Soluble Ezrin Purified from Placenta Exists as Stable Monomers and Elongated Dimers with Masked C-Terminal Ezrin−Radixin−Moesin Association Domains[†]

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Received September 26, 1995; Revised Manuscript Received November 3, 1995[⊗]

ABSTRACT: Previous work has indicated that ezrin, a membrane−microfilament linking protein, exists largely as a monomeric protein in solution. Here we purify from human placenta two cytosolic ezrin species that chromatograph differently on gel filtration, anion, and cation exchange resins. Both species contain only the ezrin polypeptide, yet they do not readily interconvert *in vitro* as determined by gel filtration analysis. Determination of the physical properties of the two species indicates that one represents the conventional monomer, whereas the other represents highly asymmetric dimers. Chemical cross-linking data support this conclusion. Purified ezrin monomers normally have a masked C-terminal domain (termed a C-ERMAD) that, upon exposure, can associate with an N-terminal domain (termed N-ERMAD) of another ezrin molecule. Here we show that purified ezrin dimers also have masked C-ERMADs. On the basis of these results, we suggest a working model for the molecular organization of ezrin monomers and dimers and propose a hypothesis that explains the stable coexistence of ezrin monomers and dimers in placenta. Since radixin and moesin, the two other members of the closely related ERM protein family, both contain N- and C-ERMADs, the results we have documented and models proposed for ezrin are likely to apply to radixin and moesin as well.

Ezrin was originally isolated as a cytoskeletal component of intestinal microvilli and found to be enriched in actin-containing cell surface structures in a wide variety of cultured cells (Bretscher, 1983; Pakkanen, 1988). Analysis of its cDNA-derived protein sequence (Gould et al., 1989; Turunen et al., 1989) revealed a relationship to the N-terminal domain of band 4.1, the microfilament−membrane linking protein of the erythrocyte cytoskeleton (Anderson & Lovrien, 1984; Marfatia et al., 1994). This relationship, and the close association of ezrin with the plasma membrane of microvilli as determined by immunoelectron microscopy (Berryman et al., 1993, 1995), strongly suggested that ezrin is also a microfilament−membrane linking protein. These data led to a hypothesis in which the N-terminal ~300 residues of ezrin specified a membrane binding domain and the remaining ~285 residues contained a region involved in attachment to the cytoskeleton. In support of this hypothesis, transfection studies revealed that constructs expressing the N-terminal domain associated with the plasma membrane *in vivo*, whereas C-terminal constructs colocalized with F-actin (Algrain et al., 1993).

Ezrin shows about 75% sequence identity with two other proteins, radixin and moesin (Funayama et al., 1991; Lankes & Furthmayr, 1991), that make up what has come to be known as the ERM[1] family (Sato et al., 1992). By analogy with band 4.1 and ezrin, all members of the ERM family are now believed to be membrane−cytoskeletal linking proteins. Indeed, Tsukita et al. (1994) have shown that these proteins can associate with the transmembrane protein CD44, and Turunen et al. (1994) and Pestonjamasp et al. (1995) have used different approaches to identify an F-actin binding site in the 34 C-terminal residues of ezrin that is highly conserved between all family members.

Although ezrin was originally isolated and characterized as a monomeric protein in solution (Bretscher, 1983; Narvanen, 1985; Ullrich et al., 1986), two groups have recovered ezrin from cultured cells in what appeared by gel filtration analysis to be two different species (Ullrich et al., 1986; Pakkanen & Vaheri, 1989). More recently, we have provided evidence that ezrin and moesin can undergo homotypic and heterotypic associations *in vivo* and *in vitro* (Gary & Bretscher, 1993, 1995).

In this study, we describe the development of a procedure for the separation and purification from placental extracts of the two distinct ezrin species. These are identified as ezrin monomers and dimers, and we show that they do not readily interconvert *in vitro*. The physiological relevance of stable ezrin monomers and dimers, their molecular organization, and the possible conversion of monomers to dimers are discussed.

EXPERIMENTAL PROCEDURES

Purification of Monomeric and Dimeric Ezrin. A modification of a previously reported procedure (Bretscher, 1989)

[†] This work was supported by NIH Grant GM36652. During part of these studies, Dr. Berryman was supported by an NIH Postdoctoral Fellowship (GM14352) and Dr. Gary was supported by an NIH Predoctoral Training Grant (GM07273).

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[⊗] Abstract published in *Advance ACS Abstracts,* December 15, 1995.

[1] Abbreviations: ERM, ezrin−radixin−moesin; ERMAD, ERM association domain; PVDF, poly(vinylidene fluoride); DSP, dithiobis(succinimidylpropionate).

