The EMBO Journal vol.9 no.7 pp.2125–2131, 1990

Protein tyrosine phosphorylation is induced in murine B lymphocytes in response to stimulation with anti-immunoglobulin

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Communicated by R.Dulbecco

Activation of both T and B lymphocytes through their membrane receptors for antigen is known to induce breakdown of inositol phospholipids. In addition, T cell activation by antigen is accompanied by increased protein tyrosine phosphorylation of components of the T cell antigen receptor. We now provide evidence that B cell activation through membrane immunoglobulin is also coupled to stimulation of protein tyrosine kinase activity. One potential candidate for a B lymphocyte protein tyrosine kinase is an 80 kd molecule that is itself phosphorylated at tyrosine residues in response to stimulation with anti-immunoglobulin antibodies.

Key words: B cell/protein tyrosine kinase/transmembrane signaling

Introduction

Mature B lymphocytes express clonally distributed antigen receptors on their cell surface in the form of membrane immunoglobulin (Ig) M and D. Ligation of membrane Ig by antigen or by certain anti-Ig antibodies results in an activating signal being delivered to the B cell, with consequent entry into the cell cycle (reviewed by Cambier and Ransom, 1987; DeFranco *et al.*, 1989).

Signal transduction through membrane Ig is accompanied by the breakdown of inositol phospholipids and generation of the second messenger molecules inositol trisphosphate and diacylglycerol (Coggeshall and Cambier, 1984; Bijsterbosch *et al.*, 1985 and reviewed by Berridge and Irvine, 1989). Indirect evidence suggests that membrane Ig is coupled to a polyphosphoinositide phosphodiesterase through one or more pertussis and cholera toxin-insensitive guanine nucleotide binding proteins (Gold *et al.*, 1987; Harnett and Klaus, 1988). Inositol phospholipid hydrolysis results in an increase in free cytoplasmic Ca^{2+} concentration (Bijsterbosch *et al.*, 1985; Ransom *et al.*, 1986; Bijsterbosch *et al.*, 1986), and activation of the Ca^{2+} and phospholipid-dependent serine/threonine kinase, protein kinase C (PKC) (Chen *et al.*, 1986; Nel *et al.*, 1986).

Despite these findings, it is not clear whether breakdown of inositol phospholipids is the only signal generated as a result of transmembrane signaling through membrane Ig. Several groups have found that signal transduction through membrane Ig can be dissociated from the activation of PKC in both human (Francois *et al.*, 1988) and murine (Mond *et al.*, 1987; Sarthou *et al.*, 1989) B lymphocytes. It is also clear that B cells may be induced to proliferate by stimulation with anti-Ig [in combination with the lymphokine, interleukin 4 (IL-4)] at doses of anti-Ig that have a minimal effect on inositol phospholipid turnover (Klaus *et al.*, 1987). Interleukin 4 itself does not appear to stimulate inositol phospholipid breakdown either alone or in combination with anti-Ig (Justement *et al.*, 1986; Mizuguchi *et al.*, 1986).

In T lymphocytes, the immediate response to activating stimuli includes both inositol phospholipid hydrolysis with subsequent activation of PKC (reviewed by Weiss *et al.*, 1986; Imboden and Inokuchi, 1989) and activation of one or more protein tyrosine kinases. This raises the possibility that stimulation of B lymphocytes through their antigen receptor might also lead to activation of one or more protein tyrosine kinases in addition to the well documented hydrolysis of inositol phospholipids.

There are few studies of protein tyrosine phosphorylation in B cells although these cells are known to be rich in tyrosine kinase activity (Earp *et al.*, 1984; Harrison *et al.*, 1984). Nel *et al.* (1984) demonstrated that the detergent-insoluble fraction of human B lymphocytes contains protein tyrosine kinase activity and that stimulation with anti-Ig increases the *in vitro* phosphorylation of 56 and 60 kd polypeptides at tyrosine residues. Given the lack of specificity of tyrosine protein kinases *in vitro*, however, it is not possible to determine whether phosphorylation of the 56 and 60 kd substrates is immediately relevant to signal transduction through membrane Ig *in vivo*.

Indirect evidence implicating protein tyrosine phosphorylation in B lymphocyte activation comes from studies using antibodies against the cell surface molecule CD45. This molecule has recently been shown to have protein tyrosine phosphatase activity (Tonks *et al.*, 1988). Treatment of B lymphocytes with anti-CD45 antibodies has been shown to inhibit anti-Ig mediated activation (Mittler *et al.*, 1987; Gruber *et al.*, 1989), suggesting that protein tyrosine phosphorylation is in some way critical to this process.

To determine whether protein tyrosine phosphorylation is directly linked to transmembrane signaling through membrane Ig on murine B lymphocytes, we have examined whether anti-Ig stimulation induces tyrosine phosphorylation in intact B cells.

