

Functional expression and purification of the ABC transporter complex associated with antigen processing (TAP) in insect cells

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Abstract Using the baculovirus expression system the gene products of human *tap1* and *tap2* were over-expressed as wild-type as well as oligohistidine fusion proteins in *Spodoptera frugiperda* (Sf9) insect cells. Both gene products were co-expressed within the same cells and were found enriched in microsomal membranes. Immunoprecipitation and immobilized metal affinity chromatography revealed complex formation between TAP1 and TAP2. The expressed TAP complex was shown to be functional by peptide translocation into microsomes of Sf9 cells. Peptide transport strictly requires TAP1 and TAP2 as well as ATP. For the first time the functional expression of the human TAP complex in insect cells has been demonstrated, indicating that additional cofactors of a highly developed immune system are not essential for peptide transport across microsomal membranes.

Key words: ABC transporter; Membrane protein; Traffic ATPase; Antigen presentation; Peptide transport; Baculovirus; Insect cell

1. Introduction

Antigen presentation by major histocompatibility complex (MHC) class I molecules requires limited proteolysis of intracellular antigens, translocation of the derived peptides into the lumen of the endoplasmic reticulum (ER), and association with the assembling MHC class I molecules. Subsequently, class I–peptide complexes are transported to the cell surface, where they can be recognized by the appropriate CD8⁺ T lymphocytes (for review see [1,2]).

Two genes, named *tap1* and *tap2*, which are essential for correct MHC class I antigen presentation, have been identified in the MHC class II gene region and sequenced for human [3–6], mouse [7,8] and rat [9,10]. Comparison of amino acid sequences revealed that TAP proteins exhibit a high degree of homology with a superfamily of proteins known as the ABC transporters [11], possessing two predicted ATP binding cassettes (ABC) and two stretches of 6–8 membrane-spanning regions [12]. TAP1 and TAP2 are thought to form a heterodimer [13,14] located in the membrane of the ER and *cis*-Golgi [15]. Other members of the family include the multidrug resistance P-glycoprotein (MDR) [16], the cystic fibrosis transmembrane conductance regulator (CFTR) [17], the oligopeptide transporter of *Salmonella typhimurium* [18] and the yeast protein Ste6 [19,20].

Two lines of evidence support the idea that the TAP1/2 complex translocates antigenic peptides into the lumen of the ER for association with MHC class I molecules: (i) restoration of correct antigen presentation in defective mutant cell lines transfected with the corresponding human [21], mouse [22] or rat [23] *tap* genes, and (ii) peptide translocation assays using permeabilized cells [24,25] or microsomes prepared from mammalian cell lines [26]. In general, these assays take advantage of core glycosylation or binding of translocated radiolabelled peptides to MHC class I molecules in the ER or microsomal lumen, respectively, preventing rapid export out of the ER. Sequence specificity, length selectivity of the peptides, as well as ATP-dependence of the translocation [27–30] were investigated in these assays. Furthermore, nucleotide binding studies to the hydrophilic, C-terminal domains of the transporter indicate a direct interaction and regulatory function of ATP [31]. For understanding the molecular architecture and further characterization of the function of the complex, possibly in an *in vitro* translocation assay with purified reconstituted TAP1/2 complex, large amounts of active protein are required.

The baculovirus expression system is well suited for the over-expression of transmembrane proteins; a wide array of them have been successfully expressed in a functionally active state, including the Na⁺/H⁺ [32] and the Na⁺/Ca²⁺ [33] antiporter, the Na⁺/glucose co-transporter [34], and the two subunit complex H⁺/K⁺-ATPase [35]. Other examples are the ABC transporters multidrug resistance P-glycoprotein (MDR) [36,37] and the cystic fibrosis transmembrane conductance regulator (CFTR) [38].

In this study, we report on the expression and formation of a functionally active TAP1/2 complex in Sf9 cells using the baculovirus expression system. Co-expression of the proteins was confirmed by immunoblotting, precipitation and fluorescence. Strategies for isolation of the complex were developed, demonstrating that the TAP complex has the stoichiometry TAP1/TAP2 of 1:1 when solubilized using the appropriate detergent. Functionality of the complex was shown to require both components of the complex and to be ATP-dependent.

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Abbreviations: ABC, ATP binding cassette; AMP-PNP, 5'-adenylylimidodiphosphate; BV, baculovirus; DM, dodecyl- β -D-maltoside; DOTAP, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammoniummethylsulfate; IMAC, immobilized metal ion affinity chromatography; mAb, monoclonal antibody; Ni-IDA, nickel iminodiacetic acid; pAb, polyclonal antibody; p.i., post infection; TAP, transporter associated with antigen processing; wt, wild-type.

