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Novel proteins associated with MHC class I antigens in cells expressing the adenovirus protein E3/19K

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Assembly of histocompatibility class I antigens (MHC) with β_2-microglobulin (β_2m) and peptide takes place in the rough endoplasmic reticulum (ER). At present, it is unclear why peptides generated in the cytosol or ER by proteolysis are not further degraded. Also, it is an open question whether assembly and/or peptide binding is self-instructive or is promoted by additional molecules, for example, chaperones. We previously demonstrated that the adenovirus glycoprotein E3/19K binds to human and some mouse MHC molecules in the ER, interfering with their transport to the cell surface. Here we show that immunoprecipitates from human cells that express transfected E3/19K and murine MHC K^d molecules not only contain MHC heavy chain, β_2m and E3/19K but also two additional proteins with apparent molecular weights of 100 kDa and 110 kDa. Biochemical characterization of these proteins, designated p100 and p110, demonstrates that they are transmembrane glycoproteins with a similar if not identical protein backbone. Consistent with a role as chaperones, we find that glucose starvation induces complex formation between p100/110 and MHC-E3/19K. Most interestingly, p100 and p110 are displaced from the complex by addition of K^d-specific peptides. Therefore, p100 and p110 might be chaperones that promote correct folding of MHC antigens and/or peptide binding to MHC.

Key words: adenovirus/chaperones/E3/19K/MHC class I antigens/peptide transfer

Introduction

Histocompatibility class I antigens are a family of cell surface glycoproteins composed of two polypeptides. A highly polymorphic transmembrane glycoprotein, encoded in the major histocompatibility complex (MHC), with an apparent molecular weight (M_r) of ~45 kDa is non-covalently associated with a light chain (M_r 12 kDa), called β_2-microglobulin (β_2m). A very remarkable feature of these MHC antigens (termed K, D and L in mouse and A, B and C, in human; Ploegh *et al.*, 1981) is their polymorphism (for review see Bjorkman and Parham, 1990) which seems to be related to their function, namely, to bind a large variety of peptides and present them to cytotoxic T cells (CTL; for reviews see Zinkernagel and Doherty, 1979; Townsend and Bodmer, 1989; Braciale and Braciale, 1991). Cells that express MHC molecules presenting a foreign peptide will be recognized and lysed by immune T cells.

It is currently believed that peptides presented by class I MHC antigens are generated by a proteolytic machinery in the cytosol (Ortiz-Navarrete *et al.*, 1991; Monaco, 1992) that degrades viral and cellular proteins to peptides. There is increasing evidence that these peptide fragments are then transported across the membrane of the rough endoplasmic reticulum (ER) by specific transporter proteins termed TAP1 and TAP2 (Monaco *et al.*, 1990; Trowsdale *et al.*, 1990; Arnold *et al.*, 1992; Momburg *et al.*, 1992; Powis *et al.*, 1992; Spiess *et al.*, 1992), although this has not been directly proven (Levy *et al.*, 1991). Peptide binding to heavy chains or heavy chain-β_2m complexes in the ER induces a conformational change within the MHC molecule that can be monitored by monoclonal antibodies (Townsend *et al.*, 1990; Elliot *et al.*, 1991). At present it is unclear how the antigenic peptides avoid complete degradation in the cytosol and ER, and what prevents their secretion from the ER. Also it is an open question whether assembly and/or peptide binding is self-instructive or is promoted by additional molecules, for example, chaperones (Degen and Williams, 1991; Neefjes *et al.*, 1991). However, only the trimolecular complex is thermodynamically stable and is transported efficiently out of the ER to the Golgi complex and the plasma membrane (Townsend *et al.*, 1989; Ljunggren *et al.*, 1990). In mutant cell lines that lack β_2m or the putative peptide transporters, the incomplete MHC complexes are transported inefficiently resulting in complete loss (β_2m^-; Ploegh *et al.*, 1981; Sege *et al.*, 1981) or very reduced MHC expression on the cell surface ($TAP1^-$; Townsend *et al.*, 1989; Hosken and Bevan, 1990; Ljunggren *et al.*, 1990).

We previously observed another mechanism whereby MHC class I antigens can be trapped inside the cell (Burgert and Kvist, 1985). Most human adenovirus (Ad) serotypes express an early non-essential glycoprotein (Pääbo *et al.*, 1986) that binds to the majority of human MHC antigens (Severinsson *et al.*, 1986; Burgert, Striebel and Ebenau-Jehle, in preparation) and also to some mouse MHC alleles (Burgert and Kvist, 1987; Cox *et al.*, 1990). Complex formation occurs in the ER (Andersson *et al.*, 1985; Burgert and Kvist, 1985) inhibiting transport of newly synthesized MHC antigens by virtue of an ER retention signal in the cytoplasmic tail of the Ad protein (Pääbo *et al.*, 1987; Gabathuler *et al.*, 1990). As a consequence, CTL recognition of $E3/19K^+$ cells *in vitro* is strongly impaired (Andersson *et al.*, 1987; Burgert *et al.*, 1987; Rawle *et al.*, 1989; Cox *et al.*, 1990). We postulated that this process contributes to the ability of this virus to persist in human beings.

Using exon shuffling of MHC molecules we demonstrated that both the α1 and α2 domains that form the peptide binding pocket are essential for complex formation with E3/19K (Burgert and Kvist, 1987). In the course of those studies we noted that each time the complex was formed, we precipitated not only MHC heavy chain, β_2m and

E3/19K, but also additional molecules with molecular weights of 100, 110 and 55–60 kDa.

Here, we have characterized the 100 and 110 kDa proteins in greater detail. We show that they are distinct molecules and not higher molecular weight forms of MHC-E3/19K. Both proteins contain N-linked carbohydrates and seem to have a transmembrane orientation. As complex formation with E3/19K is induced by glucose starvation they might belong to the group of stress proteins like chaperones. Interestingly, peptides can displace p100/110 from the MHC-E3/19K complex. Therefore, we believe that p100/110 might be involved in assembly of MHC with peptides.

