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Tyrosine Phosphatase CD45 Regulates Hydrogen Peroxide-Induced Calcium Mobilization in B Cells

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ABSTRACT

By taking advantage of established CD45-deficient DT40 cells, the roles of CD45 in oxidative stress signaling were investigated. Using *p*-nitrophenyl phosphate as substrate, it was found that CD45 constituted nearly 40% of the total protein-tyrosine phosphatase activity. Almost 90% of the phosphatase activity was rapidly inactivated upon hydrogen peroxide treatment. Hydrogen peroxide-induced tyrosine phosphorylation of cellular proteins and c-Jun N-terminal kinase activation were markedly enhanced in CD45-deficient cells relative to that in its parental cells. In comparison, hydrogen peroxide-induced inositol 1,4,5-trisphosphate production and Ca^{2+} mobilization were impaired in CD45-deficient DT40 cells. However, hydrogen peroxide-induced tyrosine phosphorylation of phospholipase Cγ2 (PLCγ2), phosphatidylinositol 3-kinase activity precipitated by anti-phosphotyrosine antibody, and activation of Bruton's tyrosine kinase appeared intact in CD45-deficient DT40 cells. This suggests that CD45 mediates the ability of hydrogen peroxide-activated PLCγ2 to hydrolyze its substrate via a mechanism independent of both tyrosine phosphorylation of PLCγ2 and phosphatidylinositol 3-kinase, as well as activation of Bruton's tyrosine kinase. Taken together, our observations demonstrated that, in addition to its negative regulatory or phosphatase activity, CD45 has a positive role in oxidative stress signaling. *Antioxid. Redox Signal.* 4, 481–490.

INTRODUCTION

CD45 is the major transmembrane tyrosine phosphatase of the immune system and is highly expressed by all hematopoietic cells (17, 48). The well described role for CD45 is that its phosphatase activity is required for activation of Src-family kinases associated with antigen receptors (48). The Src-family members have two critical tyrosine residues whose phosphorylation markedly affects their kinase activity. One of them is in the C-terminal portion of the molecule, and its phosphorylation by C-terminal Src kinase (Csk) diminishes kinase activity (3) by allowing this portion of the molecule to fold back and bind to the kinase SH2 domain to yield an inactive conformation (50, 51). On the other hand, CD45 dephosphorylates the C-terminal inhibitory site, opposing Csk action (26, 41, 52) and thereby resulting in activation of the Src-family kinases. CD45-deficient cells are unable to efficiently activate signaling in response to antigen or direct receptor crosslinking (18, 20, 52). Analysis of these cell lines demonstrates that the abnormality in antigen-receptor signaling correlates with a defect in activation of the Src-family kinases (7, 26, 41). Therefore, a rational explanation for the requirement of CD45 for antigen receptor signaling is that dephosphorylation of the inhibitory site in Src-family kinases is essential for kinase activation required to initiate signal transduction.

Reactive oxygen species (ROS) have emerged as physiological mediators of cellular responses. The production of ROS has been detected in a variety of cells stimulated with cytokines (25, 47), peptide growth factor (2, 44), or agonists of receptors with seven transmembrane spans (9). The ROS generated appeared to be required for mediating subsequent biochemical responses (2, 44). When exogenous hydrogen peroxide is applied to cells as one form of ROS, hydrogen

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peroxide can activate an array of nonreceptor-type and receptor-type protein-tyrosine kinases (PTKs) in a variety of cell systems (10, 11, 19, 32, 42, 43). Furthermore, hydrogen peroxide stimulates Ca^{2+} mobilization and tyrosine phosphorylation patterns in lymphocytes identical to those observed following antigen receptor activation (36, 37). However, roles of protein-tyrosine phosphatases (PTPs) in these processes are not totally clear. Hydrogen peroxide is a well known PTP inhibitor, and 3 m*M* hydrogen peroxide treatment can almost completely inhibit CD45 activity in intact cells (40). The molecular mechanism is believed to be through the specific and reversible oxidation of the thiolate anion of a cysteine residue in the PTP-reactive center to sulfenic acid (5), thereby blocking the formation of the phosphoryl-cysteine intermediate, which is a critical step in dephosphorylation (6). Therefore, hydrogen peroxide may function by inducing random tyrosine phosphorylation and subsequent biochemical responses as a consequence of the inhibition of PTPs. Consistent with this notion are the reports showing that CD45-deficient Jurkat cells (also called Wurzburg cells) grow faster than their parent cells (38), and hydrogen peroxide can strongly activate nuclear factor-κB (NF-κB) in Wurzburg cells, but only weakly in parental Jurkat cells (1). Moreover, hydrogen peroxide induces tyrosine phosphorylation of cellular proteins, including several PTKs, in a cell-free system, and this phosphorylation is reversible because it returns to the dephosphorylated state upon removal of hydrogen peroxide (16). In addition to its role as a PTP, CD45 may function as a docking protein, most likely via tyrosine phosphorylation. Pervanadate, a powerful PTP inhibitor, can oxidize catalytic cysteine in the catalytic center to cysteic acid, irreversibly down-regulating PTP activity (12). In a T-cell line overexpressing the activated form of Lck, pervanadate treatment leads to tyrosine phosphorylation of CD45 and its association with Lck and at least three (rasGAP, Grb2, and Vav) signaling molecules involved in the regulation of $p21^{ras}$ (21). CD45 also appears to positively regulate pervanadate-induced Ca^{2+} mobilization, which is exclusively from Ca^{2+} influx. In CD45-deficient Jurkat cells obtained by repeated fluorescence-activated cell sorting, pervanadate-induced Ca^{2+} mobilization is completely abolished, but the mechanism is not deciphered (14, 15). These cells also respond differently to hydrogen peroxide and pervanadate treatment as evidenced by NF-κB activation. CD45 inhibits hydrogen peroxide-induced NF-κB activation, but it is essential for pervanadate-induced NF-κB activation (1, 15). It should be pointed out that these CD45-deficient variants of the Jurkat T cells are not exactly CD45-deficient; rather they contain either low or no expression of CD45 cells. To avoid this disadvantage, a single CD45-deficient DT40 clone is established by targeted disruption of CD45 alleles (52). Using this CD45-deficient cell line, we investigated the role of CD45 in B-cell activation induced by hydrogen peroxide. We report here that, as expected, CD45 deficiency enhanced hydrogen peroxide-induced c-Jun N-terminal kinase (JNK) activation and tyrosine phosphorylation of cellular proteins. In comparison, hydrogen peroxide-induced inositol 1,4,5-trisphosphate (IP_3) generation and Ca^{2+} mobilization were inhibited, suggesting that CD45 has positive roles in oxidative stress signaling.

