Journal of General Virology (2001), **82**, 2131–2142. Printed in Great Britain

A measles virus glycoprotein-derived human CTL epitope is abundantly presented via the proteasomal-dependent MHC class I processing pathway

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Peptides derived from measles virus (MV) are presented by MHC class I molecules at widely divergent levels, but it is currently unknown how functional these levels are. Here, for the first time, we studied the natural occurrence and the underlying processing events of a known MV CTL epitope derived from the fusion glycoprotein (MV-F) and restricted via HLA-B*2705. Using MHC–peptide elution of MV-infected cells followed by sensitive mass spectrometry we determined the naturally occurring sequence to be RRYPDAVYL, corresponding to MV-F$_{438–446}$. Its level of expression was enumerated at approximately 1500 copies per cell, which is considered to be abundant, but lies within the range described for other viral CTL epitopes in human MHC class I molecules. We found that processing of the MV-F$_{438–446}$ epitope occurs primarily via the classic MHC class I loading pathway, since presentation to CTL depends on both the transporter associated with antigen presentation (TAP) and the proteasome. Even though it is cotranslationally inserted into the ER, a major part of MV-F is located in the cytosol, where it accumulates rapidly in the presence of proteasome inhibitors. We therefore conclude that a substantial cytosolic turnover of MV-F, together with some excellent processing features of MV-F$_{438–446}$ precursors, such as precise C-terminal excision by proteasomes, efficient TAP transport and strong HLA binding, dictate the abundant functional expression of the MV-F$_{438–446}$ CTL epitope in HLA-B*2705 at the surface of MV-infected cells.

Introduction

CTL suppress a virus infection by killing virus-infected cells upon recognition of short virus-derived peptides presented by MHC class I molecules at the cell surface (Zinkernagel & Doherty, 1979). Although T-cell-mediated immunity appears to play a central role in the clearance of established measles virus (MV) infection and protection against re-infection (Fu *et al.*, 1998), little is known about MHC class I presentation of MV peptides, and only a few human MV CTL epitopes have been described (Nanan *et al.*, 1995; van Binnendijk *et al.*, 1992). Recently, we identified three previously unknown naturally occurring MV peptides presented by human MHC class I molecules after MV infection through subtractional mass spectrometric analysis (van Els *et al.*, 2000). This technique also allowed us to estimate the number of individual peptide–MHC complexes at the surface of MV-infected cells. These varied widely between the less abundant haemagglutinin-derived peptide (less than 200 copies per cell), the relatively highly abundant matrix-derived peptide (almost 2000 copies per cell) and the exceptionally highly abundant C protein-derived peptide (more than 100000 copies per cell). This extremely diverging level of epitope expression has so far only been

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described for MV, and its relevance is not yet understood. While the natural density of a viral epitope is likely to play an important role during both the onset and maintenance of the anti-viral T cell response, only a minor portion of all known human viral CTL epitopes has been quantified, with numbers ranging from less than 1 to several thousands of copies per cell (Crotzer *et al.*, 2000; Levitsky *et al.*, 1996; Lucchiari-Hartz *et al.*, 2000; Shi *et al.*, 1997; Tsai *et al.*, 1996; Tsomides *et al.*, 1994). To gain more insight into the issue of functional epitope density in the case of MV infection, we studied the natural occurrence of a known MV epitope recognized by a CTL clone isolated from an MV patient 4 weeks after clinical symptoms. This epitope is derived from the MV fusion glycoprotein (MV-F) and is presented in the context of HLA-B*2705 (van Binnendijk *et al.*, 1992). Using mass spectrometry, we were able to identify the natural viral epitope in peptide eluates from MV-infected Epstein–Barr virus (EBV)-transformed B cells, and found it was abundantly expressed (approximately 1500 copies per cell).

We then asked which processing events were underlying such successful epitope generation, especially since for cell membrane-targeted proteins, such as MV-F, no general MHC class I processing pathway has been described. The classic MHC class I processing pathway used by proteins present in the cytosol is relatively well understood. Key events are cytosolic degradation by proteasomes, transport of the proteolytic fragments from the cytosol into the ER by the transporter associated with antigen presentation (TAP) and peptide binding to nascent MHC class I molecules in the ER followed by exocytosis of the peptide–MHC class I complexes to the cell membrane. CTL epitopes derived from transmembrane proteins have been described to exploit this classic processing pathway, requiring functional proteasomes and/or TAP for presentation (Ferris *et al.*, 1996; Hammond *et al.*, 1995; Hombach *et al.*, 1995; Mosse *et al.*, 1998). These transmembrane proteins might gain access to the cytosol as a result of faulty synthesis on free ribosomes (Hammond *et al.*, 1995) or following an ER-associated degradation route involving retrograde transport of unproperly synthesized or folded proteins back into the cytosol (Ward *et al.*, 1995; Wiertz *et al.*, 1996). Alternatively, proteasomal- and TAP-independent MHC class I presentation pathways for transmembrane proteins are described. Alternative compartments for processing of the parental protein have been suggested, for example the ER itself (Hammond *et al.*, 1995) or endolysosomes, through which cell surface expressed MHC class I molecules can recycle and where peptide reloading can take place (Grommé *et al.*, 1999; Neumeister *et al.*, 2001).

We here find that processing of the abundant MV-F CTL epitope predominantly follows the classic MHC class I pathway, requiring functional proteasomes and TAP for presentation. In addition, we further describe which favourable events in this pathway lead to the successful presentation of this human viral CTL epitope.

Methods

Virus, cell lines, infection and T cell clone. The melanoma cell line Mel-JuSo, the monocytic cell line U937 and the HLA-A*B2705-expressing EBV-transformed B cells GR and WH were all cultured in RPMI 1640 medium supplemented with antibiotics and 10% FCS. The T cell clone WH-F40 was cultured in RPMI 1640 supplemented with antibiotics, 10% human AB serum and IL2 (20 U/ml) and antigenically restimulated as previously documented (van Binnendijk *et al.*, 1992). T2 and HLA-B*2705-transfected T2 cells were cultured in Protein-Free Hybridoma Medium (PFHM) (Gibco) supplemented with antibiotics and 5% FCS.

Plaque-purified MV (Edmonston B strain), cultured in Vero cells and containing 10^7 $TCID_{50}$/ml, was used to infect GR, WH or Mel-JuSo cells at an m.o.i. of 1 in RPMI 1640 medium supplemented with antibiotics and 2% FCS.

Peptide synthesis. Synthetic peptides were prepared by standard solid-phase Fmoc chemistry using an ABIMED AMS 422 simultaneous multiple peptide synthesizer.

Isolation and HPLC fractionation of MHC-bound peptides. MHC class I molecules were immunoprecipitated from 48 h MV-infected and uninfected EBV-transformed B cells as described previously (van Els *et al.*, 2000) using the HLA-B- and -C-specific monoclonal antibody B1.23.2 (Drouet *et al.*, 1995). Peptides were eluted and fractionated on a 2·1 mm × 10 cm reversed-phase HPLC (rpHPLC) C2/C18 column (Pharmacia, SMART system) using an acetonitrile gradient (0–60%) and 0·1% trifluoroacetic acid in water (flow 100 μl/min). A part of each fraction was tested for its ability to sensitize EBV-transformed B cells to cytotoxic killing by clone WH-F40. Positive fractions were further analysed by mass spectrometry.

