The Adaptor Protein Nck1 Mediates Endothelin A Receptor-regulated Cell Migration through the Cdc42-dependent c-Jun N-terminal Kinase Pathway*

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Cell migration plays key roles in physiological and pathological phenomena, such as development and oncogenesis. The adaptor proteins Grb2, CrkII, and Nck1 are composed of only a single Src homology 2 domain and some Src homology 3 domains, giving specificity to each signal transduction pathway. However, little is known about the relationships between their adaptor proteins and cell migration, which are regulated by the G protein-coupled receptor. Here we showed that Nck1, but not Grb2 or CrkII, mediated the inhibition of cell migration induced by the endothelin-1 and endothelin A receptor. The small interference RNA and dominant negative mutants of Nck1 diminished the endothelin-1-induced inhibition of cell migration. Although overexpression of wild-type Nck1 was detected in the cytosol and did not affect cell migration, expression of the myristoylation signal sequence-conjugated Nck1 was detected in the membrane and induced activation of Cdc42 and c-Jun N-terminal kinase, inhibiting cell migration. Taken together, these results suggest that the endothelin A receptor transduces the signal of inhibition of cell migration through Cdc42-dependent c-Jun N-terminal kinase activation by using Nck1.

Cell migration plays key roles in development and oncogenesis. During development, organized cell migration is essential for proper tissue formation (1). However, unregulated cell migration is observed in pathological processes, such as oncogenesis, which involves, specifically, invasion and metastasis. The elucidation of the processes by which molecular signaling mechanisms positively and negatively regulate cell migration is critical in understanding both development and disease progression.

In many cases of the migration process, cells show directed movement (called chemotaxis) toward soluble chemoattractants. A number of chemoattractants have been identified: chemokines, lipid mediators, growth factors, and cytokines (1, 2). Chemokines bind and activate their cognate chemokine receptors, which belong to a large family of G-protein-coupled receptors (GPCRs) characteristic of the seven-transmembrane domain. As a result, Sos is translocated to the plasma membrane, leading to Ras-dependent activation of extracellular signal-regulated protein kinase (ERK), a subfamily of mitogen-activated protein kinases (MAPKs) (15, 16).

