Intracellular Localization of Tyrosine Kinase Substrates beneath Crosslinked Surface Immunoglobulins in B Cells

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Summary

Crosslinking of surface immunoglobulins (sIg) in B cells led to the accumulation of submembranal phosphotyrosine, which was followed morphologically with the PY20 antiphosphotyrosine monoclonal antibody. Phosphotyrosine was not detected before sIg crosslinking. After sIg crosslinking, phosphotyrosine-containing proteins were redistributed from scattered small clusters near the plasma membrane to a juxtanuclear region, where immunofluorescent staining decreased with time. Double immunofluorescent staining of individual cells showed accumulation of phosphotyrosine beneath crosslinked sIg molecules at the cell surface. The sIg molecules were subsequently internalized more rapidly than the phosphotyrosine-containing molecules were redistributed. Genistein, a protein tyrosine kinase (PTK) inhibitor, blocked intracellular tyrosine phosphorylations but not cell surface patching of crosslinked sIg. When polyacrylamide beads coated with anti-Ig antibodies were added to the cells, intracellular tyrosine phosphorylation occurred beneath the regions of contact with the beads. This study provides an independent line of evidence confirming recent biochemical experiments that show that crosslinking of the antigen receptor induces PTK activity in B cells, and that components of the newly described sIg complex are among the PTK substrates. The surprising finding that the bulk of the induced phosphotyrosine remains associated with crosslinked sIg for many minutes suggests a role for complex local protein interactions in phosphotyrosine-mediated signal transduction through the antigen receptor of B cells.

Crosslinking of surface immunoglobulin (sIg),[1] the B cell antigen receptor, initiates a complex cellular response. Upon crosslinking, most sIg becomes physically associated with detergent insoluble cytoskeletal components (1, 2), followed by patching, capping, and endocytosis (3). Crosslinking of sIg activates phospholipase C (PLC)-mediated hydrolysis of phosphatidyl inositol to inositol triphosphate and diacylglycerol, which lead, respectively, to increased free intracellular Ca^{2+} and activation of Ca^{2+}-regulated kinases, and to protein kinase C (PKC) activation (4–6). GTP-binding protein(s) appear to couple sIg receptors to inositol phospholipid hydrolysis (7–10). sIg associates noncovalently with one isotype-specific 32–33-kD protein and two non–isotype-specific proteins of 34 and 37–38 kD (11, 12). These proteins may be involved in coupling sIg to GTP-binding proteins (in a manner analogous to the T cell CD3 signal transducer complex) by forming a sIg receptor complex, since sIg molecules lack a cytoplasmic tail like that shared by receptors that interact directly with GTP-binding proteins.

Crosslinking of sIg also leads to very rapid tyrosine phosphorylation by protein tyrosine kinases (PTKs) of several cytoplasmic and membrane proteins (13–15). Although protein substrates are assumed to be modified functionally by the phosphorylation of tyrosine residues, the identities and functions of those substrates are not fully described. In addition, the membrane-associated protein tyrosine phosphatase (PTPase) CD45 (16) regulates signal transduction in B cells. Antibodies to CD45 inhibit an early phase in B cell activation initiated by anti-Ig antibodies and characterized by an initial rise in intracellular Ca^{2+} concentration (17), and phosphatidyl inositol breakdown (18). Thus, protein phosphorylations and dephosphorylations on tyrosine are likely to regulate signal transduction initiated by sIg crosslinking.

To study the role of protein tyrosine phosphorylation in antigen receptor signal transduction in B cells, we have characterized by immunofluorescence the appearance and subcellular location, redistribution, and disappearance of tyrosine

[1] *Abbreviations used in this paper:* PKC, protein kinase C; PLC, phospholipase C; PTK, protein tyrosine kinase; PTPase, protein tyrosine phosphatase; sIg, surface immunoglobulin(s); TRITC, tetramethyl rhodamine isothiocyanate.

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phosphorylated proteins in B cells after crosslinking sIg. Tyrosine phosphorylated proteins accumulate directly under crosslinked sIg, so it appears that the major substrates for the induced PTK activity remain associated with crosslinked sIg.

Materials and Methods

Cells and Culture Conditions. Akata cells (19) and Ramos cells (20) were grown in RPMI 1640 supplemented with 10% heat-inactivated FCS at 37°C in a humidified atmosphere of 5% CO_2 in air. Peripheral blood B cells were isolated from a healthy adult as described (21). For sIg crosslinking, cells were suspended in fresh medium at 10^6 cells/ml, and 50 μg/ml $F(ab')_2$ fragments of Ig class-specific goat anti-human IgG or IgM antibodies (Cappel Laboratories, West Chester, PA) dialyzed against PBS were added for indicated times at 37°C. Alternately, 50 μg/ml rabbit anti-human Ig Immunobeads (Bio-Rad Laboratories, Richmond, CA) were used.

