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Induction of the antigen receptor expression on B lymphocytes results in rapid competence for signaling of SLP-65 and Syk

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The binding of antigen to the B cell antigen receptor (BCR) results in the activation of protein tyrosine kinases (PTKs) such as Lyn and Syk, and the phosphorylation of several substrate proteins including HS1 and SLP-65. How these signaling elements are connected to the BCR is not well understood. Using an expression vector for a tamoxifen-regulated Cre recombinase, we have developed a method that allows the inducible expression of the BCR. Disruption of the VH leader reading frame of the immunoglobulin heavy chain by two *loxP* sites is overcome by Cre-mediated DNA recombination and results in the cell surface expression of the BCR starting 4 h after exposure of transfected B cells to tamoxifen. This method can, in principle, be employed for the inducible expression of any secreted or type I transmembrane protein. By monitoring the activation of signaling elements in pervanadate-stimulated B cells expressing different levels of the BCR, we show here that phosphorylation of SLP-65 and Syk, but not of Lyn, is strictly dependent on the expression of the BCR on the cell surface. These data suggest that the BCR reorganizes its signaling molecules as soon as it appears on the cell surface.

Keywords: Cre recombinase/inducible gene expression/ protein tyrosine kinase/PTK substrate proteins

Introduction

The B cell antigen receptor (BCR) is a multiprotein complex consisting of the membrane-bound immunoglobulin (mIg) heavy chain, the Ig light chain and the Ig-α/Ig-β heterodimer. Transport of the BCR to the cell surface is dependent on the expression and assembly of all four components. Cross-linking of the BCR leads to activation of protein tyrosine kinases (PTKs) such as Lyn and Syk, which in turn phosphorylate a number of substrate proteins, including HS1, SLP-65 and the Ig-α/Ig-β heterodimer (DeFranco, 1997; Kurosaki, 1997; Reth and Wienands, 1997). Ig-α and Ig-β are each phosphorylated at tyrosines of the immunoreceptor tyrosine-based activation motif (ITAM) (Reth, 1989; Cambier, 1995; Thomas, 1995). SLP-65 is a B cell-specific adaptor protein which forms a complex with Grb2 and other signaling elements (Wienands *et al*., 1998). This protein, also called BLNK, is a substrate of Syk and is involved in phospholipase Cγ (PLCγ) activation and the calcium response in stimulated B cells (Fu *et al*., 1998). T cells produce a similar adaptor protein called SLP-76 (Jackman *et al*., 1995; Motto *et al*., 1996). SLP-76-deficient mice have normal numbers of B cells but a complete block of pre-T cell development at the double-negative stage, indicating that this element is required for signal transduction from the pre-TCR but not the BCR (Clements *et al*., 1998; Pivniouk *et al*., 1998). A T cell line lacking the SLP-76 protein displays a reduced calcium response upon stimulation (Yablonski *et al*., 1998).

Exposure of B cells to either antigen or to the phosphatase inhibitor pervanadate induces the phosphorylation of the same PTK substrate proteins. The rapid phosphorylation of most PTK substrates detected in mIg transfectants of the J558L myeloma line upon exposure to pervanadate requires the expression of the BCR on the cell surface (Wienands *et al*., 1996). This suggests that the BCR organizes intracellular signaling elements into a transducer complex once it appears on the cell surface. One potential caveat of these experiments is that during the 4 weeks of selection following transfection of mIg heavy chain vector, the expression of signaling molecules could have been altered. Therefore, the gene expression of the BCR^+ transfectants may not be identical to that of the J558L parental line. To test the organizational role of the BCR, it would be desirable to express it in an inducible fashion on the surface of BCR-negative cells. Several systems for inducible gene expression have been established in recent years. These include the Tet-operator/repressor system (Gossen *et al*., 1994) and the hormone-regulated DNA recombinases Flp and Cre (Logie and Stewart, 1995; Metzger *et al*., 1995). Cre is a recombinase from the bacteriophage P1 which can mediate the deletion or inversion of DNA sequences lying between two *loxP* sites (Sauer, 1996), and has been used widely for tissue-specific or inducible deletion of genes from the mouse genome (Gu *et al*., 1994; Kuhn *et al*., 1995). We have constructed the expression vector pANMerCreMer encoding a chimeric Cre recombinase that is flanked on both sides (N- and C-terminal) by a mutated hormone-binding domain of the murine estrogen receptor (Mer) (Zhang *et al*., 1996). This domain no longer binds estrogen but does bind the antagonist 4-hydroxytamoxifen (OH-TAM) (Littlewood *et al*., 1995). In the absence of the ligand, the chimeric MerCreMer protein is complexed by the heat shock protein Hsp90 and retained in the cytoplasm (Figure 1A). Exposure of cells to OH-TAM releases the MerCreMer protein from Hsp90 (Scherrer *et al*., 1993). The MerCreMer protein is translocated into the nucleus where it can recognize *loxP* sites and recombine the DNA. We have used this method in J558L cells to regulate the expression of the μm heavy chain. The analysis of signaling elements upon induction of BCR expression in these cells supports the notion that the BCR organizes its signaling elements into a transducer complex as soon as it appears on the cell surface.

