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Antigen endocytosis and presentation mediated by human membrane IgG1 in the absence of the Igα/Igβ dimer

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Membrane immunoglobulin (mIg) M and D heavy chains possess minimal (KVK) cytoplasmic tails and associate with the Igα/Igβ (CD79) dimer to achieve surface expression and antigen presentation function. In contrast, the cytoplasmic tail of mIgG is extended by 25 residues (γct). We have tested the possibility that mIgG can perform antigen capture and presentation functions independently of the Igα/β dimer. We show that CD4/γct chimeras are efficiently endocytosed partially dependent on a tyrosine residue in γct. In addition, human mIgG was expressed on the surface of Igα/Igβ-negative non-lymphoid cells and mediated antigen capture and endocytosis. Antigen-specific human mIgG targeted antigen to MIIC-type vesicles in the Igα/β negative melanoma Mel JuSo and augmented antigen presentation 1000-fold, identical to the augmentation seen in Igα/β-positive B-cells expressing the same transfected mIgG. Thus, unlike mIgM, mIgG has autonomous antigen capture and presentation capacity, which may have evolved to reduce or eliminate the BCR's dependence on additional accessory molecules.
Keywords: antigen presentation/class II MHC/ endocytosis/Igα/Igβ/mIgG/

Introduction

The B-cell antigen receptor (BCR) is a membrane-anchored immunoglobulin (mIg) (for review, see Reth, 1992; Cambier *et al.*, 1994) whose expression on the cell surface is essential for normal B-cell maturation beyond the pre-B-cell stage (Kitamura *et al.*, 1991). The BCR is composed of heavy and light immunoglobulin chains and a non-covalently associated disulfide-linked dimer of Igα and Igβ proteins (Hombach *et al.*, 1988). Several features of BCR function depend on co-expression of this dimer. In its absence, mIgM cannot be expressed on the cell surface (Hombach *et al.*, 1990; Williams *et al.*, 1990; Venkitaraman *et al.*, 1991; Matsuuchi *et al.*, 1992), BCR signalling capacity is lost (Grupp *et al.*, 1993; Sanchez *et al.*, 1993; Michnoff *et al.*, 1994; Williams *et al.*, 1994) and consequently, normal B-cell maturation is disrupted (Papavasiliou *et al.*, 1995a,b; Gong and Nussenzweig, 1996; Torres *et al.*, 1996).

B-cells expressing antigen-specific BCRs can present antigens to MHC class II-restricted T-cells 10^3- to 10^4-fold more efficiently than non-specific B-cells (Rock *et al.*, 1984; Lanzavecchia, 1985). This is due to specific antigen binding, endocytosis and delivery for processing to the MHC class II-rich endosomal compartments (MIIC/CIIV) found in these cells (Peters *et al.*, 1991; Amigorena *et al.*, 1994; reviewed in Watts, 1997). The Igα/Igβ dimer has also been demonstrated to play an important role in BCR antigen presentation function. Since mIgM (μ) and mIgD (δ) have transmembrane heavy chains with only three putative cytoplasmic residues (–KVK), the Igα/Igβ dimer is thought to act as an adaptor permitting the external antigen binding site to be internalized and access the endocytic pathway. Indeed, the cytoplasmic domain of Igβ was able to confer endocytosis and antigen presentation capacity when directly fused to mIg or Fc receptors (Patel and Neuberger, 1993; Bonnerot *et al.*, 1995), although in one study only the tail of Igα appeared able to target FcR-bound antigen to newly synthesized class II MHC molecules (Bonnerot *et al.*, 1995).

Following antigen stimulation and isotype switching, B-cells continue to express membrane-anchored immunoglobulins. However, the isotype-switched immunoglobulin classes IgG (γ), IgA (α) and IgE (ε) possess extended cytoplasmic tails encoded by the M2 exon (Bensmana and Lefranc, 1990; Kinoshita *et al.*, 1991; Sun *et al.*, 1991, respectively). In the case of mIgG, the KVK sequence is extended by 25 residues to form a cytoplasmic tail (γct) which is highly conserved among γ sub-types and between species (Bensmana and Lefranc, 1990; Kinoshita *et al.*, 1991). No function for the cytosolic domains of class-switched membrane Igs has been described. We have examined the possibility that the extended heavy chain of mIgG may permit BCR cell surface expression and antigen presentation function, independent of the Igα/Igβ dimer. We demonstrate for the first time that the cytoplasmic tail of a human mIgG contains an endocytosis motif. Further, we show that the mIgG BCR can be expressed on the surface of cells lacking the Igα/Igβ dimer and that this minimal form of the BCR can provide the full 10^3- to 10^4-fold enhancement of antigen presentation characteristic of antigen-specific B-cells.

Results

The cytoplasmic tail of membrane IgG1 heavy chain can mediate endocytosis

To initiate studies on the mIgG form of the BCR we used RT–PCR to clone the heavy and light chain genes (see Materials and methods) from the Epstein–Barr Virus (EBV)-transformed human B-cell clone 11.3 which expresses membrane IgG1 specific for tetanus toxin (Lanzavecchia, 1985). The predicted γ heavy chain was 521 residues in length and, compared with membrane μ chains, was extended as expected by 25 C-terminal residues (γct; Figure 1A). In clone 11.3 cells we found an

