Assembly, Intracellular Localization, and Nucleotide Binding Properties of the Human Peptide Transporters TAP1 and TAP2 Expressed by Recombinant Vaccinia Viruses*

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The transporter associated with antigen processing (TAP) transports short peptides from the cytosol to the endoplasmic reticulum, where peptides assemble with class I molecules of the major histocompatibility complex. TAP is comprised of two subunits, termed TAP1 and TAP2. We produced recombinant vaccinia viruses that direct synthesis of the TAP subunits, either individually or together. Virus-encoded TAP is rapidly and efficiently assembled (t50 of 5 min or less) by cells and does not spontaneously assemble in detergent extracts. By confocal immunofluorescence microscopy, TAP1 when expressed alone or with TAP2 is largely, if not exclusively, localized to the endoplasmic reticulum. Metabolic labeling with [2-3H]mannose demonstrates that TAP1 (but not TAP2) possesses Asn-linked oligosaccharides, but the lack of binding of [35S]methionine-labeled TAP to concanavalin A-agarose suggests that the glycosylated form represents a minor population of TAP1. The two subunits of the assembled complex present in detergent extracts photolabeled equally with 8-azido-[α-32P]ATP. Photolabeling of the two subunits was inhibited in parallel by various di- and trinucleotides, suggesting that their nucleotide binding sites function in a highly similar manner. Incubation of detergent extracts at 37°C results in the rapid loss of TAP1 immunoreactivity, indicating either an unusual sensitivity to proteases or an irreversible conformational alteration.

CD8+ T cells (TCD8+) recognize peptides, usually 8–10 residues in length, bound to major histocompatibility complex (MHC) class I molecules. Peptides are predominantly generated from a cytosolic pool of proteins (2, 3). Class I molecules consist of a polymorphic integral membrane glycoprotein (α chain) complexed to β2-microglobulin, a soluble nonglycosylated protein. Both chains possess NH2-terminal hydrophobic sequences that target them co-translationally to the endoplasmic reticulum (ER). Most antigenic peptides, having no such ER insertion sequence, remain sequestered on the cytosolic side of the ER membrane and require a specific transporter, termed TAP (acronymic for transporter associated with antigen processing) to access class I molecules. TAP is produced by the association of two MHC-encoded subunits, termed TAP1 and TAP2 (4–7). The central importance of TAP in TCD8+ responses is most stunningly shown by the severe depletion of T cells in mice with a targeted disruption of the TAP1 gene (8).

The TAP genes are members of a large family of integral membrane transporters referred to as ABC (ATP binding cassette) proteins since each has a characteristic sequence associated with ATP binding. ATP hydrolysis is believed to drive the transport of the wide variety of substrates handled by the various family members (9). Typically, ABC transporters are comprised of a single subunit containing two cytosolic ATP binding domains of approximately 300 residues and 12 hydrophobic domains believed to traverse the membrane, with short peptides connecting the hydrophobic domains. The structure of TAP is similar to other ABC proteins, with the most notable difference being its division into two polypeptides, TAP1 and TAP2, each containing a single ATP binding domain and 6 potential transmembrane domains.

The past 2 years have witnessed rapid progress in understanding TAP-mediated translocation of peptides. Using semi-intact and cell-free systems, the basic requirements for peptide length and sequence have been defined (10–23). There are still sizable gaps, however, in our knowledge of numerous aspects of TAP, including its assembly, intracellular trafficking, and precise mechanism of function. In the present study we explore some of these issues using recombinant vaccinia viruses expressing each TAP subunit or the two subunits simultaneously.

