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Evidence that transporters associated with antigen processing translocate a major histocompatibility complex class I-binding peptide into the endoplasmic reticulum in an ATP-dependent manner

(peptide transport)

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ABSTRACT **We have investigated the role of the putative peptide transporters associated with antigen processing (TAP) by using a permeabilized-cell system. The main objective was to determine whether these molecules, which bear homology to the ATP-binding cassette family of transporters, translocate antigenic peptides across the endoplasmic reticulum membrane for assembly with major histocompatibility complex (MHC) class I molecules and β_2-microglobulin light chain. The pore-forming toxin streptolysin O was used to generate permeabilized cells, and peptide translocation was determined by measuring the amount of added radiolabeled peptide bound to endogenous class I molecules. No radiolabeled peptide was associated with MHC class I glycoproteins from unpermeabilized cells. We found that efficient peptide binding to MHC class I molecules in permeabilized cells is both transporter dependent and ATP dependent. In antigen-processing mutant cells lacking a functional transporter, uptake occurs only through a less-efficient transporter and ATP-independent pathway. In addition, short peptides (8–10 amino acids) known to bind MHC class I molecules compete efficiently with a radiolabeled peptide for TAP-dependent translocation, whereas longer peptides and a peptide derived from an endoplasmic reticulum signal sequence do not compete efficiently. This result indicates that the optimal substrates for TAP possess the characteristics of MHC-binding peptides.**

A basic requirement for the presentation of antigenic peptides by major histocompatibility complex (MHC) class I molecules to T cells is the transport of these peptides to the site of class I assembly. The breakdown of protein antigens to peptides destined for presentation by class I molecules is thought to occur in the cytosol through the degradative action of proteasomes (1). Two proteins encoded within the MHC, termed LMPs for low molecular weight proteins, share homology with proteasome subunits and may play a role in the specific degradation of MHC class I-associated antigens (2). Once the peptide is generated, it must be targeted to the correct compartment where it can bind to class I molecules. The available evidence suggests that this compartment is the endoplasmic reticulum (ER) (3). The discovery of two other proteins encoded within the MHC, termed TAP-1 and TAP-2 for transporters associated with antigen processing because of their homologies to the ATP-binding cassette family of transporters (for review, see refs. 2 and 4), has led to speculation that these proteins are responsible for transporting peptides across the ER membrane. Restoration of normal class I processing and presentation occur in mutant cell lines that lack one or both of the TAP proteins when functional TAP-encoding genes are introduced by transfection (5, 6). Two such mutant cell lines, .174 and T2, possess a large deletion in their HLA-class II region, which encompasses both the TAP- and LMP-encoding genes. As a result, class I expression and presentation of whole antigen are drastically reduced. A normal phenotype can be rescued in .174 and T2 cells by returning TAP-1 and TAP-2 genes to the cells (7, 8).

To date there is no direct evidence that antigenic peptide transport is mediated by TAP. Several groups have used purified microsomes to look at this question *in vitro* (9, 10). Others have used minigene constructs encoding class I-specific peptides and measured peptide uptake by presentation to T cells (11, 12). The results of these studies indicate that a TAP- and ATP-independent mechanism exists that allows for peptide uptake; however, evidence that a TAP- and ATP-dependent uptake mechanism exists is lacking. To show TAP-dependent uptake of antigenic peptides we developed a permeabilized-cell system using the bacterial pore-forming toxin streptolysin O (13). An advantage of using streptolysin O is that by binding it to cells at 4°C one can restrict pore formation to the cell surface, leaving internal membranes intact. Exogenously added peptides and other factors have easy access to the cell interior once pores are formed. The translocation of peptide across the ER membrane can be measured through the binding of the peptide to empty HLA class I molecules located in the ER.

In this report we use a naturally processed class I-binding peptide derived from the human immunodeficiency virus 1 nef protein (nef7B; see Table 1) and HLA-A3 as our target class I molecule. We show that in permeabilized cells radioiodinated nef7B is translocated across the ER membrane and binds to HLA-A3 in a TAP- and ATP-dependent manner, thus providing evidence for the role of TAP proteins in the transport of antigenic peptides.

