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Functional interaction between the epidermal growth factor receptor and c-Src kinase activity

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Abstract To study the relationship between the tyrosine kinase c-Src and the epidermal growth factor receptor (EGF-R), we used the breast cancer cell line ZR75-1, which was transfected with the EGF-R. The EGF-R transfected cell line expressed 60 times more EGF-R than a control cell line transfected with the empty vector. In the presence of EGF, the EGF-R over-expressing cell line grew much faster than the control cell line. Both cell lines expressed approximately equal amounts of c-Src. However, the cell line over-expressing the EGF-R showed a twofold enhancement of c-Src kinase activity after EGF stimulation. The activation of c-Src kinase by EGF was confirmed in other EGF-R expressing cell types.

Key words: c-Src; Epidermal growth factor (EGF) receptor; Breast cancer cell line

1. Introduction

The tyrosine kinase activity of growth factor receptors is critical for the signal transduction pathways required for mitogenesis, transformation and cell differentiation [1]. After the activation of growth factor receptors, a multitude of processes takes place, including dimerization of the receptors, autophosphorylation, and transduction of the signal to the interior of the cell. In the past few years, it has become increasingly clear that binding of the substrates to the activated growth factor receptors requires their SH2 (Src homology 2) domains [2]. Examples are given by the GTPase activating protein of ras [3,4], phospholipase C- γ [5,6], the 85 kDa subunit of phosphatidylinositol-3 kinase [7,8], shc [9], GRB2 [10], vav [11,12] and Src, Fyn and Yes [13]. It has been shown that the Src tyrosine kinase can associate with different growth factor receptors, for example, to the platelet-derived growth factor receptor (PDGF-R). The kinase activity of c-Src was elevated after PDGF stimulation of quiescent fibroblasts, coincident with transient association of Src with the PDGF-R [13]. Also, an interaction with the activated colony-stimulating factor-1 (CSF-1) receptor has been shown [14]. In contrast, the involvement of the Src kinase in EGF receptor signaling is less clear. Recently, evidence was presented that Src becomes associated to the activated EGF receptor in breast carcinoma cell lines [15]. In the present report, we further studied the relationship between the EGF-R and the Src kinase using the breast cancer cell line ZR75-1, which was transfected with the EGF-R, or a control ZR75-1 cell line transfected with an empty vector. We now show that EGF stimulation of the EGF-R over-expressing cells resulted in enhancement of c-Src tyrosine kinase activity. Activation of c-Src kinase by EGF appeared to be a general phenomenon and could be reproduced in other cell types.

Abbreviations: EGF(-R), epidermal growth factor (-receptor); PDGF(-R), platelet-derived growth factor (-receptor); CSF-1, colony-stimulating factor-1; SH2, Src homology 2; PTK, protein tyrosine kinase; PVDF, polyvinylidene difluoride.

2. Materials and methods

2.1. Cell culture

ZR75-1 human breast cancer cells, transfected with the EGF receptor (ZR11) or the empty vector (ZR9B11), were kindly donated by E. Valverius and have been described elsewhere [16]. HER14 cells (NIH-3T3 cells transfected with wild-type EGF receptor [17]) were provided by J. Boonstra (Utrecht, The Netherlands); A172 cells are non-manipulated glioblastoma cells expressing EGF receptors (own unpublished observation) and were obtained from the American Type Culture Collection (Rockville, MD). Cells were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM 1-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin, in a 5% CO₂ humidified atmosphere at 37°C.

2.2. EGF stimulation

For the stimulation of cells with EGF, either 4.6×10^6 (for short-term stimulation) or 1.7×10^6 cells (for long-term stimulation) were plated in culture dishes (14 cm diam.) in HEPES-buffered (pH 7.4), Phenol red-free RPMI with 10% fetal calf serum. After 72 h, the medium was replaced with RPMI containing 5% DCC ([18] dextran-treated, charcoal stripped calf serum). 48 h later, the cells were stimulated with EGF for 10 min (short-term stimulation), or for 48 h (long-term stimulation). Cell number after long-term stimulation was determined by Methylene blue staining of the cells, according to [19,20].

2.3. Sample preparation

Cells were washed three times with ice-cold PBS and scraped in to extraction buffer containing 50 mM HEPES (pH 7.5), 1.5 mM Mg-acetate, 5 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.055 TIU/ml aprotinin and $100 \,\mu$ M Na₃VO₄. The sample was sonicated twice for 5 s (4°C), centrifuged at $800 \times g$ for $10 \,\text{min}$ (4°C) to remove whole cells and nuclei, and the supernatant was clarified by centrifugation at $48,000 \times g$ for $60 \,\text{min}$ (4°C). The $48,000 \times g$ supernatant was used as the cytosolic fraction. The remaining pellet was solubilized in extraction buffer containing 1% Nonidet P-40. This membrane fraction was kept on ice for 1 h, during which it was sonicated twice for $10 \,\text{s}$ (4°C). The solubilized membrane fraction was obtained by clarification at $48,000 \times g$ for $60 \,\text{min}$ (4°C). Protein was determined according to Bradford [21].

