Src phosphorylates EAST, a novel EGF receptor-associated protein

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Abstract We have recently found and characterized EAST, a novel EGF receptor-associated protein with SH3 and TAM domains. In this study we show that EAST becomes phosphorylated by Src kinase. This, in conjunction with our earlier observations on the close association between EAST and the endocytic machinery, suggests that EAST could be involved in Src-dependent effects on EGF receptor endocytosis.

Key words: EAST; Epidermal growth factor receptor; Endocytosis; Phosphorylation; Src

1. Introduction

Binding of a ligand to the epidermal growth factor receptor (EGFR) leads to autophosphorylation of the receptor on several tyrosine residues in the cytoplasmic part of the molecule. They serve as docking sites for various signaling molecules, such as Grb2, phospholipase C-γ and Shp2 [1]. These effector molecules then, either by an altered subcellular localization or by enzymatic activity, initiate signaling pathways that lead e.g. to cell proliferation.

C-Src is a 60-kDa membrane-associated phosphoprotein with intrinsic kinase activity [2]. Numerous studies have implicated it in the control of cell growth and proliferation [2]. It is still unclear, however, how Src relates to the signal transduction from mitogenic receptor tyrosine kinases. The best studied is the case of platelet-derived growth factor receptor (PDGFR) which associates with c-Src upon stimulation by PDGF [3]. It has also been shown that a dominant-negative mutant of c-Src inhibits PDGF signaling [3]. The putative binding site for c-Src in PDGFR has also been identified [4].

We have recently cloned and characterized a novel SH3 and TAM domain-containing protein EAST (EGF receptor-associated protein with SH3 and TAM domains) which associates with and becomes phosphorylated by EGFR [5]. EAST is also associated with eps15, a substrate of EGFR and a component of the endocytic machinery [6–10]. In this study we show that EAST becomes phosphorylated by Src. The results suggest that EAST is involved in the Src-mediated events of EGFR signaling.

2. Materials and methods

2.1. Cell culture

Ts-Src MDCK cells, a variant of a parental MDCK cell line which is stably transfected with a temperature-sensitive v-Src [11] (a kind gift from Dr. J. Behrens, Max Delbrück Center for Molecular Medicine, Berlin, Germany), were maintained in Dulbecco's modified Eagle's medium (Seromed) containing 10% heat-inactivated fetal bovine serum (Hyclone) and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin sulfate, 0.25 μg/ml amphotericin B).

2.2. Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were carried out essentially as described previously [5]. Briefly, the cells were grown to 70–90% confluence, washed with ice-cold PBS and scraped in a lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 25 mM NaF, 10 μM $ZnCl_2$, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 5 μg/ml leupeptin, 5 μg/ml aprotinin and 1 mM PMSF). For immunoprecipitation, the lysates were first incubated with anti-rabbit IgG agarose beads for 2 h at 4°C followed by spinning down the beads. Antibodies (anti-Src or anti-EAST, see below) were then added to the supernatant followed by an incubation on a nutator for 2 h at 4°C. Then, anti-rabbit IgG-agarose beads were added and the incubation was continued for another 2 h at 4°C. The beads were spun down and washed three times with the lysis buffer, boiled in 2×Laemmli's sample buffer and subjected to SDS-PAGE and immunoblot analysis. The blots were developed by the ECL method. For repetitive probing, the filters were stripped for 40 min at 63°C in a stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol), washed extensively and reprobed.

2.3. Kinase assays

The in vitro immunocomplex kinase assay was performed essentially as previously described [5]. Briefly, ts-Src MDCK cells were lysed as above, and the cleared lysates were used for the immunoprecipitation using polyclonal anti-Src antibody (Santa Cruz Biotech.) and anti-rabbit IgG-agarose beads. The beads were washed three times with the lysis buffer and once with the kinase buffer (0.5% NP-40, 140 mM NaCl, 25 mM HEPES, pH 7.2, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin). They were then resuspended in 50 μl of the kinase buffer. Unlabelled ATP was added at a final concentration of 50 μM and the incubation was carried out for 20 min at 22°C. The complexes were then washed twice with the kinase buffer. 4 μg of the wild-type or mutated GST-EAST fusion protein and 10 μCi of [γ-^{32}P]dATP were added and the mixtures were incubated for 20 min at 32°C. GST-EAST was collected on glutathione Sepharose 4B beads which were added to the incubation mixture. The beads were then washed with the kinase buffer, boiled and subjected to SDS-PAGE and autoradiography.

The direct phosphorylation assay was carried out by using a purified, recombinant c-Src kinase (Upstate Biotechnology). The procedure was according to the manufacturer's instructions, with the exception that the kinase buffer was as above.

