Ezrin has properties to self-associate at the plasma membrane

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SUMMARY

Ezrin, a member of a family of proteins involved in the interaction of the microfilament cytoskeleton with the plasma membrane, plays a role in membrane translocation in gastric parietal cells (Hanzel, D., Reggio, H., Bretscher, A., Forte, J. G. and Mangeat, P. (1991). EMBO J. 10, 2363-2373). Human ezrin was expressed in and purified from Escherichia coli. It possesses all the major biophysical, immunological and physiological properties of natural ezrin. Upon microinjection in live gastric HGT-1 cells, ezrin was incorporated into the dorsal microvilli, a site where the endogeneous protein is localized. By coimmuno-precipitation and ezrin-affinity assays, two HGT-1 cell proteins of 77 and 72 kDa behaved as ezrin-binding proteins. In enriched gastric apical membranes, 125I-ezrin labelled proteins of 80, 77 and 72 kDa by overlay assay. The 80 kDa protein was identified as ezrin and the 77 and 72 kDa proteins as gastric forms of proteins structurally related to ezrin, such as radixin and moesin. In insect cells infected with a recombinant baculovirus, one-third of over-expressed ezrin accumulated at the plasma membrane. Ezrin bound a 77 kDa endogenous peripheral membrane protein, behaving as an insect counterpart of the mammalian ezrin family. In addition to the respective role of the amino- and carboxyl-terminal domains of ezrin in linking the membrane and the cytoskeleton (Algrain, M., Turunen, O., Vaheri, A., Louvard, D. and Arpin, M. (1993). J. Cell Biol. 120, 129-139), both domains interacted synergistically in a salt-dependent manner to trigger self-association of ezrin. Ezrin's self-association properties could represent another way of regulating the number of ezrin molecules bound at specific membrane sites.

Key words: radixin, moesin, membrane-cytoskeleton, gastric parietal cell

INTRODUCTION

Ezrin (cytovillin, p81) is a member of an expanding family of proteins that are involved in the interaction of the cell cytoskeleton with the plasma membrane, during signal transduction and growth control (Bretscher, 1993; Tsukita et al., 1993). The most studied protein of the family is protein 4.1, which interacts with spectrin and actin in the erythrocyte membrane skeleton (reviewed by Luna and Hitt, 1992; Mangeat, 1988). The subsequent molecular cloning of ezrin and talin (Gould et al., 1989; Turunen et al., 1989; Rees et al., 1990) indicated that both proteins present some degree of structural homology with protein 4.1 (Conboy et al., 1986). All proteins share an N-terminal domain, which, in the case of protein 4.1 and ezrin, is believed to mediate membrane binding (Leto et al., 1986; Algrain et al., 1993). A long α-helical region follows this N-terminal domain. Finally, the C-terminal domain of these proteins is more variable, although it is generally composed of a high number of charged amino acids. The spectrin binding domain of protein 4.1 was mapped in this latter domain and in part of the α-helix stretch (Correas et al., 1986). Other proteins, whose cDNAs were cloned and sequenced, joined this protein family. All these proteins possess a homologous N-terminal domain. Two of them have, in their C-terminal sequence, a domain with putative tyrosine phosphatase activity (Gu et al., 1991; Yang and Tonks, 1991). Two other proteins, moesin and radixin, are highly related to ezrin (~70% amino acid sequence homology; Lankes and Furthmayr, 1991; Funayama et al., 1991). These constitute with ezrin a subfamily of proteins, the ezrin-radixin-moesin (ERM) family (Sato et al., 1992). Recently, Merlin (schwannomin), the product of a candidate tumor suppressor gene, was added to this subfamily, since it displayed 45-47% amino acid sequence homology with ERM proteins (Rouleau et al., 1993; Trofatter et al., 1993).

Ezrin was first characterized as a growth factor/tyrosine kinase substrate (Hunter and Cooper, 1981; Gould et al., 1986; Krieg and Hunter, 1992). Independently, ezrin was localized at specific membranous sites such as intestinal brush border microvilli (Bretscher, 1983), dorsal microvilli and membrane ruffles of different cell lines in culture (Bretscher, 1983; Gould et al., 1986; Pakkanen, 1988; Everett and Nichol, 1990; Birgbauer et al., 1991), and also in gastric parietal cell microvilli (Hanzel et al., 1989, 1991). Although no function has been demonstrated for ezrin in intestinal brush border microvilli, an important function was postulated in gastric tissue. In parietal cells, ezrin is a major cytoskeletal protein, phosphorylated upon stimulation of HCl secretion (Urushidani
et al., 1987, 1989). Once phosphorylated, ezrin binds more tightly to the membrane (Hanzel et al., 1991). This observation together with its specific location and abundance in these cells showed ezrin to be a key regulatory element of membrane translocation in parietal cells. In accord with this, recent evidence demonstrated that ezrin functions as a membrane cytoskeletal linker. Using transient expression of the protein or of its truncated forms in mammalian cultured cells, Algrain et al. (1993) have shown that the N-terminal domain of ezrin is involved in membrane binding and that the C-terminal domain interacts with the actin cytoskeleton.

One major question regarding the function of ezrin is to characterize ezrin-binding proteins and its interaction with the membrane. In this paper, we used recombinant ezrin to search for the presence of such proteins in a gastric cell line and in gastric mucosa, and to study ezrin overexpression in baculovirus-infected insect cells. Experimental evidence indicates that ezrin has the ability to self-associate as well as to interact with other members of the ERM family of proteins such as radixin and moesin. This interaction involves both the N- and C-terminal domains of ezrin. In addition, when ezrin is expressed in insect cells, it associates predominantly with the plasma membrane, presumably via a protein behaving similarly to an insect counterpart of the mammalian proteins of the ERM family. These findings help us to understand how ezrin accumulates physiologically at the membrane.

MATERIALS AND METHODS

Materials

Chemicals, glutathione-agarose beads (sulfur-linkage), thrombin and antibiotics were from SIGMA Chimie (St Quentin Fallavier, France). Culture media were from GIBCO BRL (Cergy Pontoise, France). Enzymes and the transfection reagent DOTAP were from Boehringer Mannheim (Meylan, France). Na125I and [35S]Met were from NEN Products Du Pont de Nemours (Les Ulis France).

