Mechanisms of Zn\(^{2+}\)-induced signal initiation through the epidermal growth factor receptor

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Abstract

Zn\(^{2+}\) is a ubiquitous ambient air contaminant that is found as a constituent of airborne particulate matter (PM). Previous studies have associated Zn\(^{2+}\) levels in PM with health effects in exposed populations and have shown proinflammatory properties of Zn\(^{2+}\) exposure in vivo and in vitro. In the present study, we studied the mechanisms of epidermal growth factor receptor (EGFR) dimerization, phosphorylation, and kinase activity in A431 cells treated with Zn\(^{2+}\). EGF, but not Zn\(^{2+}\), induced dimerization of EGFR in A431 cells and membrane extracts. Like EGF, Zn\(^{2+}\) induced phosphorylation of EGFR at tyrosines 845, 1068, and 1173. However, unlike EGF, Zn\(^{2+}\) failed to induce detectable dimerization of EGFR. The EGFR kinase inhibitor PD153035 ablated all phosphorylation induced by EGF but none caused by Zn\(^{2+}\). PD153035 abolished EGF-induced phosphorylation of the EGFR substrate Cbl, but had no effect on levels of phospho-Cbl caused by Zn\(^{2+}\). Inhibition of EGFR kinase activity did, however, blunt Zn\(^{2+}\)-induced phosphorylation of ERK. Exposure to Zn\(^{2+}\), but not EGF, induced phosphorylation of the activating site of c-Src (tyrosine 416), and Zn\(^{2+}\)-induced phosphorylation of EGFR at tyrosines 845 and 1068 was blocked by the c-Src kinase activity inhibitor PP2. In summary, Zn\(^{2+}\) ions induce EGFR phosphorylation in a manner dependent on c-Src but not on EGFR dimerization or EGFR kinase activation, suggesting that Zn\(^{2+}\) induces EGFR transactivation by c-Src.

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Introduction

Numerous studies have associated exposure to ambient particulate matter (PM) and elevated incidence of morbidity and mortality. PM collected in varying locations contains a number of metallic compounds (Claiborn et al., 2002; Ki et al., 2002; Magari et al., 2002; Molinelli et al., 2002) that possess various toxicological properties when inhaled (Nemery, 1990). Zn\(^{2+}\) is one of the most pervasive soluble metals found associated with ambient PM (Chang et al., 2000; Claiborn et al., 2002), and recent studies have associated levels of ambient Zn\(^{2+}\) with adverse health effects (Adamson et al., 2000; Claiborn et al., 2002; Kodavanti et al., 2002).

Zn\(^{2+}\) is an essential micronutrient whose concentration in animal tissues is second only to that of iron. Interestingly, concentrations of Zn\(^{2+}\) can be as high as 300 μM in certain tissues, suggesting a physiological role for this cation in processes such as synaptic transmission, as well as a mediator of tissue injury (Chen et al., 1997; Huang, 1997). The catalytic activity of hundreds of enzymes of every type is dependent on the participation of Zn\(^{2+}\) (Falchuk, 1993; Vallee and Falchuk, 1993). The ability of Zn\(^{2+}\) to undertake multiple coordinate geometries is exploited in zinc finger proteins that interact with other proteins, nucleic acids, and enzymes.

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lipids (Vallee and Auld, 1990). Recently, a regulatory role of Zn$^{2+}$ in cell signaling has also been recognized (Maret, 2001). Hershfinkel and colleagues (2001) have reported the existence of a Zn$^{2+}$-sensing receptor that triggers calcium-dependent signaling. Evidence that Zn$^{2+}$ acts extracellularly to induce this effect, combined with the ability of Zn$^{2+}$ to organize protein domains in a multidentate manner (Vallee and Auld, 1990), raises mechanistic questions concerning the mode of interaction of the Zn$^{2+}$ ligand with its putative receptor.

