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# Forum Review

# Redox-Linked Signal Transduction Pathways for Protein Tyrosine Kinase Activation

IZUMI NAKASHIMA, MASASHI KATO, ANWARUL A. AKHAND, HARUHIKO SUZUKI, KOZUE TAKEDA, KAHLED HOSSAIN, and YOSHIYUKI KAWAMOTO

## ABSTRACT

**The signaling for activation of protein tyrosine kinases (PTKs) is usually started by binding of ligands to cell-surface receptors. However, recent evidence suggests the presence of ligand binding-independent signaling pathways that are mediated by oxidative stress. Oxidation and reduction of protein cysteine sulfhydryl (SH) groups may work as a molecular switch to start or to stop the signaling. It is known that oxidation of cysteine SH groups on protein tyrosine phosphatases switches off the action of protein tyrosine phosphatases. This event may not, however, signal for initial autophosphorylation of previously unphosphorylated PTKs, whereas it certainly prevents dephosphorylation of once-phosphorylated PTKs. We have suggested new mechanisms for oxidative stress-mediated PTK activation. First, cell-surface glycosylphosphatidylinositol-anchoring proteins and a phosphoglycolipid/cholesterol-enriched membrane microdomain termed a "raft" can be the direct targets of oxidative stress for inducing their clustering through an S-S-bonded or S-X-S-bonded crosslinking of cell-surface proteins and subsequent activation of raft-associating Src family PTKs. Second, intracellular specific cysteine SH groups on PTK proteins can be another target of oxidative stress for inducing a conformational change necessary for initial activation of PTKs. A possible relationship between cell-surface and intracellular events is that the former frequently induces superoxide production as the second messenger for the latter.** *Antioxid. Redox Signal.* 4, 517–531.

## INTRODUCTION

INTRACELLULAR SIGNAL TRANSDUCTION from the cell surface to the nucleus is most commonly started by activation of either receptor-type or nonreceptor-type protein tyrosine kinases (PTKs), which directs a number of downstream signaling molecules for cascade reactions (9, 102). Activation of receptor-type PTKs, such as epidermal growth factor receptor (EGFR), insulin receptor kinase (IRK), and RET PTK, usually occurs following interaction with their specific ligands (30, 78, 79). Binding of specific ligands to cell-surface receptors, such as T-cell receptors, Fc receptors, and cytokine receptors, also causes activation of nonreceptor-type PTKs, such as Lck, Fyn, c-Src, Syk, ZAP70, and Jak, that associate with the receptors (9, 20, 43, 64, 102). Recent evidence suggests the presence of ligand-independent pathways for activation of PTKs that are triggered by oxidative stress (50, 52, 54, 59–63, 82, 93). The oxidative agents that trigger the ligand-independent pathways for PTK activation include reactive oxygen species (ROS) such as hydrogen peroxide ($H_2O_2$) (1, 21, 22, 31, 52, 53, 80), nitric oxide (NO) (3, 45), oxidative chemicals such as diamide (6) and oxidized low-density lipoproteins (46), alkylating agents such as iodoacetamide (6), heavy metal ions such as $HgCl_2$ (2, 14, 33, 44, 56, 58, 66–69, 71, 73) and $NaAsO_2$ (25), x-ray (39), ultraviolet (UV) light (13, 23, 36, 37, 74), and osmotic stress (70, 74, 97). These agents have been shown to activate Src (3, 13, 68,69), Lck (21, 53, 58), Fyn (1), Syk (70), c-Abl (39), Ltk (6), Jak (1), EGFR (31), IRK (22, 79, 80), and RET (36, 37, 97). A common chemical reaction mediated by these agents is

Department of Immunology, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan.

thought to be oxidation of sulfhydryl (-SH) groups on protein cysteines and glutathiones, possibly producing disulfide (S-S) bonds. Oxidation of protein cysteine SH groups may induce a conformational change of the protein, particular when it forms either an intermolecular or intramolecular S-S bond (54,59–63). SH group-reactive heavy metals and alkylating reagents can produce an S-S bond-mimicking S-X-S bond (Fig. 1). Reaction of oxidative agents with glutathiones for their consumption also causes an impairment of the normal cellular redox balance.

Three-dimensional structures of whole molecules have recently been reported for c-Src and Hck (87, 105). The catalytic activity of these PTKs is normally regulated by phosphorylation/dephosphorylation at specified tyrosine residues either positively (11, 72, 98, 102) or negatively (5, 98). Many PTKs carry a tyrosine residue(s) in the activation segment of the catalytic domain that is autophosphorylated in close association with activation of the kinases (87, 105). Phosphorylation of this tyrosine residue(s) is likely to work as a local switch to induce a conformational change required for a functional kinase. This local switch has been suggested to be coupled with a global regulatory switch, possibly mediated by other domains of the kinase together (105). Src family kinases, such as c-Src, Lck, and Fyn, carry a regulatory tyrosine in the C-terminal tail, of which phosphorylation by a distinct PTK termed Crk and subsequent interaction with the Src homology 2(SH2) domain result in a closed conformation of the kinase protein for inactivation (9, 87, 102, 105). Such a closed conformation is destabilized by dephosphorylation of the regulatory phosphotyrosine, which works as a global regulatory switch to turn on the local switch. Disruption of the interaction of the Src homology 3 (SH3) domain with the linker and with the N-terminal lobe of the catalytic domain also destabilizes the closed conformation to work as another global regulatory switch (87, 105). The catalytic activity of PTKs is thus bidirectionally regulated by protein tyrosine phosphatases (PTPases) that dephosphorylate phosphotyrosines at regulatory sites (for positive regulation) or autophosphorylation sites (for negative regulation) (5, 11, 72, 98, 102).

A well known target of oxidative chemical reaction is protein phosphatases in cells (8, 12, 18, 29, 31, 40, 48, 52, 53, 65, 75, 77). The protein phosphatases contain SH groups in the catalytic domain of which oxidative modification impairs the catalytic activity (19, 91). One suggested mechanism of activation of PTKs by oxidative stress is through inactivation of regulatory PTPases. A number of recent experimental studies, however, have suggested that the mechanism of activation of PTKs by oxidative stress is more complex, including chemically induced cell-surface receptor crosslinkage (25, 58, 59, 67) and structural modification of PTK proteins (3, 36, 37, 68, 79, 80, 97). Here we review the results of relevant studies in our and other laboratories, and we propose a new oxidation and reduction (redox)-linked signaling pathway for PTK activation.

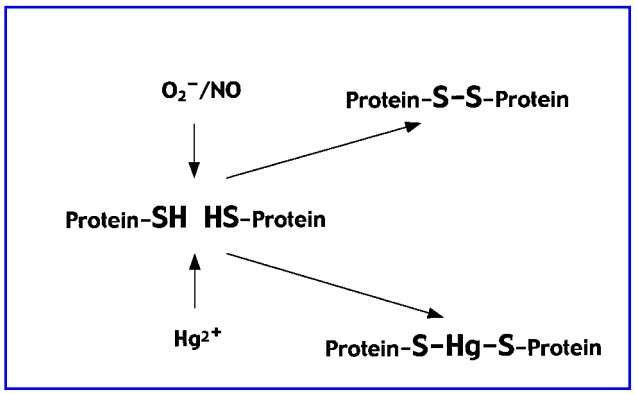


**FIG. 1. S-S-bonded or S-X-S-bonded crosslinkage of cellular proteins.** Two cysteine SH groups on cellular proteins are oxidized to form a protein-S-S-protein bond. This protein-S-S-protein bond may be mimicked by a protein-S-X-S-protein bond, which is formed by bivalent SH group-reactive chemicals (X), including $HgCl_2$ ($Hg^{2+}$).