0006-2960/95/0434-16830$09.00/0

was used. All procedures were carried out at 4 °C or on ice. About 150 g of human placenta was homogenized by blending hard in six volumes of 0.15 M NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 10 mM Tris-HCl (pH 7.5), 0.5 mM PMSF, and 0.5 mM benzamidine. The homogenate was clarified by centrifugation in a Sorvall GSA rotor at 13 000 rpm for 15 min and then again at 13 000 rpm for 30 min. Ammonium sulfate was added to 40% saturation (22.6 g/100 mL) and the precipitated material removed by centrifugation at 13 000 rpm for 15 min in the GSA rotor. The supernatant was made 65% saturated by addition of an additional 15.3 g/100 mL ammonium sulfate. This precipitate was harvested by centrifugation and resuspended in a minimal volume (about 15 mL) of dialysis buffer [20 mM NaCl, 0.5 mM DTT, 10 mM imidazole hydrochloride (pH 6.7), 0.5 mM PMSF, and 0.5 mM benzamidine]. Diisopropyl fluorophosphate was added (0.1 mL) to inactivate proteases and the extract dialyzed twice for 2 h against 2 L of dialysis buffer. During this dialysis, a small amount of precipitate formed and was removed subsequently by centrifugation at 20 000 rpm for 10 min in a Sorvall SS34 rotor. The extract was made 100 mM in potassium phosphate by the addition of one-seventh of a volume of 0.8 M K_2HPO_4–KH_2PO_4 (pH 7.0) and reclarified by centrifugation. The extract, which was typically 40 mL, was then applied to a 150 mL hyroxyapatite (HA Ultrogel, LKB Instruments Inc.) column at a rate of 1.5 mL/min. The column was first washed with 200 mL of 100 mM K_2HPO_4–KH_2PO_4 (pH 7.0) and then with a 250 mL linear gradient of 0.1 to 0.2 M K_2HPO_4–KH_2PO_4. The column was eluted at 1.5 mL/min with a 150 mL linear gradient of 0.2 to 0.8 M K_2HPO_4–KH_2PO_4, and 5 mL fractions were collected. The polypeptide composition of fractions was evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and those rich in ezrin were pooled and dialyzed vigorously over 8 h with three changes of 2 L buffer [0.15 M NaCl, 5 mM EDTA, 1 mM DTT, and 10 mM Tris-HCl (pH 7.5)]. The dialyzed material was made 63% in ammonium sulfate (38.3 g/100 mL) and the precipitated protein collected and dissolved in gel filtration buffer [20 mM Bis-tris-propane, 100 mM NaCl, 0.2 mM 1,2-bis(2-aminoethoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 0.5 mM DTT (pH 6.7)]. After clarification, the protein was applied to a 150 mL Sephacryl S300HR column which was run at 0.1 mL/min. Fractions (2 mL) were collected and ezrin dimers typically eluted in fractions 27–30 and ezrin monomers in fractions 32–36. These fractions were pooled separately, and each was chromatographed on a 1 mL Poros 10 HQ column (PerSeptive Biosystems, Inc., Cambridge, MA) in gel filtration buffer using a 20 mL gradient from 20 to 500 mM NaCl. Fractions rich in ezrin were pooled, diluted 3-fold with water, applied to a 1 mL Poros 10 SP column equilibrated with 20 mM MES, 20 mM NaCl, and 0.5 mM DTT (pH 6.7), and developed with a 20 mL 20 to 500 mM NaCl gradient in this buffer. Ezrin monomers and dimers purified by this method were stable for at least 1 week at 4 °C. Typically, 1–2 mg of ezrin monomer and 0.5 mg of ezrin dimer were recovered from 150 g of placental tissue.

Assay of N- and C-ERMAD Activities. Purified ezrin monomers and dimers were biotinylated for use as soluble probes (Gary & Bretscher, 1993). Slot blots containing immobilized glutathione *S*-transferase (GST) fusion proteins consisting of ezrin residues 1–439, which contains a functional N-ERMAD, and residues 479–585, which contains a functional C-ERMAD, were also prepared as described (Gary & Bretscher, 1995). To test whether the biotinylated probes contained accessible or masked ERMADs, the activities of native and SDS-unfolded probes were compared.

Other Techniques. Protein cross-linking with the homobifunctional reagent dithiobis(succinimidylpropionate) (DSP) was performed as follows. Gel filtration fractions were made 1 mM in DSP and placed on ice for 45 min. SDS sample buffer was added, and the samples were boiled before analysis by nonreducing SDS–PAGE. Stokes radii of the two ezrin species were determined on a Sephacryl S300HR column calibrated with bovine thyroglobulin (85 Å), equine apoferritin (67 Å), and bovine serum albumin (35.5 Å). Sedimentation coefficients were estimated in 5 to 20% sucrose gradients after velocity sedimentation in a Beckman SW60 rotor at 44 000 rpm for 16 h at 4 °C by comparison with the following standards: bovine erythrocyte aldolase (7.35*S*), bovine serum albumin (4.35*S*), and bovine erythrocyte carbonic anhydrase (3.2*S*). Polypeptide masses were determined by mass spectroscopy using a matrix-assisted laser desorption mass spectrometer (Wang & Chait, 1994). Digestion of ezrin with α-chymotrypsin was performed as described (Franck et al., 1993). SDS–polyacrylamide gels were run as described (Laemmli, 1974). For native gels, the following modifications from SDS–PAGE were made. SDS was not included in the sample, gel, or running buffer, no stacking gel was used, and the gel was run at 4 °C. Affinity-purified ezrin antibody (Bretscher, 1989) was used for immunoblotting as described (Franck et al., 1993).

RESULTS

Purification of Two Ezrin Populations from Human Placenta. Ezrin has been purified from chicken intestinal brush borders (Bretscher, 1983), human placenta (Bretscher, 1989), parietal cells (Urushidani et al., 1989), and cultured choriocarcinoma cells (Narvanen, 1985) and as a tumor antigen from methylcholanthrene-induced sarcoma cells (Ullrich et al., 1986), although for some time it was not appreciated that all these proteins were ezrin. Characterization of ezrin from many of these sources indicated that the purified protein existed largely in a monomeric form (Bretscher, 1983; Narvanen, 1985), although other species of the protein were also reported (Ullrich et al., 1986; Pakkanen & Vaheri, 1989).

