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Immunology

Evidence for a preformed transducer complex organized by the B cell antigen receptor

(tyrosine phosphatase/tyrosine kinase/signal transduction)

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ABSTRACT **The B cell antigen receptor (BCR) consists of the membrane-bound immunoglobulin (mIg) molecule and the Ig-α/Ig-β heterodimer, which functions as signaling subunit of the receptor. Stimulation of the BCR activates protein tyrosine kinases (PTKs) that phosphorylate a number of substrate proteins, including the Ig-α/Ig-β heterodimer of the BCR itself. How the PTKs become activated after BCR engagement is not known at present. Here, we show that BCR-negative J558L cells treated with the protein tyrosine phosphatase inhibitor pervanadate/H_2O_2 display only a weak substrate phosphorylation. However, in BCR-positive transfectants of J558L, treatment with pervanadate/H_2O_2 induces a strong phosphorylation of several substrate proteins. Treatment with pervanadate/H_2O_2 does not result in receptor crosslinking, yet the pattern of protein phosphorylation is similar to that observed after BCR stimulation by antigen. The response requires cellular integrity because tyrosine phosphorylation of most substrates is not visible in cell lysates. Cells that express a BCR containing an Ig-α subunit with a mutated immunoreceptor tyrosine-based activation motif display a delayed response. The data suggest that, once expressed on the surface, the BCR organizes protein tyrosine phosphatases, PTKs, and their substrates into a transducer complex that can be activated by pervanadate/H_2O_2 in the absence of BCR crosslinking. Assembly of this preformed complex seems to be a prerequisite for BCR-mediated signal transduction.**

The B cell antigen receptor (BCR) is composed of multiple subunits. Antigen bound by transmembrane immunoglobulins (mIg) of different classes transmits an activation signal to the cell interior through the mIg-associated Ig-α/Ig-β heterodimer (1, 2). The immunoreceptor tyrosine-based activation motif (ITAM) (3) plays a critical role in this process and is responsible for the communication of the BCR with cytoplasmic protein tyrosine kinases (PTKs) (4–8). The amino acid sequence of ITAM is $DEx_6D/ExxYxxLx_7YxxI/L$ and a single copy of the motif is present in the cytoplasmic part of both Ig-α and Ig-β. The current model of BCR signaling predicts that crosslinking of the BCR results in activation of the Src-related PTKs, Lyn, Blk, Fyn, and/or Lck (9–11), which first phosphorylate one or two tyrosine residues of the ITAMs of both Ig-α and Ig-β (5). This in turn leads to the SH2-mediated recruitment of the PTK Syk from the cytosol to the receptor and its subsequent activation (6–8). Thereafter, a number of additional effector molecules and PTK substrates like HS1 (12–14) and SHC (13, 15) are recruited into the complex and become activated. Indeed, the use of PTK inhibitors, coimmunoprecipitation studies, and gene targeting experiments have shown that PTK activity is essential to BCR signaling for both early and late events (16–21). However, it is a matter of debate whether the unligated BCR is already associated with one or more PTKs and which PTK is the first to become activated. A direct structural analysis of the resting and/or activated BCR is hampered by the fact that lysis of cells by detergent can disrupt important interactions between the receptor and intracellular proteins. This makes it difficult to know whether the small number of BCR molecules (<5%) that are associated with PTKs before receptor stimulation (17) reflects a low stoichiometry of association or whether this small number is a consequence of the experimental procedures.

The myeloma cell line J558L was used previously to identify the signaling components of the BCR (22–24), to functionally characterize the ITAM (5), and to analyze the role of CD45 (25). Like other myelomas, the J558L line has switched off many genes for surface markers on B cells, including CD19, CD20, CD21, CD22, CD40, and CD45. Crosslinking of the BCR on mIg/mb-1 transfectants of this line, however, still results in a rapid PTK activation and phosphorylation of substrate proteins, many of which become also phosphorylated in B lymphoma cell lines and normal B cells (26).

Treatment of living cells with pervanadate/H_2O_2 inhibits protein tyrosine phosphatases (PTPs), thereby modifying the intracellular equilibrium between dephosphorylation and phosphorylation (27–31). In the present study, we have used pervanadate/H_2O_2 to activate protein tyrosine phosphorylation in J558L and different transfectants of J558L. We describe here that only BCR-positive transfectants of this line responded to the drug with a rapid and efficient protein tyrosine phosphorylation.