2.4. Additional reagents

The production, purification and mutagenesis of the GST-EAST fusion proteins has been described previously [5]. The production and specificity of the rabbit polyclonal anti-EAST antibody raised against the NH_2-terminus of EAST has also been described elsewhere [5]. Polyclonal anti-Src (N-16) and monoclonal anti-phosphotyrosine (PY99) antibodies were purchased from Santa Cruz Biotechnology. Anti-rabbit IgG-agarose was from Sigma.

3. Results

In order to study whether EAST is a substrate of Src, we took advantage of the MDCK cells stably transfected with a temperature-sensitive mutant of v-Src (ts-Src MDCK cells [11]). In these cells, temperature shift leads to an abrupt acti-

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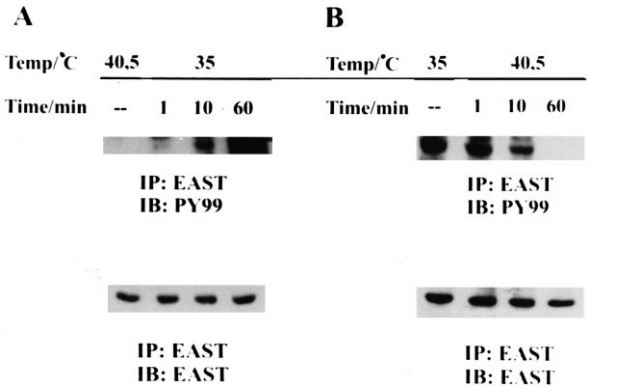

Fig. 1. Tyrosine-phosphorylation of EAST. A: Ts-Src MDCK cells were grown at the non-permissive temperature (40.5°C) and then shifted to the permissive temperature (35°C) for the indicated time periods (minutes). The anti-EAST immunoprecipitates (IP) were resolved by SDS-PAGE and analyzed by immunoblotting (IB) using the anti-phosphotyrosine antibody (upper panel). The filters were stripped and reprobed with the anti-EAST antibody (lower panel). B: Ts-Src MDCK cells were grown at the permissive temperature (35°C) and then shifted to the non-permissive temperature (40.5°C) for the indicated time periods (minutes). The anti-EAST immunoprecipitates (IP) were resolved by SDS-PAGE and analyzed by immunoblotting (IB) using the anti-phosphotyrosine antibody (upper panel). The filters were stripped and reprobed with the anti-EAST antibody (lower panel).

vation of kinase activity of Src which is accompanied by a change in the cellular phenotype [11]. When the ts-Src MDCK cells were grown at a non-permissive temperature (40.5°C), no phosphorylation of EAST was seen, as judged by immunoprecipitation with the anti-EAST antibody followed by immunoblotting with the anti-phosphotyrosine antibody. (Fig. 1A). Rapidly after a shift to the permissive temperature (35°C), however, phosphorylation of EAST was seen. It was already apparent within 1 min and reached the maximum within 1 h of the shift (Fig. 1A). Immunoblotting with anti-EAST indicates that equal amounts of EAST were present in the precipitates (Fig. 1A). A Src-dependent phosphorylation was evident also in a reciprocal experiment in which the cells were shifted from the permissive temperature to the non-permissive temperature: phosphorylation that was seen at permissive temperature waned in cells kept at the non-permissive temperature until no phosphorylated EAST was seen after 1 h of the shift (Fig. 1B). The presence of equal amounts of EAST in the precipitates was verified by reprobing the blots with the anti-EAST antibody (Fig. 1B). These results show that EAST is phosphorylated by a Src-dependent mechanism. The time scale of the EAST phosphorylation matches that reported for the changes seen in the morphology of the cells [11].

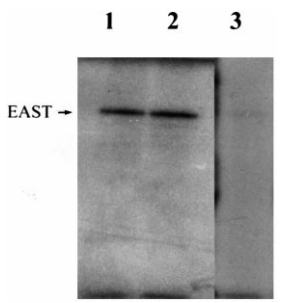

Fig. 2. In vitro immunocomplex kinase assay. Src was immunoprecipitated from the ts-Src MDCK cells grown at the permissive temperature (lanes 1 and 2) and from the ts-Src MDCK cells grown at the non-permissive temperature (lane 3). The immunocomplexes were then incubated in the presence of [γ-^{32}P]dATP with the wild-type GST-EAST fusion protein (lanes 1 and 3) and with the GST-FF-TAM (tyrosines of the TAM motif mutated to phenylalanines) fusion protein (lane 2). The proteins were separated by SDS-PAGE and visualized by autoradiography.

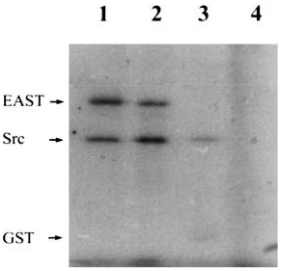

Fig. 3. Direct kinase assay with recombinant c-Src kinase. C-Src kinase was incubated in the presence of [γ-^{32}P]dATP with the wild-type GST-EAST fusion protein (lane 1), with the GST-FF-TAM fusion protein (lane 2) or with GST (lane 3). As a control, the wild-type GST-EAST fusion protein was incubated without c-Src kinase in the presence of [γ-^{32}P]dATP (lane 4).

