Erbin Is a Protein Concentrated at Postsynaptic Membranes That Interacts with PSD-95*

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Neuregulin is a factor essential for synapse-specific transcription of acetylcholine receptor genes at the neuromuscular junction. Its receptors, ErbB receptor tyrosine kinases, are localized at the postjunctional membrane presumably to ensure localized signaling. However, the molecular mechanisms underlying synaptic localization of ErbBs are unknown. Our recent studies indicate that ErbB4 interacts with postsynaptic density (PSD)-95 (SAP90), a PDZ domain-containing protein that does not interact with ErbB2 or ErbB3. Using as bait the ErbB2 C terminus, we identified Erbin, another PDZ domain-containing protein that interacts specifically with ErbB2. Erbin is concentrated in postsynaptic membranes at the neuromuscular junction and in the central nervous system, where ErbB2 is concentrated. Expression of Erbin increases the amount of ErbB2 labeled by biotin in transfected cells, suggesting that Erbin is able to increase ErbB2 surface expression. Furthermore, we provide evidence that Erbin interacts with PSD-95 in both transfected cells and synaptosomes. Thus ErbB proteins can interact with a network of PDZ domain-containing proteins. This interaction may play an important role in regulation of neuregulin signaling and/or subcellular localization of ErbB proteins.

The neuromuscular junction is a cholinergic synapse that conveys signals rapidly from motoneurons to muscle cells. The fast and accurate neurotransmission at this synapse is guaranteed by the high concentration of acetylcholine receptors in the postsynaptic membrane, which accounts for only 0.1% of total muscle surface (1, 2). Muscle fibers are multinucleated cells. Remarkably, it is only the synaptic nuclei that actively transcribe genes encoding acetylcholine receptor subunits. Such synapse-specific transcription is mediated by neuregulin, a factor used by motoneurons to stimulate acetylcholine receptor synthesis at the neuromuscular junction. Neuregulin receptor transmembrane tyrosine kinases of the ErbB family: ErbB2, ErbB3, and ErbB4. Stimulation by neuregulin of ErbB receptors leads to their tyrosine phosphorylation (3–6) and subsequent activation of multiple intracellular signaling cascades (6–9), essential for compartmental synthesis of acetylcholine receptors. In the central nervous system, neuregulin regulates expression of neuronal nicotinic acetylcholine receptor (10), N-methyl-D-aspartate receptor (11), and γ-aminobutyric acid receptor (12). Recent studies suggest that in addition to an essential role during development, neuregulin appears to regulate synaptic plasticity in the adult brain (13).

ErbB proteins are not expressed evenly on the surface of cells. On the contrary, they are localized in subcellular compartments. In the nervous system, ErbB proteins are concentrated in postsynaptic membranes both at the neuromuscular junction (3, 14–16) and in the central nervous system (13, 17). In epithelial cells, ErbB2 appears to be enriched in basolateral membranes (18). The mechanism by which ErbB proteins are localized in the subcellular compartments remains largely unknown. The intracellular portions of ErbB receptor tyrosine kinases contain large C termini in addition to kinase domains. Thus, it is conceivable that ErbBs may interact with proteins that regulate their localization, surface expression, or kinase activity. Indeed, recent studies demonstrated that ErbB4, via its C terminus, interacts with postsynaptic density (PSD)1–95 (or SAP90), a PDZ domain-containing protein (13, 17). PDZ domains are motifs of 80–90 amino acids which often bind to specific sequences at the extreme C termini of target proteins (19–22). They were originally identified in PSD-95, the Drosophila septate junction protein discs large, and the epithelial tight-junction protein zona occludens 1 (23–26). PDZ domain-containing proteins appear to coordinate the assembly of functional subcellular domains. PSD-95 uses multiple PDZ domains to cluster ion channels, receptors, and cytosolic signaling proteins in subcellular domains including synapses and cellular junctions (27). The interaction of PSD-95 with ErbB4 potentially may allow for a localized signaling complex at synapses while minimizing unwanted cross-talk. Moreover, PSD-95 could enhance neuregulin signaling probably by promoting dimerization of ErbB4 receptor tyrosine kinases (13).

