In primary embryonal fibroblasts from transgenic mice expressing H-2 genes and a miniature swine class I transgene (PD1), transformation with the highly oncogenic Ad12 results in a reduction in peptide transporter and proteasome-associated (LMP2 and LMP7) gene expression, and suppression in transport and cell surface expression of all class I antigens. The selective suppression in transport of H-2 (but not of PD1) molecules in cells reconstituted for the expression of peptide transporter and LMP genes implied that an additional factor(s) is involved in the assembly of class I complexes. Here we show that the $\beta_2m$, H-2D$^b$, and H-2K$^b$ genes are transcribed and translated in Ad12-transformed cells. However, unlike normal and E1Ad5-transformed cells, in which $\beta_2m$ is either secreted unbound or bound to class I heavy chains, in Ad12-transformed cells significant amounts of $\beta_2m$ are retained in the cell bound to the membrane, but free of class I heavy chains. This abnormal turnover of $\beta_2m$ in the Ad12-transformed cells suggests the existence of a novel $\beta_2m$-binding molecule(s) that sequesters $\beta_2m$, and this process may provide a mechanism by which transformation with Ad12 may subvert class I complex formation.

MHC class I molecules are polymorphic, integral membrane proteins that bind a diverse group of peptides derived from endogenous antigens and display these peptides for recognition by cytotoxic T-lymphocytes (1). This mechanism enables the immune system to control infectious diseases and the growth of tumor cells (2, 3). Indeed, cells infected by a variety of viruses that interfere with cell surface expression of class I antigens, as well as tumors of various origins that demonstrate suppressed levels of class I antigens, can escape immune surveillance (4).

The biochemistry and cell biology of antigen processing and presentation by class I MHC molecules has been analyzed in detail in recent years (5–8). The crucial role of $\beta_2m$ in maintaining cell surface class I MHC molecules (13–15). The existence of a substantial pool of inactive heavy chains on the cell surface, which are able to bind added $\beta_2m$ at 37 °C, has been demonstrated by Rock et al. (23), implying a physiological role for exogenous $\beta_2m$ in maintaining cell surface class I heavy chains in a state suitable for subsequent binding of exogenous peptides.

Increased levels of $\beta_2m$ can be detected both in the peripheral circulation and locally, in many abnormal in vivo conditions (24–28). Additionally, lymphoid and hematopoetic cells have been shown to produce and secrete $\beta_2m$ when stimulated with various cytokines (29, 30). If these augmented levels facilitate the accumulation of class I-bound peptides, then the regulation of $\beta_2m$ secretion may have functional consequences. Supportive of such a role are data demonstrating the strong in vivo priming of cytotoxic T-lymphocytes to class I-binding peptides if the latter are combined with $\beta_2m$ when injected into mice (31).
expression of all class I antigens (12, 32–34). Expression of these class I genes is either normal or up-regulated in cells transformed by the non-oncogenic virus Ad5. Re-expression of TAP and LMP in an Ad12-transformed cell line completely reconstituted the cell surface expression and transport of PD1 and induced the transport of 20% of the H-2Db molecules, but did not affect the transport or expression of H-2Kb molecules (34). These data, as well as the fact that in Ad12-transformed cells, H-2 molecules were not recognized by conformation-independent antibodies (33, 34), raised the possibility that either the synthesis of β2m in these cells is defective and the class I heavy chains compete for a limited amount of β2m molecules (PD1 being more competitive), or that Ad12-transformed cells are deficient in a factor(s) that facilitate the assembly of β2m with a particular set of class I heavy chains (H-2). We now present data substantiating that β2m, as well as H-2Db and H-2Kb, are transcribed and translated in Ad12-transformed cells. However, the results show an abnormal turnover of β2m molecules and suggest the existence of a novel β2m-binding molecule(s) that acts to retain β2m in the transformed cells.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—The Ad12-transformed (VAD12.79, VAD12.42, VAD12.20, VAD12.25, VAD12.36, VAD12.54, and VAD12.43), E1A5-transformed (AS01 and AS05), and TAP-transfected cell lines and the normal cell line M1 have been described previously (32–34). Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 0.1% Triton X-100, 50 mM Tris (pH 7.5), and 150 mM NaCl. For EndoH treatment, immunoprecipitates were eluted by adding 25 mM Tris (pH 7.6), 0.5 mM MgCl₂ with protein inhibitors and incubated on ice for 30 min. The cells were lysed with a Dounce homogenizer, and the tonicity of the solution was restored by adding NaCl to a final concentration of 0.15 M. The suspension was spun at 500 × g to remove the nuclei, and the supernatant was further centrifuged for 45 min at 100,000 × g at 4°C. The supernatant, which contained soluble proteins, was transferred to a separate tube, and the pellet was resuspended in lysis buffer.