MATERIALS AND METHODS

Materials

RPMI 1640 medium and fetal bovine serum were purchased from GIBCO Inc. Protein A was from Calbiochem Corp. Fura 2-AM was from Molecular Probes. Anti-phosphotyrosine antibody (4G10), polyclonal anti-Bruton's tyrosine kinase (anti-Btk) antibody, and polyclonal anti-phospholipase Cγ2 (anti-PLCγ2) antibody were from Upstate Biotechnology Inc., Pharmingen (San Diego, CA, U.S.A.), and Santa Cruz Biotechnology, respectively. Enhanced chemiluminescence reagents were from Dupont. The assay kit for IP_3 production was from Amersham.

Cell culture and harvest

Establishment of CD45-deficient DT40 was performed as described previously (52). DT40 and DT40-derived cells were maintained in RPMI 1640 medium, supplemented with 10% (vol/vol) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified 95% air/5% CO_2 atmosphere. For experiments, cells were collected by centrifugation as previously described (29). Cells were stimulated by hydrogen peroxide at 37°C.

Preparation of cell extracts

Stimulated cells (1×10^7 cells/ml) were lysed in ice-cold lysis buffer (5 m*M* EDTA, 150 m*M* NaCl, 1% Triton X-100, 100 μ*M* Na_3VO_4, 2 m*M* phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 50 m*M* Tris, pH 7.4) following a short centrifugation step. Lysates were clarified by centrifugation at 16,000 *g* for 15 min at 4°C.

Assay of PTP activity

Phosphatase activity was measured using *p*-nitrophenyl phosphates as substrate. Aliquots of the lysates, derived from 5×10^5 cells, were added to an incubation mixture containing 50 m*M* sodium acetate, pH 5.5, and 5 m*M* *p*-nitrophenyl phosphate in a final volume of 600 ml. Reaction mixtures were incubated at 37°C for 10 min and stopped by addition of 600 ml of 1 *M* NaOH. Absorbance was measured at 410 nm, and the amount of *p*-nitrophenolate anion produced was calculated using the molar absorption coefficient (1.78×10^4 M^{-1} cm^{-1}). One unit of PTP activity was defined as 1 μ*M* *p*-nitrophenolate produced per minute. Phosphatase activity is calculated as (phosphatase activity without vanadate) − (phosphatase activity with vanadate) and is reported as milliunits per 10^4 cells.

Immunoblot analysis

Cell extracts or immunoprecipitates were resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred electrophoretically onto polyvinylidene difluoride membranes, and then immunoblotted with the indicated antibodies. Immunoreactive proteins were visualized by enhanced chemiluminescence.

Measurement of free cytosolic Ca^{2+} concentration

Ca^{2+} mobilization was measured using the fluorescent indicator, Fura 2, as previously described (29).

Measurement of IP_3 levels

After hydrogen peroxide stimulation, IP_3 in chicken B-lymphocytes was extracted by perchloric acid and measured with a highly specific D-*myo*-[^{3}H]IP_3 assay system (Amersham) as described by the supplier. This assay was based on the competition between unlabeled IP_3 and a fixed quantity of a high-specific-activity tracer [^{3}H]IP_3 for a limited number of binding sites on a specific and sensitive bovine adrenal binding-protein preparation.

Phosphatidylinositol 3-kinase (PI3K) assay

The cell extracts from treated or untreated cells were incubated with anti-phosphotyrosineantibody 4G10 for 30 min, followed by further incubation with protein A–agarose for 1 h. The immunoprecipitates were washed three times with lysis buffer, twice in buffer containing 10 m*M* Tris-HCl, pH 7.5, 100 m*M* NaCl, and 1 m*M* EDTA, and once in PI3K assay buffer (20 m*M* Tris-HCl, pH 7.5, 100 m*M* NaCl, 10 m*M* $MgCl_2$, 0.1 m*M* EGTA, 100 μM vanadate, 20 μM ATP, and 200 μM adenosine). After the last wash was removed, 10 μl of sonicated phosphatidylinositol substrate (1 mg/ml in 10 m*M* HEPES, pH 7.5, 3 $\times$ 20 s) was added to each sample, and samples were incubated for 10 min on ice. The reaction was carried out at room temperature for 20 min by adding 40 μl of PI3K assay buffer containing 10 μCi of [γ-^{32}P]ATP and then quenched with 100 μl of 1 *M* HCl. Phospholipids were extracted once with 200 μl of $CHCl_3$/methanol (1:1) using a bench-top Heracus Biofuge centrifuge (8,000 rpm $\times$ 3 min), and then the lower phase (organic phase) was transferred to new microtubes and extracted once with 160 μl of 1 *M* HCl/methanol (1:1). The organic phase was dried under nitrogen gas and resuspended in 10 μl of $CHCl_3$/methanol (1:1). Phosphorylated products were resolved on oxalate-impregnated[wetted by 1.2% potassium oxalate/methanol (1:1)] Silica 60 plates using $CHCl_3$/methanol/4 *M* NH_4OH (9:7:2) as solvent for ~2 h, and the gel was air-dried for ~10 min. Autoradiogram exposure was typically for <2 days. Radioactive spots, representing phosphatidylinositol 3-phosphate, were visualized and quantitated using the PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

RESULTS

Hydrogen peroxide inhibits PTP activity in DT40 cells

Cells were sonicated and total PTP activity was assessed in the broken cell preparations with *p*-nitrophenyl phosphate as the substrate. PTP activity was significantly lower in CD45-deficient cells compared with that in wild-type DT40 cells (Fig. 1A). The total PTP activity in CD45-deficient and wild-type DT40 cells was 29.2 and 47.0 mU/10^4 cells, respectively. Thus, CD45 activity represented ~38% of total PTP activity in DT40 cells.

