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Immunology

The membrane IgM-associated proteins MB-1 and Ig-β are sufficient to promote surface expression of a partially functional B-cell antigen receptor in a nonlymphoid cell line

(membrane immunoglobulin/intracellular trafficking/signal transduction/tyrosine phosphorylation)

LINDA MATSUUCHI*†‡§, MICHAEL R. GOLD¶‖**, ADAM TRAVIS*¶††, RUDOLF GROSSCHEDL*¶††, ANTHONY L. DEFRANCO*¶‖, AND REGIS B. KELLY*†

*Department of Biochemistry and Biophysics, †Hormone Research Institute, ¶Department of Microbiology and Immunology, ‖G.W. Hooper Foundation, and ††Howard Hughes Medical Institute, University of California, San Francisco, CA 94143-0448

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ABSTRACT **The B-cell antigen receptors consist of membrane immunoglobulins (mIgs) noncovalently associated with two accessory proteins, MB-1 and Ig-β. We used transfection into a nonlymphoid cell line to test whether MB-1 and Ig-β were sufficient to promote cell surface expression of mIgM capable of signal transduction. Expression of MB-1 and Ig-β, but not MB-1 alone, allowed high-level surface expression of mIgM in the AtT20 endocrine cell line, which presumably lacks other B-cell-specific components. The reconstituted antigen receptor was capable of mediating some of the signaling reactions characteristic of mIgM in B lymphocytes. Crosslinking mIgM on transfected AtT20 cells stimulated tyrosine phosphorylation of MB-1 and Ig-β and also increased the amount of phosphatidylinositol 3-kinase activity that could be precipitated with anti-phosphotyrosine antibodies. When total cell lysates were analyzed by anti-phosphotyrosine immunoblotting, however, no induced phosphorylation of more abundant proteins was detected. Moreover, crosslinking of the receptor in AtT20 cells did not stimulate inositol phospholipid breakdown. Thus, the transfected B-cell antigen receptor could initiate some signal transduction events but AtT20 cells may lack components required for other signaling events associated with mIgM.**

The antigen receptors on B lymphocytes initiate intracellular signals that regulate B-cell growth and differentiation. Crosslinking of the B-cell antigen receptors stimulates inositol phospholipid hydrolysis (1–3) and protein tyrosine phosphorylation (4–7). The B-cell antigen receptors consist of membrane immunoglobulin (mIg) that is noncovalently associated with at least two accessory proteins. Murine mIgM is associated with the 34- to 36-kDa MB-1 protein [the product of the *mb-1* gene (8–10)], which is disulfide linked to the 37- to 40-kDa Ig-β protein [the product of the *B29* gene (11, 12)]. It is unclear whether there are additional mIg-associated proteins. The mIg-associated proteins could have two roles, facilitating cell surface expression of mIg and coupling mIg to its signal transduction pathways.

When mIgM is expressed in the J558 plasmacytoma cell line, it is retained in the endoplasmic reticulum (ER) unless MB-1 is also expressed (13). J558 cells, however, express Ig-β and may also express other lymphoid-specific proteins required for surface expression of mIgM. To investigate the functions of MB-1 and Ig-β, we expressed mIgM, MB-1, and Ig-β in a nonlymphoid cell line and asked whether these components were sufficient for cell surface expression in the absence of other B-cell-specific components and sufficient to mediate signal transduction events characteristic of mIgM in B cells.

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MATERIALS AND METHODS

Expression Vectors and DNA Transfection. A 2.1-kilobase (kb) membrane form of μ heavy chain (μ_m) cDNA (14) was ligated into the *Hin*dIII site of pRSVpLpA (gift of M. Walter and D. Standring, University of California, San Francisco). A 5.9-kb *Xba* I fragment containing a λ_1 genomic clone (15) was inserted into pRSV *Bam*HI (16). A full-length B29 cDNA was generated by polymerase chain reaction from Bal 17 B-lymphoma cDNA (gift of W. Hempel, University of California, San Francisco) and ligated into the *Hin*dIII–*Xba* I site of pRSVpLpA.

pCMV was made by ligating the 0.68-kb *Hin*dIII–*Sau*3A cytomegalovirus enhancer/promoter element (17) from pRR23 (gift of W. Schaffner) to the 2.5-kb *Sal* I–*Xho* I fragment from pμ (18). pCMVMB-1 was made by ligating an *mb-1* cDNA into the *Xho* I site of pCMV. To generate a construct containing the intact *mb-1* gene, *mb-1* cDNA sequences of pCMVMB-1 from +12 to the poly(A) tail were removed and replaced with a 5.3-kb *Xba* I–*Bam*HI *mb-1* genomic DNA fragment (19, 20).