Results

Anti-Ig stimulates protein tyrosine phosphorylation in resting splenic B cells

Small dense murine splenic B cells were stimulated with goat polyclonal antiserum specific for mouse μ chain (goat anti-μ) (5 μg/ml) and the tyrosine phosphorylation of B cell proteins was analyzed by Western blotting with affinity-purified polyclonal rabbit anti-phosphotyrosine antiserum. Low levels of phosphotyrosine were detected in unstimulated cells in polypeptides of 101, 60 and 57 kd (Figure 1). Anti-μ treatment stimulated tyrosine phosphorylation of molecules of ~80, 76, 63 and 32 kd (Figure 1). An increase in protein

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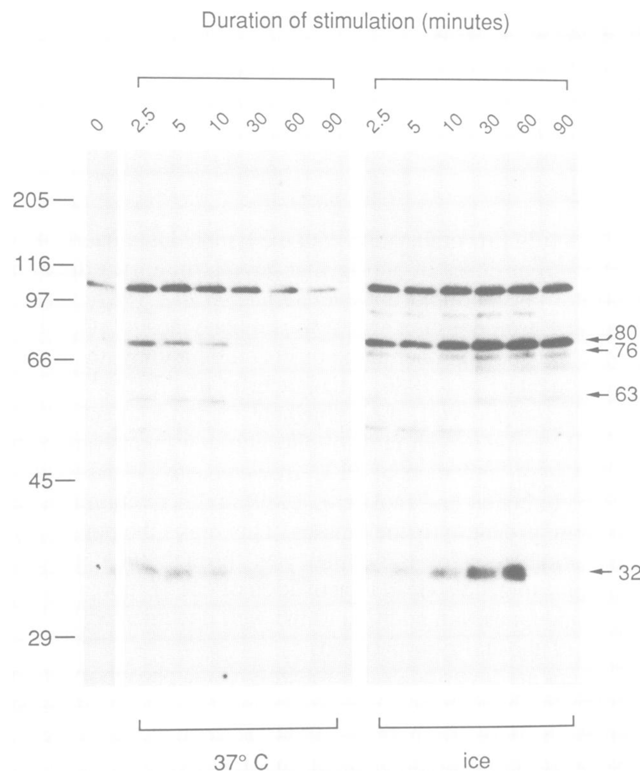

Fig. 1. Stimulation of splenic B cells with anti-μ induces protein tyrosine phosphorylation. Dense splenic B cells were stimulated with polyclonal goat anti-mouse μ antiserum (5 μg/ml) for the indicated times at 37°C or on ice. Detergent-soluble cell lysates, equivalent to 2×10^6 cells per lane, were mixed with an equal volume of 2 × reducing electrophoresis sample buffer and analyzed by electrophoresis on 15% SDS–polyacrylamide gels followed by Western blotting with a polyclonal rabbit anti-phosphotyrosine antiserum and detection with $[^{125}I]$protein A. Positions of mol. wt standards are indicated on the left-hand side of the figure. Detection of labeled bands was by fluorography with an exposure time of 48 h.

tyrosine phosphorylation could be detected as early as 30 s following stimulation with anti-Ig at 37°C (data not shown). The response peaked within 2.5 min and diminished to background levels over the following 90 min (Figure 1). In cells treated on ice, the levels of phosphotyrosine in these proteins were also increased; however, at this temperature the levels of phosphotyrosine generally remained elevated over 90 min (Figure 1). An exception to this was the 32 kd molecule, whose phosphorylation decreased to background levels over 90 min even on ice.

The increase in protein tyrosine phosphorylation was proportional to the amount of anti-μ used for stimulation (Figure 2A) and paralleled the proliferative response to anti-μ treatment as measured by uptake of tritiated thymidine (Figure 2B).

In order to determine whether this increase in protein tyrosine phosphorylation is directly related to signaling through membrane immunoglobulin, agents that activate resting B cells through receptors other than membrane Ig were examined for their ability to stimulate protein tyrosine phosphorylation.

The polyclonal B cell mitogen, lipopolysaccharide (LPS), does not bind to membrane Ig and triggers B cell proliferation through an as yet undefined pathway (reviewed by DeFranco, 1987). Stimulation of resting B lymphocytes with mitogenic doses of LPS (25 μg/ml) (Figure 3, lane c) did not induce the marked increases in protein tyrosine phosphorylation that were observed in response to treatment with anti-μ (Figure 3, lane d). Only a small increase in the apparent tyrosine phosphorylation of an 80 kd band was visible on the Western blot of cells treated with LPS (Figure 3, lane c). This band has not been observed consistently, and its significance is unclear.

Interleukin 4 has multiple effects on B lymphocyte growth and development. Treatment of resting B lymphocytes with IL-4 induces an activation state transitional between G_0 and G_1 of the cell cycle (Rabin *et al.*, 1985) which is accompanied by an increase in cell size and increased expression of membrane Ia antigens (Roehm *et al.*, 1984; Noelle *et al.*, 1984). In addition, IL-4 costimulates B cell proliferation when added in combination with submitogenic doses of anti-Ig (Howard *et al.*, 1982).