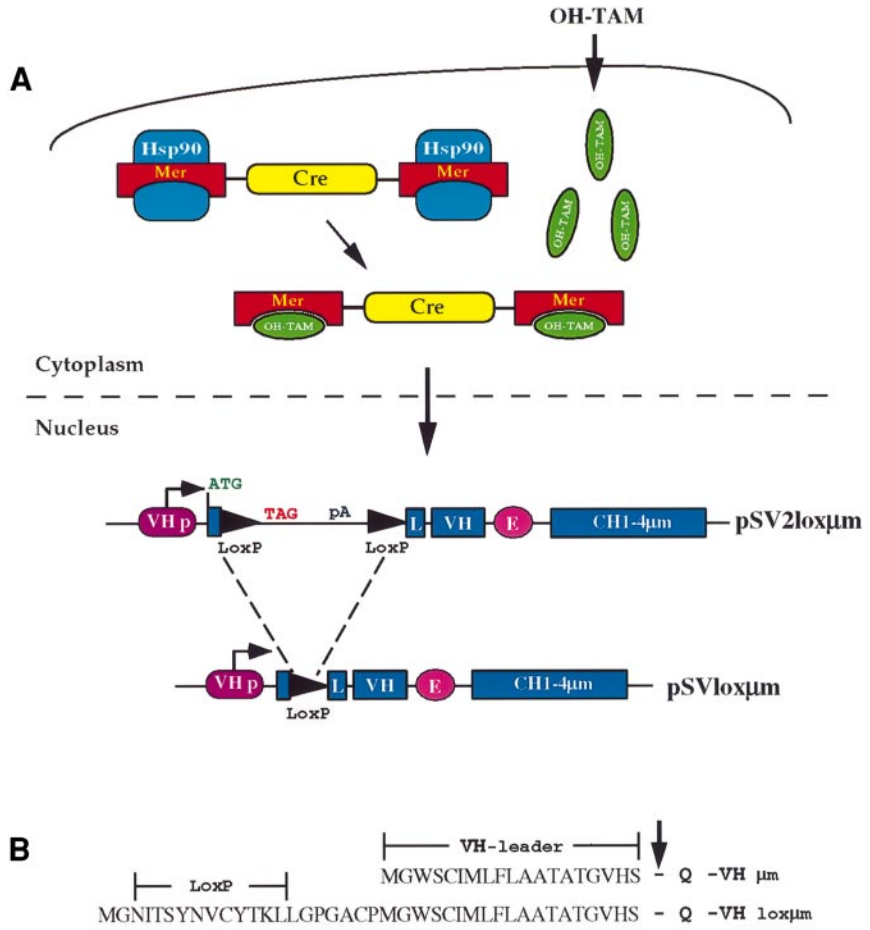

Fig. 1. Cre-mediated inducible expression of the μm heavy chain. (**A**) The B cell line J558Lmb-1 was co-transfected with the vectors pANMerCreMer and pSV2loxμm. In the latter vector, the reading frame of the VH gene is interrupted immediately after the start codon (ATG) by a *loxP*-flanked 441 bp DNA insert carrying a translational stop codon (TAG) and a poly(A) site (pA). In the absence of OH-TAM, the chimeric MerCreMer enzyme is bound by the heat shock protein Hsp90 and retained in the cytoplasm. Exposure of the transfected cells to OH-TAM results in the release of MerCreMer from the Hsp90 complex and its translocation into the nucleus. In turn, Cre recombines the two *loxP* sites of pSV2loxμm vector to generate pSVloxμm from which the μm chain can be expressed. The transcription of this vector is controlled by the VH promoter (VH p) and IgH intron enhancer (E). (**B**) The VH leader peptide sequence of the wild-type μm chain is altered in the loxμm chain by an N-terminal extension of 21 amino acids including the translated *loxP* sequence. This alteration does not prevent the targeting function of the leader which is removed (see arrow) after the loxμm chain is inserted into the ER. Thus a wild-type μm chain is produced by the pSVloxμm vector.