Immunofluorescence Procedures. Cells were washed with PBS, spotted onto a glass slide, dried, and fixed and permeabilized with methanol/acetone (1:1) at −20°C for 10 min. Cells were stained with a 1:100 dilution of PY20 mAb (ICN ImmunoBiologicals, Costa Mesa, CA) (22) specific for phosphobenzene derivatives such as phosphotyrosine and phenylphosphate, washed, and incubated with FITC-conjugated goat anti-mouse Ig antibodies (Cappel Laboratories) or with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated donkey anti-mouse Ig antibodies (Jackson Immunoresearch, West Grove, PA). For double-label immunofluorescence, FITC-conjugated $F(ab')_2$ fragments of goat anti-human Ig antibodies were used for stimulation of cells. After fixation, the slides were stained with PY20 mAb and then with TRITC-conjugated donkey anti-mouse Ig antibodies. Double-label immunofluorescence was also used for staining of cells cultured with Immunobeads. Cells were stained with PY20 mAb and then with FITC-conjugated donkey anti-rabbit Ig antibodies (Jackson Immunoresearch) and TRITC-conjugated donkey anti-mouse Ig antibodies. Stained slides were mounted in 50% glycerol in PBS and viewed under a standard microscope equipped for phase contrast and fluorescence microscopy with a Planapo 63× objective lens (Carl Zeiss, Inc., Thornwood, NY). The filter sets discriminated completely the fluorescence from the two fluorochromes. At least 250 cells at each time point were counted for classification. Photographs were taken on Kodak Tri-X film.

Other Reagents. O-phospho-L-tyrosine, calcium ionophore A23-187, dioctanoylglycerol, TRITC-conjugated wheat germ agglutinin, TRITC-conjugated Con A, and DMSO were obtained from Sigma Chemical Co., St. Louis, MO. Genistein was from ICN Immuno-Biologicals. Sodium orthovanadate was from Fisher Scientific Co., Pittsburgh, PA.

Results

Detection of Tyrosine Phosphorylation by Immunofluorescent Labeling. sIg molecules of Akata or Ramos cells were crosslinked with $F(ab')_2$ fragments of goat anti-human IgG or IgM antibodies, respectively. The prominent phosphotyrosine labeling of both cells was observed as clusters (Fig. 1, *A* and *B*). Since PY20 mAb did not stain living, intact cells, the immunofluorescent staining of phosphotyrosine was intracellular, consistent with the known location of PTKs at the inner surface of the plasma membrane or in the cytoplasm (23). The reaction of the PY20 antiphosphotyrosine mAb

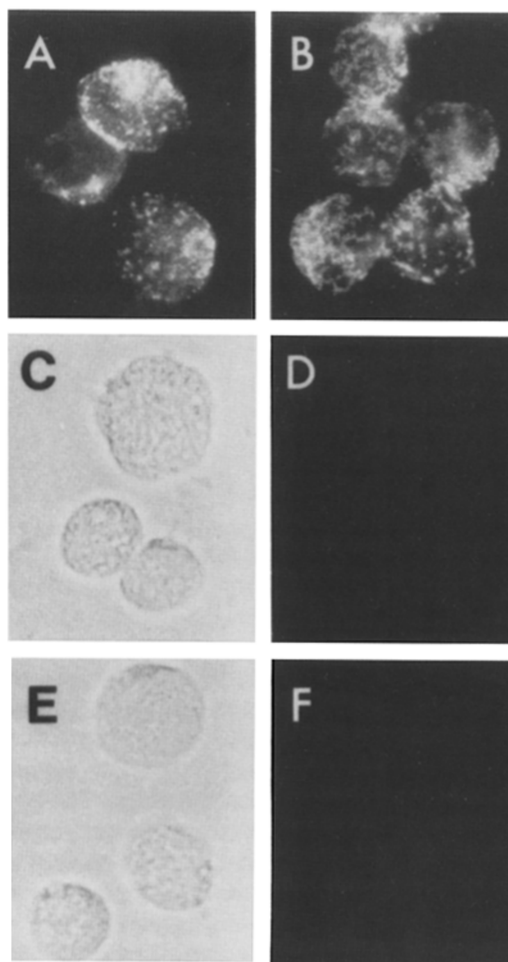

Figure 1. Phosphotyrosine staining. Cells were incubated with $F(ab')_2$ fragments of goat anti-human Ig antibodies, fixed with acetone/methanol (1:1), and stained with PY20 antiphosphotyrosine mAb followed by FITC-conjugated goat anti-mouse Ig antibodies. Fluorescent patches were observed in both Akata cells (*A*) and Ramos cells (*B*) 5 min after treatment with anti-Ig antibodies. No staining was seen with PY20 mAb blocked with phosphotyrosine (10 mM) (*D*, Akata cells). Unactivated cells were not stained (*F*, Akata cells). Phase contrast micrographs of the fields of *D* and *F* were shown in *C* and *E*, respectively (×900).

was inhibited completely by phosphotyrosine (10 mM) (Fig. 1, *C* and *D*). No immunofluorescence was seen in unactivated cells (Fig. 1, *E* and *F*). When cells were treated with the diacylglycerol analog, dioctanoylglycerol, an agonist for PKC, and A23187, a calcium ionophore, no PY20-positive cells were seen (data not shown).