In order to study whether the observed phosphorylation of EAST in ts-Src MDCK cells really is an effect of Src on EAST, and not due to some other kinase(s) activated by Src, we carried out an in vitro immunocomplex kinase assay. Src was immunoprecipitated from the ts-Src MDCK cells grown at the permissive temperature (35°C). The immunocomplex was then incubated with the bacterially produced GST-EAST fusion protein in the presence of [γ-^{32}P]ATP. As shown in the autoradiogram in Fig. 2 (lane 1), GST-EAST became phosphorylated. When the assay was repeated by using Src immunoprecipitated from the cells grown at the non-permissive temperature (40.5°C), no phosphorylation of the fusion protein was seen (Fig. 2, lane 3).

Although suggestive of a direct effect of Src on EAST, the previous experiment does not rule out the possibility that the phosphorylation is due to some other kinase(s) present in the immunocomplex. Therefore, we performed a direct kinase assay by using purified, recombinant c-Src kinase and the GST-EAST fusion protein as a substrate. Again, a distinct phosphorylation of the GST-EAST fusion protein was seen (Fig. 3, lane 1). No phosphorylation was seen in the control experiments in which GST alone was incubated with Src (Fig. 3, lane 3) or the GST-EAST was incubated in the kinase buffer devoid of Src (Fig. 3, lane 4). Taken together, these results show that EAST is a substrate for Src, and becomes phosphorylated by Src kinase in vitro.

It has been shown previously that Src-family kinases are responsible for the phosphorylation of the conserved tyrosine residues in the TAM motif e.g. in T cell receptor [12]. Due to the presence of a TAM motif in EAST [5], we decided to explore whether Src phosphorylates the corresponding tyrosines. For that purpose, we mutated the tyrosines of the EAST TAM (Tyr-359, Tyr-372) to phenylalanines and repeated the kinase assays by using the mutated EAST fusion protein (FF-TAM) as a substrate. The results indicate that both the wild-type and the mutated EAST were phosphorylated to about the same extent (Fig. 2, lane 2, and Fig. 3, lane 2) with only a slight decrease in the tyrosine phospho-

rylation of FF-TAM. This suggests that two tyrosines of the TAM motif are not, at least, the sole targets of Src kinase in EAST.

4. Discussion

In this study, we show that EAST, a novel EGFR-associated protein [5], is also a substrate for Src kinase. In MDCK cells stably transfected with a temperature sensitive mutant of Src, EAST shows a Src-dependent phosphorylation pattern. In an in vitro experiment with a recombinant Src, direct phosphorylation of EAST is seen.

EGFR is a classical example of a receptor protein tyrosine kinase (RPTK) and it serves as a model of RPTK-initiated signal transduction in general. Its signaling is based on dimerization and auto-/transphosphorylation of the receptor upon ligand binding. The phosphorylation sites, tyrosine residues in a defined sequence context, then serve as binding sites for the downstream effectors [1]. These events lead to an initiation of signaling cascades which relay specific activation signals to the nucleus and, thus, modulate transcriptional status of the target genes.

There is accumulating evidence that c-Src kinase is involved in signaling from EGFR. First, stimulation of A431 cells with EGF leads to an increased kinase activity of Src [13]. On the other hand, cells overexpressing c-Src show enhanced mitogenic response to EGF [14]. An association of Src with the EGFR was suggested on the basis of a co-immunoprecipitation of the endogenous c-Src with the tyrosine-phosphorylated EGFR [15]. Binding of the Src SH2-containing peptides to the activated EGFR suggested that binding of Src to EGFR could be mediated by the SH2 domain of Src. In another study, however, a non-SH2 binding site was proposed [13]. In the receptor, the binding site for Src was suggested to be different from any of the well-characterized autophosphorylation sites [16].

In the present study, we could unequivocally demonstrate phosphorylation of EAST by Src. In immunoprecipitation experiments we occasionally also saw co-immunoprecipitation of Src with EAST. The results were not reproducible, however. Thus, it remains to be elucidated whether there is a direct binding of Src to EAST. Except for the TAM motif, EAST does not possess any distinct binding sites known to be utilized by PTKs.

EAST is a modular protein. It contains a SH3 domain and a TAM motif which are both known to serve a function in protein-protein interactions. Moreover, in its very NH_2-terminal end, EAST has a VHS domain which is suggested to be important in membrane localization since it is found in a small group of molecules involved in endocytosis ([17], Ponting, C., Schultz, J. and Bork, P., SMART database, EMBL). Identification of EAST as a Src substrate suggests that its interactions with other proteins may be regulated by its phosphorylation status.

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