Standard CTL assay. Cell-mediated cytotoxicity was measured in a standard chromium release assay using peptide-pulsed GR cells as target cells as described previously (van Binnendijk *et al.*, 1992).

Mass spectrometry. Peptide fractions were analysed using micro-capillary-rpHPLC-electrospray ionization-mass spectrometry (μLC-ESI-MS) as previously described (van der Heeft *et al.*, 1998). The presence or absence of specific peptides was determined by the presence or absence of their masses and confirmed by comparison with synthetic analogues. The number of epitopes per infected cell was calculated by first determining the molar amount of peptide in a sample (using several dilutions of the synthetic peptide and the sample), multiplication by Avogadro's number and division by the number of cells.

Proteasomal dependency assay. At 12 h post-MV-infection the cell surface of WH cells was stripped by incubation with 4 mg/ml Pronase in the presence of 1 μg/ml DNase for 30 min at 37 °C. Membrane stripping was stopped by addition of culture medium (containing 10% FCS), followed by two washing steps with PBS. Protease inhibitors lactacystin, $CbzL_3$ or leupeptin were added at the indicated concentrations in RPMI with 2% FCS. After 6 h, cells were washed thoroughly with PBS and fixed with 1% paraformaldehyde and washed with 0·2 M glycine in PBS. These cells were then used as stimulator cells for clone WH-F40 in a TNFα release assay (Traversari *et al.*, 1992).

Peptide translocation by TAP. Peptide translocation was performed as previously described by Neisig *et al.* (1995). Briefly, WH cells were harvested and permeabilized by streptolysin O treatment. For each translocation assay, radioiodinated reference peptide (TVNTERAY) containing a glycosylation sequence, competing peptide (final concentration as indicated) and ATP were added to the permeabilized cells.

Peptide translocation was performed at 37 °C for 5 min, stopped by lysing the cells with lysis mix containing Triton X-100, and glycosylated radiolabelled reference peptide was recovered with ConA–Sepharose. Associated peptides were quantified by gamma counting, after extensive washing with lysis mix.

■ **Confocal microscopy.** For microscopical studies enzymatically dispersed Mel-JuSo cells were infected with MV and allowed to grow on 8-well Lab-Tek glass chamber slides for 24 h. Then cells were washed in serum-free PFHM medium and subsequently fixed in 2% paraformaldehyde (pH 7·6). Lactacystin (10 μM) was added 1 h before fixation. Immunostaining was done with polyclonal rabbit anti-MV-F or mouse monoclonal anti-MV-haemagglutinin (anti-MV-H) antibodies. These primary antibodies were stained with FITC-labelled $F(ab)_2$ DonkeyαRabbit-IgG and Cy5-labelled GoatαMouse-IgG (Jackson ImmunoRes Lab), respectively. After washing, slides were mounted in Vectashield and directly observed under a NIKON Optiphot 2 microscope. Fluorescence was imaged with a Bio-Rad 1024 confocal laser-scanning microscope equipped with an argon/krypton laser. Wavelengths of 488 nm and 645 nm were used to excite fluorescein and Cy5, respectively. Confocal images were recorded with a ×60 Plan Apo objective lense with identical settings between images. Images were processed with Lasersharp software (Bio-Rad). Fluorescence of fluorescein and Cy5 was pseudocoloured as green or red, respectively, and high intensity fluorescence (channels 175–255) was pseudocoloured as yellow.

■ **Proteasome purification and *in vitro* proteasomal digestions.** Partially purified proteasomes were derived from U937 cells. Aliquots of approximately 1×10^8 cells were washed twice with PBS and lysed in distilled water. After homogenization with a Potter-Elvejem homogenizer, compensation buffer was added to a final concentration of 20 mM Tris, 2 mM ATP, 5 mM $MgCl_2$, 1 mM DTT, pH 7·5. Post-nuclear supernatants (1 ml) were loaded on a sucrose gradient (10–40%, w/v, in compensation buffer) and centrifuged for 21 h at 200000 ***g***. Fractions of 0·65 ml were recovered and 10 μl of each fraction was assayed for proteolytic activity by measuring hydrolysis of Suc-LLVY-MCA (100 μM; 40 min; 37 °C; assay buffer, 50 mM Tris, 5 mM $MgCl_2$, 5 mM DTT, pH 7·5) in a fluorometric assay. Active fractions were pooled and dialysed overnight against compensation buffer followed by concentration to approximately 50 μl with a 100 kDa centrifugal concentrator, after which the activity was assayed again. Approximately 1000 U of proteasome activity (1 U = hydrolysis of 1·5 nmol Suc-LLVY-MCA per hour) was used per digestion of 10 μg of RRYPDAVYL containing polypeptide as indicated in assay buffer (50 mM Tris, 5 mM $MgCl_2$, 5 mM DTT, 2 mM ATP, pH 7·5). Digestions were performed at 37 °C for 1 and 3 h. Digest samples were subjected to rpHPLC; peptide-containing fractions were pooled, dried under vacuum and analysed by mass spectrometry. Individual peptides were identified by their molecular masses and quantified by the intensity of their peaks in the mass spectra. A proteasomal cleavage site was considered to be major if it generated peptide fragments with a relative amount above 9% of the total amount of RRYPDAVYL-containing polypeptide digested by the proteasome.

■ **HLA-B*2705 peptide-binding assay.** TAP-deficient T2 cells transfected with HLA-B*2705 were incubated overnight in 200 μl with serial dilutions of peptides in PFHM supplemented with antibiotics in V-bottom 96-wells at 37 °C. Cells were washed with cold (4 °C) FACS buffer (1% BSA in PBS) and assembled surface HLA-B*2705 molecules were detected by flow cytometry using the monoclonal antibody B1.23.2 and FITC-labelled rat anti-mouse IgG (Dako) using FACScan (Becton Dickinson). Only peptides binding to the otherwise unstable HLA-B*2705 molecules on these cells will increase their cell surface expression. In this assay, binding of a peptide is quantified as its fluorescence index (FI), FI = [(mean fluorescence in the presence of the peptide) − (mean fluorescence without the peptide)]/(mean fluorescence without the peptide). At a peptide concentration of 50 μM an FI of 0·5 or higher is considered as positive and an FI above 1·0 is indicative of high affinity binding of HLA-B*2705 to the peptide.

Results

Natural occurrence of a known MV-F-derived CTL epitope

The previously documented $CD8^+$ cytotoxic T cell clone WH-F40 recognizes synthetic peptides sharing the minimal amino acid sequence RRYPDAVYL (residues 438–446) of MV-F in the context of HLA-B*2705 (Fig. 1) (van Binnendijk *et al*., 1992). To determine the naturally occurring variant of the epitope, HLA-B*2705-binding peptides were extracted from MV-infected, HLA-B*2705-positive B lymphoblastoid GR cells. The generated crude peptide mixture was fractionated by rpHPLC, and the subsequent fractions were tested for recognition by clone WH-F40 in a standard chromium release assay (Fig. 2 A). CTL-sensitizing fractions (fractions 34 and 35) were analysed by mass spectrometry (only shown for fraction 34 in Fig. 2B–E) for the presence of peptides containing the $MV\text{-}F_{438–446}$ sequence. A peptide species with a molecular mass matching the molecular mass of synthetic $MV\text{-}F_{438–446}$, detected as its $[M+2H]^{2+}$ ion at m/z 576·7, was present in the CTL-sensitizing fractions (Fig. 2D), and not in the corresponding control fractions derived from uninfected GR cells (Fig. 2 C). The sequence of the peptide was confirmed to be RRYPDAVYL by mass spectrometry sequence analysis (data not shown). No further length variants of this epitope were detected.

