# Identification of EBP50: A PDZ-containing Phosphoprotein that Associates with Members of the Ezrin-Radixin-Moesin Family

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*Abstract.* Members of the ezrin-radixin-moesin (ERM) family of membrane–cytoskeletal linking proteins have $NH_2$- and COOH-terminal domains that associate with the plasma membrane and the actin cytoskeleton, respectively. To search for ERM binding partners potentially involved in membrane association, tissue lysates were subjected to affinity chromatography on the immobilized $NH_2$-terminal domains of ezrin and moesin, which comprise the ezrin-radixin-moesin–association domain (N-ERMAD). A collection of polypeptides at 50–53 kD from human placenta and at 58-59 kD from bovine brain bound directly to both N-ERMADs. The 50–53-kD placental proteins migrated as a major 50-kD species after phosphatase treatment, indicating that the heterogeneity is due to different phosphorylation states. We refer to these polypeptides as ERM-binding phosphoprotein 50 (EBP50). Sequence analysis of human EBP50 was used to identify an ~2-kb human cDNA that encodes a 357-residue polypeptide. Recombinant EBP50 binds tightly to the N-ERMADs of ezrin and moesin. Peptide sequences from the brain candidate indicated that it is closely related to EBP50. EBP50 has two PSD-95/DlgA/ZO-1–like (PDZ) domains and is most likely a homologue of rabbit protein cofactor, which is involved in the protein kinase A regulation of the renal brush border $Na^+/H^+$ exchanger. EBP50 is widely distributed in tissues, and is particularly enriched in those containing polarized epithelia. Immunofluorescence microscopy of cultured cells and tissues revealed that EBP50 colocalizes with actin and ezrin in the apical microvilli of epithelial cells, and immunoelectron microscopy demonstrated that it is specifically associated with the microvilli of the placental syncytiotrophoblast. Moreover, EBP50 and ezrin can be coimmunoprecipitated as a complex from isolated human placental microvilli. These findings show that EBP50 is a physiologically relevant ezrin binding protein. Since PDZ domains are known to mediate associations with integral membrane proteins, one mode of membrane attachment of ezrin is likely to be mediated through EBP50.

T HE apical aspect of polarized epithelial cells is generally studded with abundant microvilli containing a core bundle of actin filaments. To assemble and maintain the microvilli, the filaments must attach to the membrane both at the tip of the microvillus, and laterally down its length (for review see Bretscher, 1991; Mooseker, 1985). Since its discovery, ezrin has been proposed to function as a membrane–cytoskeletal linking protein that attaches the actin filaments laterally to the plasma membrane. This suggestion was based on the finding that ezrin is a component of the isolated intestinal microvillus cytoskeleton and is specifically enriched in actin-containing surface structures on cultured cells (Bretscher, 1983).

Ezrin is one member of a family of closely related proteins known as the ezrin-radixin-moesin (ERM)[1] family (Gould et al., 1989; Turunen et al., 1989; Funayama et al., 1991; Lankes and Furthmayr, 1991; Sato et al., 1992). These proteins all possess a ~300-residue $NH_2$-terminal domain that shares sequence homology with the corresponding domain of erythrocyte band 4.1, followed by an ~170-residue region predicted to be largely α-helical, and terminating in a ~100-residue domain in which an F-actin binding site resides (Turunen et al., 1994; Pestonjamasp et al., 1995; Yao et al., 1996). Further support for a mem-

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1. *Abbreviations used in this paper*: EBP50, ERM-binding-phosphoprotein-50; ERM, ezrin-radixin-moesin; ERMAD, ERM-association-domain; NHE-RF, $Na^+/H^+$ exchanger regulatory factor; PDZ, PSD-95/DlgA/ZO-1–like; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; PKA, cAMP-dependent protein kinase A; PVDF, polyvinylidene fluoride; TKA-1, tyrosine kinase activator–1.

brane–cytoskeletal linking role came from the knowledge that the NH$_2$-terminal domain of band 4.1 binds to the membrane protein glycophorin C in an association enhanced by an additional factor known as p55 (Marfatia et al., 1994, 1995).

Various laboratories have tried to identify ERM-binding proteins by using coimmunoprecipitation approaches. Using an antibody to moesin, Tsukita et al. (1994) reported that the hyaluronate receptor CD44 binds to the ERM proteins. Recently, they have shown that CD44 associates with the NH$_2$-terminal domains of all family members in a PIP$_2$-dependent manner (Hirao et al., 1996). Using a similar immunoprecipitation approach, we discovered that ezrin associates with a subpopulation of moesin in cultured cells where both are expressed. This result led to the finding that ezrin and moesin can form very tight homo- or heterotypic associations when expressed in the same cell (Gary and Bretscher, 1993).

The discovery that ezrin associates either with itself or with moesin gave rise to a paradox because the bulk of soluble ezrin in tissue homogenates exists in monomeric form (Bretscher, 1983, 1989). To help resolve this issue, ezrin's self-association domains were delineated, and their accessibility in isolated monomers was determined (Gary and Bretscher, 1995). This study revealed that ezrin contains an NH$_2$-terminal domain of ~300 residues that can bind with high affinity to a ~100-residue COOH-terminal domain. Because the NH$_2$-terminal domain can associate with the COOH-terminal domain of any ERM member, the domains were termed N- and C-ERMADs (ERM-association domain). The N-ERMAD coincides with the band 4.1 homology domain, a region of the molecule folded into a compact structure based on its relative resistance to protease (Franck et al., 1993). The C-ERMAD follows the region predicted to be largely α-helical, and is also relatively protease resistant (Gary and Bretscher, 1995; Niggli et al., 1995). Using bacterially expressed N-ERMAD as a probe, it was found that the activity of the C-ERMAD is masked in the native monomer, thus explaining why ezrin can exist as a monomeric protein in the cytoplasm. Moreover, the C-ERMAD contains the F-actin binding site, which is also expected to be masked in the isolated monomer (Gary and Bretscher, 1995). When the C-ERMAD is exposed by unfolding agents, it readily binds to an N-ERMAD. Although these results were most exhaustively shown for ezrin and to a lesser degree for moesin (Gary and Bretscher, 1995), the model likely extends to radixin. Indeed, Magendantz et al. (1995) showed that immobilized radixin N-ERMAD will bind full-length, denatured radixin where the radixin C-ERMAD is expected to be exposed. Also consistent with the concept that the C-ERMAD has activities normally masked in the intact molecule, is the finding that high level expression of this domain, but not the intact molecule or NH$_2$-terminal domain, causes the formation of long appendages on transfected cells (Henry et al., 1995; Martin et al., 1995).

Based on these studies, and the finding that isolated microvilli contain a preponderance of ezrin oligomers over monomers, we proposed that ezrin can exist in vivo in both dormant and activated states (Berryman et al., 1995; Gary and Bretscher, 1995). Activation of the monomer, perhaps by phosphorylation, induces a conformational change that exposes the masked C-ERMAD, thereby allowing self-association. In addition, activation may lead to the exposure of the COOH-terminal F-actin binding site, and possibly of a masked membrane association site (Berryman et al., 1995). Since the membrane association site might be masked in the dormant monomer, we sought to identify proteins that would bind to the isolated N-ERMAD.

In this study, we describe a protein that binds to the N-ERMADs of ezrin and moesin. The isolation, identification, and colocalization of this protein with ezrin in cell surface structures is reported here.

## Materials and Methods

### Materials

Human placenta was obtained from consenting patients at Tompkins Community Hospital (Ithaca, NY). Bovine brain was provided by Dr. W. Brown (Cornell University, Ithaca, NY). Adult female CD-1 mice were provided by Dr. M. Salpeter and M. Strang (Cornell University). Restriction enzymes and other reagents for molecular biology were purchased from GIBCO BRL (Gaithersburg, MD).

### Production and Purification of Recombinant Proteins

The cDNA sequences encoding the human ezrin and moesin N-ERMADs (amino acids 1–296) were amplified by PCR from clones F6 (Gould et al., 1989) and HEBA06 (a gift from Dr. Stachowitz, Gezentrum, Munich, Germany), respectively, using primers which generated EcoRI and Hind-III sites at their ends. These products were then subcloned into the expression vector pQE16 (QIAGEN Inc., Chatsworth, CA). The cDNA sequence encoding residues 1-357 of ERM-binding phosphoprotein 50 (EBP50) was amplified by PCR with SphI and HindIII sites at the ends and subcloned into pQE70 (QIAGEN Inc.). Vector sequences coding for the six histidine tags were absent in all of the final constructs. To make the EBP50 COOH-terminal construct, the cDNA sequence encoding residues 241–357 was amplified by PCR using primers that created HindIII and BglII sites at the ends. This product was joined with the 0.99-kb BglI/Hind-III fragment of pQE50 (QIAGEN Inc.) and the 2.42-kb BglI/BglII fragment of pQE16 in a three-arm ligation reaction to create the final His-tagged fusion construct. All recombinant sequences were determined to be free of PCR errors by nucleotide sequence analysis. Recombinant plasmids were propagated in the JM109 strain of *Escherichia coli* (Stratagene, La Jolla, CA).