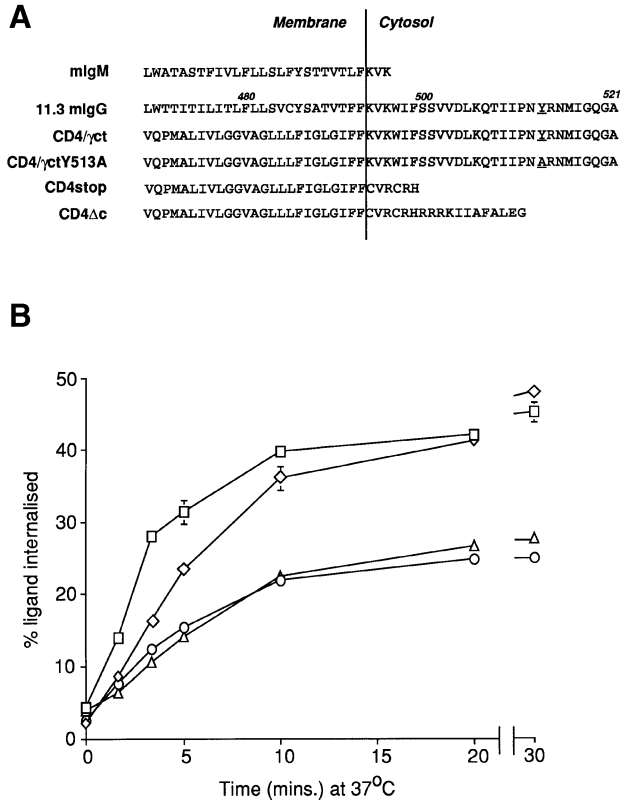

Fig. 1. (**A**) Amino acid sequences of wild-type human mIgM, B-cell clone 11.3 mIgG and CD4 chimeric transmembrane/cytoplasmic domains. Residues in 11.3 mIgG are numbered from the first residue (Gln) of the variable region. (**B**) The γct domain contains a tyrosine-based endocytosis signal. Internalization of ^{125}I mAb Q4120 bound to CD4 chimeras in transfected CHO cell lines was measured as described in Materials and methods for CD4/γct (□); CD4/γctY513A (◇); CD4stop (○); and CD4Δc (△).

asparagine residue at position 512 instead of the aspartic acid reported for other human membrane gamma chains (Bensmana and Lefranc, 1990; Kinoshita *et al.*, 1991).

We first tested the capacity of the cytoplasmic tail of mIgG (γct) to mediate endocytosis by creating chimeras in which γct was transplanted onto the extracellular and transmembrane domains of CD4 (CD4/γct; Figure 1A). We compared the endocytosis kinetics of this construct with that of two other CD4 derivatives, CD4stop and CD4Δc (Figure 1A) which show basal endocytosis rates (Ponnambalam *et al.*, 1994; Sauter *et al.*, 1996). Because γct contains a tyrosine residue (Y513) in a context (IPNYRNM; Figure 1A) resembling other tyrosine-based endocytosis signals (Trowbridge *et al.*, 1993; Naim and Roth, 1994), we mutated this residue to alanine in CD4/γct (CD4/γctY513A). All constructs were stably expressed in CHO cells and initial endocytosis kinetics measured using the CD4-specific mAb Q4120. ^{125}I-labelled Q4120 was bound at 0°C, unbound ligand removed, and the cells shifted to 37°C for different time periods. Internalized ligand was then measured by removing residual cell surface ligand with acid. Q4120 was endocytosed at an initial rate of ~2% per min in CD4stop and CD4Δc transfectants, producing a steady-state intracellular pool of ~25% after 10–15 min (Figure 1B). This is similar to the basal rates of endocytosis previously observed for CD4 mutants lacking specific endocytosis signals (Pelchen-Mathews *et al.*, 1991; Sauter *et al.*, 1996). In contrast, the CD4/γct chimera was endocytosed at a considerably enhanced rate (~8% per min) and >40% of the ligand equilibrated with an intracellular pool after 10–15 min (Figure 1B). Substitution of the tyrosine residue in γct for alanine (CD4/γctY513A) reduced the initial rate of ligand uptake ~2-fold. However, this chimera was still endocytosed faster than CD4stop or CD4Δc and eventually equilibrated with a similar-sized intracellular pool as CD4/γct (Figure 1B). These data suggest that the γct domain contains at least two endocytosis signals, one of which is based around Y513. Moreover, since the rate of ligand clearance from the cell surface and the steady-state pool size observed with the CD4/γct chimera is comparable with previously observed for specific antigen uptake via mIgG on human B-cell lines (Watts and Davidson 1988; Davidson and Watts 1989), this raised the possibility that γct might be involved in antigen endocytosis via mIgG BCRs.

Surface expression and endocytosis of mIgG in the absence of both Igα and Igβ

Several studies demonstrate that mIgM (μ) requires co-expression of the Igα/Igβ dimer in order to be exported from the endoplasmic reticulum (Hombach *et al.*, 1988; Matsuuchi *et al.*, 1992). However, the murine γ2b and γ2a isotypes, together with associated light chain, were able to reach the cell surface of J558L plasmacytoma cells which lack the Igα sub-unit (Venkitaraman *et al.*, 1991; Weiser *et al.*, 1994). We therefore tested whether the human γ1 isotype can reach the cell surface in the absence of both Igα and Igβ by expressing the light chain (κ) gene along with either wild-type or mutant γ chains in HeLa cells which are Igα/Igβ-negative (Verschuren *et al.*, 1993). When expressed alone, the γ heavy chain failed to reach the cell surface and biosynthetically labelled material remained endoglycosidase H-sensitive (Figure 2). In contrast, co-transfection with light chains resulted in association with the γ heavy chain, acquisition of endoglycosidase H-resistance and transport to the cell surface (Figure 2). Indirect immunofluorescence microscopy confirmed that the heavy/light chain complex had reached the cell surface (Figure 3A, panel b) whereas the heavy chain expressed alone did not (Figure 3A, panel a) and instead was retained in the endoplasmic reticulum (Figure 3A, panel e′). Thus, unlike mIgM, mIgG can be exported to the cell surface of non-lymphoid cells in the absence of both Igα and Igβ.

Interestingly, export of γ/κ complexes from the endoplasmic reticulum appeared to be partially dependent on γct since deletion of the 25 residue γ-specific tail H(KVK), while not affecting the level of surface expression (Figure 3A, panel d), caused significant accumulation of mIgG in the endoplasmic reticulum (Figure 3A, panels h and h′). Mutagenesis of the single tyrosine (Y513A) within γct had no detectable effect on cell surface expression of mIgG (Figure 3A, compare panels b and c).

Permeabilization of fixed γ/κ HeLa transfectants revealed the presence of numerous cytoplasmic vesicles which co-stained with both anti-light or heavy chain antibodies (Figure 3A, panels f and f′ respectively) and with biotinylated tetanus toxin antigen, demonstrating properly assembled mIgG (Figure 3A, panels i and i′).

