Erbin Inhibits RAF Activation by Disrupting the Sur-8-Ras-Raf Complex*

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Penggao Dai, Wen C. Xiong, and Lin Mei1

From the Program of Developmental Neurobiology, Institute of Molecular Medicine and Genetics, Medical College of Georgia, Augusta, Georgia 30912

Erbin is a member of the LAP (leucine-rich repeat (LRR) and PDZ domain) family. It inhibits Ras-mediated activation of ERK in response to growth factors. In this study, we investigated the mechanisms by which Erbin regulates the Ras-Raf-MEK pathway. The N-terminal LRR domain was necessary and sufficient to inhibit neuregulin-activated expression of e116-Luc, a reporter of ERK activation. On the other hand, Erbin had no effect on Ras activation, but it attenuated neuregulin-induced Raf activation, suggesting that Erbin may regulate Raf activation by Ras. Via the LRR domain, Erbin interacts with Sur-8, a scaffold protein necessary for the Ras-Raf complex. Expression of Erbin attenuated the interaction of Sur-8 with active Ras and Raf. Moreover, Erbin-shRNA, which suppressed Erbin expression at mRNA and protein levels, increased the interaction of Sur-8 with Ras and Raf, ERK activation, and neuregulin-induced expression of endogenous acetylcholine receptor ε-subunit mRNA. These results demonstrate a regulatory role of Erbin in the Ras-Raf-MEK pathway, suggesting that Erbin may inhibit ERK activation by disrupting the Sur-8-Ras/Raf interaction.

Neuregulin is a family of epidermal growth factor-containing polypeptides implicated in regulating neuron migration, neurite outgrowth, and expression of neurotransmitter receptors (1, 2). Neuregulins act by activating their receptors, the 180-kDa ErbB receptor tyrosine kinases ErbB2, ErbB3, and ErbB4 (3, 4). Upon activation, tyrosine residues in the carboxyl termini become phosphorylated and serve as docking sites for cytoplasmic signaling molecules such as Shc and Grb2 to activate various downstream signaling pathways. Grb2 brings guanyl nucleotide exchange factor (SOS) to the plasma membrane in proximity with Ras and expedites the exchange of GDP for GTP on Ras (5). Activated Ras (GTP-bound) then directly binds to Raf and allows the latter to be activated (6, 7). Active Raf triggers sequential activation of MEK2, a MAPK kinase, and ERK (8).

Erbin is an intracellular protein of the LAP family (LRR and PDZ), containing a LRR domain and a PDZ domain (9–11). Besides Erbin, LAP proteins include Densin-180 (12), Lano (13), LET-413, an Erbin orthologue in Caenorhabditis elegans (14), and Scribble in Drosophila (15). Densin-180 is a protein associated with the postsynaptic density in the rat brain, which may be involved in the localization of synaptic proteins (11, 16). LET-413 and Scribble are junctional proteins involved in establishing and maintaining epithelial cell polarity (17). Erbin is believed to play a role in basolateral targeting in epithelial cells (18, 19). It is shown to interact with various proteins via the PDZ domain including ErbB2 (9, 10, 18), p120 catenin (p0071 and δ-catenin) (20), ARVCF (21, 22), EBP0 (23), PAPIN (24), β1-integrin (25), and bullous pemphigoid antigen-1 (25). The unique region between the LRR and PDZ domains associates with PSD-95 (11) and Smad (26). The LRR domain binds to active Ras (27). We showed that Erbin inhibits growth factor-induced activation of ERK (27). Suppression of Erbin expression potentiates neuregulin-induced neuronal differentiation of PC12 cells and disrupts E-cadherin adherence junctions in Schwann cells (23). These results indicate that this protein may function as a signaling molecule in addition to being a scaffolding protein.

In this study, we investigated mechanisms of Erbin in inhibiting ERK activation. Erbin bound to Sur-8 and inhibited the interaction of Sur-8 with Ras and Raf. Suppression of Erbin expression in cells increased the interaction of Sur-8 with active Ras and Raf, ERK activation, and neuregulin-induced expression of endogenous AChR ε-subunit mRNA. Taken together, these results demonstrate a regulatory role of Erbin in the Ras-Raf-MEK pathway, suggesting that Erbin may inhibit ERK activation by disrupting the Sur-8-Ras-Raf complex.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HEK 293, COS-7, and C2C12 cells were cultured as described previously (27). COS-7 cells were using an adenovirus-aided DEAE-dextran transfection procedure (28, 29). Cells in 10-cm dishes (70–90% confluence) were incubated with 5.0 ml of Dulbecco’s modified Eagle’s medium, 1.0 ml of E1-defective adenovirus-aided DEAE-dextran, and 5 μg of plasmid DNA for 2 h at 37 °C. The cells were rinsed with phosphate-buffered saline and incubated with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum.