Redistribution of Phosphotyrosine. To study the induction of protein tyrosine phosphorylation and how the proteins were redistributed and dephosphorylated, Akata cells were stained with PY20 mAb at various times after stimulation with anti-Ig antibodies (Fig. 2). Almost all cells were stained with PY20 mAb at 2 min. Maximal induction of PY20-positive cells was constant from 2 to 30 min and then the percentage of positive cells decreased. The frequencies of three types of immunofluorescent patterns were classified as a func-

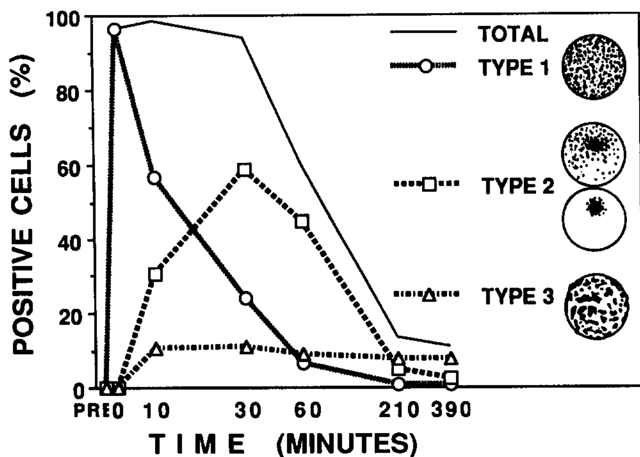

Figure 2. Time course of tyrosine phosphorylation. At indicated times of incubation with anti-Ig antibodies, Akata cells were labeled with PY20 mAb as described for Fig. 1 and classified by the illustrated criteria. Type 1, scattered dots without concentration; Type 2, accumulation of dots in a juxtanuclear region; Type 3, large patch formation without juxtanuclear accumulation. Cells of types 1, 2, and 3 are shown in Fig. 3 *A*, *a*, *c*, and *d*, respectively.

tion of time (Fig. 2). Type 1 cells had fine, punctate patches widely distributed near the cell surfaces as judged by focusing through different layers of the cell (Fig. 3 *A*, *a*). Type 2 cells showed accumulation of PY20-labeled molecules in one or more regions (Fig. 3 *A*, *c*), which were juxtanuclear, as judged by double staining with TRITC-conjugated wheat germ agglutinin, which reacted with glycoconjugates unique to the Golgi apparatus (24) (Fig. 3 *B*). In the first 30 min, type 1 cells decreased as the type 2 cells increased. The percentage of type 2 cells peaked at 30 min and then declined. Type 3 cells had large patches near the cell surface and lacked accumulations in juxtanuclear regions (Fig. 3 *A*, *d*). Their percentage remained constant during the entire time of observation.

Double Staining for sIg and Phosphotyrosine Molecules. To test how sIg and tyrosine phosphorylated proteins were associated, double staining for sIg and phosphotyrosine molecules was undertaken. Akata and Ramos cells were stimulated with FITC-conjugated $F(ab')_2$ fragments of goat anti–human Ig antibodies, fixed, and stained with PY20 mAb followed by TRITC-conjugated donkey anti–mouse Ig antibodies (Fig. 3 *A*). The locations of tyrosine phosphorylated molecules and sIg molecules overlapped closely in the majority of Akata cells (Fig. 3 *A*, *a*, *b*, *c*, and *d*) and Ramos cells (Fig. 3 *A*, *f*). PY20-labeled dots appeared exactly with the cell surface distribution of sIg. When the primary PY20 mAb was omitted from the staining procedure, no TRITC fluorescence was detected (data not shown), indicating that the secondary anti–mouse Ig antibodies do not crossreact with human B cell sIg nor with goat anti–human Ig antibodies. Furthermore, phosphotyrosine (10 mM) completely abolished staining with PY20 mAb. Therefore, both antigens were actually recognized and the apparent colocalization could not be owing to an artifact of the fluorescent antibody procedure. During microscopy, sharply focusing for one fluorochrome near the top of the cell rendered the alternate label slightly out of focus, presumably since extracellular anti-Ig antibodies were not exactly in the same plane of focus as the intracellular phosphotyrosine label.