We demonstrated that the endothelin-1 (ET-1) and endothelin A (ETA) receptors activate Cdc42 of Rho GTPases, which in turn stimulate the signaling cascade of c-Jun N-terminal kinase (JNK), a subfamily of MAPKs (17, 18). This signaling pathway is involved in the inhibition of cell migration (17, 18). To further investigate the mechanism whereby the ETA receptor inhibits cell migration, the effects of various dominant negative mutants on cell migration were assayed on the transient transfection system using human epithelial 293T cells. Here we show that the Nck1, but not Grb2 or CrkII, is a critical regulator in the chemorepellent signaling pathway coupling the ETA receptor to Cdc42-dependent activation of JNK. Additionally, membrane-targeted Nck1 inhibited cell migration. These results suggest that Nck1 functions as a mediator of the chemorepellent signaling downstream of the ETA receptor.
MATERIALS—Monoclonal antibodies against active phosphorylated JNK (Thr183/Tyr185), active phosphorylated ERK (Thr202/Tyr204), and rabbit polyclonal antibodies against JNK1 and c-Src were obtained from Cell Signaling Technology, Inc (Beverly, MA). A mouse monoclonal antibody against MBP and rabbit polyclonal antibodies against JNK1 and c-Src were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal antibodies against Cdc42 and Nck1 were purchased from Transduction Laboratories (San Jose, CA). A mouse antibody against the Aequorea victoria green fluorescent protein (GFP) was obtained from Dharmacon, Inc. (Lafayette, CO). GFP siRNA was used by pCMV-MBP. The pME-ETA receptor EGFP (0.3 μg) was cotransfected with the dominant negative mutants of pCMV-MBP. Mutations in all three Nck1 SH3 domains (designated SH3All in this paper) were carried out by sequentially repeating the above procedure. The fragments of Grb2 or SH2 lacking the SH2 domain (amino acids 60–158) and Grb2ΔSH2 lacking the SH3 domain (amino acids 1–60 and 159–218) of Grb2 were inserted into pCMV-MBP. For the myristoylated products, the 14 amino acid c-Src myristoylation signal was first synthesized by PCR (19). The PCR product was digested and cloned into pEGFP-N3 (Clontech) to make pEGFP-Nck1-N3. To produce dominant negative mutants of Nck1 and CrkII, the conserved arginine residue of the FLVRES sequence in the SH2 domain was changed to lysine, creating Nck1R308K and CrkIIR35K, or the first tryptophan residue of the characteristic tryptophan doublet of the SH3 domain was changed to lysine, creating Nck1W309K, Nck1W140K, Nck1W299K, Cdc42W309K, and CrkIIW275K. The resulting values are identical to the corresponding residues of the SH3 domain and are essential for binding to their ligands (19). The amino acid substitutions were performed by the overlap extension method based on PCR with mutant oligonucleotides. These mutants were ligated into the pCMV-MBP. Mutations in all three Nck1 SH3 domains (designated SH3All in this paper) were carried out by sequentially repeating the above procedure. The fragments of Grb2 or SH2 lacking the SH2 domain (amino acids 60–158) and Grb2ΔSH2 lacking the SH3 domain (amino acids 1–60 and 159–218) of Grb2 were inserted into pCMV-MBP. For the myristoylated products, the 14 amino acid c-Src myristoylation signal was first synthesized by PCR (19). The PCR product was digested and cloned into pEGFP-N3 (Clontech) to make pEGFP-Nck1-N3 plasmid. Nck1 was subcloned into pEGFP-Nck1-N3, pCMV-FLAG-Cdc42T19N, pCMV-FLAG-Rac1T17N, pCMV-FLAG-GRB2T20N, and the Escherichia coli expression plasmid encoding the Cdc42-binding domain (CRIB) of PakCRIB was constructed as described previously (20, 21). pUSE-CA-Src (a constitutively activated mutant of c-Src) was purchased from Upstate Biotechnology, Inc. (Charlottesville, VA). pME-ETA receptor EGFP was generously provided by Dr. T. Sakurai (Tsukuba University, Tsukuba, Japan) (22).

RESULTS

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Human epithelial 293T cells were maintained in Dulbecco’s modified Eagle’s medium containing 100 μg/ml kanamycin and 10% heat-inactivated fetal bovine serum. The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO2. Plasmid DNAs were transfected into cells by the calcium-phosphate precipitation method (18). Transfection efficiency typically exceeded 80% using the pEGFP-C1 (Clontech) as a control plasmid in 293T cells. The final amount of transfected DNA for a 60-mm dish was exceeded 80% using the pEGFP-C1 (Clontech) as a control plasmid in phosphate precipitation method (18). Transfection efficiency typically exceeded 80% using the pEGFP-C1 (Clontech) as a control plasmid in phosphate precipitation method (18). Transfection efficiency typically exceeded 80% using the pEGFP-C1 (Clontech) as a control plasmid in phosphate precipitation method (18). Transfection efficiency typically exceeded 80% using the pEGFP-C1 (Clontech) as a control plasmid in phosphate precipitation method (18).

Cell Migration Assay—Cell migration was measured using a 24-well Boyden chamber (Discovery Labware) according to the manufacturer’s protocol. Briefly, the upper wells with polyethylene terephthalate filters (8-μm pore size) were coated with 10 μg/ml extracellular matrix E-C-L (Upstate Biotechnology Inc.). Serum-starved cells (2 × 105 cells in 500 μl of Dulbecco’s modified Eagle’s medium/well) were loaded into the upper wells, which were immediately plated in a chamber containing 165 μl ET-1 (750 μl of Dulbecco’s modified Eagle’s medium/well). After incubation at 37 °C for 5 h, the upper filters were stained with a Diff-Quick staining kit (Biochemical Sciences Inc., Sterling Heights, MI). Using an optical microscope, the number of migrated cells was counted in at least three independent experiments.