MATERIALS AND METHODS

Cells and Cell Culture. C1R, T2, RMA, and RMA-S cells were stably transfected with HLA-A3 genomic DNA by electroporation, as described (20, 21). RMA and RMA-S cells were cotransfected with human β_2-microglobulin genomic DNA. The cell lines were grown in Iscove's modified Dulbecco's medium (IMDM; GIBCO) supplemented with 5% bovine calf serum (HyClone) and gentamicin at 20 μg/ml.

Peptides and Peptide Iodination. The peptides used were synthesized by the Keck Foundation Biotechnology Resource Laboratory (Yale University) or by Multiple Peptide Systems (San Diego). Each peptide was purified by HPLC

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Abbreviations: MHC, major histocompatibility complex; TAP, transporters associated with antigen processing; ER, endoplasmic reticulum.

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Table 1. Peptide sequences

Name	HLA restriction	Sequence	Ref.
nef7B	A3	QVPLRPMTYK	14
nef7X	A3	FPVTPQVPLRPMTYKAAVDLS	14
nef6	A3	QEEEEVGFPVTPQVPLRPMTYKAAV	14
K62	A2	GILGKVFTLTV (influenza matrix)	15
IP-30	A2	LLDVPTAAV	16
B27#1	B27	GRIDKPILK (ribosomal protein)	17
B27#3	B27	RRYQKSTEL (histone H3)	17
VSV	K^b	RGYVYQGL	18
NP	D^b	ASNENMETM (influenza)	19

IP-30, interferon-inducible protein; VSV, vesicular stomatitis virus; NP, nucleoprotein.

and shown to be a single species by mass spectrometry. The iodination of nef7B (specific activity of 6–8 × 10^4 cpm per pmol) was done as described (22).

Permeabilization of Cells with Streptolysin O. Cells (1–2 × 10^7) were washed once in serum-free medium, resuspended in 1 ml of serum-free medium containing 4 mM dithiothreitol, and incubated for 10 min on ice with streptolysin O (Murex, Norcross, GA) at 1 unit/ml. The cells were then washed three times in serum-free medium and resuspended in 1 ml of intracellular transport buffer (ICT) (50 mM Hepes, pH 7.0/78 mM KCl/4 mM $MgCl_2$/8.37 mM $CaCl_2$/10 mM EGTA/1 mM dithiothreitol) containing bovine serum albumin at 4 mg/ml and various concentrations of ^{125}I-labeled nef7B. Unless otherwise indicated, the cells were then incubated for 20 min at 37°C to allow for permeabilization and peptide translocation. After incubation the cells were washed once with ICT buffer and solubilized in 1 ml of buffer containing 1% Triton X-100, 0.15 M NaCl, 0.01 M Tris (pH 7.4), 5 mM iodoacetamide, 0.5 mM phenylmethylsulfonyl fluoride, and an excess of unlabeled nef7B (10 μM) to prevent postsolubilization association of labeled peptide with HLA-A3. The extracts were clarified by centrifugation at 10,000 × *g* for 5 min before immunoprecipitation.

Reconstitution of MHC Class I Transport. Cells (1 × 10^7) were metabolically labeled for 15 min with 250 μCi of Tran^{35}S-label (ICN) (1 Ci = 37 GBq) per ml of methionine-free medium at 37°C, as described (23). Cells were washed once with serum-free medium and treated with streptolysin O on ice, as indicated above. Incubation at 37°C was then carried out for 45 min in 1 ml of ICT buffer containing HeLa cell cytosol at 4 mg/ml (24), ATP in the form of an ATP-regenerating system (2.2 mM ATP/0.44 mM GTP/22.2 mM creatine phosphate/creatine phosphate kinase at 31.8 units/ml) (25), and with or without nef7B. The cells were then washed once with 1 ml of ICT buffer and solubilized in detergent, as described above.

Immunoprecipitation. For ^{125}I-labeled peptide-translocation experiments, solubilized HLA-A3 molecules containing bound peptide were immunoprecipitated from 0.5–1.0 ml of the extract with the monoclonal antibody GAP.A3 (26) (5 μg) and protein A-Sepharose beads (25 μl; Sigma). After precipitation the beads were washed three times with 1 ml of wash buffer (0.02 M Tris, pH 8.0/0.15 M NaCl/0.5% Triton X-100/0.5% deoxycholate/0.05% SDS/0.02% NaN_3). Nonspecific binding was determined by immunoprecipitation with the anti-transferrin receptor monoclonal antibody 1G12 (16) or anti-H-2K^b monoclonal antibody Y3 (27). The level of bound peptide was determined by counting the protein A-Sepharose–immunoglobulin complexes in a γ counter (LKB Compugamma CS).