The original method developed for the purification of ezrin from tissue extracts involves ammonium sulfate fractionation, followed by sequential chromatography on hydroxyapatite, anion, and cation exchangers. In preliminary experiments, partially purified ezrin obtained after the hydroxyapatite column was chromatographed on a Sephacryl S300HR gel filtration column and found to migrate as two species, one characteristic of the monomer and a second with a much larger Stokes radius. The purification procedure was then adapted to isolate the two distinct ezrin species resolved by this gel filtration column.

Elution profiles and polypeptide compositions of fractions generated during the purification of the two ezrin species from placenta are displayed in Figure 1. As in the original method, ezrin was extracted from placenta and then chromatographed on a hydroxyapatite column, which affords an

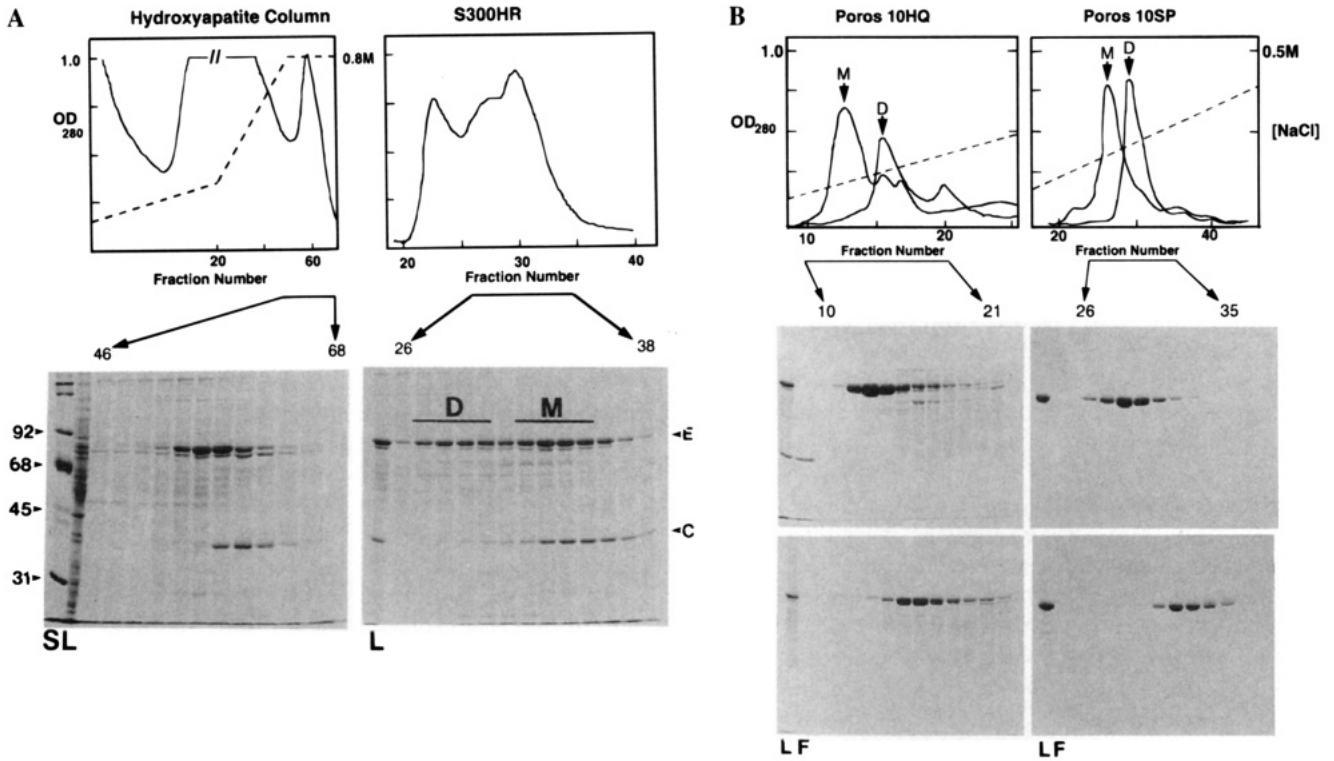

FIGURE 1: Purification of two ezrin species with different Stokes radii from placenta. (A) Elution profiles for the first two columns, hydroxyapatite and Sephacryl S300HR, are shown along with 10% SDS−PAGE analysis of the peak fractions. An extract of placenta was fractionated with ammonium sulfate (see Experimental Procedures), loaded on a hydroxyapatite column (lane L), and eluted with the phosphate gradient (dotted line) shown in the upper left panel. Fractions rich in ezrin were pooled, dialyzed, concentrated by ammonium sulfate precipitation, and applied to a Sephacryl S300HR column (load, lane L). Among the eluting proteins were two peaks of ezrin (E), corresponding to monomers (M) and dimers (D) (see below) that were pooled separately. C indicates a polypeptide identified as the heavy chain of calpactin I. Molecular mass standards (S) are shown at left in kilodaltons. (B) The pooled monomers and dimers were chromatographed separately on anion (Poros 10HQ) and cation (Poros 10SP) columns; the superimposed elution profiles together with SDS−PAGE analysis of peak fractions (upper gels, monomers; lower gels, dimers) are shown. Traces for the monomer (M) and dimer (D) pooled fractions are indicated. Samples of the column loads (L) and flow through (F) are also shown.

enormous enrichment for ezrin as well as moesin and a 35 kDa band identified as calpactin I (data not shown). Fractions rich in ezrin were pooled as indicated, concentrated by ammonium sulfate precipitation, and chromatographed on a Sephacryl S300HR sizing column. Ezrin species with small and large Stokes radii were pooled separately, dialyzed, and sequentially chromatographed on Poros 10 HQ and Poros 10 SP resins. Interestingly, on both these ion exchange resins, the larger species eluted at a higher salt concentration than the smaller species. After the four columns, each preparation contained a single polypeptide with an apparent molecular mass of 81 kDa when analyzed by SDS−PAGE. No additional polypeptides in association with ezrin could be detected when either species was analyzed on 5 to 20% gradient gels.