Results

Two novel proteins associate with the MHC K^d molecule in human embryonic kidney cells expressing the Ad E3/19K protein

We previously demonstrated that the Ad protein E3/19K binds to human and some mouse class I MHC antigens in the rough ER, thereby interfering with transport of MHC antigens to the cell surface (Burgert and Kvist, 1985, 1987). In these studies, a transfection system was used in which the *Eco*RI D fragment of Ad2 containing the E3/19K gene was introduced into the human embryonic kidney cell line, 293. To investigate which MHC antigens bind to the E3/19K protein, we supertransfected the E3/19K$^+$ cell line, 293.12, with different murine MHC genes together with the hygromycin resistance gene. In addition, the same MHC genes were introduced into 293 cells not expressing E3/19K. Drug resistant clones were selected and complex formation was monitored by immunoprecipitation of MHC antigens with monoclonal antibodies (mAbs) followed by SDS–PAGE and fluorography. A typical result for the H-2K^d antigen is shown in Figure 1. In 293 cells transfected with the H-2K^d gene (293K^{d}2), two protein bands of 46 and 12 kDa (the K^d heavy chain and β_2-microglobulin, β_2m) were detected (Figure 1, lanes 1 and 3). Immunoprecipitation of K^d from the E3/19K$^+$ transfectant, 293.12K^{d}8, revealed additional bands (Figure 1, lanes 2 and 4): as expected, the E3/19K molecule with an M_r of 25 kDa was co-precipitated, but surprisingly also two additional proteins with molecular weights of 100–105 and 110–120 kDa were detected. Often also a diffuse band between 50 and 60 kDa was visible (see Figures 4, 5 and 8); however, as the amount varied, this protein was not further studied. Upon reduction of the samples, the two high molecular weight proteins, designated p100 and p110, did not shift to lower molecular weight forms, as typical for disulfide-linked molecules, but increased in molecular weight like β_2m, E3/19K and K^d, consistent with the presence of intramolecular disulfide bridges.

We decided to investigate the nature of p100 and p110 and the potential role of these molecules in MHC assembly in more detail. First, we ruled out the possibility that co-precipitation of p100 and p110 was a peculiarity of the particular antibody used. As shown in Figure 2, all five K^d-specific mAbs tested precipitated p100 and p110. The reactivity was also not due to a cross-reacting antibody present in the ascites fluids used for immunoprecipitation, since no difference in intensity of p100/110 was seen using protein A-purified antibodies from hybridomas grown in cell culture (Figure 2, compare lanes 3 and 4). Furthermore,

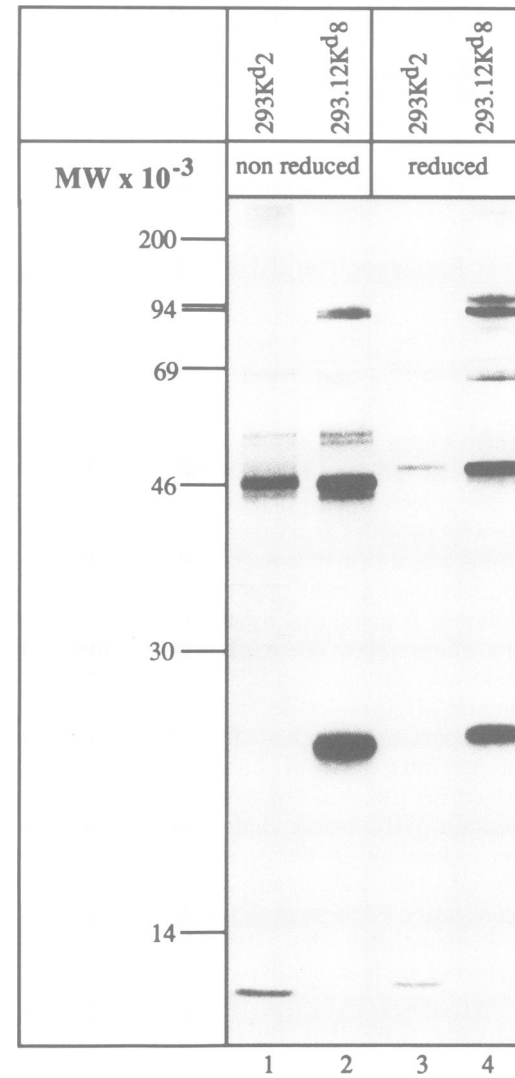

Fig. 1. SDS–PAGE analysis of MHC K^d-bound p100/110 molecules. The cell lines 293K^{d}2 (lanes 1 and 3) and 293.12K^{d}8 (lanes 2 and 4) were labeled for 1 h with 100 μCi/ml of [^{35}S]methionine and lysed, and H-2 K^d antigens were immunoprecipitated with the mAb 20-8-4. Immunoprecipitates analysed under reducing conditions (lanes 3 and 4) were treated with DTT before being loaded onto the gel. Protein bands visible at 69 kDa represent material nonspecifically bound to protein A–Sepharose. Molecular weight marker proteins were myosin (200 kDa), phosphorylase-b (~94 and 100 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa).

mAbs directed against β_2m also co-precipitated p100/110 (data not shown). Thus, all reagents that recognize the K^d molecule in these cells also co-precipitate p100/110.

Although the results shown in Figure 1 indicated that p100/110 did not consist of cysteine-linked subunits, it was still possible that p100/110 is a high molecular weight complex of K^d, E3/19K and β_2m, that is linked by other covalent bonds. To examine this, we prepared peptide maps of each protein. The immune complex was separated by SDS–PAGE, and bands corresponding to p110, p100, K^d and E3/19K were excised and treated with different amounts of V8 protease. The resulting fragments were separated on a second SDS–polyacrylamide gel (Figure 3). The peptide maps of p100/110 clearly differ from those of K^d and E3/19K (compare lanes 1–6 with lanes 7–9 and 10–12,

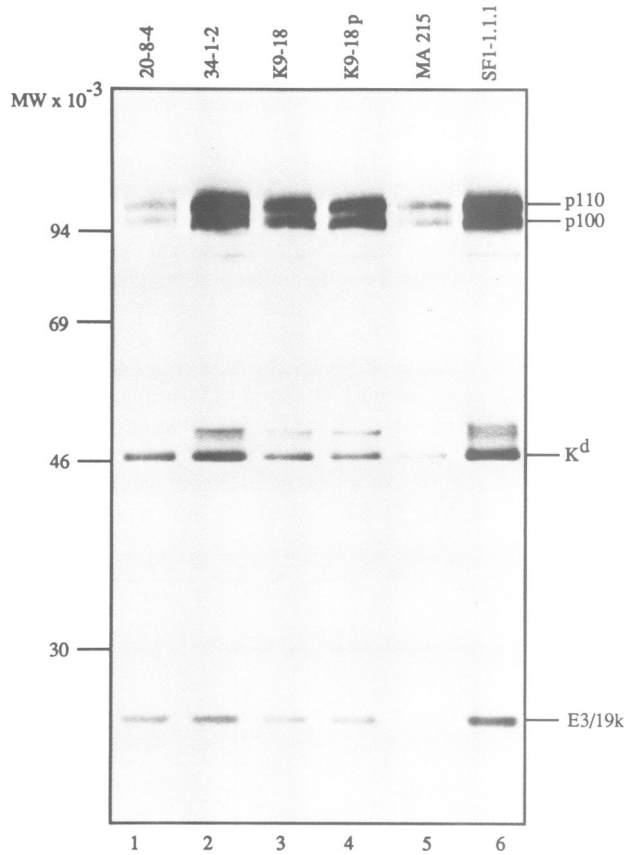

Fig. 2. Different H-2 K^d specific mAbs co-immunoprecipitate the p100/110 proteins. 293.12K^d8 cells were labeled for 1 h with 100 μCi/ml of [^{35}S]methionine and lysed, and H-2 K^d antigens and associated proteins were immunoprecipitated by mAbs 20-8-4 (lane 1), 34-1-2 (lane 2), K9-18 (lane 3), K9-18, protein A−Sepharose purified (lane 4), MA-215 (lane 5) and SF1-1.1.1 (lane 6). Only the portion of the gel ranging from 20 to 200 kDa is shown.

respectively), implying that p100 and p110 are distinct molecules and not higher molecular weight forms of K^d, E3/19K and β_2m. Furthermore, the peptide patterns of p100 and p110 are very similar, differing slightly in the lower molecular weight range (Figure 3, lanes 3 and 6), strongly suggesting that p100 and p110 have a similar if not identical protein backbone. Thus, p100 and p110 may be different post-translationally modified forms of a common precursor molecule.