Immunoprecipitation and Immunoblotting—Cell lysates (~400 μg of protein) were incubated without or with indicated antibodies 1 h at 4 °C and subsequently with protein A- or protein G-agarose beads overnight at 4 °C on a rotating platform. After centrifugation, beads were washed five times with the modified radioimmune precipitation assay buffer. Bound proteins were eluted with the SDS sample buffer, resolved by SDS-PAGE, and transferred onto nitrocellulose membranes (Schleicher and Schuell). Nitrocellulose membranes were incubated at room temperature for 1 h in blocking buffer containing Tris-buffered saline with 0.1% Tween (TBS-T) and 5% milk or 5% bovine serum albumin, followed by incubation with indicated antibodies in the blocking buffer. After being washed three times for 5 min each with TBS-T, the membrane was incubated with horseradish peroxidase-conjugated donkey anti-mouse or anti-rabbit IgG (Amersham Biosciences) followed by washing. Immunoreactive bands were visualized with enhanced chemi-
luminescence substrate (Pierce). In some experiments, the nitrocellulose filter was incubated in a buffer containing 62.5 mM Tris-HCl, pH 6.7, 100 mM β-mercaptoethanol, and 2% SDS at 50 °C for 30 min and then washed with 0.1% Tween 20 in 50 mM Tris-buffered saline at room temperature for 1 h and reblotted with different antibodies. The following antibodies were used: FLAG (M2, Sigma), Myc (9E10, Santa Cruz Biotechnology), phospho-Raf-1 (Ser-338, Upstate), phospho-MAPK (Promega), anti-Ras (clone RAS10, Upstate), GST (Biocompare), and Erbin (28). For quantitative analysis, autoradiographic films were scanned with Epson Expression 1680, and the captured image was analyzed with NIH Image software.

Luciferase Assay—C2C12 myoblasts were cotransfected with or without Myc-Erbin, plus the e-subunit promoter-luciferase transgene that contains 416 nucleotides of the 5′-untranslated region of the e-subunit gene (30). 24 h after transfection, myoblasts were incubated in the differentiation medium to induce myotube formation. Myotube formation was completed 48 h after the switch to differentiation medium. C2C12 myotubes were stimulated with neuregulin at a final concentration of 10 nm at 37 °C for 24 h. pRL-SV40, which expresses Renilla luciferase under the control of the SV40 promoter (Promega), was cotransfected as a control to monitor the transfection efficiency. 48 h after transfection, cells were lysed, and the activities of the two different luciferases were assayed with their respective substrates with a dual luciferase assay kit (Promega).

Ras Assay—Active GTP-bound Ras was precipitated by the minimal Ras-binding domain (RBD) (amino acids 51–131) of Raf (31, 32). Briefly, serum-starved HEK 293 cells were treated with neuregulin (10 nm) for 10 min and lysed in Ras-binding buffer (15% glycerol, 50 mM Tris/HCl, pH 7.4, 1% Nonidet P-40, 200 mM NaCl, 10 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 2 μg/ml aprotinin). Cleared lysates were incubated for 1 h with recombinant GST-RBD (15 μg/sample) that had been precoupled to glutathione-agarose beads. After four washes in the Ras-binding buffer, bead complexes were separated on a 12% SDS-PAGE and transferred to nitrocellulose membrane for Western blotting. Ras was detected using the rat monoclonal antibody Y13-259 followed by horseradish peroxidase-coupled goat anti-rat antiserum (Santa Cruz Biotechnology).