However, PSD-95 interacts with ErbB2 poorly and does not interact with ErbB3 (13, 17), which raises the possibility that other PDZ domain-containing protein may exist. Using a yeast two-hybrid strategy, we identified a novel PDZ domain-containing protein that interacts specifically with ErbB2, but not ErbB3 or ErbB4. This protein was named B2BP for ErbB2-binding protein. B2BP was a polypeptide of 180 kDa. It had 16 leucine-rich repeats (LRRs) in the N terminus and a PDZ domain in the C terminus. While the study was in progress, Borg et al. reported the cloning of Erbin as an ErbB2-interact-

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‡ The abbreviations used are: PSD, postsynaptic density; B2BP, ErbB2-binding protein (Erbin); LRR, leucine-rich repeat; GST, glutathione S-transferase; HEK, human embryonic kidney; PBS, phosphate-buffered saline; SPM, synaptosomal plasma membrane; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-(3-cholamidopropyl)-dimethylammonio)-1-propanesulfonic acid.
ErbBs Interacting with a Network of PDZ Proteins

The GST fusion protein containing the PDZ of Erbin (amino acids 1241–1371) was produced, affinity purified, and concentrated as described previously (29). The GST fusion protein and GST- Erbin/PDZ fusion protein to Affi-Gel 15 (Bio-Rad), respectively, according to the manufacturer’s instruction. The GST fusion protein was used in all experiments unless otherwise specified.

Immunoprecipitation, Pull-down Assays, and Immunoblotting—Cell lysates (~400 µg of protein) were incubated directly without or with indicated antibodies for 1 h at 4 °C. They were then incubated with protein A-agarose beads overnight at 4 °C on a rotating platform. After centrifugation, beads were washed four or five times with the modified RIPA buffer. Bound proteins were eluted with SDS sample buffer and subjected to SDS-PAGE. Immunoprecipitation of Erbin from rat brain P2 fraction was done as previously described (19).

The GST fusion protein containing the PDZ of Erbin (amino acids 1241–1371) was induced in BL21 cells with 1 mM isopropyl-β-D-thiogalactopyranoside and purified using glutathione-agarose beads (Roche Molecular Biochemicals, Indianapolis). Equal amounts of GST fusion protein beads (~50 µg of protein) were incubated with cell lysates overnight at 4 °C on a rotating platform. After centrifugation, beads were washed four or five times with wash buffer (150 mM sodium chloride, 10 mM sodium phosphate, 1% Triton X-100, 0.1% sodium dodecyl sulfate) and resuspended in 10 µl of 2x SDS loading buffer. They were then boiled for 5 min and subjected to SDS-PAGE and immunoblotting.

Proteins resolved on SDS-PAGE were transferred to nitrocellulose membranes (Schleicher & Schuell). Nitrocellulose blots were incubated at room temperature for 1 h in Tris-buffered saline with 0.1% Tween-20 (TBS-T) containing 5% milk followed by an incubation with 1% milk with the indicated antibodies except the anti-phosphotyrosine antibody, which required 3% bovine serum albumin in the blocking buffer and 1% bovine serum albumin in the blotting buffer. After washing three times for 15 min each with TBS-T, the blots were incubated with horseradish peroxidase-conjugated donkey anti-mouse or anti-rabbit IgG (American Pharmacia Biotech) followed by washing. Immunoreactive bands were visualized with enhanced chemiluminescence substrate (Pierce). In some experiments, after visualizing an immunoreactive protein, the nitrocellulose filter was incubated in a buffer containing 625 mM Tris/HCl, pH 6.7, 100 mM β-mercaptoethanol, and 2% SDS at 50 °C for 30 min, washed with 0.1% Tween 20 in 50 mM TBS at room temperature for 1 h, and reblotted with different antibodies.
the affinity-purified antibodies against Erbin or preimmune serum in 2% normal goat serum in PBS overnight at 4 °C. In some experiments, affinity-purified antibodies were preincubated with 10 ng GST-ErbB/PDZ overnight at 4 °C prior to immunohistochemical studies. After washing the sections five times with PBS, each for 30 min, the sections were incubated with a fluorescein isothiocyanate-conjugated anti-rabbit antibody (Zymed Laboratories Inc., San Francisco) and rhodamine-conjugated α-bungarotoxin (Molecular Probes, Eugene, OR). Fluorescent images of cells were captured on a Sony CCD camera mounted on a Nikon E600 microscope using Photoshop imaging software.

