3 EXPERIMENTAL PROCEDURES

3.1 *E. coli* protocols

3.1.1 Bacterial culture

E. coli (XL1-Blue: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*q*Z*Δ*M15* Tn*10* (Tetr)], Stratagene) was cultured in LB medium on a shaker or as single colonies on agar plates at 37° C. Ampicillin resistant *E. coli* was cultured in LB medium containing 100µg/ml ampicillin.

3.1.2 Storing of bacterial cultures

E. coli was stored in 15% glycerol (v/v) (added to fresh culture) at -80° C.

3.1.3 Preparation of competent bacteria

Fresh *E. coli* was grown in LB medium until a density of OD_{550}=0,5. The bacteria were cooled on ice and pelleted at 3000 rpm for 10 min at 4° C. The bacterial pellet was resuspended in 0,25x the culture volume ice-cold 0,1M $MgCl_2$ and incubated on ice for 10 min. The bacteria were centrifuged and the pellet was resuspended in 0,25x culture volume ice-cold 0,1M $CaCl_2$ and incubated on ice for 20 min. Again the bacteria were pelleted and resuspended in 0,05x culture volume ice-cold 0,1M $CaCl_2$ containing 12% (v/v) glycerol. The bacteria were now competent. Aliquots of 60-80µl (enough for one transformation) were snap-frozen in liquid N_2 and stored at -80° C.

3.1.4 Transformation of DNA to competent *E. coli*

Competent bacteria were thawed on ice for 10-15 min. The ice-cold DNA (about 50ng) was added to the bacteria and incubated on ice for 30 min. The bacteria were then given a heat shock for 45 sec in a 42° C water bath. The bacteria were cooled on ice for 2 min prior to addition of 1ml pre-warmed (37° C) LB-medium. The bacteria were shaken 30-60 min at 37° C and then plated on LB-agar under selectional pressure (100µg/ml ampicillin).

3.2 DNA protocols

3.2.1 DNA preparation

DNA was isolated from bacterial cultures using Qiagen mini or midi kits following manufacturers protocol. Alternatively, DNA out of mini cultures was also prepared as follows: 2ml culture was cooled on ice and bacteria were pelleted at 13000 rpm for 1 min. The pellet was resuspended in 300µl P1. 300µl P2 was added and the tubes were inverted gently 6 times and incubated at RT for 5 min. 300µl P3 was added, the suspension was again inverted and centrifuged at 13000 rpm for 10 min. The SN was applied to a fresh tube and the DNA was precipitated with 0,7 vol's of isopropanol. The suspension was vortexed and centrifuged at 13000 rpm for 25 min at 4° C. The DNA pellet was washed with 70% EtOH and centrifuged at 13000 rpm for 10 min at 4° C. The DNA pellet was dried and dissolved in 100µl 10mM Tris/HCl (pH 8,5) and stored at -20° C.

3.2.2 Cleaving and isolation of DNA fragments

Cleaving of DNA for analysis or cloning of genes or gene fragments was accomplished using type II restriction endonucleases (purchased from New England Biolabs, Fermentas or Invitrogen), which cleave the DNA within the recognition sequence. The digest reactions were performed following manufacturers protocol. The DNA fragments were separated parallel to a DNA ladder standard (1kb ladder from New England Biolabs) by agarose gel electrophoresis. The DNA was visualized in the ethidium bromide containing gel using an UV-transilluminator (312nm). For cloning, the fragments of interest were cut out of the gel and the DNA was subsequently extracted by the Qiagen QIAquick Gel Extraction Kit following manufacturers protocol.

3.2.3 Dephosphorylation of DNA fragments

If ligation of DNA fragments were not targeted, i.e. only one restriction enzyme was used, the vector would be able to religate without the insertion of DNA fragment. To avoid religation, the vector was dephosphorylated at its 5'-end. For this purpose the calf intestinal alkaline phosphatase was used. The reaction was performed as suggested by the manufacturer. The dephosphorylated DNA vector was purified using a Qiagen silica-gel–membrane (QIAquick PCR purification).

3.2.4 Ligation of DNA fragments

DNA fragments were produced by an excessive over digestion of DNA with each restriction endonuclease. The fragments and the target vector were then ligated in a reaction volume of 10-20µl. The fragment to be inserted was given in a 5-fold excess to the vector DNA, from which 50-100ng was used. The amount of insert was determined using the equation:

$$[ng]\ insert = \frac{5\ x\ [kb]\ insert\ x\ [ng]\ vector}{[kb]\ vector}$$

The ligation reaction using T4 DNA ligase was performed following manufacturers protocol at 16° C o/n or for 1h at RT.

3.2.5 PCR (polymerase chain reaction)

PCR (Mullis and Faloona, 1987) was performed for analysis of DNA, for mutagenesis or isolation of genes or gene fragments. The PCR reaction for preparative purposes was performed in 50µl containing:

100µM dNTP's (Roche)
3µM forward primer
3µM reverse primer
10-50ng template
1x manufacturers buffer (containing $MgCl_2$)
2U Expand High Fidelity PCR System

The PCR reactions for analytical purpose was performed in 25µl containing:

100µM dNTP's
3µM forward primer
3µM reverse primer
1,5mM $MgCl_2$
10-50ng template
1x manufacturers buffer (without $MgCl_2$)
0,5U Taq polymerase (Invitek Gmbh)

The PCR reaction was performed in a PCR cycler starting with a 5 min denaturation step at 95° C. After 25-30 cycles of denaturation, primer hybridization (temperature varying) and elongation (72° C) an additional elongation step followed for 10 min. The PCR products were analyzed by agarose gel electrophoresis. Fragments produced for cloning were purified using the QIAquick PCR Purification Kit (Qiagen) prior to any further procedures.

