The MHC-encoded class I molecule, H-2K^k, demonstrates distinct requirements of assembly factors for cell surface expression: roles of TAP, Tapasin and β_2-microglobulin

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Abstract

Major histocompatibility complex encoded class I (MHC-I) molecules display peptides derived from endogenous proteins for perusal by CD8$^+$ T lymphocytes. H6, a mouse hepatoma cell line, expresses low levels of surface H-2D^d but not H-2K^k. Surface H-2D^d molecules are unstable and their levels, but not H-2K^k, are induced at 22 °C. Immunoprecipitation experiments revealed that H-2K^k, H-2D^d and β_2-microglobulin (β_2m) are expressed intracellularly; however no conformed MHC-I are present. Transcriptional profiling of factors required for MHC-I assembly demonstrated greatly reduced levels of the *Transporter associated with antigen processing (Tap)2* subunit. The role of key assembly molecules in the MHC-I pathway was investigated by ectopic expression studies. Overexpression of β_2m enhanced surface H-2D^d, but not H-2K^k, levels whereas overexpression of TAP2 rescued surface H-2K^k, but not H-2D^d, levels. Interestingly, Tapasin plays a dual role: first, in quality control by reducing the induced surface expression of TAP2-mediated H-2K^k and β_2m-mediated H-2D^d levels. Secondly, Tapasin overexpression increases *Tap2* transcripts and cooperates with TAPl or human β_2m to enhance surface H-2K^k expression; this synergy is TAP-dependent as demonstrated by infected cell protein 47 (ICP47) inhibition studies. Unlike the well studied H-2 MHC-I alleles, H-2K^b, H-2D^b, H-2K^d and H-2D^d, a functional TAP is "essential" for H-2K^k cell surface expression.

Keywords: H-2 alleles; MHC-I trafficking; Folding and stability; Assembly factors; Chaperones

1. Introduction

MHC class I molecules (MHC-I) are recognized by TCRs on CD8$^+$ T lymphocytes and by NK receptors on NK cells and play an important role during the cellular immune response. MHC-I consist of a trimer: heavy chain (HC), β_2m and peptide, typically consisting of ∼8–10 aa. Genes encoding HCs are present in the MHC locus, known as *H-2* in mouse and *HLA* in humans. Mouse MHC-I genes are polygenic, including H-2K, H-2D and H-2L, and they are also polymorphic. The assembly of MHC-I occurs in the endoplasmic reticulum (ER) and involves several factors (Paulsson and Wang, 2003; Yang, 2003). β_2m is required for the proper assembly and cell surface expression of most MHC-I (Bix et al.,1992; Machold et al., 1995). Proteasomes degrade cytosolic endogenous proteins into peptides that are translocated into the ER by TAP, which is a heterodimer of TAP1 and TAP2. In cells lacking a functional TAP, low levels of unstable MHC-I are expressed on the cell surface at 37 °C. However, incubation of cells at low temperature enhances transport, stability and surface expression of these "peptide-receptive" MHC-I (Ljunggren et al., 1990; Rock et al., 1991; Van Kaer et al., 1992; Day et al, 1995; Grandea et al., 2000). Cells lacking both β_2m and TAP display a severe reduction in MHC-I maturation and surface expression (Machold et al., 1995). In addition to these key assembly factors, Tapasin aids MHC-I assembly by several mechanisms (Grandea and Van Kaer, 2001; Momburg and Tan, 2002): as a linker molecule (Ortmann et al., 1997; Grandea et al., 1998; Tan et al., 2002) by binding to TAP via its C-terminal domain (Lehner et al., 1998) and MHC-I HC via its N-terminal region (Bangia et al., 1999), retaining class I

Abbreviations: β_2m, β_2-microglobulin; hβ_2m, human β_2-microglobulin; HC, heavy chain; HSV, herpes simplex virus; ICP47, infected cell protein 47; Lmp, low molecular mass polypeptide; mβ_2m, mouse β_2-microglobulin; MHC-I, major histocompatibility complex encoded class I molecule/s; PLC, peptide loading complex; TAP, transporter associated with antigen processing; Tapasin, TAP associated glycoprotein

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molecules in the ER (Grandea et al., 2000; Schoenhals et al., 1999; Paulsson et al., 2002; Pentcheva et al., 2002) to enhance the binding of high affinity peptides to MHC-I (Peh et al., 1998; Lauvau et al., 1999; Barnden et al., 2000; Garbi et al., 2000; Myers et al., 2000; Tan et al., 2002; Williams et al., 2002; Park et al., 2003), and increasing the steady state levels of TAP and activity (Lehner et al., 1998; Raghuraman et al., 2002; Garbi et al., 2003). Purification of steady state levels of TAP associated proteins has shown that each TAP heterodimer binds four molecules of Tapasin and four molecules of MHC-I HC (Ortmann et al., 1997). This peptide loading complex (PLC) consists of multiple proteins: HC, β_2m, TAP, Tapasin, calreticulin and Erp57. The general chaperones that are involved in proper folding of MHC-I include Erp57, a thiol-dependant oxidoreductase important in isomerisation of disulphide bonds (Harris et al., 2001; Dick et al., 2002), and the mono-glucosylated N-glycoprotein binding ER chaperones, calnexin and calreticulin. However, deficiency in calnexin does not affect MHC-I expression (Scott and Dawson, 1995) whereas MHC-I expression was reduced by ~25–30% in a calreticulin deficient cell line (Gao et al., 2002).

Despite the general model of MHC-I assembly pathway, evidences in the literature suggest that various MHC-I differ in their requirements for proper assembly and/or cell surface expression. Perhaps, the most striking difference in trafficking requirements of MHC-I has been shown for human and mouse MHC-I. K^b, D^p, D^d, D^b and L^d molecules, but not most HLA molecules studied, are expressed on the cell surface of the MHC-I antigen processing mutant human cell line, T2 (Alexander et al., 1989; Anderson et al., 1993). Also, both K^b and D^b are expressed at low levels on RMA-S, the well studied mouse *Tap2* mutant cell line (Ljunggren et al., 1990; Rock et al., 1991). These observations have led to the general impression that mouse MHC-I, but not most human MHC-I are able to reach the cell surface and are expressed at low levels in the absence of a functional TAP. In addition, there are differences between binding of MHC-I HC with mouse β_2m (mβ_2m) or human β_2m (hβ_2m). Both mβ_2m and hβ_2m bind HC with the same affinity, but mβ_2m has a ~3-fold higher dissociation constant than β_2m (Pendersen et al., 1995; Shields et al., 1999). In fact, almost 30–80% of mouse MHC-I are not associated with β_2m on the cell surface whereas ~95% of human MHC-I are β_2m-associated (Edidin et al., 1997). The role of assembly factors in surface expression of different HLA alleles is well studied. In the tapasin deficient cell line 721.220, the levels of MHC-I expression are variable and allele dependent. Cell surface expression of HLA-A2, A3 and B7 are not affected whereas HLA-A1 and B8 are severely reduced in 721.220 (Greenwood et al., 1994). Polymorphic residues in HLA alleles that are important in binding to the PLC complex have been identified. HLA-B35 (Neisig et al., 1996) and -B15 (Turnquist et al., 2000) subtypes with Tyr-116 bind strongly with TAP and possess less surface expression compared to those with Ser-116. On the other hand, a single amino acid change in HLA-B4402 (Asp-116) results in a change from a Tapasin-dependent to a Tapasin-independent allele, HLA-B4405 (Tyr-116); consequently, HLA-B4402 binds to optimal peptides (Williams et al., 2002). HLA alleles containing an acidic residue in position 114 are Tapasin dependent whereas replacement with histidine makes them Tapasin independent (Park et al., 2003). It should be noted, however, that binding to the PLC complex is not essential for optimal cell surface expression and stability by some MHC-I, e.g. HLA-B2705 (Peh et al., 1998) and RT1.A^U (Knittler et al., 1998).

Differences in maturation rates of mouse MHC-I are known: K^k traffics to the cell surface in 1 h, whereas D^k reaches the surface in ~4–5 h (Williams et al., 1985) and D^b matures slower than K^b (Machold et al., 1995). The reasons for these are not known and low cell surface expression of conformed K^b and D^b are detected on cells lacking *Tap1*; however K^b surface levels are more reduced, compared to D^b, in cells lacking $\beta_2 m$ (Bix and Raulet, 1992; Machold et al., 1995), suggesting that K^b is more dependent on β_2m, compared to D^b, for surface expression. Improper folding and low expression of both K^b and D^b is also observed in mice lacking *Tapasin* (Grandea et al., 2000; Garbi et al., 2000) and K^d, K^b and L^d are expressed on the surface in the absence of Tapasin in a peptide receptive state (Myers et al., 2000). Most studies are on the K^b, D^b, K^d, D^d, and L^d alleles and studies on mice mutants lacking different assembly factors are performed primarily on the C57BL6 strain (Bix and Raulet, 1992, Van Kaer et al., 1992; Machold et al., 1995, Garbi et al., 2000; Grandea et al., 2000; Garbi et al., 2003) or with RMA-S (Ljunggren et al., 1990; Rock et al., 1991; Day et al., 1995; Powis et al., 1991; Attaya et al., 1992). The requirement of assembly factors for other mouse MHC-I alleles is not well studied. We studied the cell surface expression of MHC-I in the hepatoma cell line, H6, in which IFN-γ induces greatly the expression of proteasomal subunits, low molecular mass polypeptide (LMP)2, LMP7 and LMP10 (Nandi et al., 1996). We investigated the expression of MHC-I in H6 and found that K^k and D^d were present intracellularly but only D^d, but not K^k, was expressed on the cell surface. This observation prompted us to study, in detail, assembly factors required for optimal cell surface expression for K^k and D^d. We demonstrate that cell surface expression of K^k is strictly dependent on a functional TAP, unlike other mouse MHC-I studied. The implications of the differential requirements of assembly factors for optimal cell surface expression of MHC-I alleles with respect to the immune response are discussed.

2. Experimental procedures

2.1. Cell culture

H6 cells (hepatoma, *H-2^a)* were cultured in RPMI 1640 medium containing 25 mM HEPES (Sigma, St. Louis, MO),

5% heat-inactivated FCS (HyClone, South Logan, UT), 5 μM β-mercaptoethanol (Sigma), l00 μg/ml penicillin, 250 μg/ml streptomycin, 50 μg/ml gentamycin (HiMedia Labs, Mumbai, India) and 2 mM glutamine (Life Technologies, Gaithersburg, MD) at 37 °C in the presence of 5% CO_2 in an incubator (Sanyo, UK). Cells were induced with 50 U/ml of mouse IFN-γ (Life Technologies) for different time points, as indicated.

