MOLECULAR AND CELLULAR BIOLOGY, May 1992, p. 2315–2321
0270-7306/92/052315-07$02.00/0

Vol. 12, No. 5

Association between B-Lymphocyte Membrane Immunoglobulin and Multiple Members of the Src Family of Protein Tyrosine Kinases

MARY-ANN CAMPBELL* AND BARTHOLOMEW M. SEFTON

Molecular Biology and Virology Laboratory, The Salk Institute, P.O. Box 85800, San Diego, California 92186-5800

Received 30 December 1991/Accepted 13 February 1992

Treatment of B lymphocytes with antibodies to membrane immunoglobulin (Ig) stimulates protein tyrosine phosphorylation. We have examined the phosphorylation in vitro of proteins associated with membrane Ig. The Src family protein tyrosine kinases p53/56^{lyn}, p59^{fyn}, and p56^{lck} are associated with membrane Ig in spleen B cells and B-cell lines and undergo phosphorylation in vitro. The pattern of expression of Src family protein tyrosine kinases in B cells varied. Our studies suggest that multiple kinases can potentially interact with membrane Ig and that within any one B-cell type, all of the Src family kinases expressed can be found in association with membrane Ig. We also observed that the Ig-associated Igα protein, multiple forms of Igβ, and proteins of 100 and 25 kDa were tyrosine phosphorylated in vitro. The 100- and 25-kDa proteins remain unidentified.

Cross-linking of the B-cell antigen receptor, membrane immunoglobulin (Ig), with anti-Ig antibodies mimics the effects of antigenic stimulation (reviewed in reference 7). Activation of B cells with anti-Ig antibodies rapidly stimulates both protein tyrosine phosphorylation (11, 17) and the breakdown of phosphatidylinositol bisphosphate, the latter resulting in activation of protein kinase C and elevation of cytoplasmic Ca^{2+} (3, 15). The recent findings that phospholipase Cγ is tyrosine phosphorylated in response to anti-Ig stimulation (12) and that inhibitors of protein tyrosine phosphorylation inhibit anti-Ig-stimulated Ca^{2+} mobilization (30) suggest that protein tyrosine phosphorylation is essential for B-cell activation and operates upstream of inositol phospholipid turnover.

Membrane Ig is physically associated with at least two other glycoproteins, Igα and Igβ (21, 23, 46–48), and these associated molecules may comprise part of a signal transduction complex. In addition, membrane Ig is associated with p53/56^{lyn}, p56^{blk}, and p59^{fyn}, protein tyrosine kinases of the Src family (5, 49), and there is evidence to suggest that these kinases are activated following cross-linking of membrane Ig (5). One or more of these kinases could play a critical role in B-cell activation.

To study the protein tyrosine kinases involved in B-lymphocyte activation, we have examined the phosphorylation in vitro of proteins associated with membrane Ig. We have found that, in addition to p53/56^{lyn} and p59^{fyn}, p56^{lck} was associated with membrane Ig. The pattern of expression of Src family protein tyrosine kinases in B cells varied. Our studies suggest that multiple kinases can potentially interact with membrane Ig and that within any one B-cell type, all of the Src family kinases expressed can be found in association with membrane Ig.

MATERIALS AND METHODS

Reagents. Cells were grown in Dulbecco-Vogt modified Eagle's medium (Mediatech) supplemented with 50 μM 2-mercaptoethanol, nonessential amino acids (GIBCO), 1 mM sodium pyruvate (GIBCO), penicillin, streptomycin, and 10% heat-inactivated fetal bovine serum (HyClone). Affinity-purified goat anti-mouse IgG was obtained from Sigma. Affinity-purified rabbit anti-mouse IgG-IgA-IgM was obtained from Zymed. Goat anti-rat Ig, non-cross-reactive with mouse Ig, was obtained from GIBCO/BRL. Monoclonal antibodies MKD6 (anti-class II; IA^d) (27), 34-1-2S (anti-class I; K^d, D^d), and 30H12 (rat anti-mouse Thy 1.2) (32) were the generous gift of Bob Hyman. AMS 9.1.1.1 (anti-mouse δ, a allotype) (42), MAR18.5 (mouse anti-rat κ) (31), and 187.1 (rat anti-mouse κ) (50) were obtained from the American Type Culture Collection. b.7.6 (rat anti-mouse μ) was the generous gift of Michael Julius (25). Monoclonal antibodies were used as tissue culture supernatant (30H12 and 187.1) or as Ig purified on protein A- or protein G-Sepharose (Pharmacia). The murine B-lymphoma line WEHI-231 (4) was obtained from the American Type Culture Collection, and the hybridoma line LK was the gift of Phillippa Marrack (27). Rabbit antiserum to a TrpE-p56^{lck} (amino acids 34 to 150) fusion protein was prepared by Tamara Hurley (24). Rabbit anti-p56^{lck} peptide (amino acids 39 to 58) and the competing peptide were the generous gifts of Andrey Shaw. A rabbit antiserum raised against the C-terminal 10 amino acids of p53/p56^{lyn} was the generous gift of Toshiniko Torigoe and John Reed. Rabbit anti-*fyn*2 was the generous gift of Robert Kypta and Sarah Courtneidge. The monoclonal anti-Src antibody, GD11, was the gift of S. Parsons.

Cell preparation. Spleen cells were isolated from female BALB/c mice (Harlan Sprague-Dawley) at 8 to 10 weeks of age into medium containing antibiotics and 1% fetal bovine serum. Erythrocytes were lysed with 0.83% (wt/vol) ammonium chloride. Spleen cell suspensions were depleted of T cells by incubation with anti-Thy 1.2 (30H12) followed by incubation with rabbit complement (GIBCO). Splenic B cells were layered onto 50–60–75% Percoll (Pharmacia) discontinuous gradients and centrifuged for 30 min at 600 × *g*. Small, dense, resting B cells were recovered from the 60–75% Percoll interface.

Cell lines were harvested in log phase of growth and washed once in medium containing 1% fetal bovine serum.