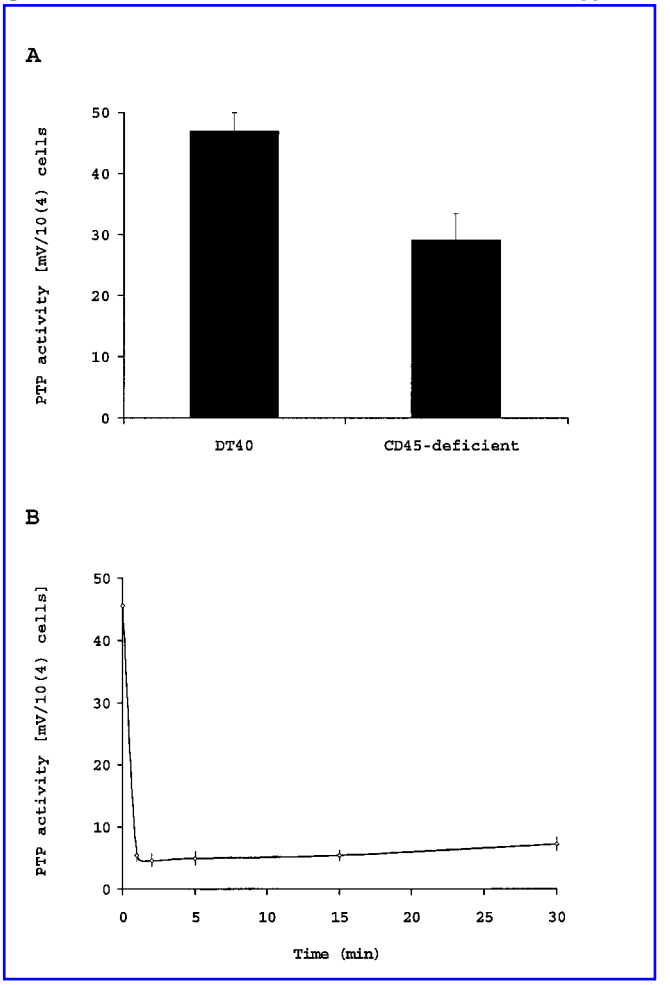

FIG. 1. Inhibition of PTPs in DT40 cells by hydrogen peroxide. (**A**) Percentage of CD45 activity in total PTP activities. Lysates from wild-type and CD45-deficient DT40 cells were assayed for PTP activity as described in Materials and Methods. (**B**) Time course of hydrogen peroxide inhibition of PTP activities. DT40 cells were stimulated with 5 m*M* hydrogen peroxide at the indicated time points. PTP was assayed as in A.

The effect of hydrogen peroxide exposure on the catalytic activity of total PTP in intact DT40 cells was evaluated. As shown in Fig. 1B, treatment of intact DT40 cells with 5 m*M* hydrogen peroxide inhibited almost 90% of the PTP activity in DT40 cells within 2 min. This inhibition remained nearly constant during the 30-min exposure.

Tyrosine phosphorylation of cellular proteins and JNK activation are enhanced in CD45-deficient DT40 cells following hydrogen peroxide stimulation

Because the steady-state level of protein tyrosine phosphorylation in intact cells is the result of the opposing activities of PTKs and PTPs, the effect of hydrogen peroxide on protein tyrosine phosphorylation in CD45-deficient DT40 cells was examined to determine if the accumulation of tyrosine-phosphorylated proteins in hydrogen peroxide-treated

cells was mediated by the inhibition of CD45 activity (Fig. 2A). Stimulation by hydrogen peroxide led to an increase in tyrosine phosphorylation in both cell types. The tyrosine-phosphorylated protein pattern resulting from hydrogen peroxide treatment appeared to be similar, but the phosphorylation intensity was markedly enhanced in CD45-deficient DT40 cells compared with that observed in parental cells. These results suggest that the stimulatory effect of hydrogen peroxide on protein tyrosine phosphorylation in intact DT40 cells was not due to the inhibition of CD45 activity; rather CD45 seemed to negatively regulate hydrogen peroxide-induced protein tyrosine phosphorylation.