**Labeling of Surface Proteins**—To label surface proteins, cells were washed with cold PBS containing 1 mm MgCl2 and 0.1 mm CaCl2 and incubated with 0.5 mg/ml sulfo-NHS-LC-biotin in the same buffer at room temperature for 30 min. The labeling reaction was quenched by incubation with 100 mm glycin for 10 min at room temperature. Cells were then lysed in the modified RIPA buffer. Lysates were incubated with streptavidin-agarose beads (Molecular Probes) overnight at 4 °C. Bound proteins were subjected to SDS-PAGE.

**Protein Assay**—The protein was assayed according to the method of Bradford (34) using bovine serum albumin as a standard.

## RESULTS

### Cloning of Erbin—The C terminus (DVPV*) of ErbB2 fits the consensus site for PDZ binding. However, whereas ErbB4 binds strongly to PSD-95, ErbB2 has little or no affinity for PSD-95 (13). To identify proteins that bind to ErbB2, we generated several bait constructs composed of the ErbB2 C terminus in various lengths. Most of the baits showed autonomous transactivation activity in various yeast strains except the one with the last 10 amino acid residues (amino acids 1251–1260). Screens using this bait of mouse muscle, mouse brain, and human heart cDNA libraries led to isolation of cDNAs all of which encoded partial sequences of an apparently same protein with a PDZ domain in the C terminus. This protein was initially named as B2BP for ErbB2-binding protein because it only interacted with ErbB2 and not ErbB3 or ErbB4 (see below). While this study was in progress, Borg et al. reported Erbin (18). Sequence analysis indicated that B2BP was the mouse homolog of Erbin. Thus, this protein will be referred as Erbin in the rest of this manuscript. Erbin showed high homology to Densin-180, a protein identified previously as a postsynaptic component (35, 36). Like Densin-180, Erbin had 16 LRR domains in the N terminus. In the C terminus, there is a PDZ domain of group I which is characterized by a conserved histidine residue (37). The homology between Erbin and Densin-180 was 73% in LRR domains, 71% in the PDZ domain, and 39% in the middle region.

**Characterization of Erbin Binding to ErbB2 in Yeast**—Nice clones were isolated from the libraries that encoded two fragments of Erbin: Erbin965 and Erbin1254 (Fig. 1A). The binding of Erbin to ErbB2 was dependent on the PDZ domain of Erbin because deletion of the PDZ domain prevented the interaction. Furthermore, the PDZ alone was sufficient to bind to ErbB2. Although Erbin showed high homology with Densin-180, ErbB2 did not interact with the PDZ domain of Densin-180 (Fig. 1A) nor did it interact with the PDZ domains of PSD-95 (Fig. 1A) and of Scribble, nNOS, or α1, β1, and β2-syntrophin (data not shown). The binding of Erbin to ErbB2 was dependent on the ErbB2 C terminus. Mutation of the valine residues at the −1 and −3 positions to alanine prevented ErbB2 from interacting with Erbin (Fig. 1B). On the other hand, Erbin interacted specifically with the C terminus of ErbB2 and did not interact with C termini of the ErbB3, ErbB4, or NR2A subunit of the N-methyl-D-aspartate receptor (Fig. 1C).

