Implication of Phosphatidylinositol 3-Kinase Membrane Recruitment in Hydrogen Peroxide-Induced Activation of PI3K and Akt

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ABSTRACT: The effect of tyrosine phosphorylation of PI3K on its enzymatic activity is quite controversial, and the molecular mechanism by which ROS trigger PI3K membrane relocation is unclear. Therefore, we investigated the regulatory mechanism of hydrogen peroxide-induced PI3K activation in DT40 cells, utilizing genetic and pharmacological approaches. Our results revealed that hydrogen peroxide induced tyrosine phosphorylation of the p110 but not the p85 subunit of PI3K in DT40 cells. This phosphorylation was intact in Btk- and Cbl-deficient DT40 cells, but was drastically suppressed in Lyn, Syk, or BCAP-deficient DT40 cells. Tyrosine phosphorylation of p110 did not alter its catalytic activity, and hydrogen peroxide stimulation did not cause an increase in the intrinsic PI3K activity; however, hydrogen peroxide stimulation did induce PI(3,4,5)P_3 accumulation and activate Akt. The activation of Akt, as monitored by its ability to phosphorylate GSK-3α/β and by its S^{473} phosphorylation, was strictly dependent on PI3K activity. Under our conditions, hydrogen peroxide-induced PI3K and Akt activation was independent of Lyn, Syk, Cbl, BCAP, or Ras when each was eliminated individually either by mutation or by a specific inhibitor. In comparison, Akt activation by B cell receptor cross-linking was dependent on BCAP. In addition, hydrogen peroxide treatment caused an increase in the amount of p85 PI3K associated with the particulate fraction. Together, these results indicate that the hydrogen peroxide-induced PI3K and Akt activation in DT40 cells was achieved through PI3K membrane recruitment to its substrate site, thereby enabling PI3K to maximize its catalytic efficiency.

Reactive oxygen species (ROS)[1] have emerged as physiological mediators of cellular responses. The production of ROS has been detected in a variety of cells stimulated with cytokines (*1*, *2*), with peptide growth factor (*3*, *4*), and with agonists of receptors with seven transmembrane spans (*5*). The generated ROS is correlated to subsequent biochemical responses (*3*, *4*). Thus, studying hydrogen peroxide-stimulated signaling should yield mechanistic information about both receptor-mediated signaling and cellular responses to oxidative stress. When exogenous hydrogen peroxide is applied to cells as one form of ROS, hydrogen peroxide can activate an array of non-receptor-type protein tyrosine kinases (PTKs), including Lyn of the Src family, Syk of the Syk family, and Btk of the Tec family in a variety of cell systems (*6*−*11*). Hydrogen peroxide stimulation leads to the initiation of downstream signaling events, such as stimulation of PLCγ2 and mitogen-activated protein kinases and activation of phosphatidylinositol 3-kinase (PI3K) (*6*−*11*). The requirements of PTKs for some of these signaling pathways, including tyrosine phosphorylation of PLCγ, calcium mobilization, and activation of mitogen-activated protein kinases (e.g., ERK and JNK), have been characterized in detail (*7*−*11*).

PI3K is a lipid kinase that phosphorylates phosphoinositides at the 3′ position of the inositol ring (*12*). Class A PI3K is characterized as a heterodimer consisting of a p85 regulatory and a p110 catalytic subunit (*13*). Tyrosine phosphorylation of the p85 regulatory subunit (*14*), the p110 catalytic subunit (*15*), or both (*16*) of PI3K is observed in B cells; however, the effect of tyrosine phosphorylation of PI3K on its enzymatic activity is quite controversial. Yuan et al. reported that ionizing radiation-induced tyrosine phosphorylation of p85 inhibits PI3K activity (*17*). In contrast, Cuevas et al. demonstrated that tyrosine phosphorylation of p85 enhanced PI3K activity, possibly through relieving the inhibitory activity of p85 on p110 (*18*). No evidence regarding the effect of tyrosine phosphorylation of p110 on PI3K activity is available. The effect of hydrogen peroxide-stimulated tyrosine phosphorylation of PI3K on its activity will be addressed in this paper.

PI3K is currently believed to be activated by binding to a phosphorylated tyrosine residue of receptors or adaptor proteins, which form the docking site for the Src homology 2 (SH2) domain of the PI3K adaptor subunit (*19*). This tyrosine-phosphorylated adaptor-mediated translocation of PI3K to receptor tyrosine kinases and their substrates apparently helps position the catalytic subunit close to the membrane where the lipid substrate of PI3K is located. The SH2 domains of p85 have a preference for binding to

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[1] Abbreviations: BCAP, B cell adaptor for phosphatidylinositol 3-kinase; Btk, Bruton's tyrosine kinase; PH, pleckstrin homology; PI3K, phosphatidylinositol 3-kinase; PI(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; PTK, protein tyrosine kinase; SH2, Src homology 2; Syk, spleen tyrosine kinase; ROS, reactive oxygen species.

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pYXXM motifs (*20*). In B cells, CD19 (*21*, *22*), Cbl (*23*), B cell adaptor for phosphatidylinositol 3-kinase (BCAP) (*24*), and Grb2-associated binder (Gab) (*25*) are tyrosine phosphorylated, and one or several of these tyrosine-phosphorylated adaptors are then involved in PI3K activation in response to B cell receptor cross-linking. These molecules likely bind the p85 regulatory subunit of PI3K via their tyrosine-phosphorylated YXXM motifs and then recruit PI3K to the plasma membrane (*21*−*24*). Alternatively, activated Ras (GTP-loaded Ras) can activate PI3K by direct binding and membrane targeting of the p110 subunit of PI3K (*26*, *27*). The Ras.GTP−p110 and tyrosine-phosphorylated adaptor−p85 interactions might synergize to give full activation of PI3K. However, the roles of PTKs (Lyn, Syk, and Btk), adaptor proteins, and Ras in hydrogen peroxide-induced activation of PI3K remain unclear.

Akt is a protein serine/threonine kinase consisting of a pleckstrin homology (PH) domain in its N-terminus, a central kinase domain, and a hydrophobic and proline-rich domain in the C-terminus (*28*, *29*). Akt can be activated by ligand binding to a diverse range of cell surface receptors (*15*, *30*) and by stresses, such as heat shock and hydrogen peroxide (*7*, *31*). Akt activation is considered a surrogate marker of PI3K activation since Akt activation is thought to occur via the binding of phosphatidylinositol 3,4,5-trisphosphate [PI-(3,4,5)P_3] to the PH domain of Akt, thereby causing a proportion of Akt to translocate to the plasma membrane, whereupon it undergoes conformational changes and is subsequently phosphorylated on Thr^{308} and Ser^{473} by PI-(3,4,5)P_3-dependent kinase (PDK)-1 and -2, respectively (*32*, *33*). Hydrogen peroxide treatment has led to PI3K-dependent activation of Akt (*7*, *31*, *34*); however, the roles of the individual PTKs and adaptor proteins in hydrogen peroxide-induced activation of Akt are elusive. Here, we took genetic and pharmacological approaches to examining the roles of Lyn, Syk, Btk, Cbl, BCAP, and Ras in mediating hydrogen peroxide-induced activation of PI3K and Akt. Our experimental results revealed that Lyn- and/or Syk-mediated hydrogen peroxide-induced tyrosine phosphorylation of the p110 subunit of PI3K seemed unlikely to affect its enzymatic activity. Hydrogen peroxide stimulation enhanced generation of PI(3,4,5)P_3, which is essential for hydrogen peroxide-triggered activation of Akt, via recruiting PI3K to membrane independent of BCAP, Cbl, and Ras.