2.2. Antibodies, peptide and β_2m

Culture supernatants from hybridomas secreting different mAbs were used to detect cell surface molecules: 11.4.1 (TIB 95; anti-$K^{k,p,q,r}$) (Oi et al., 1978), 34-2-12S (HB-87; anti-D^d, α3 domain) (Evans et al., 1982), S19.8 (anti-β_2m) (Tada et al., 1980) and J11D (anti-CD24) (Bruce et al., 1981); F23.1 (anti-Vβ8 TCR) (Staerz et al., 1985) was used as the isotype antibody control. A rabbit anti-H2, which recognizes only conformed MHC-I (Machold et al., 1995) (a gift from S. Nathenson), and rabbit anti-mβ_2m (a gift from C.-R. Wang) were used for immunoprecipitation studies. FITC-labeled goat anti-mouse or FITC-conjugated goat anti-rat secondary antibodies were obtained from Jackson Immunoresearch Laboratories, West Grove, PA. The D^d binding HTV-1 envelope protein gpl60 peptide (Suh et al., 1999), RGPGRAFVTI, was synthesized at XCyton, Bangalore, India and used for the peptide binding studies. Purified human β_2m was from Sigma.

2.3. Flow cytometric analysis

H6 cells were incubated at 37 °C or 22 °C in RPMI, in the presence or absence of FCS, for 6 h. After these treatments, ∼5 × 10^5 cells were centrifuged at ∼200 g and incubated with optimal amounts of primary mAbs for 30 min at 4 °C. Cells were then washed twice with HBSS buffer, in the absence of FCS (minus FCS treatment) or containing 0.5% FCS, and 0.01% sodium azide (FACS buffer). Cells were centrifuged at ∼200 g and the cell pellet was incubated with appropriate amounts of secondary antibodies for 30 min at 4 °C. Subsequently, cells were washed twice with FACS buffer and fixed with 0.5 ml of 1% paraformaldehyde at 4 °C. Analysis was performed on a Becton Dickinson FACScan™ flow cytometer and CellQuest (Becton Dickinson) software was used for acquisition. WinList (Verity, Topsham, ME) software was used to calculate MFl and percentage of cells staining positive with primary mAbs, after subtraction with the isotype control.

2.4. Metabolic labeling and immunoprecipitation studies

H6 cells (4 × 10^6), either untreated or induced with 50 U/ml IFN-γ for 48 h, were washed and resuspended in RPMI medium lacking methionine and cysteine (Sigma) for 30 min. Cells were labeled for 15 min in RPMI medium containing 250 μCi/ml EXPRE^{35}S-Protein labeling Mix (NEN Life Science Products, MA, USA) for 15 min at 37 °C. After extensive washing and centrifugation at ∼200 g, the cell pellets were resuspended in RPMI 5% FBS medium containing 2 mM methionine for 1 h at 37 °C. Cells were centrifuged at ∼200 g and the cell pellet was subjected to lysis in buffer containing 0.5% NP-40, 0.05 M Tris–Cl pH 8.0, 150 mM NaCl, l mM EDTA and 0.02% sodium azide for 30 min on ice. Lysates were centrifuged at 10,000 g and postnuclear supematants were precleared by incubating with ProteinG-Sepharose (Sigma) for 30 min. Equal cell equivalents of precleared lysates were immunoprecipitated with appropriate Abs bound to ProteinG-Sepharose for 20 min. Immunoprecipitates were washed twice by centrifugation at 5000 × *g* with buffer containing 0.5% NP-40, 0.1% SDS and 0.5% SDS and twice with buffer containing 0.5% NP-40 and 0.1% SDS. Pellets were resuspended, boiled in SDS sample buffer, and loaded onto a 12.5% SDS-PAGE gel. The immunoprecipitated complexes were visualized and quantified using phosphor image analysis, Image Gauge, version 3.0, FugiScience Labs, Japan.

2.5. Probes and expression constructs

The source constructs and probes used in the study were: *H-2k^b (EcoRI* digest from *H-2k^b pcDNAI)* and *H-2D^d (EcoRI* digest from *H-2D^{d}pBG36T)* (gifts from N. Shastri), *mβ$_2$m (HindIII/XbaI* excision product from *β$_2$m-pcR3)* (gift from R. Ehrlich), *Tap1 (BamHI* digest from *Tap1-pcDNAIneo), Tap2 (KpnI* excision product from *Tap2-pcDNA1neo), Tapasin (NotI/SalI* double digest from *Tapasin-pCMVsport)* (gifts from J. J. Monaco), *Gapdh (KpnI/HindIII* digest of *Gapdh/pUC19)* (gift from P. Kondaiah). The expression constructs used for transfections were *Tap1-pcDNA1neo, Tap2-pcDNA1neo* (gifts from J. J. Monaco), *hβ$_2$m-pcDNA3* and *ICP47-pcA4* (gifts from D. Johnson). *Tapasin* cDNA was excised from *Tapasin-pCMVsport2* (gift from J. J. Monaco) and subcloned in the *NotI/EcoRI* sites of *pcDNA3. mβ$_2$m* was excised from *β$_2$m-pcR3* (gift from R. Ehrlich) and subcloned in *BamHI/XhoI* sites of *pcDNA3.* Expression of *pcDNA3, pcDNA1neo* and *pcA4* is driven by the pCMV promoter.

2.6. Northern blot analysis

Total RNA was extracted by the acid guanidium isothiocynate–phenol–chloroform extraction procedure (Chomczynski and Sacchi, 1987). RNA (15 μg) from each condition was separated in 1% agarose gels containing 2.2 M formaldehyde and transferred to nylon membranes. cDNA probes were generated using restriction digests (see above), separated on 1% agarose gels, labeled using random primers and ^{32}P-ATP from NEN. RNA blots were hybridized with the labeled probes in Church buffer (1% BSA, l mM EDTA, 7% SDS and 0.5M $NaPO_4$, pH 7.5) at 58 °C in a hybridization oven (Amersham Pharmacia

Biotech, UK) for 16 h. The membrane was washed in low stringency wash buffer (2× SSC, 1% SDS) for three times at 55 °C for different time intervals. After each washing step, the counts on the membrane were monitored and, depending on the intensity of the background signal, a high stringency wash (0.2× SSC/0.1% SDS) was performed either once or twice for 20 min followed by phosphor image analysis.

2.7. Semi-quantitative RT-PCR

First strand cDNA was synthesized from total RNA (l μg) using 200 U of MMLV RT (Life Technologies) and oligo $dT_{(12–18)}$ primer (Life Technologies) in a total volume of 25 μl. cDNA (1 μl) was used as template for PCR amplification in buffer containing 0.4 μm of each primer, 500 μM of dNTP mix and 0.25 U of Taq polymerase (Bangalore Genei, India). The sequences of the primers used and the cycle conditions (Seliger et al., 2001) were as follows: *Tap1*, 5′gacaagagccgctgctatttgg3′ & 5′tgataagaagaaccgtccgaga3′; *Tap2*, 5′tatctagtcatacggagggtga3′ & 5′cctgggatacgaaaaggagacg3′; *Tapasin*, 5′gagcctgtcgtcatcaccat3′ & 5′agcaccttgaggagtccgag3′; *β_2m*, 5′atggctcgctcggtg3′; & 5′acatgtctcgatccca3′; & 5′gttggatacaggccagactttgttg3′ *Hprt* 5′tcggatatccggtcggatgggag3′. After PCR amplification (Eppendorf master cycler, Hamburg, Germany) for 25, 30, 35 or 40 cycles followed by a final extension at 72 °C for l0 min, 6 μl of each PCR product was loaded on a 1.25% agarose gels and stained using ethidium bromide. To calculate the relative abundance of transcripts in H6, untreated, induced with IFN-γ or transfectants, each band was quantified using Image Gauge software and a graph of intensity versus number of cycles was plotted to deduce slope values. After normalizing with *hprt*, the relative induction with IFN-γ or transfectants was compared with appropriate control cells, uninduced H6 or H6 transfected with vector alone.

2.8. Transfections

Plasmids were purified using a miniprep kit (Qiagen, Hilden, Germany). One microgram of DNA was transfected in H6 cells using Effectene (Qiagen). Transfected cells were selected in RPMI medium containing 5% FCS and 750 μg/ml Geneticin (Sigma) and allowed to grow to confluence in 500 μg/ml Geneticin containing medium for ∼10 days. Cell surface expression of K^k, D^d or β_2m was analyzed by FACS using appropriate Abs. Relative MFI for each cell surface molecule on the transfectants was calculated by normalizing with the vector alone transfected cells for each condition. Relative MFI is expressed as the MFI of the molecule on cells transfected with assembly molecule/s divided by the MFI for the same molecule on vector alone transfected cells. The relative MFI was expressed as mean ± S.E. on transfectants and was representative of two to eight independent transfections.

2.9. Sequence alignments and modeling of H-2k^k

Amino acid sequences of different mouse MHC-I were downloaded and multiple sequence alignment was done using ClustalW (http://www.ebi.ac.uk.edu). The K^k model was generated by SWISSMODEL (http://www.ExPASY.org) using the available known H-$2K^b$ crystal structures as template. The PDB identities of the structures used for modeling were lnanL.pdb, lkj3H.pdb, lnanH.pdb, lkj3I.pdb, lkj2I.pdb. The molecular graphics visualization tool RasMol 2.7 was used to visualize the model and mark the residues.

3. Results

3.1. H6 expresses low cell surface MHC-I which are induced by IFN-γ

Cell surface expression of MHC-I on H6 cells was studied using flow cytometry. As shown in Fig. 1A, H6 expressed low levels of cell surface D^d and β_2m. K^k was not detected using two mAbs: 11-4-1, which was used through out this study, and HB16, 16-1-11N (data not shown). However, on induction with 50 U/ml IFN-γ for 48 h (previously optimized), cell surface levels of K^k, D^d and β_2m were greatly induced. Across multiple experiments, K^k levels were increased by ∼70–100 fold whereas D^d levels were induced by ∼15–20 fold by IFN-γ (data not shown). CD24 was expressed at high levels on the surface of H6 cells and there was no enhancement with IFN-γ treatment. Thus, the IFN-γ-mediated induction was specific with respect to MHC-I.

