5 DISCUSSION

To date, biological inhibition of ABC transporters is limited to very few herpesviral proteins. gpUS6 of HCMV and ICP47 of HSV-1 represent elaborated inhibitors evolved to interrupt the catalytic transport cycle of TAP at different stages (Bauer and Tampe, 2002; Momburg and Hengel, 2002). Both inhibitors were used in this study to elucidate molecular requirements of TAP structure and function. Common features of gpUS6 and ICP47, i.e. species-restricted inactivation of TAP and recognition of preformed TAP heterodimers only, were taken advantage of. Functional chimeric human/rat intrachain TAP mutants were analyzed for binding and inhibition by gpUS6 and ICP47. Furthermore, interrupting the peptide transport cycle by mutational insertions in the Walker A domain of TAP1 and TAP2, conformational constraints on TAP could be demonstrated for gpUS6 and ICP47.

5.1 Determination of minimal binding domains on TAP for gpUS6 and ICP47 using human/rat TAP chimeras

Herpesvirus family members like HCMV and HSV have co-evolved over millions of years with a distinct natural host. As a result of selection pressure, immuno-evasive proteins have developed a high target specificity resulting in a number of cases in a species-restricted phenotype. The affinity of the HSV-1 ICP47 protein to the cytosolic surface of human TAP has been determined to be 50nM, whereas the binding affinity to mouse TAP is at least 100-fold lower (Ahn et al., 1996; Tomazin et al., 1998). At first glance gpUS6 resembles the unrelated ICP47 protein by blocking TAP in human and monkey cells but failing to inhibit of rat and mouse TAP (Halenius et al., 2005). Human and rat TAP exhibit a high degree of sequence homology, reaching about 80%. Their conserved structure is exemplified by a replaceable function, i.e. both subunits of rat TAP assemble with the complementary human subunit to form a transport-competent interspecies hybrid transporter. Thus, it is remarkable that both ICP47 and gpUS6 distinguish between human and rat TAP. This feature was the starting point for examination of minimal gpUS6 and ICP47 binding domains on TAP, testing mixed human/rat transporters and by constructing functional human/rat intrachain TAP hybrids.

5.1.1 The membrane topology of TAP as determined by gpUS6

Inhibiting the TAP heterodimer exclusively via the ER lumenal surface gpUS6 qualifies as an unique sensor recognizing distinct loops facing the ER membrane. Assembly of TAP subunits into heterodimers is absolutely required for gpUS6 recognition of both TAP1 and TAP2, since neither TAP1 nor TAP2 monomers are recognized by gpUS6 (Halenius et al., 2005; Hewitt et al., 2001). This indicates profound conformational rearrangements of the TAP1 and TAP2 TMD upon heterodimeric association. To dissect the sequence requirements on TAP1 and TAP2 subunits for gpUS6 binding, full-length human/rat intrachain TAP chimeras were analyzed. Pursuing this approach, two contact sites of gpUS6 on the TAP1 TMD could be mapped, which exhibit a different and hierarchical impact upon TAP function (gpUS6 interaction with the TAP1 chimeras are summarized in Table 5.1). The major target sequence for gpUS6 inhibition was identified at the fifth ER lumenal loop, although a weaker gpUS6 binding was also observed to the fourth ER loop. The evidence calling for the fifth ER lumenal loop is in conflict with an earlier study, which suggested that this sequence locates to the cytoplasmic peptide binding domain (Vos et al., 1999). Based on the glycosylation pattern of C-terminally truncated TAP mutants containing N-glycosylation consensus sites, it was concluded that this last C-terminal part of the TAP1 TMD is exposed to the cytosol without reaching the ER lumenal side, implying that TAP1 and TAP2 subunits would form only 8 and 7 TMSs, respectively (Reits et al., 2000; Vos et al., 1999). Lacking a stabilizing effect by the C-terminal NBD, the TMD topology of truncated TAPs may, however, not have formed regularly, and could explain why no evidence for membrane integration of the isolated TMS9-10 was obtained previously (Vos et al., 1999). In addition, since gpUS6 does not prevent peptide binding to TAP (Ahn et al., 1997; Hengel et al., 1997) the conclusion is drawn, that exposure of the fifth loop between TMS9 and TMS10 into the ER lumen represents a conformational state of TAP1 with high substrate affinity.