P100/110 are transmembrane glycoproteins

To investigate this possibility a pulse−chase analysis was carried out. 293.12K^d8 cells were labeled for 20 min with [^{35}S]methionine and then either lysed or further incubated in medium containing an excess of unlabeled methionine for the time indicated at the top of Figure 4. As can be seen in lane 1, even after a short labeling period, p100/110 are already resolved as at least two distinct bands. Thus, we were unable to detect a precursor−product relationship between p100 and p110, although we cannot rule out modifications occurring within the labeling period. However, even after pulse labeling for 10 min, two discrete bands are visible (data not shown). Also, no shift to a higher molecular weight, typical for glycoproteins undergoing processing in the Golgi apparatus, is visible (see for example the processing of the K^d molecule in the control cells, lanes 9 and 10). At around 4−8 h of chase, p100/110 separate into four or five bands.

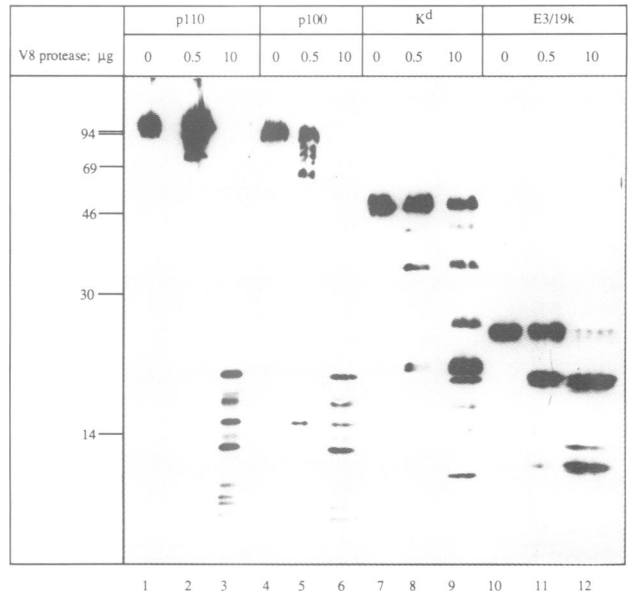

Fig. 3. The peptide maps of p100 and p110 are distinct from those of the H-2 K^d and E3/19K proteins. 293.12K^d8 cells were labeled for 1 h with 200 μCi/ml of [^{35}S]methionine and lysed, and H-2 K^d antigens and associated proteins were immunoprecipitated by the mAb 20-8-4. A proteolytic digest was performed from p110 (lanes 1−3), p100 (lanes 4−6), K^d (lanes 7−9) and E3/19K (lanes 10−12) proteins according to Cleveland *et al.* (1977). The concentration of V8 protease is indicated at the top of the figure.

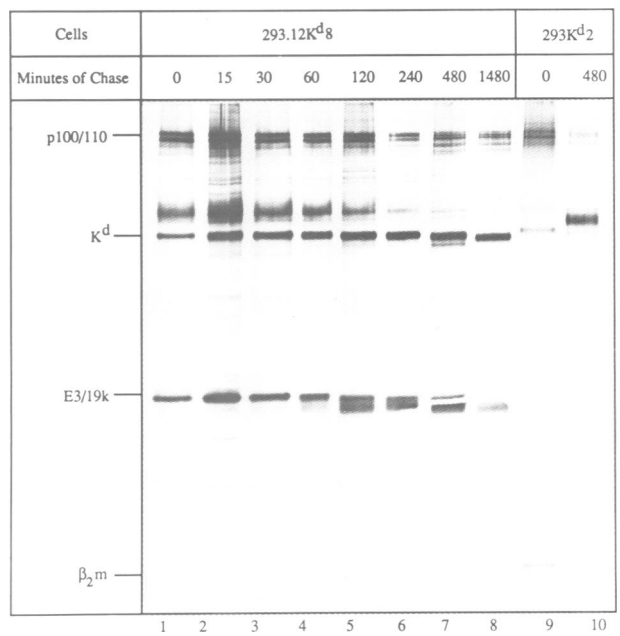

Fig. 4. Pulse−chase analysis of p100 and p110. The cell lines 293.12K^d8 and 293K^d2 were pulse-labeled for 20 min with 200 μCi/ml of [^{35}S]methionine and either lysed immediately (0) or incubated in medium containing an excess of cold methionine for the times indicated at the top of the figure and then lysed. Immunoprecipitation was done as described in Figure 2. In this particular SDS−PAGE the 55/60 kDa proteins mentioned before (above the K^d band) are rather prominent, continuously decreasing in intensity during the chase period. Their relationship to the other proteins remains unclear. Peptide mapping, however, did not reveal any similarity to p100/110 (data not shown).

Concomitantly, an increased co-precipitation of β_2m is noted. K^d and the E3/19K molecule show a higher mobility at the end of the chase period indicating trimming of their carbohydrates in the rough ER. In the control cell line, 293K^d2, (lanes 9 and 10), K^d shifts to a higher molecular weight after the chase period, indicative of transport through the medium and late Golgi compartments. Interestingly, p100/110 are also co-precipitated with K^d in 293K^d2 cells, although to a lesser degree.

We next investigated whether or not p100 and p110 are modified with N-linked carbohydrates and phosphates. Neither phosphatase treatment of [^{35}S]methionine-labeled immunoprecipitates nor labeling with [^{32}P]phosphate suggested that the proteins contained any phosphate (data not shown).

N-linked glycosylation was examined in two ways. First, 293.12K^d8 cells were labeled with [^{35}S]methionine in the absence (Figure 5, lane 2) or presence (Figure 5, lane 1) of tunicamycin, an inhibitor of N-glycosylation. Secondly, cells were labeled as usual, but the immunoprecipitates were subsequently treated with endoglycosidase F (Endo F), an enzyme that removes N-linked carbohydrates except for one, *N*-acetylglucosamine. Both treatments reduced the molecular weights of p100/110 by ~4–5 kDa. Similarly, the mobility of the other known glycoproteins, K^d and E3/19K, also increased (compare lane 3 with lane 4 and lane 1 with lane 2). Extrapolating from the molecular weight reduction seen in K^d and E3/19K (~7 kDa), we assume that p100 and p110 contain one or two N-linked carbohydrates. Hence, the two MHC-associated proteins are glycoproteins.