Constructions of bacterial expression vectors

The full-length human ezrin cDNA (plasmid pCV6; Turunen et al., 1989) was subcloned into pGEX-2T vector (Pharmacia Biotech SA, St-Quentin Yvelines, France) by a two-step procedure: pGEX-2T was digested by BamHI and EcoRI. Two overlapping complementary oligodeoxynucleotides, extending from the ATG initiation codon to the NcoI restriction site of ezrin cDNA and creating a BamHI site upstream from the ATG codon, were ligated in-frame with BamHI-digested pGEX-2T. The ligation product was digested by NcoI to eliminate the products of the oligodeoxynucleotide autoligation. The plasmid pGEX-Ez containing the fusion cDNA GST(glutathione S-transferase)-ezrin was then obtained by inserting the NcoI-EcoRI fragment of ezrin cDNA into the NcoI and EcoRI sites of the modified plasmid pGEX-2T. The plasmid pGEX-EzN containing the fusion cDNA GST-N-terminal moiety (amino acids 1-309) of ezrin was obtained by deleting the Smal-EcoRI fragment of pGEX-Ez. To express the C-terminal moiety (amino acids 280-586), pCV6 was cut by BamHI, blunt-ended, and cut by EcoRI. The BamHI-EcoRI fragment was then cloned into the Smal and EcoRI restriction sites of pGEX-2T to obtain pGEX-EzC. pGEX-Ezpept was constructed by deletion of the NcoI-EcoRI fragment from pGEX-EzC. The plasmid was then blunt-ended and ligated. The fusion product encoded for the overlapping peptide (amino acids 280-310) that is present in both the N and C constructs of ezrin.

Purification of recombinant ezrin and domains

Escherichia coli (TG1 strain) containing the pGEX-Ez vector were grown overnight at 37°C in LB medium containing 100 µg/ml ampicillin. After 60 minutes of induction with 0.5 mM IPTG, bacteria were harvested by centrifugation for 15 minutes at 4000 g, the pellet was suspended in 1/75 culture volume of ice-cold PBS supplemented with 1.5 mM EDTA and 1 mM PMSF, and sonicated. The lysate was centrifuged at 100,000 g for 20 minutes, and the supernatant was recycled overnight on a glutathione-agarose column (5 ml). The column was thoroughly washed with PBS, then equilibrated with 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2.5 mM CaCl2, for subsequent cleavage by human thrombin (250 ng protease/mg protein) into the column at 25°C for 30 minutes. Under these conditions, complete cleavage of the fusion protein was obtained. The column fractions containing ezrin were pooled and dialyzed overnight against 20 mM MES, pH 6.8, 0.02% β-mercaptoethanol, 20 mM NaCl, and then applied to a Mono-S HPLC column, which was developed with a linear NaCl gradient (20 mM to 500 mM) in the dialysis buffer. Recombinant ezrin eluted at ~200 mM NaCl and was cleared from contaminant proteolytic products. A dramatic loss of material occurred at this final stage. We routinely obtained ~2 mg of pure recombinant ezrin from 5 liters of culture.

E. coli containing pGEX-EzN or pGEX-EzC vectors were grown and lysed as described for the production of ezrin except that, for the N-terminal fragment, IPTG was added for only 30 minutes to increase the recovery of soluble fusion protein. After overnight chromatography on a glutathione-agarose column, in PBS, fusion proteins were eluted after appropriate washes with 50 mM Tris-HCl, pH 8.0, containing 5 mM glutathione. The thrombin cleavage of both fusion proteins containing the N and C forms of ezrin was never as complete as that of total ezrin. When necessary, sufficient cleaved N-terminal form could be purified in one step from the glutathione-agarose column. Additional DEAE-Sepharose chromatography was necessary to achieve the purification of the C-terminal moiety.

Purification of parietal cells and fractionation of gastric mucosa

Rabbit parietal cells were purified to 90% homogeneity using the elutriation and Nycodenz gradient procedure developed by Chew et al. (1989) as modified (Mangeat et al., 1990). Cells were homogenized in MSEP buffer (Reenstra and Forte, 1990). The membrane fraction was recovered by high-speed centrifugation from the post-nuclear supernatant. Rabbit gastric mucosa was fractionated from resting and stimulated animals to purify either enriched resting H+-K+-ATPase tubulovesicles or crude stimulated apical membranes, respectively, as described previously (Reenstra and Forte, 1990). Rat gastric membrane fractionation and immunoprecipitation of H+-K+-ATPase were performed according to Mercier et al. (1989a). Extraction of membrane proteins was performed according to the Triton X-114 procedure (Bordier, 1981) and the carbonate extraction method at pH 11 (Fujiki et al., 1982).

Moesin and radixin purification

Moesin and radixin were partially purified, respectively, from human placenta and rat liver adherens junctions, as described (Bretscher, 1989; Tsukita et al., 1989). A radixin-enriched fraction, eluted with 60 mM NaCl from a DEAE-cellulose column (Tsukita et al., 1989) was used in this study. A moesin-containing fraction was obtained from the final Mono-S column during the course of purification of placenta ezrin (Bretscher, 1989). The identity of the moesin band was confirmed by microsequencing (Matsudaira, 1990) at the Centre National de la Recherche Scientifique Facility (Service Central d’Analyse, Vernaison, France). The sequence of the first 18 amino-terminal amino acids (PKTISVYVTMMDALEFA) was unambiguously equivalent to that reported for human moesin (Lankes and Furthmayr, 1991).

SDS-PAGE, immunoblots and blot overlays

SDS-PAGE (12.5%) contained 0.1% bis-acrylamide. Transfers on nitrocellulose membranes and immunoblots were performed as
described earlier (Mercier et al., 1989a), using 125I-Protein A and autoradiography. For blot overlays, non-specific sites were blocked with 4% BSA, 0.1% gelatin in 50 mM potassium phosphate, pH 7.4. 125I-labelled ezrin (2×106 to 10×106 cpm/ml; 0.5-2 μg/ml) was incubated for 90 minutes in overlay buffer (50 mM potassium phosphate, pH 7.4, 0.8% BSA, 0.1% gelatin and 0.5% Triton X-100, in the presence of KCl when indicated). The blots were then extensively washed with overlay buffer and autoradiographed.

Antibodies
A rabbit antibody raised against human placenta ezrin (Bretscher, 1989) was used in the blot shown in Fig. 1 (lane i). An antibody against human recombinant ezrin was raised in rabbit according to standard immunization procedure. It was used for immunocytochemistry after immunopurification on an affinity column made with the fusion protein GST-ezrin. Another antibody was also made in rabbit against the purified recombinant N-terminal domain of ezrin. A mouse monoclonal antibody (mAb 146.14; Mercier et al., 1989a), specific for H+K+ATPase β chain (Martin and Mangeat, 1994) was used to stain and to immunoprecipitate the gastric proton pump.