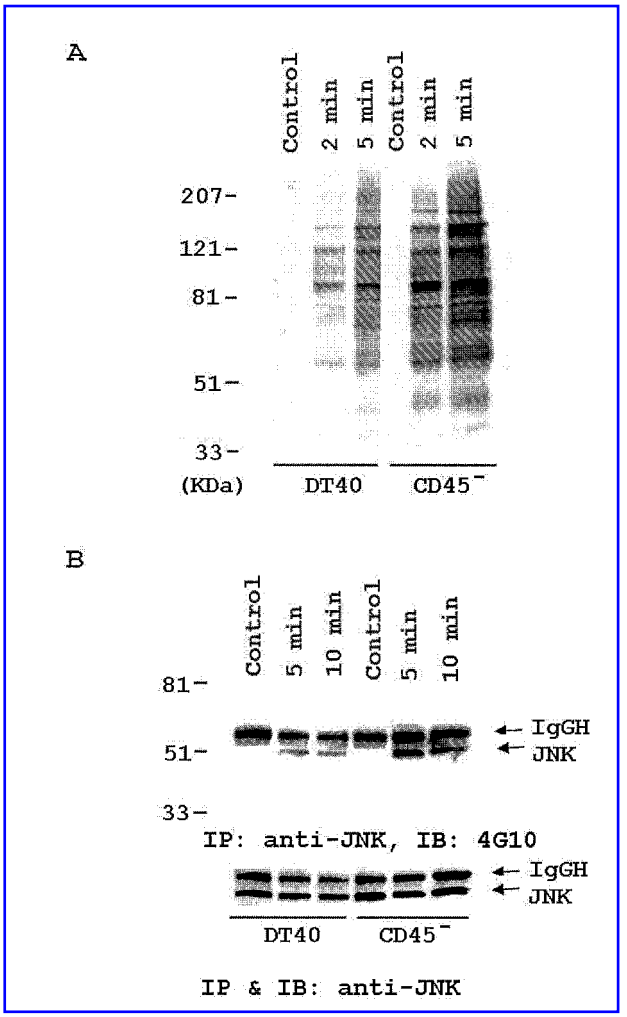

FIG. 2. Effect of CD45 deficiency on hydrogen peroxide-induced tyrosine phosphorylation of cellular proteins and JNK activation. (**A**) Enhanced tyrosine phosphorylation of cellular proteins following hydrogen peroxide stimulation in CD45-deficient DT40 cells. DT40 cells (1×10^7/ml) were stimulated with 5 m*M* hydrogen peroxide and the lysates derived from 2×10^5 cells were run on a 10% SDS-PAGE followed by immunoblotting with 4G10. Shown is a representative profile. (**B**) Enhancement of hydrogen peroxide-induced JNK activation by CD45 deficiency. Anti-JNK immunoprecipitates from wild-type and CD45-deficient DT40 cells were immunoblotted with anti-phosphotyrosine (**top**) or anti-JNK (**bottom**).

Hydrogen peroxide stimulation can activate the JNK, and this activation requires the presence of functional Syk (30). We investigated whether CD45 was involved in the regulation of hydrogen peroxide-induced JNK activation by monitoring the tyrosine phosphorylation level of JNK. As shown in Fig. 2B, hydrogen peroxide stimulated a rapid tyrosine phosphorylation of JNK. The extent of this phosphorylation declined after 10 min of incubation. In comparison, tyrosine phosphorylation of JNK in CD45-deficient DT40 cells was markedly enhanced, confirming the notion that CD45 plays a negative role in certain oxidative stress signaling.

Increase in intracellular Ca^{2+} concentration is reduced and delayed in CD45-deficient DT40 cells

Following the induction of tyrosine phosphorylation, one of the earliest activation events is an increase in the free cytosolic Ca^{2+} concentration. In DT40 cells, we observed that hydrogen peroxide induced a rise in the intracellular Ca^{2+} concentration corresponding to both a release from intracellular stores and an influx (29, 31). Thus, we analyzed the variations in Ca^{2+} mobilization following hydrogen peroxide stimulation of wild-type and CD45-deficient DT40 cells loaded with Fura 2 (Fig. 3). The Ca^{2+} response of CD45-deficient DT40 cells to hydrogen peroxide was reduced and slower compared with that observed in wild-type cells (Fig. 3A), indicating that CD45 was required for maximal Ca^{2+} response. The reduced level of Ca^{2+} concentration in CD45-deficient cells was quite similar to that in DT40 cells pretreated with the PI3K inhibitor, Wortmannin (Fig. 3B), and hydrogen peroxide-induced Ca^{2+} mobilization in CD45-deficient cells was no longer affected by Wortmannin (Fig. 3C).

CD45 deficiency impairs hydrogen peroxide-induced IP_3 generation, but does not affect tyrosine phosphorylation of PLCγ2

In DT40 cells, the Ca^{2+} release induced by hydrogen peroxide has been shown to correlate with IP_3 generation (29), and this correlation between Ca^{2+} release and IP_3 generation is also observed with Wortmannin-treated wild-type DT40 cells (31). Considering the similar effects of CD45 and Wortmannin on hydrogen peroxide-induced Ca^{2+} mobilization, we investigated whether the reduced Ca^{2+} mobilization observed with CD45-deficient cells is correlated to IP_3 production. Figure 4A shows that in CD45-deficient DT40 cells the hydrogen peroxide-induced IP_3 generation was inhibited up to ~35%, which is in agreement with the Ca^{2+} mobilization data shown in Fig. 3A.

PLCγ2 is the only isoform of PLCγ expressed in DT40 cells (45), and tyrosine phosphorylation is known to activate PLCγ (34, 45). PLCγ2 activation in CD45-deficient DT40 cells was partially inhibited as evidenced by the IP_3 production. To investigate whether this partial inhibition of IP_3 production is correlated to changes in the tyrosine phosphorylation level of PLCγ2, we quantitated the tyrosine-phosphorylated PLCγ2 precipitated by anti-PLCγ2 antibody with anti-phosphotyrosine monoclonal antibody. Upon hydrogen peroxide stimulation, induction of tyrosine phosphorylation in PLCγ2 reached a peak at 5 min and then declined after 10 min (Fig. 4B). Like

