Ezrin Contains Cytoskeleton and Membrane Binding Domains Accounting for its Proposed Role as a Membrane-Cytoskeletal Linker

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Abstract. Ezrin, a widespread protein present in actin-containing cell-surface structures, is a substrate of some protein tyrosine kinases. Based on its primary and secondary structure similarities with talin and band 4.1 it has been suggested that this protein could play a role in linking the cytoskeleton to the plasma membrane (Gould, K. L., A. Bretscher, F. S. Esch, and T. Hunter. 1989. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:4133–4142; Turunen, O., R. Winqvist, R. Pakkanen, K.-H. Grzeschik, T. Wahlström, and A. Vaheri. 1989. *J. Biol. Chem.* 264:16727–16732). To test this hypothesis, we transiently expressed the complete human ezrin cDNA, or truncated cDNAs encoding the amino- and carboxy-terminal domains of the protein, in CV-1 cells. Protein epitope tagging was used to unambiguously determine the subcellular distribution of the protein encoded by the transfected cDNA. We show that this protein is concentrated underneath the dorsal plasma membrane in all actin-containing structures and is partially detergent insoluble. The amino-terminal domain displays the same localization but is readily extractable by nonionic detergent. The carboxy-terminal domain colocalizes with microvillar actin filaments as well as with stress fibers and remains associated with actin filaments after detergent extraction, and with disorganized actin structures after cytochalasin D treatment. Our results clearly demonstrate that ezrin interacts with membrane-associated components via its amino-terminal domain, and with the cytoskeleton via its carboxy-terminal domain. The amino-terminal domain could include the main determinant that restricts the entire protein to the cortical cytoskeleton in contact with the dorsal plasma membrane and its specialized microdomains such as microvilli, microspikes and lamellipodia.

EZRIN was first characterized as a minor component of intestinal brush border microvilli (Bretscher, 1983). Related proteins were subsequently identified in various actin-based cytoskeleton structures. These include a 75-kD protein called cytovillin in microvilli of choriocarcinoma cell (Pakkanen et al., 1987; Pakkanen, 1988), an 80-kD phosphoprotein (called 80 K) in induced microvilli of gastric-stimulated parietal cells (Urushidani et al., 1989) and the p81 protein from A 431 human epidermoid carcinoma cells (Hunter and Cooper, 1981; Bretscher, 1989). Immunological studies (Gould et al., 1986; Pakkanen and Vaheri, 1989; Hanzel et al., 1991) and cDNA sequencing data (Gould et al., 1989; Turunen et al., 1989) have now established that ezrin, cytovillin, p81, and 80 K are the same protein. It is present in a wide range of cells where it is concentrated in surface protrusions (Bretscher, 1983; Pakkanen, 1988; Gould et al., 1986; Everett and Nichol, 1990).

Several observations suggested that this microvillus-associated protein could be involved in membrane-cytoskeleton interactions. Firstly, ezrin was rapidly redistributed in newly emerging microvilli on the cell surface after viral infection (Pakkanen et al., 1988), growth factor induction (Bretscher, 1989), and hormonal stimulation (Hanzel et al., 1991). Furthermore, the redistribution of ezrin in A431 cells after treatment with EGF occurred concomitantly with the phosphorylation of the protein on serine and tyrosine residues (Bretscher, 1989). Relocalization of ezrin in parietal gastric cells was also accompanied by the phosphorylation of ezrin on serine residues (Urushidani et al., 1989). However, the contribution of ezrin to the formation of these surface structures and the functional role of this phosphorylation have not been elucidated. Secondly, subcellular fractionation of EGF-treated cells, and of stimulated parietal cells, indicated that ezrin remains partially associated with the cytoskeletal fraction. Sedimentation assays revealed a weak interaction between ezrin and F-actin at low salt conditions, but none at all at physiological salt concentrations (Bretscher, 1983, 1991).

The hypothesis that ezrin plays a role in modulating the association of the cortical cytoskeleton with the membrane was further supported by the analysis of the cloned human ezrin cDNA (Gould et al., 1989; Turunen et al., 1989). The primary structure shows 37 and 23% amino-terminal domain identities with the human erythrocyte protein 4.1 (Conboy et al., 1986) and the focal adhesion protein talin, respectively (Rees et al., 1990). Futhermore, the secondary structure

prediction indicates that the carboxy-terminal region of ezrin contains an extensive α-helical region similar to that found in the carboxy-terminal domain of band 4.1 and talin. Moreover, this domain in each of these proteins, is followed by a region rich in charged amino acids (see Fig. 1 *A*). These similarities in primary and secondary structure indicate that ezrin is a member of the submembranous cytoskeleton-associated protein family, whose best characterized members are band 4.1 and talin. The role of band 4.1 in linking the spectrin–actin network to the plasma membrane in erythrocytes has been extensively studied. Band 4.1 binds to the transmembrane protein glycophorin and band 3 (Anderson and Lovrien, 1984; Anderson and Marchesi, 1985; Pasternack et al., 1985; Bennett, 1989). The glycophorin binding site in band 4.1 has been localized to the amino-terminal domain, while the cytoskeleton-binding site has been mapped in the carboxy-terminal region of the protein (Correas et al., 1986). Binding assays suggested that both the amino- and carboxy-terminal ends of talin are involved in the interaction with integrins (Horwitz et al., 1986; Simon, K. O., and K. Burridge. 1991. *J. Cell Biol.* 115:351a.), while the 190 kD carboxy-terminal fragment of talin binds to vinculin (Burridge and Mangeat, 1984).

Much less information is available for ezrin. Although its distribution in transient and stable microvillar structures and its homologies with talin and band 4.1 suggest that ezrin could also connect the cytoskeleton to the plasma membrane, no direct evidence for such interactions has been obtained so far. In particular, proteins that might interact with ezrin have not yet been identified. The identification of such components would help us to better understand the role played by ezrin in the reorganization of the cortical cytoskeleton produced by various physiological signals. In an attempt to define the functional domains of ezrin which interact with membrane components or with the cytoskeleton, we have transiently expressed the complete ezrin cDNA or truncated cDNAs encoding the amino- and carboxy-terminal domains of the protein in CV-1 cells. Here we report investigations on the subcellular distribution of these proteins by immunocytochemistry. We show that the complete ezrin and its amino-terminal domain localize underneath specialized cell surface structures containing microfilaments. In addition, its carboxy-terminal domain contains a binding site which enables it to interact directly or indirectly with the stress fibers observed near the plasma membrane at the ventral faces of cultured cells. We also discuss the advantages of using epitope protein tagging to unambiguously assess the subcellular distribution of ezrin. Indeed, recent studies report the presence in various cell types of proteins highly homologous to ezrin, such as radixin (Funayama et al., 1991), moesin (Lankes and Furthmayr, 1991), and the EM10 protein of *Echinococcus multilocularis* (Frosch et al., 1991). These recent findings have emphasized the diversity of this protein family, and suggest specific interactions with cellular membranes, the physiological relevance of which remains to be elucidated.

Materials and Methods

Materials

Cell Culture. CV1 monkey kidney cells (American type cells culture ATCC CCL 70 (American Type Culture Collection, Rockville, MD) were grown in Dulbecco's minimum essential medium supplemented with 10% FCS, at 37°C, under a 10% CO_2 atmosphere.

Antibodies. mAb (P5D4) raised against the 11-amino acid carboxy terminus of the vesicular stomatitis virus glycoprotein G was previously described (Kreis, 1986) and was kindly provided by Dr T. Kreis (European Molecular Biology Laboratory, Heidelberg, Germany). Affinity-purified polyclonal anti-ezrin antibody was obtained as previously described (Pakkanen, 1988). The rhodamine-coupled sheep anti-rabbit antibody and fluorescein-linked sheep anti-mouse IgG were purchased from Amersham Corp. (Arlington Heights, IL).