EXPERIMENTAL PROCEDURES

Cells and Viruses—The antigen processing-deficient human cell lines T2 (24) and B6 (25) were maintained in Iscove’s modified DMEM supplemented with 7.5% (v/v) fetal bovine serum. L929 mouse fibroblasts were maintained in DMEM supplemented with 7.5% (v/v) fetal bovine serum. rVV's were propagated in thymidine kinase-deficient human 143B osteosarcoma cells. rVV's expressing TAP1 (VV-TAP1) or TAP2 (VV-TAP2) were produced by cloning CDNAS encoding the respective genes behind the earlylate VV p7.5 promoter into a modified pSC11 plasmid as described (26). The human TAP1 (a allotype) and TAP2 (b allotype) genes have been described (27). A double rVV expressing TAP1 and TAP2 genes under the control of the p7.5 promoter (VV-TAP1–2) was produced by the method described by Coupal et al. (1988) (28). Briefly, the TAP2 gene fragment ending with XhoI sites was inserted into the SalI site of pTK-7.5B plasmid, which contains the herpes simplex virus type 1 thymidine kinase gene under the control of the VV promoter P-E. 143B cells infected with TAP1-rVV were transfected by the pTK-7.5B plasmid containing the TAP2 insert. Double rVV's with the TAP1 gene inserted in the VV tk gene and the TAP2 gene...
In the VV HindIII F region were selected for thymidine kinase expression by inclusion of aminopterin in the growth medium (29). rVVs encoding A/Puerto Rico/8/34 influenza virus HA and NP under the control of the VV p7.5 promoter have been described (30). rVVs encoding a secreted form of NP and mouse ICAM-1 will be described elsewhere.

Cytofluorography—T2 cells were infected for 15 h with rVV and incubated for 15 min at 0 °C with fluorescein-conjugated antibodies specific for HLA class I molecules (W6/32; Accurate, Westbury, NY), β2-microglobulin (The Binding Site, San Diego, CA), or mouse ICAM-1 (Pharmingen, San Diego, CA). After washing once, cells were resuspended in PBS supplemented with 0.1 mg/ml ethidium homodimer (Molecular Probes, Eugene, OR) and analyzed using a FACSscan (Becton Dickinson, San Jose, CA). Nonviable cells fluorescently labeled with ethidium homodimer were excluded from analysis.

Cytotoxicity Assay—Target cells were infected with VV as described (31, 32). T~CD8~ were generated from splenocytes derived from animals immunized with viruses 2–6 weeks previously by 7-day in vitro stimulation with virus-infected autologous splenocytes as described (31, 32). Microcytotoxicity assays were performed as described (31, 32). Data are expressed as percentage of specific release defined as [(experimental cpm – spontaneous cpm)/total cpm – spontaneous cpm] × 100.

Immunofluorescence—VV-infected L929 cells were prepared for immunofluorescence as described (33). Double antibody immunofluorescence was performed by incubating coverslips for 2 h at 20 °C with PBS containing 0.1% BSA and rabbit anti-TAP IgG, washed, and incubated for 2 h at 20 °C with PBS containing 1% (w/v) Texas Red-conjugated goat anti-rabbit IgG, washed, and incubated for 2 h at 20 °C with PBS containing 33% (v/v) rat hybridoma tissueculture supernant containing a mAb specific for immunoglobulin binding protein (BIP) (34). Coverslips were then washed, incubated for a further 2 h at 20 °C with PBS containing 1% (w/v) fluorescein isothiocyanate-conjugated rabbit anti-rabbit IgG, washed, and mounted in Fluorosave (Calbiochem, San Diego, CA). Images of fluorescent staining were acquired using a Bio-Rad MRC 600 confocal microscope, and hard copies were produced as described (35).

Lactin Depletion—Detergent extracts were prepared as described below. It was shown that the extraction buffer was supplemented with 5 mM Ca2+ and 5 mM Mg2+. Extracts were incubated for 2 h at 4 °C with agarose coupled to concanavalin A (ConA, Vector Laboratories, Burlingame, CA), and immunoreactive species remaining in the supernatant were collected as described below.