3.2. Detection of K^k and D^d in H6 cells

Next, we wished to study whether K^k and D^d HCs were synthesized in H6 cells. Northern blot analysis was performed to study transcript levels of K^k and D^d, in the absence or presence of IFN-γ. As seen in (Fig. 1B) K^k and D^d transcripts were expressed in H6 and were induced on IFN-γ treatment. To study protein expression of K^k and D^d, pulse-chase experiments were performed (Fig. 1C). Low levels of K^k and D^d HCs were immunoprecipitated with respective antibodies in H6 and their levels were greatly induced in the presence of IFN-γ. Both mAbs to K^k and D^d immunoprecipitated more HC relative to β_2m in untreated H6 compared to IFN-γ-induced H6, based on quantitation of the bands. β_2m was present in H6 as shown by high levels of immunoprecipitated complexes. Most importantly, in H6 cells there was no reactivity with anti-H2, which recognizes only conformed MHC-I (Machold et al., 1995). However, the amount of conformed MHC-I was greatly increased in the presence of IFN-γ. Although K^k and D^d molecules were detected in H6, they were not properly assembled. Consistent with role of IFN-γ (Boehm et al., 1997), MHC-I increased at RNA, protein and assembly levels.

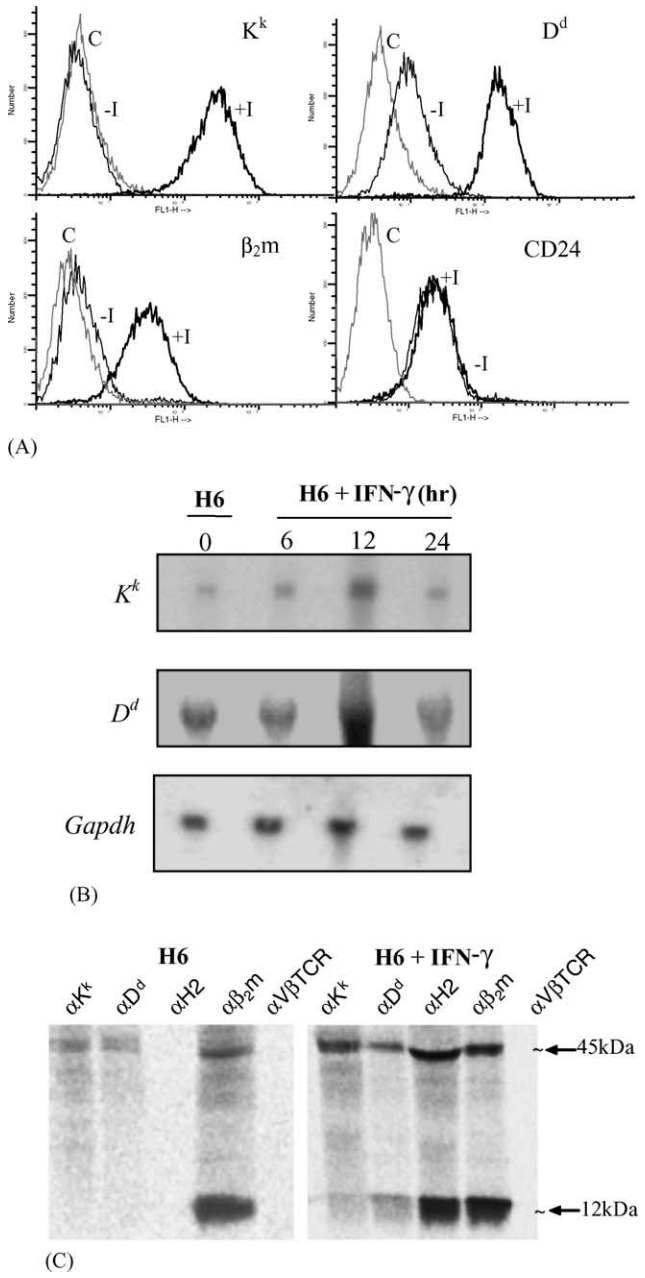

Fig. 1. Cell surface MHC-I levels and expression of heavy chains and β_2m in H6 cells. (A) Untreated H6 cells (denoted as −I) and H6 cells treated with 50 U/ml of IFN-γ for 48 h (denoted as +I) were studied for K^k, D^d, β_2m and CD24 expression, using appropriate Abs and flow cytometry. C represents staining with the isotype control. (B) H6 cells were harvested at different time intervals (0, 6, 12, 24 h) after induction with 50 U/ml of IFN-γ. RNA (15 μg) was loaded on formaldehyde-agarose gels and transcript levels were studied by Northern blot analysis using ^{32}P-labelled *H-2K* and *H-2D* cDNA probes. *Gapdh* expression levels were used as a loading control. (C) Untreated H6 and IFN-γ treated cells (50 U/ml for 48 h) were pulsed with ^{35}S-Protein labeling mix for 20 min. Cells were lysed in 0.5% NP-40 buffer and extracts were immunoprecipitated with appropriate Abs.

3.3. Role of low temperature and exogenous factors in surface MHC-I expression on H6

We investigated the expression of MHC-I on H6 at 22 °C (previously optimized) as the cell surface expression of unstable mouse MHC-I is increased on incubation of cells at low temperature (Ljunggren et al., 1990; Rock et al., 1991). In addition, we studied the expression of MHC-I in the presence and absence of FCS. Bovine β_2m present in FCS binds and stabilizes unstable MHC-I (Rock et al, 1991; Otten et al., 1992). In the absence of FCS, basal D^d surface expression was greatly reduced, suggesting a stabilizing role of bovine FCS in surface D^d expression (Fig. 2A). Both in the presence or absence of FCS, incubation of H6 cells at 22 °C increased the surface expression of D^d. However, K^k expression was not detected on the H6 cell surface at either temperature tested, in the presence or absence of FCS (Fig. 2A). We used this system to study the role of exogenous peptide and/or β_2-microglobulin in cell surface expression of D^d. Preincubation of H6 cells with a D^d-binding peptide, but not a D^b-binding peptide (data not shown), or human β_2m increased surface D^d expression at 37 °C (Fig. 2B). The combination of both exogenous D^d-binding peptide and human β_2m was additive and maximally increased surface D^d expression. Thus, D^d surface expression was increased by either peptide or β_2m but optimal expression required both.

The increased surface expression of unstable MHC-I at low temperature was used by us to study the relative stability of MHC-I molecules. As observed in Fig. 2C, there was increased expression of D^d at 22 °C compared to 37 °C both in the absence or presence of FCS. D^d expression on H6 cells induced with IFN-γ was greatly increased; however, there was no difference in D^d levels at 37 °C and 22 °C in H6 cells induced with IFN-γ, suggesting stable D^d. K^k was not detected on H6 cell surface but was greatly increased with IFN-γ induction. No difference in K^k expression was observed at 37 °C and 22 °C in H6 cells induced with IFN-γ, suggesting stable K^k expression. These experiments demonstrated that K^k and D^d were stably expressed at high levels in H6 cells induced with IFN-γ.

3.4. Expression profiling of assembly molecules involved in MHC-I pathway in H6 cells

We studied the expression of molecules known to be important in the MHC-I assembly pathway in H6 using semi-quantitative RT-PCR. As shown in Fig. 3A, *Tapl*, *Tapasin* and *β_2m* were detectable in H6 after 35 cycles of PCR amplification. However, *Tap2* expression was difficult to detect even after 40 cycles of PCR amplification. The expression of *Tap1*, *Tap2*, *Tapasin* and *β_2m* increased with IFN-γ-induction although *Hprt* levels were not modulated. The fold differences in H6 cells induced with IFN-γ compared to uninduced H6 cells were determined (slope values), by plotting the number of cycles versus intensity of the bands followed by normalizing with *Hprt* values. As shown

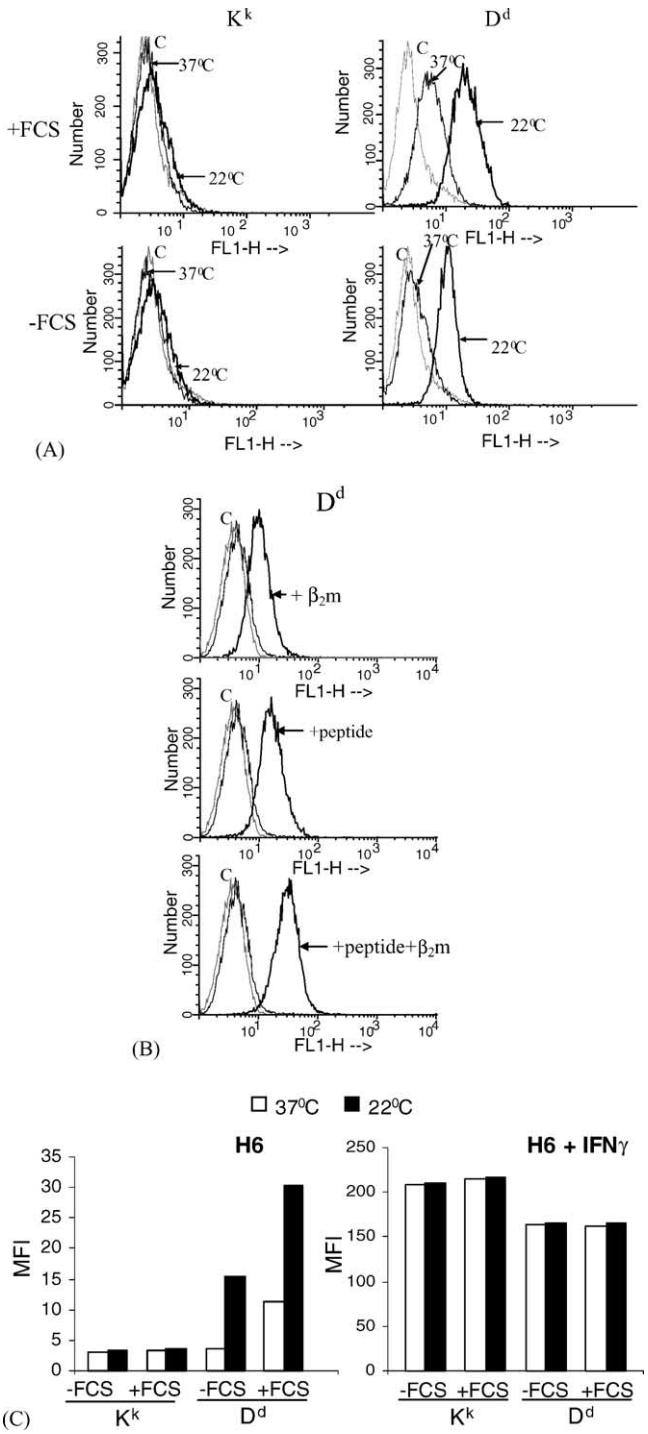

Fig. 2. Effect of low temperature and FCS on MHC-I surface levels. (A) H6 cells were cultured in RPMI, in the absence or presence of 5% FCS, and incubated at 22 °C or 37 °C for 6 h. Cells were subsequently stained with appropriate mAbs and surface MHC-I levels were analyzed by FACS. (B) H6 cells were incubated for 12 h at 37 °C in the presence or absence of a D^d binding peptide (100 μm) and/or human $\beta_2 m$ (2.5 μg/ml), either singly or together, in RPMI medium in the absence of FCS. Cells were subsequently stained with appropriate Abs and surface levels of D^d molecules were analyzed by FACS. (C) H6 cells, uninduced or induced with IFN-γ (50 U/ml for 48 h), were cultured in the absence or presence of FCS for 6 h at 37 °C or 22 °C followed by staining with appropriate Abs.