For protein expression, plasmid constructs were transformed into the *E. coli* strain M15[pRep4] (QIAGEN, Inc.). Saturated overnight cultures were inoculated at 1:20 dilution in LB medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin, and grown for 90 min at 37°C. Isopropyl β-D-thiogalactopyranoside was added to 2 mM and cells were grown for an additional 180 min. Cells were harvested by centrifugation at 8,000 *g* for 15 min. Total bacterial lysates were prepared from cells resuspended in 1 vol of Laemmli buffer (Laemmli, 1970), boiled 2 min, and then passed through a 28-gauge needle to reduce viscosity.

To purify bacterially expressed ezrin or moesin N-ERMAD, induced cells were resuspended in 6 vol of 180 mM KH$_2$PO$_4$, pH 7.0, at 4°C, containing 50 μg/ml PMSF and 75 μg/ml benzamidine, lysed by sonication (Branson Ultrasonics Corp., Danbury, CT), clarified at 45,000 *g* for 10 min, and then loaded onto a preequilibrated hydroxyapatite column (HA–Ultragel; Pharmacia Fine Chemicals, Piscataway, NJ). The column was developed using a six-column volume linear gradient of 180–800 mM KH$_2$PO$_4$, and fractions were monitored by SDS-PAGE on 15% gels. Fractions rich in N-ERMAD were pooled, dialyzed against 20 mM MES, 150 mM NaCl, pH 6.7, at 4°C, centrifuged at 45,000 *g* for 10 min, applied to a preequilibrated S-Sepharose column (Pharmacia Fine Chemicals, Piscataway, NJ), and developed with a five-column volume linear gradient of 0.15–1.0 M NaCl. Homogenous N-ERMAD eluted at ~490 mM NaCl.

To purify recombinant EBP50, induced bacterial lysates were prepared in TBS (50 mM Tris, 0.15 M NaCl, pH 7.4, at 4°C) in the presence of protease inhibitors, according to the method described above, and EBP50 affinity purified using N-ERMAD–coupled beads in a manner analogous to that used in the affinity binding assay. After washing the beads in TBS

made up to 0.5 M NaCl, bound EBP50 was eluted with 2 M NaI, or by boiling the beads in Laemmli buffer.

The His-tagged EBP50 COOH-terminal fusion product was purified on nickel nitrilo-triacetic acid resin (QIAGEN) under denaturing conditions in 8 M urea, according to the manufacturer's protocol.

## Affinity Binding Assay

Purified N-ERMAD proteins and BSA were coupled covalently to CNBr-activated Sepharose 4B (Sigma Chemical Co., St. Louis, MO) at a final concentration of 2 mg/ml. Specifically, dried beads were swelled for 15 min in 1 mM HCl at room temperature, and then washed once in ice-cold C buffer (0.1 M NaHCO$_3$, 0.5 M NaCl, pH 8.3, at 4°C). Purified protein in C buffer was immediately added to these beads and incubated for 16 h at 4°C with gentle inversion. The beads were pelleted at 3,000 $g$ for 10 s, the supernatant removed, and the remaining active groups on the beads blocked by a 16-h incubation in 0.25 M glycine, pH 8.0, at 4°C. Beads were washed five times in C buffer, and finally stored for use as a 25% slurry at 4°C in H buffer (50 mM Tris, 0.15 M NaCl, 1% Triton X-100, pH 7.4, at 4°C) made 0.2% with NaN$_3$. For each set of beads made, samples of the starting protein solution and the post-couple supernatant were compared by SDS-PAGE to determine the efficiency of the coupling reaction, which in all cases for the beads used was >95%.

Lysates were prepared from tissues that had been stored frozen at −80°C. Tissues were thawed at 4°C, and homogenized in a blender (Waring Products Div., New Hartford, CT) in 2 ml H buffer per gram (wet weight) of tissue in the presence of 50 μg/ml PMSF and 75 μg/ml benzamidine. Homogenates were clarified by centrifugation at 48,000 $g$ for 15 min at 4°C, and the resulting supernatant was then recentrifuged at 200,000 $g$ for 30 min at 4°C to yield a soluble lysate with a protein concentration of ∼5 mg/ml.

Affinity binding assays were carried out by mixing 50 μl of a 25% slurry of coupled beads with 1 ml of tissue lysate at 4°C for 2 h. For some reactions, a fourfold excess (100 μg) of soluble ezrin N-ERMAD or BSA was also included. The beads were washed six times in 1 ml H buffer made up to 0.5 M NaCl, and bound lysate proteins extracted by boiling for 2 min in Laemmli buffer.

For the large-scale affinity precipitation of EBP50 from tissues, the affinity bead-binding assay was scaled up ∼100-fold. Beads that had been incubated with lysate were transferred to a 5-ml chromatography column, and washed with five column volumes of H buffer. Bound protein was collected in 0.3-ml fractions while eluting with two column volumes of 8 M urea buffered with 50 mM Tris, pH 7.4, at 4°C.

## Phosphatase Assays

Human placental EBP50 was affinity purified on N-ERMAD beads, collected by elution in 2 M NaI, and then dialyzed against 50 mM Hepes, 1 mM MgCl$_2$, pH 7.5, at 4°C. Treatments with calf intestine alkaline phosphatase (Sigma Chemical Co.) were performed essentially according to the method described by Coligan et al. (1996). Reactions were set up using ∼3 μg EBP50 and 0.6 U phosphatase, incubated for 15 min at 30°C, and then terminated by boiling in Laemmli buffer. In control reactions, EBP50 was incubated either in buffer alone or in the presence of the phosphatase and the inhibitors 10 mM β-glycerophosphate and 100 μM Na$_3$VO$_4$.

## Antibodies

Antibodies to EBP50 were raised in rabbits and affinity purified as described (Bretscher, 1983) using purified recombinant human EBP50 COOH terminus as antigen. Affinity-purified antibodies to human placental ezrin have been described (Bretscher, 1989; Franck et al., 1993).

## SDS-PAGE, Blot Overlays, and Immunoblots

SDS-PAGE was performed according to Laemmli (1970). Gels were stained with Coomassie brilliant blue R-250, or were silver stained (Oakley et al., 1980). For blots, proteins were transferred from gels to polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Bedford, MA) using a semidry electroblotter (Integrated Separation Systems, Hyde Park, MA). All blots were developed using an enhanced chemiluminescence detection system (Amersham Corp., Arlington Heights, IL).

Biotinylated N-ERMAD and EBP50 probes were prepared and blot overlays were performed as described (Gary and Bretscher, 1993). The biotinyl probes were omitted in control experiments. Purified human placental ezrin and moesin were prepared as described (Bretscher, 1989).

Immunoblots were blocked with 10% nonfat dry milk, then probed with 0.1 μg/ml affinity-purified EBP50 or ezrin antibodies in 1% milk, followed by 0.1 μg/ml peroxidase-conjugated goat anti–rabbit IgG in 1% milk. Primary antibodies were omitted for control blots.

Murine tissue samples were obtained from adult female CD-1 mice. Total SDS-soluble lysates were prepared from fresh tissues or cells homogenized in Laemmli buffer, boiled 2 min, sonicated 15–30 s, and centrifuged 100,000 $g$ for 30 min at 20°C. The resulting supernatants were collected for analysis. Human placental microvilli were prepared as described (Berryman et al., 1995) and total SDS-soluble lysates made as above.

## Sequence Analysis

EBP50 was affinity purified from human placenta or bovine brain using N-ERMAD beads in the large-scale, affinity binding assay. Approximately 8 μg of each protein was resolved by preparative SDS-PAGE, and then blotted to PVDF. The membrane was stained with Ponceau-S (Sigma Chemical Co.) and regions containing the desired EBP50 bands were excised and then washed extensively in double-distilled water. Amino acid analysis and peptide microsequencing was performed at Harvard Microchem (Cambridge, MA). Samples were digested in situ with endoproteinase lys-C, subjected to HPLC fractionation, and the peak fractions were analyzed using matrix-assisted laser desorption time-of-flight mass spectrometry. Homogenous fractions were then chosen for automated peptide sequencing. Peptide sequences (see Figs. 4 and 5) were used to query the National Center for Biotechnology Information (Bethesda, MD) nonredundant database using the BLAST program (Altschul et al., 1990).

The Institute for Genomic Research (Rockville, MD) human cDNA database was searched using the EBP50 peptide sequences. cDNA clones that matched these query sequences were obtained from Genome Systems Inc. (St. Louis, MO). The insert sizes were determined by restriction endonuclease digestion using enzymes appropriate for the cloning sites in each library parent vector. The cDNA insert of a clone from a human infant brain library was sequenced in its entirety using a set of four oligonucleotide primers that yielded overlapping sequence information. All nucleotide sequencing was done using an automated cycle sequencer (model 373A; Applied Biosystems, Inc., Foster City, CA).