**Probes and Plasmids**—The following probes have been described previously (32–34); the actin probe was a PstI-PstI fragment from chicken β actin cDNA, the PD1-specific probe was a Sau3A-BamHI genomic fragment containing exons 2–7 of the PD1 gene, the H-2 probe was an EcoRI-HindIII fragment derived from pH-2K3 (H-2Kb), the β2m probe was a PstI-PstI fragment from β2m cDNA, and the histone probe was a BamHI genomic fragment. Plasmids containing H-2Kb cDNA and H-2Db genomic (pMo/Db) fragments (40) were used as templates for PCR reactions and were a kind gift from Dr. L. Eisenbach (The Weizmann Institute of Science, Rehovot, Israel) and from Dr. D. Pardoll (Johns Hopkins University, Baltimore, MD), respectively.

**Hybridizations**—The hybridization solution contained 4 × SSC, 50% formamide, 0.2% SDS, 0.5% polyvinylpyrrolidone (PVP), and 0.1% salmon sperm DNA. Hybridizations were carried out at 42°C, followed by washes with 2 × SSC, 0.1% SDS at room temperature, and 0.2 × SSC at temperatures ranging between 55 and 65°C. After stripping with a boiling solution of 0.1% SDS, the blots were used for additional hybridizations.

**RNA Analysis**—Cytoplasmic RNA was prepared using a modification of the White and Bancroft method (41), as described previously (34). RNA was denatured and fractionated on a 1.2% formaldehyde/formamide agarose gel, blotted onto a Hybond-N membrane (Amersham International, Little Chalfont, UK) and hybridized with the appropriate probe, which had been labeled with [α-32P]dCTP (Rotem Industries, Dimona, Israel), using a Random Priming labeling kit (U. S. Biochemical Corp.).

**Polysome Fractionation**—Polysomes were fractionated as described previously (42). Cells were grown to 80% confluence and harvested by trypsination (0.25 mM trypsin EDTA; Biological Industries, Israel). A quantity of 6 × 10⁷ cells was lysed per fractionation. The cells were washed with PBS, and cell pellets were kept at −70°C until used. Trypsin, PBS, and media contained 100 μg/ml cycloheximide (Sigma). Cells were resuspended in ice-cold RNA isolation buffer containing 10 mM NaCl, 10 mM Tris (pH 7.4), 1.5 mM MgCl₂, and 50 μl of ribonuclease vonadyl complexes (200 mM) (New England Biolabs). Cells were lysed following the addition of 50 μl of polysomal buffer containing 10% Triton X-100 and 10% deoxycholate by brief mixing and after 3 min of incubation on ice. Nuclei were pelleted by centrifugation for 3 min at 4°C, and the postnuclear supernatant was diluted with an equal volume of 25 mM Tris (pH 7.5), 10 mM MgCl₂, 25 mM NaCl, 0.14 M sucrose, 500 μg/ml heparin, 0.05% Triton X-100. The suspension was layered over 35 ml of 15–45% (w/w) sucrose gradient containing 10 mM NaCl, 10 mM Tris (pH 7.4), 1.5 mM MgCl₂, and 50 μl of ribonuclease vonadyl complexes (200 mM) (New England Biolabs). Cells were then labeled with a 2-ml cushion of 45% sucrose. The 2-ml sucrose cushion was added to the 45% sucrose gradient, the 1:1 volume of 45% sucrose was transferred, and the 1:1 volume of 15% sucrose was added to the gradient. The samples were then passed through a 26,000 rpm for 4 h at 4°C in a Beckman SW27 rotor. After centrifugation, 38 fractions of 1 ml each were collected into tubes containing 10 μl of 10% SDS. The A260 was monitored, as the RNA digestion profile was determined, and each 2–3 tubes were pooled to give a total of 8–10 tubes. RNA was extracted from pools by TCA precipitation and subpolysomal fractions as described before. Only gradients with identical polysomal profiles were compared.

**RT-PCR**—Reverse transcription of RNA was carried out by using 1 μl of 1 μl each of 25 μM oligo(dT)₁₈ primer (New England Biolabs), 1 μM nucleotide mixture, 5 mM MgCl₂, 35 units of ribonuclease inhibitor (MBI Fermentas, Vilnius, Lithuania), and 7 units of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI). The cDNA was stored at −25°C until used.

**PCR** was carried out with 1 μl of the cDNA, and 50 pmol of the following primers (40): a 5' consensus primer for H-D and H-2Kb (5'-CCG GAC GCT GCT GCG CAC AGG-3'), and 3' primers specific for H-2Db (5'-TAC AAT CTC GGA GAG ACA TT-3') or H-2Kb (5'-TAC AAT CTC GGA GAG ACA GA-3'), 40 μM nucleotide mixture, and 0.375 units of Taq DNA polymerase (Promega). Each PCR cycle included 1 min of denaturation at 94°C, 1 min of annealing at 65°C, and 1 min of
extension/synthesis at 72 °C. After the appropriate number of PCR cycles (MiniCycler, MJ Research Inc., Watertown, MA), 10 μl or less of the reaction mix were fractionated on a 1% agarose gel. The gel was soaked twice for 20 min in 1.5 M NaCl, 0.5 M NaOH at room temperature and blotted onto a Hybond-N membrane (Amersham International, Little Chalfont, UK) with transfer buffer containing 1.5 M NaCl, 0.25 M NaOH. The membrane was baked for 10 min at 80 °C, and the DNA was cross-linked to the membrane by exposure to UV.

