CD40 is a 50-kD transmembrane glycoprotein expressed on all mature B cells (1, 2). It plays a critical role in B cell survival, activation, and differentiation. Engagement of CD40 by mAb results in B cell proliferation (3, 4), homotypic cell adhesion (5, 6), and expression of bcl-2 protein in germinal center B cells (7). In synergy with IL-4, mAbs against CD40 promote long-term B cell growth in culture (8) and induce resting B cells to undergo IgE isotype switching via deletional switch recombination (9). A ligand for CD40 is expressed on T cells upon activation (10, 11). Interaction between CD40 and its ligand appears to play an important role in T cell–dependent isotype switching because a soluble form of CD40 inhibits T cell–driven isotype switching to IgE in IL-4–treated B cells (12). Furthermore, Epstein-Barr virus–transformed B cells transfected with the CD40 ligand replace T cells in synergizing with IL-4 to induce IgE synthesis in B cells (11).

B cells stimulated by anti-CD40 mAb demonstrate increased protein tyrosine kinase (PTK) activity, 1,4,5-inositol triphosphate (IP3) production, and serine–threonine kinase activity (13). Activation of PTK appears to play an important role in mediating the biologic effects of CD40 because PTK inhibitors inhibit B cell aggregation (6) and isotype switching induced by anti-CD40 mAbs (14). The predicted amino acid sequence of the CD40 cytoplasmic region does not contain a kinase domain (15), suggesting that CD40-mediated PTK activity occurs indirectly, via activation of a separate kinase. However, the identity of that putative kinase has not yet been determined.

We have investigated the CD40 signal transduction pathway, focusing on the role of src-type PTK and subsequent target substrates for CD40-mediated PTK activation. We now report that CD40 engagement results in the activation of the src-type PTK lyn and of phosphatidylinositol-3-kinase (PI-3-kinase). Furthermore, we show that both PI-3-kinase and phospholipase Cγ2 (PLCγ2) are substrates for CD40-mediated phosphorylation. Our findings suggest that these three proteins play an important role in CD40 signal transduction.
Blot analysis as previously described (20). Blots were incubated with rabbit antiserum of the appropriate specificity followed by goat anti-rabbit Ig conjugated to alkaline phosphatase, and then visualized using alkaline phosphatase developing reagent (Bio-Rad Laboratories, Hercules, CA).

Western Blot Analysis. To measure the amount of peptide in each sample, an aliquot of equal volume from each immunoprecipitate was transferred to polyvinylidene difluoride membranes for Western blot analysis as previously described (20). Blots were incubated with rabbit antiserum of the appropriate specificity followed by goat anti-rabbit Ig conjugated to alkaline phosphatase, and then visualized using alkaline phosphatase developing reagent (Bio-Rad Laboratories, Hercules, CA).

PI-3-Kinase Assays. PI-3-kinase assays were carried out as described by Gold et al. (21). After stimulation, cells (15 x 10⁶/ml) were lysed and PI-3-kinase immunoprecipitates were prepared as described above. The immunoprecipitates were then washed four times with 150 mM NaCl, 20 mM Tris HCl (pH 7.5). Kinase reaction buffer containing 0.2 mg/ml sonicated phosphatidylinositol, 10 mM ATP, 200 mM adenosine, 20 mM MgCl₂, and 20 mM Hepes, pH 7.4, and 10 μCi γ-[³²P]ATP was added to each sample. The reaction was allowed to continue for 20 min at room temperature, and terminated by addition of 450 μl 1.0 M HCl. The lipid fraction was extracted with 225 μl chloroform/methanol (1:1) and concentrated under nitrogen to a volume of 50 μl. Each sample was spotted onto a silica gel TLC plate that had been impregnated with 1% potassium oxalate, dried, and baked for 1 h at 140°C.

PI-3-Kinase Assays. PI-3-kinase assays were carried out as described above. Blots were incubated with rabbit antiserum of the appropriate specificity followed by goat anti-rabbit Ig conjugated to alkaline phosphatase, and then visualized using alkaline phosphatase developing reagent (Bio-Rad Laboratories, Hercules, CA).