The amount of naturally processed $MV\text{-}F_{438–446}$ present in CTL-sensitizing fractions was quantified by comparing the μLC-ESI-MS response of the biological fractions with known amounts of the synthetic analogue (data not shown). From 1×10^9 GR cells, 1·1 pmol of $MV\text{-}F_{438–446}$ peptide was extracted. Assuming an overall yield of 50% during isolation, fractionation and analysis in our procedures (van Els *et al*., 2000), we enumerated the number of MHC–peptide complexes per MV-infected GR cell to be 1325. Similarly, the amount of $MV\text{-}F_{438–446}$ peptide recovered from another HLA-B*2705-positive MV-infected B lymphoblastoid cell line WH corresponded to 1650 copies per infected cell. Herewith this MV epitope ranks as a relatively highly abundant human CTL epitope (Table 1).

Presentation of the TAP-dependent $MV\text{-}F_{338–346}$ epitope requires functional proteasomes

To understand why $MV\text{-}F_{438–446}$ is presented at such a high level, we first investigated which MHC class I processing pathway is involved in the presentation of this transmembrane protein-derived CTL epitope. Elsewhere, it was found that MV-infected TAP-deficient T2 cells transfected with HLA-

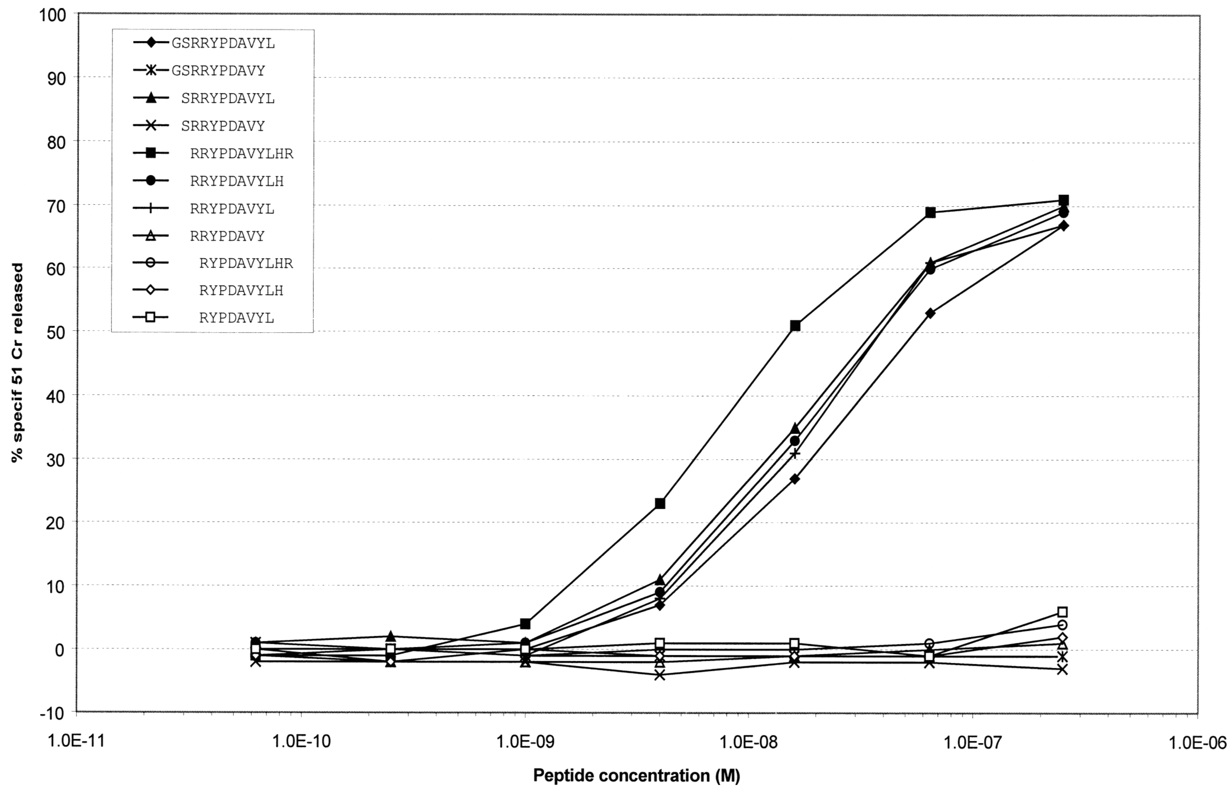

Fig. 1. Minimal epitope mapping of the HLA-B*2705-restricted, MV-specific CTL clone WH-F40. HLA-B*2705-expressing GR cells were incubated with synthetic peptides prior to use in a standard cytotoxicity assay with clone WH-F40. ◆, MV-F$_{436-446}$; ⋇, MV-F$_{436-445}$; ▲, MV-F$_{437-446}$; ×, MV-F$_{437-445}$; ■, MV-F$_{438-448}$; ●, MV-F$_{438-447}$; +, MV-F$_{438-446}$; △, MV-F$_{438-445}$; ○, MV-F$_{439-448}$; ◇, MV-F$_{439-447}$; □, MV-F$_{439-446}$.

B*2705 cells are not recognized by clone WH-F40 (van Binnendijk *et al.*, 1992), suggesting that presentation of MV-F$_{438-446}$ is TAP-dependent and might follow the classic MHC class I processing pathway. As another component of that pathway, we studied the involvement of the proteasome in the presentation of the abundant MV-F$_{338-346}$ epitope using the proteasomal inhibitors lactacystin and CbzL$_3$. Cell surface molecules were stripped from MV-infected antigen-presenting WH cells by Pronase treatment, followed by a 6 h incubation period to allow re-expression of HLA-B*2705 molecules containing the MV-F$_{438-446}$ epitope, in the presence or absence of protease inhibitors. To exclude interference of any of these substances on the activity of clone WH-F40, the antigen-presenting cells were fixed and washed before co-culture with clone WH-F40 in a TNFα release assay. The proteasomal inhibitors lactacystin and CbzL$_3$ greatly inhibited the recognition of MV-infected cells in a dose-dependent fashion (Fig. 3). In contrast, untreated cells or cells incubated with the lysosomal protease inhibitor leupeptin at concentrations described to be effective in the literature (Reis e Sousa & Germain, 1995; Martinez-Kinader *et al.*, 1995) (up to 1 mM, data partially shown) were recognized at normal levels. Pulsing of inhibitor-treated MV-infected stimulator cells with synthetic peptide restored recognition (data not shown), indicating that functional HLA-B*2705 molecules were present at the cell surface, but did not contain the MV-F$_{438-446}$ epitope. Thus, besides TAP, proteasome function is required for the presentation of this epitope from the MV-F glycoprotein in MV-infected EBV-transformed B cells, indicating that presentation follows the classic processing pathway and not an alternative proteasome- and TAP-independent pathway.

