The B Cell Antigen Receptor of Class IgD Induces a Stronger and More Prolonged Protein Tyrosine Phosphorylation than that of Class IgM

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Summary

Most mature B lymphocytes coexpress two classes of antigen receptor, immunoglobulin (Ig)M and IgD. The differences in the signal transduction from the two receptors are still a matter of controversy. We have analyzed B cell lines expressing IgM or IgD antigen receptors with the same antigen specificity. Cross-linking of these receptors with either antigen, or class-specific antibodies, results in the activation of protein tyrosine kinases and the phosphorylation of the same substrate proteins. The kinetic and the intensity of phosphorylation, however, was quite different between the two receptors when they were cross-linked by antigen. In membrane IgM-expressing cells, the substrate phosphorylation reached a maximum after 1 minute and diminished after 60 minutes whereas, in the membrane IgD-expressing cells, the substrate phosphorylation increased further over time, reached its maximum at 60 minutes, and persisted longer than 240 minutes after exposure to antigen. As a result, the intensity of protein tyrosine phosphorylation induced by cross-linking of membrane IgD was stronger than that induced by membrane IgM. Studies of chimeric receptors demonstrate that only the membrane-proximal C domain and/or the transmembrane part of membrane-bound IgD molecule is required for the long-lasting substrate phosphorylation. Together, these data suggest that the signal emission from the two receptors is controlled differently.

The B cell antigen receptor (BCR)[1] is a complex containing the membrane-bound immunoglobulin (mIg) molecule and the Ig-α/Ig-β heterodimer (for reviews see references 1–4). Ig-α and Ig-β are glycosylated transmembrane proteins encoded by the B cell–specific genes mb-1 and B29, respectively (5, 6). Signal transduction from the cross-linked BCR involves the rapid activation of two types of protein tyrosine kinases (PTK); the src-related PTKs Lyn, Fyn, Lck, and Blk (7–9) (for review see reference 10) as well as the cytoplasmic PTK Syk/PTK72 (11–14). These enzymes phosphorylate several substrate proteins in B cells, including the BCR components Ig-α and Ig-β (15). The Ig-α/Ig-β heterodimer plays an important role in the activation of these PTKs (16–22) and, after its tyrosine phosphorylation, becomes a target for src-homology-2–carrying proteins (23–25).

All classes of mIg are associated with the Ig-α/Ig-β heterodimer (26). The molecular weight of the mIgD-associated Ig-α (IgD-α) is larger than the mIgM-associated Ig-α (IgM-α) (27, 28). This size difference is due to an altered glycosylation of the extracellular domains of these proteins (26, 27). The cytoplasmic parts of the Ig-α/Ig-β heterodimer, however, are identical in the two BCR complexes. As the mIgM and mIgD molecules also have the same short cytoplasmic sequence (KVK), both BCR classes should be the same on the cytoplasmic side and, thus, are expected to communicate with the same intracellular molecules.

Most mature B cells coexpress the IgM-BCR and IgD-BCR on their cell surfaces. Whether the two BCR classes have different functions on B cells is a matter of controversy. Several studies suggest that the engagement of mIgM and mIgD induces similar B cell responses (28–30). This holds true for the early biochemical responses involving the activation of PTK (31, 32) and the hydrolysis of inositol phospholipids (33, 34). Other studies, however, show that mIgM, but not mIgD, can induce negative responses in B cells, such as anergy and apoptosis (35–40). The IgD knockout mice do not show a severe immunodeficiency, which suggests at least a partial redundancy in the function of mIgD and mIgM (41, 42).

The two BCR classes are heterogeneously expressed on normal mature B cells with IgD-BCR generally being more abundant than IgM-BCR. To compare the signaling func-

[1] *Abbreviations used in this paper:* BAP, BCR-associated proteins; BCR, B cell antigen receptor; ECL, enhanced chemiluminescence; EGFR, epidermal growth factor receptor; mIg, membrane-bound immunoglobulin; EV, enhancer + V-promotor; NGFR, neuronal growth factor receptor; NIP, 4-hydroxy-3-iodo-5-nitrophenyl; PTK, protein tyrosine kinases; TKR, tyrosine kinase receptors.