Immune Complex Kinase Assays. After stimulation, cells (10⁶/ml) were lysed using the same lysis buffer, and the lysates were preclarified by pelleting the cells and lysing with lysis buffer (1% NP-40, 150 mM NaCl, 10 mM Hepes, pH 7.4, 1 mM PMSF, 1 mM Na₃VO₄, 50 mM NaF, 25 μg/ml leupeptin, and 1 μg/ml each of chymostatin, pepstatin, and antipain). Immunoprecipitates from the cell lysates were prepared by preclarifying with normal rabbit serum and precipitating with specific antisera and protein G agarose as previously described (19). An aliquot of equal volume from each immunoprecipitate was subjected to SDS-PAGE under reducing conditions. Radiolabeled proteins were visualized by autoradiography and densitometry was performed as previously described (19).

Immune Complex Kinase Assays. After stimulation, cells (10⁶/ml) were lysed using the same lysis buffer, and the lysates were preclarified and specifically immunoprecipitated as described above. The immunoprecipitates were then resuspended in 50 μl kinase reaction buffer (0.5% NP-40, 150 mM NaCl, 10 mM MnCl₂, 10 mM MgCl₂, 20 mM Hepes, pH 7.4) with 5 μM ATP and 2.5 μCi γ-[³²P]ATP. Acid denatured enolase (2.5 μg/sample) was added as an exogenous substrate. The reaction was terminated with Laemmli sample buffer, and equal volume aliquots from each sample were analyzed by SDS-PAGE. Phosphorylation of enolase was then visualized by autoradiography and quantitated by densitometry.

Results

CD40 Engagement Results in Activation of lym Kinase. Ligation of CD40 antigen on B cell lines by mAbs results in increased PTK activity as detected by tyrosine phosphorylation of multiple proteins (13). Because the cytoplasmic domain of CD40 does not code for a kinase (15), we investigated the possibility that a src-type kinase plays a role in CD40-mediated PTK activation.

The three major src-type kinases expressed in B cells are lym, fyn, and blk (22). Due to differential mRNA splicing, two isoforms of lym exist, with molecular masses of 53 and 56 kD (23). Both isoforms are expressed in Daudi cells (24). Fyn also exists in two isoforms, with molecular masses 56 and 59 kD, but only the 59-kD isoform is expressed in B cells (25). Expression of blk by Daudi cells has not been reported. In addition to these three PTK, fgr expression has been reported in EBV transformed B cell lines, such as Daudi (26). Expression of blk in B cells and B cell lines has also been reported (27).

We first examined by an in situ phosphorylation technique the effect of CD40 engagement on the phosphorylation of lym. Fig. 1 A shows the results of a representative in situ phosphorylation experiment. Within 1 min after stimulation of Daudi cells with the anti-CD40 mAb 626.1, an increase in phosphorylation of both lym isoforms was noted. Phosphorylation peaked at 5 min and remained sustained for up to 20 min. Western blot analysis of anti-CD40-stimulated Daudi cell lysates showed equal amounts of precipitable kinase at all time points, indicating that the increase in lym phosphorylation noted after CD40 ligation was not merely due to a change in the amount of precipitated kinase (Fig. 1B). Fig. 1 C shows the pooled results from six different experiments. Phosphorylation of lym kinase is expressed as relative phosphorylation noted after CD40 ligation was not merely due to a change in the amount of precipitated kinase (Fig. 1B). Fig. 1 C shows the pooled results from six different experiments. Phosphorylation of lym kinase is expressed as relative phosphorylation noted after CD40 ligation was not merely due to a change in the amount of precipitated kinase (Fig. 1B).
Figure 1. Effect of CD40 engagement on phosphorylation of lyn kinase. (A) Daudi cells (10⁷/ml) were stimulated with anti-CD40 mAb 626.1 for the time periods (min) indicated. Phosphorylation of lyn was measured using the in situ phosphorylation assay as described in Materials and Methods. The migration positions of the 53- and 55-kD isoforms of lyn are indicated by the labeled arrow. (B) Western blot of aliquots from each immunoprecipitate sample, using lyn antisera as a probe and alkaline phosphatase as the developing reagent as described in Materials and Methods. (C) Pooled results of six separate experiments. The mean relative phosphorylation index at each time point was determined. Error bars represent 2 standard errors. The lanes labeled NRS represent immunoprecipitates prepared with control preimmune normal rabbit serum.

Figure 2. Effect of CD40 engagement on lyn kinase activity. (A) Daudi cells (10⁷/ml) were stimulated with mAb 626.1 for the time points indicated (min). Phosphorylation of enolase was detected using the immune complex kinase assay. The migration position of enolase is indicated by the labeled arrow. The lane labeled TS1/22 represents a sample of Daudi cells stimulated for 5 min with the anti-LFA-1 mAb TS1/22. The lane labeled NRS represents immunoprecipitates prepared with control preimmune normal rabbit serum. (B) Prolonged exposure of an autoradiogram of another immune complex kinase assay performed under identical conditions as described above. Autophosphorylation of lyn can be seen. (C) Pooled results of six separate experiments. The mean relative phosphorylation index of enolase at each time point was determined. Error bars represent 2 standard errors.