MV-F is mainly located in the cytosol and accumulates after lactacystin treatment

The role of the proteasome, a cytosolic protease, in the processing of the cell membrane-targeted MV-F poses a topological problem. We therefore assessed the availability of this protein for breakdown in the cytosol by studying the intracellular localization and expression of MV-F in Mel-JuSo cells 24 h after MV infection. Mel-JuSo cells can be readily infected with MV and have a larger content of cytoplasm than, for example, EBV-transformed B cells, and were therefore chosen for these experiments. Polyclonal rabbit anti-MV-F serum was used to ensure detection of all MV-F forms present in the cell, including partially synthesized or degraded proteins. We found a scattered and diffuse immuno-expression of the MV-F glycoprotein in the cytosol of multinucleated cells (Fig.

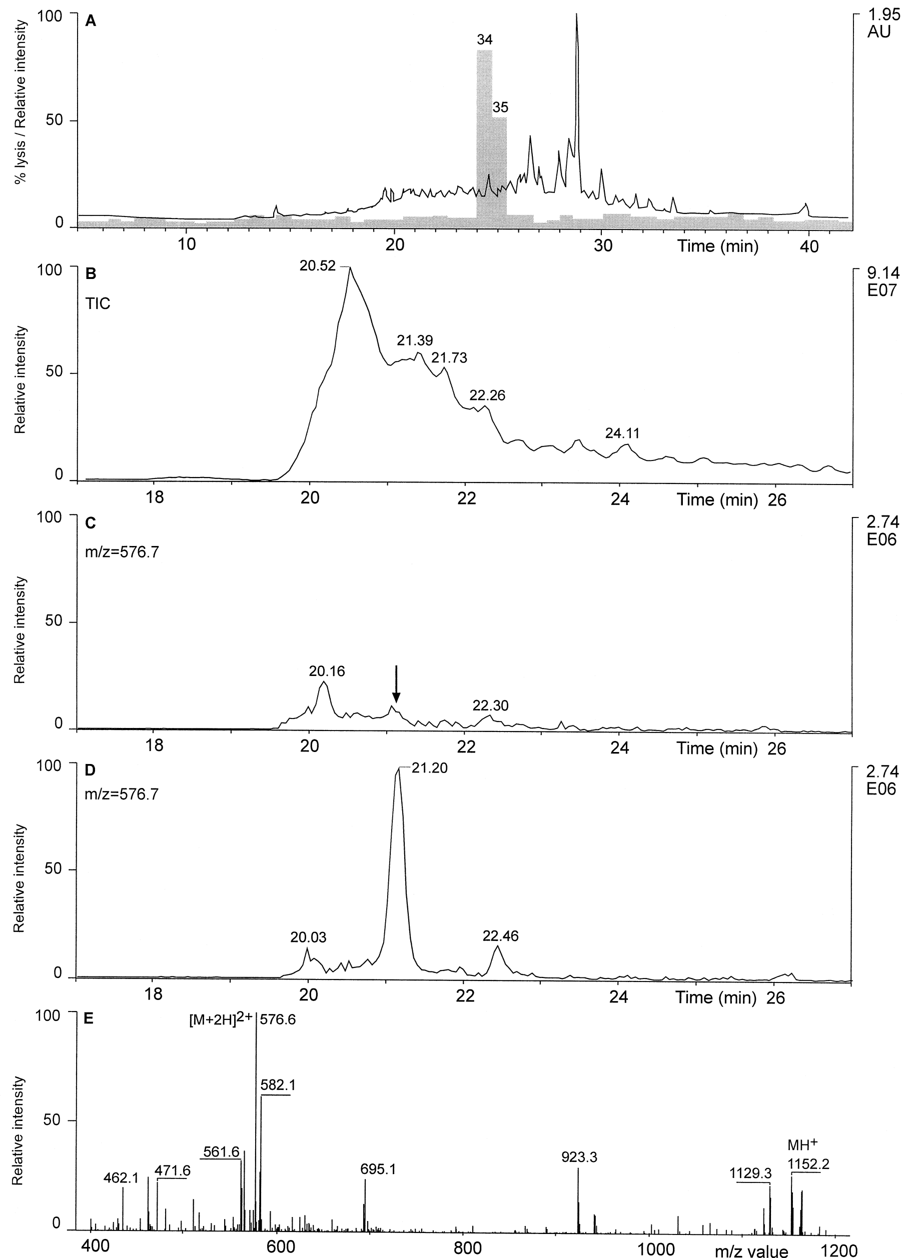

Fig. 2. Detection of the naturally processed HLA-B*2705-restricted MV CTL epitope of clone WH-F40. Peptides were isolated from MV-infected GR cells as described in Methods, and fractionated by rpHPLC. Fractions were tested in a CTL-reconstitution assay by clone WH-F40. Positive fractions were analysed by μLC-ESI-MS. Mass spectrometry spectra (*m*/*z* range 400–1500) were acquired every 3 s. (A) HPLC profile of peptide pool derived from MV-infected GR cells, absorption at 218 nm (solid line) and recognition of uninfected GR cells pulsed with individual peptide fractions by clone WH-F40 (shaded area). (B) Total ion current (as obtained by summing the signals from individual *m*/*z* in each spectrum) of positive fraction 34. (C) The extracted ion current (representing the signal of ions with a particular *m*/*z* value) of fraction 34 of uninfected cells at *m*/*z* value 576·7. (D) The extracted ion current of fraction 34 of infected cells at *m*/*z* value 576·7. (E) Mass spectrum acquired at time-point 21·20 of μLC-ESI-MS analysis of fraction 34 of the peptides derived from infected cells.

4 A). A very small fraction of MV-F was present at the cell membrane with high fluorescence intensity (Fig. 4B). This plasma membrane-bound MV-F colocalizes with the other transmembrane protein of MV, MV-H (Fig. 4A). After treatment with lactacystin for 1 h prior to fixation, the overall fluorescence intensity staining for MV-F increased, with dramatically more and more highly intense staining localized in the cytoplasm (Fig. 4D, E), indicating a strong accumulation of this protein in lactacystin-treated, MV-infected cells. In addition, after lactacystin treatment more mononucleated cells became positive for MV-F. Similar observations were made when using a monoclonal MV-F antibody (data not shown). In

Table 1. Quantified naturally occurring human viral CTL epitopes

Antigen source*	Protein	Sequence	Restriction element	Copies per cell†	Reference
MV	Fusion	RRYPDAVYL	HLA-B*2705	1325–1650[a]	This article
MV	Haemagglutinin	LMIDRPYVL	HLA-A*0201	150[a]	Nanan *et al.* (1995); van Els *et al.* (2000)
HIV	Gag	SLYNTVATL	HLA-A*0201	400[b]	Tsomides *et al.* (1994)
HIV	Reverse transcriptase	ILKEPVHGV	HLA-A*0201	12[b]	Tsomides *et al.* (1994)
EBV	EBNA-4	IVTDFSVIK	HLA-A11	350–12800[b,c]	Levitsky *et al.* (1996)
EBV	EBNA-4	AVFDRKSVAK	HLA-A11	8–760[b,c]	Levitsky *et al.* (1996)
EBV	EBNA-3C	LDFVRFMGV	HLA-B*3701	120[b]	Shi *et al.* (1997)
HBV	Nucleocapsid	YVNVNMGLK	HLA-A11	1000–5000[b]	Tsai *et al.* (1996)
EBV	EBNA-3B	RRARSLSAERY	HLA-B27	31–213[b,d]	Crotzer *et al.* (2000)
EBV	LMP2	RRRWRRLTV	HLA-B27	1–1376[b,d]	Crotzer *et al.* (2000)
EBV	EBNA-3C	FRKAQIQGL	HLA-B27	19–229[b,d]	Crotzer *et al.* (2000)
EBV	EBNA-3C	RRIYDLIEL	HLA-B27	0·1–0·8[a,b,c]	Crotzer *et al.* (2000)
HIV	Nef	PLTFGWCYKL(V)	HLA-A2	85–125[b,e]	Lucchiari-Hartz *et al.* (2000)
HIV	Nef	TPGPGVRY(PL)	HLA-B7	840–3600[b,e]	Lucchiari-Hartz *et al.* (2000)
HIV	Nef	YPLTFGWCY	HLA-B7	80[b]	Lucchiari-Hartz *et al.* (2000)

* HIV, human immunodeficiency virus; HBV, hepatitis B virus.
† Copies per cell quantified by mass spectrometry (*a*), quantified by CTL reconstitution assay (*b*), determined on different EBV-transformed B cells during persistent infection (*c*), depending on HLA-B27 subtype (*d*) and depending on the length variant (*e*).