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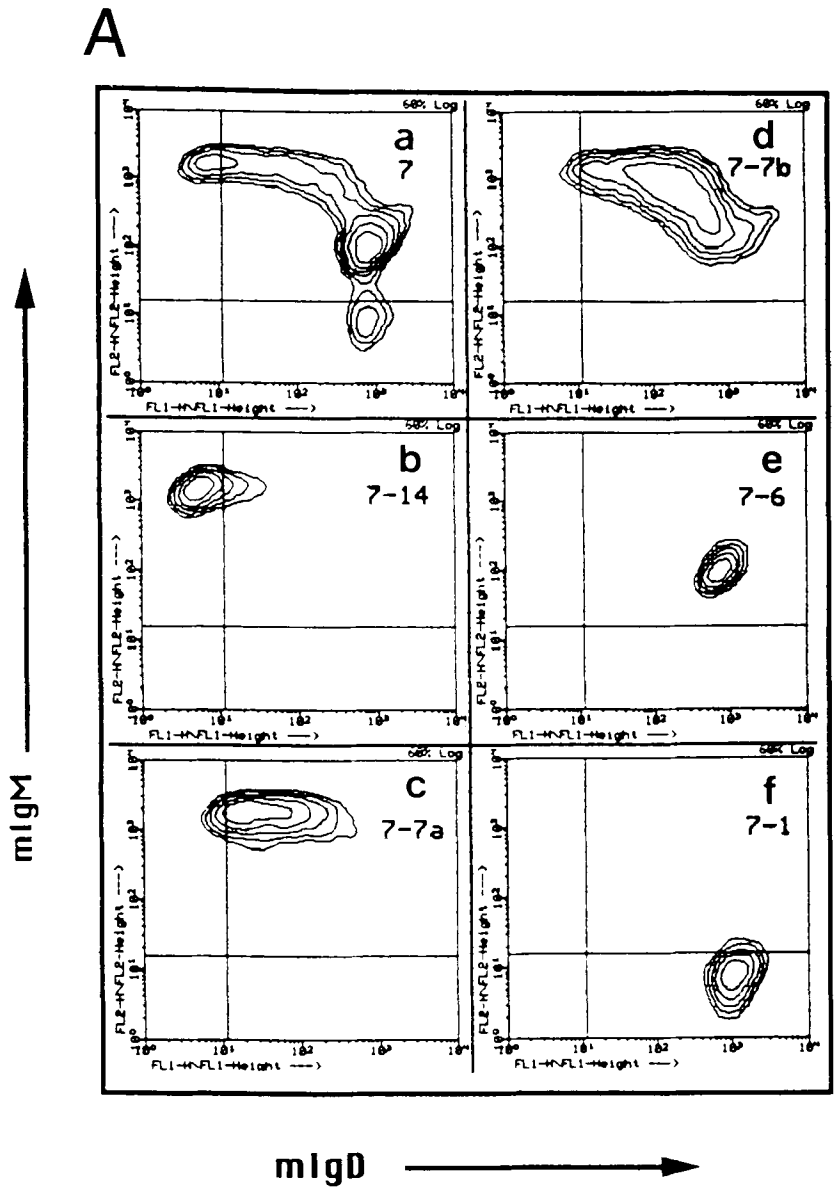
tion of these receptors we chose Ig-transfectants of J558L as an antigen-specific and homogenous cell population. J558L cells produce Ig-β protein and λ1 light chain, but neither Ig-α protein, nor Ig heavy chain (43, 44). After transfection with expression vectors for the Ig-α protein and for heavy chains carrying the B1-8 VH domain, these cells express on their surfaces large amounts of BCR with specificity for the hapten 4-hydroxy-3-iodo-5-nitrophenyl (NIP). Although these myeloma cells lack several B cell surface markers, including CD19, CD22, CD40, and CD45, their initial response, after engagement of the BCR, is quite similar to that of B lymphoma cells (45) or normal B cells (31). In particular, the same PTKs are activated, and most PTK substrate proteins seem to be identical in these different B cells. We have generated J558L myeloma transfectants expressing similar amounts of antigen-specific IgM-BCR and/or IgD-BCR on the cell surface. A functional analysis of these transfectants shows that the cross-linking of the IgD-BCR provokes a stronger and more long-lasting substrate phosphorylation than the cross-linking of the IgM-BCR.

Materials and Methods

Construction of the Chimeric Heavy Chain Expression Vectors pSVγ12μ4m and pSVγ12δ3m. A DNA fragment containing the Cμ4 exon with a single Pst site was obtained by PCR using the oligos: 5' CTTACACTCGAGTTGCAGGAC and 3' CTATATGTGTGCCTGAATGCT and the plasmid pSVu1 (46) as template DNA. The PCR fragment was cut by a XhoI and a HindIII and cloned into a variant of the plasmid pBS-KS lacking the Pst site in the polylinker. The Pst-HindIII fragment of Cμ4 was replaced by the 1.1-kb Pst-HindIII fragment γ obtained from the expression vector pSVμm5 (47). From this plasmid, we isolated the 1.5-



kb KpnI-EcoRI fragment μ4m containing the coding information for the Cμ4 domain, the transmembrane, and the cytoplasmic parts of the μm chain. The 1.3-kb BglII-KpnI fragment γ12 containing the Cγ1, hinge, and Cγ2 exons was obtained by PCR using DNA of the vector pG2am (22) as a template. The 3.8-kb EcoRI-BamHI fragment containing the VH promotor, the VH exon, and the IgH enhancer + V-promotor (EV) was described previously (48). The fragments EV, γ12, and μ4m were then cloned into the EcoRI cut pSV2gpt plasmid to obtain the expression vector pSVγ12μ4m.

The Cδ1, hinge, and Cδ3 exons reside on a 2.5-kb BglII-KpnI fragment from which we isolated the 0.4-kb SalI-KpnI fragment δ3 containing the Cδ3 exon of the δ gene. By subcloning we changed the 1.4-kb BamHI fragment carrying the two membrane exons of the δ gene into the KpnI-EcoRI fragment δm (48). The 1.3-kb γ12 fragment was obtained as the BglII-SalI fragment γ12s. To generate the expression vector pSVγ12δ3m, we cloned the fragments EV, γ12s, δ3, and δm into the EcoRI cut pSV2gpt plasmid.

Cells. J558L is a myeloma line which produces λ1 light chain, but no heavy chain (43). J558Lμm3 expresses mIgM in association with the Ig-α/Ig-β heterodimer (49). J558Lμm3/δm7 was made by the transfection of J558Lμm3 with an expression vector for the δm heavy chain (50). Cells were cultured in RPMI 1640 medium supplemented with 15% FCS, 2 mM glutamine, 5×10^{-5} M 2-mercaptoethanol, and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin).

Antibodies and Reagents. The affinity-purified antibodies: rat anti-mouse IgD, goat anti-mouse IgM, and goat anti-mouse IgG, as well as their biotinylated, or FITC-coupled, counterparts and PE-coupled streptavidin, were obtained from Southern Biotechnology Associates Inc. (Birmingham, AL). The rat anti-mouse IgM mAb b7.6 and the rat anti-mouse IgD mAb 10.4.22 were provided by Dr. M. Lamers (Max-Planck Institute for Immunobiology, Freiburg, Germany). The mouse anti-phosphotyrosine mAb 4G10 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

The antiidiotype mAb Ac146, the antigen NIP-coupled BSA (NIP_{10}-BSA), and NIP-coupled Sepharose 4B beads were provided by Dr. K. Rajewsky (Institute for Genetics, University of Cologne, Germany).