Preparation of cell lysates. Cells were resuspended in

* Corresponding author.

ice-cold lysis buffer (11) (20 mM Tris-HCl [pH 8.3], 150 mM NaCl, 200 μM sodium orthovanadate, 2 mM EDTA, 50 mM NaF) containing 10 mM 3-{(3-cholamidopropyl)dimethylammonio}-1-propanesulfonate (CHAPS) (Pierce) (14). While many workers have used digitonin for studies of membrane Ig-associated molecules (8, 14, 21), we have found that $p56^{lck}$ is poorly soluble in this detergent (11a). Studies by Chen et al. have shown that the interaction between membrane Ig and associated molecules is stable in both CHAPS and digitonin (14). Cells were lysed for 20 min on ice, nuclei were pelleted by centrifugation at 21,000 × *g* for 30 min, and detergent-soluble lysates were collected.

Immunoprecipitation. Detergent-soluble cell lysates were precleared with Pansorbin (Calbiochem) coated with control rabbit serum. In the absence of this preclearing step, we found the backgrounds in our immune complex kinase assays to be unacceptably high. Lysate from 10^7 cells was incubated with 2 μg of purified antibody, 2 μl of antiserum, or 100 μl of tissue culture supernatant followed by 30 μl of Pansorbin or Pansorbin precoated with goat anti-rat Ig (for those rat monoclonal antibodies not binding protein A). Immunoprecipitates were washed three times in lysis buffer and then twice in Tris-buffered saline (150 mM NaCl, 50 mM Tris-HCl [pH 7.2]). Immunoprecipitates were assayed for in vitro kinase activity as described previously (11) except that the reaction was terminated by addition of 1 ml of 50 mM Tris-HCl (pH 7.2) containing 150 mM NaCl, 2 mM EDTA, and 1 mM CHAPS. In reprecipitation experiments, in vitro-phosphorylated immunoprecipitates were boiled in 1 volume of 20 mM Tris-HCl (pH 8.0) containing 0.5% sodium dodecyl sulfate (SDS) and 1 mM dithiothreitol. RIPA correction buffer (4 volumes) was then added to give a final concentration of 0.1% SDS, 1% sodium deoxycholate, 1% Nonidet P-40 (NP-40), 24 mM Tris-HCl (pH 8.3), 150 mM NaCl, 200 μM sodium orthovanadate, 2 mM EDTA, 1 mM dithiothreitol, and 50 mM NaF. Reprecipitation was done by using antibodies specific for members of the Src family of protein tyrosine kinases.

Immunoprecipitates were resolved on 15% polyacrylamide gels and then electrophoretically transferred to Immobilon (Millipore). Following exposure for fluorography, individual bands were excised for phosphoamino acid analysis (26) and phosphotryptic peptide mapping (33).

RESULTS

Spleen B-cell membrane Ig is physically associated with protein tyrosine kinase activity in vitro. To study the protein tyrosine kinases associated with membrane Ig in primary cells, membrane Ig was isolated from murine resting spleen B lymphocytes by using a variety of monoclonal and polyclonal antibodies. The immunoprecipitates were then assayed for their ability to undergo autophosphorylation in vitro. Protein kinase activity was detected in immunoprecipitates of membrane Ig (Fig. 1A, lanes 1, 2, and 4) but not in immunoprecipitates of either class I or class II major histocompatibility antigens (lanes 5 and 6). Both polyclonal and monoclonal anti-mouse Ig antibodies precipitated kinase activity, but polyclonal and monoclonal antibodies specific for rat Ig did not (lanes 3 and 7). The molecules associated with membrane Ig that are phosphorylated in vitro can be considered as four groups; a broad 100-kDa band, a group of three bands of 59, 56, and 53 kDa, a group of four bands of 34 to 42 kDa, and a 25-kDa band. All of these molecules were phosphorylated principally on tyrosine in vitro (data not shown).

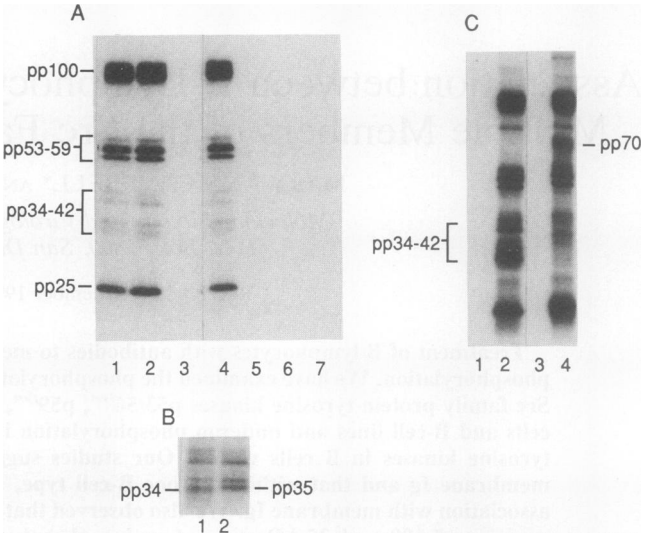

FIG. 1. Phosphorylation of spleen B-cell membrane Ig-associated molecules. CHAPS-soluble cell lysates were immunoprecipitated with various antibodies and assayed for associated in vitro kinase activity. Phosphorylated proteins were separated on SDS–15% polyacrylamide gels. (A) Spleen B-cell Ig-associated molecules. Lanes: 1, rabbit anti-mouse Ig; 2, goat anti-mouse Ig; 3, goat anti-rat Ig, non-cross-reactive with mouse Ig; 4, rat monoclonal anti-mouse κ light chain (187.1); 5, mouse monoclonal anti-class I (K^d, D^d; 34-1-2S); 6, mouse monoclonal anti-class II (IA^d; MKD6); 7, mouse monoclonal anti-rat κ light chain (MAR18.5). (B) Spleen B-cell membrane IgM and IgD-associated molecules. Lanes: 1, rat monoclonal anti-mouse μ chain (b.7.6); 2, mouse monoclonal anti-δ chain (a allotype; AMS 9.1.1.1). (C) Spleen B-cell membrane Ig-associated molecules resolved under reducing (lanes 1 and 2) and nonreducing (lanes 2 and 4) conditions. Lanes: 1 and 3, control rabbit serum; 2 and 4, rabbit anti-mouse Ig.