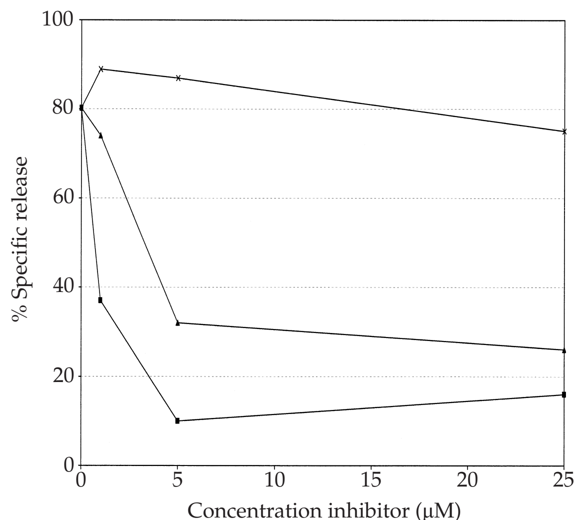

Fig. 3. Proteasomal-dependent recognition of MV-infected cells by clone WH-F40. After removal of surface MHC–peptide complexes by pronase treatment, MV-infected WH cells were incubated with protease inhibitor lactacystin (▲), CbzL$_3$ (■) or leupeptin (×) and tested for recognition by clone WH-F40 in a TNFα release assay.

marked contrast, MV-H was predominantly expressed at the cell membrane (Fig. 4A, C), and neither the intensity nor the cellular distribution pattern of MV-H was affected by lactacystin treatment (Fig. 4D, F). In conclusion, a major portion of MV-F is available in the cytosol, and lactacystin treatment induced a clear cytosolic accumulation of MV-F, indicating that under normal circumstances a substantial part of MV-F is degraded within 1 h by the proteasome.

The MV-F$_{438–446}$ CTL epitope contains a major intra-epitopic proteasomal cleavage site, but the C terminus is liberated efficiently

Next, we examined the efficiency of proteasomal liberation of MV-F$_{438–446}$. We therefore performed *in vitro* digestions of synthetic 22-mer polypeptides containing this viral CTL epitope with variable N- and C-terminal extensions of the natural flanking residues by semi-purified proteasomes. Extensive proteasomal degradation of these polypeptides could be detected after 1 h of incubation (data not shown). The cleavage sites found after 3 h of digestion are summarized in Fig. 5. The most frequently observed cleavage site was located between L$_{446}$ and H$_{447}$ at the exact C terminus of the CTL epitope. The digestion products containing H$_{447}$ as the N terminus amounted to 34–56% of the total quantity of peptide digested by the proteasome depending on the incubation time and polypeptide used. Furthermore, fragments containing L$_{446}$ as the C terminus amounted up to 8–30%. Several other major cleavage sites could be identified: one between V$_{435}$ and G$_{436}$, one between G$_{436}$ and S$_{437}$ both upstream of the epitope, one between Y$_{440}$ and P$_{441}$ within the epitope, and one between H$_{447}$ and R$_{448}$ one amino acid residue downstream of the epitope. Fragments with the proper N terminus could not be detected, indicating the necessity of further N-terminal processing of the MV-F$_{438–446}$ epitope. A similar digestion pattern was found after 1 h of incubation, and digestion could be inhibited by the proteasomal inhibitor CbzL$_3$ (data not shown). Taken together, the proteasome efficiently liberated the C terminus of MV-F$_{438–446}$, which is postulated as a prerequisite