Surface Biotinylation. Surface biotinylation was performed, as described previously (16), with some modifications. Cells (2×10^7) were washed twice with PBS and incubated in 1 ml of PBS containing 0.5 mg/ml of sulfo-*N*-hydroxysuccinimido-biotin (Pierce

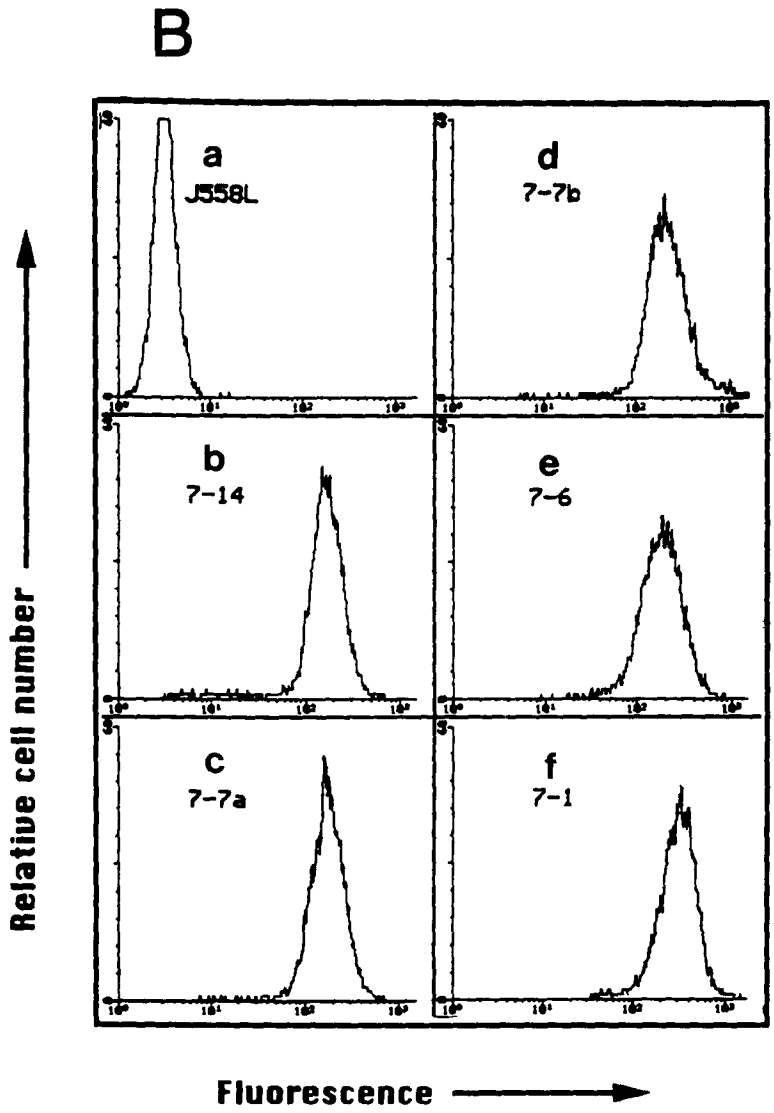


Figure 1. Expression of IgM-BCR and IgD-BCR on the surface of the J558Lμm3/δm7 transfectant and five of its subclones. (*A*) The amount of IgM-BCR (ordinate) and IgD-BCR (abscissa) on the surface of J558Lμm3/δm7 (*a*) and the subclones 7-14 (*b*), 7-7a (*c*), 7-7b (*d*), 7-6 (*e*), and 7-1 (*f*) was examined by two-color flow cytometry (FACScan®, Becton Dickinson and Co., Mountain View, CA). The mIgM molecules were detected with a combination of biotinylated goat anti-mouse IgM and PE-coupled streptavidin, while the mIgD molecules were detected with a combination of rat anti-mouse IgD and FITC-coupled anti-rat IgG antibody. (*B*) The total amount of BCR expressed on the surface of J558L (*a*), and the subclones 7-14 (*b*), 7-7a (*c*), 7-7b (*d*), 7-6 (*e*), and 7-1 (*f*) was examined by flow cytometry (FACScan®). The cells were stained with a combination of the antiidiotypic antibody Ac146 and FITC-coupled anti-mouse IgG.

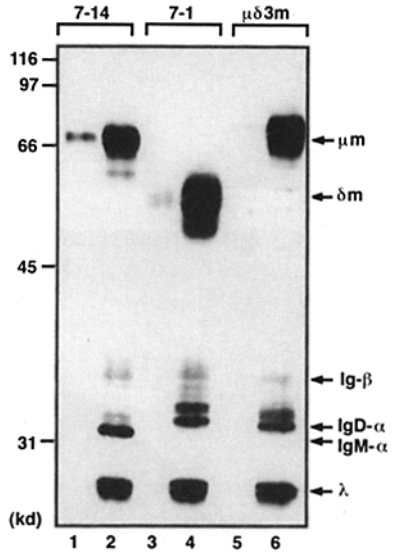


Figure 2. Biochemical analysis of the IgM-BCR (lanes *1* and *2*), IgD-BCR (lanes *3* and *4*), and a chimeric IgM/D-BCR (lanes *5* and *6*) on the surface of the subclones 7-14, 7-1, and the transfectant J558Lμδ3m, respectively. Surface biotinylated receptors were purified over NIP-coupled Sepharose and analyzed by 10% SDS-PAGE under reducing conditions. In lanes *1*, *3*, and *5*, the specific binding of the receptor to the affinity column was inhibited by 10 μg/ml of NIP-BSA. The biotinylated proteins were detected on the Western blot by the horseradish peroxidase coupled streptavidin and the ECL system (Amersham).