The family of epidermal growth factor receptor (EGFR) tyrosine kinases is comprised of four members, ErbB1 (commonly and herein referred to as EGFR), ErbB2, ErbB3, and ErbB4 (Wells, 1999). The prototype EGFR is a 170-kDa glycoprotein that is widely expressed in mammalian cells. It consists of ligand binding, transmembrane, and tyrosine kinase domains, in addition to a carboxy terminal span that contains tyrosine residues that undergo auto- and transphosphorylation during receptor activation. Activation of EGFR by its cognate ligand EGF begins with EGF binding to the extracellular domain, which promotes the formation of a homodimer consisting of two EGFR molecules or a heterodimer that includes other ErB family members (Earp et al., 1995). Dimerization results in activation of the intrinsic tyrosine kinase activity and leads to cross-phosphorylation at the autophosphorylation sites of the receptor molecules. There are at least five tyrosines that serve as sites of autophosphorylation on EGFR, i.e., three major sites, tyrosines 1068, 1173, and 1148, and two minor sites, tyrosines 992 and 1086 (Hackel et al., 1999). Additional tyrosines have been identified as sites of transphosphorylation by other kinases (Biscardi et al., 1999). The display of phosphotyrosines at these sites promotes SH2-mediated binding of the receptor by a variety of proteins, resulting in downstream signaling (Bogdan and Klambt, 2001; Wells, 1999).

We have shown that exposure to Zn$^{2+}$ ions causes EGFR phosphorylation and induces EGFR-dependent signaling, including Ras and MAPK activation, in lung cells (Wu et al., 1999). However, the mechanism through which Zn$^{2+}$ ions induce the activation of the EGFR is not known. One possibility examined in this study is that Zn$^{2+}$ ions can activate the EGFR by causing the dimerization or complexation of two or more EGFR monomers. Such a mechanism could involve oxidative cross-linking of cysteiny1 sulfhydryls, resulting in the formation of a disulfide linkage between neighboring EGFR molecules. In addition, Zn$^{2+}$-induced complexation of EGFR monomers could theoretically occur through an indirect mechanism, such as the destabilization of cysteiny1 sulfhydryls or the formation of a stabilizing Zn$^{2+}$ bridge between two EGFR monomers (Falchuk, 1993; May and Contoreggi, 1982; Vallee and Falchuk, 1993).

It is also possible that Zn$^{2+}$-induced EGFR activation occurs in the absence of dimerization, conceivably through activation of the tyrosine kinase domain by Zn$^{2+}$ or by increased phosphorylation of critical tyrosine residues on the EGFR molecule by transactivating kinases. Such an effect could be achieved through Zn$^{2+}$-induced inhibition of tyrosine phosphatases that oppose EGFR phosphorylation, such as PTP1B (Samet et al., 1999). Biscardi et al. (1999) have reported convincing evidence demonstrating that c-Src can induce the phosphorylation of EGFR at tyrosine 845 (Biscardi et al., 1999). This supports the possibility that EGFR signaling in response to Zn$^{2+}$ exposure occurs without a change in the intrinsic kinase activity in the EGFR.

To gain a better understanding of the mechanism of Zn$^{2+}$-induced EGFR signaling, in the present study we have dissected the effect of Zn$^{2+}$ exposure on EGFR activation into its three subcomponent events, dimerization, phosphorylation, and kinase activation. For comparison purposes, we also examined the effects of V$^{4+}$ and As$^{5+}$. We report that exposure to Zn$^{2+}$ ions results in phosphorylation of the EGFR molecule at multiple sites, in a manner that is independent of EGFR kinase activation and does not involve EGFR dimerization but involves transactivation by c-Src.