When B lymphocytes were treated with recombinant IL-4 alone (100 U/ml), no marked changes in protein tyrosine phosphorylation were observed (see Figure 3, lane b) although a very faint band of 80 kd, similar to that observed in LPS-treated cells, was detectable (Figure 3). Nevertheless, this treatment was sufficient to induce increased expression of Ia antigens after 18 h of culture (data not shown). When IL-4 was added in conjunction with anti-μ, this did not stimulate a further increase in tyrosine phosphorylation (see Figure 3, lanes d and e), despite the ability of this combination of stimuli to induce proliferation as measured by uptake of tritiated thymidine (data not shown). Even when cells were stimulated with very low amounts of anti-μ (0.5–1.0 μg/ml), the addition of IL-4 did not alter the pattern or levels of phosphotyrosyl proteins detected by Western blotting (data not shown).

The results of these experiments suggested that the increase in B cell protein tyrosine phosphorylation observed in response to treatment with anti-Ig antibodies might be specific for stimulation through membrane Ig, rather than representing a secondary response to B lymphocyte activation in general.

Anti-Ig treatment also stimulates protein tyrosine phosphorylation in a B cell line

Studies of the B lymphoma line, WEHI 231, provided further evidence that the increases in protein tyrosine phosphorylation observed in B lymphocytes following stimulation with anti-Ig were directly related to signaling through membrane Ig. The WEHI 231 line resembles cells at a stage in B cell maturation intermediate between that of the pre-B cell, where B lymphocytes express cytoplasmic μ chains but no surface Ig, and the fully mature, surface IgM and IgD positive, splenic B cells of normal adult mice (Ralph, 1979). WEHI 231 cells are surface IgM positive but do not express surface IgD (Ralph, 1979). These cells proliferate *in vitro* and are highly sensitive to treatment with anti-Ig (Boyd and Schrader, 1981). In direct contrast to the activation seen with more mature B cells, however, crosslinking of WEHI 231 membrane Ig leads to arrest of growth in the G_1 phase of the cell cycle (Scott *et al.*, 1985). The growth inhibition provoked by anti-Ig can be prevented if LPS is present at the time of stimulation (DeFranco *et al.*, 1987).

As in resting splenic B cells, the levels of phosphotyrosyl proteins detected by Western blotting in unstimulated WEHI 231 cells were low [Figure 4, lane a (splenic B cells) and

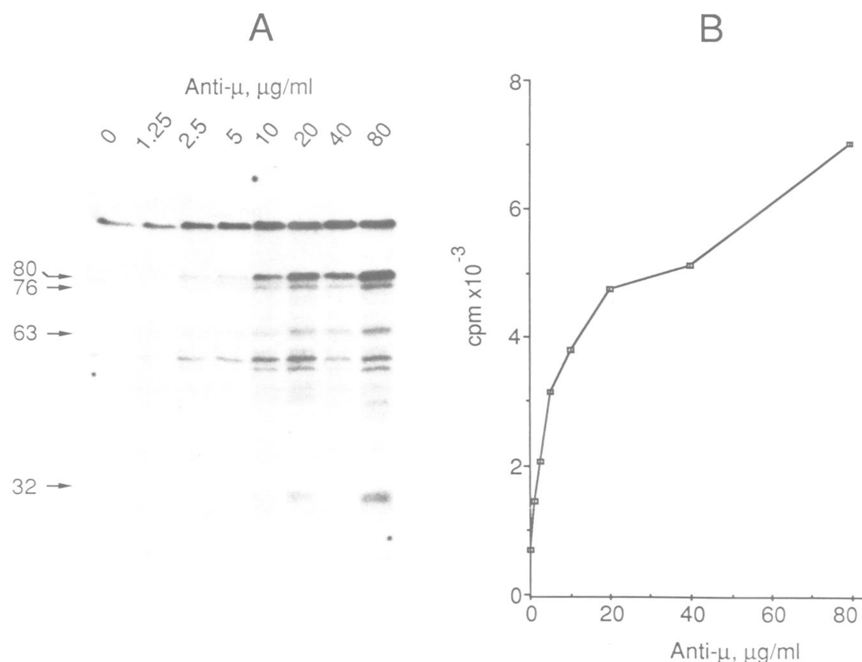

Fig. 2. B-lymphocyte protein tyrosine phosphorylation is dependent on the amount of anti-μ used for stimulation. (**A**) Dense splenic B cells were stimulated with increasing doses of anti-μ for 10 min at 37°C. Samples were prepared and analyzed by Western blotting with anti-phosphotyrosine antibodies as described in the legend to Figure 1. Exposure time was 64 h. (**B**) Uptake of tritiated thymidine in response to increasing doses of anti-μ. Small B lymphocytes were cultured in quadruplicate at 5×10^5/ml for 72 h in 200 μl of medium containing 10% FBS and increasing concentrations of goat anti-μ antibody. During the last 4 h of culture, cells were pulse-labeled with 0.5 μCi methyl-[^{3}H]thymidine. Incorporation of [^{3}H]thymidine was measured by liquid scintillation counting.

lane b (WEHI 231)]. Bands of 101 and 60 kd were constitutively phosphorylated at tyrosine residues in WEHI 231 cells, as in splenic B cells. In lysates from unstimulated WEHI 231 cells, an additional band of 29 kd was detected with anti-phosphotyrosine antibodies.

