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Dual role of the tyrosine activation motif of the Ig-α protein during signal transduction via the B cell antigen receptor

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The B cell antigen receptor (BCR) is a multimeric protein complex consisting of the ligand binding immunoglobulin molecule and the Ig-α/β heterodimer that mediates intracellular signalling by coupling the receptor to protein tyrosine kinases (PTKs). Transfection of the Ig-α deficient myeloma cell line J558Lμm with expression vectors coding for mutated Ig-α allowed us to test the function of the tyrosines in the cytoplasmic region of Ig-α in the context of the BCR. Furthermore we expressed Ig-α mutations as chimeric CD8-Ig-α molecules on K46 B lymphoma cells and tested their signalling capacity in terms of PTK activation and release of calcium. We show here that the conserved tyrosine residues in the cytoplasmic portion of Ig-α have a dual role. First, they are required for efficient activation of PTKs during signal induction and second, one of them is subject to phosphorylation by activated src-related PTKs. Phosphorylation on tyrosine in the cytoplasmic portion of Ig-α is discussed as a possible mechanism to couple the BCR to SH2 domain-carrying molecules.

Key words: B cells/immunoglobulins/protein tyrosine kinases/receptors

Introduction

The mouse B cell antigen receptor (BCR) consists of membrane-bound immunoglobulin (mIg) which is non-covalently associated with a disulfide-linked heterodimer of the two transmembrane proteins Ig-α and Ig-β (for review see Reth, 1992).

Ig-α and Ig-β are glycoproteins of M_r 34 000 and M_r 39 000, respectively (Campbell and Cambier, 1990; Hombach *et al.*, 1990; Venkitaraman *et al.*, 1991). It was shown (Hombach *et al.*, 1990; Campbell *et al.*, 1991) that Ig-α and Ig-β are encoded by the *mb-1* (Sakaguchi *et al.*, 1988) and the B29 genes (Hermanson *et al.*, 1988), respectively. Both proteins carry a glycosylated extracellular Ig-like domain, a single transmembrane region of 22 amino acids and a cytoplasmic portion of either 61 or 48 amino acids. Sequence comparison of the mouse sequence with the human counterpart of the *mb-1* gene (Flaswinkel and Reth, 1992; Ha *et al.*, 1992; Yu and Chang, 1992) and the human B29 gene (Müller *et al.*, 1992; Hashimoto *et al.*, 1993) revealed a strong conservation of the transmembrane and cytoplasmic sequences suggesting that important functions reside in these parts of the protein. Upon cross linking of BCR, several proteins are phosphorylated on tyrosine residues (Campbell and Sefton, 1990), among them the Ig-α and Ig-β proteins (Gold *et al.*, 1991). Two types of protein tyrosine kinase (PTK) are activated via the BCR and may play a role in this phosphorylation, namely the src-related PTKs: lyn, fyn, blk and lck (Burkhardt *et al.*, 1991; Yamanashi *et al.*, 1991; Campbell and Sefton, 1992; Lin and Justement, 1992), and the cytoplasmic enzyme PTK72 (Hutchcroft *et al.*, 1992) which might be the mouse homologue of syk (Taniguchi *et al.*, 1991).

It is not known at present how and in what order these PTKs become activated after cross linking of the BCR. Recently, a new method has been developed that uses chimeric transmembrane molecules to test the signalling function of cytoplasmic sequences (Irving and Weiss, 1991; Letourneur and Klausner, 1991; Romeo and Seed, 1991; Howard *et al.*, 1992; Kim *et al.*, 1993a). Using this approach it was shown that the cytoplasmic sequences of Ig-α and Ig-β can both signal independently of the BCR in B cells and that Ig-α can activate PTK much more efficiently than Ig-β (Kim *et al.*, 1993a).

Both sequences contain the conserved amino acid sequence motif D/ExxxxxxD/ExxYxxLxxxxxxxYxxL/I (Reth, 1989) which is also present in the cytoplasmic tail of the components of the T cell receptor (TCR) and of Fc receptors. This motif consists of two negatively charged amino acids and two tyrosines followed with precise spacing by either a leucine or an isoleucine residue. The position of the negatively charged amino acid is not always conserved whilst that of the tyrosines is. We refer here to the tyrosine-containing part of the motif (YxxLxxxxxxxYxxL) as the *t*yrosine-based *a*ctivation *m*otif (TAM) (Samelson and Klausner, 1992). The presence of TAM in components of the TCR is required for their function as signal-transducing molecules (Letourneur and Klausner, 1991; Romeo *et al.*, 1992; Wegener *et al.*, 1992; Irving *et al.*, 1993). So far, however, point mutations of this motif have not been tested in the context of the complete antigen receptor.

The myeloma cell line J558Lμm expresses the membrane form of IgM intracellularly but, due to the lack of Ig-α, fails to transport it on to the cell surface (Hombach *et al.*, 1988). Previous experiments have shown that upon transfection of an *mb-1* expression vector coding for Ig-α, IgM is expressed at high levels on the cell surface of J558Lμm/*mb-1* (Hombach *et al.*, 1990; Venkitaraman *et al.*, 1991). In addition, the BCR expressed on the surface of J558Lμm/*mb-1* upon transfection of *mb-1* can transduce signals into the cytoplasm by activating PTK (Kim *et al.*, 1993b), although these cells do not carry the CD45 surface marker and are not undergoing a calcium response (Justement *et al.*, 1990, 1991). The capacity to signal and the absence of endogenous *mb-1* expression in J558Lμm enabled us to test mutated Ig-α in the context of the complete BCR. We show here that mutation of both tyrosines of the TAM of Ig-α results in a