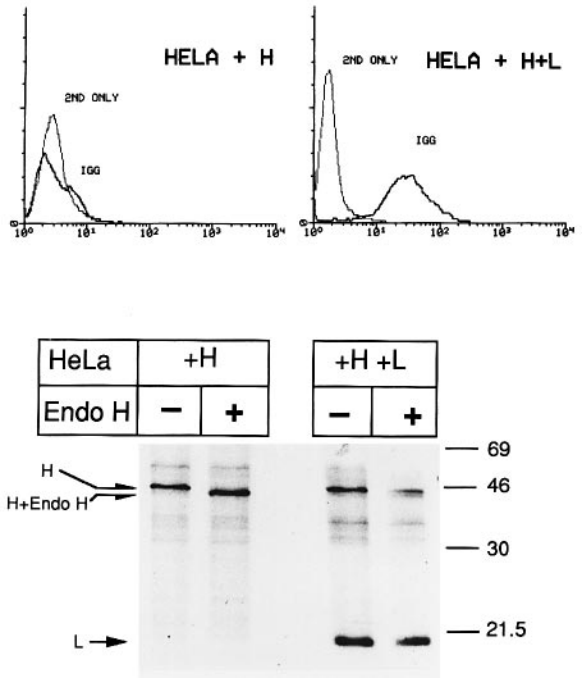

Fig. 2. Cell surface expression of mIgG in the absence of the Igα/Igβ dimer. Stable HeLa transfectants were generated expressing either Igγ heavy chain cDNA (left-hand panels) or Igγ heavy and κ light chain cDNAs (right-hand panels). Top panels show flow cytometric analysis (10^4 cells) stained with biotinylated goat anti-human IgG followed with streptavidin FITC (IGG) or with streptavidin FITC only (2ND ONLY). Bottom panels show immunoprecipitated (arrows) heavy (H) (left panel) or heavy and light (L) chains (right panel) from ^{35}S-labelled transfectants treated (+) or mock-treated (–) with endoglycosidase H.

These vesicles were also evident in the H(Y513A) mutant (Figure 3A, panels g and g′), but they were much less prominent in cells transfected with the H(KVK) γ chain where most intracellular mIgG appeared to be confined to the endoplasmic reticulum (Figure 3A, panels h and h′). To establish whether these vesicles were endocytic in origin we incubated transfected HeLa cells with biotinylated antigen at 37°C for 60 min. As expected, cells transfected with heavy chain only showed no evidence of antigen uptake (Figure 3B, panels a and a′) whereas cells transfected with heavy and light chains were clearly able to endocytose antigen (Figure 3B, panel b and b′). Taken together, these data show that, unlike mIgM, mIgG can be expressed on the cell surface and can mediate the endocytosis and intracellular accumulation of specific antigen in the absence of both components of the Igα/Igβ dimer.

mIgG mediates equivalent augmentation of antigen presentation with or without the Igα/Igβ dimer

We next assessed whether mIgG antigen presentation function requires co-expression of Igα/Igβ. We therefore introduced mIgG into both Igα/Igβ-positive B-cells (Ramos; human and A20; mouse) and into the HLA-DR- and DM-positive melanoma line Mel JuSo. This non-lymphoid cell line contains class II MHC-positive endosomes (Pieters *et al.*, 1991; Sanderson *et al.*, 1994; Tulp *et al.*, 1994) similar to the MIIC vesicles identified in B-lymphocytes and dendritic cells (Peters *et al.*, 1991; Nijman *et al.*, 1995). Importantly, the Mel JuSo transfectants were negative for the Igα/Igβ complex as assessed by staining with the CD79b (Igβ)-specific monoclonal antibody CB3-1 (Nakamura *et al.*, 1992) which detected abundant expression on the Burkitt lymphoma cell line Ramos (Figure 4A). Pilot experiments also showed that Mel JuSo cells could process and present the tetanus toxin epitope 1273–1284 to the DRw52a/c-restricted T-cell clone KT4 (Demotz *et al.*, 1989) following antigen pulsing (Figure 4B). As expected, both Ramos (Figure 5B) and A20 (not shown) expressed the intact or the tailless mIgG [H(KVK)] on the cell surface (Figure 5A). As previously seen in HeLa cells, Mel JuSo transfectants also expressed the intact or the tailless mIgG [H(KVK)] on the cell surface in the absence of Igα/Igβ (Figure 5A). In addition, immunoelectron microscopy of the Mel JuSo transfectants revealed that the MIIC-like structures previously observed in this cell line (Pieters *et al.*, 1991) also contained the transfected mIgG (Figure 5B), indicating its successful targeting to this specialized endosome population in the absence of Igα/Igβ expression.

To test antigen presentation capacity, transfectants were cultured together with graded doses of antigen in the presence of tetanus toxin-specific T-cell clones. Introduction of tetanus toxin-specific mIgG into the Ramos cell line allowed antigen presentation to the T-cell clone D1c at concentrations 10^3- to 10^4-fold lower than those required for untransfected Ramos cells (Figure 6A). This is consistent with earlier studies which demonstrated a very similar enhancement of antigen presentation in antigen-specific versus non-specific B-cells (Rock *et al.*, 1984; Lanzavecchia, 1985). We next tested the relative efficiency of antigen presentation in control Mel JuSo cells versus Mel JuSo clones expressing the same antigen-specific heavy and light chains. Remarkably, the same dramatic augmentation of antigen presentation was observed in Mel JuSo cells expressing wild-type mIgG as was observed in Ramos (Figure 6B and C). Thus, the mIgG BCR can enhance antigen presentation to the same extent in the presence or absence of the Igα/Igβ dimer. To assess the contribution that γct might make to antigen presentation via the mIgG BCR we also tested antigen presentation in Ramos B cells and Mel JuSo cells expressing a heavy chain lacking the cytoplasmic tail [H(KVK)]. With this truncated form of the BCR, Ramos transfectants presented antigen at least as well as Ramos transfected with full-length γ heavy chains (Figure 6A). Similar results were obtained in A20 murine B-cells transfected with the same mIgG BCR constructs (not shown). In these experiments we utilized a tetanus toxin/ovalbumin conjugate and the ovalbumin-specific T-cell hybridoma 3DO.548 to assay antigen presentation (data not shown). [We attribute the slight enhancement seen with the H(KVK) Ramos cells to their somewhat higher levels of BCR expression compared with cells expressing intact mIgG (Figure 5A and data not shown).] Taken together, we conclude that antigen presentation via the mIgG BCR is not measurably compromised by the absence of the γ cytoplasmic tail either in Ramos or in A20 B cells. In contrast, Mel JuSo cells expressing the H(KVK) γ chain (clones 1G9 and 1H5) presented antigen at least 10-fold less effectively compared with cells (clones 8H5 and 5G1) expressing full-length mIgG (Figure 6B and C). This demonstrates that, in the absence of Igα/Igβ, the γct domain is required for maximal