MATERIALS AND METHODS

Cell Culture. The J558L myeloma cells and its transfectants were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 units/ml penicillin, 50 mg/ml streptomycin, and 200 mM 2-mercaptoethanol. Surface IgD-positive transfectants of J558L are 558Lδm7.1, expressing wild-type IgD-BCR (32), and J558Lδm2.6, expressing a glycosylphosphatidylinositol-linked form of IgD (33). The J558Lμm15–25 cells express the mIgM molecule intracellularly but not on the cell surface (34). Surface IgM-positive transfectants are J558Lμm3, which expresses a wild-type IgM-BCR (34), and J558LM1, J558LM4, and J558LM6, which produce an IgM-BCR with a mutated Ig-α subunit (5). The IgM molecule of J558LμTM cells contains the transmembrane part of the major histocompatibilty complex class I protein and is transported onto the cell surface independently of Ig-α and Ig-β (35). The J558Lμs cells secrete the IgM molecule (34). The transfectants J558Lγ2am/mb1 and J558Lγ2am produce mIgG2a that is expressed on the

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Abbreviations: BCR, B cell antigen receptor; ITAM, immunoreceptor tyrosine-based activation motif; mIg, membrane-bound form of immunoglobulins; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; NIP, hapten 4-hydroxy-5-jodo-3-nitrophenylacetyl.

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cell surface either as wild-type IgG2a-BCR or independent of Ig-α/Ig-β (36). All mIg molecules of the different cell lines carry the antigen-binding site of the B1–8 idiotype and are thus specific for the hapten 4-hydroxy-5-jodo-3-nitrophenylacetyl (NIP).

Stimulation of Cells. For stimulation of the cells with pervanadate/H_2O_2, 1 ml of a 20 mM sodium orthovanadate stock solution was mixed with 330 μl of 30% H_2O_2 and incubated for 5 min at room temperature, yielding a solution of 6 mM pervanadate plus remaining H_2O_2. To stimulate cells with 25 μM pervanadate/H_2O_2, the prepared solution was diluted 1:5 with H_2O and 5 μl was added to 1–5 $\times$ 10^7 cells suspended in 1 ml RPMI 1640 medium without supplements. As pervanadate/H_2O_2 is unstable, a fresh solution was prepared each time. The sodium orthovanadate stock solution, which is also unstable, was titrated from time to time to ensure optimal results. BCR crosslinking was induced by incubating the cells with 10 μg/ml NIP-coupled bovine serum albumin (NIP_7-BSA). All stimulations were carried out at 37°C. Approximately 20 sec before the end of the desired stimulation time, cells were pelleted and lysed for 25 min on ice in 1 ml lysis buffer consisting of 137 mM NaCl, 50 mM Tris·HCl (pH 7.8), 10% vol/vol glycerol, 1 mM sodium orthovanadate, 2 mM EDTA (pH 8.0), 1% vol/vol Nonidet P-40, plus protease inhibitors leupeptin and aprotinin (10 μg/ml each), and 1 mM phenylmethylsulfonyl fluoride. Cell debris were spun down for 20 min at 16,500 $\times$ *g* at 4°C and cleared cellular lysates were analyzed by anti-phosphotyrosine immunoblotting using the 4G10 antibody (Upstate Biotechnology, Lake Placid, NY) as described (13).

***In Vitro* Kinase Assay.** To stimulate protein tyrosine phosphorylation in cellular lysates, 1–5 $\times$ 10^7 cells were lysed in 1 ml 50 mM Tris·HCl (pH 7.4), 5 mM $MnCl_2$, 0.1 mM $NaVO_3$, and protease inhibitors, and the lysates were cleared as described above. ATP was added to a final concentration of 250 μM and the samples were incubated at 37°C for 15 min in the presence of either recombinant PTKs obtained from baculovirus-infected insect cells or with various concentrations of pervanadate/H_2O_2. The reactions were stopped by adding Laemmli's sample buffer and boiling for 4 min at 95°C.

