Immunology 1992 **77** 539–542

Constitutive endocytosis and recycling of major histocompatibility complex class II glycoproteins in human B-lymphoblastoid cells

P. A. REID & C. WATTS *Department of Biochemistry, Medical Sciences Institute, University of Dundee, Dundee*

Accepted for publication 6 August 1992

SUMMARY

Cleavable cell surface radiolabelling reagents were used to measure the endocytosis and recycling of class II major histocompatibility complex (MHC) glycoproteins in human B-lymphoblastoid cells. It is shown that mature class II $\alpha\beta$ dimers on the cell surface are constitutively endocytosed and that recycling can be demonstrated even from small endosomal pools. Endocytosis was blocked when cellular ATP levels were depleted or when clathrin polymerization was inhibited, implicating clathrin-coated pits in the endocytic process. Taken together with earlier studies, these results suggest that mature class II MHC molecules are constitutively endocytosed and recycled from acidic peripheral endosomes which may enhance their capacity to bind and present T cell epitopes which do not require processing.

INTRODUCTION

In antigen-presenting cells such as B cells and macrophages, class II major histocompatibility complex (MHC) glycoproteins are involved in transporting peptide fragments, derived from endocytosed antigens, to the cell surface for recognition by helper T cells (reviewed in refs 1–3). These class II MHC glycoproteins consist of an α-chain (34,000 MW) and non-covalently associated β-chain (29,000 MW). Two separate populations are found in antigen-presenting cells: newly synthesized molecules undergoing transport through the biosynthetic pathway to the cell surface and mature molecules on the surface. Recent studies in human B-lymphoblastoid cells have shown that newly synthesized class II MHC molecules bind physiologically processed peptides but that exogenous peptides can bind to mature cell surface molecules.[4] Most peptides appear to be bound essentially irreversibly to class II molecules in living cells[5] suggesting that exogenous peptides most likely do not replace existing peptide/class II complexes but rather utilize a small fraction of empty class II molecules which exist on the cell surface.[6] Thus although newly synthesized class II MHC appears crucial for peptides generated during antigen processing, mature cell surface class II MHC molecules might offer some capacity for peptides that can bind without further processing, Given that peptide binding occurs optimally at mildly acidic pH[7] transient passage of the cell surface population through acidic endosomes might facilitate peptide binding to empty molecules. Using a sensitive cell surface radiolabelling protocol, earlier studies showed that in human B-lymphoblastoid cells the class II MHC molecules were internalized but that only a small intracellular pool was detected.[8] If cells were treated with primaquine, which slows the recycling of other receptors,[9,10] a larger intracellular pool was detected and this population was recycled back to the cell surface.[8] These observations have now been extended to show that constitutive endocytosis and recycling of class II MHC molecules occur even in cells which have not been treated with primaquine, demonstrating a pathway which may allow optimal binding and presentation of exogenous peptides which do not require processing.

Correspondence: Dr P. A. Reid, MRC Laboratory for Molecular Cell Biology, Medawar Building, University College London, Gower Street, London WC1E 6BT, U.K.

MATERIALS AND METHODS

Cell culture

The human B-lymphoblastoid cell A46 specific for tetanus toxoid was cultured in RPMI-1640 medium plus supplements as described previously.[11,12]

Preparation of DPSgtc

DPSgtc (Dithiopropionyl, sulphosuccimidyl, glycyl-tyrosine cholamine) was prepared and labelled with ^{125}I exactly as described previously.[8,13,14]

Detection of endocytosis using DPSgtc

These experiments were performed as described previously.[8,13,14] Briefly, cells were washed in DPBS, resuspended in 0·1 M Na_2HPO_4 and labelled on ice for 20 min with [^{125}I]DPSgtc. The cells were washed free of unincorporated reagent, resuspended in RPMI/20 mM HEPES pH 7·5/10% foetal calf serum (FCS) then aliquots either held on ice or warmed to 37° for appropriate

times. The cells were chilled and treated as appropriate with reduced glutathione (GSH) to remove the labelled reagent bound to the cell surface population. All cells were lysed in buffer containing Nonidet P-40 and iodoacetamide, centrifuged to remove nuclei and other cellular debris then airfuged at 100,000 *g* for 30 min. The class II MHC glycoproteins and transferrin receptor were then sequentially immunoprecipitated from the labelled cell lysates using DA6.231[15] and HuLy-m9 (Serotec, Oxford, U.K.) antibodies respectively, followed by affinity-purified rabbit anti-mouse immunoglobulin (Serotec) and Protein A Sepharose. The immune complexes were washed, eluted in non-reducing SDS sample buffer and run on SDS polyacrylamide gels. The dried gels were exposed to Fuji-RX film.

