophil granulocytes most probably depend also on the functions of direct F-actin-binding proteins capable to link to the plasma membrane. Indeed, a direct anchor protein: moesin turned out to be a major actin-cytoskeletal protein in porcine neutrophils in our investigations. The present results emphasize also the dynamism of the anchoring system: cell-substrate adhesion itself appears to trigger or stimulate the plasma membrane appearance of moesin and some adhesion (laminin) receptors. Further studies on the mechanism of plasma membrane targeting of these proteins may facilitate the invention of new anti-iflammatory drugs in the future.

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