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Mercuric Chloride Mediates a Protein Sulfhydryl Modification-Based Pathway of Signal Transduction for Activating Src Kinase Which Is Independent of the Phosphorylation/Dephosphorylation of a Carboxyl Terminal Tyrosine

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Abstract Little is known about the regulatory mechanism of c-Src kinase in cells except the suggested regulation through phosphorylation and dephosphorylation of its carboxyl terminal tyrosine residue (Y527). We here demonstrated that exposure of NIH3T3 cells to mercuric chloride ($HgCl_2$) induces both aggregation and activation of Src kinase protein through a redox-linked mechanism. The aggregation of Src proteins was suggested to be induced by the sulfhydryl groups-to-Hg^{2+} reaction-mediated polymerization of cell membrane proteins to which the Src proteins associate noncovalently. The possibility was ruled out that the aggregation occurred secondarily to the promotion of protein tyrosine phosphorylation. Further study revealed that the Src kinase was activated by $HgCl_2$ at least in part independent of the known Csk kinase-linked or Y527-phosphorylation/dephosphorylation-mediated control. Correspondingly, CNBr cleavage mapping of phosphopeptides for autophosphorylated c-Src protein demonstrated selective promotion of phosphorylation at Y416 in $HgCl_2$-treated cells without obvious change in the phosphorylation level at Y527. These results suggest a unique protein sulfhydryl modification-based pathway of signal transduction for activating Src kinase in NIH3T3 cells.

Key words: Src kinase, mercuric chloride, redox, sulfhydryl group, receptor polymerization

Nonreceptor protein tyrosine kinase (PTK) $p60^{c\text{-}src}$ (Src) is widely distributed in cells of various sources and is potentially involved in the signal delivery for controlling cellular growth and function [Cooper and Howell, 1993; Cantley et al., 1991; Chackalaparampil and Shalloway, 1988]. The catalytic activity of c-Src kinase has been suggested to be downregulated by phosphorylation of its carboxyl-terminal tyrosine (Y527) [Cooper et al., 1986; Kmiecik and Shalloway, 1987; Piwnica-Worms et al., 1987] by another PTK named Csk [Okada and Nakagawa, 1989; Nada et al., 1991; Thomas et al., 1991], dephosphorylation of which by phosphotyrosine phosphatase (PTPase) upregulates it [Cooper and Howell, 1993; Cooper and King, 1986]. Correspondingly, v-Src kinase, a Y527-defective mutant of c-Src, is constitutively activated, thereby working as an oncogene for unlimited cell growth [Cantley et al., 1991; Kmiecik and Shalloway, 1987; Cooper and King, 1986]. $p56^{lck}$ (Lck), another PTK of the Src family, is known to associate with transmembrane CD4/CD8 molecules in T lymphocytes, through which a signal is transmitted for regulating the kinase activity [Weiss, 1993; Anderson et al., 1994]. Such a transmembrane regulatory mechanism has not been clearly identified for c-Src kinase. However, PTKs of the Src family seem to share a common pathway of regulation through glycosylphosphatidylinositol (GPI)-anchored cell membrane proteins

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; DOC, sodium deoxycholate; DTT, dithiothreitol; FCS, fetal calf serum; GPI, glycosylphosphatidylinositol; PAGE, polyacrylamide gel electrophoresis; PTK, protein tyrosine kinase; PTPase, phosphotyrosine phosphatase; P-tyr, phosphotyrosine; SDS, sodium dodecyl sulfate; 2ME, 2-mercaptoethanol.

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which are localized in a pariticular noninoic, detergent-resistant compartment of the plasma membrane [Stefanova et al., 1991; Brown and Rose, 1992]. The signal primarily initiated at the external cell surface could thus be transmitted intracellularly through this or another pathway for regulating the Src activity.

Recent studies have demonstrated that oxidative stresses from the environment, which are provided by oxidants and ultraviolet, activate the Src family kinases [Devary et al., 1992; Nakamura et al., 1993; Hardwick and Sefton, 1995]. The molecular mechanism of this pathway of kinase activation remains unknown, but sulfhydryl modification of signal transducing elements through changes in cellular redox potential is suggested to play a key role. Mercuric ions (Hg^{2+}) are known to carry a unique property to bind sulfhydryl groups with an exceptionally high association constant, preferentially forming a stable S-Hg-S bridge which mimics the physiologically occurring S-S bond in oxidative cellular environment [Stricks and Kolthoff, 1953; Simpson, 1961; Sperling et al., 1969; Steer et al., 1974; Utschig et al., 1995]. Previously, we showed that exposure of T lymphocytes to $HgCl_2$ induced extensive phosphorylation of a number of cellular proteins at tyrosine residues and activation of Lck [Rahman et al., 1993; Nakashima et al., 1994; Katano et al., 1995]. By immunofluorescence microscopic analysis we found that $HgCl_2$ caused aggregation of multiple cell surface proteins including transmembrane CD3, CD4, and CD45 and GPI-anchored Thy-1, which accompanied intracellular aggregation of Lck and phosphotyrosine (P-tyr)-containing cellular proteins [Nakashima et al., 1994]. Further experiments showed that the $HgCl_2$-mediated signal transduction was partially regulated by the phosphatidylinositol glycan class A (PIG-A) gene which is essential for GPI anchor biosynthesis [Pu et al., 1995]. This evidenced that external cell surface proteins including GPI-anchored proteins are involved in the signal pathway for activation of Lck by $HgCl_2$, possibly as the primary target molecules of the sulfhydryl-reactive Hg^{2+} for polymerization. Here we further characterize this pathway of signal transduction in the model of Src kinase for which cell lines expressing normal (c-Src) and mutated (v-Src) kinase proteins at different levels plus or minus Csk kinase are available. The results demonstrate the Hg^{2+}-mediated activation of Src kinases which is closely linked to aggregation of the Src proteins. In this model, we provide evidence that the demonstrated aggregation-linked activation of Src kinase progresses for selective Y416 phosphorylation at least in part independent of the Csk- and Y527 phosphorylation/dephosphorylation-mediated regulation.

