Evidence for Ezrin-Radixin-Moesin-binding Phosphoprotein 50 (EBP50) Self-association through PDZ-PDZ Interactions*

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Ezrin-radixin-moesin (ERM)-binding phosphoprotein 50 (EBP50) is a versatile membrane-cytoskeleton linking protein that binds to the COOH-tail of specific integral membrane proteins through its two PDZ domains. These EBP50 binding interactions have been implicated in sequestering interactive sets of proteins into common microdomains, regulating the activity of interacting proteins, and modulating membrane protein trafficking. With only two PDZ domains, it is unclear how EBP50 forms multiprotein complexes. Other PDZ proteins increase their breadth and diversity of protein interactions through oligomerization. Hypothesizing that EBP50 self-associates to amplify its functional capacity, far-Western blotting of cholangiocyte epithelial cell proteins with EBP50 fusion protein revealed that EBP50 binds to a 50-kDa protein. Far-Western blotting of EBP50 isolated by two-dimensional gel electrophoresis or immunoprecipitation demonstrates that the 50-kDa binding partner is itself EBP50. Further, co-transfection/co-precipitation studies show the self-association can occur in an intracellular environment. In vitro analysis of the EBP50-EBP50 binding interaction indicates it is both saturable and of relatively high affinity. Analysis of truncated EBP50 proteins indicates EBP50 self-association is mediated through its PDZ domains. The ability to self-associate provides a mechanism for EBP50 to expand its capacity to form multiprotein complexes and regulate membrane transport events.

PDZ (PSD95, Dlg, ZO-1) proteins serve as central organizers of protein complexes. The 80–90-amino acid PDZ domains are most noted for their capacity to bind the COOH-tail of specific integral membrane proteins (1). Through a distinct interface, some PDZ domains also have the capacity to participate in separate PDZ-PDZ interactions. Interestingly, these properties allow a single PDZ domain to bind concurrently with both the COOH-tail of an integral membrane protein and another protein through a PDZ-PDZ interaction (2). PDZ proteins amplify their capacity and diversity of protein-protein interactions through co-expression of additional protein binding motifs (e.g. SH2 domains, SH3 domains, ankyrin repeats, ERM binding domains), expression of multiple PDZ domains within the same PDZ protein, and oligomerization with other PDZ proteins (2–7). Oligomerization can occur either through non-PDZ domain interactions or through PDZ-PDZ interactions.

ERM-binding phosphoprotein 50 (EBP50; also known as NHE regulatory factor) is a PDZ domain protein found in several epithelial cell types. EBP50 has been shown to bind a growing number of integral membrane proteins including cAMP-regulated Na+/H+ exchanger 3 (NHE3), β2-adrenergic receptor, G protein-coupled receptor kinase-6A (GRK6A), Yes-associated protein 65 (YAP65), and the cAMP-dependent cystic fibrosis transmembrane conductance regulator Cl– channel (cftf) (8–12). With only two PDZ domains, individual EBP50 proteins have a limited capacity to form multiprotein arrays. Theoretically, EBP50 oligomerization could amplify the capacity of the existing PDZ domains to sequester interactive proteins within membrane microdomains. The present study demonstrates the capacity of EBP50, both in vivo and in vitro, to self-associate with high affinity through PDZ-PDZ interactions.

EXPERIMENTAL PROCEDURES

EBP50 Fusion Protein Constructs and Expression—GST-EBP50 fusion proteins were created by polymerase chain reaction amplification of the full-length or segment rat EBP50 cDNA (GenBank™ accession number AF154336), ligation into PGEX-2T or PGEX-4T-3 vectors (Amersham Pharmacia Biotech), and transformation into Escherichia coli strain DH5α. The primer sets include (a) ggtgaattccgcagagcagatgagcgcg/ggagaattctgctcacttgctccagtccatctg (COOH-tail); (b) ggtgaattccgcagagcagatgagcgcg/ggagaattctgctcacttgctccagtccatctg (PDZ1); (c) ggtgaattccgcagagcagatgagcgcg/ggagaattctgctcacttgctccagtccatctg (PDZ2); (d) ggtgaattccgcagagcagatgagcgcg/ggagaattctgctcacttgctccagtccatctg (PDZ1,2); (e) ggtgaattccgcagagcagatgagcgcg/ggagaattctgctcacttgctccagtccatctg (PDZ1,2); (f) ggtgaattccgcagagcagatgagcgcg/ggagaattctgctcacttgctccagtccatctg (PDZ1,2); (g) ggtgaattccgcagagcagatgagcgcg/ggagaattctgctcacttgctccagtccatctg (PDZ1,2); (h) ggtgaattccgcagagcagatgagcgcg/ggagaattctgctcacttgctccagtccatctg (PDZ1,2). The truncated EBP50 proteins were expressed in bacteria and purified using glutathione-Sepharose 4B beads.