When WEHI 231 cells were stimulated with 5 μg/ml goat anti-μ for 10 min at 37°C, increased tyrosine phosphorylation was detected in polypeptides of 80, 76, 63 and ~32–35 kd (Figure 4, lane d). Treatment of WEHI 231 cells with LPS did not stimulate protein tyrosine phosphorylation (Figure 4, lane e). While LPS was able to override the negative growth signal provided by treatment with anti-μ (as determined by uptake of tritiated thymidine, data not shown), adding LPS to anti-μ-treated WEHI 231 cells did not alter the pattern of protein tyrosine phosphorylation observed (Figure 4, lane f). Interestingly, when WEHI 231 cells were stimulated at 37°C, tyrosine phosphorylation of a polypeptide of ~45 kd was observed (Figure 4, lanes d and f). Phosphorylation of this molecule was not observed in splenic B cells stimulated with anti-μ at 37°C or on ice (Figure 4, lane c and see also Figure 1) or in WEHI 231 cells maintained on ice during stimulation (data not shown).

A tyrosine-phosphorylated molecule with a mol. wt of ~32–35 kd was seen in both anti-μ-stimulated WEHI 231 and splenic B cells (Figure 4, lanes c, d and f). The two bands do not, however, comigrate precisely. It is possible that the slightly larger molecule in WEHI 231 cells represents a more highly phosphorylated form of the 32 kd molecule seen in the splenic B cells, but we have no evidence as yet to support this hypothesis.

A further difference between WEHI 231 and splenic B cells was in the intensity of staining of the 63 kd tyrosine-phosphorylated polypeptide (see Figure 4 lanes c, d and f). It would appear that this molecule is either more abundant in WEHI 231 cells or that it is more highly tyrosine-phosphorylated in response to anti-Ig treatment.

Isolation of B lymphocyte phosphotyrosyl proteins

In order to demonstrate unequivocally that protein tyrosine phosphorylation is stimulated by anti-μ, it is necessary to label cells *in vivo* with ^{32}P and characterize phosphorylated molecules by phosphoamino acid analysis. Resting B lymphocytes are exceptionally difficult to label with ^{32}P *in vivo* as they are metabolically inert. As anti-μ treatment also stimulates protein tyrosine phosphorylation in WEHI 231 cells (detected by Western blotting), WEHI 231 cells were labeled *in vivo* with ^{32}P. Phosphotyrosyl proteins were then isolated using a monoclonal anti-phosphotyrosine antibody, 1G2 (Huhn *et al.*, 1987), coupled to Sepharose.

Stimulation of radiolabeled WEHI 231 cells at 37°C for 10 min with 10 μg/ml anti-μ resulted in the appearance of two major ^{32}P-labeled bands in eluates from anti-phosphotyrosine Sepharose (Figure 5A). These bands, of ~80 and 45 kd, were subjected to phosphoamino acid analysis and were both found to contain phosphotyrosine (Figure 5B and C). In addition to phosphotyrosine, the 80 kd band contained significant levels of phosphoserine (Figure 5B) and the 45 kd band contained both phosphothreonine and phosphoserine (Figure 5C).

A tyrosine-phosphorylated protein tyrosine kinase is present in anti-Ig-activated B cells

Stimulated protein tyrosine kinases are usually phosphorylated at tyrosine residues (Hunter and Cooper, 1986), a phenomenon that has been utilized to isolate the epidermal growth factor and platelet derived growth factor receptor

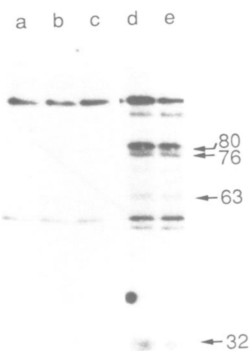

Fig. 3. LPS and IL-4 do not significantly increase B lymphocyte protein tyrosine phosphorylation. Dense splenic B cells were treated for 15 min on ice with medium (**lane a**), 100 U/ml recombinant IL-4 (**lane b**), 25 μg/ml LPS (**lane c**), 10 μg/ml goat anti-μ (**lane d**) or both 10 μg/ml goat anti-μ and 100 U/ml recombinant IL-4 (**lane e**). Samples were prepared and analyzed by Western blotting with anti-phosphotyrosine antibodies as described in the legend to Figure 1. Exposure time was 83 h.