Cell culture, [35S]Met labelling, immunoprecipitation
HGT-1 cells were routinely cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. For biosynthetic [35S]methionine labelling, subconfluent cells were grown in mixed methionine-free medium and complete medium (4:1, v/v) supplemented with serum and antibiotics and [35S]methionine at 25 μCi/ml for 20 hours before lysis. For immunoprecipitation, labelled cells were lysed by sonication in hypotonic buffer: 10 mM Tris-HCl, pH 7.5, 0.5 mM MgCl2, 1 mM EGTA, 1 mM PMSF, 0.5 mM benzamidine, 50 mM NaF. Debris and nuclei were pelleted by centrifugation for 5 minutes at 700 g. The supernatant was then adjusted to 140 mM NaCl, 0.6 M KCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, incubated for 30 minutes at 4°C, and centrifuged for 10 minutes at 12,000 g. The extract was preincubated with immobilized-fixed Staphylococcus aureus cells (10%, v/v) by rotation for 15 minutes at 4°C. The microfuged supernatant was incubated for 6 hours at 4°C with 5 μl specific serum antibody or with the respective preimmune serum. Protein A-bearing cells were then added for 30 minutes at 4°C. Immunoprecipitates were washed 5 times, eluted with Laemmli sample buffer, and analyzed on SDS-PAGE, followed by gel drying and autoradiography at −80°C.

Iodination
Recombinant ezrin, Protein A and the gastric membrane protein extract were iodinated for 2 minutes at room temperature (1 mCi Na125I/50 μg protein) using the chloramine T procedure as previously described (Hunter and Greenwood, 1962). The reaction products were separated from free iodine on a Sephadex G-50 column equilibrated in the buffer used for the appropriate applications. The iodinated material was stored at 4°C in the presence of 5 mg/ml BSA, and 0.02% NaN3, β-Mercaptoethanol (0.02%) was also added to iodinated ezrin and gastric membrane extract. On some occasions, iodination of ezrin was performed for 7 minutes using the iodogen procedure. This latter method generated iodinated ezrin of identical quality for the overlay assay.

Microinjection and immunolocalization
For microinjection, HGT-1 cells were plated on glass coverslips and 2 days later microinjected manually as described (Mangeat and Burridge, 1984) with a mixture of recombinant ezrin and an irrelevant protein as internal control; in this case it was mAb 146.14 (HGT-1 cells do not express H+K+ATPase), at a concentration of 1 mg/ml of each purified protein in 10 mM potassium phosphate buffer, pH 7.4, 75 mM KCl. Three hours post-injection, cells were fixed and processed for double-labelling immunocytochemistry. A sublimiting amount of immunopurified anti-ezrin antibody was used in order only to faintly label endogenous ezrin. Semithin and thin cryosection on fixed rat gastric and intestinal mucosa and on Sf9 cells were performed as previously described (Mercier et al., 1989b). For insect cells infected for 40 hours with ezrin-expressing baculovirus at a multiplicity of infection of 10, Sf9 cells were scraped, washed in PBS and fixed for 1 hour with 2% paraformaldehyde and 0.01% glutaraldehyde in 100 mM sodium phosphate buffer, pH 7.4. Fixed cells were pelleted, infused with 10% gelatin and further processed for sectioning. Immunocytochemistry was performed using immunopurified anti-recombinant ezrin antibody followed by purified goat anti-rabbit IgG conjugated with either rhodamine (light microscopy) or 10 nm colloidal gold particles (electron microscopy).

Ezrin affinity assay
The HGT-1 cell extract was prepared from [35S]Met-labelled cells lysed as for immunoprecipitation, except that after pelleting of nuclei, lysis buffer was supplemented with 0.5% Triton X-100 and 140 mM NaCl. GST- or GST-ezrin agarose beads (50 μl) were preincubated for 1 hour with supplemented lysis buffer containing 4% BSA to saturate non-specific binding sites. The cell extract was incubated for 6 hours at 4°C, with the beads in lysis buffer containing 0.8% BSA. The beads were extensively washed with the same buffer. Elution of bound material was performed, after equilibrium in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.5 mM CaCl2, with thrombin, which released cleaved ezrin. The gastric membrane extract was obtained from radiolabelled rabbit apical gastric membrane extracted with Triton X-144 as described by Bordier (1981). GST- or GST-ezrin agarose beads (50 μl) were preincubated with 50 mM potassium phosphate, pH 7.4, 4% BSA, 0.5 mM EDTA, 0.02% β-mercaptoethanol, 1 mM PMSF. The aqueous fraction was incubated with the beads for 1 hour in the same buffer containing 0.8% BSA. The beads were then extensively washed with the same buffer, except that BSA was 0.1%, and 0.01% Triton X-100 was added. Elution of bound material was performed with either glutathione, which released the fusion protein, or thrombin. Eluted proteins were analyzed by SDS-PAGE, and the dried gels by autoradiography at −80°C.

Construction of baculovirus expression vector
To construct a baculovirus transfer vector containing the entire ezrin coding sequence, pGEX-Ez (previously obtained) was cut with EcoRI, then partially digested with BanHI. The longest (2.2 kb) BanHI-EcoRI fragment was gel isolated, filled-in with Klenow and blunt-cloned into the Xmal site of pGmAc34T-related vector, leading to the pGmAc34T-Ez construct. In the pGmAc34T vector, the polyhedrin start codon was mutated (a T replacing the last nucleotide, G, of the triplet) and a cloning Xmal-Smal site was introduced at a deletion between nucleotides +45 and +462. This kind of vector allowed the production of high levels of non-fused recombinant protein under the control of the polyhedrin gene promoter (Roey et al., 1992).

Insect cell culture, virus propagation and infection
We used the Autographa californica nuclear polyhedrosis virus (AcMNPV) clone 1.2 (Crozier et al., 1988) as the wild-type baculovirus. Spodoptera frugiperda (Sf9) cells (ATCC no. CRL 1711) were obtained from Dr G. Devauchelle and were used to propagate wild-type and recombinant baculoviruses. Sf9 cells were grown as monolayer cultures at 27°C in TC100 medium supplemented with 10% heat-inactivated FCS, 50 μg/ml streptomycin, 125 μg/ml penicillin and all the components from Grace medium (except yeastolate) that are missing in the TC100 powder medium, according to the procedure routinely performed in Dr Devauchelle’s laboratory.