**Interaction of Erbin with ErbB2, Not ErbB3 or ErbB4**—To characterize further the interaction between Erbin and ErbB proteins, we examined the ability of Erbin’s PDZ domain to bind to ErbBs in *in vitro* pull-down assays. Lysates from HEK 293T cells transfected with ErbB2, ErbB3, or ErbB4 were incubated with GST-Erbin fusion protein immobilized on agarose beads. Bound proteins were resolved on SDS-PAGE and immunoblotted with individual anti-ErbB antibodies. Consistent with the results from yeast two-hybrid assays, GST-Erbin was only able to pull down ErbB2 (Fig. 2A). In contrast, ErbB3 or ErbB4 was undetectable in the Erbin complex. To determine whether ErbB2 interacts with Erbin in mammalian cells, we expressed ErbB proteins with or without Myc-tagged Erbin in HEK 293T cells. Lysates of transfected cells were incubated with individual anti-ErbB antibodies and the resulting immunocomplex was blotted with anti-Myc antibodies. Erbin was detected in the immunoprecipitates from cells that had been cotransfected with ErbB2 and Erbin (Fig. 2B), suggesting that ErbB2 associates with Erbin in *vivo*. In contrast, Erbin was not detected in the ErbB3 or ErbB4 immunoprecipitates (Fig. 2C).

**Expression of Erbin mRNA**—Northern blot analysis was used to study mRNA expression of Erbin. The membrane loaded with mRNAs from multiple tissues was probed with a 32P-labeled Erbin DNA fragment (encoding amino acids 1241–1371 plus the 3'-noncoding region). A major transcript at 7.5 kilobases was detected in various tissues (Fig. 3, top panel). The expression was high in the lung, heart, and kidney, moderate in the brain, skeletal muscle, and testis, and little, if any, in the spleen and liver. In contrast, expression of the Densin-180 mRNA was brain-specific as reported previously (35). The 7.4-kilobase transcript of Densin-180 was detected only in the brain, but not in any of tested periphery tissues (Fig. 3, middle panel). Note the exposure time of blots for Densin-180 (10 days) and Erbin (1 day), whereas both used a similar amount of probes (5 ng/ml, 5 ml) with same specific activity (4 × 10⁶ cpm/µg of DNA). These results suggest that the expression level of Erbin may be at least five times higher than that of Densin-180 in the brain.

**Erbin Is a Protein Tightly Associated with Membrane**—To study Erbin expression at the protein level, antibodies against Erbin were generated using as antigen the Erbin PDZ domain (amino acids 1241–1371). When affinity purified, the antibody
detected a 180-kDa protein on Western blots of HEK 293T cell lysates (Fig. 4A, large arrow). The interaction of the 180-kDa protein and the serum was specific because it could be blocked by preincubation of serum with the antigen GST-Erbin/PDZ fusion protein (Fig. 4A, lane 3). The antibodies also recognized the transfected Erbin965 recombinant protein in HEK 293T cells (Fig. 4A, small arrow), whose expression was evident by blotting with an anti-Myc antibody (Fig. 4A, lane 5). Although there is a high homology between the PDZ domains of Erbin and Densin-180, the anti-Erbin antibody did not cross-react with Densin-180 (Fig. 4B, lane 7). Taken together, these results indicate that Erbin is not a human or mouse ortholog of Densin-180.