2. Materials and methods

2.1. DNA constructs

Using PCR, human *tap1* and *tap2* cDNAs (kind gifts from Dr. J. Trowsdale [3]) were amplified as wild-type genes and in the case of *tap1* the codons for 6 histidine residues were inserted at the 3' terminus. Subsequently, the PCR products were cloned as *Bgl*II–*Xba*I fragments into the baculovirus transfer vector pVL1392 (Invitrogen, USA). Alternatively, the cDNAs of *tap1* and *tap2* were inserted into the baculovirus co-expression vector pAcUW51 (Pharmingen, USA) resulting in pAcUW51/TAP1/TAP2 and pAcUW51/TAP1–6 × His/TAP2. In parallel, the baculovirus vector pVL1393 harboring the cDNAs of *tap1* and *tap2* were used to generate recombinant baculoviruses [39]. The constructs were evaluated by restriction analysis and their correct sequences determined by sequencing of the inserts by the dideoxynucleotide method (USB Sequenase kit, USA) [40].

2.2. Cell culture and protein expression

Recombinant baculoviruses carrying the human *tap* genes were generated by co-transfection of Sf9 (*Spodoptera frugiperda*) cells with the recombinant transfer vectors together with linearized baculovirus Baculogold DNA (Pharmingen, USA) using the lipofection reagent DOTAP (Boehringer, Germany). Infection of Sf9 cells grown as a monolayer was routinely performed with a multiplicity of infection (m.o.i.) of about 3–5; culture conditions followed standard procedures [42].

Anti-TAP1 monoclonal (148.3) or polyclonal antibody raised against a C-terminal peptide of TAP1 (Wesse and Tampé, in prep.) and anti-TAP2 mAbs (435.3 and 429.3) raised against a baculovirus-expressed 280 aa C-terminal fragment of TAP2 [39] were used for immunoblotting and fluorescence. Immunoprecipitation experiments were carried out by incubating mAb 148.3 with extracts of Sf9 cells, followed by precipitation of the complex with protein A–Sepharose (Sigma, USA). Immunofluorescence was performed according to [42] using fluorescein (FITC)- and rhodamine (TRITC)-coupled goat anti-mouse and anti-rabbit antibodies (Sigma, USA).

2.3. Purification of the TAP complex

Purification of the TAP complex containing the TAP1–6 × His fusion protein was performed by immobilized metal affinity chromatography (IMAC). 10^7 Sf9 cells (60 h p.i.) were lysed in 2 ml 150 mM Na₂HPO₄, pH 8.0 containing 1.5% dodecyl- β -D-maltoside (DM; Sigma, USA). After centrifugation (10 min, 12,000 × g at 4°C) the solubilized proteins were applied to a Ni-IDA column (Pharmacia, Germany). The column was pre-equilibrated in buffer A (1 mM DM, 150 mM Na₂HPO₄, pH 8.0) and proteins were eluted using a gradient of 0–200 mM imidazole in buffer A. The TAP-containing fraction was analyzed by size-exclusion chromatography using a Superose 6 column (Pharmacia, Germany) with buffer A as running buffer at a flow rate of 0.3 ml/min. Elution of proteins was followed by absorbance (OD₂₈₀) or tryptophan fluorescence detection ($\lambda_{ex}/\lambda_{em}$ = 280/350 nm). Fractions of 1 ml were analyzed by immunoblotting with TAP1- and TAP2-specific antibodies.

2.4. Preparation of microsomes

For preparation of microsomes from Sf9 cells about 10^8 cells/ml were lysed by repeated drawing through a 26 gauge needle in cavitation buffer (250 mM sucrose, 25 mM KOAc, 5 mM MgOAc, 0.5 mM CaOAc, 50 mM Tris, pH 7.4) with proteinase inhibitor mix. After centrifugation for 5 min at 1000 rpm (4°C), the supernatant was diluted 6.4-fold with 2.5 M sucrose solution in gradient buffer (150 mM KOAc, 5 mM MgOAc, 50 mM Tris, pH 7.4) and subsequently overlaid with 2.0 M sucrose solution and 1.3 M sucrose solution (both in gradient buffer). The step gradient was centrifuged overnight (4°C, 85,000 × g) and the microsomes accumulated at the interface of the 2.0 and 1.3 M sucrose solution were washed once with PBS, 1 mM DTT and centrifuged for 2 h (4°C, 190,000 × g). After resuspending in PBS, 1 mM DTT, the microsomes were snap-frozen in liquid nitrogen and stored at –80°C.