3.2.6 DNA sequencing

Sequencing of DNA was performed as a modification of the dideoxy method by Sanger (Sanger et al., 1977). The PRISM™ Big Dye Terminators v3.0 Cycle Sequencing Kit was used in a 10µl sequencing reaction containing 750ng template and 6µM primer. The kit Premix contains reaction buffer, dNTP's, fluorescence labeled ddNTP's and the AmpliTaq DNA polymerase. The sequencing reaction was performed in a PCR cycler starting with a denaturation step at 96° C for 10 sec. 25 cycles were used with primer hybridisation at 55° C for 5 sec and elongation at 60° C for 4 min. Further treatment of the samples was performed at the sequencing service station at Robert Koch-Institute: precipitation and drying of samples and separation by the ABI PRISM™ 377 DNA Sequencer (Applied Biosystems).

3.2.7 Construction of US6 and TAP mutants

The TAP chimeras r8hT1, h8rT1, r4hT2 and h4rT2 in pBluescript II KS (Stratagene) were received from Dr. F. Momburg (DKFZ, Heidelberg) and the TAP Walker A mutants in pVL1392 (Pharmingen) from Dr. P. van Endert (Hopital Necker, Paris). All constructs were subcloned into the rVV-recombination vector p7.5k131a.

gpUS6sol (amino acids 1-139) was generated through PCR amplification of the wild type *US6* sequence in pcDNA1neo (Hengel et al., 1997) using the primers 5'-GAATTCGCCGCCATGGATCTCTTGATTCGTCTC-3' and 5'-CTAGCGAAACG-CGTTCC-3'. The amplified product was cloned into pCR2.1-TOPO (Invitrogen) and subsequently subcloned to the EcoRI sites of p7.5k131a.

For the VR amino acid substitution in the r8hT1 chimera (**r8hVRT1**) mutation primers 5'-GGAGGCTGTGGCCTATGCAG-3' and 5'-CTGCTTACAGCCCCTCTGACCACCAGC-TGCCCACC-3' (mutations in red) were used with the template r8hT1-p7.5k131a. The amplified product was used in a second PCR as a primer together with 5'-

GCTGTGATTTCCTCCATAGTTGGC-3' and again with r8hT1-p7.5k131A as template. The second PCR product was digested by the restriction enzymes SpeI (#1453 in X57522, hTAP1a) and DraIII (#1681 in X57522, hTAP1a) and ligated to r8hT1-p7.5k131A digested by same enzymes.

The conserved restriction site SanDI in hTAP1a (# 1822 in X57522) and rTAP1a (# 1590 in X57523) was used for the construction of the chimeric TAP1 cDNA **rxhT1** in p7.5k131a. The EcoRI site in p7.5k131a was used at the 5' end.

After restriction endonuclease analysis of generated constructs correct sequence was affirmed by sequencing before further analysis was continued.

3.3 Cell culture

All cell lines were grown in DMEM supplemented with 10% inactivated (30 min at 56° C) FCS, 100 units/ml penicillin, 100µg/ml streptomycin and 2mM glutamine in a 5% CO_2 atmosphere incubator at 37° C. Cell lines stably transfected with a gene of interest were selected and kept under 500µg/ml geneticin treatment. Cells contaminated with mycoplasma were treated with 10µg/ml ciprofloxacin hydrochloride. See Table 2.3 for list of cell lines and transfectants used. For storing, cells were trypsinated, washed with DMEM and resuspended in freezing medium. Cells were frozen slowly (-1°/1h) to -80° C in a freezing container and were then removed to liquid N_2.

3.4 Working with vaccinia virus

3.4.1 Vaccinia virus stock

Vaccinia virus (VV) was propagated on CV1 cells. For preparation of VV stock a T175 flask of 100% confluent CV1 cells were infected with an MOI of 0,05 (see section 3.4.3) of VV. At 2 days post infection (p.i.) cells were harvested at 3000 rpm for 10 min at 4° C. The cell pellet was resuspended in 1ml ice-cold PBS, removed to a 1,5ml tube and sonicated 3 x 10 sec. Sonication disrupts the cell membrane and releases the virus from the cell. VV possess a stable envelope that protects against sonication. After sonication the suspension was centrifuged at 4000 rpm for 3 min at 4° C and the SN1 was collected in a fresh 2ml tube. The cell pellet was resuspended once again in 1ml ice-cold PBS, sonicated 3 x 10 sec and

centrifuged at 4000 rpm for 3 min at 4° C. SN2 was pooled with SN1. The new VV stock was titrated and kept at 4° C. An aliquot (master stock) was stored at -80° C.

3.4.2 Titration of VV stock

Titration of VV stocks was performed in 24well plates. Dilution of the VV stock was made in 1ml 1/10 steps beginning with 10^{-5} and ending with 10^{-10}µl stock/ml medium. The VV dilution serial was added to 60-70% confluent CV1 cells. 36h p.i. the first reading of plaques was possible. The plaques should be read no later than 48h p.i., since satellite plaques are forming. The titer was given as number of plaque forming units (PFU) per 1ml stock.