AtT20 cells were transfected by calcium phosphate precipitation (16, 21) and stable clones were selected in medium containing 0.5 mg of G418 per ml or 0.175 mg of hygromycin per ml. Clones expressing the desired proteins were identified by metabolic labeling and immunoprecipitation or by immunofluorescence (16, 21, 22). AtT20 cells expressing the $5HT_{1c}$ serotonin receptor were produced by transfection with the pDJ201 plasmid (ref. 23; gift of D. Julius, University of California, San Francisco).

Immunofluorescence. Immunofluorescence was performed on fixed cells (22) using fluorescein- or rhodamine-coupled goat anti-mouse μ chain (10 μg/ml; Organon Teknika–Cappel). For staining of intracellular IgM, cells were permeabilized with 0.1% saponin. Live cells stained with fluoresceinated goat anti-mouse μ chain (Jackson ImmunoResearch) were analyzed by flow cytometry on a Becton Dickinson FACScan as described (24).

Cell Labeling and Immunoprecipitation. Cells were surface-iodinated using sulfosuccinimidyl 3-(4-hydroxyphenyl)propionate (Pierce) or metabolically labeled with Trans^{35}S-label (100 μCi/ml, ICN; 1 μCi = 37 kBq) as described (25, 26). Immunoprecipitation of digitonin-lysed cells with anti-MB-1 or anti-μ chain antibodies followed by two-dimensional

Abbreviations: mIg, membrane immunoglobulin; μ_m, membrane form of μ heavy chain; anti-Tyr(*P*), anti-phosphotyrosine; PI 3-kinase, phosphatidylinositol 3-kinase; ER, endoplasmic reticulum; endo H, endoglycosidase H.

‡Present address: Dept. of Zoology, University of British Columbia, Vancouver, BC, Canada V6T 1Z4.
§To whom reprint requests should be addressed at ‡.
**Present address: The Biomedical Research Centre, University of British Columbia, Vancouver, BC, Canada V6T 1Z3.

nonreducing/reducing SDS/PAGE was performed as described (25, 27). In some cases, cells were lysed in 1% Nonidet P-40/0.4% deoxycholate-containing lysis buffer (21), immunoprecipitated with a mixture of anti-μ, anti-λ, and anti-MB-1, and incubated with 2 units of endoglycosidase H (endo H) (Boehringer Mannheim) at 37°C for 20 hr in 100 mM sodium citrate (pH 5.5).

Inositol Phosphate Production. Cells grown in six-well dishes were labeled overnight with [^{3}H]inositol (5 μCi/ml; Amersham), washed with phosphate-buffered saline (PBS), and 1.5 ml of modified Hepes/saline (24) containing 10 mM LiCl was added to each well. After 15 min at 37°C, the cells were washed with PBS, and then 1.5 ml of Hepes/saline was added to each well along with the indicated stimuli. Reactions were terminated by washing the cells twice with PBS and adding 1 ml of cold 10% trichloroacetic acid. Insoluble material was removed by centrifugation and the supernatant fraction was extracted six times with cold water-saturated ethyl ether. Inositol phosphates were bound to Dowex formate columns and eluted together with 1 M ammonium formate/0.1 M formic acid (28).

