Class I MHC Is Stabilized Against Thermal Denaturation by Physiological Concentrations of NaCl[†]

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ABSTRACT: Class I MHC molecules are ternary complexes composed of an allotype specific heavy chain, a noncovalently associated protein β_2-microglobulin (β_2m), and a peptide. The complexes are assembled in the endoplasmic reticulum by a complex series of chaperones and peptide-loading mechanisms. In the absence of β_2m or peptide, very little class I heavy chain is transported to the surface of the cell. Complexes that do not contain all three parts of the protein are not made productively in vivo and not at all in vitro. The ability of the complex to withstand thermal denaturation in vitro has been shown to be related to the binding affinity of the peptide. Paradoxically, some low-affinity peptide complexes denature at or below human basal body temperatures in vitro but are effective biological agents in vivo. Here we show that these complexes are stabilized against thermal denaturation by physiological cosolvents and maximally stabilized by 150 mM NaCl. While the degree of stabilization by 150 mM NaCl is greatest for low-affinity peptide/MHC complexes, the mechanism of stabilization is independent of peptide sequence. This effect is hypothesized to occur by multiple mechanisms including increasing the affinity of β_2m for the complex and charge screening.

Class I molecules are ternary complexes that are expressed on the plasma membrane of nearly all cells in the body (*1*). These molecules are composed of a 44 kDa polymorphic heavy chain, a noncovalently associated light chain (∼11 kDa) called β_2-microglobulin (β_2m),[1] and a small peptide (*2*). In vitro studies typically only use the extracellular portion of the heavy chain (*2*−*4*) which contains the peptide-binding superdomain and an immunoglobulin domain (*5*). Both the peptide-binding superdomain and the immunoglobulin domain make extensive contacts with β_2m (*6*).

Class I MHC molecules bind small peptides (8−11 amino acids) and present them to circulating T cells at the plasma membrane. In so doing, the class I MHC molecules serve to signal to T cells the identity of the proteins being expressed within the cell. The peptides that class I MHC bind are endogenously derived from host or intracellular pathogens. They are processed by the proteasome in the cytosol and are transported into the endoplasmic reticulum by specific transporters (*7*). The peptides are actively loaded onto class I MHC molecules within the endoplasmic reticulum before export to the cell surface (*1*). These peptide/MHC (pMHC) complexes are recognized by clonotypic T cells via their T cell receptors (TCR). Upon proper binding of TCR with pMHC, the T cells lyse the cell. A properly functioning cell will present unmutated self-peptides with class I MHC. These cells are typically untouched by the immune system, because either self-reactive T cells are eliminated during T cell development or they are functionally inactivated in the periphery. Cells that are infected by a virus or transformed to a cancerous state by DNA mutation present peptides that have not been seen by the immune system. Thus, this signal of the state of the cell prevents further viral or tumor propagation by selectively destroying aberrant cells [reviewed in (*8*)].

Class I MHC molecules bind many different peptides primarily through the invariant peptidic termini. These termini dock in pockets composed of conserved amino acids found within a large cleft in a superdomain formed by the α1 and α2 domains of the heavy chain (*5*). Altering the chemical nature of the peptidic termini drastically reduces

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[1] Abbreviations: CTL, cytotoxic T cell; TCR, T cell receptor; MHC, major histocompatibility complex; pMHC, peptide/MHC complex; A2, human class I MHC HLA-A2.1; D^b, murine class I MHC molecule H-$2D^b$; K^b, murine class I MHC molecule H-$2K^b$; β_2m, β_2-microglobulin (h or m prefix refers to human or murine protein); HEL, hen egg white lysozyme; CD, circular dichroism spectropolarimetry; T_m, temperature at which 50% protein is denatured.

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the stability of the class I MHC complex as measured by thermostability experiments (*9*). Sequencing of pooled peptides eluted from class I (*10*) and determination of individual sequences (*11, 12*) showed that class I MHC restricts the use of amino acids at specific positions within the antigenic peptide. These positions within the peptides are termed anchors; a combination of anchors constitutes a peptide-binding motif (*5, 13*−*15*). The anchors are bound by the polymorphic residues within the peptide-binding superdomain of class I MHC. Because the polymorphic nature of the cleft forms the basis for the amino acids selected within the peptide, the motifs are specific for each class I allotype. These anchor residues may (*9, 16*) or may not (*17*) be used to generate significant binding free energy. It is unclear how many peptides may really be able to bind to each class I MHC. Estimates of the numbers of peptides bound to a class I MHC allotype on the cell surface have been in excess of 10^4 based on mass spectrometry (*18*), but are surely underestimated because of sampling error due to MHC preparation and vaporization for mass spectrometry.

Immunotherapies for cancer or viral infection that utilize class I molecules and peptides have been proposed (*19*). For this therapy to be successful, some critical quantity of class I molecules have to present antigenic peptide for a significant duration to signal the immune system (*20*). Understanding what makes a particular peptide bind well and another bind poorly is of critical importance to this work. Unfortunately, little is known about the mechanism of peptide binding beyond qualitative descriptions.

Poor peptide binding to class I has been postulated to be the cause for inefficient immunogenicity against tumor antigens in vivo (*21, 22*). Clearly, if a peptide does not bind well, the pMHC complex cannot be detected by circulating T cells. We examined binding of a group of peptides that are known to be recognized by cytotoxic T cells (CTL) derived from the tyrosine kinase HER-2/neu using circular dichroism (CD) spectropolarimetry and flow cytometry. Previous experiments have shown that the T_m calculated from CD thermal denaturation profiles is proportional to the peptide equilibrium binding constant (*23*). Our CD thermal denaturation experiments of class I MHC complexes with HER-2/neu-derived peptides displayed a range of T_ms. Interestingly, some of the complexes had T_ms that are at or below human basal body temperature (*24*). This result generated an interesting question. How can class I complexes fall apart at human physiological temperatures in vitro and stimulate an immune response in vivo? A hypothesis developed which states that physiological osmolytes stabilize class I MHC/peptide complexes against thermal denaturation in vitro and in vivo.

