Src-dependent Phosphorylation of the Epidermal Growth Factor Receptor on Tyrosine 845 Is Required for Zinc-induced Ras Activation*

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Previous studies have shown that exposure of cells to Zn$^{2+}$ ions induces Ras and MAPK activation through the EGF receptor (EGFR). To further determine the role of EGFR in Zn$^{2+}$-induced signaling, mouse B82L fibroblasts expressing no detectable EGFR protein (B82L-par), wild type EGFR (B82L-wt), kinase-deficient EGFR (B82L-K721M), or COOH-truncated EGFR (B82L-c’958) were tested. Exposure to Zn$^{2+}$ induced Ras activity in B82L-wt, B82L-K721M, and B82L-c’958 but not in B82L-par cells, indicating that the tyrosine kinase domain and the auto-phosphorylation sites of the EGFR were not required for Zn$^{2+}$-induced Ras activation. Zn$^{2+}$ induced Src activity in all B82L cell lines, including B82L-par, indicating that Src activation is independent of the presence of the EGFR. A Src kinase inhibitor blocked Zn$^{2+}$-induced Ras activation in all the B82L cell lines capable of this response, suggesting the involvement of Src kinase in Zn$^{2+}$-induced Ras activation via the EGFR. Zn$^{2+}$ induced the association of the EGFR with Src and specifically increased the phosphorylation of EGFR at tyrosine 845 (Tyr-845), a known Src phosphorylation site. Stably transfected B22L cells with a point mutation of the EGFR at Tyr-845 (B82L-Y845F) exhibited only basal Ras activity following exposure to Zn$^{2+}$. These data demonstrate that Src-dependent phosphorylation of the EGFR at Tyr-845 is required for EGFR transactivation and Zn$^{2+}$-induced Ras activation.

Zinc (Zn$^{2+}$), a Group Ib metal, is not only an essential micronutrient involved in structural and regulatory cellular functions (1) but also a common airborne metallic contaminant that may contribute to the health effects of ambient and occupational pollution (2–6). Recently, much attention has been attracted to Zn$^{2+}$-induced intracellular signaling (7). Effects of Zn$^{2+}$ ions on intracellular signaling molecules have been determined, including Ca$^{2+}$ (8), mitogen-activated protein kinases (2, 9), phosphatidylinositol 3-kinase (10), tyrosine kinases such as the nonreceptor tyrosine kinase Src, and EGFR1 (11–13).

The EGFR family of receptor tyrosine kinases in mammals is composed of four members: EGFR (ErbB1), ErbB2, ErbB3, and ErbB4 (14). Of these, an 1186-amino acid residue transmembrane glycoprotein (15), is the prototypal member of this superfamily and is expressed in many cell types (16). Structurally, EGFR consists of an extracellular ligand binding domain, a single α-helical transmembrane pass, an intracellular tyrosine kinase domain, and a COOH-terminal region that contains autophosphorylation sites (15, 17–20). Upon binding of specific polypeptide ligands, including EGF, transforming growth factor-α, betacellulin, heparin-binding EGF, epiregulin, and amphiregulin, EGFR undergoes homo- or heterodimerization and activation of its intrinsic tyrosine kinase activity (17, 21). These events lead to the autophosphorylation of multiple tyrosine residues in the COOH-terminal tail of the molecule that serve as binding sites for cytosolic signaling proteins containing Src homology 2 (SH2) domains and phosphoryrosine binding domains (20, 22).

Five sites of in vivo autophosphorylation have been identified in the EGFR: three major (tyrosines 1068, 1148, and 1173) and two minor (tyrosines 992 and 1086) (18, 23–25). The binding of phosphorylated EGFR tyrosines with downstream signaling proteins initiates multiple signaling cascades that culminate in cell proliferation, migration, protein secretion, differentiation, and/or oncogenesis (16). Among these signaling pathways, the Shc/Grb2/Sos/Ras/MAPK cascade is a major mitogenic signaling pathway initiated by the EGFR (26). EGFR is also part of signaling networks transactivated by stimuli that do not directly interact with this receptor. These stimuli include agonists that specifically bind to other membrane receptors, membrane depolarization agents, and environmental stressors (27). The mechanisms for the transactivation of EGFR vary with the cell type and stimuli (27).

