Ezrin was identified over twelve years ago as a minor component of the isolated intestinal microvillus cytoskeleton (Bretscher, 1983), and has since been found in the apical microvilli of a wide variety of polarized epithelial cell types within the body (Hanzel et al., 1991; Wolf et al., 1991; Berryman et al., 1993; Hofer and Drenckhahn, 1993), in the microvilli and other actin-rich surface structures of cultured cells (Bretscher, 1983, 1989; Gould et al., 1986; Pakkanen, 1988; Pakkanen et al., 1987, 1988; Sato et al., 1992; Franck et al., 1993; Soroka et al., 1993; Algrain et al., 1993; Shuster and Herman, 1995; Martin et al., 1995), and in the marginal band of chicken erythrocytes (Winckler et al., 1994).

Several lines of evidence indicate that ezrin attaches microfilaments to the plasma membrane. Ezrin contains an NH2-terminal domain that shares 37% sequence identity with the corresponding region of band 4.1 (Gould et al., 1989). This domain of band 4.1 associates with integral membrane proteins of the erythrocyte plasma membrane to mediate cytoskeletal linkages (Anderson and Lovrien, 1984; Anderson and Marchesi, 1985; Pasternack et al., 1985; Leto et al., 1986; Marfatia et al., 1994). Support for a membrane-cytoskeletal linking function for ezrin includes immunoelectron microscopy, which has revealed that ezrin is closely and specifically associated with the microvillus plasma membrane in several epithelial cell types (Berryman et al., 1993). Moreover, Algrain et al. (1993) have shown that the COOH- and NH2-terminal halves of ezrin associate with the cytoskeleton and plasma membrane, respectively. Complexes containing ezrin and CD44, a polymorphic integral membrane protein which binds to extracellular matrix proteins, have been isolated from cultured cells by co-immunoprecipitation (Tsukita et al., 1994). Finally, two laboratories have identified an F-actin binding site in the COOH-terminal tail of ezrin (Turunen et al., 1994; Pestonjamasp et al., 1995), a domain which appears to be masked in the native ezrin molecule (Gary and Bretscher, 1995).

Radixin and moesin are two independently discovered proteins that share 75% sequence identity with ezrin over the entire length of the coding region (Funayama et al., 1991; Lankes and Furthmayr, 1991). Together, these pro-
teins constitute the ezrin-radixin-moesin (ERM) family (Sato et al., 1992). Structural analysis, localization studies, and evolutionary considerations suggest related but distinct functions for ezrin, radixin, and moesin (Gould et al., 1989; Funayama et al., 1991; Sato et al., 1992; Berryman et al., 1993; Franck et al., 1993; Gary and Bretscher, 1993; Lankes et al., 1993; Amieva et al., 1994; Takeuchi et al., 1994a, b; Tsukita et al., 1994; Winckler et al., 1994; Pestonjamasp et al., 1995). Although ERM proteins are coexpressed in many cultured cell lines (Sato et al., 1992), their pattern of expression in the body is mainly cell-type specific (Berryman et al., 1993; Amieva et al., 1994). Depletion of ERM proteins by antisense oligonucleotides has provided direct evidence for their role in determining cell surface morphology (Takeuchi et al., 1994a).

In addition, ERM-related molecules which appear to play a role in cell morphology have been identified in Droso phila (Ibnsouda et al., 1993; Edwards et al., 1994) and in the sea urchin Lytechinus variegatus (Bachman and McClay, 1995). An intriguing property of ERM family members is their ability to associate in a homotypic or heterotypic fashion both in vivo and in vitro (Gary and Bretscher, 1993; Andreoli et al., 1994). These interactions appear to involve exclusively complementary binding of two nonoverlapping NH2- and COOH-terminal domains, termed N- and C-ERMADs (ezrin-radixin-moesin association domain), respectively. The C-ERMAD, which encompasses the entire F-actin binding site, is masked in native ezrin in solution, suggesting that F-actin binding as well as heterotypic ERM associations might be regulated through unmasking of this domain (Gary and Bretscher, 1995).

A correlation between the phosphorylation of ezrin and changes in cell morphology has been demonstrated in several systems. There exists a close temporal relationship between the phosphorylation of ezrin on tyrosine residues and the rapid formation of microvilli and membrane ruffles enriched in both actin and ezrin in EGF-stimulated A431 cells (Bretscher, 1989). Ezrin also becomes phosphorylated on tyrosine in many other systems (Kamps et al., 1986; Krieg and Hunter, 1992; Hansen and Moeller, 1993; Fazioli et al., 1993; Egerton et al., 1992; Thullier et al., 1994). Stimulation of gastric parietal cells with agents that elevate cAMP levels induces the phosphorylation of ezrin on serine and threonine residues concomitant with the appearance of microvilli rich in ezrin (Urushidani et al., 1989; Hanzel et al., 1989, 1991).

