Acceleration of intracellular targeting of antigen by the B-cell antigen receptor: importance depends on the nature of the antigen–antibody interaction

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The B-cell antigen receptor (BCR) internalizes bound antigen such that antigen-derived peptides become associated with emigrating major histocompatibility complex (MHC) class II molecules for presentation to T cells. Experiments with B-cell transfectants reveal that BCR confers a specificity of intracellular targeting since chimeric antigen receptors which internalize antigen by virtue of a heterologous cytoplasmic domain do not necessarily give rise to presentation. In contrast, however, previous studies have shown that antigen binding to irrelevant cell surface molecules (e.g. transferrin receptor, MHC class I) can ultimately lead to presentation. The solution to this paradox appears to be that the intracellular targeting by BCR actually reflects an acceleration of antigen delivery. Depending on the nature of the BCR–antigen interaction, this accelerated targeting can be essential in determining whether or not internalization leads to significant presentation. Physiologically, the accelerated delivery of antigen by BCR could prove of particular importance early in the immune response when antigen–BCR interaction is likely to be poor.

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phosphorylation that can drive the cell into cycle and lead to increased expression of MHC class II and T-cell co-stimulatory molecules (Janeway and Bottomly, 1994). The presence on the B-cell surface of specific peptide–MHC complexes together with the co-stimulatory molecules then leads to the recruitment of T-cell help.

Although B cells can take up antigen by fluid-phase pinocytosis, the presentation of antigen taken up through the BCR is a much more efficient process and works at some 10⁶-fold lower antigen concentrations (Chesnut and Grey, 1981; Lanzavecchia, 1985). This increased efficiency of BCR-mediated presentation is largely due to the specificity of antigen–BCR interaction. The question arises, however, as to what extent BCR is adapted to mediate particularly efficient targeting of antigen for presentation or whether presentation is an inevitable consequence of antigen internalization. This question is important not just because BCR-mediated presentation is likely to be a major step in assisting the activation of B cells during the primary and, particularly, secondary responses, but rather because presentation may play a critical role during affinity maturation where the quality of antigen–BCR interaction might in part be monitored by the efficiency of recruitment of T-cell help.

Mutational studies of BCR itself have indicated previously that its efficacy in driving presentation can be ascribed to the Ig α / β sheath as well as to the transmembrane portion of the immunoglobulin itself (Shaw *et al.*, 1990; Parikh *et al.*, 1992; Patel and Neuberger, 1993; Bonnerot *et al.*, 1995). Here, by analysing presentation through families of mutant and chimeric BCRs with different antigen specificities, we show that BCR does not merely facilitate the efficient internalization of antigen but, by accelerating the delivery of antigen for loading onto emigrating MHC class II molecules, drives intracellular targeting. This activity is essential for the presentation of some antigens but not others, with the dependence being determined by the nature of antigen–antibody interaction.

Results

Internalization is not sufficient for presentation

In previous work, we used transfectants of a mouse B-cell lymphoma to show that whereas a wild-type BCR of IgM isotype mediated the efficient internalization of antigen and presentation of derived peptides, mutant receptors which lacked the Ig α / β sheath gave only poor internalization and ineffective presentation (Patel and Neuberger, 1993). This indicated that the sheath played a role in driving internalization and therefore presentation.

We wanted to use this same system to test whether internalization *per se* is sufficient to give rise to presentation or whether there are features to the antigen receptor that, following internalization, facilitate particularly

Introduction

The B-cell antigen receptor (BCR), which is composed of membrane immunoglobulin sheathed by the non-covalently associated Ig α /Ig β heterodimer, mediates the response of B lymphocytes to antigen through two major processes: endocytosis and transmembrane signalling. Endocytosis leads to the internalization of the antigen that is bound to BCR and its subsequent degradation; peptides derived from the degraded antigen can then be transported back to the B-cell surface in association with major histocompatibility complex (MHC) class II molecules for subsequent presentation to T cells (Lanzavecchia, 1990). The transmembrane signalling initiates a cascade of protein

Fig. 1. Internalization by NIP-specific receptors. (A) Structures of the BCRs giving the amino acid sequences for the transmembrane–cytoplasmic junction of the different chimeric heavy chains. An asterisk denotes a translation stop. (B) Flow cytometric profiles of transfectants stained with biotinylated Ac38 anti-idiotope antibody and FITC–streptavidin. (C) Internalization of the various BCRs on A20 transfectants that had been surface stained with FITC-conjugated goat anti- μ and incubated for various times at 37°C. The residual mean fluorescence is expressed as a percentage of the zero time value. (D) Internalization and degradation of [125 I]NIP $_8$ -GPDH by the various transfectants. Internalized radioactivity (determined as the cell-associated pronase-resistant fraction) is expressed as the percentage of the total cell-bound radioactivity at time zero. (E) Antigen degradation was monitored by measuring the radioactivity in the cell supernatant that was not precipitable in 10% trichloroacetic acid; this is expressed as a percentage of the total cell-bound radioactivity at time zero. IgM/β (■), IgM/LDL (○), IgM/H2 (●).