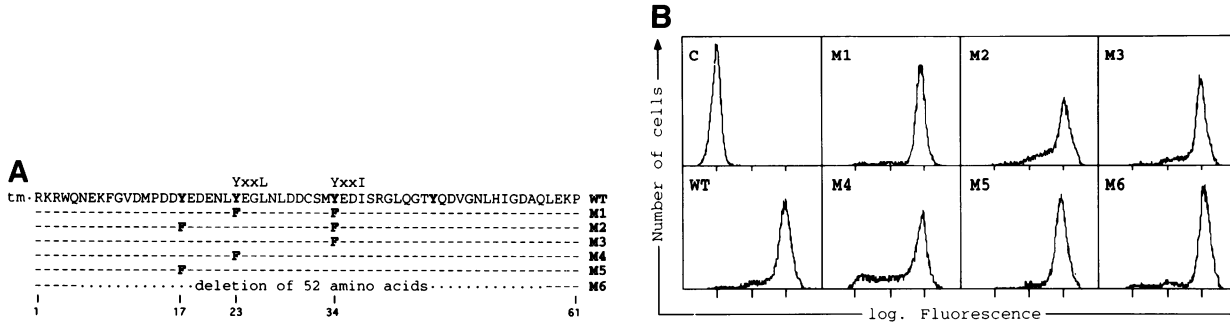

Fig. 1. (**A**) Introduced mutations of the intracellular region of the mouse Ig-α protein. By site-directed mutagenesis, three of the four tyrosines of the Ig-α protein were changed in different combinations to a phenylalanine (M1−M5). The mutant M6 carries a 52 amino acid deletion in its cytoplasmic sequence. Introduced phenylalanines are indicated by an F; the deleted amino acids in the truncations are marked by a point. A dash represents identity to the sequence above. The position of the YxxL and YxxI of TAM is shown above the sequence. Numbers below the sequence indicate the position of the amino acids in the cytoplasmic portion. The position of the adjacent transmembrane region is marked tm. (**B**) Expression of sIgM on J558Lμm cells before (C), and after transfection with either the *mb-1* WT or mutant (M1−M6) *mb-1* expression vector. The cells were stained with an FITC labelled goat anti-mouse IgM serum and surface IgM expression was determined on a FACScan.

defective PTK activation via the complete BCR and that the first tyrosine of the TAM is a dominant target of a src-related PTK. The signal transduction of mutated CD8−Ig-α chimeric receptors on K46 cells is, however, already abolished by the mutation of a single tyrosine of the TAM.

Results

Mutations in the intracellular portion of Ig-α do not interfere with surface expression of the BCR

The cytoplasmic sequence of Ig-α is comprised of 61 amino acids containing four tyrosine residues (see bold Y in Figure 1A). The first tyrosine (Y17) is surrounded by five negatively charged amino acids. A tyrosine in a similar sequence context is present in the human erythrocyte band 3 protein and has been shown to be a major target of the PTK72 enzyme (Low *et al.*, 1987; Wang *et al.*, 1991). The next two tyrosines (Y23 and Y34) are part of the TAM. With a PCR approach, we generated six mutations. In five of these (M1−M5) the three tyrosine residues are changed in different combinations into phenylalanine (see Figure 1A). In M1, both tyrosines of the TAM are mutated, whereas M3 and M4 carry a point mutation of Y34 and Y23, respectively. In M5, only the first tyrosine (Y17) is mutated and this mutation is combined in M2 with that of Y34. The mutation M6 is not a point mutation but an internal deletion of 52 cytoplasmic amino acids which includes all four tyrosines of the Ig-α tail. The *mb-1* expression vectors carrying the mutations (M1−M6) were stably transfected into J558Lμm cells. We first analysed whether the mutated Ig-α proteins were able to mediate the transport of the BCR to the cell surface. Figure 1B shows the sIgM expression on the different transfectants (M1−M6) in comparison with J558Lμm/*mb-1* expressing the wild type (WT) Ig-α and with the untransfected control J558Lμm (C). IgM surface expression on J558L transfectants producing mutated Ig-α or WT Ig-α protein is similar in all transfectants. Therefore the mutations introduced into the cytoplasmic sequence of Ig-α do not seem to interfere with cell surface expression. A similar result has been obtained in T cells, where a cytoplasmic truncation of the CD3-ε (Transy *et al.*, 1989) or ζ (Wegener *et al.*, 1992) does not prevent the transport of the TCR to the cell surface.

Next, we analysed the size of the two most drastically

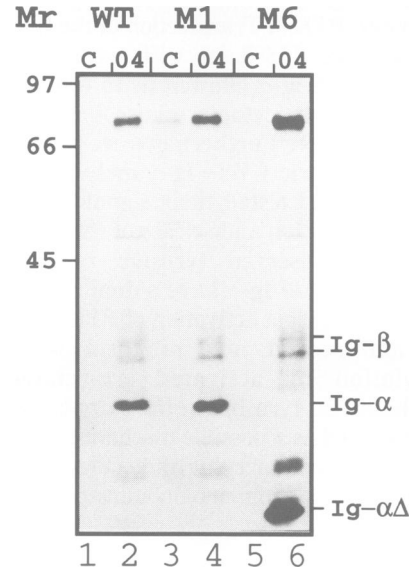

Fig. 2. Biochemical analysis of the Ig-α/Ig-β heterodimer expressed on the surface of either WT J558Lμm/*mb-1* cells (lane 2) or mutant J558Lμm cells M1 (lane 4) and M6 (lane 6). After surface biotinylation proteins were precipitated with either monoclonal anti-Ig-β (MAB29T-04) designated 04 or anti-GST MAGTT-20 designated C as control and size separated by 10% SDS−PAGE under reducing conditions. Lanes 2, 4 and 6 show precipitations with the monoclonal anti-Ig-β antibody; lanes 1, 3 and 5 are control precipitations using an isotype-matched anti-GST antibody as control. The positions of Ig-α, Ig-β and the truncated Ig-αΔ are indicated on the right. Numbers on the left indicate the $M_r \times 10^{-3}$ of the molecular size marker proteins.

