Activation of the Eck Receptor Protein Tyrosine Kinase Stimulates Phosphatidylinositol 3-Kinase Activity*

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Akhilesh Pandey, Dan F. Lazar, Alan R. Saltiel, and Vishva M. Dixit

From the Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan 48109-0602 and the
Department of Signal Transduction, Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company, Ann Arbor, Michigan 48105

The Eph/Eck subfamily of receptor protein tyrosine kinases is currently the largest subfamily of receptor protein tyrosine kinases with a dozen members (Van der Geer, P., Hunter, T., and Lindberg, R. A. (1994) Annu. Rev. Cell Biol. 10, 251–337). Using the cytoplasmic domain of Eck as bait in a yeast two-hybrid screen of mouse embryonic and T-cell cDNA libraries, it was discovered that the p85 subunit of phosphatidylinositol 3-kinase bound ECK. Further, using glutathione S-transferase fusion proteins, it was found that the C-terminal src homology 2 domain of the p85 subunit specifically interacted with Eck. Additionally, Eck coimmunoprecipitated with p85 in ligand-activated cells confirming their interaction in vivo. In keeping with the above observations, activation of Eck by its ligand, B61, increased phosphatidylinositol 3-kinase activity. This is the first description of a signal transduction pathway initiated by any member of the Eph/Eck family.

Receptor protein tyrosine kinases (RPTKs) have been shown to be important in diverse cellular functions including proliferation, differentiation and migration. Eck belongs to the Eph/Eck subfamily of RPTKs which is now the largest subfamily of RPTKs (1). While most of the members of this family are expressed only in the adult brain, Eck and Eph have a broader tissue distribution, being expressed in lung, kidney and other organs in addition to the brain (2, 3). The signal transduction pathways engaged by this family of RPTKs have not been elucidated primarily because the cognate ligands have not been identified till of late. B61, originally described as a cytokine inducible gene product, has now been shown to be the Eck RPTK ligand (4, 5). Since RPTKs associate with signal transducing molecules upon ligand activation, the availability of recombinant B61 allowed us to characterize potential downstream signaling molecules that associate with Eck upon its activation.

A recently described powerful technique for the detection of protein-protein interactions in vivo is the yeast two-hybrid system (6). The interaction of a protein of interest expressed as bait with that encoded by a transfected library plasmid (prey) results in the activation of marker genes (such as β-galactosidase) that allows for easy detection. To identify molecules that bind the cytoplasmic domain of Eck upon activation, we used it as bait in a yeast two-hybrid screen. It was assumed that the cytoplasmic domain would autophosphorylate itself (due to its intrinsic tyrosine kinase activity) as this is a prerequisite for interacting molecules to bind through their SH2 domains that specifically recognize phosphotyrosines (7). Support for such an assumption came from the finding that Src, a cytoplasmic tyrosine kinase, does undergo autophosphorylation when expressed in yeast (8) and that the cytoplasmic domain of Eck expressed either in vitro translation or following transfection of 293T cells possesses potent tyrosine kinase activity (data not shown).

Two interacting clones were isolated that encoded different regions of the β isoform of the p85 subunit of PI 3-kinase. PI 3-kinase is a heterodimer composed of a regulatory p85 subunit (α and β isoforms have been identified) and a p110 catalytic subunit (9). Binding of ligand to a multitude of receptors including the platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), interleukin (IL)-2 (IL-2), IL-4, and IL-7 receptors stimulates PI 3-kinase activity which phosphorylates the head group of phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P2) at the D-3 position to yield a highly polar membrane lipid PtdIns(3,4,5)P3 which has been postulated to act as a second messenger (10). The downstream intracellular targets of PI 3-kinase are not known, though a recent study indicated that PtdIns(3,4,5)P3 could activate a phospholipase C isoform (11). Other evidence implicating PI 3-kinase in signal transduction includes the finding that the transforming activity of the polyoma virus middle T antigen is dependent on the binding of PI 3-kinase to pp60src and that activation of PI 3-kinase is required for platelet-derived growth factor-β receptor internalization (12, 13).

The p85 subunit is a modular adapter that contains an SH3 domain (binds proline-rich sequences), two SH2 domains (specific for phosphotyrosines in a linear pYXXM motif) and a unique N-terminal region (1, 10, 14). Either or both of the SH2 domains (N-terminal (N-SH2), C-terminal (C-SH2)) could potentially mediate binding to the phosphorylated cytoplasmic domain. However, it was found that almost exclusively, the C-SH2 domain bound to the Eck cytoplasmic domain. Confirming this was the finding that the C-SH2 domain expressed as a GST fusion protein precipitated the Eck RPTK from vascular smooth muscle cell lysates. Furthermore, anti-p85 antibody coimmunoprecipitated Eck from B61-stimulated cells indicating that Eck interacts with PI 3-kinase in vivo. Finally, activa-
tion of the Eck RPTK by its cognate ligand B61 resulted in the stimulation of PI 3-kinase activity.