2.5. Peptide translocation assay

The peptide R-10-T (RYWANATRST) was synthesized by the solid-phase technique and purified by reverse-phase chromatography. Its identity was verified by mass spectrometry. Iodination of 15 μ g R-10-T

with 1 mCi Na¹²⁵I (Amersham, USA) was performed using free chloramin T [43]. Iodinated peptide (R-10-T*) was separated from free Na¹²⁵I by gel-filtration through a Sephadex G10 (Pharmacia, Germany) column. The peptide translocation assay was performed according to [24,25]. Microsomes were incubated in 150 μ l of buffer T (PBS; 0.1% dialyzed BSA, 10 mM MgCl₂, 1 mM DTT) with ~50 ng (~40 pmol) of R-10-T* (~1.5 × 10⁶ cpm) in the presence or absence of ATP, apyrase, AMP-PNP (all Sigma, USA) and a 100-fold excess of unlabelled R-10-T for 10 min at 37°C. After one washing step, the microsomes were lysed by addition of 1 ml lysis buffer (1% NP-40, 150 mM NaCl, 5 mM MgCl₂, 50 mM Tris-HCl, pH 7.5). After centrifugation (2500 × g, 5 min, 4°C) the supernatant was incubated for 2 h with 100 μ l of Concanavalin A–Sepharose (Sigma, USA) at 4°C, washed two times, and bound peptide was eluted with 1 ml 200 mM α -methylmannoside (Sigma, USA) in lysis buffer (60 min at room temperature) and quantified by γ -counting.

3. Results and discussion

3.1. Expression and localization of TAP1 and TAP2

Human TAP1 and TAP2 were expressed in Sf9 insect cells using the baculovirus expression system. After PCR amplification of the cDNAs, human *tap1* and *tap2* were inserted into the transfer vectors pVL1392 and pAcUW51. In order to establish a mild procedure for protein purification, we generated a fusion construct coding for TAP1 with a C-terminal stretch of 6 histidine residues (TAP1–6 × His). All constructs were confirmed by DNA sequencing.

The time-dependent expression of the TAP proteins in Sf9 cells infected with recombinant baculovirus containing the genes for TAP1–6 × His (rBV-TAP1–6 × His) and TAP2 (rBV-TAP2) is shown in Fig. 1. Total cellular proteins were separated by SDS-PAGE, and analyzed by immunoblotting with TAP1- and TAP2-specific monoclonal antibodies (Fig. 1a,b). TAP1–6 × His and TAP2 were detected at a molecular weight of about 75 kDa. Comparison of the recombinant proteins of all our constructs with cell extracts of different human cell lines revealed identity of the molecular weights of the wild-type TAP1/2 proteins (71/75 kDa). Due to the additional fusion tag, TAP1–6 × His runs at a slightly higher molecular weight; thus, TAP1–6 × His could not be separated from TAP2. Cell extracts from non-infected or wild-type baculovirus (BVwt)-infected Sf9 cells did not express TAP proteins (Fig. 1c). Insect cells infected with rBV-TAP1 and rBV-TAP2 were treated with tunicamycin and total cellular proteins were analyzed by SDS-PAGE. No shift in molecular weights of the TAP proteins was observed, indicating that TAP1 and TAP2 are not glycosylated (data not shown). For all constructs, the protein expression is under the control of a strong, late viral promoter (p_h or p₁₀) correlating with an expression maximum between 48 and 72 h after infection. After 72 h degradation products are visible. Therefore, cells were routinely harvested 60 h p.i.

Immunofluorescence microscopy was performed with infected Sf9 cells (rBV-TAP1/rBV-TAP2 and BVwt) using TAP1-specific pAbs or mAbs and TAP2-specific mAbs. In TAP-expressing cells a heterogeneous fluorescence pattern centered around the nucleus indicates that TAP1 and TAP2 are localized in cytoplasmic compartments, most probably in the ER (Fig. 2). TAP1 and TAP2 could not be detected in the plasma membrane or in the nucleus. By co-labelling with TAP1-specific polyclonal and TAP2-specific monoclonal antibodies both proteins could be localized within the same cell although not always in equal amounts. This reflects the fact