The pGmAe34T-Ez transfer vector was cotransfected with wild-type baculovirus DNA onto Sf9 cells using the transfection reagent DOTAP. Briefly, plasmid DNA (7 μg) and viral AcMNPV wild-type DNA (0.5-1 μg) were mixed in 1.5 ml of TC100 modified medium without serum and left at room temperature for 45 minutes. A 40 μl
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sample of transfection reagent DOTAP was added just before use to a second tube containing the same volume of medium. Sf9 cells (3×10^6 cells/25 cm² flasks) plated 1 hour before in serum-free medium, were rinsed with the same medium. DNAs and DOTAP solutions were mixed and this mixture was added to the cells. Four hours later, fresh complete medium was added, and the cells were left for 4 days at 27°C. Recombinant viruses were then purified by plaque assays as described (Summers and Smith, 1987).

For infection, cells were plated at a density of 3×10^6 cells/25 cm² flask. One hour later, cells were infected with viral stocks at a multiplicity of infection of 10 and cells were harvested at appropriate times after infection, and analyzed by SDS-PAGE and immunoblots. For cell fractionation studies, cells were lysed at 4°C by sonication in 20 mM sodium phosphate, pH 7.0, 10 mM NaCl, 0.5 mM PMSF, 0.1% β-mercaptoethanol, 1 mM EGTA, 1 mM MgCl₂. Nuclei were pelleted by centrifugation for 5 minutes at 200 g. The supernatant was collected. The same volume of lysis buffer was added to the nuclear pellets, which were treated as above. The supernatants were pooled and subjected to 1 hour of centrifugation at 100,000 g. The supernatant was considered to be the cytosolic fraction and the pellet the membrane fraction. After addition of 4× Laemmli denaturation buffer, the fractions were boiled and analyzed by SDS-PAGE and immunoblotting.

Fig. 1. Characterization of human recombinant ezrin and its domains. Upper panel: schematic drawing of the ezrin constructs. (A) Human ezrin cDNA and corresponding structural domains of the protein. The successives boxes correspond to the regions of the cDNA coding for the different structural motifs of the protein. Each number refers to the amino acid position in the ezrin coding sequence: (stippling) N-homologous domain of the ezrin family of proteins; (stripes) α-helical motif; (dots on black background) heptaproline stretch; (dots on white background) C-terminal highly charged motif. The restriction sites used for the constructs are indicated by the upper letters: B, BamHI; S, SmaI; E, EcoRI. The untranslated 3’ part of the cDNA is indicated by the thin interrupted line on the right. (B) Drawing of human ezrin and domains produced in E. coli as a fusion protein with glutathione S-transferase using pGEX-2T as vector. Due to the presence of a thrombin cleavage site incorporated between the GST and ezrin moieties, the N-terminal sequence of the different cleaved products was actually modified as follows: for ezrin 1-586 and ezrin 1-309, G-S- precede amino acid 1; for ezrin 280-586 and ezrin 280-310, G-S-P- precede amino acid 280. Because of the constructions that were used, the C-terminal side of ezrin 1-309 was extended with Q-F-I-V-T-D, and that of ezrin 280-310 with N-S-S. (C) Purification of recombinant ezrin and of N- and C-terminal domains. Lanes a-h, Coomassie Blue staining of different fractions analyzed by SDS-PAGE: (a) molecular mass standards indicated in kDa on the left; (b) E. coli soluble extract bound to a glutathione-agarose column and containing 100 kDa GST-ezrin fusion protein; (c) material released from the glutathione-agarose column by treatment with thrombin and containing 80 kDa ezrin and degradation products; (d) a successive treatment of the glutathione-agarose column with glutathione released GST (note that ezrin was completely released in the previous thrombin cleavage step); (e) purified recombinant ezrin after chromatography on a Mono-S column; (f) purified ezrin N-terminal domain after glutathione-agarose binding and thrombin cleavage; (g) purified ezrin C-terminal domain after glutathione-agarose column, thrombin cleavage and DEAE chromatography (note that the polypeptide is still contaminated with proteolytic fragments); (h) purified GST-Ezpept released from a glutathione-agarose column with glutathione. (D) Western blots incubated with: lane i, anti-natural ezrin antibody (Bretscher, 1989); lanes j-l, anti-recombinant ezrin antibody; and lanes m-r, anti-recombinant ezrin N-terminal domain antibody. Gels were loaded with 2 µg of purified: (i, l, m) recombinant ezrin; (n) N-terminal domain; (o) C-terminal domain; (p) fusion protein GST-Ezpept; (q) GST; or 50 µg of: (j) rat or (k,r) rabbit gastric mucosa membranes. Molecular mass markers are indicated on the left in kDa.
RESULTS

Characterization and purification of human recombinant ezrin and fragments

pGEX-2T vector was used to express in E. coli ezrin and the truncated forms used in this study as fusion proteins with glutathione S-transferase (Smith and Johnson, 1988) (Fig. 1). The presence of a thrombin cleavage site between the GST moiety and the recombinant protein permitted the purification of ezrin or of ezrin domains by affinity chromatography on a glutathione-agarose column, and subsequent cleavage of the fusion protein and recovery of the recombinant product with thrombin. The strategy was very efficient for ezrin purification, since close to 100% of the protein was released directly from the glutathione column (Fig. 1, lanes b-d). However, a high level of degradation products eluted with the 80 kDa protein and one chromatography step on a Mono-S column was required to remove these low molecular mass contaminants (Fig. 1, lane e). This last step resulted in a dramatic loss of material. The same strategy was applied to purify either the N- or the C-terminal domain of the overlapping ezrin peptide (Fig. 1, lanes f-h). Some difficulties were encountered in recovering in a soluble form the N-terminal domain. For the C-terminal domain, major difficulties prevented the efficient recovery of pure C-terminal domain; among which were a high degree of degradation and more importantly a lack of efficient cleavage with thrombin. In these studies we mainly used the C-terminal domain as a fusion protein or, when needed, as a purified product after purification on a DEAE column. At this stage the yield of protein was very low and the protein was generally still contaminated with proteolytic fragments (Fig. 1, lane g).

The identity of the 80 kDa recombinant product with ezrin was confirmed by western blotting with an antibody raised against natural ezrin (Fig. 1, lane i). Antibodies to recombinant ezrin were also generated in rabbits (Fig. 1, lanes j-l). As previously described by Algrain et al. (1993), anti-ezrin did not recognize the N-terminal domain (not shown). Antibodies against ezrin N-terminal domain were also produced. They specifically labelled the N-terminal domain (Fig. 1, lane n) and also ezrin (lane m) and the fusion protein containing the overlapping peptide (lane p). It is interesting to note that anti-N antibodies recognized only faintly the N-terminal domain when it was incorporated into the entire protein. Both antibodies recognized one 80 kDa band present in gastric membranes (Fig. 1, lanes j, k, r) that comigrated with recombinant ezrin. In immunofluorescence studies, anti-recombinant ezrin antibody stained structures known to contain high levels of ezrin, such as gastric apical membranes and intestinal brush borders (Fig. 2). At this stage we concluded that all recombinant constructs possessed the right coding sequence and that the correct protein products were purified.