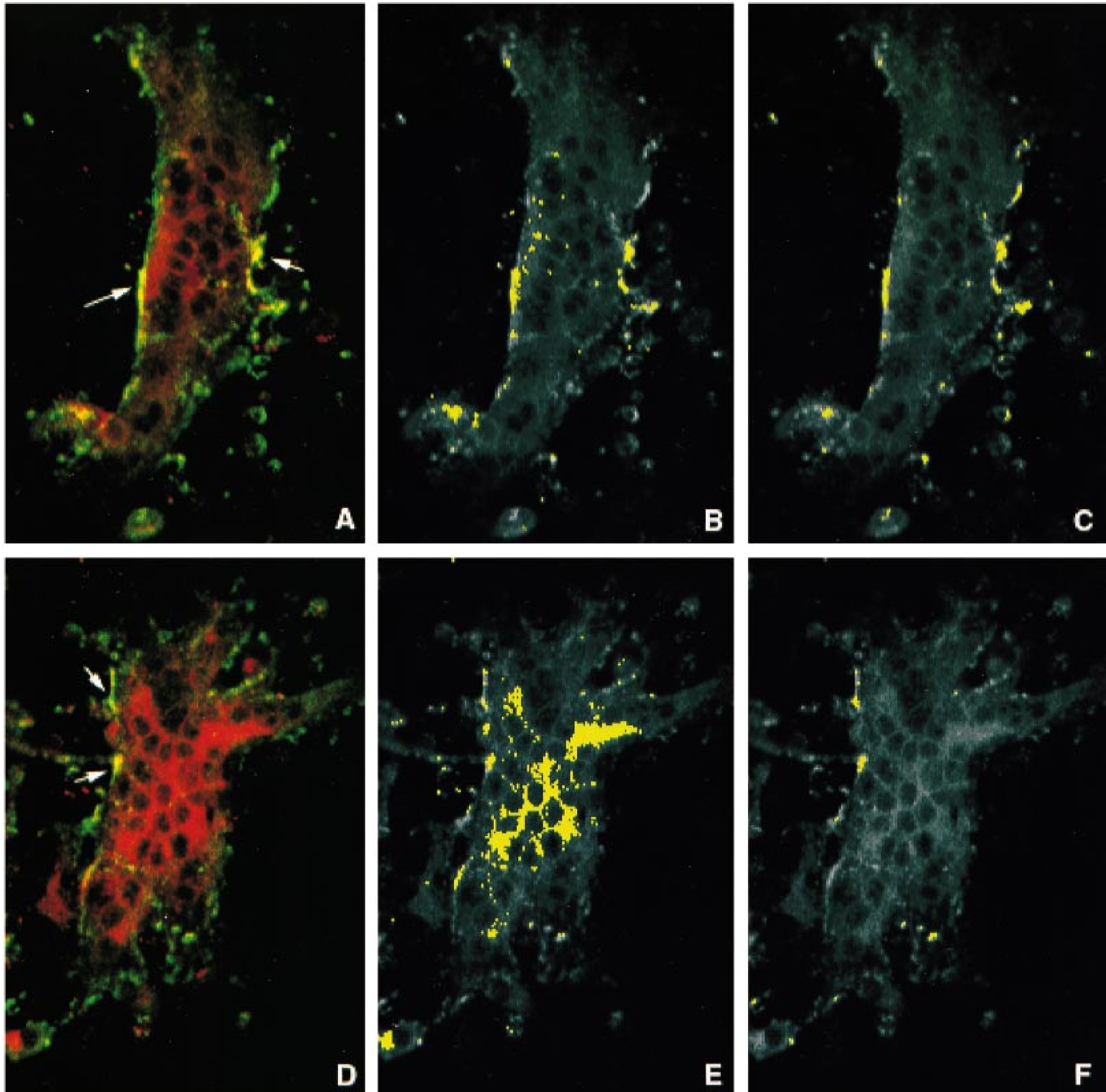

Fig. 4. MV-F but not MV-H accumulates in MV-infected Mel-JuSo cells after treatment with a proteasome inhibitor. MV-infected cells were treated with lactacystin (D, E and F) or left untreated (A, B and C). Cells were double-stained for MV-F (red) and MV-H (green), and co-localization is shown in yellow (see arrow in A and D). High intensity fluorescence (channel 175–255) was pseudocoloured as yellow for MV-F (B and E) or for MV-H (C and F).

for efficient CTL epitope presentation (Craiu *et al.*, 1997; Snyder *et al.*, 1998). At the same time it may destroy an unknown portion of potential MV-$F_{438-446}$ epitopes due to the presence of a major internal cleavage site.

N-terminally extended candidate precursors of the MV-$F_{438-446}$ CTL epitope can be transported by TAP

Once liberated by the proteasome, epitope precursors that follow the classic MHC class I processing pathway are transported by TAP into the ER lumen. *In vitro*, TAP-dependent transport can be measured by the ability of a peptide to inhibit the translocation of a radiolabelled reporter peptide (TVNTERAY) (Neisig *et al.*, 1995). In view of the claimed desirability of precise C-terminal cleavage of CTL epitopes by the proteasome (Craiu *et al.*, 1997; Snyder *et al.*, 1998), and of the fact that N-terminal trimming of precursor peptides to the naturally presented length can occur both in the cytosol and in the ER (Eisenlohr *et al.*, 1992; Roelse *et al.*, 1994;

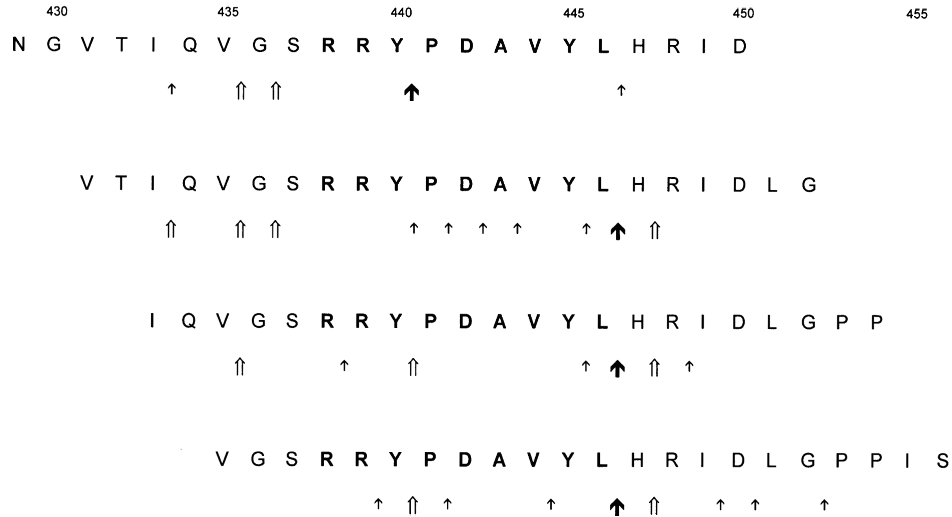

Fig. 5. Proteasomal cleavage sites of oligopeptides containing the $MV_{438-446}$ CTL epitope. Four overlapping 22-mer peptides were incubated with partially purified proteasomes for 3 h. Digest products were analysed by rpHPLC and mass spectrometry. Peptide fragments were identified by their mass. ↑ Detectable cleavage site; ⇑ major cleavage site; 🡅 most frequently observed cleavage site.

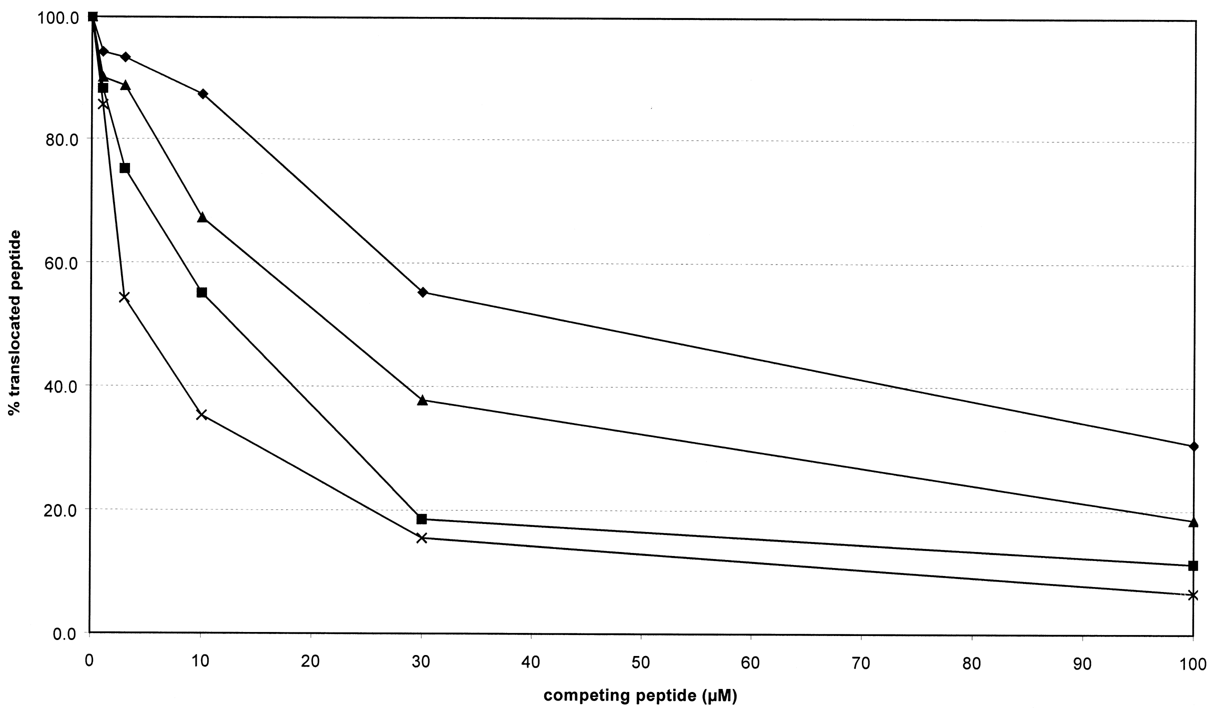

Fig. 6. $MV\text{-}F_{438-446}$ and N-terminal extended peptides are transported by TAP. TAP translocation of radiolabelled reporter peptide TVNTERAY was measured in the presence of several concentrations of unlabelled competing peptides: $MV\text{-}F_{436-446}$ (◆), $MV\text{-}F_{437-446}$ (■), $MV\text{-}F_{438-446}$ (▲) and TVNTERAY (×).