Although sIg and PY20-positive dots were associated at the cell surface, intracellular migration of PY20-stained dots appeared to differ from that of sIg in some cells (Fig. 3 *A*, *e*). To test whether dephosphorylation during internalization caused this difference, vanadate (1.0 mM), an inhibitor of PTPase (25), was added. Vanadate inhibited fading of PY20 labeling: 89.7% of cells were still positive for PY20 after 1 h incubation with anti-Ig antibodies and vanadate. In addition, after incubation with vanadate in both control and anti-Ig cultures, a small number (<5%) of diffusely PY20-stained cells appeared, presumably due to effective inhibition of PTPase activity. This treatment did not alter the distributions of either sIg or PY20-stained molecules up to 30 min. The redistribution of both sIg and PY20 mAb–recognized molecules was determined in individual cells as a function of time after sIg stimulation (Fig. 4). Cells cultured with vanadate were doubly stained. Patterns of staining of cells of types 1 and 2 were subdivided as shown in Fig. 4 to reflect apparent stages of endocytosis and redistribution from the cell surface to the juxtanuclear region. At every time point, we found that redistribution of sIg label in individual cells was more advanced than that of phosphotyrosine label. For example, in the cells shown in Fig. 3 *A*, *e*, sIg was classified as type 2b while PY20 labeling was type 1 or 2a. Since this difference in the staining patterns was not affected by vanadate, it is not likely to be owing to dephosphorylation of phosphoproteins during redistribution to the juxtanuclear region. We conclude that crosslinked sIg is internalized more rapidly than the bulk of the sIg-associated, tyrosine phosphorylated proteins. sIg and phosphotyrosine were colocalized at the cell surface in all cells.

Type 3 cells (Figs. 2 and 3 *A*, *d*), in which sIg remained on the cell surface in patches, also did not redistribute PY20-stained dots during the observation period. We then determined how vanadate affected PY20 staining on these cells (Table 1). After 1 h of stimulation in the presence of vanadate, 10% of cells were diffusely positive for PY20. The majority (80%) of type 3 cells had varying degrees of diffuse PY20 staining in addition to the PY20 staining, which was congruent to the sIg patches. In contrast, only 3% of type 1 and 2 cells showed diffuse staining.

We found that crosslinking of sIg also induces tyrosine phosphorylation beneath crosslinked sIg receptors in normal B cells. PY20 labeling was observed with patched or capped sIgM or sIgG molecules (Fig. 5), but not without stimulation with anti-Ig antibodies (data not shown). This finding is consistent with the previous studies (13–15), which showed that in both normal B cells and B cell lines crosslinking sIg stimulates phosphorylation of protein tyrosine.

Inhibition of Tyrosine Phosphorylation by Genistein. Genistein, an inhibitor of PTK (26), did not inhibit PY20 labeling when added 10 min after anti–human Ig antibodies, but it did block PY20 labeling when added 10 min before anti-Ig antibodies, (Fig. 6, *A* and *B*). The percentage of positive cells did not