In vitro interaction experiments were performed using a GST-EBP50 fusion protein immobilized on glutathione-Sepharose 4B beads. Beads were washed with 150 mM NaCl, 50 mM Tris, pH 8.0, and 1 mM EDTA, followed by two washes with 50 mM Tris, pH 8.0, and 1 mM EDTA, and finally with 50 mM Tris, pH 8.0, 150 mM NaCl. The GST fusion protein was then incubated with the purified EBP50 protein for 2 hours at 4 °C. The beads were washed extensively with 50 mM Tris, pH 8.0, 150 mM NaCl, and 1 mM EDTA, followed by two washes with 50 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Nonidet P-40. The beads were then suspended in SDS-PAGE sample buffer and boiled for 5 minutes.

SDS-PAGE and Western blotting were performed to visualize the GST-EBP50 fusion protein and the purified EBP50 protein. The blots were probed with anti-EBP50 antibodies and visualized using horseradish peroxidase-conjugated secondary antibodies and chemiluminescence.

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4 µg of total protein from normal rat cholangiocytes was solubilized in 5× PAGE buffer (5% SDS, 25% sucrose, 50 mM Tris, 5 mM EDTA, pH 8.0), electrophoresed on a 3.5–17.5% SDS-PAGE gel, and transferred to polyvinylidene difluoride (PVDF) membrane. Subsequent overlay studies blotted 10 µg of recombinant EB50 protein. Immobilized proteins were blocked with 5% nonfat dry milk in PBS-T (0.1% Tween-20) for 1 h at room temperature. Unless otherwise stated, the immobilized proteins were then incubated in blot buffer with 500 ng recombinant EB50 protein and protease inhibitors for 2 h at room temperature. In some experiments, EB50 was biotinylated (0.5 µg/mL N-hydroxysuccinimidobiotin (Pierce), 45 min, 4 °C) prior to overlaying the blots. EB50 protein was detected by Western blotting with HRP-tagged secondary antibody and enhanced chemiluminescence (Pierce). Biotinylated EB50 was detected with HRP-tagged streptavidin (Amersham, Piscataway, NJ) followed by ECL. EB50 localization and binding interaction, respectively. Following Western and far-Western blotting, the membranes were washed in TBS-Tween and then incubated in blot buffer containing 0–10 µM biotinylated EB50. Following paired colorimetric development of all blots using Opti-CN, the relative levels of bound EB50 were quantified by densitometry (IP Laboratory, National Institutes of Health).

EB50 Oligomerization and Cross-linking—Thrombin-activated recombinant EB50 (0.1 mg/mL) was brought to room temperature, incubated for 1 h, and divided into two aliquots. The first aliquot was cross-linked in 3.3 mM bis(sulfosuccinimidyl) suberate (Pierce) for 2 °C. EBP50 itself does not contain thrombin-sensitive sites. Thrombin was then inactivated by the addition of 1 mM phenylmethylsulfonyl fluoride and 1 µM leupeptin.

EB50 Domain-specific Binding—To determine the domain responsible for EB50 self-association, domain-specific EB50 fusion proteins were generated, biotinylated, cleaved from the immobile phase with thrombin, and overlaid onto blotted full-length EB50. These recombinant EB50 domains included the PDZ1,2 domain (amino acids 1–260), the post-PDZ domain COOH segment (amino acids 228–356), and COOH-tail (amino acids 1–348). Next, domain-specific EB50 proteins (COOH-tail, PDZ1,2, post-PDZ COOH segment) were blotted and overlaid with biotinylated forms of the same domain-specific form of EB50. Subsequently, the PDZ domain (amino acids 1–133) and PDZ1 domain (amino acids 133–260) were generated, biotinylated, and overlaid onto wild-type, PDZ1,2, PDZ1, and PDZ1,2 domains of EB50.

