Interaction between Protein Kinase C δ and the c-Abl Tyrosine Kinase in the Cellular Response to Oxidative Stress*

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Xiangao Sun, Frank Wu, Rakesh Datta, Surender Kharbanda, and Donald Kufe‡

From the Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115

Protein kinase C (PKC) isoforms are phosphorylated on tyrosine in the response of cells to oxidative stress. The present studies demonstrate that treatment of cells with hydrogen peroxide (H_2O_2) induces binding of the PKCδ isoform and the c-Abl protein-tyrosine kinase. The results show that c-Abl phosphorylates PKCδ in the H_2O_2 response. We also show that PKCδ phosphorylates and activates c-Abl in vitro. In cells, induction of c-Abl activity by H_2O_2 is attenuated by the PKCδ inhibitor, rottlerin, and by overexpression of the regulatory domain of PKCδ. These findings support a functional interaction between PKCδ and c-Abl in the cellular response to oxidative stress.

The protein kinase C (PKC) family of serine/threonine kinases consists of at least 12 isoforms that possess conserved catalytic domains (1, 2). The isoforms have been divided into conventional PKC, novel PKC, and atypical PKC subgroups on the basis of differences in their regulatory domains. The ubiquitously expressed PKCδ is a member of the nPKC subgroup and is activated by phorbol esters/diacylglycerol in a calcium-independent manner (3–5). Phorbol ester treatment of cells overexpressing PKCδ is associated with growth arrest (6, 7). Other studies have demonstrated that PKCδ inhibits growth by suppressing cyclin G1 expression (8). PKCδ is cleaved by caspase 3 at the third variable region (V3) to a 38-kDa regulatory domain (RD) and a 40-kDa catalytically active fragment in cells induced to undergo apoptosis (9–11). The finding that overexpression of the PKCδ catalytic fragment is associated with chromatin condensation, nuclear fragmentation, and lethality has supported a role for cleavage of PKCδ in induction of apoptosis (11). The association of PKCδ with growth arrest and apoptosis is in concert with findings that PKCδ confers a potential tumor suppressor function (12).

Diverse signals have been associated with phosphorylation of PKCδ on tyrosine. Activation of the platelet-derived growth factor or epidermal growth factor receptors induces tyrosine phosphorylation of PKCδ (13, 14). Transformation by Ras or v-Src also results in phosphorylation of PKCδ on tyrosine (15, 16). Other studies have demonstrated that PKCδ is phosphorylated on tyrosine and activated in cells treated with hydrogen peroxide (H_2O_2) (17, 18). In vitro, PKCδ is tyrosine-phosphorylated by c-Src (14, 16, 19), c-Fyn (13, 14), and growth factor receptors (13, 14). The effects of tyrosine phosphorylation on the serine/threonine kinase activity of PKCδ, however, have been conflicting. Moreover, little information is available regarding the tyrosine kinases that directly phosphorylate PKCδ in cells.

Recent work has demonstrated that the c-Abl tyrosine kinase phosphorylates and activates PKCδ in the response of cells to DNA damage (20). In the present studies, we show that treatment of cells with H_2O_2 induces binding of PKCδ and c-Abl. The results also show that PKCδ contributes to the activation of c-Abl in the oxidative stress response.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections

COS7 cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) containing 10% heat-inactivated fetal bovine serum (Sigma), 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Vectors expressing full-length FLAG-PKCδ and FLAG-PKCδR (amino acids 1–330) were generated by subcloning polymerase chain reaction-generated PKCδ fragments into the FLAG tag pcDNA3 vector. FLAG-PKCδR(K378R; designated K-R) was generated by site-directed mutagenesis (Stratagene). The FLAG-PKCδR vector was stably introduced into cells by SuperFect transfection reagent (Qiagen) and selection in 800 μg/ml G418 (Life Technologies, Inc). Transient cotransfections of FLAG-PKCδ or FLAG-PKCδR-K-R with empty pSR, pSR-c-Abl, or pSR-c-Abl(K-R) vector (21) were performed by the calcium-phosphate method.

Immunoprecipitation and Immunoblot Analysis

Cells were washed twice with ice-cold phosphate-buffered saline and lysed in lysis buffer (50 mM HEPES, pH 7.5, 0.5% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM NaF, 2 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of pepstatin, leupeptin, and aprotinin). Soluble proteins were incubated with anti-c-Abl antibody (sc-23; Santa Cruz), anti-PKCδ antibody (sc-937; Santa Cruz), or anti-FLAG (M2) antibody (Sigma) for 1 h and precipitated with protein G- or A-Sepharose beads (Santa Cruz) for an additional 1 h. The immune complexes were washed with lysis buffer, separated by SDS-PAGE, and then transferred to nitrocellulose paper. The filters were incubated with anti-c-Abl, anti-PKCδ, anti-FLAG, anti-Tyr(P) (4G10; Upstate Biotechology Inc.) or anti-PCNA (sc-56; Santa Cruz). The antigen-antibody complexes were visualized by enhanced chemiluminescence (ECL, Amersham Corp.).

