CD40 Signaling Pathway: Anti-CD40 Monoclonal Antibody Induces Rapid Dephosphorylation and Phosphorylation of Tyrosine-phosphorylated Proteins Including Protein Tyrosine Kinase Lyn, Fyn, and Syk and the Appearance of a 28-kD Tyrosine Phosphorylated Protein

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Summary

CD40 plays an important role in B cell activation, proliferation, and Ig class switching. The signal transduction pathway mediated by CD40 was studied using monoclonal antibody (mAb) 626.1 to CD40. Burkitt's lymphoma and Epstein-Barr virus–transformed B cell lines and tonsilar B lymphocytes were treated with the anti-CD40 mAb for various lengths of time. The early events triggered by CD40 were examined by monitoring the changes in tyrosine phosphorylation of cellular proteins with anti-phosphotyrosine mAb. Dephosphorylation of specific proteins ranging between 50–110 kD and the appearance of a 28-kD tyrosine phosphorylated protein were seen within 30 s in human B cell lines. The dephosphorylation was reversed and the 28-kD protein was dephosphorylated in cells stimulated for 1 min. In resting B cells, the appearance of the 28-kD phosphoprotein was observed in 30 s after the addition of the anti-CD40 mAb. The tyrosine phosphorylation of this protein persisted. The patterns of protein tyrosine phosphorylation differed from those induced by an anti-immunoglobulin M mAb. The changes in the state of tyrosine phosphorylation induced by the anti-CD40 mAb were obviated by mAb to CD45, a protein tyrosine phosphatase (PTP) or by the addition of sodium orthovanadate, a broad PTP inhibitor. They were also blocked by protein tyrosine kinase (PTK) inhibitors, herbimycin A and genistein, and PKC and protein serine/threonine kinase inhibitors, H7 and HA1004. In addition, the alteration in the tyrosine phosphorylation of PTKs Lyn, Fyn, and Syk was directly demonstrated. Engagement of CD40 for 30 s induced a transient decrease in tyrosine phosphorylation of these PTKs. These results indicate that the early events in CD40 signaling involve the complex interaction between PTP and protein kinases.

C D40 is a 47–50-kD glycoprotein expressed on B cells and some normal and neoplastic epithelial cells including follicular dendritic cells and thymic epithelium (1–8). The cDNA sequences of both human and murine CD40 show that these glycoproteins are type I transmembrane proteins and contain cysteine-rich extracellular domains followed by serine/threonine-rich regions preceding the transmembrane domain (9, 10). They have significant homology to the members of the nerve growth factor receptor family (9–11).

CD40 plays an important role in B cell activation, differentiation, and survival. Heterologous Abs and mAbs to CD40 induce B cell proliferation (1, 2, 4, 12, 13) and the engagement of CD40 by mAb provides a stimulatory signal synergistic with those delivered by IL-4 or Ab to either surface IgM (sIgM)[1] or CD20 (2, 14, 15). An anti-CD40 mAb in the presence of IL-4 promotes long-term B cell growth (16). CD40 also plays a role in the induction of homotypic adhesion (17, 18). A mAb to CD40 induces bcl-2 expression and

[1] Abbreviations used in this paper: CD40L, CD40 ligand; PTK, protein tyrosine kinase; P-Tyr, phosphotyrosine; PTP, protein tyrosine phosphatase; sIgM, surface IgM.

prevents apoptosis of germinal center B cells (19). CD40 mediates the induction of Ig class switching. Anti-CD40 mAbs induce IgE synthesis in the presence of IL-4 (20, 21) and they induce the secretion of various Ig isotypes in the presence of different cytokines (22, 23).

The recent identification and isolation of the CD40 ligand (CD40L), gp39, on activated CD4$^+$ T lymphocytes (24–26) further underscores the importance of CD40 and its ligand in T–B cell interactions. The recombinant-soluble CD40L stimulates B cell proliferation in the presence of IL-4 and induces IgE secretion in a manner similar to the engagement of CD40 by mAbs (24–28). Recently, defective expression of CD40L has been shown to be responsible for the immunodeficiency of X-linked hyper-IgM syndrome (29–33), further supporting the importance of the CD40–CD40L interaction in the humoral arm of the immune response.

The early biochemical events triggered through CD40 have not been fully delineated. Similar to sIg, cross-linking of CD40 stimulates the phosphatidyl inositol pathway (34–37). Unlike mAbs to IgM, the anti-CD40 mAb fails to stimulate a measurable increase in calcium influx in B cells (13). In addition, an anti-CD40 mAb induces an increase in serine/threonine phosphorylation within minutes of binding to CD40 and enhances tyrosine phosphorylation of several substrates (37). In light of the complex role of CD40 in B cell activation and differentiation, we have investigated the mechanism of signal transduction mediated by CD40 with particular emphasis on the early events of this process.

