Advances in Brief

Nuclear Signaling Induced by Ionizing Radiation Involves Colocalization of the Activated p56/p53<sup>lyn</sup> Tyrosine Kinase with p34<sup>cdc2</sup>

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Abstract

The Src-like protein-tyrosine kinase p56/p53<sup>lyn</sup> associates with cell membranes and transduces signals from activated cell surface receptors. In the present work, cell fractionation and confocal microscopy studies demonstrate expression of Lyn in the nucleus. We also demonstrate that exposure of intact cells to ionizing radiation is associated with selective activation of nuclear Lyn. Similar findings have been obtained following irradiation of purified nuclei. Immunoprecipitation studies of nuclear lysates demonstrate radiation-induced binding of Lyn to p34<sup>cdc2</sup>. Nuclear colocalization of Lyn with Cdc2 has been confirmed by confocal microscopy. Other studies with glutathione S-transferase-Lyn fusion proteins demonstrate that the binding of Lyn to nuclear Cdc2 is associated with inhibition of Cdc2 activity. These findings suggest that the association of activated Lyn with Cdc2 in the nucleus may contribute to regulation of a DNA damage-dependent premitotic checkpoint.

Introduction

The cellular response to IR<sup>3</sup> includes cell cycle arrest, activation of DNA repair, and lethality (1). The signaling mechanisms responsible for the regulation of these events, however, remain unclear. Recent studies have shown that exposure of eukaryotic cells to IR is associated with activation of serine/threonine protein kinases and the induction of certain early response genes that code for transcription factors (2–5). Other studies have shown that levels of the tumor suppressor p53 protein increase during IR-induced arrest of cells in G<sub>1</sub> (6, 7). IR also activates protein kinase C and PTK activities (3, 8–10). Exposure of bacteria to DNA-damaging agents activates the recA protease and a cascade of events referred to as the SOS response (11, 12). Although DNA damage and arrest of replication forks are believed to initiate the bacterial survival response, studies in mammalian cells exposed to UV light have supported the involvement of Src family PTKs (13). Because the Src-like PTKs associate with the inner surface of the cell membrane and transduce signals from activated cell surface receptors (14), these findings have suggested that mammalian cells respond to certain agents with induction of signals from the cell membrane rather than the nucleus.

A member of the Src family, p56/p53<sup>lyn</sup>, contains SH2 and SH3 domains and an amino-terminal sequence, which, when myristoylated, serves as a membrane localization signal (14). In B lymphocytes, Lyn associates with membrane-bound IgM and participates in antigen-mediated signaling (15, 16). Engagement of the B-cell antigen receptor induces activation of Lyn and its association with phosphatidylinositol 3-kinase, GTPase activating protein, and phospholipase Cγ2 (17). Other studies have demonstrated that Lyn associates with gangliosides (18) and glycoporphatidylinositol-anchored cell surface molecules (19, 20). Recent work has demonstrated that exposure of human HL-60 myeloid leukemia cells to IR is associated with activation of Lyn and its association with p34<sup>cdc2</sup> (10, 21). The activity of p34<sup>cdc2</sup> in a complex with cyclin B is required for the G<sub>2</sub>-M checkpoint (22). Although previous studies have demonstrated that IR exposure is associated with inhibition of p34<sup>cdc2</sup> activity as a mechanism of G<sub>2</sub> arrest (23), the signaling events that regulate p34<sup>cdc2</sup> in irradiated cells remain unclear.

The present studies have examined the effects of IR treatment on activation of Lyn in human HL-60 myeloid leukemia cells. Cell fractionation and confocal microscopy studies demonstrate expression of Lyn in the nucleus and the cell membrane. The results further demonstrate activation of nuclear and not membrane Lyn in irradiated cells. We also demonstrate radiation-induced binding of nuclear Lyn to p34<sup>cdc2</sup>.

Materials and Methods

Cell Culture. HL-60 myeloid leukemia cells were grown in RPMI 1640 containing 15% heat-inactivated fetal bovine serum supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1 mM nonessential amino acids. Cells in logarithmic growth phase were suspended in complete RPMI 1640 with 0.5% fetal bovine serum for 18 h before irradiation. Irradiation was performed at room temperature using a GammaCell 1000 irradiator (Atomic Energy of Canada, Ottawa, Canada) under aerobic conditions with a 137Cs source emitting at a fixed dose rate of 13.3 Gy/min, as determined by dosimetry.