Since no specific function for ezrin could be assayed in vitro, the recombinant protein was microinjected into human gastric cells in culture in order to test its behavior in living cells. Three hours after injection into human gastric HGT-1 cells, the cells were fixed and processed for indirect immunofluorescence microscopy. Since some purified monoclonal antibody against a protein not expressed in HGT-1 cells was cojected with ezrin, the injected cells were easily identified as those that expressed fluorescence specific for anti-mouse IgG antibody (Fig. 3b,d,f). In order to detect the injected recombinant protein, anti-ezrin antibody was used at a subliming dilution, which faintly revealed endogenous ezrin in un.injected cells (Fig. 3a,e). Compared to the diffuse cytosolic pattern of injected irrelevant antibody, the ezrin pattern was strikingly different. In addition to some cytoplasmic diffuse fluorescence, specific staining was found in ruffling membranes and in numerous bright fluorescence grains (microvilli) located at the dorsal surface of the cells. These structures were similar but much brighter than those observed in non-injected cells. The increase in microvillar fluorescence in injected cells indicated that exogenous ezrin was readily incorporated into the cell microvilli. This effect did not induce any noticeable change in cell morphology; in particular, microfilament reorganization was not observed (Fig. 3c).

Characterization of potential ezrin-binding proteins

Several lower molecular mass bands, mainly at 77 and 72 kDa, were specifically communoprecipitated from [35S]Met-labelled HGT-1 cells with anti-ezrin antibody (Fig. 4, lane c). Since anti-ezrin antibody recognized a single band at 80 kDa in HGT-1 cells by western blotting (Fig. 4, lane d) these lower molecular mass bands could represent proteins that were associated with ezrin. In order to test if these HGT-1 cell proteins could indeed bind ezrin in vitro, the following experiment was carried out. Ezrin adsorbed on glutathione-agarose gel as a fusion protein was incubated with a lysate of [35S]Met-labelled HGT-1 cells. After washings, cleavage and elution of ezrin from the adsorbant were performed with thrombin. Any radioactive protein associated with ezrin should, therefore, be
recovered in the eluate fraction. Two bands running at 77 and 72 kDa were specifically recovered (Fig. 4, lane f). This experiment indicated that in the conditions used for immunoprecipitation an interaction between ezrin and some partners may indeed occur. In order to detect if the same proteins were present in the gastric tissue, the same affinity assay was applied to radioiodinated proteins extracted from rabbit gastric membranes with non-ionic detergent. In this case a protein of 77 kDa coeluted specifically with ezrin (Fig. 4, lanes i, j). Since radioiodination of a membrane extract might fail to label all the proteins present, another assay was used to characterize ezrin-binding candidates in gastric tissue. Recombinant ezrin was radioiodinated and overlaid onto a protein extract prepared from purified rabbit parietal cells. Under these conditions a
triplet band of 80, 77 and 72 kDa was labelled by $^{125}$I-ezrin (Fig. 5, upper panel). The binding was very specific, since it was totally displaced in the presence of an excess of cold ezrin (Fig. 5, lower panel). Interestingly, an equal excess of ezrin N-terminal domain displaced the binding as well, whereas addition of the C-terminal domain was partially effective and addition of the overlapping peptide present in both N and C constructs was not (Fig. 5, lower panel).

**Ezrin-binding proteins are peripheral membrane proteins**

Since gastric tissue was quantitatively available and the overlay technique was found reliable and easy to use, the ezrin-binding proteins from gastric tissue were further characterized and identified by this assay. In the first set of experiments, overlays were performed on membrane and cytosolic fractions from rat and rabbit gastric mucosa. A triplet of proteins was significantly detected in rat and rabbit gastric membrane fractions whereas it was absent from the cytosolic fraction, indicating that the three proteins were associated with membrane (not shown). No band of the triplet was directly related to the H$^+$.K$^+$-ATPase $\beta$ chain, which is part of the gastric proton pump and migrates on SDS-PAGE as a highly diffuse band at 60-80 kDa. The triplet was clearly present in non-purified tubulovesicles and disappeared with purification of H$^+$.K$^+$-ATPase-containing membranes; accordingly, no band was labelled when the proton pump was specifically immunoprecipitated with anti-H$^+$.K$^+$-ATPase $\beta$ chain (data not shown). Ezrin-binding proteins were extracted by selective methods to distinguish between integral and peripheral membrane proteins. The protein triplet partitioned with peripheral membrane proteins in extraction experiments performed with either Triton X-114 or sodium carbonate at basic pH (data not shown).

**Self-association of ezrin and interaction with radixin and moesin**

One of the ezrin-binding proteins, the 80 kDa band, comigrated with ezrin, suggesting that it might be ezrin itself. Indeed, purified recombinant ezrin overlaid with $^{125}$I-ezrin was specifically labelled, whereas another cytoskeletal protein, fimbrin, was not (Fig. 6A). When overlays were performed at high ionic strength (up to 0.6 M KCl), the labelling of ezrin and that of the 80 kDa band in gastric membrane was greatly enhanced (Fig. 6B). These combined results imply that the labelled 80 kDa band detected in gastric membrane was ezrin. To analyze further the binding properties of ezrin to ezrin, $^{125}$I-ezrin was overlaid on the purified N- and C-terminal domains of ezrin (Fig. 6C). $^{125}$I-ezrin specifically bound the N-terminal domain and no salt-effect dependency was observed.

No other clue permitted the simple identification of the two other bands at 77 and 72 kDa. These bands, however, did not appear to be ezrin degradation products, since no immunoreactive forms were detected at these molecular mass values (see western blot of rabbit gastric membranes with anti-ezrin in Fig. 1, lane k). Since ezrin self-associated, there was a possibility that ezrin might also bind ezrin-related molecules such as radixin and moesin. Fractions enriched with these two proteins were prepared from rat adherens junctions and human placenta, respectively, and subjected to ezrin overlay assay. As shown in Fig. 7, $^{125}$I-ezrin specifically labelled bands at 77 and 72 kDa corresponding, respectively, to radixin and moesin. The identity of this latter 72 kDa protein with human placental moesin was confirmed by direct N-terminal microsequencing of the first 18 amino acids. These two proteins comigrated with the 77 and 72 kDa bands present in gastric membrane and...
labelled with $^{125}$I-ezrin. We thus conclude that these two bands
may correspond to the gastric forms of radixin and moesin or
to highly related proteins.

