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# Expression of $\beta_2$-microglobulin-free HLA class I $\alpha$-chains on activated T cells requires internalization of HLA class I heterodimers

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## SUMMARY

HLA class I molecules on activated T cells are expressed as heterodimers associated with $\beta_2$-microglobulin ($\beta_2$-m) and also $\beta_2$-m-free HLA class I $\alpha$-chains. Mechanisms leading to the expression of the activation associated $\beta_2$-m-free HLA class I $\alpha$-chains are poorly defined, however. Upon enzymatical removal of HLA class I $\alpha$-chains on activated T cells, re-expression is observed within minutes upon reculture, reaching half-maximal levels within 1 hr. This process is independent of *de novo* protein synthesis and of export of newly synthesized proteins. Inhibition of the formation of coated pits by potassium depletion of cells abrogated the re-expression of HLA class I $\alpha$-chains, suggesting that recycling events of HLA class I heterodimers via endosomal compartments are required for the generation of monoclonal antibody LA45-reactive $\alpha$-chains. Furthermore, the rate of $\alpha$-chain generation seems to be governed by the amount of cell surface-expressed HLA class I heterodimers. Taken together these findings suggest that $\beta_2$-m-free HLA class I $\alpha$-chains are generated during the process of class I heterodimer recycling.

## INTRODUCTION

On T cells activated *in vitro* and *in vivo*, HLA class I molecules are present in at least two discrete forms: (1) as heterodimers associated with $\beta_2$-microglobulin ($\beta_2$-m) and recognized by conformation-dependent monoclonal antibodies (mAb) like W6/32;[1] and (2) as $\beta_2$-m-free HLA class I $\alpha$-chains as recognized by mAb like LA45,[2–4] HC10,[5] L31 and M38.[6] The mechanisms responsible for the cell surface expression of the $\beta_2$-m-free HLA class I $\alpha$-chains have not been clearly solved, however.

HLA class I heterodimers, once expressed on the cell surface, behave like classical receptor molecules; they show spontaneous and rapid internalization, followed by subsequent re-expression at the cell surface—a process known as recycling.[7–11] Reid & Watts[8] presented evidence that the kinetics of the recycling process are fast and thereby keep the intracellular pool size small.[8] Recycling of HLA class I molecules via coated pits seems to display an intrinsic transport function, as shown by reports on the co-internalization of HLA class I molecules and endorphins in lymphocytes.[12]

Ligand-independent recycling of HLA class I molecules has been observed to occur in Epstein–Barr virus (EBV)-transformed B cells and leukaemic T cells resembling an activated phenotype.[8,11] However, several reports are not consistent with these experiments and could not demonstrate significant internalization of HLA class I molecules for either B or T cells.[13–15] This discrepancy may have at least two reasons. Firstly, recycling of major histocompatibility complex (MHC) molecules may be a cell-type specific, differentiation-restricted phenomenon; and secondly, detection of recycling products may depend strongly on the techniques used and their inherent thresholds. Of possible importance in this context are the observations of Hochmann *et al.*[9] who showed for activated murine T cells that it is the acidic milieu of the endosomes which leads to dissociation within minutes of internalized $\beta_2$-m from HLA class I $\alpha$-chains.[9] Thus, the cell type-specific interference of an acidic milieu[16] with putatively endocytosed HLA class I heterodimers might have influenced their association with $\beta_2$-m and consequently might have led to the failure of reactivity with mAb W6/32.

Thus, even though there is evidence for recycling of HLA class I molecules governing the traffic in distinct cells, there is also considerable uncertainty or controversy (reviewed in ref. 17). In this report, we present evidence that the recycling of HLA class I heterodimers via endosomal compartments contributes to the expression of $\beta_2$-m-free HLA class I $\alpha$-chains at the cell surface of the human T-cell line HUT-102.

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Abbreviations: $\beta_2$-m, $\beta_2$-microglobulin; BFA, brefeldin A; CHX, cycloheximide.