Many osmolytes are found in vivo as cosolvents (*25*). Salts, monosaccharides, and amino acids are typically found in micromolar to millimolar concentrations in the cellular environment. Most of these molecules have multiple functions inside the cell, but one function, which has been frequently neglected, is protein stabilization. Protein chemists have shown that specific osmolytes such as glycerol and sucrose can stabilize protein exposed to denaturing conditions at high concentrations (>1.0 M). Physiological concentrations of these agents do not typically have significant effects by themselves. We have performed numerous stability experiments with class I complexes using different cosolvents. The results were generally as expected; osmolytes stabilized class I MHC to thermal denaturation. The surprising result was that physiological concentrations of NaCl demonstrated the largest stabilization. We hypothesize that the stabilization is partly due to the increase of β_2m binding to heavy chain and partly due to stabilization by Debye−Hückel charge screening.

Table 1: Effect of 150 mM NaCl on the T_m of Class I MHC/Peptide Complexes[a]

MHC allotype	peptide	T_m (°C) (0.0 mM NaCl)	T_m (°C) (150 mM NaCl)	ΔT_m (°C)
A2	IISAVVGIL	36.2	60.0	23.8
A2	IISAVVGIV	38.8	59.8	21.0
A2	KISAVVGIL	41.6	55.9	14.3
A2	ILKEPVHGV	50.0	58.6	8.6
A2	YLKEPVHGV	56.0	61.7	5.7
A2	FLKEPVHGV	55.0	60.0	5.0
A2	KTWGQYWQV	52.7	57.7	5.0
A2	IMDQVPFSV	49.5	56.5	7.0
A2	ITDQVPFSV	45.1	53.9	8.8
A2	MLLSVPLLL	53.1	55.9	2.8
A2	ALGIVCPIC	45.3	63.4	18.1
D^b	KAVYNFATM	43.7	53.7[b]	10.0
D^b	FAPGVFPYM	41.0	65.0[b]	24.0

[a] Thermal melts were performed on multiple pMHC complexes as described in the text. [b] These values are for 100 mM NaCl.

EXPERIMENTAL PROCEDURES

Synthetic Peptides. All peptides were synthesized by the Peptide Synthesis Facility at the University of North Carolina, Chapel Hill. The sequences of the peptides are given in Table 1. All peptides were purified by reverse-phase HPLC to greater than 95% purity, and the sequences were confirmed by matrix-assisted laser desorption ionization−time-of-flight spectrometry.

Production of Class I MHC/Peptide Complexes. pMHC complexes were prepared as previously described (*26*). Briefly, the extracellular portions of class I MHC heavy chain were produced in *E. coli* as inclusion bodies. Purified inclusion bodies were rapidly diluted in the presence of β_2m, individual peptides, and a chaotropic buffer consisting of 100 mM Tris-HCl, pH 8.0, 400 mM L-arginine, pH 8.0, 6 mM glutathione (10:1 mixture of reduced/oxidized), and a cocktail of protease inhibitors. Folded pMHC complexes were concentrated by ultrafiltration (Amicon, Inc., Beverly, MA) with a YM10 membrane. The concentrated protein was purified to homogeneity by gel filtration on a BIOSEP SEC-S2000 column (Phenomenex, Torrance, CA) in a running buffer of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl and exchanged into 10 mM KH_2PO_4/K_2HPO_4, pH 7.5, using Centricon filtration devices (Amicon, Inc.). Buffer was exchanged at least 3 times (8000-fold dilution) to ensure the removal of any residual buffers or salts. For those experiments requiring β_2m alone, the β_2m inclusion body material was folded in vitro as described above for pMHC except that a 3000 MW cutoff membrane was used for concentration before purification on the gel filtration column.

K^d covalently bound to β_2m (SC-K^d) and single-chain K^d complexes (sBDH) were produced in CHO cells and purified as described previously (*27, 28*). The purified protein was concentrated and exchanged into 10 mM KH_2PO_4/K_2HPO_4, pH 7.5, using Centricon filtration devices (Amicon, Inc.).

Circular Dichroism (CD) Spectroscopy and Thermal Denaturation Measurements. Purified complexes were diluted to 4−12 μM protein with 10 mM KH_2PO_4/K_2HPO_4 with or without additional cosolvents. The pH of the resulting solution was monitored carefully due to the known shift in T_m of class I complexes at low and high pH (*29*). The thermal denaturation profile (melting curve) of class I complexes was collected by monitoring the change in the circular dichroic signal as a function of temperature as described previously (*9, 30*). Thermal scans were performed on an AVIV 62DS spectrophotometer (Aviv Associates, Lakewood, NJ) equipped with a Peltier-effect temperature controller using 0.1 cm path length cuvettes. Thermal denaturation data were typically collected at 218 nm with 1 °C intervals from 4 to 95 °C. All measurements were made at least 3 times from different preparations and averaged. Thermal denaturation curves were scaled between 0 and 1 to provide plots of the fraction unfolded versus temperature for analysis. The T_m of a class I complex is the temperature at which 50% of the molecules are unfolded. CD spectra were collected between 350 and 200 nm at 1 nm increments and 10 s averaging times. Five or more spectra were averaged for the final spectrum of each sample.