In experiments with azide and 2-deoxy-D-glucose, the cells were pretreated with 20 mM sodium azide/50 mM 2-deoxy-D-glucose for 1 hr at 37° prior to labelling with [^{125}I]DPSgtc.[12] To study endocytosis in the presence of hypertonic medium[17,18] [^{125}I]DPSgtc labelled cells were warmed to 37° in RPMI-1640/20 mM HEPES pH 7·5/10% FCS containing 0·45 M sucrose.

Detection of recycling using DPSgtc

This was performed essentially as described previously.[8,14] Aliquots of [^{125}I]DPSgtc-labelled A46 cells were held on ice or warmed to 37° prior to treatment with reduced GSH as appropriate. Cells containing a small intracellular pool of class II MHC glycoproteins were rewarmed to 37° for periods up to 10 min, rapidly chilled and retreated with GSH on ice. As a control one aliquot of cells was rewarmed but not treated a second time with GSH. After lysis of the cells, class II MHC glycoproteins were immunoprecipitated, the immune complexes run under non-reducing conditions on a SDS polyacrylamide gel and the dried gel subject to autoradiography.

RESULTS

Constitutive endocytosis of class II MHC molecules

The Epstein–Barr virus (EBV) cell line A46 was labelled with DPSgtc at 0° as previously reported and either held at 0° or incubated at 37° for various periods of time. This reagent allows radioiodination of free amino groups on cell surface proteins and is reversible under mild reducing conditions due to the presence of a disulphide linkage between the [^{125}I]tyrosine residue and the reactive ester.[8,13] Endocytosis of ^{125}I-labelled cell surface molecules consequently protects the label from a subsequent incubation with reduced glutathione which is essentially impermeant. As shown in Fig. 1a and as previously reported[13] approximately 70% of labelled cell surface transferrin receptors become protected from GSH within 5 min. Class II MHC α- and β-chains also become protected from GSH but more slowly and to a much lesser extent so that at steady state between 5 and 7% of the cell surface molecules are found inside the cells. To study the initial rate of endocytosis of the class II molecules, cells were warmed to 37° for up to 16 min and treated with reduced GSH prior to lysis and immunoprecipitation. The α-chain band was excised from the dried gels and quantitated by γ-counting. The initial rate of endocytosis was approximately 1·3% of the cell surface population/min, somewhat slower than measured previously in primaquine-treated cells.[8]

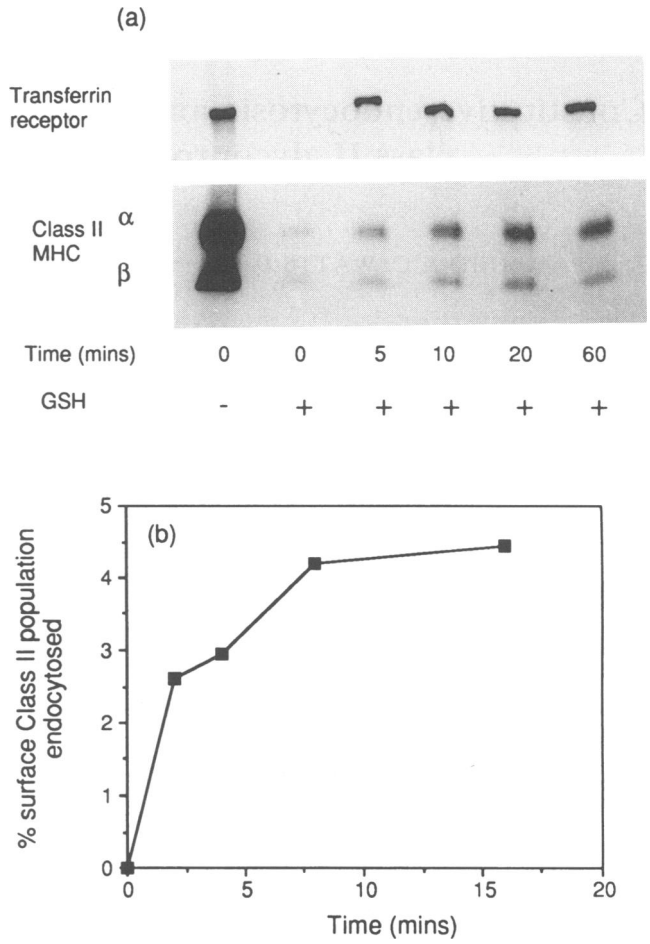

Figure 1. Endocytosis of class II MHC glycoproteins and transferrin receptor. (a) Aliquots of [^{125}I]DPSgtc-labelled A46 cells were held on ice or warmed to 37°, followed by treatment with reduced glutathione on ice as appropriate.[8,13,14] The class II MHC glycoproteins and transferrin receptor were immunoprecipitated from the cell lysates, then analysed by SDS polyacrylamide gel electrophoresis and autoradiography. (b) Bands corresponding to the class II MHC α-chain were excised from the dried gels and the size of the intracellular pool protected from glutathione reduction quantitated by γ-counting and removal of appropriate backgrounds. The means from duplicate experiments are shown.