mutated Ig-α proteins (M1 and M6) and also tested whether these mutations interfered with heterodimer formation between Ig-α and Ig-β. Using our monoclonal anti-Ig-β antibody MAB29T-04, we precipitated surface biotinylated Ig-α/Ig-β heterodimer from Triton X-100 lysates of J558Lμm transfectants and separated the two molecules of the heterodimer by reducing SDS−PAGE. Figure 2 shows that M1 Ig-α has the same size as WT Ig-α. The truncated M6 Ig-α (labelled Ig-αΔ) migrates faster due to the loss of most of its cytoplasmic residues (Figure 2, lane 6). No WT size Ig-α is detected in the M6 cell line, confirming that these cells do not express the endogenous *mb-1* gene. The two sizes of the Ig-β protein seen on the Western blot may correspond to differential glycosylation (Chen *et al.*, 1990)

or C-terminal truncation (Friedrich *et al.*, 1993) of the Ig-β protein in J558L cells. The finding that the mutated Ig-α/Ig-β heterodimer on the mutant cell lines is as efficiently precipitated as those on WT cells, indicates that the heterodimer formation is not impaired by mutation or deletion of the Ig-α cytoplasmic sequence.

Reduced activation of PTK by BCR with mutated Ig-α protein

Incubation of J558Lμm/mb-1 cells (WT) with either the antigen NIP-BSA or anti-IgM antibodies results in cross linking of the BCR and activation of PTKs which will phosphorylate several substrate proteins including Ig-α and Ig-β (Kim *et al.*, 1993b). To analyse the activation of PTK in the J558Lμm transfectants expressing M1−M6 or WT Ig-α proteins, we cross linked their BCR for 3 min with anti-IgM antibodies. Proteins in the cell lysates of unstimulated (−) and stimulated (+) cells were separated by reducing SDS−PAGE and the phosphorylated proteins were detected on a Western blot with anti-phosphotyrosine antibodies (Figure 3). The untransfected parental J558Lμm line (PL), which does not express BCR, served as a negative control. After stimulation of WT cells tyrosine phosphorylation of Ig-α, Ig-β and other, unknown substrate proteins (p55−p80) is drastically increased. The same or even an increased level of tyrosine phosphorylation is seen in activated M2 and M5 cells carrying Y17/Y34 and the Y17 mutation, respectively (Figure 3, lanes 8 and 14). In comparison with WT cells the p55, p65 substrate phosphorylation is slightly reduced in activated M3 and M4 cells carrying a Y34 and Y23 mutation, respectively, whereas substrate phosphorylation in the double mutation M1 or the deletion M6 is drastically reduced. This analysis shows that the mutation of both tyrosines of TAM in M1 has as drastic an effect on PTK activation in J558L cells as the deletion of the nearly complete Ig-α cytoplasmic sequence. No tyrosine phosphorylation of Ig-α is detected in activated M1, M4 and M6 cells which all carry a mutation or deletion of tyrosine Y23. This result defines Y23 as the major PTK phosphorylation site of Ig-α in activated J558L cells.

The signalling defect in the M1 line was further analysed over time and in a dose−response experiment (Figure 4A and B). In antigen-activated WT cells substrate phosphorylation reaches maximum levels in 40−60 s and declines after 5 min of activation (Figure 4A, lanes 1−5). The substrate proteins p66, p65, Ig-β and Ig-α are readily detected, whereas p80 is only very weakly phosphorylated under these conditions. We have previously identified (Kim *et al.*, 1993b) the p66 phosphoprotein as the tyrosine kinase PTK72/syk (Hutchcroft *et al.*, 1992). In activated M1 cells, only the substrate protein p65 becomes phosphorylated. Furthermore, in M1 cells the phosphorylation of p65 is delayed and never reaches the level of phosphorylation seen in the WT cells. The dose−response experiment shows a similar result. Whereas near maximum phosphorylation occurs in WT cells treated with as little as 0.2 μg/ml of antigen, in M1 cells phosphorylation never reaches the maximum level, even if these cells are treated with a 100-fold higher concentration of antigen (20 μg/ml). The reduced PTK activation in M1 cells is not due to a general reduced PTK activity because the treatment of WT and M1 cells with vanadate/H_2O_2 resulted in the same increase in the level of tyrosine substrate phosphorylation (data not shown).