Although ezrin purified from intestinal microvilli (Bretscher, 1983) and cultured human chorionicarcinoma cells (Narvanan, 1985) was originally characterized as a monomeric protein in solution, subsequent studies suggested the existence of oligomeric species as well (Pakkannen and Va heri, 1989; Ullrich et al., 1986). We recently reported the purification from human placenta of two stable forms of ezrin that do not readily interconvert in vitro. Chemical and physical characterization revealed that they are composed of ezrin monomers and elongated dimers (Bretscher et al., 1996). Here we show that ezrin is a major component of isolated placental microvilli where it is cytoskeletally associated and exists primarily in oligomeric form. We also document the rapid formation of ezrin oligomers with tyrosine phosphorylation of ezrin in EGF-stimulated A431 cells. Since these growth factor–induced biochemical events coincide with the appearance of newly formed microvilli and membrane ruffles (Bretscher, 1989), it is possible that ezrin oligomerization plays an important role in the biogenesis of these structures and in the development of epithelial cell polarity. Based on these results, we propose a speculative model for the involvement of ERM proteins in the assembly of cell surface structures.

Materials and Methods

Materials

Ezrin was purified from human placenta (Bretscher, 1989), obtained from consenting patients at Tompkins Community Hospital (Ithaca, NY). Affinity-purified rabbit anti-human ezrin antibodies that do not recognize moesin or radixin have been described (Franck et al., 1993). Mouse monoclonal anti-ezrin antibody Z036, raised against a synthetic peptide corresponding to the COOH-terminal 10 residues of human ezrin, was purchased from Zymed Laboratories, Inc. (South San Francisco, CA). Affinity-purified rabbit anti-radixin antibody, raised against a synthetic peptide corresponding to residues 502–509 of mouse radixin (Fazioli et al., 1993), was a generous gift from Dr. Di Fiore and Dr. Wong (National Institutes of Health, Bethesda, MD).

Preparation of Microvilli

Microvilli were prepared from fresh placental tissue essentially as described by Booth et al. (1980). All steps were performed at 4°C. The umbilical cord and membranes were removed, and the placenta was cut into 20–30 g pieces. After rinsing the pieces briefly in 0.15 M NaCl (saline) to remove excess blood, the tissue was minced and stirred in 500–600 ml saline for 1 h. The material was passed through a nylon sieve (1.0 mm mesh) and the filtrate was centrifuged 10,000 g for 10 min. The supernatant was then centrifuged 90,000 g for 30 min. Using a loose-fitting Dounce, this pellet was resuspended in 50 ml of 10 mM mannitol, 2 mM Tris, pH 7.1, 1 mM PMSF, and 0.5 mM benzamidine. Solid MgCl2·6H2O was added to 10 mM final concentration and the suspension was stirred for 15 min, followed by centrifugation at 5,000 g for 10 min. The supernatant was centrifuged 20,000 g for 30 min to pellet the microvilli. Microvilli were washed by gentle resuspension in saline and recentrifugation. Finally, microvilli were resuspended at 1–2 mg protein/ml in saline containing 0.02% sodium azide, and either used immediately or stored on ice.

Electron Microscopy

Freshly isolated microvilli were prepared for conventional thin section analysis by initial fixation in suspension with addition of an equal volume of 4% glutaraldehyde, 4% formaldehyde, and 2% tannic acid in PBS, pH 7.0. After centrifugation, the pellet was post-fixed with 2% osmium tetroxide, dehydrated in ethanol, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate.

For immuno electron microscopy, intact pieces of fresh placenta tissue were processed as previously described (Berryman et al., 1993). Isolated microvilli were fixed in suspension by addition of an equal volume of 0.5% glutaraldehyde and 2% formaldehyde in PBS, pH 7.4. Following centrifugation, the pellet was treated with 50 mM ammonium chloride, post-fixed with 1% uranyl acetate, dehydrated in ethanol, and embedded in LR Gold resin as described previously (Berryman and Rodewald, 1990). Sections were stained with 2 μg/ml affinity-purified ezrin antibody, followed by 5-nm colloidal gold-labeled secondary antibodies. Specimens were examined at 60 kV in a Philips 301 electron microscope (Philips Electronic Instruments Inc., Mahwah, NJ).