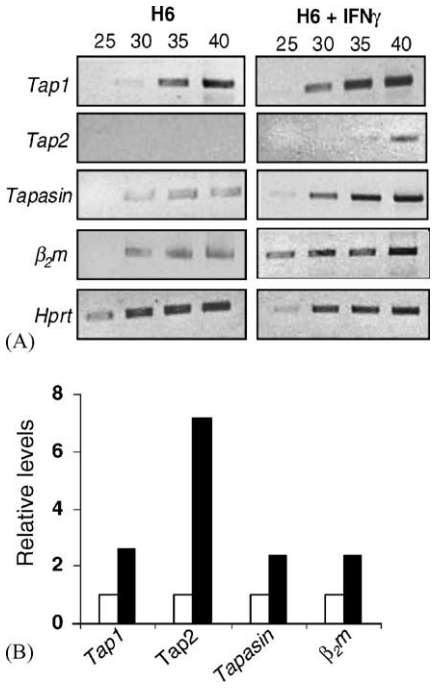

Fig. 3. Transcriptional profiling of assembly factors in H6. (A) Total RNA from H6 cells, untreated or treated with 50 U/ml of IFN-γ for 12 h, was reverse transcribed and the expression of *Tap1*, *Tap2*, *Tapasin* and $\beta_2 m$ transcripts was studied by PCR amplification for different cycles, using specific primers. *Hprt* expression was used as an internal control. (B) The relative fold increase in transcript levels of assembly factors in the presence of IFNγ was determined after normalizing with *hprt*.

in Fig. 3B, *Tap1*, *Tap2*, *Tapasin* and $\beta_2 m$ were induced by IFN-γ by 2.59, 7.19, 2.36 and 2.33 fold, respectively. Thus, H6 expressed low levels of *Tap2* and was maximally induced on IFN-γ treatment compared to *Tap1*, *Tapasin* and $\beta_2 m$.

3.5. Relative levels of assembly factors in H6 transfectants

We investigated the role of assembly factors, either singly or in combinations, by overexpressing these genes using mammalian expression vectors. To ensure that minor differences in expression of genes or selection of sub-populations of cells do not affect the overall trends, data shown is representative of several independent transfection experiments. In addition, we quantitated the expression of assembly factors in H6 cells transfected with single genes or combinations. As observed in Fig. 4, *Hprt* levels were relatively similar in different transfectants. As expected, cells transfected with *Tap1* demonstrated increased levels of *Tap1* mRNA but not other assembly factors. Similar results were observed with H6 cells transfected with *Tap2*, $m\beta_2 m$ and $h\beta_2 m$. Interestingly, overexpression of *Tapasin* increased the level of *Tapasin* transcripts but also increased the level of *Tap2* transcripts. The relative expression of assembly factors was reasonably similar in cells transfected with single genes or in combinations (Table 1).

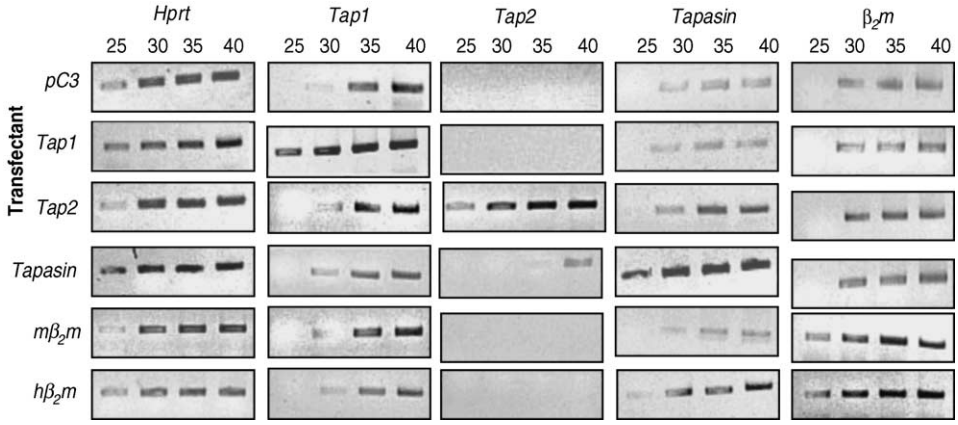

Fig. 4. Transcript levels of assembly factors in H6 transfectants. H6 cells were transfected with *pcDNA3* (v) or with *Tap1, Tap2, Tapasin, mβ2m, hβ2m, Tap1 + Tap2, Tap1 + Tapasin, Tap2 + Tapasin, mβ2m + Tapasin, hβ2m + Tapasin, mβ2m + Tap1, mβ2m + Tap2, hβ2m + Tap1 and hβ2m + Tap2* containing expression constructs. RNA was isolated from different transfectants and the expression levels of different assembly factors were determined using semi-quantitative RT-PCR.

3.6. Effect of over-expression of different assembly molecules on K^k cell surface expression

Among the single gene H6 transfectants, overexpression of TAP2 alone at 37 °C and in the presence of FCS (Fig. 5A) increased high levels of surface K^k expression. This effect was most pronounced when TAP1 and TAP2 were coexpressed, encoding both components of functional TAP. In addition, TAP2 synergized with hβ2m to induce higher levels of K^k surface expression. Interestingly, Tapasin synergized with hβ2m or TAP1 to induce surface K^k. These results were consistent both in the absence and presence of FCS (Fig. 5A and Table 2). However, overexpression of Tapasin together with TAP2 resulted in lowered surface expression of K^k compared to TAP2 alone. It is possible that Tapasin was retaining the surface expression of TAP2-induced K^k molecules. Interestingly, Tapasin overexpression alone induced low levels of surface K^k expression at 22 °C, but not at 37 °C, in an FCS dependent manner (Table 2). These results suggested that, under this condition, unstable K^k trafficked to the cell surface and was stabilized by binding bovine β2m. Under all conditions in which surface K^k levels increased, no further increase was observed at 22 °C, suggesting that surface K^k molecules were stable. Our results suggested that the translocation of peptides is limiting in H6 and overexpression of TAP2, together with existing TAP1, probably allows the formation of functional TAP, which results in translocation of K^k-binding peptides into the ER and high levels of surface K^k expression.

Table 1
Relative abundance of different assembly factor transcripts

Transfectant	Relative levels			
	Tap1	*Tap2*	*Tapasin*	*β2m*
pC3	1	1	1	1
Tap1	3.67	1.07	1.24	1.07
Tap2	1.07	33.22	1.02	0.84
Tapasin	1.07	4.45	4.48	1.29
mβ2m	1.21	0.95	0.9	3.21
hβ2m	1.17	1.27	1.02	2.91
Tap1 + Tap2	3.96	30.39	1.22	0.83
Tap2 + Tapasin	0.69	31.21	3.83	0.64
mβ2m + Tapasin	1.32	4.34	4.69	3.69
hβ2m + Tapasin	0.86	2.03	3.98	2.87
mβ2m + Tap1	3.73	0.78	1.33	3.61
hβ2m + Tap1	3.72	1.15	1.14	3.49
mβ2m + Tap2	0.89	28.51	1.11	3.63
β2m + Tap2	0.82	28.01	1.25	3.39

Data is representative of two independent transfections.

3.7. Effect of over-expression of different assembly molecules on D^d cell surface expression

The effect on D^d expression was also studied on the identical H6 transfectants described above. At 37 °C and in the presence of FCS, basal levels of D^d expression were observed. Maximal increase in D^d cell surface expression was observed on overexpression of mβ2m or hβ2m alone but not other assembly factors (Fig. 5B). However, overexpression of Tapasin together with mβ2m or hβ2m decreased D^d surface expression compared to β2m single transfectants (Fig. 5B). These effects were more pronounced in the absence of FCS (Table 2). Notably, low level of surface D^d expression, studied in the absence of FCS, was induced on overexpressing both TAP1 and Tapasin but not singly. As the basal level of D^d was high in the presence compared to the absence of FCS (Fig. 5B and Table 2), it is possible that this effect was masked in the presence of FCS. As shown before (Fig. 2), at 22 °C the expression of surface D^d molecules was increased in vector-transfected cells by 4.21 fold compared to 37 °C in the absence of FCS. While overexpression of both TAP1+TAP2 or Tapasin did not increase D^d levels, surface D^d levels were reduced to ∼2.9 from 4.2 fold at low temperature. This demonstrated a quality control role for TAP and/or Tapasin in retaining empty or low affinity peptide