Reagents. Cytochalasin D and rhodamine-labeled wheat germ agglutinin were obtained from Sigma Chemical Co. (St. Louis, MO). Restriction enzymes were purchased from Amersham Corp., SfiI from New England Biolabs, Inc. (Beverly, MA) and Ampli Taq Polymerase from Cetus Corp. (Berkeley, CA).

Recombinant DNA Constructions

Expression of the Wild-type Human Ezrin cDNA. Human ezrin cDNA containing the complete coding sequence flanked by 90 and 400 bp of the 5′ and 3′ untranslated regions, respectively, was inserted in the EcoRI sites of the Bluescript-KS plasmid (KS-pCV6). For transient expression in the CV-1 cell line the EcoRI fragment was inserted in the corresponding sites of the eucaryotic expression vector pCB6 (a kind gift of Dr. M. Roth, University of Texas, Southwestern Medical Center, Dallas, TX). In the construct, the 5′-end of the ezrin cDNA was located downstream of the cytomegalovirus promoter.

Construction of the Fusion Ezrin-G cDNA. Ezrin was tagged by incorporating an oligonucleotide encoding the 11 carboxy-terminal amino acids of the vesicular stomatitis virus glycoprotein G into the 3′ cDNA coding sequence (Kreis, 1986). This oligonucleotide is flanked at the 5′-end either by the SfiI restriction site alone or by the SmaI-SfiI restriction sites, and at the 3′-end by XbaI, BglII, and KpnI restriction sites (see Fig. 1 *B*). To ligate the 3′-end of the ezrin cDNA coding sequence with this oligonucleotide, the ezrin cDNA starting at the unique SmaI restriction site and ending upstream of the translation termination codon was amplified. The oligonucleotides used for the amplification were designed to allow the two fragments to be ligated across the SfiI restriction site and to allow the deletion of the translation termination codon. The sense primer (5′-GGCCCGGGAGGAGAAGCATC-3′) corresponded to the sequence 999–1019 and contained the unique SmaI site at the 5′-end. The reverse primer (5′-TGGGCCTGGTGGGCCCAGGGCCTCGAACTC-3′) contained an oligonucleotide complementary to the bases 1820–1834 at its 5′ end and the SfiI restriction site at its 3′ end. Amplification of the cDNA fragment was performed using a Perkin-Elmer thermal cycler (The Perkin-Elmer Corp., Norwalk, CT) with 1 μg of DNA plasmid (KS-pCV6), primers at 100 pmoles, and 2.5 U of Ampli Taq Polymerase. The cycling parameters were 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min, for 24 cycles. The amplified fragment was purified on a 1% agarose gel and digested with SmaI and SfiI endonucleases. This 1-kb fragment was then ligated with the oligonucleotide encoding the peptide G sequence, digested by SmaI and SfiI, and with the plasmid KS-pCV6 digested by SmaI–XbaI. This ligation resulted in the replacement of the 3′ region of ezrin cDNA starting at the SmaI site by the amplified fragment linked to the tag oligonucleotide sequence. The resulting plasmid, referred to as KS-pCV6-G, was checked by double-strand sequencing using the T7 Sequencing kit (Pharmacia Fine Chemicals, Piscataway, NJ). The complete cDNA fused to the oligonucleotide was then excised from this vector after digestion by HindIII–XbaI and inserted into the corresponding restriction sites of the expression vector pCB6, downstream from the cytomegalovirus promoter (see Fig. 1 *B, b*).

Construction of Mutant cDNA Encoding the Carboxy-terminal Domain of Ezrin. The 5′-untranslated region and the 5′-coding sequence were removed by digesting the plasmid KS-pCV6-G with HindIII and BamHI. This fragment was replaced by an amplified sequence containing the 5′-untranslated region of ezrin cDNA and an ATG initiation codon. The amplification was performed according to the conditions described above. The sense primer (5′-ATAAGCTTGGGGTTGGGACA-3′) was located in the untranslated region (1–12) and was flanked at the 5′ end by HindIII restriction site. The reverse primer (5′-AACCGAAAATGCGGATCCTG-3′) contained the sequence upstream from the initiation codon (69–76), an initiator ATG and a BamHI site in frame with the coding sequence located downstream from the BamHI site (see Fig. 1 *B, d*).

Construction of Mutant cDNA Encoding the Amino-terminal Domain of Ezrin. The amino-terminal domain of ezrin was obtained by removal of the 3′-coding region 1002–1834 by digestion of the vector KS-pCV6 with

SmaI and XbaI. This fragment was replaced by the SmaI–XbaI-digested oligonucleotide encoding the tag sequence (see Fig. 1 *B*, *c*).

Transient cDNA Expression in CV I Cells

Exponentially growing CV-1 cells were seeded 24 h before DNA transfer on 3.5-cm plastic culture dishes. DNA transfer was performed following the procedure of Chen and Okayama (1987).

Cells were first incubated in the presence of the calcium-phosphate DNA precipitated for 18 h under 3% CO_2 at 37°C, washed twice with PBS containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Dulbecco's minimum essential medium supplemented with 10% FCS was then added. Cultures were analyzed for production of proteins encoded by the transfected DNA after 18- or 20-h incubation under 10% CO_2 at 37°C.

Immunoblotting

The transfected cells plated on 3.5-cm-diameter dishes were washed three times with PBS, scraped off with a rubber policeman in the presence of PBS and 1 mM PMSF and centrifuged for 10 min at 300 *g* at 4°C. After removal of the supernatant, the pellet was resuspended in Laemmli buffer (Laemmli, 1970). The samples were heated to 100°C for 2 min, then sonicated and 10 mM DTT added. Proteins were separated by electrophoresis on a 7% polyacrylamide gel in the presence of SDS. Electrotransfer to nitrocellulose and immunolabeling of the proteins were performed essentially according to the method described by Coudrier et al. (1983).

Indirect Immunofluorescence

Transfected CV-1 cells were fixed with 3% paraformaldehyde and permeabilized with 0.2% Triton X-100. Endogenous ezrin and ezrin encoded by the transfected DNA were analyzed by indirect immunofluorescence. Staining was performed in two sequential incubation steps. First, cells were incubated with polyclonal anti-ezrin or monoclonal anti-peptide G antibodies depending upon the experiment. Second, cells were incubated with fluorescent anti-IgG antibodies (conjugated to either rhodamine or fluorescein). These reagents were rabbit IgG specific in the case of polyclonal anti-ezrin antibodies or mouse IgG specific in the case of mAbs.

For fluorescent double labeling of the cell surface and ezrin, living cells on cover slips were washed with PBS at 20°C, cooled on ice, and externally labeled for 5 min at 4°C with fluorescent wheat germ agglutinin (conjugated to either rhodamine or fluorescein 10 μg/ml). After three washes with cold PBS, 3% paraformaldehyde was added and cells were allowed to warm up to 20°C. After fixation, cells were permeabilized and processed for ezrin labeling as described above.

For fluorescent double-labeling of F-actin and ezrin, cells were incubated with a mixture of fluorescent phalloidin (conjugated to either rhodamine or fluorescein, 2 U per dish) and the anti-peptide G antibody.

Detergent Extraction

Two approaches were used to define the association of the proteins encoded by the transfected cDNAs with the cytoskeleton following the procedure described by Kreis (1987).