Spleen B cells simultaneously express two classes of membrane Ig, IgM and IgD, and some studies have suggested these two molecules differ subtly in the signals that they transmit to B cells (1, 6, 44). The in vitro phosphorylation of proteins associated with membrane IgM and IgD was next compared (Fig. 1B). The recovery of in vitro-phosphorylated proteins associated with membrane IgM was lower than that of IgD-associated proteins, consistent with the lower level of expression of IgM than of IgD on resting spleen B cells (36). The only qualitative difference between the phosphotyrosine-containing molecules associated with membrane IgM and those associated with membrane IgD was in the size of the smallest band in the 34- to 42-kDa group: 35 kDa in association with IgD and 34 kDa in association with IgM (Fig. 1B). Lane 1 in Fig. 1B contains IgM immunoprecipitated from 10^7 cells; lane 2 contains IgD immunoprecipitated from 5×10^6 cells. The 34/35-kDa band most likely corresponds to Igα, an Ig-associated cell surface glycoprotein that has previously been shown to be phosphorylated on tyrosine (8, 18). Membrane IgMα is approximately 1 kDa smaller than membrane IgDα, and this difference in size probably reflects differences in glycosylation (9). The pp36, pp39, and pp42 associated with both IgM and IgD probably correspond to forms of Igβ. Igβ is a group of related, Ig-associated glycoproteins encoded by the B29 gene (22) that are disulfide linked to Igα and are tyrosine phosphorylated (10, 18). On polyacrylamide gels run under nonreducing conditions, the in vitro-phosphorylated, Ig-associated 34- to 42-kDa bands were no longer seen (Fig.

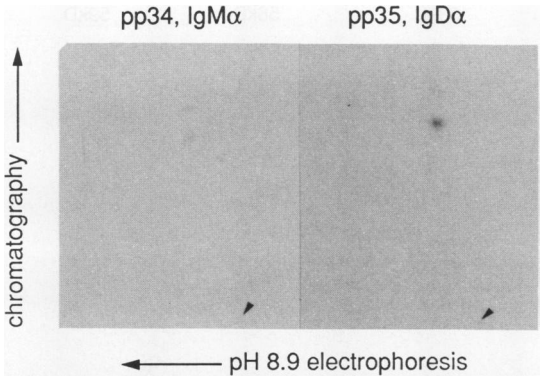

FIG. 2. Two-dimensional phosphotryptic peptide maps of in vitro-phosphorylated pp34 and pp35. Membrane IgM and IgD from spleen B cells were immunoprecipitated with monoclonal antibodies to μ and δ heavy chains and subjected to phosphorylation in vitro. Following SDS-PAGE and transfer to Immobilon, individual Ig-associated bands corresponding to the sizes shown were cut out and digested with trypsin. Digests were then resolved by electrophoresis at pH 8.9 in the first dimension and by chromatography in the second dimension. The position of the origin is indicated by an arrowhead.

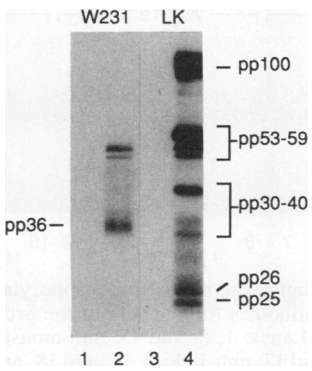

FIG. 4. Phosphorylation of B-cell line membrane Ig-associated molecules. CHAPS-soluble cell lysates were immunoprecipitated with various antibodies and assayed for associated in vitro kinase activity. Phosphorylated proteins were separated on SDS–15% polyacrylamide gels. WEHI-231, lanes 1 (nonspecific rabbit serum) and 2 (rabbit anti-mouse Ig); LK, lanes 3 (nonspecific rabbit serum) and 4 (rabbit anti-mouse Ig).

1C). Instead, a band of approximately 70 kDa was observed. On two-dimensional, diagonal nonreducing/reducing polyacrylamide gels, the 70-kDa molecule was resolved into bands of 34 to 36 and 39 to 42 kDa (data not shown). The 34- to 42-kDa molecules, therefore, are part of disulfide-linked dimers and almost certainly correspond to Igα and -β.

The sites of in vitro protein tyrosine phosphorylation on the Igα and -β molecules were studied by phosphotryptic peptide mapping. The in vitro-phosphorylated, IgM-associated pp34 was compared with the IgD-associated pp35 (Fig. 2). In each case, one major and one minor phosphorylated peptide were detected. Peptides from the two proteins comigrated exactly (data not shown); thus, IgM-associated pp34 and IgD-associated pp35 are phosphorylated on identical tryptic peptides in vitro.

Phosphotryptic peptide mapping of pp36, pp39, and pp42 is shown in Fig. 3. A single phosphotryptic peptide was detected in digests of both pp36 and pp39, and mixing experiments showed that the pp36 phosphopeptide was identical to the pp39 phosphopeptide. pp42 yielded two phosphopeptides and therefore is phosphorylated on at least two sites in vitro. Furthermore, mixing experiments revealed that the peptide with the greatest chromatographic mobility in pp42 (indicated in Fig. 3 by an asterisk) is identical to the phosphopeptide present in pp36 and pp39. These experiments show that pp36, pp39, and pp42 are highly related.

Membrane Ig isolated from B-cell lines is associated with in vitro protein tyrosine kinase activity. To determine whether membrane Ig in established B-cell lines is associated with similar protein tyrosine kinase activity, we examined immunoprecipitates of membrane Ig from the cell lines LK and WEHI-231 for associated kinase activity in vitro (Fig. 4). LK is an IgG-expressing hybridoma derived from a fusion between spleen cells and a B-lymphoma cell line (27). WEHI-231 is an IgM-positive B-lymphoma cell line that has many characteristics of immature B cells (34).