Results

A system for inducible BCR expression

To regulate the expression of the μm heavy chain, we constructed the vector pSV2loxμm in which the μm reading frame is interrupted by a 441 bp fragment inserted immediately after the ATG start codon of the VH leader sequence. This insert, called *loxP*-stop-pA-*loxP*, carries two *loxP* sites flanking a translational stop codon and a polyadenylation site (Figure 1A). Therefore, μm chains cannot be expressed from the pSV2loxμm vector unless a Cre-mediated recombination deletes the insert, leaving behind a *loxP* sequence which restores the μm reading frame. The recombined vector (pSVloxμm) expresses the μm chain with a 21 amino acid extended VH leader which is cleaved off once the protein is transported into the endoplasmic reticulum (ER) lumen (Figure 1B). Thus, a wild-type μm chain is produced from this vector.

The vector pSV2loxμm, in combination with the expression vector pANMerCreMer (Zhang *et al*., 1996) encoding the tamoxifen-regulated chimeric Cre enzyme, can be employed to express the μm chain in an inducible, tamoxifen-dependent fashion. The efficiency of this system was tested in the myeloma cell line J558Lmb-1 which expresses the λ light chain, Ig-β and Ig-α, but no Ig heavy chain. J558Lmb-1 cells were co-transfected with the pSV2loxμm and pANMerCreMer vectors. The transfectants were cultured for 3 days in the presence of 200 nM OH-TAM and analyzed by FACScan for surface expression of IgM-BCR. Thirteen out of 44 transfectants stained positive for IgM, and one transfectant (2loxμm-3) was chosen for further analysis.

Time course of Cre-mediated recombination and BCR expression

The 2loxμm-3 cells were cultured with 200 nM OH-TAM. At different time points, the cells were harvested and their genomic DNA was extracted. Cre-mediated recombination was detected by nested PCR with primers lying 5′ and 3′ of the two *loxP* sites of pSV2loxμm. The sizes of the unrecombined and recombined PCR products are 986 and 612 bp, respectively. The Cre-mediated recombination product was detected as early as 1 h after exposure to

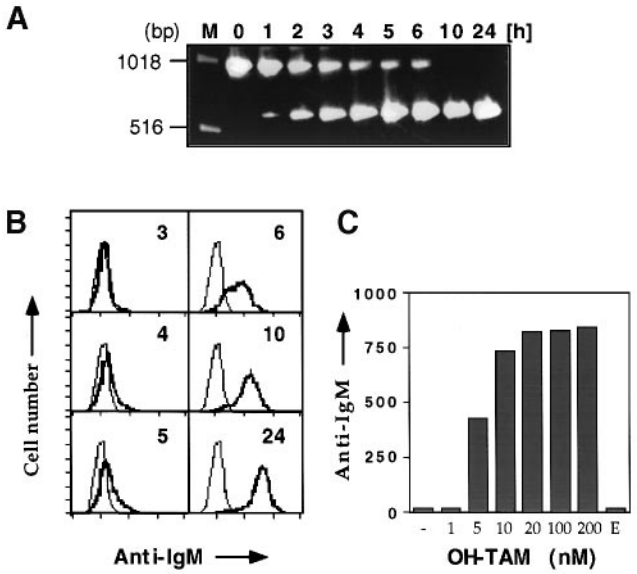

Fig. 2. Time course and dose–response of the MerCreMer-mediated BCR expression on the surface of 2loxμm-3 cells. (**A**) The extent of MerCreMer-mediated DNA recombination of the pSV2loxμm vector was determined by nested PCR. (**B**) The amount of BCR on the surface of 2loxμm-3 was assayed by flow cytometry using fluoresceinated goat anti-mouse Ig antibodies. The thick and thin lines show the cells cultured in the presence and absence of 200 nM OH-TAM, respectively, and for the indicated times (h) shown at the upper right corner of each profile. (**C**) Dose–response of the induced expression of the IgM-BCR on the surface of 2loxμm-3 cells cultured for 3 days with the indicated concentrations of OH-TAM. As a control, the cells were cultured either without OH-TAM (dash) or in medium containing 100 nM estrogen (E). For each culture, the mean value of IgM-BCR expression was determined by flow cytometry.