Immunodepletion and Immunoprecipitation

Microvilli (0.5 mg protein) were pelleted by centrifugation, and then extracted by resuspension in 1 ml ice-cold RIPA buffer: 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 0.15 M NaCl, 2 mM EDTA, 25 mM Tris, pH 7.4, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM PMSF, and 0.5 mM DSP, dithiobis-succinimidyl-propionate (DSP, dithiobis-succinimidyl-propionate; ERM, ezrin-radixin-moesin; ERMAD, ezrin, radixin, moesin association domain; PVDF, polyvinylidene fluoride.)
benzamidine. The extract was clarified by centrifugation at 100,000 g for 20 min. The insoluble pellet was resuspended to the same volume as the supernatant and aliquots of these fractions were boiled in Laemmli sample buffer under reducing conditions. Immunodepletion of ezrin was performed by incubation of 400 μl of soluble extract with 15 μl of ezrin antiserum that had been pre-adSORbed to 25 μl protein A-Sepharose beads (Sigma Chemical Co., St. Louis, MO). Quantitative removal of ezrin was accomplished by two sequential incubations with the immunobeads. The immunoprecipitates were washed, eluted by boiling in Laemmli sample buffer, and then pooled.

Preparation of Cytoskeletons
Microvilli in suspension (2 mg/ml) were extracted for 15 min at room temperature by addition of an equal volume of 2% Triton X-100, 75 mM KCl, 10 mM Hepes, pH 7.4, 1 mM DTT, 1 mM EGTA, 2 mM PMSF, and 1 mM benzamidine. The extract was centrifuged 20,000 g for 15 min to pellet the cytoskeletons. The pellet was washed by gentle resuspension in buffer containing 0.1% Triton X-100 and then recentrifuged. In some experiments, washed cytoskeletons were resuspended in various extraction buffers, centrifuged, and equal volumes of the soluble and insoluble fractions analyzed on stained gels and by Western blots.

Western Blots, Blot Overlay, and Lectin Blot
Proteins were resolved by SDS-PAGE (Laemmli, 1970), and then transferred to Immobilon polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Bedford, MA) using a semi-dry electroblotter (Integrated Separation Systems, Hyde Park, MA).

Western blots were blocked with 10% nonfat dry milk, and probed directly with 0.1 μg/ml peroxidase-conjugated anti-phosphotyrosine antibody (Transduction Laboratories, Lexington, KY), or probed with 0.1 μg/ml ezrin antibody followed by 60 ng/ml protein A-peroxidase (Sigma Chemical Co.).

Biotinylated ezrin was prepared and blot overlays were performed according to the method of Gary and Bretscher (1993). For control blots, either the biotinyl-ezrin probe was omitted or a 10-fold excess of unlabeled purified human ezrin was mixed with the biotinyl-ezrin to compete for the biotinyl-ezrin probe was omitted or a 10-fold excess of unlabeled ezrin.

Cell Culture and EGF Treatment
A431 cells were harvested between 10% confluence and 80% confluence. A431 (human epidermoid carcinoma) cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. Cells were grown to near confluence in 100-mm dishes and then incubated in serum-free medium for 10 h before treatment at 37°C with 100 ng/ml EGF (Upstate Biotechnology Inc., Lake Placid, NY) for various times. Next, cells were rinsed briefly with ice-cold PBS and scraped into the appropriate extraction buffer. Following brief trituration, the extracts were clarified by centrifugation at 100,000 g for 20 min, and then subjected to gel filtration chromatography or chemical cross-linking, as described below.

In some experiments, cells were treated with the indicated concentrations of tyrosphin AG1478 (Calbiochem-Novabiochem Corp., San Diego, CA) or phenylarsine oxide (Aldrich Chem. Co., Milwaukee, WI) in dimethyl sulfoxide during the last 3 h or 30 min, respectively, of serum starvation and during stimulation with EGF. Control cells were incubated with dimethyl sulfoxide alone.

Gel Filtration Chromatography and Quantitation
Placental microvilli were extracted for 10 min on ice with 1% Triton X-100, 0.6 M KCl, 10 mM Hepes, pH 7.4, 1 mM EDTA, and protease inhibitors, and then were centrifuged 100,000 g for 20 min. The supernatant was collected onto a Superose 6 HR 10/30 column (Pharmacia Fine Chemicals) and developed with a 40 ml gradient in this buffer with 20-1,000 mM NaCl. The peak ezrin-containing fractions were pooled and dialyzed against 150 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 7.4, and then made 33% in ammonium sulfate. The precipitate was collected by centrifugation at 13,000 g for 15 min and immediately dissolved in a minimal volume of 100 mM NaCl, 1 mM EDTA, 10 mM Hepes, pH 7.4. The material was filtered and run on a Superose 6 HR 10/30 column equilibrated with the same buffer. Fractions containing pure ezrin were pooled, cross-linked, and then analyzed by Cooamassie blue staining, Western blotting, and mass spectroscopy.

Mass Spectroscopy
Ezrin purified from placental microvilli was cross-linked with 1 mM DSP, followed by the addition of 1/20 volume of 1 M glycine, 1 M Tris, pH 6.8 to quench the reaction. The material was dialyzed extensively against deionized water, lyophilized, and then redissolved in deionized water. The masses of the cross H-linked species were determined on a matrix-assisted laser desorption mass spectrometer (Mock et al., 1991; Wang and Chait, 1994).