Crude Membrane Preparation—Briefly, the harvested cells were homogenized in an ice-cold buffer (5 mM Tris-HCl (pH 7.5), 250 mM sucrose, and 1 mM MgCl2). Nuclei and unbroken cells were separated from the cell extract by a centrifuge (700 × g, 4 °C, 10 min). The supernatant was sonicated and centrifuged (150,000 × g, 4 °C, 30 min). The supernatant was used as the postnuclear cytosol fraction, and the pellet was used as the crude membrane fraction (24).

Statistical Analysis—Values shown in the figures represent the mean ± S.E. from at least three separate experiments. A Student’s t test was carried out for intergroup comparisons with the control (*, p < 0.01).

The ETA Receptor Induces the Inhibition of Cell Migration via Cdc42/JNK—Because we previously suggested that the ETA receptor inhibits cell migration through the JNK signaling pathway (17, 18), using the dominant negative mutant of JNK activator, MKK4K95R, we examined the effect of a JNK-specific inhibitor on cell migration. As shown in Fig. 1, A–C, ETA receptor-induced inhibition of cell migration was blocked only by SP600125 (JNK inhibitor) but not by U0126 (MEK1/2 inhibitor) or SB203580 (p38 MAPK inhibitor). Next, we investigated the involvement of Rho GTPases in this signaling pathway. As shown in Fig. 1D, the dominant negative mutant of Cdc42, but not of RhoA or Rac1, attenuated ETA receptor-induced inhibition, indicating that Cdc42 functions downstream of the ETA receptor. Taken together with previous data (18), these results indicated that the Cdc42/JNK pathway plays a key role in the inhibition of cell migration induced by the ETA receptor.

The ETA Receptor Induces the Inhibition of Cell Migration through Nck1 but Not through Grb2 or CrkII—A growing number of studies have suggested that adapter proteins play an important role in mediating the signaling pathway from receptor tyrosine kinases to the Rho family-dependent regulation of the actin cytoskeleton (25, 26). Cell migration is a complex cellular process that is regulated by a number of regulatory proteins and is driven by cytoskeletal reorganization. To ex-
plore the potential involvement of adaptor proteins in the ETA receptor-induced inhibition of cell migration, we transiently transfected the dominant negative mutants of these adaptor proteins into cells. The cells transfected with the plasmid encoding Grb2/H9004SH2 or CrkII/R38K had no effect on the ETA receptor-induced inhibition of cell migration (Fig. 2, A and B).

In contrast, various SH3 domain-deficient mutants (Nck1/W38K, W143K, W229K, and SH3All) and an SH2-deficient mutant (Nck1/R308K) of Nck1 rescued the negative effect (Fig. 2C). These results suggest that Nck1, but not Grb2 or CrkII, is necessary for the inhibition of cell migration induced by the ETA receptor.

**JNK Activation Induced by an ETA Receptor Is Mediated by Nck1 but Not by Grb2 or CrkII**—To examine whether an ETA receptor activates JNK through adaptor proteins, endogenous JNK was immunoprecipitated from the cell lysates and immunoblotted with an anti-phosphorylated JNK antibody, which recognizes the active state of JNK. As shown in Fig. 3, ETA receptor-induced JNK activation was blocked by co-transfection with the dominant negative mutants of Nck1 (Nck1/W38K, W143K, W229K, SH3All, and R308K). However, co-transfection with dominant negative mutants of Grb2 (Grb2/H9004SH3 and H9004SH2) or CrkII (CrkII/W169K, W275K, and R38K) had no effect on JNK activation elicited by the ETA receptor (Fig. 3, B and C). On the other hand, Grb2/H9004SH2 inhibited EGF-induced ERK phosphorylation, and CrkII/R38K blocked EGF-induced JNK phosphorylation, in agreement with earlier findings (27, 28) (Fig. 3, D and E). However, the dominant negative mutants of Nck1 had no effect on either EGF-induced JNK (Fig. 3F) and ERK (data not shown) activation. These results indicate that
Nck1, but not Grb2 or CrkII, is a specific component of the signaling pathway from the ETA receptor to JNK. The ETA Receptor Increases the GTP-bound Form of Cdc42 through Nck1 but Not through Grb2 or CrkII—Next, using a pull-down assay, we investigated whether the ETA receptor activates Cdc42 through adaptor proteins. Following stimulation with ET-1, the GTP-bound active form of Cdc42 was dramatically increased, and this effect was blocked by the dominant negative mutants of Nck1 (Fig. 4A). As shown in Fig. 4B and C, the dominant negative mutants of Grb2 and CrkII had no effect on the ETA receptor-induced activation of Cdc42. Taken together, these results suggest that Nck1 mediates the ETA receptor-induced inhibition of cell migration involving Cdc42 and JNK.

