Supporting Information

Baldauf et al. 10.1073/pnas.1001308107

SI Text

SI Materials and Methods. ***Materials.*** Antibodies specific for the transporter associated with antigen processing (TAP) were monoclonal anti-TAP1 (mAb 148.3) and anti-TAP2 (mAb 435.3) (1, 2). Fluorescein-5-maleimide (FM), 5-iodoacetamidofluorescein (5-IAF), *N*-ethylmaleimide (NEM), and 1,10-phenanthroline were ordered from Molecular Probes (Invitrogen). Methanethiosulfonate (MTS) reagents were purchased from Toronto Research Chemicals. Peptides were synthesized by Fmoc [N-(9-fluorenyl) methoxycarbonyl] solid-phase chemistry and purified by reversed-phase C_{18} HPLC (3). The protein concentration was determined by the micro bicinchoninic acid assay (Pierce). All other reagents were of reagent grade and obtained from commercial sources.

Cloning and expression. Based on human *tap1* and *tap2*, combinations of Cys-less (CL) and wild-type (WT) TAP subunits were generated (4).The *tap1CL* gene was cloned into the *Bam*HI and *Hind*III sites of pFastBacDual™ (Invitrogen) downstream of the polyhedrin promoter and combined with *tap2*. The *tap2* was cut from p46TAP2WT via *Not*I (treated with Klenow) and *Nsi*I and cloned into pFastBacDual.TAP1CL via *Xho*I (treated with Klenow) and *Nsi*I (5). The resulting plasmid was termed pFastBacDual.TAP1CL/TAP2WT. The combination of *tap1* and *tap2CL* was generated by cloning *tap2CL* into the pFastBacDual.TAP1WT plasmid via *Xho*I and *Nsi*I. The resulting plasmid was termed pFastBacDual.TAP1WT/TAP2CL.

To generate a TAP complex containing cysteine residues only in the N-terminal region (1–265) of TAP2, first a 5′ *Xho*I/*Dra*I fragment of *tap2* was combined in a single ligation step with a 3′ *Dra*I/*Nsi*I fragment of *tap2CL* in pFastBacDual.TAP1CL, which was opened with *Xho*I and *Nsi*I. The resulting plasmid was termed pFastBacDual.TAPCL/TAP2CL(1-265WT). Single cysteines were reintroduced into *tap2CL* at the original position by site-directed mutagenesis to generate CL/single-Cys complexes. The following sense oligonucleotides were used to introduce cysteines at the indicated positions: TAP2A197C, 5′-CTTCTTCATGTGCCTGTTCTCCTTCG-3′; TAP2A209C, 5′-CTGTCCGCTGGTTG CAGAGGCGGTTC-3′; TAP2S213C, 5′-CAGAGGCGGTTGTTTCACCTAC AC-3′; TAP2S353C, 5′-GAACACGAAGTCTGTAGATACAAGGAAGCTCTG-3′; TAP2S362C, 5′-GCTCTGGAACAGTGTAGACAGC; TAP2V394C-3′, 5′-TGATGCTGTCCTGCGGT CTGCAAC-3′ (exchanged nucleotides are underlined). All constructs were confirmed by sequencing. Single-Cys constructs of *tap2* were combined with *tap1CL* resulting in plasmids encoding always both subunits (pFastBacDual.TAP1CL/TAP2single-Cys).

Recombinant baculovirus and cell culture. Bacmid DNA was produced by transformation of DH10Bac cells with pFastBacDual.-TAP (Invitrogen). Insect cells (*Spodoptera frugiperda*, *Sf9*) were grown in Sf900II medium (Invitrogen) following standard procedures. Recombinant baculovirus was generated and used for infection of insect cells as described previously (4).

Membrane preparation. For crude membrane preparation, 2.0×10^6 insect cells per milliliter were resuspended in Tris-buffer (10 mM Tris · HCl, 1 mM DTT; pH 7.4) and homogenized with a tight glass Dounce homogenizer (Wheaton). Nuclei and cell debris were removed by centrifugation at $200 \times g$ for 4 min followed by $700 \times g$ for 8 min at 4 °C. Membranes were harvested at $100,000 \times g$ for 30 min at 4 °C, washed, and resuspended in PBS (pH 7.4) to a final concentration of 5 mg protein per milliliter. Aliquots were frozen in liquid nitrogen and stored at −80 °C. All buffers used for preparation were supplemented with protease inhibitors [50 μg/mL 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 μg/mL aprotinin, 150 μg/mL benzamidine, 10 μg/mL leupeptin, 5 μg/mL pepstatin]. The preparation of microsomes was performed as described previously (1).