Metabolic Labeling—For metabolic labeling with [35S]methionine (Amersham Corp.), L929 cells were infected as described (36). Four hours post-infection, cells were incubated for 30 min at 37 °C in serum-free, methionine-free DMEM and then, after adjusting cells to a concentration of 1 – 2 × 106/ml for 5–15 min at 37 °C in 1 ml of the same medium supplemented with 400 μCi of [35S]methionine. Cells were then pelleted and chased at 37 °C in DMEM containing 1 mg/ml methionine. For labeling with [2-3H]mannose (American Radiolabeled Chemicals, St. Louis, MO), 1–2 × 106 cells were incubated in serum-free, glucose-free medium for 15 min at 37 °C in 1 ml of the same medium supplemented with 1 μCi of [2-3H]mannose. For all radiolabeling experiments, cells were shifted to 0 °C after appropriate incubation periods until detergent-extracted by 15 min of incubation at 0 °C in a mixture of 150 mM NaCl, 1 mM EDTA, 50% Nonidet P-40, 0.5% MEG9, 1 mM phenylmethylsulfonyl fluoride, 10 mM Tris, pH 7.4. Following centrifugation at 15,000 g, supernatants were harvested and precluded by overnight incubation with protein A-agarose preloaded with rabbit serum specific for an irrelevant antigen. Supernatants were then incubated for 1 h with protein A-agarose preloaded with the affinity-purified rabbit anti-peptide antisera RING4 produced by immunizing rabbits with a synthetic peptide corresponding to the COOH-terminal 15 residues of TAP1 (5). After extensive washing, proteins were eluted from beads by boiling in SDS-PAGE sample buffer and analyzed by SDS-PAGE using the conditions of Laemmli (37). Digestion of samples with endo-β-N-acetylglucosaminidase H (endo H) was performed as described (36). After acid fixing and staining with Coomassie Blue to ensure that equivalent amounts of antibody were recovered from samples, gels were dried at 80 °C under vacuum. For samples labeled with [2-3H]mannose, gels were incubated with Amplify® (Amersham) prior to drying. Fluorographs of tritiated samples were produced by exposing dried gels to preflashed film at –70 °C. Dried gels containing [35S]methionine-labeled proteins were exposed to PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA), which were imaged and quantitated on a dedicated hardware and software. Fluorographs were digitized using a flatbed scanner and quantitated using the PhosphorImager software. Hard copies of digital images were produced using Adobe Photoshop software and a Kodak XL7700 printer.

Mice—Six–to eight-week-old BALB/dbyj (H-2d) mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were immunized with 106 plaque forming units of VV by intravenous injection.

Phase Partitioning—VV-infected cells were labeled with [35S]methionine and detergent extracted as described above with 1% Triton X-114 (Pierce) replacing Nonidet P40 and MEG9. Following centrifugation at 15,000 × g at room temperature the aqueous phase and detergent phase (yellow droplet at the bottom of the tube) were removed separately and diluted 5 times with normal extraction buffer, and species reactive with anti-TAP1 antisera were collected as described above.

Photolabeling—Photolabeling was performed according to Hobson et al. (39). 3.5 h post-infection, cells were incubated for 30 min in serum-free, glucose-free medium containing 1 mM carbonyl cyanide m-chlorophenylhydrazide to deplete ATP. Extracts were prepared by incubating cells for 15 min at 0 °C in 250 mM sucrose, 50 mM KCl, 2 mM MgCl2, 2 mM EGTA, 1% Triton X-100 (1v), 1 mM phenylmethylsulfonyl fluoride, apotinin, leupeptin, 10 mM Tris, pH 6.8 and centrifuging at 15,000 × g for 30 min. 100-µl aliquots of the supernatant were placed in the wells of a 96-well flat bottom microtiter plate, and 2 µl of 8-azido-[35S]ATP were added (ICN Biomedicals, Costa Mesa, CA). Samples were irradiated for 2 h at 245 nm (1250 microcrockets/cm2) and then at 365 nm (1050 microcrockets/cm2), with a 1-min interval to prevent excessive heating. Approximately 3 × 106 cells were used for each labeling reaction. For competition experiments, the same conditions were used, but samples were preincubated on ice with various nucleotides. Samples were diluted to 1 ml with the extraction buffer used for radiomuno-affinity columns, and the reactive species were analyzed by SDS-PAGE as described above.