RESULTS

Characterization of GST-EB50 Fusion Proteins—GST-EB50 fusion proteins were generated for use in far-Western detection of binding partners (Fig. 1A). Following isolation of GST proteins, total protein staining (left panel) detected no protein from control bacteria (lane 1), a single protein consistent with 26-kDa GST protein (lane 2), and two prominent protein bands of ~ 80 kDa consistent with GST-EB50 (lane 3). Western blotting with anti-GST antibody (middle panel) confirmed the single protein in lane 2, and the doublet in lane 3 contained GST protein. Western blotting with anti-EB50 antibody (right panel) confirmed that the protein doublet in lane 3 contained EB50. The lower band of the doublet is a likely partial proteolytic product of the intact protein. Thrombin treatment of the intact GST-EB50 resulted in site-specific cleavage (Fig. 1B). Total protein staining (left panel) showed that thrombin treatment cleaved the intact protein (lane 1) and liberated a 50-kDa protein (lane 2). GST was not eluted from the immobile phase following thrombin digestion and would not be predicted to be present in lane 2. Western blotting with EB50 antibody (right panel) confirmed that the thrombin-activated protein was indeed EB50. Similar approaches were utilized to generate truncated forms of EB50 (see below).

Recombinant EB50 Is Capable of Binding with a 50-kDa Protein in Cholangiocytes—Thrombin-activated recombinant EB50 was used in two different far-Western blotting protocols to demonstrate EB50 is capable of binding an endogenous 50-kDa protein from NRC cells. First, Western-blotted proteins from NRC cells, which express endogenous EB50, were overlaid with recombinant EB50. Paired blots without overlaid recombinant protein served as the control. Immunodetection of
EBP50, which detects both endogenous and recombinant EBP50, was then performed (Fig. 2; A-1 and A-2). In all paired samples tested ($n=5$), the 50-kDa band on the blot that was overlaid with recombinant EBP50 (A-1) was more intense than the 50-kDa band that was not overlaid with recombinant EBP50 (A-2). This increased EBP50 signal intensity in the overlaid sample suggests that EBP50 binds either to itself or another protein of similar mass.

To specifically detect only the overlaid EBP50, recombinant EBP50 was biotinylated before being overlaid onto the blotted NRC proteins and detected with horseradish peroxidase-labeled neutravidin. The overlaid biotinylated EBP50 again detected a protein at 50 kDa. Its relative intensity is lower when compared with other EBP50-bound proteins (Fig. 2; B-1). This lower relative intensity would be predicted because endogenous EBP50 is not detected by this assay. Paired blots of NRC proteins that were not overlaid with biotinylated EBP50 (negative control; B-2) showed no signal when detected with neutravidin-HRP. These two observations indicate that recombinant EBP50 specifically interacts with either endogenous EBP50 or a protein(s) that co-migrates at 50 kDa.

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Recombinant EBP50 Is Capable of Self-associating with EBP50 from NRC Cells—To directly determine if the endogenous 50-kDa binding partner is indeed EBP50, three distinct approaches were employed. First, in a similar overlay approach, paired NRC protein samples (Triton-insoluble fraction) were separated by two-dimensional gel electrophoresis and transferred to nitrocellulose. The first two-dimensional blot was developed by standard Western blotting to identify the migration pattern of EBP50. EBP50 (Fig. 3A, panel 1, arrow)
Both HA-EBP50 and FLAG-EBP50 were co-transfected. Together, these vectors only/FLAG-EBP50, FLAG-EBP50 was readily detected when Western blotting for EBP50 was performed. Immunoprecipitation of FLAG-EBP50 was assessed by Western blotting against the HA-EBP50 was immunoprecipitated from cell lysates, and co-precipitated EBP50 was readily detected in a region consistent with its demonstrated migration at 50 kDa and predicted pl value of 6.2. Protein staining with colloidal gold shows the relative position of EBP50 to neighboring proteins (panel 2). In the paired blot, these proteins were used to confirm the location of EBP50 (panel 4). When paired blots were overlaid with biotinylated recombinant EBP50, there was specific binding between recombinant EBP50 and endogenous EBP50 (arrow; panel 3).

These observations were mirrored in overlays of immunoprecipitated EBP50 (Fig. 3B). Western blotting (left panel) shows that EBP50 was specifically immunoprecipitated from NRC cell lysates. In contrast to either precipitates obtained in the absence of EBP50 antibody or albumin controls, paired blots show that biotinylated EBP50 readily binds to immunoprecipitated EBP50 protein, again consistent with EBP50 self-association.

**EBP50 Self-association Occurs in an in Vivo Environment**—To determine if the observed in vitro EBP50-EBP50 binding interaction can occur in an intracellular environment, a co-transfection/co-precipitation approach was employed (Fig. 3C). HA- and FLAG-tagged EBP50 were co-transfected into HEK-296 cells, and the co-precipitation of FLAG-EBP50 with immunoprecipitated HA-EBP50 was assayed. In contrast to cells co-transfected/co-precipitated with FLAG-EBP50 and HA-vector only, cells co-transfected with FLAG-EBP50 and HA-EBP50 showed FLAG-EBP50 present in the washed HA-immunoprecipitate fraction (Fig. 3C). Other control co-transfections/co-precipitations (FLAG-vector only/HA-vector only; FLAG-vector only/HA-EBP50) were also negative.