Results
Ezrin Is a Major Protein of Isolated Placental Microvilli
Human placenta is known to be a rich source of ezrin (Bretscher, 1989) that is concentrated in the microvilli of...
the syncytiotrophoblast (Berryman et al., 1993). High-resolution immunoelectron microscopy reveals in appropriately cut structures (Fig. 1A, arrows), that ezrin is closely associated with the microvillus plasma membrane of these cells. Remarkably, ezrin is essentially absent from nonmicrovillus regions of the apical plasma membrane, indicating that in these and other polarized cells (Berryman et al., 1993), ezrin is highly specific for actin-containing surface structures.

Given the abundance and distribution of ezrin in placental microvilli, we reasoned that these structures might be an excellent system for biochemical analysis of ezrin function. We therefore purified placental microvilli using a modification of established procedures (Booth et al., 1980), resulting in a preparation of microvilli that resembled their counterparts on intact cells. By thin section electron microscopy, the isolated structures appeared as right-side-out, spherical and elongated microvillus vesicles (Fig. 1B). Contamination with other organelles was minimal: for example, very few nuclei or mitochondria were seen. Immunoelectron microscopy revealed that >80% of the structures contained ezrin (Fig. 1C). In negative stain preparations of glutaraldehyde-fixed microvilli, we observed a network of fine filaments within the interior of many structures, indicating that the integrity of the cytoskeleton was at least partially preserved. Negative staining also revealed the presence of some clathrin coated vesicles, which was not surprising since placenta is a classic source for their purification (Pearse, 1982).

SDS-PAGE analysis of the overall composition of isolated microvilli (Fig. 2A) showed a reproducible, simple polypeptide profile, in good agreement with earlier studies.
Among the most abundant polypeptide components are several members of the annexin family of proteins (Edwards and Booth, 1987), actin, a 70-kD doublet, ezrin, and α-actinin (Vanderpuye et al., 1986). Treatment of the microvilli with RIPA buffer, which is often used to solubilize cells for immunoprecipitation studies, extracted surprisingly little of the actin, about half of the ezrin, and little of the α-actinin. The 81-kD band extracted by RIPA buffer was identified as ezrin because it could be removed quantitatively from the extract by immunoprecipitation with ezrin antibody (Fig. 2 A). Since about half of the 81-kD polypeptide was extracted from these structures with RIPA buffer, we questioned whether the 81-kD polypeptide in the residual insoluble material was also ezrin. Western blot analysis of serial dilutions of SDS-solubilized RIPA-soluble and RIPA-insoluble fractions showed that both contained about the same amount of ezrin polypeptide (Fig. 2 B). Therefore, most, if not all, of the material at 81-kD is ezrin, of which about half can be extracted with RIPA buffer. By comparison with known amounts of purified protein, we estimate that ezrin comprises ~5% of the total protein mass of isolated microvilli. The subunit molar abundance of actin/ezrin/α-actinin, as determined by densitometry of Coomassie blue-stained gels, was 10:2:4:1.

Western blots of total microvillus proteins probed with antibodies specific for moesin (Franck et al., 1993) and radixin (Fazioli et al., 1993) revealed variable but minor amounts of these related proteins. This was expected for moesin since it is enriched in stromal endothelial cells and not the syncytiotrophoblast (Berryman et al., 1993). The localization of radixin has not been reported in placenta, so the possibility that a small amount of the material in our preparations at ~81 kD is radixin cannot be excluded.

Placental Microvilli Contain Multiple Covalent Ezrin Adducts

In addition to the major 81-kD ezrin band, several polypeptides with a mobility ranging from ~180–220 kDa were recognized by affinity-purified ezrin antibody in Western blots of total microvillus proteins (Fig. 3 A). As compared with p81 ezrin, the high molecular weight species were extracted poorly by RIPA buffer, suggesting a very tight association with the cytoskeleton. We refer collectively to these novel ezrin-immunoreactive species as covalent adducts, because several criteria established that they contain the ezrin polypeptide. First, the adducts were recognized specifically by three different ezrin antibodies, including the anti-peptide monoclonal Z036. A banding pattern similar to the Western blot in Fig. 3 A is representative of the species seen in a number of different microvillus preparations. No significant qualitative or quantitative changes in this banding pattern were apparent after storage of microvilli for two weeks on ice, ten freeze–thaw cycles before or after boiling in Laemmli buffer, increasing the concentration of reducing agent, or boiling for various times (0–180 s) in Laemmli buffer. Although the individual adduct species appeared minor in abundance relative to the major 81-kD ezrin band, and were difficult to see on stained gels, collectively they represented a significant proportion of the total population of ezrin-immunoreactive species. Second, the small amount of adducts solubilized by RIPA buffer were precipitated by ezrin antibody (Fig. 3 A, lane IP*). Third, p81 and the adduct bands were recognized directly by biotinylated ezrin in blot overlays (Fig. 3 B). Earlier work has shown that ezrin specifically interacts with itself and other ERM family members in this assay (Gary and Bretscher, 1993). Although the relative staining intensity varied among individual adducts both within and between the two blotting methods we used (compare Total in Fig. 3, A and B), analysis of Western blots and blot overlays exposed for various times revealed identical banding patterns comprised of up to twelve distinct adduct species. The specificity of the blot overlay was confirmed by inclusion of excess unlabeled purified ezrin to compete with biotinyl-ezrin binding sites on the blot, resulting in a loss of adduct staining and a decreased signal at 81 kDa.