In Vivo Kinase Assays

For c-Abl kinase assays, cell lysates were subjected to immunoprecipitation with anti-c-Abl as described (22). The protein complexes were washed and incubated in kinase buffer (20 mM HEPES, pH 7.4, 10 mM MgCl₂, 10 mM MnCl₂) containing 2.5 μCi of [γ-32P]ATP and GST-Crk-(120–225) for 20 min at 30 °C. PKCδ activity was assayed by incubating anti-PKCI immunoprecipitates in PKC kinase buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 20 μM ATP, 2 μCi of [γ-32P]ATP, and 200 μg/ml histone H1 (17) for 5 min at 30 °C. The reaction products were analyzed by SDS-PAGE and autoradiography.

In Vitro Kinase Assays

Phosphorylation of PKCδ by c-Abl—Kinase-active c-Abl prepared from baculovirus-infected insect cells (23) was incubated with heat-inactivated purified PKCδ (Calbiochem) and [γ-32P]ATP in kinase buffer for 20 min at 30 °C. Phosphorylated proteins were separated by
6% SDS-PAGE and analyzed by autoradiography. The PKCδ fragment encoding amino acids 473–537 was generated by polymerase chain reaction and ligated into the GST pGEX4T3 vector. The Tyr^512 and Tyr^523 sites were mutated to Phe by site-directed mutagenesis (Stratagene). The wild-type and mutant GST-PKCδ (473–537) fusion proteins were incubated with kinase-active c-Abl and [γ-32P]ATP. The products were analyzed by 12% SDS-PAGE and autoradiography.

Phosphorylation of c-Abl by PKCδ—Kinase-active PKCδ was incubated with heat-inactivated c-Abl and [γ-32P]ATP in kinase buffer. Phosphorylated proteins were separated by 6% SDS-PAGE and analyzed by autoradiography.

Activation of c-Abl by PKCδ—Kinase-active c-Abl was incubated with kinase-active PKCδ, GST-Crk (120–225), and [γ-32P]ATP in kinase buffer. Phosphorylated proteins were separated by 12% SDS-PAGE and analyzed by autoradiography.

RESULTS AND DISCUSSION

Previous work has demonstrated that c-Abl interacts with PKCδ in the cellular response to genotoxic stress (20). To assess whether the oxidative stress response involves an interaction between c-Abl and PKCδ, we cotransfected COS7 cells with FLAG-tagged PKCδ and c-Abl. Analysis of anti-FLAG immunoprecipitates by immunoblotting with anti-c-Abl demonstrated little association of PKCδ and c-Abl in control cells (Fig. 1A). By contrast, treatment with H2O2 resulted in the coprecipitation of FLAG-PKCδ and c-Abl (Fig. 1A). To confirm these findings, anti-c-Abl immunoprecipitates from the transfected cells were analyzed by immunoblotting with anti-PKCδ. The results demonstrate that H2O2 treatment induces the binding of PKCδ and c-Abl (Fig. 1B). Similar studies were performed on nontransfected cells to assess the interaction between endogenous PKCδ and c-Abl. Analysis of anti-PKCδ immunoprecipitates by immunoblotting with anti-c-Abl demonstrated binding of PKCδ and c-Abl in H2O2-treated, but not untreated, cells (Fig. 1C). Further support for H2O2-induced binding of PKCδ and c-Abl was obtained by immunoblot analysis of anti-c-Abl immunoprecipitates with anti-PKCδ (Fig. 1D). These results demonstrate that H2O2 induces the association of PKCδ and c-Abl.

To determine whether the kinase functions of PKCδ or c-Abl are involved in H2O2-induced binding of these proteins, we cotransfected cells with c-Abl and kinase-active FLAG-PKCδ or kinase-inactive FLAG-PKCδ(K-R). The results demonstrate that, although H2O2 treatment is associated with binding of c-Abl to PKCδ, the interaction is substantially decreased with PKCδ(K-R) (Fig. 2A). Likewise, H2O2-induced binding of FLAG-PKCδ to wild-type c-Abl was greater than that found with kinase-inactive c-Abl(K-R) (Fig. 2B). These results indicate that activation of both PKCδ and c-Abl contributes to the association of these proteins in the response to H2O2. To determine whether H2O2 induces c-Abl-mediated tyrosine phosphorylation of PKCδ, we cotransfected cells with FLAG-PKCδ and empty vector (pSR), c-Abl, or c-Abl(K-R). As indicated, H2O2-induced binding of FLAG-PKCδ to c-Abl was obtained by immunoblot analysis of anti-c-Abl immunoprecipitates with anti-PKCδ (Fig. 2C). These results demonstrate that H2O2 induces the association of PKCδ and c-Abl.
Previous studies have demonstrated that H$_2$O$_2$ induces tyrosine phosphorylation of histone H1 was increased by incubating PKC. In addition, phosphorylation with that found with c-Abl alone (Fig. 3). These findings collectively support an interaction between PKC and c-Abl Tyrosine Kinase.