Since both genes yield an RT-PCR product that is identical in size, two positive and two negative controls were included in each set of reactions. A plasmid containing a genomic fragment from H-2Dβ was amplified with H-2Dβ primers and with H-2Kb primers, and a plasmid containing H-2Kβ cDNA was amplified with H-2Kβ primers and with H-2Dβ primers. Since the yield of the PCR product is proportional to the starting amount of the template, only under conditions in which PCR amplification proceeds exponentially at a constant efficiency (43, 44) is the PCR reaction quantitative. A titration curve for amplified cDNA from each cell line and for each gene was plotted to ensure that the number of amplification cycles was below the plateau level. Detailed analysis of the results is presented under “Results.”

Quantitation of Radioactive Signals—Dried radioactive gels or blots were exposed to a phosphoimager screen and analyzed with a phosphoimager (Fuji BAS1000, Tokyo, Japan). The data are presented as phosphostimulated luminescence (PSL) units. In some cases, following scanning densitometric analysis of the x-ray films was performed.

RESULTS

Steady State Level of Class I and β2m mRNA in Ad12-transformed Cells—Due to post-transcriptional interference with class I assembly and transport (32, 33), most of the Ad12-transformed cell lines express very low levels of class I antigens despite having near normal levels of class I mRNA. Fig. 1 (A and B) substantiates previous data from our laboratory (12, 32), demonstrating that nearly all the Ad12-transformed cell lines express normal or elevated levels of the miniature swine class I transgene (PD1) mRNA, and a normal level or less than a 2-fold reduction in H-2 mRNA, as compared with that in the normal cell lines as exemplified by M1. The level of class I mRNA in E1Ad5-transformed cells is either normal or enhanced. Full restoration of cell surface expression level and transport of PD1 was achieved by re-expression of peptide transporter molecules in the Ad12-transformed cell line VAD12.79 (12) but the expression level of H-2Dβ was only partially restored, and the expression level of H-2Kβ remained very low in these TAP-reconstituted and in TAP-LMP-reconstituted cells (12, 34). In order to examine whether the selective suppression of H-2Kβ expression was mediated by H-2Kβ-specific gene regulatory elements, the mRNA levels of the individual H-2 genes were analyzed in the transformed cells. Since efficient detection and quantitation of H-2 mRNA by Northern blot analysis requires a probe that does not distinguish H-2D from H-2Dm mRNA, we utilized a quantitative RT-PCR assay in order to determine the relative amounts of mRNA encoded by each of these genes. A total of 5–30 PCR cycles were performed for each of the cDNAs that were reverse-transcribed from total RNA. The PCR products were fractionated on agarose gels, blotted, hybridized to an H-2 probe, and the radioactive signal was quantitated, following various exposure periods. Fig. 2A shows the results of the analyses of H-2 cDNA in one of the transformed cell lines, using specific primers for H-2Kβ and H-2Dβ. The primers were complementary to the same region in the two genes, thus minimizing possible differences that could

![Fig. 1. Steady state level of class I heavy chains and β2m mRNA in normal and Ad-transformed cell lines.](http://www.jbc.org/)
Regulation of $\beta_{2m}$ in Ad12-transformed Cells

**Fig. 2. Comparison of steady state level of H-2Kb and H-2Db mRNA as determined by RT-PCR in normal and Ad-transformed cell lines.** RT-PCR was carried out using primers specific for H-2Kb and H-2Db. PCR products were fractionated on agarose gels, blotted onto nylon membranes, and hybridized with an H-2 probe. Each blot was exposed for various time intervals, assuring that all test points were at the linear detection range of the phosphoimager. A response curve for amplified cDNA from each cell line and for each gene (radioactive signals are presented as log PSL), was plotted as a function of the number of PCR cycles. Representative response curves are shown in A. A representative gel, which includes samples from a normal cell line (M1), E1Ad5-transformed cell line (A5O1 and A5O5) and Ad12-transformed cell lines (VAD12.79 and VAD12.42), is shown in B. The blot was exposed for various intervals, and the results of the best exposure times are displayed. Specificity controls using plasmid DNAs as templates are shown in the first four lanes. The ratio between H-2Db and H-2Kb signals in three individual experiments is summarized in Table I. D$^+$-g, a plasmid containing a genomic fragment of H-2Db. K$^+$-c, a plasmid containing a cDNA fragment of H-2Kb. RT-PCR prod., RT-PCR products.

RT-PCR results were supported by data obtained with TAP-reconstituted VAD12.79 and VAD12.42, Ad12-transformed cell lines. The reduced levels of $\beta_{2m}$ mRNA in Ad12-transformed cells might limit $\beta_{2m}$ synthesis to a level whereby competition for this molecule by different class I heavy chains results in inefficient assembly and transport of particular class I complexes.

