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Proteasome subunits encoded by the major histocompatibility complex are not essential for antigen presentation

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MAJOR histocompatibility complex (MHC) class I molecules bind and deliver peptides derived from endogenously synthesized proteins to the cell surface for survey by cytotoxic T lymphocytes. It is believed that endogenous antigens are generally degraded in the cytosol, the resulting peptides being translocated into the endoplasmic reticulum where they bind to MHC class I molecules. Transporters containing an ATP-binding cassette encoded by the MHC class II region seem to be responsible for this transport[1–8]. Genes coding for two subunits of the '20S' proteasome (a multicatalytic proteinase) have been found in the vicinity of the two transporter genes in the MHC class II region, indicating that the proteasome could be the unknown proteolytic entity in the cytosol involved in the generation of MHC class I-binding peptides[9–13]. By introducing rat genes encoding the MHC-linked transporters into a human cell line lacking both transporter and proteasome subunit genes, we show here that the MHC-encoded proteasome subunits are not essential for stable MHC class I surface expression, or for processing and presentation of antigenic peptides from influenza virus and an intracellular protein.

TABLE 1 Presentation of the minor histocompatibility antigen HA-2 by T2 cells transfected with transporter genes

	Effector cells					
	Anti-HLA-A2.1 allo		Anti-HA-2		Q66.9	
Target cells	10:1	1:1	10:1	1:1	10:1	1:1
X	71	57	68	43	1	1
Y	72	60	55	26	0	0
T1	96	82	71	64	10	5
T2	100	87	0	1	2	1
T2/TAP1	100	83	0	3	0	1
T2/TAP2	81	61	5	5	3	1
T2/TAP1+2	82	75	57	26	0	0

T2 cells transfected with rat *TAP1*[a] and *TAP2*[a] cDNAs were tested for their capacity to present the endogenously synthesized minor histocompatibility antigen HA-2 to an HLA-A2.1-restricted minor histocompatibility antigen HA-2-specific CTL clone. T2 cells transfected with both transporter genes but not the untransfected T2 could present the HA-2 antigen. An alloreactive CTL clone specific for HLA-A2.1 and the influenza matrix-specific CTL clone Q66.9 (see Table 2*a*) were included as controls. The HA-2-specific CTL clone, designated HA-2 (ref. 25), and the HLA A2.1 alloreactive CTL clone[26] were mixed with chromium-labelled targets at effector-to-target ratios of 10:1 and 1:1, and specific lysis was measured in a 4-h chromium release assay; values represent per cent specific lysis. X and Y are lymphoblastoid cell lines transformed by Epstein–Barr virus and obtained from HLA-A2.1-positive healthy individuals.

TABLE 2 Presentation of influenza matrix protein M1 by T2 cells transfected with transporter genes

(*a*)

	Q66.9 Effector cells							
	Influenza virus infected				M1 peptide 58–66 added			
	(expt 1)		(expt 2)		0		1 μg ml^{-1}	
Target cells	5:1	0.5:1	5:1	0.5:1	5:1	0.5:1	5:1	0.5:1
X	28	14	nt	nt	3	0	23	14
Y	33	15	nt	nt	0	0	30	12
JY	nt	nt	31	7	3	0	23	14
T1	50	18	62	17	0	0	68	19
T2	7	4	8	3	7	3	71	21
T2/TAP1	8	3	12	5	6	4	62	31
T2/TAP2	4	1	5	2	6	0	70	25
T2/TAP1+2	26	7	33	11	0	0	73	29

(*b*)

	4-30 Effector cells					
	M1-vac infected		M1 peptide 58–66 added			
	(expt 1)		0		2.5 μg ml^{-1}	
Target cells	10:1	0.6:1	10:1	0.6:1	10:1	0.6:1
JY	72	32	8	1	80	24
T1	78	22	11	3	68	24
T2	4	1	8	0	80	21
T2/TAP1	14	6	2	2	67	14
T2/TAP2	7	2	1	0	77	21
T2/TAP1+2	49	16	7	2	75	17