MATERIALS AND METHODS

Materials. RPMI-1640 medium and fetal bovine serum were purchased from GIBCO Inc. Protein A and manumycin A were from Calbiochem Corp. (San Diego, CA). The anti-phosphotyrosine antibody (4G10), anti-PtdIns 3-kinase p85 sera (catalog no. 05-212 for Western blotting and catalog no. 06-195 for immunoprecipitation), and the Ras activation assay kit were from Upstate Biotechnology Inc. (Lake Placid, NY). The phospho-Akt (Ser473) antibody, the anti-Akt antibody, and the Akt kinase assay kit were obtained from Cell Signaling Technology (Beverly, MA). Enhanced chemiluminescence reagents were from Dupont. The polyclonal anti-Cbl antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The goat anti-chicken IgM antibody was from Bethyl Laboratories (Montgomery, TX). The DT40 cell lines were purchased from Riken Cell Bank (generated by Dr. Kurosaki, Osaka, Japan).

Cell Culture. DT40 and DT40-derived cells were maintained in RPMI-1640 medium, supplemented with 10% (v/v) fetal bovine serum, 100 units/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 and stimulated by hydrogen peroxide at 37 °C (*11*).

Preparation of Cell Extracts. Stimulated cells (1×10^7 cells/mL) were lysed in ice-cold lysis buffer [5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 100 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride, 10 mg/mL leupeptin, and 50 mM Tris (pH 7.4)] after a short centrifugation step. Lysates were clarified by centrifugation at 16000*g* for 15 min at 4 °C.

Immunoblot Analysis. Cell extracts or immunoprecipitates were resolved on SDS−PAGE, transferred electrophoretically onto PVDF membranes, and then immunoblotted with the indicated antibodies. Immunoreactive proteins were visualized by enhanced chemiluminescence.

Akt Assay. Cell extracts from stimulated or unstimulated DT40 cells were immunoprecipitated with the anti-Akt antibody, and the kinase assay was then carried out following the manufacturer's instructions. Briefly, Akt-catalyzed phosphorylation of GSK-3 was monitored with Western blotting using the phospho-GSK-3α/β antibody.

PI3K Assay. The cell extracts from treated or untreated cells were incubated with anti-phosphotyrosine antibody 4G10, anti-p85, or anti-Cbl antibody for 30 min followed by further incubation with protein A−agarose beads for 1 h. The immunoprecipitates were washed three times with lysis buffer, twice with buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 1 mM EDTA, and once with PI3K assay buffer [20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EGTA, 100 μM vanadate, 20 μM ATP, and 200 μM adenosine]. After the last washing buffer had been removed, 10 μL of in vitro PI3K substrate phosphatidylinositol [1 μg/μL in 10 mM Hepes (pH 7.5) by sonicating for 3×20 s] was added to each sample, and samples were incubated for 10 min on ice. Reactions were 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 N HCl. Phospholipids were extracted once with 200 μL of a $CHCl_3$/MeOH mixture (1:1) and centrifuged at 8000 rpm for 3 min using a benchtop Biofuge centrifuge made by Heracus, and then the lower phase (organic phase) was transferred to a new microtube and extracted once with 160 μL of a 1 N HCl/MeOH mixture (1:1). The organic phase was dried under nitrogen gas and resuspended in 10 μL of a $CHCl_3$/MeOH mixture (1:1). Phosphorylated products were resolved on oxalate-impregnated [wetted with a 1.2% potassium oxalate/MeOH mixture (1:1)] Silica 60 plates using a $CHCl_3$/MeOH/4 M NH_4OH mixture (9:7:2) as a solvent (~2 h), and the gel was air-dried for ~10 min. Autoradiogram exposure typically lasted for less than 2 days. Radioactive spots, representing PI3P, were visualized and quantitated by using the PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Membrane Translocation of PI3K. Wild-type or mutant DT40 cells (3×10^7 cells/mL) were stimulated at 37 °C with 3 mM (1 mM/10^7 cells) hydrogen peroxide. Cells were resuspended in 1 mL of hypotonic lysis buffer [10 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM KCl, 1 mM EGTA, 1

mM EDTA, 1 mM vanadate, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 50 mM NaF, and 1 mM PMSF] and lysed by 100 strokes of a Dounce homogenizer. Nuclei and unbroken cells were removed by centrifugation at 800*g* for 10 min. Soluble and particulate fractions were obtained by ultracentrifugation of the cleared lysates at 100000*g* for 30 min. Pellets were washed twice with lysis buffer and resuspended in lysis buffer containing 1% Triton X-100. These resuspensions were centrifuged at 12000*g* for 10 min, and the resulting supernatants were assayed for protein concentration (Bio-Rad). Equivalent amounts of the protein were subjected to anti-PI3K immunoprecipitation. The immunoprecipitated proteins were then separated with an 8% SDS−PAGE gel and blotted with the anti-PI3K antibody.

Ras Activation Assay. Stimulated or unstimulated DT40 cells were extracted with Ras lysis buffer [25 mM Hepes (pH 7.5), 10 mM NaF, 10 μg/mL leupeptin, 150 mM NaCl, 1% Nonidet P-40, 10 mM $MgCl_2$, 1 mM PMSF, 1 mM sodium vanadate, and 10% glycerol] for 20 min at 4 °C with gentle rotation. Clarified cell lysates were incubated for 2 h at 4 °C with the GST−Raf-1 RBD conjugate (residues 1−149) precoupled to glutathione−agarose beads (50% slurry). Only GTP-loaded Ras will bind to beads, so activated protein can be pelleted with beads by quick centrifugation. Beads were boiled in SDS−PAGE sample buffer for 5 min, and the proteins were separated on a 4 to 20% SDS−PAGE gel. Proteins were transferred to PVDF, immunoblotted with the Ras antibody, and visualized by chemiluminescence. Equal protein loading was verified by staining a transferred PAGE gel with Coomassie Blue stain.