N-linked glycosylation of p100 and p110 implies that they are either luminal or transmembrane glycoproteins. To distinguish between these possibilities, microsomes were isolated from radioactively labeled cells and incubated with or without proteinase K. The results are shown in Figure 6.

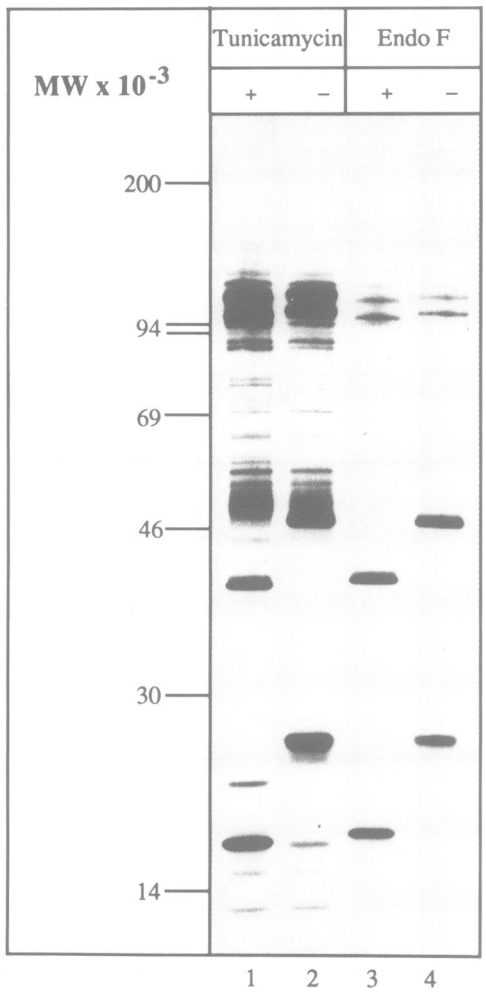

Fig. 5. p100 and p110 contain N-linked carbohydrates. 293.12K^d8 cells were labeled as described in Figure 2. Cells were either treated with 7 μg/ml tunicamycin for 1 h prior to and during the labeling period (lane 1) or proteins were cleaved with 0.5 U of Endo F after immunoprecipitation (lane 3). As controls for the tunicamycin and Endo F treatment, respectively, cells (lane 2) or immunoprecipitates (lane 4) were mock treated. The slightly higher molecular weight of the Endo F-treated proteins compared with that after tunicamycin treatment is due to the residual GlcNAc that is not removed by Endo F. The protein bands below p100/110, also visible in Figures 1, 2 and 4, might represent calnexin although we have not directly tested this (see Discussion).

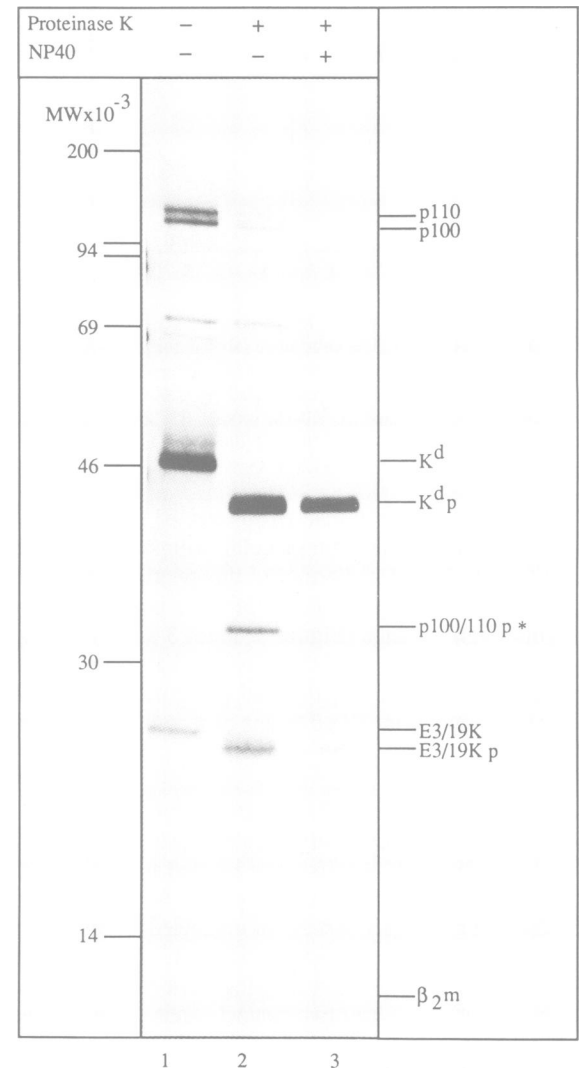

Fig. 6. Transmembrane topology of the p100/110 proteins. 293.12K^d8 cells were labeled for 1 h with 100 μCi/ml of [^{35}S]methionine and microsomes were prepared as described in Materials and methods. Treatment of microsomes with proteinase K or NP40 is indicated at the top of the figure. Immunoprecipitation was carried out with mAb 20-8-4. The symbols are as follows: K^dp, E3/19Kp and p100/110p* represent the proteinase K cleaved forms of these proteins. The other symbols are as in Figure 4.

Upon treatment with proteinase K, most of the p100/110 proteins are degraded and an additional band appears with an M_r of 32 kDa (Figure 6, lane 2, p100/110p*). This band is seen neither in the control sample lacking protease (Figure 6, lane 1) nor in immunoprecipitates of 293K^{d}2 cells treated with the protease (data not shown). Addition of detergent leads to a complete destruction of p100/110 including the 32 kDa band (Figure 6, lane 3). The transmembrane proteins, K^d and E3/19K, are shortened to the length expected after removal of their cytoplasmic exposed tail (K^{d}p and E3/19Kp), whereas the position of the luminal protein, β_2m, was unaltered, indicating a proper sealing of the microsomes (Figure 6, lane 2). Thus a portion of p100/110 is protected by the ER membrane indicating a transmembrane orientation of p100/110.

P100/110 are induced by glucose starvation

All the biochemical data suggest that p100/110 are two transmembrane glycoproteins that interact with K^d–E3/19K complexes in these cells. What is their function? P100/110 could be proteins that remove complexes/aggregates or misfolded proteins in the ER. However, we found no evidence that the K^d–E3/19K complexes are misfolded as they can be recognized by any mAb tested. Alternatively, they could have an MHC-specific function. For example, a chaperone may exist that promotes correct folding and assembly of MHC or peptide binding (Neefjes *et al.*, 1991).

