Ligand Activation of ELK Receptor Tyrosine Kinase Promotes Its Association with Grb10 and Grb2 in Vascular Endothelial Cells*

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ELK is a member of the Eph-related tyrosine kinase family that includes receptors signaling axonal guidance, neuronal bundling, and angiogenesis. We recently identified ELK expression in human renal microvascular endothelial cells and sought to identify intracellular proteins through which it signals responses. The cytoplasmic domain of ELK was used as “bait” in a yeast two-hybrid screen to identify interactive proteins expressed from a randomly primed embryonic murine library (E9.5–10.5). Among interactive products of 76 cDNAs characterized, 10 nonidentical, overlapping clones encoded the SH2 domain of the recently reported B61, its ligand, AL-1 (RAGS) (1, 2, 4).

Other data have shown that Eph family receptors are important in vascular biology. The endothelial cell product, B61, is expressed GST-ELKcy fusion protein bound Grb10 and Grb2. A self-phosphorylated recombinant, baculovirus-expressed GST-ELKcy fusion protein bound Grb10 and Grb2 from human renal microvascular endothelial cell extracts, while the unphosphorylated fusion form did not. Site-directed mutation identified Tyr-929 as a putative phosphorylation site required for Grb10, but not Grb2, interaction in yeast and recombinant protein assays. The ELK ligand, LERK-2/Fc, stimulated tyrosine phosphorylation of ELK, and recruitment of Grb10 and Grb2 to endothelial ELK receptors recovered by wheat germ agglutinin lectin and immunoprecipitation. These findings define ligand-activated interaction between ELK and the SH2 domains of Grb2 and the newly identified Grb10 protein that shares homology with a Cae

Members of the Eph-related receptor tyrosine kinase family have been assigned important roles in signaling axonal guidance, neuronal bundling, and, more recently, angiogenesis (1–4). There are now at least 13 distinct family members that are expressed in species ranging from Xenopus laevis to humans, in cell-type and tissue-restricted patterns that suggest that they signal targetting or differentiation (5, 6). This idea was supported by recent evidence that the Mek4 receptor targets retinal neurons to their posterior tectal projections in response to its ligand, AL-1 (RAGS) (1, 2, 4).

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‡E. Stein and T. O. Daniel, unpublished results.

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EXPERIMENTAL PROCEDURES

Construction of Recombinant Fusion Proteins and Baits—Fusion plasmids were constructed to permit shuffling of ELK-encoding inserts from the baculovirus expression vector pAC-GST to the yeast two-hybrid “bait” LexA fusion plasmid pBTM116. Parent sequences were derived from the predominant human ELK cDNA recovered from a human renal microvascular endothelial cell library (HRMEC) (HuELKI), and amino acid designations refer to that sequence.1

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amplification, using oligonucleotides containing the PCR/ligation-independent cloning (LIC) sequences (underlined) representing ELKc sequences (oligonucleotide 1, 5′-primer: 5′-CTG GTT CCG GCG ATC CCG GGG AGG AAA CGG GAT TAT AGC-3′ and oligonucleotide 2, 3′-primer: 5′-CTC GTG CCG GCG AGG TCG AGC TGA TGC CAT TGC GGT TGG-3′). PCR product was gel purified and ligated into pAC-GST-LIC (Pharmingen). Constructs encoding regional domain deletions of the ELK cytoplasmic domain as GST fusions generated in a similar manner and the catalytic domain construct, pAC-GST/ELKcyΔJM (C-terminal HisELKcy amino acids 618–883); 2) pAC-GST-ELKcyΔCterm (amino acids 566–883); and 3) pAC-GST-ELKcyΔJM (amino acids 618–984). The LexA-ELKcy bait plasmids were constructed by digesting the respective pAC-GST-ELKcy plasmids with Smal and SalI, recovering ELKcy sequences encoding the NH2-terminal domain (amino acids 618–984) from the P1 lysate, and 3 days later, cells were lysed in triple detergent lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% Tween 20, 1 mM PMSF, 2 mg/ml aprotinin) for 30 min on ice. Lysates were cleared by centrifugation; the preclariﬁed lysates were incubated for 2 h at 4°C with glutathione-agarose beads (Sigma) washed twice with lysis buffer, followed by washes with in vitro kinase buffer (20 mM HEPES, 50 mM NaCl, 0.1% Triton X-100, 1 mM PMSF) at 4°C. As indicated in the legend to Fig. 3, glutathione-agarose beads were incubated in the presence or absence of 30 μM ATP for 30 min at 30°C (25).