Complex kinase assays, CD40-mediated lyn autophosphorylation was also observed, the intensity of which paralleled the enzymatic activity of lyn upon the enolase substrate (Fig. 2 B). The results from six different experiments were pooled and shown in Fig. 2 C. Activity of lyn kinase is expressed as relative enolase phosphorylation index. This index is the ratio of the densitometric value of enolase in stimulated vs. unstimulated cells (time 0). The mean increase in enolase phosphorylation was 2.8-fold at 1 min, 3.7-fold at 5 min, and remained elevated 2.2-fold 20 min poststimulation.

The effect of anti-CD40 mAbs on lyn was specific, because stimulation of Daudi cells with an isotype-matched mAb against LFA-1 (mAb TS1/22) had no effect on lyn kinase activity (Fig. 2 A). CD40-mediated activation of lyn kinase did not require engagement of Fc receptors on Daudi cells, since biotinylated Fab fragments of mAb 626.1 cross-linked with

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avidin elicited equivalent degrees of lyn phosphorylation and enzymatic activity as intact antibody (data not shown).

In contrast to the effect of CD40 engagement on lyn, treatment of Daudi cells with anti-CD40 mAb resulted in markedly different effects on other src-type PTK. Minimal phosphorylation of fyn was observed in mAb 626.1-stimulated Daudi cells (Fig. 3 A). In three experiments, maximal fyn phosphorylation was 1.7-fold at 5 min poststimulation. There was also no significant increase in fyn autophosphorylation or kinase activity towards the enolase substrate after CD40 engagement (Fig. 3 B). In four experiments, maximal fyn kinase activity was 1.3-fold at 1 min poststimulation. Western blot analysis showed no change in precipitable fyn after stimulation with anti-CD40 mAb (Fig. 3 C). Fig. 4 shows that CD40 engagement resulted in a minimal, transient phosphorylation of fgr at 1 min (Fig. 4 A) and in no detectable change in kk phosphorylation (Fig. 4 B). fgr and kk enzymatic activity were barely detectable using our immune complex kinase assay; no significant change was seen after stimulation of Daudi with anti-CD40 mAbs, although in the same experiment CD40-mediated lyn activation was clearly evident (data not shown). Western blots confirmed that equal amounts of kinase were detected in each lane (Fig. 4, B and D).

These results suggest a differential role for B cell src type kinases in CD40 signal transduction.

**CD40 Engagement Results in Phosphorylation and Activation of PI-3-Kinase.** Engagement of cell surface receptors that de-

Liver mitogenic signals, such as platelet-derived growth factor receptor (PDGFR) (28), sIg (21), and IL-2R (29), is associated with activation of the enzyme, PI-3-kinase, which catalyzes the phosphorylation of phosphoinositols on the 3' moiety (30). PI-3-kinase appears to play a key role in mitogenesis, because abrogation of PI-3-kinase activation via PDGF receptor results in loss of PDGFR-induced mitogenesis (28). PI-3-kinase is a heterodimer consisting of a 85-kD regulatory subunit (p85) and a 110-kD catalytic subunit (p110) (30). Tyrosine phosphorylation of the p85 chain is thought to be important in the regulation of enzyme activity (21, 30, 31).

Stimulation of B cells with anti-CD40 mAb 626.1 or with cells transfected with CD40 ligand results in their proliferation (4, 11). To examine the capacity of CD40 engagement to phosphorylate PI-3-kinase, Daudi cells were stimulated with mAb 626.1 and phosphorylation of PI-3-kinase was assessed by the in situ phosphorylation technique, using a rabbit antibody directed against the p85 subunit. Fig. 5 A shows that p85 phosphorylation peaked by 1 min after CD40 ligation and returned to baseline by 10 min. Western blot analysis of the cell lysates showed no change in the amount of precipitable PI-3-kinase after anti-CD40 stimulation, indicating that increased p85 phosphorylation was not due to an increased amount of protein (Fig. 5 B). Similar results were obtained in two other experiments. CD40 engagement had a minimal effect on the phosphorylation of the p110 subunit of PI-3-kinase, which coprecipitates with p85 (32). A 90-kD phosphoprotein was also seen in the PI-3-kinase immunoprecipitates and displayed minimal changes in phosphorylation after CD40 engagement.