As chaperones belong to the group of stress proteins which can be induced by a number of experimental conditions, such as heat shock, glucose starvation and calcium ionophore treatment (Welch, 1991; Rothman, 1989; Gething and Sambrook, 1992), we tested whether p100/110 are inducible by these treatments. 293K^{d}2 and 293.12K^{d}8 cells were mock-treated or heat shocked for 0.5 h and 2 h. After labeling of the cells with [^{35}S]methionine, the K^d molecule was precipitated as described above. No induction of p100/110 was noted in either cell line (Figure 7A, lanes 1–6), whereas a several-fold induction of the control heat shock protein (HSP) 72/73 protein was detected (Figure 7A, lanes 7–12). HSP72/73 is an internal control as it is co-precipitated with K^d at higher temperatures. Although detection of p100/110 depends on binding to the MHC class I molecule and thus the amount precipitable (along with the K^d molecule) may be limited, we should have seen an increase in co-precipitating material in 293K^{d}2, if they were HSPs. Furthermore, analysis of total lysates did not reveal

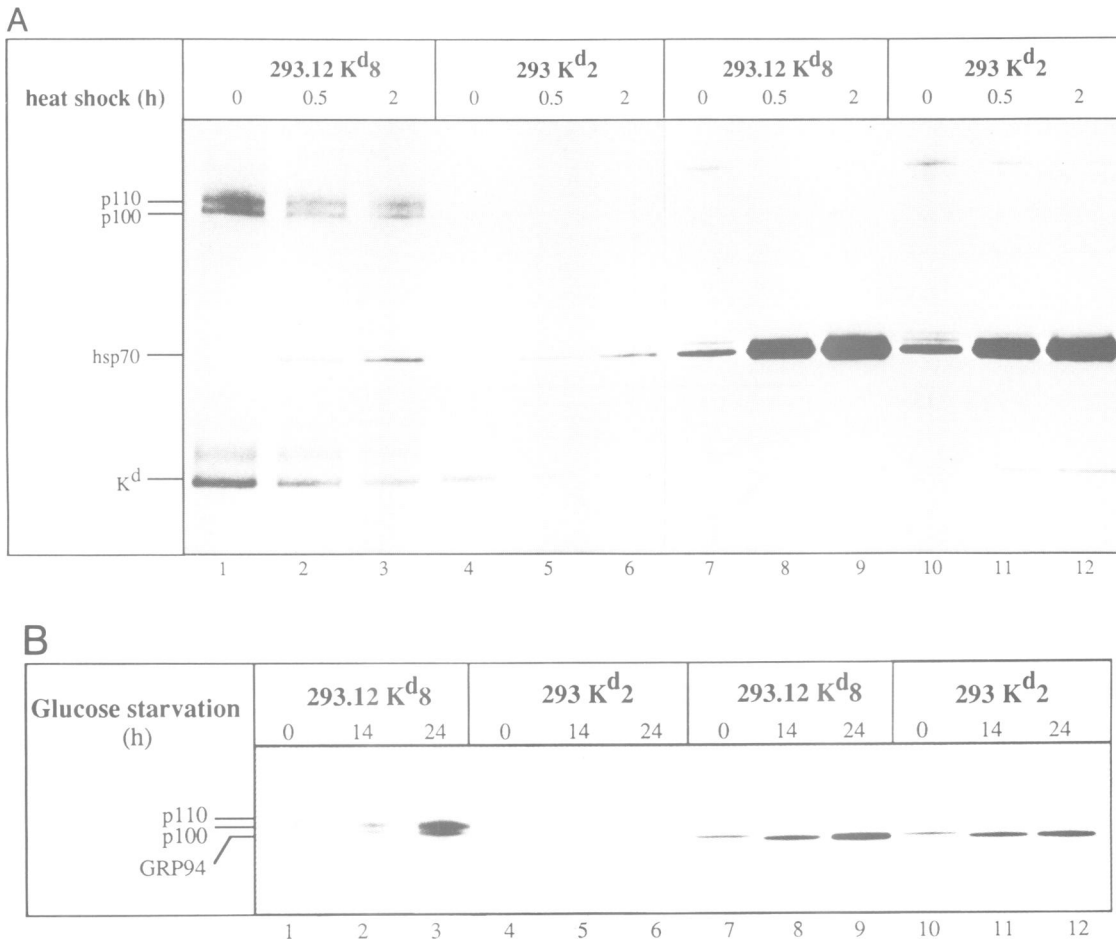

Fig. 7. p100/110 are induced not by heat shock but by glucose starvation. **(A)** 293.12K^{d}8 and 293K^{d}2 cells were heat shocked at 42°C for 0.5 (lanes 2, 5, 8 and 11) or 2 h (lanes 3, 6, 9 and 12), labeled for 1 h with 100 μCi/ml of [^{35}S]methionine and lysed. Immunoprecipitation was carried out with mAbs 20-8-4 (lanes 1–6) or W27 (anti-HSP 72/73, lanes 7–12). At higher temperatures HSP72/73 seems to co-precipitate with K^d. **(B)** 293.12K^{d}8 and 293K^{d}2 cells were deprived of glucose for 14 (lanes 2, 5, 8 and 11) or 24 h (lanes 3, 6, 9 and 12) and labeled as above. Immunoprecipitation was carried out with the mAb 20-8-4 (lanes 1–6) or a polyclonal antiserum against GRP94 (lanes 7–12), kindly provided by M.Green, St Louis, USA. Only a part of the gel (molecular weight range: 65–200 kDa) is shown. Immunoprecipitations were done with the same amount of cells. Background bands and e.g. that for β_2-microglobulin did not increase in intensity during glucose starvation.

an increased synthesis of p100/110, whereas HSP72/73 and presumably HSP90 were readily detectable after heat shock (data not shown). Therefore, p100/110 are not typical HSPs.

We next examined whether p100/110 belong to the group of chaperones up-regulated by glucose starvation, such as the glucose regulated protein GRP78 (BiP) and GRP94 (Gething and Sambrook, 1992; Mazarella and Green, 1987). Therefore, 293K^{d}2 cells and 293.12K^{d}8 cells were deprived of glucose by incubation in glucose-free medium for 14 and 24 h prior to immunoprecipitation. As seen in Figure 7B (lanes 1–3), increased amounts of p100/110 are found in K^d immunoprecipitates of 293.12K^{d}8 cells upon glucose starvation, very similar to the induction of GRP94 seen in those cells (Figure 7B, lanes 7–12). These data suggest that