2.4. Immunoprecipitation

Immunoprecipitations were performed as described previously [22]. Src was precipitated from cytosolic fractions (0.5 mg of protein) and solubilized membranes (0.1 mg of protein) with 5 μ g/ml #327 anti-Src [23] (kindly donated by J.S. Brugge, Philadelphia, PA). Incubations with non-relevant mouse immunoglobulins (Sigma) were performed as negative controls.

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2.5. Immunoblotting

Polyclonal rabbit anti-Src cst.1 antibodies were kindly donated by S.A. Courtneidge (Heidelberg, Germany) and have been described elsewhere [24]. Monoclonal mouse antibodies to phosphotyrosine (PY-20) were from ICN (Cleveland, OH), and rabbit polyclonal anti-EGF receptor antibodies directed against amino acids 984–996 of the human EGF receptor were kindly donated by L.H. Defize (Utrecht, The Netherlands). Immunoprecipitates were washed three times with radio-immunoprecipitation assay (RIPA) buffer and subjected to SDS-PAGE and immunoblotting [22]. Reacting antibodies were visualized by immuno-gold silver staining [25].

2.6. Immune complex kinase assay

Src immunoprecipitates were washed three times with RIPA buffer, once with HNEN buffer (50 mM HEPES, pH 7.5, 140 mM NaCl, 5 mM EDTA, 1% Nonidet P-40), twice with HNE buffer (HNEN without Nonidet P-40) and once with phosphorylation buffer (20 mM HEPES, pH 7.5, and 5 mM MgCl₂). Src autophosphorylation and phosphorylation of acid-denatured rabbit muscle enolase (Boehringer-Mannheim, Germany), prepared according to Cooper et al. [26], was carried out in 20 μ l phosphorylation buffer supplemented with 3 mM MnCl₂ and 2 μ g enolase. The reaction was started by the addition of 50 μ M [32P]ATP (New England Nuclear, Berkely, CA; 3.33 Ci/mmol). After 5 min at 30°C, $25 \mu l$, $2 \times$ concentrated sample buffer was added and the samples were heated at 95°C. Proteins were separated by reducing 8% SDS-PAGE and electroblotted to a polyvinylidene diffuoride filter. Marker proteins were visualized by staining with Coomassie blue. Src protein was detected by immunoblotting as described above with cst.1 or #327 anti-Src. Phosphate incorporation was analysed in a PhosphorImager coupled to ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Alternatively, ³³P-labeled ATP was used in the kinase assay and Src protein on the filters was subsequently visualised with 125 I-labeled protein A. The application of these isotopes allows precise imaging of both the kinase activity and the amount of Src protein on the same filter.

3. Results and discussion

The relative expression of functional EGF receptors in ZR9B11 and ZR11 cells was determined by stimulating growth factor-deprived cells with EGF for 10 min, scraping off the cells and analyzing the amount of EGF-R and its phosphorylation

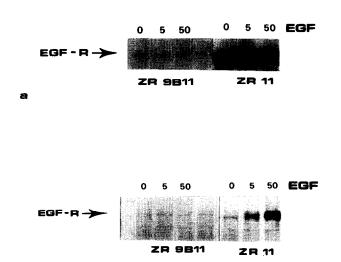


Fig. 1. Expression and autophosphorylation of the EGF-R. ZR11 cells and ZR9B11 cells were growth factor-deprived for 48 h, and stimulated with 0, 5 or 50 ng/ml EGF for 10 min. Immunoblots of whole cell lysates were probed with either anti-EGF-R antibodies (a) or anti-phosphotyrosine antibodies (b). Antibodies were visualized by immuno-gold silver staining [25].

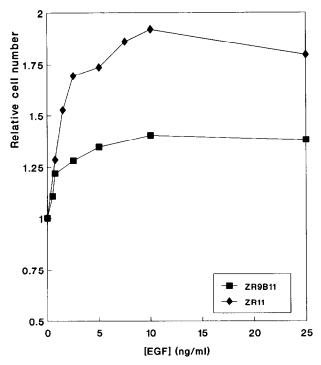


Fig. 2. Proliferation of ZR11 and ZR9B11 cells. ZR11 cells and ZR9B11 cells were deprived of serum for 48 h and stimulated with various concentrations of EGF for another 48 h. The increase in cell count with respect to non-stimulated cells is given.

by immunoblotting with anti-EGF-R and anti-phosphotyrosine. In accordance with the results of Valverius et al. [16] we found that in the ZR11 cells, an approximately 60-fold larger amount of EGF-R was present compared to the ZR9B11 cell line (Fig. 1a). When the parallel immunoblots were treated with anti-phosphotyrosine, essentially the same increase in autophosphorylation could be detected (Fig. 1b).

