Nck Recruitment to Eph Receptor, EphB1/ELK, Couples Ligand Activation to c-Jun Kinase*

Elke Stein, Uyen Huynh-Do, Andrew A. Lane, Douglas P. Cerretti, and Thomas O. Daniel§

From the Departments of Pharmacology, Cell Biology, and Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee 37232 and ImmunoCorporation, Seattle, Washington 98101

Eph family receptor tyrosine kinases signal axonal guidance, neuronal bundling, and angiogenesis; yet the signaling systems that couple these receptors to targetting and cell-cell assembly responses are incompletely defined. Functional links to regulators of cytoskeletal structure are anticipated based on receptor mediated cell-cell aggregation and migratory responses. We used two-hybrid interaction cloning to identify EphB1-interactive proteins. Six independent cDNAs encoding the SH2 domain of the adapter protein, Nck, were recovered in a screen of a murine embryonic library. We mapped the EphB1 subdomain that binds Nck and its Drosophila homologue, DOCK, to the juxtamembrane region. Within this subdomain, Tyr594 was required for Nck binding. In P19 embryonal carcinoma cells, activation of EphB1 (ELK) by its ligand, ephrin-B1/Fc, recruited Nck to native receptor complexes and activated c-Jun kinase (JNK). Transient overexpression of mutant EphB1 receptors (Y594F) blocked Nck recruitment to EphB1, attenuated downstream JNK activation, and blocked cell attachment responses. These findings identify Nck as an important intermediary linking EphB1 signaling to JNK.

Eph family receptor tyrosine kinases transmit signals that direct cell migration, cell targeting, and cell-cell aggregation (1–5). These receptors are functionally subdivided into two subclasses (EphA or EphB) based on their overlapping affinities for either glycerolphosphatidylinositol-linked (ephrins A1–A6) or transmembrane (ephrins B1–B3) protein ligands (6, 7). Many Eph family receptor tyrosine kinases and their ligands are reciprocally compartmentalized during development, consistent with their roles in directing migration and organization of specialized cell-cell interactions (2, 8–10). For example, tectal gradients of the EphA3 (Mek4) ligand, ephrin A2 (ELF-1), direct developmental targeting of retinal axons expressing EphA3 receptor (11, 12). Similarly, axonal migratory paths of spinal motor neurons expressing Eph receptors, EphB4 and EphB3 (HTK/HEK2), are directed by segmental expression of the EphB4 ligand, ephrin B2 (5). In a reconstituted system, 32D cells transfected with EphB3 (Hek2) receptors aggregate with cells transfected with the EphB3 ligand, ephrin B1 (4).

The intracellular mediators of these targeting responses are incompletely defined. EphA2 (Eck) and EphB1 (ELK) are prototypic examples of these respective subclasses that signal through distinct cytoplasmic mediators. Ligand-activated EphA2 binds the p85 subunit of phosphatidylinositol 3-kinase and activates phosphatidylinositol 3-kinase (13). A novel Src homologous adapter protein, SLAP, also binds ligand-activated EphA2 (14). EphA4 interacts with the Src family kinase, pp55ɣc, through the major phosphorylation site at position Tyr402 (15).

We recently showed that ligand-activated EphB1 recruits two different adapter proteins, Grb2 and Grb10, through their respective SH2 domains (16). Two distinct EphB1 subdomains are involved. The Grb10 SH2 domain binds EphB1 through Tyr292 (17), a residue within the conserved, carboxyl-terminal sterile α motif that is shared among all Eph family receptors and a wide range of other signaling proteins (18). In contrast, Grb2 binds residues within the catalytic domain (16).

Here we used a yeast two-hybrid interaction screen to identify a third SH2 containing adapter protein, Nck (19), as one that interacts with EphB1 upon ligand activation. Unlike Grb10 and Grb2, the Nck SH2 domain binds EphB1 at a juxtamembrane tyrosine residue that is required for ligand activation of c-Jun kinase. Functional studies identify an important role for this residue in mediating cell attachment to fibronectin.

MATERIALS AND METHODS

Construction of Recombinant Fusion Proteins, Bait, and Expression Constructs—Fusion plasmids were constructed to permit shuttling of EphB1-encoding inserts from the pACT-DEST1 (Pharmingen, San Diego, CA) expression vector to the yeast two-hybrid “bait” LexA fusion plasmid pBTM116 (20) to the carboxyl terminus of the hEphB1 cDNA.

Site-directed Mutagenesis—Overlap extension PCR was used to generate the EphB1Y mutations, EphB1Y594F and EphB1Y600F (21). Oligonucleotide 1 (5′ primer, 5′-CTGTTTCCGATCCGCTCAGAG-3′) or Y600F (5′ primer Y600Frev, 5′-GGGTTCAATGAAAGATCTTCC-3′) were used to generate the 5′ PCR product; oligonucleotide 2 (3′ primer, 5′-CTCGCTCCGGGGAG-3′) or Y600Frev (5′ primer Y600Frev, 5′-GAGGATCCCAAC-3′) were used to generate the 3′ PCR product. Site-directed mutagenesis was performed with the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions.