efficient intracellular targeting for class II presentation. We sought to construct a sheathless chimeric receptor that would internalize antigen by use of a cytoplasmic domain that is not itself usually implicated in antigen presentation. To this end, we constructed a plasmid directing the expression of an IgM/low density lipoprotein (LDL) chimera in which an IgM λ molecule specific for the hapten

5-iodo-4-hydroxy-3-nitrophenacetyl (NIP) was linked to an MHC class I transmembrane portion [which allows it to be transported to the surface without the Ig α /β sheath (Williams *et al.*, 1990)] and the cytoplasmic domain of the mouse LDL receptor (Figure 1A).

This plasmid, as well as controls encoding analogous chimeras containing the cytoplasmic domain of either the

Fig. 2. Antigen presentation by NIP-specific receptors. Presentation by the various A20 transfectants of I-A^d/OVA³²³⁻³³⁹ to the 3DO-54.8 T-cell hybridoma as measured by IL2 production following a 24 h co-culture in the presence of various concentrations of NIP₁₇-OVA. Unconjugated OVA was used to monitor fluid-phase presentation. The results are representative of four experiments carried out with at least two A20 transfectants for each plasmid construct. IgM(α/β) (□), IgM/β (■), IgM/LDL (○), IgM/H2 (●).

Igβ sheath polypeptide or MHC class I H2K^b, were transfected into the A20 mouse B-cell lymphoma and, for each construct, at least two independent clones were selected that expressed similar amounts of NIP-specific receptors. Internalization of the receptors was then compared by incubating the cells with fluorescein isothiocyanate (FITC)-conjugated goat anti-μ antiserum and, following incubation at 37°C, determining the decrease in cell-associated fluorescence (Figure 1B and C). The FITC fluorescence rapidly diminishes with the acidification that accompanies endocytosis. The IgM/LDL chimera (like the IgM/β chimera) internalizes well. A similar conclusion was reached when the kinetics of internalization of bound antigen were followed. Cells were incubated with a radiolabelled NIP-glyceraldehyde-3-phosphate dehydrogenase conjugate ([¹²⁵I]NIP₈-GPDH; GPDH is a protease-sensitive carrier). The bound NIP₈-GPDH was internalized by the cell (becoming resistant to exogenously added extracellular proteases) and was then degraded by intracellular proteases with the resultant return of radioactivity to the medium. Again, the IgM/LDL (but not the IgM/H2) chimera showed good internalization characteristics (Figure 1D) and led to degradation of internalized antigen such that low molecular weight radioactivity was returned to the medium (Figure 1E).

The ability of the different antigen receptors to mediate the presentation of specific antigen was compared by incubating the transfectants with different concentrations of NIP-ovalbumin (NIP₁₇-OVA) and measuring the amount of interleukin-2 (IL2) produced following a 24 h co-culture with the T-cell hybridoma 3DO-54.8 (specific for the OVA peptide, OVA³²³⁻³³⁹ in the context of I-A^d). Unlike the wild-type receptor and IgM/β chimera, clones expressing the IgM/LDL chimera were clearly defective in specific presentation (Figure 2). This same deficiency in mediating specific presentation was also evident if a distinct T-cell hybridoma recognizing the same OVA peptide (3DO-11.10) was used for readout; the various transfectants were, however, all matched with respect to their ability to mediate non-specific fluid-phase presentation as judged by carrying out the assays in the presence of high concentrations of unconjugated ovalbumin.

Requirement for targeting is dependent on the quality of antigen-BCR interaction

Thus the cytoplasmic domain of the LDL receptor confers efficient internalization but not efficient presentation, consistent with there being more to specific presentation than a mere endocytosis and degradation of antigen. These findings are consistent with some previous studies indicating that the antigen receptor mediates intracellular targeting of endocytosed antigen (Mitchell *et al.*, 1995; Song *et al.*, 1995). However, such trafficking to meet MHC class II cannot be a property unique to BCR and other professional antigen-presenting receptors since the binding of antigen to cell surface molecules that have no biological role in antigen internalization can also lead to presentation (Casten and Pierce, 1988; Snider and Segal, 1989; McCoy *et al.*, 1993; Niebling and Pierce, 1993).