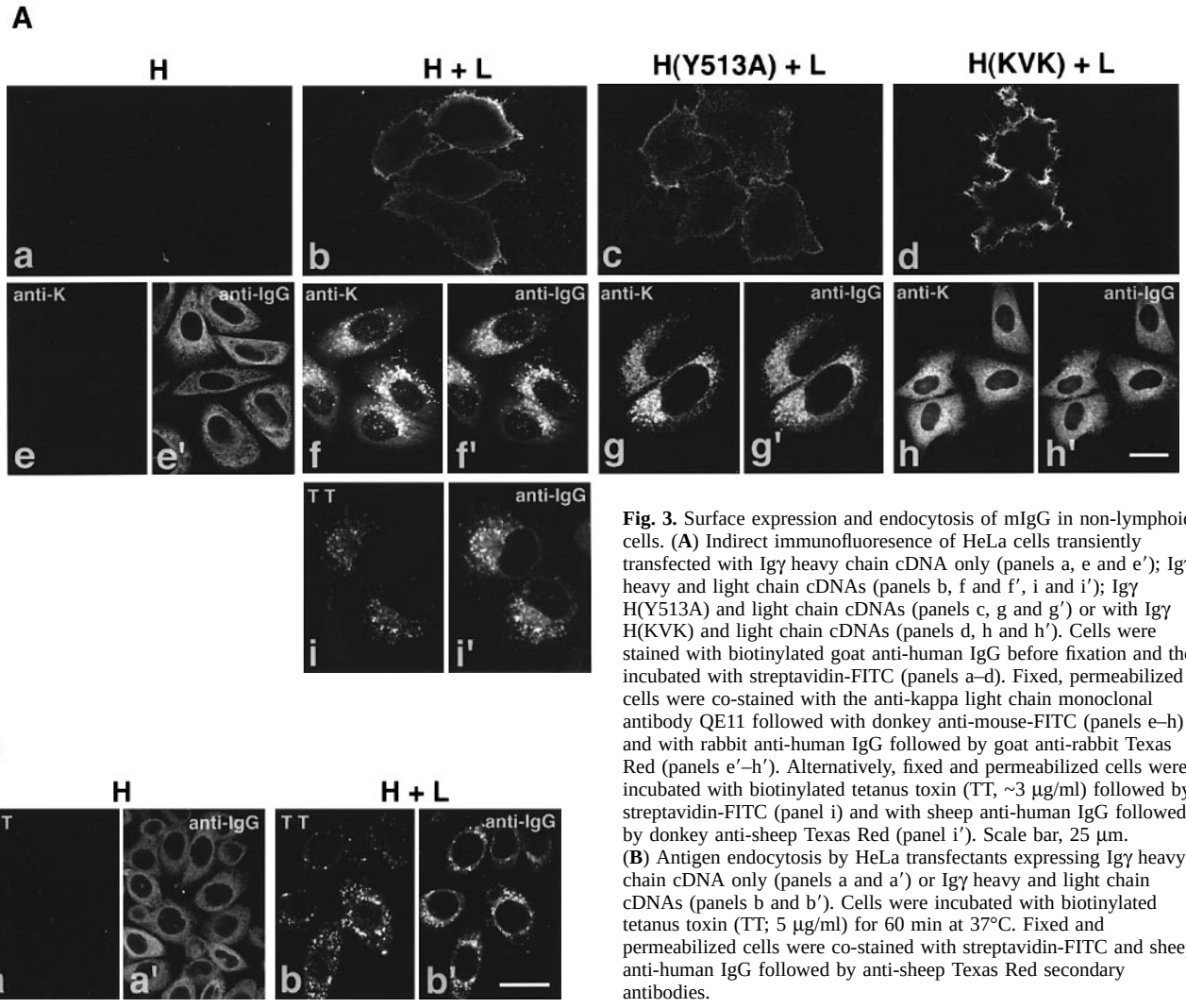

Fig. 3. Surface expression and endocytosis of mIgG in non-lymphoid cells. (**A**) Indirect immunofluoresence of HeLa cells transiently transfected with Igγ heavy chain cDNA only (panels a, e and e′); Igγ heavy and light chain cDNAs (panels b, f and f′, i and i′); Igγ H(Y513A) and light chain cDNAs (panels c, g and g′) or with Igγ H(KVK) and light chain cDNAs (panels d, h and h′). Cells were stained with biotinylated goat anti-human IgG before fixation and then incubated with streptavidin-FITC (panels a–d). Fixed, permeabilized cells were co-stained with the anti-kappa light chain monoclonal antibody QE11 followed with donkey anti-mouse-FITC (panels e–h) and with rabbit anti-human IgG followed by goat anti-rabbit Texas Red (panels e′–h′). Alternatively, fixed and permeabilized cells were incubated with biotinylated tetanus toxin (TT, ~3 μg/ml) followed by streptavidin-FITC (panel i) and with sheep anti-human IgG followed by donkey anti-sheep Texas Red (panel i′). Scale bar, 25 μm. (**B**) Antigen endocytosis by HeLa transfectants expressing Igγ heavy chain cDNA only (panels a and a′) or Igγ heavy and light chain cDNAs (panels b and b′). Cells were incubated with biotinylated tetanus toxin (TT; 5 μg/ml) for 60 min at 37°C. Fixed and permeabilized cells were co-stained with streptavidin-FITC and sheep anti-human IgG followed by anti-sheep Texas Red secondary antibodies.

mIgG-mediated antigen presentation. Nonetheless, cells expressing the H(KVK) form of the γ chain still presented antigen substantially better than cells lacking antigen-specific BCRs (Figure 6B and C; also see Discussion). Interestingly, Mel JuSo transfectants expressing the H(Y513A) mutant heavy chain showed a similar loss of antigen presentation efficiency as seen in cells expressing the H(KVK) heavy chain (Figure 6B). Taken together, these results clearly demonstrate, first, that the mIgG BCR delivers equivalent augmentation of antigen presentation in the presence or absence of the Igα/Igβ dimer and, secondly, that the cytoplasmic tail (and in particular Y513) of the γ heavy chain is necessary for full Igα/Igβ-independent antigen presentation capacity.