Reverse Transcription-PCR—Total RNA was extracted by TRIzol (Invitrogen) and first-strand cDNA prepared using the SuperScript III First-strand Synthesis Kit (Invitrogen) according to the manufacturer’s instruction. PCR parameters were 25 cycles (94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s) followed by a final extension cycle at 72 °C for 7 min. PCR product was resolved on 6% acrylamide gels and visualized by ethidium bromide. Primers were as follows: 5′-TGA TGC TGA AAG TGG CCC ACC AGC C-3′ and 5′-TGA AGA AAC TTC TCG TAC AAT GAT G-3′ for Erbin; 5′-CCA TGT CCC CGC GGC TGC GC-3′ and 5′-GAG CCC ACG CTG AAG AGC AC-3′ for the AChR e-subunit; and

FIGURE 1. Erbin inhibition of neuregulin-induced activation of Raf. A, effect of Erbin on neuregulin-induced Ras activation. HEK 293 cells were transfected with HA-Ras with or without Myc-Erbin or Myc-LRR and were stimulated with neuregulin (NRG) 36 h after transfection. Cell lysates were incubated with the GST fusion protein of Raf-RBD immobilized on agarose beads. Bound proteins were resolved by SDS-PAGE and blotted with an anti-Ras antibody (top). Similar amounts of transfected proteins in lysates were revealed by immunoblotting (IB) with the indicated antibodies. B, quantitative analysis of data in A. C, inhibition of Raf activation by Erbin. HEK 293 cells were transfected with FLAG-Raf and Myc-Erbin or Myc-LRR and were stimulated as in A. Cell lysates were incubated with an anti-FLAG antibody to purify FLAG-Raf. Active Raf was revealed by immunoblotting with anti-phospho-Raf antibody. Expression of Myc-Erbin and Myc-LRR was revealed by immunoblot with anti-Myc antibody. IP, immunoprecipitate. D, quantitative analysis of data in C. Shown are mean ± S.E., n = 3; p < 0.01.
5′-GCT CGTCGA CAA CGG CTC-3′ and 5′-CACAAT- GATCT GGG TCA TCT TCT C-3′ for β-actin.

Inhibition of Erbin Expression by Small Hairpin RNA (shRNA)—
A representative experiment in duplicates, which was repeated three times with similar results. & the LRR domain is necessary and sufficient to inhibit neuregulin-induced e416-Luc expression. C2C12 cells were transfected with e416-Luc, pRL-TK, and the indicated Erbin constructs (structures are shown below the histogram). Luciferase activities were assayed as in A, *p < 0.01.

FIGURE 2. The LRR domain is required for Erbin inhibition of neuregulin induction of e416-Luc. A, Erbin inhibition of e416-Luc expression by active Ras but not active Raf or MEK1. C2C12 myoblasts were transfected with e416-Luc with various amounts of expression constructs of active Ras, Raf, or MEK1. A Renilla luciferase plasmid, pRL-TK, was cotransfected as a control to monitor the transfection efficiency and sample handling. After differentiation, cells were lysed, and firefly and Renilla luciferase activities were assayed. Cells transfected with an empty vector were taken as 100%. Shown is a representative experiment in duplicates, which was repeated three times with similar results. B, the LRR domain is necessary and sufficient to inhibit neuregulin-induced e416-Luc expression. C2C12 cells were transfected with e416-Luc, pRL-TK, and the indicated Erbin constructs (structures are shown below the histogram). Luciferase activities were assayed as in A, *p < 0.01.

RESULTS AND DISCUSSION

Erbin Inhibits Activation of Raf but Not Ras—In an earlier report, we showed that Erbin inhibits ERK activation (27). The site of action was mapped to be between Raf and Ras using phospho-ERK as the readout, because Erbin inhibits ERK activation by active Ras but not by active Raf. Raf is a serine-threonine kinase that phosphorylates and activates the dual specificity kinase, MEK (MAPKK) (33) which, in turn, phosphorylates and activates ERK. Although these observations suggest that Erbin may inhibit Raf activation, it is possible that Ras activation may also be a target of regulation. To address these questions, we studied the effects of Erbin on Ras and Raf activation in HEK 293 cells. Tagged Ras and Raf were cotransfected with Erbin or LRR. After stimulation with neuregulin, active Ras was purified by the Ras-binding domain of Raf immobilized on beads. The RBD binds to active Ras with an affinity 3 orders of magnitude higher than inactive GDP-bound Ras and has been used widely to purify active Ras (31, 32, 34). Purified active Ras was revealed by immunoblotting with anti-Ras antibody. As shown in Fig. 1, A and B, active Ras was increased by neuregulin. However, the increase was not altered by Erbin or LRR, suggesting that Erbin may have little effect on Ras activation.