MATERIALS AND METHODS

Cells and Reagents

The NIH3T3 mouse fibroblast cell line, in which c-Src kinase was overexpressed, was kindly provided by Dr. D. Shalloway (Pennsylvania State University, University Park, PA). v-Src–expressing NIH3T3 cells were from our own stock. Wild-type (T24E, Csk^+) and mutant (T29E, Csk^-) embryonic cell lines were kindly provided by Dr. P. Soriano (Fred Hutchinson Cancer Research Center, Washington, DC). T29E was originally established by Imamoto and Soriano [1993] from mouse embryo in which the Csk gene had been disrupted. These cell lines were maintained in plastic plates with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) at 37°C in a 5% CO_2 incubator. For experimental use, cells were resuspended with DMEM containing 10% FCS in plastic plates for a further 20–24 h incubation. Before use, the cells were rinsed in fresh modified Eagle's medium (MEM) twice and incubated in MEM at 37°C for 15–30 min. The chemicals, such as $HgCl_2$, dithiothreitol (DTT), and staurosporine, were from Sigma (St. Louis, MO).

Immunoprecipitation

$HgCl_2$-treated and untreated control cells were washed twice with cold phosphate buffered saline, pH 7.2, and then lysed in lysis buffer (10 mM Tris-HCl, pH 8.0, 0.1% Triton X-100, 150 mM Tris-HCl, 1 mM EDTA, 0.5 mM Na_3VO_4). By centrifugation at 15,000 rpm for 30 min, we obtained supernatant (sup) and pellet (pel) fractions of the lysate, the former of which should normally contain cytosolic and plasma membrane proteins including Src. The pellet fraction from the $HgCl_2$-treated cells, which contained the Src aggregate, was resuspended in the lysis buffer added with 10% 2-mercaptoethanol (2ME) and kept on ice for 15 min with several occasional vortexes. The solubilized fraction was obtained by centrifugation at 5,000 rpm for 15 min. From both the supernatant fraction and the 2ME-solubilized pellet fraction, Src proteins were immunoprecipitated as previously described

[Hamaguchi and Hanafusa, 1987]. Briefly, samples were incubated with anti-Src monoclonal antibody (mAb327) (donated by Dr. J.S. Brugge, ARIAD Pharmaceuticals Inc., Cambridge, MA) [Lipsich et al., 1983] for 2 h at 4°C, followed by another 1 h incubation with immobilized protein A on Sepharose beads (Pierce, Rockford, IL) which had been conjugated with rabbit antimouse IgG antibody (MBL, Nagoya, Japan). The beads bearing immunoprecipitated c-Src were washed three times with lysis buffer for either immunoblot analysis or in vitro kinase assay.

Immunoblot Analysis

Fractions of cell lysates in lysis buffer with or without ionic detergents (0.1% sodium dodecyl sulfate (SDS) and 1% sodium deoxycholate [DOC]) were obtained by centrifugation as above. Whole cells and fractions of the cell lysates were boiled for 30 min in sample buffer (2×; 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerin) with or without 5% (v/v) 2ME. They were loaded on SDS/10% polyacrylamide gels as described [Hamaguchi et al., 1988]. The proteins separated by this SDS-polyacrylamide gel electrophoresis (PAGE) were electrophoretically transferred to polyvinylidene difluoride microporous membrane (Millipore, Bedford, MA), which was incubated in blocking buffer (3% ovalbumin in washing buffer containing 100 mM Tris, 0.5% Tween 20, 9% NaCl, 0.2% NaN_3) overnight at 4°C. Blocked membrane was probed with anti-P-tyr polyclonal rabbit antibody [Hamaguchi et al., 1988] or with mAb327 specific to Src protein followed by treatment with rabbit antimouse IgG antibody. They were then labeled with ^{125}I-protein A (ICN, Irvine, CA) and exposed to Fuji X-ray film at −80°C. The P-tyr specificity of the protein bands developed by use of the anti-P-tyr antibody was repeatedly confirmed [Hamaguchi et al., 1988; Nakashima et al., 1991]. The molecular sizes of the stained proteins were estimated by comparison with protein molecular mass (Mr) standards (GIBCO, Gaithersburg, MD).

In Vitro Kinase Assay

In vitro kinase assay was performed as previously described [Hamaguchi et al., 1993]. Briefly, the immunoprecipitated Src proteins were washed three times with kinase buffer (10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$) and suspended in the kinase buffer with 1.5 μg acid-denatured enolase (Sigma) as an exogeneous substrate and radiolabeled [γ-^{32}P]ATP (370 kBq) (NEN, Wilmington, DE). This mixture was incubated at 30°C for 20 min. The kinase reaction was terminated by adding SDS sample buffer with 2ME. The immunoprecipitates were then boiled for 3 min and subjected to SDS-PAGE as described above. Gels were dried and exposed to Fuji X-ray film.

In Vivo Radiolabeling and Phosphopeptide Mapping of Src Protein

NIH3T3 cells were labeled in vivo with phosphorous-32 (H_3PO_4, 10 mCi/ml; NEN) in phosphorous-free medium with 2% FCS at a final concentration of 1 mCi/ml for 16 h basically as described previously [Jove et al., 1987; Thomas et al., 1991]. Phosphorous-32–containing medium was discarded, and cells were washed three times with MEM.