Materials and Methods

Cell Preparation. B lymphocytes were isolated from tonsils obtained from routine tonsillectomy following the method of Gruber et al. (13). Mononuclear cells were separated by ficoll-hypaque centrifugation. T cells were depleted by rosetting with 2-aminoethylisothiouronium bromide treated sheep red blood cells. The resultant B cells were separated by discontinuous Percoll gradient centrifugation (Pharmacia, Piscataway, NJ). Dense B lymphocytes were collected at the interface of 60 and 70% Percoll. This B cell population was typically 96% CD20 positive as determined by FACS® analysis (Becton Dickinson and Co., Mountain View, CA).

Cell Lines. Burkitt's lymphoma and EBV-transformed B cell lines were maintained in RPMI 1640 supplemented with 10% FCS supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. The Burkitt's lymphoma cell lines Raji (μ^+), Daudi (μ^+,δ^+), Ramos (μ^+), and EBV-transformed cell line 32a1 (α^+) were used in our studies.

mAb and Antisera. The anti-CD40 mAb 626.1 and the anti-CD45 mAb A1.1 were generated in our laboratory as previously described (13). A second anti-CD45 mAb BCw, and anti-LFA mAb 60.3 were generously provided by Dr. J. A. Hansen (Fred Hutchinson Cancer Research Center, Seattle, WA). The anti-IgM–producing hybridoma HB57 was obtained from American Type Culture Collection (Rockville, MD). The anti-phosphotyrosine (P-Tyr) mAb PT113.4 was characterized previously (38). The antisera to the unique NH₂-terminal regions of Lyn and Fyn were a generous gift from Drs. J. Bolen and A. Burkhardt (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ) (39). Additional antisera to Lyn and Fyn peptides were also purchased from Upstate Biotechnology Inc. (Lake Placid, NY). The antisera to Syk was

kindly provided by Dr. R. L. Geahlen (Purdue University, West Lafayette, IN) (40).

Anti-CD40 Stimulation and Detection of Tyrosine Phosphorylation. Several B cell lines and tonsillar B cells were stimulated with mAb 626.1 for various amounts of time. The cells were lysed in 10 mM Tris-HCl, pH 8, 150 mM NaCl, 1% NP-40, 1% glycerol, and 1 mM EDTA containing 1 mM PMSF, 1 mM Na₃VO₄, 1 μg/ml leupeptin, 1 μg/ml trypsin inhibitor, and 0.2 U/ml aprotinin. In certain experiments, 0.1% SDS was included in the extraction buffer. Western blots were performed using biotinylated anti-P-Tyr mAb PT113.4, which recognizes P-Tyr with little reactivity to P-Ser or P-Thr. The blots were counter-stained with avidin conjugated with horseradish peroxidase and developed with the enhanced chemiluminescence kit (ECL; Amersham Corp., Arlington Heights, IL). The anti-P-Tyr mAb 4G-10 (Upstate Biotechnology Inc.) was used during the early stage of the investigation. mAb PT113.4 and 4G-10 yielded similar results. A similar experimental procedure was adopted after stimulation with the anti-IgM mAb HB57. Equal loading in each well was determined by protein quantification before loading and by staining the nitrocellulose filters with ponceau S after electrophoresis.

Kinase Inhibitors. The cell lines were incubated in the presence of herbimycin A (7 μg/ml), genistein (30 μg/ml), 1-(5-isoquinolinesulfonamide)-2-methylpiperazine (H7, 12 μg/ml), or N-(2-guanidinoethyl)-5-isoquiniolinesulfonamide (HA1004, 12 μg/ml) obtained from Calbiochem-Behring Corp. (San Diego, CA) for 1 h at 37°C. The cells were then stimulated as above.

Immunoprecipitation. The Raji and Ramos cell lines were stimulated with anti-CD40 for the specified period of time. The cells were washed in cold PBS containing 1 mM sodium orthovandate and then lysed as mentioned above. The cell lysates from 10⁷ cells were cleared by centrifugation at 13,000 g, and incubated with the anti-Lyn, anti-Fyn, or anti-Syk sera followed by protein A–Sepharose beads (Pharmacia) for 4 h at 4°C.

Results

Rapid Alteration in Tyrosine Phosphorylation Induced by the Anti-CD40 mAb 626.1. The anti-CD40 mAb 626.1 was used to stimulate B cells for various amounts of time. The resulting protein tyrosine phosphorylation was examined by immunoblot analysis using the anti-P-Tyr mAb. Treatment of the B cell lines with the anti-CD40 mAb 626.1 resulted in a time-dependent alteration of the tyrosine phosphorylation pattern of several proteins (Fig. 1).