3.4.3 VV infection for analysis

Newly plated and attached cells are more easily infected than cells grown for a day or more. Therefore, cells were splitted to an adequate culture dish 3-6h prior to infection. The cells were infected in a minimal volume of medium (e.g. 600µl for a 6well) with an MOI (multiplicity of infection: number of PFU/cell) of 4-6 of VV and incubated at RT for 15-30 min. Medium was added and the infected cells were incubated in a humidified atmosphere at 37°C 6-16h depending on the cell type and analytical method to be used. VV expression inhibits host macromolecular synthesis. Therefore, only a short infection time is possible if endogenously expressed proteins are important for the analytical method. The p7.5k promoter that drives the expression of inserted genes in rVV results in a clearly detectable gene expression already 5-6h p.i. Often co-infection with 2 or 3 viruses was performed, in these cases the cells were infected with an MOI of 3-6 with each virus.

3.5 Recombinant vaccinia virus

3.5.1 Preparation of VV DNA

For construction of recombinant vaccinia viruses, wt VV DNA had to be prepared. 40 T175 flasks of CV1 cells were infected with wt VV (Copenhagen strain) as for preparation of VV stock. At 2 days p.i. the cells were harvested and sonicated twice in PBS as for normal VV stock. SN1+2 were pooled and centrifuged in a SW28 rotor at 20000 rpm for 1h at 4° C. The pellet was resuspended in 10ml PBS. 500µg DNase (Roche) and 100µl 1M $MgCl_2$ were added and the suspension was incubated for 30 min at 37° C. 1mg trypsin was added and the suspension was incubated another 60 min at 37° C. The suspension was applicated carefully

to a 20ml 36% sucrose cushion and centrifuged in a SW28 rotor at 20000 rpm for 1h at 4° C. The pellet was resuspended in 10ml PBS and the DNase/trypsin treatment was repeated. After the second centrifugation, the virus pellet was resuspended in 7,2ml buffer I and 800µl 20% sarcosyl (sodium laurel sarcosinate) was added. The suspension was incubated at 37° C with frequent shaking until suspension became viscous and was then removed to 2ml tubes (a' 1ml). The DNA was extracted with 1ml phenol/chloroform, by vortexing and centrifugating for 15 min at 13000 rpm. The extraction was repeated until the aqueous phase became clear. The DNA was precipitated from the clear aqueous phase by 0,3M NaAc (pH 4,8) and 2 vol's abs. EtOH. The suspension was vortexed, incubated o/n at -20° C and centrifuged at 13000 rpm for 25 min at 4° C. The DNA pellet was dried and dissolved in 20-50µl 10mM Tris, pH 8,5. The DNA was pipetted only with cut tips to avoid shearing. The DNA was pooled and concentration was measured. A HindIII restriction endonuclease digest of the new VV DNA was performed parallel with an old aliquot of VV DNA. The DNA fragments were separated for 13h by 60V on a 0,5% 0,5x TBE-agarose gel (Agagel Maxi, 20 x 20 cm^2) and compared for correct band pattern. The DNA was finally aliquoted, so that one aliquot corresponded to the amount of DNA (usually 0,5µg, but has to be controlled) needed for one recombination sample and stored at -20° C.

3.5.2 Preparation of rVV

Genes of interest in the recombination vector p7.5k131a are transferred to the vaccinia virus genome through homologous recombination disrupting the thymidine kinase (tk) gene. Therefore, recombinant viruses can be selected by BrdU in thymidine kinase negative (tk^-)-cells. Because the DNA of VV is not infective and does not produce virus particles, essential VV enzymes are added as ts7 (temperature sensitive mutant) VV particles. The ts7 virus replicates only at 33° C and is inactivated at 39,5° C.

ts7 infection

CV1 cells were grown on a 6well dish until 60-70% confluent. The cells were infected with the VV mutant ts7 with an MOI of 0,005-0,008 (titer determined at 33° C), or an amount of virus that results in only a few plaques after incubation at 39,5° C for 2 days. The ts7 VV was given to the cells in 500µl medium and incubated at RT for 1h (plates were shaken now and then). 2ml medium was added and the cells were incubated for 2h in a humidified atmosphere at 33° C.

Transfection mix:
(sterile solutions)

62µl 2M $CaCl_2$
0,5µg wt VV DNA
1µg p7.5k131a recombination plasmid
ad 500µl H_20

A tube containing 500µl 2xHBS (pH 7,14) was kept on a vortexer (~ 600 rpm) while pipetting the transfection mix drop by drop to the tube. The suspension was incubated at RT for max. 10 min and then 500µl of the mix was added directly to the medium of the ts7 infected CV1 cells and incubated at 39,5° C for 3-4h. The cells were washed with 4 ml PBS and 3ml fresh medium was added. The CV1 cells were incubated at 39,5° C for 2 days or until plaques were visible. At that point the CV1 6well plates were put in –20° C for 1h or longer. The cells were thawed and 2 ml of the cell/medium suspension was put to a 2ml tube and stored at 4° C. For release of virus from the cells the suspension was sonicated 3x10 sec.