The software programs, EDITSEQ and MEGALIGN (DNASTAR Inc., Madison, WI), were used for DNA and protein sequence editing, and protein sequence alignments, respectively.

## Immunofluorescence and Immunoelectron Microscopy

Cryosections of human placenta were prepared and stained as described (Berryman et al., 1993). Murine intestinal epithelial cells were prepared and stained as described (Bretscher and Weber, 1978). Affinity-purified EBP50 antibodies were used at 3 to 5 μg/ml. Tissue sections were viewed using a Zeiss Axioskop fluorescence microscope (Carl Zeiss Inc., Thornwood, NY) and images were recorded on Kodak T-Max 400 film (Eastman Kodak Co., Rochester, NY).

JEG-3 cells obtained from the American Type Culture Collection (Rockville, MD) were grown on glass coverslips in MEM supplemented with 10% FCS and stained for microscopy as described in Franck et al. (1993), using 3 μg/ml of affinity-purified EBP50 antibodies. Cells were viewed with a Zeiss Axiovert 100-TV fluorescence microscope (Carl Zeiss Inc.), and images were acquired using Metamorph imaging software (Universal Imaging Corp., West Chester, PA).

Immunoelectron microscopy was performed as described (Berryman et al., 1993).

## Immunoprecipitation

Human placental microvilli were prepared, and radioimmunoprecipitation assay (RIPA) extracts were made as described (Berryman et al., 1995). Ezrin and EBP50 were immunoprecipitated from 300 μl of soluble extract of microvilli using 7 μl of antiserum and 25 μl of protein A–Sepharose beads (Sigma Chemical Co.). In some experiments, 50 μg of purified human ezrin was included in the reaction mixture as competitor. Immunoprecipitates were washed with RIPA buffer and eluted by boiling in Laemmli sample buffer. The immunoprecipitates were run on SDS-PAGE, blotted to PVDF, and probed with biotinylated EBP50 or ezrin antibodies. Avidin peroxidase (ExtrAvidin; Sigma Chemical Co.) was used as a secondary detection reagent.

**A** Ezrin N-ERMAD     **B** Moesin N-ERMAD
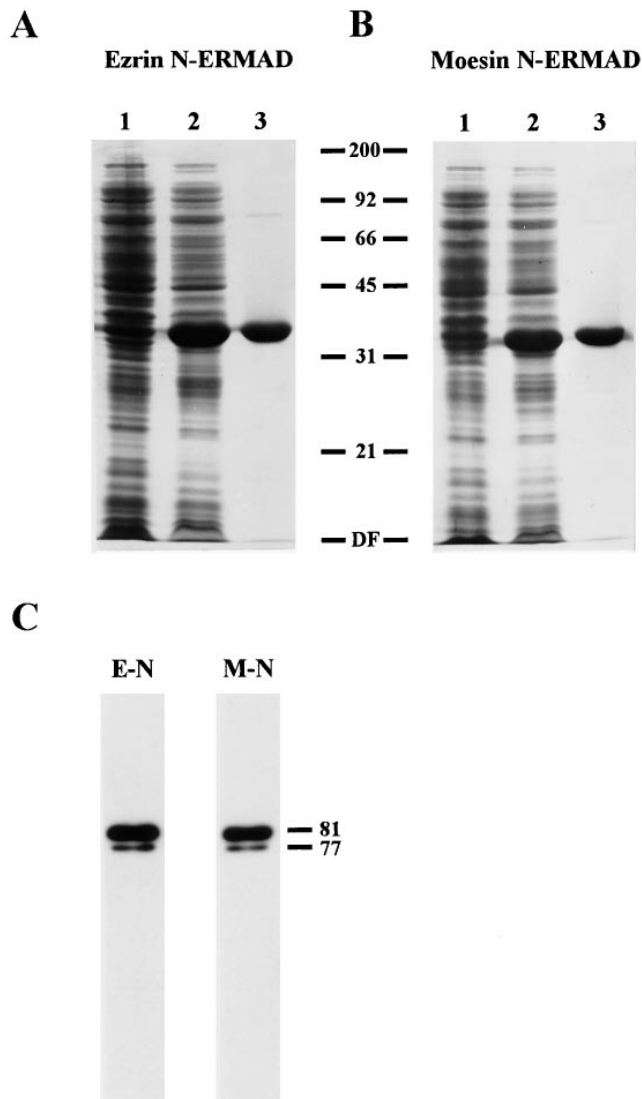
*Figure 1.* Purification and characterization of ezrin and moesin N-ERMADs. (*A* and *B*) show the purifications. Samples were run on a 15% SDS gel and stained with Coomassie blue. Lane *1*, total extract of uninduced bacteria; lane *2*, total extract of induced bacteria; lane *3*, purified proteins. (*C*) The recombinant proteins exhibit ERMAD activity. Blot overlays using biotinyl ezrin N-ERMAD (*E-N*) and biotinyl moesin N-ERMAD (*M-N*) probes on a mixture of ezrin and moesin are shown. The mobilities of molecular mass standards and of placental ezrin (81) and moesin (77) are indicated in kD. *DF*, dye front.

## Results

### Identification of Ezrin and Moesin N-ERMAD Binding Proteins

The N-ERMADs of human ezrin and moesin (residues 1–296) were expressed as soluble, untagged proteins in bacteria, and purified to homogeneity (Fig. 1, *A* and *B*). Because N-ERMADs require a native conformation for their activity in a blot overlay assay (Gary and Bretscher, 1995), we tested the ability of these bacterially expressed products to bind purified ezrin and moesin. Both recombinant ERMADs bound specifically to human placental ezrin and moesin that had been electrophoresed and blotted to

a membrane (Fig. 1 *C*). These results suggested that they were native in conformation and therefore suitable for use in the search for binding proteins.

An affinity binding assay was used in which the native N-ERMADs were immobilized on agarose beads, mixed with detergent-soluble tissue lysates, washed extensively, and then any binding proteins were eluted by boiling in SDS. A set of beads to which an identical amount of BSA was coupled served as a control. Using this assay, lysates of human placenta were found to contain a group of polypeptides of apparent molecular mass 50–53 kD that bound specifically to the ezrin and moesin N-ERMAD beads (Fig. 2 *A*, lanes *5* and *6*), but not to the control beads (Fig. 2 *A*, lane *4*). Similarly, lysates of bovine brain contained polypeptides of apparent molecular mass 58–59 kD that bound specifically to both sets of N-ERMAD beads (Fig. 2 *A*, lanes *10* and *11*). The presence of a small amount of ezrin, as confirmed by immunoblot analysis (data not shown), was also seen in both of the N-ERMAD eluates from placenta (Fig. 2 *A*, lanes *5* and *6*). This ezrin was probably recovered due to association between the immobilized N-ERMAD and a small amount of soluble ezrin having an exposed C-ERMAD, or by virtue of being bound, either directly or indirectly, to the placental N-ERMAD–binding candidates.

The specificity of binding between the N-ERMADs and the placental and brain candidates was examined further. The ability of the moesin N-ERMAD beads to bind these proteins from lysates containing a fourfold excess of uncoupled ezrin N-ERMAD was tested. Under these conditions, the entire series of placental and brain candidate bands, as well as the small amount of ezrin precipitated from placenta, was specifically competed away (Fig. 2 *A*, lanes *7* and *12*). In mock competitions where a fourfold excess of uncoupled BSA was used, the recovery of the candidate proteins and ezrin was unaffected (Fig. 2 *A*, lanes *8* and *13*). These results indicate that the presence of the N-ERMAD in solution can prevent the binding of the candidates and ezrin to the beads. Since the soluble ezrin N-ERMAD diminished the binding of the candidates to the moesin N-ERMAD beads, it is likely that the candidates associate with homologous sites on the ezrin and moesin N-ERMADs.

To determine if the interaction between the candidates and the N-ERMAD might be direct, biotinylated ezrin N-ERMAD was used as a probe in a blot overlay assay on the samples shown in Fig. 2 *A*. Fig. 2 *B* shows that the biotinylated N-ERMAD bound not only to the 50–53-kD placental polypeptides (Fig. 2 *B*, lanes *5*, *6*, and *8*) but also to the 58–59-kD brain polypeptides (Fig. 2 *B*, lanes *10*, *11*, and *13*). The candidate proteins were also detected in samples of the starting lysates (data not shown). The ezrin in the placental precipitates was specifically recognized on the blot because of the association between its exposed C-ERMAD and the N-ERMAD probe (Fig. 2 *B*, lanes *5*, *6*, and *8*). In those instances where soluble ezrin N-ERMAD competitor was used in the binding assay, neither the candidates nor ezrin was detected, corroborating the specificity of the affinity binding assay (Fig. 2 *B*, lanes *7* and *12*). These results demonstrate a direct association between the placental and brain candidates and the N-ERMADs of ezrin and moesin.