RESULTS AND DISCUSSION

Function of rVV-encoded TAP1 and TAP2—The function of rVV-produced TAP1 and TAP2 was examined by testing the ability of rVVs to rescue the ability of T2 cells (which express neither TAP subunit) to present VV antigens to mouse H-2Kd restricted, VV-specific T~CD8~. To enable T~CD8~ recognition, cells were co-infected with a rVV expressing Kd α chains. As previously reported (31, 40), presentation of Kd-restricted VV antigens by T2 cells is minimal (Fig. 1A). Co-infection of cells with VV-TAP1+2 greatly increased lysis, despite the fact that co-infection with an additional rVV would be expected to decrease the rate of Kd biosynthesis. Target cell lysis was Kd restricted since cells infected by VV-TAP1+2 alone were not lysed. Co-infection of VV-Kd infected cells with either VV-TAP1 or VV-TAP2 alone failed to restore antigen presentation. Triple infection of cells with all three recombinants, however, enabled T~CD8~ recognition. The decreased restoration relative to double infection with Kd and VV-TAP1+2 can be attributed to both competition for gene expression between rVVs and the decreased likelihood of triple infection versus double infection. Notably, co-infection of VV-Kd infected cells with either VV-TAP1 or VV-TAP2 alone failed to rescue antigen presentation. This indicates that neither subunit alone is able to rescue antigen presentation. In additional experiments using antigen processing-deficient cells lacking one of the two subunits, infection with the appropriate rVV expressing a single TAP subunit was able to restore presentation of either VV antigens or a number of foreign antigens to the appropriate T~CD8~ population (data not shown).

The number of class I peptide complexes necessary for lysis by T~CD8~ in the cytotoxicity assay employed in this study is not known but is possibly under 1000 complexes/cell (41). To more quantitatively test the ability of rVVs expressing TAP subunits to restore class I assembly, T2 cells were infected overnight with rVVs at 37 °C, and the cell surface expression of native endogenous class I molecules was determined by flow cytometry. T2 cells cultured at 37 °C express considerable amounts of HLA-A2 due to its association with peptides derived from ER
insertion sequences (42, 43). Class I expression is increased, however, by TAP expression (25). As shown in Fig. 1B, the level of endogenous class I expression detected by fluorescein-conjugated W6/32 mAb specific for native class I molecules, fluorescein-conjugated sheep anti-human \( \beta_2 \)-microglobulin, or fluorescein-conjugated rat anti-mouse ICAM-1 mAb. Cells were analyzed in a cytofluorograph, and the log10 mean channel fluorescence of viable cells (gated by exclusion of ethidium homodimer) is shown.

![Characterization of TAP Assembly and Function](image)

Fig. 1. *Function of VV-encoded TAP subunits.* A, T2 cells were infected with the indicated rVVs for 3 h before testing in a 6-h cytotoxicity assay using secondary in vitro restimulated VV-specific TCD8 +. B, T2 cells infected overnight with the rVVs indicated were tested for binding to saturating quantities of fluorescein-conjugated W6/32 mAb specific for native class I molecules, fluorescein-conjugated sheep anti-human \( \beta_2 \)-microglobulin, or fluorescein-conjugated rat anti-mouse ICAM-1 mAb. Cells were analyzed in a cytofluorograph, and the log10 mean channel fluorescence of viable cells (gated by exclusion of ethidium homodimer) is shown.

Fig. 2. *Assembly of VV-encoded TAP subunits.* A, L929 cells infected for 4 h with the indicated rVVs were labeled with [\(^{35}\)S]methionine for 15 min, and material in detergent extracts reactive with anti-TAP1 antibodies was collected and analyzed by SDS-PAGE. Only the region of the gel containing TAP1 and TAP2 is shown. B, as in A, except extracts were mixed prior to exposure to anti-TAP1 antibodies (middle), or cells were mixed prior to exposure to extraction buffer (EB) (right panel). On the left are reactive species present in cells infected with VV-TAP1, VV-TAP2, or both rVVs. C, as in A, except cells were labeled for 1 min and then placed on ice or incubated for 5 or 15 min at 37°C prior to detergent extraction and collection of antibody reactive species.

systems that both subunits are required for peptide transport into the ER above background levels (10, 18, 19) or formation of a functional peptide binding site (21).