Conﬂuent monolayers of HRMECs (~5 × 107 cells/P100) were washed twice with phosphate-buffered saline, 1 mM PMSF and then infected with 200 μl of buffer D (150 mM NaCl, 300 mM NaF, 0.1% Triton X-100, 1 mM PMSF). Lysates were cleared of particulate by centrifugation at 4°C and incubated overnight at 4°C with ~50 μg of glutathione-agarose-immobilized (phosphorylated or unphosphorylated) GST/ELKcy, GST/ELKcyΔJM, or GST/ELKcyY929F fusion proteins, as indicated in the legend to Fig. 3. Immobilized GST/fusion protein complexes were washed three times with buffer D, omitting bovine albumin from the last wash. Bound proteins were resolved on 15% SDS-polyacrylamide gel electrophoresis gels and detected by Western blot analysis using the ECL system (Amersham, Arlington Heights, IL).

Copurification of Ligand-stimulated ELK with Grb10 and Grb2—Plates of 100–100% confluent HRMECs were washed and then incubated in OPTI-MEM medium (Life Technologies, Inc.) supplemented with 0.5 mM sodium suramin (Calbiochem) for 16 h to dissociate any endogenous ligand from ELK receptors. For 60 min prior to stimulation, 10 μM sodium o-vanadate was added to the media. Cells were then exposed for 37°C to 500 ng/ml of highly puriﬁed Fc fusion forms of either the best characterized ligand for ELK receptors, LERK-2Fc (10), or an unrelated open reading frame protein, ORF/Fc, and then exposed for 16–18 h at 4°C; recovered; and then washed extensively on protein A-Sepharose beads. Eluates or immunoprecipitates were separated on 15% SDS-polyacrylamide gel electrophoresis gels, and Western blots were incubated with previously characterized rabbit antisera containing antibodies against the extracellular domain of rat ELK (10, 26), or Grb10 (27), the murine monoclonal antibodies against Grb2 (Upstate Biotechnology Inc., Lake Placid, NY) or phosphotyrosine antibody, 4G10 (Transduction Laboratories, Lexington, KY).

RESULTS

Interaction Screening of an Embryonic Mouse Library with the Cytoplasmic Domain of ELK (ELKcy)—To identify EIPs expressed in an embryonic (E9.5–10.5) murine library (15), we utilized a yeast two-hybrid system. The yeast reporter strain L40 was initially transformed with the pLexA-ELKcy sequences encoding the entire ELK cytoplasmic domain (ELKcy-bait) protein fused to the LexA DNA-binding domain (LexBD). We determined that this bait did not transactivate and express the HIS3 gene product in the presence of 1.5 mM 3-aminoazonole (data not shown). On the basis of our previous observation that ELK is expressed in E9.5 murine embryos, we screened a murine embryonic library (E9.5–10.5) for EIPs, anticipating that developmental expression of EIPs would coincide with ELK expression.

Yeast from a single transfected colony carrying the bait were selected on Trp plates, made competent, and then cotrans-
fected with the E9.5–10.5 embryonic murine cDNA library encoding potential prey products as fusion proteins with the VP16 transcription activation domain (Fig. 1). Formation of a complex between LexA-ELKcy and VP16-EIPs should confer histidine auxotrophy and β-galactosidase activity. Among 2 × 10^7 yeast transformants screened, 320 colonies grew on His^−^ plates; among these, 290 displayed β-galactosidase activity. Plasmid DNA was prepared from 80 of the colonies surviving both selections, and of these, 4 were shown to transactivate as cotransfectants with either pLexA-Lamin fusion protein or pBTM116 alone. The remaining 76 were subjected to sequence analysis.

Among these 76 potential EIPs, multiple distinct cDNAs encoding at least 19 different previously described proteins and 3 novel proteins were identified. Among the most frequently represented cDNA sequences recovered in this screen were those encoding SH2 domains of Grb10 (10 independent clones) and Grb2 (3 independent clones). Fig. 1B depicts the comparative positions of the peptides encoded by the partial cDNA sequences compared with the parent proteins.

**ELK Self-phosphorylation Is Required for Its Interaction with Grb10 and Grb2**—To determine whether the ELKcy interactions with Grb10 and Grb2 require ELK self-phosphorylation, we inactivated the tyrosine kinase of the ELKcy fusion proteins by site-directed mutagenesis of the ATP-binding lysine (K652R). Fig. 2 shows that the kinase-inactive form of the ELKcy bait does not support interaction with Grb10 or Grb2 SH2 domain interactors. This finding implies that tyrosine phosphorylation of the ELKcy domain plays a critical role in its interaction with Grb10 and Grb2, as is predicted by the phosphotyrosine-binding function of SH2 domains (28).