PI(3,4,5)P_3 Assay. PI(3,4,5)P_3 generation was monitored as described by Okada et al. (*24*). Briefly, DT40 cells (10^7 cells/mL) were radiolabeled in calcium buffer [containing 10 mM Hepes (pH 7.4), 135 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5.6 mM glucose] with 1 mCi/mL [^{32}P]orthophosphate (Amersham) for 1 h. ^{32}P-labeled cells were pelleted by centrifugation, then washed once with calcium buffer, and resuspended in the same buffer. After stimulation (8×10^6 cells/time point), the reactions were quenched by addition of 600 mL of a methanol/1.2 N HCl mixture (1:1), and lipid was extracted with 480 μL of chloroform. Dried samples were spotted onto a silica gel plate (Sigma) pretreated with potassium oxalate and developed in a chloroform/acetone/methanol/acetic acid/water mixture (40:15:13:12:7). The radioactivity of PI(3,4,5)P_3 spots was quantified by using a Storm 860 (Molecular Dynamics) PhosphorImager analyzer.

RESULTS

Lyn- or Syk- but Not Btk-Dependent Tyrosine Phosphorylation of PI3K Induced by Hydrogen Peroxide. Our previous data demonstrated that hydrogen peroxide stimulates an increase in anti-phosphotyrosine antibody-immunoprecipitable PI3K activity (*9*). Utilizing the respective tyrosine kinase-deficient DT40 cells, we investigated the role of protein tyrosine kinases Lyn, Syk, and Btk in hydrogen peroxide-induced tyrosine phosphorylation of PI3K or PI3K-associated protein(s). The PI3K activity associated with the anti-phosphotyrosine antibody was dramatically reduced in Lyn- and Syk-deficient DT40 cells, whereas in Btk-deficient DT40 cells, PI3K activity was elevated to a level higher than

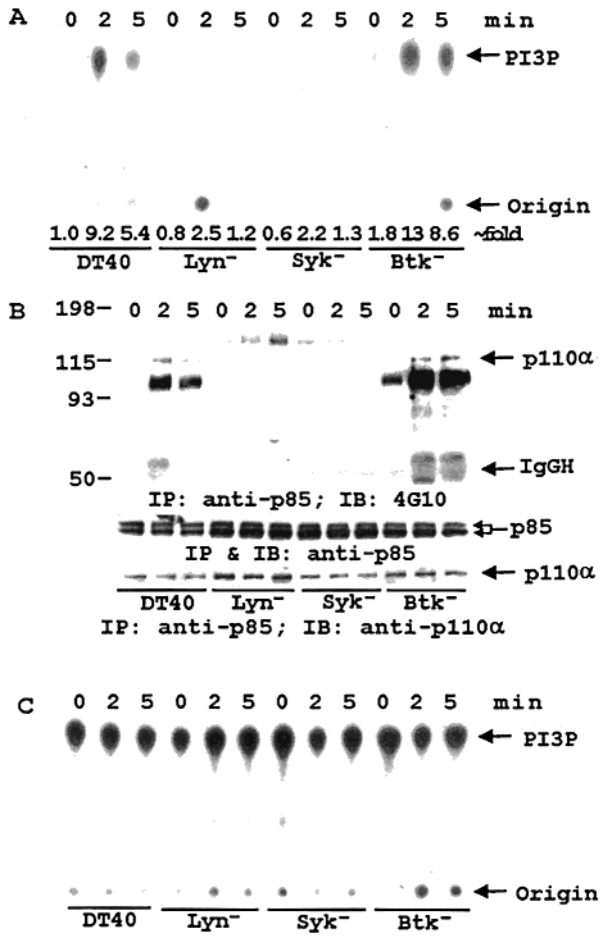

FIGURE 1: Regulation of hydrogen peroxide-induced tyrosine phosphorylation of PI3K by Lyn, Syk, and Btk. (A) Decreased anti-phosphotyrosine antibody immunoprecipitable PI3K activity in Lyn- and Syk- but not Btk-deficient DT40 cells upon hydrogen peroxide stimulation. Cells were stimulated with 1 mM hydrogen peroxide for the indicated time points. Lysates were precipitated with anti-PTyr and assayed for PI3K activity as described in Materials and Methods. The position of phosphatidylinositiol 3-phosphate (PI3P) is indicated. (B) Indispensability of Lyn and Syk but not Btk for hydrogen peroxide-induced tyrosine phosphorylation of PI3K. Lysates from wild-type or Lyn-, Syk-, or Btk-deficient DT40 cells treated with or without 1 mM hydrogen peroxide were immunoprecipitated with the polyclonal antibody against the p85 subunit of PI3K, which also coprecipitates with the p110 catalytic subunit of PI3K. Anti-p85 immunoprecipitates were probed with either anti-PTyr (top), anti-p85 (middle), or anti-p110α (bottom). The doublet bands detected in the anti-p85 immunoblot were presumed to be p85α and p85β isoforms: IP, immunoprecipitation; IB, immunoblot. (C) No change in anti-p85 immunoprecipitable PI3K activity upon hydrogen peroxide stimulation. Lysates from various DT40 cells with or without 1 mM hydrogen peroxide stimulation were precipitated with anti-p85, and the immunoprecipitates were assayed for PI3K activity.

that observed with the wild-type (WT) DT40 cells (Figure 1A). To test whether deficiency of either Lyn or Syk affected hydrogen peroxide-induced tyrosine phosphorylation of PI3K subunits in DT40 cells, immunoprecipitates of the anti-p85 regulatory subunit of PI3K, which also coprecipitates the p110 catalytic subunit of PI3K, were subjected to immunoblotting with anti-phosphotyrosine antibody 4G10. Figure 1B (top) shows that hydrogen peroxide induced rapid and transient tyrosine phosphorylation of two major proteins having approximate molecular masses of 115 and 98 kDa, while tyrosine phosphorylation of the p85 subunit was not

observed in these hydrogen peroxide-stimulated cells. DT40 cells predominantly express the α isoform among p110α, p110*β*, and p110*δ* (*35*). Reprobing the filter with anti-p110α revealed that the 115 kDa band was p110α (Figure 1B, bottom). Similar results were observed by immunoblot analysis of anti-p110α immunoprecipitates with 4G10 and anti-p110α (data not shown). Hydrogen peroxide-induced tyrosine phosphorylation of the p110 catalytic subunit was not observed in Lyn- or Syk-deficient DT40 cells, suggesting that Lyn and Syk were necessary for the hydrogen peroxide-induced tyrosine phosphorylation of the p110 catalytic subunit. In comparison, in Btk-deficient DT40 cells, hydrogen peroxide-induced tyrosine phosphorylation of the p110 subunit was maintained or somewhat enhanced. Thus, Btk was dispensable for hydrogen peroxide-induced tyrosine phosphorylation of the p110 subunit in DT40 cells. Nevertheless, comparable amounts of the p85 and p110 subunits of PI3K were detected in the immunoprecipitates from the wild-type and mutant DT40 cell lines (Figure 1B, middle and bottom). The doublet bands detected in the anti-p85 immunoblot were thought to be p85α and p85*β* isoforms.

To investigate whether tyrosine phosphorylation of the p110 catalytic subunit affects its enzymatic activity, lysates from DT40 cells and various DT40 mutants treated with or without hydrogen peroxide were immunoprecipitated with the same polyclonal anti-p85 antibody, and then the precipitates were analyzed for PI3K activity. As shown in Figure 1C, a high basal PI3K activity was detected in anti-p85 immunoprecipitates, and this activity was independent of both hydrogen peroxide treatment and deficiency in either Lyn, Syk, or Btk.