**EBP50 Self-association Is Saturable and of Relative High Affinity**—Using isolated recombinant EBP50 protein, the binding characteristics of the EBP50-EBP50 interaction were evaluated. First, EBP50 self-association was confirmed by binding of biotinylated recombinant EBP50 to immobilized GST-EBP50 (Fig. 4A). Albumin and GST-only served as negative controls. Coomassie Blue staining of albumin, GST-only, and GST-EBP50 in paired gels demonstrate that equivalent amounts of protein were added (left panel). Biotinylated EBP50, after being overlaid on a paired blot, bound only to the GST-EBP50 protein (right panel).

Using albumin as a negative control, the EBP50-EBP50 interaction was evaluated further by overlaying immobilized EBP50 with varying concentrations of biotinylated EBP50 (Fig. 4B). Quantitation of the resultant binding showed that the EBP50-EBP50 interaction was both saturable and of relatively high affinity.

**EBP50 Self-association Can Result in the Formation of Multimers**—EBP50 self-association could generate simple dimerization or lead to the formation of more complex oligomers. To determine if oligomerization can occur, recombinant EBP50 was incubated in suspension, covalently cross-linked, and assayed for the presence of multimers by Western blotting (Fig. 5). Whereas denatured EBP50 in non-cross-linked samples ran almost exclusively at 50 kDa, a significant fraction of EBP50 in paired cross-linked samples appeared at higher molecular masses. The apparent mass of these proteins (~100 kDa and ~150 kDa) is consistent with oligomer formation. Although the predominant oligomer was the dimer, a trimer was also readily observed, and a tetramer was seen at longer exposures (data not shown).

**EBP50 Self-associates through PDZ-PDZ Interactions**—To evaluate which regions of EBP50 participate in the self-association interaction, specific domains of EBP50 were synthesized.
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**Fig. 4.** *In vitro* binding analysis demonstrates avid EBP50 binding and multimer formation. Biotinylated recombinant EBP50 bound specifically to GST-EBP50. A, equivalent amounts of albumin (Alb) (negative control), GST-only (negative control), and GST-EBP50 were blotted onto nitrocellulose (left panel). When overlaid with biotinylated EBP50 only the GST-EBP50 protein had detectable EBP50 (right panel), further indicating that EBP50 is capable of self-association. B, paired blots of recombinant EBP50 (10 μg) were overlaid with biotinylated EBP50 at varied concentrations (1–5000 nM). Blotted albumin (10 μg) served as a negative control. The binding was relatively high in affinity and saturable. A representative experiment from five separate experiments is shown.

**Fig. 5.** EBP50 is capable of forming trimers and tetramers. To assess if EBP50 binding is limited to dimer formation or is capable of forming larger multimers, recombinant EBP50 was allowed to self-associate in solution and subsequently covalently cross-linked and assayed by Western blotting. While EBP50 dimers were the most prevalent oligomer observed, both trimers and tetramers (at longer exposures) were observed.

(Fig. 6), biotinylated, and overlaid onto immobilized full-length EBP50 (Fig. 7A). These EBP50 domains include 1) full-length EBP50, 2) COOH-tailless EBP50, 3) PDZ_{1,2} domains, 4) post-PDZ_{1,2} COOH segment, 5) PDZ₁ domain, and 6) PDZ₂ domain.

The COOH-tailless EBP50 proteins bound with an avidity that was indistinguishable from the full-length EBP50 protein. This observation was reiterated by comparing the self-association of COOH-tailless to immobilized COOH-tailless and full-length EBP50 (Fig. 7B). Again, the degree of association was not notably different between the two groups, indicating that the COOH-tail was not involved in EBP50 self-association.

The COOH segment EBP50 protein showed little or no detectable binding (Fig. 7A). This indicates the post-PDZ COOH segment is not capable itself of binding EBP50. Likewise, no detectable binding was observed when the COOH segment was overlayed onto itself (data not shown). In contrast, the PDZ₁,₂ domain showed a greater degree of binding to EBP50 than the full-length EBP50 (Fig. 7A). This occurred consistently across three separate preparations of recombinant EBP50 proteins. The avid binding indicates that the PDZ₁,₂ domain is involved in the EBP50-EBP50 self-association reaction. To verify that the PDZ₁,₂ domain binding interaction occurred with the PDZ₁,₂ domain of the immobilized EBP50, the binding of biotinylated PDZ₁,₂ to blotted PDZ₁,₂ was examined (Fig. 7B). PDZ₁,₂ showed avid binding to PDZ₁,₂, demonstrating that the EBP50-EBP50 binding interactions occurred through a PDZ₁,₂ domain.