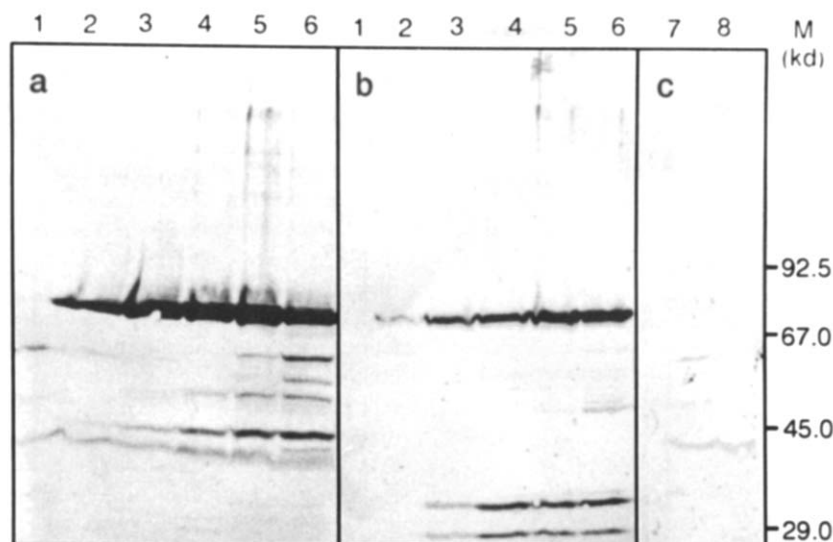


Fig. 1. Time-dependent co-expression of TAP1 and TAP2 in Sf9 cells. Immunoblot of SDS-PAGE (8–18%) of Sf9 insect cells infected with recombinant baculovirus containing the genes of TAP1–6 × His and TAP2. Total cellular proteins of $\sim 5 \times 10^5$ cells harvested 12 h (lane 1), 36 h (lane 2), 48 h (lane 3), 60 h (lane 4), 72 h (lane 5) and 94 h (lane 6) after infection were treated with the TAP1 specific mAb 148.3 (a) and the TAP2 specific mAb 435.3 (b). Lanes 7 and 8 show total cellular proteins of non-infected and wild-type baculovirus infected Sf9 cells, respectively, harvested 72 h after infection and treated with both monoclonal antibodies (c).

that the cells shown in Fig. 2 are infected with rBV-TAP1 and rBV-TAP2 but it may be that not every single cell is co-infected at the same time. Comparative immunoblots of total cell extracts, membrane fractions and subcellular organelles reveal that TAP proteins are found in membrane fractions enriched in the microsomes corresponding to the rough and smooth ER (data not shown). These results are in agreement with the observed localization of TAP in mammalian cells by immunogold labelling [15].

3.2. Complex formation

To address the question of whether TAP1 and TAP2 over-expressed in insect cells associate with each other, a number of

detergents were tested for effective solubilization of the TAP proteins. Dodecyl- β -D-maltoside proved to be very suitable for mild, but effective, solubilization of the TAP complex. Subsequent immunoprecipitation with our TAP1-specific mAb demonstrated that both TAP proteins are still associated under these conditions, since TAP2 co-precipitates together with TAP1 (data not shown). For comparison with affinity chromatography using TAP-specific antibodies, which usually includes rather harsh elution conditions, a method for mild isolation and purification of the TAP complex was developed. Solubilized proteins of Sf9 cells infected with rBV-TAP1–6 × His/TAP2 were separated by metal chelate affinity chromatography (Fig. 3). Weakly bound proteins were removed by washing