Methods

Materials. Human epidermoid A431 cells were obtained from the Lineberger Cancer Research Center Tissue Culture Facility at the University of North Carolina (Chapel Hill, NC). Phosphate-buffered saline, tissue culture media, and reagents were purchased from GibcoBRL (Gaithersburg, MD); bis[sulfosuccinimidyl]suberate (BS$^3$) was obtained from Pierce (Rockford, IL), protease inhibitors, PD153035, PP2, and EGF were purchased from Calbiochem (San Diego, CA); detergents, metals salts, and common laboratory reagents were from Sigma Chemical Co. (St. Louis, MO). Luminescence reagents were obtained from Amersham (Boston, MA). Tissue culture flasks were purchased from Falcon (Fisher Scientific, Raleigh, NC). Polyacrylamide was obtained from Roche (Indianapolis, IN). Electrophoresis supplies were from BioRad (Richmond, CA). Pan-specific and phosphorylation-state-specific antibodies directed against EGFR phosphorylated at tyrosines 1068, 1173, or 845, ERK2, ERK1/2 phosphorylated at threonine 202, and tyrosine 204, c-Src, and c-Src phosphorylated at tyrosine 416 were purchased from Cell Signaling Technology (Beverly, MA).

Analysis of EGFR dimerization in intact cells. EGFR dimers were measured by using a modification of a previously described method (Canals, 1992). A431 cells were cultured on plastic flasks in Dulbecco’s minimum essential medium (DMEM) with high glucose supplemented with 10% fetal bovine serum and gentamicin (5 $\mu$g/ml). For stimulation of intact cells with metals or EGF, subconfluent cells grown in T75 flasks were deprived of serum for 12–18 h in 8 ml of DMEM. Stimuli were then added in 2 ml of DMEM containing 5× the desired final concentration of
NaAsO₃, VOSO₄, ZnSO₄, or EGF. In some experiments, cells were pretreated with 1 μM PD153035 for 90 min prior to stimulation. Cells were then immediately washed with ice-cold phosphate-buffered saline (PBS) and treated with 1 ml of 2.5 mM BS³ in PBS for 30 min at room temperature. The cross-linking reaction was stopped by incubating with PBS containing 20 mM Tris, pH 7.5, for 15 min, and the cells were scraped into 1 ml of PBS and centrifuged at 1000 × g for 5 min at 4°C. Protein extracts were then prepared as described below (cell pellets resuspended in 50 μl for lysis) and subjected to Western blotting using a mouse anti-human-EGFR antibody cocktail that recognizes the extracellular domain of the EGFR (antibody 14) (Neomarkers, Fremont, CA).

Assay of dimerization in cell extracts. A431 extracts were prepared following a previously published procedure (Tanner and Kyte, 1999). Confluent A431 cells totaling 3000 cm² in area were harvested by scraping into ice-cold PBS and pelleting at 1000 × g for 25 min at 4°C. The cell pellet was then taken up in an equal volume (~500 μl) of 20 mM HEPES containing 1 mM dithothreitol (DTT), 1 mM EGTA, and a protease inhibitor cocktail mix [final concentrations: 1 mM AEBSF, 800 nM aprotonin, 50 nM bestatin, 15 μM E-64, 20 μM leupeptin hemisulfate, and 10 μM pepstatin A]. The cells were then lysed by the addition of an equal volume of 2% Triton in 20 mM HEPES and shearing in a Dounce homogenizer or by passing through a 21-gauge syringe needle. The resulting detergent was clarified by centrifuging at 100K × g for 30 min at 4°C in a Beckman TL-100 benchtop ultracentrifuge (Fisher, Raleigh, NC). Samples were then either mixed with 1 volume of 2× Laemmli loading buffer for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) or stored at −80°C for subsequent analyses.

The assay for dimerization of EGFR in A431 extracts was carried out by using 45 μl of protein extract (representing 75 cm² of A431 cells) to which a final concentration of 200 ng/ml of EGF or 500 μM metal was added in a total reaction volume of 50 μl. BS³ was prepared immediately before use in dimethyl sulfoxide (DMSO) and added to a final concentration of 2.5 mM for 30 min at room temperature. The reaction was stopped with 50 mM glycine (final) and prepared for SDS-PAGE by mixing with sample loading buffer without heating.