Indirect Immunofluorescence of Detergent-Extracted CV-1 Cells. In this case, cells were briefly washed with PBS containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$. The coverslip was then dipped four times for 5 sec in four different beakers each containing 30 ml of extraction buffer MES (50 mM MES, 3 mM EGTA, 5 mM $MgCl_2$, 0.5% wt/vol Triton X-100, pH 6.4). Cells were then fixed immediately with a 3% paraformaldehyde solution.

Cell Fractionation into Detergent-Soluble and Insoluble Fractions. CV-1–transfected cells (3.5-cm dishes) were extracted on the dish for 40 sec at room temperature with 300 μl of the extraction buffer (MES) described above. Detergent-soluble fractions were precipitated 4 h in 85% acetone at −20°C and the pellets recovered after centrifugation 10 min at 300 *g* at 4°C. Detergent-insoluble material was scraped off with a rubber policeman in the presence of PBS and 1 mM PMSF and centrifugated for 10 min at 300 *g* at 4°C. Pellets containing either the detergent-soluble or insoluble material were resuspended in the same volume of Laemmli buffer (Laemmli, 1970) before separation on a 7% SDS-PAGE.

Cytochalasin D Treatment

Cells seeded on 3.5-cm dishes were treated with 2.5 μM cytochalasin D for 20 min at 37°C. After washing with Dulbecco's minimum essential medium, the cells were rapidly fixed with a 3% paraformaldehyde solution.

Results

Construction of cDNAs Coding for the Wild-type Human Ezrin and Its Amino- and Carboxy-terminal Domains

To study the subcellular distribution of ezrin and its putative functional domains, three recombinant plasmids encoding the entire human ezrin or the amino- and carboxy-terminal domains were inserted in the pCB6 eucaryotic expression vector under the control of the cytomegalovirus promoter.

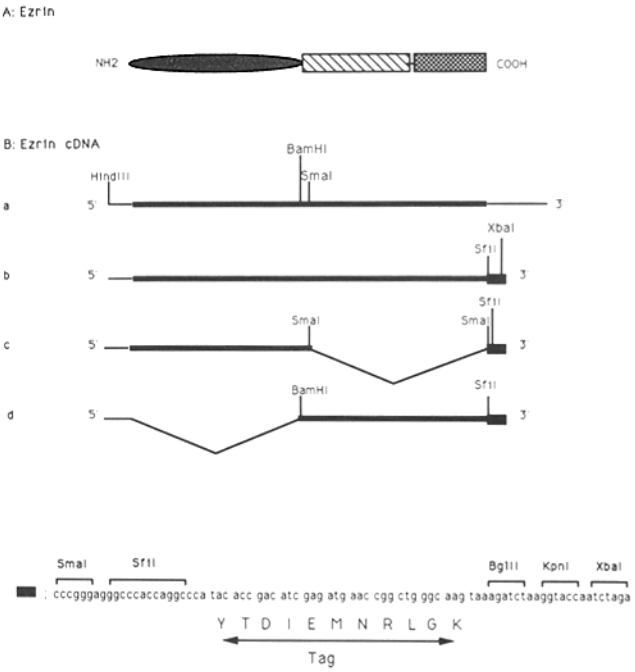

Figure 1. Representation of ezrin domains and cDNA constructs. (*A*) Model of the predicted structural organization of ezrin deduced from its primary structure (Gould et al., 1989; Turunen et al., 1989). The amino-terminal domain (*shaded oval*) is followed by a α-helical domain (▨) and by a region rich in charged amino acids (▩). (*B*) cDNA constructs for production of human ezrin and its mutants in CV-1 cells. Details of the cDNA constructs are described in Materials and Methods. For expression in the CV1 cells, three constructs (*b*, *c*, and *d*) were prepared from the human ezrin cDNA (*a*) and were inserted into the eukaryotic expression vector pCB6 under the control of the cytomegalovirus promoter. (*a*) Ezrin cDNA. Only the restriction enzyme sites that were used for the cDNA constructs are indicated. (*b*) The ezrin cDNA was fused through the SfiI site to the oligonucleotide encoding the eleven amino acids of the vesicular stomatitis virus glycoprotein tail, referred to as the tag and represented by a black box. (*c*) cDNA coding for the ezrin amino-terminal domain. The 3′-coding sequence starting at the SmaI site was deleted. The cDNA encoding the amino-terminal domain was fused to the tag sequence through the SmaI site. (*d*) cDNA coding for the ezrin carboxy-terminal domain. After deletion of the 5′-sequence by digestion with HindIII and BamHI of the ezrin cDNA construct shown in (*b*), the 5′-noncoding sequence and the ATG initiation codon were fused through the BamHI site to the cDNA encoding the carboxy-terminal domain of ezrin.

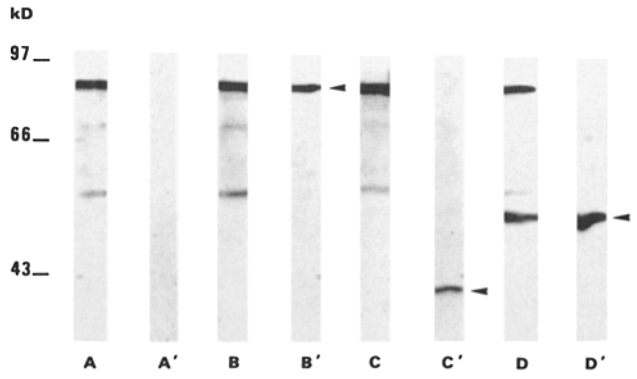

Figure 2. Immunoblot analysis of protein lysates from cells transfected with cDNA encoding the wild-type ezrin and its amino- and carboxy-terminal domains. CV-1 cells were transfected with wild-type or deleted ezrin cDNA and cell lysates were analyzed 48 h after transfection by SDS-PAGE, on a 7% polyacrylamide gel. Proteins were transferred onto nitrocellulose filters and the presence of endogenous ezrin and proteins encoded by the transfected cDNA were revealed by immunoblotting, with polyclonal anti-ezrin antibody (*A*, *B*, *C*, and *D*) and monoclonal anti-peptide G antibody (*A'*, *B'*, *C'*, and *D'*). (*A* and *A'*) Lysates from nontransfected cells. (*B* and *B'*) Lysates from cells transfected with the complete wild-type ezrin cDNA. (*C* and *C'*) Lysates from cells transfected with the ezrin cDNA encoding the amino-terminal domain of the protein. (*D* and *D'*) Lysates from cells transfected with the ezrin cDNA encoding the carboxy-terminal domain of the protein. Proteins encoded by the transfecting cDNA were detected with the anti-peptide G antibody (*B'*, *C'*, and *D'*) and are indicated by arrows. Polyclonal anti-ezrin antibody recognizes, in addition to ezrin (80 kD), two minor protein bands which could be breakdown products (*A*, *B*, *C*, and *D*).

The two domains corresponding to the amino- and carboxy-terminal ends of the protein were previously defined on the basis of their similarities to the primary and secondary structures of band 4.1 and talin (Fig. 1 *A*). Deletion of the 3′ cDNA sequence resulted in a truncated protein corresponding to the amino-terminal domain of ezrin (amino acids 1–309). The carboxy-terminal domain (amino acids 280–585) was obtained by deleting the 5′ coding sequence of ezrin cDNA. According to secondary structure predictions, this domain has extensive α-helical structure, as do talin and band 4.1. The resulting truncated proteins overlap by 30 amino acids (280–309).