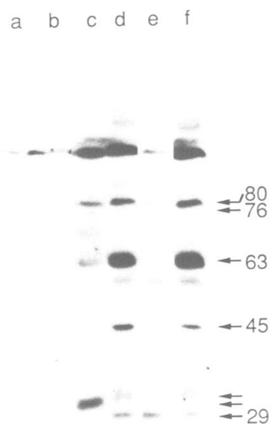

Fig. 4. Anti-μ-stimulated protein tyrosine phosphorylation in a B cell line is similar to that seen in dense splenic B cells. WEHI 231 cells and dense splenic B cells were stimulated for 10 min at 37°C, and protein phosphorylation was analyzed by Western blotting with anti-phosphotyrosine antiserum as described in the legend to Figure 1. WEHI 231 cells were treated with medium (**lane b**), with 5 μg/ml goat anti-μ (**lane d**), with 25 μg/ml LPS (**lane e**) or with both 5 μg/ml goat anti-μ and 25 μg/ml LPS (**lane f**) (lysate equivalent to 5×10^5 WEHI 231 cells per lane). Splenic B cells were treated with medium (**lane a**) or with 5 μg/ml goat anti-μ (**lane c**) (lysate equivalent to 2×10^6 B cells per lane). Exposure time was 60 h.

protein tyrosine kinases on immobilized anti-phosphotyrosine antibodies (Frackelton *et al.*, 1983, 1984). This approach was used in an attempt to identify kinases responsible for the increased protein tyrosine phosphorylation observed in anti-Ig-stimulated B cells.

Splenic B cells and WEHI 231 cells were incubated for 15 min on ice with or without goat anti-μ (10 μg/ml) and detergent-soluble cell lysates were prepared. Phosphotyrosyl proteins were then isolated by adsorption to 1G2-Sepharose in the presence or absence of the competing hapten, phenyl phosphate. Immunoprecipitates were washed and assayed for *in vitro* kinase activity.

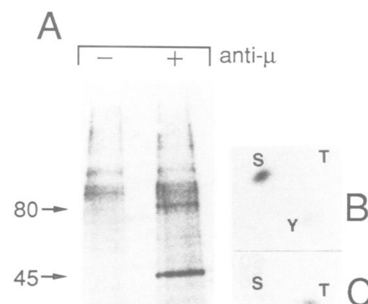

Fig. 5. B lymphocyte phosphotyrosyl proteins can be isolated from cells labeled *in vivo* with ^{32}P. (**A**) WEHI 231 cells were labeled *in vivo* with ^{32}P and treated for 10 min at 37°C with medium or 10 μg/ml goat anti-μ. Phosphotyrosine-containing proteins were isolated by affinity chromatography on anti-phosphotyrosine Sepharose, analyzed by electrophoresis on a 15% SDS−polyacrylamide gel (reducing conditions) (lysate equivalent to 4×10^6 cells per lane) and visualized by fluorography. Exposure time was 10 h. (**B**) Phosphoamino acid analysis by 2-dimensional electrophoresis of the ^{32}P-labeled, 80 kd band from *in vivo* labeled, anti-μ-stimulated WEHI 231 cells. Positions of non-radioactive phosphoamino acid standards are indicated. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. Exposure time was 4 days. (**C**) Phosphoamino acid analysis of the ^{32}P-labeled, 45 kd band from *in vivo* labeled, anti-μ-stimulated WEHI 231 cells. Positions of non-radioactive phosphoamino acid standards are indicated. S, phosphoserine, T, phosphothreonine; Y, phosphotyrosine. Exposure time was 4 days.

A single major band of ~80 kd was phosphorylated *in vitro* in the phosphotyrosine-enriched fraction derived from anti-μ-activated B cells (Figure 6A). This band comigrated on polyacrylamide gel electrophoresis with the 80 kd, phosphotyrosine-containing molecule isolated on anti-phosphotyrosine Sepharose from *in vivo* ^{32}P-labeled, anti-μ stimulated WEHI 231 cells (data not shown).

The binding of kinase activity to anti-phosphotyrosine Sepharose was inhibited in the presence of the phosphotyrosine analog, phenyl phosphate (ϕP) (Figure 6A). Significant kinase activity was not recovered from unstimulated cells (Figure 6A). Partial proteolytic mapping of the 80 kd band using *Staphylococcus aureus* V8 protease suggested that this molecule is phosphorylated *in vitro* at the same sites when isolated from splenic B cells and WEHI 231 cells (data not shown). Phosphoamino acid analysis of the *in vitro* phosphorylated 80 kd band (Figure 6B) showed that tyrosine is the major phosphoamino acid present in this polypeptide from both WEHI 231 and splenic B cells.

Discussion

The data presented in this paper provide the first direct evidence that activation of murine B lymphocytes through their antigen receptor, membrane Ig, is accompanied by an increase in protein tyrosine phosphorylation *in vivo*.

Three lines of evidence support the hypothesis that the protein tyrosine phosphorylation stimulated by treatment with anti-Ig antibodies is directly coupled to transmembrane