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## MATERIALS AND METHODS

*Monoclonal antibodies and cell lines*

The LA45 mAb (IgG1) reacts with free HLA class I heavy chains[2,3] carrying the amino acids arginine and asparagine at residues 62–63 of the $\alpha_1$-domain.[4,18] The mAb W6/32 (IgG2a) reacts with a conformation-dependent epitope of heterodimeric

HLA class I molecules; mAb BBM.1 reacts with $\beta_2$-m.[1] The latter two hybridomas were obtained from ATCC (Rockville, MD). Monoclonal antibodies VI-AP (IgG1; calf intestine alkaline phosphatase specific), used as an isotype-specific negative control, and LA45[2] were generated at the Institute for Immunology (University of Vienna, Austria). The HUT-102 cell line used in this study was obtained from ATCC. Cells were maintained in RPMI-1640 (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin.

*Reagents*

Cycloheximide was purchased from Boehringer Mannheim (Boehringer Mannheim, Mannheim, Germany) and was used at a final concentration of 100 μM. Brefeldin A, chloroquine, monensin and subtilisin (Sigma Chemicals, St Louis, MO) were used at concentrations of 5 μg/ml, 140 μM, 10 μM and 0·5 mg/ml, respectively. $NH_4Cl$-containing medium (50 mM) and $NH_4OH$-medium (5 mM) were prepared by the addition of the respective chemicals to RPMI-1640/10% FCS medium.

*Enzyme treatment of cells and re-expression experiments*

The general protocol for all re-expression experiments was firstly to exchange the standard medium (RPMI-1640/10% FCS) to specific medium (containing the respective chemical or drug) and to reculture the cells for 45 min in the incubator at 37°. Trypsinization with 0·25% trypsin w/v (Sigma Chemicals) was performed in specific serum-free media for 10 min at 37° in the incubator. Papain treatment was achieved by incubating cells with 155 U/ml papain from *Carica papaya* (Serva, Heidelberg, Germany) for 1 hr at 37° in RPMI-1640/10% FCS medium. When removal of W6/32-reactive heterodimers was required, the medium was supplemented with 10 mM cysteine to efficiently activate the protease activity.[19] Subsequently, enzyme-treated cells were washed three times at 4° and recultured in the respective media for the indicated time periods at 37° in a humidified atmosphere containing 5% $CO_2$.

*Inhibition of coated pit formation*

Potassium depletion was performed according to Larkin *et al.*[20] by 'hypotonic-shock' treatment. Briefly, HUT-102 cells were incubated for 10 min in 145 mOsmolar medium at 37°. Subsequently, cells were recultured in $K^+$-deficient simplified medium (SM: 145 mM NaCl, 1 mM $Na_2HPO_4$, 1 mM $CaCl_2$, 0·5 mM $MgSO_4$, 5 mM glucose, 10 mM HEPES, pH 7·40) for 45 min. Hypertonic treatment according to Heuser & Anderson[21] was achieved by culture of cells in SM containing 0·45 M sucrose. Intracellular acidification was achieved by the 'ammonia prepulse' technique described by Heuser.[22] Briefly, cells were exposed to saline medium containing 25 mM $NH_4Cl$ for 15 min at 37°. Subsequently, pretreated cells were cultured in $Na^+$-deficient/$Ca^{2+}$-low medium, promoting an outward diffusion of ammonia and thus leaving $H^+$ behind, which results in a rapid acidification of the cytoplasm.

*Immunofluorescence analysis*

The binding of primary mAb (10 μg/ml) was visualized by using sheep $F(ab')_2$ anti-mouse immunoglobulin–fluorescein isothiocyanate (FITC) (SAM; Grub, Scandic, Vienna, Austria) as a second-step reagent. As a negative control an irrelevant isotype-matched control mAb (VI-AP) was used as the primary antibody. Membrane fluorescence was analysed on a FACStar Plus® flow cytometer supported by the LYSIS II® software (Becton Dickinson, San Jose, CA). Cell viability was determined by standard ethidium bromide exclusion.