For phosphopeptide analysis, the in vivo phosphorous-32–labeled cells were lysed, and Src proteins were immunoprecipitated. The collected Src proteins were subjected to SDS/10%-PAGE. The band for c-Src was excised from the dried gel and digested with 50 mg/ml cyanogen bromide (CNBr) (Sigma) in 70% formic acid at room temperature for 1 h. Products were washed with distilled water and lyophilized at −60°C until the pH became neutral. CNBr cleavage products were finally separated on a 24% acrylamide–0.054% bisacrylamide gel [Schuh and Brugge, 1988; Imamoto and Soriano, 1993]. Gels were dried and exposed to film as described above. Molecular sizes of cleaved peptides were determined by comparing with protein Mr standards (GIBCO).

RESULTS

$HgCl_2$ Induces Aggregation of Src Proteins in NIH3T3 Cells

NIH3T3 cells overexpressing c-Src were briefly exposed to 0.05–0.5 mM $HgCl_2$. These cells were lysed in lysis buffer containing nonionic Triton X-100 but not ionic SDS/DOC and separated into supernatant and pellet fractions by centrifugation for demonstration of the Src protein by SDS-PAGE followed by immunoblot. As shown in Figure 1A, 0.5 mM $HgCl_2$ induced heavy aggregation of Src proteins (lower panel, lane 3), whereas the action of 0.05 mM $HgCl_2$ for this effect was marginal. The aggregation of Src proteins was prevented by adding DTT before $HgCl_2$ (Fig. 1B, lane 4 compared with lane 2 as positive control). This suggested that reaction of Hg^{2+} to sulfhydryl groups underlies the mechanism of the Src aggregation.

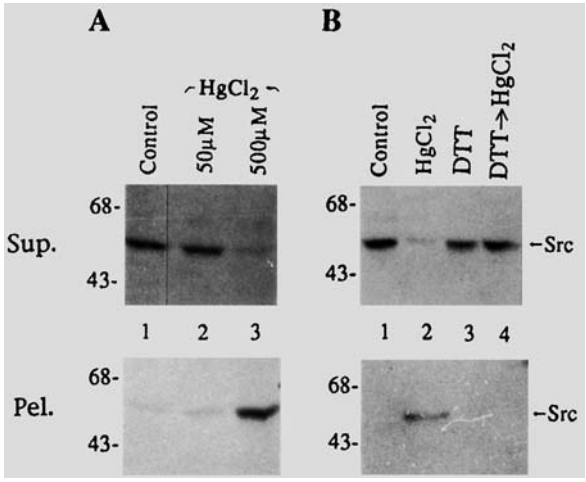

Fig. 1. Exposure of NIH3T3 cells to sulfhydryl-reactive $HgCl_2$ induces production of nonionic, detergent-resistant aggregate of Src proteins. **A:** NIH3T3 cells were incubated at 37°C for 2 min with 0.05 mM (*lane 2*) or 0.5 mM (*lane 3*) $HgCl_2$ and were then lysed in lysis buffer. *Lane 1:* No $HgCl_2$ control. Supernatant (Sup.) and pellet (Pel.) fractions were obtained from the lysate by centrifugation at 15,000 rpm for 30 min for development of the Src band by immunoblot. **B:** Cells were incubated with 0.5 mM $HgCl_2$ (*lane 2*) or 5 mM DTT (*lane 3*) or with DTT followed by $HgCl_2$ (*lane 4*) and were lysed. *Lane 1:* No treatment control. Supernatant and pellet fractions were obtained by centrifugation for development of the Src band. The positions of Mr markers and Src protein are indicated.

The $HgCl_2$-Induced Src Aggregate Is Labile to Both 2ME and SDS

Sensitivity of the Hg^{2+}-induced Src aggregate to both reducing reagent 2ME and ionic detergents SDS/DOC was tested. As shown in Figure 2A, a definite amount of Src protein was released from the aggregate in the pellet fraction by the treatment with 2ME (lane 2 of middle panel, compared with lane 2′ for no 2ME control). Figure 2B shows that the Src proteins in both supernatant and 2ME-solubilized pellet fractions were able to be collected by immunoprecipitation. These results confirmed that a redox-linked mechanism mediates the $HgCl_2$-induced Src aggregation.

Next we examined the sensitivity of the $HgCl_2$-induced Src aggregate to ionic detergents. As shown in Figure 2C, the majority of the Src proteins from the $HgCl_2$-treated cells was dissolved in the lysis buffer added with SDS/DOC. Moreover, the Src proteins in the pellet fraction from the $HgCl_2$-treated cells ran as monomers in SDS-PAGE under either reducing (Fig. 2D, left panel) or unreducing (Fig. 2D, right panel) condition. These results suggest that the Src proteins associated with the $HgCl_2$-induced aggregate through ionic detergent-sensitive bonds.

$HgCl_2$ Induces Tyrosine Phosphorylation and Aggregation of Multiple Cellular Proteins

When NIH3T3 cells were exposed to 0.5 mM $HgCl_2$, tyrosine phosphorylation was promoted extensively on multiple cellular proteins (Fig. 3, lane 2). Some of these P-tyr–containing proteins formed aggregates in lysis buffer and were pelleted together with the Src protein by centrifugation (Fig. 3, right panel).

Accelerated Protein Tyrosine Phosphorylation Does Not Underlie the Mechanism for the Src Aggregation by $HgCl_2$

A possibility arose that Src proteins were aggregated secondarily to the $HgCl_2$-induced acceleration of protein tyrosine phosphorylation. To test this possibility, we examined the effects of addition of PTK inhibitor staurosporine before $HgCl_2$ on the levels of both protein tyrosine phosphorylation and Src aggregation. As shown in Figure 4A, the staurosporine treatment almost completely inhibited induction of tyrosine phosphorylation of cellular proteins by $HgCl_2$ (lane 4 compared to lane 3). In contrast, the same treatment never prevented aggregation of Src proteins by $HgCl_2$ (Fig. 4B, lane 4 compared to lane 3). This result ruled out the possibility above described, thereby suggesting the concern of the Src aggregation as a primary event of the Hg^{2+} action.

