Identification of a Lysosomal Peptide Transport System Induced during Dendritic Cell Development

Özlem Demirel, Zoe Waibler, Ulrich Kalinke, Frank Grünebach, Silke Appel, Peter Brossart, Andrej Hasilik, Robert Tampé, and Rupert Abele

From the Institute of Biochemistry, Biocenter, Goethe-University Frankfurt, Max-von-Laue-Strasse 9, Frankfurt/M. D-60438, the Division of Immunology, Paul-Ehrlich-Institut, Paul-Ehrlich-Strasse 51–59, Langen D-63225, the Department of Hematology, Oncology and Immunology, Eberhard-Karls-University Tübingen, Otfrid-Müller-Strasse 10, Tübingen D-72076, and the Institute of Physiological Chemistry, Philipps-University Marburg, Karl-von-Frisch-Strasse 1, Marburg D-35032, Germany

The delivery of protein fragments to major histocompatibility complex (MHC)-loading compartments of professional antigen-presenting cells is essential in the adaptive immune response against pathogens. Apart from the crucial role of the transporter associated with antigen processing (TAP) for peptide loading of MHC class I molecules in the endoplasmic reticulum, TAP-independent translocation pathways have been proposed but not identified so far. Based on its overlapping substrate specificity with TAP, we herein investigated the ABC transporter ABCB9, also named TAP-like (TAPL). Remarkably, TAPL expression is strongly induced during differentiation of monocytes to dendritic cells and to macrophages. TAPL does not, however, restore MHC class I surface expression in TAP-deficient cells, demonstrating that TAPL alone or in combination with single TAP subunits does not function as a transport complex required for peptide loading of MHC I in the endoplasmic reticulum. In fact, by using quantitative immunofluorescence and subcellular fractionation, TAPL was detected in the lysosomal compartment co-localizing with the lysosome-associated membrane protein LAMP-2. By in vitro assays, we demonstrate a TAPL-specific translocation of peptides into isolated lysosomes, which strictly requires ATP hydrolysis. These results suggest a mechanism by which antigenic peptides have access to the lysosomal compartment in professional antigen-presenting cells.

In the adaptive immune system, T-lymphocytes monitor the peptide repertoire presented in complex with major histocompatibility complex (MHC) molecules on the cell surface to identify exogenous or endogenous pathogens. In the classical MHC class I-mediated immune response, TAP is an essential factor for transporting peptides, generated in the cytosol primarily by proteasomal degradation, into the ER lumen (1). These peptides bind to MHC class I and are shuttled in complex to the cell surface. Exogenous antigens are taken up via the endocytic pathway, and, after fragmentation in lysosomal compartments, peptides are loaded onto MHC class II (2). In professional antigen-presenting cells like macrophages, dendritic cells, and B-cells, a process termed cross-presentation exists, in which MHC class I presents exogenous antigens, whereas cytolysically generated peptides of endogenous origin are displayed by MHC class II on the cell surface (3, 4). In both cases, a TAP-independent pathway has been proposed but not yet identified (5–7).

Most recently, TAPL was uncovered as an ATP-dependent peptide transporter, sharing some degree of overlap in substrate specificity with TAP (8). TAPL transports a very broad spectrum of peptides ranging from 6- up to 59-mers (8), whereas TAP prefers peptides with a length of 8–12 amino acids (9). Both transporters belong to the superfamily of ABC proteins (10). Members of this family are found in all three phyla of life, where they translocate a very broad range of solutes across membranes. Functional complexes are built up either by heterodimers of TAP1 and TAP2, or homodimers of TAPL (8, 11–13). However, whether TAPL can assemble with TAP1 or TAP2 into a functional transport complex is still an open issue (14). TAPL and TAP share a similar exon organization and a high degree of sequence similarity (15–17). Because TAP is an essential factor in the MHC class I pathway, cells lacking TAP1 or TAP2 show a strongly impaired surface expression of MHC class I molecules (18, 19).