Inhibition of endocytosis

To establish that the GSH-resistant population of class II MHC molecules has become resistant due to endocytosis and not, for example, due to migration into GSH-inaccessible sites on the cell surface, the labelled cells were exposed to conditions known to block endocytosis but which would not be expected to block surface redistribution. The cells were pretreated for 1 hr at 37° with 20 mM sodium azide and 50 mM 2-deoxy-D-glucose to deplete cellular ATP levels, labelled at 0° with [^{125}I]DPSgtc, incubated at 37° and treated with GSH. As shown in Table 1, the protection of the class II MHC α- and β-chains is reduced by at least threefold in ATP-depleted cells. The endocytosis of the transferrin receptor, immunoprecipitated from the same labelled cells as a control, was also reduced by a factor of three (data not shown). It was then asked if the acquisition of glutathione resistance could be blocked by hyperosmotic condi-

Table 1. The effect of hypertonic medium, primaquine and azide/2 deoxy-D-glucose on the size of the intracellular pool of class II MHC glycoproteins. Aliquots of [^{125}I]DPSgtc-labelled A46 cells were held on ice or warmed to 37° for 30 min in RPMI-1640/20 mM HEPES pH 7·5/10% FCS containing 0·45 M sucrose and/or 300 μM primaquine, followed by treatment on ice with reduced glutathione as appropriate.[8] In one experiment a pretreatment with 20 mM sodium azide/50 mM 2-deoxy-D-glucose for 1 hr at 37° preceded labelling with [^{125}I]DPSgtc. Bands corresponding to class II MHC α- and β-chains were excised from dried gels and the intracellular pool quantitated by γ-counting and removal of appropriate backgrounds[8]

Treatment	Class II α-chain % endocytosed	Class II β-chain % endocytosed
1 Warmed to 37°	5·9	5·3
2 Warmed to 37° + 0·45 M sucrose	1·8	0·3
3 Warmed to 37° + 300 μM primaquine	20·9	19·2
4 Warmed to 37° + primaquine + 0·45 M sucrose	2·9	1·1
5 Warmed to 37°	8·6	11·2
6 20 mM azide/50 mM 2-deoxy-D-glucose pretreatment	1·8	2·3

tions known to paralyse the clathrin lattice and block endocytosis.[17,18] Labelled cells were incubated at 37° in the absence or presence of medium containing 0·45 M sucrose. As shown in Table 1, hyperosmotic media reduced the protection of class II MHC glycoproteins almost fivefold, providing strong evidence that the GSH-resistant population had indeed been endocytosed. Hyperosmotic treatment also blocked endocytosis in primaquine-treated cells reducing the steady-state pool sevenfold (Table 1).

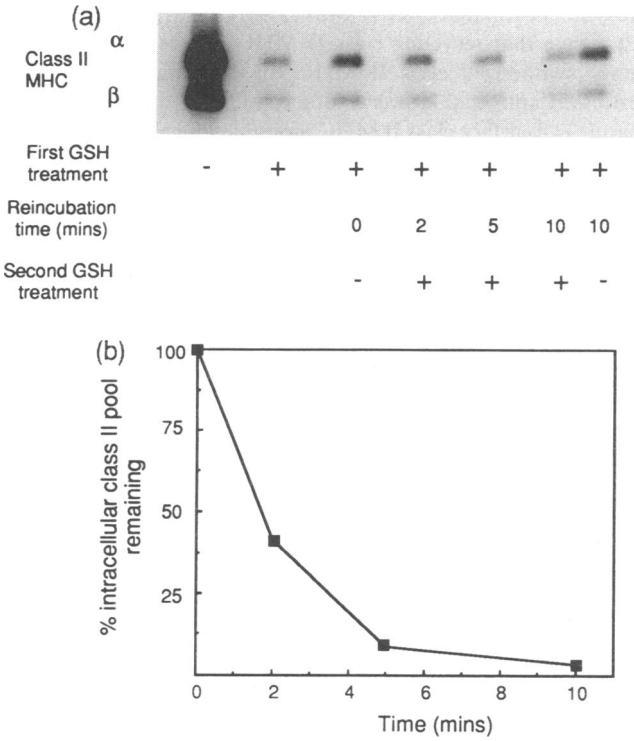

Figure 2. The recycling of endocytosed class II MHC glycoproteins back to the cell surface. (a) Aliquots of [^{125}I]DPSgtc-labelled A46 cells were held on ice or warmed to 37° for 1 hr then treated with reduced GSH on ice. Cells were then rewarmed to 37° for up to 10 min, chilled and further treated with reduced GSH on ice. A control cell aliquot was rewarmed to 37° but not treated further with GSH. Class II molecules were immunoprecipitated, run under non-reducing conditions on a SDS polyacrylamide gel and the dried gel autoradiographed. (b) Bands corresponding to the class II MHC α-chain were excised from the dried gel and the per cent of the endocytosed pool which remained inaccessible to GSH (i.e. was still found inside the cell) after various times of exocytosis plotted.