Aggregated c-Src Proteins From $HgCl_2$-Treated Cells Are Extensively Activated

We next asked the question whether aggregation of Src proteins would connect to change in their kinase activity. Supernatant and 2ME-solubilized pellet fractions were obtained from NIH3T3 cells treated with 0.05–0.5 mM $HgCl_2$ for 5 s to 20 min. The Src proteins immunoprecipitated from these fractions were tested for their kinase activity in vitro. The results are shown in Figure 5. When cells were exposed to 0.5 mM $HgCl_2$, catalytic activity of c-Src kinase was greatly elevated from 5–30 s after exposure and then declined during incubation for 2–20 min. Noticeably, however, most of the elevated kinase activity during 30 s to 20 min after the $HgCl_2$ exposure was demonstrated in the 2ME-solubilized pellet fraction (Fig. 5, bottom panel, lanes 5, 7). Together with the results presented

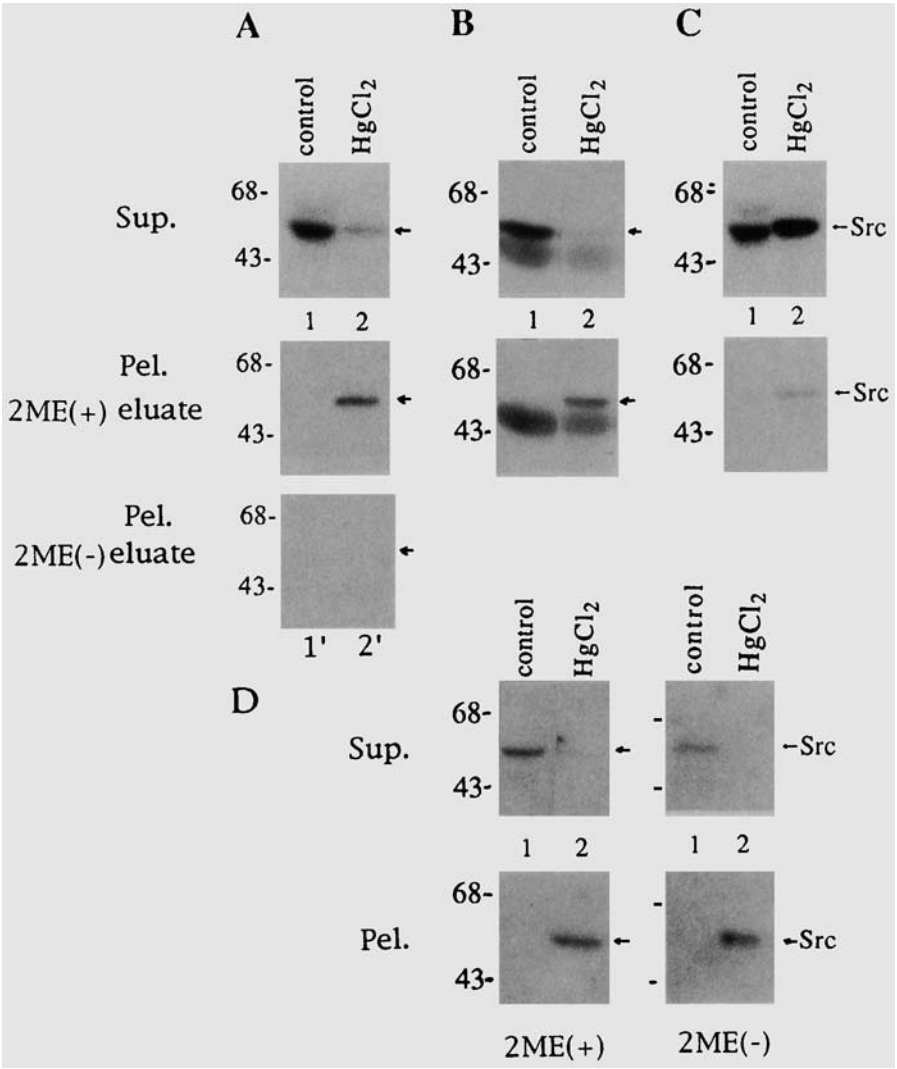

Fig. 2. Sensitivity of the $HgCl_2$-induced Src aggregate to the reducing reagent and ionic detergents. NIH3T3 cells were incubated for 2 min with (*lane 2*) or without (*lane 1*) 0.5 mM $HgCl_2$ and were lysed in lysis buffer. **A:** The pellet (Pel.) fraction of the lysate after centrifugation was treated with the lysis buffer added with 10% 2ME. The eluate was separated by centrifugation for development of the Src band by immunoblot. The bottom picture (*lanes 1 and 2*) shows no 2ME eluate control. **B:** Supernatant (Sup.) and 2ME-solubilized pellet fractions obtained as in A were immunoprecipitated with anti-Src antibody for development of the Src band from the precipitate by immunoblot. **C:** Cell lysis was done by use of lysis buffer added with 0.1% SDS and 1% DOC. Supernatants and pellets of the lysates after centrifugation were examined for the Src band by immunoblot. **D:** Supernatant and pellet fractions of the lysate were subjected to SDS-PAGE under both reducing (with 2ME) and unreducing (without 2ME) conditions and examined for the Src protein. The positions of Mr markers and Src protein are indicated.

in Figures 1 and 2, these data suggest a close connection of activation of Src kinase to its aggregation in $HgCl_2$-treated cells. By exposure of cells to 0.05 mM $HgCl_2$, which barely produced heavy aggregate of Src proteins, less marked activation of Src kinase occurred with a peak at 2 min after exposure (Fig. 5, lane 6) and subsided by 20 min (Fig. 5, lane 10).

Parallel examination of cell viability by a dye exclusion test showed that, after exposure to 0.5 mM $HgCl_2$, numbers of dead cells increased during 2 min (5–10%) to 20 min (50–70%), whereas almost all cells were alive by 30 s when peak kinase activity was attained. In addition, only 5–10% of cells were dead at 20 min after exposure to 0.05 mM $HgCl_2$. These results showed that cell membrane was grossly intact at an early but not a late stage after exposure to 0.5 mM $HgCl_2$. Consequently, the upregulation of the kinase activity at the early stage but not the downregulation at the late stage could be an event occurring in live cells.