Cellular Src functions as a co-transducer of transmembrane signals emanating from a variety of growth factor receptors, including EGFR (28–30). Evidence indicates that EGFR and

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1 The abbreviations used are: EGFR, EGF receptor; EGF, epidermal growth factor; MAPK, mitogen-activated protein kinase; Grb2, growth factor receptor-bound protein 2; DMEM, Dulbecco’s modified Eagle’s medium; HRP, horseradish peroxidase; RIPA, radioimmune precipitation buffer; PBS, phosphate-buffered saline; RBD, Ras binding domain; SOS, son of sevenless; SH2, Src homology 2; PP1, 4-amino-5-(4-methylphosphino)-7-(t-butyl)parazonol,3,4-dipyrimal; She, Src homology and collagen protein.

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the nonreceptor tyrosine kinase Src cooperate in both mitogenesis and transformation (28–32). Novel Src-phosphorylated tyrosine residues on the EGFR have been identified (33–35).

Our previous study showed that Zn\(^{2+}\) ions induced EGFR signaling in human airway epithelial cells (12). In this study, we examined the mechanisms involved in Zn\(^{2+}\)-induced EGFR-mediated signaling, especially the possible cooperation of EGFR with Src. We found that an intact tyrosine at 845 of EGFR (Tyr-845) was necessary for Zn\(^{2+}\)-induced Ras activation. However, the tyrosine kinase and autophosphorylation sites within EGFR were dispensable. Furthermore, Zn\(^{2+}\) was found to activate Src kinase and induce its association with EGFR. These results indicate that Ras activation triggered by Zn\(^{2+}\) is dependent on Src-mediated phosphorylation of EGFR at Tyr-845.

**EXPERIMENTAL PROCEDURES**

**Materials and Reagents—**American Chemical Society-grade metal salt zinc sulfate was obtained from Sigma Chemical Co. (St. Louis, MO). SDS-PAGE supplies such as molecular mass standards, polyacrylamide, ready gels, and buffers were from Bio-Rad (Richmond, CA). Phospho-EGFR (Tyr-845), phosphoryo-EGFR (Tyr-1068), and phospho-Src (Tyr-416) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Monoclonal anti-EGFR antibody LA22, human recombinant EGFR, and anti-Src antibody (clone GD11) were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-EGFR antibody (1005), horseradish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti-mouse IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-EGFR antibody AB14 was obtained from Neomarkers (Fremont, CA). PP1 was purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). LipofectAMINE transfection reagents were purchased from Invitrogen.

**Cell Culture and Exposure—**The B82L, parental fibroblasts (B82L-par) and B82L fibroblasts expressing the human wild type EGFR (Stable Transfection with the EGFR) (B82L-K721M), and the COOH-terminally truncated EGFR at Tyr-958 (B82L-c'958) have been described previously (36, 37). B82L-par fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) containing 10\% fetal bovine serum. A mutant dihydrofolate reductase gene provides a dominant selectable marker for the B82L cells overexpressing the EGFR. H9262, a mutant dihydrofolate reductase gene provides a dominant selectable marker for the B82L cells overexpressing the EGFR.

A solution of 100 mM zinc sulfate was prepared in distilled water and used as a stock for dilution into serum-free DMEM. The final concentration of Zn\(^{2+}\) was 500 \(\mu\)M, and that of EGFR was 100 ng/ml.

**Stable Transfection with the EGFR (Y845F) Construct—**The EGFR (Y845F) construct was described previously (34, 35). The B82L-par cells were grown to 80% confluence and transfected with the EGFR (Y845F) construct using LipofectAMINE (Invitrogen). Specifically, 10 \(\mu\)g of the plasmid DNA was incubated with 60 \(\mu\)l of LipofectAMINE reagent for 30 min at room temperature. Transfection mixtures were diluted into Opti-MEM I reduced serum medium (Invitrogen), and the diluted complex solution was overlaid onto the subconfluent cells in 100-mm culture dishes. After incubating with B82L-par cells for 6 h at 37 \(^\circ\)C, the transfection mixture was removed and replaced with fresh DMEM containing 10% bovine serum. The transfected cells were passaged in DMEM containing 1000 \(\mu\)g/ml Geneticin (Invitrogen). After five passages, several colonies were picked and characterized for EGFR protein expression and phosphorylation using specific antibodies.