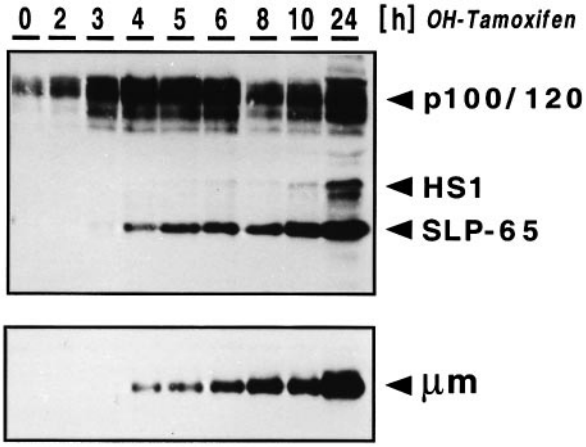

Fig. 3. Western blot analysis of PTK substrate phosphorylation and μm chain expression in pSV2loxμm cells cultured for the indicated time in the presence of 200 nM OH-TAM. The cells were then stimulated for 2 min with 10 μg/ml of the antigen NIP-BSA, followed by a 30 s treatment with 25 μM pervanadate. After size separation by SDS–PAGE on a 10% gel, the PTK substrate proteins and μm chain in the total cellular lysates were detected using the anti-phosphotyrosine antibody 4G10 (upper panel) and anti-μ antibody (lower panel). The location of the μm chain and of the dominant PTK substrate proteins p100, HS1 and SLP-65 is indicated.

OH-TAM (Figure 2A). After 3 h in the presence of OH-TAM, >50% of the pSV2loxμm vector DNA was recombined and after 10 h the recombination was complete. The 2loxμm-3 cells were analyzed in parallel for expression of the IgM-BCR on the cell surface (Figure 2B). The first BCR-positive cells were detected 4 h after exposure to OH-TAM. After 6–10 h of culture, more cells became BCR positive, expressing increasing amounts of the BCR on their surface. The high efficiency of the Cre-mediated recombination is indicated by the fact that after a 24 h culture with OH-TAM, nearly all 2loxμm-3 cells are BCR positive. Not all pSV2loxμm and pAN-MerCreMer co-transfectants of J558L/mb-1 showed this rapid and complete Cre-mediated recombination. The efficiency of this process seems to depend on the amount of MerCreMer expression (data not shown).

The amount of Cre-mediated recombination and subsequent BCR expression is not only dependent on the time in culture but also on the dose of OH-TAM, as indicated by the analysis of 2loxμm-3 cells cultured for 3 days with different doses of OH-TAM (Figure 2C). A similar level of BCR expression was detected in cultures with 20–200 nM OH-TAM, whereas partial and no BCR expression was seen in the 5–10 and 1 nM OH-TAM cultures, respectively. Identical results were obtained in the cells cultured with 200–1600 nM OH-TAM (data not shown). No BCR was detected on the surface of 2loxμm-3 cells cultured in 100 nM of estrogen, confirming that estrogen does not bind the mutated hormone-binding domains of MerCreMer (Figure 2C). In the following experiments, 200 nM OH-TAM was used to activate MerCreMer.

The pervanadate-induced tyrosine phosphorylation of SLP-65 is tightly coupled to BCR expression

We previously have identified SLP-65 as one of the most rapidly phosphorylated PTK substrate protein in antigen- or pervanadate-stimulated J558Lμm3 cells (Kim *et al.*, 1993; Wienands *et al.*, 1996). Furthermore, SLP-65 phosphorylation occurs only in stable BCR-positive transfectants of J558L (Wienands *et al.*, 1998). To demonstrate that this phosphorylation was directly dependent on the expression of BCR, we next analyzed the PTK substrate phosphorylation in 2loxμm-3 cells at different time points of BCR expression. The 2loxμm-3 cells were first cultured for different times in OH-TAM-containing medium and then stimulated with a combination of antigen and pervanadate. PTK substrate proteins were detected in the total cellular lysates of these cells by anti-phosphotyrosine immunoblotting (Figure 3, upper panel).

The analysis of PTK substrate proteins in stimulated 2loxμm-3 cells showed that phosphorylation of SLP-65 is tightly coupled to μm and BCR expression in these cells. The SLP-65 phosphorylation can be detected already after 4 h of exposure to OH-TAM when only minor amounts of the BCR are produced. Other PTK substrate proteins in 2loxμm-3 cells, such as the 80 kDa HS1 protein (Yamanashi *et al.*, 1993) and Ig-α (data not shown), require a much stronger expression of the BCR for their antigen- and pervanadate-stimulated phosphorylation (Figure 3, upper panel, 10 and 24 h lanes). The same Western membrane was also developed with anti-μ antibodies (Figure 3, lower panel). The μm chain is first detected in a 4 h OH-TAM culture of 2loxμm-3 cells, and its expression increases over the next 24 h. This data correlated well with the flow cytometric analysis (Figure 2B).