As observed with ErbB2, Erbin was present only in membrane, but not soluble, fractions (Fig. 5A). To determine how tightly Erbin associates with the membrane fraction, we treated brain membrane (P2) fractions with high concentrations of salt, high pH, or various detergents to solubilize Erbin. Solubilized or insolubilized fractions were immunoblotted with anti-Erbin antibodies. As shown in Fig. 5A, Erbin was resistant to wash with 1 M NaCl or high pH buffer (0.1 M NaCO₃), which disrupted protein interactions and extracted mainly peripheral membrane proteins, respectively. Except for partial solubilization by 3% Nonidet P-40, Erbin was virtually insoluble in 2.5% CHAPS or 1% Triton, conditions under which many membrane proteins were solubilized. However, Erbin could be solubilized...
Fig. 5. Erbin was a membrane-associated but not integral protein. A, Erbin was tightly associated with membrane fractions in the brain. Rat brain synaptic plasma membranes (P2, 100 μg of protein) were resuspended in PBS containing the indicated buffers with high concentration of salt, high pH, or various detergents for 30 min on ice and spun at 10,000 × g for 15 min. The resulting pellets (P) and supernatant (S) were resolved on SDS-PAGE and subjected to immunoblotting with the respective antibodies. B, Erbin was not an integral protein. HEK 293T cells were labeled with sulfo-NHS-LC-biotin and lysed as described under “Experimental Procedures.” Lysates (20 times of input) were incubated with streptavidin-agarose beads to pull-down biotinolated proteins or those associated with biotinolated proteins. Isolated proteins were subjected to Western blot for the indicated proteins. The left panel shows blots from a representative experiment; the right panel shows the results of densitometric analysis (mean ± S.D. of three different samples) of autoradiograms, which were scanned and analyzed with NIH Imaging. C, dependence of Erbin presence in pull-down complexes on the PDZ domain. Cells were transfected with green fluorescent protein (GFP)-Erbin or GFP-ErbinPDZ and labeled with sulfo-NHS-LC-biotin and lysed as described in B. Pull-down complexes were probed for transfected proteins with the indicated antibodies.

Fig. 6. Expression of Erbin in muscle cells and at the neuromuscular junction. A, Erbin was expressed in C2C12 and primary myoblasts and myotubes. Homogenates (50 μg of protein) of rat brain, skeletal muscles and lysates of C2C12, and rat primary muscle cells were resolved on SDS-PAGE and subjected to immunoblotting with antibodies against Erbin or ErbB2. MB, myoblasts; MT, myotubes; Primary, primary muscle cells. B, C, and D, colocalization of Erbin with α-bungarotoxin (αBTX) in skeletal muscles. Mouse diaphragm sections were incubated with affinity-purified anti-Erbin antibody (B), preimmune serum, or the anti-Erbin antibody preabsorbed with the immunogen. Rhodamine-conjugated α-bungarotoxin was added to label the acetylcholine receptor. The Erbin immunoreactivity was visualized by fluorescein isothiocyanate-conjugated secondary antibody. Small arrows indicate acetylcholine receptor clusters; large arrows indicate sarcolemma.

because the hydrophobicity profile of the Erbin amino acid sequence did not predict the presence of a transmembrane domain (data not shown). We speculated that Erbin was associated with membranes by interacting with integral proteins such as ErbB2. To test this hypothesis, we biotinylated surface proteins in HEK 293T cells, which were then isolated with streptavidin-agarose beads. As shown in Fig. 5B, only 8.7% of total Erbin was present in the complex pulled down with the beads. In contrast, biotin labeled 35% of total ErbB2, a protein known to have a transmembrane domain. As a control, the amount of ERK1, a cytoplasmic kinase, in the pull-down complex was barely detectable. These results suggest that Erbin may not be as accessible to surface biotinylation in intact cells as the transmembrane ErbB2. Only a minimal amount of Erbin was present in the streptavidin-pull-down complex. To determine whether the presence of Erbin in the streptavidin-pull-down complex was caused by interaction with other proteins, we expressed Erbin and a mutant with deletion of the PDZ domain. As shown in Fig. 5C, the presence of transfected Erbin...
in the streptavidin-pull-down complex was dependent on the PDZ domain. Deletion of the PDZ domain in Erbin, which blocked binding to ErbB2 (Fig. 1), abolished its presence in the complex. These results suggested to us that Erbin may be a protein in the cytoplasm. It may be tightly associated membranes in a manner dependent on the PDZ domain, probably via interaction with ErbB2. These results, however, were unable to exclude the possibility that Erbin is a transmembrane protein with the extracellular domain somehow inaccessible to biotin labeling.