Hydrophobicity analysis and sequence alignments with the MDR transporter P-gp resulted in an alternative model of TAP topology which predicts a core TMD with 2x6 TMSs and additional 4 and 3 N-terminal TMSs for TAP1 and TAP2, respectively (Abele and Tampe, 1999). According to this model, the last C-terminal loop of TAP1 is predicted to be formed by about 9 aa between TMS9 and 10, which differ at two positions between human and rat. Therefore, it was tempting to hypothesize that exchanging the nonconserved human threonine and serine residues by the rat valine and arginine would suffice to disrupt gpUS6 recognition. At least two explanations are possible for why gpUS6 binding was maintained. The

hydrophobicity of TMS9 and TMS10 is relatively moderate compared with that of TMS1-8 (see Appendix I, Fig 6.3), raising the possibility that the sequence of the TMSs 9 and 10 is relatively mobile within the ER membrane and become exposed to the ER lumen and accessible for gpUS6 at a certain step during the peptide transport cycle. In the flanking sequences of the last loop further sequence heterogeneity between human and rat TAP1 is found, which may account for the observed species-restricted binding of gpUS6. Alternatively, distantly located *cis*- or *trans*-acting rat sequences may prevent the gpUS6 target sequence from being formed by the rat TAP1 TMD. Notably, the induction of this gpUS6 target structure in human TAP1 critically depends on ATP binding sequences present in the Walker A motif of TAP2 (see following text, 5.2), suggesting that this loop is not constitutively present on TAP1 but rather coupled to signaling from the cytosolic NBD. These data indicating the presence of TMS9 and TMS10 are in line with the lateral diffusion of TAP that was measured using a FRAP (fluorescence recovery after photobleaching)-technique (Reits et al., 2000). The diffusion of integral membrane proteins is not dependent on their mass in the luminal part, but on the size and number of TMSs (Livneh et al., 1986). Depletion of ATP led to fast diffusion of TAP, which is consistent with a model of only 8 constitutive TMSs on TAP1 or at least incomplete incorporation of TMSs 9 and 10. gpUS6 binding to TAP reduced the rate of TAP diffusion, consistent with an arrested TAP1 structure with 10 TMSs. In addition, this model offers an explanation for why Vos and co-workers (Vos et al., 1999) did not find the last TMS9-10. Since the formation of the gpUS6 binding domains are dependent on ATP binding to TAP2, also the truncation mutants by Vos et al. would not be able to form these domains because of a lacking C-terminal NBD.

TABLE 5.1

Interaction of gpUS6 with human/rat TAP1 chimeras

TAP1	human parts			gpUS6	
	sequence	TMSs	lumenal loops	inhibition	binding
hT1	1-748	1-10	1-5	+	+
rT1	–	–	–	–	–
h6rT1	1-309	1-6	1-3	–	–
h8rT1	1-376	1-8	1-4	–	+
r6hT1	310-748	7-10	4-5	+	+
r8hT1	377-748	9-10	5	+	+
rxhT1	539-748	–	–	–	–
r8hVRT1	377-434; 437-748	9-10	5 (humT435V; humS436R)	+	+

5.1.2 gpUS6 interference with TAP2

At first sight the TAP2 dependent binding and inhibition results by gpUS6 are puzzling. The data delimit binding of gpUS6 to TAP2 to the lumenal N-terminal TMD (human aa 1-177). This region of TAP2 has two predicted parts in the ER lumen, the N-terminal tail (aa 1-7) and the first loop, predicted to encompass aa 76-97. It appears unlikely that gpUS6 recognizes TAP2 at its small N-terminal tail, and the first lumenal loop is a more expedient target. This site is part of the TMD that was thought to form the pore domain which is build in a head-head/tail-tail orientation (Reits et al., 2000; Vos et al., 2000). Recently, Koch et al. (Koch et al., 2003) demonstrated this part of TAP2 being dispensable for peptide transport in insect cells, but required for tapasin association. Tapasin is, however, not required for the inhibitory function of gpUS6 (Hengel et al., 1997). Therefore, it is surprising that gpUS6 interaction is more efficient with the N-terminal TMD of TAP2 than with its C-terminal portion, given that the former interaction does not suffice to mediate TAP inhibition. rTAP1 combined with TAP2 chimeras containing a rat N-terminal and a human C-terminal TMD were not precipitated by gpUS6, but were blocked for peptide translocation (data summarized in Table 5.2). At least two scenarios are possible to reconcile these findings: Although dispensable for peptide transport, the N-terminal TMD of hTAP2 gains a regulatory function onto peptide transport when directly complexed with gpUS6. Hence, this regulation is secondary and not manifest in the absence of the gpUS6 interaction with the C-terminal TMD. It was not possible to demonstrate direct binding between gpUS6 and the C-terminal TMD of TAP2, which implies that only a transient interaction takes place. Consequentially, a stabilizing