CD40-mediated phosphorylation of the p85 subunit of PI-3-kinase was accompanied by increased enzymatic activity.
Figure 5. Effect of CD40 engagement on phosphorylation of PI-3-kinase. Daudi cells (10^7/ml) were stimulated with mAb 626.1 for the times (min) indicated. (A) Phosphorylation of PI-3-kinase was measured using the in situ phosphorylation assay. (B) Western blot of aliquots from each of the immunoprecipitates using anti-PI-3-kinase antisem and developed using alkaline phosphatase system. The position of the p85 subunit of PI-3-kinase is indicated by the labeled arrow.

Figure 6. Effect of CD40 engagement on PI-3-kinase activity. Daudi cells (15 x 10^6/ml) were incubated in for 5 min with mAb 626.1 or with goat anti-IgM for 2 min, as indicated. PI-3-kinase assays were performed as described in Materials and Methods, and PI-3-P formation was detected by TLC. The migration position of PI-3-P is indicated by the labeled arrow. The origin (labeled with arrow) contains residual γ-[32P]ATP that was present in the lipid extracts, the absolute amount of which is not relevant.

Fig. 6 shows the enzymatic activity of PI-3-kinase immunoprecipitates isolated from Daudi cells stimulated for 5 min with anti-CD40 mAb 626.1. PI-3-kinase immunoprecipitates from unstimulated cells and from cells stimulated with goat anti-human IgM were used as negative and positive controls, respectively. The results show that CD40 engagement resulted in a significant increase in PI-3-kinase activity, as evidenced by increased PI-3-P formation.

Taken together, these results identify PI-3-kinase as a target for CD40-mediated phosphorylation and activation.

CD40 Engagement Results in Phosphorylation of PLCγ2. The enzyme PLC hydrolyzes 4,5-inositol bisphosphate to form IP3 and diacylglycerol (33). The results of prior investigations suggest that the activity of PLCγ isoforms is regulated by tyrosine phosphorylation. Epidermal growth factor-mediated tyrosine phosphorylation of PLCγ1 results in increased catalytic activity of the enzyme (34). Conversely, mutations in PLCγ1 that result in loss of tyrosine phosphorylation sites prevent PDGF-mediated activation of the enzyme (35). More importantly, ligation of antigen receptors on T and B cells results in tyrosine phosphorylation of PLCγ and in IP3 generation, and both events are abrogated by treatment with PTK inhibitors (36-38).

Stimulation of B cell lines with anti-CD40 mAbs results in the generation of IP3 (13). This is inhibited by tyrosine kinase inhibitors, suggesting that PLCγ may be a substrate for CD40-triggered PTK activity in B cells. Human B cells express two isoforms of PLCγ, PLCγ1, and PLCγ2. The PLCγ2 isoform is expressed in much greater abundance than the PLCγ1 isoform in B cells, including Daudi cells (39). Phosphorylation of PLCγ was measured by in situ phosphorylation. Fig. 7A shows that CD40 ligation on Daudi cells caused increased phosphorylation of PLCγ2 which peaked by 1 min after stimulation and returned to baseline by 20 min. Western blot analysis (Fig. 7B) demonstrated that equivalent amounts of PLCγ2 were present in each sample. Only minimal amounts of PLCγ1 were detected in Daudi cell ly-
sates by Western blot, as previously reported, and no changes in its phosphorylation could be detected after CD40 ligation (data not shown).

Discussion

In this report we demonstrate that CD40 ligation results in the phosphorylation and activation of the src-type PTK lyn in B cells. We show that both PI-3-kinase and PLCγ2 are target substrates for CD40-mediated phosphorylation. PI-3-kinase phosphorylation was accompanied by activation of its enzymatic activity. PLCγ2 phosphorylation is consistent with the previously observed CD40-mediated generation of IP3.

We demonstrated increased phosphorylation of lyn within 1 min of CD40 engagement. The regulation of src-type kinases by tyrosine phosphorylation is complex. Dephosphorylation of a COOH-terminal phosphotyrosine results in increased enzymatic activity, leading to a net increase in tyrosine phosphorylation via autophosphorylation (26). Under the conditions of our in situ phosphorylation assay, we could not assess the possibility that lyn undergoes a transient, early dephosphorylation event. However, our studies of lyn activity in immune complex kinase assays provide direct evidence for CD40-mediated lyn activation.