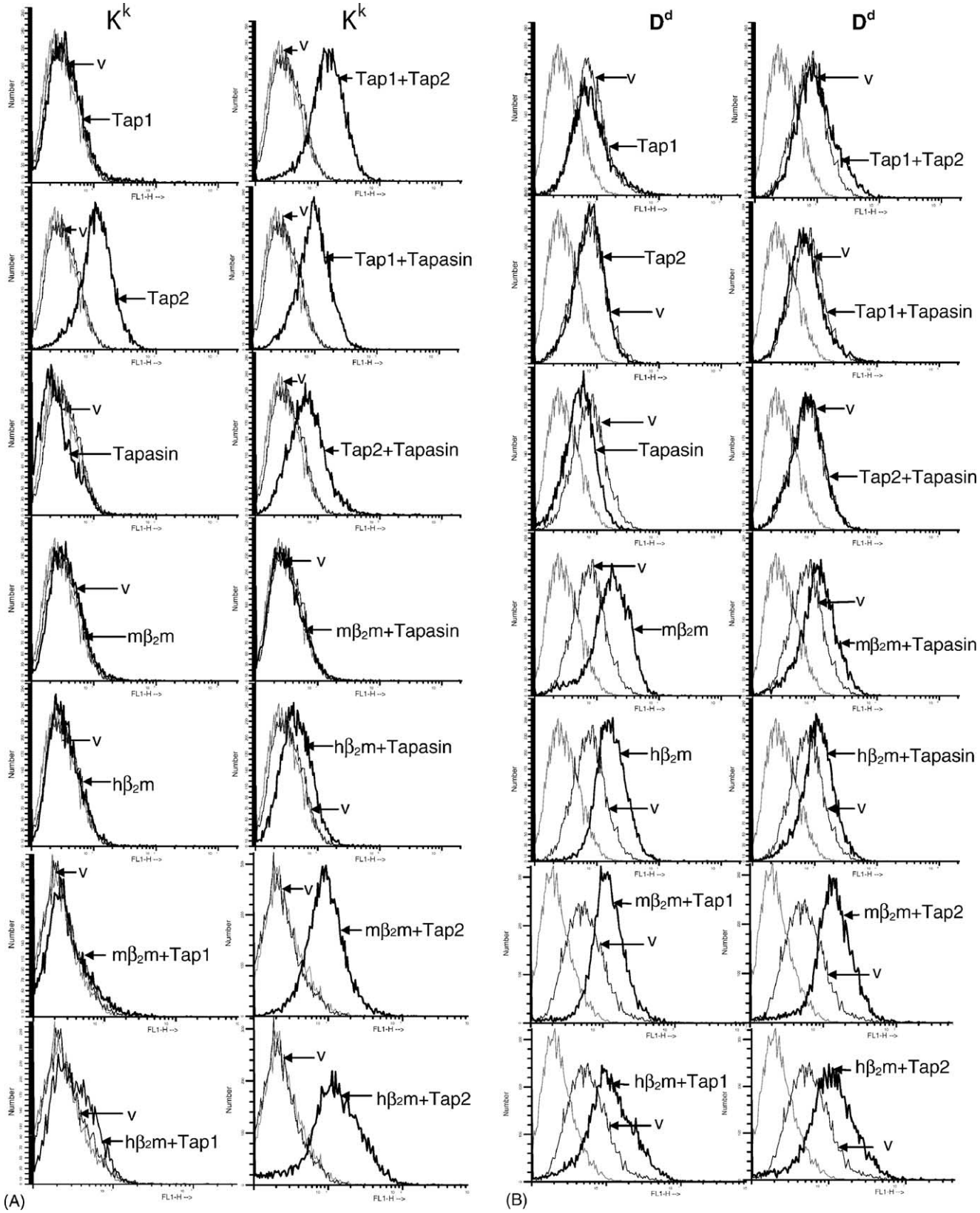

Fig. 5. Surface expression of MHC-I on different transfectants in the presence of FCS and at 37 °C. Surface expression of K^k (A) and D^d (B) molecules on H6 cells transfected with different assembly factors, either singly or in combinations, (indicated by arrows in each histogram) was analyzed by FACS after staining with appropriate Abs. Thin grey lines represent staining with F23.1, an isotype control Ab.

Table 2
Relative MFI of K^k and D^d under different conditions

Transfectant	K^k				D^d			
	+FCS		−FCS		+FCS		−FCS	
	37 °C	22 °C	37 °C	22 °C	37 °C	22 °C	37 °C	22 °C
pC3	1	1.02 ± 0.02	1	1.02 ± 0.02	1	2.87 ± 0.18	1	4.21 ± 0.01
Tap1	1.03 ± 0.05	1.05 ± 0.05	0.93 ± 0.06	1.06 ± 0.02	0.98 ± 0.05	2.64 ± 0.18	1.06 ± 0.02	4.52 ± 0.01
Tap2	2.79 ± 0.22	3.29 ± 0.39	3.09 ± 0.29	3.67 ± 0.33	1.17 ± 0.07	2.23 ± 0.22	1.12 ± 0.06	3.88 ± 0.03
mβ2m	0.96 ± 0.03	1.05 ± 0.06	1.11 ± 0.07	1.17 ± 0.05	1.99 ± 0.07	3.73 ± 0.01	2.68 ± 0.24	5.36 ± 0.12
hβ2m	1.05 ± 0.07	1.16 ± 0.09	1.14 ± 0.04	1.20 ± 0.08	2.40 ± 0.13	4.05 ± 0.14	3.31 ± 0.48	7.39 ± 0.24
Tapasin	1.11 ± 0.02	2.57 ± 0.17	1.02 ± 0.08	1.14 ± 0.05	0.96 ± 0.05	1.51 ± 0.07	0.91 ± 0.03	2.70 ± 0.02
Tap1 + Tap2	4.98 ± 0.48	6.86 ± 0.42	5.11 ± 0.59	6.04 ± 0.38	1.14 ± 0.08	1.90 ± 0.56	1.05 ± 0.04	2.96 ± 0.02
Tap1 + mβ2m	1.23 ± 0.05	1.40 ± 0.02	1.12 ± 0.05	1.31 ± 0.24	2.05 ± 0.06	2.84 ± 0.43	2.09 ± 0.50	4.28 ± 0.35
Tap1 + hβ2m	1.26 ± 0.05	1.47 ± 0.02	1.06 ± 0 08	1.38 ± 0.14	2.22 ± 0.12	3.55 ± 0.04	2.30 ± 0.24	3.96 ± 0.17
Tap2 + mβ2m	3.62 ± 0.12	3.63 ± 0.10	3.83 ± 0.10	3.65 ± 0.17	2.39 ± 0.03	3.24 ± 0.07	2.43 ± 0.02	3.65 ± 0.02
Tap2 + hβ2m	5.23 ± 0.08	5.48 ± 0.06	5.08 ± 0.47	4.46 ± 0.63	2.07 ± 0.05	2.92 ± 0.04	3.84 ± 0.11	5.05 ± 0.08
Tap1 + Tapasin	3.06 ± 0.03	3.93 ± 0.27	2.20 ± 0.35	2.12 ± 0.47	1.15 ± 0.08	1.62 ± 0.04	2.38 ± 0.10	3.56 ± 0.07
Tap2 + Tapasin	2.08± 0.10	2.62 ± 0.58	2.13 ± 0.25	1.93 ± 0.04	0.98 ± 0.01	1.37 ± 0.05	0.96 ± 0.18	2.60 ± 0.13
mβ2m + Tapasin	1.08 ± 0.52	0.94 ± 0.08	1.14 ± 0.03	1.11 ± 0.08	1.93 ± 0.04	2.57 ± 0.04	1.71 ± 0.21	2.36 ± 0.12
hβ2m + Tapasin	2.17 ± 0.15	2.41 ± 0.09	2.02 ± 0.09	2.08 ± 0.20	1.95 ± 0.26	2.68 ± 0.25	1.83 ± 0.20	2.45 ± 0.11

bound-D^d inside the cell. Overexpression of mβ2m or hβ2m increased surface D^d levels at 37 °C but 22 °C-mediated surface D^d levels were enhanced by ∼2 fold, suggesting that increased surface expression of D^d molecules did not confer sufficient stability. The difference between surface D^d levels at 37 °C and 22 °C was the least (less than 1.5 fold) for cells overexpressing TAP2 + mβ2m/hβ2m, TAPl+Tapasin and Tapasin + mβ2m/hβ2m (Table 2). Consistent with other studies (Otten et al., 1992; Anderson et al., 1993; Suh et al., 1996; Suh et al., 1999; Paquet and Williams, 2002), these results demonstrated that D^d required both peptide and β2m for "stable" surface expression.

3.8. Surface K^k is bound to endogenous β2m in H6 transfectants

Next, we wished to address the levels of endogenous β2m expressed on the cell surface on H6 transfectants expressing different assembly factors. As shown in Fig. 6, in the absence of FCS and at 37 °C, surface expression of K^k, D^d or β2m was not detected on H6 cells transfected with vector alone. Overexpression of mβ2m or hβ2m increased surface D^d and β2m expression, but not K^k. However, on overexpressing TAP2, either singly or in combination with TAP1, K^k surface expression was enhanced as was β2m. Therefore, it is most

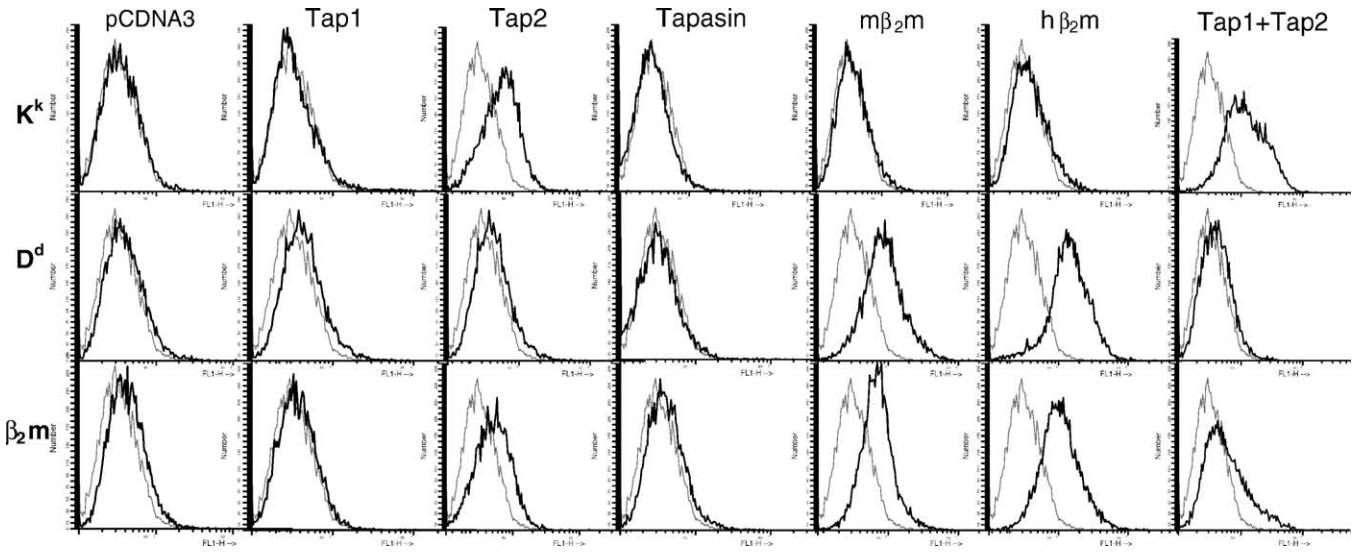

Fig. 6. Comparison of MHC-I and β2m surface levels on different transfectants in the absence of FCS. Cells transfected with different constructs, as indicated, were cultured in the absence of FCS for 6 h at 37 °C and surface levels of K^k, D^d and β2m were analyzed by FACS after staining with the appropriate Abs as indicated. Thick black lines represent surface expression on transfected cells. Thin grey lines represent staining with the isotype control Ab.

likely that K^k was bound to endogenous β_2m and the trimeric complex of K^k HC-peptide-β_2m was stably expressed on the cell surface.