Assembly of rVV-encoded TAP—To biochemically characterize TAP, affinity-purified rabbit antibodies raised to a peptide corresponding to the 15 COOH-terminal residues of TAP1 were used to recover TAP1 from detergent extracts. Following 15 min of labeling with [\(^{35}\)S]methionine of cells infected for 4 h with VV-TAP1, we detected a protein with a \( M_r \) of 69,000 in SDS-PAGE (Fig. 2A). This is less than the predicted \( M_r \) of 75,000 but in accord with previously reported values (44, 45).

The mobility of TAP1 was unchanged over a 2-h chase period (see Fig. 5B). The absence of this protein from cells infected with a control VV confirms its identity as TAP1 (not shown). Following infection with VV-TAP(1+2), or co-infection with VV-TAP1 and VV-TAP2 a new band with the expected mobility of TAP2 (\( M_r \) 74,000) appeared (Fig. 2A). To determine whether assembly of TAP occurred in detergent extracts, cells infected with VV-TAP1 or VV-TAP2 were mixed and detergent-extracted, and the species reactive with anti-TAP1 antibody were collected (Fig. 2B). This resulted in the recovery of TAP1 only. Similarly, TAP(1+2) heterodimers were not detected when detergent extracts from cells infected with either VV-TAP1 or VV-TAP2 were mixed. Adding radiolabeled TAP2 in a 5-fold excess over TAP1 still did not result in detectable complex formation. Based on these findings, we conclude that under the conditions employed for detergent extraction, all TAP heterodimers detected are formed in cells.

Following double infection of cells with VV-TAP1 and VV-TAP2, TAP1 was always recovered in higher amounts using the anti-TAP1 antiserum. Even if assembly occurred with 100% efficiency this would be expected, since it is statistically inevitable that some cells will be infected with a greater number of VV-TAP1 virions (and vice versa). Following infection with VV-TAP(1+2), enhanced recovery of TAP1 relative to TAP2 was observed in approximately half of the experiments. Since
we expect that TAP1 and TAP2 are translated at the same rate in VV-TAP(1+2)-infected cells, this implies that a pool of unassembled TAP1 (and possibly TAP2) exists. In the other experiments, however, using the identical stock of VV-TAP(1+2), the ratio of TAP1 to TAP2 was close to 1, indicating that TAP assembly can be quite efficient, even when unnaturally overexpressed in the absence of up-regulation of other gene products normally regulated in parallel with TAP. The variability in the ratio of TAP1:TAP2 recovered might reflect true variability, the efficiency of TAP assembly in vivo, or artefactual variability in the preservation of the heterodimers in biochemical procedures following detergent extraction. The former possibility would be consistent with TAP assembly being a regulated process.

We next examined the rate of assembly of TAP heterodimers by labeling VV-TAP(1+2)-infected L929 cells for 1 min with [35S]methionine and chasing cells for 5 or 15 min (Fig. 2C). The amount of TAP1 recovered increased 3.4-fold within 5 min of initiating the chase. Since protein synthesis in mammalian cells occurs at a rate of approximately 10 residues/s, synthesis of TAP is likely to require between 1 and 2 min. This probably accounts for most of the increase in TAP1 recovery over the chase period, although we cannot rule out the occurrence of intrinsic alterations in TAP structure or the association of TAP1 with molecular chaperones that limits antibody access to the COOH terminus. Most notably, there was little increase in the ratio of TAP1:TAP2 recovered in either of the chase periods relative to the pulse. This indicates that complex formation occurs extremely rapidly. These findings were confirmed using the antigen-deficient human cell line B6, which expresses only the TAP1 subunit. Following infection with VV-TAP2, complex formation was again detected within 5 min (not shown).

TAP assembly is remarkable in that it occurs as swiftly as any oligomeric membrane proteins we are aware of (46). This indicates that the complicated topology of multispansing membrane proteins such as TAP need not limit their rate of assembly. Due to the high levels of rVV expression, the present findings may represent an upper limit for the rate of TAP assembly, which could be slower under normal conditions when the concentration of newly synthesized subunits in the ER is lower. Note that the experimental design precludes determining the extent to which newly synthesized subunits pair with new versus old subunits.