Tyrosine Phosphorylation. Cells were washed and incubated with stimuli in Hepes/saline as above except that LiCl was omitted. Reactions were terminated by washing the cells with cold PBS containing 1 mM Na_3VO_4. Cells were lysed in 1% (wt/vol) Triton X-100 lysis buffer (4). Detergent-insoluble material was removed by centrifugation and protein concentrations were determined as in ref. 25. Anti-phosphotyrosine [anti-Tyr(*P*)] immunoblot analysis of total cell lysates (4) or anti-MB-1 immunoprecipitates (25) was performed as described using the 4G10 monoclonal anti-Tyr(*P*) antibody (29).

Phosphatidylinositol 3-Kinase (PI 3-Kinase) Assays. Cell lysates were prepared as for tyrosine phosphorylation assays and immunoprecipitated with anti-Tyr(*P*). Immune complexes were washed twice with lysis buffer and three times with 10 mM Tris·HCl (pH 7.4). PI 3-kinase assays (30, 31) were carried out for 15 min at 20°C. Labeled phosphatidylinositol 3-phosphate was detected by autoradiography and quantitated using a phosphor imager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

The murine AtT20 pituitary cell line was used to reconstitute the B-cell antigen receptor because the assembly and intracellular trafficking of secreted forms of Ig expressed in these cells closely resemble that in B cells (ref. 16; L.M. and R.B.K., unpublished results). In the absence of MB-1 and Ig-β, transfected μ_m and λ light chain remained in the ER of AtT20 cells as $\mu_2\lambda_2$ oligomers (data not shown). To test if MB-1 and Ig-β were the only additional proteins required for surface expression of mIgM, these proteins were coexpressed with μ_m and λ in AtT20 cells. Seventeen independent clones (69 examined) expressed mIgM on the cell surface. These clones also expressed MB-1 as well as Ig-β, whose presence was indicated by disulfide-linked, higher molecular mass oligomers containing MB-1 (detected by immunoblotting, not shown). The other 52 clones failed to express at least one transfected gene product and were not surface mIgM positive.

Cells expressing all four chains were analyzed by immunofluorescence. Fixed, nonpermeabilized cells stained with fluoresceinated anti-μ chain antibodies showed bright surface fluorescence outlining individual cells (Fig. 1 *A* and *B*). Immunofluorescence was also performed on 8-bromoadenosine 3′,5′-cyclic monophosphate-treated AtT20 cells, which flatten and extend processes. Bright fluorescence was seen on the surface of the cell body and along the process extensions (Fig. 1*C*). To examine the intracellular distribution of mIgM, cells were permeabilized with saponin. Bright staining of a perinuclear region was seen (Fig. 1*E*), a pattern similar to that observed for other secreted proteins in AtT20 cells (21, 22). Flow cytometry on live cells (Fig. 1*G*) showed that one clone, R98, had 20% as much mIgM on the surface as a strongly surface mIgM-positive B lymphoma, WEHI 231. Two other clones, R71 and R138, expressed 16% as much mIgM as WEHI 231 cells. Thus, MB-1 and Ig-β were sufficient for surface expression of mIgM in nonlymphoid cells.