**A**

| Buffer | Placenta | Brain |

**B**

1 2 3 4 5 6 7 8 9 10 11 12 13
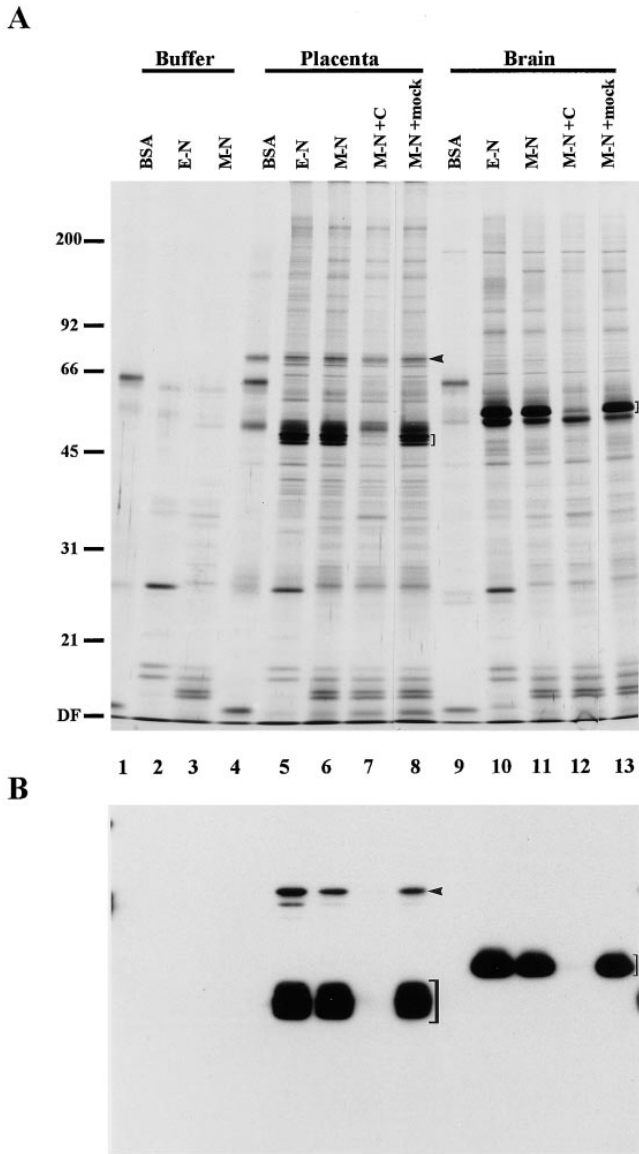
*Figure 2.* Identification of N-ERMAD binding proteins. (*A*) Lysis buffer, or placental extracts, or brain extracts were mixed with BSA–agarose (*BSA*), ezrin–N-ERMAD-agarose (*E-N*), or moesin–N-ERMAD-agarose (*M-N*), and then were washed extensively in 0.5 M NaCl and bound proteins eluted and resolved on a 6–20% silver stained gradient SDS gel. For some reactions, a fourfold molar excess of competitor ezrin N-ERMAD (*+ C*) or mock competitor BSA (*+ mock*) was added to the extract before mixing with moesin–N-ERMAD-agarose. (*B*) One quarter the amount of the same samples shown in *A* were resolved on a 10% gel, transferred to PVDF, and probed with biotinylated ezrin–N-ERMAD. Brackets indicate the N-ERMAD binding proteins, and arrowheads indicate ezrin. The mobilities of standard proteins are indicated in kD. *DF*, dye front.

## Sequence Analysis of the Binding Candidates Reveals Homologous Proteins with PDZ Domains

A scaled-up version of the affinity binding assay was used to acquire sufficient amounts of each candidate for sequence analysis. The placental 50–53-kD bands were significantly enriched in the peak fractions (Fig. 3 *A*). Under these conditions, three major placental polypeptide bands,
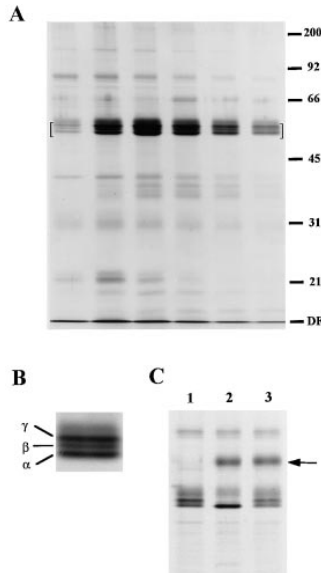


*Figure 3.* Isolation and characterization of the human placental N-ERMAD binding candidates. (*A*) An affinity binding assay similar to that shown in Fig. 2, was scaled up 100-fold, and bound proteins were eluted with urea. A silver-stained 12% gel of the peak fractions is shown; the region in which the binding proteins migrate is bracketed. (*B*) Enlarged view to show resolution of the placental candidates into three species: α, β, and γ. (*C*) Binding protein heterogeneity is due to phosphorylation. The proteins were recovered from ezrin–N-ERMAD agarose beads by elution with NaI, treated with alkaline phosphatase, and then analyzed on a 10% gel. Lane *1*, untreated sample; lane *2*, phosphatase-treated sample; lane *3*, phosphatase-treated sample in the presence of phosphatase inhibitors. The arrow indicates the migration position of alkaline phosphatase. *DF*, dye front.

which we designate α, β, and γ, were resolved (Fig. 3 *B*). Amino acid analysis of each of these bands indicated essentially identical compositions, suggesting that they might be posttranslationally modified species of the same polypeptide. Although antiphosphotyrosine immunoblots indicated that the heterogeneity was apparently not the result of tyrosine phosphorylation (data not shown), treatment of the placental candidates with calf intestinal alkaline phosphatase resulted in a collapse of most or all of these species into a major polypeptide band migrating at 50 kD (Fig. 3 *C*, lane *2*). Control experiments in which the enzyme was omitted, or phosphatase inhibitors were included, showed no detectable change in the migration of p50 α, β, or γ (Fig. 3 *C*, lanes *1* and *3*). Therefore, most or all of the heterogeneity of the placental species is due to varying degrees of serine and/or threonine phosphorylation of a 50-kD polypeptide. We refer to this collection of polypeptides as EBP50.

Two peptide sequences were derived from γ-EBP50, KGPNGYGFHLHGEK, and KRAPQMDWSK. Database searches revealed that closely related sequences are present in rabbit protein cofactor (Weinman et al., 1995) and that a sequence related to the first peptide is present in human tyrosine kinase activator–1 (TKA-1) (these sequence data are available from EMBL/GenBank/DDBJ under the accession number Z50150). Protein cofactor, also known as NHE-RF (Na$^+$/H$^+$ exchanger regulatory factor) is a 358-residue protein that is involved in the cAMP-dependent protein kinase A (PKA) regulation of the rabbit renal brush border Na$^+$/H$^+$ ion exchanger (Weinman et al., 1995; Yun et al., 1997). Information submitted to GenBank (K. Seedorf and A. Ullrich, April 1996) indicates that TKA-1 may be a novel cellular tyrosine kinase–binding protein that activates the signaling potential of the PDGF receptor. In addition, Yun et al. (1997) recently showed that TKA-1, renamed E3KARP,

## Figure 4 (left column sequence)

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                        CTTGGCACGAGGGGATTGGTCTGTGCTCCTCTCTCGGCTCCTCGCGGCTCGCGGC  54
GGCCGACGGTTCCTGGGACACCTGCTTGCTTGGCCCGTCCGGCGGCTCAGGGCTTCTCTGCTGCGCTCCCGGTTCGCTG  133
GACGGGAAGAAGGGCTGGGCCGTCCCGTCCCGTCCCCATCGGAACCCCAAGTCGCGCGCTGACCCGTCGCAGGGCGGAG  212

ATG AGC GCG CAC GCA GCG GCC GGG GCG CCC CTG CCC CGG CTC TGC TGC CTG GAG AAG GGT  272
 M   S   A   D   A   A   A   G   A   P   L   P   R   L   C   C   L   E   K   G   20

CCG AAC GGC TAC GGC TTC CAC CTG CAC GGG GAG AAG GGC AAG TTG GGC CAG TAC ATC CGG  332
 P   N   G   Y   G   F   H   L   H   G   E   K   G   K   L   G   Q   Y   I   R   40

CTG GTG GAG CCC GGC TCG CCG GCC GAA AAA GCG GGG CTC CTG GCG GGG GAC CGG CTG GTG  392
 L   V   E   P   G   S   P   A   E   K   A   G   L   L   A   G   D   R   L   V   60

GAA GTG AAC GGG GAG AAC GTG GAG AAG GAG ACC CAC CAG CAG GTG GTG AGC CGC ATC CGC  452
 E   V   N   G   E   N   V   E   K   E   T   H   Q   Q   V   V   S   R   I   R   80

GCC GCA CTC AAC GCC GTG CGC CTG CTG GTG GTC CAC CCC GAA ACG GAC GAG CAC TGC AGA  512
 A   A   L   N   A   V   R   L   L   V   V   D   P   E   T   D   E   H   C   R  100

AGC CGG CGT CCA GGT CCG AGA GGA GCT GCT CGC CGC CCA GGA ACG CCG GGG CAG GCC GAG  572
 S   R   R   P   G   P   R   G   A   A   R   R   P   G   T   P   G   Q   A   E  120