To identify protein domains through which ELKcy interacts with Grb10 and Grb2, we examined their interactions with truncations of the original ELKcy bait that deleted the juxtamembrane domain (pLexA-ELKcy_DJM), the COOH-terminal domain (pLexA-ELKcy_DCterm), or both (pLexA-ELKcy_DJM/Cterm), as described under “Experimental Procedures.” The catalytic domain was retained in each of these constructs to facilitate the tyrosine self-phosphorylation required to provide phosphotyrosine-containing SH2 binding sites. We found that while Grb2 retained interaction with the isolated ELKcy tyrosine kinase catalytic domain (pLexA-ELKcy_DJM/Cterm) (not shown here), Grb10 interaction required that ELKcy constructs retain the COOH-terminal domain (amino acids 883–984). This finding indicates that the sites of ELK interaction with Grb10 and Grb2 are distinct.

Because data were not available to define SH2 binding consensus sites for Grb10, we proceeded with site-directed muta-

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4 E. Stein and T. O. Daniel, manuscript in preparation.
ELK Receptor Binds Grb10 and Grb2

**Fig. 2. A tyrosine kinase-active form of ELKcy is required for interaction with Grb10 and Grb2.** The reporter strain L40 was cotransformed with the indicated plasmids, plated on medium lacking tryptophan, leucine, lysine, and uracil, and grown for 3 days at 30°C. Growth of the same yeast cotransfectants was compared on medium containing (+) or lacking (−) histidine (H). Plates were photographed after 3 days at 30°C.

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**Fig. 3. Binding of recombinant GST-ELKcy fusion proteins to Grb10 and Grb2 requires tyrosine self-phosphorylation.** Recombinant GST-ELKcy, GST-ELKcyΔJM, and GST-ELKcyY929F fusion proteins were purified by adsorption to GSH-agarose beads and repeated washing and then incubated for 30 min in kinase buffer containing (+) or lacking (−) ATP, as described under “Experimental Procedures.” Immobilized, adsorbed phosphorylated, or unphosphorylated, GST-ELKcy fusion proteins were incubated with HRMEC extracts, washed extensively, and analyzed by Western blot, using monospecific antibodies against GST, Grb10, or Grb2, as indicated. Bound antibodies were detected using the ECL chemiluminescent system.

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The capacity to interact with endothelial Grb10 and Grb2, biologically significant interactions should be promoted by ligand activation of endogenous ELK receptors in intact cells. To explore this issue, we exposed HRMEC to the ELK ligand, LERK-2Fc, or an irrelevant open reading frame Fc fusion, ORF/Fc, and recovered ELK receptors by lectin affinity chromatography (Fig. 4A) or immunoprecipitation (Fig. 4B). As shown in Fig. 4A, LERK-2Fc-stimulated tyrosine phosphorylation of a wheat germ agglutinin-lectin-recovered protein that comigrated with ELK receptors identified by Western analysis (Fig. 4A, upper panels); increased amounts of Grb2 and Grb10 immunoreactive proteins were recovered in the wheat germ agglutinin-agarose eluate from LERK-2Fc-stimulated cells, compared with cells exposed ORF/Fc at the same concentration.

To confirm that ELK receptors form coprecipitable complexes with Grb2 and Grb10 following ligand activation, we immunoprecipitated ELK receptors from cells exposed to either ORF/Fc or the ELK ligand, LERK-2Fc, and analyzed immunoprecipitates for coprecipitating Grb2 and Grb10. In Fig. 4B (left), ELK immunoprecipitates recovered immunoreactive Grb2 (23 kDa) following stimulation of cells with LERK-2Fc, but not ORF/Fc. The prominent, nonspecific 25-kDa band in each lane represents the immunoglobulin light chain of the precipitating anti-ELK or preimmune antibodies. In parallel (Fig. 4B, right), ELK antisera coprecipitated Grb10-immunoreactive material from LERK-2Fc, but not ORF/Fc, stimulated cells. Again, the common, 50-kDa band at the bottom of the figure represents precipitating immunoglobulin heavy chain. The multiple forms of immunoreactive Grb10 protein, ranging in size from 65 to 80 kDa, were recovered in ELK immunoprecipitates from LERK-2Fc, but not ORF/Fc, stimulated cells. These multiple Grb10 forms represent proteins generated in transfected cells through use of alternative translation start sites and modified by phosphorylation (27).
for high affinity ELKcy interaction with these particular SH2 covered sequences. We surmise this reflects some preference Grb2; in aggregate, these clones account for some 20% of re-

ping, clones that encode the SH2 domains of both Grb10 and activated, self-phosphorylated cytoplasmic domain sequences Grb2 and Grb10, as interactive partners that bind to ligand-

activated properties of ELKcy subdomain deletions were evaluated in both two-hybrid (data not shown) and direct binding assays (Fig. 3). In aggregate, these data provide strong support for ligand-coupled assembly of complexes that associate tyrosine-phosphorylated ELK with Grb10 and Grb2 at independent sites of interaction.