For MHC class I-transport experiments, [^{35}S]methionine-labeled cellular extracts (1 ml) were first preadsorbed overnight in the presence of 5 μl of rabbit anti-human IgG (Boehringer Mannheim) with 50 μl of protein A beads and then immunoprecipitated with monoclonal antibody GAP.A3, as described above. The HLA-A3 molecules were eluted from the protein A beads in SDS, digested with endoglycosidase H, as described (23), and analyzed by SDS/PAGE (10%) and autoradiography with Kodak X-Omat AR film.

RESULTS AND DISCUSSION

^{125}I-Labeled nef7B Translocation into the ER in Permeabilized C1R.A3 and T2.A3 Cells. Fig. 1*A* shows dose–response curves comparing the levels of ^{125}I-labeled nef7B bound to HLA-A3 at various concentrations of labeled peptide in permeabilized wild-type C1R.A3 and mutant T2.A3 cells. In permeabilized C1R.A3 cells, peptide translocation is readily demonstrated, with significant uptake occurring at the lowest concentrations of peptide (half-maximal translocation at ≈100 nM peptide). No binding of labeled peptide to HLA-A3 was detected when unpermeabilized cells were used. In T2.A3, however, significant translocation occurs only at the higher concentrations of peptide and even then at ≈10-fold less the levels attained in C1R.A3 cells. Although the mutant cell lines used express low levels of HLA-A3 on their surface (16, 21), they do possess similar levels of "empty" HLA-A3 molecules as their wild-type counterparts, as assessed by nef7B binding to HLA-A3 molecules in cell extracts (data not shown). A time course of ^{125}I-labeled nef7B translocation in permeabilized C1R.A3 cells is shown in Fig. 1*B*. Labeled nef7B is taken up rapidly, reaching almost maximum levels after 3 min of incubation at 37°C; this represents a minimum rate of peptide uptake because streptolysin O pores are being simultaneously formed during the 37°C incubation. Together these data suggest that an efficient uptake mechanism for nef7B, almost certainly TAP-dependent, exists in the wild-type cell. In the mutant cell TAP-independent uptake occurs by a secondary and inefficient pathway. That such a pathway exists is consistent with published peptide-translocation studies in which purified microsomes were shown to incorporate exogenous peptides in a TAP- and ATP-independent manner (9, 10). The precise mechanism for this form of peptide uptake is unknown. However, it may be of some biological relevance because certain viral epitopes can still be presented in mutant cells that lack functional TAP proteins (28, 29).

TAP-Dependent Translocation of ^{125}I-Labeled nef7B in C1R.A3 Requires ATP. Because TAP-1 and -2 belong to the ATP-binding cassette family of transporters (4) we determined whether the TAP-dependent peptide-uptake mechanism requires ATP. Fig. 2*A* shows that when ATP is depleted from the system by preincubation with apyrase, ^{125}I-labeled nef7B translocation is dramatically reduced in permeabilized C1R.A3 cells. However, ^{125}I-labeled nef7B uptake in T2.A3 cells is unaffected by apyrase addition, indicating that TAP-independent peptide translocation and binding to HLA-A3 molecules does not require ATP, again consistent with previous results (9, 10). In addition, Fig. 2*B* shows that after ATP-depletion ^{125}I-labeled nef7B translocation can be restored in permeabilized C1R.A3 cells to near-original levels