CCG CCG GCC GCC GCC GAG GTG CAG GGG GCT GGC AAC GAA AAT GAG CCT CGC GAG GCC GAC  632
 P   P   A   A   A   E   V   Q   G   A   G   N   E   N   E   P   R   E   A   D  140

AAG AGC CAC CCG GAG CAG CGG GAG CTT CGG CCT CGG CTC TGT ACC ATG AAG AAG GGC CCC  692
 K   S   H   P   E   Q   R   E   L   R   P   R   L   C   T   M   K   K   G   P  160

AGT GGC TAT GGC TTC AAC CTG CAC AGC GAC AAG TCC AAG CCA GGC CAG TTC ATC CGG TCA  752
 S   G   Y   G   F   N   L   H   S   D   K   S   K   P   G   Q   F   I   R   S  180

GTG GAC CCA GAC TCC CCG GCT GAG GCT TCA GGG CTC CGG GCC CAG GAT CGC ATT GTG GAG  812
 V   D   P   D   S   P   A   E   A   S   G   L   R   A   Q   D   R   I   V   E  200

GTG AAC GGG GTC TGC ATG GAG GGG AAG CAG CAT GGG GAC GTG GTG TCC GCC ATC AGG GCT  872
 V   N   G   V   C   M   E   G   K   Q   H   G   D   V   V   S   A   I   R   A  220

GGC GGG GAC GAG ACC AAG CTG CTG GTG GTG GAC AGG GAA ATC GAC GAG TTC TTC AAG AAA  932
 G   G   D   E   T   K   L   L   V   V   D   R   E   I   D   E   F   F   K   K  240

TGC CGA GTG ATC CCA TCT CAG GAG CAC CTG AAT GGT CCC CTG CCT GTG CCC TTC ACC AAT  992
 C   R   V   I   P   S   Q   E   H   L   N   G   P   L   P   V   P   F   T   N  260

GGG GAG ATA CAG AAG GAG AAC AGT CGT GAA GCC CTG GCA GAG GCA GCC TTG GAG AGC CCC 1052
 G   E   I   Q   K   E   N   S   R   E   A   L   A   E   A   A   L   E   S   P  280

AGG GCA GCC CTG GTG AGA TCC GCC TCC AGT GAC ACC AGC GAG GAG CTG AAT TCC CAA GAC 1112
 R   P   A   L   V   R   S   A   S   S   D   T   S   E   E   L   N   S   Q   D  300

AGC CCC CCA AAA CAG GAC TCC ACA GCG CCC TCG TCT ACC TCC TCC TCC GAC CCC ATC CTA 1172
 S   P   P   K   Q   D   S   T   A   P   S   S   T   S   S   S   D   P   I   L  320

GAC TTC AAC ATC TCC CTG GCC ATG GCC AAG GAG AGG CGC CAC CAG AAA CGC AGC AGC AAA 1232
 D   F   N   I   S   L   A   M   A   K   E   R   R   H   Q   K   R   S   S   K  340

CGG GCC CGG CAG ATG GAC TGG AGC AAG AAA AAC GAA CTC TTC AGC AAC CTC TGA 1286
 R   A   P   Q   M   D   W   S   K   K   N   E   L   F   S   N   L   *  357

GCGCCCTGCTGCCACCCAGTGACTGGCAGGGCCGAGCCAGCATTCCACCCCACCTTTTTCCTTCTCCCCAATTACTCCC 1365
CTGAATCAATGTACAAATCAGCAACCCACATCCCCTTTCTTGACAAATGATTTTTCTAGAGAACTATGTTCTTCCCCTGAC 1444
TTTAGGGAAGGTGAATGTGTTCCCGTCCTCCCGCAGTCAGAAAGGAGACTCTGCCTCCCTCCTCCTCACTGAGTGCCTC 1523
ATCCTACCCGGGTGTCCCTTTGCCACCCTGCCTGGGAACCTGCTGGAACCTGCACCATGCCCAGGATCATGGGACCAGGCG 1602
AGAGGGCACCCTCCCTTCCTCCCCCATGTGATAAATGGGTCCAGGGCTGATCAAAGAACTCTGACTGCAGAACTGCCGC 1681
TCTCAGTGGACAGGGCATCTGTTATCCTGAACCTTGGCAGACACGTCTTGTTTTCATTTGATTTTGTTAAGAGTGCAGT 1760
ATTGCAGAGTCTAGAGGAATTTTTGTTTCCTTGATTAACATGATTTTCCTGGTTGTTAATCCAGGGCATGGCAGTGGCC 1839
TCAGCCTTAAACTTTTGTTCCTACTCCCACCCTCAGCGAACTGGGCAGCACGGGGAGGGTTTGGCTACCCCTGCCCATC 1918
CCTGAGCCAGGTACCACCATTGTAAGGAAACACTTTCAGAAATTCAGCTGGTTCCTCCAAACCAAAAAAAAAAAAAAA 1997
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*Figure 4.* Nucleotide and derived protein sequence of human EBP50 cDNA. Residues matching the two placental γ-EBP50 peptide sequences are underlined. These sequence data are available from EMBL/GenBank/DDBJ under accession number AF015926.

binds to the NHE3 $Na^+/H^+$ exchanger and subjects it to PKA regulation.

The human expressed sequence tag (EST) database was found to contain a cDNA clone encoding the EBP50 peptide sequences. The 2.0-kb insert contained an open reading frame of 357 residues, with a predicted molecular mass of 38.6 kD (Fig. 4). An alignment of the EBP50 protein sequence with that of rabbit protein cofactor and TKA-1 is shown in Fig. 5. EBP50 exhibits 84 and 48% overall sequence identity to rabbit protein cofactor and TKA-1, respectively. In contrast to EBP50 and protein cofactor, which align very well over their entire lengths, the sequence of TKA-1 diverges after G261 in EBP50. These findings suggested that the correct cDNA had been obtained using the placental candidate peptide sequences, and that human EBP50 might be a homologue of rabbit protein cofactor, and a relative of human TKA-1.

Inspection of the deduced EBP50 protein sequence revealed the presence of two ~90-residue repeats in the NH$_2$-terminal half of the molecule between L11-E97 and L149-E236. These repeats, which share 74% sequence identity, are also found in nearly identical versions in both protein cofactor and TKA-1 (Fig. 5). Database searches using the isolated sequences from each EBP50 repeat yielded, in addition to the expected matches with protein

## Figure 5 (right column alignment)

*Figure 5.* Human EBP50 has homology to rabbit protein cofactor and human TKA-1. Identities are boxed. Sequences corresponding to the ~90-residue PDZ domains in each of these proteins are underlined with thick lines. The locations of sequences of the three peptides derived from the bovine N-ERMAD binding protein are indicated by thin lines. The protein cofactor and TKA-1 sequence data are available from EMBL/GenBank/DDBJ under accession numbers U19815 and Z50150, respectively.

cofactor and TKA-1, a list of more distantly related proteins including: the tumor suppressor product of the *Drosophila discs large* gene; the synapse-associated proteins PSD-95/SAP90, chapsyn 110, SAP97, and SAP102; the human tyrosine phosphatase PTPL1; the *Drosophila* InaD protein; and the human tight junction protein zonula occludin–1, among others. The common feature among these proteins is the presence of one or more PDZ domains (Fig. 6). Thus, EBP50, rabbit cofactor, and TKA-1 all have two closely related PDZ domains followed by a 120–220-residue COOH-terminal tail.

Sequence analysis of peptides derived from the 59-kD bovine brain N-ERMAD binding candidate yielded three sequences (KVGQYIRLVEPGSPAEK, KETHQQVVN-RIRA, and KLLVVDRETDEFFK) that are almost identical to sequences in the PDZ containing regions of EBP50 (Fig. 5). Therefore, the brain candidate is probably the bovine homologue of EBP50 or a very closely related protein.