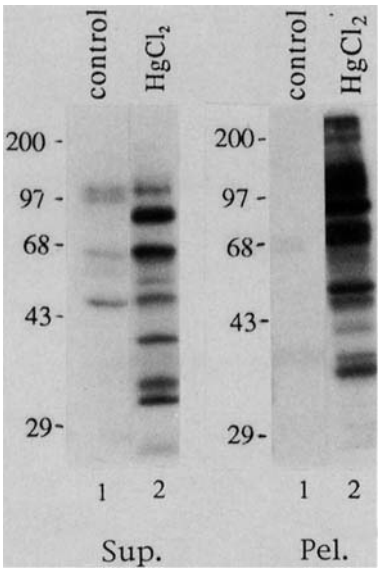

Fig. 3. Multiple cellular proteins are phosphorylated at tyrosine residues in cells exposed to $HgCl_2$. NIH3T3 cells were incubated for 2 min with (*lane 2*) or without (*lane 1*) 0.5 mM $HgCl_2$ and were lysed in lysis buffer. Supernatant (Sup.) and pellet (Pel.) fractions of the lysate were obtained by centrifugation for demonstration of P-tyr–containing proteins by immunoblot. The positions of Mr markers are indicated.

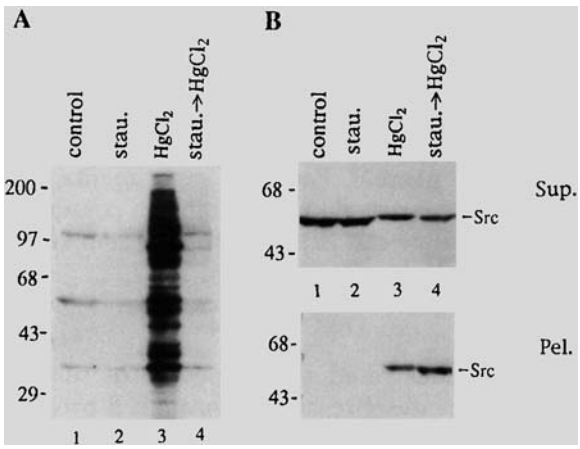

Fig. 4. The $HgCl_2$-induced Src aggregation is not an event secondary to promotion of protein tyrosine phosphorylation. NIH3T3 cells were incubated for 5 min with 2 μg/ml of staurosporine (stau.) (*lane 2*) or for 2 min with 0.5 mM $HgCl_2$ (*lane 3*) or with staurosporine followed by $HgCl_2$ (*lane 4*). *Lane 1:* Medium alone control. These cells were lysed in sample buffer for demonstration of P-tyr–containing proteins (**A**) or in lysis buffer followed by separation by centrifugation into supernatant (Sup.) and pellet (Pel.) fractions for development of the Src band (**B**). The positions of Mr markers and Src protein are indicated.

Action of $HgCl_2$ on Csk^- Mutant Cells

In order to define the relation between the $HgCl_2$ pathway for Src activation and the known regulatory mechanism of the Src kinase through Csk kinase-dependent phosphorylation of Y527 and its dephosphorylation by PTPase, we examined the $HgCl_2$ effect on Csk kinase-defective mutant cells for promotion of tyrosine phosphorylation of cellular proteins and for activation of the Src kinase. The results are shown in Figure 6. Unstimulated Csk^+ T24E cells and Sck^- T29E cells showed distinct patterns of distribution of P-tyr–containing proteins in the immunoblot analysis, probably due to lack of Csk kinase activity, and Csk-dependent regulation of Src family kinases in the latter cells. By the exposure of cells to $HgCl_2$, the overall level of protein tyrosine phosphorylation was further increased in not only Csk^+ T24E cells but also Csk^- T29E cells, although the increase of the phosphorylation level in the latter cells was less extensive than that in the former. This suggested that the presence of Csk kinase is not essential for the $HgCl_2$ effect to promote tyrosine phosphorylation of cellular proteins.

Corresponding to the original report by Imamoto and Soriano [1993], the amount of Src protein in Csk^- mutant cells was about one-fifth of that in control Csk^+ cells when measured by immunoblot for Src protein (data not shown). Nevertheless, the catalytic activity of the Src kinase in Csk^- cells was comparable with that of the kinase in Csk^+ cells (Fig. 6B, lanes 1). This result confirmed that specific catalytic activity of the Src kinase in the mutant cells is elevated. Interestingly, the kinase activity of Src proteins in both Csk^+ and Csk^- cells was definitely promoted by exposure to $HgCl_2$ (lanes 2). This suggested that activation of c-Src kinase in $HgCl_2$-treated cells could occur independent of the Csk kinase activity or the Csk kinase-linked regulatory mechanism.

The Action of $HgCl_2$ on v-Src–Expressing NIH3T3 Cells

Study was further conducted to examine the effect of $HgCl_2$ on v-Src–expressing cells for potential promotion of protein tyrosine phosphorylation and activation of Y527-defective v-Src kinase. As expected, in v-*src*–transfected NIH3T3 cells multiple cellular proteins were heavily phosphorylated at tyrosine (Fig. 7A, lane 1). Exposure of cells to $HgCl_2$ further promoted tyrosine phosphorylation of multiple cellular proteins (Fig. 7A, lane 2), and catalytic activity of v-Src kinase was elevated (Fig. 7B, lane 2 compared with lane 1). These results, together with