MATERIALS AND METHODS

Yeast Two-hybrid Screen and cDNA Isolation —The cytoplasmic domain of the human Eck RPTK was obtained by polymerase chain reaction (PCR) using a full-length Eck cDNA (2) as template and custom oligonucleotide primers. The sense primer, including a custom SfiI site (underlined) had the sequence: CTGGCCATGAGGGCCCGCCGAGCAGAGGAGAACCCAG and the antisense primer including a custom SalI site had the sequence: CGACTGTCGACTCAGATGCGGAGATCCCCACCATGTTGCAG. The amplified fragment was cloned into the yeast two-hybrid vector pAS1CYH2 to be expressed as a hybrid gene consisting of an GAL4 (15) domain of the p85 subunit of PI 3-kinase.

In Vitro Transcription and Translation —The plasmid pAS1CYH2 was amplified by PCR using an upstream primer that included a custom T7 promoter site (lower case) and library plasmid (PACT) sequence: CTACCAGACATCCCATCGGCGGATC- CCCACATGTTGCAG. To obtain the deleted form of the Eck cytoplasmic domain, a custom SalI site was included in the upstream primer containing a custom EcoRI site: ATAGCTAGCTACGGAGAAGGAGAAGCAGCCACATGTTGCAG. The downstream primer corresponded to sequences in the ADH terminator of the library plasmid: CGACTGTCGACTCAGATGGGGATCCCAGTGCAGTCCATGATCATGTTGCAGTCCA. This amplified fragment was used for coupled in vitro transcription and translation using the TNT T7 coupled reticulocyte lysate system (Promega, Madison, WI) and (35)Smethylionine (Amersham Corp.) according to the manufacturer's instructions.

Production of GST Fusion Proteins —The Eck RPTK cytoplasmic domain (amino acids 559–976) was obtained by PCR employing Eck cDNA as template and an upstream primer containing a custom EcoRI site: GGAATTCACCGAGAGGAGAAACAGCAGCCACATGTTGCAG. This was used as bait to detect interacting proteins from mouse T-cells (21). They were brought to quiescence by maintaining in serum-free medium (Eagle's minimum essential medium containing 1% bovine serum albumin (BSA)) for 48 h and then metabolically labeled with 100 μCi/ml of (35)Smethylionine (ICN) for 8 h. Cells were (Fig. 3A) treated with 1 μg/ml B61-Ig' for 5 min and then lysed in lysis buffer, boiled in SDS sample buffer (containing 8% mercaptoethanol), and resolved on a 10% SDS-polyacrylamide gel. Bound proteins were visualized by autoradiography.

Metabolic Labeling and Immunoprecipitations—Rat vascular smooth muscle cells (SMCs) were isolated and passaged as described previously (21). They were brought to quiescence by maintaining in serum-free medium (Eagle's minimal essential medium containing 1% bovine serum albumin (BSA)) for 48 h and then metabolically labeled with 100 μCi/ml of (35)Smethylionine and cysteine (Translabel, ICN) for 8 h. Cells were (Fig. 3A) treated with 1 μg/ml B61-Ig' for 5 min and then lysed in lysis buffer containing 50 μM Tris pH 7.6, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 10% glycerol and 1 mM sodium deoxycholate, 1 mM sodium azide, and protease inhibitors. The cell lysates were incubated with 5 μg/ml of anti-Eck antibody and processed as described below.

Western Blot Analysis—Precipitated immune complexes were resolved by SDS-PAGE transferred to nitrocellulose by electroblotting, blocked with 1% BSA in Tris-buffered saline containing 0.1% Tween (TBS-T) overnight at 4 °C and then incubated with 4G10 anti-phosphotyrosine monoclonal antibody (UBI, Lake Placid, New York) was done as described previously (20).

RESULTS AND DISCUSSION

The cytoplasmic domain (amino acids 559–976) of the human Eck RPTK, which when expressed in 293T cells displayed constitutive tyrosine kinase activity (data not shown), was fused in frame to the GAL4 DNA-binding domain in the yeast vector pAS1CYH2. This was used as bait to detect interacting proteins encoded by library cDNAs fused to the GAL4 or VP16 activation domains that were generated from mouse T-cells or mouse 10.5 day embryonic tissue, respectively. A total of 106

FIG. 1. Eck cytoplasmic domain specifically binds C-SH2 GST in vitro. A, (35)Smethylionine-labeled in vitro translated Eck cytoplasmic domain was incubated with N-SH2 GST, GST only, or C-SH2 GST bound to agarose beads. After incubation, the beads were washed and the bound material was eluted with SDS sample buffer, resolved by SDS-PAGE, and subjected to autoradiography. B, (35)Smethylionine labeled in vitro translated p85 subunit was incubated with GST only, ΔEck GST or Eck GST bound to agarose beads and processed as described previously (20).
transformants were screened by expression in a yeast strain harboring lacZ and HIS3 genes under control of the GAL4 upstream activating sequence. Only transformants in which HIS3 is activated will grow in the presence of 3-amino-1,2,4-triazole, an inhibitor of the HIS3 gene product. These colonies were in turn screened for lacZ expression. Library plasmids recovered from such positive colonies were then subjected to another round of cotransformation with either Eck or other heterologous baits. In this manner, library plasmids that specifically interacted with Eck were identified and marked for further characterization.