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1 The abbreviations used are: GST, glutathione S-transferase; HRMEC, human renal microvascular endothelial cells; HA, hemagglutinin; PCR, polymerase chain reaction; JNK, c-Jun kinase; GTPyS, guanosine 5′-3′-(thio)triphosphate.

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...and pSR analysis, and the SmaI fragment of EphB1cyY594F was substituted for the EphB1cy pBTM116/EphB1cy fragment that had been digested before ligation with the DOCK interaction requires both intact EphB1 tyrosine kinase function and Tyr594.

Shown in Fig. 1B, an intact EphB1cy tyrosine kinase function was required for Nck binding. Mutation of the ATP-binding cleft (K652R) disrupted the two-hybrid interaction. This finding is consistent with role of SH2 domains in binding phosphotyrosine-containing peptides (31).

Further define the site at which Nck binds EphB1, we created a series of EphB1cy domain deletions, including removal of the juxtamembrane domain (pLexA-EphB1cyJM), the carboxyl-terminal domain (pLexA-EphB1cyΔCterm), or both (pLexA-EphB1cyJMΔCterm) (16). In each case, the tyrosine kinase catalytic domain was retained to permit generation of an SH2 binding site through tyrosine self-phosphorylation. The juxtamembrane domain (amino acids 556–617) of EphB1cy was required for Nck interaction (Fig. 1B). Based on published data showing that the Nck-SH2 domain preferentially binds tyrosine phosphopeptides with the sequence (Tyr(P)-hydrophilic-Ac) as described (28). GST-c-Jun (1–135) was used as a substrate for the DOCK interaction requires both intact EphB1 tyrosine kinase function and Tyr594.

To evaluate the significance of these yeast two-hybrid results, we tested whether EphB1cy bait in the yeast two-hybrid system. Like Nck, the DOCK interaction requires both intact EphB1 tyrosine kinase function and Tyr594.

Ligand-activated EphB1 Recruits Nck—To evaluate the significance of these yeast two-hybrid results, we tested whether EphB1 cy-cooperates with EphB1 following ligand activation in cells that express homologous EphB1, HRMEC (23), and P19 embryonal carcinoma cells (33). EphB1 binds and is activated by ephrin B1/Fc (LERK-2/Fc) (34, 35). Because previous reports have demonstrated differences in response to ephrin B1/Fc, depending upon whether it is presented as a dimer or as an anti-Fc clustered multimer (5), both were evaluated. As shown in Fig. 2A, ephrin B1/Fc (dimeric and clustered multimer) stimulated tyrosine phosphorylation of EphB1. Nck was recovered in EphB1 complexes following ligand activation.
Based on the yeast two-hybrid data presented above, we proceeded to evaluate the role of EphB1-Tyr 594 in the Nck interaction. Shown in Fig. 2B, we expressed HA epitope-tagged versions of either wild type or mutant (Y594F or Y600F) EphB1 in Cos-1 cells. Ephrin B1/Fc stimulated tyrosine phosphorylation of transiently expressed EphB1. Qualitatively similar EphB1 tyrosine phosphorylation was observed in EphB1 wild type and mutants (Y594F and Y600F), consistent with our data showing that a number of cytoplasmic domain tyrosine residues undergo phosphorylation upon ligand activation (16). Nck (KLUTH) growth was assessed and pictures were taken after 3 days at 30 °C. The DOCK interaction requires a tyrosine kinase active form of EphB1cy and tyrosine phosphorylation of EphB1 cyY594F.
Ephrin B1/Fc Activates c-Jun Kinase (JNK) through EphB1-Nck Interaction—We anticipated that cytoskeletal rearrangements are a necessary feature for cell-cell aggregation and targeting functions subserved by Eph receptors. In addition, recent work showed that Nck binds a serine threonine kinase, NIK, that serves as an upstream regulator of the JNK signaling pathway (36). Based on these observations, we evaluated the potential for Nck to couple EphB1 with JNK activation. Shown in Fig. 3A, c-Jun kinase was activated when P19 cells were exposed to ephrin B1/Fc. Similar results were obtained in HRMEC (not shown). As with the recruitment of Nck (Fig. 2), dimeric or multimeric ephrinB1/Fc evoked similar JNK activation responses. These effects were seen at ephrin B1/Fc concentrations greater than 125 ng ml\(^{-1}\), and activation was not stimulated by human IgG, which is used as a control for Fc domain effects (not shown). JNK activity increases of 2–3-fold were typically seen within 10 min and in some experiments increased to 5-fold by 120 min (Fig. 3A, right panel). This timing pattern is consistent with that of Nck recruitment to ligand-activated EphB1. Nck is found in EphB1 complexes as early as 7 min and persists beyond 30 min.\(^2\) A similarly delayed JNK activation response has been observed in response to transforming growth factor-\(\beta\), where persistent increases in activity are evident between 2 and 12 h (37).