These data suggest that the two conserved tyrosines of the

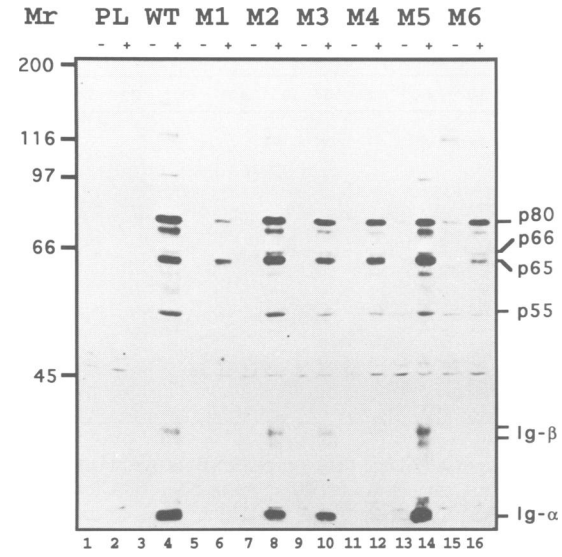

Fig. 3. Comparison of tyrosine-phosphorylated substrate proteins in J558Lμm cells transfected with either the WT or mutant *mb-1* gene (M1−M6). The surface IgM negative J558Lμm recipient cells (PL) were analysed as control. Unstimulated cells (−) and those stimulated by anti-IgM antibody (+) were lysed in Triton X-100 buffer. Proteins in the lysate of 2×10^5 cells were size separated by reducing 10% SDS−PAGE and transferred to nitrocellulose. The position of Ig-α and Ig-β as well as that of dominant substrate proteins is indicated on the right. Numbers on the left indicate the $M_r \times 10^{-3}$ of the molecular size marker proteins.

Ig-α TAM play an important role in the activation of PTK via the BCR. The mutation of both of the tyrosines results in defective signalling. The finding that signalling via the M1 or M6 BCR is not completely abolished is presumably due to the presence of unmutated Ig-β which carries its own TAM.

The signalling capacity of CD8−Ig-α chimeric molecules is abolished if one of the tyrosines of the TAM is mutated

K46 cells expressing CD8−Ig-α chimeric molecules with the mutations M1−M6 of the Ig-α cytoplasmic sequence allowed us to study the signalling capacity of mutated Ig-α independently of Ig-β. All transfectants (WT, M1−M6) expressed similar levels of chimeric CD8−Ig-α homodimer (data not shown). The cross linking of the CD8−Ig-α chimeric molecules on the different K46 transfectants with anti-CD8 antibodies results in increased substrate phosphorylation only in the K46WT and K46M5 cells (Figure 5A, lanes 2 and 12). In agreement with the data from the J558L cells the M5 mutation of Ig-α seems to be even more potent in PTK activation than the unmutated (WT) Ig-α. All other CD8−Ig-α mutations (M1−M4 and M6) fail to increase substrate phosphorylation in K46 B cells upon their cross linking. The K46 B lymphoma line expresses an endogenous IgG2a BCR whose cross linking results in the rapid release of calcium ions (Justement *et al.*, 1990). A similar response is also detected in K46 transfectants after cross linking of their CD8−Ig-α or CD8−Ig−β chimeric molecules (Kim *et al.*, 1993a). The analysis of mutated CD8−Ig-α molecules in this assay shows that only the M5 mutation can signal calcium release at a similar or even slightly higher level than the WT CD8−Ig-α molecule. The mutations M3 and M4 (Figure 5B) as well as all other mutations (data not shown) did not activate any calcium release.

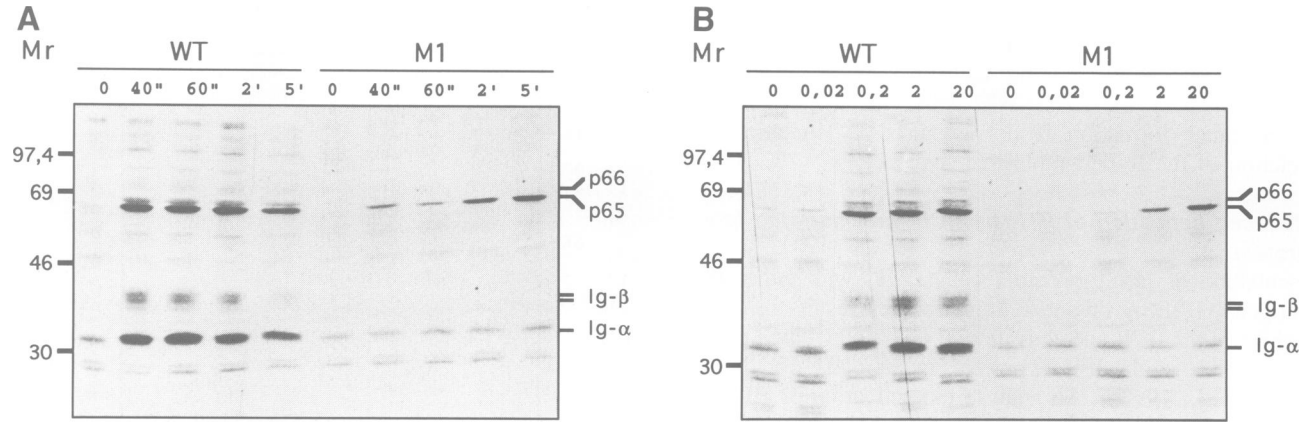

Fig. 4. Time course (**A**) and dose–response (**B**) of tyrosine phosphorylation in J558Lμm cells transfected with either the WT or mutant *mb-1* gene (M1). Cells were stimulated with 20 μg/ml of NIP–BSA for different periods of time (**A**) or with different concentrations of antigen for 3 min (**B**). Analysis of phosphorylated proteins was performed as described for Figure 3.