Cell Surface Class I Measurements. Measurements of cell surface class I molecules loaded with endogenous peptides were performed as previously described (*31*). Briefly, T2 cells (ATCC no. CRL-1992), that are defective in antigen presentation (*32*), were loaded with the peptide of interest by incubating the cells in the presence of 50 μM each of peptide and β_2m. Peptide-pulsed T2 cells were cultured in serum-free AIM V media in a 5% CO_2 environment with and without additional salts. Flow cytometry was performed on a Becton Dickinson FACScan (Lincoln Park, NJ). Propidium iodide insensitive cells were used for all experiments to be sure only live cells were analyzed. The conformationally specific antibodies BB7.2 (HLA-A2 specific) (*33*), W6/32 (class I α3 chain/β2m specific) (*34*), and HbV (HLA-A2/HIV peptide specific; Dr. Ralph Kubo, personal communication) were used as the markers of folded class I structure.

RESULTS

During our examination of the thermal stability of peptides recognized by CTL from the tyrosine kinase HER-2/neu, we found some of the peptide/MHC (pMHC) complexes have denaturation midpoints that are at, or below, human basal body temperature (*24*). Knowing that these complexes bind to their cognate TCR and elicit an immune response in vivo, we sought to determine what factors might stabilize the class I MHC complexes in vitro to extrapolate to in vivo conditions. Physiological osmolytes are well-known as stabilizers (or destabilizers) of protein structure (*35*) as such were reasonable candidates as stabilizers of class I MHC against thermal denaturation. Therefore, the effect of cosolvents on the thermal stability of class I MHC molecules was examined.

Various osmolytes including polyalcohols such as glucose and PEG, amino acids, and amino acid derivatives such as glycine and betaine, and salts such as NaCl were each added to purified recombinant proteins, and the thermal stability was measured by circular dichroism. Since the yield of

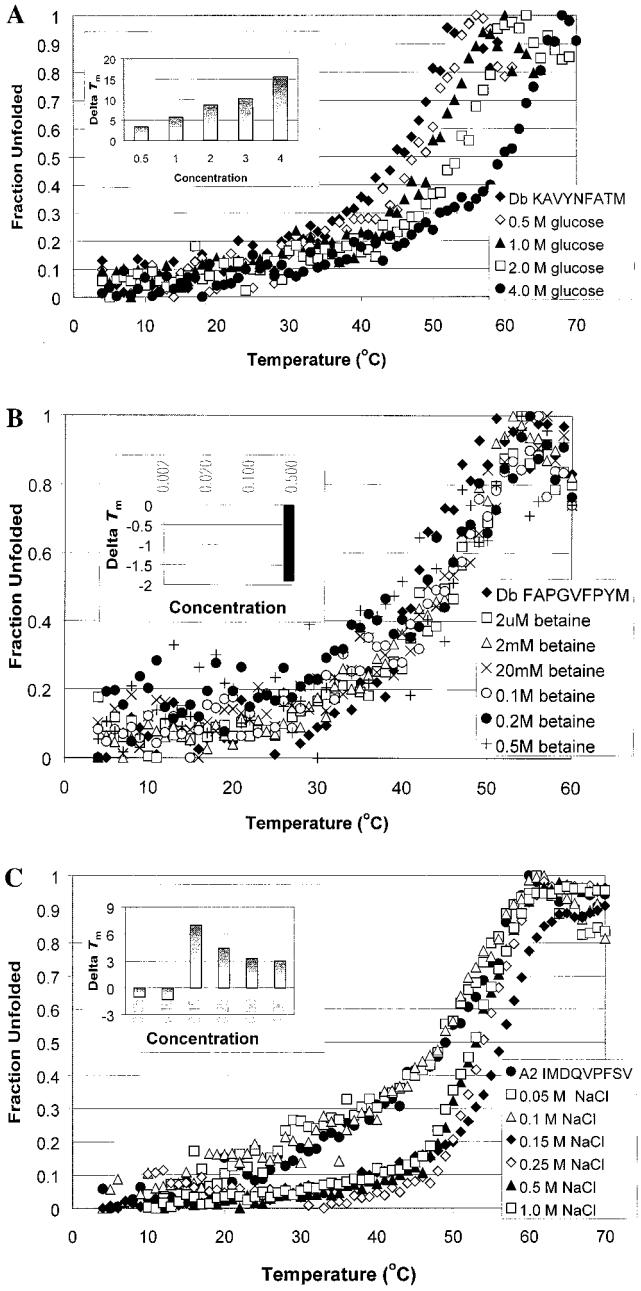

FIGURE 1: Class I MHC is stabilized against thermal denaturation by cosolvents. Thermal denaturation profiles of class I MHC in the presence of the indicated concentration of cosolvent. (A) Effect of added glucose. D^b KAVYNFATM folded in vitro was denatured in the presence of the indicated concentrations of glucose. (B) Effect of the amino acid analogue betaine. D^b FAPGVFPYM was thermally denatured in the absence of the indicated concentrations of betaine. (C) Effect of NaCl. A2 IMDQVPFSV was thermally denatured in the presence of the indicated concentrations of NaCl. The inset panels describe the difference in the calculated T_m from the T_m observed in the absence of added cosolvent. Each curve is the average of three independent experiments using 4−12 μM protein.

protein folded in vitro depends on the affinity of the peptide [(*36*) and unpublished observations], we used complexes that contained moderate- to high-affinity peptides for most analytical tests. Figure 1 shows representative results for glucose, betaine, and NaCl. Glucose shows a typical stabilization to thermal denaturation at increasing concentration (Figure 1A). Sucrose shows a similar stabilization at half the concentration. That observation is consistent with its

2-fold increase in volume relative to glucose (data not shown). Betaine showed very little effect at low concentration and a destabilizing effect at higher concentrations (Figure 1B). Glycine shows a similar destabilizing phenotype (data not shown). Interestingly, NaCl did not appear to give an easily interpretable result (Figure 1C).