Discussion

Switching of immunoglobulin class, affinity maturation of the antigen binding site and the establishment of immunological memory are all familiar characteristics of the developing antibody-mediated immune response. Less familiar and largely unexplored is a striking change in the structure of the B-cell antigen receptor. Whereas the heavy chains of membrane IgM/D molecules expressed on naive B-cells lack cytoplasmic domains, the class-switched isotypes express heavy chains extended by 25 (γ and ε) or 11 (α) residues. No function or characterization of these cytoplasmic domains has previously been described. The tailless IgM isotype depends on co-expression of the Igα/Igβ disulfide-linked dimer both for expression on the cell surface (Hombach *et al.*, 1990; Williams *et al.*, 1990; Venkitaraman *et al.*, 1991; Matsuuchi *et al.*, 1992) and for antigen presentation function (Patel and Neuberger, 1993; Bonnerot *et al.*, 1995). Mutant BCRs which can reach the cell surface in the absence of the Igα/Igβ dimer (Williams *et al.*, 1990) still lack antigen uptake and presentation capacity (Patel and Neuberger, 1993), demonstrating that the Igα/Igβ dimer fulfils distinct functions for mIgM. Here, we show that surface expression, endocytosis and antigen presentation mediated by the class-switched IgG BCR can occur in the absence of the Igα/β dimer and that under these conditions the cytoplasmic domain (γct) is important for IgG BCR function.

CD4/γct chimeras expressed in CHO cells were endocytosed 4-fold faster than control CD4 constructs and showed 2-fold greater steady-state levels of accumulation. Mutation of the single tyrosine residue in γct reduced the initial endocytosis kinetics of the CD4/γct chimera 2-fold, but the steady-state distribution between cell surface and intracellular compartments was not affected. Thus, γct

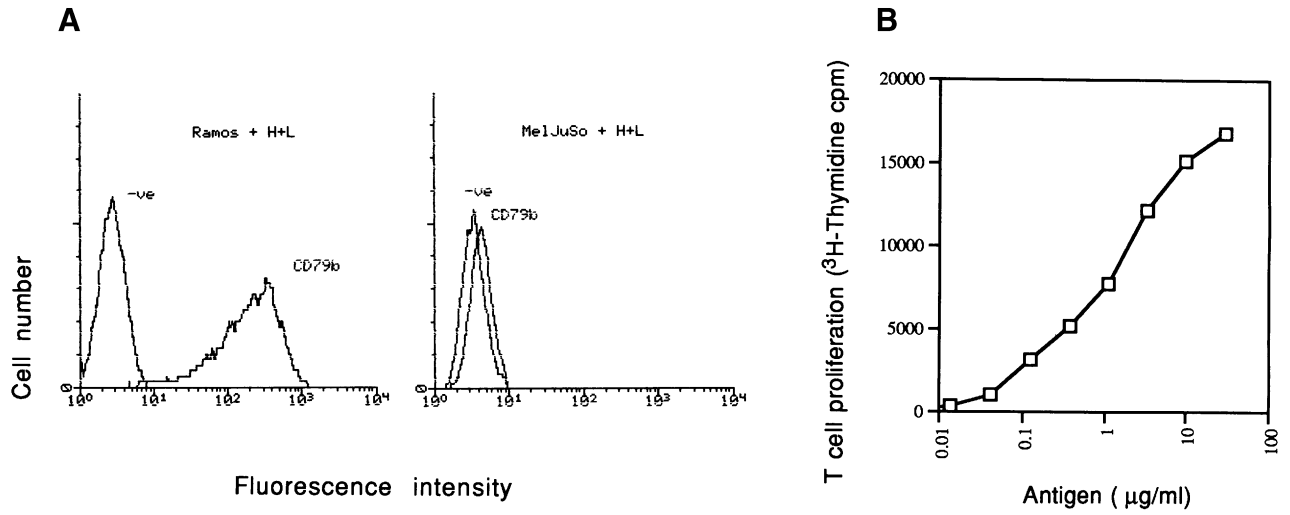

Fig. 4. Mel JuSo cells are negative for surface expression of CD79b (Igβ) and can present antigen to the tetanus toxin-specific T-cell clone KT4 (tetanus toxin 1273–1284; Demotz *et al.*, 1989). (**A**) Expression of Igα/β (CD79b) on the surface of Ramos (H+L) and Mel JuSo (H+L) transfectants. Igγ heavy and light chain cDNA transfectants (Ramos or Mel JuSo) were stained with the anti-CD79b mAb, CB3-1 (Nakamura *et al.*, 1992) followed with goat anti-mouse FITC or with the goat anti-mouse FITC antibody only (–ve). (**B**) Untransfected Mel JuSo cells were incubated with graded amounts of tetanus toxin C fragment domain in the presence of the KT4 T cell clone. Proliferation of the clone, measured by [^{3}H]thymidine incorporation (c.p.m.), was measured after 48 h.