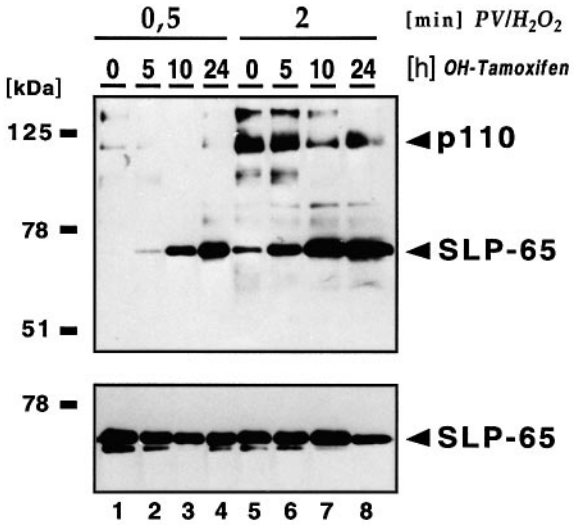

Fig. 4. Reorganization of intracellular signaling elements in IgM-BCR-expressing pSV2loxμm cells. The cells were cultured for the indicated times in the presence of 200 nM OH-TAM and stimulated for either 0.5 or 2 min with 25 μM pervanadate. Western blot analysis of Grb2-binding proteins was performed using the anti-phosphotyrosine antibody 4G10 (upper panel) or anti-SLP-65 antibody (lower panel). The proteins were purified from 0.5% NP-40 lysates of 2×10^7 cells using glutathione-coupled beads and GST fusion protein with the N-terminal SH3 and SH2 domains of Grb2.

Reorganization of intracellular signaling elements upon BCR expression

SLP-65/BLNK is constitutively associated with the adaptor protein Grb2 (Fu *et al*., 1998; Wienands *et al*., 1998). We employed a Grb2–GST fusion protein to purify Grb2-binding proteins from the NP-40 lysates of 2loxμm-3 cells cultured for various times with OH-TAM and stimulated with pervanadate for either 0.5 or 2 min. The purified proteins were size-separated by SDS–PAGE and analyzed for tyrosine phosphorylation (Figure 4, upper panel). In addition, SLP-65 protein was detected by Western blotting using anti-SLP-65 antibodies (Figure 4, lower panel). After 0.5 min of pervanadate stimulation, SLP-65 is the only detectable PTK substrate protein and its phosphorylation depends on the amount of BCR expression (Figure 4, upper panel, lanes 1–4). The 2 min pervanadate stimulation of 2loxμm-3 cells also reveals other PTK substrate proteins in the range 90–120 kDa. Interestingly, and in contrast to SLP-65, phosphorylation of these proteins is induced by pervanadate more strongly in 2loxμm-3 cells which do not produce BCR and declines with increasing BCR expression (Figure 4, upper panel, lanes 5–8). The 90/120 kDa proteins have not been detected previously in the analysis of total cell lysates of pervanadate-stimulated 2loxμm-3 cells because other, non Grb2-bound, PTK substrate proteins exist in this size range whose phosphorylation does not depend on expression of the BCR (Figure 3). The anti-SLP-65 Western blot shows that the total amount of SLP-65 protein in 2loxμm-3 cells does not change upon BCR expression (Figure 4, lower panel). However, this analysis revealed a smaller form of SLP-65, the amount of which decreases with increasing BCR expression. The disappearance of this form may be due to an altered migration of SLP-65 upon phosphorylation.

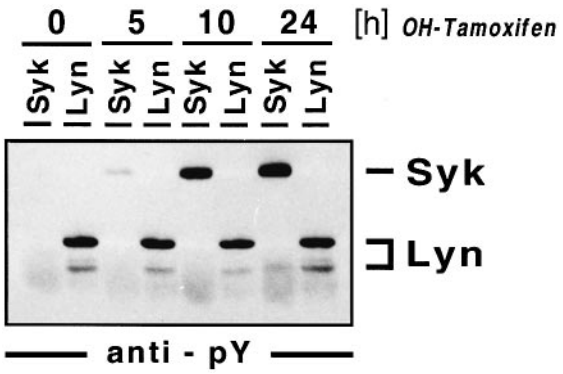

Fig. 5. Tyrosine phosphorylation of Syk but not of Lyn depends on BCR expression. The pSV2loxμm cells were cultured for the indicated times in the presence of 200 nM OH-TAM. The cells were then stimulated for 2 min with 25 μM pervanadate and lysed in 0.5% NP-40. The Syk and Lyn kinase subsequently were purified from the lysates using specific antibodies and size separated by SDS–PAGE on a 10% gel. The phosphorylated kinases were detected by Western blotting using the anti-phosphotyrosine antibody 4G10.