## CELL-SURFACE EVENTS FOR PTK ACTIVATION

Cell-surface proteins can be the primary target of oxidative agents from the environment that do not easily enter cells, such as heavy metal ions. Exposure of murine thymocytes to $HgCl_2$ was shown to cause aggregation of a number of cell-surface proteins, such as glycosylphosphatidylinositol (GPI)-anchoring Thy-1 and transmembrane CD4 and CD3 (58) (Fig. 2A, bottom). This aggregation seems to occur through an S-Hg-S-bonded crosslinkage of cell-surface proteins, because the addition of a reducing agent, dithiothreitol (DTT), before $HgCl_2$ prevented aggregation of these proteins. Aggregation of cell-surface proteins accompanied co-clustering of intracellular phosphorylated proteins, including Lck (Fig. 2A, top). Both aggregation of cell-surface proteins and tyrosine phosphorylation of cellular proteins occurred as promptly as 5 s after exposure of cells to a high concentration of $HgCl_2$. Quick and extensive activation of Lck kinase was confirmed by an *in vitro* kinase assay of the immunoprecipitated Lck proteins from cell lysates (Fig. 2B).

The crucial role of the cell-surface event that occurred after exposure of cells to $HgCl_2$ in intracellular signal transduction was demonstrated by the following experiments (67) (Fig. 2C). A mutant of murine T-cell lymphoma cells that has a PIG-A gene deficiency shows no cell-surface expression of GPI-anchoring proteins such as Thy-1, because the PIG-A gene is indispensable for synthesizing GPI anchors. These mutant cells did not respond well to exposure to $HgCl_2$ for promotion of protein tyrosine phosphorylation, but transfection of the PIG-A gene into these mutant cells restored the responsiveness. These results demonstrate the crucial role of cell-surface proteins or GPI-anchoring proteins in the mechanism of $HgCl_2$-mediated promotion of protein tyrosine phosphorylation or PTK (Lck) activation.

GPI-anchoring proteins, such as Thy-1, CD14, CD55, and CD59, are localized on a special membrane microdomain termed a raft, which is enriched with glycosphingolipid and cholesterol and is insoluble in the presence of nonionic detergents (88, 101). Thy-1 and other GPI-anchoring proteins have long been known to transduce an intracellular signal to activate Src family PTKs that also bind to the raft with the myristoylated glycine residue or palmitoylated cysteine residues at the N-terminus of these PTKs (47, 55, 57, 90). Recently, more

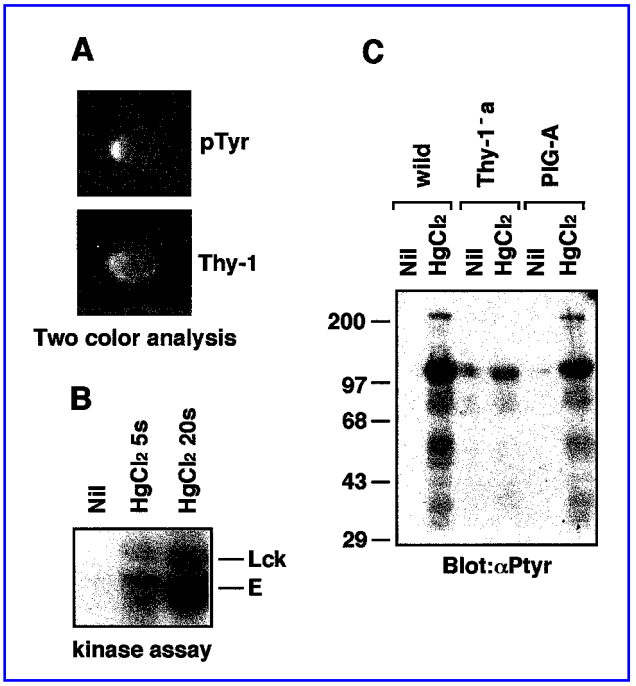


**FIG. 2. Heavy metal ($HgCl_2$)-induced PTK activation is cell-surface protein crosslinkage-linked.** (**A**) Cell-surface GPI-anchoring Thy-1 aggregation, which is induced by exposure of murine thymic T-lymphocytes to $HgCl_2$, was shown by analysis using "the two-color fluorescence antibody technique" to occur in close association with promotion of protein tyrosine phosphorylation (pTyr). (**B**) *In vitro* kinase assay for immunoprecipitated Lck protein from $HgCl_2$-treated cells showed a quick (in 5 s or 20 s) and drastic increase in catalytic activity for autophosphorylation and exogenously added substrate (enolase: E) phosphorylation. (**C**) Wild murine T-lymphoma cells (wild), but not PIG-A$^-$ mutant cells defective in GPI-anchoring proteins (Thy-1$^-$a), responded well to $HgCl_2$ for promotion of protein tyrosine phosphorylation, as demonstrated by immunoblot assay (Blot) with anti-phosphotyrosine (Ptyr) antibody. The mutant cells regained normal activity by transfection of the PIG-A gene (PIG-A). The positions of molecular mass marker proteins (kDa) are indicated on the left. Modified reproduction from Nakashima *et al.* (58) and Pu *et al.* (67) with permission.

attention has been paid to the special function of the raft as a platform of a number of signal-transducing molecules (26, 88, 89, 101). For example, in T-cells, the raft contains transmembrane CD4/CD8 and LAT as an adaptor protein (106), and interaction of T-cells with antigen-presenting cells induces the recruitment of T-cell receptor/CD3 to the raft from other parts of the cell membrane, which triggers the intracellular signal transduction for T-cell activation (24, 51, 104).

We showed that exposure of T-cells to $HgCl_2$ caused aggregation of Thy-1 (58). Through visualization of the rafts by staining with fluorescence-labeled cholera toxin, which specifically binds to sphingomyelin in rafts, Akhand *et al.* (unpublished data) showed that Thy-1-anchoring rafts are clustered following crosslinkage of Thy-1 on rafts with $HgCl_2$. Clustering of Thy-1 and rafts on T-cells and subsequent intracellular signaling for protein tyrosine phosphorylation were also demonstrated after exposure of cells to $NaAsO_2$ (25). Intriguingly, the As-induced Thy-1 clustering and subsequent signaling were prevented by the treatment of cells with β-cyclodextrin, which destroys the raft structure by extracting cholesterol from the raft (Fig. 3A and B). It is therefore likely that clustering of Thy-1 and other cell-surface receptors and subsequent intracellular signaling require membrane raft integrity in addition to chemical reaction of heavy metals with SH groups on cell-surface proteins.

What then is the mechanism of intracellular signal transduction after cluster formation of membrane rafts and raft-associating cell-surface proteins? Clustering of rafts induces accumulation of Src family PTKs that bind to rafts and intracellular domains of some transmembrane proteins such as CD4. This event promotes interaction of individual kinase proteins for mutual phosphorylation and activation. Another possible raft-linked signal pathway is through change in the intracellular redox level. Some oxidative agents, such as $H_2O_2$ and UV light, that easily penetrate the cell membrane may consume the intracellular glutathione pool and induce production of superoxide/ROS (17). It has been reported that cell-surface events, such as binding of inflammatory cytokines or growth factors to their receptors (7, 41, 49, 92) and binding of HIV glycoprotein 120 to CD4 (84), induce superoxide production in cells, possibly in a region downstream of the Lck activation (84). Superoxide production has also been demonstrated to occur in cells that are exposed to cell-surface proteins/raft-aggregating chemicals such as arsenite (10, 25) and mercury (27, 85). We actually demonstrated that the $NaAsO_2$-mediated ROS production is abolished by pretreatment of cells with β-cyclodextrin and is thereby raft-integrity-dependent (Fig. 3C). All of these observations suggest the presence of a signal transduction pathway that is associated with membrane raft integrity-dependent ROS production, as a potential second messenger of the cell-surface event. Figure 4 illustrates such a new signal transduction pathway for PTK activation.