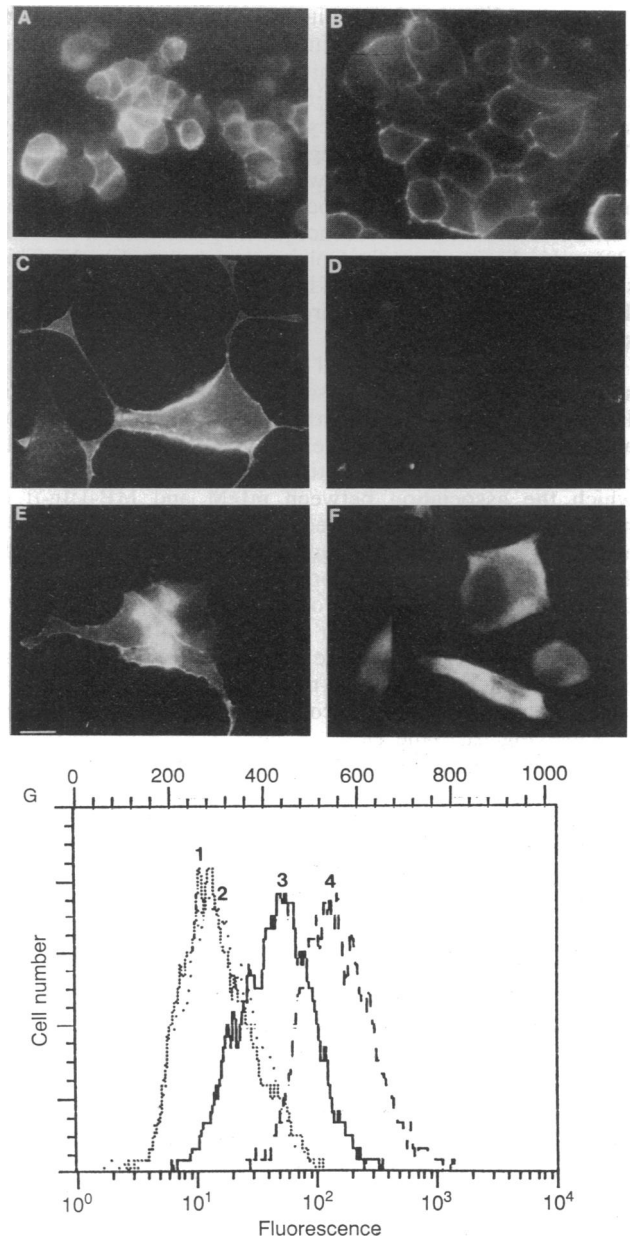

FIG. 1. Surface expression of mIgM on transfected AtT20 clones. (*A*) Clone R71 grown on poly(D-lysine)-coated coverslips and stained with fluoresceinated goat anti-μ chain antibodies. (*B*) Clone R138 grown as in *A*, stained with rhodamine-coupled goat anti-μ. (*C*) Clone R98 grown 5 days in medium containing 5 mM 8-bromoadenosine 3′,5′-cyclic monophosphate and stained with rhodamine-coupled goat anti-μ. (*D*) Negative control cell line, clone R142 (expressing only μ_m, λ_1, and MB-1), grown and stained as in *C*. (*E*) Clone R98, grown as in *C*, permeabilized with saponin, and stained with rhodamine-coupled goat anti-μ. (*F*) Clone R142, treated as in *E*. (Bar = 20 μm for all panels except *B*, where bar = 12.5 μm.) (*G*) Clone R98 stained with 35 μg of fluoresceinated goat anti-μ per ml (peak 3) or 35 μg of fluoresceinated goat anti-γ per ml (peak 2) or not stained (peak 1) and analyzed by flow cytometry. Peak 4, WEHI 231 B-lymphoma cells stained with fluoresceinated goat anti-μ.

To determine if MB-1 by itself could confer surface expression of mIgM, we examined several AtT20 clones that expressed μ_m, λ, and MB-1 but not Ig-β. In these clones, μ_m and MB-1 remained trapped in the ER and MB-1 remained monomeric. No surface fluorescence with anti-μ was seen by fluorescence microscopy (Fig. 1*D*) or by flow cytometry (data not shown). The mIgM was readily detected, however, in the ER of these cells after permeabilization (Fig. 1*F*). This pattern of immunofluorescence is characteristic of the ER (22) and is distinct from the Golgi pattern seen in Fig. 1*E*. This suggests that in the absence of Ig-β, mIgM fails to exit the ER. Thus, Ig-β as well as MB-1 are required for surface expression of mIgM.

In the clones expressing all four chains, the mIgM on the surface of the cells was associated with MB-1 and Ig-β. Immunoprecipitation with either anti-μ chain (Fig. 2*A*) or anti-MB-1 (Fig. 2*B*) antibodies from surface-iodinated cells precipitated all four polypeptides from digitonin lysates, in which the association between mIgM and MB-1:Ig-β is maintained (9). Disulfide-linked heterodimers of 200–210 kDa and 68–75 kDa under nonreducing conditions were observed. The larger molecular mass complex contained μ_m and λ as a $\mu_2\lambda_2$ complex, whereas the lower molecular mass complex contained MB-1 and Ig-β. In contrast, no mIgM, MB-1, or Ig-β was detected by surface iodination of R142 cells that express μ_m, λ, and MB-1 but not Ig-β, although the intracellular forms of these chains could be detected by metabolic labeling (Fig. 2*C*, lane 1).