## RESULTS

### $\beta_2$-m-free HLA class I molecules are sensitive to trypsin treatment

Myers *et al.*[23] reported on the susceptibility of distinct $\beta_2$-m-free MHC class I molecules, H-2 $L^d$ molecules, to proteolytic cleavage upon treatment with trypsin. Their finding prompted us to test whether a similar sensitivity to trypsin cleavage can be observed for HLA class I α-chain analogues recognized by mAb LA45. Figure 1a shows the fluorescence-activated cell sorter (FACS) profiles of native HUT-102 cells stained with mAb LA45, recognizing discrete $\beta_2$-m-free MHC class I α-chains, or with mAb W6/32 recognizing HLA class I heterodimers in comparison to the isotype-matched, non-binding control mAb, VI-AP. Treatment of HUT-102 cells with the protease trypsin for 10 min lead to a reduction of the amount of cell surface-expressed LA45-reactive molecules to nearly background levels (Fig. 1b). Upon recultivation of protease-treated cells in complete medium, re-expression of $\beta_2$-m-free HLA class I molecules was observed within minutes, reaching half-maximal

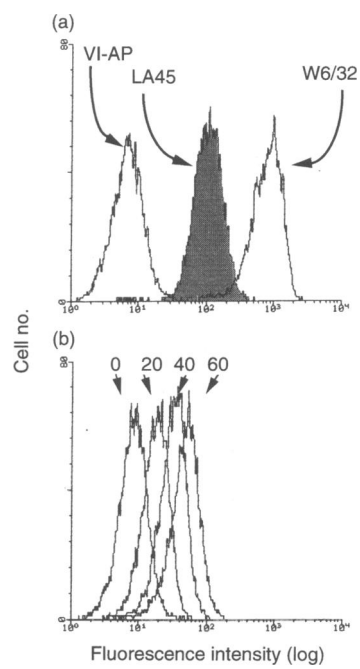


**Figure 1.** Expression of $\beta_2$-m-free HLA class I α-chains on HUT-102 cells. (a) HUT-102 cells were reacted with saturating concentrations of mAb W6/32 (HLA class I heterodimer specific), LA45 ($\beta_2$-m-free HLA class I α-chain specific) and VI-AP (non-binding isotype control) as first-step reagents. (b) Re-expression pattern of $\beta_2$-m-free HLA class I α-chains as detected by mAb LA45 upon trypsinization and reculture of HUT-102 cells in complete medium. Binding of mAb was visualized by a SAM $F(ab')_2$-FITC conjugate, and green fluorescence intensity was analysed on a FACStar Plus® flow cytometer. Time periods of reculture after protease treatment of cells are indicated in minutes.

levels after 1 hr (Fig. 1b). The amount of HLA class I heterodimers detected by mAb W6/32 was not affected by trypsin treatment (data not shown).

### Effects of weak bases and ionophores on the re-expression dynamics of LA45-reactive molecules

HLA class I molecules may recycle via endosomal compartments where the acidic milieu might influence the association with $\beta_2$-m and/or specifically bound peptides.[7–9] Weak bases and ionophores neutralize the pH of endocytic vesicles,[24,25] thereby stabilizing protein–protein interactions otherwise disrupted by the acidic milieu. Thus, we were interested to study the effects of these compounds on re-expression dynamics of LA45-reactive molecules.

Incubation of HUT-102 cells under the conditions described by Maxfield[25] produced partial but reproducible inhibition of LA45 expression with two out of the four protocols (Fig. 2). Ammonium chloride and ammonium hydroxide did not show any inhibition in re-expression rates of LA45-reactive molecules (Fig. 2c, d). Chloroquine blocked re-expression rates by approximately 40%, as observed after 60 min upon recultivation (Fig. 2a), whereas the ionophore monensin showed an intermediate blocking activity (Fig. 2b).