## REGULATION OF PTPASE ACTIVITY AND PTK ACTIVATION

As already mentioned in the Introduction, one of the major target molecules of oxidative stress is PTPase. For example, CD45, which is present ubiquitously on hematopoietic cells as a transmembrane PTPase, can be the main target of oxidative stress on the cell surface. CD45 is normally excluded from the raft and is considered to positively or negatively regulate the activity of Src family PTKs at different steps of PTK activation (5, 98). First, CD45 dephosphorylates the regulatory phosphotyrosine in the C-terminal tail of Src family PTKs, such as $Tyr^{505}$ of Lck, which is phosphorylated by Crk. The Lck $Tyr^{505}$ dephosphorylation results in destabilization of the closed conformation of Lck for activation. Second, CD45 also dephosphorylates phosphotyrosines at autophosphorylation sites of PTKs for down-regulation of the kinase activity. The $HgCl_2$-induced clustering of rafts involved some CD45 molecules (58), but this did not seem to promote dephosphorylation of the regulatory phosphotyro-

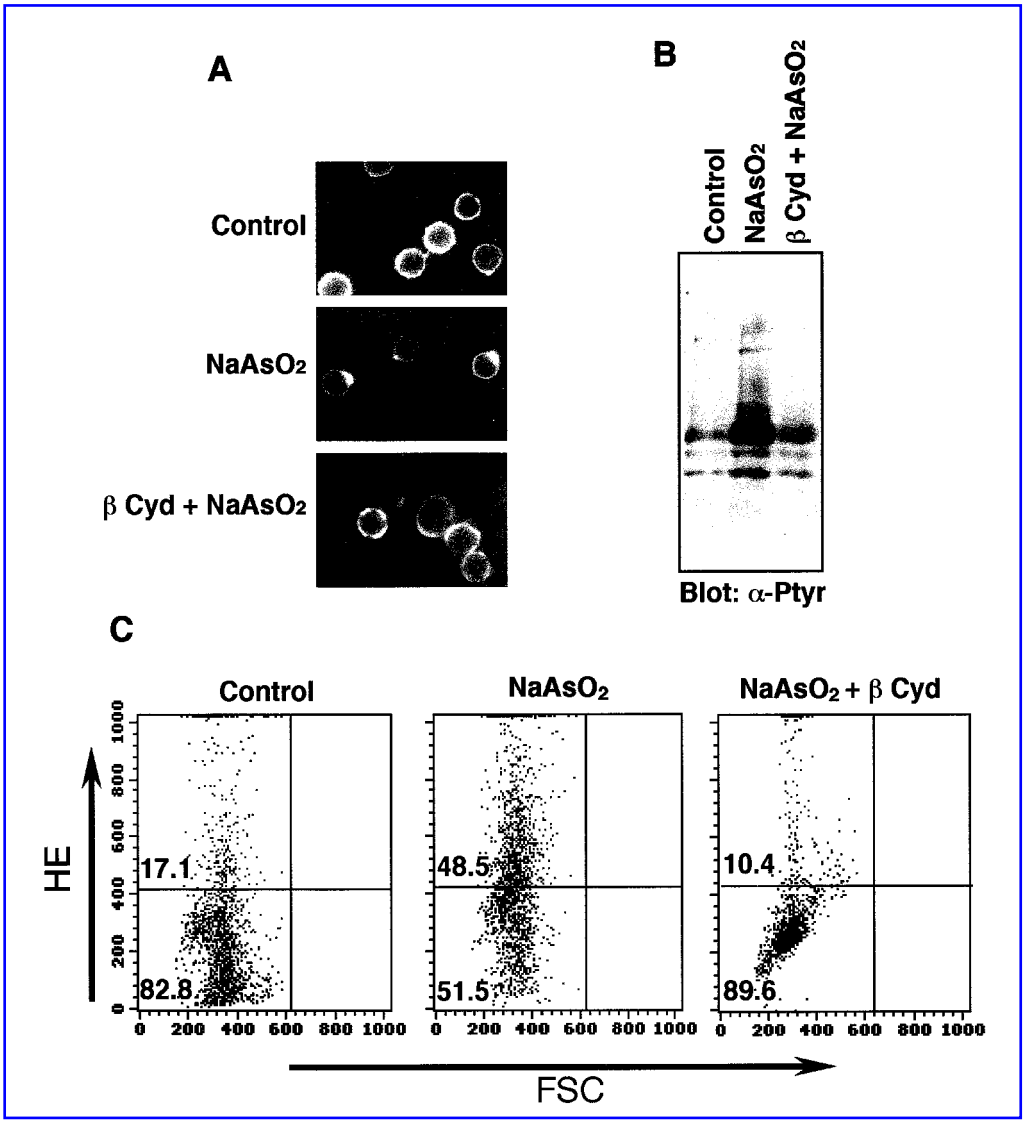


**FIG. 3. Heavy metal-induced cell-surface protein aggregation, PTK activation, and ROS production are membrane raft integrity-dependent.** Thy-1 aggregation (**A**), protein tyrosine phosphorylation promotion (**B**), and ROS production (**C**), induced by exposure of murine thymic T-lymphocytes to $NaAsO_2$, were all impaired by treatment of the cells with membrane cholesterol-extracting β-cyclodextrin (β Cyd) prior to $NaAsO_2$. FSC, forward scattered light; HE, hydroethidine fluorescence as an indicator of ROS production. Percentage of fluorescence positive (**top**) or negative (**bottom**) cells is shown as the number in the figure. Modified reproduction from Hossain *et al.* (25) with permission.

sine in the C-terminal tail of Lck or c-Src (68, 69). $HgCl_2$ may penetrate the cell membrane and inactivate CD45, which otherwise down-regulates the PTK activity. Our observation that $HgCl_2$-induced PTK activation is GPI-anchoring protein-dependent (67) argues against such a simplistic view. It could still be that ROS produced in response to metal/GPI-anchoring protein (25, 27) interaction mediates this pathway of regulation. However, tyrosine at the regulatory site, but not at the autophosphorylation site, is normally phosphorylated detectably in unstimulated cells (3, 69). Oxidation-mediated inactivation of PTPases is not likely to trigger the signaling for initial phosphorylation of tyrosine at the autophoshorylation site, whereas it definitely prevents dephosphorylation of already autophosphorylated (activated) PTKs. Because treatment of cells with $HgCl_2$ promptly induces phosphorylation of otherwise unphosphorylated tyrosine at the autophosphorylation site (69), we speculate that a mechanism(s) other than inactivation of PTPases, possibly linked to oxidation of PTK molecules, operates in the initial step of autophosphorylation/activation of PTKs and that inactivation of PTPases may play a role in keeping once autophosphorylated/activated PTKs continuously autophosphorylated/active.