Recycling of endocytosed class II MHC back to the cell surface

To show directly that the steady-state intracellular pool of class II MHC molecules returns to the cell surface on further rewarming to 37°, advantage was taken of the cleavability of the labelling reagent. [^{125}I]DPSgtc-labelled cells were allowed to endocytose class II MHC molecules for 1 hr at 37° and those remaining on the cell surface were removed by glutathione treatment at 0°. The cells were then reincubated at 37° for up to 10 min and treated a second time with glutathione at 0°. If recycling occurs, class II molecules returning to the cell surface during the rewarming period will be susceptible to the second treatment with GSH. The experiment is technically difficult because of the small intracellular pool size for class II MHC and a background of surface labelling which is insensitive to cleavage by GSH. Nonetheless, it was possible to show that recycling of virtually all class II MHC molecules takes place within a few minutes of reincubation at 37° (Fig. 2).

DISCUSSION

These experiments demonstate constitutive endocytosis and recycling of class II MHC molecules in human B-lymphoblastoid cells even in the absence of agents which block recycling such as primaquine. Studies using murine cells have also suggested recycling of class II MHC[19,20] but in human lymphoblastoid cells it has been a controversial issue[21,22] although it is clear that the intracellular pool size is very small compared, for example, to the transferrin receptor (Fig. 1).[8,13] The pool size reflects the relative rates of endocytosis versus recycling and small steady-state size is not necessarily an indicator of the importance of the recycling process. Other receptors, for example, the LDL receptor also maintain small (~15%) intracellular pools.[13]

Recent biochemical studies show that newly processed peptides from endocytosed tetanus toxin were loaded onto newly synthesized class II MHC molecules[4] and not to any significant extent onto the recycling population demonstrated here. In fact the rapid kinetics of recycling suggest that class II molecules only penetrate as far as early endosomes and may not reach compartments involved in antigen processing and class II MHC maturation.[3,22] Additional recent studies on these cells show that most peptides are bound irreversibly to class II MHC in living cells,[5] indicating that recycling does not lead to peptide displacement. These results, together with others on the

biosynthetic itinary of newly synthesized class II MHC (e.g. ref. 22), argue that recycling class II MHC cannot substitute for newly synthesized class II MHC for presentation of newly processed antigens. However other studies[4,6] show that some mature cell surface class II MHC molecules are able to bind and present to T cells exogenous peptides which do not require processing. These exogenous peptides do not displace existing peptides[5] but rather bind to a small proportion of 'empty' class II molecules which presumably did not acquire a stably bound peptide during biosynthesis. Independent evidence for the existence of 'empty' cell surface class II molecules derives from the fact that only a proportion of this population is stable in SDS, a property which has recently been shown to correlate with peptide occupancy.[23,24] It seems likely that during constitutive cycling these 'empty' molecules pass through an acidic intracellular compartment, since primaquine, which accumulates and perturbs such compartments, induces trapping of a much larger class II MHC pool.[8] Since mildly acidic pH is known to enhance binding of peptides to class II MHC,[7] we suggest that loading of exogenous peptides into 'empty' sites may be enhanced by transient endocytosis through a peripheral endocytic compartment. Further studies will be required to establish if class II recycling occurs in human macrophages and in B cells isolated *ex vivo*. In lymphoblastoid cells class II MHC probably enters invaginating endocytic vesicles passively since the endocytic rate is low (1–2%/min) compared with receptors which are actively recruited into coated pits such as the transferrin receptor (~15%/min).

Thus two types of T-cell epitope might be envisaged, which differ in the degree of processing required for binding to class II MHC: (1) a majority which require proteolytic processing and appear to bind only to newly synthesized class II molecules and (2) a minority which may be able to bind directly or following limited unfolding of antigen[25–27] and may be able to take advantage of empty sites whose capacity to become occupied may be enhanced during recycling through low pH endosomes. Presentation of the latter type of epitope should be relatively insensitive to protein synthesis inhibitors and inhibitors of biosynthetic class II traffic but, if our prediction is correct, sensitive to inhibitors of recycling traffic.

ACKNOWLEDGMENTS

We are grateful to A. Lanzavecchia, M. S. Bretscher and K. Guy for cells and reagents. We thank our colleagues for helpful discussions. This work was supported by the Medical Research Council and the Wellcome Trust and by a MRC Postgraduate Research Studentship to P.A.R.

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