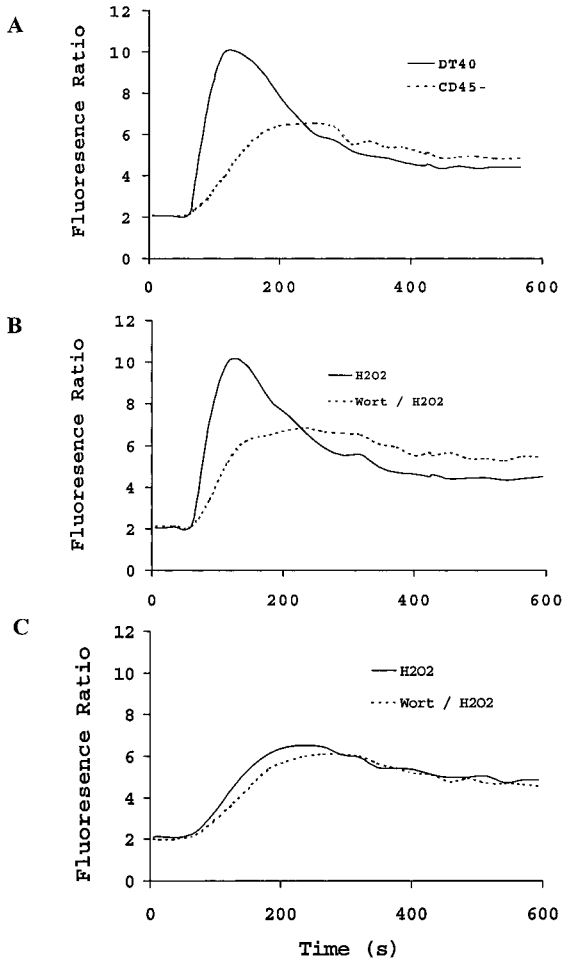

FIG. 3. Impairment of hydrogen peroxide-induced Ca^{2+} mobilization in CD45-deficient DT40 cells. (**A**) Delayed and reduced Ca^{2+} mobilization in CD45-deficient DT40 cells following hydrogen peroxide stimulation. Wild-type and CD45-deficient DT40 cells loaded with Fura 2 were stimulated with 5 m*M* hydrogen peroxide. (**B**) Partial inhibition of hydrogen peroxide-induced Ca^{2+} mobilization by Wortmannin. Wild-type DT40 cells loaded with Fura 2 were preincubated with dimethyl sulfoxide (DMSO) vehicle (0.06%) or 100 n*M* Wortmannin in DMSO for 10 min and then stimulated with 5 m*M* hydrogen peroxide. (**C**) No inhibition of hydrogen peroxide-induced Ca^{2+} mobilization in CD45-deficient DT40 cells by Wortmannin. CD45-deficient DT40 cells loaded with Fura 2 were preincubated with DMSO vehicle (0.06%) or 100 n*M* Wortmannin in DMSO for 10 min and then stimulated with 5 m*M* hydrogen peroxide. The results are expressed as relative fluorescence ratio.

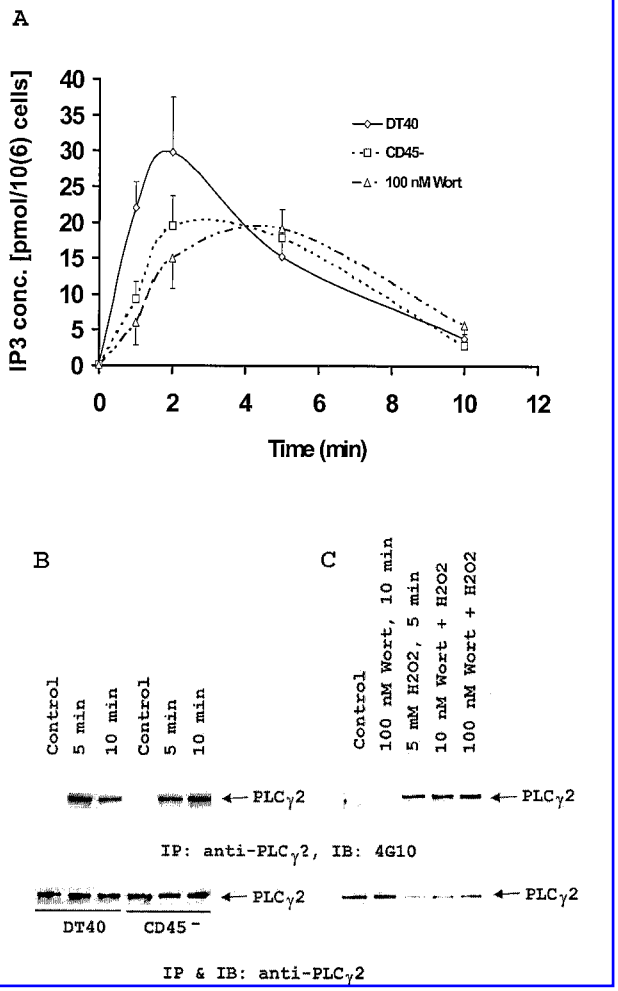

FIG. 4. Effect of CD45 on hydrogen peroxide-induced IP_3 production and tyrosine phosphorylation of PLCγ2. (**A**) Partial inhibition of hydrogen peroxide-induced IP_3 production by Wortmannin and CD45 deficiency. DT40 cells were pretreated with 100 n*M* Wortmannin for 10 min and then stimulated with 5 m*M* hydrogen peroxide. IP_3 concentrations in the extracts were assayed as described in Materials and Methods. The data presented are the mean values of three independent measurements. (**B**) Dispensability of CD45 for hydrogen peroxide-induced PLCγ2 tyrosine phosphorylation. After hydrogen peroxide stimulation, anti-PLCγ2 immunoprecipitates were immunoblotted with anti-phosphotyrosine (**top**) or anti-PLCγ2 (**bottom**). (**C**) No effect of Wortmannin on hydrogen peroxide-induced PLCγ2 tyrosine phosphorylation. DT40 cells were pretreated with or without 100 n*M* Wortmannin for 10 min and then stimulated with 5 m*M* hydrogen peroxide. Anti-PLCγ2 immunoprecipitates were immunoblotted with anti-phosphotyrosine (**top**) or anti-PLCγ2 (**bottom**).