3.9. Role of TAP in surface expression of K^k and D^d

We wished to address the role of binding of endogenous TAP-translocated peptides in surface expression of D^d and K^k in cells transfected with different assembly factors. Therefore, we transfected H6 cells with a mammalian expression vector containing ICP47, a herpes simplex virus (HSV) encoded TAP inhibitor (Ahn et al., 1996; Tomazin et al., 1996). Although HSV is a human pathogen, ICP47 is functional in mouse cells and HSV lacking ICP47 demonstrates less virulence in vivo in a mouse model of infection (Goldsmith et al., 1998). Increased surface expression of K^k was found on overexpression of TAP2, TAP1 + TAP2, TAPl + Tapasin and hβ_2m + Tapasin. Under all these conditions, both in the presence and absence of FCS, overexpression of ICP47 reduced K^k surface expression (Fig. 7A, and Table 3).

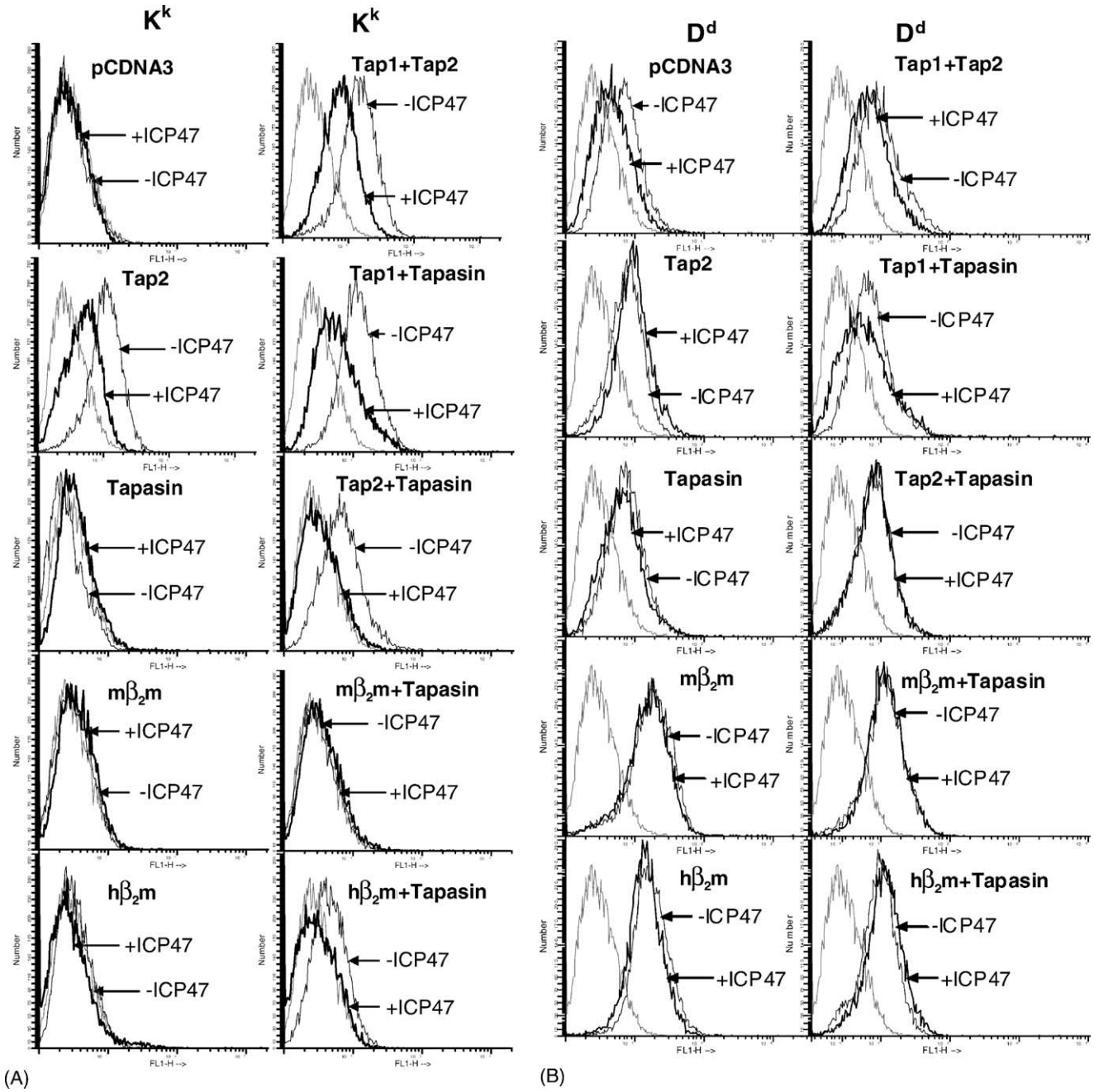

Fig. 7. Role of TAP on K^k and D^d cell surface expression in different transfectants. Cells were transfected with different expression constructs in the presence or absence of *ICP47* expression (as indicated on each histogram) and the cell surface levels of K^k (A) and D^d (B) in the presence of FCS were studied by FACS.

Table 3
Effect of ICP47 over expression on K^k and D^d surface levels in presence of FCS

Transfectant	K^k		D^d	
	−ICP47	+ICP47	−ICP47	+ICP47
pC3	1	1	1	0.76 ± 0.06
Tap2	3.25 ± 0.10	1.71 ± 0.18	1.07 ± 0.05	1.20 ± 0.21
Tapasin	0.96 ± 0.01	1.01 ± 0.06	0.88 ± 0.07	0.75 ± 0.10
mβ2m	1.13 ± 0.07	1.05 ± 0.01	2.14 ± 0.28	2.04 ± 0.17
hβ2m	1.09 ± 0.07	1.07 ± 0.03	2.24 ± 0.25	2.16 ± 0.06
Tap1 + Tap2	4.49 ± 0.24	2.54 ± 0.14	1.23 ± 0.13	1.01 ± 0.01
Tap1 + Tapasin	2.70 ± 0.20	1.62 ± 0.41	1.01 ± 0.02	0.94 ± 0.07
Tap2 + Tapasin	1.99 ± 0.09	1.06 ± 0.04	1.02 ± 0.02	1.02 ± 0.02
mβ2m + Tapasin	1.01 ± 0.09	1.12 ± 0.08	1.48 ± 0.08	1.48 ± 0.12
hβ2m + Tapasin	1.70 ± 0.13	1.06 ± 0.03	1.43 ± 0.03	1.42 ± 0.08

In additon, the data demonstrates that the Tapasin-mediated, together with TAP1 or hβ2m, increased K^k surface expression was TAP-dependent. Although mβ2m or hβ2m was responsible for the major increase in surface D^d expression, this was not reduced by ICP47 (Fig. 7B, and Table 3). These results further demonstrate that a functional TAP is essential for K^k surface expression but not for D^d.

4. Discussion

We studied the expression profile of molecules involved in MHC-I assembly and the requirements for cell surface expression of MHC-I, K^k and D^d, in the hepatoma cell line, H6. The specificity of the mAbs used is of key importance in interpreting the data. The mAb to K^k, 11-4-1, used in this study recognizes free K^k HC and conformed K^k molecules (Williams et al., 1985). Similarly, 34-2-12S recognizes free D^d HC and conformed D^d molecules, including the peptide-induced conformational epitope (Otten et al., 1992). Both mAbs immunoprecipitated free HC and HC-β2m complexes (Fig. 1C). Although K^k and D^d transcripts and proteins were detected (Fig. 1B and 1C), K^k was not expressed on the surface but unstable D^d molecules were detected (Fig. 2A). In addition, no conformed MHC-I (Fig. 1C) or β2m (Fig. 6), in the absence of FCS, was detected. Incubation of H6 cells at low temperature resulted in higher surface expression of D^d (Fig. 2A), probably due to facilitation of post-golgi transport, better folding of MHC-I, less endocytosis and less degradation (Ljunggren et al., 1990; Rock et al., 1991; Van Kaer et al., 1992, Machold et al., 1995). Thus, using multiple readouts, stably folded MHC-I were not present on the H6 cell surface and post-translational mechanisms played the major role in modulating surface MHC-I expression in H6.

The expression of assembly factors in H6 revealed an interesting phenotype: low levels of a functional TAP, which is mediated by lower *Tap2* relative to *Tap1* transcripts (Fig. 3). Despite an abundance of β2m which was readily detected at transcript and protein levels (Figs. 1C and 3A) there was no binding of β2m with K^k or D^d during assembly (Fig. 1C) and no endogenous β2m was detected on the cell surface in the absence of FCS (Fig. 6). Together these results demonstrate that H6 cells expressed β2m but not a functional TAP, which revealed the +β2m, -peptide phenotype (Fig. 8). Under this condition, K^k was present intracellularly whereas D^d HC egressed to the cell surface and bound to exogenous bovine β2m present in FCS, leading to low levels of surface expression. Consequently, no detectable D^d cell surface expression was found by incubating H6 in the absence of FCS (Fig. 2A); however, exogenous peptide and/or hβ2m increased cell surface expression of D^d in the absence of FCS (Fig. 2B), which is consistent with a previous study (Otten et al., 1992). In the presence of IFN-γ, all components of the MHC-I pathway, including HCs and key assembly factors (Figs. 2 and 4), were induced and binding of endogenous β2m with K^k and D^d was observed (Fig. 1C) in H6 cells. This resulted in high and stable surface levels of K^k, D^d and β2m in H6 (Fig. 1). In addition, K^k and D^d were expressed in a "stable" manner in H6 cells treated with IFN-γ, i.e. surface expression levels were FCS independent and not induced at 22 °C (Fig. 2C). The surface expression levels levels of D^d and K^k on IFN-γ-treated cells (Fig. 1) were much higher than those achieved in individual transfectants (Fig. 5). It should be noted that overexpression of assembly factor/s in-