To determine if there was a difference in growth rate between the two cell lines after stimulation with EGF, we plated the cells at a low density, deprived them of growth factors for 48 h, and then stimulated the cells with EGF for another 48 h. The cells were counted by Methylene blue staining. Fig. 2 shows that in the presence of increasing concentrations of EGF, the ZR11 cells grow much faster than the ZR9B11 cells, which are hardly responsive to EGF. In the absence of EGF the cells remained growth-suppressed. Thus, also in growth assays, the difference between the ZR11 and ZR9B11 cell lines was apparent.

To ensure that c-Src kinase was present in the cell lines, we performed immunoprecipitations with anti-Src #327. The precipitates were subjected to electrophoresis and transferred to PVDF filters, which were probed with anti-Src cst.1. In both cell lines, a prominent band at a molecular weight of 60,000 Da can be seen, both in the cytosol and in the membrane fraction (Fig. 3), showing that there was considerable expression of the Src kinase in these cell lines. The amount of c-Src present in both cell lines was approximately the same. A relatively large amount of c-Src, about 50%, was recovered in the cytosol. This was not due to our extraction procedure, as in other cell types we found most of the c-Src to be attached to membranes.

To study whether there was a functional interaction between the EGF-R and the Src kinase, we performed short-term stim-

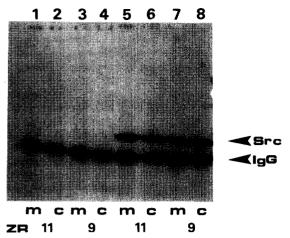


Fig. 3. Presence of Src in ZR11 and ZR9B11 cells. Cytosolic and membrane fractions of ZR11 cells and ZR9B11 cells were immunoprecipitated with anti-Src antibody #327 (lanes 5–8) or non-relevant control antibodies (lanes 1–4). Precipitates were analyzed by immunoblotting with anti-Src cst.1 antibodies.

ulations with EGF, extracted cytosolic and membrane fractions, made immunoprecipitates with anti-Src #327, and, after extensive washing, subjected the precipitates to an immune complex kinase assay using acid-denatured enolase as an exogenous substrate. A representative result is shown in Fig. 4. After stimulation of the cells with 100 ng/ml EGF, the activity of the membrane-bound Src kinase in the ZR11 cells was markedly increased. The specific Src activity could be calculated by dividing the amount of radioactivity towards enolase (determined by quantification in the PhosphorImager) by the amount of c-Src present on the immunoblot probed with cst.1, which was determined by densitometric scanning of the appropriate immunoreactive band. In the ZR11 cells, which over-express the EGF-R, the membrane-bound specific Src kinase activity was found to be increased 2-fold after EGF stimulation (2.03, S.E.M. = 0.11, mean of three independent experiments) compared to non-stimulated cells. In the ZR9B11 cells, no such elevation could be found when comparing EGF-stimulated to non-stimulated cells (0.97-fold increase, S.E.M. = 0.21, n = 3). In the cytosol of both cell lines no significant increase in the activity towards enolase was found.

To exclude the possibility that the activation of membrane-

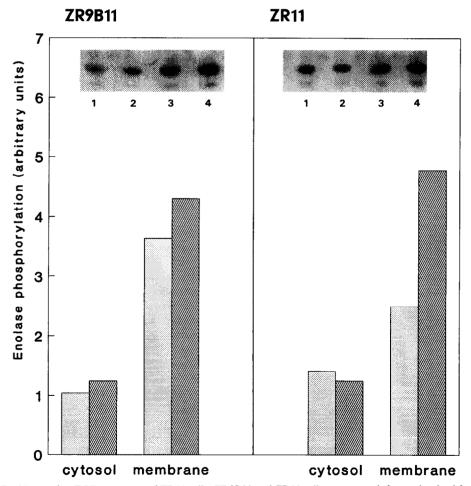


Fig. 4. Stimulation of Src kinase after EGF treatment of ZR11 cells. ZR9B11 and ZR11 cells were growth factor-deprived for 48 h and treated with 100 ng/ml EGF for 10 min (hatched bars) or left unstimulated (dotted bars). Cytosolic and membrane proteins were extracted and immunoprecipitated with anti-Src #327 antibodies. Immune complex kinase assays were performed and incorporation of phosphate into enolase was quantified with a PhosphorImager. Inset: phosphorylation of enolase by cytosolic (lanes 1 and 2) or membrane (lanes 3 and 4) anti-Src precipitates from EGF-stimulated (even lanes) or control cells (odd lanes), detected with a PhosphorImager.