Unlike the results seen with polyols and amino acids, titrations with NaCl from 5 μM to 1 M show that there is an optimal concentration of NaCl (150 mM for human class I MHC, 100 mM for murine class I MHC) for thermal stabilization. The titration shows an initial spike of stabilization at low concentration, followed by a relative destabilization, finishing with stabilization again at very high concentrations. The stabilization at high concentrations of NaCl (>0.5 M) is what has been seen many times previously for other proteins (*35*). The large increase in thermal stability at low salt concentration has not been observed previously. These effects were consistently seen for A2 and HLA-Aw68. The results seen for polyols, amino acids, and salts are not constrained to a specific MHC with a specific peptide; they are found with different peptides and different MHC molecules (data not shown). The degree of stabilization changes, but the concentration trends are the same for any class I MHC with one exception. The murine class I MHC molecule D^b showed maximum stabilization at 100 mM NaCl, not 150 mM as was seen for the human pMHC (data not shown).

We typically examine denaturation of these complexes at 218 nm. An uninteresting explanation for this effect would be if the shift in denaturation of all these pMHC complexes were due to artifactual wavelength-specific stabilization. Therefore, wavelength scans of class I MHC/peptide complexes with and without 150 mM NaCl were performed at different critical temperatures. There were no significant differences between the CD spectra of pMHC complexes at 4, 37, and 50 °C with or without 150 mM NaCl present (data not shown). There were small differences between the two conditions at 4 or 95 °C, and this observation was attributed to the solubility differences of these denatured complexes at the elevated temperatures. Denatured class I MHC complexes in the absence of NaCl are slightly soluble, while those with NaCl are not soluble as evidenced by a white precipitate at the bottom of the cuvette. These data suggest that there are no gross rearrangements of the secondary structure as a function of the NaCl concentration, and the effect is due to protein denaturation at different temperatures (data not shown). Additionally, the thermal denaturation of pMHC is reversible by the following criteria. Protein that has been heated to 90 °C for 5 min and cooled retains the same spectra as the native protein. Furthermore, the resulting protein gives the same T_m if denatured again. The denaturation is locally reversible as the T_m does not shift if a scan rate twice as long is used. Three-fold differences in pMHC concentration had no effect on T_m. However, it is not fully reversible. Much of the protein is not recovered from the first denaturation curve, and the solution must be filtered to remove the insoluble material before the second experiment is performed.

We also sought to confirm that this thermal stabilization is not artifactual by using an entirely different assay. We examined the condition of class I MHC on the surface of cells in the presence of different concentrations of NaCl by flow cytometry. T2 cells were chosen because they do not have functional peptide transporters, and thus a large fraction of the class I complexes bind exogenous peptide on the cell surface (*32*). NaCl could not be removed from the medium because the cells would die, but it could be added to the media in small amounts. Based on the data described above (Figure 1C, inset panel), NaCl concentrations above 150 mM reduce the T_ms relative to 150 mM NaCl. The hypothesis was that additional NaCl would lower the T_ms of class I on the surface of cells, resulting in fewer available complexes. First, we tested how much salt the cells could tolerate. Additional NaCl was added in 25 mM increments to AIM V serum-free medium and T2 cell viability assessed. The cells tolerated up to an additional 75 mM NaCl added to the medium over the course of the experiment (~24 h). Next, cell surface complexes of A2 were measured as a function of added NaCl. As can be seen from Figure 2, the additional NaCl reduced the number of A2 complexes on the surface of T2 cells. Different peptides bound in the pMHC showed different degrees of destabilization on the T2 cells depending on the concentration of added NaCl (Figure 2). The most affected were cells incubated with IISAVVGIV, and the least affected were cells incubated with MLLSVPLLL. Therefore, the magnitude of the effect on the surface of T2 cells correlated directly with the magnitude of the effect on T_m as measured by CD (Table 1). The effect was also not due to the hydrophobicity of the peptide, because these two peptides are very similar hydrophobicities. The shifts in fluorescence were also not due to a reduction in affinity of the BB7.2 antibody for HLA-A2, because similar effects were seen with two different antibodies that bind in distinctly different locations on the class I molecule (antibodies W6/32 and HbV, data not shown).

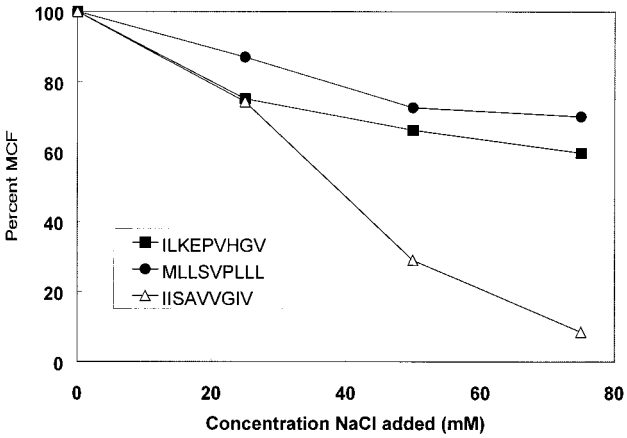

FIGURE 2: Destabilizing effect of NaCl above 150 mM is also seen on the surface of cells. Cell surface assembly assays of A2 were performed on T2 cells with the indicated peptides in the presence of 25, 50, and 75 mM NaCl added to the cell culture medium. As additional NaCl was not perfectly tolerated by the cells, live cells were analyzed by gating on propidium iodide negative cells.