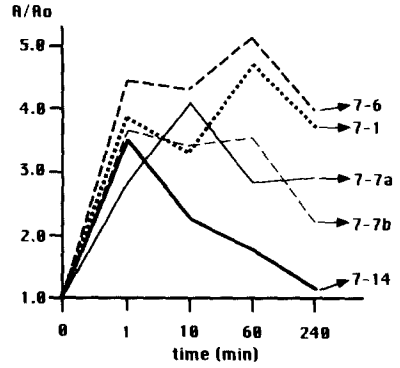


Figure 4. Difference in the kinetic of tyrosine phosphorylation of the PTK substrate protein p65 in five subclones of J558Lμm3/δm7. The cells were incubated for the indicated times with 10 μg/ml of NIP10-BSA. Tyrosine-phosphorylated p65 was detected by Western blotting and the ECL system. The intensity of the p65 signal on the film was measured with a laser densitometer (2222-020, Ultrascan XL; Pharmacia LKB Biotechnology, Inc., Uppsala, Sweden). The change in p65 phosphorylation is given as the ratio of absorbance before (*Ao*) and after (*A*) stimulation of the BCR for the indicated times.

Chemical Co., Rockford, IL) at room temperature for 15 min. Free succinimide groups were blocked by the addition of 5 ml of non-supplemented medium at room temperature for 5 min. The cells were washed twice with PBS and lysed in 1 ml of ice-cold Triton X-100 lysis buffer. After incubation for 10 min on ice, the supernatant was cleared by centrifugation at 10,000 *g* for 10 min at 4°C, and specific mIg was precipitated with NIP-coupled Sepharose beads.

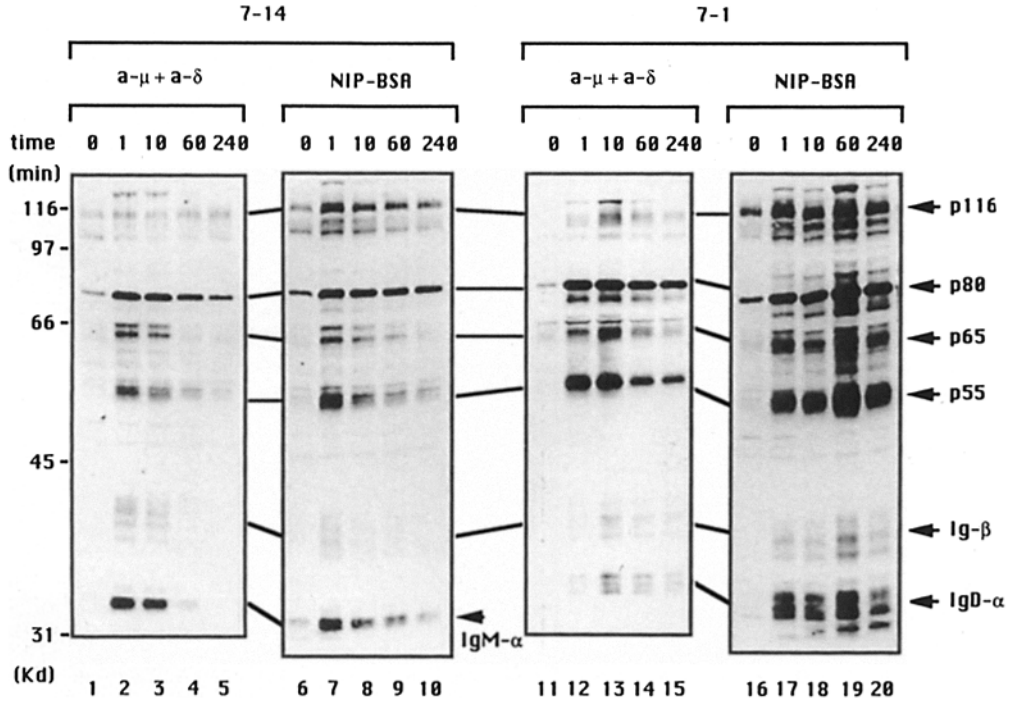


Figure 3. Comparison of the kinetics of PTK substrate phosphorylation in stimulated IgM-BCR or IgD-BCR–expressing B cells. The mIgM-producing subclone 7-14 (lanes *1–10*) and the mIgD-producing subclone 7-1 (lanes 11–20) were incubated for the indicated times, either with 40 μg/ml of a one to one mixture of the anti-μ antibody b7.6 and the anti-δ antibody 10.4.22 (lanes *1–5*, *11–15*), or with 10 μg/ml of NIP_{10}-BSA (lanes *6–10*, *16–20*). Tyrosine-phosphorylated proteins were detected on the Western blot by the mAb 4G10. The positions of molecular weight markers (*Kd*) and of major substrate proteins are indicated on the left and the right sides of the figure, respectively.

cleared by centrifugation at 10,000 *g* for 10 min at 4°C, and specific mIg was precipitated with NIP-coupled Sepharose beads.