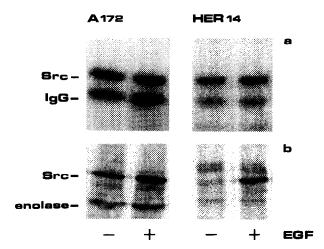


Fig. 5. EGF-induced stimulation of Src kinase in other cell types. A172 glioblastoma cells and HER14 fibroblasts were stimulated with EGF (+) or left unstimulated (-) and Src was precipitated from the membrane fractions. Kinase assays were performed with [32P]ATP and after SDS-PAGE and transfer to PVDF filters, precipitates were twice analysed in a PhosphorImager for (a) Src protein by immunoblotting with anti-Src #327 and 125I-labeled protein A, and (b) tyrosine kinase activity. Kinase activities were measured using enolase as a substrate (A172) or in an autokinase assay (HER14).

bound Src by EGF was cell type restricted, we repeated the experiments with other EGF receptor expressing cell lines. Stimulation of both A172 glioblastoma cells and HER14 fibroblasts with EGF resulted in an increase in Src kinase activity in the membrane fraction (Fig. 5). Quantification of these data in the PhosphorImager showed a 1.8-fold (A172) and 4.6-fold (HER14) enhancement of Src activity.

These results show directly a functional interaction between the EGF-R and the Src kinase. The relationship between the PDGF receptor and Src has been established before. In a paper by Kypta et al., a physical association between Src (and also Yes and Fyn, two other Src family members) and the PDGF-R was shown after stimulation of quiescent fibroblasts with PDGF [13]. In addition, the Src kinase activity was elevated 2–3 times. The association of Fyn with the activated PDGF receptor was mediated by the SH2 domain of Fyn and resulted in phosphorylation of its amino-terminal part [27]. Also, the activation of the Src family members Src, Fyn and Yes, by stimulation with CSF-1, and their association with the CSF-1 receptor has been shown recently [14]. The amount of Src activation by PDGF, or CSF-1 stimulation corresponds well with the amount of activation we see after stimulation with EGF.

Up to now, an interaction between the Src kinase and the EGF-R has been indicated only indirectly. Over-expression of c-Src in murine fibroblasts caused a 2- to 5-fold enhancement in DNA synthesis in response to EGF [28]. Kinase-defective Src, or Src lacking a signal for myristylation or an intact SH2 domain, were unable to elicit this enhanced response to EGF in the cell lines over-expressing these mutants [29]. These findings suggest that the presence of elevated levels of c-Src augments normal EGF-induced signaling pathways. Another report which points to a relationship between the EGF receptor and the Src kinase deals with the identification of a cytoskeleton-associated EGF-sensitive Src substrate, p75 [30]. Over-expression of c-Src in murine fibroblasts leads to an increase in

the basal tyrosine phosphorylation level of p75, yet p75 tyrosine phosphorylation is further enhanced upon EGF treatment [30]. Recently it was shown that in human breast carcinoma cell lines the Src SH2 domain binds to the activated EGF-R [15]. In addition, endogenous Src was found to co-precipitate with tyrosine-phosphorylated EGF-R [15]. These results suggest that Src may become physically associated to the receptor upon EGF stimulation. Our data now show that EGF stimulation actually results in an activation of the membrane-bound Src tyrosine kinase.

The fact that many oncogenes encode for tyrosine kinases points to a role for these enzymes in tumorigenesis. Consistent with this, we have shown that in all breast cancers, the protein tyrosine kinase (PTK) activity is enhanced compared to normal breast tissue, and that the increase in PTK activity may be of prognostic significance [22,31]. We identified the majority of these PTKs as the product of the c-src proto-oncogene [22]. In breast cancer, a prognostic role for the elevated expression of Neu, a growth factor receptor tyrosine kinase that is highly homologous to the EGF-R, has also been proposed [32-35]. Recently it has been demonstrated that mammary tumors expressing the neu proto-oncogene possess elevated Src kinase activity [36]. In addition, direct complex formation between Neu and Src was observed. Also the amplification or overexpression of the EGF receptor itself has clearly been implicated in the pathogenesis of a group of breast cancers [37-40]. A functional interaction between the Src kinase and the EGF receptor put these observations in a new perspective, as this may point to an important role for the two tyrosine kinases in the tumorigenesis of at least a subtype of breast cancers.

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