The involvement of TAP in antigen presentation is also mirrored by the localization of its genes in the MHC class II locus (20), whereas tapl is not clustered with genes relevant for the immune system (21). Based on phylogenetic analyses, TAPL appears to be the ancestral peptide transporter present even in...
jawless vertebrates lacking an adaptive immune system (17). In contrast to TAP, which is expressed in all somatic cells, TAPL is strongly expressed in testes, whereas moderate expression levels are found in brain, spinal cord, and heart (15, 22). The subcellular localization of TAPL is a matter of debate, because it was identified in lysosomes (15) as well as in the ER (14, 21).

In this study, we investigated the role of TAPL in the adaptive immune system. We observed a strong up-regulation of TAPL expression during maturation of dendritic cells and macrophages. By using quantitative immunofluorescence and subcellular fractionation, TAPL was found in the lysosomal compartment. In agreement with this, TAPL is not involved in the peptide delivery into the classical MHC class I loading compartment, because TAPL did not restore MHC class I cell surface expression of TAP-deficient cells. By establishing an in vitro assay, we demonstrated for the first time a TAPL-specific ATP-dependent translocation of peptides into isolated lysosomes. The identification of a new transport system in the lysosomal compartment as well as its strong up-regulation during maturation of DCs and macrophages suggest that TAPL is part of an alternative pathway of antigen presentation in professional antigen-presenting cells.

EXPERIMENTAL PROCEDURES

Cloning—Human TAPL was cloned into pEGFP-N3 and pIRE52-EGFP (both BD Biosciences) resulting in TAPL with a C-terminal EGFP and TAPL co-translated with EGFP, respectively. Furthermore, TAPL containing a C-terminal myc tag was cloned into pDNA3.1 (+) (Invitrogen). These constructs were PCR-amplified using the primer pairs pDNA3.1(+)-TAPL-myc (5'-CATATGGTCAGGCCTTGTGGAAG-3' and 5'-CGATTAGCTAGCATGCGGCTGTGGAAGG-3'), pEGFP-N3-TAPL (5'-CGATTTGAGTGGTCAGGCCTTGTGGAAG-3'), and pIREs2-TAPL-EGFP (5'-CGATTAGCTAGCATGCGGCTGTGGAAG-3' and 5'-CGATTAGTGGTCAGGCCTTGTGGAAGG-3') and cloned via NheI and BamHI sites into the vectors. For cloning of TAPL into the retroviral vector pLPCX (Clontech), Clal and BglII restriction sites were introduced by PCR via the primer pairs 5'-GAAGATCTTCCGACCATGCAGGTAGTGGGAGG-3' and 5'-ACGCTATGTCAGGCTTGTGACTGCC-3', TAPL-K545A/H699A was generated by Ligase Chain Reaction using the primers 5'-CGGGCGTTGGGGCGAGCTTCTGTGTC-3' and 5'-CTCCATCATCCGCGGCTTGAGGAC-3' (mutated bases are underlined). p46.TAP1wt DNA was used for transient expression of human TAP1 in BRE-169 cells (23).

Cell Lines and Culture—BRE-169 or STF1-169 cells are TAP1- or TAP2-deficient skin fibroblast cell lines, respectively (19). HeLa, BRE-169, STF1-169, and 293T cells were cultured in Dulbecco's modified Eagle's medium (PAALaboratories) containing 10% fetal calf serum (Biochrom AG), 1 m sodium pyruvate, 2 m/l glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin. For BRE-169 and STF1-169 cells, 50 μg/ml hygromycin was supplemented. For stably TAP2-transfected STF1-169 cells, 500 μg/ml neomycin was added to the medium (23). Human Burkitt lymphoma cells (Raji cells) were cultured as reported previously (24). THP-1 cells were grown in RPMI 1640 medium (PAALaboratories) containing 10% fetal calf serum and were stimulated with Escherichia coli as described elsewhere (25).