BrdU selection of rVV

HU143 tk^- cells were grown on 6well plates until 60-70% confluent. The sonicated viral cell/medium suspension was diluted in steps of 1:10 (3 steps are enough) in 500µl medium containing 100µg BrdU/ml. The HU143 tk^- cells were infected with the virus dilutions and incubated at RT for 15-30 min. 3ml fresh medium containing 100µg BrdU/ml was added and the cells were incubated at 37° C for 2 days or until plaques were visible. As plaques became visible for the eye, 3-4 plaques were picked from the well with least plaques. The medium was removed and the plaque was picked with a pipette containing 50µl PBS. The tip of the pipette was put on the plaque and the PBS was slightly pushed out and then sucked in again. The PBS was given to a tube and sonicated carefully (3x3 sec). The plaque was now ready for a second BrdU selection on HU143 tk^- cells. The second selection was performed as the first one, only this time all the PBS containing one plaque was given to the first dilution step. When the second round of plaques became visible they were picked and sonicated.

rVV mini stock

A mini stock of the rVV plaque obtained by double selection steps was prepared. CV1 cells were grown in 6cm dishes until 60-70% confluent. The cells were infected with the rVV

plaque in 1ml medium. The cells were incubated at RT for 15-30 min, 3ml fresh medium was added and the cells were incubated in a humidified atmosphere at 37°C until the CV1 cells showed 100% CPE (cytopathic effect). The cell/medium suspension was collected, sonicated (3x10sec) and stored at 4° C. The titer of a VV mini stock is usually around 10^7 pfu/ml. For analysis of the rVV clones PCR or/and WB were performed. When the correct clone was confirmed aliquots from the mini stocks were stored at -80° C and a normal VV stock as described above was prepared.

3.5.3 Isolation of VV DNA for PCR analysis

A Proteinase K digest was performed to release the DNA from the VV particles. To 100µl of ministock or 10µl of normal VV stock, 100µl 4x Proteinase K buffer and 10µl Proteinase K (20mg/ml) was added and the suspension was filled up to 400µl with H_2O. The Proteinase K digest was incubated at 56° C o/n. For extraction 800µl phenol/chloroform was added and the suspension was vortexed and centrifuged at 13000 rpm for 3 min. 300µl of the aqueous phase was collected and the DNA was precipitated with 0,3M NaAc (pH 4,8) and 2 vol's of abs. EtOH. The tube was vortexed and incubated at -20° C for at least 30 min. The tube was centrifuged at 13000 rpm for 25 min at 4° C and the pellet was washed with 70% EtOH and centrifuged another 15 min at 13000 rpm and 4° C. The DNA pellet was dried (mostly not visible) and dissolved in 20µl 10mM Tris (pH 8,5). For analytical PCR 3-5µl VV DNA was used.

3.5.4 Sequencing of the p7.5k131a insert

To confirm the correct recombination into the VV genome PCR and sequencing of the rVV genome (or the p7.5k131a vector) was performed. Primers that bind to the disrupted tk-gene were designed, called tk-11 and tk-12. The product of tk-11 and tk-12 was around 450 bp if no insert was present. If the gene of interest was present the PCR product length was 450 bp plus the size of the insertion. For analysis of inserted mutations two additional sequencing primers were designed, that bind the disrupted tk-gene within the PCR product produced with the primers tk-11 and tk-12. With the sequencing primers seq-1 and seq-2, the PCR product by tk-11 and tk 12 was easily sequenced (Fig. 3.1), while the low yield of VV DNA extracted was not possible to sequence directly.

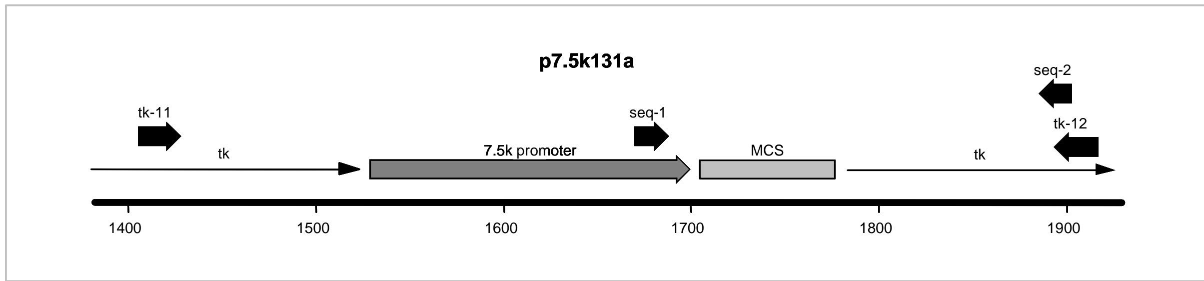

Fig. 3.1 Positions of sequencing and amplification primers for confirmation of correct recombination into the VV genome.

The gene of interest was inserted in the MCS (multiple cloning site) of p7.5k131a.

3.5.5 Human and rat specific TAP primers

For identification of the different human/rat TAP1 and TAP2 chimeras by PCR, species-specific PCR primers were designed. They were designed so that the forward primer bound upstream of the cloning site, whereas the reverse primer bound downstream, thereby making it possible to amplify chimeric proteins. An example of rVV clones of the TAP2 chimeras r4hT2 and h4rT2 is shown in Fig. 3.2.

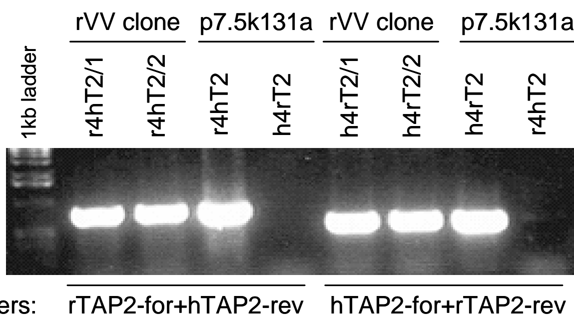

Fig. 3.2 Example of species-specific TAP PCR.

rVV clones (r4hT1/1-2 and h4rT1/1-2) and corresponding chimeric genes in p7.5k131a as positive and negative controls were amplified by species-specific TAP primers as indicated.