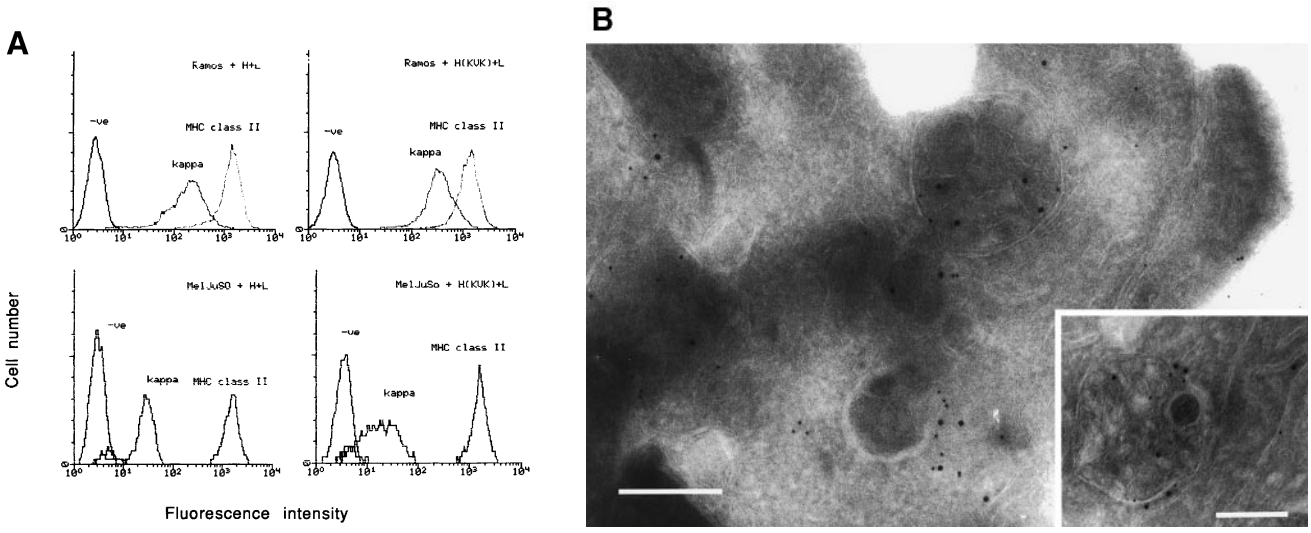

Fig. 5. Cell surface expression and MIIC targeting of mIgG in Igα/Igβ positive (Ramos) and negative (Mel JuSo) transfectants. (**A**) Expression of mIgG and MHC class II on the surface of Ramos (H+L), Ramos (H(KVK) +L), Mel JuSo (H+L) and Mel JuSo (H(KVK)+L) transfectants. Igγ heavy and light chain cDNA transfectants (Ramos or Mel JuSo) were stained with the QE11 (anti-human kappa) and L243 (anti-MHC class II) monoclonal antibodies followed with second layers as in Figure 4A. (**B**) mIgG is found in MIIC-like multivesicular compartments. Mel JuSo transfectants were processed for immunoelectron microscopy as described (West *et al.*, 1994; Lucocq *et al.*, 1995). Cryosections were sequentially labelled for class II MHC using DA6.231 mAb followed by rabbit anti-mouse and small (6 nm) gold, and with sheep anti-human IgG followed by rabbit anti-sheep and large (12 nm) gold. Bars, 200 nm. Note that labelling for mIgG was frequently observed on internal membranes (main figure and inset).

contains at least one tyrosine-based endocytosis motif and perhaps other determinants which control endocytosis/recycling kinetics.

Remarkably, expression of a minimal BCR consisting of only immunoglobulin heavy and light chains boosted antigen presenting capacity at least 1000-fold in the HLA-DR- and DM-positive non-lymphoid cell Mel JuSo, equivalent to the augmentation seen when the same receptor was expressed in Igα/Igβ-positive B-cells. Deletion of γct or mutation of its single tyrosine residue reduced the efficiency of antigen presentation 10-fold in the Igα/Igβ-negative cells. Thus, the mIgG BCR can mediate highly efficient antigen presentation in the absence of Igα/Igβ and the γct domain, and Y513 in particular is required for its full antigen presentation capacity.

Several lines of evidence suggest that endocytosis alone is not sufficient for targeting of antigen (Neibling and Pierce, 1993) or invariant chain complexes (Swier and Miller, 1995) to the compartments where antigen processing and class II MHC maturation take place (reviewed in Watts, 1997). Receptor oligomerization (Mellman and Plutner, 1984; Swier and Miller, 1995) or other targeting mechanisms (Bonnerot *et al.*, 1995; Mitchell *et al.*, 1995) appear necessary for efficient delivery to the processing

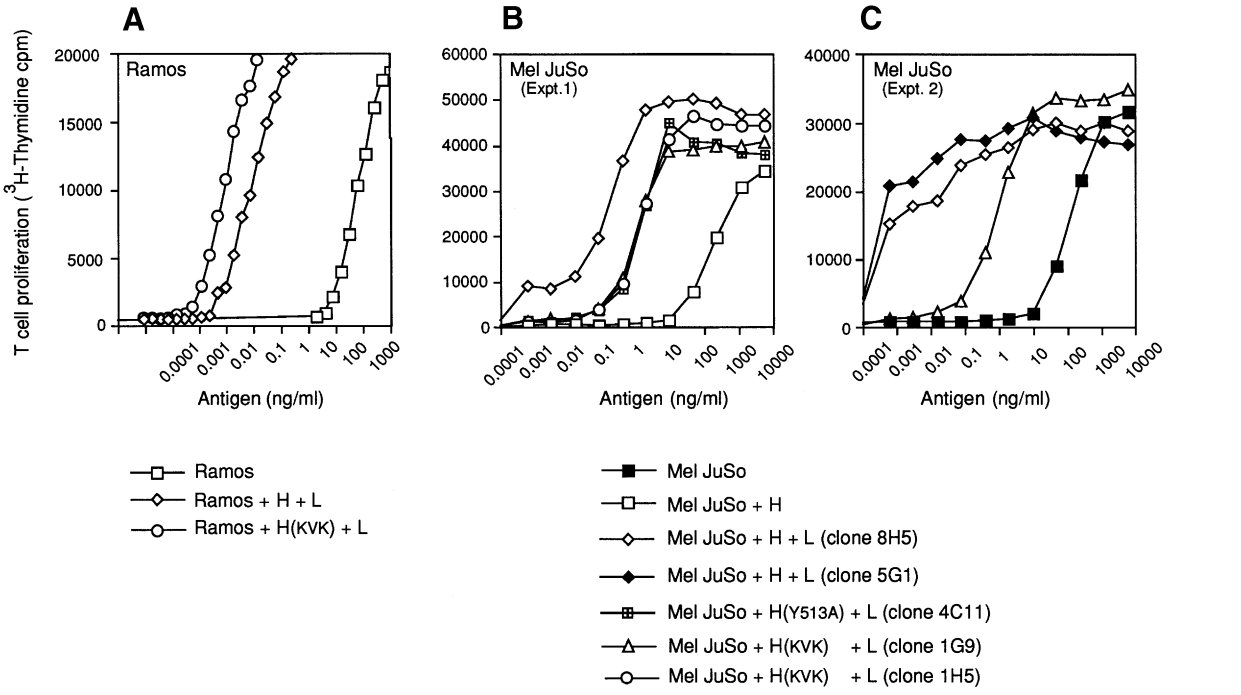

Fig. 6. mIgG augments antigen presentation to the same extent in both Igα/Igβ-positive and -negative cells. 2×10^4 irradiated Ramos (**A**) or Mel JuSo (**B** and **C**) clones expressing wild-type or mutant heavy chains plus light chains were co-cultured with 2×10^4 T-cells in the presence of graded doses of antigen in 96-well plates. T-cell clones D1c (tetanus toxin 947–966) and KT4 (tetanus toxin 1273–1284; Demotz *et al.*, 1989) were used to detect antigen presentation by Ramos and Mel JuSo respectively. Experiments shown are representative of several and illustrate similar antigen presentation capacity in independently isolated clones expressing the same cDNAs.