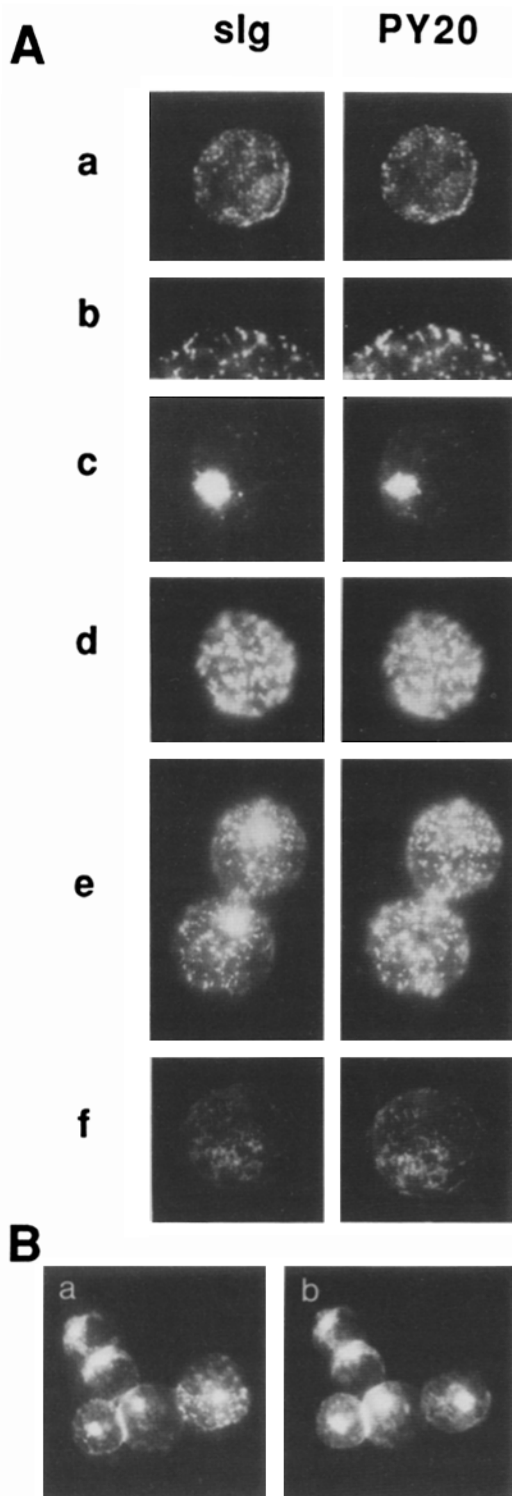

Figure 3. Double staining for sIg and phosphotyrosine molecules (*A*) and for wheat germ agglutinin and phosphotyrosine (*B*). (*A*) Cells were incubated with FITC-conjugated $F(ab')_2$ fragments of goat anti–human Ig antibodies, fixed, and stained with PY20 mAb followed by TRITC-conjugated donkey anti-mouse Ig antibodies. Akata cells were treated with anti-Ig antibodies for 10 min (*a* and *b*) and for 30 min (*c*, *d*, and *e*). Ramos cells treated for 30 min (*f*). PY20 labeling and sIg overlapped at cell surfaces but juxtanuclear accumulation of sIg was not always accompanied by PY20-labeled proteins (*e*). (*a*, *c–f*) ×900; (*b*) ×1,500. (*B*) Akata cells were incubated with anti-IgG for 15 min and fixed. They were then labeled with PY20 mAb followed by FITC-conjugated goat anti-mouse Ig antibodies (*a*) and TRITC-conjugated wheat germ agglutinin (*b*). The PY20-labeled proteins accumulated in the juxtanuclear region which was also stained by wheat germ agglutinin (×500).

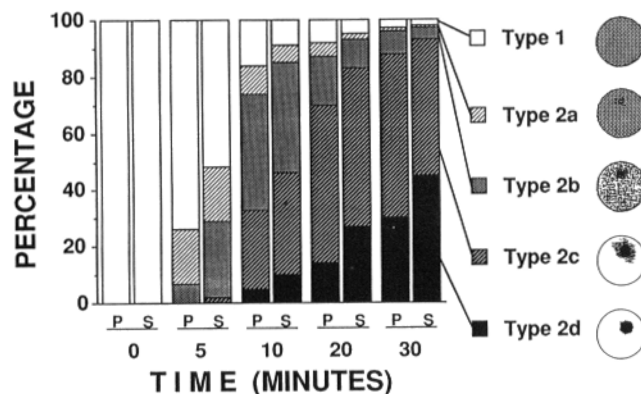

Figure 4. Comparative redistributions in time of sIg and PY20-labeled molecules. Akata cells were incubated with FITC-conjugated $F(ab')_2$ fragments of goat anti–human IgG in the presence of 1 mM vanadate, fixed, and stained as described for Fig. 3. They were examined for sIg (FITC) (marked S) and for PY20 (TRITC) (marked P). Cells were classified according to the illustrated criteria and the percentages of cells of each type were determined. Immunofluorescence was scattered on the cell surface without (type 1) or with a slight concentration (type 2a), or with an apparent concentration (type 2b) of immunofluorescence in the juxtanuclear region. Immunofluorescence was not observed over the whole cell, but was concentrated in the juxtanuclear region with (type 2c) or without (type 2d) cytoplasmic vesicles. Type 3 cells (Fig. 2 and 3 *A*, *d*) were excluded.

Table 1. *Vanadate Induces Diffuse PY20 Staining in Type 3 Akata Cells*

	PY20-positive (percent of total cell number)		
Cell type	Total	Without diffuse staining	With diffuse staining
Type 1, 2	79.7	77.3	2.4
Type 3	10.0	2.0	8.0

Akata cells were incubated for 1 h with FITC-conjugated anti-Ig antibodies and sodium vanadate (1.0 mM). Cells were fixed and stained with PY20 mAb followed by TRITC-conjugated donkey anti–mouse antibodies. 1,000 cells were classified as described in Fig. 2. Diffuse PY20 staining in type 3 cells was significantly more frequent than that in type 1 or 2 cells ($p < 0.001$, as determined by the χ^2 test).

decrease at 10 and 30 μg/ml, but decreased markedly at 100 μg/ml. The intensity of immunofluorescence of the positive cells did not change at 10 μg/ml, but was moderately reduced at 30 μg/ml (data not shown). The DMSO that was used to dissolve the genistein had no effect by itself. In doubly labeled cells, which were incubated with FITC-conjugated anti-Ig antibodies in the presence of genistein (100 μg/ml), sIg molecules patched on the cell surface in the absence of any PY20-stained dots (Fig. 6 *B*). We conclude that sIg patch