Generation of DCs—DCs were generated in two different ways. In the first method, human peripheral blood monocytes were isolated by Ficoll density gradient centrifugation and monocytes isolated using anti-CD14 micro beads according to the manufacturer's instructions (Miltenyi Biotech). 1 × 10^6 monocytes per 24-well plate were cultured in DC medium (CellGro) supplemented with 1000 units/ml GM-CSF (Biomedical Laboratories) and 1000 units/ml IL-4 (Peprotech) for 5 days to obtain immature DCs. For differentiation into mature DCs, immature DCs were additionally stimulated from days 5 to 8 with a maturation mixture (26): 10 ng/ml IL-1β (Peprotech), 10 ng/ml TNF-α (Peprotech), 1000 units/ml IL-6 (Peprotech), and 1 μg/ml progesterone (E_2). In the second method, monocyte-derived DCs were generated as described previously (27). To inhibit differentiation of monocytes, IL-10 (10 ng/ml, R&D Systems) was added together with GM-CSF and IL-4. The medium was replenished with cytokines every 2 or 3 days. For maturation, DCs were further cultured with additional stimuli as TNF-α (10 ng/ml, R&D Systems), lipopolysaccharides (LPS, 100 ng/ml, Sigma), Pam3Cys (5.0 μg/ml, EM C Microcollection), poly I:C (50.0 μg/ml, Sigma), R848 (2.0 μg/ml, InvivoGen), or CD40L (100 ng/ml, Bender Medsystem) for 24 h.

RT-PCR—RT-PCR was performed using two different protocols. With the first method, total RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. DNA was removed by treatment with DNase (Roche Applied Science) for 15 min at 37°C, and cDNA was prepared before using SuperScript II (Invitrogen) according to the manufacturer's instructions. PCRs were performed using the following primer pairs: TAPL, 209 bp (5'-GCTCTTGGAGAAGCCCTCTTC-3' and 5'-GAGCGCAAGGACAGACGC-3'), TAPL, 538 bp (5'-CAAGTACTACAGAGACGCTTCAAG-3' and 5'-GGGACATCTGCTGAGGACTG-3'), GAPDH (5'-ACCACAGTCCATGGCATTAC-3' and 5'-TCCACACCCACCTTGGTGA-3'), and β₂m (5'-GGGTTTCCATTCCGACAT-3' and 5'-GATGGTCTTACATGTCTC-3'). The mRNA content was normalized by RT-PCR analysis with GAPDH-specific primers. PCRs using RNA before reverse transcription as template confirmed the absence of genomic DNA in the samples. With the second method, total RNA was isolated from cell lysates using Qiagen RNAeasy Mini anion-exchange spin columns, including on-column DNase digestion (Qiagen), according to the instructions of the manufacturer. Total RNA (1μg) was subjected to a 20.0-μl cDNA synthesis reaction (Transcriptor First Strand cDNA Synthesis Kit, Roche Applied Science) using random hexamer primers. 1.0 μl of cDNA was used for PCR amplification. To control the integrity of RNA and the efficiency of cDNA synthesis, 1.0 μl of cDNA was amplified by an intron-spanning primer pair for the β₂-microglobulin gene.

Transfection—BRE-169 and STF1-169 cells (2 × 10^6 cells) or HeLa cells (1 × 10^7 cells) were electroporated in 400 μl of electroporation buffer (20 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7
Lyososomal Translocation System

mm Na₂HPO₄, 6 mm dextrose, pH 7.05) in the presence of 6 μg or 30 μg DNA, respectively. Electroporation was performed with a GenePulsor II (Bio-Rad, gene pulser cuvettes (0.4-cm electrode gap), 260 V, 350 microfarads, and 50 Ω). The cells were diluted in 10 ml of recovery medium (culture medium containing 3 mM EGTA) for 30 min at 37 °C. Cells were subsequently transferred to fresh culture medium.

Retroviral Transduction—Recombinant retroviruses were obtained by co-transfection of pCL-Ampho (generous gift of Peter Cresswell) (28), and the retroviral constructs pLPCX-TAPL or pLPCX-TAPL-K545A/H699A into 293T cells with Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. Supernatants were collected 30 h after transfection and filtered through a 0.45-μm pore size filter. For transduction, 1 × 10⁶ Raji or THP-1 cells were resuspended in 2 ml of viral supernatant supplemented with 8 μg/ml Polybrene (Sigma). Cells were transferred into 6-well plates and centrifuged at 1250 × g for 90 min at 32 °C. After 15 h, cells were transferred to normal medium, and after recovery for 6–8 h, the transduction was repeated. 24 h after, cells were selected in medium containing 0.5 μg/ml puromycin (Sigma).