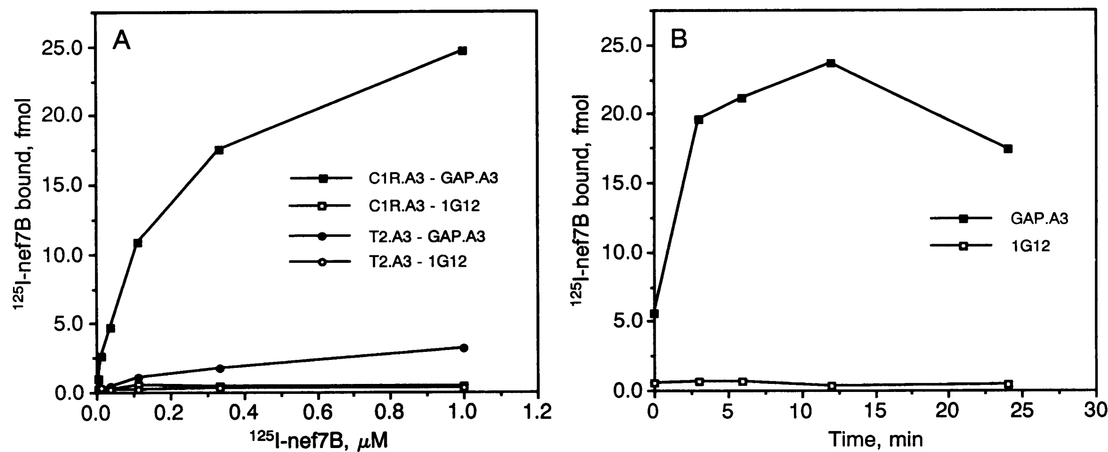

FIG. 1. Nef7B is efficiently translocated across the ER membrane in C1R.A3 cells. (*A*) Translocation of ^{125}I-labeled nef7B and its subsequent binding to HLA-A3 in permeabilized C1R.A3 and T2.A3 cells is shown as the amount of ^{125}I-labeled nef7B peptide bound to HLA-A3 (indicated as monoclonal antibody GAP.A3) at different concentrations of added peptide. Cells were permeabilized with streptolysin O, incubated with labeled peptide for 20 min at 37°C, and processed as described. As a negative control the level of ^{125}I-labeled nef7B associated with transferrin receptors (1G12) from each extract was determined. (*B*) Time course of ^{125}I-labeled nef7B uptake in C1R.A3 cells. The experimental procedures were similar to those above; however, at various times cell aliquots were removed, and the level of HLA-A3-associated nef7B was determined. The concentration of ^{125}I-labeled nef7B was constant at 150 nM throughout.

by adding back ATP (compare column 1 with column 4). When GTP is added instead of ATP, nef7B translocation is restored slightly (column 5), but this result could be from trace levels of ATP produced by the ATP-regenerating components also included during incubation (see legend). These data strongly suggest that TAP requires ATP to function and confirm experimentally the anticipated structural and functional homologies between TAP and the ATP-binding cassette family of transporters.

Addition of Nef7B to Permeabilized Cells Induces Transport of MHC Class I Molecules Out of the ER. To confirm that exogenous peptides in the permeabilized-cell system bind to newly synthesized HLA-A3 molecules in the ER, we determined whether nef7B binding could induce the intracellular transport of HLA-A3. For these experiments, C1R.A3 and T2.A3 cells were pulse-labeled with [^{35}S]methionine, permeabilized with streptolysin O, and then chased in the presence of unlabeled nef7B. To achieve reconstitution of class I transport, ATP and cytosol were added to the permeabilized cells, as described for transport in other permeabilized-cell systems (25, 30). The results (Fig. 3) indicate that the addition of nef7B peptide induces transport of HLA-A3 molecules in both C1R.A3 and T2.A3 cells. In C1R.A3 a basal transport level exists in the presence of ATP and cytosol that presumably reflects the translocation of naturally processed peptides. In response to nef7B, HLA-A3 transport is further stimulated 3- to 4-fold (lanes 6 and 8). In T2.A3 cells, however, there is no basal transport level, and the induction of HLA-A3 transport by nef7B is easily seen (lane 8). However, the induction of HLA-A3 transport in T2.A3 cells occurs only at high concentrations of nef7B (100 μM); whereas, for C1R.A3 cells induction can be seen at 10 μM (lane 6) and at concentrations as low as 100 nM (data not shown). These data confirm that peptide translocation is occurring, and the induction of HLA-A3 transport indicates that binding to MHC class I molecules occurs in a pre-medial