To evaluate functional consequences of Nck recruitment upon EphB1-mediated responses, we used a dominant negative strategy to undermine the effects coupled to endogenous receptor activation. By expressing Nck binding-defective mutant EphB1 receptors at sufficiently high levels in P19 cells, we could evaluate changes in JNK activation and attachment to fibronectin-coated plates. Our transfection methods achieve high efficiency transfection of P19 cells (60–70%), and the fibronectin-coated plates. Our transfection methods achieve high efficiency transfection of P19 cells. Using this approach, we assessed dominant effects of wild type, kinase defective (K652R) or Nck binding defective (Y594F) EphB1 upon downstream JNK activation and cell attachment. Shown in Fig. 3B, ephrin B1 stimulated 2–2.5-fold increases in JNK activity in cells transfected with either vector control (pSRa) or wild type EphB1 (pSRa-EphB1/Ha). In contrast, ephrin B1 failed to increase JNK activity in cells transfected with either kinase defective (K652R) or Nck binding defective (Y594F) mutant receptors. In multiple experiments, we consistently observed a lower activation of JNK in transfected (2–2.5-fold), compared with nontransfected cells (3–5-fold). This appears to reflect differences in basal JNK activation, depending upon the transfection protocol.

Correlating with the JNK activation results, identically treated transfected P19 cells showed marked increases in attachment to fibronectin-coated dishes when transfected with either vector alone or wild type EphB1 expression plasmid (Fig. 3B). Yet, high level expression of kinase defective (K652R) or Nck binding defective mutant (Y594F) EphB1 receptors eliminated ephrin B1-promoted attachment. In aggregate, our findings support a role for Nck in JNK activation and attachment responses downstream of EphB1 activation.

**DISCUSSION**

Nck is an EphB1-interactive protein that is recruited to EphB1 signaling complexes, apparently through binding of its SH2 domain with EphB1 residue Tyr\(^{594}\). This interaction is stimulated by ligand activation in P19 cells and HRMEC expressing native receptors. EphB1 receptor activation stimulates JNK, an effect requiring the recruitment of Nck to EphB1. Mutation of the site at which Nck binds EphB1 attenuates downstream JNK activation and EphB1-coupled attachment responses.

Identification of Tyr\(^{594}\) as the site of interaction in the yeast two-hybrid system was somewhat surprising. Based on its affinity for small phosphopeptides, a consensus recognition bind-
ing site for the Nck SH2 domain, pYDEP, was determined (31). This consensus is different from the residues adjacent to Tyr\(^{594}\) (YIDP) and Tyr\(^{600}\) (YEDP) in the EphB1 sequence. Both of these motifs are shared between EphB1 and EphB2 (15). We have confirmed the role of Tyr\(^{594}\) in Nck binding in both yeast and mammalian cell systems through experiments that included independent review of the sequences of each construct shown in Fig. 2B. A previous report identified a platelet-derived growth factor \(\beta\) receptor peptide sequence, pYVPVL, as a Nck binding site (38), suggesting that some flexibility exists in the binding requirements. It is noteworthy that the sequence, pYY(V/D)IP, is conserved at this position in all the Eph family receptors (15). The previous finding that Nck does not bind EphA4 (15) suggests that factors other than primary amino acid sequence are likely determinants of the site of tyrosine phosphorylation in this subdomain.

A recent report by Holland et al. provided evidence for an indirect role of Nck in signaling downstream of activated EphB2 (39). They found that EphB2 activation caused tyrosine phosphorylation of a 62–64-kDa protein (p62dok) (40, 41), which in turn formed a complex with the Ras GTPase-activation protein (RasGAP) and Nck. We have re-evaluated our EphB1 immunoprecipitates following ligand activation and were unable to demonstrate co-precipitation of either p62-dok or RasGAP with EphB1 recovered from P19 (data not shown).

Despite the structural similarities of the EphB subclass receptor cytoplasmic domains, it appears that remarkable differences exist in the signal transmission machinery. In particular, EphB2 (39) displays functional similarities to Nck in its capacity to bind mPak3, a Ste20 family serine/threonine kinase, to the Ste20 family serine/threonine kinase, and to the Wiskott Aldrich syndrome protein (WASP, a forerunner of the WASP family) (42). Thus, EphB2 activation causes tyrosine phosphorylation of mPak3, a Ste20 family serine/threonine kinase activation (45).

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