Having concluded that the NaCl effect on pMHC is not an artifact of the CD experiment, we reexamined the effect of NaCl on a well-studied protein, hen egg white lysozyme (HEL), and also β_2m. Figure 3 shows the change in T_m for HEL and β_2m in the presence of increasing concentrations of NaCl. There are relatively small, nearly monotonic positive changes in T_m up to 500 mM NaCl for HEL and larger, positive changes at 1.0 and 2.0 M NaCl. There was no peak of stabilization, and the observed stabilization of HEL at 150

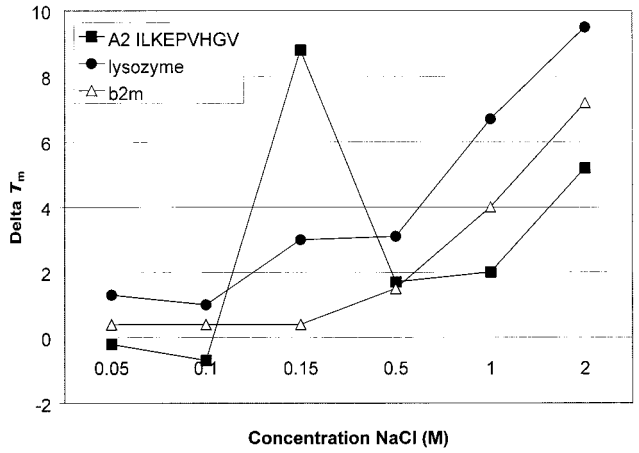

FIGURE 3: Effect of NaCl on class I MHC is not generalizable to other proteins. The indicated amounts of NaCl were added to samples of A2 ILKEPVHGV, lysozyme, or β_2m and the thermal denaturation profiles determined. Delta T_m is the calculated T_m in the absence of NaCl subtracted from calculated T_m at the indicated concentration.

mM NaCl (~2 °C) was not nearly as large as most of the effects on class I MHC (Table 1). There was no observed change in the T_m of β_2m until 0.5 M NaCl was added.

The effects of other salts on class I MHC were also examined. Figure 4A demonstrates that KCl shifts the T_m of complexes to greater temperatures than NaCl. However, the maximum was observed to be 150 mM, suggesting that ionic strength may be part of the reason the protein is stabilized. Figure 4B shows the effects of Na_2SO_4 on class I complexes. At an equivalent point of ionic strength, the effects of NaCl, KCl, and Na_2SO_4 are very similar, but different in intensity. Additionally, some salts shifted the T_ms of complexes to lower values. For example, at 150 mM NaSCN, the A2 ILKEPVHGV complex was so disrupted an accurate T_m could not be assessed. Also, LiBr at 150 mM shifted the T_m of the A2 ILKEPVHGV complex by approximately −20 °C (data not shown). In a survey of other biologically important ions, we tested the effect of 5 mM Ca^{2+} ($CaCl_2$, estimated to be the physiological concentration of Ca^{2+} in the ER) on the thermal denaturation of pMHC [in MES buffer due to the precipitation of $Ca_2(PO_4)_3$]. There was no effect on the T_m of pMHC with 5 mM Ca^{2+} (data not shown). NaCl does not change the size, shape, or oligomeric state of pMHC as determined by dynamic light scattering and sedimentation velocity experiments (data not shown).

Determining how NaCl could exert its effects on class I MHC is made difficult because of the nature of the complex. Most experiments looking at cosolvent effects were performed on small single-domain proteins. If we consider the pMHC ternary complex as a single unit, the effect would be due to changes in protein solvation, discrete ion binding to the protein, or charge screening on the protein. However, we may consider class I MHC to be a heavy chain with two ligands (peptide and β_2m). If we examine the molecule that way, the stabilization effect could be due to stabilization of either peptide binding or β_2m binding to the heavy chain as each ligand has been shown to be required for complex formation [(*37*, *38*) and unpublished data].

Does the nature of the peptide affect the degree of thermal stabilization by NaCl? A summary of different peptides and different complexes tested for thermal stability by CD is

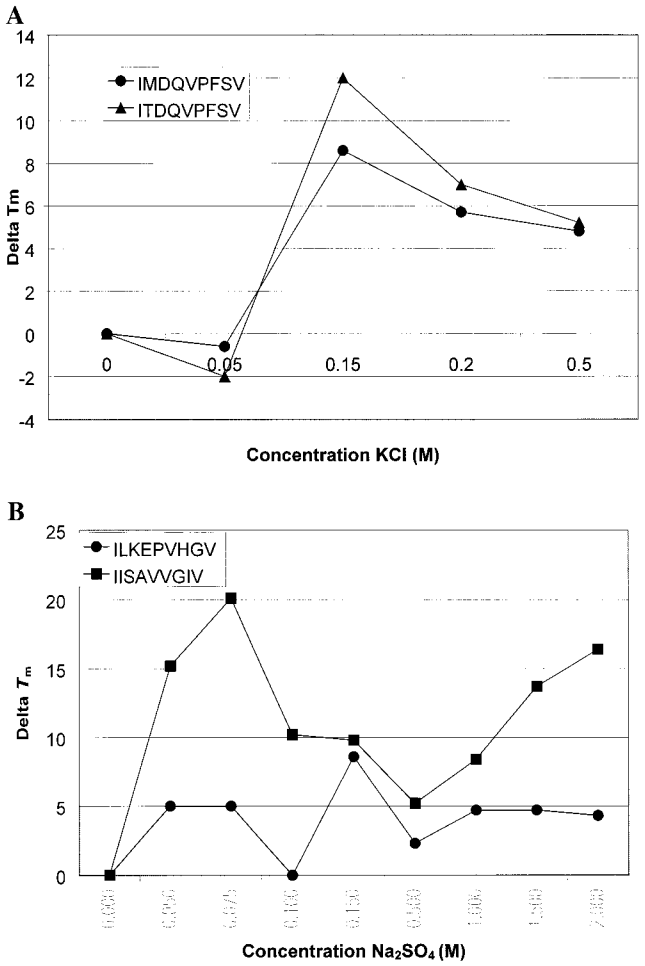

FIGURE 4: Effect generally correlates with the Hofmeister series for anions. Change in melting temperature of the class I complexes versus concentration of KCl and Na_2SO_4. (A) KCl stabilization of class I complexes of A2 bound to IMDQVPFSV or ITDQVPFSF. (B) Effect on thermal stabilization of pMHC (A2 and ILKEPVHGV or IISAVVGIV) in the presence of the indicated amounts of Na_2SO_4.