**Translation of Class I Heavy Chains and $\beta_{2m}$ mRNA in Ad12-transformed Cells—As we were not able to immunoprecipitate significant amounts of H-2 molecules, even with antibodies directed against the $\alpha_2$ domain or the cytoplasmic tail of class I antigens (33, 34), the possibility that the class I heavy chains and $\beta_{2m}$ transcripts were poorly translated was considered. Polysome-associated RNA from normal and Ad12-transformed cells was fractionated and hybridized with several probes. The control probes were chosen to represent mRNAs of 1.5-kilobase mRNAs (class I heavy chain mRNA was compared with actin mRNA), and of 0.5-kilobase mRNAs ($\beta_{2m}$ mRNA was compared with histone mRNA). Fig. 3 shows a representative experiment in which hybridization signals obtained with polysomal RNA from VAD12.79 and M1 cell lines were compared. The distribution of PD1, H-2, actin, and histone mRNAs was identical in the two cell lines. The maximal level of class I and actin mRNA was in fractions 2–4, and that of histone was in fractions 5 and 6. These results are in agreement with the mRNA sizes of these genes. The distribution of $\beta_{2m}$ mRNA showed a slight shift toward the lighter polysome fraction in VAD12.79 (fractions 3–5 in VAD12.79, compared with fractions 2–4 in M1). While these results were consistent in the VAD12.79 cell line, we did not detect a similar shift in other Ad12-transformed cell lines (data not shown). Therefore we conclude that both class I heavy chains and $\beta_{2m}$ mRNA are efficiently translated in Ad12-transformed cell lines.

**Overexpression of $\beta_{2m}$ in TAP-reconstituted VAD12.79 Cells Enhances Assembly and Transport of PD1 but Does Not Affect Assembly of H-2 Molecules—Since the intracellular concentration of $\beta_{2m}$ might affect the efficiency of assembly of class I complexes, we attempted to induce assembly and transport by overexpression of $\beta_{2m}$ molecules. TAP-reconstituted VAD12.79 cells were transfected with a vector expressing mouse $\beta_{2m}$ from an actin promoter. Several individual clones that expressed levels of $\beta_{2m}$ identical to the normal cell line were grown, and the transport of PD1 and H-2 molecules was analyzed by immunoprecipitation with antibodies directed against PD1 or against the H-2 heavy chains, respectively. Fig. 4 (A and B) shows two representative pulse-chase experiments performed with two of the transfected clones (clones 2 and 9). The transport rate of PD1 was enhanced in both clones, as evidenced by the rate of acquisition of Endo H resistance especially following the 60-min chase period. This augmentation was not seen with H-2 molecules analyzed in parallel (data not shown). These results were supported by data obtained with TAP-reconstituted Ad12-transformed cells infected with recombinant vac-

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**Table I**

| Exp. | M1 | A5O5 | A5O1 | VAD12.79 | VAD12.42 |
|------|----|------|------|----------|----------|
| H-2Db/H-2Kb (PSL) | 1.0 | 1.1 | 0.7 | 0.8 | 2.9 |
| 1 | 1.2 | 1.1 | 0.7 | 0.8 | 2.9 |
| 2 | 0.6 | 0.7 | 1.7 | 1.4 | 1.4 |
| 3 | 1.0 | 1.1 | 0.5 | 0.5 | 1.1 |

| Cell line | M1 | A5O5 | A5O1 | VAD12.79 | VAD12.42 |
|-----------|----|------|------|----------|----------|
| PSL | 1.0 | 1.1 | 0.7 | 0.8 | 2.9 |
| 1 | 1.2 | 1.1 | 0.7 | 0.8 | 2.9 |
| 2 | 0.6 | 0.7 | 1.7 | 1.4 | 1.4 |
| 3 | 1.0 | 1.1 | 0.5 | 0.5 | 1.1 |
cinia viruses expressing β₂m, and with cell extracts to which excess purified human β₂m was added (data not shown). In all three cases, an excess of β₂m resulted in augmented assembly and transport of PD1/β₂m, but did not affect the assembly of H-2/β₂m. Thus, even in VAD12.79, in which the β₂m levels are comparable or exceed levels in normal cells, it somehow was not accessible for assembly of H-2 class I complexes.

Turnover of β₂m in Normal and Ad12-transformed Cells—Since increased amounts of newly synthesized β₂m resulted in augmented assembly and transport of PD1/β₂m, but did not affect the assembly of H-2/β₂m. Thus, even in VAD12.79, in which the β₂m levels are comparable or exceed levels in normal cells, it somehow was not accessible for assembly of H-2 class I complexes.