In the R98 clone, which expressed all four chains, μ_m was endo H resistant, showing that it had traversed the Golgi complex (Fig. 2*C*, lanes 3 and 4). In contrast, the μ_m heavy chain in the Ig-β-negative-clone, R142, remained endo H sensitive (Fig. 2*C*, lanes 1 and 2), indicating that it remained trapped early in the secretory pathway, probably in the ER.

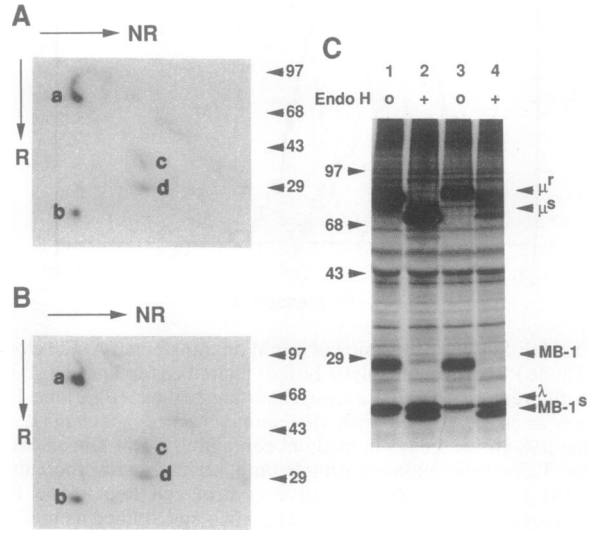

FIG. 2. Surface and intracellular forms of the mIgM receptor complex in transfected AtT20 cells. Clone R98 cells were surface iodinated, lysed in digitonin, and immunoprecipitated with either anti-μ chain antibodies (*A*) or anti-MB-1 antibodies (*B*). Immunoprecipitates were analyzed by two-dimensional nonreducing (NR) (10% polyacrylamide)/reducing (R) (12% polyacrylamide) SDS/PAGE. The labeled spots are μ_m (spot a), λ (spot b), Ig-β (spot c), and MB-1 (spot d). Molecular masses are in kDa. (*C*) Endo H sensitivity of mIgM. Metabolically labeled surface mIgM-negative R142 cells (lanes 1 and 2) or surface mIgM-positive R98 cells (lanes 3 and 4) were lysed and immunoprecipitated with a mixture of anti-μ chain and anti-MB-1 antibodies. Immunoprecipitates were incubated without (lanes 1 and 3) or with (lanes 2 and 4) 2 units of endo H. μ^r, Endo H-resistant μ_m; μ^s, endo H-sensitive μ_m; MB-1, undigested MB-1; MB-1^S, endo H-sensitive MB-1; λ, λ light chains. Molecular masses are in kDa.

It was difficult to identify the endo H-resistant MB-1 forms in R98 due to its glycosylation state or to the relative abundance of the surface pools compared with the intracellular pools of MB-1 in the ER (L.M. and R.B.K., unpublished results).

We next examined whether the mIgM:MB-1:Ig-β complex expressed on the surface of AtT20 cells was capable of signal transduction. Crosslinking mIgM on clones R98 and R138 stimulated tyrosine phosphorylation of MB-1 and Ig-β (Fig. 3*A*), similar to that described in B cells (25). The identity of the 33-kDa polypeptide as MB-1 was confirmed by reprobing the blots with anti-MB-1 and by its comigration with MB-1 from Bal 17 B-lymphoma cells (25). No tyrosine phosphorylation of these proteins was seen in the Ig-β-negative clone R142, which did not express mIgM on the cell surface.