PI3K-Dependent but PTK-Independent Akt Activation by Hydrogen Peroxide. Akt activation is recognized as a surrogate marker of PI3K activation, and PTKs are required for the activation of Akt induced by B cell receptor engagement and by hydrogen peroxide stimulation (*15*, *36*−*38*). We next tested whether hydrogen peroxide could activate Akt in DT40 cells and whether Lyn, Syk, or Btk was involved in the regulation of hydrogen peroxide-induced Akt activation. Because Akt activation induced by stimulus requires phorphorylation on both Thr^{308} and Ser^{473} (*32*), Akt activation was first measured indirectly by examining Akt phosphorylation on Ser^{473} using a phospho-specific Ser^{473} anti-Akt antibody. Hydrogen peroxide treatment rapidly induced Ser^{473} phosphorylation of Akt as detected by immunoblotting using a phosphoserine 473-specific anti-Akt antibody (Figure 2A). Akt phosphorylation on Ser^{473} was maintained in Lyn-, Syk-, and Btk-deficient DT40 cells, while comparable amounts of Akt proteins were detected in wild-type and various PTK-deficient cells relative to wild-type cells. These observations suggested that neither Lyn, Syk, nor Btk was essential for hydrogen peroxide-induced Akt phosphorylation on Ser^{473}.

To demonstrate whether hydrogen peroxide-induced Ser^{473} phosphorylation of Akt was correlated with its enzymatic activity, we further tested Akt activity by an in vitro kinase assay using a GST fusion protein that contains a sequence from glycogen synthase kinase (GSK)-3 that is phosphorylated by Akt as a substrate. As shown in Figure 2B, Ser^{473} phosphorylation of Akt was well associated with its increased kinase activity, evidenced by its ability to phosphorylate GSK-3α/*β* fusion protein. Again, hydrogen peroxide-stimulated Akt activity was independent of either Lyn, Syk, or Btk.

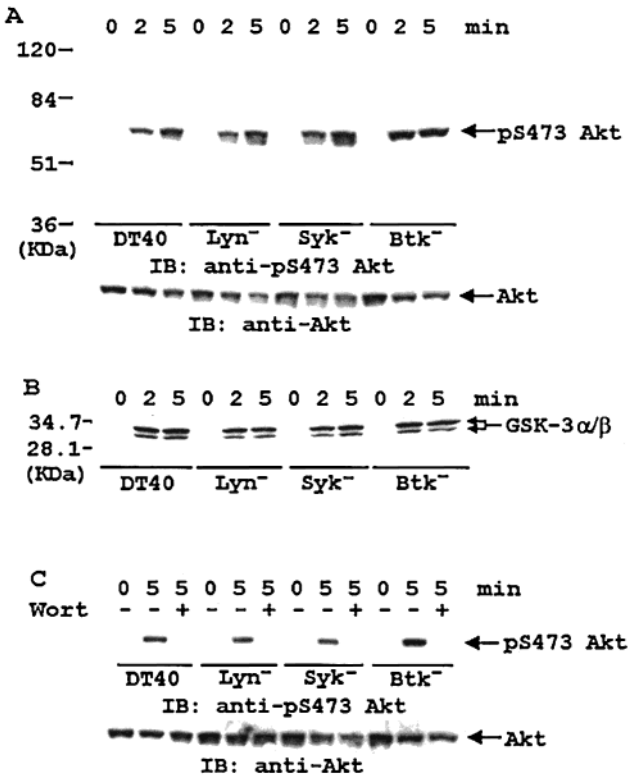

FIGURE 2: Dispensability of Lyn, Syk, or Btk for hydrogen peroxide-induced Akt activation. (A) No requirement of either Lyn, Syk, or Btk for hydrogen peroxide-induced Ser^{473} phosphorylation (pS473) of Akt. The wild type and various DT40 mutants were treated with 1 mM hydrogen peroxide for the indicated reaction times. Whole cell lysates were resolved with 8% SDS−PAGE and immunoblotted with either anti-phosphoserine 473-specific (top) or anti-Akt (bottom) antibodies. (B) No involvement of Lyn, Syk, or Btk in hydrogen peroxide-induced Akt activation. Cell lysates from stimulated or unstimulated DT40 cells were immunoprecipitated with the anti-Akt antibody, and the kinase assay was carried out as described in the manufacturer's protocol. (C) PI3K in hydrogen peroxide-induced Akt activation. Various DT40 cells were pretreated with 100 nM Wortmannin for 15 min prior to stimulation with 1 mM hydrogen peroxide. Whole cell lysates were run in 8% SDS−PAGE and immunoblotted with either anti-phosphoserine 473-specific (top) or anti-Akt (bottom) antibodies.

The role of PI3K in the activation of Akt by hydrogen peroxide was evaluated utilizing a potent inhibitor of PI3K, Wortmannin, which covalently modifies Lys^{802} of the catalytic subunit of the PI3K that is located at its active site (*39*). Akt activation induced by hydrogen peroxide was abolished by Wortmannin pretreatment in all cells (Figure 2C). This clearly indicates that PI3K activity is essential for hydrogen peroxide-induced activation of Akt.

Hydrogen Peroxide-Induced PI(3,4,5)P_3 Generation and Membrane Recruitment of p85. The observation that hydrogen peroxide stimulates PI3K-dependent Akt activation but fails to alter PI3K activity suggests that hydrogen peroxide might merely trigger membrane translocation of PI3K and enable it to access its substrate PI(4,5)P_2, thereby enhancing its catalytic efficiency for PI(3,4,5)P_3 production. To verify this possibility, we examined the in vivo production of PI(3,4,5)P_3. Radiolabeled DT40 cells were stimulated by hydrogen peroxide, and extracted phospholipids were separated by thin-layer chromatography. Figure 3A showed that

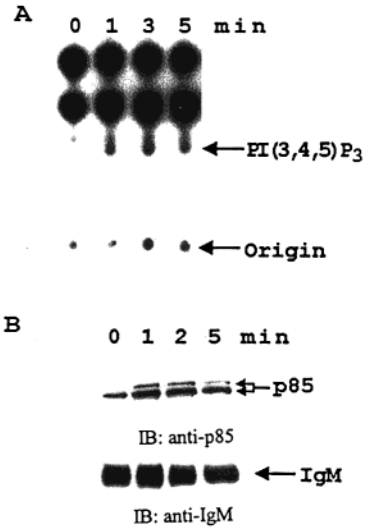

FIGURE 3: Hydrogen peroxide-induced in vivo accumulation of PI-(3,4,5)P_3 and membrane recruitment of p85. (A) Hydrogen peroxide-triggered PI(3,4,5)P_3 production. [^{32}P]Orthophosphate-loaded DT40 cells stimulated with or without 1 mM hydrogen peroxide for the indicated time. Extraction and separation of phospholipids were carried out as described in Materials and Methods. The position of PI(3,4,5)P_3 is indicated with an arrow. (B) Hydrogen peroxide-stimulated membrane translocation of p85. Particulate fractions prepared from DT40 cells treated with or without hydrogen peroxide were separated via 8% SDS−PAGE, transferred, and immunobloted with the anti-p85 antibody (top) or with goat anti-chicken IgM (bottom). The doublet bands detected with the anti-p85 antibody are presumably due to p85α and p85β.

hydrogen peroxide stimulation did induce a 2−3-fold increase in ^{32}P-labeled PI(3,4,5)P_3 content in DT40 cells. Consistent with the in vivo accumulation of PI(3,4,5)P_3, immunoblot analysis of the particulate fraction revealed that, upon hydrogen peroxide stimulation, the quantity of p85, in particular the higher-molecular mass isoform, in particulate fraction was elevated (Figure 3B, top). Equivalent loading was confirmed by blotting with goat anti-chicken IgM (Figure 3B, bottom).