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Regulation of β₂m in Ad12-transformed Cells

VAD12.79 there was minimal turnover of the β₂m during this time period. Identical results to that observed in VAD12.79 were obtained with two additional Ad12-transformed cell lines (VAD12.42 and VAD12.36; data not shown). Since the ratio of the heavy chain to the light chain did not change during the chase periods, we feel that the rapid turnover rate of free β₂m in the normal and in E1Ad5-transformed cell lines represents the true estimation of β₂m half-life and does not result from an exchange between labeled and non-labeled β₂m molecules. Thus, our data clearly demonstrate that both assembly of class I/β₂m (Refs. 12 and 33; see Fig. 7, B1) and secretion or degradation of free β₂m were both abrogated in Ad12-transformed cells.

In order to determine whether poor assembly and the factors retarding β₂m turnover are related, we determined the half-life

FIG. 3. Translation efficiency of class I heavy chains and β₂m in normal and a Ad12-transformed cell lines. Polysome-associated mRNA was isolated from a normal cell line (M1) and an Ad12-transformed cell line (VAD12.79) and fractionated on a formamide/formaldehyde gel. The gel was blotted and hybridized with PD1, H-2, β₂m, actin-, and histone-derived probes. L, light; H, heavy.

FIG. 4. Transport of PD1 in β₂m-transfected TAP-reconstituted VAD12.79 (12.79/TAP2) cells. Transport of PD1 was analyzed in the normal cell line (M1), a mixture of hygromycin-transfected clones (12.79/TAP2 C/Mix), and β₂m-transfected clones (2 in A and 9 in B), following immunoprecipitation and Endo H treatment. m, mature; imm, immature.

FIG. 5. Half-life of β₂m in normal and Ad-transformed cell lines. A representative pulse-chase experiment demonstrating the turnover of β₂m in normal (M1), E1Ad5-transformed (ASOS), Ad12-transformed (VAD12.79), TAP1±2 and TAP2-VAD12.79 cells is shown in A. B and C summarize the results of three pulse-chase experiments; cell lysates were immunoprecipitated either with antibodies directed against β₂m (total β₂m, B and C), or with antibodies directed against PD1 (PD1-assembled β₂m, B). The mean ± S.D. of the quantitated signals obtained for β₂m was calculated as the percent of the signal observed immediately after the 15-min pulse (% of time zero), and is presented as a function of the chase period. Total β₂m, β₂m precipitated with antibodies directed against β₂m. PD1-assembled β₂m, PD1-associated β₂m in M1 cells precipitated with antibodies directed against PD1.
Regulation of $\beta_2$-m in Ad12-transformed Cells

**Panel A**

| Cell line | M1 | VAD12.79 |
|-----------|----|----------|
| Chase (min) | 0 | 120 | 180 | 300 |

**Panel B**

![Graph showing half-life of $\beta_2$-m in Ad12-transformed cell lines following treatment with interferons.](https://www.jbc.org/)

**Figure 6.** Half-life of $\beta_2$-m in Ad12-transformed cell lines following treatment with interferons. A representative pulse-chase experiment demonstrating the turnover of total $\beta_2$-m in normal (M1), VAD12.79, and VAD12.79 cells pretreated with $\alpha/\beta$ or $\gamma$-interferons is shown in A. Panel B summarizes the results of three experiments. The mean $\pm$ S.D. of the quantitated signals obtained for $\beta_2$-m was calculated as the percent of the signal observed immediately after the 15-min pulse (% of time zero), and is presented as a function of the chase period. Total $\beta_2$-m, $\beta_2$-m precipitated with antibodies directed against $\beta_2$-m.

- of total $\beta_2$-m in TAP-reconstituted VAD12.79 cells, which can assemble and transport PD1 molecules (12, 34). Fig. 5 (A and C) shows that in these cells, the half-life of total $\beta_2$-m was comparable with that of PD1-assembled $\beta_2$-m, and significantly longer than that of total $\beta_2$-m in the normal cell line. Pretreatment of Ad12-transformed cells with interferons, which reconstitutes almost completely the assembly and transport of both the PD1 and H-2 molecules (34), enhanced the turnover of $\beta_2$-m molecules but did not fully restore their normal turnover rate (Fig. 6, A and B). These results imply that in Ad12-transformed cells there is a mechanism that acts to retain $\beta_2$-m in the cells and that this retention is only partially alleviated by reconstituting the assembly of class I complexes.

- $\beta_2$-m Molecules Are Associated with the Membrane Fraction of Ad12-transformed Cells—In order to localize the compartment where $\beta_2$-m is retained in Ad12-transformed cells, we separated cell lysates into two fractions: one enriched for soluble proteins, and the other for cell membranes. The latter fraction was solubilized by detergent treatment, and both fractions were immunoprecipitated with antibodies directed against $\beta_2$-m. The results of two experiments are depicted in Fig. 7. In the first (A), the cells were pulsed for 30 min in order to be able to detect molecules with short and long half-lives. In the second (B), the cells were labeled for 15 h to enrich for molecules that have a long half-life. In order to directly determine which fraction was enriched with heavy chain-associated $\beta_2$-m molecules, we immunoprecipitated both total (B2) and PD1-assembled $\beta_2$-m (B1). The immunoprecipitates were reduced or left untreated before fractionation by polyacrylamide gel electrophoresis. The data were quantitated and are summarized in Table II. The results of both experiments show that the membrane fraction of the two cell lines was enriched for $\beta_2$-m. Whereas in M1, $\beta_2$-m molecules are associated with class I heavy chains and, there-
TABLE II
Expression of β₂m in the soluble and membrane fractions of normal and Ad12-transformed cell lines