Control cells and T2 cells transfected with rat *TAP1*[a] and *TAP2*[a] were infected overnight with influenza virus Hongkong 68 and then tested for HLA-A2.1-mediated presentation of the influenza matrix antigen using the CTL clone Q66.9. The results from two representative experiments are shown (*a*). In additional experiments using recombinant vaccinia virus producing matrix protein (M1-vac) and the M1-specific CTL line 4-30, similar results were obtained (*b*). Table 2 also shows that all cell lines function as targets after preincubation with exogenously added influenza matrix peptide M58-66 (sequence ILGFVFTLTV) (*a*). Where indicated, target cells were infected overnight with influenza virus Hongkong 68. Q66.9 is a CTL clone recognizing the influenza matrix-derived peptide M58–66 restricted by HLA-A2.1. This clone was raised against the synthetic peptide M58–66 (H.S., unpublished). Lysis was determined in a 4-h chromium-release assay at effector-to-target ratios of 5:1 and 0.5:1. For presentation of exogenously added peptide, target cells were preincubated with M58–66 peptide at 1 μg ml^{-1} for 2 h before addition of the CTL clone Q66.9 (*b*). Target cells were infected with M1-vac[27] (20 PFU per cell) for 3 h. 4-30 is a fresh CTL line from an HLA-A2.1-positive donor raised against the synthetic peptide M55–73 (P.W., unpublished). Lysis was determined in a 5-h chromium-release assay at effector-to-target ratios ranging from 10:1 to 0.3:1. M58–66 peptide was added as a control 15 min before addition of CTL at 2.5 μg ml^{-1}.

The 20S proteasome consists of about 20–30 subunits with M_rs between 15,000 and 30,000 that are encoded by distinct sets of genes[14]. We used the human lymphoblastoid B cell-derived mutant T2 line to investigate whether or not the MHC-linked proteasome subunits are essential for MHC class I expression and antigen presentation. T2 has a large homozygous deletion of the MHC class II region[15], which encompasses the genes for the two ATP-binding cassette (ABC) transporter polypeptides, TAP1 and TAP2, and the two genes for the MHC-encoded proteasome subunits, Lmp2 and Lmp7. In the absence of transporters, most HLA class I molecules are devoid of the peptides