## ACTIVATION OF SUBCELLULAR SRC KINASE

Earlier studies demonstrated that exposure of subcellular c-Src and Lck proteins to oxidative or alkylating agents such

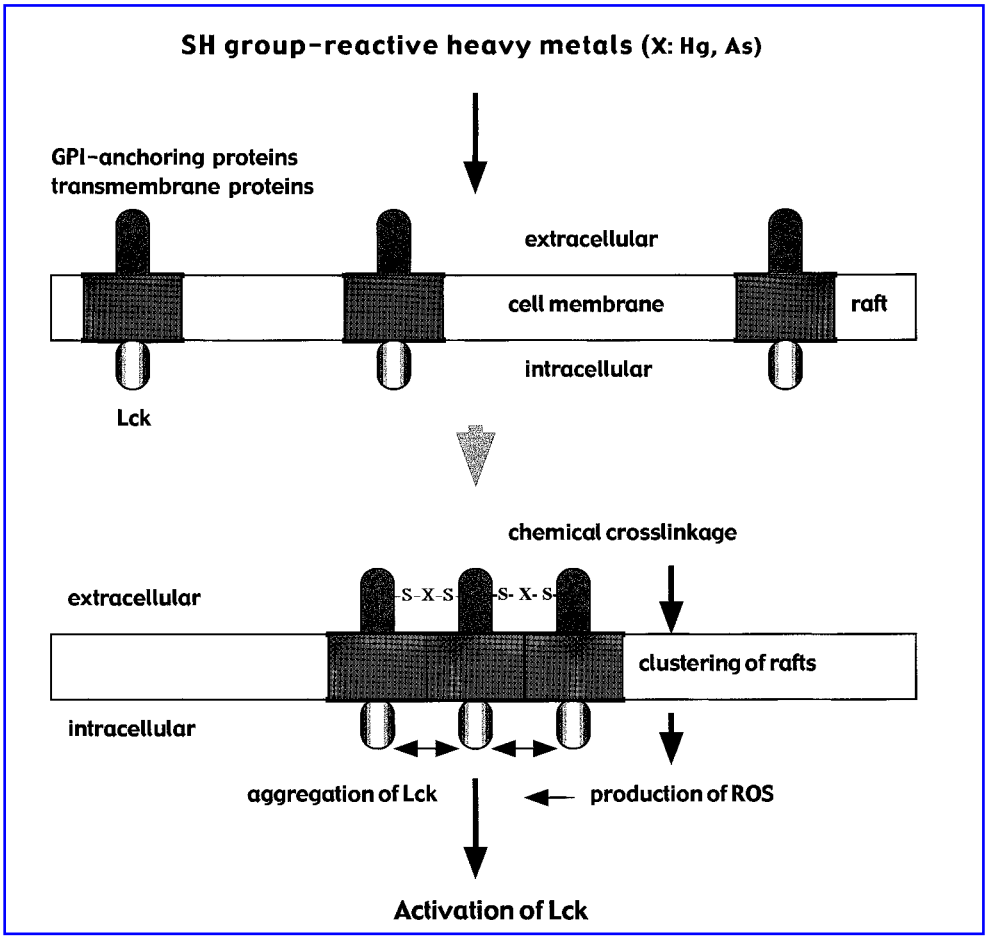


**FIG. 4. A suggested redox-linked, membrane raft integrity-dependent signal transduction pathway for PTK activation.** S-X (X = Hg, As)-S bonds between GPI-anchoring/transmembrane proteins at cysteine residues induce clustering of rafts as the station of a number of signaling molecules, including Src family PTKs (Lck, c-Src, etc.), for promotion of molecular interaction and activation. Clustering of rafts may induce ROS production, which in turn would regulate the catalytic activity of PTKs.

as $H_2O_2$ (53) and *N*-ethylmaleimide (99) impairs their catalytic activity. Exposure of cells to NO or other thiol oxidants was also shown to inhibit autokinase activation of Jak2 (15). On the other hand, Bauskin *et al.* (6) reported that exposure of COS cells to oxidative or alkylating agents such as diamide, *N*-ethylmaleimide, and iodoacetamide induces activation of Ltk as a receptor PTK that is exclusively located on rough endoplasmic reticulum. They found that activation of the kinase accompanies S-S-bonded polymerization of the kinase proteins, possibly mediated by protein disulfide isomerase in the oxidative microenvironment. More recently, we have found that exposure of immunoprecipitated Src proteins to low concentrations of $HgCl_2$ does increase the catalytic activity of the kinase (68). Phosphoamino acid analysis of the phosphorylation-promoted c-Src proteins showed that exposure to $HgCl_2$ preferentially induces phosphorylation at tyrosine residues. Peptide mapping of the radiolabeled phosphorylated Src proteins after cyanobromide cleavage revealed that phosphorylation at $Tyr^{416}$ is preferentially promoted. Analysis of *in vivo* radiolabeled c-Src proteins after exposure to $HgCl_2$ *in vitro* also showed that dephosphorylation at the regulatory $Tyr^{527}$ is not promoted by the treatment, suggesting that the mechanism of activation of c-Src kinase by $HgCl_2$ is $Tyr^{527}$-independent. $Tyr^{527}$ independency of $HgCl_2$-mediated Src kinase activation was also shown by an experiment in which $HgCl_2$ induced activation of not only c-Src, but also $Tyr^{527}$-defective v-Src or c-Src from Csk-defective cells, which were constitutively activated. This latter finding suggests that both genetic and environmental elements together induce a second-step activation (superactivation) of the kinase. Addition of *N*-acetylcysteine as a reducing agent before $HgCl_2$ abolished all the activity of $HgCl_2$ for Src kinase activation. These results suggest that structural modification of Src proteins with $HgCl_2$, which reacts with free SH group(s) of cysteine(s) on Src proteins, causes their activation through a $Tyr^{527}$-independent mechanism.

More direct evidence of a redox-linked chemical modification-dependent Src kinase has been obtained from experiments testing the actions of NO-releasing chemicals on Src kinase (3). Exposure of immunoprecipitated c-Src proteins to *S*-nitroso-*N*-acetylpenicillamine (SNAP) or sodium nitroprusside (SNP) clearly promoted the catalytic activity of the kinase for both autophosphorylation at $Tyr^{416}$ and phosphorylation of enolase as an exogenously added substrate (Fig. 5A, left). The SNAP/SNP-mediated Src kinase activation was proved to be mediated by NO, because addition of hemoglobin and homo-

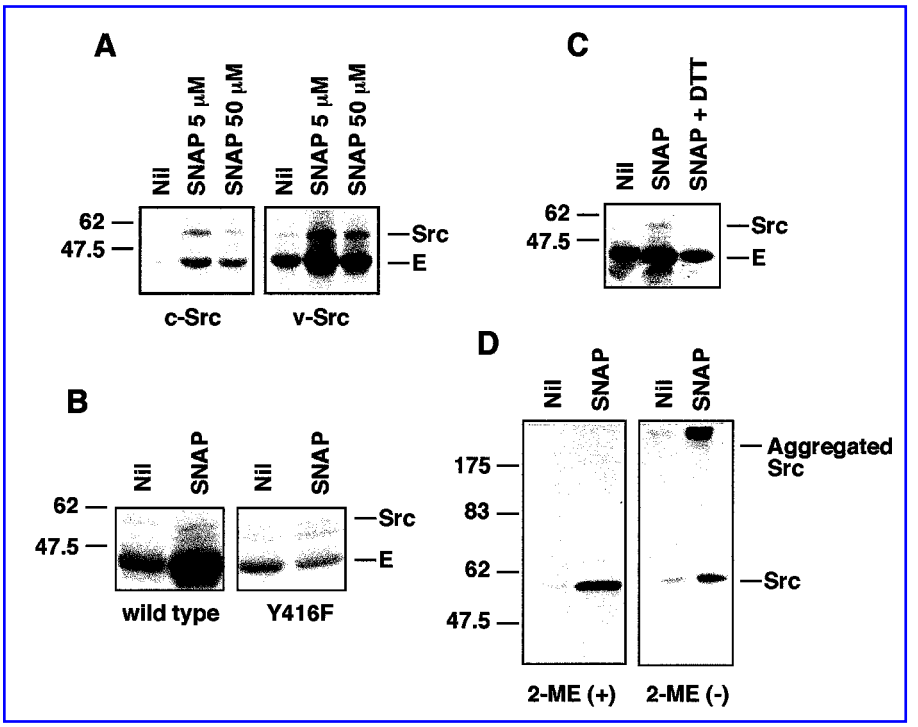


**FIG. 5. SNAP induces activation of Src kinases *in vitro* by a regulatory Tyr[527]-independent and protein cysteine SH group-oxidation-dependent mechanism.** Exposure of immunoprecipitated c-Src (**A**, left) or Y527-defective v-Src (**A**, right; **B**, left) proteins, but not the Y416F mutant of v-Src (**B**, right), to NO-releasing SNAP induced activation of the kinases for both autophosphorylation and phosphorylation of enolase (E). Treatment of SNAP-activated c-Src with a reducing reagent, DTT, reduced the catalytic activity to the background level (**C**). SNAP treatment of c-Src proteins induced their S-S-bonded polymerization in close association with autophosphorylation promotion, as demonstrated by analysis of Src proteins after an *in vitro* kinase assay under nonreducing (2ME−) conditions (**D**). 2ME, 2-mercaptoethanol. The positions of molecular mass marker proteins (kDa) are indicated on the left. Modified reproduction from Akhand *et al.* (3) with permission.