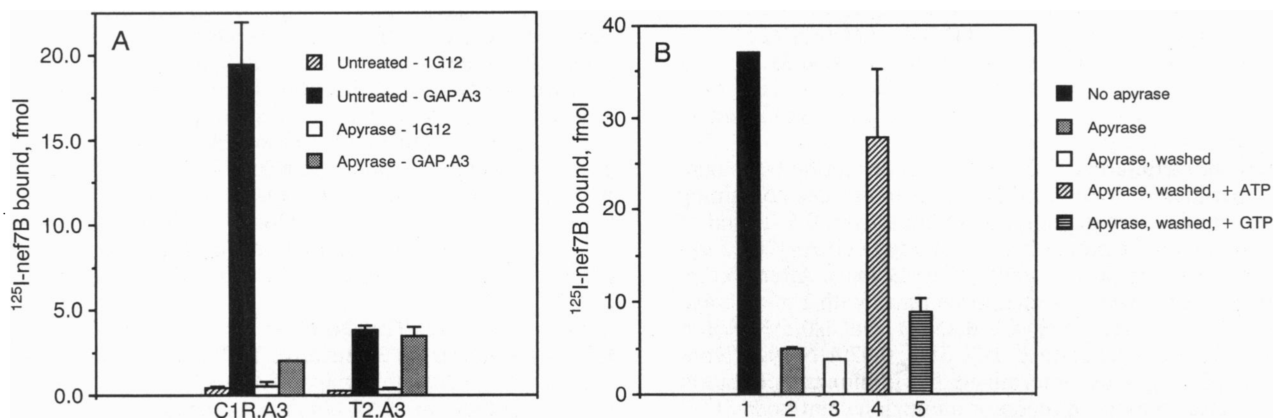

FIG. 2. (*A*) ^{125}I-labeled nef7B translocation is inhibited by apyrase treatment of permeabilized C1R.A3 cells but is not inhibited in T2.A3 cells. The levels of ^{125}I-labeled nef7B bound to HLA-A3 from permeabilized C1R.A3 and T2.A3 cells with and without apyrase are shown. Before addition of labeled peptide, cells were preincubated with or without apyrase (Sigma) at 50 units/ml for 10 min at 37°C. ^{125}I-labeled nef7B was then added, and the incubation was continued at 37°C for an additional 20 min. The concentrations of ^{125}I-labeled nef7B used for C1R.A3 and T2.A3 cells were 100 nM and 1 μM, respectively. (*B*) Transport of ^{125}I-labeled nef7B can be restored in apyrase-treated C1R.A3 cells by ATP addition. Permeabilized cells were treated essentially as described above. However, after apyrase treatment, three sets of cells (columns 3–5) were washed once with 1 ml of ICT buffer and resuspended in fresh buffer; the incubation was then continued at 37°C in the presence of labeled peptide (150 nM) and 1 mM ATP (column 4) or 1 mM GTP (column 5) together with the ATP-regenerating-system components creatine phosphate and creatine phosphate kinase. Values for amount of peptide bound represent the mean of duplicate points. GAP.A3, monoclonal antibody GAP.A3. 1G12, monoclonal antibody 1G12.

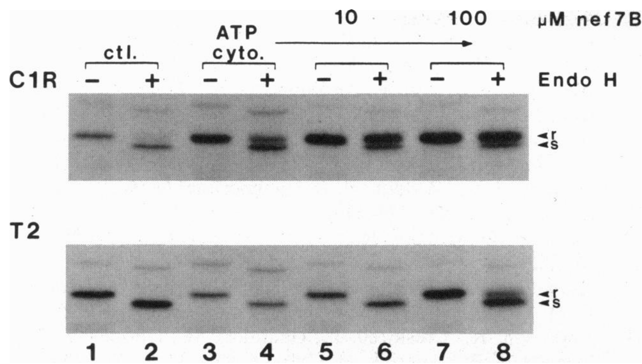

FIG. 3. Nef7B induces transport of HLA-A3 out of the ER in permeabilized C1R.A3 and T2.A3 cells. SDS/gel analysis shows the levels of HLA-A3 transport, measured by endoglycosidase H (Endo H)-resistance, in response to different concentrations of unlabeled nef7B. The control (ctl.) lanes 1 and 2 represent cells incubated only in ICT buffer/bovine serum albumin at 4 mg/ml with no further additions. Lanes 3–8 represent cells incubated in ICT buffer/4 mg of cytosol (cyto.) per ml, with ATP, and without (lanes 3 and 4) or with 10 μM (lanes 5 and 6) or 100 μM (lanes 7 and 8) nef7B. (*Upper*) C1R.A3 cells. (*Lower*) T2.A3 cells. r, Endo H resistant; s, Endo H sensitive.