Because ezrin is present in a large variety of cell lines, and its synthesis varies from cell to cell, epitope tagging was necessary to discriminate ezrin encoded by the transfected cDNA from endogenous ezrin. The eleven carboxy-terminal amino acids of the vesicular stomatitis virus glycoprotein G, preceded by a proline-rich secondary structure breaker (GPPGP), were linked in frame to the carboxy terminus of either wild-type ezrin or its deletion mutants, upstream of the stop codon (Fig. 1 *B*). Cells transfected with any of these three constructs synthesized a fusion protein detectable with antibodies raised against either wild-type ezrin or against the tag corresponding to the carboxy-terminal end of the glycoprotein G.

Synthesis of Wild-type Ezrin or its Amino- and Carboxy-terminal Domains in Transfected CV-1 Cells

Proteins in CV-1 cells transfected with DNA constructs coding for wild-type ezrin or its amino- or carboxy-terminal domains were monitored by immunoblotting, using polyclonal anti-ezrin or monoclonal anti-peptide G antibodies.

The protein lysates from untransfected cells and CV-1 cells transfected with the wild-type ezrin cDNA were analyzed by immunoblotting with the polyclonal antibody directed against ezrin (Fig. 2, *A* and *B*) or with the monoclonal anti-peptide G antibody (Fig. 2, *A'* and *B'*). In transfected cells (Fig. 2 *B'*), the monoclonal anti-peptide G antibody reacted with a protein having the same apparent molecular mass (80 kD) as endogenous ezrin (Fig. 2 *A*).

When the lysate from CV-1 cells transfected with the cDNA encoding the tagged amino-terminal domain was analyzed with anti-peptide G antibody a protein of 38 kD was recognized (Fig. 2 *C'*). This is the molecular mass of the amino-terminal domain (38,287 D) calculated from the human ezrin cDNA sequence. The anti-ezrin antibody only recognized the 80-kD polypeptide corresponding to endogenous ezrin (Fig. 2 *C*). The fact that different antibodies raised against the entire ezrin produced in *Escherichia coli* (C. Andreoli and P. Mangeat, personal communication), or purified from human cells, failed to detect this amino-terminal domain (Fig. 2 *C*) indicates that they react mainly with the carboxy-terminal domain of ezrin.

Cells transfected with the cDNA coding for the carboxy-terminal domain of ezrin produced, in addition to the endogenous protein, a protein recognized by both polyclonal antibody against ezrin and monoclonal antibody against peptide G (Fig. 2, *D* and *D'*). This protein is ~46 kD, while the calculated molecular mass of the carboxy-terminal domain deduced from the cDNA sequence is 35,339 D. The same abnormal electrophoretic migration was observed with the whole protein. Ezrin contains 585 amino acid residues, which give it a molecular mass of 69,290 D, ~12 kD smaller than its apparent molecular mass. The reduced mobility on SDS-PAGE of the entire protein, or of its carboxy-terminal domain, may be due to the presence of the seven consecutive prolines in this domain (residues 469–475) or to the high number of acidic amino acids (Turunen et al., 1989; Gould et al., 1989).

Intracellular Distribution of Wild-type Ezrin in CV-1 Cells

Transient transfections of CV-1 cells with wild-type ezrin cDNA with or without tag confirmed that this protein was produced in large amount in transfected cells compared with nontranfected cells as indicated by immunofluorescence staining with anti-ezrin antibody or anti-peptide G antibody.

To study the subcellular location of ezrin, double fluorescence staining of CV-1 cells transfected with tagged ezrin cDNA was performed with anti-peptide G antibody and rhodamine-labeled wheat germ agglutinin, which allowed observation of cell surface organization (Fig. 3, *A* and *A'*). This experiment indicated that ezrin was predominantly distributed underneath the plasma membrane and highly concentrated in all actin-containing membrane protrusions (e.g., microspikes, filopodia, and microvilli). In addition, ezrin was also detected in ruffles and leading edges (data not shown). Overexpression of ezrin cDNA did not induce striking modifications of the overall cell surface specializations. Finally, it is important to notice that the addition of the tag at the carboxy-terminal end of ezrin did not modify its subcellular localization, as compared with the oversynthesized