Detection of Tyrosine Phosphorylated Proteins by Western Blotting. Tyrosine phosphorylated proteins were detected, as previously described (16, 45), with some modifications. 4×10^6 cells were resuspended in 0.5 ml of RPMI 1640 medium and stimulated in 24-well culture plates. After washing twice with ice-cold PBS containing 1 mM sodium orthovanadate (Sigma Chemie GmbH, Deisenhofen, Germany), cells were lysed with 100 μl of Triton X-100 lysis buffer containing 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 10 μg/ml leupeptin (Sigma), 10 μg/ml aprotinin (Sigma), 1 mM PMSF (Sigma), and 1 mM sodium orthovanadate. After incubation for 10 min on ice, the supernatant was cleared by centrifugation at 10,000 *g* for 15 min at 4°C. Subsequently, 20 μl of supernatant was subjected to 12% SDS-PAGE and Western blotting. The nitrocellulose membrane was incubated with the antiphosphotyrosine mAb 4G10 (Upstate Biotechnology, Inc.) and with horseradish peroxidase–coupled anti–mouse Ig antibody (Amersham Buchler GmbH, Braunschweig, Germany). The bound antibodies were visualized by the enhanced chemiluminescence (ECL) detection system (Amersham).

Results

Characterization of Five Subclones of the Cell Line J558Lμ m3/δm-7 which Coexpress mIgM and mIgD. The Ig-α–positive Ig-transfectant J558Lμm3 expresses large amounts of mIgM on the cell surface in association with the Ig-α/Ig-β heterodimer (49). The J558Lμm3/δm-7 cell line was generated by the transfection of J558Lμm3 cells with an expression vector encoding the mIgD heavy chain (50). Both the IgM and the IgD antigen receptors of the J558Lμm3/δm-7 cells bind the hapten NIP. A flow cytometric analysis revealed a heterogeneous expression of mIgM and mIgD on the surface of the J558Lμm3/δm-7 cells (Fig. 1 *A*, *a*). J558Lμm3/δm-7 cells with strong mIgD expression had less mIgM on the cell surface, suggesting a competition of the two Ig classes for limiting quantities of the Ig-α/Ig-β heterodimer or another as yet unknown factor. By cell sorting and/or limiting dilution we have isolated the five subclones 7-14, 7-7a, 7-7b, 7-6, and 7-1 (Fig. 1 *A*, *b–f*). Each of these subclones has a more homogeneous mIg expression than that of the parental population, and the phenotype is stable during prolonged culture of these cells. The subclone 7-7b has an intermediate expression of both classes whereas the subclones 7-14 and 7-1 express almost exclusively mIgM and mIgD, respectively. In spite of these differences, all subclones carry on their cell surfaces roughly the same amount of total mIg (Fig. 1 *B*) as detected by the antiidiotypic antibody Ac146 (51), which binds to the identical hapten binding site of the mIgM and mIgD molecules.

The composition of the IgM antigen receptor on the cell surfaces of the subclones 7-14 and 7-1 was analyzed by surface biotinylation and the affinity purification of the receptor complexes over NIP-coupled Sepharose. The SDS-PAGE analysis showed that the IgM–BCR (Fig. 2, lane *2*) contained roughly the same amount of the Ig-α/Ig-β heterodimer as the IgD-BCR (Fig. 2, lane *4*). The two receptor complexes differ, however, in the size of their Ig-α component, which is due to stronger glycosylation of the IgD-associated Ig-α (IgD-α). The analysis of a chimeric antigen receptor (μδ3m) showed that the more extensive glycosylation of IgD-α requires only the most membrane proximal C-domain and the transmembrane domain of the δm heavy chain (Fig. 2, lane *6*).

Activation of PTK by Cross-linking of mIgM and mIgD with Anti-Ig Antibodies or the Specific Antigen NIP_{10}-BSA. The two J558Lμm3/δ subclones 7-14 and 7-1 were used to analyze the activation of PTKs upon engagement of the IgM- and IgD-BCR, respectively. The two subclones were incubated for different lengths of time (1–240 min) in the presence of either a mixture of the two monoclonal antibodies (mAb) 10.4.22 (anti-IgD) and b7.6 (anti-IgM) (Fig. 3, lanes *1–5* and *11–15*), or the polyvalent antigen NIP_{10}-BSA (Fig. 3, lanes *6–10* and *16–20*). The PTK activation in these cells was monitored by the increase of tyrosine phosphorylation of PTK substrate proteins in total Triton X-100 cell lysates. The proteins p116, p80, p66, p65, p55, p39, and p34 are major substrate proteins in Ig-transfectants of J558L. The identities of most of these substrates are unknown at present, except those of p66, p39, and p34 which we have identified as PTK72/syk, Ig-β, and Ig-α, respectively (45). Note that due to glycosylation differences, IgD-α has a size (35 kD), which differs by 1 kD from that of IgM-α. Cross-linking the IgM-BCR or IgD-BCR, results in the phosphorylation of the same substrate proteins, but with a different intensity. The IgD-BCR induces a stronger phosphorylation than that of the IgM-BCR, especially when it is cross-linked by antigen (Fig. 3, lanes *16–20*) rather than by anti-Ig antibody (Fig. 3, lanes *11–15*). Furthermore, the kinetics of phosphorylation differ between the two receptors. After the IgM-BCR were cross-linked, the substrate phosphorylation reached its maximum in 1 min and decreased thereafter (Fig. 3, lanes *1–10*). A similar kinetic has also been found in other mIgM- or mIgG-expressing transfectants of the myeloma line J558L or the B lymphoma line K46 (22, 45). In contrast, in the mIgD-producing cell 7-1, the substrate phosphorylation still increases after 1 min of stimulation and reaches a maximum 10 and 60 min after cross-linking of the IgD-BCR with the anti-IgD antibody and the antigen NIP_{10}-BSA, respectively.

To exclude the possibility that the observed difference in PTK substrate phosphorylation was due to variation between transfectants rather than to the class of the BCR, we incubated all five J558Lμm3/δm7 subclones for varying times with the antigen NIP_{10}-BSA. We then monitored by densitometry (Fig. 4) the increase in tyrosine phosphorylation of p65, a substrate which becomes most rapidly and strongly phosphorylated after BCR cross-linking (45). All IgD-BCR–expressing subclones showed a stronger and more persistent p65 phosphorylation than the IgM-BCR–producing line 7-14. Indeed, the two subclones (7-6 and 7-1) with the highest IgD-BCR expression, displayed the same intensity and kinetics of p65 phosphorylation with a maximum 60 min after exposure to antigen.