Wortmannin treatment (Fig. 4C), CD45 deficiency did not significantly affect hydrogen peroxide-induced tyrosine phosphorylation of PLCγ2 (Fig. 4B). These observations indicate that CD45 and PI3K do not regulate the catalytic efficiency of PLCγ2 by varying the extent of tyrosine phosphorylation of PLCγ2. In comparison, hydrogen peroxide-induced tyrosine phosphorylation and activation of PLCγ2 was abrogated in Syk-deficient or Btk-deficient DT40 cells and led to the loss of the majority of Ca^{2+} mobilization (28, 31).

Activation of Btk by hydrogen peroxide is PI3K-but not CD45-dependent

Phosphatidylinositol 3,4,5-trisphosphate (PI-3,4,5-P3), a lipid product of PI3K, has been shown to bind to the pleckstrin homology domain of Btk, supporting its association to the membrane where it is phosphorylated and activated by a

Src-family kinase (4, 22). We showed that Wortmannin treatment inhibited hydrogen peroxide-induced Ca^{2+} release independent of tyrosine phosphorylation of PLCγ2 (31). Pasquet *et al.* further revealed that PI-3,4,5-P3 and Btk regulated collagen-related peptide-induced Ca^{2+} influx independent of tyrosine phosphorylation and catalytic activity of PLCγ2 (27). We examined if CD45 deficiency affected the hydrogen peroxide-induced activation of Btk and PI3K, as monitored with anti-phosphotyrosine precipitatable PI3K activity. Lysates from DT40 cells treated with or without hydrogen peroxide were immunoprecipitated with anti-phosphotyrosine antibody, and the precipitates were analyzed for PI3K activity. Treatment with hydrogen peroxide resulted in a rapid and sustained increase in anti-phosphotyrosine precipitatable PI3K activity. When DT40 cells were pretreated with 100 n*M* Wortmannin for 10 min, the hydrogen peroxide-induced PI3K activity was inhibited by 53% and 91% at 2 min and 5 min of stimulation, respectively. However, CD45 deficiency did not apparently alter hydrogen peroxide-stimulated PI3K activity in DT40 cells (Fig. 5A).

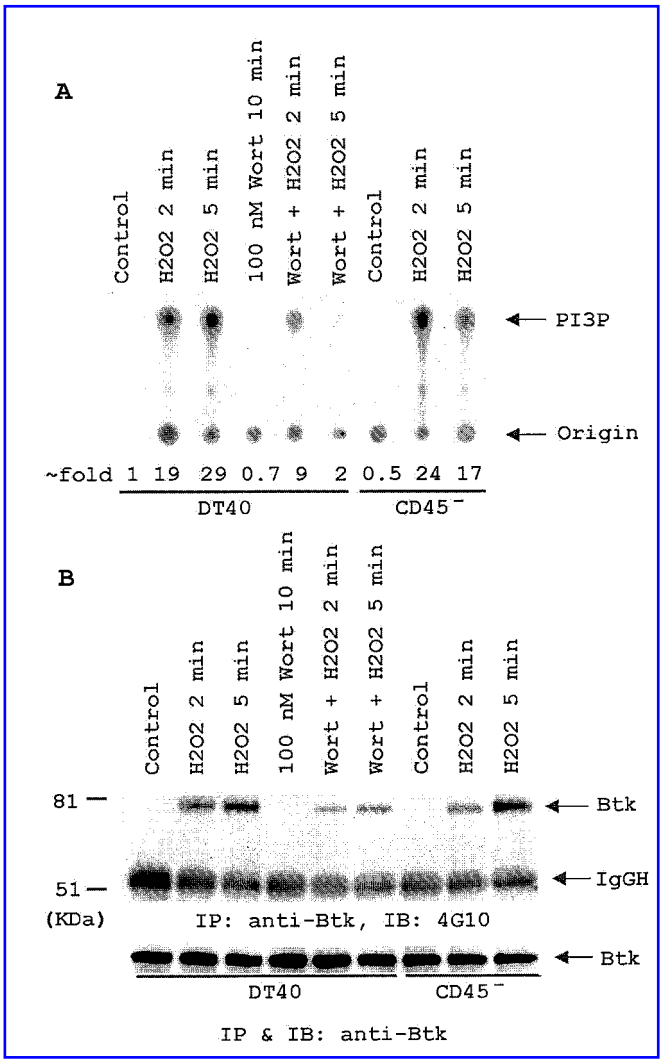

FIG. 5. Differential effects of Wortmannin and CD45 on hydrogen peroxide-induced activation of PI3K and Btk. (**A**) Inhibition of hydrogen peroxide-induced PI3K activation by Wortmannin, but not by CD45 deficiency. Cells were preincubated with dimethyl sulfoxide or 100 n*M* Wortmannin for 10 min and then stimulated with 5 m*M* hydrogen peroxide at the indicated time points. Lysates were precipitated with anti-phosphotyrosine and assayed for PI3K activity as described in Materials and Methods. Position of phosphatidylinositiol 3-phosphate (PI3P) is indicated. (**B**) Inhibition of hydrogen peroxide-induced Btk activation by Wortmannin, but not by CD45 deficiency. Anti-Btk immunoprecipitates from DT40 cells or CD45-deficient DT40 cells were immunoblotted with anti-phosphotyrosine (**top**) or with anti-Btk (**bottom**).

In B cells, activation of the B-cell receptor leads to tyrosine phosphorylation of Btk, which is well correlated with its tyrosine kinase activity (4, 24). Therefore, we examined Btk activation in DT40 cells by monitoring the tyrosine phosphorylation level of Btk. Significant tyrosine phosphorylation of Btk was detected after cells were incubated with 5 m*M* hydrogen peroxide for 2 min and reached a peak at 5 min (Fig. 5B, 30). Preincubation of cells with 100 n*M* Wortmannin resulted in ~50% reduction in hydrogen peroxide-induced tyrosine phosphorylation of Btk. Again, CD45 deficiency failed to significantly alter the hydrogen peroxide-induced Btk activation, although a slightly lower level of tyrosine-phosphorylated Btk was observed at an earlier time point (Fig. 5B). These data indicate that CD45 does not regulate hydrogen peroxide-induced Ca^{2+} flux by altering the activation of PI3K and Btk.