Ezrin Is a Component of the Detergent-insoluble Cytoskeleton

Extraction of microvilli with 1% Triton X-100 under physiological salt conditions to yield classic cytoskeletons showed that ~50% of the total ezrin at 81 kDa was associated with
the detergent-insoluble fraction (Fig. 4 B), similar to that seen in extracts prepared with RIPA buffer (Fig. 3 A). The adducts were even more resistant to extraction under these conditions (~80% insoluble), suggesting a tighter association with the cytoskeleton. The 81-kD ezrin and the adducts both remained completely insoluble when cytoskeletons were extracted under low salt (10 mM KCl) conditions, and only minor amounts were extracted by millimolar concentrations of divalent cations (data not shown). Extraction of cytoskeletons with 1 M KCl solubilized ~75% of the 81-kD ezrin and a small proportion of the adducts. The adducts required extraction with 6 M urea to achieve greater than 50% solubility.

In addition to ezrin, the detergent-insoluble material was enriched in the cytoskeletal markers actin and α-actinin (Fig. 4 A). The lectin blot in Fig. 4 C shows that the detergent-soluble fraction was highly enriched in membrane glycoproteins, demonstrating that efficient solubilization of membrane proteins was achieved under these conditions.

**Ezrin Exists Primarily As Noncovalent Oligomers in Placental Microvilli**

We recently described the purification and characterization of ezrin monomers and noncovalent dimers from human placenta (Bretscher et al., 1996). To determine which species are present in placental microvilli, we analyzed high salt detergent extracts of microvilli by gel filtration chromatography on a Superose 6 column previously calibrated with conventional standards as well as purified ezrin monomers (Stokes radius 41 Å) and dimers (72 Å).

Under these extraction conditions, ~70% of the ezrin was solubilized. As shown in the Coomassie blue-stained gel and Western blot in Fig. 5, A and B, ezrin exhibited an unusually broad elution profile compared with other proteins, and was resolved into two peaks corresponding to the monomer (M; fractions 35–37) and the dimer (D; fractions 31–32). A substantial amount of ezrin eluted ahead of the dimer (fractions 25–30), perhaps reflecting higher order ezrin oligomers. Evidence to support this notion is presented in Fig. 5 C. We have shown previously by SDS-PAGE that chemical cross-linking of purified ezrin monomers and dimers yields broad bands at ~81 and ~180 kD, respectively (Bretscher et al., 1996). In Western blots of fractions subjected to chemical cross-linking with DSP, we observed several high molecular weight bands, each of which exhibited a characteristic mobility during SDS-PAGE that correlated well with its relative position of elution during chromatography. The relative mobilities of the cross-linked monomer and dimer were identical to that seen with the purified proteins (Bretscher et al., 1996). At least two additional higher order oligomeric species were resolved both by gel filtration (fractions 25–30) and SDS-PAGE of cross-linked ezrin. The Stokes radius of the putative trimer was determined to be 85 Å. Quantitative analysis revealed that the oligomers (dimer and higher) account for ~70% of the total ezrin mass in the microvillus extract. These noncovalent oligomers are remarkably stable in high salt: extracts of microvillus cytoskeletons prepared and chromatographed in the presence of 2 M KCl yielded results similar to those shown in Fig. 5. However, all the noncovalent oligomers were completely reduced to monomers in microvillus extracts prepared and fractionated by gel filtration in the presence of 4 M urea.

To determine whether or not the ezrin-containing bands generated by cross-linking consist entirely of ezrin, we purified ezrin from placental microvilli by sequential chromatography on hydroxyapatite and Mono S cation exchange resins, ammonium sulfate fractionation, and gel filtration. The purified ezrin migrated as a single 81-kD band in Coomassie blue-stained gels (Fig. 6 A) under both reducing and nonreducing conditions, as expected. Cross-linking of purified ezrin with DSP yielded two broad bands corresponding to the relative mobilities of cross-linked ezrin monomer and dimer (Fig. 6 B). The masses of the purified cross-linked monomer and dimer, as determined by mass spectroscopy, were 71,162 and 141,789 D, respectively, consistent with the predicted molecular masses of these species after chemical modification (Gould et al., 1989; Bretscher et al., 1996). Although at least two additional high molecular weight bands were detected with longer exposures of the blot shown in Fig. 6 B, the masses of these higher order oligomers were not determined due to insufficient quantities.