The reason for the contrast between these sets of results is not readily apparent. It could lie in the nature of the antigen-receptor pairs studied (affinities of interaction; mode of receptor internalization; pattern of intracellular proteolysis of the antigen; nature of the MHC class II-peptide complex monitored), in the sensitivity of the T-cell readouts or in whether the various studies were performed comparing receptors matched for their ligand interaction properties.

We sought to address some of these issues by extending our experiments using a set of antigen receptors specific for a different antigen. To this end, we created a family of plasmids that directed the expression of either wild-type or chimeric receptors specific for the antigen hen egg lysozyme (HEL). The V_H and V_K regions used derive from those of the mouse monoclonal anti-HEL antibody D1.3 (Amit *et al.*, 1986). The V_H region was linked to the various μ_m, μ/H2, μ/β and μ/LDL C-regions, and the V_K was linked to a rat C_K exon (Figure 3A). We also constructed a chimera containing the cytoplasmic domain of the Igα sheath polypeptide. Transfectants of A20 were established and clones expressing matched levels of receptor were identified by staining with anti-μ, anti-rat κ and anti-idiotypic antibodies (Figure 3B) as well as by their ability to bind radiolabelled HEL (not shown). With regard to internalization, these HEL-specific receptors behaved in a parallel fashion to their NIP-specific counterparts (Figure 3C).

Presentation assays were performed by co-culturing the HEL-specific B-cell transfectants for 24 h with the T-cell hybridoma 1E5.111 (specific for HEL¹⁰⁸⁻¹¹⁶ in the context of I-E^d) in the presence of various concentrations of HEL. Turkey egg lysozyme (TEL), which contains the same T-cell epitope but is not recognized by the D1.3 antibody, served as a control for fluid-phase presentation. The results were dramatically different from those obtained with the NIP-specific transfectants (Figure 4A). All receptors were effective in potentiating HEL presentation. Similar results were obtained with independent clones of B-cell transfectants for each construct as well as when using another, though less sensitive, HEL¹⁰⁸⁻¹¹⁶-specific T-cell hybridoma for readout (hybridoma 1H11.3). To confirm that the IgM/LDL chimera could also efficiently potentiate presentation of other HEL epitopes apart from HEL¹⁰⁸⁻¹¹⁶/I-E^d, we established transfectants of the LK-35.2 B-cell hybridoma (which is of H-2^{k/d} haplotype) since this would allow us to read out the presentation of HEL⁴⁶⁻⁶¹ and HEL²⁵⁻⁴³.

Fig. 3. Internalization by HEL-specific receptors. (A) Structures of the HEL-specific BCRs and maps of the genes encoding their immunoglobulin heavy and light chains. μ_m , the heavy chain of membrane IgM; μ/χ_s , the heavy chain of the IgM chimeras. A, *Apal*; B, *Bam*HI; Bc, *Bcl*I; Bs, *Bst*EII; P, *Pst*I; R, *Eco*RI; S, *Sac*I; X, *Xho*I. (B) Flow cytometric profiles of transfectants stained with biotinylated E5.2 anti-idiotype antibody and FITC-streptavidin. (C) Internalization of the HEL-specific BCRs as monitored by the decrease in mean fluorescence with time at 37°C of A20 transfectants that have been surface stained with FITC-conjugated goat anti- μ . IgM/ α (Δ), IgM/ β ($\blacksquare$), IgM/LDL ($\circ$), IgM/H2 ($\bullet$).

epitopes on I-A^k. Whilst the LK-35.2 and A20 transfectants were well matched for expression of the chimeric receptors, the LK-35.2 transfectants did not in fact trigger the same magnitude of IL2 production as that achieved with the corresponding A20 transfectants when monitoring HEL^{108–116} on I-E^d (Figure 4B, left hand panel); this presumably reflects a diminished processing capacity or MHC class II loading. Nevertheless, it is evident (Figure 4B) that the IgM/LDL chimera could efficiently mediate presentation of other HEL epitopes tested in this 24 h assay.

To ascertain whether the difference between the results obtained in the NIP and HEL systems could be a consequence of a difference in the T-cell readouts used, we exploited a *Pichia pastoris* expression system to prepare a recombinant form of HEL in which the OVA T-cell epitope had been fused to the HEL carboxy-terminus (Figure 5A). The tagged (as well as matched wild-type) recombinant protein was purified by ion exchange chromatography from the yeast culture supernatant and

used for presentation assays. When the HEL/OVA^{322–342} recombinant protein was internalized by HEL-specific B-cell transfectants, good presentation was achieved. This applied whether (i) the HEL-specific BCRs carried an Ig β , an LDL receptor or an H2K^b cytoplasmic domain and (ii) HEL^{108–116}- or OVA^{323–339}-specific T-cell hybridomas were used for readout (Figure 5B). Thus, the ability of IgM/LDL and IgM/H2 chimeric BCRs to potentiate presentation using HEL as the antigen but not using NIP–OVA reflects a difference in the antigen–BCR pairing rather than in the peptide epitope monitored in the T-cell readouts.