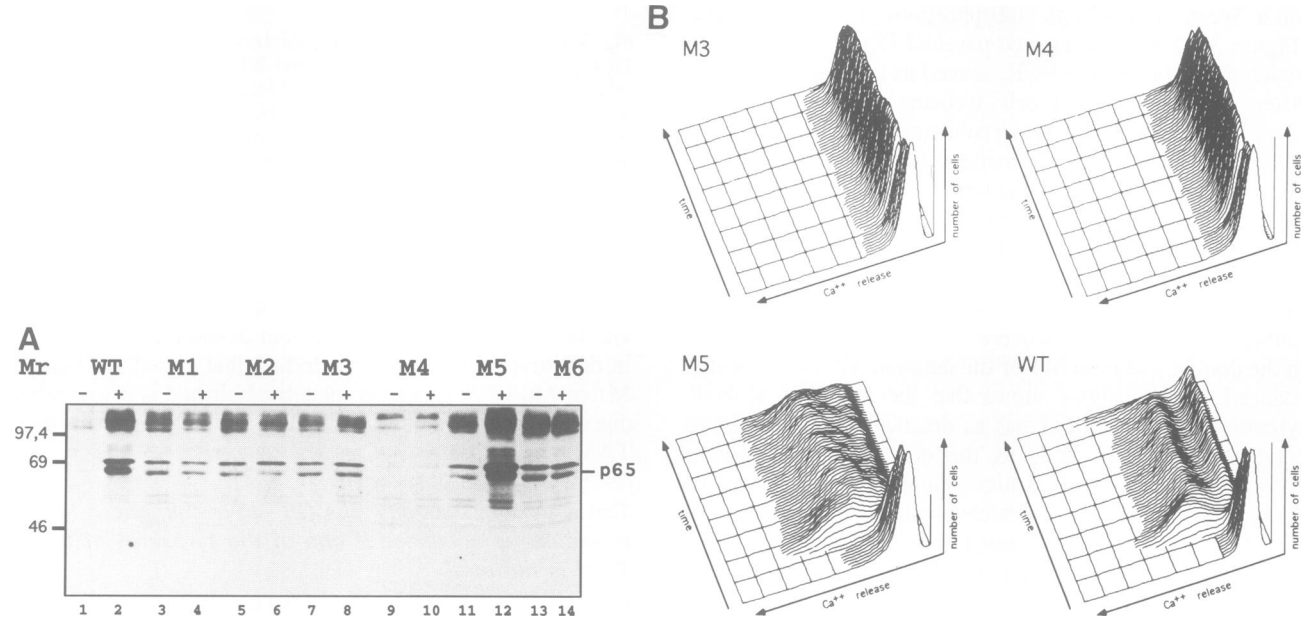

Fig. 5. Activation of substrate phosphorylation and release of calcium in K46CD8/Ig-αWT and M1–M6 cells. (**A**) Substrate phosphorylation of unstimulated cells (−) and those stimulated with 20 μg/ml of anti-CD8 antibody (Tib105) (+) was analysed as described in Figure 3. Numbers on the left indicate the $M_r \times 10^{-3}$ of the molecular size marker proteins. (**B**) Increase of intracellular free calcium ions in K46 after cross linking of CD8–Ig-α chimeric molecules with 25 μg/ml of rat anti-mouse CD8 antibody. Release of calcium was measured for a total period of 512 s. Cells were stimulated 70 s after the start of the measurement.

Thus the analysis of PTK activation and release of calcium in K46 transfectants yield the same result and show that signalling via chimeric CD8–Ig-α molecules requires the presence of both tyrosines of the TAM.

Tyrosine 23 of Ig-α is phosphorylated by the src-related tyrosine kinase fyn

From the four src-related PTKs (lyn, blk, fyn and lck) implicated in BCR signalling only fyn and lyn are expressed in J588L cells. The cross linking of the BCR on J558Lμm WT cells also results in phosphorylation and presumably activation of the cytoplasmic tyrosine kinase PTK72/Syk (Kim *et al.*, 1993b). Which of these PTKs are responsible for the observed phosphorylation of Ig-α in J558Lμm is at present unknown. We have employed an *in vitro* kinase assay to test whether tyrosines of the Ig-α cytoplasmic sequence are substrates for one of the src-related PTKs expressed in J558Lμm. For this purpose, fusion proteins consisting of the glutathione-*S*-transferase (GST) and cytoplasmic portions of the WT or M1–M6 Ig-α proteins produced in bacteria, were incubated for 30 min in a kinase buffer with human fyn kinase expressed from a recombinant baculovirus. After the kinase reaction, fusion proteins were separated by SDS–PAGE and the phosphorylated proteins were detected on a Western blot by anti-phosphotyrosine antibodies (Figure 6A). The total amount of fusion proteins (F) was determined on the same Western blot by a monoclonal anti-GST antibody (Figure 6B). Whilst the fusion proteins carrying the cytoplasmic portion of WT, M2, M3 or M5 Ig-α show a strong tyrosine phosphorylation, there is no detectable phosphorylation in fusion proteins carrying the cytoplasmic portion of M1, M4 or M6 Ig-α. Thus, the tyrosine phosphorylation of GST–Ig-α fusion proteins by fyn is strictly dependent on the presence of Y23 in the Ig-α tail. The