RESULTS

The Pervanadate/H_2O_2-Induced Phosphorylation of Most PTK Substrates in J558L Requires BCR Expression. As previously shown (27–31), treatment of cells with pervanadate/H_2O_2 inhibits PTPs and results in an increased tyrosine phosphorylation of PTK substrate proteins. When BCR-negative (−) J558L myeloma cells are incubated for 2 min with increasing doses of pervanadate/H_2O_2 (Fig. 1*a*) only two proteins, p100 and p120, become tyrosine phosphorylated. This phosphorylation event is first detected at a concentration of 25 μM pervanadate/H_2O_2 (Fig. 1*a*, lane 7) and increases slightly with higher doses of pervanadate/H_2O_2 (lanes 9, 11, and 13). In the IgD-BCR positive (+) transfectant J558Lδm7.1, even low doses of pervanadate/H_2O_2 activate tyrosine phosphorylation and additional substrates were detected. Apart from p100 and p120, substrate proteins of 80, 72, 65, 55, and 35 kDa are phosphorylated only in the transfectant. Using PTK specific antibodies, p72 and p55 were identified as Syk and Lyn, respectively (data not shown). The p35 phosphoprotein was recognized by the tandem SH2 domains of Syk (data not shown), which are known to bind specifically to tyrosine-phosphorylated Ig-α (7). Note that a dose as low as 5 μM pervanadate/H_2O_2 induces weak phosphorylation of p65 (lane 4), whereas phosphorylation of p100, p120, and p80 requires 10 μM (lane 6) and that of Syk, Lyn, and Ig-α requires 25–50 μM of pervanadate/H_2O_2 (lanes 8 and 10). Phosphorylation of all of the substrates was further enhanced at higher doses of pervanadate/H_2O_2 (lanes 12 and 14). This shows that substrates in the BCR-positive J558Lδm7.1 transfectants are more sensitive to PTK phosphorylation induced by pervanadate/H_2O_2 than the BCR-negative J558L parental cells.

A time course experiment reveals further differences between the two cell lines studied (Fig. 1*b*). In J558L cells incubated with 25 μM pervanadate/H_2O_2, the phosphorylation of p100 and p120 requires an incubation time of at least 2 min (lane 9), whereas in J558Lδm7.1 cells, phosphorylation of p100, p120, and p65 is already visible 10 sec after stimulation (lane 4). Phosphorylation of the other PTK substrates in J558Lδm7.1 such as p80, Syk, Lyn, and Ig-α requires a longer incubation with pervanadate/H_2O_2 (lanes 6, 8, and 10). Maximal phosphoprotein content is reached 3 min poststimulation (lane 12) and gradually declines thereafter (lanes 14, 16, and 18). Together, these results demonstrate that in pervanadate/H_2O_2-treated J558L cells, PTK substrate proteins are phosphorylated either very inefficiently (in the case of p100 and p120) or not at all (in the case of p65, p80, Syk, Lyn, and Ig-α) unless these cells express a BCR on their cell surface. Thus,