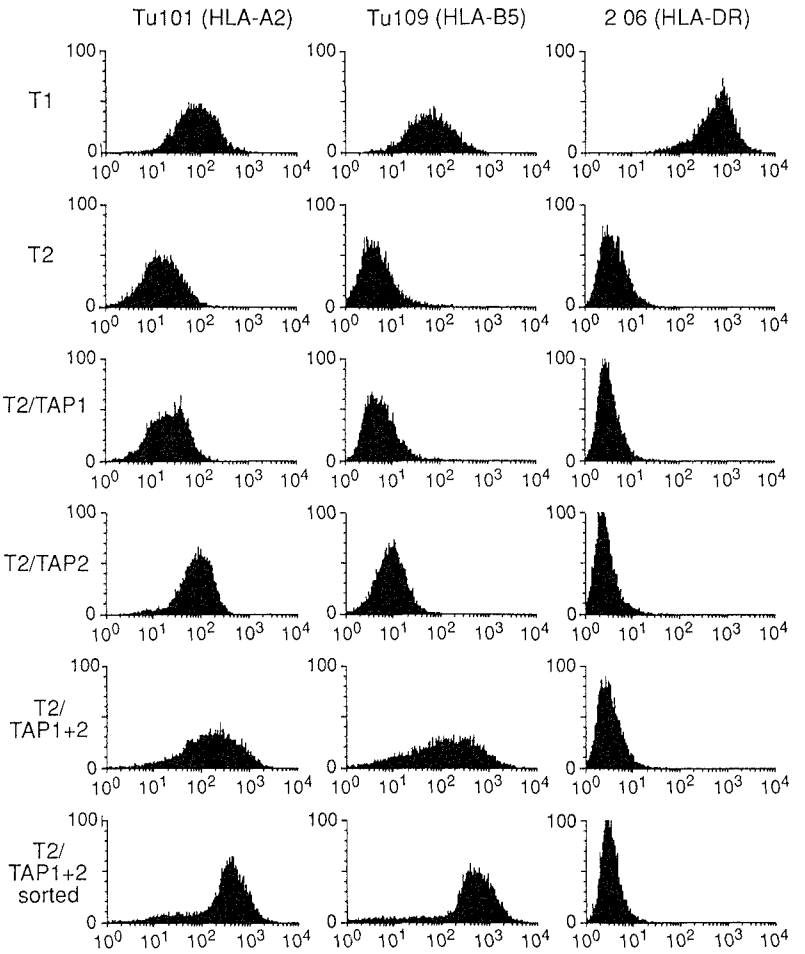


FIG 1 Restoration of HLA class I expression on T2 cells transfected with transporter genes The cDNAs for the two rat transporter chains *TAP1a* and *TAP2a* were used individually or as a mixture to transfect T2 cells HLA cell-surface expression was measured by cytofluorometry In five independent cotransfections with *TAP1* and *TAP2* we always obtained a substantial fraction of cells expressing high levels of HLA class I, similar to the example shown here METHODS In each experiment, 10^7 T2 cells were transfected by electroporation with ~2 μg each of rat cDNAs *TAP1*^{a} (*mtp1*^{a}, clone 510-15) or *TAP2*^{a} (*mtp2*^{a}, clone 441-11) in the pHβAPr-1-neo expression vector[15,21] Both plasmids contain the β-actin promoter and the neomycin-resistance gene Selection was in 1 mg ml^{-1} G418 (Gibco) After 4 to 6 weeks, cytofluorometry was done with the bulk cultures using a FACScan (Becton and Dickinson) Antibodies used were Tu101 against HLA-A2 (ref 28), Tu109 against HLA-Bw4 (ref 29), and 2 06 against HLA-DR, -DP and -DQ (ref 30), detected by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma) Staining with antibodies Tu48 (HLA-Bw4, ref 31) and BB7 2 (HLA-A2; ref 32) yielded comparable results, except that BB7 2 stained HLA-A2 antigens on untransfected T2 cells more strongly than antibody Tu101 The unseparated bulk culture of T2 cotransfected with both transporters yielded a broad peak containing both negative and positive cells Bottom row shows staining profiles of T2/TAP1 + 2 enriched by cell sorting for high expression of HLA-B5 using antibody Tu109 and FACStar Plus cell sorter

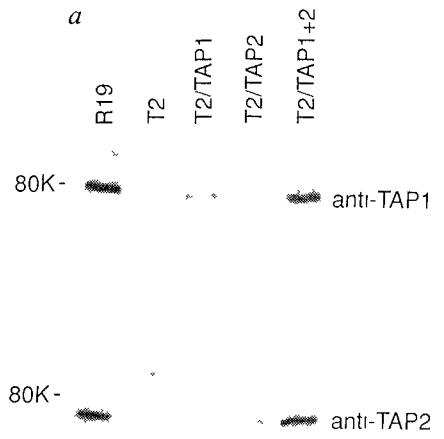
necessary for stable assembly of the class I heavy chain with β_2-microglobulin and for expression at the cell surface[16–18]. Therefore the T2 cell line shows strongly decreased expression of HLA-B5 and partially decreased expression of HLA-A2.1 molecules and is deficient in antigen presentation[19,20].

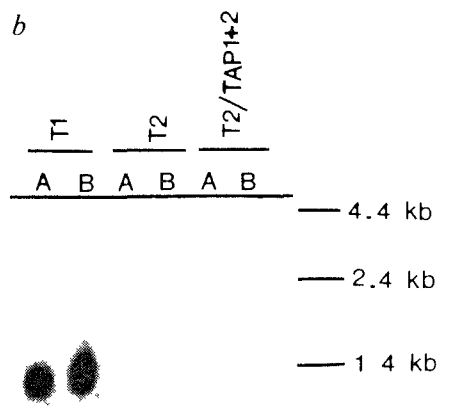
We transfected the T2 cells with the rat ABC transporter complementary DNAs rat *TAP1*^{a} and rat *TAP2*^{a}, previously named *mtp1*^{a} and *mtp2*^{a}, respectively[1,5,21]. The resulting transfectants were analysed for class I cell-surface expression. Parental T1 cells, from which T2 cells were derived, were positive for HLA-A2.1 and -B5 and also for HLA class II molecules, whereas T2 cells did not stain for HLA-B5 and only weakly for HLA-A2.1 (Fig. 1). Transfection with rat *TAP1*^{a} alone did not alter HLA expression. Transfection with *TAP2*^{a} resulted in a slight increase of HLA-A2 and HLA-B5 expression, which we are investigating at present. But when both *TAP1*^{a} and *TAP2*^{a} rat transporter genes were transfected into T2 cells, HLA-A2.1 and HLA-B5 expression was restored to levels two to three times higher than on T1 cells. Expression of class I in this bulk culture was improved by sorting for cells expressing high levels of HLA-B5 (bottom panel).