Subcellular Fractionation. Subcellular fractionation was performed as described (24). HL-60 cells were washed twice with cold PBS and resuspended in 1 ml hypotonic lysis buffer (1 mM EGTA, 1 mM EDTA, 10 mM β-glycerophosphate, 0.5 mM sodium orthovanadate, 2 mM MgCl<sub>2</sub>, 10 mM KCl, 1 mM DTT, 40 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin, pH 7.2). After swelling on ice for 30–45 min, the cells were disrupted by Dounce homogenization (25 strokes). The homogenate was layered onto 1 ml of 1 M sucrose in lysis buffer and centrifuged at 1600 × g for 15 min to pellet the nuclei. The supernatant above the sucrose cushion was collected and centrifuged at 150,000 × g for 30 min at 4°C to collect the soluble or cytoplasmic fraction. Purity of the fractions was monitored by immunoblot analysis with anti-PDGF receptor (SC-78, Santa Cruz Biotechnology, Santa Cruz, CA) and antihistone H1 (1492–519, Boehringer-Mannheim) antibodies.

Preparation of Cytoplasts. Enucleated cells were prepared as described (25). In brief, a discontinuous density gradient consisting 25, 19, 18, 17, 16, 15, 12.5 and 10% Ficoll was prepared from Ficoll stock solution (50% w/v in water) containing 10 μg/ml cytochalasin B and RPMI 1640. Cells (2 × 10<sup>6</sup>) were washed twice with PBS, resuspended in 2 ml of 10% Ficoll containing cytochalasin B, loaded onto the gradients, and spun at 25,000 rpm at 30°C for 1 h. Cytoplasts recovered from the 15–16% Ficoll interface were washed three times with fresh medium and incubated for 1 h at 37°C before treatment.
Cytoplasm purity was assessed by Wright-stained cytoblast preparations and propidium iodide staining of fixed cytoblasts. The cytoblast preparations were greater than 95% free of whole cells.

**PTK Autophosphorylation Assays.** Cells (2–3 × 10^5) were washed twice with ice-cold PBS and lysed in 2 ml of lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and 10 μg/ml of leupeptin and aprotinin). After incubation on ice for 30 min, insoluble material was removed by centrifugation at 14,000 rpm for 10 min at 4°C. Soluble proteins were precleared by incubation with 5 μg/ml rabbit antine IgG for 1 h at 4°C and then addition of protein A-Sepharose for 30 min. The supernatants were incubated with 2 μL of antihuman Lyn antibody (UBI, Lake Placid, NY) for 1 h at 4°C followed by 30 min of incubation with protein A-Sepharose. The immune complexes were washed three times with lysis buffer, once with kinase buffer (20 mM HEPES, pH 7.0, 10 mM MgCl₂ and 10 mM MnCl₂), and resuspended in 30 μl of kinase buffer containing 1 μCi/μl [γ-32P]ATP (3000 Ci/mmol; DuPont NEN, Boston, MA). The reaction was incubated for 10 min at 30°C and terminated by the addition of 2X SDS sample buffer. The proteins were resolved in 10% SDS-polyacrylamide gels, dried, and analyzed by autoradiography.

**Confocal Microscopy.** HL-60 cells were collected on coverslips and fixed with ice-cold methanol for 5 min. After incubation with polyclonal anti-Lyn antibody (Santa Cruz Biotechnology) followed by biotinylated-antirabbit antibody (Biomeda, Foster City, CA), cells were labeled with streptavidin-fluorescein (Biomeda). Finally, cells were stained with the DNA dye Hoechst 33258 (Molecular Probes, Eugene, OR) and viewed by the LSM 410 confocal laser scanning microscope (Zeiss, Germany). The 488- and 647-nm lines of the argon-krypton lasers were used for excitation.

**Production of GST-Lyn Fusion Protein.** The plasmid encoding a GST-Lyn (amino acids 1–243) fusion protein was provided by John Cambier (National Jewish Center for Immunology, Denver, CO; Ref. 17). The pGEX construct was transfected into Esherichia coli DH5α and the fusion protein was purified by affinity chromatography using glutathione-Sepharose beads (Pharmacia) as described (17) and equilibrated in lysis buffer.