The Two Ezrin Species Are Stable Monomers and Dimers. The fact that the two ezrin species had distinct chromatographic elution patterns on gel filtration, anion, and cation exchange columns suggested that they are very stable. To examine this further, the purified proteins were rechromatographed on a Superose 6 analytical gel filtration column 1 week after their initial separation by gel filtration (Figure 2). The two purified preparations behaved as distinct stable species of ezrin, with apparent Stokes radii of 41 Å (panel A) and 72 Å (panel B). Aliquots of each fraction from the Superose 6 column were then subjected to chemical cross-linking using the homo-bifunctional reagent DSP and analyzed by nonreducing SDS−PAGE. The smaller ezrin species reacted with the reagent, but this did not significantly change its electrophoretic mobility (panel C), consistent with this species being an ezrin monomer. In contrast, the larger ezrin species was quantitatively converted to a species of lower mobility (panel D), suggesting that it is an oligomeric form of ezrin.

Analysis of the two purified proteins by electrophoresis on 7.5% native polyacrylamide gels revealed that each preparation migrated as a uniform species with a characteristic and distinct mobility (Figure 3A). That the proteins in these preparations contained ezrin was demonstrated by immunoblotting with ezrin-specific antibody (Figure 3B). Moreover, a time course of digestion of the native proteins with α-chymotrypsin gave almost identical polypeptide profiles for the two forms (Figure 4). If in fact the ezrin species with the larger Stokes radius consisted of one ezrin subunit complexed with another protein that comigrated with it on SDS−PAGE, we would expect to see additional bands in the cleavage pattern of the dimer. However, no evidence for additional bands was seen, indicating that both species consist entirely of the ezrin polypeptide.

Several physical properties of the two ezrin species were evaluated (Table 1). Their apparent Stokes radii were determined on a calibrated gel filtration column, their sedimentation coefficients were determined in 5 to 20% sucrose gradients by comparison with standards, and their subunit molecular masses were determined by mass spectroscopy. The data for the species having a smaller Stokes radius are consistent with previous reports on the physical properties of ezrin monomers (Bretscher, 1983). The native