Nucleotide Binding Properties of TAP—As members of the ABC transporter family, TAP1 and TAP2 possess putative ATP binding domains. ATP was previously demonstrated to bind to the nucleotide binding domain of human TAP1 expressed in Escherichia coli and to the COOH-terminal domains of mouse TAP1 and TAP2 expressed in Drosophila melanogaster cells (47, 48). Binding of ATP to intact TAP has not, however, been reported.

We first attempted to detect binding of [35S]methionine-labeled, detergent-solubilized TAP1 (expressed alone or with TAP2) to Affi-Gel Blue Sepharose (which binds many ATP-binding proteins) or ATP-agarose by eluting bound material with ATP and collecting TAP1 using anti-TAP1 antibodies. Both matrices bound cellular or viral proteins that were released by ATP, but we failed to detect TAP1 in either eluate (not shown). Since similar failures have been reported for other ABC transporter family members, we used 8-azido-[α-32P]ATP to photolabel TAP. Cell extracts prepared from L929 cells co-infected with VV-TAP1 and VV-TAP2 were UV-irradiated in the presence of 8-azido-[α-32P]ATP, and species reactive with TAP1 antiserum were analyzed by SDS-PAGE. This revealed labeling of both TAP subunits (Fig. 3). The specificity of labeling is demonstrated by the failure to recover labeled TAP in the absence of UV irradiation (not shown) or when 1 mM unlabeled ATP was added to samples prior to irradiation (Fig. 3).
Fig. 4. Immunofluorescence localization of TAP. VV-TAP[1-2]-infected L929 cells were paraformaldehyde-fixed and detergent-permeabilized prior to activity with antibodies specific for TAP or BiP, and suitable secondary anti-Ig reagents were conjugated to fluorescein or Texas Red. Fluorescence was simultaneously detected by confocal scanning laser microscopy.

control VV was at the low levels observed when the rabbit antibody was omitted (not shown). By contrast, staining of VV-TAP1 or VV-TAP[1-2] was quite intense, yielding a classical ER pattern (Fig. 4). The ER localization of TAP1 in VV-TAP[1-2]-infected cells was confirmed by its nearly perfect co-localization with a rat antibody specific for mouse BiP detected using rabbit anti-rat IgG fluorescein-conjugated antibodies (Fig. 4). The ER localization of TAP is consistent with previous data indicating that peptide association occurs in an early secretory compartment (35) and with findings that peptides transported via TAP into microsomes are glycosylated (10). It differs somewhat from the report that considerable amounts of TAP are present in the cis-region of the Golgi complex (53). Additional experiments are required to determine whether a minor portion of the intense TAP staining co-localizes with the Golgi complex. It is possible, however, that transport of TAP to the Golgi complex occurs too slowly for detection under the transient conditions employed (5 h of infection). Also, it is worth noting that interpretation of the immuno-EM images required a correction for background staining to TAP-deficient cells and that the specificity of staining of the Golgi complex was less certain than staining of the ER (53).