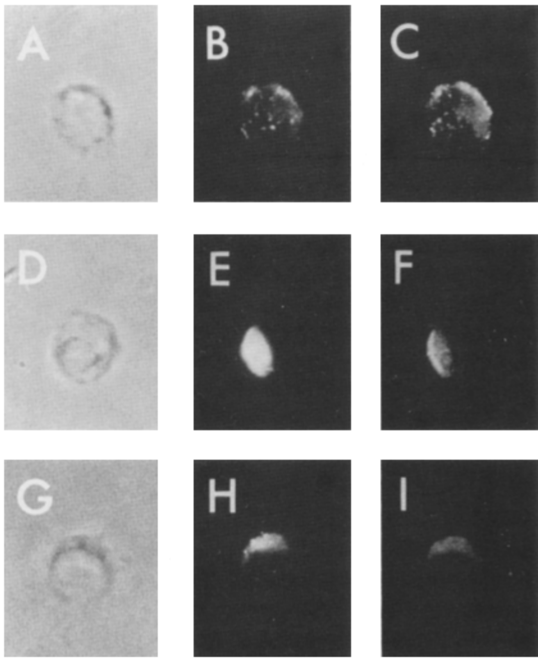

Figure 5. Double staining for sIg and phosphotyrosine molecules in normal B cells. B lymphocytes from peripheral blood were incubated with FITC-conjugated $F(ab')_2$ fragments of goat anti–human IgM or IgG antibodies and stained for phosphotyrosine as described for Fig. 3: sIg labeling (*B*, *E*, and *H*), phosphotyrosine labeling (*C*, *F*, and *I*); and the corresponding phase contrast pictures (*A*, *D*, and *G*). Patching of sIg (*B*) after 5 min of incubation with anti-human IgM antibodies accompanies phosphotyrosine staining (*C*). Capping after 15 min of incubation with anti–human IgM (*E*) or IgG (*H*) antibodies also accompanies coordinate redistribution of phosphotyrosine labeling (*F* or *I*, respectively) (×950).

formation was independent of tyrosine phosphorylation in these cells. However, we did not see internalization of sIg label in the presence of genistein. When genistein-treated, anti-IgG crosslinked cells were washed after 20 min and resuspended in genistein-free medium, full levels of phosphotyrosine formation were found just after genistein removal (data not shown).

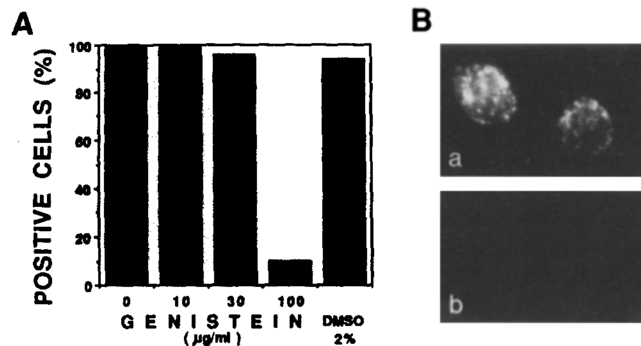

Figure 6. Genistein blocks tyrosine phosphorylation. (*A*) Akata cells were treated with genistein (10, 30, or 100 μg/ml) for 10 min, stimulated with anti–human Ig antibodies for 10 min, and stained with PY20 mAb as described for Fig. 1. (*B*) Double immunofluorescence labeling for sIg and phosphotyrosine molecules after incubation with genistein (100 μg/ml). Patching of sIg (*a*) was not accompanied by phosphotyrosine staining (*b*) (×600).

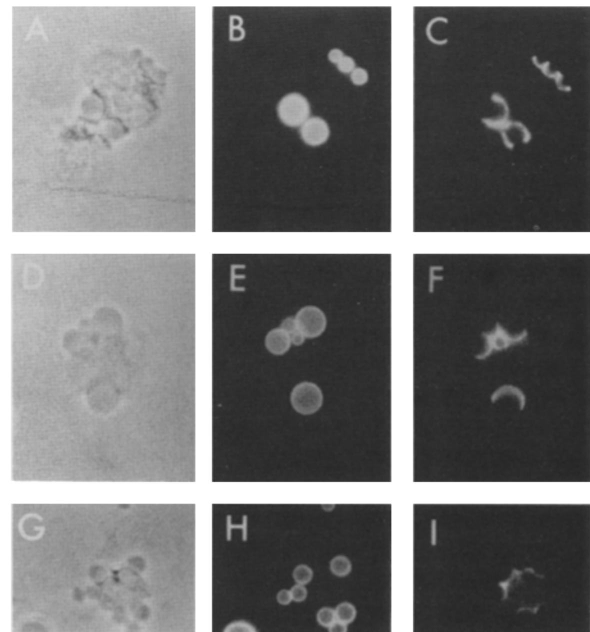

Figure 7. PY20 mAb staining of cells cultured with polyacrylamide beads bearing anti-Ig antibodies. Akata cells (*A*, *B*, and *C*), and Ramos cells (*D*, *E*, and *F*), and peripheral blood B cells (*G*, *H*, and *I*) were stimulated with beads for 30 min and stained as described for Fig. 3. Phosphotyrosine was localized to the cell membrane attached to beads (*C*, *F*, and *I*). Phase contrast micrographs (*A*, *D*, and *G*), and staining with FITC-conjugated donkey anti–rabbit Ig antibodies (*B*, *E*, and *H*) of the same fields. Phosphotyrosine appeared where the cells were bound to beads (×700).