Flow Cytometry—BRE-169 and STF1-169 cells were harvested 48 h after electroporation, washed once in FACS buffer (phosphate-buffered saline, 2% fetal calf serum), and blocked with bovine serum albumin buffer (FACS buffer containing 5% bovine serum albumin). Afterward, 5 × 10⁵ cells were stained with a phycoerythrin (PE)-conjugated anti-human HLA-ABC (W6/32, eBioscience) or PE-conjugated mouse IgG₁ isotype control antibody (eBioscience) and analyzed by flow cytometry with a FACSaria cell sorter (BD Biosciences) controlled by FACSDiva software (BD Biosciences). Routinely, 30,000 cells were counted for one measurement. The data were analyzed with FCS-Express software (De Novo Software). Monocytes as well as immature and mature DCs were washed twice with phosphate-buffered saline and immunostained in the presence of polygolgyn for 20 min at 4 °C using the following monoclonal antibodies diluted to the optimal concentration: anti-CD14-Pacific blue (BD Pharmingen), anti-HLA-DR-APC-Cy7 (Biolegend), anti-CD83-APC (BD Pharmingen), and anti-CD86-PE (Biolegend). Cells were analyzed by flow cytometry using LSR II with DIVA and/or WinList software.

Quantitative Immunofluorescence—Stably transduced and stimulated THP-1 cells or HeLa cells grown on poly-L-lysine-coated coverslips were fixed for 12 min at room temperature in 2% paraformaldehyde in phosphate-buffered saline and permeabilized with 0.1% Triton X-100 for 20 min at room temperature. After blocking with 5% bovine serum albumin, cells were stained with the primary antibody (rabbit anti-calnexin polyclonal antibody (Stressgen), mouse anti-calnexin (Dianova), rabbit anti-EEA1 polyclonal antibody (Calbiochem), mouse anti-EEA1 (Abcam), anti-myc monoclonal antibody (BD Biosciences), anti-LAMP-2 monoclonal antibody (2D5) (29), epitope-purified, rabbit anti-TAPIpolycional antibody (8)) followed by fluorescence-labeled secondary antibody (Cy3 donkey anti-rabbit antibody, Cy3 donkey anti-mouse antibody, Alexa Fluor 488 goat anti-mouse antibody (Dianova)) for 30 min each at room temperature. Preparations were mounted in 0.1 ml Tris/HCl, pH 8.5, 25% (w/v) glycerol, 10% (w/v) Mowiol (Calbiochem), and 2.5% 1,4-diazabicyclo[2.2.2]octane (Sigma). Samples were analyzed with a confocal laser-scanning microscope (LSM 510, Zeiss) using the argon laser line at 488 nm for Alexa Fluor 488 or EGFP excitation and a 543 nm HeNe laser for excitation of Cy3. Fluorescence was detected through a 505–530 nm band pass filter and a 560 nm long pass filter for the argon laser and HeNe laser excitation, respectively. 512 × 512 pixels per image were recorded with a Plan-Apochromat 63 × oil immersion objective (numerical aperture, 1.4). To reduce blur, improve resolution, and reduce background, blind decon- volution with the program Auto Deblur (Bitplane AG, Switzerland) was performed. The restored images were used for the co-localization analysis with the software package Imaris (Bit- plane AG). The threshold was set to 50 to eliminate influence of background for co-localization. For each pair of co-localization analysis, the number of co-localized voxels was determined from 2 to 10 images.

Preparation of lysosomes—2 × 10⁶ Raji or stimulated THP-1 cells were harvested, washed once in HEPES buffer (10 mM HEPES, 1 mM EDTA, 250 mM sucrose, pH 7.4), and homogenized in a Dounce homogenizer. The homogenate was centri- fuged at 1,000 × g for 5 min, and the post-nuclear supernatant was layered on 31% (v/v) Percoll underlayed with a cushion of 27.6% Nycodenz solution. The gradient was centrifuged at 40,000 × g for 1 h. 14 fractions were collected and analyzed by SDS-PAGE and immunoblotting using TAPL (8), LAMP-2 (29), and tapasin-specific antibodies (30). Fraction 14 corre- sponding to the dense pool of lysosomes was used for the peptide translocation assay.