Peptide binding. Peptides were radiolabeled with $Na^{125}I$ using the chloramin T method (6). Peptide binding to TAP was performed using filter assays. Filter plates (MultiScreen plates with a glass fiber filter, pore size 1 μM; Millipore) were preincubated with 0.3% polyethyleneimine. TAP-containing membranes (20 μg of total protein) were incubated with 1 μM or with various concentrations of radiolabeled peptides (RRYQKSTEL or RYWANATRSX, where X represents Phe, Lys, Val, or Thr) in 50 μL of binding buffer (PBS with 5 mM $MgCl_2$; pH 7.0) for 20 min on ice. Subsequently, the membranes were transferred to filter plates and washed twice with 100 μL of ice-cold binding buffer. Filter-associated radioactivity was quantified by γ-counting. Unspecific binding was determined in the presence of a 400-fold molar excess of unlabeled peptide (RRYQKSTEL) or in the presence of the TAP specific Herpes Simplex viral inhibitor ICP47 (50 μM) (7). Specifically bound peptides were plotted against the total peptide concentration and fitted to a Langmuir (1:1) binding model:

$$B = B_{\max} \times \frac{[C]}{K_D + [C]}, \qquad \textbf{[S1]}$$

where B represents the bound peptide, $B_{\max}$ the maximal amount of bound peptide, $[C]$ the peptide concentration, and K_D the dissociation constant, respectively.

The affinities of the peptide libraries were determined by a competition assays in order to determine in the half-maximal inhibitory concentration (IC_{50}). TAP-containing membranes (20 μg of protein) were incubated with 1 μM of the radiolabeled peptide RYWANATRST and various concentrations of the peptide library and treated as described above. To calculate the IC_{50}, the amount of specifically bound peptides was plotted against the peptide concentration and fitted with the following equation:

$$B = \frac{B_{\max} - B_{\min}}{1 + 10^{[C] - \lg\ IC_{50}}} \qquad \textbf{[S2]}$$

where B corresponds to the bound peptide, $B_{\max / \min}$ to the amount of bound peptide (maximal and minimal), $[C]$ to the peptide concentration, and IC_{50} to the half-maximal inhibitor concentration, respectively.

For TAP inhibition, microsomes were incubated with NEM (500 μM), FM (250 μM), 5-IAF (250 μM), [2-{trimethylammonium)ethyl]methanethiosulfonate bromide (1 mM), (2-sulfonatoethyl)methanethiosulfonate (10 mM), or (2-aminoethyl) methanethiosulfonate (2.5 mM), respectively, for 15 min at 4 °C (8). After washing with PBS, peptide binding was measured using 1 μM of radiolabeled peptide as described above. To reverse MTS labeling, samples were incubated with 100 mM of β-mercaptoethanol (β-ME) for 30 min on ice. After washing with PBS, peptide-binding assays were performed as described above. The MTS-labeling efficiency was determined via 5-IAF alkylation (250 μM) for 15 min at 4 °C in the dark. Samples were washed twice prior to the analysis by SDS-PAGE (10%) and immunoblotting.

Relative amounts of 5-IAF-labeled TAP were determined by in-gel fluorescence with a Lumi Imager F1 (Roche). In order to determine the maximal labeling capacity, TAP was denatured with 2% of SDS for 20 min at room temperature and then labeled with 250 μM of 5-IAF for 3 min prior to the analysis by SDS-PAGE (10%) and immunoblotting.

1. Meyer TH, van Endert PM, Uebel S, Ehring B, Tampé R (1994) Functional expression and purification of the ABC transporter complex associated with antigen processing (TAP) in insect cells. *FEBS Lett* 351:443–447.
2. van Endert PM, et al. (1994) A sequential model for peptide binding and transport by the transporters associated with antigen processing. *Immunity* 1:491–500.
3. Uebel S, et al. (1995) Requirements for peptide binding to the human transporter associated with antigen processing revealed by peptide scans and complex peptide libraries. *J Biol Chem* 270:18512–18516.
4. Schrodt S, Koch J, Tampé R (2006) Membrane topology of the transporter associated with antigen processing (TAP1) within an assembled functional peptide-loading complex. *J Biol Chem* 281:6455–6462.
5. Heintke S, et al. (2003) Functional cysteine-less subunits of the transporter associated with antigen processing (TAP1 and TAP2) by de novo gene assembly. *FEBS Lett* 533:42–46.
6. Neumann L, Tampé R (1999) Kinetic analysis of peptide binding to the TAP transport complex: Evidence for structural rearrangements induced by substrate binding. *J Mol Biol* 294:1203–1213.
7. Neumann L, Kraas W, Uebel S, Jung G, Tampé R (1997) The active domain of the herpes simplex virus protein ICP47: A potent inhibitor of the transporter associated with antigen processing. *J Mol Biol* 272:484–492.
8. Loo TW, Bartlett MC, Clarke DM (2004) The drug-binding pocket of the human multidrug resistance P-glycoprotein is accessible to the aqueous medium. *Biochemistry* 43:12081–12089.