cysteine as scavengers of NO or $N_2O_3$ prevented the activation. The NO-mediated Src kinase activation also seemed to be Tyr[527]-independent, because NO induced a superactivation of Tyr[527]-defective v-Src (Fig. 5A, right). Moreover, v-Src with the Tyr[416] → Ala mutation was unable to be activated by NO, suggesting the absolute requirement of Tyr[416] in the mechanism of NO-mediated Src activation (Fig 5B). The fact that treatment of the SNAP-activated c-Src kinase with a reducing agent, DTT, abolished the promoted catalytic activity of the kinase (Fig. 5C) indicated that the SNAP-induced activation of Src kinase was redox-linked. Corresponding to this, we noticed that a small portion of Src proteins are normally polymerized through S-S bond formation between molecules and that NO promotes the S-S-bonded polymerization of Src proteins in close association with autophosphorylation promotion (Fig. 5D). It is speculated from these results that NO induced activation of Src kinase in association with an S-S-bonded crosslinkage of the kinase proteins.

## ACTIVATION AND SUPERACTIVATION OF RET KINASES

RET is a receptor PTK, of which the gene was originally isolated by Takahashi *et al.* (96) as a hybrid gene between c-*RET* and *RFP* from NIH3T3 cells that had been transfected with the DNAs isolated from human lymphocytic leukemia cells. c-RET is preferentially expressed on neural cells and plays an important role in neural cell development (81, 94). Physiologically, it is activated by glial-derived neurotrophic factor (GDNF) as a ligand of a complex of GDNF family receptors and c-RET (30). Germ-line point mutation or rearrangement of c-*RET* causes a number of hereditary or nonhereditary diseases, such as multiple endocrine neoplasia type 2A (MEN2A) and type 2B (MEN2B), papillary thyroid carcinoma (PTC), and Hirshsprung's disease, either promoting or impairing the RET kinase activity. RET-MEN2A has an amino-acid exchange at the cysteine in the cysteine-rich region of the extracellular domain of the RET protein. Asai *et al.* (4) and Santoro *et al.* (76) showed that RET proteins with MEN2A mutation are dimerized through intermolecular S-S bond formation and that this dimerization is associated with activation of RET kinase. On the other hand, RET proteins with RET-MEN2B mutation are thought to have altered conformation that is needed for activation of RET.

Recently, we have found that UV irradiation to c-*RET*-bearing NIH3T3 cells induces activation of c-RET (36, 37). Interestingly, RET proteins with RET-MEN2A or RET-MEN2B mutation that are constitutively activated due to genetic mutation were also affected by UV irradiation for a second-step activation (superactivation). This finding suggests that, in addition to the well known activity causing damage in DNA and the cell membrane, UV light modifies the function of the protein (RET) as an oncogene product, and this may be involved in the

mechanism of multistep tumor development. Human hereditary cancers, such as MEN and PTC, develop after a long latent period, even though mutant *RET* oncogenes exist in germ lines. We have established a metallothionein/*RET*-transgenic mouse line in which severe melanosis, benign melanocytic tumor, and malignant melanoma develop sequentially (28, 37). Again, in this model, it takes quite a long time for initial tumor development (2–4 months) and for the subsequent transition of the tumor to malignant melanoma (~10 months). Oncogenic mutant *RET* genes display activities to transform cells immediately upon transfection into cells cultured *in vitro*. This observation suggests that the oncogenic activity of mutant *RET* is suppressed *in vivo* by a yet unclarified regulatory mechanism, which is intact at an early age but fails with ageing. The ageing-dependent activity of environmental elements that causes failure of the regulatory mechanism may include damage to DNAs for recruiting other oncogenes or tumor suppressor genes in oncogenesis (107) or impairment of immunological defense against once-transfomed cells (42). We speculate that ageing-dependent stepwise superactivation of oncogene products (kinases) by environmental elements, such as UV light, can in part contribute to oncogenesis through failure of the oncogene-regulatory mechanism. In support of this hypothetical view, the expression and activation levels of oncogene *RET* in *RET*-transgenic mice, which rapidly increased soon after birth, probably in response to delivery stress, and became undetectably low 10–20 days after birth (35), recovered a few months later, when benign melanocytic tumors developed, and reached a peak level ~1 year after birth, when the tumors had become malignant. When these RET-transgenic mice received a single dose of 30 kJ/m$^2$ of UV-B light, the levels of both protein expression and specific catalytic activity per a constant protein amount of RET were increased, suggesting that superactivation of RET by UV irradiation occurs not only *in vitro,* but also *in vivo* (38). The line 192 of *RET*-transgenic mice regularly develops benign melanocytic tumors, but none of them become malignant melanoma. We gave repeated doses of a total of 4,950 kJ/m$^2$ of UV-B over a period of 28 weeks to the transgenic mice of this line in which benign melanocytic tumors had developed, and we examined the effects of the treatment on tumor growth and RET-linked signal transduction. We found that repeated UV irradiation caused transition of the benign melanocytic tumors to malignant melanoma and that this phenotypic change was associated with a marked increase in both protein expression and activation levels of RET kinase that accompanied extensive activation of mitogen-activated protein family kinases and transcriptional factor c-Jun as the elements potentially downstream of the activated RET (38). These results support the view that a new possible action mechanism of UV irradiation, in addition to other known mechanisms for oncogenesis, is oxidative chemical reaction-mediated superactivation of PTK whose catalytic activity is originally activated due to genetic mutation.

## MOLECULAR MECHANISMS OF RET KINASE ACTIVATION

We have examined molecular mechanisms of UV irradiation-mediated RET kinase activation. We noticed that a small percentage of RET proteins exist as S-S-bonded dimers in cells under normal condition and that UV irradiation to the cells promotes S-S-bonded dimerization of RET proteins (36, 37). Interestingly, the level of autophosphorylation per a constant protein amount of dimer RET was several times higher than that of monomer RET, suggesting a close relation between dimerization and activation of RET proteins. Once the S-S bond had been broken by the addition of the reducing reagent DTT, the level of catalytic activity of the kinase actually decreased to the background level. More intriguingly, extracellular domain-defective mutant RET (RET-PTC-1) proteins, a small percentage of which normally form dimers through an S-S bond, were also affected by UV irradiation for promotion of dimer formation (Fig. 6A, bottom), in association with an increase in the autophosphorylation level (Fig. 6A, top). It is speculated from these results that RET dimers are produced through S-S bond formation at the cysteine residues on the intracellular domain of the RET protein and that these cysteines are the target of UV irradiation for dimerization promotion.