To confirm this, we used chemical cross-linking to form an NIP₁₇–OVA/HEL heteroconjugate that could be internalized by either family of receptors, with presentation being monitored by T-cell hybridomas specific for both HEL and OVA peptides. The results (Figure 6) demonstrate that, on incubation with the heteroconjugate, the IgM/LDL chimera can give rise to both HEL^{108–116} and

Fig. 4. Antigen presentation by HEL-specific receptors. (A) Presentation by the various A20 transfectants of I-E^d/HEL¹⁰⁸⁻¹¹⁶ to the 1E5.111 T-cell hybridoma as measured by IL2 production following a 24 h co-culture in the presence of various concentrations of HEL. Turkey lysozyme (TEL) was used to monitor fluid-phase presentation. The results are representative of four experiments carried out with at least two A20 transfectants for each plasmid construct. (B) Presentation by the various LK-35.2 transfectants of I-A^k/HEL⁴⁶⁻⁶¹, I-A^k/HEL²⁵⁻⁴³ and I-E^d/HEL¹⁰⁸⁻¹¹⁶ epitopes to the 1C5.1, 2B6.3 and 1E5.111 T-cell hybridomas respectively in a 24 h co-culture assay. IgM(α/β) ($\square$), IgM/ β ($\blacksquare$), IgM/LDL ($\circ$), IgM/H2 ($\bullet$).

OVA³²³⁻³³⁹ epitopes so long as the antigen is internalized by virtue of HEL binding to the HEL-specific BCR; neither T-cell epitope is generated effectively if the same antigen is internalized through NIP binding to the NIP-specific BCR. Thus, there is something about the quality of antigen–receptor interaction that determines whether antigen binding to the non-professional antigen receptor (IgM/LDL or IgM/H2) leads to effective presentation. This same discrimination with regard to the quality of antigen binding is not apparent with the IgM/ β antigen receptor.

A possible explanation for these observations was that whereas the IgM/ β receptor might accelerate the intracellular delivery of antigen for processing for presentation, the IgM/H2 and IgM/LDL receptors might take a more circuitous route such that only antigens that bound tightly to these non-canonical receptors would be delivered efficiently to an appropriate processing compartment. We therefore compared the different receptors with respect to the kinetics with which they delivered antigen for presentation.

BCR accelerates delivery of antigen to emigrating MHC class II

A pulse assay was used, fixing the B-cell transfectants after various times of incubation with antigen, to compare

the kinetics of delivery of HEL peptide following HEL internalization by either the IgM/ β or IgM/LDL chimeras. The results were dramatic. The IgM(α/β) as well as IgM/ α and IgM/ β receptors gave rise to surface-expressed class II/HEL¹⁰⁸⁻¹¹⁶ complexes within 20 min, whilst presentation by the IgM/LDL and IgM/H2 took considerably longer (Figure 7A). However, at longer time points, it is clear that the IgM/LDL could confer antigen presentation comparable with that mediated by the IgM(α/β), IgM/ α and IgM/ β , whilst the H2 tail was only marginally less potent. Fluid-phase presentation took considerably longer still (Figure 7B). Despite the rapidity of the presentation mediated by the canonical BCR, the peptide is still apparently loaded onto *de novo* synthesized MHC class II molecules since rapid presentation was inhibited if the incubation for antigen internalization was performed in the presence of cycloheximide (Figure 7C).

Having shown that the canonical BCR accelerates delivery of antigen to MHC class II, we wondered whether we could detect differential trafficking by these receptors using confocal microscopy. The B-cell transfectants were incubated with a goat anti- μ antiserum that had been conjugated with biotin through a cleavable disulfide linker. After incubation for various times at 37°C, cells were treated with cysteine at 4°C to strip the biotin from complexes remaining on the cell surface and, following

Fig. 5. Presentation assays using recombinant HEL/OVA³²²⁻³⁴². **(A)** Structure of the HEL and HEL/OVA inserts in the *P.pastoris* expression vector and SDS-PAGE analysis of the purified recombinant HELs. **(B)** Presentation by the various anti-HEL A20 transfectants of I-A^d/OVA³²³⁻³³⁹ and I-E^d/HEL¹⁰⁸⁻¹¹⁶ epitopes as measured by IL2 production following a 24 h co-culture with the 3DO-54.8 or 1E5.111 T-cell hybridomas in the presence of various concentrations of HEL/OVA³²²⁻³⁴². No fluid-phase presentation by untransfected A20 occurred in the antigen concentration range used. IgM/ β (■), IgM/LDL (○), IgM/H2 (●).

fixation, internalized complexes were revealed with FITC-streptavidin. The results (Figure 8A) reveal that both IgM/ β and IgM/LDL are internalized rapidly, although the pattern of staining obtained with the two transfectants differed in that large foci of fluorescence are detectable earlier with IgM/ β than with IgM/LDL. Co-staining with an antibody against the lysosomal protein Lamp-1 indicates that the internalized IgM/ β complexes are targeted more rapidly to Lamp1-containing compartments than the IgM/LDL complexes (Figure 8B).