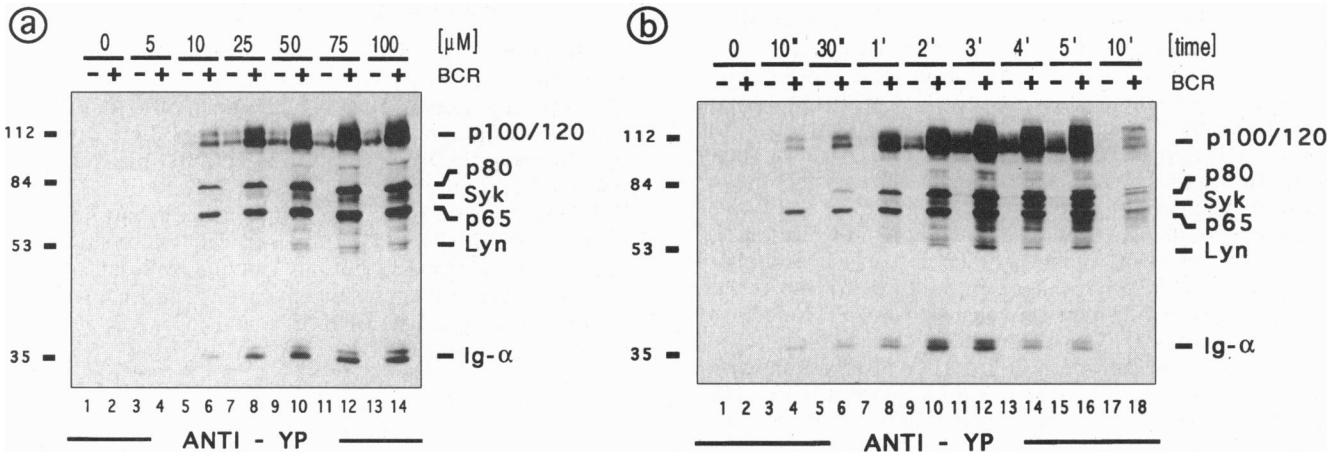

FIG. 1. Pervanadate/H_2O_2 induces a strong and rapid PTK substrate phosphorylation only in BCR-expressing J558L cells. (*a*) Dose response of pervanadate/H_2O_2-induced protein tyrosine phosphorylation in the BCR-negative (−) J558L parental cells (lanes 1, 3, 5, 7, 9, 11, and 13) and in the IgD-BCR positive (+) transfectant J558Lδm7.1 (lanes 2, 4, 6, 8, 10, 12, and 14). Approximately 1 $\times$ 10^7 cells per ml in RPMI 1640 medium without supplements were stimulated for 2 min at 37°C with the indicated amounts of pervanadate/H_2O_2 and than lysed in 1% Nonidet P-40. Cleared cellular lysates were analyzed by anti-phosphotyrosine immunoblotting. Each lane contains proteins obtained from 5 $\times$ 10^5 cells. Numbers on the left indicate the relative molecular mass in kDa. Prominent PTK substrate proteins are indicated on the right. (*b*) Time course of pervanadate/H_2O_2-induced protein tyrosine phosphorylation in J558L (lanes 1, 3, 5, 7, 9, 11, 13, 15, and 17) and J558Lδm7.1 (lanes 2, 4, 6, 8, 10, 12, 14, 16, and 18). The cells (1 $\times$ 10^7 cells per ml) were stimulated for the indicated times with 25 μM pervanadate/H_2O_2 at 37°C.

expression of a BCR in J558L seems to result into a reorganization of PTKs and their substrate proteins. This would mean that there is a transducer complex organized by the BCR, which can be rapidly activated by pervanadate/H_2O_2 only in BCR-positive J558L cells. It is worth noting that we did not find any biochemical evidence for a direct effect of pervanadate/H_2O_2 on the BCR itself. First, incubation of BCR-positive B cells with different doses of pervanadate/H_2O_2 did not result in patching or capping of the BCR, which was easily detected after crosslinking of the BCR with anti-μ antibodies (data not shown). Second, upon incubation of soluble IgM or IgG with doses of pervanadate/H_2O_2 up to 100-fold higher than those used in our stimulation protocol, no covalent linkage between the antibody molecules could be detected by a subsequent Western blot analysis (data not shown). Thus, pervanadate/H_2O_2 does not lead to a linkage of BCR molecules that could account for receptor crosslinking and aggregation.

The Efficient Response to Pervanadate/H_2O_2 Depends on Ig-α/Ig-β Expression and the ITAM Sequence. The phenomenon of BCR-dependent substrate phosphorylation was analyzed in different Ig-transfectants of J558L (Table 1) stimulated for 2 min with 100 μM pervanadate/H_2O_2 (Fig. 2). The same set of BCR-dependent substrate proteins efficiently phosphorylated in the IgD-BCR-positive J558Lδm7.1 (lane 4) are also seen in the IgM-BCR-positive J558Lμm3 cells (lane 8). However, other Ig transfectants of J558L that lack Ig-α expression are similarly to the J558L parental cells (lane 2) in that only p100 and p120 become phosphorylated. These transfectants are J558Lδm2.6 (lane 3), which express a glycosylphosphatidylinositol-linked IgD molecule, J558Lμs (lane 5), expressing the secreted form of IgM, J558Lμm15–25 (lane 6), which produces mIgM molecules that are not transported to the cell surface due to the lack of Ig-α, and J558LμTM (lane 7), which expresses a cell surface chimeric mIgM protein containing the transmembrane part of the H2-K protein. The mIgG2a molecule can be expressed either alone or as an IgG2a-BCR in association with the Ig-α/Ig-β heterodimer on the cell surface of J558L (24, 36). The two forms of IgG2a are expressed on the transfectants J558Lγ 2am and J558Lmb1/γ 2am, respectively. After treatment of these transfectants with pervanadate/H_2O_2, only the latter shows phosphorylation of p80, p65, and Ig-α (lane 13). This confirms that the Ig-α/Ig-β heterodimer is required for the pervanadate/H_2O_2-induced phosphorylation of these substrate proteins.