The addition of the anti-CD40 mAb to the cell line Raji, for as little as 30 s, resulted in rapid and transient dephosphorylation of several proteins ranging between 50 and 110 kD. This was accompanied by a marked increase in tyrosine phosphorylation of an ∼28-kD protein (Fig. 1A). By 1 min there was a marked increase in the tyrosine phosphorylation of proteins in the 50–60- and 70–80-kD regions and a decrease in the 28-kD protein phosphorylation. Increased phosphorylation was also observed on several proteins in the region between 30 and 46 kD. A minor 120-kD band was also transiently dephosphorylated. These results were confirmed by densitometry scanning of the films. These proteins remain tyrosine-phosphorylated after treatment with anti-CD40 for up to 30 min (data not shown). These results were consistently observed in two additional experiments. Similar results
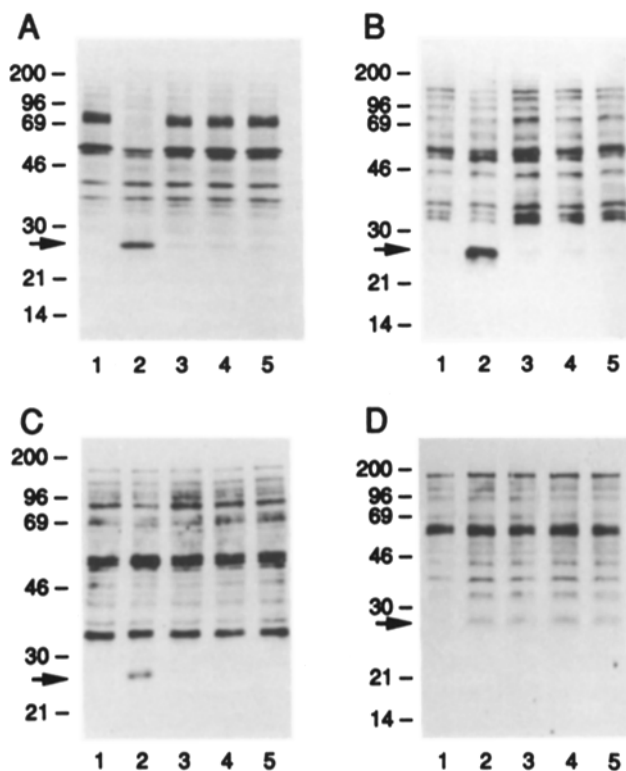
Figure 1. Anti-CD40–induced protein tyrosine dephosphorylation and phosphorylation. The human Burkitt's lymphoma cell lines Raji (*A*), Daudi (*B*), and Ramos (*C*), and dense tonsillar B cells (*D*) were treated with the anti-CD40 mAb 626.1. Lane *1* is untreated cells. Lanes *2–5* were cells treated with mAb 626.1 for 30 s, 1, 5, or 10 min, respectively. The reaction was stopped with cold PBS containing 1 mM Na$_3$VO$_4$. The cells were lysed in 1% NP-40 buffer supplemented with 0.4 mM EDTA, 1 mM PMSF, 1 mM Na$_3$VO$_4$, 1 μg/ml leupeptin, 1 μg/ml trypsin inhibitor, and 0.2 U/ml aprotinin. The cell lysates (20 μg/lane) were electrophoresed by SDS-PAGE, transferred to nitrocellulose, and then analyzed by Western blot using the anti-P-Tyr mAb PT113.4 followed by ECL chemiluminescence detection. These results are representative of three experiments.

were obtained in two experiments with another anti-P-Tyr mAb, 4G-10.

Two other Burkitt's lymphoma cell lines, Daudi (IgM$^+$) and Ramos (IgM$^+$) were also studied (Fig. 1, *B* and *C*). Their phosphorylation and dephosphorylation patterns differ from that of Raji cells. In addition to the transient appearance of the tyrosine-phosphorylated 28-kD protein, the cell line Daudi exhibited a reversal of the intensities of two tyrosine-phosphorylated bands of ~56 and 59 kD during the first 30 s of stimulation. There was a transient increase in phosphorylation of proteins in the range of 70–80 and 30–40-kD which peaked at 1 min. In the case of the cell line Ramos, treatment with the anti-CD40 mAb for 30 s induced the increased phosphorylation of proteins of ~50–53 kD accompanied simultaneously by the appearance of tyrosine-phosphorylated 28-kD proteins. The apparent increase in phosphorylation in this region differs from the rapid dephosphorylation observed in the Raji cells. Although not marked, there was transient dephosphorylation of a 80-kD protein. After 1-min of incubation with the anti-CD40 mAb, the tyrosine-phosphorylated 28-kD protein was not detected and there was increased

staining of substrates ranging between 90 and 120 kD by the anti-P-Tyr mAb. Treatment of an IgA producing EBV-transformed B cell line 32a1 with the anti-CD40 mAb yielded a similar pattern of dephosphorylation and rephosphorylation as in Daudi cells (data not shown). Anti-CD40 stimulation of dense tonsillar B lymphocytes induced a sustained and marked increase of phosphorylation of 28- and 32-kD proteins (Fig. 1 *D*). There was also an increase in tyrosine phosphorylation of 38- and 42-kD protein substrates. However, dense tonsillar B cells failed to demonstrate the transient dephosphorylation described above in the B cell lines.