**Accumulation of overexpressed ezrin at the plasma
membrane of insect cells**

Recombinant ezrin was also prepared using a baculovirus
expression vector. Three independent viruses were isolated and
amplified. All three expressed similarly high levels of a protein
running at the expected apparent $M_r$ for ezrin that was
immunoreactive to anti-ezrin antibody. One of these viruses
was used in subsequent experiments. A subfractionation study (Fig. 8)
as well as immunological localization at the light microscopy level (Fig. 9A)
indicated that a prominent fraction of the expressed protein appeared to be
bound to the membrane. In the particulate fractions, a high $M_r$
form of immunoreactive ezrin was also detected (Fig. 8),
presumably corresponding to dimeric ezrin. In order to document
unambiguously the ezrin accumulation at the Sf9 cell
membrane, we performed an immunological characterization
by electron microscopy. As shown (Fig. 9C-D), in addition to
diffuse cytoplasmic and nuclear staining, gold particles
labelled intensively a subplasma area of electron-dense
material that did not exist in control uninfected cells or in wild-
type baculovirus-infected cells (not shown). This electron-
dense material therefore indicated that a very high number of
ezrin molecules were recruited at the membrane, demonstrat-

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**Fig. 5.** Characterization of ezrin-binding proteins from rabbit gastric
parietal cells by $^{125}$I-ezrin overlay assay. (A) Lanes m-a, Coomassie
Blue staining showing molecular mass markers (m) indicated on the
left in kDa and a membrane fraction from 90% enriched rabbit
gastric parietal cells (a); (a’) autoradiogram of a nitrocellulose replica
of (a) overlaid with $^{125}$I-ezrin. (B) Competition overlay assay.
Autoradiograms of a nitrocellulose replica of rabbit gastric
membrane proteins incubated with 1 µg/ml $^{125}$I-ezrin alone (a) or in
the presence of 100 µg/ml of ezrin (b), N-terminal domain of ezrin
(c), C-terminal domain of ezrin (d), or ezrin peptide 288-310 (e).
Note that identical results were obtained whatever the source of cold
competitor (purified recombinant protein or enriched fusion protein).

**Fig. 6.** The 80 kDa ezrin-binding protein is ezrin. The binding is dependent on ionic strength. (A) m, a-c, Coomassie Blue staining of a 12.5%
SDS-PAGE showing (m) molecular mass markers indicated on the left in kDa; (a) crude rabbit gastric apical membranes, (b) purified
recombinant ezrin, (c) purified recombinant fimbrin. (a’-c’) Autoradiograms of nitrocellulose replicas of gel lanes a-c shown on the left overlaid
with $^{125}$I-ezrin. In (b’ and c’) 5 times less purified proteins have been loaded as compared with lanes b and c, respectively. (B) Autoradiograms
of nitrocellulose replicas of gel lanes a and b shown on the left part of A overlaid with $^{125}$I-ezrin in the presence of different concentrations
of KCl as indicated. The autoradiogram was exposed for a 4-fold shorter period of time compared with that of A. (C) Left lane, CB, Coomassie
Blue staining of (E) purified recombinant ezrin, (C) purified recombinant C domain of ezrin, (N) purified recombinant N-terminal domain of
ezrin. Right lanes: autoradiograms of nitrocellulose of gels of ezrin, C- and N-terminal domains overlaid with $^{125}$I-ezrin in the presence of the
indicated concentrations of KCl.
Self-association of ezrin at the membrane

To characterize this site, a membrane fraction of uninfected Sf9 cells was prepared and overlaid with 125I-ezrin. As shown in Fig. 10, although no cross-reactive form of ezrin could be detected with an anti-human ezrin antibody, a single band of 77 kDa band was labelled with 125I-ezrin. Interestingly, the binding increased at higher ionic strength. In addition, this 77 kDa band was characterized as a peripheral membrane protein, since most of the protein was recovered in the supernatant fraction after carbonate extraction at pH 11.

DISCUSSION

Properties of human recombinant ezrin

We made use of the pGEX-2T vector to express human ezrin and different domains of the protein in bacteria. Production of soluble forms required the bacterial culture conditions and the IPTG induction time to be set to empirical values specific for each recombinant product. Ezrin was easily and quantitatively recovered after the thrombin cleavage step, whereas cleavage was not complete for the N and especially for the C-terminal domain. Ezrin appeared very susceptible to proteolysis as is the case for the natural protein. This is a property recovered entirely in the C-terminal domain, which was very difficult to obtain in a pure non-degraded form. In contrast, the N-terminal domain was very stable with time. Since ezrin function is not known, except that it is expected to play the role of a membrane cytoskeletal linker (Hanzel et al., 1991; Algrain et al., 1993), several criteria were examined to determine if the recombinant protein was functionally active. Recombinant ezrin behaves like the natural product, by the following criteria. From the physico-chemical point of view, it runs on SDS-PAGE gels as an apparent 80 kDa band like the natural product, whereas its actual molecular mass is 69 kDa. The retarded migration is a property of the C-terminal domain as predicted from its structure (Turunen et al., 1989; Gould et al., 1989) and as observed by Algrain et al. (1993). From the immunological point of view, when antibodies were produced against the recombinant protein, they presented similar properties to those antibodies made against the human protein. In particular, they did not react against the N-terminal domain, like antibodies raised against human placenta ezrin and rabbit gastric ezrin (not shown) or human cytovillin (Algrain et al. 1993). The lack of epitopes located in the N-terminal part of the molecule is not the result of poor antigenicity of this domain, since pro-
duction of antibodies against the purified N-terminal domain was very easy in rabbits (Fig. 1). This indicates that the N-terminal domain might be located more internally than the C-terminal domain in the three-dimensional structure of ezrin. Overall, this implies that the conformation of recombinant ezrin must be rather similar to the natural product in order to show similar antigenic properties. From the physiological point of view, when ezrin was injected into living cells, it accumulated at membrane locations such as dorsal microvilli, where the endogenous protein is also found, without inducing biogenic effect. These results are in agreement with the transient transfection experiments of Algrain et al. (1993), where ezrin was shown to be incorporated into microvilli structures without generating gross morphological modifications in the cell structure. In the present study, we used chemically unmodified recombinant protein for microinjection. This clearly prevented us from distinguishing unambiguously between exogenous and endogenous ezrin in injected cells. However, the results were adequate to demonstrate the ability of the recombinant molecule to be incorporated into microvilli. Preliminary attempts to microinject fluorescently labelled or biotinylated recombinant ezrin failed. These modified molecules were not recovered in specific membrane structures (not shown). How ezrin accumulates at the membrane remains unknown at this stage. One should consider, however, that either spare sites on microvilli are occupied by additional ezrin molecules, or, alternatively, ezrin self-accumulates on pre-existing occupied sites. Overall, the above considerations allow us to conclude that recombinant ezrin exhibits the major properties of human ezrin.