**Fusion Protein Binding Assays and Immunoblotting.** Nuclear cell lysates were incubated with 5 μg immobilized GST or GST-fusion proteins for 2 h at 4°C. The protein complexes were washed three times with lysis buffer and boiled for 5 min in SDS sample buffer. The complexes were then separated by electrophoresis in 10% SDS-polyacrylamide gels and transferred to nitrocellulose paper. After blocking in 5% dry milk/PBS-0.05% Tween 20 for 1 h at room temperature, the filters were incubated with 1 μg/ml of a mouse anti-p34^{*}p^* monoclonal antibody, which is unreactive with other cyclin-dependent kinases (sc-54; Santa Cruz Biotechnology). After washing twice with PBS-0.05% Tween 20, the blots were incubated with antirabbit IgG peroxidase conjugate (Sigma Chemical Co., St. Louis, MO) and developed with the ECL detection system (Amersham, Arlington Heights, IL).

**Results and Discussion**

Previous studies have shown that treatment of myeloid leukemia cells with diverse classes of DNA-damaging agents is associated with activation of the Lyn tyrosine kinase (10, 26, 27). To determine the subcellular localization of IR-induced Lyn activity, we analyzed cytoplasmic, membrane, and nuclear fractions from control and irradiated cells. Immunoblot analysis with anti-Lyn antibody revealed expression of 56- and 53-kDa Lyn proteins in the cell membrane fraction and little if any Lyn in the cytoplasm (Fig. 1A). Reactivity of the anti-Lyn antibody with 56- and 53-kDa proteins was also detected in nuclei from both control and irradiated cells (Fig. 1A). This reactivity was completely blocked by preincubating anti-Lyn with a peptide used as an immunogen to generate the antibody (data not shown). To confirm the detection of Lyn in the nucleus, we used two other techniques for isolation of nuclei (24, 28) and obtained similar results (data not shown). To assess purity of the subcellular fractions, we used PDGF receptor as a membrane marker and histone H1 as a marker for the nuclear fraction. The absence of any detectable PDGF receptor in the nuclear histone H1-positive fraction supported the lack of contamination (data not shown; Ref. 29). We also used confocal microscopy of intact cells to avoid potential contamination of nuclear isolates during cell fractionation procedures. Using this approach, there was significant staining of cell membranes, as well as nuclei, with the anti-Lyn antibody (Fig. 1B). The localization of Lyn to the nucleus is not limited to HL-60 cells because this PTK has also been detected in the nuclei of U-937, HeLa, and Rat-2 cells (29, 30). Because these findings collectively supported nuclear expression of Lyn, we asked whether cell membrane- or nuclear-associated Lyn is activated by IR exposure. Anti-Lyn immunoprecipitates from control and IR-induced cell fractions were analyzed for increases in phosphotyrosine by immunoblotting with an anti-P-Tyr antibody (Fig. 1C). The results demonstrate an increase in tyrosine-phosphorylated Lyn in nuclei of irradiated cells (Fig. 1C). In other studies, anti-Lyn immunoprecipitates were incubated with [γ-32P]ATP and phosphorylated protein was analyzed by 10% SDS-PAGE and autoradiography. There was no detectable increase in IR-induced autophosphorylation of Lyn associated with the cell membrane or cytoplasmic fractions (Fig. 1D). In contrast, there was a 3-fold increase in Lyn activity in nuclei from irradiated cells as compared to control cells (Fig. 1D).

Other than the cell membrane-associated forms of Lyn, little is known about the intracellular localization of this PTK. The available evidence indicates that, with the exception of -Abl, the c-Src-related kinases are predominantly attached to the inner surface of the cell membrane, although lower levels may associate with the endoplasmic reticulum, juxtanuclear membrane, and nuclear matrix (30, 31). In contrast, the c-Abl PTK contains additional DNA-binding sequences and is found primarily in the nucleus (32, 33). Because these sequences are not present in Lyn, nuclear localization of this PTK appears to be unique among Src-like kinases. Whatever the mechanism for nuclear expression of Lyn in irradiated cells, IR-induced activation of this kinase could occur in an extra-nuclear location and involve translocation to the nucleus. To address this possibility, we assayed cytoplasts and purified nuclei for IR-induced Lyn activation. Lysates from control and irradiated cytoplasts were subjected to immunoprecipitation with anti-Lyn, and the immunoprecipitates were assayed for autophosphorylation. Under these conditions, analysis of irradiated cytoplasts failed to demonstrate an increase in Lyn activity (Fig. 2A). However, purified nuclei that were exposed to IR exhibited a 3–4-fold increase in Lyn autophosphorylation and no detectable change in Lyn protein (Fig. 2B). These findings suggested that the nuclear form of Lyn is activated by IR exposure.