Crosslinking mIgM on B cells also increases the amount of PI 3-kinase activity that can be immunoprecipitated with anti-Tyr(*P*) antibodies (31). This response presumably reflects increased tyrosine phosphorylation of this enzyme or an associated protein. PI 3-kinase is regulated by many tyrosine kinases and is thought to be involved in growth control (32). Anti-μ chain antibody treatment of clones R98 and R138 for 3–15 min caused a 2- to 2.5-fold increase in the amount of PI 3-kinase activity associated with anti-Tyr(*P*) immunoprecipitates (Fig. 4). These increases were significant and reproducible, although substantially less than those seen

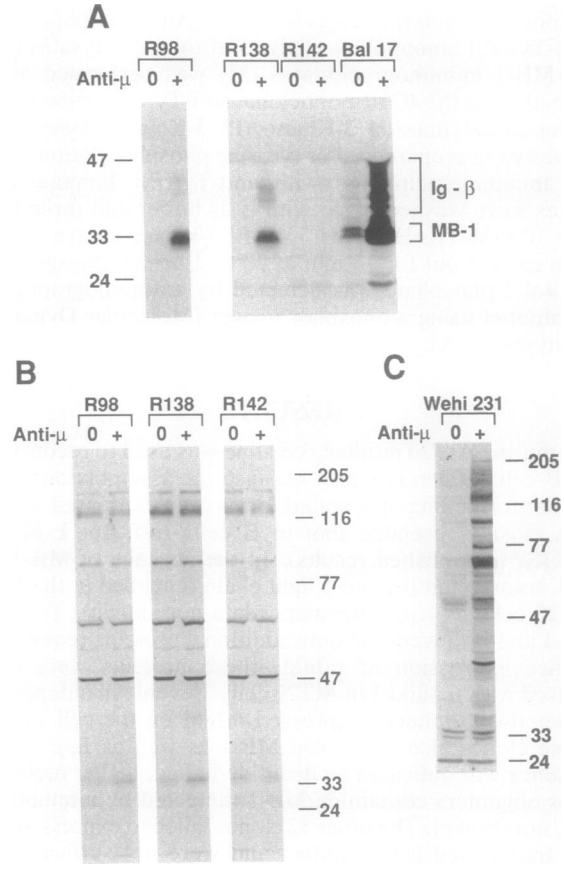

FIG. 3. Anti-μ chain antibodies stimulate tyrosine phosphorylation of MB-1 and Ig-β in transfected AtT20 cells but do not stimulate tyrosine phosphorylation of more abundant proteins. (*A*) Anti-Tyr(*P*) immunoblot of anti-MB-1 immunoprecipitates from 1.2 mg of AtT20 cell lysate or 1 mg of Bal 17 B-lymphoma cell lysate. (*B*) Anti-Tyr(*P*) immunoblot of 50 μg of total cell lysate from the same experiment. Cells were incubated without or with 20 μg of goat anti-μ chain per ml for 5 min before being lysed. (*C*) Anti-Tyr(*P*)-immunoblot of 40 μg of total cell lysate from WEHI 231 B-lymphoma cells incubated 5 min with or without 10 μg of goat anti-μ chain per ml. Molecular masses of standards are in kDa.

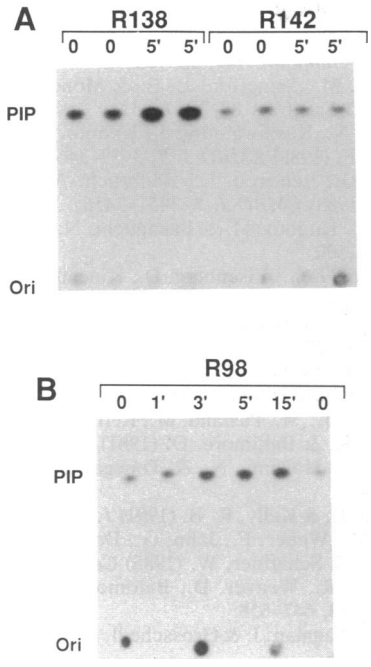

FIG. 4. Anti-μ chain antibodies increase the amount of PI 3-kinase activity immunoprecipitated with anti-Tyr(*P*) antibodies. Transfected AtT20 cells were incubated without or with 20 μg of goat anti-μ chain per ml for the indicated times and then lysed. The cell lysate (250 μg) was immunoprecipitated with anti-Tyr(*P*) antibodies and the precipitates were assayed for PI 3-kinase activity. Labeled phosphatidylinositol 3-phosphate (PIP) was separated from the labeled ATP by thin-layer chromatography and visualized by autoradiography for 4 hr. Ori, origin, containing labeled ATP that was not removed by extraction.

in B cells (31). No increase was observed in the surface mIgM-negative R142 clone.