binding to TAP2 is plausible. Alternatively, the gpUS6 binding site at the N-terminus of TAP2 may not be directly involved in the gpUS6-mediated TAP inhibition, but in stabilization of the peptide loading complex (PLC). The PLC is composed of chaperones (calreticulin, calnexin, ERp57) and the MHC class I complex bridged to TAP via tapasin in a transient association. Stabilization of the PLC by the presence of gpUS6 has been observed (Hengel et al., 1997) (see text below, 5.1.3). The N-terminal gpUS6 binding to TAP2 proximal to the tapasin binding site might prevent components of the PLC from dissociation. This way gpUS6 would not only inhibit peptide translocation, but also hinder PLC components from being recruited to other PLCs still not arrested by gpUS6. In this model, inhibition of peptide translocation would be operated from the C-terminal TMDs of both TAP1 and TAP2, whereby the interaction with TAP2 would only be transient, however, required for efficient inhibition.

TABLE 5.2

Interaction of gpUS6 with huma/-rat TAP2 chimeras

TAP2	human parts			gpUS6	
	sequence	TMSs	lumenal loops	inhibition	binding
hT2	1-703	1-9	1-4	+	+
rT2	–	–	–	–	–
h4rT2	1-177	1-4	1(-2)*	–	+
h7rT2	1-363	1-7	1-3	–	+
r4hT2	178-703	5-9	(2)*3-4	+	–
r7hT2	364-703	8-9	4	+	–

*only half of the predicted second lumenal loop is human

5.1.3 Model for gpUS6 inhibition and binding

As outlined, the data above demonstrate a non-analogous gpUS6 interaction with the transporter subunits. The findings appear consistent with a model of TAP function that assumes coordinated but non-synchronous conformational rearrangements of TAP subunits in which TAP2 is always one step ahead (van Endert et al., 2002). In Appendix I a sequence and hydrophobicity comparison between the lipid A transporter MsbA and TAP was implemented. A sequence similarity of 29% and similar pattern of loop lengths and hydrophobicity was observed. Based on these characteristics a tentative model of TAP heterodimers is set up (Fig. 5.1). The core TMSs (TMS5-10 of TAP1 and TMS4-9 of TAP2) of TAP1 and TAP2 are placed in a “head to tail”-orientation, forming the translocation pore.

The remaining TMSs are positioned outside the pore to form the docking sites for the PLC via tapasin. As indicated by the analysis of the TAP1 chimeras, gpUS6 interaction with TAP1 takes place at the two most C-terminal short lumenal loops. Whereas binding to the last, still undefined, fifth loop is relevant for inhibition, binding to the fourth loop may exert stabilization on the gpUS6-TAP interaction. In addition, gpUS6 interacts with the C-terminal TMD of TAP2, a cryptic interaction relevant for inhibition, but not detectable by co-immuno-precipitation. A stable binding to TAP2, however, was found to the N-terminal part of TAP2. In the TAP dimer model the N-terminal binding site is outside of the translocation pore and presented as a stabilization of the PLC components. Support of this idea was found by analysis of the PLC in HCMV infected cells (Halenius, Stindt and Hengel, data not shown). Neither anti-tapasin nor anti-TAP1/2 antibodies were able to co-precipitate PLC components from cells infected with HCMV for 72h. Precipitation of gpUS6-immunocomplexes on the other hand, revealed the presence of all PLC components. While the former finding calls for a further non-identified HCMV inhibitor of the MHC class I antigen presenting pathway, destabilizing the PLC 72h p.i., the latter observation suggests that gpUS6 traps PLC components in stable complexes with TAP.