We therefore attempted to identify the positions of cysteine residues in the intracellular domain of the RET protein as the target of UV irradiation. It is known that $Cys^{376}$ and $Cys^{365}$ of RET-PTC-1 or $Cys^{987}$ and $Cys^{976}$ of c-RET are widely conserved among a number of receptor-type and nonreceptor-type PTKs and some serine/threonine kinases (95, 100) (Fig. 7). We prepared mutant RET-PTC-1 or c-RET cDNAs of which the codons for $Cys^{376}$ ($Cys^{987}$), $Cys^{365}$ ($Cys^{976}$), or both had been replaced with Ala, and we transfected NIH3T3 cells with the mutant cDNAs (37, 97). Cells transfected with RET-PTC-1 with the codon $Cys^{365} \rightarrow$ Ala mutation (Fig. 6B1) or with c-RET with the codon $Cys^{976} \rightarrow$ Ala mutation (data not shown), which expressed RET proteins at the same levels as those of parental RET-PTC-1 or c-RET, displayed nearly normal kinase activity and responded to UV irradiation for promotion of dimerization and activation of RET proteins to almost the same extents as cells transfected with original RET-PTC-1 or c-RET did. In contrast, cells transfected with RET-PTC-1 with the codon $Cys^{376} \rightarrow$ Ala mutation (Fig. 6B2) or with c-RET with the codon $Cys^{987} \rightarrow$ Ala mutation (data not shown) had lower expression and activation levels of RET proteins than did those transfected with parental RET-PTC-1 or c-RET. The former cells barely responded to UV irradiation for dimerization promotion or activation. These results suggest that $Cys^{376}$ of RET-PTC-1 or $Cys^{987}$ of c-RET is crucial for the maintenance of normal levels of dimerization and activation of RET kinase and for the promotion of dimerization and activation in response to UV irradiation.

The RET proteins were also demonstrated to be promoted for S-S-bonded dimerization and activation by osmotic stress (97). In earlier experiments, Qin *et al.* (70) showed that osmotic stress-induced Syk activation requires receptor aggregation due to cell shrinkage and accumulation of ROS. We attempted to identify the submolecular target of RET to osmotic stress-induced ROS by use of mutant RET cDNAs-transfected cells. We found that osmotic stress preferentially affected $Cys^{376}$ of RET-PTC-1 or $Cys^{987}$ of c-RET for promotion of both dimerization and activation of the kinase. Unlike UV irradiation, however, osmotic stress provoked a second mechanism for RET kinase activation that is independent of $Cys^{376}$ of RET-PTC-1, possibly involving down-regulation of regulatory PTPases.

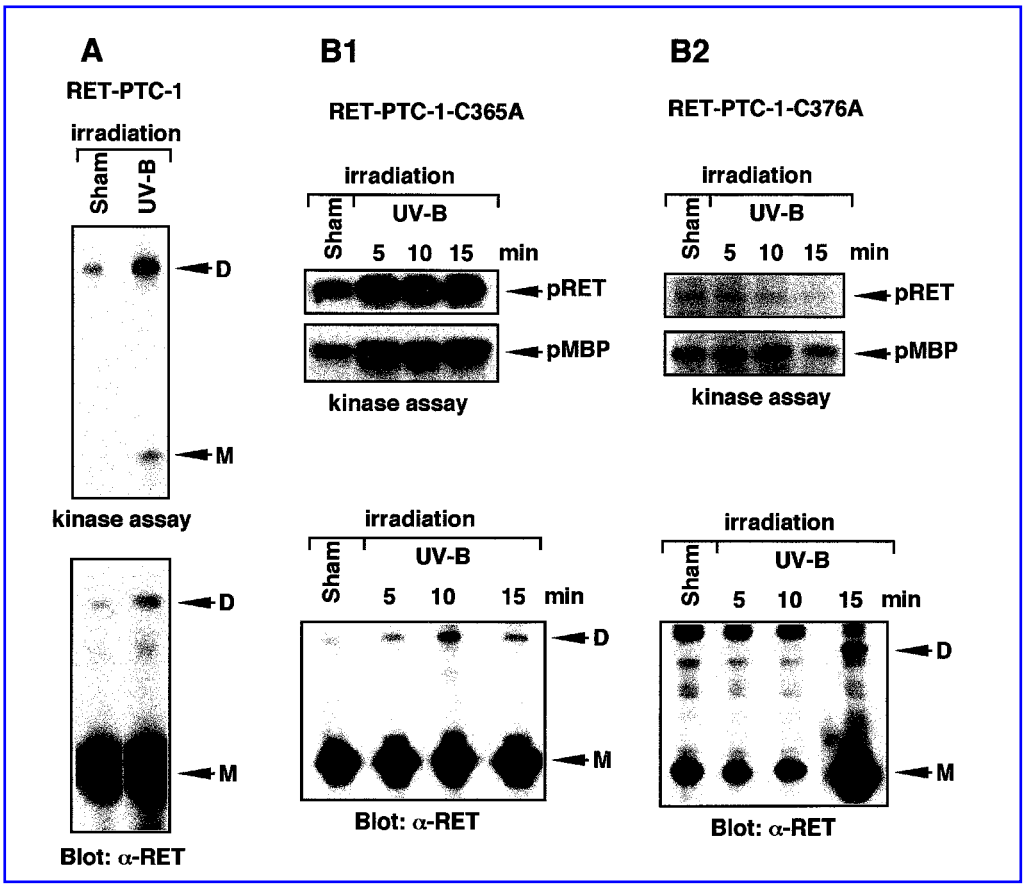


**FIG. 6. UV irradiation induces activation of RET kinases by targeting conserved cysteines in the C-terminal region of the catalytic domain for S-S bonded dimerization.** UV (UV-B) irradiation induced activation of the extracellular domain-deleted mutant RET-PTC-1. RET-PTC-1 proteins normally exist in the cell in an equilibrium of a large amount of monomers with free-cysteine SH groups and a small amount of S-S-bonded dimers (less than a few percent), as demonstrated by immunoblot assay with anti-RET antibody (**A**, bottom, sham irradiation). UV irradiation of the cells carrying RET-PTC-1 promoted S-S-bonded dimerization of RET proteins (**A**, bottom, UV-B irradiation) in close association with an increase in the autophosphorylation level in *in vitro* kinase assay (**A**, top). Note that the increase in the relative autophosphorylation level of dimers was >50 times greater than that of monomers. UV irradiation of cells carrying RET-PTC-1-C365A (**B1**), but not of cells carrying RET-PTC-1-C376A (**B2**), induced both activation in *in vitro* kinase assay (top) and S-S-bonded dimerization in immunoblot assay (bottom) of RET proteins. M, monomer; D, dimer; pRET, phosphorylated RET; pMBP, phosphorylated myelin basic protein. Modified reproduction from Kato *et al.* (37) with permission.

All of these experimental results suggest that a common mechanism operates for UV irradiation-induced and osmotic stress-mediated activations of RET kinase, through oxidation of the SH group on a specific cysteine that is located in the C-terminal region of the catalytic domain of RET.

In addition, v-Src, a nonreceptor-type PTK, carries 10 cysteines, and $Cys^{498}$ and $Cys^{487}$ of v-Src correspond to $Cys^{987}$ and $Cys^{976}$ of c-RET, respectively. Intriguingly, Senga *et al.* (83) showed that v-Src with a single mutation at $Cys^{498}$, but not at any of the other nine cysteine residues, showed a much lower catalytic activity than did the original. This suggested a general role of the specific conserved cysteine residue in the C-terminal region of the kinase domain in the catalytic activity of PTKs.

## TYPE-2 GLOBAL REGULATORY SWITCH

The results of our recent studies described above, in connection with the determined three-dimensional structures of Src family kinases (87, 105), suggest that changes in molecular conformation regulate the catalytic activity of PTKs either positively or negatively. Phosphorylation and dephosphorylation at specific tyrosine residues are known to act an on and off switches of catalytic activity of PTKs, respectively (5, 72, 98, 102). Redox-mediated structural modification might operate as another switch for protein function. Just like phosphorylation and dephosphorylation, the redox reaction may cause a reversible conformational change in proteins dynamically. Unlike the former, which is based on complementarity of two surface structures, however, the latter is linked to chemical reaction on SH groups of protein cysteines, potentially producing S-S bonds between two peptides or peptide portions bearing cysteine SH groups in the vicinity.