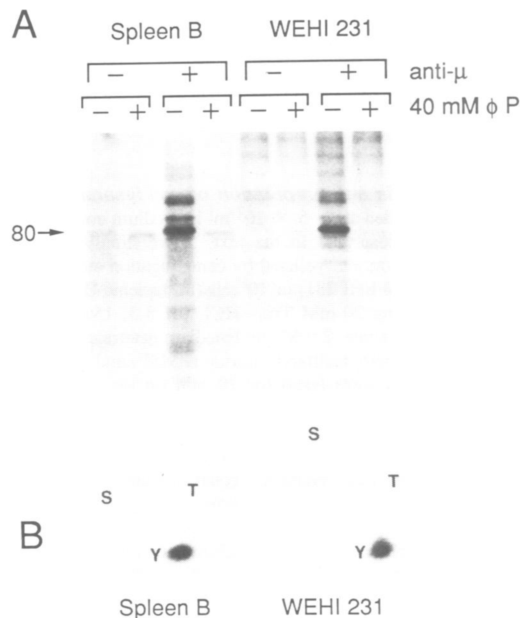

Fig. 6. Protein tyrosine kinase activity can be isolated from anti-μ-stimulated B lymphocytes by affinity chromatography with antibodies to phosphotyrosine. (**A**) Phosphotyrosyl proteins were isolated from unstimulated and anti-μ-treated (10 μg/ml for 15 min on ice) splenic B (lysate equivalent to 2.5×10^6 cells per lane) and WEHI 231 cells (lysate equivalent to 5×10^5 cells per lane) in the presence or absence of competing hapten, 40 mM phenyl phosphate (ϕP), and assayed for *in vitro* kinase activity. Incorporation of [γ^{32}P]ATP *in vitro* into phosphotyrosyl proteins was analyzed by electrophoresis on a 15% SDS–polyacrylamide gel (reducing conditions) followed by fluorography. Exposure time was 40 min. (**B**) Phosphoamino acid analysis of the 80 kd phosphoprotein phosphorylated *in vitro* in anti-phosphotyrosine isolates from anti-μ-stimulated B lymphocytes. Positions of non-radioactive phosphoamino acid standards are indicated: S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. Exposure time was 15 h.

signaling through membrane Ig, rather than occurring secondarily to signal transduction. First, the response is very rapid, being detectable within 30 s of stimulation. Secondly, agents that activate B cells through receptors other than membrane Ig, such as IL-4 and LPS, were found to have minimal effects on protein tyrosine phosphorylation. Finally, anti-Ig antibodies stimulated protein tyrosine phosphorylation in both mature, resting splenic B lymphocytes, which are induced to proliferate, and in a B cell line, WEHI 231, whose growth is profoundly inhibited by anti-Ig treatment. These results suggest that the increases in protein tyrosine phosphorylation in molecules of 80 and 76 kd observed in both cell types are directly associated with transmembrane signaling through membrane Ig rather than secondary phenomena linked to the final response of the cells.

When anti-phosphotyrosine Western blots of anti-Ig-stimulated splenic B and WEHI 231 cells were compared, a number of differences in the pattern and intensity of staining were observed. The 63 kd band was stained more intensely in WEHI 231 cells, a 45 kd band was only observed in WEHI 231 cells stimulated at 37°C and the 32 kd molecule present in splenic B cells was either absent or present in a modified form in WEHI 231 cells. These subtle differences in anti-Ig-stimulated protein tyrosine phosphorylation may reflect the distinctly different end responses seen when WEHI 231 and splenic B cells are stimulated with anti-Ig antibodies, this is, growth inhibition and growth stimulation, respectively.

B lymphocyte membrane Ig is non-covalently associated with a series of membrane glycoproteins. A 38–40 kd Igβ chain forms a covalently linked heterodimer with the 34 kd *mb-1* gene product, Ig$M\alpha$, in IgM expressing cells (Hombach *et al.*, 1988, Hombach *et al.*, 1990) and with a 35–36 kd molecule, Ig$D\alpha$, in IgD-expressing cells (Wienands *et al.*, 1990). These molecules appear to interact with membrane Ig to form an antigen receptor complex in a manner analogous to the association between the T cell receptor and the CD3 complex (Wienands *et al.*, 1990). The relationship between these membrane Ig-associated molecules, the 29 kd molecule that is constitutively tyrosine-phosphorylated in WEHI 231 cells and the 32–35 kd molecules that are phosphorylated on tyrosine in response to anti-Ig stimulation in both splenic B and WEHI 231 cells remains to be determined. Interestingly, Campbell and Cambier (1990) recently found that components of the membrane Ig receptor complex could be immunoprecipitated by anti-phosphotyrosine antibodies from B cells treated with aluminium fluoride.

Some clues to the identity of the other B lymphocyte phosphotyrosyl proteins noted here are suggested by studies of protein tyrosine phosphorylation in other cell types. In fibroblasts, treatment with epidermal growth factor stimulates the tyrosine phosphorylation of a cytosolic, 42 kd molecule, pp42 (Nakamura *et al.*, 1983). A recent study suggests that pp42 is the same as a serine/threonine kinase originally identified in insulin-stimulated 3T3-L1 cells (Ray and Sturgill, 1987) and known as microtubule-associated protein-2 kinase (MAP-2 kinase) (Rossomando *et al.*, 1989). MAP-2 kinase is phosphorylated at both tyrosine and threonine residues (Ray and Sturgill, 1988). The possibility that the 45 kd phosphotyrosine- and phosphothreonine-containing protein identified in anti-Ig-stimulated WEHI 231 cells is MAP-2 kinase is presently under investigation.