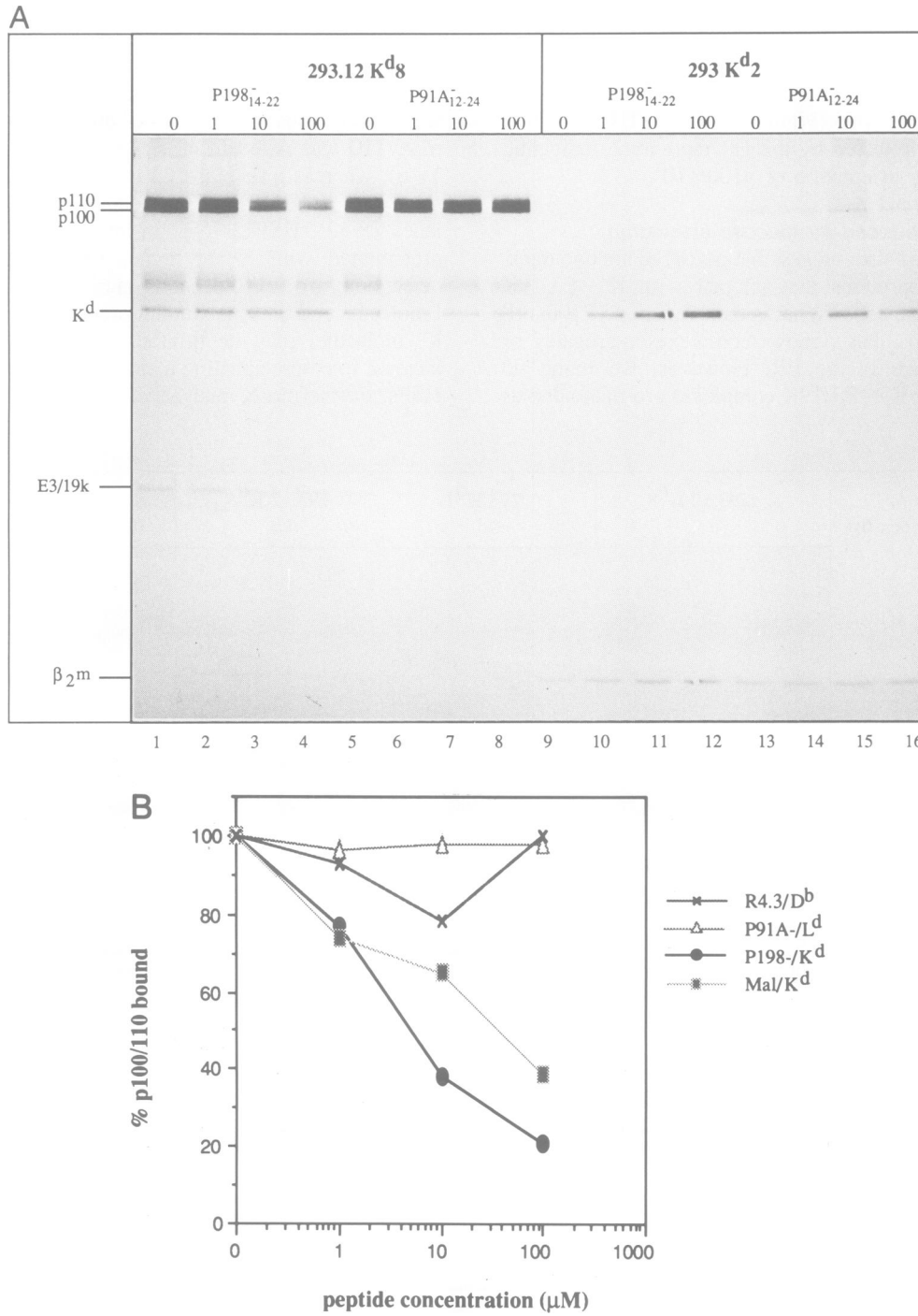

Fig. 8. K^d-specific peptides displace p100/110 from the complex. (**A**) 293.12K^{d}8 and 293K^{d}2 cells were metabolically labeled for 40 min. Microsomes were prepared as described in Materials and methods and incubated for 1 h at 37°C with different amounts of the K^d-specific peptide, P198$^-_{14-22}$, (lanes 1–4 and 9–12), or with a peptide that does not bind to K^d, P91A$^-_{12-24}$ (lanes 5–8 and 13–16). Thereafter lysis buffer was added and immunoprecipitation was carried out with mAb 20-8-4. Films were exposed for 30 h (lanes 1–8) or 4 days (lane 9–16), respectively. (**B**) Quantitative analysis of p100/110 binding to the K^d–E3/19K complex. The radioactivity of the p100/110 bands was determined using either a Phosphoimager (Molecular Dynamics, Krefeld, Germany; P198 and P91A) or by conventional densitometry of two fluorographic exposures (Mal and R4.3). Quantification was carried out from the experiment shown in Figure 8A (P198 and P91A) and from another experiment (Mal and R4.3; data not shown). The data were normalized by setting the amount of p100/110 co-precipitated without addition of peptides as 100.

p100/110 might be stress proteins whose association with MHC−E3/19K can be induced by glucose starvation. In addition, they show that p100/110 are not identical to GRP94, a resident ER stress protein with a luminal location which interacts with MHC class II molecules in the absence of the invariant chain (Schaiff *et al.*, 1992).

P100/110 can be specifically displaced by peptides

Assembly of MHC heavy chains with peptides and/or β_2m induces a conformational change in the MHC heavy chain (Townsend *et al.*, 1990; Elliot *et al.*, 1991) which in turn might induce the release of a putative chaperone-like molecule (Neefjes *et al.*, 1991; Degen *et al.*, 1992). We therefore investigated whether peptide binding displaces p100/110 from the K^d−E3/19K complex.

To test this, microsomes from metabolically labeled cells were incubated with increasing amounts of peptides and K^d molecules were subsequently immunoprecipitated. Two peptides derived from tumor rejection proteins were used: $P198^{-}{}_{14-22}$, previously shown to bind specifically to K^d, and $P91A^{-}{}_{12-24}$ which binds not to K^d but to L^d (Lurquin *et al.*, 1989; Sibille *et al.*, 1990). This specificity of peptide binding was confirmed in our system using 293K^d2-derived microsomes. Incubation with increasing amounts of K^d-specific peptide increased the amount of K^d precipitable by a conformation-specific antibody, whereas the L^d-specific peptide had no significant effect on K^d precipitation (Figure 8A, lanes 9−16). When microsomes of 293.12K^d8 cells were incubated with these peptides, co-precipitation of p100/110 was drastically reduced with increasing concentrations of the K^d-specific peptide (Figure 8A, lanes 1−4), but not with the L^d-specific one (Figure 8A, lanes 5−8). Densitometry of p100/110 (Figure 8B) reveals at the highest peptide concentration a reduction of p100/110 by 80%. Displacement of p100/110 was also seen when the K^d-specific peptide Mal (from malaria circumsporozoite protein) was used, while the D^b-specific peptide R4.3, derived from the Ad5 E1A protein (Kast *et al.*, 1989), lacked this capacity (Figure 8B). We conclude that K^d-restricted peptides compete with p100/110 binding.

This effect is also visible in 293K^d2 cells. Overexposure of the film from Figure 8A reveals bands at the position of p100/110 that can be specifically displaced by peptide addition (data not shown). Therefore, even in the absence of the Ad protein, a small amount of p100/110 is associated with the MHC K^d antigen and is displaced by K^d-specific peptides.