Thus far, the experiments with anti-CD40 stimulation were carried out with bivalent IgG. Further cross-linking of biotinylated mAb 626.1 with avidin accelerated the changes observed in all of the cell lines and tonsillar B cells during the 30 s and 1-min time points (data not shown). This acceleration made it difficult to observe the dephosphorylation and the appearance of phosphotyrosinated 28-kD protein in some experiments involving the B cell lines.

sIgM-mediated Tyrosine Phosphorylation Differs from That through CD40. Events induced after stimulation through sIgM have been extensively investigated (34, 35, 39, 41, 42). Our above results suggest that the early events of signal transduction mediated by CD40 differ from those mediated through sIg. This was directly demonstrated in cell lines, Ramos and Daudi, which have readily demonstrated surface IgM. Treatment of Ramos with the anti-IgM mAb HB57 resulted in the rapid tyrosine phosphorylation of multiple proteins, some of which were identified as 42, 56, 68, 72, 100, 140, and 180 kD within 30 s (Fig. 2 *A*). This phosphorylation pattern persisted throughout the experimental period of 20 min. Treatment of the B cell line Daudi with this anti-IgM mAb resulted in tyrosine phosphorylation of 68-, 72-, 100-, 140-, and 180-kD proteins and the increased phosphorylation of 34-, 42-, 46-, 48-, 56-, and 60-kD proteins within 30 s of the addition of the mAb although other phosphorylation changes were also discernable (Fig. 2 *B*). These changes were persistent through the 20 min of incubation. These results are in agreement with those published by other investigators
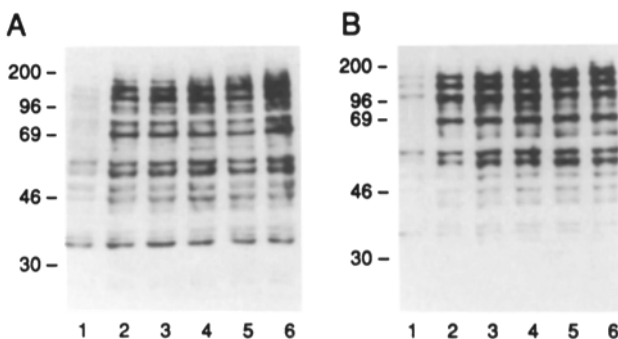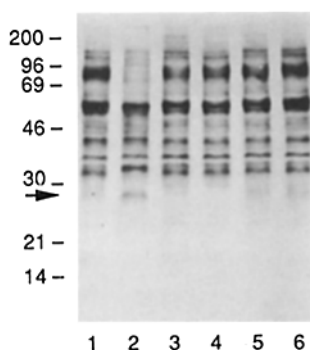

Figure 2. IgM-induced tyrosine phosphorylation. The human B cell lines Ramos (*A*) and Daudi (*B*) were untreated (lane *1*) or treated with the anti-IgM HB 57 for 30 s, 1, 5, 10, or 20 min (lanes *2–6*). The tyrosine-phosphorylated proteins were analyzed by anti-P-Tyr immunoblotting as detailed in Fig. 1. Similar results were obtained in two additional experiments.
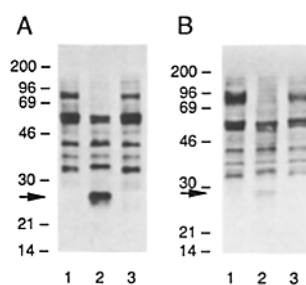
(39, 41). The transient dephosphorylation observed with anti-CD40 stimulation was not evident in the anti-IgM stimulated B cells. In addition, the anti-IgM mAb did not induce transient tyrosine phosphorylation of the 28-kD protein (compare Figs. 1 and 2). The anti-IgM mAb did not induce detectable changes in tyrosine phosphorylation of cellular proteins in Raji cells. This may be due to a low level of surface IgM expression in this cell line (data not shown).