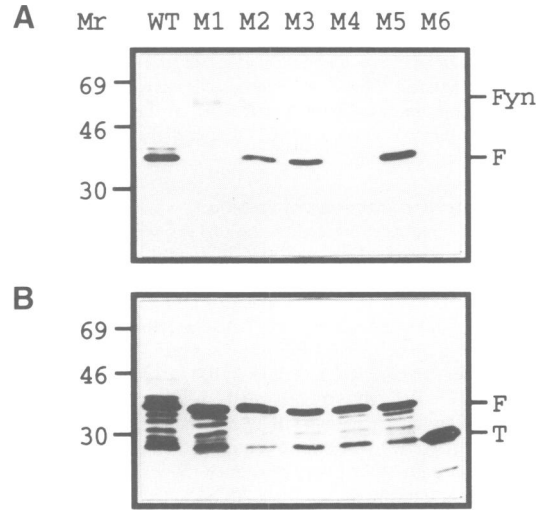

Fig. 6. *In vitro* kinase assay of GST−Ig-α fusion proteins carrying either the WT or mutated (M1−M6) Ig-α sequences. After 10% SDS−PAGE the fusion proteins (F) were detected on the same Western blot either by anti-phosphotyrosine antibody 4G10 (**A**) or monoclonal anti-GST antibody (**B**). The T indicates the position of the fusion protein with the Ig-α truncation. For the *in vitro* kinase assay, fusion protein bound to glutathione−Sepharose was incubated for 30 min with 5 μl of a baculovirus extract containing human fyn kinase.

mutation of the second tyrosine of the TAM (Y34) in M2 and M3 results in a slight decrease in the GST−Ig-α phosphorylation. Therefore, although Y34 is not (a/the) target of phosphorylation by fyn, it seems to promote the phosphorylation of Y23. The same result was obtained using the PTK src expressed from a recombinant baculovirus (data not shown). Together, the *in vitro* and *in vivo* analyses gave the same result and showed that Y23 of Ig-α is the dominant target for phosphorylation by PTK.

Discussion

By expression of mutations of the cytoplasmic sequence of Ig-α in the context of either the complete BCR or as part of a chimeric CD8−Ig-α molecule we were able to compare the signalling function of mutated Ig-α sequences under two different conditions. The data from the CD8−Ig-α are clear cut. The signalling function of these molecules is completely abolished as soon as one of the two tyrosines (Y23 or Y34) of TAM is mutated to phenylalanine. The mutation of the tyrosine Y17, however, improves rather than prevents signalling via the CD8−Ig-α molecule. The data from mutated Ig-α protein in the complete BCR are more complex. A BCR with one mutated tyrosine of the Ig-α TAM shows only a slightly reduced PTK activation. Presumably the presence of a WT Ig-β in the Ig-α/Ig-β heterodimer can overcome the signalling defect of Ig-α. If, however, both tyrosines of Ig-α TAM are mutated, the signalling defect of the BCR becomes much more pronounced and Ig-β for example is no longer phosphorylated. Our data show also that phosphorylation of Ig-α is strictly dependent on the presence of the first tyrosine (Y23) of TAM and we have thus defined this tyrosine as a major substrate for a src-related PTK.

The coupling between receptor and PTKs has been extensively studied in the case of the TCR. In the TCR complex, TAM is present once in each CD3 component (CD3-ε, CD3-γ, CD3-δ) and 3-fold in the ζ component. Signalling in a mutant TCR carrying a deletion of all three TAMs of the ζ chain is defective (Wegener *et al.*, 1992). However, even in this case signalling is not completely abolished as TAMs of the CD3 components (ε,γ,δ) may functionally replace the missing ζ chain. The signalling function of the cytoplasmic part of the TCR has also been studied in the context of chimeric protein carrying the extracellular part of CD4, CD8, CD16 or TAC and the cytoplasmic part of either ζ of CD3-ε (Chan *et al.*, 1991; Irving and Weiss, 1991; Letourneur and Klausner, 1991, 1992; Romeo and Seed, 1991). The deletion or point mutation of the TAM prevents signalling via these chimeric molecules. Furthermore, it has been shown that TAM is required for the association of the cytoplasmic ζ sequence with the N-terminal part of fyn (Timson-Gauen *et al.*, 1992) and with ZAP70 (Irving *et al.*, 1993), a T cell specific cytoplasmic PTK with a structure very similar to syk.

Our study of point mutations of TAM in Ig-α is compatible with data from the TCR. Like ζ in the TCR, Ig-α chains seem to be the dominant signalling subunit of the BCR (Kim *et al.*, 1993a; Sanchez *et al.*, 1993). This conclusion is also supported by studies showing that the cytoplasmic sequence of Ig-α binds to PTK more efficiently *in vitro* than Ig-β (Clark *et al.*, 1992). The signalling difference between Ig-α and Ig-β is surprising, given the fact that both chains have a TAM. However, it is conceivable that other amino acids besides the ones of the TAM may influence the signal function of the motif. In comparison with Ig-α, the Ig-β protein is more heavily serine/threonine phosphorylated (Van Noesel *et al.*, 1990; Clark *et al.*, 1992) and this phosphorylation may also exert a negative control over PTK activation.

The tyrosine phosphorylation of Ig-α demonstrates a high sequence specificity; indeed, of the four tyrosines of Ig-α, only Y23 becomes heavily phosphorylated in activated J558Lμm cells. The same tyrosine is also specifically phosphorylated *in vitro* by the src-related PTKs fyn and src. Only two src-related PTKs, namely lyn and fyn, are expressed in J558L (Kim *et al.*, 1993b). Therefore one of them might be the PTK responsible for the specific phosphorylation of Ig-α. The function of the specific Ig-α phosphorylation is unknown at present. Yet it seems clear that it does not play an immediate role in the PTK activation via the BCR. First, in a time course experiment with activated J558Lμm cells the phosphorylation of Ig-α occurs later than that of the dominant substrate proteins p65 and p80 (Kim *et al.*, 1993b). Second, in the M4 mutation there is still substantial PTK activation in the absence of detectable Ig-α phosphorylation. The phosphorylation of Ig-α may rather be part of a negative feedback control which terminates the PTK activation via Ig-α by modifying the conserved tyrosine of its TAM. Alternatively, the phosphorylated TAM may be a binding site for SH2 domain-containing proteins like src-related PTK, syk, crk and phospholipase C gamma (PLCγ). In this way the BCR could become connected to components of the cytoskeleton or to other signalling pathways like ras activation or hydrolysis of phosphatidylinositol (Buday and Downward, 1993; MacNicol *et al.*, 1993; Oliver *et al.*, 1993; Simon *et al.*, 1993). Indeed, the TAM sequence shows striking similarities to that of phosphopeptides known to bind to SH2 domains (Songyang *et al.*, 1993). Besides the phosphotyrosine-binding groove, an SH2 domain has a second binding site for the third amino acid