Fig. 9. Membrane association of human ezrin expressed in insect cells. (a,b) Semi-thin frozen section of Sf9 cells infected for 40 hours at a multiplicity of infection of 10 with a baculovirus, expressing human ezrin labelled with affinity-purified anti-recombinant ezrin antibody followed by fluorescein-conjugated goat anti-rabbit IgG antibody; (b) phase-contrast of the fluorescent field shown in a; (c,d) thin frozen sections of Sf9 cells stained with affinity-purified anti-recombinant ezrin antibody followed by goat anti-rabbit IgG antibody conjugated to 10 nm colloidal gold particles. Note that the electron-dense material at the membrane is heavily labelled with gold particles. In contrast, the cytoplasmic unlabelled dense structure corresponds to aggregated virally encoded p10 polypeptide. The cell in c shows the typical average intensity of all the infected cells examined. The cell in d is an example of maximal labelling. Bars: a, 50 μm; c and d, 0.5 μm.

Properties of ezrin associated with ERM proteins

Immunoprecipitation of ezrin in stringent buffer conditions revealed that some specific HGT-1 cell proteins coprecipitated with ezrin. Among them were entities of 77 and 72 kDa. These were not ezrin degradation products or proteins precipitated by cross-immunoreactivity, since ezrin antibodies only recognized one protein (ezrin) of 80 kDa by western blotting. That ezrin was able to interact with cellular proteins in such high-stringency conditions was assessed by a reciprocal experiment where labelled cellular proteins were incubated on an ezrin affinity column. Proteins with similar Mr values to those that coprecipitated were eluted specifically from the column. Interestingly, using a similar affinity assay, a protein of 77 kDa was also characterized in a membrane extract prepared from a tissue, rabbit gastric mucosa, likely to contain enriched ezrin-binding protein(s). In this latter assay, radioiodination of the membrane extract could represent a limitation of the technique. An overlay assay used as a complementary method detected three potential ezrin-binding proteins in gastric parietal cells of 80, 77 and 72 kDa. They are peripheral membrane proteins, according to two different extraction criteria, and are expressed in different tissues and species. The 80 kDa component is ezrin, since: (i) it comigrated with purified ezrin; (ii) 125I-ezrin bound purified ezrin; (iii) both ezrin and the 80 kDa gastric protein were labelled by 125I-ezrin with a similar ionic strength dependency. The full recovery of the 80 kDa band in membrane fractions is in agreement with the properties of gastric ezrin (Hanzel et al., 1989, 1991). Since ezrin interacted with itself, one would expect that it might also bind ezrin-related proteins. Using enriched fractions of radixin from rat
such as coimmunoprecipitation and affinity assays. Some related molecules was supported by complementary methods, tic role in ezrin self-association. The association of ezrin with (Fig. 5). Therefore, both domains play a critical and synergis -
results showing that both domains competed for ezrin binding
dependency required the presence of both the N- and C-
primarily in the N-terminal domain (Fig. 6C), the ionic strength
bic type of interaction. Although the binding site is located
adhesion plaques that interacts with talin (Burridge and
etal molecules such as vinculin (Fig. 7), a component of
muscles, or fi mbrin (Fig. 6A), a protein that colocalizes with
the ERM gene family is remarkably conserved during
membrane protein, and the binding was salt-dependent. Since
molecular mass of 77 kDa, partitioned as a peripheral
the 77 and the 72 kDa gastric proteins, we conclude that
showed that ezrin was indeed associated with these two related
proteins. Since radixin and moesin comigrated, respectively,
expressed ezrin. Instead, as much as one third of the exogenous
accumulated at the membrane (Fig. 8). This amount is
much more important than the amount that is necessary to
account for the saturation of a binding site and suggests that
further self-accumulation of ezrin has occurred. This can be
visualized in the extreme case of the infected cell shown in Fig.
6D, where a very thick accumulation of ezrin occurred at the
membrane (thicker than 0.5 μm). Ezrin accumulation at the
membrane did not result from a side effect of overexpression,
since no inclusion bodies were observed in the cytoplasm, but
was the consequence of a specific association event. The
presence of an endogenous SF9 cell protein that binds ezrin was
characterized. It behaved similarly to one of the ezrin-binding
gastric proteins detected previously, i.e. it possessed a similar
molecular mass of 77 kDa, partitioned as a peripheral
membrane protein, and the binding was salt-dependent. Since
the ERM gene family is remarkably conserved during
evolution, two highly related genes having been detected in the
nematode, Caenorhabditis elegans (Waterston et al., 1992),
and in the parasitic cestode, Echinococcus multilocularis
(Frosh et al., 1991), the SF9 cell 77 kDa protein is probably
an insect counterpart of one of the mammalian ERM proteins.
How does ezrin accumulate at the membrane of SF9 cells?
Ezrin probably binds the endogenous 77 kDa protein or
competes with it to gain access to some unidentifi ed membrane
anchor. The binding triggers ezrin assembly in a hydrophobic
environment and further self-association accounts for the huge
amount of ezrin found at the membrane. More evidence of
ezrin oligomerization was suggested biochemically by the
detection of a high Mr form of immunoreactive ezrin (pre-
sumably dimers) in membrane and nuclear fractions (Fig. 8).
Ezrin immunolabelling in the nucleus of infected cells might
reflect a link with the reorganization of actin microfilaments
that occurs in the nucleus of infected cells and is required for
viral replication (Volkman et al., 1992).

![Image](https://via.placeholder.com/150)

**Fig. 10.** Ezrin binds a 77 kDa peripheral membrane protein in insect cells. Panel 1: Coomassie Blue staining of a 12.5% SDS-PAGE showing: (m) molecular mass markers indicated in kDa on the left; (a) purified recombinant ezrin; (b) membrane fraction from uninfected SF9 cells. Panel 2: overexposed autoradiogram of nitrocellulose replicas of lanes a and b of the gel shown in panel 1 labelled with anti-human ezrin followed by 125I-Protein A. Note that no endogenous SF9 membrane protein was found to cross-react with anti-human ezrin although the autoradiogram was very overexposed. Panel 3: autoradiogram of nitrocellulose replicas of lane b of the gel shown in panel 1 overlaid with 125I-ezrin in the presence of the indicated concentrations of KCl. Panel 4: autoradiogram of nitrocellulose replicas of gels of (c) particulate and (d) soluble fractions of uninfected SF9 membrane obtained after pH 11 carbonate extraction and overlaid with 125I-ezrin.

liver adherens junctions and moesin from human plaencta, we
showed that ezrin was indeed associated with these two related
proteins. Since radixin and moesin comigrated, respectively,
with the 77 and the 72 kDa gastric proteins, we conclude that
these latter forms might be the gastric counterparts of radixin
and moesin or related ERM proteins.