### Inhibition of coated pit formation blocks the re-expression of LA45 molecules

The partial inhibitory effects observed for acidotropic agents prompted us to ask whether the inhibition of coated pit formation or of the budding off of coated pits would result in a perturbation of the re-expression dynamics of $\beta_2$-m-free HLA class I molecules.

$(K^+)_i$ depletion, according to the method described by Larkin *et al.*[20] also recently applied in experiments with human lymphocytes[26] was used to accomplish inhibition of coated pit formation. Figure 3 demonstrates that trypsinized HUT-102 cells, depleted for $(K^+)_i$ and re-cultured in $K^+$-deficient medium, did not re-express LA45-reactive molecules, as determined after the time-points indicated. However, replacement of the $K^+$-deficient medium by medium containing 5 mM $K^+$ resulted in proper re-expression of the LA45-reactive molecules. Trypsinized control cells cultured in $K^+$-containing medium showed a typical re-expression pattern of $\beta_2$-m-free HLA class I molecules. Importantly, the amount of HLA class I heterodimers, as detected by mAb W6/32, was neither affected by trypsin treatment nor by the $(K^+)_i$ -depletion procedure (data not shown).

Two other regimens known to interfere with the formation of coated pits, cultivation of cells in hyperosmolar medium[21] or intracellular acidification by the 'ammonia prepulse' procedure,[22] were also found to inhibit re-expression of $\beta_2$-m-free HLA class I molecules on trypsinized HUT-102 cells to an identical extent (data not shown).

### The amount of cell-surface HLA class I heterodimers determines the re-expression rate of $\beta_2$-m-free HLA class I $\alpha$-chains

The data described above were suggestive of recycling events of HLA class I heterodimers via endosomal compartments being required for the generation of LA45-reactive $\beta_2$-m-free HLA class I $\alpha$-chains. This assumption was supported by the observation that a 1 hr pretreatment of protease-treated cells with either cycloheximide (CHX) or brefeldin A (BFA) was not able to influence the re-expression dynamics of LA45-reactive moieties (data not shown), indicating that the pool of cell surface-expressed heterodimers is the source for free $\alpha$-chains.

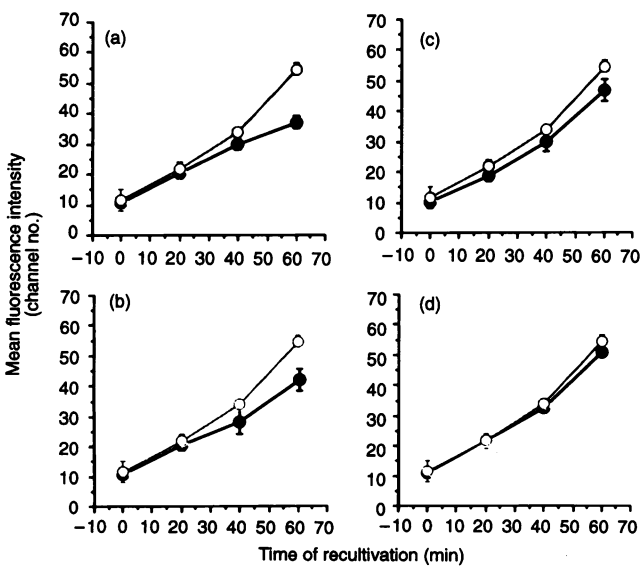


**Figure 2.** Effects of weak bases and ionophores on the re-expression kinetics of LA45-reactive molecules. Re-expression pattern of $\beta_2$-m-free HLA class I $\alpha$-chains as detected by mAb LA45 upon trypsinization and reculture of HUT-102 cells in complete medium (open circles) compared to media containing chloroquine (140 $\mu$M) (a), monensin (10 $\mu$M) (b), $NH_4Cl$ (50 mM) (c) or $NH_4OH$ (5 mM) (d) (closed circles). Expression of LA45-reactive molecules is indicated as mean fluorescence channel number of three independently performed experiments ± SD, as determined by flow cytometry. Final concentration of compounds used are shown in parentheses.