The cytoplasmic domain of ELK includes 17 tyrosine residues, 14 of which are represented at analogous sites in other Eph family kinases. Moreover, ELK shares striking sequence identity with mammalian Eck, Erk, and Ehk, as representatives of other family members. Of the 428 ELK cytoplasmic amino acids, 202 are identical among these receptors, and 79 are conserved. To date, information about intracellular proteins that mediate responses to Eph family receptors has been limited to data on Eck interactive proteins (29, 30). Pandey et al. (29) identified Eck interaction with phosphatidylinositol 3-kinase (p85 subunit) in a yeast two-hybrid screen of a T lymphocyte library and confirmed that ligand activation of the Eck receptor activates PI3K in intact endothelial cells. More recently, they identified a novel c-src homologous protein they named SLAP as an interactive protein that binds Eck on ligand activation, similar to the PI3K findings (30).

Among the 76 sequenced EIP clones that we have evaluated to date, we did not recover either phosphatidylinositol 3-kinase or SLAP. This may reflect differences in expression of these and other transcripts in the two cDNA libraries that were screened; however, it appears more likely that sequence differences or tyrosine phosphorylation sites within cytoplasmic domain sequences of ELK and Eck define distinct SH2 recognition domains. ELK lacks a consensus sequence for binding the phosphatidylinositol 3-kinase p85 subunit (Y-hydrophobic-X-hydrophobic), while Eck and Ehk both have these motifs (28).

The Grb2 protein has been assigned important roles in signaling cell proliferation through the p21-RAS pathway as an adapter molecule binding activated platelet-derived growth factor, epidermal growth factor, and other receptors directly (31). However, Grb2 has also been recognized to participate in nerve growth factor-mediated differentiation of PC12 cells, where nerve growth factor receptor activation promotes assembly of SHC-Grb2-SOS complexes (32–34). We have ascertained that ELK receptor activation does not stimulate proliferation of the microvascular endothelial cells used in this study (data not shown). In this context, it appears that Grb2 binding to ELK is likely to signal nonproliferative downstream responses, such as cell-cell aggregation behavior.

Grb10 is a recently identified adapter protein family member with high level sequence similarity to Grb7, a known interactor with HER2 and epidermal growth factor receptors (27). Grb10 and Grb7 share COOH-terminal SH2 domains and a central domain of ~350 amino acids that is encoded by the C. elegans gene, F10E9.6 (27). That locus is mutated in mig-10 worms and is implicated in their defects in embryonic neural migration. At present, it is attractive to speculate that Grb10, through its interaction with ELK, may participate similarly in neural cell targeting during embryonic mammalian central nervous system development, because ELK is highly expressed in human fetal brain (7). Grb10 participation in endothelial cell migration and targeting mediated through ELK activation is now accessible for study, using this cultured microvascular endothelial system.

Previous data have identified interactions between Grb10...
and c-ret, a receptor tyrosine kinase involved in renal and enteric neuron development, thyroid papillary carcinomas, and multiple endocrine neoplasia (types 2A and 2B) (35). However, sufficient data are not yet available to identify the sequence preferences for Grb10 SH2 domains. Comparison of the primary amino acid sequences surrounding theputative Tyr-929 binding site in ELK to those surrounding cytoplasmic tyrosine residues of c-ret do not yet readily identify a consensus for Grb10 SH2 interaction (data not shown). Nevertheless, our data using ELKΔy deletion and site-directed mutagenesis (Fig. 3) lead us to conclude that Tyr-929 is the site for phosphorylation-dependent binding of ELK to the Grb10 SH2 domain. Although tyrosine residues are conserved at that position among multiple Eph family receptors, including Eck, only 4 of 12 residues adjacent to Tyr-929 are shared between ELK and Eck. Eck has been shown not to bind Grb10 under conditions in which the c-ret kinase does (35). These limited findings suggest that, although receptors of the Eph family share striking cytoplasmic domain sequence identity, significant differences in coupled responses may be expected as individual receptors interact with distinct intracellular signaling partners.

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