Cross-linking of BCR with an Antiidiotypic Antibody. Cross-linking of the IgD-BCR with the antigen NIP_{10}-BSA resulted in a stronger and more persistent substrate phosphory-

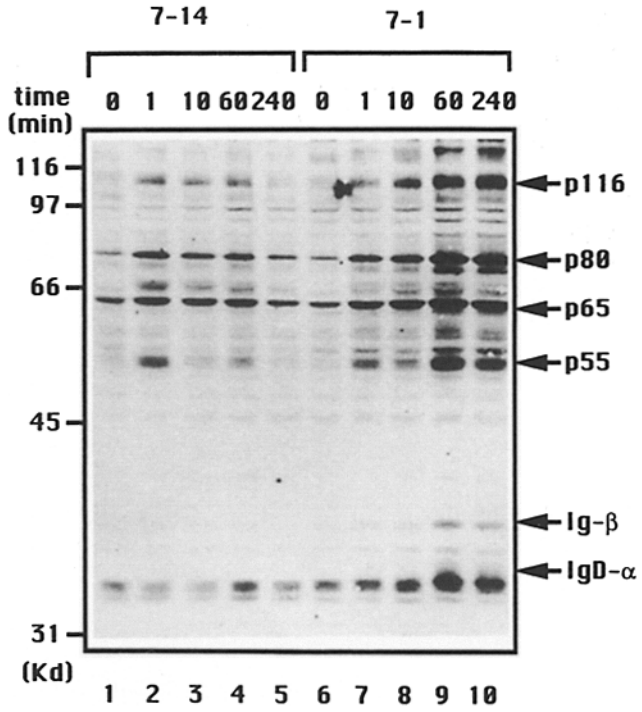


Figure 5. Kinetics of tyrosine phosphorylation of substrate proteins induced by the antiidiotype antibody Ac146. The mIgM-producing subclone 7-14 (lanes *1–5*) and the mIgD-producing subclone 7-1 (lanes *6–10*) were incubated for the indicated times with 20 μg/ml of the antiidiotype antibody Ac146. Tyrosine-phosphorylated proteins were detected by Western blotting. Molecular weight markers (*Kd*) and the major substrate proteins are indicated on the left and right sides of the figure, respectively.

lation than cross-linking with the monoclonal anti-IgD antibody. This could be due to the fact that the multivalent antigen NIP_{10}-BSA can induce a larger BCR aggregation on the B cell surface than the divalent anti-IgD antibody. Alternatively, this difference may be due to the different orientations with which these two cross-linking reagents bind to the receptor. The anti-IgD antibody is directed against the CH1 domain and should bind the BCR from the side, whereas the antigen is occupying the hapten-binding site at the top of the receptor. To distinguish between these possibilities, we used the antiidiotypic antibody Ac146 as a cross-linking reagent. The binding of Ac146 to the BCR is inhibited by the free hapten NIP-cap (51). The antibody Ac146 is, thus, complementary to the hapten-binding site and should cross-link the BCR with the same orientation as the antigen NIP_{10}-BSA, but divalently. Cross-linking the mIgD-BCR with Ac146 resulted in a strong PTK substrate phosphorylation, which reached its maximum at 60 min (Fig. 5, lanes *6–10*) and, thus, was similar to the phosphorylation invoked by antigen. This result indicates that not only the extent, but also the mode of cross-linking, is influencing the signaling output from the IgD-BCR. Engagement of the IgM-BCR with Ac146 resulted in a PTK substrate phosphorylation which reached its maximum at 1 min (Fig. 5, lanes *1–5*), stressing again the difference in signal transduction from the two classes of antigen receptors upon exposure to the same cross-linking reagent.

Analysis of Chimeric BCRs with the Membrane Proximal C Domain and the Transmembrane Region of Either mIgD or mIgM. The mIgD and mIgM molecules expressed on the surfaces of J558L transfectants possess the same light chain and the same variable domain of VH but differ in their heavy chain constant and transmembrane regions. The constant re-