Tyrosine phosphorylation of Syk but not Lyn requires BCR expression

Lyn and Syk are PTKs which phosphorylate substrate proteins in antigen- or pervanadate-stimulated B cells (Kurosaki *et al*., 1994; Takata *et al*., 1994). These PTKs are themselves targets for tyrosine phosphorylation and, in the case of Syk, this phosphorylation correlates with its activity (Kimura *et al*., 1996). To test for BCR-dependent phosphorylation of these PTKs, we immunopurified them from the lysates of 2loxμm-3 cells cultured for different times with OH-TAM and stimulated for 2 min with pervanadate. The tyrosine phosphorylation of the two isoforms of Lyn (p53 and p56) in pervanadate-stimulated cells does not depend on the expression of the BCR (Figure 5). The reason for this may be that in the CD45-negative J558L cells, Lyn is constitutively phosphorylated at the C-terminal tyrosine, Y508, and neither recruited to the BCR nor activated upon antigen stimulation (Pao and Cambier, 1997). The phosphorylation of Syk, however, is absent in the BCR-negative 2loxμm-3 cells but becomes visible (Figure 5) after 5 h of exposure of the cells to OH-TAM, when the 2loxμm-3 cells hardly express BCR on their surface (Figure 2B). This shows that, as for SLP-65, the phosphorylation (and presumably activation) of Syk is also critically dependent on the expression of the BCR on the cell surface. It should be noted that phosphorylation of Syk cannot be seen in the analysis of the total cellular lysate (Figure 3) as Syk is only weakly expressed in J558L and its detection requires immunopurification.

Discussion

Efficiency of the Cre-mediated inducible expression system

We show here that the pervanadate-induced phosphorylation of intracellular signaling molecules such as SLP-65 and Syk requires expression of the BCR on the J558L cell surface. This analysis was made possible by the use of a hormone-regulated Cre enzyme controlling the expression of the BCR. Several methods for inducible activation of genes have been developed in recent years.

These are, for example, transcriptional repressors or activators such as the tetracycline (Tet) repressor of *Escherichia coli* (Gossen *et al*., 1994; Shockett and Schatz, 1996). With this system, genes can be tightly regulated and their transcription is induced $>10^5$-fold. However, expression of large amounts of the bacterial Tet repressor or activator is not tolerated by all eukaryotic cells, and especially mature B-lymphocytes seem to be particularly sensitive to these proteins (unpublished observation). Other methods use site-specific DNA recombinases such as Flp or Cre to control the expression of genes targeted with specific recognition sites (*Frt* or *loxP*) (Kuhn *et al*., 1995; Logie and Stewart, 1995; Akagi *et al*., 1997). In comparison with the Tet system, the latter has the disadvantage that DNA recombination in most cases is not reversible and therefore can be used only once to either switch on or switch off a targeted gene.

Cre is the most widely used site-specific DNA recombinase, preferred over Flp for its reliable activity at 37°C. Using promoters regulated by interferon-γ, metal ions or dexamethasone, the Cre transgene can be expressed in an inducible fashion (Kuhn *et al*., 1995). Other methods that control the activity rather than the expression of Cre employ fusion proteins of the enzyme with the hormone-binding domains of the estrogen receptor (Feil *et al*., 1996; Zhang *et al*., 1996; Schwenk *et al*., 1998). One advantage of a post-translational control of Cre is that it can be combined with the regulated expression by a tissue-specific promoter (Gu *et al*., 1994). In our analysis of MerCreMer transfectants of J558L, we noticed, however, that an efficient site-specific DNA recombination occurred only in those cells expressing high amounts of the Cre enzyme (data not shown). Apparently, a threshold exists under which there are too few Cre molecules in the nucleus to find the *loxP* sites of a targeted gene. This limits the tissue-specific expression of Cre to those promoters which ensure a high level expression of the Cre enzyme.

The Cre system is often used for the deletion of exons or whole genes which are flanked by two *loxP* sites. It can, however, also be employed for the activation of a gene whose reading frame is disrupted by an insert flanked by *loxP* sites (Ayral *et al*., 1998). A disadvantage of the latter method is that after a Cre-mediated recombination, a *loxP* sequence is left behind in the reading frame of the gene. This only allows the expression of an altered form but not of the wild-type protein. We have overcome this problem by targeting the sequence encoding the VH leader peptide of the μm heavy chain for Cre-mediated recombination. The VH leader with an N-terminal extension of 21 amino acids was functional and cleaved off after the transport of the μm chain into the ER lumen. Thus, the wild-type μm chains are produced from the recombined pSVloxμm vector. Our method could be used to control the expression of any secreted or type I membrane protein with an N-terminal leader sequence. However, we noticed that not all leader peptides remained functional after the insertion of a *loxP* sequence (unpublished observation). In these cases, one could use the VH leader described here to control expression of these proteins.