### Expression and Purification of Recombinant EBP50

Recombinant full-length EBP50 was generated to allow for additional characterization of its interaction with the N-ERMADs. Although the protein was expressed poorly in *E. coli*, soluble EBP50 could be purified to homogeneity by affinity chromatography on either ezrin N-ERMAD or moesin N-ERMAD beads (Fig. 7 *A*). The expressed protein, predicted to have a mass of 38.6 kD, migrated anoma-

```
EBP50:     1    LEKGPNGYGFHLHGEKG      KLGQYIRLVEPGSPAEKAG  LLAGDRLVEV     NGENVEKETHQQVVSRIRAALNAVRLLV
           2    MKKGPSGYGFNLHSDKS      KPGQFIRSVDPDSPAEASG  LRAQDRIVEV     NGVCMEGKQHGDVVSAIRAGGDETKLLV
Cofactor:  1    LEKGPNGYGFHLHGEKG      KVGQYIRLVEPGSPAEKAG  LLAGDRLVEV     NGENVEKETHQQVVSRIRAALNAVRLLV
           2    MKKGPNGYGFNLHSDKS      RPGQFIRAVDPDSPAEASG  LREQDRIVEV     NGVCVEGKQHGDVVTAIKAGGDEAKLLV
TKA-1:     1    LVRGEQGYGFHLHGEKG      RRGQFIRVEPGSPAEAAA   L AGDRLVEV     NGVNVEGETHHQVVQRIKAVEGQTRLLV
           2    RRGQFIRVEPGSPAEEAAA   L AGDRIVEV     NGVNVEGETHHQVVQRIKAVEGQTRLLV
DlgA:      1    LERGNSGLGFSIAGGTDNPHIGTDTSIYITKLISGGAAAADGRLSINDIIVSV    NDVSVVDVPHASAVDALKKAGNVVVKLHV
           2    LVKGGKGLGFSIAGGIGNQHIPGDNGIYVTKLTDGGRAQVDGRLSIGDKLIAVRTNGSEKNLENVTHELAVATLKSITDKVTLII
           3    IQKGPQGLGFNIVGGEDGQ      GIYVSFILAGGPADLGSELKRGDQLLSV    NNVNLTHATHEEAAQALKTSGGVVTLLA
Chapsyn:   3    LHKGSTGLGFNIVGGEDGE      GIPVSFILAGGPADLSGELQRGDQILSV    NGIDLRGASHEQAAAALKGAGQTVTIIA
PSD-95:    3    IHRGSTGLGFNIVGGEDGE      GIFISFILAGGPADLSGELRKGDQILSV    NGVDLRNASHEQAATALKNAGQTVTIIA
PTPL1:     2    LAKNDNSLGISVTGGVNTS   VRHGGIYVKAVIPQGAAESDGRIHKGDRVLAV    NGVSLEGATHKQAVETLRNTGQVVHLLL
           4    IKSEKASLGPTVTKGNQ      RIGCYVHDVI QDPAKSDGRLKPGDRLIKV    NDTDVTNMTHTDAVNLLRAASKTVRLVI
```

*Figure 6.* Alignment of the two PDZ domains of human EBP50, rabbit cofactor and human TKA-1 with PDZ domains of selected other proteins: *Drosophila* Dlg-A (these sequence data are available from EMBL/GenBank/DDBJ under accession number M73529), human chapsyn-110 (accession number U32376), murine PSD-95 (accession number D50621), and human PTPL1 (accession number X80289). The domains have been aligned to optimize the conserved residues, shown in bold, as proposed by Ponting and Phillips (1995). A more complete alignment of other PDZ domains by Ponting and Phillips can be found at: http://biop.ox.ac.uk/www/dhr.html

lously by SDS-PAGE with an apparent mass of 50 kD, which is the same mobility seen for placental EBP50 after phosphatase treatment (Fig. 7 *B*). The similar mobilities of the bacterially expressed open reading frame and the de-
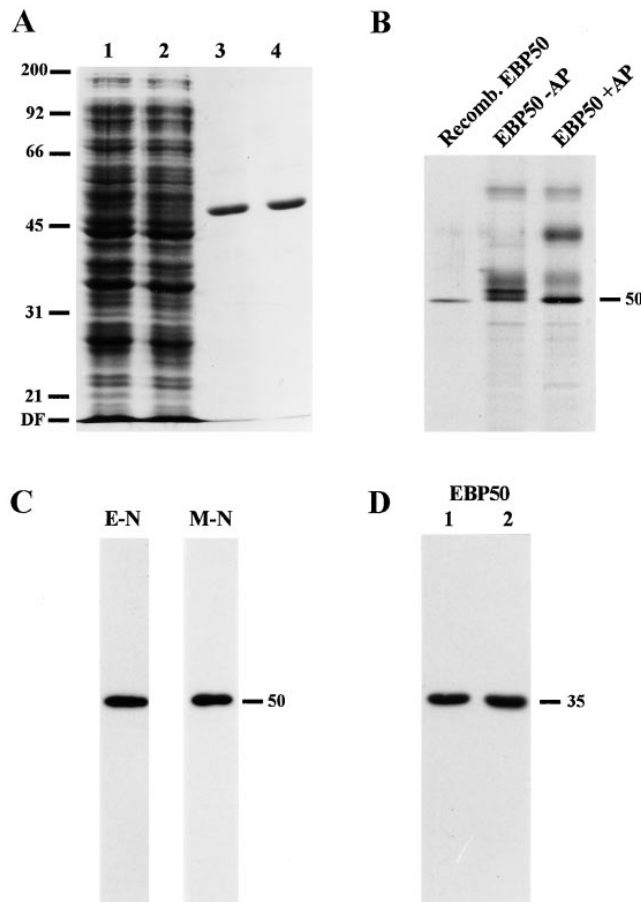


*Figure 7.* Expression, purification, and characterization of recombinant EBP50. (*A*) Expression and purification of EBP50. Samples were run on an 11.5% SDS gel and stained with Coomassie blue. Lane *1*, total extract of uninduced bacteria; lane *2*, total extract of induced bacteria; lane *3*, EBP50 purified on immobilized ezrin N-ERMAD; lane *4*, EBP50 purified on immobilized moesin N-ERMAD. (*B*) Recombinant EBP50 comigrates with phosphatase-treated EBP50 (+*AP*) and migrates faster than untreated EBP50 (−*AP*). The latter two lanes are the same as those shown in Fig. 3 *C*. (*C*) Biotinylated ezrin N-ERMAD (*E-N*) or moesin N-ERMAD (*M-N*) binds to recombinant EBP50 by blot overlay. (*D*) Biotinylated recombinant EBP50 binds to ezrin and moesin N-ERMADs by blot overlay. Samples in panels *C* and *D* were resolved on 10 and 15% SDS gels, respectively, and blotted as in Fig. 2. The migration positions of the isolated N-ERMADs (35 kD) and of EBP50 (50 kD) are shown. *DF*, dye front.

phosphorylated tissue EBP50 polypeptide indicate that the initiator methionine shown in Fig. 4 is used in vivo.

Purified recombinant EBP50 was recognized by either of the N-ERMAD probes by blot overlay (Fig. 7 *C*). These results confirmed that the human cDNA we obtained encodes a protein that binds with high affinity to the ezrin and moesin N-ERMADs and is therefore almost certainly the same cDNA that encodes the human placental candidate originally identified.

The ability to purify soluble EBP50 also afforded us the opportunity to study the results of the converse blot overlay. As shown in Fig. 7 *D*, when biotinylated recombinant EBP50 was used as a probe it bound to both N-ERMADs after SDS-PAGE, demonstrating that bidirectional association between EBP50 and the N-ERMADs is possible with this assay.

### Distribution and Localization of EBP50 in Tissues and Cultured Cells

To explore the tissue distribution and cellular localization of EBP50, a polyclonal antibody was raised to its COOH-terminal 117 residues, since this region is divergent from human TKA-1. Affinity-purified antibodies were used to probe a blot of SDS-soluble lysates from an assortment of murine and human tissues (Fig. 8). EBP50 was found to varying extents in almost all the tissues examined, except for heart and skeletal muscle. It was also found in cultured JEG-3 human choriocarcinoma cells. In addition to the information gathered from this immunoblot, EST database searches revealed that cDNA clones for EBP50 were also present in breast, white blood cells, and embryo.
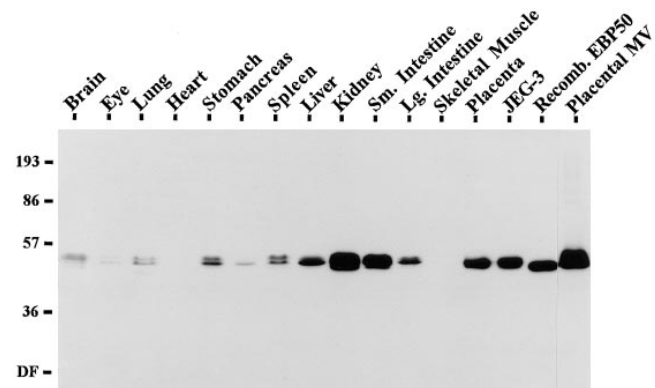


*Figure 8.* Immunoblot of EBP50 in tissues and cells. 25 μg of total proteins from murine tissues, human placenta and JEG-3 cells, 10 ng of recombinant EBP50, and 2 μg of total proteins of isolated human placental microvilli were resolved on a 11.5% gel, transferred to PVDF, and probed with affinity-purified antibody to EBP50.

The EBP50 detected on immunoblots of tissue lysates existed as a series of multiple bands, similar to those seen in our earlier affinity binding experiments on the N-ERMADs (Figs. 2 and 3). This further supports the notion that the multiple forms are the result of posttranslational phosphorylation events.

EBP50 is enriched in tissues possessing extensive, polarized epithelia. These include kidney, small intestine, placenta, and liver (Fig. 8). Since each of these tissues contains significant amounts of one or more of the ERM family members, particularly within the abundant microvilli of their epithelia (Berryman et al., 1993; Amieva et al., 1994), we sought to determine whether EBP50 might also be present in these specialized cell surface structures. Immunoblotting of proteins from isolated human placental microvilli showed that EBP50 is substantially enriched in these structures (Fig. 8).