Peptide Transport—In vitro transport assays were performed with the dense pool of lysosomes in the presence of 2 μM fluoro- rescin-labeled peptide RRY(C)(PK)KSTEL (F indicates fluores- cin coupled via a cysteine residue) in a total volume of 150 μl of HEPES buffer containing 10 mM MgCl₂. For competition or inhibition, 500 μM non-labeled peptide or 1 mM orthovanadate was added, respectively. Transport was performed in the presence of ATP or AMP (3 mM) for 30 min at 32 °C. Thereafter, the transport was stopped with stop buffer (HEPS buffer with 10 mM EDTA and 50 μM non-labeled peptide RRYKSTEL). After 15-min incubation on ice, samples were transferred to micro- filter plates preincubated with 0.3% polyethyleneimine (Multi- Screen plates, Durapore membrane, 1-μm pore size, Millipore). Filters were washed three times with 250 μl of ice-cold HEPES buffer containing 10 mM EDTA. Subsequently, the filters were incubated with 250 μl of elution buffer (phosphate- buffered saline, 1% SDS) for 10 min. Fluorescent peptides were quantified with a fluorescence plate reader (excitation and emission at 485 and 520 nm, respectively, BMG, Polarstar Galaxy).

RESULTS

TAPL Expression Is Strongly Induced during Differentiation of Monocytes to Dendritic Cells—On the transcriptional level, TAPL expression has been found in different tissues (15, 22). However, TAPL protein expression was shown only in Sertoli cells (15). Because the supply and cellular compartmentaliza- tion of antigenic peptides is of key importance for manifesta- tion of an adaptive immune response, we examined TAPL...
expression in professional antigen-presenting cells. Human CD14+/ peripheral blood monocytes were isolated and cultured in medium containing GM-CSF and IL-4. After 5 days of incubation, monocytes differentiated to immature DCs as indicated by the loss of CD14 surface expression and the up-regulation of MHC class II, CD83 and CD86 on the cell surface (Fig. 1A). Incubation for further 3 days with a cytokine mixture containing IL-1β, TNF-α, IL-6, and prostaglandin E2 (26) resulted in the maturation of DCs as indicated by further increase in MHC class II, CD83, and CD86 surface expression.

First, mRNA levels of TAPL were studied using primers binding to exons 2 and 4 of tapl, resulting in a PCR product of 209 bp (Fig. 1B). In monocytes, no mRNA for TAPL was detected by RT-PCR, whereas differentiation of monocytes with GM-CSF and IL-4 to immature DCs strongly increased the mRNA level of TAPL. Further maturation of DCs did not significantly change the transcriptional level of TAPL. RT-PCR analysis with primers binding to exons 5 and 8 gave similar results. Here, a single PCR product of 538 bp was detected for all donors analyzed, excluding the presence of the splice variant reported previously, lacking exon 7 (expected PCR product of 409 bp) (15).

The mRNA level may not necessarily reflect the expression level of TAPL. We therefore investigated TAPL expression on the protein level by immunoblotting using an antibody specific for the splice variant 12A of TAPL (Fig. 1C), which is active in ATP-dependent peptide transport (8). In monocytes isolated from various donors, TAPL expression was not detectable. However, after differentiation to immature and mature DCs, expression of TAPL was strongly induced. TAPL was detected as a single band at an apparent molecular mass of ~72 kDa. TAPL runs faster than the theoretical mass of 84 kDa. This behavior is typical for very hydrophobic proteins (8).

We next examined the regulation of TAPL mRNA levels upon stimulation of immature DCs in more detail (Fig. 1D). Monocytes treated with GM-CSF and IL-4 in combination with IL-10, which inhibits DC development (31–33), expressed very low amounts of TAPL mRNA. Furthermore, immature DCs stimulated by specific ligands, such as Pam3CSK4, poly(I:C), or LPS, strongly decreased TAPL transcript, whereas TNF-α, CD40L, and R848 showed only a moderate decrease. Noteworthy is that this down-regulation on mRNA level was not detected on the protein level (data not shown), implying that TAPL has a long half-life.