3.6 Purification of antibodies

Purification of polyclonal rabbit antiserum was performed for production of immunoprecipitation with less background. For this purpose the Amersham Pharmacia biotech HiTrap NHS-activated 1ml affinity columns were used.

Ligand coupling

The column was operated with a 2ml syringe. First, the columns were washed with 3x2ml ice-cold 1mM HCl to remove isopropanol. The flow-rate ½ drop/sec was not exceeded at any point. 1ml ligand solution (5mg/ml peptide, against which the antiserum was raised, was diluted in coupling buffer) was loaded immediately after and the column was sealed and incubated for 30 min at RT.

Washing and deactivation

Excess of active groups were deactivated and washed out by the following procedure (all solutions ice-cold):

3x2ml Buffer A was injected
3x2ml Buffer B was injected
3x2ml Buffer A was injected
column was incubated 30 min at RT
3x2ml Buffer B was injected
3x2ml Buffer A was injected
3x2ml Buffer B was injected

Finally the column was stored at 4° C in 50mM Na_2HPO_4, 01% (w/v) NaN_3, pH 7.

Antibody purification

The column was washed with 10ml ice-cold start buffer, 5ml Buffer C and 5ml Buffer D. 1ml serum (filtered through a 0,45µm filter) was loaded on the column and the column was incubated for 1h at RT. The loading procedure can be repeated up to five times and should be repeated at least one time. The column was then washed with ice-cold 6ml start buffer. The purified antibody was eluted in 3ml elution buffer. The first fraction of 800µl was eluted in a tube containing 200µl 1M Tris pH 8 to neutralize the low pH of the elute. The second fraction of 1600µl was eluted in a 2ml tube containing 400µl 1M Tris pH8. A third fraction was eluted after a 15 min incubation at RT. The third fraction was eluted as the second. The column was now washed with 10ml start buffer, 5ml Buffer C , 5ml Buffer D and again 10ml start buffer and was stored at 4° C in start buffer containing 0,1% NaN_3. The eluted fractions were tested and stored at 4° C.

3.7 Quantification of total protein

Quantification of total protein concentration in cell lysates was especially needful for testing of new antiserum in WB. The BCA protein assay kit based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein was used. The assay was performed following manufacturers protocol, using BSA as protein standard.

3.8 Immuno methods

3.8.1 RIPA lysate

For analysis by WB RIPA lysates of cells (infected or mock treated) were prepared. Cells from a 6well plate were trypsinated if mock treated or resuspended in PBS if VV-infected and removed to 1,5ml tubes. Cells were washed twice with ice-cold PBS (centrifugation of cells at 4000 rpm, 3 min at 4° C) and after the last washing step the SN was removed completely. The cell pellet from one 6well (-10^6 cells) was resuspended in 60-100µl RIPA lysis buffer and incubated on ice for 20 min. The lysate was cleared from cell debris by centrifugation at 13000 rpm for 30 min at 4° C. The SN was removed to a new tube, WB sample buffer was added and the proteins were incubated at 95° C for 5 min. The lysate was cooled before separating by SDS-PAGE or stored at -20° C.

3.8.2 Denaturing SDS-PAGE

Electrophoretic separation of proteins was performed on the basis of their apparent molecular weight using SDS-PAGE (Laemmli, 1970). Discontinuous gels consisting of a stacking and a separation gel were poured. The separating gel was first introduced to the gel apparatus. A little bit less than 1cm space was left to the lower part of comb and a layer of isopropanol was given to the unpolymerized separation gel to keep the gel surface flat. After polymerization the isopropanol was poured off, the stacking gel was introduced and the comb was inserted. After complete polymerization the gels were inserted to the electrophoresis chamber. The comb was removed and the wells were washed. Of the protein samples, 5-25µl for mini gels and up to 50µl for midi gels were introduced to the wells using a Hamilton syringe (50µl capacity). A protein marker (Kaleidoscope Prestained Standards) was loaded to one well. The mini gels were run on 20mA per gel for 45-60 min and the midi gels on 30mA per gel for 3-5h.

3.8.3 SDS-PAGE, nonreducing conditions

Nonreducing SDS-PAGE was performed as above, only the reducing agent β-mercaptoethanol was not added to the sample buffer.

3.8.4 Blotting

Transfer of proteins from the polyacrylamide gel to a nitrocellulose membrane was performed using a semi-dry blotter. Before preparing the blotting sandwich the gel was equilibrated in blotting buffer to remove rests of the running buffer. All filter papers and the membrane were pre-wetted in blotting buffer before making the sandwich. The sandwich started with three sheets of filter paper (Whatman 3MM) on the anode before the membrane was placed on the sandwich. The gel was placed on top of the membrane and the sandwich was finished with three more pre-soaked filter papers. The cathode was placed onto the stack. The proteins were transferred 30-40 min at 14-16 volt depending on the size of the proteins. Successful transfer of proteins was confirmed by reversible staining of the membrane for total protein by Ponceau S for 1 min. Transferred proteins were visualized by washing the membrane with water.

3.8.5 Immunodetection

Once the proteins were transferred to the nitrocellulose membrane, detection of specific proteins proceeded by use of antibodies. The blotted membrane was coated with a blocking agent, 2-5% (w/v) milkpowder in TBST to prevent nonspecific binding of the antibody. The blocking step was performed o/n at 4° C or for 1h at RT. The blocking agent was shortly rinsed from the membrane with TBST and the primary antibody was added in the appropriate dilution in TBST. The antibody was incubated with the membrane for 1h at RT or at 4° C o/n. The primary antibody was mostly collected and used again. Prior to incubation with secondary antibody, the membrane was shortly washed in TBST. The secondary antibody recognizes the Fc-part of the primary antibody. Diluted in TBST, the secondary antibody was incubated with the membrane for 45-60 min at RT. The secondary antibody was labeled with horse radish peroxidase (HRP), which oxidate the cyclic diacylhydrazide, a compound in the detection kit used. The kit produces a chemiluminescent reaction, detected by autoradiography film.