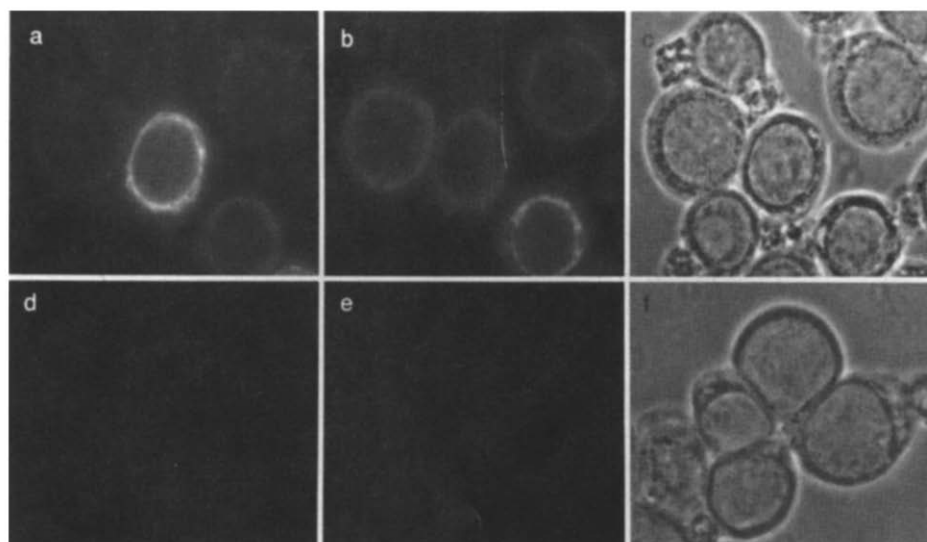


Fig. 2. Localization of TAP1 and TAP2 in Sf9 cells. Sf9 cells infected with rBV-TAP1/rBV-TAP2 (a–c) and BVwt (d–f) were visualized by immunofluorescence of fluorescein (a,d) or rhodamine (b,e) and phase contrast (c,f) 48 h p.i. Cells were co-labelled with the pAb specific against TAP1 (1:50) and mAb 429.3 specific against TAP2 (1:25) followed by incubation with a goat anti-rabbit fluorescein-coupled antibody (FITC-IgG) and goat anti-mouse rhodamine-coupled antibody (TRITC-IgG).

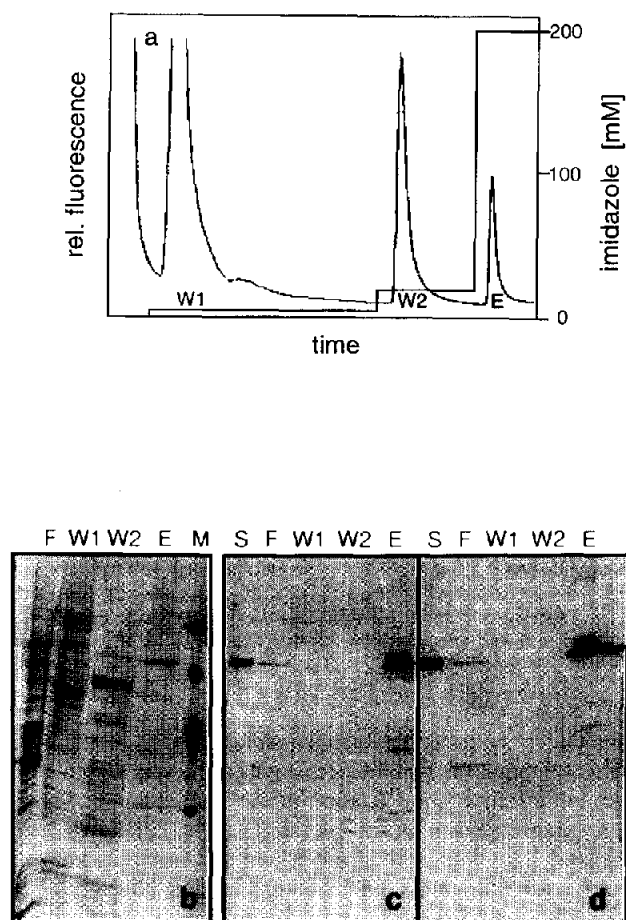


Fig. 3. Purification and complex formation of TAP1 and TAP2. Sf9 cells expressing the TAP complex containing TAP1-6 × His and TAP2 were lysed 60 h p.i. and solubilized proteins were applied onto a Ni-IDA Sepharose column. (a) Chromatogram of the Ni-IDA column run. Proteins were eluted with buffer A containing 10 mM imidazole (W1), 40 mM imidazole (W2) and 200 mM imidazole (E). The elution was detected by tryptophan fluorescence ($\lambda_{ex}/\lambda_{em} = 280/350$ nm). (b) Proteins in total cell extract (S), flow through (F), wash 1 (W1), wash 2 (W2) and elution (E) were separated by SDS-PAGE (8–18%). Coomassie staining (b) and immunoblotting with the TAP1-specific mAb 148.3 (c) and the TAP2-specific mAb 435.3 (d) are shown. Lane M contains marker proteins (92.5, 67.0, 45.0 and 29.0 kDa).

steps with 10 mM and 40 mM imidazole, respectively. Specifically bound proteins were eluted with 200 mM imidazole (Fig. 3a). SDS-PAGE confirmed that this protein peak consisted of purified protein with a molecular weight of about 75 kDa (Fig. 3b). By immunoblotting this band was identified to be a mixture of both TAP proteins (Fig. 3c,d). To answer the question of whether the TAP complex is a heterodimer or multimer, the IMAC-purified TAP proteins were analyzed by size-exclusion chromatography. Under the solubilization conditions used for purification, the exclusion volume of the TAP complex corresponds to a molecular weight of about 200 ± 50 kDa. Assuming that the molecular weight of an average detergent micelle of DM is about 50 kDa, the TAP1/2 complex has the stoichiometry of 1:1 (150 kDa) under these solubilization conditions.