Ig-α and Ig-β both carry an ITAM sequence that mediates the interaction between the BCR and intracellular signaling molecules and that may thus be involved in the organization of the BCR transducer complex. We therefore analyzed the transfectants J558LM1, J558LM4, and J558LM6, which produce a mutated Ig-α subunit. J558LM1 and J558LM4 express an Ig-α protein in which either both or only the first of the two ITAM tyrosines were changed to phenylalanine, respectively. In J558LM6, the Ig-α protein has an almost complete deletion of its cytoplasmic part. The mutated Ig-α proteins of these cells are expressed together with wild-type Ig-β as part of an IgM-BCR that is transported to the cell surface in amounts similar to the wild-type IgM-BCR in J558Lμm3 (data not shown). In response to pervanadate/H_2O_2, all BCR-dependent substrate proteins (apart from Ig-α) are still phosphorylated in the mutant cell lines (lanes 9–11). In J558LM1 cells, however, phosphorylation seems to be less efficient than in J558Lμm3 cells (compare lanes 8 and 9). To analyze the difference between the wild-type (w) and M1-mutated (m) IgM-BCR in more detail, we compared the kinetics of per-

Table 1. Phenotype of different J558L transfectants

J558L cell line	BCR components	Surface expression	Response to pervanadate/H_2O_2
Parental	λ1-light chain, Ig-β,	–	–
δm2.6	δ-heavy chain, λ1-light chain, Ig-β	Glycosylphosphatidylinositol-linked IgD	–
δm7.1	δm-heavy chain, λ1-light chain, Ig-α, Ig-β	Wild-type IgD-BCR	+
μs	secreted μ-heavy chain, λ1-light chain, Ig-β	–	–
μm15–25	μm-heavy chain, λ1-light chain, Ig-β	–	–
μTM	μ-heavy chain with transmembrane part of MHC-I, λ1-light chain, Ig-β	IgM without Ig-α/Ig-β	–
μm3	μm-heavy chain, λ1-light chain, Ig-α, Ig-β	Wild-type IgM-BCR	+
M1	μm-heavy chain, λ1-light chain, Ig-αM1,* Ig-β	IgM-BCR	Delayed
M4	μm-heavy chain, λ1-light chain, Ig-αM4,† Ig-β	IgM-BCR	+§
M6	μm-heavy chain, λ1-light chain, Ig-αM6,‡ Ig-β	IgM-BCR	+§
γ2am	γ2am-heavy chain, λ1-light chain, Ig-β	IgG2a without Ig-α/Ig-β	–
mb-1/γ2am	γ2am-heavy chain, λ1-light chain, Ig-β	Wild-type IgG2a-BCR	+

*Both tyrosine residues of the Ig-α ITAM are mutated to phenylalanine.
†First tyrosine residue of the Ig-α ITAM is mutated to phenylalanine.
‡Ig-α mutant, lacking 52 amino acids of its cytoplasmic part including the ITAM.
§Kinetic of tyrosine phosphorylation not tested.