Discussion

The results presented here show that, as monitored in a presentation pulse assay, the canonical BCR as well as the IgM/ α and IgM/ β chimeras not only drive antigen internalization but also accelerate peptide delivery onto class II. Whilst rapid internalization can also be achieved by an IgM chimera containing the cytoplasmic tail of the LDL receptor, peptide delivery onto class II in this case is much slower. Differential trafficking of the IgM/ β

and IgM/LDL receptors is also revealed by confocal microscopy, where both receptors are seen to transit rapidly into early endosomes but the IgM/ β receptor is speedier in its targeting to compartments in which it co-localizes with the lysosomal marker Lamp-1.

In other studies, peptide loading onto emigrating MHC class II molecules has been found to take place in a specialized compartment, although unanimity has not been reached concerning the nature of this compartment (Amigorena *et al.*, 1994; Qiu *et al.*, 1994; Tulp *et al.*, 1994; West *et al.*, 1994). Indeed, there are also data indicating that loading can occur widely throughout the endocytic pathway (Castellino and Germain, 1995; Pinet *et al.*, 1995). Our finding that the wild-type BCR specifically accelerates antigen targeting does not discriminate whether this targeting is to a compartment specifically devoted to class II loading. Our experiments with the IgM/ α and IgM/ β chimeras reveal that they behaved like intact BCR, with the provision of either the Ig α or the Ig β cytoplasmic domain being sufficient to accelerate antigen targeting to *de novo* synthesized class II. This

Fig. 6. Presentation of both HEL and OVA epitopes by both NIP- and HEL-specific A20 transfectants incubated with a NIP₁₇-OVA/HEL heteroconjugate. Presentation of HEL¹⁰⁸⁻¹¹⁶ (A and C) and OVA³²³⁻³³⁹ (B and D) epitopes by NIP-specific (A and B) and HEL-specific (C and D) A20 transfectants that had been cultured for 24 h with the relevant T-cell hybridoma in the presence of various concentrations of the NIP₁₇-OVA/HEL heteroconjugate was monitored by measuring the IL2 production. No fluid-phase presentation by untransfected A20 occurred in the antigen concentration range used. IgM/β (■), IgM/LDL (○), IgM/H2 (●).

differs from results obtained using a set of FcγRII-based chimeric receptors where Bonnerot *et al.* (1995) found that an FcR/Igβ chimera targeted internalized antigen to load onto recycling MHC class II molecules whereas an FcR/Igα chimera could solely load antigen onto *de novo* synthesized MHC class II. The reason for this discrepancy is unclear, although it obviously cautions that great care needs to be exercised in extrapolating from the behaviour of chimeric receptors to that of intact BCR. Nevertheless, from the experiments performed here, it is evident that the intact BCR accelerates antigen delivery onto class II in a manner that is inhibitable by cycloheximide, suggesting that, from the point of view of the HEL¹⁰⁸⁻¹¹⁶/I-E^d epitope at least, the delivery is only onto *de novo* synthesized MHC class II.

Whereas the experiments in the HEL system indicate that the canonical BCR merely accelerates antigen delivery for class II loading, the results with the NIP-specific BCRs reveal that this acceleration can make the difference as to whether presentation is in fact detected at all. This demonstration that the need for accelerated intracellular targeting depends on the quality of antigen-BCR interaction could explain the paradox that whereas several studies have revealed that BCR is particularly effective at mediating presentation (Shaw *et al.*, 1990; Parikh *et al.*, 1992; Patel and Neuberger, 1993; Bonnerot *et al.*, 1995; Mitchell *et al.*, 1995; Song *et al.*, 1995), others have shown that targeting of antigen to cell surface receptors that have no physiological role in antigen internalization (e.g. transferrin receptor, CD45, MHC class I and II) can nevertheless lead to effective presentation (Casten and