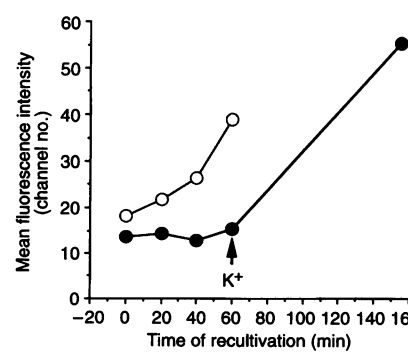


**Figure 3.** Inhibition of coated pit formation blocks the re-expression of LA45 molecules. HUT-102 cells depleted for intracellular $K^+$ were trypsinized and recultured in $K^+$-deficient medium and cell-surface LA45 reactivity was tested by indirect immunofluorescence technique and flow cytometry after the time periods indicated (closed circles). Trypsinized HUT-102 cells cultured in complete medium were monitored by the same way (open circles). Replacement of the $K^+$-deficient medium by medium containing 5 mM $K^+$ is indicated by an arrow.

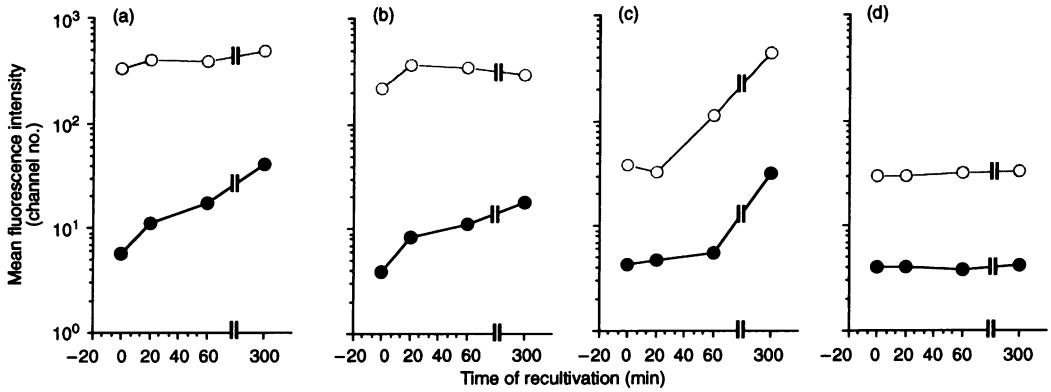


**Figure 4.** Re-expression rates of HLA class I heterodimers and $\beta_2$-m-free HLA class I α-chains upon treatment of HUT-102 cells with papain. HUT-102 cells in (a) and (b) were treated with the protease papain in standard RPMI-1640 medium to remove $\beta_2$-m-free HLA class I α-chains only; cells in (c) and (d) were also stripped of their HLA class I heterodimers by appropriate activation of papain due to the addition of 10 mM cysteine. Cells in (b) and (d) were treated with a combination of CHX and BFA 45 min prior to the protease treatment, and during the protease treatment and the recultivation period. Open circles represent mean fluorescent intensity of HLA class I heterodimers (W6/32 reactive), closed circles the mean fluorescent density of $\beta_2$-m-free HLA class I α-chains (LA45 reactive), determined after the indicated time intervals by indirect immunofluorescence technique and flow cytometry, of one representative experiment.

To strengthen the 'recycling-hypothesis', HUT-102 cells were treated with the protease papain in standard medium to remove $\beta_2$-m-free HLA class I α-chains only, or in papain-containing medium supplemented with 10 mM cysteine to activate the enzyme efficiently and thereby to strip off both $\beta_2$-m-free HLA class I α-chains and HLA class I heterodimers. Prior to enzyme treatment, aliquots of these two cell samples were treated with a combination of CHX and BFA, to rule out the contribution of biosynthesis and/or exocytic transport to the re-expression of LA45-reactive α-chains.