shown in Table 1. All of the peptides conform to the known peptide-binding motif for its allotype. Therefore, differences are not due to specific interactions with the peptide-binding cleft. However, peptide hydrophobicity could play a role. One can imagine that the addition of salt to the solvent would drive the association of peptide with the heavy chain. If peptide release is an important early step in thermal denaturation, then the stabilization would be greatest for nonpolar peptides and least for more polar peptides. Figure 5A shows a plot of the calculated hydrophobicity of a series of peptides versus the observed increase in T_m. There is clearly not a simple relationship suggesting that the hydrophobicity of the peptide does not affect the stability of the complex and that peptide release is not affected by the polarity of the solvent. This has been seen before for HLA-Aw68 (*39*). Additionally, if peptide dissociation were to affect the thermal stability of the protein, the pMHC in the cuvette should show a higher T_m in the presence of excess additional peptide. Addition of 5 μM IISAVVGIL excess peptide to A2 IISAVVGIL does not change the T_m (data not shown). Therefore, thermal denaturation is not keyed to peptide dissociation. Figure 5B shows a plot of the initial T_m versus the degree of stabilization. It is clear from the 77% correlation coefficient that the degree of stabilization

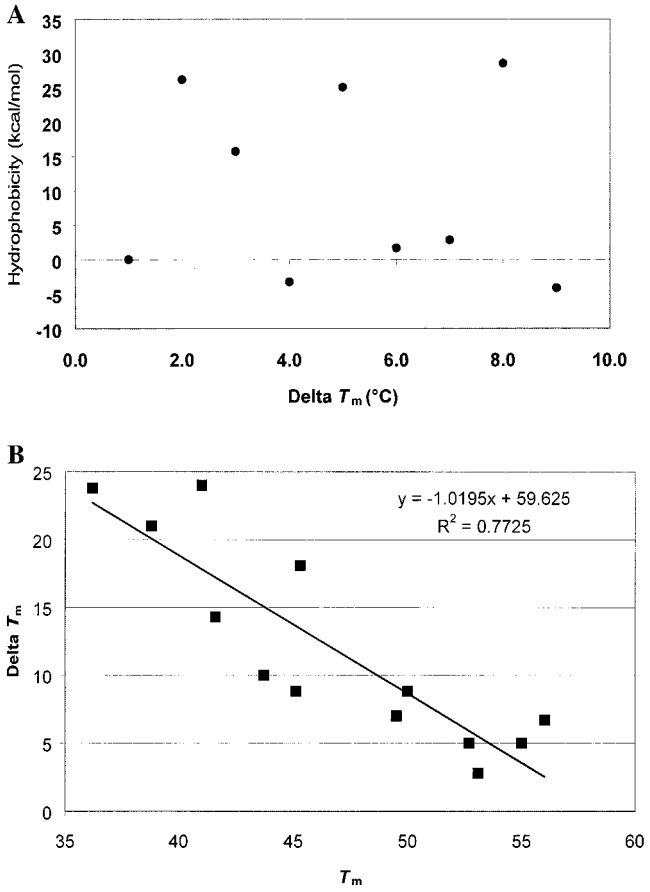

FIGURE 5: NaCl-induced change in T_m depends on the initial T_m and not on the hydrophobicity of the peptide ligand. (A) The plot of peptide hydrophobicity versus the delta T_m demonstrates that the change in T_m of class I MHC complexes is not dependent on the peptide sequence. Hydrophobicities are calculated from the sum of the free energies of transfer of the individual amino acids from cyclohexane to water (*50*). (B) Plot of the change in T_m versus initial T_m shows that the change in T_m is dependent on the initial T_m. The peptides and their respective T_ms are listed in Table 1.

is maximal for the poorest binding peptides. These data suggest that the stabilization by NaCl is not primarily due to any effect of the composition of the peptide, but it is related to how well the complex is held together.

Another ligand-binding scenario is that NaCl stabilizes the interaction of β_2m with the complex and thereby increases the thermal stability. The dissociation of β_2m has been described as a good measure of the dissociation of the peptide (*40*). Thus, it appears that peptide and β_2m dissociate from the heavy chain in a dependent fashion. The affinity of β_2m does not have a great effect on the T_m. Human β_2m has a 3-fold greater affinity for murine heavy chain than does murine β_2m (*41*). A murine class I MHC complex D^b folded with human β_2m and a low-affinity peptide FAPGVFPYM has a slightly higher T_m than the complex folded with murine β_2m (47 versus 42 °C) (Figure 6A). If β_2m dissociation were the first step in pMHC complex denaturation, addition of excess β_2m should shift the equilibrium to higher T_ms. However, if an excess of β_2m is added to the cuvette that contains the pMHC to be denatured, it does not increase the T_m (Figure 6B). Addition of NaCl to a mixture of pMHC and a 2-fold molar excess of β_2m and 100 mM NaCl stabilizes the protein to the degree seen in the absence of excess β_2m. To examine how a very large increase in affinity