Fig. 7. Kinetics of HEL¹⁰⁸⁻¹¹⁶ presentation by HEL-specific A20 transfectants. (A) Antigen pulse assay giving the IL2 production from the I-E5.111 T-cell hybridoma co-cultured with fixed A20 transfectants that had been pre-incubated with HEL (100 μg/ml) for various times. IgM(α/β) (□), IgM/α (Δ), IgM/β (■), IgM/LDL (○), IgM/H2 (●). (B) Comparison of the kinetics of receptor-mediated presentation [by A20(IgM/β) transfectants] and fluid-phase presentation (by untransfected A20 cells). (C) HEL¹⁰⁸⁻¹¹⁶ presentation is blocked by cycloheximide. Presentation of HEL¹⁰⁸⁻¹¹⁶ by A20 transfectants that had been cultured in the presence of 10 μg/ml cycloheximide (CHX) during the pulse as well as during a 3 h pre-incubation was compared. IgM/χ_s denotes IgM(α/β), IgM/α, IgM/β, IgM/LDL and IgM/H2. As a positive control, presentation by fixed A20(IgM/β) cells (■) pulsed with HEL but not pre-treated with CHX is also shown. To exclude non-specific toxicity of the drug, presentation was also monitored by A20(IgM/β) cells that had been pulsed with HEL for 40 min prior to 3 h CHX treatment [IgM/β+CHX (post-Ag)] (○).

Pierce, 1988; Snider and Segal, 1989; McCoy *et al.*, 1993; Niebling and Pierce, 1993).

More important, however, is the physiological relevance of the BCR-mediated acceleration of presentation. In the germinal centre, where B cells are undergoing very rapid proliferation, it might be necessary to achieve rapid peptide loading within each cell cycle so as to monitor whether the hypermutated BCR still retains specificity for an antigen carrying the relevant helper T-cell epitope. Furthermore, our experiments with the NIP system suggest that the BCR-mediated acceleration of antigen delivery might sometimes be essential to achieve detectable peptide delivery. What is it then about the quality of NIP interaction with the anti-NIP BCR (but not of HEL with the anti-HEL BCR) that makes successful antigen presentation dependent on the acceleration of intracellular trafficking? Experiments with different NIP-OVA conjugation ratios as well as with other hapten-protein conjugates (using transfectants specific for 2-phenyl-5-oxazolone; data not shown) suggest that the issue is not one of valency. Rather, we suspect it is either the rapid off-rate or pH sensitivity of NIP-BCR interaction (spectrophotometric titration reveals

Fig. 8. Comparison of receptor internalization by confocal microscopy. (A) Antigen receptors on the various A20 transfectants were labelled with biotin-S-S-goat anti-μ and, following various times of culture at 37°C, the surface biotin was stripped and the intracellular complexes revealed with FITC-streptavidin. (B) Co-localization of Texas red-conjugated goat anti-mouse IgM internalized by the various A20 transfectants with an anti-Lamp-1 mAb (green) was monitored after various times of incubation at 37°C. Coincident staining of optically merged images appears yellow.

the pK_a of NIP-caproate to be ~5.5). Thus, if internalized by the IgM/LDL chimeric receptor, much of the NIP-OVA antigen (possibly in a partially degraded state) may not remain attached to its receptor for sufficiently long to be escorted to a site for class II loading. Whilst further experiments using a range of B-cell transfectants with different antigen-BCR interaction parameters will be needed to address this issue, a major physiological message remains that quite apart from the fact that the antigen specificity of BCR allows B cells to take up their cognate antigens, the acceleration of intracellular targeting of these antigens can also be essential to achieve class II loading and the recruitment of T-cell help. Without such acceleration, some antigens (exemplified by NIP) might be invisible to the immune system, as monitored by T-dependent responses.

Materials and methods

B-cell transfectants

The A20 B-cell lymphoma [H-2^d; IgG2a,κ (Kim *et al.*, 1979)] was cultured in Dulbecco's modified Eagle's medium (DMEM)/10% fetal calf serum (FCS)/50 μM 2-mercaptoethanol whereas LK-35.2 [H-2^dk; IgG2a,κ (Kappler *et al.*, 1982)] was grown in supplemented RPMI. Plasmids directing the expression of NIP-specific IgM, IgM/β and IgM/H2 receptors have been described previously (Williams *et al.*, 1990; Patel and Neuberger, 1993). For the heavy chains of the IgM/LDL chimera, the HindIII-BamHI fragment containing the region encoding the β cytoplasmic tail of pSV2gpt-μ/β (Patel and Neuberger, 1993) was replaced by a PCR-generated fragment encoding the murine LDL

receptor cytoplasmic domain (see Figure 1A). An EcoRI fragment containing a transcription unit driving the expression of mouse λ1 light chain (Patel and Neuberger, 1993) was inserted into the unique EcoRI site of the resultant plasmids.