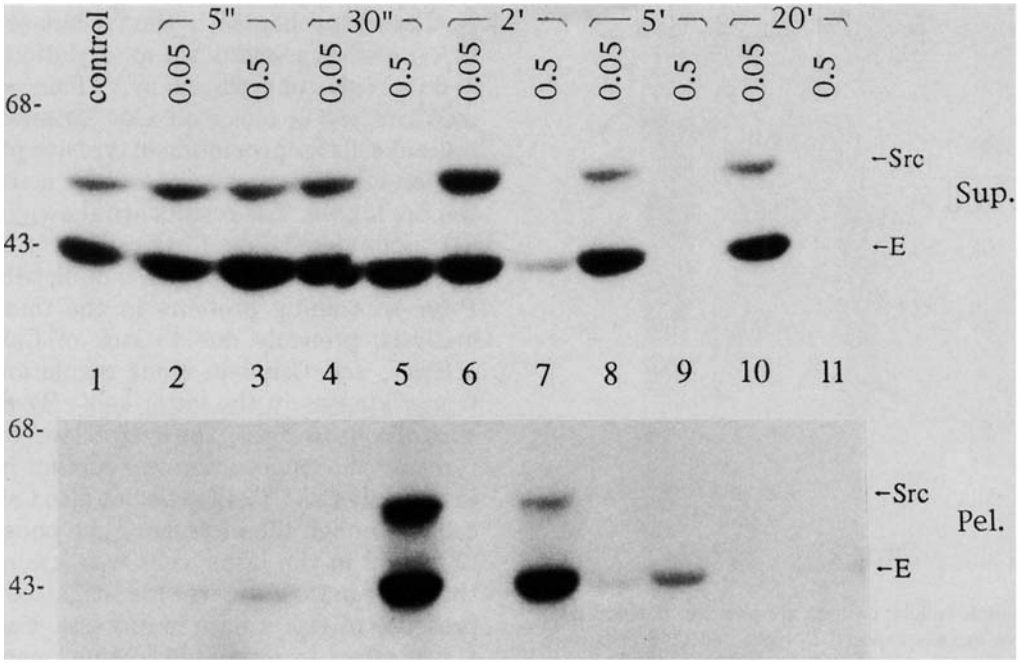

Fig. 5. Aggregation-linked activation of c-Src kinase by $HgCl_2$. NIH3T3 cells were incubated with 0.05 mM (*even-numbered lanes*) or 0.5 mM (*odd-numbered lanes* except *lane 1* as no $HgCl_2$ control) $HgCl_2$ for 5 s, 30 s, 2 min, or 20 min (from left to right) and were then lysed in lysis buffer. Supernatant (Sup.) and 2ME-solubilized pellet (Pel.) fractions of the cell lysates after centrifugation were prepared, and the Src protein from both fractions was immunoprecipitated for in vitro kinase assay (see Materials and Methods and Fig. 2B). As controls, cells incubated without $HgCl_2$ for 5 s, 2 min, and 20 min were tested with invariable results, and the data for cells incubated for 2 min are shown as the representative (*lane 1*). The positions of Mr markers, Src protein, and enolase (E) are indicated. Note the highest kinase activity in the pellet fraction in *lane 5*.

those obtained on Csk^- mutant cells (Fig. 6), suggest that the kinase activity of Src protein is upregulated in $HgCl_2$-treated cells at least in part independent of Y527-mediated regulation.

CNBr Cleavage Mapping of Phosphopeptides for Autophosphorylated Src Protein

Lastly, we conducted experiments to define which tyrosine residue or residues in the Src protein were phosphorylated in the cells treated with $HgCl_2$. NIH3T3 cells were first labeled in vivo with phosphorous-32 and were then treated with $HgCl_2$. The Src protein collected by immunoprecipitation was subjected to SDS-PAGE, and the Src band was cut out and digested by CNBr for phosphopeptide mapping on SDS-PAGE. The result is shown in Figure 8. In vivo labeling with phosphorous-32 occurred selectively on the 4 kDa peptide for the Src protein from untreated control cells. For the Src protein from the $HgCl_2$-treated cells, additional labeling developed exclusively on the 10 kDa peptide. This result suggests that autophosphorylation of Y416 but not of Y527 was promoted by the $HgCl_2$ treatment, excluding the possibility of activation of Csk kinase by the treatment. In addition, the $HgCl_2$ treatment did not extensively decrease the level of phosphorylation of the 4 kDa peptide. Repeated experiments confirmed this conclusion, although the possibility remained that the Src activation accompanied low grade dephosphorylation of Y527.

DISCUSSION

We demonstrated that exposure of cells to $HgCl_2$ activates Src kinase through a protein-sulfhydryl modification-based mechanism at least in part independent of the known Csk kinase-linked and carboxyl terminal tyrosine phosphorylation/dephosphorylation-mediated regulation. Extensive activation of Src kinase by exposure of cells to 0.5 mM $HgCl_2$ accompanied production of heavy aggregate of Src proteins which is resistant to nonionic detergent. This aggregate was easily collected in the pellet fraction by relatively low speed (15,000 rpm for 30 min) centrifugation (Fig. 1), although less heavy aggregate, which might be produced in cells treated with a lower concentration of $HgCl_2$, mostly remained in the supernatant fraction. Our results apparently correlate to the previous observation by Hamaguchi and Hanafusa (1987)