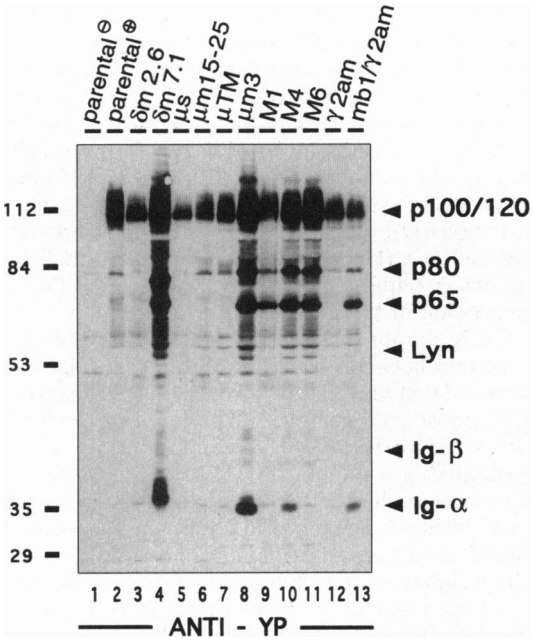

FIG. 2. The pervanadate/H_2O_2-induced phosphorylation of the BCR-dependent PTK substrates in J558L requires the expression of the Ig-α/Ig-β heterodimer. Substrate phosphorylation was induced with 100 μM pervanadate/H_2O_2 for 2 min at 37°C in J558L parental cells (lane 2) and different mb-1 and/or mIg transfectants of J558L (lanes 3–13). The transfectants express a wild-type or mutant BCR of different classes. Lanes: 3, glycosylphosphatidylinositol-linked mIgD; 4, wild-type IgD-BCR; 5, secreted form of IgM; 6, cytoplasmic mIgM without Ig-α; 7, mutated IgM with the extracellular part of IgM fused to the transmembrane part of MHC class I [H2-K^k]; 8, wild-type IgM-BCR; 9, IgM-BCR containing an Ig-α subunit with two Y>F mutations; 10, IgM-BCR containing an Ig-α subunit with one Y>F mutation; 11, IgM-BCR containing a deletion mutant of Ig-α that lacks the cytoplasmic part; 12, surface IgG2a without Ig-$\alpha\beta$; 13, wild-type IgG-BCR. For methods, see Fig. 1.

vanadate/H_2O_2-induced protein phosphorylation in J558Lμm3 and J558LM1 cells (Fig. 3). The substrate p65 is tyrosine phosphorylated within 10 sec of stimulation of J558Lμm3 cells (lane 3). Other substrate proteins become

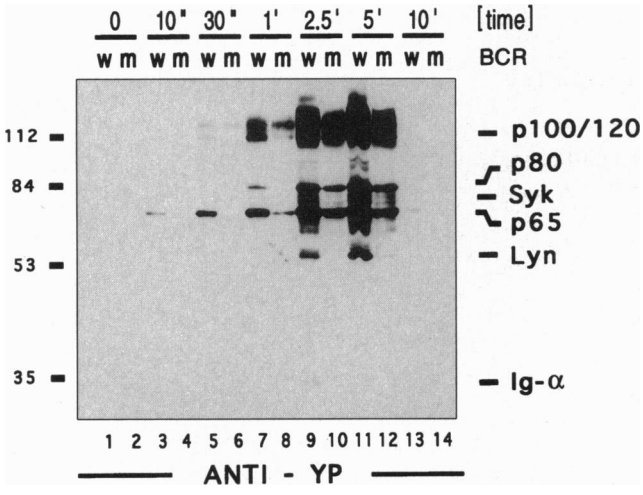

FIG. 3. A Y>F mutation of the two tyrosines of the Ig-α ITAM sequence delays the pervanadate/H_2O_2-induced substrate phosphorylation. J558Lμm3 cells expressing the wild-type IgM-BCR (w) (lanes 1, 3, 5, 7, 9, 11, and 13) and J558LμmM1 cells expressing an IgM-BCR with a mutated Ig-α (m) (lanes 2, 4, 6, 8, 10, 12, and 14) were stimulated with 25 μM pervanadate/H_2O_2 at 37°C for the indicated times. For methods, see Fig. 1.

phosphorylated after 30 sec (lane 5) and 1 min (lane 7). The substrate phosphorylation peaks at 2.5 to 5 min (lane 9 and 11) and declines with longer incubation periods (lane 13). This time course is very similar to that observed in J558Lδm7.1 cells (see Fig. 1*b*). The response of the J558LM1 mutant is significantly delayed in that p100/p120 is first phosphorylated after 30 sec (lane 6) and the phosphorylation of p65 and p80 requires 1 min (lane 8). These data show that an efficient pervanadate/H_2O_2-induced phosphorylation of BCR-dependent substrates require the wild-type sequence of the Ig-α ITAM. We thus conclude that the tyrosine residues of the Ig-α ITAM (and probably also those of the Ig-β ITAM) not only serve as PTK substrates after BCR triggering, but also have a function in the organization of the BCR transducer complex.

The Induction of BCR-Regulated Substrate Phosphorylation Requires Cellular Integrity. We next asked whether the phosphorylation difference between J558L and BCR-positive J558Lδm7.1 cells could also be observed when detergent lysates of these cells were incubated with pervanadate/H_2O_2. After incubation of intact cells (Fig. 4, lanes 3 and 8) and Nonidet P-40 lysates of these two cell lines (Fig. 4, lanes 4 and 9) with 100 μM of pervanadate/H_2O_2 for 10 min, most BCR-dependent PTK-substrates did not become phosphorylated in the cell lysates. In particular, phosphorylation of p120, p100, p65, and Ig-α is induced by pervanadate/H_2O_2 treatment in living J558Lδm7.1 cells but not in cell lysates (Fig. 4, compare lanes 8 and 9). However, one of the substrates (p80) was more strongly phosphorylated by postlysis pervanadate/H_2O_2 treatment of J558L and J558Lδm7.1 cell lysates than by treatment of the intact cells. Total cellular lysates of both cell lines were also subjected to an *in vitro* kinase assay using a