Effect of Protein Kinase Inhibitors on CD40-mediated Signaling. To further elucidate the mechanism of signal transduction through CD40, the effect of several kinase inhibitors, i.e., herbimycin A, genistein, H7, and HA1004 was studied. Incubation of Raji cells with these inhibitors alone under our experimental conditions did not induce detectable changes in the tyrosine phosphorylation pattern of cellular proteins. Treatment of Raji cells with the protein tyrosine kinase (PTK) inhibitors, herbimycin A or genistein, prevented the anti-CD40–induced phosphorylation of the 28-kD protein and resulted in a marked inhibition of the anti-CD40–induced dephosphorylation (Fig. 3, lanes *3* and *4*). Treatment of the Raji cell line with the PKC inhibitor, H7, and cAMP- and cGMP-dependent protein kinase inhibitor, HA1004, partially inhibited the anti-CD40–induced tyrosine phosphorylation of the 28-kD protein (Fig. 3, lanes *5* and *6*), whereas the anti-CD40–induced dephosphorylation was prevented completely. These results are consistent with previous studies (36, 37) demonstrating a role of PTK, PKC, and serine/threonine kinases in the CD40-mediated signaling pathway. In addition, interaction between these kinases and PTP is also evident.

Regulation of CD40-mediated Events by Protein Tyrosine Phosphatase. Because of our observation that anti-CD40–induced proliferation of resting B cells (13) is inhibited by the addition of anti-CD45 mAb, the role of CD45 in the early events of CD40 signaling was investigated. The simultaneous addition of anti-CD45 mAb A1.1 and anti-CD40 to the Raji cell line blocked the initial events mediated by CD40 (Fig. 4 *A*). Two additional experiments yielded similar results. A similar inhibition was observed in the cell lines Daudi, Ramos, and 32a1 as well as in tonsillar B cells (data not shown). These

Figure 4. Effect of anti-CD45 mAb and sodium orthovanadate on CD40-mediated signal transduction. (*A*) Anti-P-Tyr immunoblotting of cell lysates from untreated Raji cells (lane *1*), from cells stimulated with the anti-CD40 mAb alone for 30 s (lane *2*) or stimulated simultaneously with anti-CD40 and anti-CD45 mAb for 30 s (lane *3*). (*B*) Anti-P-Tyr immunoblotting of cell lysates from untreated Raji cells (lane *1*), from cells stimulated with the anti-CD40 mAb for 30 s (lane *2*) and from cells treated with Na₃VO₄ (400 μM) for 5 min, followed by the addition of the anti-CD40 mAb for 30 s (lane *3*). Similar results were obtained in two other experiments.

results were confirmed with another anti-CD45 mAb BCw, generously provided by Dr. J. A. Hansen. Both the HLA and LFA molecules are highly expressed in B lymphocytes in general and in Raji in particular. Treatment of Raji cells with the anti-CD40 mAb and the mAb to either HLA or LFA failed to inhibit the anti-CD40–mediated effects (data not shown).

The participation of protein tyrosine phosphatase(s) (PTP) in the events triggered by the anti-CD40 mAb was further supported by the inhibitory effect of the PTP inhibitor, sodium orthovanadate, on anti-CD40–stimulated Raji cells. Treatment with sodium orthovanadate blocked the appearance of the phosphorylated 28-kD protein and reduced the extent of dephosphorylation initially observed 30 s after the addition of the anti-CD40 mAb (Fig. 4 *B*). These results indicate that the early events in CD40 signaling are dependent on the coordinated interaction of PTK and PTP.

Anti-CD40 mAb-induced Changes in Tyrosine Phosphorylation in Lyn, Fyn, and Syk. The activation of PTKs after receptor binding has been reported. Some of these receptors are the TCR–CD3 complex, the IgM complex, and the PDGF receptor, and several of these PTK have been identified to be members of the src family (39–46). In addition, the sIg and the TCR have been shown to be associated with non-src PTK, namely Syk and Zap-70, respectively (40, 47). The dephosphorylation of proteins in the ranges of 50–60 and 70–80 kD as a result of CD40 engagement suggests that some of these proteins may be Lyn (56/53 kD), Fyn (60 kD) and Syk (72 kD). Thus, heterologous antisera directed against Lyn, Fyn, and Syk (39, 40) were used to immunoblot cell lysates separated by SDS-PAGE. Anti-CD40-stimulated and -unstimulated cells were lysed in buffer containing NP-40 (Fig. 5 *A*). The cell lysates were analyzed for the expression of Lyn, Fyn, and Syk by immunoblots. The relative concentrations of the Lyn and Fyn proteins remained unaltered in Raji during the 30 s and 1 min of stimulation with the anti-CD40 mAb. These results were confirmed using additional antisera to Lyn and Fyn. In the case of Syk, the staining intensity of 72-kD bands was maximal in NP-40 cell lysate of Raji cells stimulated with the anti-CD40 mAb for 30 s and the intensity decreased in the 1-min sample. This varia-