The cellular response to IR includes activation of DNA repair and arrest at G2-M phase. The transition of cells from G2 to M phase requires the activity of a complex between p34^{*}p^* and cyclin B (22). Regulation of the G2-M transition by Cdc2 activity is important for the repair of DNA damage before entry into mitosis (34). However, the precise mechanisms responsible for the regulation of Cdc2 in the presence of DNA damage remain unclear. Recent studies have demonstrated that IR induces tyrosine phosphorylation of Cdc2 (21) and that Lyn associates with Cdc2 in irradiated cells (10). Cdc2 resides in the cytoplasm and translocates to the nucleus after association with cyclin B (35). The association between activated Lyn and Cdc2 could therefore occur in the cytoplasm, although the present findings that Lyn is activated in the nucleus suggests that the binding of Lyn to Cdc2 occurs as a nuclear event. To address this issue, we used...
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Fig. 1. Localization of Lyn in HL-60 cells. A. HL-60 cells were treated with 200 cGy IR and harvested at 5 min. Equal amounts of protein from cytosol, membrane, and nuclei were immunoprecipitated with affinity-purified anti-Lyn antibody. The immunoprecipitated proteins were resolved by 10% SDS-PAGE, transferred to nitrocellulose, and subjected to immunoblotting with anti-Lyn. Reactivity was determined by chemiluminescence. B. HL-60 cells were fixed with ice-cold methanol for 5 min. After incubation with polyclonal anti-Lyn antibody followed by biotinylated antirabbit antibody, cells were labeled with streptavidin-fluorescein. Cells were then stained with the DNA dye Hoechst 33258 and viewed by the LSM 410 confocal laser scanning microscope (×256). C. anti-Lyn immunoprecipitates from cytosolic (Cyto), membrane (Mem), and nuclear (Nuc) fractions of control and IR-treated (200 cGy) cells were subjected to 10% SDS-PAGE and assayed for reactivity with anti-P-Tyr. D. anti-Lyn immunoprecipitates from cytosolic, membrane, and nuclear fractions were washed three times with lysis buffer and resuspended in kinase buffer containing 1 μCi/μL [γ-32P]ATP. The reaction was incubated for 10 min at 30°C and terminated by the addition of sample buffer. The proteins were resolved by 10% SDS-PAGE and analyzed by autoradiography.

confocal microscopy to ask whether Lyn and Cdc2 colocalize in the nucleus. Staining of irradiated cells with anti-Lyn demonstrated nuclear reactivity, which was blocked by preincubation of the antibody with peptide used as the immunogen (Fig. 3, A and B). In addition, reactivity of anti-Lyn and anti-Cdc2 demonstrated nuclear colocalization of the two signals (Fig. 3C). Similar findings were obtained in control and irradiated cells. Thus, although Lyn and Cdc2 appear to colocalize in the nucleus, a signal induced by IR treatment may be required for the association between these molecules.

To confirm nuclear interaction of Lyn and Cdc2, we subjected nuclear lysates from control and irradiated cells to immunoprecipitation with anti-Cdc2. Immunoblot analysis of the immunoprecipitates from control cells demonstrated a low level of anti-Lyn reactivity, whereas detection of the 56- and 53-kDa proteins was increased in the nuclear extracts from irradiated cells (Fig. 4A). Reprobing the filter with anti-P-Tyr demonstrated IR-induced increases in tyrosine phosphorylation of the 56- and 53-kDa proteins as compared to that obtained for control cells (data not shown). This interaction between Lyn and Cdc2 was further examined with a GST-Lyn fusion protein. Analysis of the adsorbates from nuclear extracts of control and irra-

Fig. 2. Activation of nuclear Lyn by IR. A. cytoplasts from HL-60 cells were irradiated (200 cGy), lysed, and subjected to immunoprecipitation with anti-Lyn antibody. The immunoprecipitates were assayed for autophosphorylation in the presence of [γ-32P]ATP. B. nuclei from HL-60 cells were irradiated with 200 cGy and harvested at 5 min. Lysates from control and IR-treated nuclei were immunoprecipitated with anti-Lyn antibody. One aliquot of the immunoprecipitates was monitored for autophosphorylation (upper panel). The other aliquot was subjected to immunoblotting with anti-Lyn (lower panel).
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Fig. 3. Nuclear localization of Lyn. A. HL-60 cells were layered on coverslips by cytospin, fixed, and incubated with affinity-purified anti-Lyn antibody followed by incubation with biotinylated antirabbit antibody and streptavidin-fluorescein. B. anti-Lyn antibody was incubated with a 20-fold excess of Lyn peptide (residues 195-215) at 37°C for 1 h. Immunofluorescence was then assayed with this antibody-peptide mixture. C. For double labeling, cells were incubated with a mixture of polyclonal anti-Lyn and monoclonal anti-Cdc2 antibodies followed by secondary antibodies (antirabbit-Cy5 conjugated and biotinylated antimouse) and streptavidin-fluorescein. Lyn staining is shown in red and Cdc2 staining in green. Colocalization appears in yellow. Bar, 10 μm.