In cryosections of human placenta, specific EBP50 staining was seen in the apical region of the syncytiotropho-blast (Fig. 9 A). Double labeling with rhodamine phalloidin showed that EBP50 was colocalized with actin in areas with abundant microvilli (Fig. 9, A and B, arrowheads). In addition, the distribution of EBP50, like ezrin, was highly polarized to the microvilli of the intestinal epithelial brush border (Fig. 9, C and D). The specific localization of EBP50 in surface microvilli was most clearly revealed by immunofluorescence microscopy of human JEG-3 cells (Fig. 10 A). The pattern of EBP50 staining in microvilli was very similar to that seen for ezrin (Fig. 10 B).

In the human placental syncytiotrophoblast, immuno-electron microscopy shows that EBP50, like ezrin (Berryman et al., 1993, 1995), specifically associates with the microvilli (Fig. 11).

### EBP50 and Ezrin Associate In Vivo

To assess whether EBP50 and ezrin associate in vivo, lysates of isolated human placental microvilli were subjected
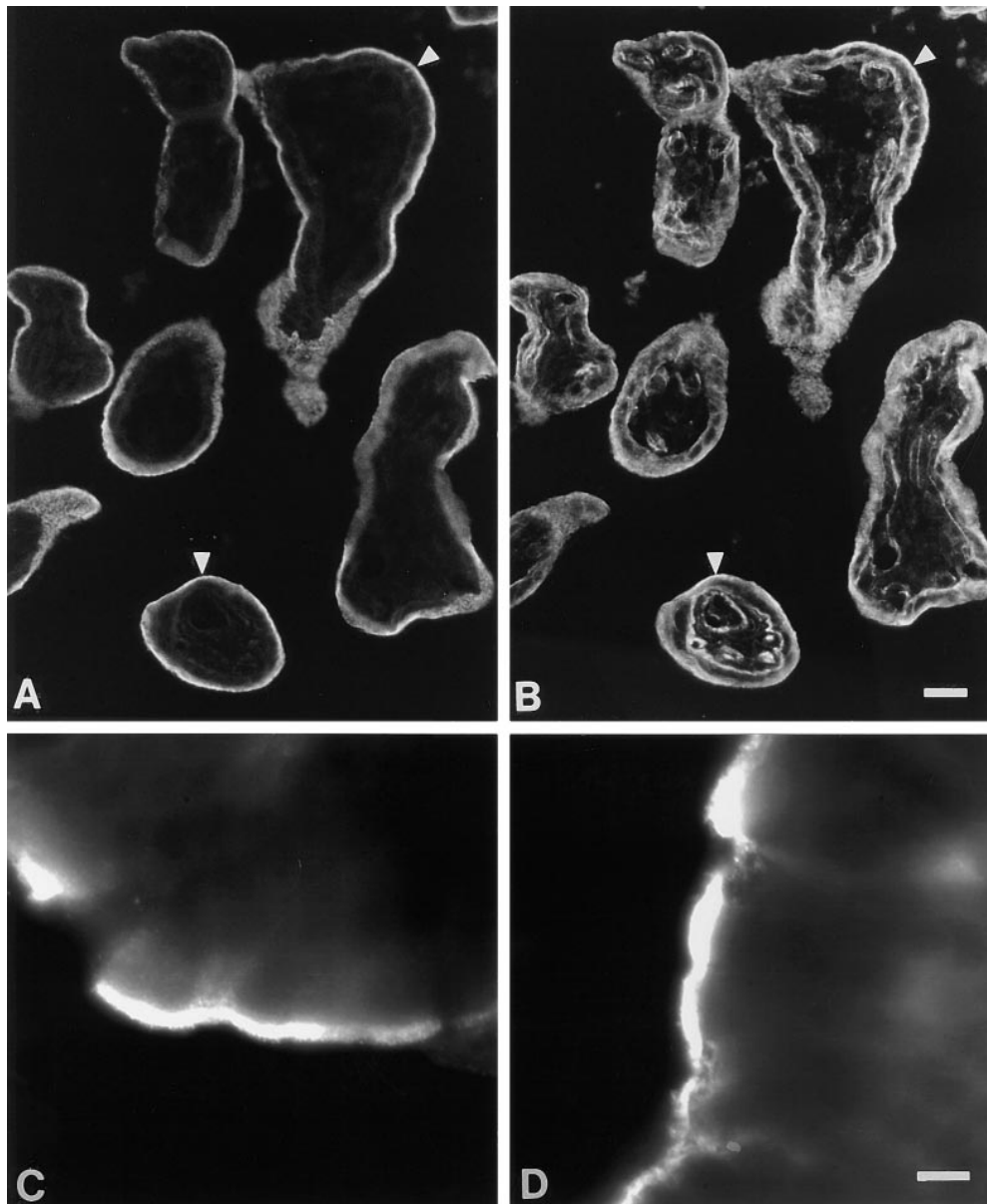


*Figure 9.* Localization of EBP50 in tissues. In human placenta (*A* and *B*), EBP50 (*A*) colocalizes with actin (stained with rhodamine phalloidin in *B*) in the microvilli-rich apical regions (*arrowheads*) of the syncytiotrophoblast. In groups of murine intestinal epithelial cells (*C* and *D*), EBP50 (*C*) is highly concentrated in the microvilli of brush borders, which are also rich in ezrin (*D*). Bars: (*A* and *B*) 20 μm; (*C* and *D*) 5 μm.
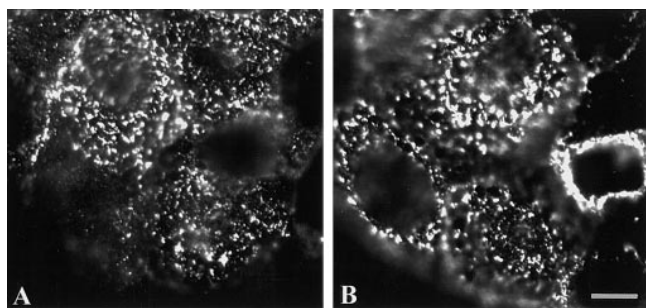
*Figure 10.* Localization of EBP50 (*A*) and ezrin (*B*) in human JEG-3 cells. The plane of focus was adjusted to the microvilli-rich apical surface of the cells. Bar, 10 μm.

to immunoprecipitation with EBP50 and ezrin antibodies and the immunoprecipitates examined for the presence of ezrin and EBP50, respectively (Fig. 12). Immunoblot analysis showed that ezrin was present in the EBP50 immunoprecipitate (Fig. 12 *A*, lane *3*). In the converse experiment, EBP50 was evident in the ezrin immunoprecipitate, although it was difficult to discern precisely which of the multiple species (α, β, or γ) was present (Fig. 12 *B*, lane *3*). Neither ezrin nor EBP50 was detected in the corresponding protein A control precipitates (Fig. 12 *A* and *B*, lane *2*) or preimmune serum control precipitates (not shown). Additional support for the existence of an ezrin–EBP50 complex came from the ability to compete away the EBP50 in ezrin immunoprecipitates by the addition of excess purified human ezrin to the reaction (Fig. 12 *B*, lane *4*).

## *Discussion*

We have identified a phosphoprotein, EBP50, that associates with high affinity and specificity with the N-ERMADs of ezrin and moesin. The binding between EBP50 and the N-ERMADs is direct: this was revealed both by blot overlays and by the binding between the N-ERMADs and recombinant EBP50. Since binding to moesin N-ERMAD can be competed by ezrin N-ERMAD, and since the ERM family members demonstrate high sequence identity over this region, EBP50 probably also binds to radixin.
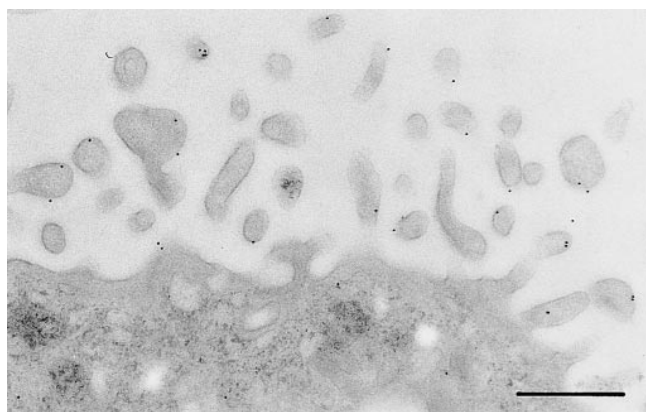