TAPL Is Not Involved in the Classical MHC Class I Presentation Pathway—Because TAPL is strongly up-regulated in professional antigen-presenting cells, we analyzed its function in the classical pathway of peptide loading of MHC class I molecules in the ER lumen. Two scenarios are conceivable, in which TAPL alone or in complex with TAP1 or TAP2 translocates peptides into the ER lumen. To address this issue, skin fibroblasts derived from a lymphocyte syndrome type I patients deficient in TAP1 or TAP2 (BRE-169 and SFTI-169 cells, respectively) (18, 19, 23) were transfected with human TAPL. TAPL expression was demonstrated by immunoblotting. Conspicuously, TAPL was not detected in non-transfected cells (Fig. 2A). Subsequently, MHC I cell surface expression reflecting successful peptide loading of MHC class I molecules in the ER lumen was monitored by flow cytometry using a PE-conjugated anti-HLA-ABC antibody (W6/32) (Fig. 2B). TAP-deficient fibroblasts showed a slightly higher fluorescence signal compared with the isotype control, resulting from TAP-independent peptide supply to MHC class I molecules (34, 35). Transfection of TAP1- or TAP2-deficient cells with TAPL did not affect MHC class I surface expression. By contrast, TAP1 or TAP2 restored peptide supply into the ER lumen in cells lacking the corresponding TAP subunit, reflected by an increase in MHC I surface expression. The transfection efficiencies of TAP and TAPL...
Lysosomal Translocation System

FIGURE 2. TAPL is not involved in the classical pathway of peptide loading of MHC I molecules in the ER lumen. A, expression of TAPL in TAPL−/− BRE-169 and TAP2−/− STF1-169 cells. TAP-deficient cells were transiently transfected with pRES2-TAPL-EGFP. TAPL expression was analyzed by SDS-PAGE and immunoblotting using an epitope-purified anti-TAPL antibody (8). As control, non-transfected cells were analyzed. An equal amount of protein per lane was verified by anti-actin staining. B, FACS analysis of MHC class I cell surface expression. For TAPL expression, TAP1-deficient BRE-169 and TAP2-deficient STF1-169 were transiently transfected with pRES2-TAPL-EGFP. TAP1 expression was restored in BRE-169 cells by transient transfection. TAP2 was stably transfected into STF1-169 cells. Cell surface expression of MHC class I was analyzed by flow cytometry using PE-conjugated anti-HLA-ABC and PE-conjugated mouse IgG, as an isotype control. Non-transfected cells were used as control.

were similar (25%) as monitored by the number of cells with elevated MHC class I level for TAP1 and the co-expression of EGFP for TAPL. In conclusion, TAPL alone or in combination with single TAP subunits does not complement the cellular function of the TAP complex in peptide loading of MHC class I molecules in the ER lumen.

TAPL Is Localized in the Lysosomal Compartment—Because the subcellular localization of TAPL is still under debate (14, 15, 21), we investigated TAPL localization by quantitative immunofluorescence confocal laser-scanning microscopy. To establish the quantification analysis, HeLa cells were transfected with human TAPL containing a C-terminal myc tag or EGFP. Two days after transfection, TAPL-myc and TAPL-EGFP were detected by immunoblotting as single bands differing by 25 kDa due to the EGFP fusion (supplemental Fig. S1A). Notably, intrinsic TAPL expression was not observed in these cells. Both TAPL constructs are functional in peptide transport into intracellular compartments using semi-permeabilized cells (see below).