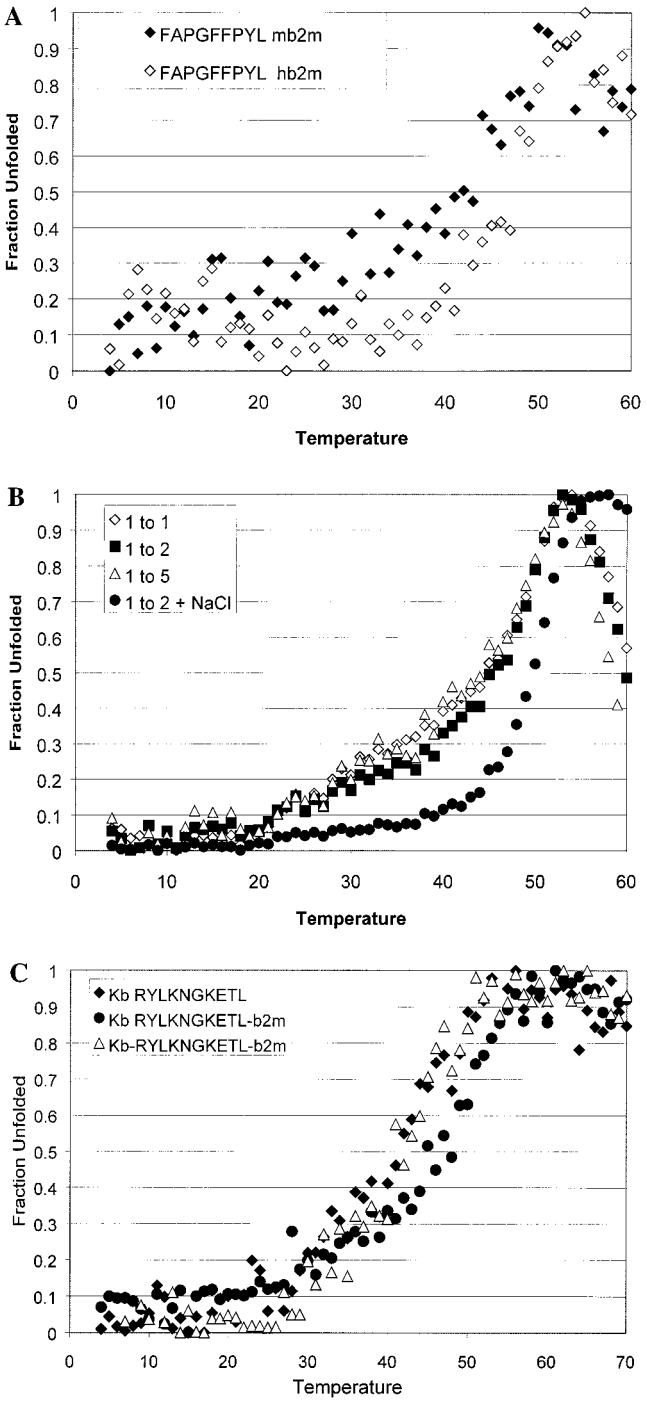

FIGURE 6: Increased association of β_2m increases the thermal stability, but not to the levels seen by the addition of NaCl. (A) D^b FAPGFFPYL has a higher T_m with hβ_2m bound than with mβ_2m. (B) The addition of molar excess of β_2m does not increase the T_m of pMHC. D^b KAVYNFATM was thermally denatured in the in the absence or presence of 1:1, 2:1, or 5:1 molar excess of mβ_2m added or in the presence of 2-fold molar excess β_2m plus 100mM NaCl. (C) Covalent addition of β_2m to the protein (Kb RYLKNGKETL-β_2m) increases the thermal stability of pMHC. However, covalent addition of peptide to make a fully covalent complex (Kb-RYLKNGKETL-β_2m) is not as thermally stable as the β_2m-attached and peptide-"free" complex.

would affect the thermal stability, we looked at protein that has β_2m covalently attached to the heavy chain (*42*). As can be seen in Figure 6C, the protein has a higher T_m compared to the noncovalently associated material (shift of ~5 °C). As the covalently linked protein has been produced in

mammalian cells and is glycosylated, we cannot exclude that the protein may be stabilized by the glycosylation. However, a complex that has covalently linked peptide and covalently linked β_2m (*43*) has a nearly identical T_m to that of the noncovalently associated ternary complex folded from material made in *E. coli* (Figure 6C). The fully covalent complex is most likely destabilized with respect to the covalent β_2m because the antigenic peptide extends from the peptide-binding groove. This has already been shown to be destabilizing due to the disruption of a conserved hydrogen bond with the peptide carboxyl terminus (*3*).

DISCUSSION

Class I MHC proteins have a simple role: to bind peptides in the endoplasmic reticulum and transport them to the cell surface. Differentiation of immunogenic versus ignored peptides is performed by TCR on the surface of circulating $CD8^+$ T cells. Most previously identified immunogenic viral epitopes bind to class I MHC well. However, peptides that bind poorly to class I MHC in vitro are also recognized by $CD8^+$ T cells in vivo (*24*). To resolve the paradox of the in vivo biological activity (i.e., T cell activation) with these in vitro physical measurements, we looked for other molecules that could stabilize proteins in vivo. This led to the examination of osmolytes as stabilizing agents and to the surprise observation that class I MHC stabilization was keyed to 150 mM NaCl.

When we began these studies, we expected to see modest increases in stability at high concentrations that we would extrapolate back to physiological conditions. As was anticipated, class I MHC molecules are stabilized by polyols such as glucose (Figure 1A) and sucrose (data not shown) at high concentrations. Other polyols, such as glycerol and poly(ethylene glycol), are destabilizing (data not shown). Amino acids (glycine data are not shown) and amino acid derivatives (Figure 1B) are destabilizing. NaCl (Figure 1C) and KCl (Figure 4A) are stabilizing at molar amounts compared to the absence of NaCl as is seen for other proteins. However, in a manner not seen before, class I MHC is maximally stabilized to thermal denaturation at physiological concentrations of added NaCl.