(Y+3) after the tyrosine. This second site may be the reason for the conservation of the leucine or isoleucine at the Y+3 position of the TAM sequence. Both tyrosines of the TAM sequence have the conserved Y+3 position although only the first tyrosine is phosphorylated in J558L. Other PTKs not expressed in J558Lμm may nevertheless specifically phosphorylate the second conserved tyrosine of TAM. In summary, our data show that the TAM has an important function in coupling the BCR to the PTK activation. TAM may interact with PTKs in three different ways. In resting B cells src-related PTKs seem to bind TAM, presumably through their N-terminal sequence (Timson-Gauen *et al.*, 1993) and to become activated by cross linking of the BCR. The activated signal cascade can then feed back and phosphorylate one or both tyrosines of TAM. A third and yet hypothetical interaction may then be the binding of phosphorylated TAM by a src-related or syk-like PTK via their SH2 domains. This third interaction has been found in the TCR where ZAP binds the ζ chain via its SH2 domain (Wang *et al.*, 1993; Weiss, 1993).

Materials and methods

Site-directed mutagenesis

Site-directed mutagenesis of the *mb-1* cDNA was performed using PCR as described (Stappert *et al.*, 1992). The template for the PCR was single strand DNA of the plasmid pTZ19-PW35.1-7 carrying the *mb-1* cDNA. (Sequences of the used *mb-1* oligonucleotides carrying the mutations are available upon request.) PCR fragments were cut with *Pvu*II and used to replace the wild type 393 bp *Pvu*II fragment of the expression vector pEVMB-1neo (Hombach *et al.*, 1990). The single base pair substitutions in cloned PCR fragments were confirmed by dideoxy sequencing. For the production of the GST−Ig-α tail fusion proteins and CD8 chimeric expression vector, a *Bam*HI and *Sma*I site were introduced into the WT or mutated *mb-1* sequence by PCR and the PCR fragment cloned into the GST vector pRP261 and CD8 vector pLVLY2Hy, respectively. The vector pLVLY2Hy is based on the vector pLVLy2-Hy (Kim *et al.*, 1993a) and modified to allow insertion of *Bam*HI−*Sma*I fragments. PCR products ligated into the GST and CD8 expression vectors were again sequenced to confirm the correct reading frame (GST-GGG ATC CGG TGG CAA . . .) and (CD8-AGG ATC CGG TGGCAA . . .) of the GST and CD8 chimeric molecules, respectively.

Transfection and culture conditions

Expression vectors of WT or mutant *mb-1* were linearized with the enzyme *Pvu*I and introduced into the myeloma cell line J558Lμm which intracellularly expresses IgM and λ light chains by electroporation as described previously (Potter *et al.*, 1984; Reth *et al.*, 1987). The same method was used to transfect expression vectors coding for chimeric CD8−Ig-α M1−M6 into the B lymphoma K46 which expresses an endogenous IgG2a/κ BCR on the surface, except that in this case the vectors were linearized with the enzyme *Hin*dIII. Cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated calf serum, 2 mM L-glutamine, 50 U/ml penicillin, 50 U/ml streptomycin, 10 mM HEPES buffer and 2×10^{-5} M β-mercaptoethanol. Selection was carried out in the presence of 500 μg/ml G418 (Gibco) and 750 U/ml of hygromycin (Calbiochem). K46CD8−Ig-αWT was a gift from K.M.Kim.

Generation of monoclonal antibody

Mice were immunized with GST−Ig-β fusion protein. After fusion of the spleen cells and selection in HAT medium, cells were screened using GST−Ig-β protein, GST protein, GST−Ig-α protein and γ2b−Ig-β fusion protein. Monoclonal anti-GST antibodies reacted only with fusion proteins containing GST but not with γ2b−Ig-β fusion protein. MAB29T-04 (IgG1/κ) reacted only with fusion proteins containing the intracellular portion of Ig-β but not with any of the other fusion proteins. The specificity of the antibody was controlled using purified Ig-α/β heterodimer (a gift from J.Wienands).

FACS analysis

For FACS analysis, 10^6 cells were stained with FITC labelled goat anti-mouse IgM antibody or with FITC labelled anti-CD8 antibody (Becton Dickinson, Mountain View, CA). Stained cells were analysed on a FACScan (Becton Dickinson, Mountain View, CA) in the presence of 1 μg/ml propidium iodide to exclude dead cells. For each diagram 10 000 cells were analysed on a logarithmic scale.