*Figure 11.* Immunoelectron microscopic localization of EPB50 in human placental syncytiotrophoblast. Note the association of EPB50 specifically with the membrane of the abundant microvilli. Bar, 0.5 μm.
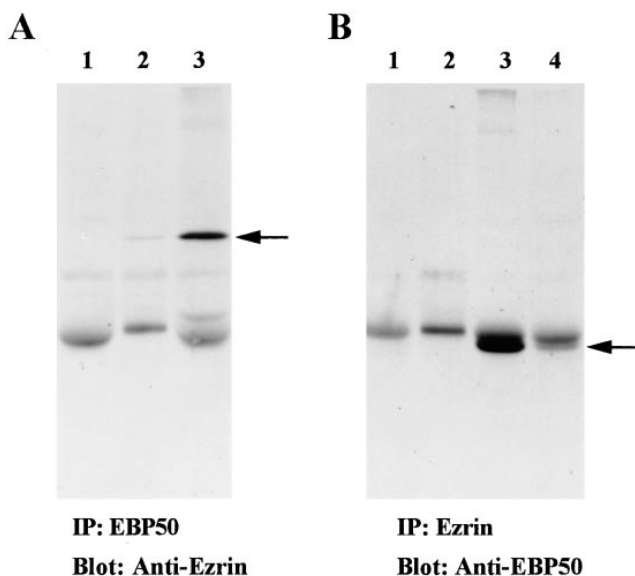


*Figure 12.* Coimmunoprecipitation of EBP50 and ezrin from human placental microvilli. Extracts of isolated microvilli were subjected to immunoprecipitation with antibodies to EBP50 (*A*) and ezrin (*B*). Lanes *1* show a control with antibody but no extract; lanes *2* show a control with extract but no specific antibody; and lanes *3* show the complete reaction. The immunoprecipitates were resolved on a 10% gel, transferred to PVDF, and then probed with biotinylated ezrin antibody (*A*) or biotinylated EBP50 antibody (*B*), followed by avidin-peroxidase. In *B*, lane *4*, excess ezrin was added to the lysate immediately before immunoprecipitation. The migration positions of ezrin (*A*) and EBP50 (*B*) are indicated by arrows.

EBP50 is widely distributed, being particularly rich in liver, kidney, small intestine, and placenta—tissues with polarized epithelia and known to contain significant amounts of ERM family members (Berryman et al., 1993; Amieva et al., 1994). Since liver contains only trace amounts of ezrin and moesin, but is rich in radixin, these results are also consistent with radixin being a ligand for EBP50. Localization studies in cultured cells and tissue sections showed a pattern of staining in cell surface microvilli indistinguishable from that of ezrin. Moreover, in the placental syncytiotrophoblast, immunoelectron microscopy reveals that EBP50, like ezrin, is specifically associated with the microvilli. Immunoprecipitation of EBP50 from extracts of highly purified placental microvilli coprecipitates some of the ezrin, and vice versa. Thus, EBP50 colocalizes with ezrin in structures containing a supporting actin bundle, and exists as a complex with ezrin in solubilized microvillar cytoskeletons. We conclude that EBP50 is a physiologically relevant ezrin-binding protein.

The most striking feature of the 357-residue EBP50 sequence is the presence of two NH$_2$-terminal, ~90-residue domains that show 74% identity to each other and homology to PDZ domains (Fig. 6). Single or multiple PDZ domains (also known as DHR domains) have been identified in a number of cortical proteins (for review see Ponting and Phillips, 1995; Saras and Heldin, 1996). These domains appear to be involved in the formation of multiprotein complexes under the plasma membrane. A well-studied example is PSD-95, which consists of a membrane-associ-

ated guanylate kinase (MAGUK) domain fused to three PDZ domains and an SH3 domain. This protein binds to the COOH terminus of the Shaker-type $K^+$ channel or to subunits of the NMDA receptor through its PDZ domains (Kim et al., 1995; Kornau et al., 1995). Another particularly relevant example is p55 of the red blood cell, which has a single PDZ domain and an SH3 domain followed by a MAGUK domain. p55 binds to the COOH-terminal region of glycophorin C, perhaps through the PDZ domain, and to the $NH_2$-terminal domain of band 4.1 through another region (Marfatia et al., 1995). Interestingly, some protein tyrosine phosphatases within the band 4.1 superfamily, such as PTPH1 (Yang and Tonks, 1991), also contain PDZ domains, suggesting that functions analogous to those provided by ezrin/EBP50 and band 4.1/p55 are combined into a single polypeptide. With this knowledge of PDZ-containing proteins, and especially the erythrocyte glycophorin C/p55/band 4.1 model, human EBP50 is most likely an adaptor molecule between ezrin and an integral membrane protein. Among the proteins that coimmunoprecipitate with moesin from BHK cells are CD44 and a 55-kD polypeptide (Hirao et al., 1996). If this 55-kD protein is EBP50, perhaps a CD44/EBP50/moesin complex might exist, reminiscent of the glycophorin/p55/band 4.1 complex (Marfatia et al., 1994, 1995). A different complex must exist in ERM-rich cell types that lack CD44 (Berryman et al., 1995; Hirao et al., 1996).

Human EBP50 is related (84% identity) over its entire length to rabbit NHE-RF (Weinman et al., 1995) and to human TKA-1 over the region encompassing the two PDZ domains (Fig. 5). Very recently it has been shown that transfection of NHE-RF or TKA-1 (also called E3KARP) into cultured cells confers PKA regulation on the NHE3 $Na^+/H^+$ exchanger (Yun et al., 1997). Moreover, it was shown that a region encompassing the second PDZ domain of TKA-1 binds the cytoplasmic domain of NHE3, so it is tempting to speculate that in intestine and kidney, the only tissues that express NHE3 (Tse et al., 1992), EBP50 might provide a link between NHE3 and ERM family members.

The identification of EBP50 as a protein that binds the N-ERMADs of ezrin, moesin, and probably radixin raises a number of interesting questions. The first relates to the details of the molecular associations between EBP50 and ERM family members. There is no obvious sequence similarity between the C-ERMAD of ezrin and any region of EBP50 that would suggest a common binding site. In fact, there is a clear biochemical distinction between the nature of the N-ERMAD/EBP50 and N-ERMAD/C-ERMAD interactions. The ability of the N-ERMAD to bind to a C-ERMAD is very sensitive to denaturation (Gary and Bretscher, 1995), whereas the N-ERMAD is recognized by EBP50 even after it has been denatured and subjected to electrophoresis. Whether EBP50 and a C-ERMAD can bind the same N-ERMAD simultaneously, or whether they compete for binding, remains an important question.

Several observations suggest that EBP50 may be subject to regulation. The earliest information is derived from studies on the role of its putative homologue, rabbit protein cofactor, in the regulation of the kidney brush border $Na^+/H^+$ exchanger (Weinman et al., 1990). Activation of PKA by parathyroid hormone reduces the activity of the exchanger in a reaction requiring a crude fraction containing a protein cofactor (NHE-RF). Analysis of partially purified fractions identified a polypeptide that was a substrate for PKA (Morell et al., 1990; Weinman et al., 1990). Partial sequence analysis was used to generate a peptide antibody that recognized a 55-kD protein in kidney brush border membranes, which appeared to be a substrate for PKA (Weinman et al., 1993). Using the same peptide sequence to design an oligonucleotide probe, Weinman et al. (1995) were able to clone a rabbit cDNA that can confer PKA regulation of NHE3 in transfected cells (Yun et al., 1997). Other evidence supporting the possible regulation of EBP50 by PKA is provided by the finding that the regulatory subunit of PKA binds ezrin (Dransfield et al., 1997); whether or not this interaction recruits the kinase to phosphorylate associated EBP50 remains to be investigated. The presence of at least three differentially phosphorylated species of EBP50 in placenta is also suggestive of regulation by phosphorylation. That these polypeptides are modified forms of EBP50 is supported by the fact that they have similar amino acid compositions, are recognized by our antibody to recombinant EBP50, and bind ezrin N-ERMAD both on the affinity column and in blot overlays. Thus, an attractive scenario is that phosphorylation regulates the association between EBP50 and a membrane protein. EBP50 has a potential PKA site (Ser338) and two potential cdc2 sites (Ser279 and Ser301). It will be important to identify the kinase(s) responsible for EBP50 phosphorylation to see in which signal transduction pathways they lie.

In addition to the putative regulation of EBP50–membrane protein association, it is also likely that the interaction between EBP50 and ezrin is regulated. We have provided evidence that ezrin can exist in a dormant and activated state, and postulated that activation induces membrane–cytoskeletal associations (Berryman et al., 1995). A possible scenario is that dormant ezrin and EBP50 do not associate, but upon activation a cascade of protein interactions occurs driving the membrane protein/EBP50/ezrin/F-actin linkage. What membrane proteins might be bound, or how the associations are regulated, both at the level of EBP50 and in terms of the regulation of ERM proteins through conformational changes, are questions for future studies.

*Note Added in Proof.* Reinspection of the TKA-1 cDNA sequence, together with the newly deposited sequence for human E3KARP (these sequence data are available from EMBL/GenBank/DDBJ under accession number AF004900), suggests an error in the TKA-1 cDNA sequence that changes the reading frame at residue 310. In the new frame, TKA-1/E3KARP has 337 residues and shows 55% indentity to EBP50 throughout the protein.

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