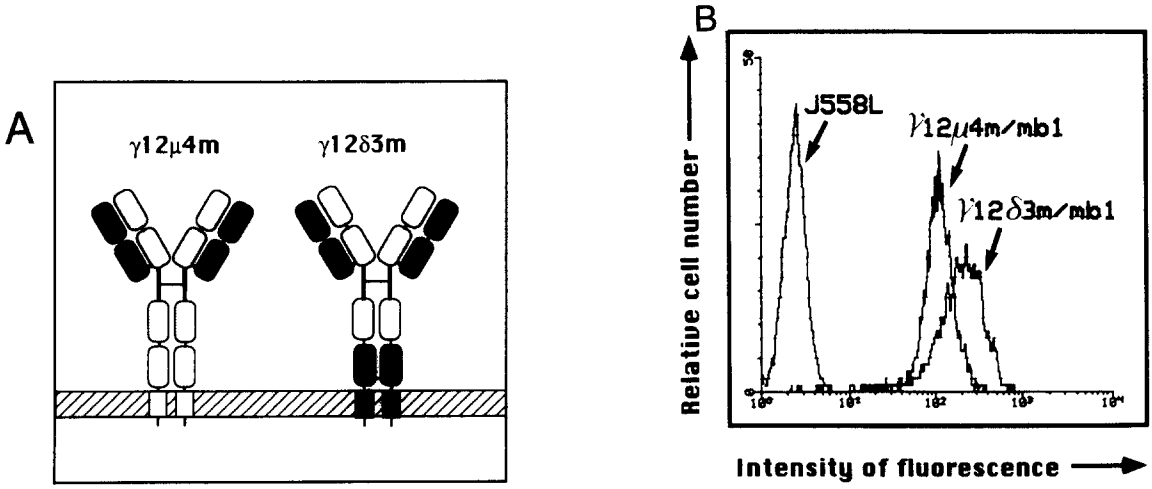


Figure 6. Expression of the chimerica IgG/M-BCR and IgG/D-BCR on the cell surface of J558L myeloma transfectants. (*A*) Domain architecture of the chimeric mIgG/M and mIgG/D molecules. The membrane proximal CH domain + transmembrane region of the μm and δm chain are drawn as white and black squares, respectively. The NH_2-terminal domains of the γ2a heavy chain and the two domains of the light chain are shown as grey and dark striped boxes, respectively. (*B*) The cell lines J558Lγ12μ4m/mb-1 and J558Lγ12δ3m/mb-1 were transfected with the chimeric heavy chain expression vectors pSVγ12μ4m and pSVγ12δ3m, respectively. The transfectants were incubated with FITC-coupled anti-mouse IgG antibodies and the amount of chimeric receptors on the surface of these cells determined by flow cytometry (FACScan®). Untransfected J558L cells were analyzed as controls in parallel.

gion of the μm chain contains four C domains (Cμ1–Cμ4), whereas the murine δm chain contains two C domains (Cδ1 and Cδ3) separated by a long hinge region. To determine whether these differences in the composition of the C domains are responsible for the different signaling kinetic of the IgM- and IgD-BCR, we constructed the expression vectors pSVγ12μ4m and pSVγ12δ3m. These vectors encode chimeric heavy chains consisting of the NIP-specific VH domain, the CH1 and CH2 domains of the γ2a heavy chain followed by the membrane proximal CH domain Cμ4 or Cδ3, and the transmembrane region of either μm or δm. The assembly of the chimeric heavy chains with the λ1 light chain and the Ig-α/Ig-β heterodimer results in the expression of antigen receptors with identical overall domain architecture (Fig. 6 *a*). The two chimeric BCRs are expressed on the cell surfaces of the transfectants J558Lγ12μ4m/mb-1 and J558Lγ12δ3m/mb-1 in similar amounts (Fig. 6 *B*). Incubation of J558Lγ12δ3m/mb-1 cells with 10 μg of the NIP_{10}-BSA for various times resulted in a PTK substrate phosphorylation which reached its maximum at 10 min and persisted well over 240 min (Fig. 7, lanes *6–10*). Under the same experimental condition, the PTK substrate phosphorylation in J558Lγ12μ4m/mb-1 cells reached its maximum in 1 min and declined after 60 min (Fig. 7, lanes *1–5*). A similar kinetic of substrate phosphorylation was observed when the two different transfectants were incubated with an anti-IgG antiserum instead of antigen (data not shown). Thus, although the chimeric IgD- and IgM-BCRs induce a less intensive tyrosine phosphorylation than their wild-type receptors. This result shows that the sequence differences in the membrane proximal C domain and/or transmembrane region, rather than the different C domain architecture, is responsible for the signaling differences between IgM- and IgD-BCR.

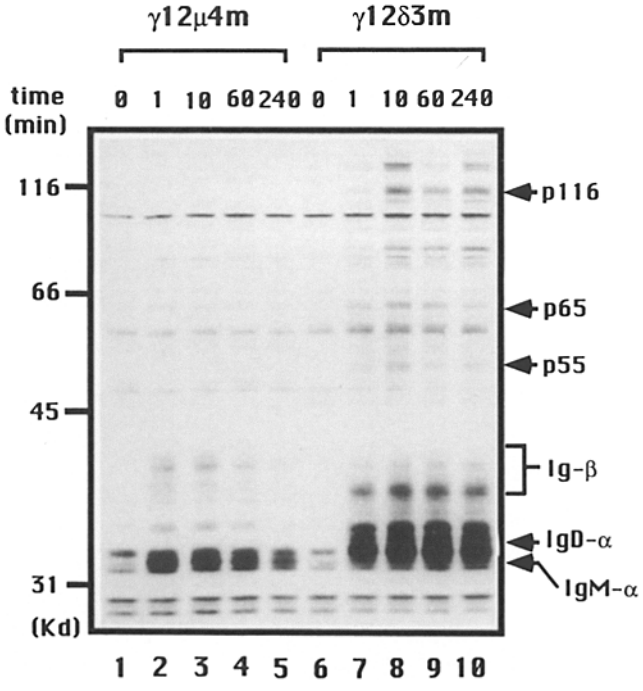


Figure 7. Kinetics of tyrosine phosphorylation of substrate proteins induced by the antigen-mediated cross-linking of the chimeric IgG/M-BCR and IgG/D-BCR. The transfectants J558Lγ12μ4m/mb-1 (lanes *1–5*) and J558Lγ12δ3m/mb-1 (lanes *6–10*) were incubated for the indicated times with 10 μg/ml of NIP10-BSA. Tyrosine-phosphorylated proteins were detected by Western blotting. Molecular weight markers (*Kd*) and the major substrate proteins are indicated on the left and the right sides of the figures, respectively.

Discussion

Our analysis of B cell lines carrying the IgM-BCR or IgD-BCR with the same antigen specificity showed that, while the initial response from these receptors is quite similar, the duration of this response differs between the two receptors. More specifically, cross-linking of both receptors results in the activation of the same PTKs as indicated by the phosphorylation of the same substrate proteins. The phosphorylation induced via the IgM-BCR, however, reaches its maximum 1 min after exposure to antigen and declines soon thereafter, whereas under the continuous exposure to antigen, IgD-BCR remains active and can signal for a prolonged period.