One or more of the tyrosine-phosphorylated proteins could be a tyrosine protein kinase, as enzymes of this class are phosphorylated at tyrosine residues (Hunter and Cooper, 1986). A number of *src*-related protein tyrosine kinases are expressed in B lymphocytes including $p56^{lck}$ (Marth *et al.*, 1985; Voronova and Sefton, 1986) and the *blk* (Dymecki *et al.*, 1990), *lyn* (Yamanashi *et al.*, 1989), *hck* (Quintrell *et al.*, 1987, Ziegler *et al.*, 1987) and *fyn* (Semba *et al.*, 1986) gene products. The 63 kd B lymphocyte phosphotyrosyl protein seen in both WEHI 231 and splenic B cells does not comigrate with $p56^{lck}$ (data not shown). It is possible, however, that this 63 kd phosphotyrosyl protein is related to another *src* family gene product. The 63 kd molecule is not recognized by 1G2, so its isolation will present some difficulties.

A preliminary attempt has been made to identify kinases responsible for anti-Ig-stimulated protein tyrosine phosphorylation. Membrane IgM, whilst not itself a protein tyrosine kinase, may associate with a protein tyrosine kinase in B lymphocytes in a manner similar to that described for the interaction between CD4 and $p56^{lck}$ in T lymphocytes (Rudd *et al.*, 1988). Using conditions that allow co-isolation of CD4 and $p56^{lck}$ (Rudd *et al.*, 1988), there is no protein tyrosine kinase activity associated with membrane Ig (data not shown).

Phosphotyrosyl proteins were isolated from anti-Ig-

activated splenic B cells and WEHI 231 cells by affinity chromatography on immobilized anti-phosphotyrosine and were assayed for kinase activity *in vitro*. Tyrosine kinase activity was isolated from both cell types after anti-Ig stimulation. Only one major band of 80 kd was phosphorylated in these preparations. This 80 kd molecule could be one of the tyrosine kinases responsible for the increased protein tyrosine phosphorylation observed in response to anti-Ig, becoming labeled *in vitro* by autophosphorylation. A 75 kd protein tyrosine kinase has been isolated from rat liver cytosol by Wong and Goldberg (Wong and Goldberg, 1983, 1984). We do not know whether the 80 kd B lymphocyte tyrosyl phosphoprotein identified in the current study is similar to this molecule. We cannot exclude however, that the 80 kd molecule is merely a substrate for an activated protein tyrosine kinase which is not itself labeled *in vitro*. An 80–85 kd tyrosyl phosphoprotein is present in anti-phosphotyrosine antibody immunoprecipitates from fibroblasts stimulated with platelet derived growth factor or transformed by polyoma middle T antigen (Kaplan *et al.*, 1987). These immunoprecipitates also contain a phosphatidylinositol 3-kinase activity (Kaplan *et al.*, 1987, Whitman *et al.*, 1987, 1988) which copurifies with the 80–85 kd phosphoprotein (Kaplan *et al.*, 1987). The possibility that the 80 kd tyrosyl phosphoprotein identified in anti-Ig-stimulated B lymphocytes is related to the fibroblast phosphatidylinositol 3-kinase is currently under investigation.

In conclusion, activation of B lymphocytes through membrane IgM stimulates phosphorylation of a number of molecules at tyrosine residues, and this increased tyrosine phosphorylation appears to be directly linked to signal transduction through membrane Ig. Preliminary experiments have suggested that one protein tyrosine kinase responsible for this phosphorylation may be an 80 kd molecule that is itself phosphorylated at tyrosine residues in response to anti-Ig stimulation.

Materials and methods

Reagents

Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Cellgro, Mediatech) supplemented with 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, non-essential amino acids (Gibco), 1 mM sodium pyruvate and heat-inactivated fetal bovine serum (FBS) (Hyclone). The batch of serum used for B cell experiments was pre-screened for its ability to support B cell activation *in vitro*. Affinity-purified goat polyclonal antiserum specific for mouse μ chain (goat anti-μ), LPS (from *Escherichia coli* O55:B5) and phenyl phosphate were obtained from Sigma. Recombinant IL-4 (Genzyme) was used at a final concentration of 100 U/ml.

Cell preparation

Spleen cells were obtained from female BALB/c mice at 4–8 weeks of age (bred at the Salk Institute from stock originally obtained from NIH). Spleen cell suspensions were depleted of T cells by incubation with a monoclonal rat anti-mouse Thy-1 antibody, NIMR-1 (Chayen and Parkhouse, 1982) (a generous gift from Dr R.M.E.Parkhouse, NIMR, London) and guinea pig complement (Gibco). Erythrocytes were lysed with 0.83% (w/v) ammonium chloride. Splenic B cells were layered onto four-step discontinuous Percoll (Pharmacia) gradients, densities 1.064, 1.076, 1.082 and 1.094 g/ml and centrifuged for 30 min at 485 *g*. Small B cells of density $\geq$ 1.082 g/ml were recovered from the gradients. These cells did not express Thy-1, CD4 or CD8, and 80–90% were positive for membrane Ig and Ia antigens as determined by immunofluorescence. The small B cells were at least 95% viable by trypan blue exclusion.

The murine B lymphoma line WEHI 231 (Boyd and Schrader, 1981) was obtained from the ATCC and maintained in medium containing 10% FBS.