Lyn Deficiency Inhibits Hydrogen Peroxide-Dependent Cbl Tyrosine Phosphorylation and Association with p85 PI3K without Affecting Hydrogen Peroxide-Dependent Activation of Akt. In view of the fact that Cbl has been implicated in mediating PI3K activation by B cell receptor signaling (*37*) and interleukin-4 receptor signaling (*40*), we investigated whether Cbl plays a role in the membrane recruitment of PI3K in response to oxidative stress signaling. We first analyzed tyrosine phosphorylation of Cbl as a function of PTKs. DT40 cells were stimulated, and cell lysates were immunoprecipitated with anti-Cbl antibodies. The resulting proteins were separated by SDS−PAGE and immunoblotted with anti-phosphotyrosine or anti-Cbl antibodies. As shown in panels A and B of Figure 4, 1 mM hydrogen peroxide, which significantly triggered Lyn- and/or Syk-dependent tyrosine phosphorylation of the p110 subunit of PI3K, induced a transient and moderate increase in the level of Cbl tyrosine phosphorylation. However, with higher concentrations of hydrogen peroxide, the level of Cbl tyrosine phosphorylation was dramatically increased. Lyn deficiency resulted in nearly total elimination of hydrogen peroxide-induced Cbl tyrosine phosphorylation, while Cbl tyrosine phosphorylation in Syk- or Btk-deficient DT40 cells remained at the same level or was somewhat enhanced compared to that observed in wild-type DT40 cells. These data clearly indicated that Lyn is the major tyrosine kinase responsible for hydrogen peroxide-induced Cbl tyrosine phosphorylation.

Once tyrosine-phosphorylated, Cbl will provide the docking site for the SH2 domain of p85 subunit of PI3K, leading to tyrosine phosphorylation-dependent complex formation. To verify this, anti-Cbl immunoprecipitates were subjected to separation by SDS−PAGE and probed with anti-p85 antibodies. Figure 4C showed that in Syk- or Btk-deficient and wild-type DT40 cells a relatively stable complex between Cbl and the p85 subunit of PI3K was formed in a hydrogen peroxide concentration-dependent manner. However, in comparison, Lyn deficiency almost abrogated hydrogen peroxide-induced association of Cbl with p85 of PI3K. In agreement with the association of Cbl with p85 of PI3K, hydrogen peroxide stimulated a 2−5-fold increase in Cbl-associated PI3K activity in wild-type DT40 cells under the same conditions (Figure 4D). However, deficiency in Lyn resulted in ~50% reduction in Cbl-associated PI3K activity following hydrogen peroxide stimulation. Cbl-associated PI3K activity in Syk- and Btk-deficient DT40 cells was slightly lower than and comparable to that observed in wild-type DT40 cells (Figure 4D), respectively.

Deficiency in Lyn inhibits hydrogen peroxide-induced tyrosine phosphorylation of Cbl, association of Cbl with p85 of PI3K, and Cbl-associated PI3K activity; thus, we investigated whether Cbl is involved in mediating hydrogen peroxide-induced activation of the PI3K and Akt pathway. Immunoblot analysis of anti-p85 immunoprecipitates with the anti-phosphotyrosine antibody (4G10) revealed that, relative to the wild type, Cbl-deficient DT40 cells exhibited a reduction in the level of tyrosine phosphorylation of p110 of PI3K in response to 1 mM hydrogen peroxide stimulation. However, this reduction was abrogated when the hydrogen peroxide concentration was increased to 5 mM (Figure 4E). Nevertheless, activation of Akt by hydrogen peroxide, assayed by phosphorylation of the GSK-3α/β fusion protein in vitro (Figure 4F), appeared to be independent of Cbl. This finding is confirmed by the results showing that hydrogen peroxide-induced PI(3,4,5)P_3 production is also independent of Cbl (Figure 5B). Thus, the overall data suggest that Cbl is not essential for hydrogen peroxide-induced activation of PI3K and Akt in DT40 cells.

No Requirement of BCAP for Hydrogen Peroxide-Induced Activation of PI3K and Akt. The B cell adaptor for PI3K, BCAP, has been demonstrated to be required for the B cell receptor-triggered activation of PI3K and Akt. In this pathway, the tyrosine-phosphorylated BCAP is involved in membrane recruitment of PI3K (*24*). Therefore, we examined if BCAP mediated hydrogen peroxide-induced activation of the PI3K and Akt pathway utilizing BCAP-deficient DT40 cells. Figure 5A showed that BCAP deficiency abolished hydrogen peroxide-induced tyrosine phosphorylation of p110 of PI3K, while tyrosine phosphorylation of the identity-unknown protein was maintained in the BCAP-deficient DT40 cells. Nevertheless, the PI3K-catalyzed PI(3,4,5)P_3 generation induced by hydrogen peroxide in the wild-type and BCAP-deficient DT40 cells was comparable, though somewhat transient, in BCAP-deficient cells (Figure 5B). Akt kinase assay also revealed that deficiency in BCAP did not affect hydrogen peroxide-induced activation of Akt (Figure