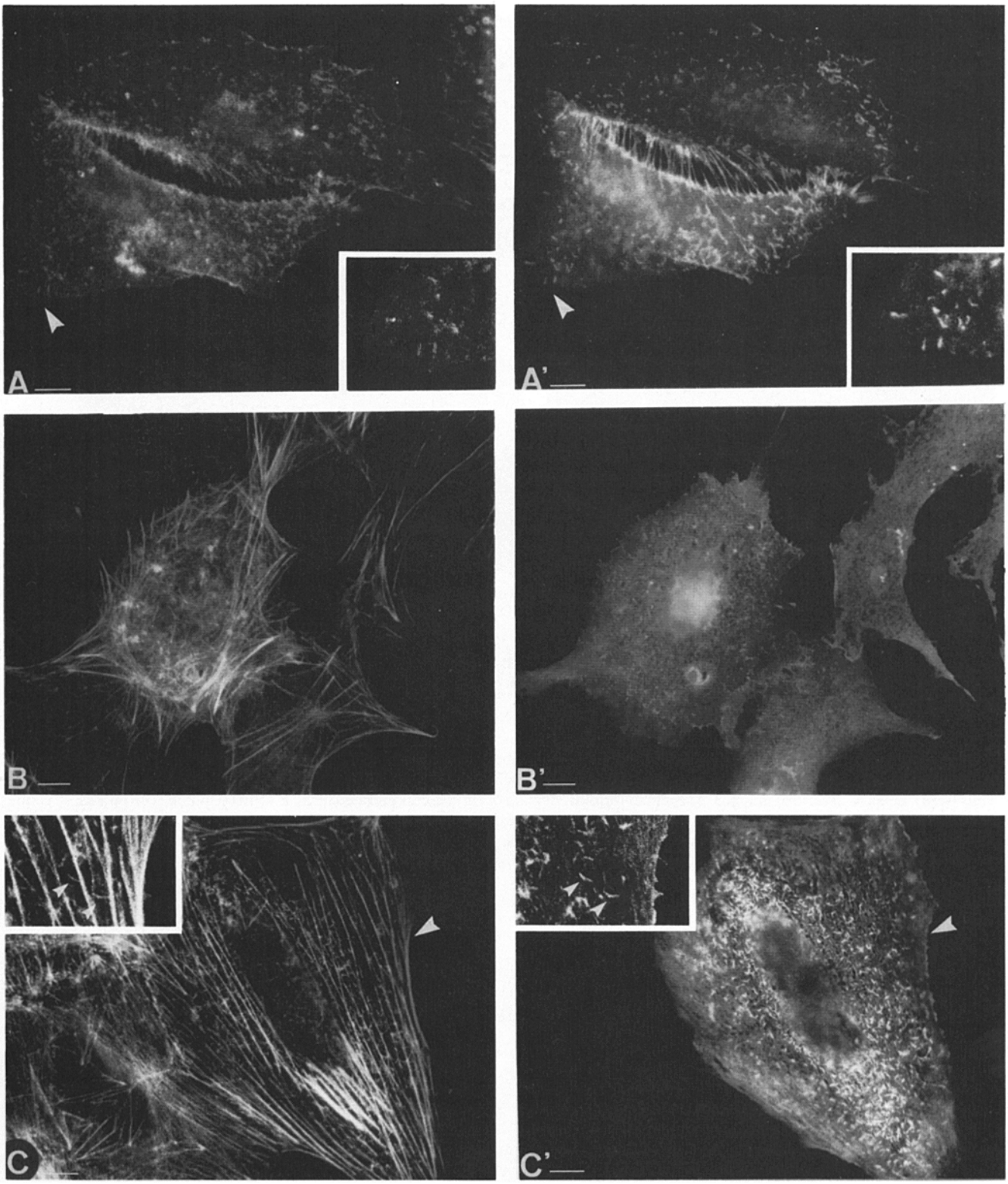

Figure 3. Localization of the wild-type ezrin in transfected cells. CV-1 cells were transfected with the tagged wild-type ezrin cDNA and analyzed by double fluorescence labeling 48 h after transfection. Double-fluorescent labeling of CV-1 cells was performed with fluorescent wheat germ agglutinin (*A*) and anti-peptide G antibody (*A'*). Living cells were labeled with rhodamine-coupled wheat germ agglutinin at 4°C for 5 min. After fixation and permeabilization of the cells with detergent (0.2% Triton X-100 for 5 min), ezrin was immunostained with anti-peptide G antibody and fluorescein-coupled anti–mouse IgG antibody. A focal plane on the dorsal face of the cell is shown. Insets correspond to twice the magnification of the area indicated by arrows. They illustrate the localization of ezrin in microvilli covering the cell surface. Double labeling of F-actin (*B*) and ezrin (*B'*). Paraformaldehyde-fixed and detergent-permeabilized cells were double labeled with anti-peptide G mAb, followed by incubation with fluorescein-coupled second antibody, and for F-actin using rhodamine-coupled phalloidin. The ezrin label is concentrated in F-actin containing cell surface structures. Ezrin does not colocalize with stress fibers. Double labeling of F-actin (*C*) and ezrin (*C'*). Cells were extracted with nonionic detergent before fixation and labeled as described above. Ezrin remains associated with the F-actin microvilli after extraction. Insets correspond to twice the magnification of the area indicated by arrows. The small arrows indicate the colocalization of ezrin with microvilli containing F-actin. Bar, 10 μm.

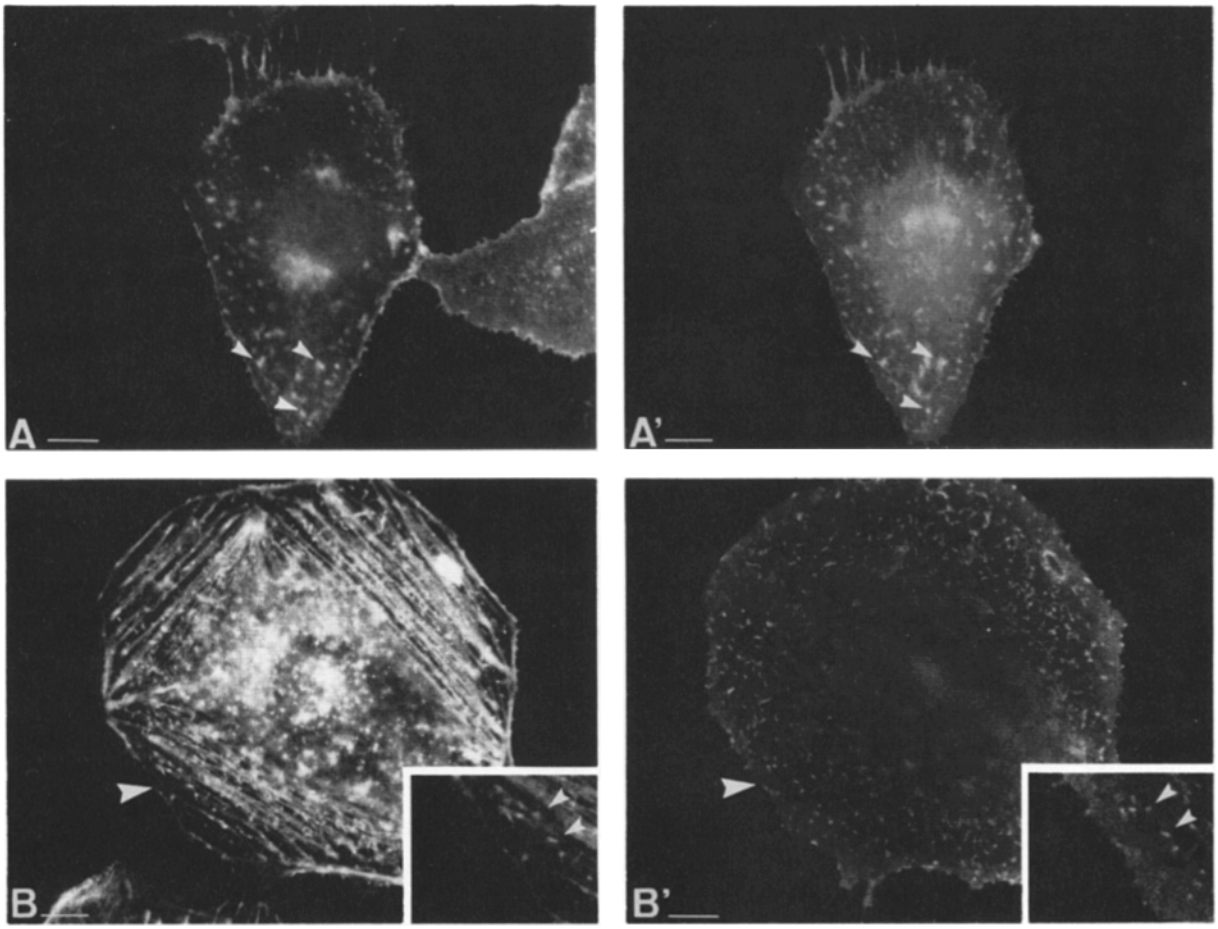

Figure 4. Localization of the ezrin amino-terminal domain. CV-1 cells were transfected with the mutant ezrin cDNA coding for the amino-terminal domain and analyzed 48 h after transfection. Double-fluorescent labeling of CV-1 cells with rhodamine-labeled wheat germ agglutinin (*A*) and anti-peptide G antibody (*A'*) was performed as described in Fig. 3. The labeled amino-terminal domain of ezrin is located in microvilli present on the cell surface (see arrows). Double-labeling of F-actin (*B*) and the amino-terminal domain of ezrin (*B'*). The cells were labeled as described in Fig. 3. The amino-terminal domain of ezrin is concentrated in the F-actin containing cell surface structures but does not appear to be associated with stress fibers. Insets correspond to twice the magnification of the area indicated by arrows. The small arrows indicate the colocalization of labeled amino-terminal domain of ezrin with microvilli containing F-actin. Bar, 10 μm.

protein encoded by the wild-type ezrin cDNA (Fig. 1 *B*, *a*) (data not shown).

To determine whether ezrin colocalized with the actin-based cytoskeleton, CV-1–transfected cells were analyzed by fluorescent double staining with anti-peptide G antibody and fluorescent phalloidin. As shown in Fig. 3, *B* and *B'* ezrin labeling and phalloidin staining were observed in the same dorsal plasma membrane structures. The diffuse stain that was also observed in these cells may be due to an excess of the synthesized protein distributed throughout the cytoplasm. However, no ezrin labeling was found associated with stress fibers, nor was there any obvious reorganization of actin-containing structures. This result suggests that ezrin can interact directly or indirectly with the cortical actin cytoskeleton. To check this possibility, we used a mild detergent extraction of living cells before fixation or cell fractionation, following a procedure described by Kreis (1987). It is well established that this extraction procedure solubilizes membrane proteins and releases cytosolic proteins, but maintains cytoskeletal integrity. Double fluorescence staining with anti-ezrin antibody and phalloidin was performed after extraction and fixation of the cells (Fig. 3, *C* and *C'*). Under these conditions, ezrin remained associated with the actin filaments which compose the cortical cytoskeleton. This observation was confirmed by fractionation into detergent-soluble and insoluble material of CV-1 transfected cells. Immunoblotting performed with the anti-peptide G antibody on soluble (see Fig. 6 *A*) or insoluble fractions (see Fig. 6 *A'*) indicated that the protein encoded by the ezrin cDNA was present in both fractions. A large fraction of synthesized protein was recovered into the soluble fraction probably due to its overproduction in transfected cells. However, a significant fraction of protein remained associated with the insoluble material, in agreement with our immunofluorescence studies performed on transfected cells treated in the same conditions (Fig. 3, *C* and *C'*).