Western blotting. In some experiments, cells were pretreated with 1 μM PD153035 for 60–90 min, or with 5 μM PP2 for 30–60 min prior to stimulation. After normalization for protein content (50–100 μg/lane), cell lysates or extracts were subjected to SDS-PAGE (Laemmli, 1970) on 15 × 15-cm 6% polyacrylamide gels, electroblotted onto nitrocellulose, and then blocked with 3% casein in Tris-buffered saline, pH 8.0. Detection was accomplished using horseradish peroxidase-conjugated secondary antibodies and chemiluminescence reagents on film. In some cases, blots were stripped and rebotted using a commercially available stripping reagent (Chemicon International, Temecula, CA). Blots were digitized using a Kodak EDAS 120 System (Rochester, NY). Western blotting results shown are representative of three or more experiments.

Results

Zn²⁺ ions do not induce EGFR dimerization in detergent extracts of A431 cells

We first investigated the possibility that exposure to metal ions such as Zn²⁺, V⁴⁺, or As⁵⁺ can cause dimerization of the EGFR receptor through a cross-linking mechanism in a cell-free system. Detergent extracts of A431 cells were treated with 500 μM Zn²⁺, V⁴⁺, or As⁵⁺, or 200 ng/ml EGF for 20 min at room temperature and immediately exposed to the cross-linking reagent BS³ or vehicle alone. After quenching of the BS³ with glycine, the reaction mixture was subjected to SDS-PAGE followed by Western blotting detection using anti-EGFR antibodies. As shown in Fig. 1, EGF caused the dimerization of EGFR dimers, as detected by the formation of a high molecular weight (>200 kDa) immunoreactive band. In contrast, there was no evidence of dimer formation following exposure of A431 detergent extracts to Zn²⁺, V⁴⁺, or As⁵⁺ either in the presence or absence of BS³ treatment. These results suggested that heavy metal ions, including Zn²⁺, do not cause the formation of EGFR dimers through a direct cross-linking mechanism.

Zn²⁺ ions do not induce the formation of EGFR dimers in intact A431 cells

We next determined whether exposure to Zn²⁺ ions can induce EGFR dimerization indirectly by initiating a signaling mechanism in intact cells. A431 cell cultures were
incubated with 500 μM ZnSO₄ or 200 ng/ml EGF for 1–20 min. As seen in the detergent extracts, EGF treatment was effective in inducing EGFR dimers in intact A431 cells. EGF-induced dimer formation was rapid and persistent, reaching an apparent maximum in intensity by 1 min and remaining detectable for at least 20 min (Fig. 2). However, Zn²⁺ exposure did not induce detectable dimerization in whole A431 cell cultures. This finding expanded the results obtained using detergent extracts of these cells by establishing that Zn²⁺ ions do not induce dimerization of the EGFR either directly or indirectly.

**Zn²⁺ ions induce phosphorylation of EGFR at multiple sites in A431 cells**

We reported previously that exposure of human airway epithelial cells to Zn²⁺ ions results in EGFR phosphorylation (Wu et al., 1999). We therefore determined whether Zn²⁺-induced phosphorylation of the EGFR in A431 cells occurs in the absence of evidence of EGFR dimerization. Western blotting using phosphorylation-state specific antibodies showed that, relative to untreated (control) A431 cells, treatment with 500 μM Zn²⁺ ion for 20 min induces marked phosphorylation of EGFR at the known autophosphorylation sites Tyr1068 and Tyr1173, as well as the transphosphorylation site Tyr845 in A431 cells (Fig. 3). In subsequent experiments, phosphorylation at Tyr1068 was used as an indicator of EGFR autophosphorylation while Tyr845 was monitored as a potential site of EGFR transphosphorylation. As expected, the positive control, EGF, also induced a pronounced phosphorylation of the EGFR at all three Tyr sites. However, exposure to As³⁺ or V⁴⁺ ions had no effect on Tyr phosphorylation at the Tyr sites examined (Fig. 3). Virtually the same results were obtained with A431 cells exposed to 500 μM Zn²⁺, As³⁺ or V⁴⁺ ions or EGF for 5 min (data not shown).