Cell labelling and immunoprecipitation

Labelling of proteins expressed on the surface was done according to the procedure described by Kim *et al.* (1993b) with some minor modifications. For surface biotinylation 4×10^7 cells were washed three times with PBS and then incubated on ice for 15 min with 1 ml PBS containing Sulfo-NHS-Biotin 0.5 mg/ml (Pierce, Rockford, IL). The cells were then washed once with RPMI and three times with PBS. Cells were lysed for 15 min on ice in lysis buffer containing 1% Triton X-100, 20 mM Tris−HCl pH 6.8, 137 mM NaCl, 10% glycerol and 2 mM EDTA. The protease inhibitors aprotinin (10 μg/ml), leupeptin (10 μg/ml) and PMSF (1 mM) were added directly before lysis. Lysates were centrifuged for 15 min at 12 000 r.p.m. at 4°C. Lysates precleared with protein G−Sepharose were split into two 1.5 ml tubes and incubated for 1 h at 4°C with either monoclonal antibody against the cytoplasmic portion of Ig-β MAB29T-04 or with isotype matched anti-GST antibody, as a control. The antigen−antibody complex was incubated with protein G−Sepharose for a further 2 h at 4°C. Following centrifugation for 2 min at 1000 r.p.m., the precipitate was washed three times with 750 μl lysis buffer and boiled in 1 × SDS sample buffer. Proteins in the sample buffer were size separated by 10% SDS−PAGE and blotted on to nitrocellulose (Amersham, UK). Blotting was done for 2 h using Tris−glycine blotting buffer. Non-specific binding sites on the membrane were blocked by a 1 h incubation at room temperature with PBS containing 0.1% Tween 20 (PBS-T), 5% skim milk, and 0.1% NaN_3. Blocked membranes were washed three times with PBS-T and susequently incubated for 1 h with horseradish peroxidase (HRP)−conjugated streptavidin (Amersham, UK). The membrane was subsequently washed three times with PBS-T and biotinylated proteins on the membrane were visualized with the ECL Western blot detection system (Amersham, UK).

Stimulation of transfected cells and phosphotyrosine analysis

The method is based on the procedure described by Kim *et al.* (1993a) with some minor modifications. Prior to cross linking of their antigen receptors, the cells were incubated in 0.5 ml medium at a density of 2×10^6 cells/ml in a 24 well plate for at least 8 h. The cells were incubated for 3 min with 25 μg/ml of goat anti-mouse IgM serum (Sigma, St Louis) and the reaction was stopped with 1 ml of ice cold PBS containing 1 mM sodium vanadate (PBS-V). Alternatively, the cells were incubated with 10 μg/ml of 4-hydroxy-5-iodo-nitrophenyl-acetyl coupled to bovine serum albumin at a rate of 10:1 (NIP−BSA). The cells were transferred to 1.5 ml tubes and washed twice with PBS-V. Cell lysis, SDS−PAGE and Western blotting were performed as described for the immunoprecipitation, except that the lysis buffer was supplemented with 1 mM sodium vanadate. Blocked filters were incubated for 1 h at room temperature with 0.2 μg/ml of monoclonal anti-phosphotyrosine antibody 4G10 (UBI, Lake Placid, NY). Filters were washed three times and incubated for 1 h at room temperature with 0.1 μg/ml of HRP labelled goat anti-mouse κ serum (Southern Biotechnology Associates, Birmingham, AL). Following three washes in PBS−T, phosphorylated proteins were visualized with the ECL Western blot detection system (Amersham, UK).

Measurement of calcium release

K46CD8−Ig-αWT and M1−M6 were incubated for 45 min at 37°C in the dark with Iscove's medium containing 1% heat-inactivated fetal calf serum, 2 μM indo-1-acetooxymethylester and 0.02% (w/v) pluronic F-127 (both from Molecular Probes) (Owen, 1989; Alber *et al.*, 1993). Cells were washed once, diluted in medium, and kept on ice until tested. For measurement on a FACStar Plus cell sorter (Becton Dickinson) the cells were further diluted and warmed to 37°C. After 70 s monoclonal rat anti-mouse CD8α was added to a final concentration of 25 μg/ml and the mean fluorescence ratio of calcium bound to calcium-free indo-1 (FL4 ratio, a measure of the relative calcium concentration) recorded. Tracings were monitored for 512 s using the CHRONYS program. In each case loading of the cells and responsiveness to stimuli were controlled using goat anti-mouse IgG to a final concentration of 25 μg/ml.

In vitro kinase assay

Expression of the different GST−Ig-α fusion proteins (WT, M1−M6) in the transfected bacteria was induced with IPTG (Sigma, St Louis, MO).

After 2 h of incubation bacteria were pelleted and sonicated. Fusion proteins were purified with glutathione-conjugated Sepharose (Pharmacia, Freiburg, Germany). The amount and quality of the fusion proteins bound to Sepharose were assessed by SDS−PAGE and Coomassie blue staining. Aliquots of fusion protein bound to Sepharose beads were washed once in kinase buffer containing 50 mM Tris−HCl pH 7.6 and 10 mM $MnCl_2$. Baculovirus extract (Alber *et al.*, 1993) containing human fyn (a kind gift of Sara Courtneidge) was diluted 1:20 in kinase buffer containing 1 mM ATP and mixed with the fusion protein. Following 30 min of incubation at 30°C the reaction mixture was washed once with PBS-V. SDS sample buffer containing 1 mM sodium vanadate was added, proteins were separated by SDS−PAGE and blotted on to nitrocellulose. Phosphorylated tyrosines were visualized in the same way as described for the phosphotyrosine analysis of cell lysates. The filter was then incubated overnight in blocking buffer containing 0.1% NaN_3 which binds to HRP and in this way inhibits the signal from the phosphotyrosine analysis. The filter was subsequently incubated with a monoclonal antibody specific for GST as first, and HRP labelled goat anti-mouse κ serum as second antibody. The amount of the respective fusion proteins was visualized using the ECL detection system.

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