For quantitative immunofluorescence, cells expressing TAPL-myc were stained with anti-myc and affinity-purified anti-TAPL antibodies. The deconvoluted micrographs of stained cells, their merged pictures, and the merge after the co-localization analysis are shown in Fig. 3A. A co-localization with a Pearson correlation coefficient of 0.73 ± 0.08 was determined (Table 1). A coefficient between zero and −1 indicates no co-localization, whereas a value of 1 attests a perfect co-localization, which can only be reached in theory (36). Similar co-localization coefficients (0.66 ± 0.04) were detected after analysis of TAPL-EGFP with anti-TAPL antibody. As negative control, the double staining of calnexin, an ER-resident protein, and the lysosome-associated membrane protein 2 (LAMP-2) resulted in a Pearson coefficient of 0.04 ± 0.06.
The subcellular localization of TAPL in professional antigen-presenting cells was analyzed in THP-1 cells, a promonocytic cell line widely used as a model system for monocyte to macrophage differentiation. Upon stimulation with LPS or intact E. coli, THP-1 cells differentiate into macrophages followed by morphology changes, up-regulation of antigen presentation, and processing elements (37). For immunofluorescence, stably transduced THP-1 cells showing high TAPL expression (supplemental Fig. S2) were stimulated with E. coli and stained with anti-TAPL antibody. As shown in Fig. 3B, TAPL clearly colocalizes with the lysosomal marker LAMP-2 (Pearson coefficient of 0.639 ± 0.089) but is neither found in the ER (−0.049 ± 0.082) nor in early endosomes (0.075 ± 0.044), as demonstrated by the marker proteins calnexin and early endosome antigen 1 (EEA1), respectively. Similar results and Pearson coefficients were obtained with HeLa cells expressing TAPL-myc or TAPL-myct (Table 1 and supplemental Fig. S1, B and C). Based on these data, we conclude that TAPL is exclusively found in the lysosomal compartment, but not in the ER or early endosomes.

To formally rule out that the observed lysosomal localization is due to overexpression of TAPL, we stimulated THP-1 cells with E. coli and performed a subcellular fractionation using a Percoll density gradient. Upon stimulation of monocytes to professional antigen-presenting cells, TAPL expression is highly up-regulated (supplemental Fig. S3). Remarkably, TAPL is highly enriched in the dense fraction of the Percoll gradient co-migrating with the lysosomal marker protein LAMP-2 (Fig. 3C). Noticeably, this fraction does not contain ER-resident marker proteins, such as tapasin, showing that endogenously expressed TAPL also localizes in lysosomal compartments.

**TAPL-specific Peptide Translocation into Lysosomes**—After analyzing TAPL in dendritic cells and macrophages, we examined TAPL function in the lysosomal compartment of B-cells. We used human Burkitt’s lymphoma (Raji) cells as a model system. After retroviral transduction, TAPL and the Walker A/H-loop mutant K545A/H699A were detected by immunoblotting as single bands (Fig. 4A). To uncover TAPL function, lysosomes were purified via Percoll density gradient and peptide translocation was studied using the dense fraction, which is strongly enriched in the lysosomal marker LAMP-2 but deficient in the ER-marker tapasin. Peptide transport is detected only in lysosomes containing wild-type (6-fold above background) but not mutant TAPL. Peptide transport is inhibited by orthovanadate, which traps a post-hydrolysis state (38). In addition, the transport is peptide-specific, because it is inhibited by an excess of non-labeled peptide. Similar results were obtained with HeLa cells transiently transfected with TAPL-EGFP or TAPL-myc. 3-Fold transport activity above background was observed with saponin semi-permeabilized cells, confirming that the function of TAPL is not affected by the C-terminal tags and is independent of the cell and expression system used. Taken together, we identified a novel ATP-dependent peptide translocation system in lysosomes encoded by the ABC transporter TAPL.

**DISCUSSION**

For a perfect interplay between different cells mediating the immune response, it is a prerequisite that the antigens are efficiently presented on the surface of professional antigen-presenting cells. TAP-independent access of cytosolic peptides into endolysosomal compartments was reported (5, 7); however, the molecular mechanisms underlying this process are poorly understood. In this study, we demonstrate by using quantitative immunofluorescence and subcellular fractionation that TAPL is found in the lysosomal compartment in professional antigen-presenting cells. TAPL is strongly up-regu-
Lysosomal Translocation System

lated during maturation of peripheral blood monocytes into immature and mature DCs and during differentiation of monocytes to macrophages. We further established an in vitro TAP-dependent peptide translocation assay into isolated lysosomes. Consistent with these results, TAPL was found not to be involved in the classical pathway of MHC I presentation, because it alone or in combination with either TAP1 or TAP2 does not complement TAP function in peptide loading of ER-resident MHC I molecules.