Snyder *et al*., 1994; Stoltze *et al*., 2000), we reasoned that besides $MV\text{-}F_{438-446}$ itself, only N-terminally extended length variants of this epitope, such as those found after proteasomal degradation, are candidates for TAP translocation. So, N-terminally extended synthetic variants of $MV\text{-}F_{438-446}$ as well as the natural epitope were tested as competitor peptides in the TAP translocation assay. As shown in Fig. 6, all studied length variants competed for transport with the reference peptide, with similar, intermediate efficiencies. Thus, after proteasomal degradation, the N-terminally extended intermediates as well as the $MV\text{-}F_{438-446}$ epitope itself can be readily transported by TAP.

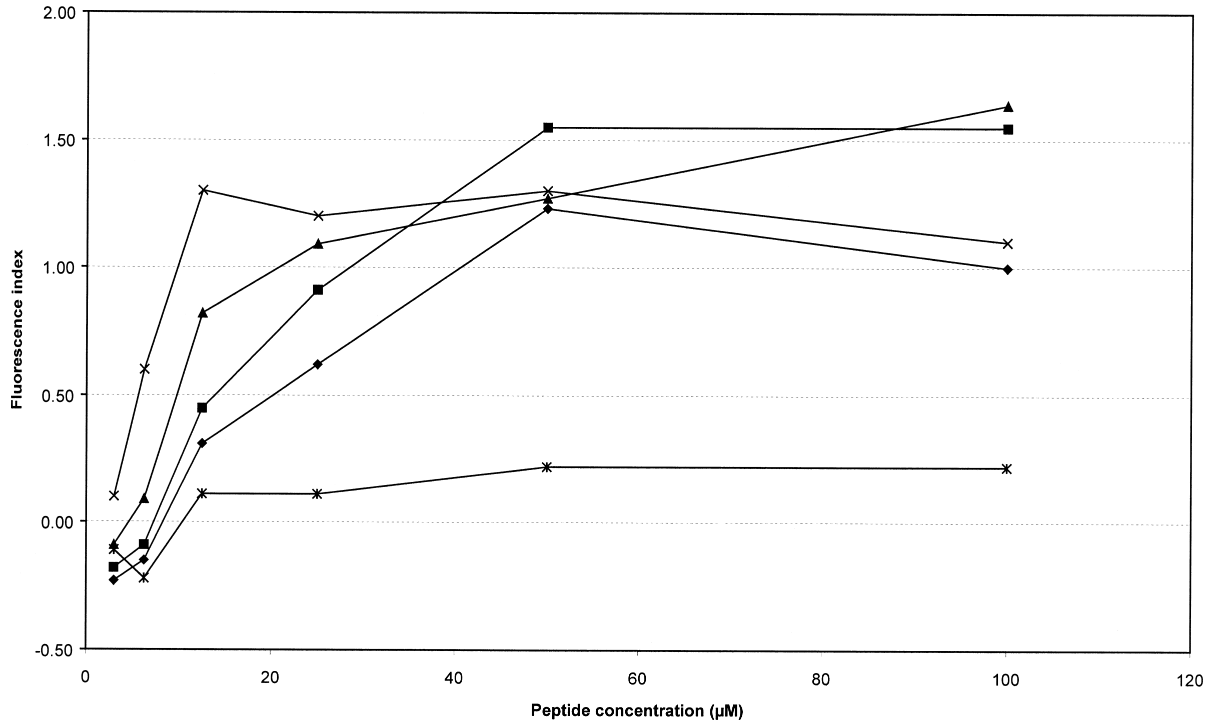

Fig. 7. The MV-$F_{438-446}$ peptide binds to HLA-B*2705 with similarly high affinity as other known viral HLA-B*2705-restricted CTL epitopes. HLA-B*2705-expressing T2 cells were incubated overnight with increasing amounts of peptide, and peptide binding was measured as the expression of stable HLA-B*2705 by indirect fluorescence. Results are given as the fluorescence index (see Methods). ◆, RRYPDAVYL (MV-$F_{438-446}$); ■, RRIYDIEL (EBV-EBNA3$C_{258-266}$) (Brooks *et al.*, 1993); ▲, SRYWAIRTR (Influ-$NP_{383-391}$) (Huet *et al.*, 1990); ×, KRWIILGLNL (HIV-$Gag_{263-272}$) (Huet *et al.*, 1990); and ⁎, HLA-A*0201-restricted CTL epitope TLGIVCPI (HPV16-$E7_{86-93}$, negative control).

Peptide binding to HLA-B*2705 is a very efficient step in the formation of the relatively abundant HLA-B*2705-MV-$F_{438-446}$ complex

After generation and TAP transport of the naturally presented MV-$F_{438-446}$ epitope, it must be rescued from further degradation by binding to its restriction element, HLA-B*2705. After virus infection, thousands of viral and self-peptides compete for binding to nascent MHC class I molecules in the ER. The efficiency of peptide binding depends on the extent to which a peptide sequence fulfils the binding motif of a particular MHC class I molecule and thus can be anticipated. When screening all protein sequences of MV (the MV proteome), using an HLA-B*2705 peptide-binding algorithm (http://bimas.dcrt.nih.gov/molbio/hla_bind/) (Parker *et al.*, 1994), the naturally occurring MV-$F_{438-446}$ sequence (RRYPDAVYL) was predicted as one of two most stable HLA-B*2705-binding peptides amongst all potential MV-derived 9- and 10-mer epitopes.

To determine the affinity of binding of the MV-$F_{438-446}$ epitope to its restriction element, we used an assay based on the capacity of a peptide to stabilize the cell surface expression of empty unstable HLA-B molecules on HLA-B*2705-transfected T2 cells. The measured affinity was high, and comparable to that of other known HLA-B*2705-restricted CTL epitopes (Fig. 7). Based on these findings we conclude that formation of the HLA-B*2705–MV-$F_{438-446}$ complex is efficient and is probably highly favoured in the ER during MV infection.

Discussion

In this study we examined the natural sequence, abundance and processing events of a known functional MV epitope derived from one of the two viral envelope proteins of this negative-stranded RNA virus and presented to CTL by HLA-B*2705. In contrast to most MHC class I molecules, members of the HLA-B27 family can accommodate peptides longer than the normal 8–11 amino acids (Rammensee *et al.*, 1997; Urban *et al.*, 1994). We found that several synthetic length variants of the MV-F epitope, when loaded on HLA-B*2705-positive target cells, can activate CTL clone WH-F40. However, only the shared minimal epitope, RRYPDAVYL corresponding to amino acid residues 438–446, located in the luminal domain of MV-F, could be identified in peptide eluates from MV-infected cells by sensitive mass spectrometric analysis. The average copy number was determined to be approximately 1500 MHC–peptide complexes per cell. Herewith the MV-$F_{438-446}$ epitope ranks as an abundant naturally occurring viral CTL epitope in humans (Table 1). Notably, a naturally processed HLA-A*0201-binding CTL epitope derived from the second glycoprotein from MV, MV-H (Nanan *et al.*, 1995; van Els *et*

al., 2000), was presented at a tenfold lower level (van Els *et al*., 2000).