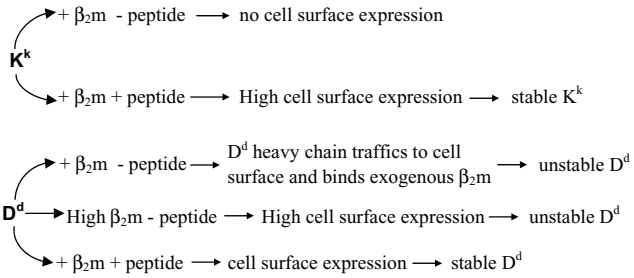

Fig. 8. A model depicting the requirements of assembly factors for stable cell surface expression of MHC-I, K^k and D^d. H6 expresses β2m but not TAP2, leading to the +β2m-peptide phenotype. Expression of TAP2, i.e. a functional TAP, leads to the stable cell surface expression of K^k-peptide-endogenous β2m trimeric complex. On the other hand, D^d egresses to the cell surface and binds exogenous bovine β2m present in FCS, in the absence or presence of TAP2 overexpression. Binding of both β2m and high affinity peptide is required for stable D^d surface expression.

duced surface expression of the "constitutive levels" of K^k and D^d HCs present in H6. In case of IFN-γ, transcription of K^k and D^d HCs were induced (Fig. 1B) as were other assembly factors (Fig. 3); consequently, K^k and D^d were maximally induced and expressed on the surface with IFN-γ (Boehm et al., 1997). The phenotype of H6 is distinct from RMA-S: Both K^b and D^b in RMA-S are expressed at low levels compared to RMA. Low levels of D^b surface expression is observed, in the absence or presence of FCS, which increases on peptide binding (Rock et al., 1991). It is possible that D^b in RMA-S is bound to endogenous β_2m and requires either exogenous peptide (Ljunggren et al., 1990; Rock et al., 1991) or functional TAP activity (Powis et al., 1991; Attaya et al., 1992) for higher surface expression.

To study the roles of important assembly molecules in surface expression of K^k and D^d, we transfected *Tap1*, *Tap2*, *Tapasin*, *mβ_2m* and *hβ_2m* containing expression constructs, either singly or in combinations, in H6. Importantly, we studied the expression of K^k and D^d on "identical" H6 transfectants, under different conditions. This system allowed us to dissect the distinct requirements of assembly factors for expression and stability of K^k and D^d molecules. Perhaps, the most stringent condition to study MHC-I cell surface expression is at 37 °C and in the absence of FCS: unstable MHC-I are rapidly endocytosed at 37 °C and only stable cell surface MHC-I will be expressed. In the absence of FCS, HC binding to endogenous β_2m will be required for high levels of cell surface expression (Ljunggren et al., 1990; Rock et al., 1991; Machold et al., 1995). The results of overexpression of assembly factors in H6 at 37 °C and in the absence of FCS is summarized in Table 4. Maximal levels of surface K^k required high expression of TAP2 or coexpression of TAP1 and TAP2 (Table 2). The absence of FCS did not diminish K^k expression (Table 2) as K^k was bound to endogenous β_2m (Fig. 6). Peptide binding to K^k HC probably induced the proper assembly of the trimeric complex of K^k HC bound to β_2m and peptide, which was "stably" expressed on the cell surface (Fig. 8). Consequently, overexpression of β_2m, in the absence of a functional TAP, did not result in K^k surface expression. Transfection of K^d and K^k, into BHK, a hamster kidney cell line, results in cell surface expression of only K^d but not K^k. It appears that rat TAP,

Table 4
Effect of over expression of assembly factors on K^k and D^d cell surface expression

Assembly factor/s	Possible mechanism/s involved	Surface expression at 37 °C, −FCS	
		K^k	D^d
Vector/TAP1	K^k present intracellularly due to lack of functional TAP. Unstable D^d egressed to the cell surface and required binding to bovine β_2m	−	−
TAP2	Compensates for low TAP2 level in H6, leading to a functional TAP, and cell surface expression of K^k HC bound to endogenous β_2m and peptide. Unstable D^d egressed to the cell surface and required binding to bovine β_2m	++	−
TAP1 + TAP2	High levels of TAP led to stable surface expression of K^kHC-endogenous β_2m-peptide. TAP retained empty D^d as induction at 22 °C was reduced	+++	−
Tapasin	Unstable K^k egressed to surface at 22 °C (TAP dependent) and required binding to bovine β_2m Tapasin retained empty D^d as induction at 22 °C was reduced	−	−
TAP1 + Tapasin	This combination increased TAP, leading to surface K^k bound to peptide and β_2m. Increased surface expression and stability of D^d at 37 °C, as induction at 22 °C was reduced.	+	++
TAP2 + Tapasin	Tapasin reduced TAP2-induced surface K^k levels probably allowing surface expression of only high affinity peptides bound to K^k HC-endogenous β_2m complexes. This combination retained empty D^d as induction at 22 °C was reduced.	+	−
mβ_2m/hβ_2m	K^k present intracellulary due to lack of a functional TAP. Increased intracellular levels of mβ_2m feed to surface expression of D^d-β_2m, which were unstable (induced at 22 °C) probably due to the lack of peptide.	−	++
TAP1 + mβ_2m/hβ_2m	K^k present intracellularly due to lack of a functional TAP. Increased intracellular levels of hβ_2m bound to D^d and these dimers were expressed on the surface; D^d were unstable (induced at 22 °C) probably due to the lack of peptide.	−	++
TAP2 + mβ_2m/hβ_2m	Functional TAP led to high surface K^k levels and hβ_2m was better compared to mβ_2m in synergizing with TAP2 to enhance surface K^k levels. Increased levels of D^d were observed due to β_2m expression. However, there was less 22 °C-mediated D^d expression probably due to D^d HC-β_2m-peptide complex; hβ_2m was better compared to mβ_2m in conferring stable D^d surface expression.	++	++
mβ_2m + Tapasin	There was no increase of K^k surface expression on overexpression of mβ_2m. Reduced mβ_2m-induced surface D^d levels. D^d stability was enhanced probably due to D^d HC -high affinity peptide-mβ_2m surface expression, as induction at 22 °C was reduced.	−	+
hβ_2m + Tapasin	This combination increased surface K^k levels and demonstrated that hβ_2m was functionally different from mβ_2m. This combination reduced hβ_2m-induced surface D^d levels and enhanced D^d stability probably due to D^d HC-high affinity peptide-hβ_2m surface expression, as induction at 22 °C was reduced.	+	+

but not hamster TAP, is able to rescue cell surface expression of K^k in BHK (Lobigs et al., 1995). Notably, K^k cell surface expression in BHK was studied using HB16 (Lobigs et al., 1995), which is different from the mAb used to detect K^k in this study. Together, these observations revealed that a functional TAP was essential for cell surface expression of K^k but not for most other H-2 alleles studied, K^b, D^b (Ljunggren et al., 1990; Rock et al., 1991; Powis et al., 1991; Attaya et al., 1992; Anderson et al., 1993; Machold et al., 1995; Day et al., 1995; Grandea et al., 2000), K^d (Anderson et al., 1993, Lobigs et al., 1995), D^d (Anderson et al., 1993) and D^p (Alexander et al., 1989).

Tapasin overexpression in H6 induced *Tap2* mRNA expression (Fig. 4). As *Tap2* levels are greatly reduced in H6, it is possible that the use of this sensitive system allowed for detection of this novel role of Tapasin. A previous study has shown that Tapasin overexpression increases cell surface HLA expression and *Lmp2* and *Lmp7* transcripts in the Hep G2 cell line (Matsui et al., 2001). Further studies are required to investigate the mechanisms by which Tapasin increases *Tap2* transcript levels. Tapasin expression is known to increase steady state levels of TAP (Lehner et al., 1998; Raghuraman et al., 2002; Garbi et al., 2003). Also, in Drosophila SC-2 cells, transfection with Tapasin increased amounts of TAP2 that coimmunoprecipitated with TAP1 (Schoenhals et al., 1999). Overexpression of Tapasin increased surface K^k at 22 °C, but not at 37 °C, in a FCS-dependant (Table 2) and ICP47-sensitive manner (data not shown), suggesting TAP-dependence. Tapasin overexpression increased TAP levels, probably by increasing TAP2, in H6 allowing for translocation of K^k binding peptides. However, K^k molecules were unstable following Tapasin overexpression alone and required binding to β_2m for stability (Table 2). For stable expression of endogenous K^k, higher levels of TAP2 were required compared to that induced by Tapasin overexpression (Table 1). However, Tapasin synergized with TAP1 or hβ_2m to increase stable surface K^k levels and this increase was TAP dependent as it was reduced by ICP47 (Fig. 7). The combination of Tapasin+TAPl led to expression of surface levels of both D^d and K^k. In this condition, surface K^k were induced via TAP (Fig. 7); however, the mechanisms by which stable surface D^d levels were increased are unclear. Tapasin overexpression also reduced surface levels of the TAP2-induced K^k, suggesting that Tapasin was retaining MHC-I to probably enhance the binding of high affinity K^k-binding peptides. This retaining role by Tapasin was most evident in case of D^d. Tapasin reduced the mβ_2m or hβ_2m-mediated increase in D^d cell surface expression at 37 °C (Table 2), suggesting a quality control role for Tapasin. Incubation of H6 cells transfected with vector alone at low temperature resulted in increased surface expression of D^d. However, overexpression of TAP1 & TAP2 or Tapasin reduced the 22 °C-mediated increase in D^d surface expression in the absence or presence of FCS (Table 2). Together these results demonstrated a role for TAP-Tapasin complex to reduce cell surface expression of peptide receptive D^d molecules. These studies are consistent with previous reports that have shown that the TAP-Tapasin complex is the main anchor retaining empty MHC-I and enhancing the binding of high affinity peptides to MHC-I in the ER (Suh et al., 1996; Neisig et al., 1996; Knittler et al., 1998; Peh et al., 1998; Schoenhals et al., 1999; Lauvau et al., 1999; Barnden et al., 2000; Garbi et al., 2000; Grandea et al., 2000; Myers et al., 2000; Paulsson et al., 2002; Pentcheva et al., 2002; Tan et al., 2002; Williams et al, 2002). However, Tapasin synergised with TAP1 or hβ_2m to enhance cell surface expression of K^k via TAP, demonstrating that it plays dual roles.