Membrane Ig isolated from LK cells was associated with a broad 100-kDa band, a group of three bands of 59, 56, and 53 kDa, a group of three bands of 30 to 40 kDa, and 26- and 25-kDa bands, all of which were phosphorylated in vitro (Fig. 4, lane 4). Phosphotryptic peptide mapping showed that the 100-kDa band associated with membrane Ig in LK was the same as that seen in spleen B cells, whereas the 25-kDa band was different (data not shown). Membrane IgM from

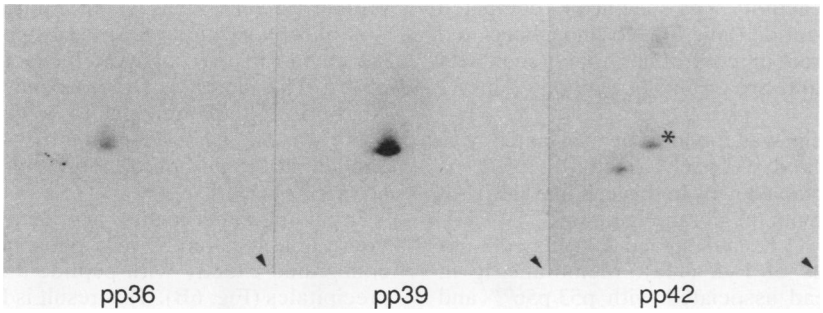

FIG. 3. Two-dimensional phosphotryptic peptide maps of in vitro-phosphorylated pp36, pp39, and pp42. Membrane Ig from spleen B cells was immunoprecipitated with rabbit anti-mouse Ig and subjected to phosphorylation in vitro. Following SDS-PAGE and transfer to Immobilon, individual Ig-associated bands corresponding to the sizes shown were cut out and digested with trypsin. Digests were then resolved by electrophoresis at pH 8.9 in the first dimension and by chromatography in the second dimension. The position of the origin is indicated by an arrowhead.

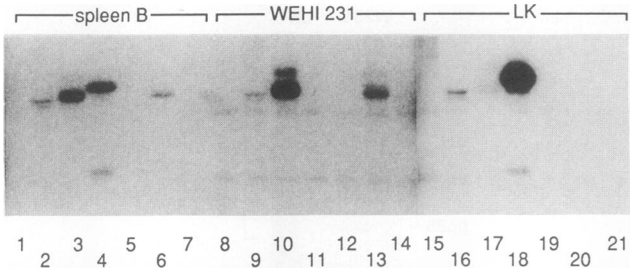

FIG. 5. Reprecipitation of in vitro-phosphorylated, Ig-associated molecules with antibodies to members of the Src family of protein tyrosine kinases. Lanes: 1, 8, and 15, anti-mouse Ig; 2, 9, and 16, anti-Lyn; 3, 10, and 17, anti-Lck; 4, 11, and 18, anti-Fyn; 5, 12, and 19, anti-Src; 6, 13, and 20, anti-Lck, amino acids 39 to 58; 7, 14, and 21, anti-Lck, amino acids 39 to 58 plus competing peptide.

WEHI-231 cells was also associated with protein tyrosine kinase activity. The molecules detected differed from those observed in spleen B cells and in LK cells in that only Ig-associated molecules of 56, 53, and 36 kDa were observed to undergo phosphorylation in vitro (lane 2).

We have yet to determine whether the 30- to 40-kDa and 26/25-kDa in vitro-phosphorylated molecules associated with membrane Ig in LK cells are related to Igα or -β. Likewise, we do not yet know whether the in vitro-phosphorylated, Ig-associated 36-kDa phosphoprotein in WEHI-231 cells is related to Igα or -β.

Protein tyrosine kinases associated with membrane Ig. Previous studies have indicated that the Src family protein tyrosine kinases Lyn, Fyn, and Blk are physically associated with membrane Ig (5, 49). We therefore wished to determine whether the 53-, 56-, and 59-kDa phosphotyrosine-containing, membrane Ig-associated molecules corresponded to members of the Src family. In vitro-phosphorylated anti-Ig immunoprecipitates were dissociated by boiling in SDS and then reprecipitated with antibodies to Lyn, Fyn, Lck, and Src (Fig. 5).

The 59-kDa phosphoprotein associated with membrane Ig in spleen B cells was reprecipitated by anti-Fyn antibody (Fig. 5, lane 4), confirming the findings of Burkhardt et al. (5). Anti-Lyn antibody reprecipitated a doublet of 53 and 56 kDa (lane 2), which probably correspond to the two splicing variants of Lyn, b and a (43, 51). Surprisingly, however, the majority of the 56-kDa band associated with membrane Ig in spleen B cells was not reprecipitated by anti-Lyn antiserum but was instead precipitated by anti-Lck antiserum (lane 3). This result was confirmed using a second anti-Lck peptide antibody (lane 6) whose activity was specifically competed for by the appropriate peptide (lane 7). No phosphorylated molecules were recovered in control immunoprecipitates obtained with use of an anti-Src or anti-Ig antibody (lanes 1 and 5).

Membrane Ig in LK cells was found to be associated with $p59^{fyn}$ (Fig. 5, lane 18) and $p53/p56^{byn}$ (lane 16). $p56^{lck}$ is expressed at barely detectable levels in this cell line (11a) but was found in association with membrane Ig (lane 17). WEHI-231 cells, in contrast to LK and spleen B cells, do not express significant levels of Fyn (11a). Membrane Ig in WEHI-231 cells is instead associated with $p53/p56^{byn}$ and with $p56^{lck}$ (lanes 9, 10, 13, and 14).