In light of the inability of Erbin to inhibit Ras activation and active Raf-mediated ERK activation, we determined whether Erbin inhibits Raf activation. FLAG-Raf1 was cotransfected with Myc-Erbin or LRR in HEK 293 cells. FLAG-Raf1 was immunoprecipitated with anti-FLAG antibody and blotted with anti-phospho-Raf antibody. Neuregulin elicited Raf1 activation in control HEK 293 cells (4 ± 0.2-fold above basal, mean ± S.E., n = 3) (Fig. 1, C and D). In cells co-expressing Erbin and LRR, Raf1 activation was inhibited by 40% and 45% (2.4 ± 0.2 and 2.2 ± 0.2, respectively; mean ± S.E., n = 3). These observations indicate that Erbin inhibits Raf activation but has no apparent effect on Ras activation.

The LRR Domain Is Necessary and Sufficient to Inhibit e416-Luc Expression—Erbin has three domains: the N-terminal LRR domain, an LAP-specific domain that is C-terminal to the LRR, and the C-terminal PDZ domain (13). The LRR domain has been shown to be sufficient to inhibit ERK activation (27). To exclude the possible involvement of other domains, it was important to determine whether the LRR domain is required for the inhibitory effect. To this end, we characterized the effects of ΔLRR, a deletion mutant without the LRR domain, on neuregulin-induced expression of e416-Luc (Fig. 2B). e416-Luc is a reporter transgene containing a 416-bp 5′-flanking region of the AChR β-subunit gene and the luciferase gene, which responds to neuregulin stimulation in skeletal muscle cells (30, 32, 35). Expression of constitutively active Ras(V12), Raf(BXB), or Mek1(ddMek1) increased the promoter activity of e416-Luc. Expression of constitutively active Ras and Raf increases expression of the e416 transgenes (30). As shown in Fig. 2A, co-expression of Erbin suppressed RasV12-induced activation of e416-Luc. In contrast, however, Erbin appeared to be unable to inhibit RafBXB- or ddMek1-induced e416-Luc expression. These results confirm our early report that Erbin may function downstream or at the level of Ras and, at the same time, validate the reporter for a reliable readout. Erbin, LRR, or ΔLRR was cotransfected with e416-Luc in C2C12 myoblasts, and the resulting myotubes were stimulated with neuregulin and assayed for luciferase activity as described under “Experimental Procedures.” Although Erbin and LRR attenuated neuregulin-induced expression of e416-Luc, the deletion of LRR (ΔLRR) prevented Erbin from inhibiting neuregulin-induced expression. These results suggest that the LRR domain is necessary and sufficient for inhibition of e416-Luc expression.

Erbin Interaction with Sur-8—Sur-8 is believed to be a positive regulator of the Ras-Raf-MEK pathway, functioning as a scaffold that enhances ERK activation by facilitating the interaction between Ras and Raf (33, 36). Sur-8 is composed almost entirely of 18 LRRs that show homology to the LRR domain of Erbin. Interestingly, like Erbin, Sur-8 interacts with active RasV12 but not with inactive RasN17 (36). We reasoned that Erbin may interact with Sur-8 and thus disrupt its interaction with Ras to inhibit ERK activation. First we tested whether Erbin
Figure 3. Interaction between Erbin and Sur-8. A, co-immunoprecipitation of Sur-8 and Erbin. COS-7 cells were transfected with mammalian expression constructs of GST-Sur-8 or/and Myc-Erbin. Top panels, cell lysates were incubated with glutathione-conjugated beads to purify GST-Sur-8. The resulting complex was probed with anti-Myc antibody. Lysates were also blotted with anti-GST or anti-Myc antibodies to show expression of transfected proteins. Bottom panels, cell lysates were incubated with anti-Myc antibody and subsequently protein G beads to precipitate Myc-Erbin. The complex was probed with anti-GST antibody. The presence of transfected proteins in lysates was revealed by immunoblotting (IB) with indicated antibodies. IP, immunoprecipitate; PD, pull down. B, the LRR domain in Erbin is necessary and sufficient to interact with Sur-8. COS-7 cells were transfected with mammalian expression constructs of GST-Sur-8 together with Myc-Erbin, Myc-LRR, or Myc-H904LRR. Cell lysates were incubated with glutathione-conjugated beads to purify GST-Sur-8, and the resulting complex was probed with anti-Myc antibody. Expression of GST-Sur-8 was shown in the lower panel. C, interaction of endogenous Erbin and Sur-8. COS-7 lysates were incubated with anti-Sur-8 antibody or control serum and subsequently with protein A beads to precipitate Sur-8. The resulting complex was probed with anti-Erbin antibody (upper panel) or anti-Sur-8 antibody (lower panel).