Transport of the BCR

In B cells producing a BCR constitutively, not all receptor molecules are expressed on the cell surface. A substantial amount is also found in intracellular compartments: ER, Golgi and lysosomes. The generation of these intracellular stores and the maintenance of a flow of receptor molecules between these compartments are not well understood. It was interesting, therefore, to analyze whether, after their *de novo* production, the BCR molecules would first fill up the internal stores or whether they would be transported directly to the cell surface. Our experiments suggest that the latter is the case. In fact, after 6 h of OH-TAM incubation, IgM-BCR is clearly detected on the cell surface by FACScan, although its expression level is only ~10% of that detected in the J558L transfectants constitutively expressing BCR. The levels of surface and total IgM increase in a comparable way from 6 to 24 h of OH-TAM incubation and reached maximal levels 2 days following exposure to OH-TAM (data not shown). These data suggest that the BCR is transported on the cell surface as soon as it is assembled in the ER and that the intracellular stores of this receptor accumulate only after a prolonged expression of the BCR in these cells. A comparison of the time course of μm gene recombination and BCR expression suggests that it takes 1–2 h from the transcription of the μm gene to the expression of the BCR on the cell surface. These data are consistent with pulse–chase experiments in radioactively labeled B cells showing that after a 2 h chase, BCR is expressed on the cell surface (Brouns *et al*., 1995).

The organizing role of the BCR

Many receptors must associate with intracellular proteins in order to signal. The lag time between the *de novo* expression of a receptor and its assembly into a signaling-competent complex is not very well studied. Using an inducible system, we found that the BCR already becomes signaling competent 1–2 h after its production, at a time when only minor amounts of this receptor are found on the cell surface. These experiments also showed that the phosphorylation of SLP-65 and Syk in prevanadate-stimulated 2loxμm3 cells is critically dependent on the expression of the BCR in these cells. This suggests that the phosphorylation of these proteins is regulated directly by the BCR. Interestingly, the SLP-65/BLNK adaptor protein is a specific substrate of Syk (Fu *et al*., 1998). The expression of these two proteins does not differ in BCR^+ and BCR^- J558L cells. Why, then, can Syk not phosphorylate SLP-65 in the absence of the BCR? Syk is activated upon binding of its two SH2 domains to the doubly phosphorylated ITAM (pITAM) sequence of Ig-α or Ig-β (Rowley *et al*., 1995). One possible scenario is, therefore, that only pITAM-bound Syk can recruit and phosphorylate SLP-65. However, in time course experiments, we have found that phosphorylation of the ITAM tyrosines occurs later (Kim *et al*., 1993; Wienands *et al*., 1996) and requires higher amounts of BCR on the cell surface than the phosphorylation of SLP-65. Furthermore, in BCR mutant J558L cells with a deletion of the cytoplasmic tail of Ig-α, no phosphorylation of the ITAM tyrosines of Ig-β can be detected, yet SLP-65 is still phosphorylated by Syk, albeit less efficiently (Flaswinkel and Reth, 1994). These observations may indicate that

Syk and SLP-65 can associate with the BCR in a pITAM-independent manner, but the clarification of this issue requires further studies.

In our analysis of Grb2-binding proteins isolated from 2loxμm-3 cells induced to express the BCR and stimulated for 2 min with pervanadate, we noticed substrate proteins in the range of 90–120 kDa, the phosphorylation of which declined with increasing BCR expression (Figure 4, upper panel). These proteins, which in comparison with SLP-65 require longer exposure of cells to (or higher doses of) pervanadate for their phosphorylation, thus behaved in an opposite manner to Syk and SLP-65. In analogy to SLAP 130/Fyb, a PTK substrate protein which inhibits SLP-76 activation (Musci *et al*., 1997), one or more of these proteins may sequester Syk and possibly also SLP-65 in an inactive complex in the absence of BCR. To test this hypothesis, these proteins must be identified and characterized functionally. As exemplified by our study, the use of methods for inducible gene expression may result in a better understanding of the organization and dynamic changes of signaling elements.