**EGF Stimulates the Formation of Ezrin Oligomers In Vivo**

Since placental microvilli contain abundant ezrin dimers, we reasoned that stimulation of A431 cells with EGF,
which induces microvillus formation, might result in an increase in the proportion of ezrin dimers in vivo. Although about half of the ezrin in A431 cells is cytoskeletally associated when they are prepared under very gentle conditions (Gould et al., 1986), >95% of the ezrin can be extracted when cells are solubilized with Triton X-100 and 0.6 M KCl (Fig. 7 A). High salt detergent extracts from cells treated with or without EGF were fractionated by gel filtration and analyzed by Western blotting (Fig. 7 B). In contrast to placental microvilli, ~95% of the ezrin in unstimulated A431 cell extracts was monomeric. However, about a twofold increase in the amount of ezrin migrating at the position of the dimer was seen in extracts prepared just two minutes after stimulation with EGF.

To determine the relationship between phosphorylation of ezrin on tyrosine and oligomer formation as a function of time after EGF treatment, we devised an assay in which cell extracts were subjected to chemical cross-linking with a relatively low concentration of DSP and then analyzed by Western blotting. As shown in Fig. 8 A, cross-linking resulted in the appearance of a high molecular weight ezrin species which increased in abundance within 1 min after addition of EGF; no further increase was seen with 4 or 10 min of EGF treatment. This result is consistent with the rapid formation of ezrin dimers described in Fig. 7 B. In Fig. 8 A, the cross-linked dimer has an apparent mobility in excess of 180 kD; however, we have found that the apparent mobility of this species is sensitive to the acrylamide concentration. Although it is difficult to prove that the cross-linked species seen in Fig. 8 A actually represents ezrin dimers, they were found to migrate with a mobility characteristic of a cross-linked dimer. In the same assay, ezrin was seen as a major EGF-induced phosphotyrosine-containing band in blots probed with phosphotyrosine antibody (Fig. 8 B). The identity of this 81-kD phosphotyrosine band as ezrin was confirmed by immunodepletion (data not shown) and immunoprecipitation experiments (Fig. 8 C).

The correlation between EGF-induced ezrin tyrosine phosphorylation and oligomerization was extended through the use of a tyrosine kinase inhibitor and a tyrosine phosphatase inhibitor (Fig. 9). Treatment of cells with tyrphostin AG1478, a potent inhibitor of the EGF receptor kinase (Fry et al., 1994; Osherov and Levitski, 1994; Ward et al., 1994), before and during stimulation with EGF, blocked both tyrosine phosphorylation of ezrin and de novo forma-
Chemical cross-linking of ezrin purified from placental microvilli. (A) Purified microvillar ezrin was run on 10% SDS-PAGE in the presence of reducing agent, and visualized by Coomassie blue staining. Ezrin appears to be devoid of any major protein contaminants and migrates as a single band at 81 kD. (B) The sample shown in A was run on 6% SDS-PAGE in the absence of reducing agent before and after cross-linking with DSP, then transferred to a PVDF membrane and probed with ezrin antibody. The products of the cross-linking reaction consist of two broad bands at ~81 and ~180 kD, corresponding to the monomeric and dimeric forms of ezrin, respectively. As expected, a single band is seen at 81 kD in the absence of cross-linking. Equal amounts of protein were loaded for all three samples.

Discussion

In this report we have examined the molecular structure of ezrin in microvilli isolated from the placental syncytiotrophoblast. These microvilli were previously known to contain ezrin, based on Western blotting and immunocytochemistry (Berryman et al., 1993). The protein composition of cytoskeletons from placental microvilli is quite distinct from that of the intestinal brush border (Bretscher, 1991), presumably reflecting differences in cytoskeletal architecture, morphology, and function between these two polarized epithelial cell types. In isolated intestinal microvilli, where ezrin is a relatively minor constituent, the actin filaments are bundled together by fimbrin and villin, and this bundle is attached laterally to the plasma membrane by brush border myosin I (Bretscher, 1991). In contrast, isolated placental microvilli lack fimbrin and villin, but do contain α-actinin and ezrin as two major components of their actin-based cytoskeletons. Structurally, the intestinal brush border is characterized by densely packed microvilli of uniform size and shape, which have a highly ordered microfilament core bundle that inserts into a well-structured terminal web. In contrast, the syncytiotrophoblast brush border displays less dense packing of microvilli, which vary in size and shape and are often bridged by clathrin-coated pits at their bases, and whose apparently disorganized cytoskeletons are supported by a loosely organized terminal web. These ultrastructural features correlate with the relatively high rates of endocytic uptake and membrane recycling at the apical surface of the syncytiotrophoblast. Our immunoelectron microscopy studies reveal a high concentration and close association of ezrin with the microvillus plasma membrane, but little or none in coated pits or other invaginations of the apical membrane. These observations are consistent with the proposed membrane–cytoskeletal linking function of ezrin, and also suggest the possibility that ezrin may serve a tethering role which limits or prevents the diffusion of certain resident membrane proteins into coated pits, thereby helping to maintain cell polarity.