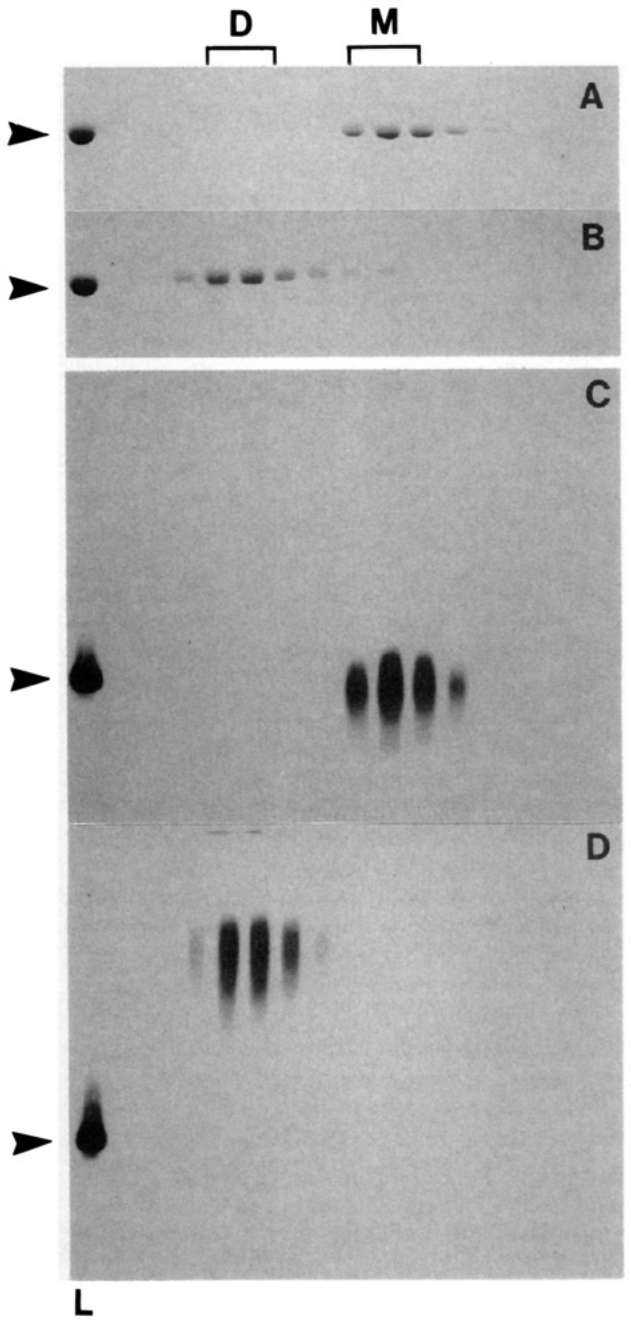

FIGURE 2: Ezrin monomers and dimers are distinct stable species. Samples of purified monomeric and dimeric ezrin were separately analyzed on a calibrated Superose 6 gel filtration column. (A and B) Coomassie blue-stained SDS−PAGE of the polypeptide compositions of each fraction (A, monomers; B, dimers). (C and D) The chemical cross-linking reagent DSP was added to aliquots of each fraction, and the resulting products were resolved by nonreducing SDS−PAGE, transferred to PVDF membranes, and processed for immunoblotting with ezrin antibody (C, cross-linked monomer fractions; D, cross-linked dimer fractions). Note that the cross-linking reagent reacted with the monomers, as indicated by band broadening, but did not appreciably change their electrophoretic mobility, whereas the dimers were quantitatively cross-linked to yield a product with a much slower electrophoretic mobility. The protein load (L) for each sample is shown. Arrows at left for each panel indicate the migration position of unmodified ezrin. The elution positions of ezrin monomers (M) and dimers (D) are indicated at the top of the figure.

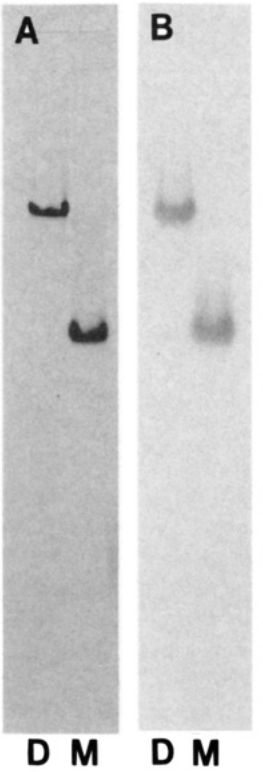

FIGURE 3: Native 7.5% PAGE of ezrin species. (A) Coomassie blue-stained gel of ezrin monomers (M) and dimers (D). (B) Immunoblot with ezrin antibodies of a duplicate of panel A.

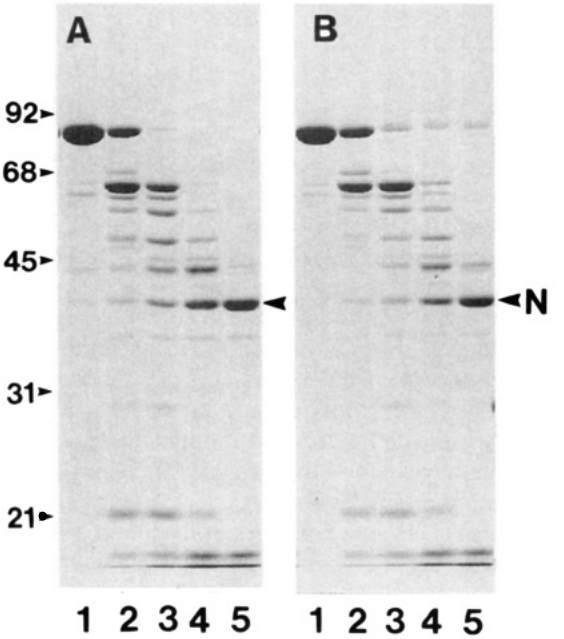

FIGURE 4: Proteolytic digestion of ezrin monomers (A) and dimers (B). α-Chymotrypsin (10 μg/mL) was added to purified monomers or dimers to give a final mass ratio of 30:1 (ezrin:protease) and the mixture incubated at 18 °C. At various time points, samples were withdrawn and SDS gel samples made. SDS−PAGE of the digests is shown. Time points are as follows: lanes 1−5, 0, 2, 10, 40, and 120 min of digestion, respectively. The protease resistant N-terminal domain of ezrin, previously identified (22), is indicated by N. Migration of molecular mass standards in kilodaltons is shown at left.

molecular masses of the two species calculated from the Stokes radius and sedimentation coefficient (Siegel & Monty, 1986) are 69 000 and 139 000 Da, respetively, consistent with their assignment as monomers and dimers. Mass spectroscopy revealed that both preparations consisted of a single polypeptide of about 69 100 Da, which is very close to the molecular mass predicted by the ezrin cDNA sequence. Mass spectroscopy on the DSP cross-linked species, displayed in

Table 1: Physical Properties of Ezrin Monomers and Dimers

property	monomer	dimer
apparent subunit molecular mass (SDS−PAGE)	81 000 Da	81 000 Da
calculated subunit molecular mass (from cDNA sequence)	69 286 Da	69 286 Da
subunit mass (mass spectrometry)	69 167 Da	69 037 Da
subunit mass after chemical cross-linking (mass spectrometry)	72 242 Da	144 127 Da
sedimentation coefficient	3.9*S*	4.5*S*
Stokes radius (gel filtration)	41 Å	72 Å
frictional ratio f/f_o[a]	1.5	2.1

[a] Calculated from $f/f_o = R_s(4\pi N/3\bar{v}M_r)^{1/3}$, where R_s is the experimentally determined Stokes radius, N is Avogadro's number, $\bar{v}$ is the partial specific volume (taken to be 0.74 cm^3/g), and M_r is the calculated molecular mass.