N-Linked Glycosylation of TAP—The ER is the site of addition of Asn-linked oligosaccharides. TAP1 has three potential sites for N-linked glycosylation (TAP2 has none). Using hydrophathy plots to identify the transmembrane regions of TAP1 and the resulting ER versus cytosolic orientation of the inter-transmembrane regions, one Asn (Asn229) is predicted to be lumenerally oriented and a potential target for Asn-linked glycosylation. To investigate Asn-linked glycosylation of TAP1, VV-TAP1- or VV-TAP[1-2]-infected L929 cells were pulse-radiolabeled with [2-3H]mannose, and the radiolabeled species reactive with anti-TAP1 antisera were analyzed by SDS-PAGE. [2-3H]Mannose is the most specific metabolic radiolabel for N-linked oligosaccharides (54), and the pulse labeling conditions should further minimize its conversion to other metabolites. As seen in Fig. 5A, [2-3H]Mannose was incorporated into influenza virus hemagglutinin (HA) collected from cells infected with a rVV containing the HA gene. HA is an integral membrane glycoprotein containing five N-linked oligosaccharides. All of the label was removed from pulse-labeled HA by endo H, whose specificity for digestion of immature high mannose oligosaccharides is shown by its failure to digest a slower migrating form of HA in the chase known to contain oligosaccharides modified by Golgi complex-associated enzymes into...
proximately 1⁄6 the level of HA, which suggests that only a subpopulation is labeled. While endoH-induced a large shift in the mobility of H-2K^d (an integral membrane glycoprotein with three N-linked oligosaccharides) treated in parallel, it had no effect on the mobility of TAP1 in this experiment or on the mobility of TAP1 or TAP2 from VV-TAP1[1-2]-infected cells (not shown). This finding is consistent with the prior observation that the electrophoretic mobility of TAP produced by insect cells was unaffected by tunicamycin inhibition of Asn-linked glycosylation (19). The lack of effect of endo H on TAP1 mobility has two plausible explanations: the shift in M, resulting from detachment of a single oligosaccharide is too small to resolve by the SDS-PAGE conditions utilized, or only a minor, undetected population of TAP is glycosylated. In an attempt to maximize mobility difference associated with the presence of N-linked oligosaccharides, we labeled cells treated with inhibitors (bromoconduritol or deoxynojirimycin) that prevent the removal of the glucose residue present on the oligosaccharide initially transferred (55). This failed to alter the mobility of TAP1 in SDS-PAGE (not shown). Finally, we incubated cell extracts prior to radio-immuno-collection with ConA-agarose. ConA binds proteins containing Asn-linked oligosaccharides, particularly those with high mannose oligosaccharides. As seen in Fig. 6, incubation with ConA-agarose did not remove [35S]methionine-labeled TAP1 from detergent extracts. Under the same conditions a secreted form of influenza virus NP containing a single Asn-linked oligosaccharide was nearly completely depleted from extracts. Based on these findings, we provisionally conclude that Asn-linked glycosylation of TAP is limited to a subpopulation of molecules.

**Characterization of TAP Assembly and Function**

**Fig. 5. [2-3H]mannose labeling TAP1.** A, L929 cells were labeled for 30 min with [2-3H]mannose 3.5 h post-infection with VV-TAP1 or VV-HA and incubated at 0 °C (P) or 37 °C (C) for 3 h. Species reactive with anti-TAP1 antibodies or the anti-HA mAb H28-E23 (56) were collected, digested with endo H (+) or mock-digested, and analyzed by SDS-PAGE. B, L929 cells were labeled for 10 min with [35S]methionine 3.5 h post-infection with VV-TAP1 or VV-K^d and incubated on ice or at 37 °C for 120 min. TAP1 or K^d was collected from detergent extracts, digested with endo H (+) or mock-digested, and analyzed by SDS-PAGE.

**Fig. 6. Absence of TAP1 binding to lectins.** L929 cells were labeled for 15 min with [35S]methionine 4 h post-infection with VV-TAP1 or VV-IS-NP (influenza virus NP with an ER insertion sequence). Detergent extracts were incubated with agarose coupled to ConA, and immunoreactive TAP or IS-NP present in supernatants was analyzed by SDS-PAGE.

**Fig. 7. TX114 phase partitioning of TAP[1-2].** TX114 extracts from VV-TAP1 or VV-TAP[1-2]-infected cells were incubated on ice or at 37 °C for 5 min and then partitioned into detergent (Det.) or aqueous phases (Aq.), and immunoreactive species were analyzed by SDS-PAGE.
Nonidet P-40 extracts were incubated at 37 °C (not shown). Since these detergents phase partition only at 50 °C, the loss of immunoreactive TAP is related to temperature and not phase partitioning per se. There are two explanations for these findings. First, the COOH terminus of TAP1 (against which anti-peptide TAP1 antibody is directed) may be cleaved by a protease insensitive to the inhibitors used. It is notable that under the same conditions, influenza virus NP, which is very sensitive to proteolysis, was not digested (not shown). Thus, if loss of immunoreactive TAP reflects proteolysis, a highly specific protease may be involved. Second, elevated temperatures may induce an irreversible conformational alteration or association with other factors in the extract, resulting in diminished accessibility of the antibody to its determinant. Regardless of the precise mechanism, the temperature-dependent decrease in immunoreactive TAP may reflect a process that modulates TAP function in vivo.

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