compartments. Because mutation of the tyrosine residue in γct reduced the efficiency of antigen presentation 10-fold but did not—at least in the CD4/γct chimera—affect equilibration with peripheral recycling endosomes, we suggest that this residue may also form part of a signal important for post-endocytic targeting of IgG-BCR-bound antigen to the processing and class II MHC loading compartment(s).

We expected that Mel JuSo cells expressing specific antigen receptors lacking the γct domain would still present antigen better than cells lacking BCRs altogether since the latter cells depend solely on fluid phase pinocytosis for antigen entry into the endocytic pathway. However, the degree of enhanced antigen presentation observed in cells expressing the H(KVK) form of the heavy chain was somewhat surprising and raises the interesting possibility that γct is not the sole determinant of antigen targeting in mIgG.

Deletion of the cytoplasmic tail of membrane IgG had no obvious effect on antigen presentation in the two B-cell lines we tested. Presumably, the Igα/Igβ dimer expressed in these cell lines can provide both endocytosis and antigen presentation function for the tailless mIgG as it does for mIgM. This raises the question of the role of γct in class-switched B-cells. Given the high degree of sequence conservation of the cytoplasmic tail among γ sub-types and between species (Bensmana and Lefranc, 1990; Kinoshita *et al.*, 1991) and the fact that it can augment antigen presentation to an extent equivalent to that provided by Igα/Igβ, it seems very likely that this capacity is utilized *in vivo* following switching to the IgG class. One possibility is that certain IgG-positive B-cells may lose expression either of Igα/Igβ or some other component which interacts with Igα/Igβ to deliver full antigen presentation function to the IgM BCR on naive B-cells. In fact, there is evidence that additional components besides the Igα/Igβ dimer may be important even for IgM class BCRs. Mutation of a tyrosine residue in the transmembrane region of mIgM heavy chain abolished antigen presentation function (Shaw *et al.*, 1990) but did not affect antigen uptake or degradation (Mitchell *et al.*, 1995). Since this mutation did not disrupt association with the Igα/Igβ dimer, these studies indicate that other unidentified components may exist which are crucial for the fidelity of antigen targeting to MIIC/CIIV via the IgM BCR. Thus, although expression of the Igα/Igβ dimer apparently persists in IgG-positive B-cells (Venkitaraman *et al.*, 1991; van Noesel *et al.*, 1992) other putative components of the BCR sorting machinery may be poorly expressed or absent in isotype-switched B-cells. Consequently, the switched BCR isotypes may therefore require autonomous antigen presentation capacity as demonstrated here.

Materials and methods

Cell lines and culture conditions

Chinese Hamster Ovary (CHO), HeLa, and Mel JuSo cells were routinely grown at 37°C in Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL), supplemented with 10% fetal calf serum (FCS), 100 μg/ml kanamycin and 2 mM glutamine. Ramos (human mIgM$^+$, λ$^+$ B-lymphoma) and A20 (mouse mIgG2a$^+$, κ$^+$, B-lymphoma) cells were grown in RPMI 1640 (Gibco-BRL) supplemented with 10% FCS, 100 μg/ml kanamycin, 2 mM glutamine, 1 mM pyruvate, non-essential amino acids and 50 μM β-mercaptoethanol. Tetanus toxin-specific T-cell clones, KT4 (donor KK) and D1c (donor PDS) were maintained as previously described (Lanzavecchia, 1985).

mIgG cloning and mutagenesis

5 μg of RNA from the EBV-transformed tetanus toxin-specific B-cell clone 11.3 (Lanzavecchia, 1985) was reverse-transcribed with AMV reverse transcriptase (Pharmacia), specifically primed with oligonucleotides (γct 5′-CTGGGATCCCTAGGCCCCCTGTCCGATCAT-3′ and κc 5′-CTGGAATTCCTAACACTCTCCCCTGTTGAA-3′) complementary to the last 18 bp of the human γ1 membrane exon (M2) and the κ constant region respectively (Hieter *et al.*, 1980; Bensmana and Lefranc, 1990; Kinoshita *et al.*, 1991). The primers contained a stop codon and a restriction site. The resulting cDNAs were amplified by PCR using conditions described (Love *et al.*, 1990) with degenerate primers based on γ and κ leader sequences (Larrick *et al.*, 1989). PCR products were cloned into the mammalian expression vectors pMCFR–HphI, pMCFR–NI or pMCFR–pac (kind gifts from Drs Denzin, Cresswell and Novak); their subsequent sequencing confirmed that these products encoded the membrane γ1 heavy and κ light chain products respectively. For the light chain construct, detectable expression was obtained only after replacing the PCR-amplified leader sequence with that from the γ heavy chain. In addition, residue 512 of the γ1 heavy chain from the 11.3 EBV clone (Asn) differed from that previously reported (Asp; Bensmana and Lefranc, 1990; Kinoshita *et al.*, 1991); however, mutating Asn to Asp had no effect on the endocytosis rates of the CD4/γct chimera (data not shown).

Utilizing a unique *Nsi*I site within the 11.3 γ1 heavy chain cDNA, a 276 bp *Nsi*I–*Bam*HI fragment encoding the entire transmembrane and cytoplasmic domains was subcloned into pSL1180 (Pharmacia). Mutagenesis of Y513 to A513 was subsequently performed on pSL1180/276 bp using a PCR-based method described by Mikaelian and Sergent (1992) using the following γ-specific primer: γctY513A 5′-ACCATCATCCCCAACGCCAGGAACATGATCGG-3′. Once the mutation was confirmed by sequencing the resultant fragment was re-cloned back into the 11.3 heavy chain cDNA.