Figure 4. Disruption of the Sur-8-Ras interaction by Erbin. A and B, HEK 293 cells were transfected with mammalian expression constructs of FLAG-Ras (V12) and GST-Sur-8 without or with Myc-Erbin at a single concentration (A) or at various concentrations (B). GST-Sur-8 was purified as described for Fig. 3, and the resulting complex was probed with anti-Ras antibody. Expression of transfected proteins was revealed by immunoblotting (IB) with the indicated antibodies. C, disruption of the Sur-8-Raf interaction by Erbin. Experiments were the same as in A and B, except FLAG-Raf was transfected. PD, pull down.
and Sur-8 interact in cells. GST-Sur-8 and Myc-Erbin were cotransfected into HEK 293 cells. Cell lysates were incubated with glutathione-conjugated agarose beads to pull down Sur-8, and the resulting precipitates were blotted with anti-Myc antibody. As shown in the top panels of Fig. 3A, Myc-Erbin was detected in the precipitates of GST-Sur-8. In a reciprocal experiment, GST-Sur-8 was present in immunoprecipitates of Myc-Erbin (Fig. 3A, bottom panels). These results suggest that Erbin interacts with Sur-8 in cells. Further analyses showed that Erbin interaction with Sur-8 requires the LRR domain. Deletion of this domain prevented Erbin from interacting with Sur-8 (Fig. 3B). To determine whether the interaction occurs between the two proteins at endogenous levels, HEK 293 cell lysates were incubated with anti-Sur-8 serum, and the resulting precipitates were probed with anti-Erbin antibodies (Fig. 3C). Erbin was detected in Sur-8 precipitates, suggesting that endogenous Erbin and Sur-8 may interact in cells.

Disruption of the Sur-8-Ras and -Raf Interaction by Erbin—To determine whether Erbin regulates the Sur-8-Ras interaction, HEK 293 cells were transfected with GST-Sur-8, FLAG-RasV12, and Myc-Erbin. Cell lysates were incubated with glutathione-conjugated agarose beads to pull down Sur-8, and the resulting precipitates were blotted for RasV12 with anti-FLAG antibody. Sur-8 and RasV12 co-precipitated (Fig. 4A), in agreement with earlier studies (36). However, co-expression blocked the interaction of Sur-8 with RasV12. This effect was dose-dependent (Fig. 4B). The amount of RasV12 interacting with Sur-8 decreased as the concentration of Erbin increased. These results demonstrated that Erbin may be able to disrupt the Sur-8-Ras complex. In addition, Erbin expression appeared to
inhibit the interaction between Sur-8 and Raf (Fig. 4C). These observations are in agreement with the notion that Erbin inhibits ERK activation by disrupting Sur-8 interaction with Ras and Raf.

Increased Sur-8-Ras and -Raf Interaction in Cells in Which Expression of Erbin Was Suppressed—To further study the role of Erbin in regulating ERK activation, we explored the consequences of suppression of Erbin expression by using a small interfering RNA approach, which diminishes the expression of a specific gene in cells. RNA interference has been shown recently to specifically suppress the expression of endogenous and heterologous genes in mammalian cell lines (37–39). The Erbin DNA sequence was analyzed by a Web-based program (BLOCK-iT™ RNAi Designer) for putative siRNA sequences. Three distinct sequences were chosen that are conserved in human, mouse, and rat and have no homology to any DNA sequence in the NCBI sequence bank. They were subcloned in pENTR/U6 and tested for the ability to suppress endogenous Erbin expression in HEK 293 cells. Two clones, designated 2583 and 4049, suppressed expression of Erbin but not Densin-180, a protein that is homologous to Erbin (Fig. 5, A and B). Because of apparent higher efficiency, Erbin 4049 was used in the following experiments except when indicated otherwise.

If Erbin inhibits the interaction of Sur-8 with Ras and Raf, suppression of Erbin expression is expected to increase the interaction. To test this hypothesis, HEK 293 cells were transfected with Erbin 4049 to knock down Erbin expression; cell lysates were incubated with antibodies against Ras or Raf, and the resulting precipitates were probed for Sur-8. As shown in Fig. 5C, the amount of Ras and Raf precipitated with Sur-8 was increased in cells in which Erbin expression was suppressed (in comparison with cells transfected with the control vector pENTR that encodes LacZ shRNA), in line with the notion that Erbin disrupts Sur-8 interaction with Ras and Raf. Notice that the interaction of endogenous Raf with Sur-8 was barely detectable in LacZ shRNA-transfected cells but increased dramatically when Erbin was depleted, suggesting that Erbin may act by preventing Raf recruitment to the Ras-Sur-8 complex.