Intracellular Distribution of the Amino- and Carboxy-terminal Domains of Ezrin in CV-1 Cells

To assess the contribution of ezrin domains to its specific localization and to its association with the cytoskeleton, we transfected CV-1 cells with the cDNAs encoding the two ezrin domains.

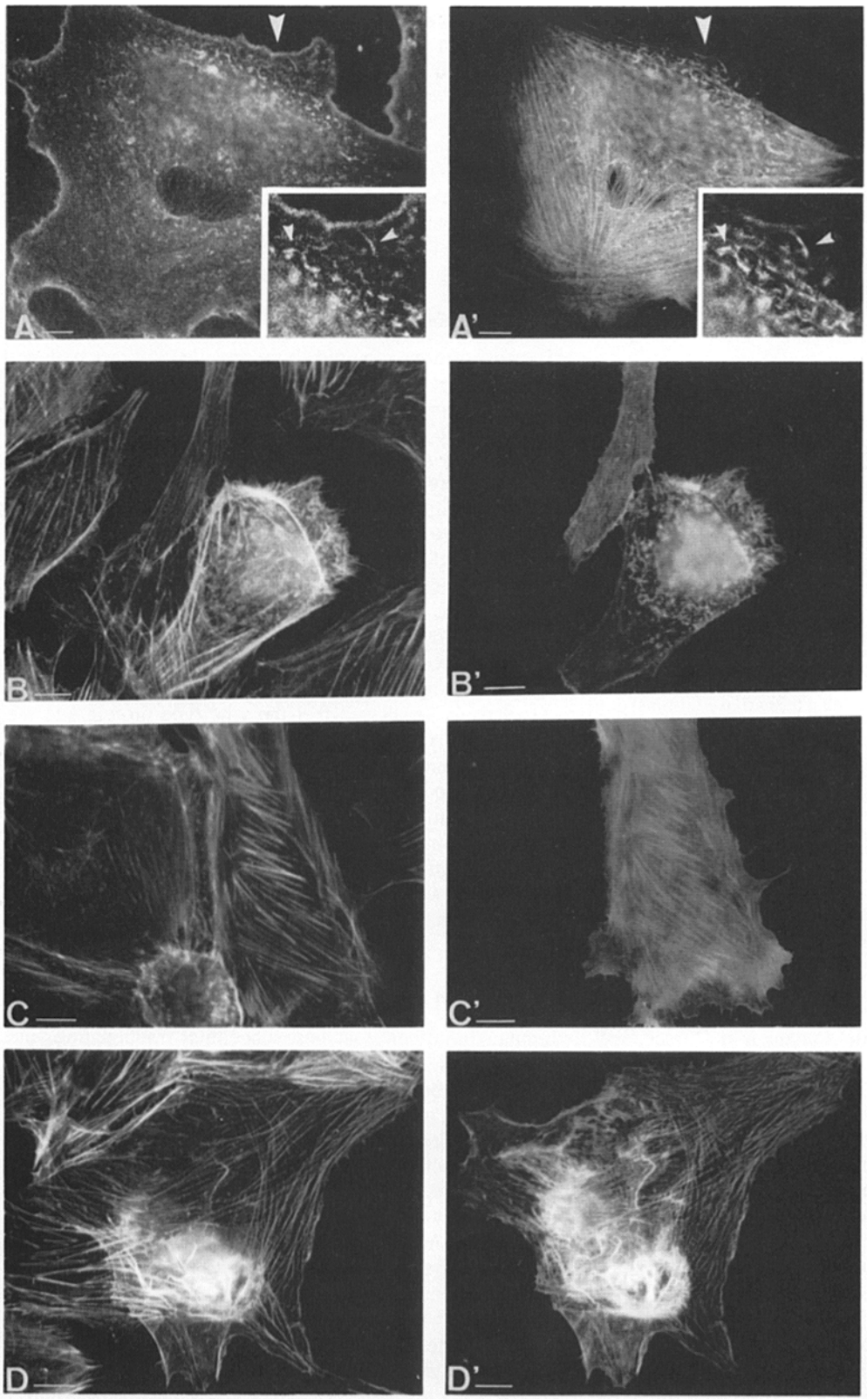

Figure 5. Localization of the carboxy-terminal domain of ezrin. CV-1 cells were transfected with the mutant ezrin cDNA coding for the carboxy-terminal domain. Double fluorescent labeling of CV-1 cells with rhodamine-labeled wheat germ agglutinin (*A*) and anti-peptide G antibody (*A'*) was performed as described in Fig. 3. Insets correspond to twice the magnification of the area indicated by arrows. The small arrows indicate the colocalization of labeled carboxy-terminal ezrin with stained microvilli present on the cell surface. Double labeling of F-actin (*B* and *C*) and the carboxy-terminal domain of ezrin (*B'* and *C'*). Cells were labeled as described in Fig. 3. The focal plane (*B* and *B'*) on the dorsal face of a positive cell is shown. The carboxy-terminal domain is concentrated in microvilli. The focal plane (*C* and *C'*) shows the bottom of the cell where stress fibers can be clearly seen. Note the colocalization of the label of the carboxy-terminal domain with that of stress fibers. Double labeling of F-actin (*D*) and the carboxy-terminal domain of ezrin (*D'*) was performed on cells extracted with non ionic detergent, before fixation, as described in Fig. 3. The images are focused at the bottom of the cells. The association between the carboxy-terminal domain and stress fibers is maintained after the extraction procedure. Bar, 10 μm.

The Amino-terminal Domain. CV-1 cells transfected with the recombinant cDNA coding for the amino-terminal domain were analyzed by a double-fluorescence procedure with the anti-peptide G antibody and rhodamine-labeled wheat germ agglutinin. A pattern of localization similar to that observed with the wild-type protein was obtained. This domain was concentrated in cell surface protrusions (Fig. 4, *A* and *A'*). Moreover, it colocalized with actin filaments present in these structures, as shown by double fluorescence labeling with rhodamine-labeled phalloidin and anti-peptide G antibody (Fig. 4, *B* and *B'*). This colocalization was restricted to the cortical actin cytoskeleton since no labeling was found