This finding may shed light on the ongoing controversy whether or not these two classes of BCR differ in their signal transduction capabilities. It has been shown that the early events in signal transduction like the PTK activation (31, 32, 37) and the release of Ca^{2+} ion (52) are quite similar between the two receptors. However, the cellular responses of activated B cells, such as tolerance induction, apoptosis, or interleukin secretion, can be quite different depending on whether the cells were stimulated via the IgM-BCR or the IgD-BCR. Specifically, engagement of IgM on immature B cells results in their deletion, whereas engagement of the IgD-BCR on these cells does not have this effect (40). Similarly, data from δm-transfectants of the WEHI231 (36) or CH33 (53) cell lines showed that cross-linking of the IgM-BCR, but not of the IgD-BCR, can activate an apoptosis program in these cells. It is conceivable that the apoptotic pathway in these B cells is inhibited by the more prolonged PTK activity which we have detected after engagement of the IgD-BCR. Recently, Roes and Rajewsky (41) reported that, in mIgD knockout mice, the production of high affinity antibodies against T cell–dependent antigens is delayed in the early primary response. The long-lasting signals from the mIgD-BCR may, therefore, also be required for the efficient selection of high affinity B cells.

The different behavior of the IgM and IgD antigen receptors on B cell lines is reminiscent of that which is observed in tyrosine kinase receptors (TKR) on the neuronal cell line PC12. These cells express two types of TKR, namely the epidermal growth factor receptor (EGFR) and the neuronal growth factor receptor (NGFR). Signal transduction from the two receptors involves the same signaling pathway; yet, the outcome of this signal is different, resulting either in growth or in neural differentiation. How the two receptors could achieve these differential responses was a puzzle (54). Two recent publications (55, 56) provide an answer to this riddle by showing that the activation of the NGFR results in a stronger and more prolonged microtubule-associated protein 2 kinase ac-

tivation than that resulting from the activation of the EGFR. Furthermore, after overexpression of the EGFR or mutation of serines in the cytoplasmic part of the EGFR involved in feedback regulation the signal from this receptor no longer resulted in growth but in the neuronal differentiation of PC12, thus giving the same result as signal transduction via the NGFR. These experiments clearly demonstrate that an increase and prolongation of signals can dictate the outcome of the signal.

The different kinetics of PTK activity observed in IgM-BCR or IgD-BCR–expressing B cells cultured in the presence of antigen suggest that the IgM-BCR and the IgD-BCR are under a different feedback control. Such a control can operate either via the downmodulation of the receptor and/or via the silencing of the receptor. A flow cytometric analysis of J558L transfectants cultured for 2 h in the presence of antigen showed a similar (three- to fivefold) reduction of the IgM-BCR as well as of the IgD-BCR on the surface of these cells (data not shown). The differences in signal transduction from the two classes of BCR are, therefore, not due to a preferential downmodulation of one of the two receptors. This leaves the silencing of the IgM-BCR as a possible mechanism for the short-term activation of this receptor. The molecular details of this silencing process are not clear at present. It is possible that the serine/threonine phosphorylation which occurs at the cytoplasmic tail of Ig-α and Ig-β (57) is involved in this process. A model for such a feedback regulation is the TKR c-kit and EGFR whose signal emission is altered by protein kinase C-mediated serine phosphorylation (58, 59).

Our data could be explained by a scenario whereby antigen-mediated cross-linking of the IgM-BCR results in a defined BCR aggregation, which allows the signaling and silencing process to operate, whereas the aggregated IgD-BCR can activate only the former process. How the two BCRs which have identical cytoplasmic parts can form functionally different aggregates remains to be elucidated. By the study of chimeric BCRs, we have excluded the possibility that the different domain architecture of the δm and μm heavy chain is responsible for this different behavior, and have shown that only the last (membrane proximal) C domain and/or the transmembrane domain is required for the class-specific function of these receptors. The same structures have previously been implicated as contact sites between the heavy chain and the Ig-α/Ig-β heterodimer. It is, thus, possible that mIgM and mIgD interacts in different ways with the Ig-α/Ig-β heterodimer and that this alters the aggregation and signal transduction of the two receptors. Alternatively, proteins other than Ig-α and Ig-β may interact with the two BCRs in a class-specific manner and influence their signal transduction. Recently, four new BCR-associated proteins (BAP) were identified (48, 60): BAP37 and BAP32, which are exclusively associated with the mIgM molecule, and BAP29 and BAP31, which are preferentially associated with the mIgD molecule. These novel membrane proteins are associated with the transmembrane part of the mIgM or mIgD molecule. At present, however, it is not clear whether the BAP/mIg complexes are formed only intracellularly or also on the cell surface. If the latter is true, the BAPs may influence the signal transduction from the BCR in a class-specific manner.

The functional difference between the IgM-BCR and IgD-BCR is more obvious when the two receptors are cross-linked by antigen or antiidiotypic antibody than by class-specific antibodies. This may be the reason why these differences have remained undetected so far since most previous studies used the latter reagents. Our results are in line with studies from other receptors which show that most antireceptor antibodies can only imperfectly mimic the ligand–receptor interaction (61). Apparently, for efficient signal induction to occur, the BCR has to be cross-linked with a precise orientation. For the antigen or for idiotype-specific antibodies which bind the receptor from the top, this criterium may be easier to fulfill than for isotype-specific antibodies which bind the receptor from the side. Indeed, from the three anti-δ mAb we tested, only one was able to cross-link the IgD-BCR in such a way that PTKs were induced. Clearly, more remains to be learned about the precise topology of the signal-emitting BCR aggregate and about the differences between the IgM-BCR and IgD-BCR complexes.

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