MHC-I are highly homologous and the percent identity of K^k and K^b is 85% and that of K^k and D^d is 78%. The differential requirement of assembly factors for K^k and D^d surface expression led us to analyze the possible residues that may play an important role. Multiple sequence alignments of different mouse MHC-I sequences were performed and the residues important for β_2m and peptide binding were studied based on crystal structures of K^b (Fremont et al., 1992) and D^d (Achour et al., 1998). In addition, the role of residues in D^b α3 domain (219–233) (Kulig et al., 1998 and D^d (Glu-222) that binds to TAP (Suh et al., 1996) and Tapasin (Suh et al., 1999; Paquet & Williams, 2002) were studied. In particular, we studied residues that are present in K^k (TAP-dependent) but are not conserved in other alleles, K^b, K^d, D^b and D^d, that are expressed at low levels on the cell surface in the absence of a functional TAP. As shown in Fig. 9A, no major difference was observed in the residues known to be important for TAP or Tapasin binding between K^k and the other alleles studied. Out of 18 residues important in β_2m binding, a single difference was observed between K^k (32Q) and K^b (32E) or D^d (32E). However, out of 20 residues important in peptide binding in K^b, there were differences in 9 residues between K^k and K^b, D^d & K^d, respectively. As expected, most of the polymorphisms were in the residues important for peptide binding compared to those involved in β_2m binding. A total of 13 residues are not conserved between K^k and the other alleles studied and 5/13 residues are important in peptide binding in the following positions, 9, 63, 66, 73 and 77. Modeling studies (Fig. 9B) revealed that residue nine was present in the β sheet whereas the other residues involved in peptide binding were present in one α helix. The other eight positions in K^k that are not conserved are: 19, 49, 55, 56, 114, 173, 180 and 191. In position 49, the amino acid change is from V in K^k to A in others; however, in other positions, the changes are significant. Residue 191 is present in the α3 domain whereas 114 is present in the β sheet and residue 180 is present in a turn localized in between the αl/α2 and α3 domains. It is possible that residues important in peptide binding play an important role because the α2 region is important in determining the efficiency of assembly HLA-B35 (Hill et al., 1993). Indeed, a single residue 97 has been shown to be responsible for higher cell surface expression of L^q (Arg-97) compared to L^d (Trp-97) (Smith et al.,

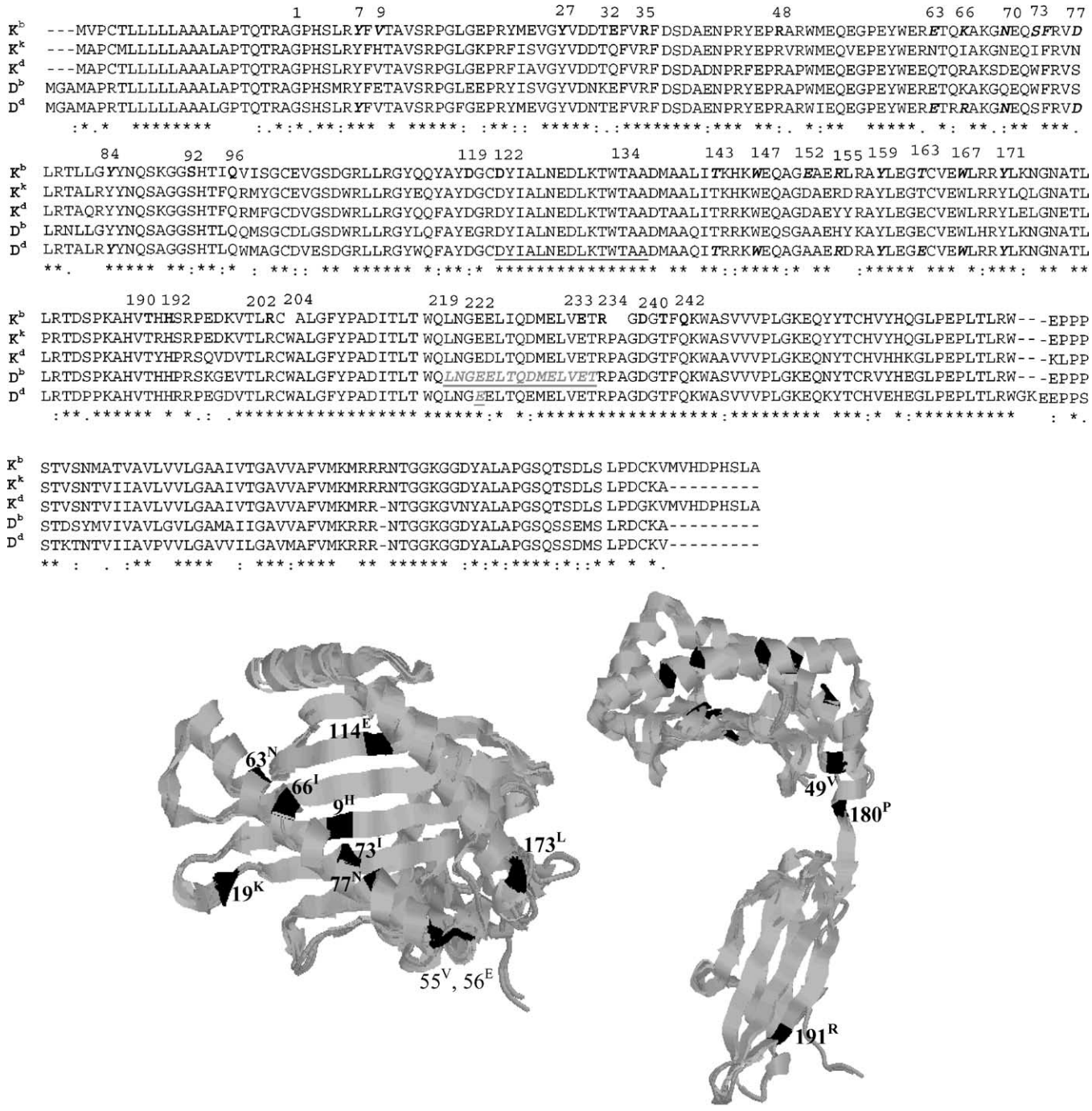

Fig. 9. Multiple sequence alignment of different MHC-I HCs and a model of K^k. (A) Amino acid sequences of different mouse MHC-I were subjected to multiple sequence alignment using ClustalW. The accession numbers of different genes are as follows. K^b (P01901), K^k (AAB17608), K^d (P01902), D^b (P01901), and D^d **(**P01902**)**. The amino acid positions are marked as present in the mature protein and based on the crystal structures of K^b and D^d molecules. The residues important in binding to β_2m are denoted in bold whereas bold and italicised letters denote the residues important in interaction with peptides. Bold, italicised and underlined residues in D^b (219–233) are involved in TAP association whereas the underlined residue E222 in D^d is also important for association with Tapasin. (B) The location of polymorphic residues in K^k (strictly TAPdependent) compared to other H2 alleles, K^b, K^d, D^b and D^d, which are expressed on cells lacking a functional TAP was modeled using SWISSMODEL and depicted using Ras Mol 2.7.

2002). However, we cannot overlook the potential roles of the eight residues that are different between K^k and other alleles (K^b, K^d, D^b and D^d) but are not involved in binding to peptide/β_2m/TAP/Tapasin based on the existing literature. Further studies are required to evaluate the role of residues in K^k that are responsible for its unique TAP-dependence for cell surface expression compared to K^b, K^d, D^b and D^d.

There are several possible implications for the distinct requirement of assembly factors for optimal surface expression of MHC-I alleles. First, it is well known that the sur-

face expression pattern of MHC alleles after viral infections is affected. Infection of L929 cells with pseudorabies virus reduces expression of D^k but enhances expression of K^k (Sparks-Thissen and Enquist, 1999). Also, viruses have evolved strategies to lower MHC-I expression and MCMV encodes m4/gp34 that binds K^b, but not D^b, which is expressed on the cell surface and blocks TCR-MHC interactions and CTL induction (Kavanagh et al., 2001). However, peptides derived from m4/gp34 are recognized by CTLs in the context of D^b (Holtappels et al., 2000). Different trafficking requirements may allow for cell surface expression of at least some types of MHC-I when one type is targeted for down regulation during infection. Second, binding to TAP or the lack of binding may be functionally relevant during disease progression. A functional role for TAP-MHC binding has been found in case of patients with HLA-B3503 (Phe-116) which associates with TAP well but results in faster AIDS progression. However, HLA-B3501 which contains (Ser-116) associates poorly with TAP and may be beneficial as there is slower progression to AIDS (Gao et al., 2001). Third, in two humans lacking TAP activity, NK activity was not detected; however, $CD8^+$ T lymphocytes were observed in the older child, which suggests involvement of TAP-independent mechanisms for low levels of MHC-I expression and modulation of the immune response (de la Salle et al., 1994). Fourth, peptide-receptive MHC-I (e.g. D^d) may bind peptides outside cells, following endocytosis and recycling back to the cell surface or bind exogenous β_2m. Some of these mechanisms may result in presentation of peptides derived from exogenous antigens on peptide-receptive class I. In fact, MHC-I from *Tapasin*$^{-/-}$ and *Tap1*$^{-/-}$ mice bind to exogenous peptides although they are deficient in the vacuolar alternate cross presentation pathway due to enhanced susceptibility of unstable MHC-I to acidic pH (Chefalo et al., 2003). The fifth and final point to be noted is that H6 is a tumor cell line and reduction in MHC-I is a mechanism to evade the anti-tumor CTL response (Johnsen et al., 1999; Seliger et al., 2000; Seliger et al., 2001; Qin et al., 2002). It is possible that H6 represents a tumor survival strategy to evade an anti-K^k CTL response. However, expressing of empty D^d molecules reduces the risk of an anti-D^d CTL response; more importantly, expression of D^d may protect tumors from lysis by NK cells. Multiple mechanisms for MHC-I expression allows for greater flexibility for cell surface expression of these molecules that play a critical role during cellular defense. Studying the requirements of surface expression and stability of different MHC-I alleles may help in devising strategies to optimize MHC-I antigen processing and presentation to generate an efficient $CD8^+$ T cell response.

Acknowledgements

This study was funded by grants from the Council of Scientific and Industrial Research and The Indian Council for Medical Research, Government of India. We appreciate the generosity of the members of the MHC-I scientific community who gifted us cDNAs and Abs. We thank O. Joy and H. Krishnan for help with FACS and P. Sadhale, S. Das and P. Kondaiah for help with technical queries and reagents. We are grateful to S. Rath, R. Manjunath and E. Hermel for their comments on this study. Finally, this study was possible due to the “active” encouragement and generosity by J. J. Monaco.

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