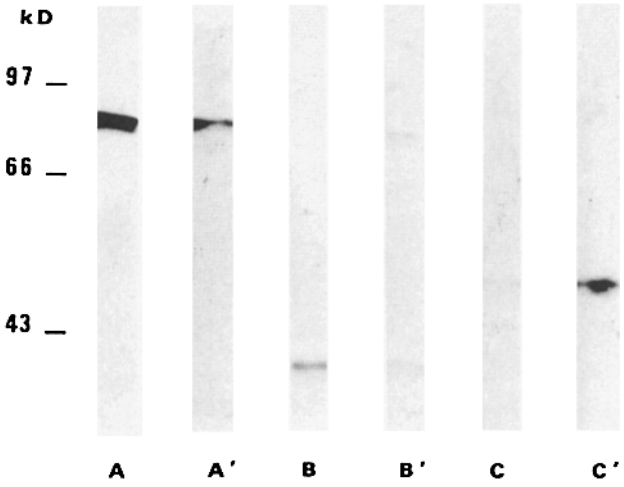

Figure 6. Immunoblot analysis of cell fractions obtained by detergent extraction. CV-1 cells transfected with the wild-type or deleted ezrin cDNAs were detergent extracted (see Materials and Methods). Stoichiometric amounts of the detergent soluble (*A*, *B*, and *C*) and insoluble fractions (*A'*, *B'*, and *C'*) were separated on a 7% polyacrylamide gel and the proteins were transferred onto nitrocellulose filters. Proteins encoded by the transfected cDNA were probed with the monoclonal anti-peptide G antibody. (*A* and *A'*) Lysates from cells transfected with the complete ezrin cDNA. (*B* and *B'*) Lysates from cells transfected with the ezrin cDNA encoding the amino-terminal domain of ezrin. (*C* and *C'*) Lysates from cells transfected with the ezrin cDNA encoding the carboxy-terminal domain of ezrin.

associated with stress fibers. However, a major difference was observed in the behavior of the wild-type protein and the amino-terminal domain. While the wild-type protein remained associated with the actin filaments present in membrane protrusions after detergent extraction, the amino-terminal fragment was fully extracted (data not shown). Immunoblotting analysis of the detergent-soluble (see Fig. 6 *B*) and insoluble (see Fig. 6 *B'*) fractions indicated that the amino-terminal fragment was mainly present in the soluble fraction. This suggests that this domain is not associated or is only weakly associated with the actin cytoskeleton. Whether the extracted protein is associated or not with extracted plasma membrane proteins remains to be elucidated.

The Carboxy-terminal Domain. When immunostaining was performed with the anti-peptide G antibody and rhodamine-labeled wheat germ agglutinin on cells transfected with the truncated ezrin cDNA coding for the carboxy-terminal domain (Fig. 5, *A* and *A'*), we detected this protein fragment in all cell surface structures. Moreover, double-fluorescence labeling performed with anti-tag antibody and rhodamine-labeled phalloidin showed a colocalization of this protein fragment with actin-containing structures underneath the plasma membrane (Fig. 5, *B* and *B'*). But, in contrast with what was observed with the wild-type protein or with the amino-terminal domain, images focused at the ventral faces of cells also showed a colocalization of the carboxy-terminal domain with stress fibers (Fig. 5, *C* and *C'*). The carboxy-terminal domain appeared to be distributed along the stress fibers, but excluded from focal contacts (data not shown). In a few cells transfected with this construct, we also observed that stress fibers were disorganized and aggregated (Fig. 5 *D'*). However, concomitant disorganization of microtubules or intermediate filaments was not seen in any samples (data not shown).

Double-fluorescence labeling performed on the detergent-extracted cells allowed us to confirm the tight association of the carboxy-terminal domain with the dorsal cortical microfilament network, as well as with stress fibers of ventral faces (Fig. 5, *D* and *D'*). Furthermore, after detergent extraction and fractionation, most of the carboxy-terminal fragment was found in the insoluble fraction as shown by immunoblotting analysis (Fig. 6, *C* and *C'*).

Cytochalasin D Treatment of Transfected Cells

The observations reported above provided indirect evidence that ezrin plays a role in linking the actin cytoskeleton to the plasma membrane. However such a direct interaction of ezrin with actin filaments was not found in in vitro binding assays (Bretscher, 1983). Our observations on the association of the carboxy-terminal domain of ezrin suggest the presence of an actin-binding site in this region of the molecule. In an attempt to further analyze this association and to determine whether the disorganization of the actin filaments would also lead to a redistribution of the carboxy-terminal domain, a concentration of cytochalasin D known to disrupt the stress fibers (2.5 μM) was applied for 20 min to cultured cells. Double-fluorescence labeling with anti-peptide G antibody and fluorescent phalloidin performed on cells treated with cytochalasin D showed that the wild-type protein and the amino-terminal domain remained evenly distributed underneath the membrane (Fig. 7, *A*, *A'*, *B*, and *B'*). We did not observe a codistribution of these proteins with actin foci resulting from the cytochalasin D action on the stress fibers. In contrast, in cells transfected with the cDNA coding for the carboxy-terminal domain and treated with cytochalasin D, we observed a codistribution of the carboxy-terminal domain and the actin patches (Fig. 7, *C* and *C'*).

Discussion

Immunofluorescence studies, subcellular fractionation, and analysis of the primary structure of ezrin have suggested that this protein belongs to a class of proteins able to link the cytoskeleton to the membrane. However, no direct evidence for such a function has yet been reported. In an attempt to map the functional domains of this protein, we expressed wild-type and truncated human ezrin cDNAs in CV-1 cells. In this paper, we show that transient transfection of human ezrin cDNA in these cells led to a production of the protein, which displayed a specific distribution pattern. The ezrin label was concentrated in the dorsal microvillar surface structures, all of which contain actin filaments. This is in agreement with the localization of endogenous ezrin reported in a wide range of cells by immunofluorescence studies (Bretscher, 1983, 1989; Pakkanen and Vaheri, 1989; Hanzel et al., 1989).

In contrast to what was previously observed upon overproduction of two actin-binding proteins, microvillus induction by villin (Friederich et al., 1989) and stress fiber disruption by gelsolin (Finidori et al., 1992), high level production of ezrin did not induce changes in cell surface organization or modification of the cytoskeleton.