Although mIgM crosslinking stimulated tyrosine phosphorylation of MB-1, Ig-β, and PI 3-kinase in R98 and R138, no increases in tyrosine phosphorylation were observed when total cell lysates were analyzed by anti-Tyr(*P*) immunoblotting (Fig. 3*B*), even when cells were pretreated with the tyrosine phosphatase inhibitor Na_3VO_4 (data not shown). In contrast, mIg crosslinking on WEHI 231 B-lymphoma cells stimulated tyrosine phosphorylation of a number of proteins (Fig. 3*C*). This assay detects tyrosine phosphorylation of only the most abundant proteins. mIgM crosslinking may stimulate tyrosine phosphorylation of less abundant proteins, in addition to MB-1 and Ig-β, which can be detected only after immunoprecipitation.

Although crosslinking mIgM on AtT20 cells stimulated protein tyrosine phosphorylation, no stimulation of inositol phospholipid breakdown was observed in either R98 or R138 (Table 1). Inositol phosphate production was stimulated by a serum component, and AtT20 cells expressing a transfected 5-HT_{1c} serotinin receptor produced large amounts of inositol phosphates in response to serotonin. These cells were therefore capable of receptor-stimulated inositol phospholipid breakdown. Thus, the transfected B-cell antigen receptor consisting of mIgM, MB-1, and Ig-β could stimulate some, but not all, of the signaling events characteristic of mIgM.

DISCUSSION

Transport of membrane protein complexes to the cell surface usually requires the presence of all subunits. Partially assembled complexes are retained intracellularly or targeted for degradation (33). For example, the T-cell antigen receptor requires the CD3 complex and two ζ chains for complete

Table 1. Production of inositol phosphates by transfected AtT20 cells

Exp.	Clone	Stimulus	Time, min	Total inositol phosphate cpm	%
1	R98	None	15	4,333 ± 196	
		Anti-μ	15	4,553 ± 121	5*
		FCS	15	5,752 ± 20	33
	R98	None	60	4,852 ± 181	
		Anti-μ	60	4,494 ± 87	0
		FCS	60	8,586 ± 436	77
2	R138	None	15	886 ± 34	
		Anti-μ	15	832 ± 31	0
		FCS	15	1,502 ± 54	70
3	R138	None	60	9,779 ± 785	
		Anti-μ	60	9,324 ± 348	0
		FCS	60	15,983 ± 44	63
4	SR1	None	15	1,717 ± 109	
		Serotonin	15	10,111 ± 246	489
		FCS	15	2,821 ± 15	64

Transfected AtT20 cells were incubated with 20 μg of goat anti-μ chain per ml, 10% (vol/vol) dialyzed fetal calf serum (FCS), or 1 μM serotonin for the indicated times. LiCl was included to prevent the conversion of inositol phosphates to inositol and allow the inositol phosphates produced to accumulate. Data are presented as the average ± range of total inositol phosphates produced by duplicate wells. The majority of the inositol phosphates produced by untreated cells was produced during the overnight labeling and not during the course of the experiment (data not shown). The percent increase over the unstimulated control is also given.
*Not significantly different than control.