Localization of Erbin at the Neuromuscular Junction—As with in the brain, Erbin was expressed as a 180-kDa protein in the skeletal muscle, C2C12 mouse muscle cells, and muscle cells in primary culture (Fig. 6A). Expression of Erbin was at similar level in myoblasts and in myotubes, suggesting that differentiation of muscle cells had little effect on its expression. ErbB proteins including ErbB2 are concentrated in the postsynaptic membrane of the neuromuscular junction (31). The immunoreactivity of Erbin was visualized with a fluorescein isothiocyanate-conjugated secondary antibody. On the right, an image of overlays. Small arrows indicate acetylcholine receptor clusters; large arrows indicate sarcolemma.

To determine that the Erbin staining signal at the neuromuscular junction was from the postsynaptic instead of presynaptic components, we studied the effect of denervation on Erbin expression. Denervation of the skeletal muscle causes rapid degeneration of presynaptic nerves. Until the development of pathological conditions (such as atrophy and inflammation), expression of postsynaptic proteins is normal or elevated to compensate the loss of presynaptic input (44). As shown in Fig. 7A, the level of Erbin protein was increased in denervated muscles, and so was that of ErbB2 as observed previously (5). These results suggest that Erbin expression may be regulated by electric activity. Remarkably, Erbin was detectable at the neuromuscular junction in denervated muscles (Fig. 7B), supporting the notion that Erbin was present in the postsynaptic membrane because postsynaptic components degenerate in denervated muscles.

Erbin Is a Component of PSD in the Central Nervous System—Expression of Erbin was at similar levels in the cerebral cortex, hippocampus, cerebellum, and brain stem (Fig. 8A). To determine whether Erbin was present in the PSD fraction, we performed subcellular fractionation studies. As shown in Fig. 8B, ErbB2 and Erbin were present in synaptosomes and copurified into PSD fractions. The degree of Erbin enrichment in the PSD fraction correlated strongly with that seen with ErbB2. These biochemical studies demonstrate that Erbin is present in the PSD fraction and suggest that it is appropriately localized to form a protein complex in vivo with ErbB2. Next we determined whether Erbin interacts with ErbB2 in the central nervous system. The interaction between ErbB2 and Erbin was examined in rat brain synaptosomes. Synaptosomes were solubilized with 1% deoxycholate and incubated with antibodies against Erbin. As shown in Fig. 8C, immunoprecipitation of Erbin resulted in coimmunoprecipitation of ErbB2. In the lane where antibodies were missed, ErbB2 was absent in the precipitates (Fig. 8C). Furthermore, preabsorption of Erbin antibodies with the antigen blocked the coimmunoprecipitation of ErbB2 (data not shown). These results suggest that Erbin is associated with ErbB2 in vivo.

Erbin Interacted with PSD-95 in Synaptosomes and in Transfected Cells—Both ErbB2 and ErbB4 are proteins in the central nervous system synapses (13, 17) and at the neuromuscular junction (40). Considering that PSD-95 binds to ErbB4 (13, 17), we determined whether Erbin and PSD-95 were in the same complex in the central nervous system. Deoxycholate-solubilized synaptosomes were incubated with anti-Erbin antibodies to isolate Erbin immunocomplexes. As shown in Fig. 9A, PSD-95 was detected in Erbin immunoprecipitates, suggesting an interaction of Erbin with PSD-95 in vivo. To identify the domains in PSD-95 and Erbin which are required for the in-
Interaction, HEK 293T cells were cotransfected with various constructs (Fig. 9B). The interaction of PSD-95 with Erbin was dependent on the PDZ domains of PSD-95 (Fig. 9D); in fact, the first and second PDZ domains were sufficient for interaction with Erbin (Fig. 9C). On the other hand, the Erbin interaction with PSD-95 did not appear to require Erbin’s PDZ domain (Fig. 9E). Further analysis suggested that Erbin may interact with PSD-95 via the region between amino acids 965 and 1241 because an Erbin mutant with a deletion of the N-terminal 1–964 amino acid residues was able to interact with PSD-95 (Fig. 9F).