3.8.6 Immunoprecipitation of non-labeled proteins

Immunoprecipitation of non-labeled proteins was detected by WB. Depending on the expression rate of the protein to be precipitated the amount of cells had to be considered. In this work only rVV-expressed proteins or ubiquitous endogenous proteins were precipitated and therefore one 6well (1-2x10^6 cells) of cells was enough for one precipitation.

The cells were washed twice and lysed in the cell culture dish in 600µl IP lysisbuffer for 20 min on ice. The cell lysate and cells were removed thoroughly from the culture dish to 1,5ml tubes. The lysates were cleared from cell debris at 13000 rpm for 30 min at 4° C. After centrifugation the SN was removed to fresh tubes and the specific antibody was added (0,5-1,5µg/sample or 5-10µl of antiserum/sample). The immunoprecipitation was incubated o/n at 4° C in an overhead shaker. The protein A or G sepharose (selected depending on the antibody used, see Table 3.1) was washed once in wash buffer B and then mixed 1:1 in wash buffer B. 40µl of the sepharose mix was added to the samples (using cut tips) and incubated for 1h at 4° C in the overhead shaker. The samples were now washed twice with wash buffer B, once with wash buffer C and once with wash buffer D. Between the washing steps the sepharose was precipitated by centrifugation for 20 sec at 13000 rpm. After the last step of washing as much as possible of the buffer was removed from the sepharose pellet. To the dry pellet 14µl of 1x SB (WB) was added and the sepharose was incubated for 5 min at 95° C. The samples were cooled on ice and then centrifuged for 1 min at 13000 rpm. The samples were loaded to SDS-polyacrylamide mini gels by a Hamilton syringe.

3.8.7 Immunoprecipitation of metabolically labeled proteins

Immunoprecipitation (IP) of metabolically labeled proteins makes it possible to isolate specific proteins from cell lysates, to follow and analyse their maturation, degradation, interaction partners and modifications.

Cells were prepared in 6well plates (1 well for one sample). Prior to labeling the cells were carefully washed twice with warm (37° C) PBS containing 3% FCS. 1ml starving medium (RPMI without cysteine and methionine) was added and the cells were incubated for 1h in a humified atmosphere at 37° C. The medium was removed and 0,7ml fresh starving medium was added to the cells together with ~150µCi L-[^{35}S]-methionine and L-[^{35}S]-cysteine. The cells were labeled for 30-60 min at 37° C. Then the cell dishes were put on ice and washed carefully three times with ice-cold PBS. After the last step of washing the PBS was removed

completely and the cells were lysed for 20 min on ice in 1ml lysis buffer. The cell lysate and cells were removed thoroughly from the culture dish to 1,5ml tubes. The lysates were cleared from cell debris at 13000 rpm for 30 min at 4° C. After centrifugation the SN was removed to fresh tubes, leaving about 100µl left in the old tube, avoiding too strong background. To the lysates the specific antibody was added (0,5-1,5µg/sample or 2-10µl of antiserum/sample). The precipitation was incubated in an overhead mixer for 1h at 4° C. The protein A or G sepharose was washed twice in wash buffer B and finally diluted 1:1 in wash buffer B. 40µl of the sepharose-buffer mix was added to the tubes (using cut tips) and incubated another hour in the overhead mixer at 4° C. The sepharose was now washed three times with wash buffer B, twice with wash buffer C and once with wash buffer D. Between the washing steps the sepharose was precipitated by centrifugation for 20 sec at 13000 rpm. After the last step of washing as much as possible of the buffer was removed from the pellet. To the dry pellet 40µl of 1x SB was added and the sepharose was incubated for 5 min at 95° C. The samples were cooled on ice and 8µl 0,5M IAA was added and the samples were centrifuged for 6 min at 13000 rpm, 4° C. The samples were now ready to be loaded to the IP-gel together with an [^{14}C]-labeled protein weight marker (Amersham Pharmacia).

TABLE 3.1

Protein A/G affinities for monoclonal and polyclonal antibodies

Monoclonal antibodies		Polyclonal antibodies	
Human IgG_1	PAS and PGS	Human	PAS and PGS
Human IgG_2	PAS and PGS	Mouse	PAS and PGS
Human IgG_3	PGS	Rabbit	PAS and PGS
Human IgG_4	PAS and PGS	Rat	PGS (weak)
Mouse IgG_1	PGS	Hamster	PGS (weak)
Mouse IgG_{2a}	PAS and PGS	Sheep	PGS (weak)
Mouse IgG_{2b}	PAS and PGS	Goat	PGS (weak)
Mouse IgG_3	PGS	Cow	PGS
Rat IgG_1	PGS (weak)	Pig	PAS and PGS
Rat IgG_{2a}	PGS	Guinea pig	PAS
Rat IgG_{2b}	PGS (weak)	Horse	PGS
Rat IgG_{2c}	PGS (weak)	Chicken	PGS (weak)

3.8.8 Endoglycosidase H cleaving of high mannose structures

Endoglycosidase H (Endo H) cleaves the chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins. It is possible to determine if a protein has entered the ER by analysing the glycans by Endo H digestion. If the glycoprotein has passed the transgolgi network it becomes Endo H resistant. Therefore, three forms of a glycoprotein

can exist: the nonglycosylated form, the Endo H sensitive glycosylated form and the Endo H resistant high glycosylated form. These modifications of glycoproteins can be analysed by Endo H.