Complex formation between p100/110 and K^d is not restricted to 293.12K^d8 cells but is also observed in Jurkat cells and, remarkably, in murine SV40-transformed embryonic BALB/c fibroblasts (B7) expressing E3/19K and K^d molecules (data not shown). Thus, p100/110 are detectable in association with the MHC K^d antigen in mouse and human cells and the presence of E3/19K strongly induces or prolongs this binding.

Discussion

In this study, we have shown that the murine MHC K^d molecule expressed in 293 cells is associated with two proteins, designated p100 and p110, with M_rs of 100 and 110 kDa. Co-expression of the Ad protein E3/19K strongly induces this interaction. As a first step towards understanding their function, we have characterized these proteins biochemically. Peptide mapping studies (Figure 3) and SDS−PAGE under reducing conditions (Figure 1) indicate that p100 and p110 are distinct molecules and not higher molecular weight forms of K^d, E3/19K and β_2m. P100/110 have a similar if not identical protein backbone, are N-glycosylated and contain intramolecular disulfide bonds (Figures 5 and 1). Protease digestion experiments of microsomes (Figure 6) suggest that p100 and p110 are transmembrane proteins.

At present, we do not know the structural relationship between p100 and p110. All attempts to detect a differential post-translational modification, such as N-glycosylation or phosphorylation (Figures 4 and 5; data not shown), were unsuccessful. Therefore, the difference in molecular weight may be the result of differential splicing.

Both the biochemical and the functional data indicate that p100 and p110 are newly discovered proteins that specifically interact with MHC class I molecules. Although a number of proteins in the microsomal fraction with similar M_r are known, none of these has properties identical to those ascribed to p100/110. We ruled out the possibility that p100 and p110 are identical to β-COP, the coat protein of the Golgi stack and Golgi-derived vesicles which has an M_r of 110 kDa (Duden *et al.*, 1991; Serafini *et al.*, 1991). First, β-COP is not a transmembrane protein and second, in our gel system β-COP has a higher mobility than p100/110 (data not shown). We were also unable to show any similarity with a 90 kDa protein that is immunoprecipitated with a mAb against E3/19K and is considered to be a cellular homolog of E3/19K (Cox *et al.*, 1991; data not shown).

We believe that the co-precipitation of p100/110 is specific since co-precipitation does not occur with major resident ER proteins, such as GRP78 (BiP; Gething and Sambrook, 1992) and ERp99/GRP94 (Mazzarella and Green, 1987). GRP94 shares a number of properties with p100 and p110 but differs in its molecular weight (Figure 7B) and in its location in the ER lumen (Schaiff *et al.*, 1992; M.Green, personal communication). Most remarkably, p100 and p110 are not identical to calnexin (p88), another abundant ER membrane-protein that can be cross-linked to murine MHC molecules (Degen and Williams, 1991; Ahluwalia *et al.*, 1992; Degen *et al.*, 1992) and is found complexed to human HLA class I molecules but also to a number of other molecules (Höchstenbach *et al.*, 1992). Calnexin is a phosphoprotein, is non-glycosylated and has a different molecular weight from p100/110 (data not shown). Thus, p100/110 seem to be novel proteins not formerly described. However, they might be related to the ~105 kDa molecules found by Townsend and colleagues in a reciprocal system in association with D^b molecules of RMA-S lysates containing an excess of human β_2m (Townsend *et al.*, 1990). The authors suggested that the 105 kDa molecule may bind specifically to empty class I molecules or may catalyze MHC assembly or both. The existence of such a molecule has been suggested previously by Cresswell and colleagues (Alexander *et al.*, 1990). We have not analyzed D^b in our system as it does not bind to E3/19K.

P100/110 seem also to be unique regarding their functional activity. Their specific release from the MHC−E3/19K complex by addition of K^d-specific peptides makes them attractive candidates for the chaperonin proposed to take part in MHC folding, assembly and/or peptide binding (Neefjes *et al.*, 1991). Consistent with such a role, we find increasing amounts of p100/110 in K^d immunoprecipitates upon

glucose starvation. Initially, the 88–90 kDa protein (calnexin) was considered to be an MHC-specific chaperone (Degen and Williams, 1991; Ahluwalia *et al.*, 1992; Degen *et al.*, 1992) but recently it became obvious that it is associated with a number of other proteins (Höchstenbach *et al.*, 1992). As the association of calnexin with MHC is prolonged in cells that lack the putative peptide transporters and is found throughout the lifetime of an MHC molecule in β_2m-deficient cells, this molecule may serve a more general chaperone function rescuing e.g. incompletely assembled MHC molecules (Degen *et al.*, 1992). In our system, the K^d molecule appears not to be in an aberrant conformation recognized by these more general chaperones (Figure 7). This is supported by the data from Figure 2 showing that K^d molecules are in a rather native conformation detectable by all conformation-dependent K^d-specific antibodies tested.

Other authors hypothesized that HSPs like gp96 may function in transferring peptides from tumor antigens to MHC class I molecules (Srivastava and Maki, 1991). However, direct evidence, such as co-precipitation with MHC or peptide binding is lacking. P100/110 might be related to gp96 but are distinct with regard to their location in the ER membrane (rather than ER lumen) and their resistance to heat shock.

The specific release of p100/110 from the MHC–E3/19K complex by addition of K^d-specific peptides is a novel finding for an MHC-associated molecule. We believe that p100 and p110 function in peptide binding to MHC in general, although only the K^d molecule was investigated. We postulate that in normal cells, the interaction between p100/110 and MHC molecules is short-lived due to efficient peptide binding and, therefore, a complex is not detectable in immunoprecipitations. In 293K^d2 cells, peptide loading seems to take longer presumably due to peptide shortage or inefficient β_2m binding. Therefore, interaction of p100/110 with MHC is prolonged and can be monitored. The presence of E3/19K in 293.12K^d8 cells seems to stabilize this interaction, possibly by inhibiting further transport or peptide binding.

Supporting this view we find a lack of K^d-specific peptides in 293K^d2 cells. Most of the K^d molecules cannot be detected by K^d-specific mAbs unless K^d-specific peptides are added (Figure 8A, lanes 9–12). Using an antiserum against the cytoplasmic tail of K^d, we confirmed that a large proportion of K^d molecules is not fully assembled and therefore is not precipitated with mAbs (data not shown). This phenomenon is reminiscent of that observed in RMA-S cells and embryo-derived cell lines (Bikoff *et al.*, 1991), which lack expression of functional peptide transporters. Further work is necessary to clarify whether this is also the case in 293 cells which are embryo-derived cells or is a more general phenomenon of a heterologous system.

The opposite is true of the E3/19K^+ 293.12K^d8 cells where nearly all K^d molecules are in a conformation detectable by K^d-specific mAbs (D.Feuerbach and H.-G.Burgert, unpublished results). Presumably, E3/19K binding induces a conformational change similar to peptide binding. This might explain why we do not see an increase in precipitable K^d molecules after addition of peptides to 293.12K^d8-derived microsomes.