Binding of ezrin to itself and to related molecules is specifi c.
Ezrin did not bind, as shown by overlay assay, other cytoskel-
etal molecules such as vinculin (Fig. 7), a component of
adhesion plaques that interacts with talin (Burridge and
Mangeat, 1984), another member of the ezrin superfam-
y of proteins, or fimbri (Fig. 6A), a protein that colocalizes with
ezrin in membrane ruffles and in intestinal brush border
cytoskeleton. The binding was not washed out by increasing
the ionic strength of the incubation buffer. On the contrary,
the association was dramatically enhanced, revealing a hydropho-
bic type of interaction. Although the binding site is located
primarily in the N-terminal domain (Fig. 6C), the ionic strength
dependency required the presence of both the N- and C-
terminal domains in the molecule. This is consistent with other
results showing that both domains competed for ezrin binding
(Fig. 5). Therefore, both domains play a critical and syner-
gistic role in ezrin self-association. The association of ezrin with
related molecules was supported by complementary methods,
such as coimmunoprecipitation and affinity assays. Some
authors have previously noticed that ezrin (Pakkanen and
Vaheri 1989; Ulrich et al., 1986; Bretscher, 1993) and radixin
(Funayama et al., 1991) possess some tendency to oligomerize and
that stable oligomers may exist in solution (Gary and
Bretscher, 1993). Recently, the interaction of ezrin and moesin
has been documented in A431 cells, where both proteins colo-
ralized in membrane ruffles (Franck et al., 1993) and were
coprecipitated by specific antibodies raised against each other
protein, arguing for their interaction (Gary and Bretscher,
1993). Using overlay assays, the authors showed that ezrin
binds moesin and that, reciprocally, moesin binds ezrin. In
contrast to our results they did not detect any interaction with
radixin-related molecules.

**Accumulation of overexpressed human ezrin at the plasma membrane of insect cells**

Our study demonstrates that ezrin has the capacity to bind
proteins of the same family already associated with the
membrane. This was particularly clear in the case of the
expression of ezrin in insect cells, where a huge amount accu-
mulated at the plasma membrane of infected SF9 cells. This
result is surprising, since the endogenous membrane binding
site should be already occupied by an endogenous related
molecule. If human ezrin is able to compete on this site, the
site should be readily saturated with a small percentage of
expressed ezrin. Instead, as much as one third of the exogenous
ezrin accumulated at the membrane (Fig. 8). This amount is
much more important than the amount that is necessary to
account for the saturation of a binding site and suggests that
further self-accumulation of ezrin has occurred. This can be
visualized in the extreme case of the infected cell shown in Fig.
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Ezrin immunolabelling in the nucleus of infected cells might
reflect a link with the reorganization of actin microfilaments
that occurs in the nucleus of infected cells and is required for
viral replication (Volkman et al., 1992).
Physiological implications of the self-association properties of ezrin

Recent studies (Sato et al., 1992; Furthmayr et al., 1992; Berryman et al., 1993; Gary and Bretscher, 1993) support the concept that ERM proteins are coexpressed in cultured cells, whereas the situation is very different in differentiated tissues, where the proteins are differentially expressed. Ezrin concentrates at the apical microvilli membrane of epithelial cells, whereas moesin is preferably expressed in endothelial cell types (Berryman et al., 1993). A clear distribution of radixin compared to the two other proteins is not known, although radixin appears to be mainly localized at the cell adherens junctions (Tsukita et al., 1989) and in the clavage furrow of dividing cells (Sato et al., 1991). At first glance, these data appear somehow contradictory to ours, since we detected the presence of three ERM proteins in gastric parietal cells. The possibility cannot be excluded that some of these proteins originated from other cell types. However, if this was the case, during the course of purification of parietal cells, an increase in the signals for these ezrin-related proteins would have been found in cell fractions depleted of parietal cells. Clearly, that was not the case (not shown). The triplet bands appeared to be present along the different gastric cell fractions obtained during the cell purification procedure without any significant change in band intensity. On the basis of immunolocalization using a moesin-specific antibody, moesin is absent in parietal cells but present in adjacent connective tissue (Berryman et al., 1993).

If radixin and moesin are absent in parietal cells, then the detection of their presence in membrane fractions originating from 90% enriched parietal cells is very sensitive, and is significant for the specificity of the binding, and raises once again the question of the physiological meaning of this binding.

Considering that the expression and sublocalization of the various ERM proteins is different in different tissues, it is unlikely that a heteroaasociation exists in fully differentiated cells. Our data would indicate, rather, that homo-oligomerization of the proteins might occur at specific locations in cells and would explain how the protein can accumulate in a hydrophobic environment without the need for the stoichiometric proximity of a membrane binding site. This might explain in part why it is so difficult to characterize ezrin-membrane anchors. Once an anchor site is saturated, self-aggregation might be a way of further concentrating a protein at a specific location, which was proposed for the cortical protein vinculin in adhesion plaques (Avnur et al., 1983).

Finally, in addition to the well documented linker role of the N- and C-terminal domains of ezrin with the cell membrane and the actin cytoskeleton, respectively (Algrain et al., 1993), self-association of ezrin, or of other ERM proteins, might be a mechanism, among others, by which to regulate the recruitment of these molecules, from a cytoplasmic pool, at strategic membrane sites, as a consequence of an external cell stimulus.

We thank Drs Monique Arpin, Marianne Algrain and Daniel Louvard for help in the recombinant ezrin constructs and for discussion and communication of results prior to publication; Dr Antti Vaheri for providing human ezrin cDNA; Dr Anthony Bretscher for anti-ezrin antibody; Drs Martine Cerutti and Gérard Devauchelle for the pGmAc34T vector and the SI9 cell line, and the conditions of cell culture and cell transfection. This work was completed in the Centre CNRS-INSERM de Pharmacologie-Endocrinologie in Montpellier, France. Laurent Charvet is thanked for the photographic work and Anne Cohen-Solal for taking care of the animals. This work was supported in part by grants from l’Association pour la Recherche sur le Cancer (contract 6844 to P.M. and fellowship to C.A.), la Fondation pour la Recherche Médicale et l’Association Française contre les Myopathies.

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