Further studies were aimed at the processing events leading to the abundant presentation of this MV-$F_{438-446}$, since no general MHC class I processing pathway for glycoproteins is known. Previously, van Binnendijk *et al*. (1992) suggested that the presentation of the MV-$F_{438-446}$ epitope requires TAP, based on the absence of WH-F40 CTL recognition of MV-infected, HLA-B*2705-transfected and TAP-deficient T2 cells. In contrast, Grommé *et al*. (1999), recently reported a residual WH-F40 recognition of the same target cells, suggesting that expression of this epitope in HLA-B*2705 is not fully dependent on TAP. In addition they reported that recognition was inhibitable by treatment with NH_4Cl, a lysosomotropic agent. Therefore these authors propose that alternative processing of MV-$F_{438-446}$ may occur in endolysosomal compartments followed by presentation by MHC class I molecules, which recycle from the cell surface through these endolysosomes. In contrast, we find only residual recognition of another TAP-deficient cell line (BM36.1; Urban *et al*., 1994) which was transfected with HLA-B*2705 and infected with MV by clone WH-F40 (K. Stittelaar & C. Herberts, unpublished observations). Moreover, we found that presentation of the HLA-B*2705-associated MV-F epitope is profoundly hampered by proteasomal inhibitors and not an inhibitor of lysosomal proteases. From these combined data we conclude that, even though a small portion of the MV-$F_{438-446}$ epitope may be processed via an alternative MHC class I loading pathway, the majority is formed through the classic proteasome- and TAP-dependent pathway.

While examining the processing pathway of the MV-$F_{438-446}$ epitope, we found some typical processing features which might explain its abundance and its outnumbering of the MV-H-derived MHC class I CTL epitope (Table 1). Immunostaining experiments indicated that MV-F products were not predominantly localized at the cell surface, but were mainly present in the cytosol. In contrast, MV-H proteins were highly associated with the cellular membrane. Furthermore, we found a strong cytosolic accumulation of MV-F, but not of MV-H, within 1 h of lactacystin treatment, indicating extensive proteasomal breakdown of MV-F. So the cytosol appears to be more readily accessible for MV-F than MV-H, implying that the availability for proteasomal degradation also differs markedly for these two transmembrane proteins. Recently, it was postulated that a considerable part of all newly synthesized proteins (up to 30%) is targeted for proteasomal degradation, because of errors in translation or in post-translational processes necessary for proper protein folding (Schubert *et al*., 2000). Some proteins may be more predisposed than others towards becoming these so-called defective ribosomal products (DRiPs), on the basis of size or inherent difficulties in folding or assembly. We like to propose that the abundant cytosolic MV-F forms are in fact DRiPs, failing to attain their correct structure, and that the biogenesis and assembly of MV-F are more sensitive to errors than those of MV-H. While both proteins require folding and glycosylation for full maturation, MV-F also needs to be cleaved postranslationally into two disulphide-linked subunits, F_1 and F_2, to generate the functional form of this transmembrane protein.

In addition to the cytosolic availability of MV-F, we studied the efficiency of several processing steps further downstream in the classic MHC class I pathway. *In vitro* proteasomal liberation of the *precise* MV-$F_{438-446}$ epitope from MV-F precursor polypeptides did not occur, but an assumed important requirement for the generation of CTL epitopes, proteasomal cleavage at the exact C terminus of the epitope (Craiu *et al*., 1997; Snyder *et al*., 1998), was met. In fact, the C terminus was liberated very efficiently by the proteasome. So, N-terminally extended versions of the MV-$F_{438-446}$ epitope are the presumed *in vivo* post-proteasomal processing intermediates. The fact that the proteasome did not generate the exact N terminus of the CTL epitope is not likely to limit its abundant presentation since redundant trimming systems acting downstream of the proteasome have been described both in the cytosol and in the ER (Craiu *et al*., 1997; Roelse *et al*., 1994; Snyder *et al*., 1994; Stoltze *et al*., 2000), and the epitope itself as well as the extended length variants are transported by TAP with comparable efficiencies.

In addition to the major C-terminal proteasomal cleavage site we also found a major cleavage site within the MV-$F_{438-446}$ epitope. In contrast to some CTL epitopes where the presence of an internal cleavage site was found to abolish presentation altogether (Luckey *et al*., 1998; Niedermann *et al*., 1995; Ossendorp *et al*., 1996), here this still allows abundant expression of the MV-$F_{438-446}$ epitope. One explanation could be that once the N-terminal length variants of the epitope are formed by cleavage at the major cleavage sites, C-terminal and upstream of the epitope, the internal cleavage site is ignored by the proteasome *in vivo*. Alternatively, the presence of an intra-epitopic cleavage site may be of little significance for the presentation of viral CTL epitopes generated during acute infection, due to the high level of viral protein synthesis and subsequent turnover during lytic infection. Overall, from the observed digestion pattern, we conclude that proteasomal degradation is an efficient step in the generation of the MV-F-derived CTL epitope. Similar assumptions, with respect to the relevance of proteasomal cleavage sites, were made by Kessler *et al*. (2000), who successfully identified four novel naturally presented CTL epitopes derived from the tumour antigen PRAME utilizing an improved 'reverse immunology' strategy, including the analysis of proteasomal digestions. Candidate CTL epitopes were primarily selected on the basis of efficient liberation of their precise C terminus, while intactness of the candidate epitope was evaluated as a secondary factor and no significance was assigned to exact N-terminal excision of the epitope.

The next two processing events following proteasome digestion, i.e. transport by TAP and peptide binding to HLA-

B*2705, were efficient and probably non-limiting in the generation of MHC class I–MV-$F_{438-446}$ complexes. Moreover, given the high affinity binding of synthetic MV-$F_{438-446}$ to HLA-B*2705 and the unique score of this epitope when screening the whole MV proteome using a peptide-binding algorithm, we propose that, once in the ER, the MV-$F_{438-446}$ epitope does not encounter any strong competition from other potential MV-derived 9- or 10-mers for binding to HLA-B*2705.

Altogether, we found that this particular glycoprotein-derived CTL epitope is predominantly processed and presented via the classic MHC class I loading pathway, and that due to the unexpected extensive presence and turnover of its precursor protein in the cytosol, in combination with multiple other excellent intrinsic processing features, the MV-$F_{438-446}$ epitope reaches a relatively high level of cell surface expression when compared to other examples of human viral CTL epitopes (Table 1). In general, viral glycoproteins, including MV-F and MV-H, appear to be a major source of target proteins for antiviral CTL responses (Jaye *et al*., 1998; Rammensee *et al*., 1997). Therefore more insight into their individual processing requirements and their naturally occurring MHC class I epitopes can be instrumental in the development of new generations of viral vaccines.

We would like to thank Dr W. Ovaa for his help with the proteasomal digestion assay, Dr A. Neisig from the Department of Cellular Biochemistry of The Netherlands Cancer Institute for her help with the TAP translocation assay, Dr P. Hoogerhout for the peptide synthesis, M. van der Grift and M. van Wijk for excellent biotechnical skills, Dr R. van Binnendijk for the gift of clone WH-F40, and Drs G. van Bleek and C. Boog for critically reading the manuscript.

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Received 27 February 2001; Accepted 30 April 2001