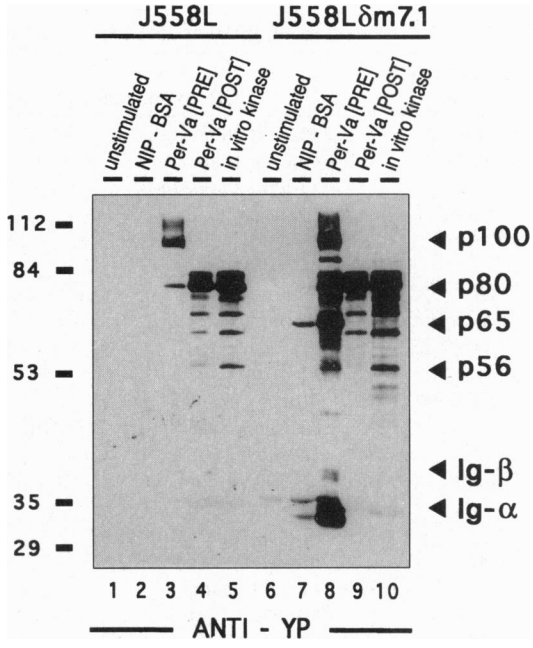

FIG. 4. Lysis of the cells by detergent prevents the pervanadate/H_2O_2 induced phosphorylation of most PTK substrates in J558L. J558L parental cells (lanes 1–5) and IgD-BCR expressing J558Lδm7.1 cells (lanes 6–10) were left untreated (lanes 1 and 6) or were stimulated for 2 min at 37°C with either 10 μg/ml NIP_7-BSA (lanes 2 and 7) or with 100 μM pervanadate/H_2O_2 (lanes 3 and 8). Alternatively, cleared cellular lysates from J558L cells (lanes 4 and 5) or from J558Lδm7.1 cells (lanes 9 and 10) were incubated for 2 min at 37°C with 100 μM pervanadate/H_2O_2 (lanes 4 and 9) or in an *in vitro* kinase assay for 10 min at 37°C in the presence of 100 μM ATP with a mixture of the baculovirus-expressed PTKs: Syk, Lyn, and Blk (lanes 5 and 10). The phosphoproteins from all samples were affinity-purified by Glutathion beads loaded with 10 μg/ml GST–SH2 fusion protein containing the SH2 domain of Src. For methods, see Fig. 1.

mixture of the baculovirus produced PTKs Syk, Lyn, and Blk. In this assay, nearly the same pattern of phosphoproteins was found as upon postlysis stimulation with pervanadate/H_2O_2 (Fig. 4, compare lanes 5 and 10 with lanes 4 and 9). In summary, using two different assays, we did not detect a difference in phosphorylation between the cell lysates of J558L and J558Lδm7.1. These experiments indicate that PTKs, and most of their substrates, are separated from each other in the lysate suggesting that the BCR-organized transducer complex is disrupted upon exposure to detergents. This may explain why such a complex has not been detected by a commonly used method, i.e., immunoprecipitation. Fig. 4 also shows that upon treatment of J558Lδm7.1 cells either with the antigen NIP-BSA (Fig. 4, lane 7) or with pervanadate/H_2O_2 (Fig. 4, lane 8), the same substrate proteins become phosphorylated, although the antigen-induced phosphorylation is much weaker than that induced by pervanadate/H_2O_2.

DISCUSSION

Using pervanadate/H_2O_2 to change the PTP-PTK balance inside living cells, we have found a striking difference between J558L parental cells and BCR-positive transfectants. Only BCR-positive cells respond to the drug with a rapid and efficient protein tyrosine phosphorylation. Thus, surface expression of the BCR may cause a redistribution of relevant PTKs and their substrates, presumably by organizing these proteins into a BCR transducer complex. In the absence of a BCR, these proteins seem to be separated from each other, thereby preventing a successful activation of the phosphorylation cascade. Because pervanadate and H_2O_2 act inside the cells without crosslinking the BCR, our results suggest that some of these signaling components are already associated with the resting (i.e., the unligated) BCR and constitute a preformed BCR-associated transducer complex.