Endo H cleavage was performed on metabolically labeled and precipitated proteins. After the last washing step with wash buffer D, 30µl of the Endo H buffer was added to the dry sepharose. The sepharose was incubated at 95° C for 5 min and then cooled on ice. 6µl 1M β-mercaptoethanol and 0,01 units of Endo H were added. The reaction was incubated at 37° C for 4h or o/n. Then 14µl 4x SB (IP) was added and the samples were incubated for 5 min at 95° C. The samples were cooled on ice and centrifuged for 6 min at 13000 rpm and 4° C. The samples were loaded to the IP gel.

3.8.9 Big scale SDS-PAGE

Pouring the gel

The gel apparatus for IP SDS-PAGE was designed in our laboratory. The vertical gel system has a separating gel length of 22cm (30 cm width). In most cases a gradient gel was poured covering a range of 13-10% polyacrylamide. The gel composition of the IP gel was the same as for normal SDS-PAGE, with the exceptions that in case of a gradient gel only 0,03% (w/v) APS was used and the solution with higher acrylamide amount contained 15% (w/v) sucrose. For pouring the gradient gel a gradient former was used. The running buffer for IP gels had the composition of 2x Laemmli running buffer. The proteins were separated for about 14h by 20mA.

Fixing and drying a polyacrylamide gel

After gel electrophoresis the gel was fixed. The gel was removed from the glass plates carefully by slowly letting it slide to the fixing solution. The gel was fixed for 1h on a shaker at very low rpm and was then washed three times with water. A 3MM filter paper was slipped under the gel (lying in water) and put on a second piece of filter paper. The gel was covered with cling film and dried for 2h at 80° C. The dried gel was put into a film cassette and the labeled proteins were detected by autoradiography film.

3.8.10 FACS

FACS (fluorescence activated cell sorting) was performed for analysis of cell membrane protein expression. FACS can also be used for intracellular staining of proteins but that procedure was not performed within this thesis and therefore only extracellular FACS will be described here. The principle of the FACS method is specific staining of proteins in/on intact cells. The FACS reader measures multiple parameters of cells as they pass through a light source, including the antibody staining (e.g. FITC - fluorescein isothiocyanate) of specific proteins.

Cells grown on 6well plates ($1x10^6$ cells enough for 2-3 samples) were washed with PBS and trypsinated. The cells were removed to a 15ml tube with 5ml medium. By centrifugation at 1200 rpm for 5 min at 4° C the cells were pelleted and resuspended in 2ml FACS buffer. Cells corresponding to one 6well were split up on two 1,5ml tubes. The tubes were centrifuged at 4000 rpm for 3 min at 4° C. The cell pellet was resuspended in 50µl FACS buffer (negative control) or in 50µl primary antibody diluted in FACS buffer and incubated for 25 min on ice. The cells were washed twice with 1ml FACS buffer and centrifuged as above. The cell pellets were resuspended in 50µl of the secondary antibody diluted in FACS buffer and incubated 25 min on ice. Because the FITC-labeled secondary antibody is sensitive to light the last incubation was performed in dark. After the incubation the cells were washed twice in FACS buffer as above and finally resuspended in 500µl FACS buffer. The cells were now ready for reading. 10000 cells per sample were read and using 15mg/ml propidium iodide dead cells were excluded from the evaluation.

3.9 Peptide translocation assay

The peptide translocation assay measures the amount of peptide translocated from the cytosol to the ER lumen. The peptides used in the assay contain an N-glycosylation site and are labeled by ^{125}I chloramine-T-catalyzed iodination (the labelled peptides were a kind gift of Dr. F. Momburg, DKFZ, Heidelberg, Germany). Therefore, it is possible to quantify the amount of glycosylated (i.e. peptides that entered the ER lumen) peptides (Fig. 3.3).

Preparation of cells, permeabilization

For one assay $2x10^6$ cells (TAP deficient CMT 64.5) were used. Cells were cultured in 100mm culture dishes ($4\text{-}5x10^6$ cells, enough for dual measurement). Cells were infected

with 5 MOI of one TAP1 expressing rVV and 5 MOI of one TAP2 expressing rVV. 10h p.i. the cells were washed once with PBS and trypsinated. The cells were resuspended in 5ml ice-cold PBS supplemented with 3,0% FCS. The cells were given to a 15ml falcon tube with another 5ml PBS-FCS, on ice. Cells were counted and adjusted to $5x10^6$ cells/tube. The tubes were centrifuged for 5 min at 1200 rpm and 4° C. The cell pellets were resuspended in 1ml ice-cold PBS and transferred to a 1,5ml tube. Again, the cells were centrifuged for 3 min at 4000 rpm and 4° C. The pellet was resuspended in 1ml TB buffer and centrifuged at 4000 rpm, 3 min and 4° C. As much as possible of the SN was discarded. The cells were now permeabilized by 200µl SLO for 20 min at 37° C. SLO has to be reduced before using it: 13,2mg SLO was dissolved in 1ml TB buffer and 4µl 1M DTT was added (→ 2,2U SLO/ml). After incubation with SLO the tubes were put on ice. A 1-2µl aliquot from 1 or 2 probes was mixed with 6µl trypan blue stain (0,4%) and incubated for about 5 min at RT. The cells were analyzed through the microscope. Cells colored blue were successfully permeabilized. If cells were not blue additional 50-100µl SLO was added and the cells were incubated for another 10-15 min at 37° C. After permeabilization 1ml ice-cold TB buffer was added to the tubes and they were centrifuged for 3 min at 4000 rpm and 4° C. Now the cells were resuspended in TB with 0,1% BSA and again cells were centrifuged as above. As much as possible of the SN was discarded and the cells were resuspended in 45µl TB with 0,1% BSA (this gives a total volume of ~65µl cell suspension, which is enough for two reactions).