**Inhibition of EGFR kinase activity does not block Zn-induced phosphorylation of EGFR**

The data shown above indicating Zn²⁺-induced phosphorylation of EGFR in the absence of dimerization suggested that the intrinsic kinase activity in the EGFR may not be involved in Zn²⁺-induced signaling. To test this possibility, we pretreated A431 cells with the selective EGFR kinase inhibitor PD153035 (1 μM for 90 min) prior to stimulation with 500 μM metal ions or 200 ng/ml EGF for 20 min. Western blotting demonstrated that, consistent with a complete blockade of EGFR kinase activity, PD153035 effectively abolished the pronounced EGFR phosphorylation induced by EGF treatment at Tyr1068 and Tyr845 (Fig. 4). In striking contrast, this effective inhibition of EGFR kinase activity had no discernible effect on the phosphorylation of Tyr1068 or TyrY845 in A431 cells treated with Zn²⁺ ions (Fig. 4). As seen earlier, As³⁺ or V⁴⁺ exposure did not induce EGFR phosphorylation at either Tyr position. These results indicated that Zn²⁺-induced phosphorylation of EGFR is independent of EGFR kinase activity.

**Zn²⁺-induced phosphorylation of Cbl is independent of EGFR kinase activity**

To confirm that the activation of EGFR induced by Zn²⁺ does not require its intrinsic kinase activity and to assess whether Zn²⁺-induced EGFR phosphorylation results in functional downstream signaling in A431 cells, we exam-
As shown in Fig. 5, treatment of A431 cells with 500 μM Zn$^{2+}$ or 200 ng/ml EGF for 20 min caused increased levels of phosphorylated Cbl as detected by Western blotting using a phospho-specific Cbl antibody. Exposure to As$^{5+}$ also caused clear increases in P-Cbl, while the effect of V$^4+$ was minimal at this time point (Fig. 5). Inhibition of EGFR kinase activity with PD153035 suppressed basal levels and ablated increases in P-Cbl caused by treatment with As$^{5+}$, V$^4+$, or EGF. However, pretreatment with the EGFR kinase inhibitor did not have any effect on Zn-induced phosphorylation of Cbl in A431 cells (Fig. 5).

Zn$^{2+}$-induced EGFR phosphorylation is dependent on c-Src activity

The data presented above suggested that Zn$^{2+}$-induced EGFR phosphorylation is initiated through transactivation of EGFR. The nonreceptor tyrosine kinase c-Src has been reported to phosphorylate EGFR at tyrosine 845 (Biscardi et al., 1999). We therefore examined the effect of the c-Src kinase inhibitor PP2 on EGFR phosphorylation in A431 cells exposed to Zn$^{2+}$ or EGF. As seen earlier, treatment with 500 μM Zn$^{2+}$ or 200 ng/ml EGF for 20 min caused marked phosphorylation of EGFR at Tyr845 as well as Tyr1068 (Fig. 6). Pretreatment with PP2 blocked Zn$^{2+}$-induced phosphorylation of EGFR at Tyr845 and Tyr1068 (Fig. 6). PP2 also had an inhibitory, albeit lesser, effect on EGF phosphorylation in A431 cells stimulated with EGF.

To determine the effect of Zn$^{2+}$ or EGF treatment on c-Src activation in A431 cells, we next measured levels of activated c-Src in A431 cells. Western blotting using a phospho-specific antibody against Tyr416-c-Src showed that 500 μM Zn$^{2+}$ treatment for 20 min resulted in a marked increase in active c-Src kinase, whereas 200 ng/ml EGF had no discernible effect (Fig. 7). Pretreatment with PP2 also inhibited Zn$^{2+}$-induced phosphorylation of Tyr416 c-Src (Fig. 7).