We have demonstrated that ezrin is highly abundant in placental microvilli, and that much of it is associated with the cytoskeleton. Extraction of placental microvilli with detergent and physiologically relevant buffers resulted in the solubilization of about half the ezrin together with
Figure 8. Time course of EGF stimulation shows a correlation between the onset of ezrin oligomer formation and the phosphorylation of ezrin on tyrosine. A431 cells were treated with EGF for various times (0, 1, 4, and 10 min) and scraped into solubilization buffer. After centrifugation to remove insoluble material, the extracts were subjected to cross-linking with DSP, and the products of the reaction resolved by 7% SDS-PAGE under nonreducing conditions. Proteins were transferred to PVDF membranes, and probed with either ezrin antibody (A) or anti-phosphotyrosine antibody (B). As seen in A, the addition of cross-linking reagent resulted in the appearance of a high molecular weight ezrin species, which presumably represents the dimeric form resolved by gel filtration in Fig. 7 B. This band increases in intensity within 1 min after addition of EGF and shows little further increase at 4 or 10 min. In B, uncross-linked ezrin is seen as a major EGF-induced 81-kD phosphotyrosine-containing protein in cells stimulated for 1 min with EGF. The level of tyrosine phosphorylation of ezrin is maximal at 1 min and progressively decreases at 4 and 10 min after growth factor treatment. The addition of DSP resulted in a correspondingly diminished phosphotyrosine signal at each time point. Ezrin was immunoprecipitated from cross-linked extracts and analyzed on a Western blot probed with phosphotyrosine antibody (C). The time course of phosphotyrosine staining of 81-kD ezrin parallels what is seen in total extracts in B.

some of the α-actinin and actin. Increasing amounts of ezrin and other cytoskeletal components could be titrated from the cytoskeletons with increasing salt concentrations. Surprisingly, ~10% of the ezrin, and a significant proportion of the actin, was resistant to extraction even by 2 M KCl. Gel filtration analysis of intact microvilli solubilized by 0.6 M KCl or of cytoskeletons extracted with 2 M KCl gave similar ezrin elution profiles with the bulk of the ezrin migrating as oligomers, while most of the actin eluted as a monomer and α-actinin as an homogeneous dimer. In contrast, in 4 M urea, all the ezrin eluted as a monomeric species yet the α-actinin still remained a dimer. These observations show that much of the ezrin is very tightly associated with the cytoskeleton, and that the extracted material contains multiple ezrin species.

An unanticipated finding was that the bulk of the ezrin extracted from placental microvilli exists as dimeric and higher oligomeric forms. It seems reasonable to predict that the assembly hierarchy of the oligomers is monomers to dimers, to trimers, to tetramers, etc. This prediction is based on the physical properties of purified monomers and dimers, and the existence of exclusively complemen-
tary association domains in the ezrin polypeptide known as N-ERMADs and C-ERMADs (Bretscher et al., 1996; Gary and Bretscher, 1995). The interactions that stabilize the dimer likely involve the association of the N-ERMAD of one molecule with the C-ERMAD of a second subunit. Since the C-ERMAD is masked in soluble monomeric ezrin, thereby precluding dimer formation, some regulatory step must be required to activate or otherwise expose this domain to form a dimer (Gary and Bretscher, 1996). Because the dimer also has a masked C-ERMAD (Bretscher et al., 1996), we hypothesize that multiple activation events are required for the formation of trimers and higher order structures. These monomers, dimers, trimers, etc. that we can extract from placental microvilli in vitro apparently represent distinct and stable ezrin populations that coexist in vivo.

In addition to these noncovalent oligomers, placental microvilli contain a minor, yet perhaps significant, population of covalently cross-linked ezrin species. A plausible explanation for their existence is that noncovalent ezrin oligomers are converted into covalent adducts by some endogenous cross-linking activity (e.g., a transglutaminase). This cross-linking presumably occurs in vivo, since the adducts did not increase in abundance after prolonged storage of isolated microvilli for up to two weeks on ice or after incubation at 37°C for 30 min, nor do they appear to be artifacts induced by sample preparation. The variable mobility of the adducts during SDS-PAGE may result from the introduction of topologically different cross-links, or the cross-linking of ezrin to some other protein. Although minor, and therefore technically difficult to work with, the adducts might be of considerable importance as they are much more tightly associated with the microvillus cytoskeleton than the rest of the ezrin.