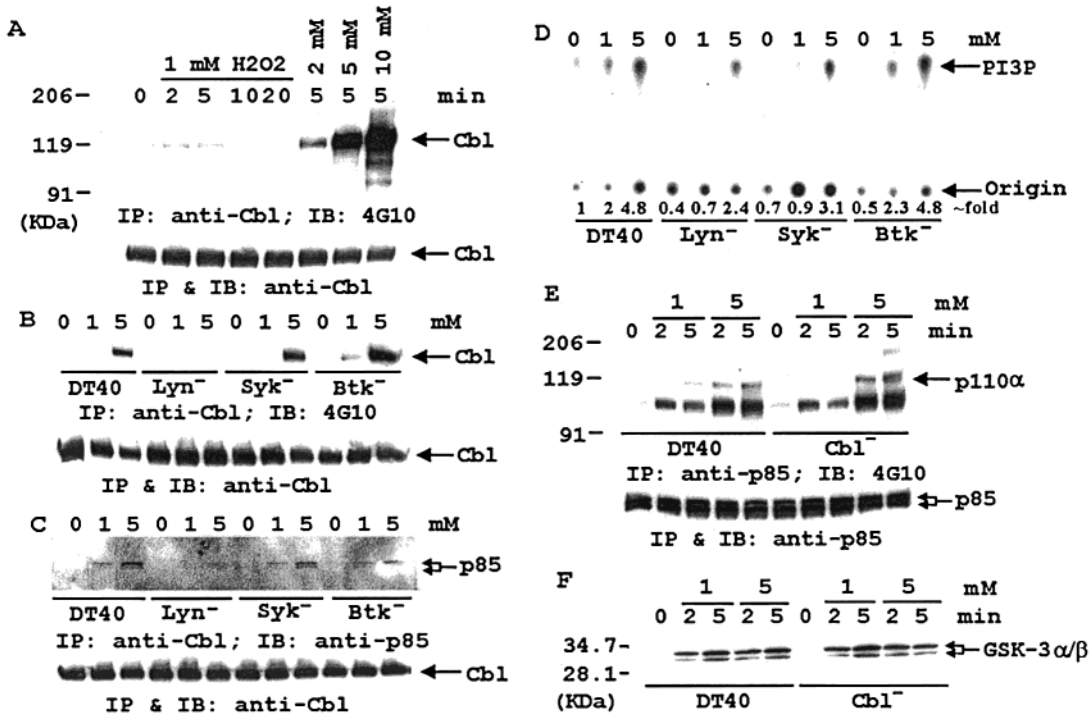

FIGURE 4: In vivo tyrosine phosphorylation-dependent association of Cbl with the p85 subunit of PI3K. (A) Time- and dose-dependent tyrosine phosphorylation of Cbl.DT40 cells were stimulated with hydrogen peroxide for the indicated reaction times or doses. Anti-Cbl immunoprecipitates were resolved via 8% SDS−PAGE and immunoblotted with either anti-phosphotyrosine (top) or anti-Cbl (bottom) antibodies. (B) Lyn-dependent tyrosine phosphorylation of Cbl. Lysates from wild-type or Lyn-, Syk-, or Btk-deficient DT40 cells with or without hydrogen peroxide stimulation for 5 min were immunoprecipitated with anti-Cbl. Anti-Cbl immunoprecipitates were probed with either anti-PTyr (top) or anti-Cbl (bottom). (C) H_2O_2-dependent association of Cbl with the p85 subunit of PI3K. Lysates from wild-type or Lyn-, Syk-, or Btk-deficient DT40 cells with or without stimulation (5 min) with the indicated concentrations of hydrogen peroxide were immunoprecipitated with anti-Cbl. Anti-Cbl immunoprecipitates were probed with either anti-p85 (top) or anti-Cbl (bottom). (D) Cbl-associated PI3-kinase activity. Lysates from wild-type or Lyn-, Syk-, or Btk-deficient DT40 cells with or without hydrogen peroxide stimulation were immunoprecipitated with anti-Cbl. PI3K activity was assayed as described in Materials and Methods using phosphatidylinositol as an in vitro substrate. (E) No effect of Cbl on hydrogen peroxide-induced tyrosine phosphorylation of the p110 subunit of PI3K. Lysates from wild-type and Cbl-deficient DT40 cells with or without hydrogen peroxide stimulation were immunoprecipitated with the polyclonal antibody against the p85 subunit of PI3K, which also coprecipitates the p110 catalytic subunit of PI3K. Anti-PI3K immunoprecipitates were probed with either anti-PTyr (top) or anti-p85 (bottom). (F) No requirement for Cbl in hydrogen peroxide-induced activation of Akt. Cell lysates from wide-type and Cbl-deficient DT40 cells with or without hydrogen peroxide stimulation were immunoprecipitated with the anti-Akt antibody, and the kinase assay was carried out as described by the manufacturer's protocol.

5C). In contrast, anti-IgM-induced PI3K-dependent Akt activation in DT40 cells was BCAP-dependent (Figure 5D).

No Apparent Role of Ras in Hydrogen Peroxide-Induced PI(3,4,5)P_3 Accumulation and Akt Activation. Redox stress is shown to activate Ras, probably through oxidation of cysteine 118, and the activated Ras targets the p110 subunit of PI3K to the plasma membrane, leading to apparent activation of PI3K (*41*, *42*). Hydrogen peroxide-induced Ras activation was assayed using the Ras binding domain of Raf fused to GST, since activated Ras was able to bind the Ras binding domain on Raf. As shown in Figure 6A, Ras activation by 1 mM hydrogen peroxide occurred at 2 min and was maintained within 5 min. Ras activation by hydrogen peroxide was inhibited by pretreatment with 10 μM manumycin A, which is a potent and selective farnesyltransferase inhibitor (*43*). A role for Ras in mediating hydrogen peroxide-induced activation of PI3K and Akt was next tested by using the Ras inhibitor manumycin A. Figure 6B illustrates that pretreatment of DT40 cells with manumycin A failed to inhibit hydrogen peroxide-induced PI(3,4,5)P_3 accumulation, as compared to that observed in manumycin A-untreated DT40 cells. Furthermore, PI3K-dependent Akt activation by hydrogen peroxide was also not affected by manumycin A treatment, since Akt activity in manumycin A-treated and untreared DT40 cells was comparable (Figure 6C).

DISCUSSION

Tyrosine phosphorylation of either the p85 subunit, the p110 subunit, or both subunits of PI3K has been reported in different cell systems in response to a variety of ligands (14−16). Panchamoorthy et al. (*44*) also showed that neither of the PI3K subunits was tyrosine-phosphorylated upon B cell receptor engagement. The effect of tyrosine phosphorylation of PI3K subunits on enzymatic activity was elusive and quite controversial. Tyrosine phosphorylation of p85 has been shown to inhibit or stimulate PI3K activity (*17*, *18*). With the DT40 system, we did observe hydrogen peroxide-induced tyrosine phosphorylation of p110 (Figure 1B). Taking advantage of PTK-deficient DT40 cells, we demonstrated that Lyn and/or Syk was responsible for hydrogen peroxide-induced p110 tyrosine phosphorylation. Tyrosine phosphorylation of p110 seemed to play little or no role in regulating its own enzymatic activity. Supporting evidence includes the following. (1) Deficiency in Lyn or Syk that was essential for hydrogen peroxide-induced tyrosine phosphorylation of the p110 subunit neither changed PI3K activity nor inhibited hydrogen peroxide-induced PI3K-dependent activation of Akt