The Membrane-bound Form of Nck1 Stimulates the Inhibition of Cell Migration by Increasing the Activities of Cdc42 and JNK—To examine whether Nck1 can inhibit cell migration through the Cdc42/JNK signaling pathway, we made a membrane-bound mutant of Nck1. We fused the myristoylation signal sequence of c-Src to the N terminus of Nck1 (Fig. 5A). To verify the effectiveness of the myristoylation signal sequence, transfected cells were homogenized and fractionated into crude membrane, cytosol, and nucleus. Wild-type Nck1 was detected in the cytosol fraction, and myr-Nck1 was mainly found in the membrane fraction (Fig. 5A). As shown in Fig. 5B, myr-Nck1 significantly inhibited cell migration; however wild-type Nck1 did not. Additionally, myr-Nck1 increased the activities of JNK and the GTP-bound form of Cdc42 (Fig. 5, C and D). Taken together, these results suggest that Nck1 mediates the inhibition of cell migration involving Cdc42 and JNK.
siNck1 inhibits the ETA receptor-induced inhibition of cell migration through the JNK pathway. To confirm the requirement of Nck1 in the pathway from the ETA receptor to the inhibition of cell migration, we then carried out RNA interference-mediated gene silencing using a synthetic 21-mer oligonucleotide RNA duplex (siRNA) of Nck1. As shown in Fig. 6A, a pair of oligonucleotides (siNck1–1 or siNck1–2) corresponding to the sequence of human Nck1. Transfection of siNck1–1 or siNck1–2 into the cells suppressed the expression of endogenous Nck1, but not tubulin, in a dose-dependent manner (Fig. 6A). Because we observed a prominent silencing effect of siNck1–1 on endogenous Nck1, we used siNck1–1 in the following experiments.

We investigated whether siNck1–1 affects the endogenous JNK activity stimulated by the ETA receptor. As shown in Fig. 6B, ETA receptor-induced JNK activation was inhibited by siNck1–1. In contrast, the control siRNA (siGFP) had no effect on the JNK activation. Additionally, the distinct signaling, i.e., the EGF-induced phosphorylation of ERK, was not affected by siNck1–1 (data not shown). In parallel with JNK activity, increase of the active form of Cdc42 induced by the ETA receptor was inhibited by siNck1–1 (data not shown). Finally, we examined the effect of siNck1–1 on cell migration. As shown in Fig. 6C, silencing of Nck1 partially rescued the ETA receptor-induced inhibition of cell migration. These results strongly suggest that Nck1 has an essential role in the ETA receptor-stimulated inhibition of cell migration through Cdc42 and JNK.

Src Kinase Suppresses Cell Migration via the Nck1/Cdc42/JNK Pathway. We demonstrated previously that Src kinase acts upstream of the Cdc42 and JNK in the ETA receptor signaling pathway (17, 18). To investigate the involvement of Nck1 in the CA-Src-induced inhibition of cell migration, the dominant negative mutants of Nck1 were co-transfected with CA-Src. As shown in Fig. 7A, the CA-Src-induced inhibition of cell migration was completely rescued by Nck1SH3All or Nck1R308K. Additionally, CA-Src-induced JNK (Fig. 7B) and Cdc42 (data not shown) activation was also inhibited by these mutants.