| Exp. | Cell line | M1 | VAD12.79 |
|------|-----------|----|----------|
|      |           | Sol. Mem. | Sol. Mem. |
| 1    | β₂m       | 45 211   | 95 284 |
| 2    | β₂m-deg.  | 91 363   | 341 1023 |
|      | β₂m-PD1   | 71 0     | 0 0 |

of the same cells (B1, lane 3 compared with B2, lane 3). This result indicates that most of the β₂m residing in the membrane fraction of M1 cells is bound to class I heavy chains. Unlike M1 in which all membrane-bound β₂m can be accounted for by class I heavy chain associations, in VAD12.79 some other membrane component must be playing this role. Moreover, the association of this membrane component with β₂m is more stable than the β₂m-association with class I heavy chains, as evidenced by the fact that after 15 h of labeling the amount of both soluble (B2, lanes 1 and 2) and membrane β₂m (B2, lanes 3 and 4), in VAD12.79 cells was 3-fold higher than in the M1 cells, whereas after a short pulse these signals in the membrane fraction were almost identical in both cell lines (A, lanes 3 and 4). The binding between this yet to be identified membrane component and β₂m is not via covalent disulfide bonds, since the same results were obtained whether or not the immunoprecipitates were reduced before fractionation (Fig. 7).

The fact that VAD12.79 cells appear to possess a unique membrane β₂m-binding component was further substantiated by the finding that in the soluble fraction of the M1 cells, partial degradation of β₂m molecules was evident (B2, lanes 1), while no such degradation was detected in the soluble fraction of VAD12.79 cells (B2, lanes 2), despite the fact that the β₂m signal in these cells was 3.5-fold stronger. Degradation products of β₂m molecules were also detected in immunoprecipitates of total cell extracts from M1 cells, but not from VAD12.79 cells (B2, lanes 5 and 6, respectively).

DISCUSSION

The selective suppression in transport and cell surface expression of H-2 molecules in Ad12-transformed cells that were reconstituted for the expression of peptide transporter and LMP genes implied that there was an additional factor(s) involved in the assembly and transport of the class I complex. The following possibilities were considered: (a) differences in the steady state level of H-2Kb and H-2Db mRNA, leading to differences in the magnitude of their expression; (b) differences in the translation efficiency of class I transcripts, resulting in differences in the amounts of proteins produced; (c) mutations affecting the conformation of the endogenous class I molecules and, thus preventing their transport through cell organelles; and (d) competition among class I heavy chains for limited amounts of β₂m or inaccessible β₂m molecules, leading to selective enrichment in particular class I complexes.

Our data show that equal amounts of H-2Kb and H-2Db genes were expressed (Fig. 2). We also found that PDI and H-2 mRNAs were translated efficiently (Fig. 3). In one Ad12-transformed cell line (VAD12.79), we observed some inhibition in the translation of β₂m mRNA as compared with that in the normal cell line M1. However, even in this cell line, β₂m mRNA was associated with the polysomal fraction and the slight shift of its mRNA toward association with lighter polysomes was not detected in other Ad12-transformed cell lines. Thus, aberrant transcription or translation of the class I heavy chains or of β₂m molecules cannot account for the low level of assembled and transported H-2 molecules in the TAP and LMP-reconstituted Ad12-transformed cells.

In order to determine whether inefficient assembly of specific class I heavy chains resulted from mutations in the endogenous genes which affect their conformation, we previously overexpressed either H-2 or β₂m molecules via infection with recombinant vaccinia viruses and analyzed their assembly and transport (34). The results were comparable with those obtained with the endogenous molecules, i.e. the transport of H-2 molecules was inefficient, while the transport of PD1 was completely restored. In the present study we also found that TAP-reconstituted VAD12.79 cells stably transfected with a β₂m gene show enhanced assembly and transport of PD1, but not of H-2 molecules (Fig. 4).