Reactive oxygen species have been implicated in the regulation of cell growth and apoptosis (25, 26). The intracellular signals that are activated by reactive oxygen species, however, are largely unknown. Certain insights have been made available from the finding that H$_2$O$_2$ treatment is associated with phosphorylation of PKCδ in vivo (17). The present studies indicate that c-Abl is in part responsible for tyrosine phosphorylation of PKCδ in the response to H$_2$O$_2$. In this context, H$_2$O$_2$ induces the binding c-Abl and PKCδ. In addition, H$_2$O$_2$-induced tyrosine phosphorylation of PKCδ is attenuated in cells expressing the kinase-inactive c-Abl(K-R). The findings also demonstrate that c-Abl-mediated phosphorylation of PKCδ activates the PKCδ kinase function and that this effect occurs in the absence of lipid cofactors. The phosphorylation of PKCδ on Tyr512 and Tyr523, has been shown to be important for H$_2$O$_2$-induced activation (17). Our findings demonstrate that c-Abl phosphorylates PKCδ on Tyr512, but not Tyr523. Taken together, these findings indicate that PKCδ is phosphorylated by c-Abl and at least one other tyrosine kinase.

Previous studies have demonstrated that the c-Abl kinase is activated by DNA-damage (22). The present work demon-

![Fig. 3](image)

**Fig. 3.** Coactivation of PKCδ and c-Abl by kinase-dependent mechanisms in vitro. A, c-Abl, heat-inactivated (HI) c-Abl, PKCδ and heat-inactivated PKCδ were incubated as indicated with [γ-32P]ATP. The reaction products were analyzed by 6% SDS-PAGE and autoradiography. B, c-Abl and PKCδ were incubated as indicated with [γ-32P]ATP. The reaction products were analyzed by 6% SDS-PAGE and autoradiography. C and D, c-Abl and/or PKCδ were incubated as indicated in the presence of [γ-32P]ATP and GST-Crk-(120–225) (left) or histone H1 (right). The reaction products were analyzed by 12% SDS-PAGE and autoradiography.

![Fig. 4](image)

**Fig. 4.** PKCδ activates c-Abl in the response to H$_2$O$_2$. A, cells were treated with 3 mM H$_2$O$_2$ for 15 min. Anti-PKCδ immunoprecipitates were incubated with histone H1 and [γ-32P]ATP. The reaction products were analyzed by 12% SDS-PAGE and autoradiography. B, cells were pretreated with 10 μM rottlerin for 10 min and then exposed as indicated to 3 mM H$_2$O$_2$ for an additional 15 min. Anti-c-Abl immunoprecipitates were incubated with GST-Crk-(120–225) and [γ-32P]ATP. Reaction products were analyzed by 12% SDS-PAGE and autoradiography. C, COS7 cells were stably transfected to express the empty pcDNA3-FLAG vector or FLAG-PKCδRD. Lysates were subjected to IB with anti-FLAG and, as a control, anti-PCNA. D, the indicated cells were treated with 3 mM H$_2$O$_2$ for 15 min. Anti-c-Abl immunoprecipitates were incubated with GST-Crk-(120–225) and [γ-32P]ATP. Reaction products were analyzed by 12% SDS-PAGE and autoradiography.
strates that c-Abl is also activated by H₂O₂ and that this response is dependent on the PKCδ kinase function. The results support a model in which c-Abl is activated by PKCδ in response to oxidative stress, and in a potential feedback loop, c-Abl phosphorylates and thereby further activates PKCδ. PKCδ, like c-Abl, has been linked to the induction of apoptosis in the response of cells to genotoxic stress (9–11, 27). Other studies have shown that PKCδ is activated by serum stimulation and that phosphorylation of the PKCδ activation loop is mediated by PDK1 (28). Thus, PKCδ may be functional in both pro- and anti-apoptotic pathways. In this context, PKCδ could represent a switch that determines cell fate, such that PKCδ confers anti-apoptotic signals following PDK1-mediated phosphorylation and pro-apoptotic signals as a consequence of c-Abl-mediated phosphorylation.

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