DISCUSSION

This study was aimed at investigating the role of CD45 in oxidative stress signal transduction in DT40 cells utilizing CD45-deficient clones generated from chicken B cells by homologous recombination. To achieve maximal biochemical response, we used 5 m*M* hydrogen peroxide to investigate the molecular mechanisms of redox-dependent signaling events. Our results revealed that in CD45-deficient DT40 cells, hydrogen peroxide-induced tyrosine phosphorylation of total cell lysates and JNK activation are induced more strongly than that observed in the parent cells. However, hydrogen peroxide-induced Ca^{2+} mobilization in these cells is reduced by 45% relative to that in the parent cells. This impaired Ca^{2+} mobilization is well correlated with the inhibition of PLCγ2 activation as evidenced by the IP_3 production. The molecular mechanism by which CD45 regulates hydrogen peroxide-induced Ca^{2+} mobilization appears not to be through either inhibiting tyrosine phosphorylation of PLCγ2 or altering the activation of PI3K and Btk.

Hydrogen peroxide stimulates Ca^{2+} mobilization and tyrosine phosphorylation patterns in lymphocytes, identical to those observed following antigen receptor activation (36, 37). The mechanism by which hydrogen peroxide stimulates lymphocyte activation events most likely involves the inhibition of multiple PTP activities including CD45. PTPs are susceptible to oxidative stress because the cysteine at their catalytic centers can be oxidized and then inactivate the enzymic activ-

ity (5). Indeed, treatment of intact DT40 cells with hydrogen peroxide abolishes >90% of total PTP activity. Consequently, tyrosine phosphorylation of cellular proteins is induced and reaches peak at ~5 min after hydrogen peroxide treatment under the conditions used. CD45 plays an important role in this regulatory process because CD45-deficient cells exhibit higher sensitivity to hydrogen peroxide-mediated tyrosine phosphorylation of cellular proteins and JNK activation relative to that observed in wild-type cells.

CD45 is associated with a variety of proteins, including the cytoskeletal polypeptide fodrin (13), the ζ chain of CD3 (8), and 30–32-kDa phosphoproteins [termed CD45-associated phosphoproteins (CD45-AP) or lymphocyte phosphatase-associated phosphoproteins (LPAP)] that also complex with Lck (39, 46). Lee *et al.* also identified that CD45 can form complexes with certain signaling proteins, including Lck and members of the Ras pathway, Grb2, mSOS, rasGAP, and Vav, upon pervanadate treatment in a mouse T-cell hybridoma cell line overexpressing an activated form of Lck (21). These data suggest that, in addition to its PTP activity, CD45 may also function as a membrane docking site for certain signaling molecules during lymphocyte activation.

When intracellular Ca^{2+} mobilization induced by hydrogen peroxide in CD45-deficient DT40 cells was investigated, we found that CD45 deficiency causes a partial inhibition of hydrogen peroxide-induced Ca^{2+} mobilization. To search for the molecular basis for this inhibition, the hydrogen peroxide-induced IP_3 generation by the CD45-deficient cells was also monitored. Figure 4A shows that hydrogen peroxide-induced IP_3 production is reduced by 35% in CD45-deficient DT40 cells compared with that found in wild-type DT40 cells. Together, these reveal that CD45 deficiency affects hydrogen peroxide-stimulated activation of PLCγ2. PLCγ2 is the only isoform of PLCγ expressed in DT40 cells, and tyrosine phosphorylation is known to activate the PLCγ (34, 45). Thus, tyrosine phosphorylation of PLCγ2 upon hydrogen peroxide stimulation presumably results in the observed IP_3 production. However, as shown in Fig. 4B, tyrosine phosphorylation of PLCγ2 was found to be comparable in both CD45-deficient and parent DT40 cells. These results indicate that CD45 may regulate hydrogen peroxide-induced activation of PLCγ2 via a mechanism other than mediating its tyrosine phosphorylation level.

It is interesting to note that the effects of CD45 on hydrogen peroxide-induced tyrosine phosphorylation of PLCγ2, IP_3 production, and Ca^{2+} mobilization are quite similar to those observed in the presence of the PI3K inhibitor, Wortmannin (Figs. 3 and 4). This led us to investigate the effect of CD45 deficiency on the activities of PI3K and Btk. A kinase assay of PI3K *in vitro* revealed that, although Wortmannin pretreatment almost abolished anti-phosphotyrosine precipitatable PI3K activity induced by hydrogen peroxide, PI3K activity was intact in CD45-deficient cells (Fig. 5A). Immunoblot analysis also demonstrated that Wortmannin pretreatment inhibited hydrogen peroxide-induced Btk activation by 50%, while activation of Btk by hydrogen peroxide appeared to be similar in both CD45-positive and CD45-deficient DT40 cells (Fig. 5B). These observations indicate that CD45 mediates hydrogen peroxide-stimulated IP_3 production and Ca^{2+} mobilization via a mechanism that is independent of both tyrosine phosphorylation of PLCγ2 and activation of Btk, as well as the anti-phosphotyrosine precipitatable PI3K activity.