Tyrosine Phosphorylation of Cells Stimulated by Anti-Ig Bound to Polyacrylamide Beads. To examine further the spatial rela-

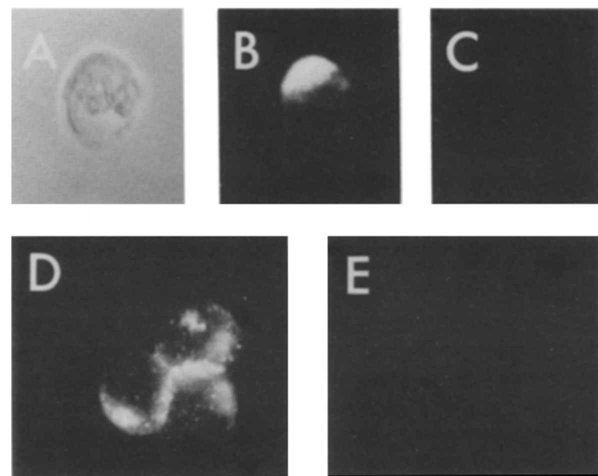

Figure 8. Phosphotyrosine staining of cells with crosslinked Con A receptors. Peripheral blood B cells (*A*, *B*, and *C*) or Akata cells (*D* and *E*) were incubated with TRITC-conjugated Con A (30 μg/ml) for 30 min at 37°C, fixed, and stained for phosphotyrosine as described Fig. 3. Tyrosine phosphorylation (*C* and *E*) is not observed beneath patched or capped Con A receptors (*B* and *D*, respectively). (*A*) the phase contrast micrograph of the same cell in *B* and *C*. (*A*, *B*, and *C*) ×1,200; (*D* and *E*) ×750.

tionship between sIg and phosphotyrosine-containing molecules, anti-Ig bound to polyacrylamide beads was used to stimulate Akata, Ramos, and peripheral blood B cells. PY20 mAb labeling was observed only in the cellular regions adhering to the beads (Fig. 7). No phosphotyrosine immunofluorescence was seen in the rest of the cytoplasm or the nucleus. Most beads remained on the cell surface even after 18 h in culture, when PY20 labeling was still observed.

Con A Does Not Induce Tyrosine Phosphorylation beneath Crosslinked Receptors. To examine whether tyrosine phosphorylation beneath crosslinked sIg is a general phenomenon that accompanies any ligand-induced surface receptor-cytoskeleton interaction, we examined the distribution of phosphotyrosine during patching and capping of Con A surface glycoprotein receptors (Fig. 8). Incubation of peripheral blood B cells or Akata cells with Con A at 37°C induced patching, capping, and internalization of Con A receptors. In addition, Con A induced marked agglutination of Akata cells and accumulated at their contact area. Phosphotyrosine formation could not be detected in Con A–treated cells at any time during the observation period of 120 min, indicating that tyrosine phosphorylation beneath crosslinked receptors is specifically associated with sIg crosslinking.

Discussion

We have analyzed tyrosine phosphorylation induced by crosslinking sIg in B cells by immunofluorescent staining with an antiphosphotyrosine mAb. The staining was specific for phosphotyrosine (Fig. 1 *D*). Induction of the phosphorylation was inhibited in cells treated with the tyrosine kinase inhibitor, genistein (Fig. 6). Tyrosine phosphorylation was induced rapidly, and the phosphorylated substrates patched and were redistributed in association with the crosslinked sIg. Tyrosine phosphorylation was observed in both normal B cells and neoplastic B cell lines and in both $sIgM^+$ cells and $sIgG^+$ cells, indicating that colocalization of anti-Ig-induced tyrosine phosphorylated proteins with sIg is a general phenomenon that accompanies the crosslinking of sIg molecules.