Fig. 4. Association of nuclear Lyn with Cdc2. A, nuclear lysates from control and IR-treated cells were subjected to immunoprecipitation with preimmune rabbit serum (PIRS) or anti-Cdc2. The proteins were resolved by 10% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-Lyn. B, nuclear lysates from control and IR-treated cells were incubated with GST (Lanes 1 and 2) or GST-Lyn (full length; Lanes 3 and 4) immobilized on glutathione-Sepharose. The adsorbed proteins were separated by 10% SDS-PAGE and subjected to immunoblotting with anti-Cdc2. C and D, HL-60 cell lysates were immunoprecipitated with anti-Cdc2. The immune complexes were incubated with GST or GST-Lyn for 30 min at 4°C. After washing, the immune complexes were suspended in kinase buffer and incubated with 0.1 μg/μl histone H1 (C) or GST-pp60c-src (amino acids 1-258; D) and [γ-32P]ATP. Phosphorylation was assessed by SDS-PAGE and autoradiography (upper panels). The presence of equal amounts of substrate was assessed by Coomassie blue staining of the gel (lower panels).

Fig. 4B. A peptide used to generate the anti-Cdc2 antibody blocked detection of the 34-kDa signal (data not shown). The effects of Lyn binding to Cdc2 were examined by incubating anti-Cdc2 immunoprecipitates with GST-Lyn, washing, and then assaying for Cdc2 kinase activity. Using this approach, phosphorylation of histone H1 by Cdc2 was inhibited by interaction of Cdc2 and GST-Lyn (Fig. 4C). Similar findings were obtained when the NH2-terminal region (amino acids 1-258) of pp60c-src, which contains three Cdc2 phosphorylation sites (36), was used as the substrate (Fig. 4D).

The Src-like kinases are regulated by phosphorylation of carboxy-terminal tyrosine residues (37). Dephosphorylation of these sites by tyrosine phosphatases activates certain members of the Src family (38). Other studies have indicated that phosphorylation of pp60c-src by Cdc2 at three amino-proximal serine/threonine residues stimulates carboxy-terminal tyrosine dephosphorylation and mitotic activation of c-Src (36). Similar Cdc2-dependent mechanisms could be responsible for activation of Lyn in nuclei of irradiated cells, although there are no consensus sequences for Cdc2 phosphorylation in the amino-proximal region of Lyn. Indeed, binding of Lyn to Cdc2 appears to be sufficient to inhibit Cdc2 activity, and thus, the association of these proteins may block Cdc2-mediated signals. Cdc2 activity is inhibited by phosphorylation on Tyr-15 (39, 40), and recent studies have demonstrated that this site serves as a substrate for Lyn in vitro (10, 26). Alternatively, binding of Lyn to Cdc2 may prevent the interaction of Cdc2 with other proteins, such as pp60c-src, that participate in mitosis. The association of Lyn and Cdc2 in nuclei of irradiated cells could in these or potentially in other contexts contribute to regulation of a DNA damage-dependent premitotic checkpoint.
The signal responsible for IR-induced activation of Lyn and binding of Lyn to Cdc2 is presumably DNA damage. Although IR also induces formation of reactive oxygen intermediates, the findings that 
a) the radical scavenger N-acetylcysteine has no detectable effect, and 
b) hydrogen peroxide treatment is not associated with activation of Lyn, support a reactive oxygen intermediate-independent mechanism (10). 
Moreover, the finding that diverse alkylating agents induce activation of Lyn (26) supports a common mechanism involving DNA damage. 
Other studies have indicated that the activation of Lyn by genotoxic agents is not confined to cells in G2. In this context, the arrest of cells in G1-S phase by ara-C is associated with the interaction of activated Lyn with Cdc2 (27). Recent work has demonstrated that the c-Ab1 tyrosine kinase is also activated in the stress response to IR and certain other DNA-damaging agents (3, 41, 42). The findings that Lyn and c-Ab1 are detectable in a nuclear complex^ and that both are activated by DNA damage has supported a potential role for these PTKs in coordinating responses to genotoxic stress.

References

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