The 53-, 56-, and 59-kDa Ig-associated phosphoproteins from spleen B cells were further analyzed by phosphotryptic peptide mapping. The 59-kDa Ig-associated band yielded a single major phosphorylated tryptic peptide (Fig. 6A). This

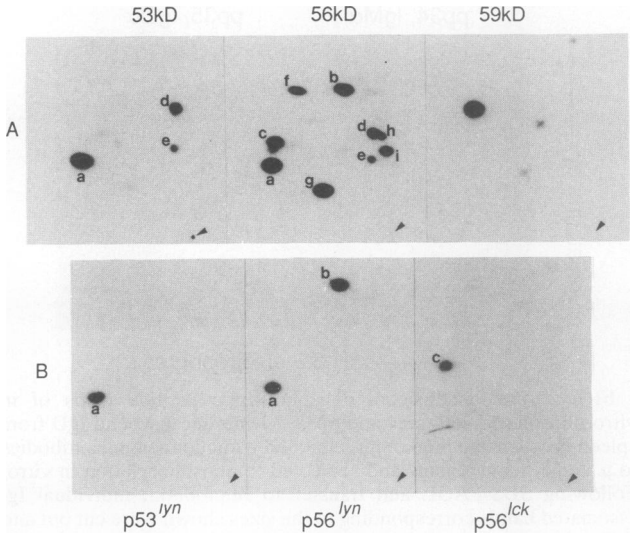

FIG. 6. Two-dimensional phosphotryptic peptide maps of in vitro-phosphorylated, spleen B-cell membrane Ig-associated molecules. (A) Membrane Ig from spleen B cells was immunoprecipitated with rabbit anti-Ig and subjected to phosphorylation in vitro. Following SDS-PAGE and transfer to Immobilon, individual Ig-associated bands corresponding to the sizes shown were cut out and digested with trypsin. Digests were then resolved by electrophoresis at pH 8.9 in the first dimension and by chromatography in the second dimension. The position of the origin is indicated by an arrowhead. (B) $p53/p56^{byn}$ and $p56^{lck}$ were immunoprecipitated from spleen B cells and autophosphorylated in vitro. Bands corresponding to the autophosphorylated kinases were then cut out and digested with trypsin as described for panel A.

finding strongly suggests that the 59-kDa, Ig-associated band contains only $p59^{fyn}$. The 53-kDa Ig-associated band resolved into one major and two minor phosphopeptides, and the 56-kDa Ig-associated band resolved into a complex pattern containing nine major phosphopeptides (Fig. 6A).

To identify the multiple phosphopeptides observed in digests of the Ig-associated 53- and 56-kDa molecules, these phosphopeptides were compared with those of $p53^{byn}$, $p56^{byn}$, and $p56^{lck}$ immunoprecipitated directly from spleen B cells with specific antisera (Fig. 6B).

Spleen B-cell $p53^{byn}$ was phosphorylated in vitro on a single tryptic peptide (Fig. 6B) which probably contains the major autophosphorylation site (tyrosine 397 in Lyn a and tyrosine 376 in Lyn b, by analogy with other Src-related protein tyrosine kinases). In contrast, spleen B cell $p56^{byn}$ was phosphorylated on at least two sites. The first peptide (spot a in Fig. 6B) comigrated exactly with that observed in $p53^{byn}$. The identity of the second site of phosphorylation (spot b in Fig. 6B) remains to be determined. Spleen B-cell $p56^{lck}$ was phosphorylated in vitro on a single tryptic peptide containing the autophosphorylation site, tyrosine 394 (Fig. 6b, spot c) (13).

In mixing experiments, peptide a derived from the membrane Ig-associated 53-kDa phosphorylated band (Fig. 6A) comigrated exactly with peptide a from anti-Lyn immunoprecipitates (Fig. 6B). This result is further evidence that the Ig-associated 53-kDa protein is $p53^{byn}$. Phosphopeptides d and e probably represent sites phosphorylated by a kinase(s) other than Lyn, as they are not observed in direct immunoprecipitates of Lyn (Fig. 6B).

The complex pattern of phosphotryptic peptides seen in

digests of the membrane Ig-associated 56-kDa phosphorylated protein suggests that this band consists of several molecules that are not resolved by SDS-polyacrylamide gel electrophoresis (PAGE). In mixing experiments, phosphopeptides a and b comigrated with a and b from $p56^{lyn}$ (Fig. 6B). Likewise, phosphopeptides d and e from the Ig-associated 56-kDa protein comigrated with d and e from the Ig-associated 53-kDa protein (Fig. 6A), suggesting that the Ig-associated form of $p56^{lyn}$ is also phosphorylated at these additional sites. Phosphopeptide c of the Ig-associated 56-kDa protein comigrated with peptide c seen in maps of $p56^{lck}$ (Fig. 6B).

These phosphotryptic peptide mapping experiments therefore confirm the findings of our reprecipitation studies, i.e., that $p53^{lyn}$, $p56^{lyn}$, $p56^{lck}$, and $p59^{fyn}$ are all associated with membrane Ig in spleen B cells. We have yet to identify the phosphotryptic peptides corresponding to f, g, h, and i in Fig. 5A; Burkhardt et al. have reported that $p56^{blk}$ is associated with membrane Ig (5); therefore, one or more of these peptides may correspond to in vitro-phosphorylated, Ig-associated $p56^{blk}$.

DISCUSSION

We have found that membrane Ig in B lymphocytes is associated with a number of molecules that are tyrosine phosphorylated in vitro. In addition to $p53^{lyn}$, $p56^{lyn}$, $p56^{lck}$, and $p59^{fyn}$, Igα, Igβ, and unidentified molecules of 25 and 100 kDa all undergo tyrosine phosphorylation in vitro. One or more of the protein tyrosine kinases associated with membrane Ig may be instrumental in the increased tyrosine phosphorylation observed in B cells following antibody-mediated cross-linking of membrane Ig. Studies by Burkhardt et al. have shown that Blk, Lyn, and Fyn kinase activity is slightly increased in B cells following anti-Ig stimulation (5). It remains to be determined whether the phosphorylation in vitro of Ig-associated molecules is also stimulated by anti-Ig antibodies. This problem will prove difficult to address, as membrane Ig rapidly becomes associated with the detergent-insoluble cytoskeleton following anti-Ig antibody-mediated cross-linking (40).