Enhanced Activation of Raf and ERK When Erbin Expression Was Suppressed—We next examined the effect of Erbin suppression on Raf and ERK activation by neuregulin. As shown in Fig. 6, transfection of HEK 293 cells with Erbin 2583 and 4049 decreased Erbin protein by 60 and 80%, respectively. However, Raf or ERK expression in cells transfected with LacZ shRNA or the Erbin shRNA constructs remained similar (Fig. 6), demonstrating specific suppression of Erbin expression. The basal levels of active Raf and ERK, as indicated by respective phospho-counterparts, appeared to be increased in the absence of neuregulin. Nevertheless, stimulation with neuregulin increased levels of both phospho-Raf and -ERK. In comparison with control cells (transfected

FIGURE 7. Suppression of Erbin expression enhanced neuregulin induction of AChR promoter activity and mRNA. A, increase in neuregulin induction of the AChR promoter activity in C2C12 myotubes infected with Erbin-shRNA viruses. Myoblasts were transfected with e146-Luc and pRL-TK and infected with Erbin-shRNA 4049 viruses (titers 2 × 10⁶ transducing units/ml) 12 h later. The resulting myotubes were lysed and assayed for firefly and Renilla luciferase activities. The relative firefly/Renilla activity in cells infected with the control virus was taken as 100%. Shown was a representative experiment in duplicates, which was repeated three times with similar results. B and C, reciprocal relationship between Erbin protein level and neuregulin-induced AChR e-subunit mRNA. C2C12 myoblasts were infected with Erbin-shRNA 4049 as in A. Resulting myotubes were subjected to analysis of AChR, Erbin, and actin mRNA by reverse transcription-PCR. Representative gels are shown in B, and quantitative analyses are shown in C.
Neuregulin-induced expression of the AChR—
Neuregulin has been implicated in local AChR synthesis at the synapse (30). To study the role of Erbin in regulating ERK activation in vivo, we generated lentiviral Erbin 4049, the more potent form of two shRNAs (Fig. 5). Infection of C2C12 myotubes with the Erbin-shRNA virus inhibited expression of endogenous Erbin (Fig. 7B) and appeared to have little, if any, effect on muscle differentiation (data not shown). Neuregulin-induced expression of the e416-Luc transgene, however, was increased in muscle cells infected with the Erbin-shRNA virus (Fig. 7A), in line with the notion that Erbin functions as a negative regulator of neuregulin signaling. To determine whether inhibition of Erbin expression affects the expression of endogenous AChR mRNA level, virus-infected C2C12 myotubes were stimulated with neuregulin, and expression of the AChR ε-subunit mRNA was analyzed by reverse transcription-PCR. As shown in Fig. 7, B and C, viral infection decreased the mRNAs levels of Erbin but not β-actin. Remarkably, the AChR ε-subunit mRNA increased in a concentration-dependent manner. The reciprocal relationship between the levels of Erbin and ε-subunit mRNA indicates a role of Erbin in regulating neuregulin-induced AChR expression.

These observations demonstrate that Erbin inhibits Raf activation possibly by disrupting the interaction of Sur-8 with Ras and Raf. Convincing evidence is provided by studies using small interference RNA techniques. First, Erbin 4049 reduced Erbin expression at the mRNA and protein level. The decrease in Erbin expression correlated negatively with readouts of the proposed function of Erbin: increase in Sur-8 interaction with Ras and Raf (Fig. 5), enhanced activation of Raf and ERK by neuregulin (Fig. 6), and elevated expression of AChR ε-subunit mRNA and promoter activity (Fig. 7). Second, the effect of Erbin shRNA was concentration-dependent (Fig. 7). A reciprocal relationship of Erbin expression and AChR ε-subunit mRNA was demonstrated. Third, Erbin shRNA was gene-specific and had no effect on expression of Densin-180, a protein that is homologous to Erbin (Fig. 5). Finally, a similar effect was observed with two different Erbin shRNA constructs targeted to different sites in the Erbin mRNA. Both Erbin 2583 and 4049 suppressed Erbin expression and increased Raf and ERK activation by neuregulin in transfected cells (Fig. 6).

The results of this study and an earlier report (27) suggest that Erbin may serve as a negative feedback molecule to down-regulate the Ras-Raf-MEK pathway. Because Erbin interacts with various proteins, some of which appears to be scaffold components, it may play a role in fine-tuning the local signaling strength in a subcellular domain.

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