Figure 4 demonstrates that only cells with high, i.e. constitutive, expression of HLA class I heterodimers, as detected by mAb W6/32, were able to re-express LA45-reactive molecules (Fig. 4a, b, c). On W6/32$^{high}$ cells treatment with CHX and BFA was not able to inhibit LA45 re-expression significantly (Fig. 4b). A certain threshold of HLA class I heterodimer expression seemed to be required to allow the initiation of the re-expression process of $\beta_2$-m-free HLA class I α-chains, as a marked delay in the re-expression kinetics was observed for cells with an initial W6/32$^{low}$-expression pattern (Fig. 4c). Where HLA class I heterodimer cell surface expression was low and protein synthesis and export were blocked, no LA45-reactive molecules were expressed (Fig. 4d).

Thus, even though W6/32$^{high}$ expression seems to be necessary for proper re-expression rates of $\beta_2$-m-free HLA class I α-chains, it can be deduced from the experiments inhibiting coated pit formation that this is not sufficient. Consequently, the combined view of the above described experimental settings provides circumstantial evidence for the prerequisite of heterodimer recycling in the generation of free HLA class I heavy chains.

## DISCUSSION

Expression of $\beta_2$-m-free heavy chains is a common feature of both human T and B cells activated *in vitro*.[3,4] Schnabl *et al.*[3] showed that reactivity with mAb LA45 is not an *in vitro* artefact, as *in vivo*-activated T cells derived from mononucleosis patients revealed clear-cut expression of $\beta_2$-m-free HLA class I α-chains. That LA45-reactive HLA class I heavy chains do not associate with $\beta_2$-m has been shown previously by the failure of coprecipitation of $\beta_2$-m from surface iodinated cells.[3] Irrespective of whether activated cell were grown in human serum or FCS-supplemented medium, constant mAb LA45 reactivity and absence of β2-m association with LA45-reactive α-chains was the consistent finding in those experiments. Thus, β2-m-free HLA class I heavy chain expression seems to be a physiological feature of activated lymphocytes.

In this study, we investigated the mechanisms involved in the generation of $\beta_2$-m-free HLA class I α-chains. We found that the formation of coated pits, being responsible for the import of the majority of classical receptor molecules, is critically involved in this process.[27,28] We show here that in HUT-102 cells deprived of their functional internalization machinery governed by the formation of clathrin-coated vesicles, an almost complete absence of newly expressed LA45-reactive molecules became evident. A very similar observation was made when the cells were stripped of both their HLA class I heterodimers and their $\beta_2$-m-free HLA class I α-chains by papain treatment: re-expression of LA45-reactive α-chains did not start before a certain degree of HLA class I heterodimers were already expressed. This lag period in re-expression kinetics underscores the 'substrate-like' nature of cell surface-expressed HLA class I heterodimers for the generation of LA45-reactive α-chains.

In addition, the experiments presented in Fig. 4 exclude the possibility that free heavy chains are the consequence of newly expressed molecules coming along the secretory pathway. The experimental setting clearly shows that under the pressure of CHX and BFA, only when heterodimers were present (Fig. 4b) at the cell surface, but not where they are almost completely lacking (Fig. 4d), was ongoing free heavy chain expression detectable.