Collectively, the data suggested that one of the components (β₂m or peptides) of the class I complex was not available for assembly and led us to compare in greater detail the fate of β₂m in the normal versus the transformed cells. Based on our previous immunoprecipitation data and on other studies (45–49), it seemed likely that in normal cells most of the β₂m molecules would be associated with class I heavy chains and free molecules would either be degraded or secreted. Several studies have shown that free β₂m is secreted from cells and that its secretion can be enhanced by various cytokines, growth factors, and growth promoters (47–49). Dragmont et al. (45) demonstrated that free β₂m was secreted into the culture media of cell lines independent of cell surface expression of class I heavy chains. β₂m has also been shown to be secreted from endomtrial cells (46), leukemic B cells (47), fibroblastas (48), and hepatocytes (49). Much less is known about the degradation pathway of free β₂m within the cells. Since in Ad12-transformed cells there is no assembly with class I heavy chains, it seemed likely that most of the β₂m would be secreted from the cells or rapidly degraded. However, surprisingly, unlike normal and E1Ad5-transformed cells in which free β₂m was either secreted or degraded, and class I-assembled β₂m was transported to the cell membrane, in Ad12-transformed cells, free β₂m was retained in the cells (Fig. 5). Furthermore, we found that most of the β₂m molecules in the transformed cells resided in the membrane-enriched fraction (Fig. 7) and were more efficiently retained, following an overnight labeling period, than in the normal cell line. These data suggest that in the absence of detectable assembly with class I heavy chains, β₂m molecules could bind other membrane-associated molecules, resulting in the retention of these molecules within the cell. We cannot completely rule out the possibility of the existence of denatured class I heavy chains in Ad12-transformed cells that have lost all epitopes recognized by the antibodies used for immunoprecipitations and still have the potential to bind β₂m efficiently and to be retained in a stable form in the ER. Nevertheless, this possibility seems highly unlikely in view of the fact that none of the panel of antibodies directed against epitopes on the α₂ domain of H-2Db (33), H-2Db (34), PD1 (33, 34), and antisera against peptide 8 of the H-2Kb molecules (data not shown) detected significant assembly of the relevant heavy chain with β₂m.

Since it has been documented that γ-interferon modulates the secretion of β₂m (47, 49) and the same cytokine also reconstitutes the transport of H-2 molecules in Ad12-transformed cells (Ref. 34, and data not shown), we pretreated the transformed cells with interferons and determined the half-life of β₂m. Indeed, interferon treatment significantly shortened the half-life of β₂m, but it was still longer than in normal or...
Regulation of β2m in Ad12-transformed Cells

E1Ad5-transformed cells. Thus, we can conclude that in Ad12-transformed cells, most β2m is retained in the cells via a membrane-bound component and that it can be partially released by assembly with a class I heavy chain/peptide or by interferon treatment.

β2m is known to bind at least two other non-MHC molecules that have low significant homology with class I heavy chains: the neonatal Fc receptor (50, 51) and the CD1 class I-like molecule (51, 52). Neonatal Fc receptors mediate transfer of maternal IgG to the newborn, providing the neonate with humoral immunity before the development of a fully functional immune system. The neonatal Fc receptor shows similarity to the class I heavy chains in the organization of domains and their sequence, especially in the α3 domain (53), a fact that explains its binding to β2m molecules. On the other hand, the non-MHC-encoded CD1 family of molecules present antigens entirely distinct from MHC class I, class II, or related molecules (51). The molecules are abundantly expressed on professional antigen-presenting cells and are associated with β2m, and their expression and function is independent of the expression of peptide-transporter molecules (52). Thus, it is clear that β2m can bind class I-related proteins with apparently diverse functions. It is tempting to speculate that β2m can function as a scaffold for multiple proteins and promote their correct folding, as suggested by Solheim et al. (54) for class I heavy chains.

In the latter case β2m would participate in class I transport not only by stabilization of the class I heavy chains, but also by induction of a heavy chain conformation that enables it to transit to the cell surface. The same group also demonstrated (55) that β2m was associated with TAP in a class I negative cell line, supporting the hypothesis that β2m can bind to multiple proteins within the cell, either directly or via another molecule(s).

Since β2m could function to stabilize and maintain a variety of proteins, the constitutive secretion of β2m by a variety of cells may have an important physiological role. Indeed, free β2m has been shown to have some unexpected biological activities. β2m produced by synovial fibroblasts stimulated by phorbol esters induced collagenase synthesis in these cells (56), β2m also increased the number of insulin-like growth factor I transcripts and polypeptides, as well as their receptors, on cultured bone marrow cells (57). These data suggested that β2m could be involved in the modulation of connective tissue breakdown and bone remodeling. β2m might also play a role in the migration of hematopoietic cell precursors from their site of emergence, the bone marrow, to their site of differentiation, the thymus, since it is produced by a thymic epithelial cell line and is able to induce oriented migration of immature lymphoid bone marrow cells in vitro (58). Cytokines and growth factors that were reported to enhance β2m secretion possibly could act via two mechanisms: enhancement of β2m transcription and induction of β2m release from specific cellular proteins that might be similar to the β2m-binding proteins described in this paper.