Figure 2C,D, revealed that the larger species had a molecular mass twice that of the DSP-modified monomer (Table 1). These data therefore indicate that the dimers consist of a stable association of two monomeric ezrin subunits. The frictional coefficients f/f_o of the monomer and dimer are calculated to be 1.5 and 2.1, respectively. The high degree of asymmetry of the dimer is reflected by its relatively low sedimentation coefficient and large Stokes radius. Over the period of experimentation, no dissociation of the dimers into monomers or association of monomers into dimers was seen (Figure 2A,B). Moreover, concentration of the monomer species 10-fold did not induce the formation of dimers as determined by native gel electrophoresis (data not shown).

To assess further whether the monomer had the potential to assemble into a dimer, a small amount of biotin-tagged monomer was added to unlabeled dimer to give an approximate molar ratio of 1:20, and the mixture was placed at 4 °C for 28 h and then subjected to gel filtration chromatography. The fractions were run on SDS−PAGE and analyzed by Coomassie blue staining and avidin blotting to show the migration position of the dimer and the location of the biotinylated material, respectively. The bulk of the protein eluted at a position characteristic of dimers (Figure 5A), consistent with the persistence of the complex after mutiple rounds of gel filtration as noted earlier. If dimers readily dissociate to monomers and then reassociate into dimers, the dimer fraction should incorporate most of the biotin-labeled material. However, if dimers are very stable, then the biotinylated material would remain as a monomer, indicating little or no subunit exchange between the two populations. The elution profiles of the biotinylated monomer mixed with dimer, or mixed with bovine serum albumin as a control, are shown in Figure 5B,C. Only a very small amount (<5%) of the biotinylated material comigrated with the dimer during gel filtration, and none did in the presence of bovine serum albumin. Thus, very little subunit exchange between monomers and dimers occurred over a period of 1 day. Although this experiment assumes that biotinylated monomers are functionally interchangeable with unmodified monomers, this is likely to be true because biotinylated monomers can bind with high affinity to unfolded immobilized monomers through ERMAD associations (Gary & Bretscher, 1995; see below). These results show that monomers and dimers consist of the same polypeptides and do not readily interconvert *in vitro*.

Native Ezrin Monomers and Dimers Each Have Extant N-ERMAD Activity and Latent C-ERMAD Activity. Soluble

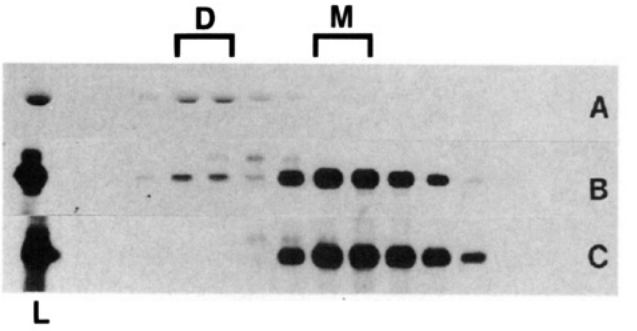

FIGURE 5: Ezrin monomers do not readily exchange into ezrin dimers *in vitro*. Monomeric ezrin was biotinylated and mixed with purified dimer at a 1:20 molar ratio, respectively. The mixture was placed on ice for 28 h and then chromatographed on a Superose 6 column. (A) The Coomassie blue-stained gel of each fraction shows that the majority of the protein elutes at the position characteristic of the dimer (D). (B) Biotinylated material was detected by subjecting samples of each fraction to SDS−PAGE, transferring to a PVDF membrane, and identifying biotinylated proteins with streptavidin peroxidase. The bulk of the biotin-tagged protein remained as a monomer (M), although a very small amount migrates at the position of the dimer. (C) A mixture of biotinylated monomeric ezrin and 1.0 mg/mL bovine serum albumin was chromatographed, and fractions were analyzed as for panel B. All the biotinylated material migrated in the position of the ezrin monomer. The load after reduction (L) of each column is shown at left.

biotinylated monomeric ezrin associates with high affinity and specificity with ezrin that has been through SDS−PAGE and transferred to PVDF membranes (Gary & Bretscher, 1993). In such blot overlay assays, the minimal region necessary for the interaction has been localized to the C-terminal residues 479−585 of the immobilized ezrin. The soluble probe that recognizes this C-terminal domain requires residues 1−296 in a native conformation for its activity. The N-terminal domain and the C-terminal domain, referred to as N- and C-ERMADs (ezrin−radixin−moesin association domains), bind to each other but do not bind to themselves (Gary & Bretscher, 1995). Native ezrin monomers display N-ERMAD activity as they will bind to an immobilized C-ERMAD, although it is not yet clear if all or just a subpopulation of native monomers possesses this activity. However, native monomers clearly have a masked C-ERMAD and cannot bind to an immobilized native N-ERMAD; this masked C-ERMAD can be exposed by unfolding the protein with SDS, after which it can then bind to an immobilized N-ERMAD (Gary & Bretscher, 1995).