We showed by subcellular fractionation that endogenous and overexpressed TAPL is localized in the dense pool of lysosomes. This result was confirmed by quantitative immunofluorescence microscopy. By image restoration, the background is strongly reduced and co-localization of signals with different intensities can be detected, which is only hardly recognized by conventional overlay. In contrast to previous reports, our data indicate that TAPL is found in lysosomal compartments but not in early endosomes or the ER (14, 21). These results are in agreement with localization studies using stably transfected human ovarian carcinoma cells (15). However, in this previous study, a partial overlap with endosomal staining was visible, which may originate from different cell lines or different markers that have been used for analysis. Because TAP and TAPL are localized in different subcellular compartments, it is not very surprising that no TAPL-TAP heterodimers are formed.

DCs are the most potent antigen-presenting cells and are critical for the initiation of CD4+ and CD8-positive T-cell response (39). In the peripheral tissue, immature dendritic cells are specialized in capturing antigens. After stimulation with the appropriate maturation signal, mature dendritic cells migrate to T-cell-rich zones of secondary lymphoid organs and become potent T-cell activators. Interestingly, DCs possess a broad plasticity in response to pathogens and their components, which is reflected in differential gene expression patterns depending on the acquired stimuli (40–42). Stimulation of immature dendritic cells with a mixture composed of TNF-α, IL-16, IL-6, and prostaglandin E2 did not significantly change TAPL expression. However, the stimulation of immature dendritic cells by specific factors decreased TAPL expression to different mRNA levels. It is well established that, for example, LPS and TNF-α stimulate the gene expression in immature DCs differentially (43, 44), which is also reflected in a distinct pattern of TAPL expression. LPS seems to initiate the differentiation of the immature to mature DCs. In contrast, the gene expression pattern after treatment with TNF-α resembles more that of untreated cells. Under these conditions, the down-regulation of TAPL is accompanied with the decrease of MHC II transcripts during maturation of DCs in the presence of LPS (43, 44). The high level of TAPL protein in the mature dendritic cells may result from the long half-life of the ABC transporter as it was shown for MHC class II molecules upon treatment of cells with LPS (45).

Professional antigen-presenting cells have the ability of cross-presenting exogenous antigens internalized by pinocytosis or endocytosis on MHC class I molecules by at least two different pathways (for review see Refs. 3, 46). In the TAP-dependent pathway, internalized antigens are delivered to the cytosol where they are processed by the proteasome and subsequently transported by TAP into the ER or phagosomes for loading onto MHC class I (47–49). However, there also exists a TAP-independent pathway, in which exogenous antigens are found in endosomes and degradation products of these antigens are loaded on MHC class I molecules in endolysosomes (50–52).

As a paradigm, MHC class II molecules present exogenously delivered antigens. During cross-presentation, MHC II can stimulate CD4+ T-lymphocyte response to endogenous antigens in tumor and viral infected cells (53–55). Moreover, the delivery of cytoplasmic or nuclear antigens to MHC class II molecules was recently shown (56, 57). This process is dependent on cytoplasmic proteolysis but is clearly TAP-independent (5, 58). Based on the peptide transport system in the lysosomal compartment identified in this study, we hypothesize that TAPL is involved in cross-presentation of cellular or nuclear proteins on MHC class II molecules or in peptide delivery into endolysosomes for presentation of exogenous antigens on MHC I as described above.

In addition, chaperone-mediated autophagy provides a selective mechanism for the delivery and degradation of soluble cytosolic polypeptides in lysosomes (59). In this process, cytosolic proteins are recognized via LAMP-2a and Hsc70 and subsequently translocated into the lumen of the lysosome. It has been reported that this pathway is involved in the presentation of cytosolic autoantigens (60). The transport system within the pathway has nevertheless not been identified so far. It is hard to imagine that the single transmembrane-spanning protein LAMP-2a and the associated chaperone Hsc70 alone can form a translocation complex. We speculate that the lysosomal TAPL membrane translocation system is the key link missing in the chaperone-mediated autophagy and a novel route discharging cytosolic antigens in lysosomal compartments induced in professional antigen-presenting cells.

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