3.3. TAP- and ATP-dependent peptide translocation

To investigate whether the expressed TAP proteins are functional, peptide translocation assays were carried out using microsomes prepared from infected insect cells (60 h p.i.). As a reporter peptide we used R-10-T* which bears an N-glycosylation sequence. Assuming that glycosylation targeting in insect and mammalian cells is comparable, transported peptides should be glycosylated in microsomes and subsequently recoverable by specific binding to Concanavalin A (Con A)-Sepharose. Our results demonstrate that microsomes containing TAP1 and TAP2 are active in ATP-dependent peptide accumulation (Fig. 4). Peptide transport into microsomes strictly requires expression of both proteins, since microsomes from Sf9 cells infected only with rBV-TAP1 (Fig. 4), rBV-TAP2 or BVwt (data not shown) do not accumulate labelled peptides. TAP-dependent peptide translocation is an active transport process requiring the hydrolysis of ATP, which is shown by a 35-fold increase in R-10-T* (28,600 cpm, 1.5% of the total radioactivity) eluted from Con A-Sepharose in the presence of 10 mM ATP. Blocking of the ATP-binding domain by incubation with the non-hydrolyzable ATP-analogue AMP-PNP resulted in no recovery of glycosylated peptide. Translocation of radioactive peptides was completely inhibited by competition with a 100-fold excess of unlabelled peptide (R-10-T) in the presence of 10 mM ATP. Assays with microsomes containing TAP complexes tagged with an oligohistidine sequence revealed the same results.

In this paper we demonstrate for the first time that peptide translocation into the ER of insect cells infected with recombinant baculovirus containing human *tap1* and *tap2* could be proven, and that other cofactors of a highly developed cellular immune system are not essential. Over-expressed TAP proteins assemble to form a stable complex highly enriched in the ER membrane. Using insect cell microsomes over-expressing TAP proteins we have also demonstrated direct binding of peptide substrates to the TAP complex, providing additional evidence for the functional state of insect cell-expressed TAP proteins [39]. Therefore, microsomal membranes of infected Sf9 cells are highly suitable for peptide binding and translocation assays as

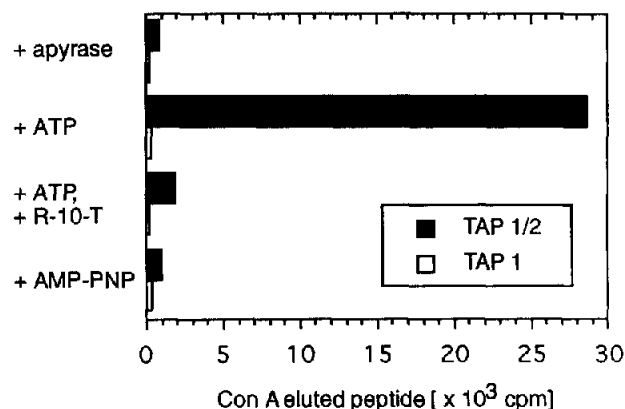


Fig. 4. Peptide translocation into microsomes. Microsomes of infected Sf9 cells containing both TAP proteins (TAP1/2) or only TAP1 were incubated with radioactive peptide (R-10-T*) in the presence or absence of apyrase (20 U/ml), ATP (10 mM), AMP-PNP (10 mM) and a 100-fold excess of non-labelled R-10-T for 10 min at 37°C. The radioactivity of specifically eluted R-10-T* from the Con A-Sepharose represents the amount of transported and glycosylated peptide.

well as for isolation of the transporter complex in large quantities. Strategies were developed for purification of the solubilized complex. From the translocation assays described, strong, although only indirect, evidence for the essential function of the peptide transporter for antigen processing was found. Isolation and functional reconstitution of the TAP complex into artificial membranes is a promising approach to study the mechanism of substrate recognition and transport as well as the structure of the ABC transporter.

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