Figure 3. Effect of protein kinase inhibitors on CD40-mediated signal transduction. The Raji cell line was incubated in medium alone (lanes *1* and *2*), or in medium containing herbimycin A (7 μg/ml, lane *3*), genistein (30 μg/ml, lane *4*), H7 (12 μg/ml, lane *5*), or HA1004 (12 μg/ml, lane *6*) for 1 h. The cells were then stimulated with the anti-CD40 mAb for 30 s (lanes *2–6*). The cell lysates were separated by SDS-PAGE and analyzed by Western blotting using the anti-P-Tyr mAb PT113.4. Similar results were obtained in three separate experiments.
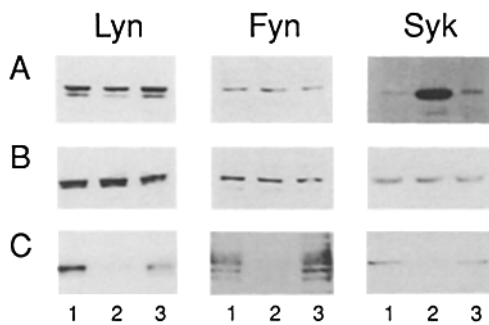
Lyn **Fyn** **Syk**

A

B

C

1 2 3   1 2 3   1 2 3

Figure 5. Involvement of Lyn, Fyn, and Syk in CD40 signal transduction. (*A*) NP-40 lysates from untreated Raji cells (lane *1*) and cells stimulated with the anti-CD40 mAb for 30 s and 1 min (lanes *2* and *3*) were analyzed by immunoblotting with antisera to Lyn, Fyn, and Syk. (*B*) Lysates from treated and untreated cells were prepared in the presence of SDS, and analyzed by immunoblotting as above. (*C*) Cell lysates from untreated Raji cells (lane *1*) and from cells stimulated with the anti-CD40 mAb for 30 s or 1 min (lanes *2* and *3*) were immunoprecipitated with antisera to Lyn, Fyn, and Syk. The precipitates were electrophoresed under nonreducing conditions and analyzed by Western blotting using the biotinylated anti-P-Tyr mAb. These results are representative of the experiments. Similar results were obtained in two other experiments, using different antisera to Lyn and Fyn.

tion of staining intensities did not correlate with the protein concentrations of these bands as determined by Ponceau S staining. The possibility that the Syk protein redistributes after stimulation of the lymphocytes with the anti-CD40 mAb was considered. Lysates of Raji cells were made in the presence of SDS and Western blot analysis with these heterologous antisera was carried out. As shown in Fig. 5 *B*, the total concentrations of Lyn, Fyn, and Syk did not change as a result of anti-CD40 stimulation, indicating that redistribution of Syk into a NP-40–extractable compartment resulted from the anti-CD40 stimulation.

To assess the tyrosine phosphorylation states of Lyn, Fyn, and Syk proteins, cell lysates of untreated and anti-CD40 treated Raji cells (10⁷) were immunoprecipitated with these antisera. The inhibitors genistein (20 μM) and sodium orthovanadate (1 mM) were added to the lysis buffer to inhibit in vitro kinase and phosphatase activities after extraction. The immunoprecipitates were electrophoresed, blotted to nitrocellulose paper, and analyzed with anti-P-Tyr mAb. As shown in Fig. 5 *C*, less P-Tyr staining was observed in immunoprecipitates by these antisera from Raji cells stimulated for 30 s with the anti-CD40 mAb. By 1 min, the tyrosine phosphorylation of Lyn, Fyn, and Syk appeared to return to the levels approximately observed in the untreated samples. In the case of Fyn, there were other phosphorylated proteins in the blot. These proteins also appeared in other two experiments. It remains to be determined whether these proteins are specifically associated with Fyn. Because of the observed anti-CD40–induced redistribution of Syk, it appears that the NP-40–extractable Syk underwent transient dephosphorylation and that the redistributed Syk was not tyrosine phosphorylated. However, the tyrosine-phosphorylation state of the Syk protein, not extractable in NP-40 buffer, cannot be ascertained in these experiments.

Thus, Lyn, Fyn, and Syk appear to undergo a marked de-

phosphorylation during the initial 30 s of treatment of B cells with the anti-CD40 mAb to be followed by rapid rephosphorylation of these kinases. Preliminary experiments suggest that the rapid dephosphorylation of Lyn at 30 s was accompanied by an increase in in vitro kinase activity in the anti-Lyn–precipitated immune complexes (data not shown).