Many receptor-type and nonreceptor-type PTKs have a conserved core structure in the catalytic domain (87, 105). Intriguingly, the distance between the conserved tyrosine in the active segment of the kinase domain as the major autophosphorylation site and two conserved cysteines with a 10-

amino-acid interval in the C-terminal region of the kinase domain is the same for a large number of PTKs, including c-Src, Lck, Hck, IRK, and RET (Fig. 7). It is therefore possible that these conserved cysteines play a common role in the mechanism for PTK activation. The major autophosphorylation site ($Tyr^{416}$ of c-Src, for example) works as a local switch, phosphorylation of which alters the three-dimensional structure (conformation) of the activation segment of the kinase domain to make it suitable for transferring ATP from the ATP-binding site to tyrosine residue(s) on the substrate. The mechanism of destabilization of the closed conformation of the whole kinase protein is thought to work as a global regulatory switch that starts the initial phosphorylation of the tyrosine at the major autophosphorylation site as the local switch. In addition to known protein–protein interaction-dependent mechanisms for turning on or off the global regulatory switch (which we call type 1), which are based on protein-surface complementarity, we propose that oxidization of conserved cysteine residues in the kinase domain also induces destabilization of the closed conformation of the kinase protein, working as another type (type 2) of global regulatory switch (Fig. 8).

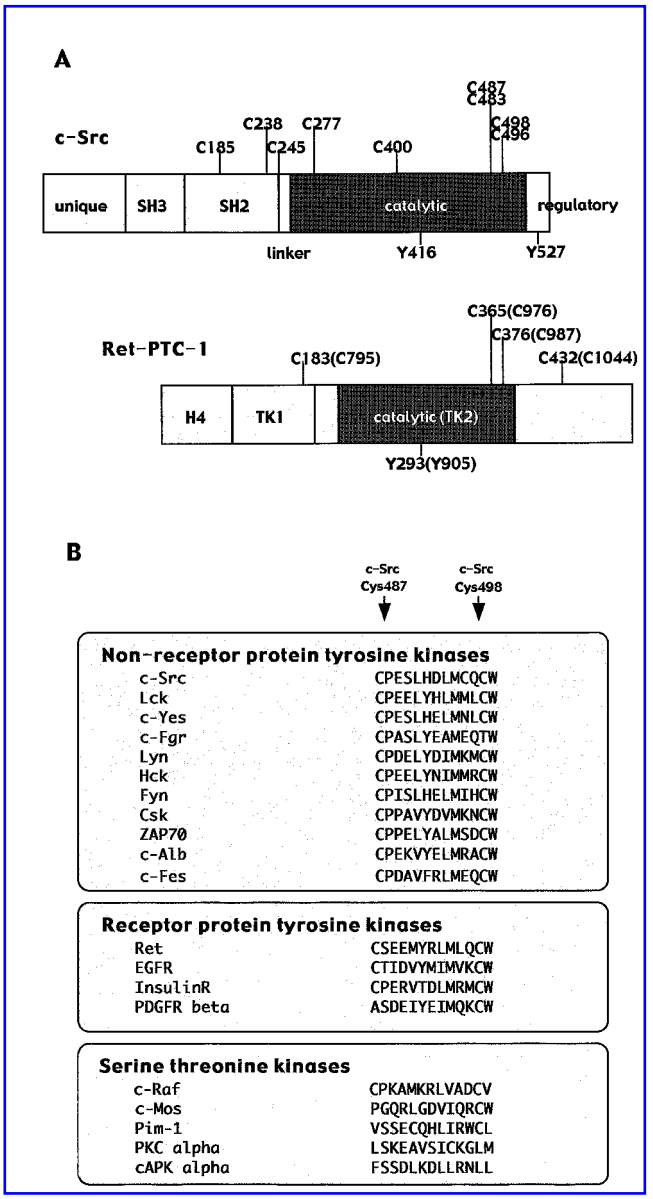


**FIG. 7. Structures of c-Src and RET-PTC-1 and positions of conserved cysteine residues on them.** (**A**) Structures of c-Src and RET-PTC-1 are illustrated together with the positions of all cysteine residues and tyrosine residues at the major autophosphorylation sites and at regulatory sites. Note that the distance in the amino-acid sequence between $Cys^{987}$ and $Tyr^{905}$ of c-RET is the same as that between $Cys^{498}$ and $Tyr^{416}$ of c-Src. (**B**) Distributions of two conserved cysteines ($Cys^{487}$ and $Cys^{498}$ of c-Src and equivalents) among various nonreceptor-type or receptor-type PTKs or serine/threonine kinases are shown. Modified illustrations from Veillette *et al.* (100) and Nakashima *et al.* (63).

Our conclusion is somewhat related to that of Schmid *et al.* regarding IRK (79, 80). They recently reported that signaling by insulin requires autophosphorylation of IRK at $Tyr^{1,158}$, $Tyr^{1,162}$, and $Tyr^{1,163}$ and that nonphosphorylated IRK is relatively inactive because the ATP-binding site of IRK is blocked by its activation loop. They further showed that phosphocreatine in combination with $H_2O_2$ serves as an alternative phosphate donor for ATP through a distinct binding site and that conversion of any of the four cysteine residues, $Cys^{1,056}$, $Cys^{1,138}$, $Cys^{1,234}$, and $Cys^{1,245}$, into sulfonic acid produces conformational changes that bring $Tyr^{1,158}$ into contact with $Asp^{1,083}$ and render the catalytic sites at $Asp^{1,132}$ and $Tyr^{1,162}$ accessible. The tyrosine residue corresponding to $Tyr^{1,158}$ of IRK is missing in RET or c-Src. Therefore, possibly related but not identical redox-linked global regulatory switches may work for activation of RET/c-Src and IRK. Our conclusion that a redox mechanism turns on or off the type 2 global regulatory switch in the PTK activation pathway corresponds to related observations that some PTK inhibitors are SH group-reactive (16) or have antioxidant activities (32, 86, 103).

## CELLULAR REDOX BALANCE AND SIGNAL CONTROL

It is known that the microenvironment inside a cell is normally maintained in a reducing condition by a high concentration of glutathione (GSH) in equilibrium with a low concentration of glutathione disulfide (GSSG), whereas the environment outside the cell is more oxidative. The structures of proteins to be secreted are normally completed in the rough endoplasmic reticulum by necessary S-S bond formation in the oxidative microenvironment (6). Protein SH groups might also be the primary target of a number of oxidative chemicals, alkylating agents, and heavy metals, occasionally producing an S-S bond or an S-X-S bond, potentially for major conformational changes in proteins that mimic the tertiary structure complementarity-dependent protein–protein (receptor–ligand) interaction. Many oxidative agents may induce a reduction in the cellular glutathione level through either direct consumption or induction of superoxide production. A reduction in the glutathione level would cause an increase in the ratio of the oxidized form to the reduced form of cellular protein cysteines, just as was observed with c-RET proteins after UV irradiation (36, 37). Because the level of superoxide production, which is low in normal cells, can be changed by attacks of ox-