**Immunoprecipitation and Immunoblotting—**Confluent B82L cells pretreated with vehicle or kinase inhibitor were treated with 500 \(\mu\)M Zn\(^{2+}\) or 100 ng/ml EGFR for 20 min. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed with RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS, pH 7.4) containing 0.1% vanadyl sulfate and protease inhibitors (0.5 mg/ml leupeptin, 0.5 mg/ml aprotinin, 5 mg/ml trans-epoxysulfide-o-(4-guanidino)butane (E-64), 0.5 mg/ml pepstatin, 0.5 mg/ml bestatin, 10 mg/ml chymostatin, and 0.1 mg/ml leupeptin). After normalization for protein content, cell extracts were subjected to SDS-PAGE on 11% gradient PAGE gels or 4–15% Tris-HCl ready gels from Bio-Rad (Richmond, CA) with a Tris-glycine-SDS buffer. Proteins were electrophoretically transferred onto nitrocellulose membrane. The blots were blocked with 3% nonfat milk, washed briefly, incubated with phospho-specific antibodies in 3% bovine serum albumin at 4 \(^\circ\)C overnight, and then incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Bands were detected using chemiluminescence reagents on film as described previously (2).

**Ras Activation Assay—**A Ras activation assay kit was purchased from Upstate Biotechnology (Lake Placid, NY). Activity was determined according to the supplier’s instruction. B82L cells treated with zinc sulfate were lysed with 5 \times 10^{-5} M lysis/wash buffer (MLB) (125 mM HEPES, pH 7.5, 750 mM NaCl, 5% Nonidet P-40, 50 mM MgCl\(_2\), 5 mM EDTA, and 10% glycerol). 500–1000 \(\mu\)g of cell lysate was preclatured with glutathione agarose. 5 \(\mu\)l of a 50% slurry of Ras RBD (Ras binding domain)-agarose was incubated with the lysate at 4 \(^\circ\)C for 30 min. The agarose was collected by centrifugation and washed with MLB three times and once with cold PBS, then boiled in 25 \(\mu\)l of reducing sample loading buffer. GTP-bound Ras protein was resolved by electrophoresis and transferred to nitrocellulose before being probed with a mouse monoclonal anti-Ras (clone Ras10) antibody (1 \(\mu\)g/ml). Protein bands were visualized using a goat anti-mouse secondary antibody conjugated to HRP and an enhanced chemiluminescence detection system (12).

**Co-immunoprecipitation of the EGFR with Src—**Confluent B82L cells were treated with zinc sulfate for 20 min and lysed with RIPA lysis buffer. The lysates were pre-cleared with Protein A-Sepharose and immunoprecipitated with agarose-conjugated anti-EGFR antibody for 1 h at 4 \(^\circ\)C. Immunocomplexes were washed twice with RIPA buffer and once with cold PBS. The immunoprecipitates were suspended in 25 \(\mu\)l of 4X sample loading buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.7 M β-mercaptoethanol, 0.05% bromphenol blue) and boiled for 5 min before separation on 4–15% Tris-HCl ready gels. The protein was transferred to a nitrocellulose membrane and incubated with anti-Src mouse monoclonal IgG, at 4 \(^\circ\)C overnight. The EGFR-associated Src bands were detected with HRP-conjugated goat anti-mouse secondary antibody using ECL chemiluminescence reagents as described above.

**RESULTS**

**EGFR Mutants—**Schematic representations of human wild type EGFR and three EGFR mutants are shown in Fig. 1. The three EGFR mutants include a construct encoding a kinase-inactive receptor that contains a lysine to methionine substitution at position 721 (B82L-K721M), which was involved in ATP binding, a construct encoding a kinase-active receptor truncated at Y958 (B82L-c'958), which lacks all five EGFR
autophosphorylation sites, and a construct encoding a mutant EGFR at Tyr-845 where the tyrosine residue is replaced with phenylalanine (34, 35). No endogenous EGFR protein was detected in B82L-par cells while other transfected B82L cells express abundant EGFR protein (data not shown), which is consistent with previous studies (37, 39, 41).

Zn\(^{2+}\)-induced Ras Activation in B82L Cells—To assess the functional activation of EGFR, we used Ras as a downstream marker of EGFR signaling. Ras activation assays showed that exposure of B82L-par cells to Zn\(^{2+}\) or to EGF did not increase GTP-bound Ras after 20 min (Fig. 2A). In contrast, Zn\(^{2+}\) induced a significant increase in GTP-bound Ras in B82L-wt, B82L-c958, or B82L-K721M cells (Fig. 2B-D), confirming the requirement for EGFR, as suggested by our previous study. Interestingly, Zn\(^{2+}\) increased GTP-bound Ras in cells expressing kinase-inactive and COOH-truncated EGFR demonstrating that neither the tyrosine kinase nor the autophosphorylation sites of EGFR were required for Zn\(^{2+}\)-induced Ras activation. These results suggested that EGFR was transactivated in Zn\(^{2+}\)-treated cells. In contrast, no increase in GTP-bound Ras was detected in Zn\(^{2+}\)-treated B82L-Y845F cells (Fig. 2E), indicating that EGFR tyrosine 845, which is phosphorylated by c-Src (35, 42), is required for Zn\(^{2+}\)-induced Ras activation. EGFR increased GTP-bound Ras in wt, kinase-inactive K721M and to a lesser extent in c958 EGFR-expressing cells as previously repeated (39, 43, 44). EGFR Tyr-845 was not required for EGF-induced Ras activation (Fig. 2E).