Tyrosine phosphorylation of Lyn, Fyn, and Syk was also examined in Ramos cells stimulated with either anti-CD40 or anti-IgM mAb for 30 s and 1 min. As shown in Fig. 6 *A*, the anti-CD40 mAb induced rapid and transient dephosphorylation of the Lyn protein. In contrast, the anti-IgM mAb induced a time-dependent increase in tyrosine phosphorylation of this protein. There was no redistribution of Lyn due to either anti-CD40 or anti-IgM stimulation (Fig. 6 *B*). Similarly, transient anti-CD40–induced dephosphorylation involving Fyn was also observed (data not shown). In the case of Syk, the anti-IgM mAb induced readily detectable redistribution of Syk although this redistribution was much less marked in comparison with the effect induced by the anti-CD40 mAb (Fig. 6 *B*). In contrast to the anti-CD40 stimulation, the anti-IgM mAb induced no dephosphorylation and there was a time-dependent increase in the tyrosine phosphorylation in the redistributed and NP-40–extractable Syk. Similar results were obtained in an additional experiment. These observations further underscore the differences between the signal transduction pathways via CD40 and IgM.

A   **CD40**   **IgM**

Lyn

Syk

B   SDS

Lyn −

     +

Syk −

     +

1 2 3   1 2 3
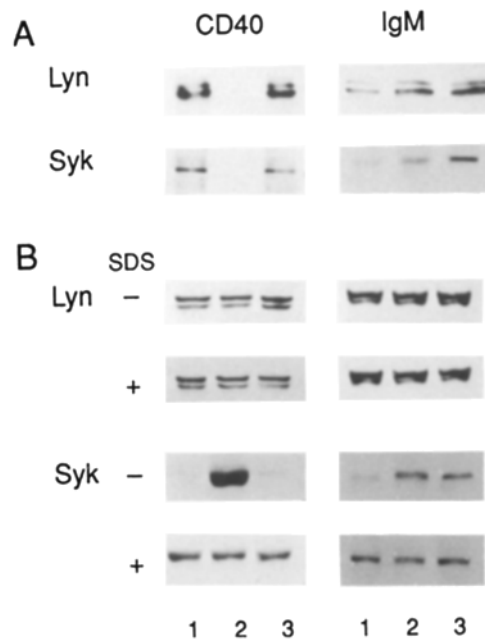
Figure 6. Different patterns of Lyn and Syk phosphorylation (*A*) and redistribution (*B*) in anti-CD40 and anti-IgM–stimulated cells. (*A*) Ramos cells were stimulated with either anti-CD40 or with anti-IgM mAb for 0, 30 s, or 1 min (lanes *1–3*). Cell lysates were immunoprecipitated with anti-Lyn and anti-Syk antisera and analyzed by immunoblotting with the anti-P-Tyr mAb. (*B*) Untreated or stimulated Ramos cells were lysed in buffer containing either NP-40 or SDS. The cell lysates were analyzed by immunoblotting with antisera to Lyn and Syk.

Discussion

In this study, we have investigated the early events mediated by the engagement of CD40. Treatment of several Burkitt's lymphoma and EBV-transformed cell lines with the anti-CD40 mAb 626.1 for 30 s induced a rapid dephosphorylation of proteins ranging between 50 and 110 kD and the phosphorylation of a 28-kD protein. Prolonged stimulation with the anti-CD40 mAb allowed the increased tyrosine phosphorylation of proteins at 50–60 and 70–80 kD, whereas it resulted in a decrease in 28-kD protein phosphorylation. There were variations in the degrees of transient dephosphorylation among these cell lines. However, even in the cell line Ramos in which transient dephosphorylation was not readily evident, transient dephosphorylation of certain PTK was demonstrated. Thus, the anti-CD40–induced transient dephosphorylation of certain PTKs and the transient appearances of a tyrosine-phosphorylated 28-kD protein appear to be coupled in early events in CD40 signaling in human EBV–transformed B cell lines. These events are unique to the CD40 signal transduction pathway. In contrast, treatment of dense tonsillar B lymphocytes with anti-CD40 induced the sustained tyrosine phosphorylation of 28-kD protein. This difference may be due to the inherent differences between resting B cells and EBV-transformed B cell lines, or to the different activation stages of the resting B cells.

The heterogeneity of phosphorylation patterns in different cell lines and B cells as a result of CD40 engagement has been observed by Uckun et al. (37). However, our results differ significantly from theirs in that an earlier time point (30 s) was included and the experiments were carried out without the addition of secondary cross-linking agents such as anti–mouse Ig Abs and PMA. These differences in experimental conditions enable the detection of the early events mediated by CD40, including transient dephosphorylation and the appearance of 28-kD phosphotyrosinated protein.

PTK and serine/threonine protein kinase inhibitors have been used in the present investigation to implicate their involvement in early events of CD40 signaling. In vitro kinase assay results using the immunoprecipitated CD40 complex also provide support for the involvement of both PTKs and other protein kinases (data not shown). These results are in agreement with those of Uckun et al. (37).