Erbin Increased Surface Expression of ErbB2—As an initial step to identify the function of Erbin, we investigated the effect of Erbin on ErbB2 surface expression. HEK 293T cells were transfected with Myc-tagged Erbin or a PDZ domain deletion mutant. As shown in Fig. 10A, expression of Erbin had no effect on the total amount of ErbB2 in transfected cells. In contrast, Erbin increased the amount of biotin-labeled ErbB2. Such an increase was dependent on the intact C terminus of Erbin. In cells transfected with the Erbin mutant with deletion in the PDZ domain, the amount of biotin-labeled ErbB2 was similar to that in mock transfected cells (Fig. 10, A and B). These results suggest a possible role of Erbin in the regulation of ErbB2 surface expression.

**DISCUSSION**

The major findings of this study are the following. First, the novel PDZ domain-containing protein Erbin interacts specifically with ErbB2, not ErbB3 or ErbB4. Second, Erbin may not be an integral protein but is tightly associated with membranes. Third, this protein, like ErbB2 receptor tyrosine kinase, is enriched both in the postjunctional membrane at the neuromuscular junction and in the PSD of the brain. Fourth, in addition to ErbB2, Erbin also interacts with PSD-95, another PDZ domain-containing protein that interacts with ErbB4. Last, expression of Erbin increases the amount of biotin-la-
proteins. Shown were blots from a representative experiment. Erbin
PDZ, or an empty vector. Protein complexes were pulled down
transfected cells. Cells were transfected with Myc-Erbin, Myc-
pressing Erbin. A sitometric analysis of data in
cells. Data were three experiments (in mean
in lysates) were calculated and normalized to the mock-transfected
(intensity of ErbB2 signals in pull-down complexes/intensity of signals
lysates) were calculated and normalized to the mock-transfected
cells. Data were three experiments (in mean ± S.D.). * p < 0.05.
beled ErbB2 in mammalian cells, suggesting that Erbin was able to increase ErbB2 surface expression. Together with a recent study that suggests a role of Erbin in basolateral localization of ErbB2 in epithelial cells (18), our results suggest that ErbB receptor tyrosine kinases interact with a network of PDZ domain-containing proteins. The interaction between ErbBs and the intracellular PDZ domain-containing proteins may be essential for localized neuregulin signaling in a subcellular compartment including the neuromuscular junction and central synapses. Moreover, the PDZ domain-containing proteins, via interacting with ErbBs, may regulate neuregulin signaling. Erbin belongs to a unique family of PDZ domain-containing proteins. These proteins include Densin-180; LET-413, an ortholog in Caenorhabditis elegans (45); and Scribble, a Drosophila protein essential for epithelial integrity (46). In addition to the PDZ domain in the C termini, the members of this family contain 16 LRRs in the N termini and have thus been named as LAP (for LRR and PDZ) proteins (18, 45). Among the members of the LAP family, Densin-180 shares a high homology with Erbin in amino acid sequence and overall primary structure (18). Densin-180 is also a postsynaptic component and enriched in the PSD of the brain (35). However, the PDZ domain of Densin-180 does not interact with ErbB2, indicating substrate specificity of PDZ domains of Densin-180 and Erbin and suggesting a different role of these proteins in the brain. The C. elegans ortholog of Erbin, LET-413, is critical for normal assembly of adherens junctions. In LET-413 mutants, adherens junctions are abnormal, cell polarity is affected, and actin cytoskeleton is disorganized (45). Our results suggest that Erbin may play an important role in regulation of neuregulin signaling. Expression of Erbin increased biotin-labeled ErbB2 in transfected cells, indicating that Erbin can promote ErbB2 surface expression. This effect was specific in that it relied on the presence of the PDZ domain, through which Erbin interacted with ErbB2. The Erbin mutant without the PDZ domain did not increase ErbB2 surface expression.