Translocation

The translocation reaction (100µl) contains:

10µl ATP (100mM) (100mM EDTA for negative control)
30µl cell suspension ($2x10^6$ cells)
60µl ^{125}I-labeled peptide diluted in TB with 0,1% BSA

ATP was first given to the translocation tubes and kept on ice. Then the ^{125}I-peptide was diluted in TB with 0,1% BSA and 60µl was added to the tubes. Finally 30µl cell suspension was introduced to the translocation reaction and the tubes were incubated for 20 min at 37° C. The tubes were put on ice and 1ml lysis buffer was added. The tubes were incubated for 30 min on ice and then centrifuged at 13000 rpm for 10 min at 4° C. After the centrifugation 10µl aliquots from 3-4 samples were collected in PCR tubes for estimation of amount of input peptide. The SN was transferred to new tubes containing 30µl Concanavalin A sepharose

(Con A sepharose) and incubated in overhead mixer at 4° C o/n. Then the Con A sepharose was washed three times with lysis buffer. The sepharose was pelleted at 6000 rpm for 2 min. After the last step of washing the buffer was removed and 100µl lysis buffer was added to the Con A sepharose. The sepharose was now removed to PCR tubes by pipetting with cut tips. The counts of the sepharose were measured with a gamma counter.

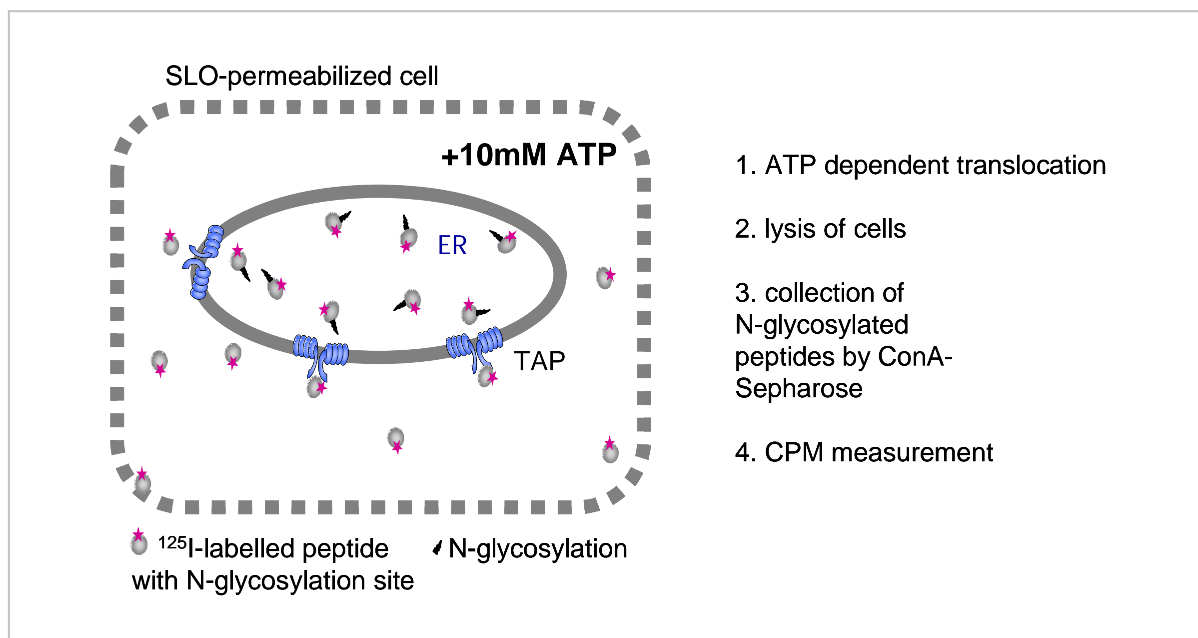

Fig. 3.3 Scheme of ATP dependent peptide translocation by TAP.

3.10 ATP binding assay

ATP binding by TAP can be assessed by ATP-agarose. For this purpose cells were grown on 6well plates. At 6h p.i. cell dishes were put on ice and washed twice with ice-cold PBS. After the last step of washing the PBS was removed completely and the cells were lysed for 20 min on ice in 600µl ATP binding buffer. The cell lysates were removed thoroughly from the culture dish to 1,5ml tubes. The lysates were cleared from cell debris at 13000 rpm for 10 min at 4° C. After centrifugation the SN was removed to fresh tubes and 20µl ATP-agarose was added. For binding of TAP to the ATP-agarose tubes were rotated for 30 min at 4° C. The ATP-agarose was washed three times with ATP binding buffer containing 0,1% digitonin. After the last step of washing as much as possible of the buffer was removed from the ATP-agarose pellet. To the dry pellet 14µl of 1x SB (WB) was added and the ATP-agarose was incubated for 5 min at 95° C. The samples were cooled on ice and then centrifuged for 1 min

at 13000 rpm. The samples were loaded to SDS-PAGE mini gels by a Hamilton syringe and proteins were detected by Western blotting.