assembly and surface expression (34, 35). Similarly, previous studies had shown that surface expression of mIgM in J558L plasmacytoma cells requires MB-1 (13) and that transfection of Ig-β into this cell, which already expressed Ig-β, increases the level of surface expression (12). However, those experiments did not address whether other B-cell-specific components were also required for surface expression. Our results demonstrate that MB-1 and Ig-β are the only B-cell-specific components required for cell surface expression of mIgM in a nonlymphoid cell line. In addition, we showed that Ig-β is required for cell surface expression of mIgM since AtT20 clones expressing MB-1 but lacking Ig-β (e.g., R142) failed to express mIgM on the surface. The level of mIgM surface expression that we achieved in AtT20 cells by cotransfection of MB-1 and Ig-β was comparable to that of many B-cell lines, making it unlikely that other components are required. Recently, Venkitaraman *et al.* (36) have also shown that MB-1 and Ig-β are the only B-cell-specific proteins required for cell surface expression of mIgM, mIgA, and mIgE. Interestingly, they found that although mIgD and mIgG2b can associate with MB-1 and Ig-β, these isotypes can be expressed on the surface in the absence of these accessory proteins.

mIgM expressed on the surface of AtT20 cells was capable of initiating some of the signaling reactions characteristic of mIgM in B lymphocytes. Crosslinking mIgM on the AtT20 transfectants stimulated tyrosine phosphorylation of MB-1, Ig-β, and PI 3-kinase. The magnitude of the PI 3-kinase response was smaller than that observed in B cells, but AtT20 cells may express low levels of PI 3-kinase. Alternatively, mIg crosslinking may regulate PI 3-kinase inefficiently in AtT20 cells. As no other stimuli that cause tyrosine phosphorylation have been identified for AtT20 cells, we could not test this possibility. Serum, which contains growth factors that activate receptor tyrosine kinases, did not stimulate protein tyrosine phosphorylation or regulate PI 3-kinase in AtT20 cells (data not shown). In any case, our results clearly argue that the complex of mIgM, MB-1, and Ig-β can activate

a tyrosine kinase in the absence of other B-cell-specific components.

Crosslinking mIgM on AtT20 cells did not, however, stimulate tyrosine phosphorylation of more abundant proteins, in contrast to what is observed in B cells (refs. 4–7; Fig. 3*C*). It is not clear why significant tyrosine phosphorylation of MB-1 and Ig-β would occur without tyrosine phosphorylation of other proteins. In B cells, mIgM crosslinking activates multiple tyrosine kinases, including *lyn*, *blk*, and *fyn* (37). If these tyrosine kinases phosphorylate different substrates, it is possible that AtT20 cells express the kinase that phosphorylates MB-1 and Ig-β but not the kinases responsible for phosphorylation of other proteins. Alternatively, differences in expression of tyrosine phosphatases or tyrosine kinases may be responsible for partial, rather than full, signal transduction by mIgM in these cells.

mIgM crosslinking also did not stimulate inositol phospholipid breakdown in the transfected AtT20 cells. AtT20 cells may lack an additional component of the B-cell antigen receptor that has yet to be identified or another protein required for activation of phospholipase C. mIgM-stimulated inositol phospholipid breakdown in B cells involves an unidentified G protein (28, 38) that could be missing in AtT20 cells. The serotonin receptor, which stimulated inositol phosphate production when transfected into AtT20 cells, is a seven-transmembrane-domain receptor and presumably activates phospholipase C via a G protein that is present in AtT20 cells. This G protein, however, may not interact with the B-cell antigen receptor. Serum components also stimulated inositol phospholipid breakdown in AtT20 cells, although the relevant receptor and the mechanism by which it activated phospholipase C are not known. Activation of phospholipase C by the B-cell antigen receptor may also require tyrosine phosphorylation of the enzyme. This has been shown to occur in B cells (39, 41) but may not occur in AtT20 cells. Expression of the CD45 tyrosine phosphatase is also required for mIgM-induced inositol phospholipid hydrolysis (40). Since CD45 is normally expressed only in hematopoietic cells, it is likely that AtT20 cells do not express this molecule.

Surface expression of the mIgM receptor complex in a nonlymphoid cell provides an important tool for studying the role of individual components in the functioning of this receptor. It is now possible to dissect how MB-1 and Ig-β interact with mIgM and with the tyrosine kinase(s) that phosphorylates them. In addition, we can now test whether cotransfection of other molecules such as tyrosine kinases, CD45, or newly discovered components of the B-cell antigen receptor will reconstitute the signaling functions that are not carried out by AtT20 cells expressing only mIgM, MB-1, and Ig-β.

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