While membrane IgM in WEHI-231 cells is associated with Lck and Lyn, Fyn kinase activity is undetectable in these cells. Membrane IgG from LK cells is associated with Fyn, Lyn, and extremely low levels of Lck kinase activity. In contrast, in spleen B cells in which Lck, Lyn, and Fyn are all expressed, each of these kinases is found in association with membrane Ig. It may be that any Src-related kinase, if expressed in B cells, is capable of associating with membrane Ig. Cross-linking of membrane Ig in spleen B cells, LK, and WEHI-231 cells stimulates protein tyrosine phosphorylation of an essentially similar set of substrates (11, 11a, 17); this finding implies that the expression of neither $p56^{lck}$ nor $p59^{fyn}$ is absolutely required for this phosphorylation. It is possible that no single protein tyrosine kinase is essential for signalling through membrane Ig but rather that the Lyn, Lck, and Fyn kinases are each able to interact with membrane Ig and phosphorylate largely similar sets of substrates. Soriano et al. have recently reported that targeted deletion of $pp60^{src}$ by homologous recombination is not lethal, suggesting that other, related protein tyrosine kinases might substitute for $pp60^{src}$ function in these mice (41). Our findings are consistent with the possibility that kinases of the Src family are able to substitute functionally for one another.

The finding that $p56^{lck}$ is associated with membrane Ig was completely unexpected. Burkhardt et al. were not able to detect $p56^{lck}$ kinase activity in spleen B cells (5). This might be due to the use of digitonin to prepare cell lysates, as we have found that $p56^{lck}$ is poorly soluble in digitonin. We are confident that $p56^{lck}$ is expressed in B cells, as it is detected even in highly purified spleen B-cell preparations that have been positively selected for membrane Ig expression by fluorescence-activated cell sorting. In addition, $p56^{lck}$ is expressed in a number of murine B-cell lines, including WEHI-231 (Fig. 4), WEHI-279, CH33, and CH31 (data not shown). We have determined that the association between $p56^{lck}$ and membrane Ig does not occur following cell lysis. Membrane Ig isolated from mixed lysates of membrane Ig-positive LK cells (which express very low levels of $p56^{lck}$) and NK cells (an Ig-negative cell line that expresses significant levels of $p56^{lck}$) was not associated with increased levels of $p56^{lck}$ kinase activity (data not shown).

How might protein tyrosine kinases interact with the B-cell antigen receptor complex? Src-related kinases can interact with other cellular proteins by a variety of mechanisms. In T cells, $p56^{lck}$ associates with the cell surface molecules CD4 and CD8 (35, 45) through cysteine residues in its unique amino-terminal domain (38, 39). It is unlikely that the N termini of the Src kinases play a defining role in the interaction with membrane Ig, since they are unrelated in sequence. It is more probable that one or more conserved domains of these kinases are involved.

Members of the Src family of protein tyrosine kinases all contain a highly conserved region immediately amino terminal of the catalytic domain known as the SH-2 (*src*-homology 2) domain (reviewed in reference 28). This domain has been shown to bind directly to phosphotyrosine and so facilitate intermolecular interactions. The Src, Yes, and Fyn kinases may associate with the activated, tyrosine-phosphorylated form of the platelet-derived growth factor receptor through the SH-2 domain (29). Such an interaction is unlikely to explain the association with the B-cell antigen receptor complex, since surface IgM-associated molecules are not detectably phosphorylated in unstimulated spleen B cells (18), yet membrane Ig from unstimulated cells is associated with protein tyrosine kinase activity.

Recently $p56^{lck}$ was shown to interact with the β chain of the interleukin-2 receptor (20). This interaction appears to involve the catalytic domain of $p56^{lck}$, a region that is conserved among kinases of the Src family, and is therefore probably not an interaction unique to $p56^{lck}$. It is possible that the association between Src-related kinases and the B-cell antigen receptor complex is also through the kinase catalytic domain.

We have found that Igα and -β undergo tyrosine phosphorylation in vitro. These molecules have been shown to undergo tyrosine phosphorylation in vivo following cross-linking of membrane Ig (18). Tryptic peptide mapping showed that IgMα and IgDα undergo phosphorylation in vitro at identical sites. This finding is consistent with recent data suggesting that they are probably differentially modified forms of the same polypeptide (9). We observed three other Ig-associated molecules of approximately 36 to 42 kDa that were present as disulfide-linked dimers. These molecules almost certainly correspond to the Igβ and Igγ bands described by Campbell and Cambier (8). Tryptic peptide mapping showed that all three bands underwent phosphorylation in vitro at similar sites, consistent with recent data suggesting that Igβ and Igγ are related in sequence (10).

What component of the B-cell antigen receptor complex interacts with protein tyrosine kinases? All of the known

interactions between Src-related kinases and their associated molecules are stable in the presence of NP-40. This is in contrast to the association between membrane Ig and Src-related kinases, which is not observed in NP-40 cell lysates (5, 11, 49). Indeed, we have found that NP-40 dissociates the kinase-membrane Ig complex (37a). One possible explanation for this finding is that the kinase is actually associated with another component of the B-cell antigen receptor complex such as Igα or -β. The interaction between membrane Ig and Igα/β is known to be unstable in NP-40 (14).

We have observed phosphorylation in vitro of two molecules, pp100 and pp25, that are associated with membrane Ig in spleen B cells. This association has not been reported previously, possibly because of differences in techniques used in our study compared with those used previously (5, 8). We have not yet been able to identify pp25 and pp100. We were particularly interested to find that pp100 and pp25 are not phosphorylated in WEHI-231 cells. This cell line resembles the immature B-cell stage of development and has been used as a model in studies of the mechanisms of B-cell tolerance (34, 37). Cross-linking of membrane Ig on WEHI-231 cells with anti-Ig antibodies results in cell death by apoptosis (2, 19); this effect is in marked contrast to the response of mature spleen B cells, which are activated by anti-Ig treatment (reviewed in reference 7). Despite this difference in end response, spleen B cells and WEHI-231 cells are very similar in their patterns of inositol phospholipid turnover and total protein tyrosine phosphorylation following anti-Ig stimulation (3, 11, 16). It will be interesting to determine whether the phosphorylation of membrane Ig-associated pp25 and pp100 in vitro is regulated developmentally in B cells.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants CA-14195 and CA-17289. M.-A.C. was supported by EMBO and Schering-Plough.