The mechanism of class I MHC stabilization to thermal denaturation by NaCl is not clear. There are two different levels of potential stabilization to consider. The first treats the trimolecular complex as the sum of its parts. In that scheme, peptide and β_2m may be considered as ligands binding to the heavy chain. Enzyme stability has been shown to be increased by increases in ionic strength. This phenomenon has been attributed to an increase in the affinity of the ligand for the protein due to the hydrophobic effect. The observation that the increased thermal stability in the more polar environment does not correlate with peptide hydrophobicity suggests that the release of peptide is not the critical event in thermal denaturation of this complex. Similarly, changes in β_2m affinity can increase thermal stability, but the effect is very small and does not account for the large change in thermal stability in the presence of salt. Last, in both of these instances, if the effect were due to a change in partitioning of the parts of the protein due to polarity of the environment, we would expect the effect to saturate, but not peak and decline as we observe here.

The second potential mechanism of stabilization treats the trimolecular complex as a single unit. This is not necessarily a poor assumption because the thermal denaturation is two-state. If the protein is a single unit, there are three potential explanations for this stabilization by salts: changes in protein solvation, discrete ion binding, and Debye−Hückel charge screening. 150 mM NaCl is considered to be a dilute aqueous salt solution and is not considered to contribute significantly to the protein solvation (hydrophobic) effect (*44*, *45*). However, if protein solvation is a factor here, anions should dominate, and the effect should follow the Hofmeister series (*46*). If discrete ion binding plays a role, the effect should follow the electroselectivity series of anions binding to ion-exchange resins (*47*). In the absence of clear evidence of either of the other two possibilities, we would propose that Debye−Hückel screening is important, but it is normally seen in solutions below 0.1 M (*48*).

All of these factors probably contribute to the observed phenotype of class I MHC. While it is difficult to dissect the contributions from each of these factors from these experiments, we can make the following statements. The effect of the various ions does not appear to follow the electroselectivity series. It does follow the Hofmeister series, but as stated above, this effect is not typically described for dilute solutions. The most reasonable explanation is Debye−Hückel screening because stabilization (a) roughly follows ionic strength (compare NaCl versus Na_2SO_4 effects), (b) is observed at low concentrations of salts, and (c) is primarily associated with the cation (KCl stabilizes more than an equimolar amount of NaCl). Therefore, at concentrations up to 150 mM NaCl, a relatively long-range destabilizing charge within the protein is shielded (Debye length ~8 Å). At concentrations above 150 mM (shorter Debye length), the situation is reversed, and a stabilizing charge is shielded. This would make the protein less stable.

Thermal denaturation studies have been shown to be an accurate way to evaluate peptide binding to class I MHC molecules (*9*, *30*), and the derived T_ms have been shown to be proportional to the peptide equilibrium dissociation constant (*23*). We have performed many experiments comparing T_ms and relative binding constants on the surface of T2 cells. The relative binding constant that we derive has shown an excellent correlation with T_m as long as there are no cosolvents present in the CD buffer (unpublished data). However, this correlation must be qualified. As demonstrated here, it clearly does not hold true under all conditions. In the absence of salts, measurement of the T_m of the class I ternary complex by CD spectroscopy is indicative of the inherent affinity of the peptide for class I receptors (*23*). In the presence of salts, the T_m no longer reflects the peptide affinity for the class I receptor but represents the overall thermal stability of the class I ternary complex. Therefore, it seems likely that the different experiments are measuring two different processes. In the absence of salt, the denaturation of the complex is tied to the affinity of the peptide. In the presence of salts, the T_m is not related to peptide binding affinity. As can be seen in Table 1, the T_m is basically the same (60 °C) for each complex independent of the peptide bound (the error in T_m is roughly 1 °C). Thus, the process measured by CD in the presence of NaCl is different from the process measured during equilibrium peptide binding measures. The CD experiment in the presence of NaCl

apparently evaluates the inherent protein stability independent of the peptide bound.

Class I molecules have a difficult role in the adaptive immune response. They must bind a diverse set of peptides with different affinities and low specificity. Anchor residues reduce the number of peptides that may bind and limit the repertoire of presentable peptides. An examination of the structure of class I MHC shows that the peptidic termini are buried deep in pockets in the peptide-binding groove. It appears likely that a large conformational change would be required to release peptide. Some evidence of this phenomenon has been seen with specific antibodies (*49*). Here we show that class I MHC is less soluble in the presence of 150 mM NaCl than it is in the absence of NaCl. This may be an important aspect of the role of class I MHC in the immune system. Class I MHC must hold potentially antigenic peptides for long half-lives in order to allow the clonotypic T cells time to engage the ligands. $CD8^+$ T cells that recognize these complexes lyse the cells presenting the peptide ligand on class I MHC. Thus, it is critical that the viral or cancer indicating peptide be only presented on the infected or cancerous cells; therefore, peptide exchange from one class I molecule to another at the cell surface is undesirable. Here we show that class I molecules denature in the absence of peptide, which makes them incapable of binding another peptide. Additionally, this is another mechanism to fully exploit the available repertoire of peptides; the class I MHC molecules take advantage of the cellular environment. In particular, we have shown salts can stabilize the class I MHC ternary complex against thermal denaturation. Therefore, researchers measuring peptide-binding affinity by CD for development of immunotherapeutics should be careful to observe that the link between affinity and T_m does not hold true in the presence of NaCl. However, these insights allow the selection of peptides once classified as unusable because of their low T_m. These results resolve the dilemma of how class I MHC molecules loaded with extremely low-affinity peptides can have subphysiological T_ms yet are able to stimulate T cells in the immune system.

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