Another system that is similar in several aspects to that described above is the association of surrogate immunoglobulin light chains with glycoproteins in cells that do not express the immunoglobulin heavy chain (59). The complex of μ heavy chain/surrogate light chain is expressed on pre-B cell lines and has been found to transmit biochemical signals to the cells (60, 61). However, the surrogate light chain can be expressed on the cell surface in the absence of rearranged heavy chain. Several proteins were suggested as potential candidates for binding non-covalently the surrogate light chain and carrying it to the surface of pre-B and pro-B cell lines (59). Unlike the surrogate light chains, some classical immunoglobulin light chains, in the absence of heavy chains, are retained and degraded within the ER (62). The half-life of these molecules depends on their binding and dissociation from the chaperone BiP. Our data suggest that, like the surrogate light chains, β2m molecules can bind other molecules in the absence of efficient assembly with class I heavy chains. However, such molecules either have long half-life (>5 h) and/or they are not transported to the cell surface and remain associated with β2m molecules in the cells. Whether these β2m-binding molecules are expressed in normal cells, or are known chaperons such as BiP or calnexin that bind misfolded proteins, or are novel proteins, which are overexpressed in Ad12-transformed cells, remains to be determined.

The fact that in Ad12-transformed cells the membrane fraction is enriched with β2m suggests that at least one binding protein is a membrane-bound protein such as calnexin. However, calnexin is commonly thought to be a chaperone that binds mostly glycoproteins (63), although a different mechanism for its association with class I heavy chains and class II invariant chains has been suggested (64, 65). Both of these molecules associated with calnexin even when their glycosylation was inhibited, and in the latter case, calnexin association retained the invariant chain in the ER and prevented its degradation. Thus, it is conceivable that β2m binds calnexin or another molecule via a mechanism similar to that involved in class II invariant chain association.

If Ad12-transformed cells overexpress a β2m-binding protein, it might interfere with the assembly of class I heavy chain/β2m/peptide. The ability of this protein to successfully compete with class I heavy chains for β2m binding could explain our inability to fully restore the assembly of the H-2 complex in TAP-LMP-reconstituted cells. Thus, Ad12-transformed cells appear to acquire a novel membrane-associated mechanism for sequestering β2m. This mechanism may be unique to these cells or may be induced in cells that do not express fully conformed class I complexes.

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REFERENCES

1. Zinkernagel, R., and Doherty, P. C. (1979) Adv. Immunol. 22, 51–377
2. Goodenow, R. S., Vogel, J. M., and Linck, R. L. (1980) Science 206, 777–783
3. Hammerling, G. J., Klar, D., Pulm, W., Mombourg, F., and Moldenhauer, G. (1987) Biochim. Biophys. Acta 1117, 245–259
4. Ehrlich, R. (1995) Immunol. Rev. 14, 77–97
5. Yewdall, J. W., and Bennink, J. R. (1992) Adv. Immunol. 52, 1–123
6. Germain, R. N., and Margulies, D. H. (1993) Annu. Rev. Immunol. 11, 403–450
7. Spies, T., and DeMars, R. (1991) Nature 351, 323–324
8. Attaya, M., Biskoff, E., Martinez, C. R., Hermel, E., Aldrich, C., Forman, J., Fischer Lindahl, K., Bevan, M. J., and Monaco, J. J. (1992) Nature 355, 647–649
9. Van Kaer, L., Ashton-Rickardt, P. G., Ploegh, H. L., and Tanegawa, S. (1992) Cell 71, 1205–1214
10. Arnold, D., Driscoll, J., Androwiescz, M., Hughes, E., Cresswell, P., and Spies, T. (1992) Nature 359, 171–174
11. Restifo, N. P., Esquivel, F., Kawakami, Y., Yewdell, J. W., Mule, J. J., Rosenberg, S. A., and Benacerraf, B. (1993) J. Exp. Med. 177, 265–272
12. Rotem-Yehudar, R., Winograd, S., Sela, S., Coligan, J. E., and Ehrlich, R. (1994) J. Exp. Med. 178, 477–488
13. Williams, D. B., Barber, B. H., Flavell, R. A., and Allen, H. (1989) J. Immunol. 142, 2786–2806
14. Zijlstra, M., Bix, M., Simister, N. E., Loring, J. M., Rauh, D. H., and Jaenisch, R. (1990) Nature 344, 742–746
15. Koller, B. H., Marrack, P., Kappler, J. W., and Smithies, O. (1990) Science 241, 1227–1230
16. Rock, K. L., Rothstein, L. E., Gamble, S. R., and Benacerraf, B. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 7537–7521
17. Vitiello, A., Potter, T. A., and Sherman, L. A. (1990) Science 250, 1423–1426
18. Rock, K. L., Gamble, S., Rothstein, L., and Benacerraf, B. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 301–304
19. Kozlowski, S., Taksanita, T., Beohnecke, W.-H., Takahashi, H., Boyd, L. F., Germain, R. N., Berzofsky, J. A., and Margulies, D. H. (1991) Nature 349, 74–77
20. Boyde, L. F., Kozlowski, S., and Margulies, D. H. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2242–2250
21. Ottgen, G. R., Bikoff, E., Ribaudo, R. K., Kozlowski, S., Margulies, D. H., and Germain, R. (1992) J. Immunol. 148, 3723–3732
22. Abdel Motal, U. Zhou, M., Siddiqui, A. R., and Jondal, M. (1993) Scand. J. Immunol. 38, 395–409
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