REFERENCES

1. **Ales-Martinez, J. E., G. L. Warner, and D. W. Scott.** 1988. Immunoglobulins D and M mediate signals that are qualitatively different in B cells with an immature phenotype. Proc. Natl. Acad. Sci. USA **85:**6919–6923.
2. **Benhamou, L. E., P.-A. Cazenave, and P. Sarthou.** 1990. Anti-immunoglobulins induce death by apoptosis in WEHI-231 B lymphoma cells. Eur. J. Immunol. **20:**1405–1407.
3. **Bijsterbosch, M. K., C. J. Meade, G. A. Turner, and G. G. B. Klaus.** 1985. B lymphocyte receptors and polyphosphoinositide degradation. Cell **41:**999–1006.
4. **Boyd, A. W., and J. W. Schrader.** 1981. The regulation of growth and differentiation of a murine B cell lymphoma. ii. The inhibition of WEHI 231 by anti-immunoglobulin antibodies. J. Immunol. **126:**2466–2469.
5. **Burkhardt, A. L., M. Brunswick, J. B. Bolen, and J. J. Mond.** 1991. Anti-immunoglobulin stimulation of B lymphocytes activates *src*-related protein tyrosine kinases. Proc. Natl. Acad. Sci. USA **88:**7410–7414.
6. **Cambier, J. C., and J. G. Monroe.** 1984. B cell activation. v. Differentiation signalling of B cell membrane depolarization, increased I-A expression, G_0 to G_1 transition, and thymidine uptake by anti-IgM and anti-IgD antibodies. J. Immunol. **133:** 576–581.
7. **Cambier, J. C., and J. T. Ransom.** 1987. Molecular mechanisms of transmembrane signaling in B lymphocytes. Annu. Rev. Immunol. **5:**175–199.
8. **Campbell, K. S., and J. C. Cambier.** 1990. B lymphocyte antigen receptors (mIg) are non-covalently associated with a disulfide linked, inducibly phosphorylated complex. EMBO J. **9:**441–448.
9. **Campbell, K. S., E. J. Hager, and J. C. Cambier.** 1991. α-chains of IgM and IgD antigen receptor complexes are differentially N-glycosylated MB-1-related molecules. J. Immunol. **147:**1575–1580.
10. **Campbell, K. S., E. J. Hager, R. J. Friedrich, and J. C. Cambier.** 1991. IgM antigen receptor complex contains phosphoprotein products of B29 and *mb-1* genes. Proc. Natl. Acad. Sci. USA **88:**3982–3986.
11. **Campbell, M.-A., and B. M. Sefton.** 1990. Protein tyrosine phosphorylation is induced in murine B lymphocytes in response to stimulation with anti-immunoglobulin. EMBO J. **9:**2125–2131.

11a. **Campbell, M.-A., and B. M. Sefton.** Unpublished data.

12. **Carter, R. H., D. J. Park, S. G. Rhee, and D. T. Fearon.** 1991. Tyrosine phosphorylation of phospholipase C induced by membrane immunoglobulin in B lymphocytes. Proc. Natl. Acad. Sci. USA **88:**2745–2749.
13. **Casnellie, J. E.** 1987. Sites of in vitro phosphorylation of the T cell tyrosine protein kinase in LSTRA cells and their alteration by tumour-promoting phorbol esters. J. Biol. Chem. **262:**9859–9864.
14. **Chen, J., A. M. Stall, L. A. Herzenberg, and L. A. Herzenberg.** 1990. Differences in glycoprotein complexes associated with IgM and IgD on normal murine B cells potentially enable transduction of different signals. EMBO J. **9:**2117–2124.
15. **Coggeshall, K. M., and J. C. Cambier.** 1984. B cell activation. viii. Membrane immunoglobulins transduce signals via activation of phosphatidylinositol hydrolysis. J. Immunol. **133:**3382–3386.
16. **Fahey, K. A., and A. L. DeFranco.** 1987. Cross-linking membrane IgM induces production of inositol trisphosphate and inositol tetrakisphosphate in WEHI-231 B lymphoma cells. J. Immunol. **138:**3935–3942.
17. **Gold, M. R., D. Law, and A. L. DeFranco.** 1990. Stimulation of protein tyrosine phosphorylation by the B lymphocyte antigen receptor. Nature (London) **345:**810–813.
18. **Gold, M. R., L. Matsuuchi, R. B. Kelly, and A. L. DeFranco.** 1991. Tyrosine phosphorylation of components of the B-cell antigen receptors following receptor crosslinking. Proc. Natl. Acad. Sci. USA **88:**3436–3440.
19. **Hasbold, J., and G. G. B. Klaus.** 1990. Anti-immunoglobulin antibodies induce apoptosis in immature B lymphomas. Eur. J. Immunol. **20:**1685–1690.
20. **Hatakeyama, M., T. Kono, N. Kobayashi, A. Kawahara, S. D. Levin, R. M. Perlmutter, and T. Taniguchi.** 1991. Interaction of the Il-2 receptor with the *src*-family kinase $p56^{lck}$: identification of novel intermolecular association. Science **252:**1523–1528.
21. **Hombach, J., L. Leclercq, A. Radbruch, K. Rajewsky, and M. Reth.** 1988. A novel 34-kd protein co-isolated with the IgM molecule in surface IgM-expressing cells. EMBO J. **7:**3451–3456.
22. **Hombach, J., F. Lottspeich, and M. Reth.** 1990. Identification of the genes encoding the IgMα and Igβ components of the IgM antigen receptor complex by amino terminal sequencing. Eur. J. Immunol. **20:**2795–2799.
23. **Hombach, J., T. Tsubata, L. Leclerq, H. Stappert, and M. Reth.** 1990. Molecular components of the B-cell antigen receptor complex of the IgM class. Nature (London) **343:**760–762.
24. **Hurley, T. R., and B. M. Sefton.** 1989. Analysis of the activity and phosphorylation of the *lck* protein in lymphoid cells. Oncogene **4:**265–272.
25. **Julius, M. H., C. H. Heusser, and K.-U. Hartmann.** 1984. Induction of resting B cells to DNA synthesis by soluble monoclonal anti-immunoglobulin. Eur. J. Immunol. **14:**753–757.
26. **Kamps, M. P., and B. M. Sefton.** 1989. Acid and base hydrolysis of phosphoproteins bound to Immunobilon facilitates the analysis of phosphoamino acids in gel-fractionated proteins. Anal. Biochem. **176:**22–27.
27. **Kappler, J., J. White, D. Wegmann, E. Mustain, and P. Marrack.** 1982. Antigen presentation by Ia^+ B cell hybridomas to *H-2*-restricted T cell hybridomas. Proc. Natl. Acad. Sci. USA **79:**3604–3607.
28. **Koch, C. A., D. Anderson, M. F. Moran, C. Ellis, and T. Pawson.** 1991. SH2 and SH3 domains: elements that control

interactions of cytoplasmic signalling proteins. Science **252:**668–674.