CD40 has a long cytoplasmic domain, which lacks tyrosine and contains three serine and three threonine residues (9, 10). The protein is constitutively phosphorylated. In an in vitro model, an anti-CD40 mAb inhibits the growth of CD40 transfectants of murine M12 (B cell) and EL-4 (T cell) lines. Mutational analysis has revealed that Thr^{234} is essential for signal transduction in this system (48). Although this is a limited analysis, the results indicate that phosphorylation of CD40 by serine/threonine protein kinases is essential for CD40 signaling.

The tyrosine phosphorylation of PTKs Lyn, Fyn, and Syk immunoprecipitated by specific antisera was assessed by immunoblotting with anti-P-Tyr mAb. The transient dephosphorylation of these PTK was observed after 30 s of treatment with the anti-CD40 mAb. Preliminary data in our laboratory indicate an increase of kinase activity in immunoprecipitated dephosphorylated PTK Lyn from Raji cells stimulated by the anti-CD40 mAb for 30 s. This observation is of interest and is consistent with the recent observations described by Nada et al. (49) and Okada et al. (50). In their studies, Csk, a novel cytoplasmic PTK has been shown to down regulate the src family kinases. In mouse embryo that lack Csk, the src family kinases, $p60^{c-src}$, $p59^{fyn}$, and $p53/56^{lyn}$ have increased kinase activities with a decrease in tyrosine phosphorylation. It would be of interest to determine if dephosphorylation involves the negative regulatory tyrosine residue.

Anti-CD40 stimulation of B cell lines induced rapid redistribution of Syk. Without stimulation, Syk was not extractable by a buffer containing NP-40. With stimulation, a considerable amount of this protein became extractable. Thus, redistribution of Syk to a different cellular component is a feature of early events in CD40 signaling. In this investigation, redistribution of Syk, though less marked, was also shown to be a feature of the IgM-mediated signal transduction. In the case of the IgM pathway, there was no transient dephosphorylation of Syk, further underscoring the differences between the signaling pathways between CD40 and IgM.

Because Syk is not extractable in a buffer containing NP-40, it appears that the phosphorylated 72–80-kD band in unstimulated Raji in Fig. 1 A represents protein(s) other than Syk. The recently described B cell–specific PTK, Atk/Bpk (51, 52), is likely to migrate in this region. The involvement of this kinase in CD40 signaling is supported by our unpublished finding that CD40 signaling is deficient in EBV-transformed B cell lines from patients with X-linked agammaglobulinemia, in whom, Atk/Bpk deficiency has been demonstrated.

Transient early dephosphorylation appears to be a feature of the CD40-signaling pathway. CD45 is the predominant receptor-associated protein tyrosine phosphatase expressed on lymphocytes (53). The CD45 family constitutes a functionally essential participant in receptor signaling in both T and B lymphocytes (44, 45, 54–56). T and B cells lacking CD45 fail to transduce signals via TCR complex or sIg complex upon activation. Transfection of either the CD45 cDNA or the cDNA encoding a receptor molecule containing the cytoplasmic portion of CD45, was sufficient to restore TCR signal transduction in the CD45⁻ mutant T cells (44, 45, 57–60). Our previous finding of anti-CD45 mAb inhibition of anti-CD40–induced B cell proliferation (13) and the present finding of anti-CD45 mAb inhibition of anti-CD40–induced early dephosphorylation and the transient appearance of the 28-kD phosphoprotein would support the thesis that CD45 plays a major role in CD40 signaling. This hypothesis is in general agreement with the overall role of CD45 in T and B lymphocyte activation. Definitive experiments to show the direct participation of CD45 in CD40 signaling await the availability of CD45⁻ mutants of Raji cells.

As discussed above, kinase activity has been demonstrated to be associated with the immunoprecipitated CD40 com-

plex (data not shown). Multiple proteins in the complex are phosphorylated in an in vitro assay. Preliminary studies in our laboratory have identified several substrates associated with CD40, including the PTK Lyn, phospholipase C γ1, phosphatidylinositol 3-kinase, and GTPase activating protein. These findings are compatible with the recent observation that signal transduction via CD40 involves an activation of Lyn, phosphatidylinositol 3-kinase, and phospholipase Cγ2 (61).

The CD40 cytoplasmic region lacks a tyrosine kinase domain. Thus, the association of CD40 with PTKs occurs through adaptor molecules. The rapid tyrosine phosphorylation of the 28-kD protein in response to CD40 signaling suggests that p28 may be such a adaptor molecule. The appearance of this phosphorylated protein provides an additional feature distinguishing the signaling of CD40 from that of IgM. Further characterization of this molecule will provide further insights in the CD40 pathway.

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