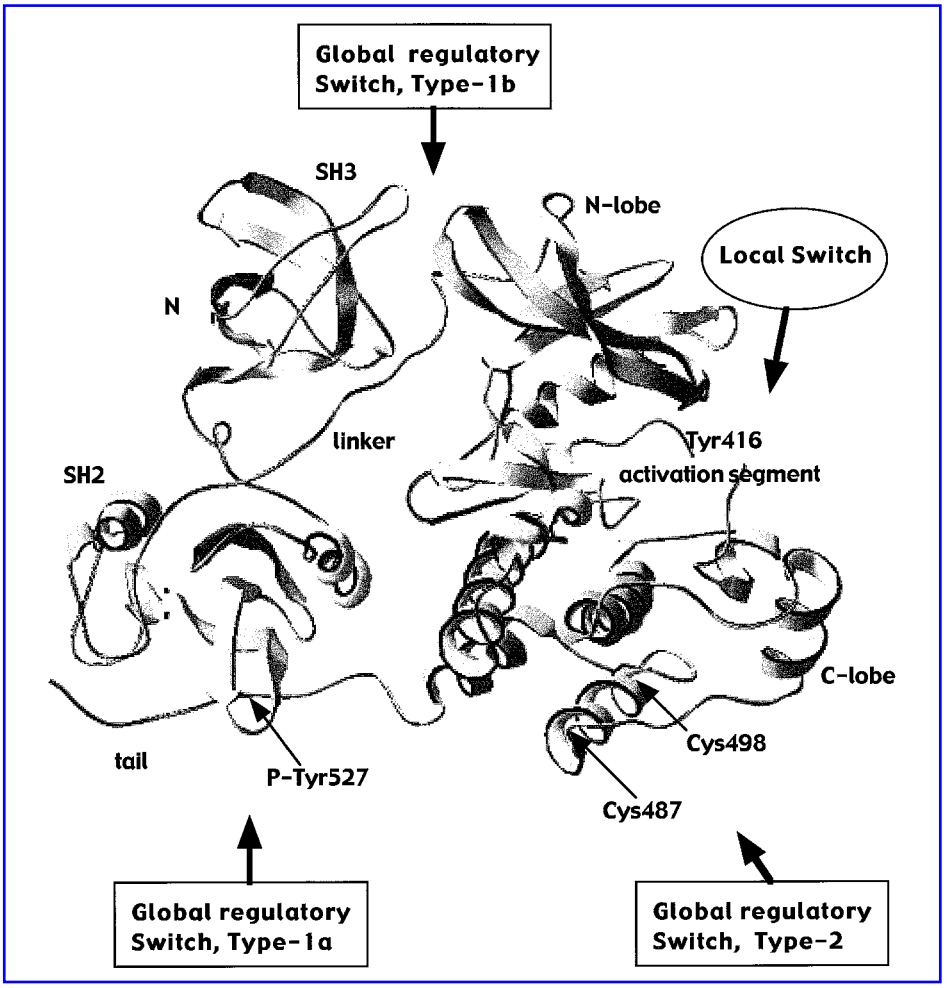


**FIG. 8. The three-dimensional structure of c-Src with the potential sites of speculative local and global regulatory switches.** Positions of $Tyr^{527}$, $Cys^{498}$, $Cys^{487}$, and $Tyr^{416}$ are indicated in the three-dimensional structure of c-Src originally reported by Xu *et al.* (105). According to Xu *et al.*, phosphorylation/dephosphorylation of $Tyr^{416}$ works as a local switch for activation/inactivation of the kinase, which is regulated by either of two global switches, one by phosphorylation/dephosphorylation of $Tyr^{527}$ (type 1a) and another by association/dissociation between the SH3 domain and the linker/N-terminal lobe of the catalytic domain (type 1b), either of which regulates the stability of the closed conformation of the molecule and thereby works as a global regulatory switch. It is speculated that oxidation of cysteine residues, particularly that of $Cys^{498}$ of c-Src or an equivalent, works as another category of global regulatory switch (type 2), again regulating the stability of the closed conformation of the molecule through a redox mechanism.

idative environmental elements, it could work as an environmental element-dependent signal that alters or modifies genetically regulated signal transduction. The environmental element-dependent signal seems to turn on or off the type 2 molecular switch, which could work independently of or in cooperation with the genetically regulated type 1 switch.

## CONCLUDING REMARKS

The suggested pathways of oxidative stress-mediated signal transduction are illustrated in Fig. 9. Both cell-surface proteins (Thy-1, for example, in the figure) and intracellular domains of PTKs (Src/Lck, RET) and PTPases can be the targets of oxidative molecular modification. Oxidative modification of cell-surface proteins triggers a membrane raft integrity-dependent mechanism for PTK activation accompanying ROS ($O_2^-$) production, which may affect intracellular domains of PTKs and PTPases. Oxidation of some conserved cysteine(s) of PTKs induces conformational change that may turn on a new (type 2) global regulatory switch at the first stage, leading to phosphorylation of tyrosine at the autophosphorylation site as the local switch. Inactivation of PTPases by oxidation may not turn on the local switch by itself, but probably works at the second stage to keep the level of autophosphorylation/activation of once-autophosphorylated/activated PTKs at the first stage.

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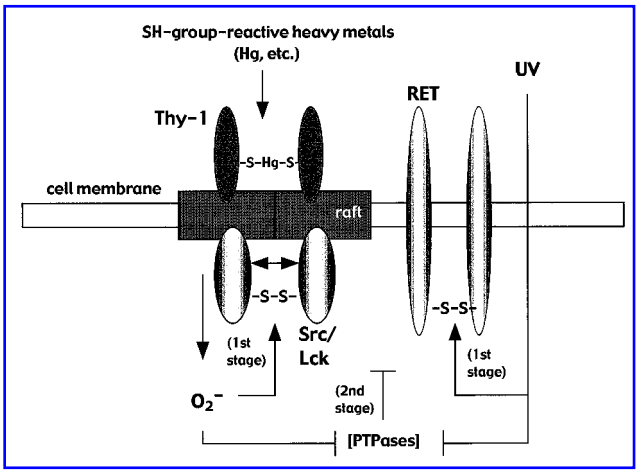


**FIG. 9. Summary of the possible mechanisms of oxidative stress-mediated activation of PTKs.** At least two target sites of oxidative stress for signal-transducing structural modification are postulated on the basis of recent study results. One is cysteine SH groups on GPI-anchoring and transmembrane cell-surface proteins, and crosslinking of two proteins through an S-S bond or S-X-S bond induces clustering of membrane rafts, which promotes interaction between PTK proteins for mutual phosphorylation and activation. Another is cysteine SH groups on PTKs and/or PTPases, the oxidation of which induces modification of the molecular conformation for autophosphorylation/activation of PTKs at the first stage and inactivation of regulatory PTPases for keeping the once-elevated autophosphorylation/activation levels of PTKs at the second stage. These intracellular cysteine SH groups are targeted directly by oxidative agents that penetrate the plasma membrane or by ROS produced as a second messenger following cell-surface receptor/membrane raft clustering.

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## ABBREVIATIONS

DTT, dithiothreitol; EGFR, epidermal growth factor receptor; GDNF, glial-derived neurotrophic factor; GPI, glycosylphosphatidylinositol; $H_2O_2$, hydrogen peroxide; IRK, insulin receptor kinase; MEN2A and MEN2B, multiple endocrine neoplasia type 2A and type 2B, respectively; NO, nitric oxide; PTC, papillary thyroid carcinoma; PTK, protein tyrosine kinase; PTPase, protein tyrosine phosphatase; redox, oxidation and reduction; ROS, reactive oxygen species; SH, sulfhydryl; SH2 and SH3, Src homology 2 and 3, respectively; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; S-S, disulfide; UV, ultraviolet.

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Address reprint requests to:
*Izumi Nakashima*
*Department of Immunology*
*Nagoya University Graduate School of Medicine*
*65 Tsurumai-cho*
*Showa-ku*
*Nagoya 466-8550, Japan*

*E-mail:* inakashi@med.nagoya-u.ac.jp

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