To construct γct/KVK, pSL1180/276 bp was amplified using the M13-20 primer (Stratagene) and a 3′ primer complementary to the transmembrane and first three residues (KVK) of the 11.3 γ1 cDNA (γct/KVK: 5′-CTGGGATCCCTACTTCACCTTGAAGAAGGTGAC-3′). Following sequencing, the resulting PCR product was then recloned back into the 11.3 cDNA as described above.

CD4/γct chimeric receptor construction

To construct the CD4/γct chimeras, plasmids containing either the wild-type or mutant Y513A 11.3 γ1 cDNAs were mixed with human CD4 cDNA (gift from Dr Mark Marsh) and amplified with the following three oligonucleotides as described (Yon and Fried, 1989): CD4 *Ava*I: 5′-GCCTCGGGACAGGTCCTGCTG-3′; CD4/γct: 5′- GAGAAGATCCACTTCACCTTGAAGAAGATGCCTAGCCCAA-3′ and γct (see above). PCR products were digested with *Ava*I and *Bam*HI and used to replace the wild-type *Ava*I–*Bam*HI fragment in the CD4 cDNA. Both the CD4stop (Sauter *et al.*, 1996) and the CD4Δc constructs (Ponnambalam *et al.*, 1994) have been described elsewhere. All constructs were confirmed by sequencing and cloned into the mammalian expression vector pSG5 (Invitrogen).

Transfections

Plasmids encoding CD4, CD4/γct, CD4/γctY513A, CD4stop, CD4Δc, 11.3 γ-heavy chain, mutants of γ as indicated and 11.3 light chain were transfected into CHO, Mel JuSo and HeLa cells using calcium phosphate precipitation. For the generation of stable clones, ~2.5×10^5 adherent cells were transfected with 10 μg of each plasmid before cloning by limiting dilution with the appropriate selection. Drug-resistant colonies were picked and analysed for expression using immunofluoresence microscopy, FACS analysis or ^{125}I-labelled tetanus toxin binding. Clones expressing similar levels of transfected product were chosen for subsequent analysis.

For B-cell transfections, 10^7 cells were washed and resuspended in 0.8 ml of PBS containing 100 μg of linear DNA and electroporated at 960 μF, 300 V. After 10 min at room temperature the cells were recultured in RPMI growth medium for 60 h. Cells were then resuspended in 50 ml RPMI growth medium containing the appropriate drug selection and plated out into four 24-well plates (Costar). Clones were screened for surface expression using both FACS analysis and ^{125}I-labelled tetanus toxin binding. Transfectants used in experiments were representative of several and showed similar levels of cell surface IgG expression.

FACS analysis and immunofluorescence

For FACS analysis, cells were harvested with PBS/10 mM EDTA, washed into PBS/2% FCS and stained with the following. Primary antibodies: biotinylated goat anti-human IgG (Southern Biotech. Assoc. Inc.); L243 (anti-MHC class II; Lampson and Levy, 1980); QEII (anti-human κ chain; Serotec); CB3-1 (anti human CD79b; Nakamura *et al.*, 1992). Secondary antibodies: streptavidin FITC; goat anti-mouse FITC (Southern Biotech. Assoc. Inc.). 10 000 events were recorded on a Becton Dickinson FACSort.

Transient transfection of HeLa cells was performed as described (Ponnambalam *et al.*, 1994). Briefly, cells were grown to 5–10% confluency on sterile coverslips in 6-well plates (Costar) before transfection with calcium phosphate (~10–15 μg of DNA). 24 h after transfection, cells were washed three times and recultured for a further 48 h before analysis by immunofluorescence microscopy (Ponnambalam *et al.*, 1994). Antibodies were as above and in addition the following primaries: rabbit anti-human IgG (Southern Biotech. Assoc. Inc.); sheep anti-human IgG (Scottish Antibody Production Unit). Secondaries: as above and in addition: goat anti-rabbit Texas Red (Vector); donkey anti-sheep Texas Red and donkey anti-mouse FITC (Jackson). Microscopy was performed on a Nikon Microphot-SA attached to an MRC 600 series confocal scanning microscope.

Internalization assays

Approximately 2.5×10^5 CHO transfectants/well were plated in 6-well plates (Costar) 48 h before the assay. The CD4-specific mAb Q4120 (gift from the MRC AIDS Directed Programme Reagent Project) was labelled to a specific activity of 7.5×10^4 c.p.m./ng using water-soluble ^{125}I-labelled Bolton Hunter reagent (Amersham). Cells were incubated with 2–3 nM ^{125}I-labelled Q4120 (1.5×10^6 c.p.m.) in RPMI/HEPES/1% FCS for 1 h at 4°C. After washing, cells were incubated at 37°C for various times in 1 ml of media. At the end of each time point the percentage of ligand endocytosed was quantitated (LKB Multigamma 1261) following two cycles of acid-stripping (1 ml of 150 mM HCl/150 mM NaCl for 3 min at 4°C). Attached cells were harvested with 0.2 M NaOH for 3 min.

Metabolic labelling

HeLa transfectants were pulsed for 15 min (0.1 mCi ^{35}S-trans-labelled methionine/cysteine; ICN) and chased for 2 h in the presence of 5 mM unlabelled methionine/cysteine before lysis and immunoprecipitation using biotinylated rabbit anti-human IgG (Southern Biotech. Assoc. Inc.) followed by streptavidin coupled to agarose beads (Sigma). After washing, one half of each sample was treated with 1 mU endoglycosidase H (Boehringer-Mannheim) for 16 h at 37°C before SDS–PAGE analysis.

Antigen presentation assays

2×10^4 irradiated Mel JuSo or Ramos cells were co-cultured with 2×10^4 T-cells in the presence of graded doses of antigen (His-tagged tetanus toxin C-fragment domain; residues 865–1315) in 96-well flat-bottomed plates as described (Lanzavecchia, 1985). T-cell proliferation was measured after 48 h by [^{3}H]thymidine (Amersham) pulsing (16 h; 1 μCi) and liquid scintillation counting. T-cell clones KT4 (tetanus toxin 1273–1284; Demotz *et al.*, 1989) and D1c (tetanus toxin 947–966) were used to detect antigen presentation by Mel JuSo and Ramos cells respectively.

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