Western blots using sera raised against rat TAP1 and TAP2 showed that the T2/TAP1 + 2 transfectant expressed the TAP polypeptides in amounts comparable to rat strain PVG.R19 lymphoblasts stimulated by concanavalin A (Fig. 2*a*). The absence of the MHC-encoded proteasome subunits in the T2 transfectants was confirmed by immunoprecipitation (not shown) and messenger RNA analysis (Fig. 2*b*). It is possible that in the absence of Lmp2 and Lmp7, peptides of inappropriate length are generated, which would result in a decreased stability of the assembled class I molecules. Lysates of biosynthetically labelled cells were incubated at 37 °C for different times. Class I molecules were then immunoprecipitated with monoclonal antibody W6/32, which only recognizes class I molecules associated with β_2-microglobulin. Class I molecules devoid of peptides are unstable under these conditions and lose the epitope recognized by W6/32 (ref. 22). HLA-A2 and B5 were separated by one-dimensional isoelectric focusing (Fig. 2*c*). Most HLA-A2 molecules expressed in T2 cells are unstable to exposure at 37 °C, whereas most HLA-A2 molecules in T1 cells and T2 cells reconstituted with TAP1 and TAP2 are stable over a 4-h exposure to 37 °C. A more drastic effect of temperature on the stability of HLA-B5 is evident. HLA-B5 in T2 cells is unstable at 37 °C, whereas it is stable both in T1 cells and in T2/TAP1 + 2 cells. Thus, expression of TAP1 and TAP2 in T2 cells results in proper stabilization of both HLA-A2 and -B5 molecules. These results demonstrate that expression of transporter polypeptides alone, in the absence of the MHC-encoded proteasome subunits, is sufficient for apparently normal and stable class I expression.

Next, T2 cells transfected with rat *TAP1*^{a} and rat *TAP2*^{a} were investigated for their ability to process and present endogenous proteins. For this purpose T2 transfectants were tested with an HLA-A2.1-restricted cytotoxic T lymphocyte (CTL) clone recognizing a minor histocompatibility antigen (HA-2). Control cells, including T1, were lysed, but not T2 cells or T2 transfected with only one transporter gene (Table 1). In contrast, T2 cells transfected with both transporter genes were lysed efficiently. An HLA-A2.1 alloreactive CTL clone included as a positive control strongly lysed all transfectants, including the parental T2 cells. As the HLA-A2.1 molecules on T2 cells appear to carry only a limited variety of peptides derived from signal sequences[23,24], our data suggest that the anti-HLA-A2.1 CTL clone recognizes such a signal sequence-derived peptide.