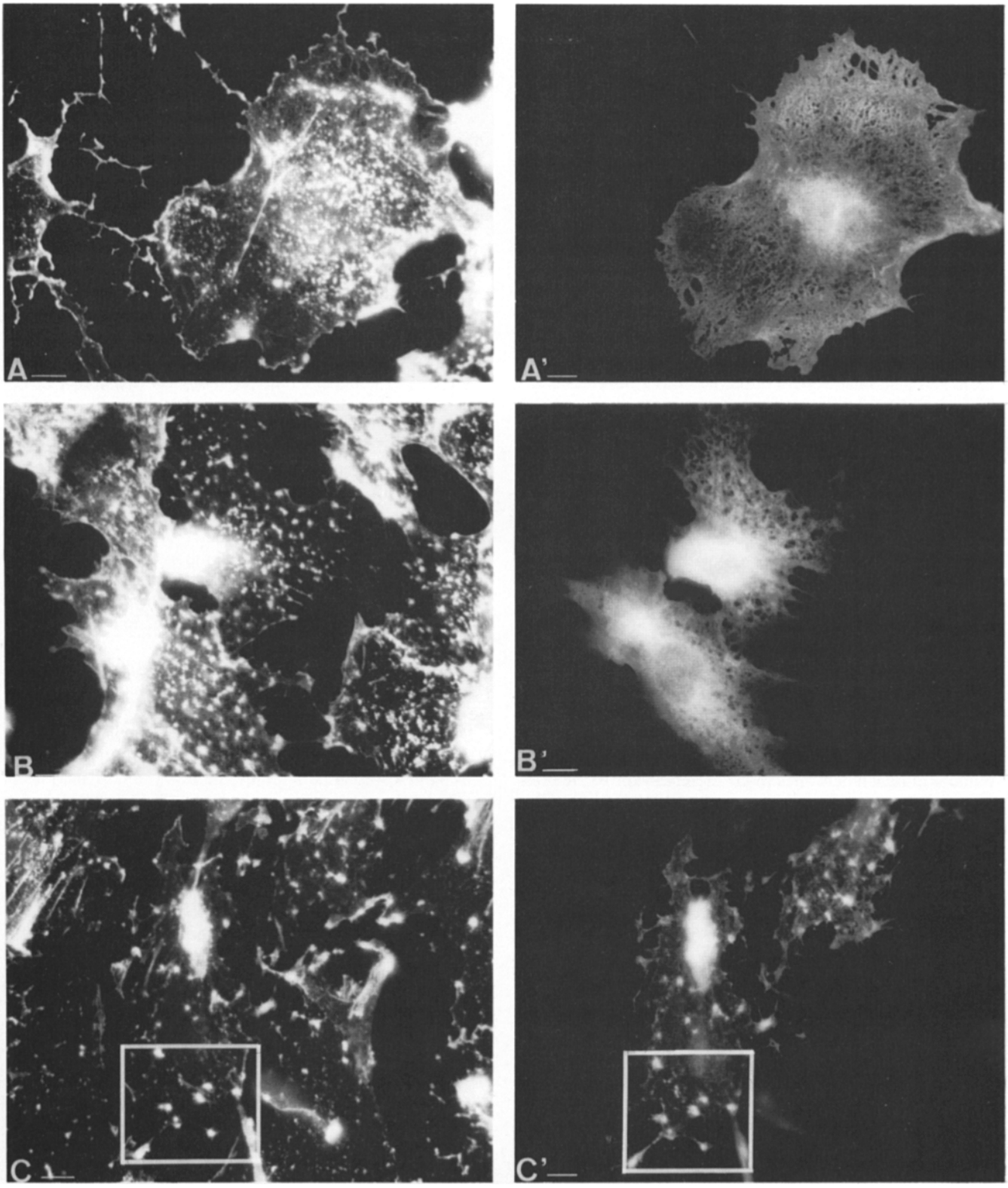

Figure 7. Localization of wild-type ezrin and its domains after disruption of stress fibers by cytochalasin D. Cells were transfected with the complete ezrin cDNA (*A* and *A'*), the cDNA coding for the amino-terminal domain (*B* and *B'*), or the cDNA coding for the carboxy-terminal domain (*C* and *C'*). Cells were incubated with 2.5 μM cytochalasin D for 20 min at 37°C, subsequently fixed, and double labeled for F-actin with rhodamine–phalloidin (*A*, *B*, and *C*) and for recombinant ezrin with anti-peptide G antibody (*A'*, *B'*, and *C'*) as described in Fig. 3. Note the colocalization of the carboxy-terminal domain with the F-actin patches (clearly visible in the frame), while wild-type ezrin and its amino-terminal domain remain localized underneath the plasma membrane. Bar, 10 μm.

Considering the structural relationship between ezrin, talin, and band 4.1, we investigated the subcellular distribution of ezrin domains. The amino-terminal domain of ezrin, which shares sequence homology with the amino-terminal domain of band 4.1 and talin, is located in the same parts of the cell as the complete protein, primarily underneath the dorsal membrane. Neither the wild-type nor the amino-terminal domain of ezrin appeared on ventral membrane surfaces. Interestingly, no preferential labeling was observed in the focal contacts where talin concentrates (Burridge and Connel, 1983). The carboxy-terminal domain of ezrin was also distributed in a similar pattern to ezrin and its amino-terminal domain; it was detected underneath the dorsal face of the cell membrane in actin-containing cell surface structures. Somewhat surprisingly, this domain also accumulated along the stress fibers, where the entire protein is not normally detected. The same overall patterns of distribution of ezrin and its domains were observed in 3T3 fibroblast cells (data not shown).

The colocalization of ezrin with actin-containing structures suggested that this protein is associated with actin filaments. To determine whether only one or both of the two domains were involved in the interaction with the cytoskeleton, we examined, by immunofluorescence and cell fractionation the behavior of the proteins encoded by the transfected cDNA in living CV-1 cells treated with nonionic detergent. Immunofluorescent analysis and biochemical fractionation into detergent-soluble and insoluble fractions demonstrated that ezrin and the carboxy-terminal domain remained associated with the cytoskeleton, while the amino-terminal domain was readily solubilized. But if the carboxy-terminal domain was found almost exclusively in the detergent-insoluble fraction, a significant proportion of the wild type protein encoded by the cDNA was found in the soluble fraction. This may suggest that there exists, for this protein, a saturable number of attachment sites to the membrane or to the cytoskeleton. Unlike the complete protein, the carboxy-terminal domain does not discriminate between the F-actin structures present in the fibroblast and its association along the stress fibers provides an increased number of binding sites.

The association of the carboxy-terminal domain alone with the microfilaments was confirmed using cytochalasin D. While the submembranous location of ezrin and its amino-terminal domain was not significantly affected by this treatment, the carboxy-terminal domain colocalized with the patches resulting from the disorganization of stress fibers. Moreover, we have shown, using different expression constructs, that the carboxy-terminal region of ezrin is capable of binding to actin in cell extracts (Turunen and Vaheri, manuscript in preparation). Altogether, these results show that the entire protein and its carboxy-terminal domain have at least one site that contributes to a direct or an indirect association with the F-actin cytoskeleton. Whether the complete protein and its carboxy-terminal domain interact with the actin cytoskeleton through the same site remains to be determined. For instance, a cryptic actin-binding site in the carboxy-terminal domain that is unmasked in the ezrin variant lacking the amino-terminal domain may account for this observation.

Altogether, these data suggest an association of the complete ezrin by its carboxy-terminal domain with the cytoskeleton, and favor the idea that the amino-terminal domain could interact with membrane components. Although the homologous amino-terminal domain of talin and band 4.1 have been assigned a role in the binding of these proteins to membrane proteins (Anderson and Lovrien, 1984; Anderson and Marchesi, 1985; Pasternack et al., 1985; Horwitz et al., 1986), a recent report indicates that this association may not be restricted to this domain (Simon, K. O., and K. Burridge. 1991. *J. Cell Biol.* 115:351a). Our experiments do not allow us to exclude the presence of a low affinity cytoskeleton binding site in the amino-terminal domain or the association of the carboxy-terminal domain with the membrane, as has been shown for talin in recent studies (Simon, K. O., and K. Burridge. 1991. *J. Cell Biol.* 115:351a). Moreover, the different behavior of complete ezrin and its carboxy-terminal domain stress the importance of sequences located throughout the complete protein for correct localization.

To further understand the physiological function of ezrin, the molecular nature of its interaction with both actin filaments and with membrane components remains to be clarified. Our experiments show that ezrin interacts only with F-actin present in the cortical cytoskeleton. Whether this specificity of interaction is due to a particular organization of these actin filaments or to the proteins associated with them has to be elucidated. Another unanswered question concerns the role of membrane components in this localization of ezrin on cortical cytoskeleton. Finally, the possibility that a posttranslational modification of ezrin changes its capacity to interact with the plasma membrane and the cytoskeleton has to be considered.

Protein tagging allowed us to analyze unambiguously the subcellular distribution of ezrin and its domains, and to discriminate between the proteins encoded by transfected cDNA and the endogenous gene, as well as related proteins. Indeed, three other members have recently been included in this band 4.1-ezrin-talin family: moesin (Lankes and Furthmayr, 1991), radixin (Funayama et al., 1991), and EM10 protein (Frosch et al., 1991), which share 72, 74.9, and 42.6% identities with ezrin, respectively. Ezrin, radixin, and moesin are coexpressed in various cell types and concentrated in regions where actin filaments are associated with the plasma membrane (Sato et al., 1992). However precise localization of these proteins requires the obtention of antibodies specific to each member. Each of these proteins may be restricted to a specific compartment of polarized cells. If this is the case, it will be important to understand which features determine their specific association with specialized cell surface structures.

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