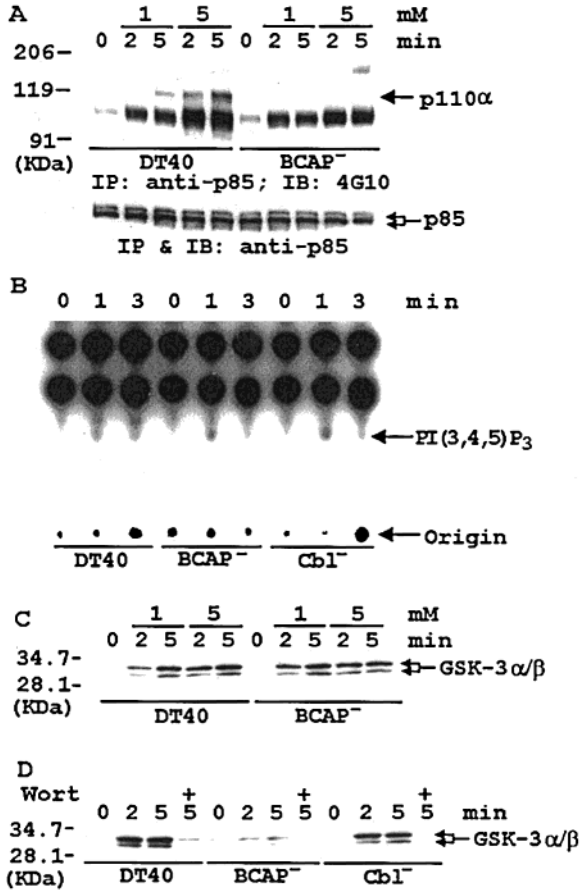

FIGURE 5: Distinctive effect of BCAP on hydrogen peroxide and anti-IgM-triggered activation of Akt. (A) Effect of BCAP on hydrogen peroxide-induced tyrosine phosphorylation of the p110 subunit of PI3K. Lysates from wild-type and BCAP-deficient DT40 cells with or without hydrogen peroxide stimulation were immunoprecipitated with the polyclonal antibody against the p85 subunit of PI3K, which also coprecipitates its p110 catalytic subunit. Anti-PI3K immunoprecipitates were probed with either anti-PTyr (top) or anti-p85 (bottom). (B) BCAP and Cbl were not essential for hydrogen peroxide-induced generation of PI(3,4,5)P_3. [^{32}P]Orthophosphate-loaded wild-type, BCAP-deficient, and Cbl-deficient DT40 cells were stimulated with or without 1 mM hydrogen peroxide for 1 or 3 min. Extraction and separation of phospholipids were carried out as described in Materials and Methods. The position of PI(3,4,5)P_3 is indicated with an arrow. (C) No requirement for BCAP on hydrogen peroxide-induced activation of Akt. Cell lysates from wild-type and BCAP-deficient DT40 cells with or without hydrogen peroxide stimulation were immunoprecipitated with the anti-Akt antibody, and the kinase assay was carried out as described by the manufacturer's protocol. (D) Requirement for BCAP but not Cbl for anti-IgM-induced activation of Akt. Cells were preincubated with either 100 nM Wortmannin or an equal volume of solvent (DMSO) for 20 min and then stimulated with 5 μg/mL anti-IgM for the indicated reaction times. Cell lysates from wild-type and BCAP-deficient DT40 cells were immunoprecipitated with the anti-Akt antibody, and the kinase assay was carried out following the manufacturer's instructions.

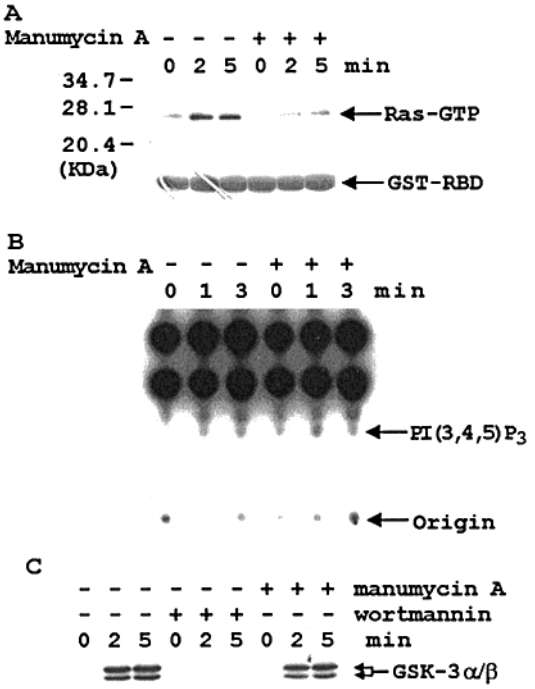

FIGURE 6: No role of Ras activation in hydrogen peroxide-induced activation of PI3K and Akt. (A) Inhibition of hydrogen peroxide-induced Ras activation by manumycin A. DT40 cells (1×10^7 cells/mL) were pretreated with or without 10 μM manumycin A for 60 min and then stimulated with 1 mM hydrogen peroxide for 2 or 5 min. Cell lysates were incubated for 2 h with the GST−RBD conjugate coupled to glutathione−agarose beads. Bound proteins were separated via 4 to 20% SDS−PAGE, transferred to a PVDF membrane, and immunoblotted with anti-pan-Ras antibody (Clone RAS10) (upper). The lower band shows the GST−RBD conjugate and confirms equal protein loading as assayed by Coomassie Blue staining of the proteins remaining on the SDS−PAGE gel after transfer. (B) No effect of manumycin A on hydrogen peroxide-induced generation of PI(3,4,5)P_3. [^{32}P]Orthophosphate-loaded DT40 cells were preincubated with or without 10 mM manumycin A for 60 min and then stimulated with or without 1 mM hydrogen peroxide for 1 or 3 min. Extraction and separation of phospholipids were carried out as described in Materials and Methods. The position of PI(3,4,5)P_3 is indicated with an arrow. (C) No effect of Ras inhibition on hydrogen peroxide-induced Akt activation. DT40 cells were pretreated with or without 10 μM manumycin A or 100 nM Wortmannin for 60 min and then stimulated with or without 1 mM hydrogen peroxide. The cell lysates that were obtained were immunoprecipitated with the anti-Akt antibody, and the kinase assay was carried out as described by the manufacturer's protocol.

(Figures 1C and 2), and (2) although BCAP was required for hydrogen peroxide-induced tyrosine phosphorylation of p110 (Figure 5A), hydrogen peroxide-induced activation of PI3K and Akt was almost intact in BCAP-deficient DT40 cells (Figure 5B,C). Intriguingly, deficiency in Btk led to an increase in anti-phosphotyrosine antibody immunoprecipitated PI3K activity upon hydrogen peroxide stimulation (Figure 1A). The molecular basis for this observation is being investigated, and the results will be published elsewhere (manuscript in preparation).