Uptake of tritiated thymidine

Small B lymphocytes were cultured in quadruplicate at 5×10^5/ml for 72 h in 200 μl of medium containing 10% FBS. Goat anti-μ, LPS and IL-4 were added as described in the text. During the last four hours of culture, cells were pulse-labeled with 0.5 μCi methyl-[^{3}H]thymidine (NEN, 6.7 Ci/mmol). Incorporation of [^{3}H]thymidine was measured by liquid scintillation counting.

Stimulation of cells and preparation of cell lysates

Cells were resuspended at $1-5 \times 10^7$/ml in medium containing 1% FBS and stimulated as described in the text. After stimulation, cells were immediately chilled on ice, pelleted by centrifugation and resuspended at 2.5×10^7cells/ml (WEHI 231) or 10^8cells/ml (splenic B cells) in ice-cold lysis buffer containing 20 mM Tris–HCl, pH 8.3, 150 mM NaCl, 200 μM sodium orthovanadate, 2 mM ethylenediaminetetraacetic acid, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 3% (v/v) Nonidet P-40 (NP-40). Cells were lyzed for 20 min on ice, nuclei pelleted by centrifugation at 21 000 *g* for 30 min and detergent-soluble lysates collected.

Western blotting

Detergent-soluble cell lysates containing equal amounts of protein were mixed with an equal volume of 2 × electrophoresis sample buffer, resolved by PAGE in the presence of SDS [15% (w/v) gels, reducing conditions] and transferred electrophoretically to Immobilon (Millipore) membranes for 60 min at 60 V. Membranes were stained with an affinity-purified polyclonal rabbit anti-phosphotyrosine antiserum followed by [^{125}I]protein A (NEX-146L, NEN) as described (Kamps and Sefton, 1988). Blots were exposed to preflashed Kodak XAR film plus intensifying screens.

In vivo labeling

WEHI 231 cells were washed in Tris-buffered saline and resuspended at 5×10^6/ml in phosphate-free DMEM containing 10% (v/v) dialyzed FBS and 1mCi/ml $^{32}P_i$ (carrier free) (ICN). After 6 h incubation at 37°C, cells were either stimulated for 10 min with 10 μg/ml goat anti-μ or left untreated. Cells were then lysed as described above.

Affinity purification of phosphotyrosyl proteins

The monoclonal anti-phosphotyrosine antibody, 1G2 (Huhn *et al.*, 1987), was purified from ascites by ammonium sulfate precipitation followed by affinity purification on phosphotyramine-Sepharose as described (Frackelton *et al.*, 1983). Affinity purified antibody was coupled to Sepharose at 10 mg/ml. For isolation of WEHI 231 phosphotyrosyl proteins, lysate from 2.5×10^7 WEHI 231 cells was passed through a 100 μl column of 1G2-Sepharose. Unbound material was removed by washing with 1 ml of lysis buffer followed by 1 ml of elution buffer containing 3.3 mM Tris–HCl, pH 7.5, 50 mM NaCl, 200 μM sodium orthovanadate and 1% (v/v) NP-40. Phosphotyrosine-containing proteins were eluted with 40 mM phenyl phosphate in elution buffer, analyzed by PAGE (reducing conditions) and visualized by fluorography.

For phosphoamino acid analysis, gels were electrophoretically transferred to Immobilon, and the phosphoamino acid content of individual bands was determined as described (Kamps and Sefton, 1989).

In vitro kinase assay

Detergent-soluble cell lysates containing similar amounts of total cell protein (2×10^6 cell equivalents of WEHI 231 or 1×10^7 cell equivalents of splenic B cells) were incubated with 5 μl 1G2-Sepharose at 4°C for 1 h with continuous rotation. The Sepharose beads were then washed three times with lysis buffer, once with buffer containing 50 mM Tris–HCl, pH 7.2 and 150 mM NaCl, and resuspended in 40 mM piperazine-*N*, *N'*-bis[2-ethanesulfonic acid] (PIPES) buffer, pH 7.0, containing 10 mM $MnCl_2$, (Hurley *et al.*, 1989). Samples were incubated at 30°C for 10 min with 5 μCi [γ^{32}P]ATP (4500 Ci/mmol, ICN) and 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 0.1% (v/v) NP-40. The samples were microfuged, and the beads resuspended in electrophoresis sample buffer for resolution on 15% (v/v) polyacrylamide gels (reducing conditions) followed by exposure for fluorography.

Acknowledgements

We are grateful to Tony Hunter, Tamara Hurley, Kunxin Luo and Pam Reynolds for critically reading the manuscript. M.A.C. is supported by an EMBO longterm fellowship (ALTF 262-1988). This work was supported by US Public Health Service Grants CA-14195 and CA-17289.

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Received on February, 13, 1990; revised on March 29, 1990.

Note added in proof

Gold,M.R., Law,D.A. and DeFranco,A.L. have also found that stimulation of B lymphocytes with anti-immunoglobulin antibodies induces protein tyrosine phosphorylation (*Nature*, in press).