Requirement of Src Kinase Activity for Zn\(^{2+}\)-induced Ras Activation—To investigate whether Src kinase mediated transactivation of EGFR in Zn\(^{2+}\)-exposed cells, we exposed cells to PP1, a potent and selective Src kinase inhibitor (45). Pretreatment with PP1 significantly blocked Zn\(^{2+}\)-induced Ras activation in B82L-wt (Fig. 3), whereas PP1 had no inhibitory effect on EGF-induced Ras activation. These results indicate that Src activity is required for Zn\(^{2+}\)- but not for EGF-induced Ras activation. Similar results were obtained with B82L-c958 or B82L-K721M cell lines used in this study (data not shown).

To exclude the possibility that PP1 affected the intrinsic kinase activity of EGFR, EGF-induced receptor autophosphorylation was examined in B82L-par cells that were pretreated with PP1. Consistent with previous reports (41, 46), PP1 had no significant effect on EGF-induced EGFR autophosphorylation (data not shown).

Zn\(^{2+}\)-induced Phosphorylation of Src on Y416—To determine whether Zn\(^{2+}\) affected c-Src, we examined the effects of Zn\(^{2+}\) on Src using a phospho-specific anti-Src (Y416) antibody. Phosphorylation of Src Tyr416 is part of the enzyme activation mechanism (47). As shown in Fig. 4, exposure to Zn\(^{2+}\) for 20 min induced the phosphorylation of Tyr416 on Src in B82L-par (Fig. 4A), B82L-wt (Fig. 4B), B82L-c958 (Fig. 4C), and B82L-K721M (Fig. 4D) cell lines. These results further support the hypothesis that Src activation is involved in EGFR phosphorylation. Src phosphorylation was also detected in the B82L-Y845F cell line (Fig. 4E) wherein the known Src phosphorylation site Tyr-845 on EGFR was replaced with phenylalanine. These data indicated that activation of Src occurred independent of the existence of EGFR Tyr-845. In some cell types (B82L-wt, B82L-K721M, or B82L-Y845F) EGF also increased phosphorylation of Src, and the magnitude of the increase was always less than those induced by Zn\(^{2+}\) (Fig. 4).

Zn\(^{2+}\)-induced Association of the EGFR with Src—Next, we determined whether Zn\(^{2+}\) induces a physical association between EGFR and Src in B82L-wt (Fig. 5A), B82L-c958 (Fig. 5B), B82L-K721M (Fig. 5C), and B82L-Y845F (Fig. 5D) cell lines. Immunoprecipitation of EGFR followed by immunoblotting with anti-Src antibody revealed that treatment with Zn\(^{2+}\) for 20 min caused a co-localization of EGFR with Src in these cell lines. In contrast, treatment with EGF for 20 min did not result in an interaction between Src and EGFR (Fig. 5).

Zn\(^{2+}\)-induced Phosphorylation of EGFR at Tyr-1068—The finding that mutation of EGFR Tyr-845 prevented Zn\(^{2+}\)-induced Ras activation suggested that phosphorylation of this site was essential for transactivation of EGFR in cells exposed to Zn\(^{2+}\). To further test this hypothesis we examined the phosphorylation of this site using a phospho-specific antibody to Tyr-1068 of the EGFR. Zn\(^{2+}\)-induced phosphorylation of EGFR at Tyr-1068 in B82L-wt (Fig. 6A), B82L-c958 (Fig. 6B), and B82L-K721M (Fig. 6C) cells. EGF treatment also increased the phosphorylation of Tyr-845 on EGFR in these cell lines. To determine whether the phosphorylation of Tyr-845 is related to Src activation induced by Zn\(^{2+}\), the effect of PP1 on Zn\(^{2+}\)-induced phosphorylation of EGFR Tyr-845 was also examined in these cell lines. PP1 significantly blocked Zn\(^{2+}\)-induced phosphorylation of EGFR Tyr-845 in all of the cell lines. In contrast, PP1 only had a slight inhibitory effect on EGF-induced EGFR phosphorylation at Tyr-845 in B82L-wt and B82L-
K721M cells. As expected, both Zn$^{2+}$ and EGF failed to induce phosphorylation of EGFR Tyr-845 in B82L-Y845F cells. Additionally, the phosphorylation of EGFR Tyr-1068, a major EGFR autophosphorylation site (23), was also examined in these cell lines stimulated with Zn$^{2+}$ or EGF. As expected, no phosphorylation of Tyr-1068 in B82L-c958 cells was observed (Fig. 7B), in addition, Zn$^{2+}$ failed to induce the phosphorylation of EGFR Tyr-1068 in B82L-wt (Fig. 7A), B82L-K721 (Fig. 7C), and B82L-Y845F (Fig. 7D) cells. In comparison, EGF induced phosphorylation of EGFR Tyr-1068 in each cell type (Fig. 7, A, C, and D).