For the construction of the HEL-specific receptors, the region of plasmid pβG-μ_m (Williams *et al.*, 1990) extending from the PstI site in V_{NP} to the BamHI site downstream of J_H2 was replaced by a PCR-generated PstI-BstEII fragment containing codons 4–110 of the V_H segment of the D1-3 anti-HEL antibody (McCafferty *et al.*, 1990) joined to a PCR-generated BstEII-BamHI fragment extending 210 nucleotides from a BstEII site created in J_H2 through to the BamHI site in the intron flanking its 3' side (see Figure 3A). The resultant plasmid was used to create derivatives encoding V_HD1-3 linked to various chimeric μ heavy chains as described for the V_{NP} family by exchanging ApaI fragments. The C_H region of the HEL-specific IgM/α chimera was assembled as described for the IgM/LDL chimera above. This IgM/α chimera differs from the previously described NIP-specific version (Patel and Neuberger, 1993), which did not reach the cell surface, at the border of the transmembrane portion and cytoplasmic domain. The light chain of the HEL-specific transfectants was encoded by a plasmid derived from LK-VβG which confers *neo*^r (Yelamos *et al.*, 1995) in which the SacI-XhoI β-globin fragment was replaced by a PCR-generated SacI and XhoI fragment extending from codon 3 to 104 of the D1-3 V_κ (McCafferty *et al.*, 1990).

Following electroporation with plasmid DNA (10–20 μg), B cells were cultured in 96-well plates in the presence of mycophenolic acid and/or G418 (Gibco-BRL). Cell surface expression of transfected immunoglobulin genes was analysed by immunofluorescence using FITC-conjugated goat anti-mouse λ, anti-rat κ, anti-mouse μ (Southern Biotechnology) or biotinylated anti-idiotypic antisera [mAb Ac38 (Reth *et al.*, 1979) for anti-NIP BCRs and mAb E5.2 (Fields *et al.*, 1995) for anti-HEL BCRs] and FITC-streptavidin (Amersham). Homogeneously staining populations were obtained from independent clones, sometimes after cell sorting.

Antigens

NIP₁₇-OVA was prepared by mixing NIP-caproate-*o*-succinimide ester (Genosys Biotechnologies) with ovalbumin in 3% sodium bicarbonate and purifying the conjugate by gel filtration and dialysis. HEL was purchased from Sigma. Recombinant lysozymes were purified by chromatography on CM-Sepharose (eluting with 0.5 M sodium acetate, pH 6) from the culture supernatant of *P.pastoris* transformants grown for 72 h in methanol-supplemented minimal buffered medium (Invitrogen manual). The plasmid directing recombinant HEL expression was assembled by cloning a PCR-generated cDNA fragment that included the signal and mature HEL coding sequence between the *Bam*HI and *Eco*RI sites of pPIC3 (Invitrogen). Expression of HEL/OVA³²²⁻³⁴² was similarly achieved by introducing into pPIC3 a synthetic PCR-generated gene in which codons 322-342 of ovalbumin followed by a translation stop codon had been tagged onto the HEL C-terminus. The NIP₁₇-OVA/HEL conjugate was prepared by a heteroconjugation strategy analogous to that used by Niebling and Pierce (1993). Sulfo-MBS-modified HEL [made by mixing HEL with a 10-fold molar excess of sulfo-MBS (Pierce)] and thiolated NIP₁₇-OVA [made by incubating NIP₁₇-OVA with a 10-fold molar excess of 2-iminothiolane (Pierce)] were purified by gel filtration, concentrated and mixed in an ~2:1 molar ratio for 2 h at room temperature. The products were subjected to gel filtration on Sephacryl S-200 and the fractions analysed by SDS-PAGE.

Antigen presentation assays

Triplicate samples of the A20 or LK-35.2 B-lymphoma transfectants (10⁵ cells) were cultured for 24 h with the relevant T-cell hybridomas (10⁵ cells) in the presence of various concentrations of antigen, and presentation was monitored by the production of IL2 in the culture medium. Fluid-phase presentation was monitored by use of unconjugated OVA or TEL as appropriate. Controls were also carried out to check for IL2 production when antigen, B cells or T cells were individually omitted or when untransfected B-lymphoma cells were used in place of the transfectants. Cross-linking of BCR (or of the IgM/β and IgM/α but not IgM/H2 and IgM/LDL chimeras) on A20 (but not LK-35.2) cells does itself lead to a low level of IL2 production (~1–5 U/ml of IL2 at the maximum antigen concentration tested, contrasting with the T-cell production of ~100–150 U/ml in the presentation assay). However, the concentration of antigen needed to trigger this low-level IL2 production from the B cell was always at least two to three orders of magnitude greater than that needed to trigger IL2 production in the presentation assay. IL2 was measured by providing samples (50 μl) of the culture supernatant (or dilutions thereof) to the HT2 IL2-dependent cell line (2.5×10⁴ cells in 0.1 ml DMEM/10% FCS/50 μM 2-mercaptoethanol) and monitoring the viability of the HT2 cells after 20 h using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma) colorimetric assay (Mossman, 1983). The assay was calibrated using purified recombinant IL2 (Boehringer Mannheim). 1E5.111, 1H11.3, 2B6.3 and 1C5.1 T-cell hybridomas have been described (Adorini *et al.*, 1993). 3DO-54.8 and 3DO-11.10 hybridomas were kindly provided by Dr D. Wraith.