Zn$^{2+}$ induces phosphorylation of ERK1/2 through an EGFR kinase-dependent mechanism

To evaluate the effect of Zn$^{2+}$ exposure on signaling downstream of the EGFR, levels of phospho-ERK1/2 were detected in A431 cells treated with 500 μM Zn$^{2+}$ for 20 min. As shown in Fig. 8, Zn$^{2+}$ treatment increased levels of the phosphorylated forms of ERKs 1 and 2, with the magnitude of this effect being comparable to that induced by 100 ng/ml EGF. Pretreatment of the cells with the EGFR kinase inhibitor PD153035 reduced the intensity of the ERK1/2 phosphorylation induced by both Zn$^{2+}$ and EGF. In contrast, inhibition of c-Src with PP2 did not affect Zn$^{2+}$- or EGF-induced ERK1/2 phosphorylation (Fig. 8).
Discussion

We previously reported that exposure to PM metal ions such as Zn\textsuperscript{2+} causes marked activation of EGFR phosphorylation and EGFR-mediated signaling in airway epithelial cells (Wu et al., 2002b). The present study was undertaken to distinguish between possible mechanisms through which Zn\textsuperscript{2+} ions might cause the activation of the well-characterized growth factor receptor EGFR. The data we present herein show that Zn\textsuperscript{2+}-induced phosphorylation of EGFR occurs at multiple tyrosine sites, in the absence of EGFR dimerization and without the involvement of EGFR kinase activity, and that, unlike EGFR-induced activation, Zn\textsuperscript{2+}-induced EGFR activation, involves c-Src kinase activation.

The regulatory role of Zn\textsuperscript{2+} ions in the activation of cell signaling has received increased scrutiny following the recent report of a putative extracellular Zn\textsuperscript{2+} receptor that controls intracellular Ca\textsuperscript{2+} mobilization (Hershfield et al., 2001). Unlike other transition metal ions (e.g., Cu\textsuperscript{2+}, V\textsuperscript{4+}, and Fe\textsuperscript{2+}), Zn\textsuperscript{2+} is not redox active and is, therefore, incapable of directly generating reactive oxygen species through a Fenton mechanism. However, treatment with Zn\textsuperscript{2+} has been reported to induce H\textsubscript{2}O\textsubscript{2} production in adipocytes by catalyzing sulhydryl autoxidation of extracellular proteins (May and Contoreggi, 1982). The possibility that Zn\textsuperscript{2+} ions could induce an oxidative cross-linking of sulphydryls neighboring EGFR monomeric molecules led us to investigate and determine that Zn\textsuperscript{2+} does not cause EGFR dimerization in A431 cells. However, in spite of our finding that Zn\textsuperscript{2+} exposure does not result in EGFR dimer formation, it is not possible to rule out other oxidant-dependent mechanisms in Zn-mediated EGFR activation such inactivation of tyrosine phosphatase activity. As such, inhibition of tyrosine phosphatases is a relevant biological property of Zn\textsuperscript{2+} in the activation of EGFR signaling. We have previously shown that exposure to Zn\textsuperscript{2+} ions inhibits tyrosine phosphatases in human lung cells (Samet et al., 1999), including PTP1B, a tyrosine phosphatase that may be associated with the dephosphorylation of EGFR itself (Liu and Chernoff, 1997). Thus, it is possible that Zn\textsuperscript{2+}-induced phosphorylation of EGFR is secondary to the inhibition of a tyrosine phosphatase that opposes the activity of EGFR or a transactivating kinase that phosphorylates EGFR. However, the involvement of such a mechanism would have to be reconciled with the fact that neither V\textsuperscript{4+}, the defining tyrosine phosphatase inhibitor (Fauman and Saper, 1996), nor As\textsuperscript{3+}, a putative selective phosphatase inhibitor (Cavigelli et al., 1996), exerted observable EGFR phosphorylation in A431 cells.