Hydrogen peroxide stimulated a PI3K-dependent activation of Akt without altering PI3K activity. This may result from hydrogen peroxide-stimulated membrane recruitment of PI3K, thereby enabling PI3K to catalyze PI(3,4,5)P_3 production. Alternatively, inhibition of the PI3K-antagonizing phosphatase PTEN by hydrogen peroxide could lead to PI(3,4,5)P_3 accumulation. TLC analysis of radiolabeled phospholipids revealed that hydrogen peroxide treatment induced PI(3,4,5)P_3 accumulation (Figure 3A). In DT40 cells, a role of PTEN in mediating hydrogen peroxide-induced PI(3,4,5)P_3 accumulation was excluded because there is no detectable expression of PTEN in DT40 cells (*36*). Immunoblot analysis showed that the amount of p85 present in the particulate fraction was elevated following hydrogen peroxide stimulation (Figure 3B). Furthermore, p85 membrane relocation and PI(3,4,5)P_3 accumulation were not affected by either Syk or

Btk deficiencies, since the amount of p85 in the membrane fraction upon hydrogen peroxide stimulation was comparable in wild-type, Syk-deficient, and Btk-deficient DT40 cells (data not shown). This observation also suggested that the identity-unknown, strongly tyrosine-phosphorylated, and PI3K-associated 98 kDa protein (see Figures 1B, 4E, and 5A) was unlikely to play a critical role in mediating hydrogen peroxide-induced p85 membrane relocation. Thus, membrane recruitment of PI3K in response to hydrogen peroxide stimulation was responsible for the accumulation of PI(3,4,5)-P_3. PI3K could be targeted to membrane via tyrosine-phosphorylated adaptor proteins that provide docking sites for the SH2 domains of p85 or activated Ras that directly binds to the p110 subunit of PI3K or both. How does PI3K translocate to the plasma membrane in response to hydrogen peroxide stimulation? To elucidate this pathway, we found that hydrogen peroxide induced tyrosine phosphorylation of Cbl via a Lyn-dependent mechanism (Figure 4A,B). Hydrogen peroxide stimulation led to the association of Cbl with p85 and increased PI3K activity in anti-Cbl immunoprecipitates (Figure 4C,D). Deficiency in Lyn partially abolished hydrogen peroxide-induced association of Cbl with p85 and the PI3K activity coprecipitated by the anti-Cbl antibody. However, Cbl appeared not to be essential for hydrogen peroxide-induced PI(3,4,5)P_3 production (Figure 5B) and Akt activation (Figure 4F), since the levels of PI(3,4,5)P_3 production and Akt activation were comparable in wild-type and Cbl-deficient DT40 cells.

Another adaptor protein to be considered is BCAP. Although BCAP was essential for the activation of PI3K and Akt induced by B cell receptor signaling (ref *24* and Figure 5D), it was not required for hydrogen peroxide-induced activation of PI3K and Akt, since PI(3,4,5)P_3 production (Figure 5B) and Akt activity (Figure 5C) in BCAP-deficient DT40 cells were comparable to those in wild-type DT40 cells. CD19 and Gab are two other known adaptor proteins involved in PI3K activation in B cells. Expression of mouse CD19 in DT40 cells revealed that cross-linking CD19 activated PI3K via tyrosine phosphorylation of BCAP (*45*). However, the existence of the chicken homologues of CD19 and Gab is not known. We tried commercially available anti-Gab antibodies, and unfortunately, none of them recognized chicken Gab if it existed. Overall, our data obtained with 1−5 mM H_2O_2 indicate that hydrogen peroxide-induced activation of PI3K and Akt is independent of either Lyn, Syk, Btk, BCAP, or Cbl. These results suggest that >1 mM hydrogen peroxide can activate multiple pathways, leading to the activation of PI3K and Akt. In this regard, Cbl was shown to be required for 1 mM, but not 5 mM, hydrogen peroxide-induced tyrosine phosphorylation of the p110 subunit (Figure 4E). Ding et al. (*46*) reported that Akt activation by 0.1 mM, but not by 1 mM, hydrogen peroxide was dependent on functional Syk.

In addition to adaptor proteins, activated Ras has been shown to activate PI3K by directly binding and targeting the p110 subunit to the plasma membrane (*41*, *42*). We took a pharmacological approach to defining the role of Ras in regulating the hydrogen peroxide-induced activation of PI3K and Akt. In DT40 cells, hydrogen peroxide treatment was able to activate Ras, and this activation was inhibited by pretreatment with a Ras-specific inhibitor, manumycin A (Figure 6A). However, PI(3,4,5)P_3 production and Akt activation were not affected by pretreatment of DT40 cells with manumycin A (Figure 6B,C), suggesting that in our system Ras is not involved in mediating the hydrogen peroxide-induced activation of PI3K and Akt.

Akt activation is widely considered by lipid binding to the PH domain that mediates the recruitment of Akt to the plasma membrane, thereby promoting the phosphorylation of Akt on Thr^{308} and Ser^{473} by PDK-1 and -2, respectively (*32*, *33*). Recently, tyrosine phosphorylation was reported to be essential for the full activation of PDK1 and Akt. Pervanadate or hydrogen peroxide treatment induced tyrosine phosphorylation of PDK1 on three tyrosine residues and increased its enzymatic activity, likely through phosphorylation of Tyr^{373} (*47*, *48*). Akt activation by the epidermal growth factor also required phosphorylation of Akt on Tyr^{315} and Tyr^{326} (*49*). We tried to examine whether hydrogen peroxide-induced activation of Akt was regulated by tyrosine phosphorylation in our system. Immunoblot analysis showed that no tyrosine phosphorylation of Akt was detected following hydrogen peroxide stimulation (data not shown). Taken together with the fact that hydrogen peroxide-induced activation of Akt was not affected in Lyn-, Syk-, or Btk-deficient DT40 cells (Figure 2), the tyrosine phosphorylation pathway plays little or no role in hydrogen peroxide-triggered activation of Akt.

It is worth noting that Halstead et al. demonstrated an alternative pathway for in vivo production of PI(3,4,5)P_3 catalyzed by murine type 1α phosphatidylinositol 4-phosphate 5-kinase, an enzyme acting as an endogenous PI(3,4)-P_2 5-kinase in response to oxidative stress. PI(3,4,5)P_3 synthesis by this pathway was completely insensitive to Wortmannin (*50*). Hydrogen peroxide induced PI(3,4,5)P_3 production in our DT40 cells; however, it was almost eliminated by Wortmannin pretreatment (data not shown). Therefore, the observed hydrogen peroxide-induced PI-(3,4,5)P_3 production was not likely to be catalyzed by type 1α phosphatidylinositol 4-phosphate 5-kinase, although we could not completely rule out its contribution.

In summary, our genetic data described above demonstrate that hydrogen peroxide stimulation did not trigger an increase in intrinsic PI3K activity. Hydrogen peroxide-induced tyrosine phosphorylation of the p110 catalytic subunit of PI3K did not alter its enzymatic activity, since PI3K activity was not changed in Lyn- or Syk-deficient DT40 cells and the two cell lines failed to induce tyrosine phosphorylation of p110. The apparent induction of PI(3,4,5)P_3 production and Akt activation by hydrogen peroxide was achieved through PI3K membrane recruitment, thereby enabling PI3K to phosphorylate its substrate. Hydrogen peroxide-triggered membrane recruitment of PI3K was independent of either adaptor proteins, BCAP and Cbl, or small G protein Ras.

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