A good candidate for a BCR transducer element is p65. It is the first PTK substrate to become phosphorylated after ligation of the BCR by antigen (26) as well as after treatment of BCR-positive J558L cells with pervanadate/H_2O_2. Its phosphorylation requires the presence of the Ig-α/Ig-β signaling subunits of the BCR. Furthermore, in cells expressing a BCR with a mutated Ig-α ITAM, the phosphorylation of p65 is delayed and less efficient. The residual phosphorylation of p65 in these cells may be due to the presence of endogenous Ig-β with its own ITAM sequence. Another putative BCR transducer element is p80 that, like p65, becomes tyrosine phosphorylated earlier than the PTKs Lyn and Syk. The late phosphorylation of Lyn and Syk in our assay suggests that these enzymes are secondary response elements rather than the primary transducer of the BCR. This is also supported by data from Lyn- or Syk-deficient cell lines (18) or knockout animals (19–21) showing that signal transduction from the BCR is not completely abolished by deletion of either of these molecules.

The finding that pervanadate/H_2O_2 induces BCR-dependent substrate phosphorylation only in living cells, but not in cell lysates, indicates that association between the BCR and its presumed transducer complex is not resistant to detergents. We have tested many different detergents, including digitonin, and none of them preserved the BCR-dependent substrate phosphorylation. Most strikingly, only a weak tyrosine phosphorylation of Ig-α and Ig-β could be detected, even in the presence of recombinant Lyn, Blk, and Syk. This may be the reason why a BCR-transducer complex could so far not be copurified with the BCR. However, pervanadate/H_2O_2 still induces some PTK substrate phosphorylation in the lysate, in particular p80 becomes more prominently phosphorylated in the lysate than in intact cells irrespective of whether or not the lysed cells express a BCR.

The BCR-dependent substrate phosphorylation observed in our experiments seems to contradict previous results in T-cell lines showing that the pervanadate/H_2O_2-induced tyrosine phosphorylation does not require surface expression of a T cell receptor TCR (28, 29). We have been able to reproduce these published results in our laboratory using both a TCR-positive and a TCR-negative variant of the human T cell line Jurkat (data not shown). Beside the TCR, however, the T cells studied may have several receptors whose transducer complex may become activated by pervanadate/H_2O_2. In contrast, the J558L myeloma line seems to lack many such receptors. Many B cell surface markers, such as CD19, CD20, CD22, CD23, and CD45, are not expressed on these cells and while all of the T cell lines as well as a number of different B cell lines respond to fetal calf serum treatment with the induction of protein tyrosine phosphorylation, no such response is observed in J558L cells (data not shown). The BCR-positive transfectants of J558L are thus useful cells to study the signaling function of the BCR without interference from other receptors.

The rapid onset of tyrosine-phosphorylation observed in activated B cells cannot easily be explained by diffusion and/or stimulation-dependent recruitment of signaling proteins to the BCR. If, however, most or all BCR-regulated signaling elements are already connected with the unligated BCR, their activation should be more rapid and more specific. Our concept of a preformed BCR transducer complex would thus fit with the observed kinetic of BCR signaling. Other receptors on the B cell surface might not be able to organize a transducer complex by themselves, but could use the BCR transducer complex once it is assembled by the BCR. For example, signaling through CD38 on B cells requires surface expression of a BCR together with Ig-α/Ig-β (37). Furthermore, in immature B cell lines, anti-CD38 antibodies induce tyrosine phosphorylation and activation of a subset of proteins similar to that induced with anti-IgM antibodies (38). Conversely, in some cases CD2, Thy-1 and Ly-6 require expression of the TCR on the T cell surface to transduce an activation signal (39).

The PTP1C tyrosine phosphatase has been implicated in negative feedback control to terminate signaling from the antigen-ligated BCR (40–42). Our finding that the PTP inhibitor pervanadate/H_2O_2 activates the transducer complex of the unligated BCR suggests that this complex is also under negative control of PTPs. This would imply that inhibition of PTPs is critically involved in the onset of BCR signaling while turning off the signal involves the activation of PTPs. Indeed, one or more PTP may be an integral component of the transducer complex as we could coprecipitate a PTP activity with the BCR from unstimulated J558Lμm3 cells (data not shown).

In summary, our studies suggest that our understanding of the initial events of BCR signaling is still fragmentary and that the role of PTKs and PTPs has to be critically reevaluated.

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