Complex formation between p100/110 and K^d molecules is not restricted to 293 cells but is also observed in Jurkat cells expressing E3/19K and K^d molecules. Most strikingly, however, SV40-transformed embryonic fibroblasts from BALB/c mice, naturally expressing K^d MHC antigens are associated with proteins of similar M_r as p100/110. Again, increased amounts of these 100 kDa proteins are detectable when E3/19K is present in these cells (H.-G.Burgert and C.Ebenau-Jehle, data not shown). Thus, p100 and p110 are also observed in a homologous system.

In summary, complex formation between p100/110 and K^d is found to some extent in all K^d-expressing cells tested, and seems to be amplified by binding of E3/19K to the MHC molecule. At present, it is unclear whether other MHC molecules that bind E3/19K will associate with p100/110 when peptides are lacking. As we have no evidence for an increased expression of p100/110 in E3/19K^+ cells, it is open if E3/19K promotes p100/110 binding by inhibiting peptide binding to MHC or the transport of MHC molecules, or by locking MHC molecules in a conformation whereby p100/110 cannot be released.

Alternatively, p100/110 may function as MHC-dependent proteases that may cleave the N-terminus of MHC-bound peptides to the correct size (octamer or nonamer; Eisenlohr *et al.*, 1992; Rötzschke and Falk, 1991). Future experiments will aim to distinguish between these possibilities and to obtain sufficient material for microsequencing and the generation of antibodies.

Materials and methods

Cell culture

Cell lines were derived and propagated as previously described (Burgert and Kvist, 1987). Cells were maintained in DMEM containing 10% FCS and antibiotics. For routine culture, the medium of 293K^d2 and 293.12K^d8 cells was supplemented with 200 μg/ml G418 (Gibco BRL, Eggenstein, Germany). Medium for the latter contained in addition 100 μg/ml hygromycin (Calbiochem, San Diego, USA).

Monoclonal antibodies and antisera

The following antibodies were used in this study: 34-1-2 (anti-H-2 K^d, D^d; Ozato *et al.*, 1982); 20-8-4 (anti-K^d, K^b, Qa; Ozato and Sachs, 1981); K9-18 (anti-H-2K^d, D^b; Arnold *et al.*, 1985); SF1-1.1.1. (anti-K^d; ATCC HB 159); MA-215 (anti-K^d; Hasenkrug *et al.*, 1987); W6/32 (anti-HLA-A,B,C; Barnstable *et al.*, 1978); W27 (anti-HSP72/73, Oncogene Science, Manhasset, USA). The polyclonal antiserum, anti-HS1, directed against the N-terminal peptide (DDEVDVDGTVEEDLGK) of GRP94 was kindly provided by M.Green, St Louis, USA.

Cell labeling, tunicamycin treatment, immunoprecipitation and SDS–PAGE

Labeling of cells with [^{35}S]methionine, tunicamycin treatment, immunoprecipitation and SDS–PAGE have been described in detail previously (Burgert and Kvist, 1985, 1987).

Endoglycosidase F treatment

After immunoprecipitation of antigens with the mAb 20-8-4, protein A–Sepharose-bound material was treated with 0.5 U of Endo F (Boehringer Mannheim, Germany) for 20 h at 37°C. Incubation was done in 0.2% NP40, 10 mM Tris (pH 7.8), 0.15 M NaCl and 2 mM EDTA (pH 8.0). After incubation, the beads were washed once with 10 mM Tris–HCl (pH 7.4) before release of the bound material with SDS sample buffer.

Preparation of microsomal membranes and incubation with peptides

$2–7 \times 10^6$ cells were trypsinized, pelleted by centrifugation at 300 *g* and resuspended in Dounce buffer (10 mM Tris pH 7.4, 10 mM KCl, 5 mM $MgCl_2$). After 10 min swelling on ice, the cells were homogenized with 20 strokes of a tightly fitting Dounce homogenizer. Intact cells and debris were removed by centrifugation at 800 *g*. The supernatant was centrifuged at 100 000 *g* for 60 min. The pellet containing the microsomal membranes was resuspended in 0.2 ml phosphate buffered saline.

For the peptide incubation assays, the peptides were added to a final concentration of 1–100 μM and incubated for 1 h at 37°C. Thereafter, microsomes were centrifuged again at 100 000 *g* or directly solubilized and

processed for immunoprecipitation. The following peptides were used: P198$-^{14-22}$, KYQAVTTTL (Sibille *et al.*, 1990); P91A$-^{12-24}$, ISTQNHRALDLVA (Lurquin *et al.*, 1989); MalCS 253–260, YIPSAEKI (Luescher *et al.* 1991); and R4.3 Ad5 E1A 234–243, SGPSNTPPEI (Kast *et al.*, 1989).

Proteinase K treatment of microsomes

For the experiment shown in Figure 6 the microsomal pellet was resuspended in 0.2 ml Dounce buffer followed by addition of proteinase K to a final concentration of 20 μg/ml. Digestion was performed for 1 h on ice and terminated by addition of PMSF to a final concentration of 100 μg/ml. After 10 min on ice, immunoprecipitation was carried out as described by Burgert and Kvist (1987).

Peptide mapping

Peptide mapping experiments were performed essentially as described by Cleveland *et al.* (1977). The H-2K^d molecule was immunoprecipitated from 1–2 × 10^7 [^{35}S]methionine-labeled 293.12K^{d}8 cells with the mAb 20-8-4. Immunoprecipitates were separated on SDS–polyacrylamide gels. The unfixed gel was dried and subjected to autoradiography. Bands corresponding to p110, p100, K^d and E319/K were excised and transferred into the slots of a new gel. The gel slice was overlaid with 0.1% SDS, 20% glycerine, 1 mM EDTA, 2.5 mM DTT, 0.066 mM Tris (pH 6.8), 0.01% bromophenol blue and 0.5–10 μg of *Staphylococcus aureus* V8 protease (Boehringer-Mannheim, Germany). The gel was run until the bromophenol blue had migrated about two-thirds of the distance into the stacking gel. Then electrophoresis was stopped for 30 min to allow proteins to be digested.

Glucose starvation and heat shock of tissue culture cells

Cells were incubated at 37°C in glucose-free DMEM containing 10% dialyzed fetal calf serum for 12 or 22 h. The medium was then changed to glucose-free and methionine-free DMEM. After 60 min incubation, cells were labeled with the same medium containing 100 μCi/ml of [^{35}S]methionine.

For the heat shock experiment, cells were grown in small (25 cm^2) tissue culture flasks. Heat shock was effected by addition of fresh culture medium prewarmed to 42°C and supplemented with 30 mM HEPES pH 7.2. Cell culture flasks were submerged in a 42°C water bath. At the end of the appropriate stress period (times indicated in the figures), methionine starvation was carried out as described above. Then cells were labeled at 37°C with 100 μCi/ml of [^{35}S]methionine.

Other reagents

Protein A–Sepharose was from Pharmacia (Uppsala, Sweden). [^{35}S]methionine was purchased from Amersham, Braunschweig, Germany.

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