We demonstrated previously that the Gq-coupled receptor induces the JNK activation in a Src-dependent manner (29). Additionally, ETA receptor-induced inhibition of cell migration was blocked by co-transfection of the Gq inhibitors (regulator of G protein signaling (RGS) 4 or N-terminal domain of β-adrenergic receptor kinase 1 (βARKnt)) (data not shown). Taken together, these results indicate that the ETA receptor regulates cell migration mediated through Nck1 with Gq, Src, Cdc42, and JNK (Fig. 8).

**DISCUSSION**

During embryogenesis, complex patterns of cell migration are essential for proper tissue formation. Bladt et al. (30) recently demonstrated that the inactivation of Nck genes (Nck1 and Nck2) results in profound defects in mesoderm-derived
Fig. 7. The Src kinase-induced inhibition of cell migration and activation of JNK involves Nck1. Cells were co-transfected with CA-Src and dominant negative mutants of Nck1. After incubation for 5 h, the cells attached to the filters were stained and counted (A). The phosphorylated JNK was analyzed 20 min after stimulation with ET-1 (B). Expression of CA-Src and the dominant negative mutants of Nck1 is shown.

Fig. 8. Schematic model for the signaling pathway coupling the ETA receptor to the inhibition of cell migration. Details are described under “Discussion.”

protein in the regulation of cell migration downstream of GPCR. It is possible that the abnormal tissue morphogenesis observed in the ETA receptor-deficient mice depended, at least in part, upon Nck1.

Cell migration includes multiple processes that are coordinately modulated by a number of regulatory proteins and driven by changes in the actin cytoskeleton (35, 36). Therefore, Nck1 could participate in the control of cell migration by binding and regulating signaling proteins involved in the rearrangement of the actin cytoskeleton. A genetic study on Drosophila indicates that Dreadlocks (Dock), which is structurally related to the mammalian Nck genes (37), links tyrosine kinases to the actin cytoskeleton (38). Furthermore, in mammalian cells, it is also likely that Nck1 functions to couple the phosphotyrosine signals to the actin cytoskeleton (26, 39). For example, the SH2 domain of Nck1 binds the receptor tyrosine kinases, such as platelet-derived growth factor, EGF, and Eph, and the SH3 domain (30, 34, 39). On the other hand, two Nck1 SH3 binding proteins, N-WASP and Pak1, regulate the actin cytoskeleton (26, 39). In the present study, we suggested that Nck1 links Src kinase to the Cdc42/JNK cascade, which may be involved in the reorganization of the actin cytoskeleton. It remains to be investigated whether these known binding partners with Nck1 are involved in the ETA receptor signaling pathway in a Src/Cdc42/JNK-dependent manner.

Rho GTPases act as molecular switches between active (GTP-bound) and inactive (GDP-bound) states (40). Their activities are controlled positively by GEFs, which catalyze the replacement of GDP with GTP (41). GEFs may be the missing link between Nck1 and Cdc42 in the GPCR/JNK signaling pathway. Zhao et al. (42) showed that the second SH3 domain (SH3(2)) of Nck1 associates with Pak, NIK, and Wip through their conserved motif PXXPXXXS. Recently, we identified a new signaling molecule, FRG, which functions as a specific GEF for Cdc42 (18). We thus analyzed whether FRG contains this Nck1 SH3(2)-binding motif. It was shown that FRG possesses a nearly identical sequence to the Nck1 SH3(2)-binding motif. It would be interesting to examine whether FRG is a binding partner with the SH3 domain of Nck1 in the ETA receptor signaling pathway.

In this study, we demonstrated that the ETA receptor inhibits cell migration through the Src/Nck1/Cdc42/JNK pathway. On the basis of these findings, we summarized the proposed signaling pathway in Fig. 8. A challenge for the future will be to define the roles of Nck1 in regulating the potential for cross-talks among the various signaling pathways involving FRG in the control of cell migration. Such studies might promote our understanding of the GPCR-regulated mechanism of the early process of development as well as oncogenesis.

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