Do ezrin dimers have active N- and C-ERMADs? The presence of an active ERMAD in a protein can be assayed by its ability to bind to ERMADs of the complementary type in native slot blots (Gary & Bretscher, 1995). Monomeric and dimeric ezrin were biotinylated and then rechromatographed on a Superose 6 gel filtration column; these probes eluted at the positions characteristic of monomers and dimers, respectively (Figure 6A), confirming that biotinylation did not cause the dimer to dissociate. The ezrin monomers and dimers were then tested for their ability to bind to N- and C-ERMADs using a native slot blot assay (Figure 6B). Both native biotinylated ezrin species bound to the immobilized C-ERMAD, whereas neither bound to the immobilized N-ERMAD. However, the ability of both species to bind to the immobilized N-ERMAD was revealed by unfolding these probes with SDS. After SDS treatment, the biotinylated probes bound to the immobilized N-ERMAD but not

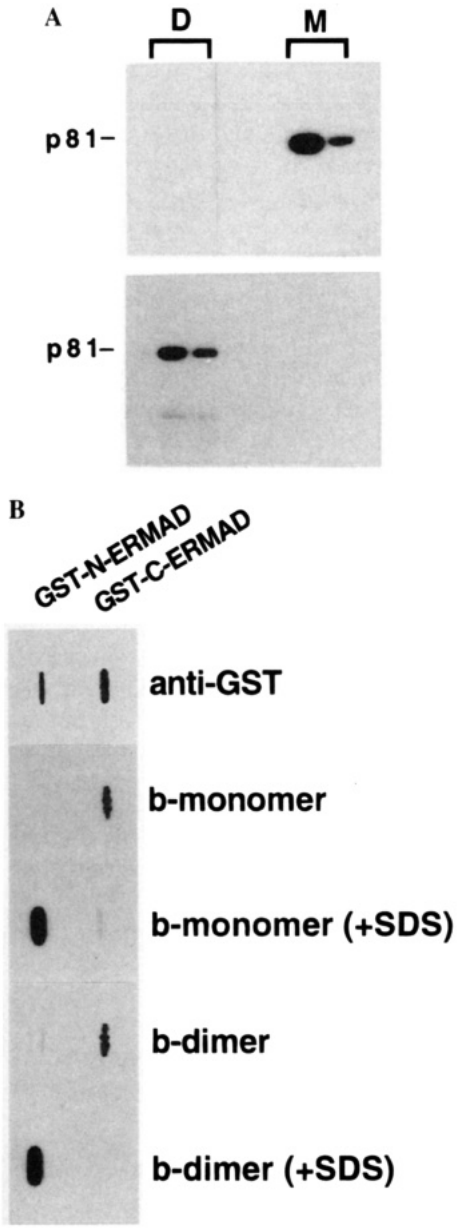

FIGURE 6: Monomeric and dimeric ezrin both have an active N-ERMAD and a masked C-ERMAD. Samples of purified monomeric and dimeric ezrin were biotinylated and then chromatographed separately on a Superose 6 gel filtration column. (A) Elution positions of the biotinylated proteins as detected by analysis of fractions by SDS−PAGE, transfer to PDVF, and incubation with streptavidin-peroxidase. The migration positions of unlabeled ezrin monomers (M) and dimers (D) are indicated. The upper panel is biotinylated monomers and lower panel biotinylated dimers. (B) The biotin-tagged proteins were used in a native slot blot assay to test their ability to bind to a GST fusion protein containing an N-ERMAD (GST-ezrin-1−439) or a C-ERMAD (GST-ezrin-428−585). The amount of GST fusion protein is indicated using GST antibody (anti-GST). Both native biotin-tagged proteins (b-monomer and b-dimer) bound only to the GST C-ERMAD, indicating the presence of a functional N-ERMAD in native monomers and dimers. After SDS treatment (+SDS) to unfold the biotinylated probes, the monomer and dimer both lost the ability to bind to the GST C-ERMAD but now gained the ability to bind to the GST N-ERMAD.

the C-ERMAD, consistent with earlier reports that this treatment inactivates N-ERMAD activity (Gary & Bretscher, 1995). These results indicate that in the native state both monomers and dimers display a functional N-ERMAD but not a functional C-ERMAD. As with the monomer, the dimer has a latent C-ERMAD that can be unmasked by agents such as SDS that alter the native conformation.

DISCUSSION

In this report, we show that ezrin from placenta can be isolated in two distinct and stable forms, which have been identified as ezrin monomers and dimers. The availability of the purified ezrin species has allowed us to begin to characterize them separately, resulting in several important findings.

First, the monomer is a relatively globular molecule, whereas the dimer is highly elongated, having a frictional coefficient of about 2.1 and an apparent Stokes radius of 72 Å. By comparison, smooth muscle α-actinin, which is a rod-shaped dimeric molecule about 35 nm in length (Meyer & Aebi, 1990) and consisting of two 100 kDa chains, has a Stokes radius of 77 Å (Suzuki et al., 1976). The presence of a more globular monomer and an elongated dimer suggests that the dimer, in contrast to the monomer, might be more likely to play a physiologically relevant structural role. Support for such a possibility is discussed below.

Second, our data show that there is very little interconversion between the monomeric and dimeric species. One explanataion for this stability is that the monomer polypeptides may have to be modified before they associate to form dimers. An alternative possibility is that the dimer polypeptides are chemically identical to the monomer but that a large activation energy must be overcome to assemble two monomers into a dimer or dissociate dimers into monomers. This latter possibility is consistent with the large conformational change that must accompany the conversion of globular monomers into elongated dimers, as also predicted from consideration of masked and exposed ERMAD activities. A definitive answer to this question will require the development of methods or the isolation of enzymatic activities for the conversion of monomers to dimers.