29. **Kypta, R. M., Y. Goldberg, and S. A. Courtneidge.** 1990. Association between the PDGF receptor and members of the *src* family of tyrosine kinases. Cell **62:**481–492.
30. **Lane, P. J. L., J. A. Ledbetter, F. M. McConnell, K. Draves, J. Deans, G. Schieven, and E. A. Clark.** 1991. The role of tyrosine phosphorylation in signal transduction through surface Ig in human B cells. J. Immunol. **146:**715–722.
31. **Lanier, L. L., G. A. Gutman, D. E. Lewis, S. T. Griswold, and N. L. Warner.** 1982. Monoclonal antibodies against rat immunoglobulin kappa chains. Hybridoma **1:**125–131.
32. **Ledbetter, J. A., and L. A. Herzenberg.** 1979. Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol. Rev. **47:**63–90.
33. **Luo, K., T. R. Hurley, and B. M. Sefton.** 1990. Transfer of proteins to membranes facilitates both cyanogen bromide cleavage and two-dimensional proteolytic mapping. Oncogene **5:**921–924.
34. **Ralph, P.** 1979. Functional subsets of murine and human B lymphocyte cell lines. Immunol. Rev. **48:**107–121.
35. **Rudd, C. E., J. M. Trevillyan, J. D. Dasgupta, L. L. Wong, and S. F. Schlossman.** 1988. The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp58) from human T lymphocytes. Proc. Natl. Acad. Sci. USA **85:**5190–5194.
36. **Scher, I., J. A. Titus, and F. E. Finkelman.** 1983. The ontogeny and distribution of B cells in normal and mutant immune-defective CBA/N mice: two parameter analysis of surface IgM and IgD. J. Immunol. **130:**619–625.
37. **Scott, D. W., J. Tuttle, D. Livnat, W. Haynes, J. P. Cogswell, and P. Keng.** 1985. Lymphoma models for B-cell activation and tolerance. ii. Growth inhibition by anti-μ of WEHI-231 and the selection and properties of resistant mutants. Cell. Immunol. **93:**124–131.

37a. **Sefton, B. M., and L. Yurchak.** Unpublished data.

38. **Shaw, A. S., K. E. Amrein, C. Hammond, D. F. Stern, B. M. Sefton, and J. K. Rose.** 1989. The *lck* tyrosine protein kinase interacts with the cytoplasmic tail of the CD4 glycoprotein through its unique amino-terminal domain. Cell **59:**627–636.
39. **Shaw, A. S., J. Chalupny, A. Whitney, C. Hammond, K. E. Amrein, P. Pavathas, B. M. Sefton, and J. K. Rose.** 1990. Short related sequences in the cytoplasmic domains of CD4 and CD8 mediate binding to the amino-terminal domain of the $p56^{lck}$ tyrosine protein kinase. Mol. Cell. Biol. **10:**1853–1862.
40. **Snow, E. C., and R. J. Noelle.** 1987. Thymus-dependent antigenic stimulation of hapten-specific B lymphocytes. Immunol. Rev. **99:**173–192.
41. **Soriano, P., C. Montgomery, R. Geske, and A. Bradley.** 1991. Targeted disruption of the c-*src* proto-oncogene leads to osteopetrosis in mice. Cell **64:**693–702.
42. **Stall, A. M., and M. R. Loken.** 1984. Allotypic specificities of murine IgD and IgM recognized by monoclonal antibodies. J. Immunol. **132:**787–795.
43. **Stanley, E., S. Ralph, S. McEwan, I. Boulet, D. A. Holtzman, P. Lock, and A. R. Dunn.** 1991. Alternatively spliced murine *lyn* mRNAs encode distinct proteins. Mol. Cell. Biol. **11:**3399–3406.
44. **Tisch, R., C. M. Roifman, and N. Hozumi.** 1988. Functional differences between immunoglobulins M and D expressed on the surface of an immature B-cell line. Proc. Natl. Acad. Sci. USA **85:**6914–6918.
45. **Veillette, A., M. A. Bookman, E. M. Horak, and J. B. Bolen.** 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase $p56^{lck}$. Cell **44:**301–308.
46. **Venkitaraman, A. R., G. T. Williams, P. Dariavach, and M. S. Neuberger.** 1991. The B cell antigen receptor of the five immunoglobulin classes. Nature (London) **352:**777–781.
47. **Weinands, J., J. Hombach, A. Radbruch, C. Reisterer, and M. Reth.** 1990. Molecular components of the B cell antigen receptor complex of class IgD differ partly from those of IgM. EMBO J. **9:**449–455.
48. **Weinands, J., and M. Reth.** 1991. The B cell antigen receptor of class IgD can be expressed on the cell surface in two different forms. Eur. J. Immunol. **21:**2373–2378.
49. **Yamanashi, Y., T. Kakiuchi, J. Mizuguchi, T. Yamamoto, and K. Toyoshima.** 1991. Association of B cell antigen receptor with protein tyrosine kinase *lyn*. Science **251:**192–194.
50. **Yelton, D. E., C. Desamord, and M. D. Scharf.** 1981. Use of monoclonal anti-mouse immunoglobulin to detect mouse antibodies. Hybridoma **1:**5–11.
51. **Yi, T. L., J. B. Bolen, and J. N. Ihle.** 1991. Hemopoietic cells express two forms of *lyn* kinase differing by 21 amino acids in the amino terminus. Mol. Cell. Biol. **11:**2391–2398.