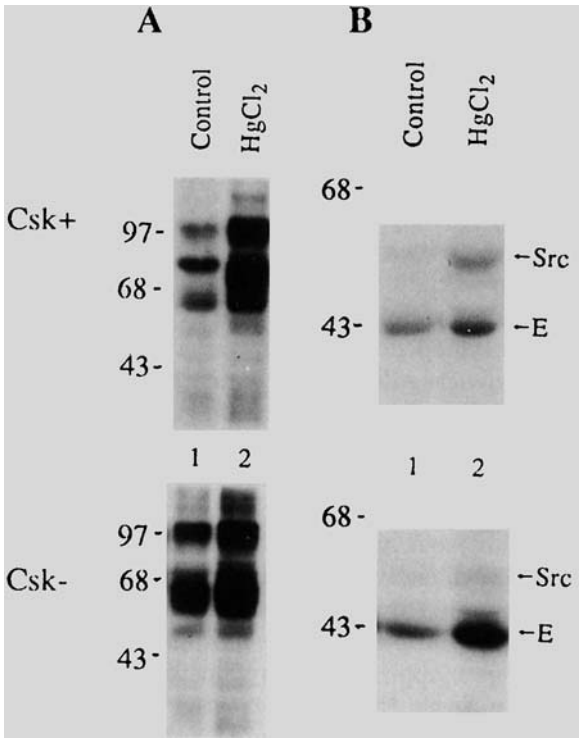

Fig. 6. The $HgCl_2$-mediated-promotion of protein tyrosine phosphorylation and Src activation develop in Csk^- mutant cells. Csk^+ T24E cells (**upper panel**) and Csk^- T29E cells (**lower panel**) were incubated for 2 min with (*lane 2*) or without (*lane 1*) 0.5 mM (**A**) or 0.05 mM (**B**) $HgCl_2$. These cells were lysed in sample buffer for demonstration of P-tyr–containing proteins (A) or in lysis buffer for in vitro kinase assay on immunoprecipitated Src proteins (B). Shown is a representative of three experiments with consistent results. The positions of Mr markers, Src protein, and enolase (E) are indicated.

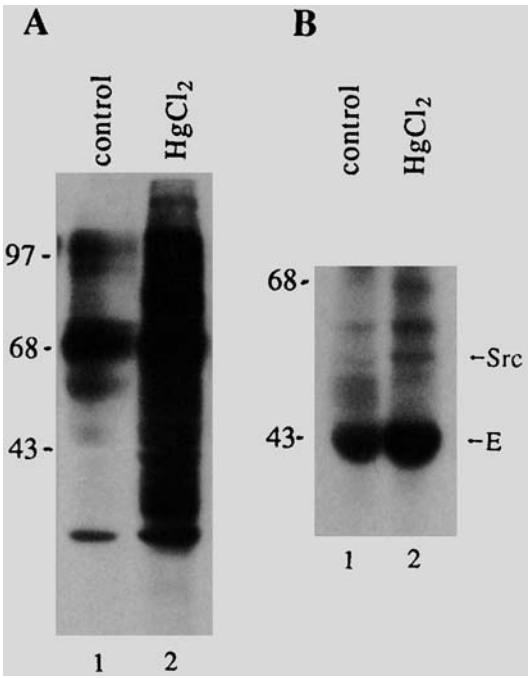

Fig. 7. The $HgCl_2$-mediated promotion of protein tyrosine phosphorylation and Src activation develop in cells expressing v-Src. v-Src–expressing NIH3T3 cells were incubated for 2 min with (*lane 2*) or without (*lane 1*) 0.5 mM (**A**) or 0.05 mM (**B**) $HgCl_2$. They were lysed in sample buffer for demonstration of P-tyr–containing proteins (A) or lysis buffer for in vitro kinase assay on immunoprecipitated Src proteins (B). Several protein bands developed in addition to those of Src and enolase due to coimmunoprecipitation of v-Src-associating cellular proteins. Shown is a representative of three experiments with consistent results. The positions of Mr markers, Src protein, and enolase (E) are indicated.

and Hamaguchi et al. [1993] that, whereas inactive c-Src kinase is soluble in nonionic detergent, constitutively activated v-Src kinase is resistant to the detergent, although the v-Src protein-membrane complex was smaller than that of the $HgCl_2$ (0.5 mM)-induced c-Src aggregate and was not pelleted by the centrifugation described above. Our present results therefore support the current view that activation of Src kinase is linked to its association with non-ionic, detergent-resistant membrane fraction.

We showed that exposure of cells to 0.5 mM $HgCl_2$, which induced extensive aggregation and activation of Src kinase, greatly accelerated tyrosine phosphorylation of multiple cellular proteins (Fig. 3). Some though not all of these proteins could be the internal substrates of the Src kinase. Such accelerated protein tyrosine phosphorylation might underlie the mechanism of Src aggregation, because protein tyrosine phosphorylation should promote interaction between P-tyr–containing proteins and proteins bearing the SH2 domain [Koch et al., 1991]. However, addition of staurosporine before $HgCl_2$, which totally inhibited promotion of protein tyrosine phosphorylation, never prevented Src aggregation (Fig. 4). This evidenced that the Src aggregation is not an event resulting from the kinase activation but is primarily mediated by direct action of sulfhydryl-reactive Hg^{2+}. Neutralization of the $HgCl_2$ action with sulfhydryl-donating DTT (Fig. 1B) and release of Src proteins from the $HgCl_2$-induced aggregate by reducing 2ME (Fig. 2A,B) supported this conclusion. However, we further provided evidence that, in $HgCl_2$-treated cells, Src proteins themselves may not be directly bonded with Hg^{2+} but indirectly associate with the $HgCl_2$-induced aggregate noncovalently (Fig. 2C,D). Taken together, our results suggest that the cell surface acting Hg^{2+} probably polymerize cell surface proteins through a redox-linked or sulfhydryl-based mechanism, to which Src proteins associ-