For antigen pulse assays, transfected A20 cells (2×10⁶ cells) were incubated for various times with 100 μg/ml of HEL, washed extensively to remove free antigen and fixed in 1% paraformaldehyde/phosphate-buffered saline (PBS) at room temperature. After washing, fixed cells (2×10⁵) were co-cultured for 24 h with the 1E5.111 T-cell hybridoma (10⁵ cells) prior to determining IL2 production. For cycloheximide inhibition, parallel samples of B cells were incubated with the drug (10 μg/ml) during the HEL pulse as well as for a 3 h pre-incubation.

Internalization assays

To monitor internalization of FITC-conjugated goat anti-mouse μ (Southern Biotechnology), cells were stained on ice in PBS/1% FCS and washed and resuspended in DMEM/10% FCS/50 μM 2-mercaptoethanol prior to warming to 37°C for various times. After rapid chilling and washing with ice-cold PBS, fluorescence was analysed using a Becton-Dickinson FACScan.

To monitor the binding, internalization and degradation of specific antigen, cells [10⁷ in 0.25 ml PBS/30% bovine serum albumin (BSA)] were incubated for 30 min at 4°C with 20 μg of [¹²⁵I]NIP₈-GPDH (1.5×10⁶ c.p.m./μg) that had been prepared by iodobead-catalysed radioiodination (Pierce) of *Bacillus stearothermophilus* GPDH (gift from John Walker) that had been conjugated with NIP-caproate-*o*-succinimide. The cells were washed and resuspended in ice-cold PBS to measure specific binding. Triplicate aliquots (4×10⁵ cells in 0.2 ml) were then incubated for various times at 37°C, diluted with 1 ml ice-cold PBS and pelleted. The counts in the supernatant provided a measure of the

radioactivity returned to the medium. To measure intracellular radioactivity, the cell pellets were resuspended in 1 ml DMEM/1 mg/ml pronase and incubated on ice for 1 h prior to re-pelleting. Counts were measured in the supernatant (pronase-susceptible cell-associated radioactivity) and pellet (taken as intracellular radioactivity) for each sample. Internalization is measured by expressing the pronase-resistant counts for each time point as a percentage of the total radioactivity initially bound by the cells.

Confocal microscopy

To visualize specifically internalized BCR complexes, cells that had been washed with ice-cold PBS were incubated in PBS/1% FCS/1% BSA on ice for 30 min with 10 μg/ml of goat anti-mouse μ antibody (Southern Biotechnology) that had been biotinylated by use of NHS-S-biotin (Pierce). Cells were washed extensively with ice-cold PBS/1% BSA prior to warming to 37°C in DMEM/10% FCS/20 mM HEPES (pH 7.2) for various times (0–90 min). After washing with ice-cold PBS and, if required, stripping cell surface biotin by incubation with 100 mM cysteine for 30 min (Bretscher, 1992), washed cells were allowed to adhere to pre-cooled glass slides for 1–2 h at 4°C. Following fixation (4% paraformaldehyde in PBS for 10 min at room temperature), free aldehyde groups were quenched with 0.1 M glycine/PBS and cells permeabilized using saponin (0.05% in DMEM/10% FCS). The biotinylated complexes were revealed using FITC-conjugated streptavidin and visualized using an MRC-600 laser scanning confocal microscopy (Bio-Rad).

To examine co-localization of internalized BCR complexes with the lysosomal marker Lamp-1, cells that had been incubated with 10 μg/ml Texas red-conjugated goat anti-mouse IgM (Calbiochem) for 30 min on ice were warmed to 37°C for various times. After fixing and washing as described above, Lamp-1 was detected using the rat mAb 1D4B (Developmental Studies Hybridoma Bank, Iowa) followed by biotinylated mouse anti-rat IgG Fc (Jackson laboratories) and FITC-streptavidin. Confocal analysis, using sequential 0.5 μm optical sections, was used to derive a 3-D picture of the cells. Co-localization was visualized using the 488 and 568 nm emissions from an Ar-Kr mixed gas laser, but the analysis was also carried out using the two excitation lines separately to rule out bleedthrough.

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