Monomeric ezrin has two association domains, referred to as N- and C-ERMADs, that interact in an exclusively complementary manner (Gary & Bretscher, 1995). When associated *in vitro*, the affinity and specificity of an N-ERMAD for a C-ERMAD is quite exceptional. A biotinylated probe containing a native N-ERMAD specifically binds to C-ERMAD-containing proteins in crude cell extracts separated by SDS−PAGE and transferred to PVDF membranes (Gary & Bretscher, 1993, 1995). Moreover, the signal is only reduced about 2-fold in blots washed for several days, indicating an off rate that is extremely slow, even by comparison with, for example, many antigen−antibody interactions.

What is the nature of the intermolecular associations that stabilize the dimer? A plausible hypothesis is that dimer formation involves intermolecular N- and C-ERMAD associations. Our results show that the N-ERMADs are functional whereas the C-ERMADs are masked in both monomers and dimers. This is not surprising, because, if monomers displayed both N- and C-ERMADs, they would be expected to oligomerize spontaneously. Similarly, if dimers had an exposed C-ERMAD, they would also be expected to self-associate or associate with the N-ERMAD of a monomer. However, we have demonstrated that monomers and dimers are very stable in the presence of one another. Therefore, the masking of the C-ERMAD in each

is consistent with the observed behavior of the two species. Perhaps the activation energy necessary for monomer to dimer conversion involves the unmasking of a C-ERMAD.

These considerations led us to propose two simple working models for the association of monomers into dimers. In the first model, dimers are envisaged as having a single intermolecular connection involving the N-ERMAD of one ezrin molecule associating with the C-ERMAD of the other. In the second model, both the N- and C-ERMADs of the first molecule are associated with the complementary C- and N-ERMADs of the second molecule to create two symmetrical intermolecular connections. Which of these models is likely to be correct? In the first model, the dimer has an accessible N-ERMAD and a masked C-ERMAD in addition to the N/C-ERMAD connection that holds the subunits together. In the second model, the N-ERMADs of both subunits are associated with the complementary C-ERMADs and therefore would be unlikely to have N-ERMAD activity. Thus, on the basis of the observed N-ERMAD activity of the dimer, we currently favor the first model involving a single intermolecular N/C-ERMAD connection between the two subunits. Moreover, this model leaves open the possibility for assembly into not only dimers but also higher order structures by the addition of appropriately activated subunits. In support of this idea is the finding that isolated microvilli contain higher order ezrin oligomers in addition to ezrin dimers (Berryman et al., 1995). In addition, vast overexpression of ezrin in insect cells causes what appears to be massive self-association under the plasma membrane (Andreoli et al., 1994).

Recently, a high-affinity F-actin binding site has been identified in the terminal 34 residues of ezrin (Turunen et al., 1994; Pestonjamasp et al., 1995) which is contained within the C-ERMAD (Gary & Bretscher, 1995). Ezrin monomers purified from placenta do not readily bind F-actin in solution, apparently because this domain is masked in the purified monomer (Gary & Bretscher, 1995). Likewise, purified ezrin dimers bind poorly to F-actin *in vitro* (unpublished data), consistent with our results which show that they too have a masked C-ERMAD. If ezrin monomers and dimers use this domain to bind F-actin *in vivo*, then ezrin has to be activated to display the F-actin binding site. In support of this, Hanzel et al. (1991) found an increase in the percentage of cytoskeletally associated ezrin after stimulation to induce the formation of surface microvilli in gastric parietal cells. Moreover, in cytoskeletons of isolated placental microvilli, much of the ezrin is resistant to extraction by high salt and requires disocciation with at least 4 M urea to release it from actin (Berryman et al., 1995). Therefore, we suspect that a very tight F-actin binding site can be unmasked by some regulatory process, presumably through a conformational change, to harness this aspect of ezrin function as needed.

What is the significance of ezrin monomers and dimers? The majority of the ezrin in cultured cells is monomeric, as determined by gel filtration studies (Ullrich et al., 1986; Pakkanen & Vaheri, 1989; Berryman et al., 1995), whereas a considerable fraction of the ezrin purified from placenta, in which the syncytiotrophoblast has abundant ezrin-containing microvilli, is dimeric. Moreover, the bulk of the ezrin in isolated placental microvilli is in an oligomeric form (Berryman et al., 1995). This suggests that monomeric ezrin can be converted *in vivo* to dimers and that the abundance of dimers is correlated with the presence of surface microvilli. These observations suggest that the cytoplasm contains a reservoir of monomeric ezrin that represents a dormant form of the molecule. Apparently, this dormant form must be activated in a regulated process to generate dimers and/or display the masked F-actin binding site. A prediction from this model is that much of the ezrin in cells should be freely soluble and that stimulation of cells to form microvilli should increase the abundance of dimers and F-actin-associated ezrin. The localization of ezrin is consistent with this hypothesis; in cells with few surface structures, much of the ezrin appears to be evenly distributed throughout the cytoplasm, whereas in cells with abundant surface structures, it is highly enriched in these structures (Franck et al., 1993). Moreover, stimulation of A431 cells with EGF, which increases the number of actin-containing surface structures, results in an increase in the proportion of ezrin-containing molecules that are most likely dimers (Berryman et al., 1995).

One intriguing avenue for future studies will be determination of the factors that activate monomeric ezrin to associate into dimers and understanding how the accessibility of the C-ERMAD and F-actin binding site of the ezrin monomer and dimer are regulated. The methods described here for the purification of monomers and dimers, as well as the assays for their detection, should greatly facilitate the development of approaches for investigation of these conversions *in vitro*.

ACKNOWLEDGMENT

We are very grateful to Janet Krizek for assistance with some of these experiments.

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BI952302X