It has been demonstrated that Lyn is a physiological target of CD45 in B-cell receptor signaling (52). It is also possible that CD45 regulates hydrogen peroxide-mediated Ca^{2+} mobilization via Lyn. Although Lyn is essential for hydrogen peroxide-induced tyrosine phosphorylation of cellular proteins, IP_3 production, and Ca^{2+} release, no increase in Lyn activity was detected following hydrogen peroxide stimulation in DT40 cells (29). Thus, we are unable to evaluate whether

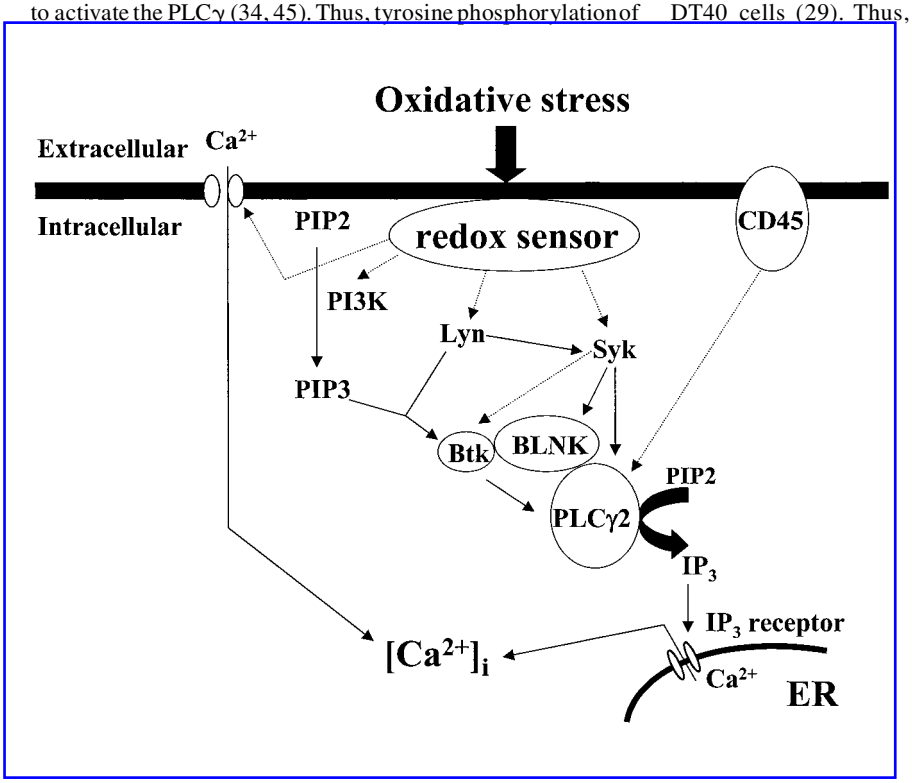

FIG. 6. Current model for oxidative stress-induced Ca^{2+} signaling in DT40 cells. The experimental evidence for a putative sensor in DT40 cells has yet to be fully demonstrated. Dotted lines are used where the molecular interactions have not been revealed. BLNK, B-cell linker protein; ER, endoplasmic reticulum; PIP2, phosphatidylinositol bisphosphate; PIP3, phosphatidylinositol trisphosphate.

CD45 is involved in the regulation of Lyn activity upon hydrogen peroxide treatment.

We have shown that inhibition of hydrogen peroxide-mediated Ca^{2+} mobilization by Wortmannin is most likely derived from the reduction of PI-3,4,5-P3, which is needed to target the activated PLCγ2 to its substrate site for maximal catalytic efficiency (31). Our data indicate that CD45 may also play a role in targeting the activated PLCγ2 for maximal catalytic efficiency. In this context, CD45-AP/LPAP have been found only in CD45-expressing cells because they are degraded in the absence of their binding partner, CD45 (39, 46), and pervanadate treatment results in increased tyrosine phosphorylation of LPAP (39). These proteins also appear to possess properties characteristic of molecules involved in protein–protein interactions (23, 39, 46, 49). These features make them critical for CD45 function. Whether losing these proteins in the absence of CD45 expression is responsible for less efficient hydrolysis of phosphatidylinositol 4,5-bisphosphate (PI-4,5-P2) by PLCγ2 is a subject for further investigation.

In summary, Btk membrane translocation is an essential step for its activation. Btk translocation is believed to be mediated by the interaction between its pleckstrin homology domain and membrane-localized PI-3,4,5-P3, which is generated by activated PI3K. This interaction targets Btk to plasma membrane, puts it in close proximity to Src-family PTKs, and initiates its activation via the tyrosine phosphorylation of Y551 by the latter (22, 33, 35). Hydrogen peroxide-activated Syk and Btk work in concert to phosphorylate specific tyrosine residues in PLCγ2, leading to the activation of PLCγ2 because a deficiency of either Syk or Btk completely abolishes hydrogen peroxide-induced IP_3 production in DT40 cells (28, 31). The whole scenario of hydrogen peroxide-triggered Ca^{2+} mobilization in DT40 cells appears to be that Syk and Btk-dependent tyrosine phosphorylation of PLCγ2 leads to its activation, but for maximal enzymatic activity a PI3K-dependent signal is required to target activated PLCγ2 to plasma membrane in close proximity with its substrate, PI-4,5-P2, for efficient hydrolysis. CD45 can affect the ability of PLCγ2 to hydrolyze PI-4,5-P2 via an unknown mechanism that is independent of alteration of tyrosine phosphorylation levels of PLCγ2 (Fig. 6).

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ABBREVIATIONS

Btk, Bruton's tyrosine kinase; CD45-AP, CD45-associated proteins; Csk, C-terminal Src kinase; IP_3, inositol 1,4,5-trisphosphate; JNK, c-Jun N-terminal kinase; LPAP, lymphocyte phosphatase-associated proteins; NF-κB, nuclear factor-κB; PI3K, phosphatidylinositol 3-kinase; PI-4,5-P2, phosphatidylinositol 4,5-bisphosphate; PI-3,4,5-P3, phosphatidylinositol 3,4,5-trisphosphate; PLCγ2, phospholipase Cγ2; PTK, protein-tyrosine kinase; PTP, protein-tyrosine phosphatase; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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