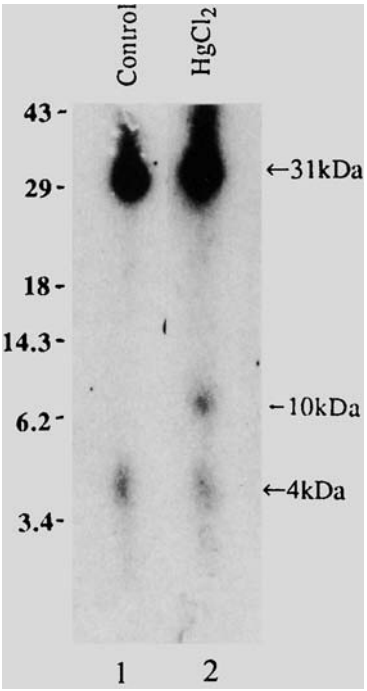

Fig. 8. CNBr cleavage mapping of phosphopeptides for autophosphorylated c-Src protein. In vivo phosphorous-32–prelabeled NIH3T3 cells were incubated for 2 min with (*lane 2*) or without (*lane 1*) 0.05 mM $HgCl_2$. These cells were lysed in lysis buffer, and Src proteins were collected by immunoprecipitation. The collected Src proteins were subjected to SDS-PAGE, and the protein band for in vivo labeled Src was excised for CNBr digestion. The digested fragments were mapped on 24% SDS-PAGE (*lanes 1–2*). The 31 kDa, 10 kDa, and 4 kDa fragments which should contain serine 17, Y416, and Y527 as major phosphorylation sites [Schuh and Brugge, 1988; Thomas et al., 1991] are indicated. Note selective in vivo labeling of the 4 kDa peptide from control cells (*lane 1*) and additional labeling of the 10 kDa peptide from the $HgCl_2$-treated cells. Shown is a representative of three experiments with consistent results. The positions of Mr markers and peptides are indicated.

ate across plasma membrane through a noncovalent but tyrosine phosphorylation-independent bond. It might be argued that the sulfhydryl-based conjugation of cellular proteins with Hg^{2+} might be labile to SDS. The resistance of the stable Hg^{2+}-mediated bond to SDS has been confirmed, however, for interaction between Hg^{2+} and cell surface proteins such as immunoglobulins [A.A. Akhand et al., unpublished observation]. We do not know which cell surface proteins on NIH3T3 cells are crosslinked by Hg^{2+} for Src aggregation. These may include some GPI anchored proteins which associate with both the nonionic, detergent-resistant compartment of plasma membrane [Stefanova et al., 1991] and Src family kinases [Brown et al., 1992], as we previously suggested for the mechanism of activation of Lck kinase in T lymphocytes by $HgCl_2$ [Pu et al., 1995]. Whatever the direct molecular target of Hg^{2+} for Src aggregation is, extensive molecular interaction among Src proteins and other proteins in the aggregate should play an important role in activating the Src kinase, because we demonstrated the highest kinase activity in the aggregated Src (Fig. 5).

Interestingly, the proposed protein sulfhydryl modification-linked activation of Src kinase by Hg^{2+} developed at least in part independent of the known regulatory mechanism through phosphorylation of carboxyl-terminal tyrosine (Y527) by Csk and its dephosphorylation by PTPase. This was demonstrated by the results of further upregulation by $HgCl_2$ of the catalytic activity of c-Src kinase from Csk^- mutant cells (Fig. 6) and of Y527-defective v-Src kinase (Fig. 7), each accompanied by the augmented protein tyrosine phosphorylation. Correspondingly, the CNBr cleavage mapping of phosphopeptides for c-Src protein from the $HgCl_2$-treated cells demonstrated selective promotion of Y416 phosphorylation, without obvious change in the Y527 phosphorylation level (Fig. 8). This result partially corresponds to the recent report by Hardwick and Sefton [1995] that Y394 as an autophosphorylation site of Lck kinase was preferentially phosphorylated in T lymphocytes exposed to H_2O_2, although they did not examine whether or not the carboxyl-terminal tyrosine-mediated regulation is involved in the activation mechanism. This suggests that a common pathway may work for activation of Src family kinases by $HgCl_2$ and H_2O_2, although a more extensive study will be needed for a conclusion on this matter.

It might be that protein sulfhydryl modification-mediated polymerization of cell surface proteins compelled the intracellular Src proteins close together to mutually catalyze the phosphorylation at Y416, although this could be only one of potentially multiple mechanisms of the Hg^{2+}-mediated Src activation. Possibly related to our results, an S-S bond-mediated dimerization of the cytokine signal transducing gp130 [Murakami et al., 1993] has been recently reported to be a critical step for activation of intracellular PTK activity. It is reported that Hg^{2+} binds two adjacent sulfhydryl groups in proteins forming an S-Hg-S bond, which successfully replaces the S-S bond for protein modification [Sperling et al., 1969; Steer et al., 1974]. The Hg^{2+}-mediated signal pathway could therefore provide a model for sulfhydryl-

based receptor dimerization which should develop physiologically for receptor activation.

In the present study we have not characterized the molecular nature of each of many tyrosine phosphorylated proteins developed in the $HgCl_2$-treated cells. Some of them should probably be linked to activation of the Src kinase. However, it is also likely that, in addition to the Src kinase, some other PTKs were subject to the $HgCl_2$ action for activation, thereby phosphorylating a number of substrate proteins. What could then be the significance of the potentially multifunctional protein sulfhydryl modification-based signal transduction pathway in cell biology? The concentration of $HgCl_2$ we used for many experiments (0.5 mM) was toxic to cells for quick death. Because this action of $HgCl_2$ was partially inhibited by addition of PTK inhibitors before $HgCl_2$ [Akhand et al., unpublished data], the demonstrated signal pathway may actively work for cell death induction. However, lower concentrations (0.001–0.01 mM) of $HgCl_2$ were previously shown to promote DNA synthesis and cytokine production of T lymphocytes, and these concentrations are weakly but definitely effective for activating Lck [Nakashima et al., 1994] and Src (data not shown). Therefore, principally the same but less extensive signal transduction could be involved in such low concentrations of $HgCl_2$-mediated promotion of cell activation. For the same reason, the demonstrated signal transduction pathway might potentially underlie the process of the low concentrations of $HgCl_2$-mediated development of lymphoproliferative autoimmune diseases in animals [Goldman et al., 1991] and related immune disorders in man caused by exposure to polluted air [Takafuji et al., 1989].

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