The lack of specificity in the site of phosphorylation of EGFR in Zn\textsuperscript{2+}-exposed A431 cells also leaves open the possibility of a signaling mechanism that also involves other EGFR family members, such as ErbB2. ErbB2, a preferred partner of EGFR (ErbB1) heterodimerization, has a very active kinase domain and is considerably less susceptible to inhibition by PD153035 (Bos et al., 1997). While it is possible that the methodology is not sufficiently sensitive to detect them, the assay used in this study would be expected to detect the formation of not only EGFR homodimers but also EGFR-ErbB2 heterodimers, yet no dimers were observed in our measurements in response to Zn\textsuperscript{2+} treatment of A431 cells.

Regarding the need for EGFR kinase activity in Zn\textsuperscript{2+}-induced signaling, the data presented in this study are predominantly consistent with a mechanism of Zn-induced EGFR activation that does not involve activation of the EGFR kinase domain. The evidence for this is as follows: (1) Although Zn\textsuperscript{2+} induces phosphorylation of EGFR at established autophosphorylation sites, it also causes phosphorylation at a known transphosphorylation site, Tyr845, which supports the involvement of another kinase(s). (2) Activation of the EGFR kinase domain is believed to be an event that is preceded by homo- or heterodimerization of EGFR congeners, yet dimerization was not observed in either intact cells or membrane extracts in response to Zn\textsuperscript{2+} treatment. (3) The phosphorylations of EGFR induced by EGF were completely ablated by the demonstrated inhibition of EGFR kinase activity with PD153035, but this potent EGFR kinase inhibitor had no effect on any of the EGFR phosphorylations induced by Zn\textsuperscript{2+}. (4) Although PD153035 effectively prevented EGF-induced phosphorylation of Cbl, a known EGFR substrate, it had no effect on Cbl phosphorylation caused by Zn\textsuperscript{2+} exposure. And (5) EGFR phosphorylations induced by Zn\textsuperscript{2+}, but not those induced by EGF, were accompanied by activating phosphorylation of c-Src and could be inhibited by a c-Src kinase activity inhibitor. However, that inhibiting of EGFR kinase activity with PD153035 blunted Zn\textsuperscript{2+}-induced ERK1/2 phosphorylation, while inhibition of c-Src activity with PP2 did not have an effect, would support the activation of multiple EGFR-dependent signaling pathways in A431 cells treated with Zn\textsuperscript{2+}.

Our finding that c-Src is activated by Zn\textsuperscript{2+} exposure of A431 cells and that its activity is necessary for Zn\textsuperscript{2+}-
induced EGFR phosphorylation is consistent with a previous report by Biscardi et al. (1999), which showed that EGFR Tyr845 is a substrate of c-Src kinase. In addition, our own recent work has demonstrated that c-Src is a transactivating kinase of EGFR Tyr845 in Zn²⁺-stimulated B82 fibroblasts (Wu et al., 2002a). In the present study we find that inhibition of c-Src activity blocks EGFR phosphorylation not only on Tyr845 but on the autophosphorylation site Tyr1068 as well. This result likely reflects differences in mechanisms regulating EGFR signaling among different cell types and suggests that, at least in A431 cells, there is a broader role for c-Src in EGFR transactivation induced by Zn²⁺.

C-Src has been previously reported to phosphorylate Cbl (Yokouchi et al., 2001). Therefore, that inhibition of EGFR kinase activity failed to block Zn²⁺-induced Cbl phosphorylation in our study suggests that it is possible that phosphorylation of Cbl is carried out by c-Src or through a tyrosine kinase in our study. This result not only on Tyr845 but on the autophosphorylation site Tyr1068, as well as the kinetics of Cbl phosphorylation, suggests that it is possible that phosphorylation of Cbl is carried out by c-Src or through a tyrosine kinase. The implication of this study is that metallic environmental contaminants such as Zn²⁺ can elicit cellular effects through a corruption of physiological signaling networks in a surprisingly speci

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