Since the discovery of ezrin (Bretscher, 1983), there have been a number of puzzling findings surrounding the study of this microfilament-associated protein. First, although apparently a cytoskeletal molecule, ezrin is generally very easy to extract from cultured cells and tissues (Bretscher, 1983, 1989; Narvanen, 1985; Hanzel et al., 1991). However, our results show that ezrin behaves as a bona fide cytoskeletal protein in isolated placental microvilli, since cytoskeletonally associated ezrin remained largely insoluble over a wide range of salt concentrations. Second, although the native intact molecule binds poorly to F-actin in vitro (Bretscher, 1983), Algrain et al. (1993) have nevertheless demonstrated that the COOH-terminal half of ezrin has the capacity to interact with F-actin in vivo. Furthermore, two independent laboratories have recently shown that the COOH-terminal region of ezrin is capable of direct association with F-actin in vitro (Turunen et al., 1994; Pestonjamasp et al., 1995). Third, purified ezrin monomers and dimers can coexist as stable independent populations and show little or no subunit exchange in vitro (Bretscher et al., 1996). Recent results from our laboratory have provided evidence that the F-actin binding domain, which is contained within the C-ERMAD, is inaccessible in the native ezrin monomer (Gary and Bretscher, 1995). Thus, it appears that the intermolecular interactions which govern F-actin binding and ezrin oligomerization are both subject to regulatory controls that affect the conformation of the C-ERMAD. This notion is further supported by studies in which ectopic expression of the COOH- and NH2-terminal halves, but not full-length ERM proteins, produce distinct morphogenetic effects in transfected cells (Algrain et al., 1993; Martin et al., 1995; Henry et al., 1995).

How can we account for all these diverse findings to explain the different structural forms of ezrin found in vivo and relate them to function? As shown in the schematic diagram in Fig. 10, our hypothesis starts with the idea that a dormant form of monomeric ezrin exists in the cytoplasm; in many cultured cell lines this may represent the bulk of the ezrin present. In response to a signal to remodel its cortical cytoskeleton to form actin-containing surface structures, dormant ezrin becomes activated, perhaps by some localized membrane-associated activator. Two types of activation can be envisaged: unmasking of the C-ERMAD for association with the N-ERMAD of another molecule to induce oligomerization, or unmasking of the COOH-terminal domain to reveal the F-actin binding site. The relationship between these two types of unmasking events remains to be determined. Once bound to F-actin, ezrin binds tenaciously, thus explaining its tight association with F-actin in placental microvilli and its ability to bind F-actin in overlay assays (Pestonjamasp et al., 1995). In addition to F-actin binding, ezrin must be able to associate with some membrane protein, either as a result of concomitant unmasking of an appropriate site, or possibly by the enhanced avidity afforded by the multivalency of the oligomeric species. Although the membrane binding partner of placental ezrin is currently unknown, Tsukita et al. (1994)
have reported the identification of the hyaluronate receptor CD44 as a membrane receptor for ERM proteins in some cells; however this does not appear to be relevant to placental microvilli because they apparently lack this protein (our unpublished observations; St. Jacques et al., 1993). Finally, additional proteins might be involved in the morphogenesis of microvilli such as the F-actin cross-linking protein depicted in Fig. 10. What might the ezrin activation process be? We have demonstrated that treatment of A431 cells with EGF induces a rapid (<1 min) increase in tyrosine phosphorylation of ezrin and in the formation of ezrin oligomers, suggesting that tyrosine phosphorylation and oligomerization might play a critical role in microvillus assembly. Although tyrosine phosphorylation may represent one step in a pathway leading to ezrin oligomerization or F-actin binding, our results indicate that stable oligomers can persist following a decrease in tyrosine phosphorylation. It is interesting to note that ezrin also becomes phosphorylated on tyrosine in a variety of growth factor–stimulated cells, virus-infected cells, and transformed cell lines (Bretscher, 1989; Gould et al., 1986; Egerton et al., 1992; Fazzioli et al., 1993; Kamps et al., 1986; Thullier et al., 1994), and on serine and threonine in stimulated gastric parietal cells (Urushidani et al., 1989). In many of these cases, the phosphorylation of ezrin correlates with the formation of ezrin-containing surface microvilli or membrane ruffles. The notion that phosphorylation of ezrin might regulate oligomerization is now further substantiated by the fact that these two events have been shown to occur together within the same signal transduction pathway. We speculate that activation or unmasking of the C-ERMAD in ezrin monomers allows for oligomer formation, F-actin binding, and membrane association to occur either simultaneously or in rapid succession to generate microvilli. The challenge now is to determine what factors control oligomer formation and how this process is integrated into the circuitry that modulates the architecture of the cortical cytoskeleton.

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