While our results present clear evidence for the involvement of lyn in CD40 signal transduction, we cannot rule out the possibility that other PTKs are also involved and contribute to CD40-mediated PTK activity. We observed minimal changes in the phosphorylation and activity of fgr and fgr, but no detectable change in blk, after stimulation of Daudi cells with anti-CD40 mAbs for 1–20 min, suggesting that these kinases do not participate in CD40-mediated PTK activity over this time interval. Further studies are required to ascertain the functional roles of lyn, lyn, and fgr in CD40 signal transduction. We could not rule out activation of the B cell–specific PTK blk, because the human blk sequence is not known and antiserum to human blk was not available. It remains to be established whether CD40 engagement could also activate cytoplasmic PTK, such as syk (40, 41) and the recently described B cell–specific PTK, atk/bkp. (42, 43).

In several systems, src-type kinases which are activated by cell surface receptors have been found to associate physically with the receptor complex. Examples include the association of lyn, lyn, and blk (25) with sIg, of lyn with the TCR (44), and of blk with CD4 and CD8 (45). We have been unable to date to coprecipitate lyn with CD40 from cell lysates prepared with a limited panel of detergent buffers (data not shown). This does not preclude a physical association between lyn and CD40. Such an association may be tenuous and easily disrupted, even in the presence of mild detergents. Alternatively, lyn may transiently associate with CD40 only upon engagement of the receptor.

In the case of several cell receptors on immune cells, including the TCR, sIg, FcεRI, and FcγRIII (CD16), src-type kinases bind to receptor-associated molecules that belong to a protein family including CD3 γ, δ, ε, mb-1, B29, FcεRIβ, and FcγRIγ (46). The cytoplasmic domains of these chains share a common tyrosine-containing motif (D/E-X7-D/E- X7-Y-X7-L-X7-Y-X7-L/I) which is thought to be important in establishing a physical association between the SH2 domains of src-type kinases and these proteins (46). For CD4, a cysteine-rich cytoplasmic domain has been shown to be critical for the binding of this receptor to p56lk (47). The cloned CD40 cDNA codes for neither of these two motifs, suggesting that association of lyn with CD40 may require different interactions between these two proteins.

We identified PI-3-kinase as a target for phosphorylation after CD40 engagement in Daudi cells. More importantly, the enzymatic activity of PI-3-kinase was increased after CD40 ligation. Given the importance of PI-3-kinase in mitogenesis and cell proliferation, activation of this enzyme may play a critical role in B cell proliferation in response to anti-CD40 mAbs and to the CD40 ligand expressed on activated T cells.

Another target of CD40-mediated phosphorylation was PLCγ2, the predominant PLCγ isofrom in human B cells. We could not rule out PLCγ1 phosphorylation after anti-CD40 stimulation because of the low level of PLCγ1 expression in Daudi cells. Tyrosine phosphorylation of PLCγ by antigen receptor ligation has been reported to be associated with activation of this enzyme (36, 38). We did not directly measure PLCγ2 activity after anti-CD40 stimulation. However, it is likely that PLCγ2 is responsible for CD40-mediated generation of IP3 which is blocked by PTK inhibitors.

Although activation of lyn, PI-3-kinase, and PLCγ are common features of signal transduction via sIg and CD40, engagement of these two receptors results in distinctly different biologic responses. While cross-linking of sIg on immature B cells results in the induction of apoptosis, CD40 ligation prevents cell death (7, 48), and engagement of CD40, but not of sIg, synergizes with cytokines to induce isotype switch recombination (9). These differences in biologic activity suggest differences in components of the signal transduction pathways employed by these two B cell specific receptors.

There is evidence to suggest that CD40-mediated PTK activation is functionally relevant. The PTK inhibitor genistein inhibits CD40-mediated B cell adhesion (49) and CD40-mediated IgE synthesis in B cells pretreated with IL-4 to express optimal levels of e germ line transcript (14). Engagement of the transmembrane protein tyrosine phosphatase CD45 has been shown to inhibit CD40-mediated B cell proliferation (4). Furthermore, in preliminary experiments, cross-linking of CD45 to CD40 inhibited IgE synthesis by IL-4–treated B cells, with negligible effect on the induction of e germ line transcripts (Loh, R., C. L. Ren, and R. S. Geha, manuscript in preparation). These observations suggest that activation of PTK plays an important role in CD40-mediated biologic responses.
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