One interacting clone from the embryonic cDNA library encoded only the C-terminal SH2 domain of the β isoform of the p85 subunit of PI 3-kinase (amino acids 579–724), while another from the T-cell library encoded both the N- and C-terminal SH2 domains of the β isoform of the p85 subunit (amino acids 335–724). To obtain independent confirmation of the interaction, a cDNA encoding the cytoplasmic domain of the Eck RPTK was in vitro transcribed and translated using a rabbit reticulocyte lysate system and a protein of the predicted molecular weight (46 kDa) obtained that specifically bound the C-SH2 domain of the p85 subunit expressed as a GST fusion protein (Fig. 1A). No detectable binding was observed with GST alone or with the N-SH2 domain expressed as a GST fusion protein despite the ability of the N-SH2 GST to precipitate EGFR and PDGFR (20). To further validate this finding, the same experiment was performed in reverse. A library plasmid that encoded both the N- and C-terminal SH2 domains of p85 was in vitro transcribed and translated and incubated with either a GST-fusion of the Eck cytoplasmic domain (Eck GST) or a GST fusion of a deleted (amino acids 559–642) form of the p85 subunit. This clone (amino acids 579–724) was precipitated by C-SH2 GST but not by N-SH2 GST. This finding is in agreement with other in vitro binding studies that indicate that high affinity binding in solution is entirely dependent on the C-SH2 domain (23). However, in vivo, both SH2 domains may be required for stable binding (24). To prove that the Eck RPTK associates with p85 in vivo, lysates from unactivated or activated SMCs were immunoprecipitated with anti-p85 antibody prior to immunoblotting with anti-Eck antibody. As shown in Fig. 1B, addition of B61 induced the association of p85 with the Eck RPTK.

To determine if activation of the Eck RPTK by its cognate ligand resulted in the stimulation of PI 3-kinase activity, quiescent SMCs were treated for 5 or 10 min with B61-Ig and cell lysates immunoprecipitated with an antiphosphotyrosine antibody to capture all tyrosine phosphorylated proteins. PI 3-kinase activity was then measured in the precipitated immune complexes and found to be increased 8-fold over basal activity within 5 min of activation and 4-fold over basal level by 10 min (Fig. 4).

To confirm the presence of Eck, the SMCs were metabolically labeled and subjected to immunoprecipitation with an anti-Eck antibody. As shown in Fig. 2A, a protein of the expected molecular mass of 130 kDa was specifically precipitated by the anti-Eck antibody but not by control serum. In addition, the Eck ligand B61 expressed as an Ig chimera (B61-Igβ), but not a control-Ig (22) chimera, induced autophosphorylation of Eck in these cells (Fig. 2B, top panel). The bottom panel shows the same blot reprobed with anti-Eck antibody.
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Fig. 4. Activation of Eck by its ligand results in stimulation of PI 3-kinase activity. PI 3-kinase assay was carried out as described under "Materials and Methods." SMCs were either untreated or treated with B61-Ig for 5 or 10 min or with 10% fetal bovine serum for 5 min. PIP, PI 3-phosphate.

Taken together, these data indicate that the Eck RPTK is capable of binding to the C-terminal SH2 domain of the p85 subunit of PI 3-kinase. Furthermore, a deletion mutant of Eck (ΔEck) that removed the entire catalytic domain was incapable of binding to the p85 subunit. This is consistent with the removal of two YXXM motifs in this deletion mutant as this motif has previously been shown to mediate p85 subunit binding (25). Finally and most importantly, the activation of Eck by its ligand B61 stimulated PI 3-kinase activity. Recently, we have found that activation of Eck by B61 results in chemotaxis. The relevance of this may lie in the finding that activation of PI 3-kinase is required for platelet-derived growth factor-induced membrane ruffling and chemotaxis (26) and that D-3 phosphorylated phosphoinositides may be involved in cytoskeletal alterations (27). Consistent with this is the observation that the p85 subunit has GTPase-activating protein activity toward Rac, which is intimately involved in membrane ruffling (28). It is therefore tempting to speculate that a similar role is being fulfilled by Eck-induced stimulation of PI 3-kinase.

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