Golgi compartment. This result is consistent with the reported localization of TAP to the ER and cis Golgi by immunoelectronmicroscopic studies (3).

^{125}I-Labeled Nef7B Is Translocated by a TAP-Dependent Mechanism in RMA.A3 Cells. The genetic deletion is large enough in T2 cells that one cannot rule out the possibility that MHC-linked genes other than the TAP genes are involved in the translocation of peptides in normal cells. To address this possibility we asked whether nef7B could be translocated in permeabilized wild-type RMA and mutant RMA-S cells transfected with *HLA-A3* and human β_2-microglobulin genes. Although the phenotypes of both T2 and RMA-S cells are similar with regard to class I expression, the responsible mutation is more restricted in RMA-S cells, which lack a functional TAP-2 protein due to a point mutation in the TAP-2-encoding gene (31). Fig. 4 shows ^{125}I-labeled nef7B uptake in permeabilized RMA.A3 and RMA-S.A3 cells. Clearly there is a significant level of peptide translocation in RMA.A3, whereas the level of translocation in RMA-S.A3 is ≈5-fold less. This result argues that TAP is the major system

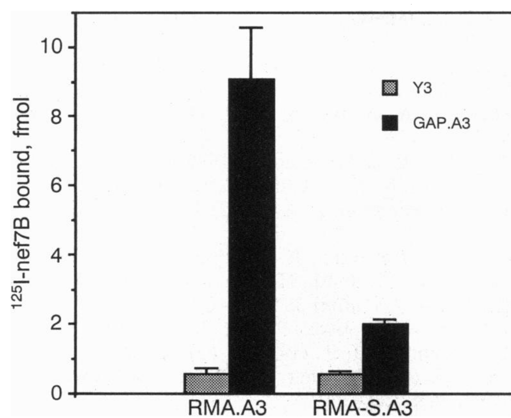

FIG. 4. Nef7B is readily translocated in permeabilized RMA.A3 cells but not in mutant RMA-S.A3 cells. The amounts of ^{125}I-labeled nef7B bound to HLA-A3 from permeabilized RMA.A3 and RMA-S.A3 cells are shown. Control histograms indicate the amount of labeled nef7B bound to H-2K^{b} (monoclonal antibody Y3) from the same extracts. The concentration of ^{125}I-labeled nef7B used for both cell lines was 500 nM. Values shown for the amount of labeled nef7B bound represent the mean of duplicate points.

involved in peptide translocation, in line with the numerous studies showing TAP-dependence of MHC class I-restricted antigen processing (5–8).

Peptide Specificity of the Translocation System. To examine the peptide specificity of the ER translocation system we performed competition experiments using a panel of unlabeled peptides of various sequences and lengths (see Table 1). ^{125}I-Labeled nef7B translocation experiments were done in permeabilized C1R.A3 cells with different concentrations of unlabeled competing peptides. The peptides used in this competition study did *not* compete for the binding of ^{125}I-labeled nef7B to HLA-A3 molecules in cellular extracts derived from C1R.A3 cells (data not shown). It is possible that any competition seen in permeabilized cells may occur at an undefined intermediate step in HLA class I molecule–peptide association, such as an obligatory transient association with a peptide-binding molecular chaperone. It seems likely, however, that the assay reflects competition for TAP-dependent translocation.

Fig. 5 shows that the level of competition of the various peptides can be loosely divided into three categories: highly competitive [peptides B27#1 and B27#3 (see Table 1)], moderately competitive [K62 (see below), VSV, and NP], and weakly competitive (IP-30, nef7X, and nef6). The competition curve for unlabeled nef7B is not shown because even