Furthermore, because of the apparent lack of LA45 expression by protease-treated, LA45-negative cells upon cultivation under conditions prohibiting receptor internaliza-

tion via coated pits, we must assume that our experiments argue against a simply passive dissociation of $\beta_2$-m from cell surface-expressed HLA class I heterodimers.[29] If dissociation of $\beta_2$-m from cell surface-expressed heterodimers is the dominant process operative in our test system, one would expect $(K^+)_i$-depleted cells to rapidly re-express LA45-reactive molecules upon recultivation in $K^+$-free medium, as these cells do express constitutively (high) amounts of W6/32-reactive heterodimers on their cell surface, with the opportunity to release weakly bound $\beta_2$-m and/or peptide. We are aware of the fact that the potassium shock protocol might affect parameters of cell function different from coated pit formation.[30] However, similar inhibitory effects were observed with two additional protocols known to interfere with coated pit function, further supporting the notion that internalization of heterodimers probably plays a role in the generation of free heavy chains and represents a requirement for the subsequent re-expression of $\beta_2$-m-free HLA class I $\alpha$-chains.

If, in accordance with the observation made by Reid & Watts[8] concerning HLA class I heterodimers, the recycled, i.e. endosomal, pool size of free heavy chains is at any one time small, the velocity of re-expression of $\beta_2$-m-free $\alpha$-chains obviously reflects the rapid recycling and subsequent dissociation of HLA class I heterodimers expressed on the cell surface in huge amounts, even on trypsin-treated cells.

Our failure to demonstrate biochemically significant amounts of recycled, $\beta_2$-m-associated $\alpha$-chains having converted to newly expressed free heavy chains might be due to the small pool size of these molecules within the recycling compartments.

Is the quality to become expressed as a $\beta_2$-m-free HLA class I $\alpha$-chain a discrete feature of every cell surface-expressed HLA class I heterodimer? In other words, what determines the apparent loss of $\beta_2$-m during the recycling process? The most obvious scenario in this respect would be the combination of unstably associated HLA complexes due to the lack of proper bound, i.e high affinity, peptides[31] together with the acidic milieu of endocytic vesicles.[9,16] This setting would then lead to the dissociation of $\beta_2$-m and favour unfolding of the $\alpha$-chain. Results presented in this paper obtained with HUT-102 cells cultured in the presence of the lysosomotropic agent chloroquine, reported to neutralize the pH in endosomal/lysosomal compartments,[16,24] support this hypothesis. Acidification of endosomes, however, might not be the only mechanism in $\beta_2$-m dissociation, as considerable dissociation occurs even in the presence of the inhibitors chloroquine and monensin.

Human free heavy chains, once expressed on the cell surface do degrade spontaneously, giving rise to a soluble serum pool of these molecules.[18] We and others have shown that it is the additional activation of protein kinase C that is necessary to allow an efficient and strongly enhanced release of free heavy chains from the cell membrane (W. F. Pickl, W. Holter, J. Stöckl, O. Majdic & W. Knapp, unpublished observations).[32] Most important, these studies showed that only a fraction (50%) of surface-expressed molecules is released rapidly under conditions activating protein kinase C. This finding might point to a more stable conformation of part of the molecules, possibly involving intact folding of the $\alpha_3$-domain of the molecule.[33]

What might the functional role of $\beta_2$-m-free HLA class I $\alpha$-chains be? One could speculate that LA45 reactive $\beta_2$-m free HLA Class I $\alpha$-chains expressed at the cell surface function as receptors for putative extracellular peptides (and $\beta_2$-m)[34,35] being subsequently introduced to the HLA class I-restricted pathway of antigen presentation, which is normally, according to the current working hypotheses, a delicate feature of HLA class II molecules. This assumption is strengthened by *in vitro* observations of Benjamin *et al.*[36] showing that 'functionally empty' HLA class I molecules, susceptible for allele-specific peptide loading, do exist at the cell surface of human cells. Experiments performed with $^3$H-tagged LA45 mAb, showing internalization of a discrete fraction of free heavy chains, perfectly fit in with this hypothesis (W. F. Pickl *et al.*, unpublished observations).

If, however, cell surface-expressed $\beta_2$-m-free $\alpha$-chains are not able to encounter an appropriate 'ligand' or to refold to ternary complexes, their fate might be to contribute to the soluble pool of HLA class I molecules,[18,37] thereby acting as a putative soluble scavenger system for peptides and $\beta_2$-m.

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