Because the nature of the HA-2 antigen is not yet known, we





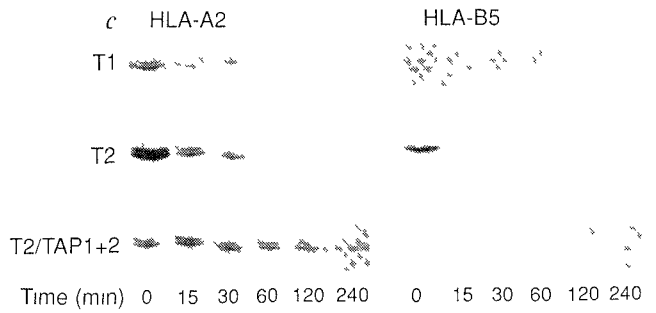


FIG 2 Expression of TAP1 and TAP2 in T2 cells results in stabilization of HLA-A2 and HLA-B5 molecules *a*, Expression of ABC transporters in transfectant cells Rat PVG R19 lymphoblasts stimulated with concanavalin A and lysed in NP40 detergent, and T2 and the single and double transfectant cells were probed with rabbit antisera raised against synthetic peptides of the carboxy termini of rat TAP1[a] and TAP2[a] The antisera fail to recognize material in T2 cells but detect rat TAP products in the transfectants Cell lysis and western blotting were done as described[5], each track contains lysate equivalent to 20,000 cells Antisera recognizing rat TAP1 (ref 5) and a new, previously undescribed antiserum raised against the rat TAP2 C-terminal sequence EQDVYAHLVQQRLEA were used at a 1/1,000 dilution *b*, Northern blot analysis was used to verify that the T2 cells expressing rat TAP1[a] and TAP2[a] transporters were indeed negative for expression of the MHC encoded subunit Lmp7 Lanes show 20 μg (lanes A) or 40 μg (lanes B) total RNA extracted from each cell line and probed with a cDNA specific for Lmp7 (isolated by Uwe Graf and V O N, unpublished) The absence of the MHC-encoded proteasome subunits was also verified by immunoprecipitation (not shown) using a rabbit serum against the 20S proteasome[11] *c*, Stability of MHC molecules was assayed by exposing cell lysates to 37 °C for different times Stable class I molecules were recovered with the monoclonal antibody W6/32 Both HLA-A2 and -B5 molecules are stabilized by expressing TAP1 and TAP2 in T2 cells and regain a similar stability as class I molecules in T1 cells We labelled 14×10^6 T1, T2 or T2/TAP1+2 cells for 15 min with 200 μCi ^{35}S-methionine and cysteine, respectively Cells were lysed in NP40 lysis mixture and their nuclei removed Lysates were precleared with normal rabbit serum and equal amounts incubated at 37 °C for the times indicated After preclearing again with normal rabbit serum, class I molecules were immunoprecipitated with monoclonal antibody W6/32 Immunoprecipitates were analysed by one-dimensional isoelectric focusing[22], only HLA-A2 and -B5 molecules are shown

analysed the processing of a well-defined antigen, influenza virus matrix protein. Cells were infected overnight with influenza virus and then used as targets for the CTL clone Q66.9, which recognizes the influenza matrix-derived peptide epitope M58-66 restricted by HLA-A2.1. Representative experiments in Table 2*a* show that infected T1 cells, the HLA-A2.1-positive cell line JY, and X and Y targets were killed, but not T2 cells or T2 transfected with the individual transporter genes. In contrast, the T2/TAP1+2 cells expressing both transporter polypeptides were lysed. This lysis (26 and 33% at a 5.1 effector to target ratio) was weaker than the lysis of T1 cells (50% and 62%, respectively) but with the other influenza virus-infected cell lines, JY, X and Y, only 28 to 33% lysis was again obtained. Results were comparable when target cells were infected with a recombinant vaccinia virus making the influenza M1 matrix protein (M1-vac) and the M1-specific CTL line 4-30 was used (Table 2*b*). Lysis of M1-vac-infected T2/TAP1+2 cells was again less than lysis of M1-vac-infected T1 cells. All transfectants were able to present the synthetic matrix peptide M58-66 when it was added exogenously. It is clear from these data that the capacity to form and present this influenza epitope has been returned to the T2/TAP1+2 transfectant, although it is possible that this capacity is suboptimal.

We conclude that the heterodimeric MHC-encoded transporter is alone sufficient for MHC class I-mediated antigen presentation, at least for the two antigens tested here. Thus, there does not seem to be an absolute requirement for the MHC-linked proteasome subunits Lmp2 and Lmp7. Our observations could mean that the presence of genes encoding two of the proteasomes in the MHC may be fortuitous and that the 20S proteasome does not play a role in antigen presentation. It is also possible however that the proteasome is only one of several proteolytic enzymes with the capacity to digest cytosolic antigen into class I-binding peptides. A proteasome lacking the MHC-encoded subunits may well have adequate proteolytic activity and could be the source of peptides assembled in our T2/TAP1+2 transfectant. The MHC-encoded proteasome subunits, which are inducible by interferon-γ, may nevertheless function to increase the overall proteolytic activity or the range of peptides generated by the proteasome from antigens in the context of an immune response, to a virus for example. Investigation of T2 cells restored with transporters and of the MHC-encoded proteasome subunits will help to clarify this issue. □

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