Materials and methods

Construction of pSV2loxμm

The *Eco*RI fragment carrying a VDJ sequence of the Ig heavy chain from the expression vector pSVμm5 (Reth *et al*., 1987) was subcloned into pBluescript II KS+/– lacking the *Not*I site to obtain the plasmid pBS-E-VDJ. After removing the *Bam*HI fragment, the vector was opened by *Nco*I–*Pst*I double digestion and a DNA fragment generated by annealing the oligonucleotides (5′-CATGGGAAATATAACTTCGTATAATGTATGCTATACGAAGTTATTAGGCGGCCGCTAAACTATCTGCA-3′ and 5′-GTAAGTTTAGCGGCCGCCTAATAACTTCGTATAGCATACATTATACGAAGTTATATATTTCC-3′) containing one *loxP* site (underlined) was inserted to create pBS-E-dB-N/P-linker. A *Not*I–*Pst*I fragment carrying the stop-pA-*loxP* sequence from the construct of plox-leader (unpublished data) was introduced into the *Not*I–*Pst*I sites of pBS-E-dB-N/P-linker to generate pBS2lox-E-dB. The *Bam*HI fragment recovered from the plasmid pBS-E-VDJ was ligated into pBS2lox-E-dB. From the yielded plasmid, the *Eco*RI restriction fragment was cloned back to the *Eco*RI site of the pSVμm5 expression vector to obtain pSV2loxμm. In the pSV2loxμm vector, the *loxP*-stop-pA-*loxP* segment was inserted immediately after the ATG start codon. The vector in addition contains the xanthine guanine phosphoribosyl transferase and ampicillin resistance genes.

Cell lines and stable transfection

J558Lmb-1 is an mb-1 transfectant of the J558L myeloma cell line. The cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 5×10^{-5} M 2-mercaptoethanol and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin). A stock solution of 1 mM OH-TAM (Sigma) was prepared in 100% EtOH. Before use, it was diluted in RPMI-1640. The stock solution of 17β-estradiol (Sigma) was prepared in a similar way. The J558Lmb-1 cells were washed once with phosphate-buffered saline (PBS), resuspended in PBS at 1.5×10^7 cells/ml, and subjected to electroporation in the presence of 8 μg of pSV2loxμm and 25 μg of pANMerCreMer plasmids in a final volume of 0.8 ml. The transfectants were selected with 1 μg/ml mycophenolic acid (Reth *et al*., 1987). Resistant cells were incubated for 72 h either in the absence or presence of 200 nM OH-TAM. The expression of IgM on the cell surface was analyzed by FACScan with fluorescein isothiocyanate (FITC)-conjugated anti-IgM antibodies (Southern Biotech. Assoc.), or by anti-IgM immunoblotting.

Cell stimulation and immunoprecipitation

The cells were harvested at the indicated time points of exposure to OH-TAM, washed once with PBS and adjusted to 1.5×10^7 cells/ml of RPMI medium without supplements. Stimulation with either antigen (NIP-BSA, 10 μg/ml), or pervanadate/H_2O_2 (25 μM) was performed as previously described (Wienands *et al*., 1996). Subsequently, cells were lysed in 1 ml of 137 mM NaCl, 50 mM Tris–HCl (pH 7.8), 10% (v/v) glycerol, 1 mM sodium orthovanadate, 2 mM EDTA (pH 8.0), 1% (v/v) NP-40, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride. Post-nuclear supernatants were subjected to sequential immunoprecipitations with anti-Syk and anti-Lyn antibodies (both purchased from Santa Cruz Biotechnology Inc.) immobilized with protein G–Sepharose (Pharmacia). Affinity purifications with a GST fusion protein encompassing the N-terminal SH3 and SH2 domain of Grb2 [Grb2(SH3–SH2)] were described previously (Wienands *et al*., 1998). Purified proteins, boiled in 150 μl of Laemmli buffer, and aliquots from postnuclear supernatants were separated by SDS–PAGE and analyzed by anti-phosphotyrosine (4G10, UBI, Lake Placid, USA) or anti-SLP-65 (Wienands *et al*., 1998) immunoblotting using the enhanced chemo-luminescence detection system (ECL, Amersham).

FACScan analysis

The stable J558L transfectants were exposed to OH-TAM at the indicated concentrations and for the indicated times. Thereafter, 1×10^5 cells were stained with FITC-conjugated goat anti-mouse IgM antibodies and analyzed for expression of μ chain on the cell surface by FACScan (Becton Dickinson). Cells not exposed to OH-TAM were used as a control.

PCR

To analyze the MerCreMer-mediated excision of the *loxP*-stop-pA-*loxP* segment, the genomic DNA was amplified by a nested PCR. Primers used for the first round of PCR are μm5′-1 (5′-AGGATCTTACCGCTGTGAGAT-3′) and 3′-1 (5′-GTGGCCTTGGTCTTGAACTT-3′). In the second round of PCR, the primers are μm5′-2 (5′-TAGGACTGGGGCTTCAGAATC-3′) and 3′-2 (5′-CGCAGAGTCTCAGATGTCAG-3′). The two sets of PCR primers flank the *loxP*-stop-pA-*loxP* sequence of pSV2loxμm. The reactions were carried out for 30 cycles, each consisting of denaturation at 95°C for 40 s, annealing at 58°C for 40 s and extension at 72°C for 50 s. A 100-fold dilution of the first-round PCR product was used as the template for the second-round PCR.

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