Rapid protein tyrosine phosphorylation of a number of substrates in sIg-crosslinked, human and murine B cells has been demonstrated by others by immunoblotting of SDS-PAGE-separated proteins with antiphosphotyrosine antibodies or by polyacrylamide gel electrophoresis of ^{32}P-labeled molecules isolated on antiphosphotyrosine Sepharose (13–15). The phosphorylated proteins were found in the cytoplasm and membrane proteins that were solubilized with Triton X-100, and in the Triton X-100-insoluble fraction of cells (13). We do not know exactly which protein substrates for PTK were principally detected in our immunofluorescence assay. While several proteins were reported in immunoblots to be phosphorylated on tyrosine residues without anti-Ig stimulation (13–15), phosphorylation was detected by immunofluorescence only in stimulated cells (Fig. 1 *F*). Thus, not all PTK substrates may be detected by immunofluorescent staining. After incubation with vanadate, a PTPase inhibitor, some cells diffusely stained by PY20 mAb appeared, presumably due to the accumulation of phosphotyrosyl residues. These diffusely distributed, phosphorylated substrates were not lost during fixation, but smaller amounts in a diffuse distribution might have eluded detection by immunofluorescent staining. Alternatively, the presence of vanadate in the cell fractionation procedures used by others may have led to the accumulation of phosphotyrosine above the levels found in unmanipulated cells. In contrast, sIg crosslinking induced PY20-labeled dots that were redistributed in a manner similar to sIg receptors. Therefore, the major substrates detected in this study were, like crosslinked sIg, associated physically with the cytoskeletal components.

In our work, tyrosine phosphorylation was demonstrated initially in regions submembranal to crosslinked sIg. Upon crosslinking of sIg molecules by soluble anti-Ig antibodies, the tyrosine phosphorylated molecules appeared rapidly as numerous tiny dots and then redistributed to the juxtanuclear region in the majority of the Akata cells. Double staining analyses demonstrated that the sIg and tyrosine phosphorylated molecules were closely associated at the cell surface (Fig. 3), but sIg appeared to move more rapidly to the juxtanuclear region than did phosphorylated substrates (Figs. 3 *A*, *e*, and 4). When cells were stimulated by anti-Ig antibodies bound to beads, the tyrosine phosphorylated substrates appeared only near the cell surface beneath the beads where they remained for hours. These results are consistent with the view that activation of PTK takes place where sIg receptors are crosslinked and may reflect a role of PTK as a transducer in the signaling pathway.

Since tyrosine phosphorylation was detected at the sites where sIg formed patches or caps, some PTK substrates colocalize with the sIg. Calmodulin and several cytoskeletal proteins such as actin, myosin, vimentin, α-actinin, and fodrin accumulate beneath sIg patches or caps (reviewed in reference 27). Some of these molecules could be components of a "signal transduction complex" beneath the sIg patches, while others may function in patching and endocytosis of antigen. Whether such cytoskeletal proteins were actually substrates for PTK in our experiments is not clear. In Rous sarcoma virus–transformed chick cells, vinculin, a cytoskeletal protein localized in adhesion plaques, is a substrate for $p60^{v-src}$ PTK (28). In cell-free systems, several cytoskeletal proteins are phosphorylated on tyrosine residues by receptor tyrosine kinases, while none were reported to be tyrosine phosphorylated in intact cell systems (29). CD19, which co-caps with sIg (30), forms a complex with CD21 (31). Both have tyrosine residues in their cytoplasmic domains and are thus candidates for PTK substrates. Anti-Ig also induces redistribution and colocalization of $p21^{ras}$ in B lymphocytes (32). A GTP-binding protein has been implicated in coupling of sIg to inositol phospholipid hydrolysis (7–10), but as yet, no role for $p21^{ras}$ in signal transduction or capping has been established. Tyrosine phosphorylation is implicated in the activation of PLC after receptor tyrosine kinase activation (reviewed in reference 33). Tyrosine phosphorylation is suggested to be an early, essential step in PLC activation in T cells (reviewed in reference 34) and B cells (35). Human B cells have a 146-kD PLC (36), and some substrates for PTK in B cells have a similar molecular mass (13, 14).

Likely candidates for phosphorylated substrates detected by immunofluorescence are PTK itself and potential members of a sIg signal transduction complex. Several PTKs of the *src* gene family are expressed as cytoplasmic membrane-associated proteins in B cells (37, 38). As PTKs are phosphorylated on tyrosine in cells (39), one or more could be detected by immunofluorescent staining, if they coalesce beneath sIg patches by physical association with a member of the sIg signal transfer complex in a manner analogous to the association of CD4/CD8-p56^{lck} complexes with the antigen receptor in T cells (40, 41). This is supported by the recent study (42) that showed that the *lyn* protein and its kinase activity could be coimmunoprecipitated with IgM from B cell lysates. CD4-CD4 crosslinking in T cells induces phosphorylation of three substrates that are also physically associated with CD4-p56^{lck} complex as an oligomer (43). The ζ chain of the TCR-CD3 complex is phosphorylated on tyrosine upon activation (44), and the γ, δ, and ϵ chains of the CD3 complex are tyrosine phosphorylated by the CD4/CD8-p56^{lck} complex in an in vitro assay (41). Similarly, components of the sIg receptor complex could be immunoprecipitated by antiphosphotyrosine antibodies in B cells after GTP-binding protein stimulation (12). It is possible that these B cell elements were also seen by our immunofluorescence assay.

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