Finally, to determine if one or both of the two PDZ domains of EBP50 are involved in the self-association reaction, the binding of biotinylated PDZ₁ and PDZ₂ domains to immobilized full-length EBP50 as well as the individual PDZ₁ and PDZ₂ domains was assessed (Fig. 7C). Interestingly, both PDZ₁ and PDZ₂ bound EBP50. When the specific PDZ domain interactions were evaluated, both PDZ₁ and PDZ₂ could bind to either PDZ domain. Their apparent interactions, however, were not equivalent. For both PDZ domains, the homologous binding interactions predominated such that PDZ₁ binding was greater with PDZ₁ than with PDZ₂ and PDZ₂ binding was greater with PDZ₂ than with PDZ₁.
EBP50, the binding interaction between tailless EBP50 and immobilized full-length EBP50 was also analyzed. It also failed to show any difference in either its apparent affinity or binding capacity (Fig. 7B). Consequently, the EBP50-EBP50 interaction is unlikely to occur in a head-to-tail fashion.

PSD95, one of the originally described PDZ proteins, oligomerizes through a non-PDZ segment of its protein (4). The post-PDZ1,2 COOH segment has not been fully characterized but, as a minimum, has the capacity to bind members of the ezrin-radixin-moesin family (7). Given its known protein binding capacity, its potential involvement in EBP50 self-association was examined. Run in parallel with full-length EBP50, no oligomerization of EBP50-COOH segment was observed (Fig. 7A). Taken together, these findings indicate that the COOH segment is unlikely to account for the EBP50-EBP50 binding.

The capacity of proteins to oligomerize through PDZ-PDZ interactions is demonstrated by the association of nNOS with syntrophin and PSD95 and the dimerization of InaD (2, 5). To determine if EBP50 self-association occurs through its PDZ domains, the binding of the PDZ1,2 construct to immobilized EBP50 was compared with that of wild type EBP50. In three separate preparations, there was greater binding of PDZ1,2 than full-length EBP50 (Fig. 7). This strongly supports the hypothesis that EBP50 self-association occurs through PDZ-PDZ interactions and suggests that the COOH segment could modify the binding characteristics. Unlike the dimerization of InaD, which occurs between two distinct PDZ domains within InaD, the most prominent PDZ-PDZ domain interactions of EBP50 occurred between homologous PDZ domains (i.e. PDZ1 to PDZ1 and PDZ2 to PDZ2; Fig. 7C). Thus, EBP50 self-association is apparently mediated through a novel interaction involving homologous PDZ domains.

**Functional Significance of EBP50-EBP50 Self-association**

The functional significance of EBP50 self-association remains speculative at this point. In other systems, PDZ domain-dependent protein-protein interactions have been described (2, 5). PDZ-PDZ interactions can occur between disparate proteins (5) or between PDZ domains of the same protein (2). In the case of nNOS-syntrophin binding, both proteins contain only a single PDZ domain, and the PDZ-PDZ binding interaction is considered a mechanism to allow nNOS to affiliate with the dystrophin complex (5). In contrast, InaD has five distinct PDZ domains which enable InaD to sequester and regulate receptor, signaling, and effector proteins of the rhodopsin phototransduction system of *Drosophila*. InaD is central to the spatiotemporal orchestration of events that culminate in photoreceptor activity and subsequent desensitization. Oligomerization of InaD through the third and fourth PDZ domains of separate InaD proteins is considered to amplify the capacity and complexity of InaD sequestered proteins. EBP50 by itself, with only two PDZ domains, has a limited capacity to sequester arrays of proteins into membrane microdomains. In the renal proximal tubule, where EBP50 has been studied in greatest detail, EBP50 can bind at least three discrete proteins to its PDZ domains (8, 10, 13). This includes the concurrent binding and activity modulation of β-adrenergic receptor or GRK6A with NHE3 (10, 13). As evidence for multiple protein interactions between EBP50, regulatory proteins, and effector proteins continues to emerge, a better understanding of the molecular mechanisms that could coordinate these interactions is required. Accordingly, the capacity of EBP50 to oligomerize into trimers and tetramers, rather than simple dimers (Fig. 5), further extends the potential of EBP50 to sequester interactive proteins within membrane microdomains. In this regard, EBP50 self-association seems more likely to resemble the InaD paradigm, serving as a mechanism...
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to congregate and integrate interactive signaling, regulatory, and effector proteins.