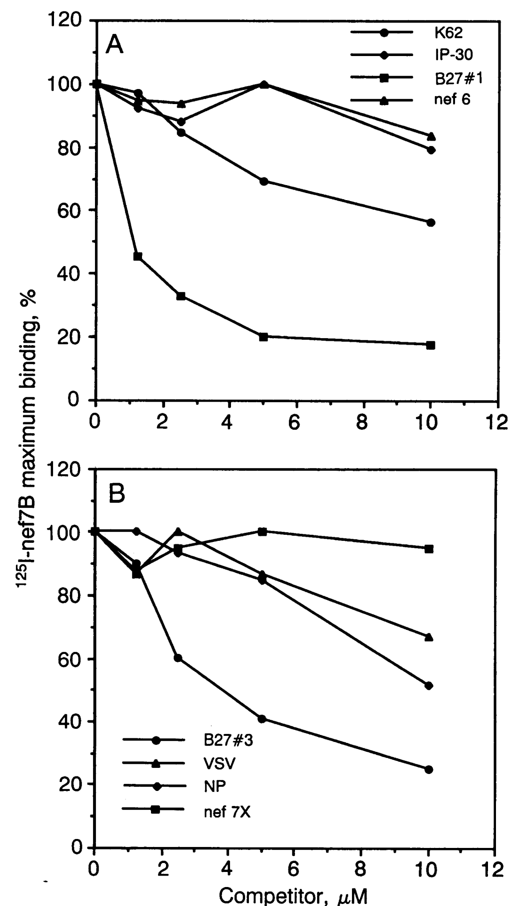

FIG. 5. Competition analysis of ^{125}I-labeled nef7B translocation with unlabeled peptides. ^{125}I-labeled nef7B translocation and binding to HLA-A3 in permeabilized C1R.A3 was done as described; however, various amounts of unlabeled peptides were added to the reaction mixture before the addition of labeled peptide and incubation at 37°C. The concentration of ^{125}I-labeled nef7B was constant at 150 nM. The percentage of maximum binding (*y* axis) indicates the amount of labeled peptide bound to HLA-A3; the level of peptide bound without competing peptides was normalized to 100%. VSV, vesicular stomatitis virus; NP, nucleoprotein.

at the lowest unlabeled peptide concentration, the binding of ^{125}I-labeled nef7B is completely abolished due to competition at both the level of translocation and the level of post-translocation binding to HLA-A3. B27#1 and B27#3 are defined peptides found associated with the HLA-B27 molecule (17) and therefore are naturally generated and translocated in human cells. K62 is a soluble variant of a suboptimal 11-residue HLA-A2 epitope from influenza matrix protein in which Phe-62 has been altered to lysine (15). Thus, K62 is not a natural epitope, which may be the reason it does not compete more effectively. The natural influenza matrix epitope is highly insoluble and could not be used in the assay. The vesicular stomatitis virus and nucleoprotein peptides are naturally processed epitopes in the mouse (18, 19), but they do not compete as well as their human counterparts for TAP transport. Possibly this reflects the fact that peptides associated with human class I molecules are drawn from a pool naturally selected for efficient translocation by human TAP. Even though the results in Fig. 4 show that mouse TAP can translocate nef7B, we do not know how this level of transport would compare with that of natural mouse class I-restricted peptides in RMA cells. Therefore, this does not rule out the possibility that TAP proteins from different species may preferentially transport different peptides. Such variation is clearly seen within a species; for example, rat TAP-2 polymorphism results in the isolation of distinct sets of peptides from rat class I molecules (32). However, RMA-S cells reconstituted with human TAP-2 show no differences in antigen processing, as measured by susceptibility to virus-specific, HLA-class I-restricted cytotoxic T lymphocytes (33). Furthermore, another group has shown a lack of species specificity in peptide loading using cells infected with vaccinia virus recombinants encoding influenza nucleoprotein or hemagglutinin antigens (34). However, it is conceivable that in this study a TAP-independent mechanism of HLA-class I-restricted epitope generation may be involved, as has been demonstrated in other systems (12, 28, 29).

The longer peptides (nef7X and nef6) appear to be poor substrates for TAP. This result suggests that the major proteolytic processing events occur in the cytosol and that transport of longer peptides via TAP into the lumen of the ER and subsequent trimming may not occur or is of limited importance. Furthermore, the finding that the HLA-A2-binding peptide derived from the IP-30 signal sequence does not compete well in this system argues that not all MHC class I-binding peptides are good substrates for TAP-mediated peptide transport and is consistent with the idea that signal-sequence-derived peptides do not require TAP for their ER localization.

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