**DISCUSSION**

Cross-communication between heterologous signaling systems is essential to integrate a variety of extracellular stimuli into a limited number of signaling pathways (48, 49). EGFR has been identified as a key element in the complex signaling network that is transactivated by G protein-coupled receptors, cytokine receptors, estrogen receptors, integrins, ion channels, or stress-inducing agents (49–52). Accumulated evidence indicates that Src kinases, calcium, the Ca$^{2+}$-regulated focal adhesion kinase family kinase Pyk2, protein kinase C, Janus, and tyrosine kinase Jak2 play crucial roles in the process of EGFR transactivation (48, 53, 54). Moreover, the recent identification of Zn$^{2+}$-dependent metalloproteinases and transmembrane growth factor precursors as critical elements in G protein-coupled receptor-induced EGFR transactivation pathways has defined new components of a cellular communication network of rapidly increasing complexity (51, 55, 56).

In the present study, the mechanisms for Zn$^{2+}$-induced EGFR signaling were examined in B82L cells expressing wild type, null, or modified versions of EGFR. These results show that Zn$^{2+}$ ions induce Ras activation through a mechanism that requires EGFR but not EGFR tyrosine kinase activity or autophosphorylation sites. Furthermore, an intact tyrosine at Tyr-845 on EGFR was required for Zn$^{2+}$-induced Ras activation.

Zn$^{2+}$-induced phosphorylation of the EGFR Tyr-845 residue
requires Src activation. These data suggest that Zn$^{2+}$-induced EGFR transactivation occurs through an Src-dependent pathway.

Src phosphorylation of EGFR has been mapped to most of the five autophosphorylation sites as well as other novel sites (50). Tyr-845 in the EGFR is not a known autophosphorylation site and has been shown to be phosphorylated by Src both in vivo and in vitro (34, 57). Our data support a direct phosphorylation of EGFR by c-Src for the following reasons: First, Zn$^{2+}$ ions induce co-localization of EGFR with Src and Src phosphorylation of Tyr-845 has been reported to occur in an EGFR-Src complex (57). The c-Src SH2 domain can bind activated Src specifically and directly (58–60), which suggests other kinases may not be necessary to mediate the phosphorylation of Tyr-845 (34). Other Src-dependent or -independent residues on EGFR may act as docking sites for Src or facilitate the association of EGFR with Src, leading to Src phosphorylation of Tyr-845 (33, 34). However, phosphorylation of Tyr-845 is only one of multiple Src effectors, because Zn$^{2+}$ still induced phosphorylation of Src in the absence of Tyr-845 in B82L-Y845F cells. Second, Src activity is required for the phosphorylation of Tyr-845 as demonstrated with the use of a pharmacological kinase inhibitor, suggesting that constitutive Src activity may be responsible for the basal activation of Ras. The phosphorylation of Tyr-845 induced by Zn$^{2+}$ ions, but not that induced by EGF, is dependent on Src activity. Therefore, Src activation and subsequent phosphorylation of Tyr-845 are crucial steps in the process of Zn$^{2+}$-induced Ras activation. Other tyrosine phosphorylation sites play a significant role in EGF-induced Ras activation. We have observed a baseline of GTP-bound Ras in all B82L cell lines, which could be suppressed by the Src kinase inhibitor, suggesting that constitutive Src activity may be responsible for the basal activation of Ras.

In summary, this study describes a novel mechanism for metal-induced EGFR transactivation, which is likely to be mediated by Src through the phosphorylation site of Tyr-845 on EGFR.

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