At the neuromuscular junction, proteins essential for neurotransmission are densely packed at the postsynaptic membrane (1, 2, 47). This is caused and maintained at least in part by active transcription in synaptic nuclei. Neuregulin is a molecule from motoneurons which stimulates acetylcholine receptor synthesis (48). In fact, one initially identified isoform of neuregulin is ARIA (for acetylcholine receptor inducing activity) (4). Neuregulin is synthesized in motoneurons (4) released from motoneurons and deposited in the synaptic cleft (49, 50), activates ErbB receptors (3, 6, 51) and subsequent multiple signaling pathways (6, 9, 52, 53) in the skeletal muscle to increase acetylcholine receptor expression. Results from studies of neuregulin-1 and ErbB2 mutant mice indicate that neuregulin is essential for the formation and maintenance of the neuromuscular junction (43, 54). In support of this hypothesis are findings that ErbB protein tyrosine kinases and downstream signaling molecules are concentrated at the neuromuscular junction (3, 15, 16, 40, 55, 56). However, the mechanisms underlying clustering of ErbB proteins in muscle cells remain unclear. We believe that ErbBs are clustered at the neuromuscular junction by interaction via the C termini with a network of anchoring proteins. Of the family of membrane-associated guanylate kinase-like proteins (MAGUK), PSD-95, SAP97, and SAP102 interacted with ErbB4 (13).2 They are expressed in skeletal muscle cells.2 Moreover, earlier studies suggested that SAP97 (57) and PSD-95 (58) may be localized at the neuromuscular junction. These proteins may play a role in clustering ErbB4 at the neuromuscular junction. Erbin may be a protein that anchors ErbB2 at the neuromuscular junction. The immunoreactivity of Erbin was enriched at the neuromuscular junction. Furthermore, denervation that destroyed presynaptic components had no apparent effect on Erbin staining in the skeletal muscle, suggesting that Erbin is present in the postsynaptic membrane of the neuromuscular junction where ErbB2 is localized. The hypothesis was supported further by results from the recent study of ErbB2 expression in polarized epithelial cells. ErbB2 is localized at the basolateral side of epithelial cells (18). This localization is dependent on the intact C terminus, the site of interaction with Erbin.

Another interesting finding of this paper is that PSD-95 interacts with Erbin. PSD-95 is a well characterized protein with three PDZ domains in the N terminus, an inactive guanylate kinase domain in the C terminus, and a SH3 domain in between (19–22). PSD-95, via distinct PDZ domains, interacts with various proteins. In addition, it can form head-to-head multimers via disulfide linkage of its N terminus (41). The interacting proteins of PSD-95 include N-methyl-D-aspartate receptors, potassium channels, neurelin, SynGap, and CRIP (42). Thus it is believed that PSD-95 is important for the assembly and maintenance of anatomic and/or functional synaptic complex. In this study, PSD-95 interacted with Erbin not only in the heterologous expression system but also in synaptosomes. The interaction of Erbin with PSD-95 did not depend on Erbin’s PDZ domain, but a region between amino acids 965 and 1241, suggesting that Erbin may interact simultaneously with ErbB2 and PSD-95. Together with previous observations that PSD-95 interacts with ErbB4 (13, 17), these results suggest that ErB B proteins may interact with a network of PDZ domain-containing proteins. It will be interesting to determine whether ErbB clustering requires both Erbin and PSD-95 or a PSD-95-like protein in central nervous system synapses and at the neuromuscular junction.

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REFERENCES
1. Hall, Z. W., and Sanes, J. R. (1993) Cell 73, 99–121
2. Sanes, J. R., and Lichtman, J. W. (1999) Annu. Rev. Neurosci. 22, 389–442

2 Y. Z. Huang, Q. Wang, W. C. Xiong, and L. Mei, unpublished observation.
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