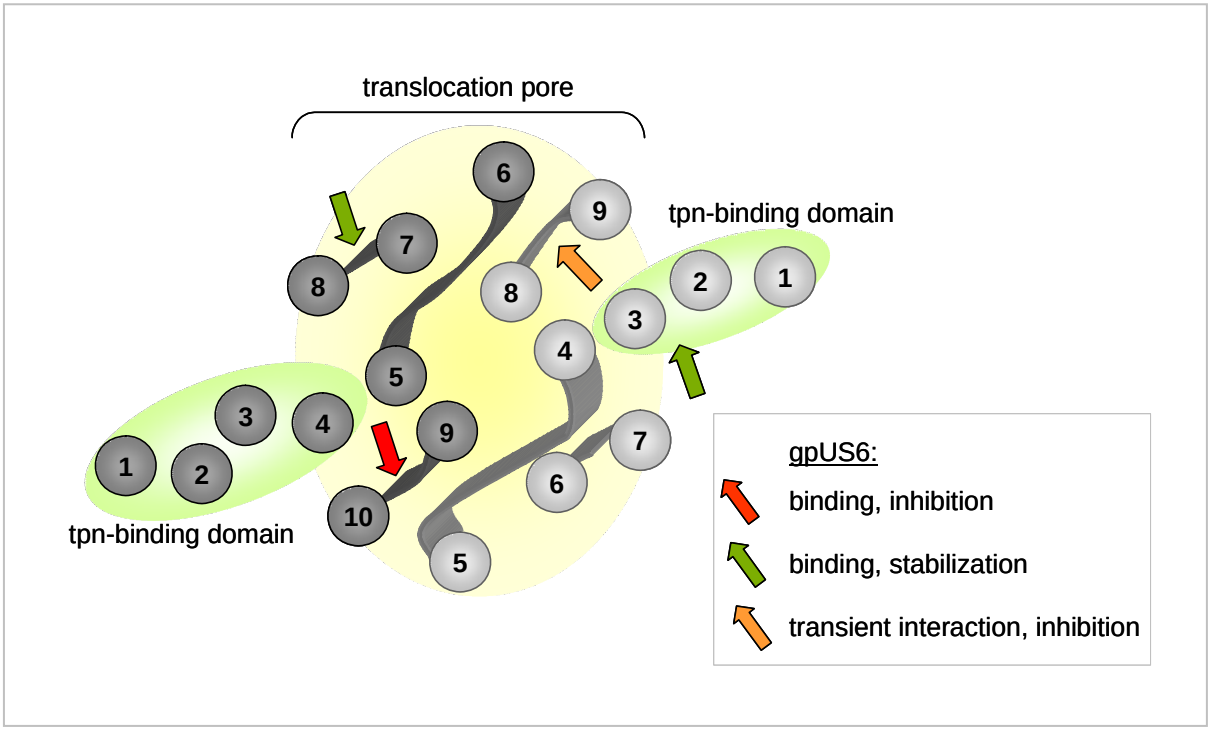

Fig. 5.1 Model of gpUS6 interaction with TAP in a closed conformation.

In a birdview (from the lumenal side vertical to the ER membrane) based on the MsbA crystal structure (Appendix I) the predicted TMSs of TAP1 and TAP2 are shown as dark and light grey circles, respectively. The lumenal loops forming the peptide translocation pore are shown in light and dark grey as well. The tapasin binding domains are highlighted by a green background, the translocation pore by a yellow background. gpUS6 interaction (binding and inhibition) domains are indicated by arrows.

5.1.4 Structural requirements of gpUS6

gpUS6, a lumenal type I transmembrane protein comprising one TMS and a single glycan, has been shown to retain functionality, i.e. ability to inactivate TAP, when lacking both the TMS and the N-glycosylation (Ahn et al., 1997; Hewitt et al., 2001; Kyritsis et al., 2001). As depicted above, at least four independent gpUS6 contact sites must be present on TAP, two located on the TAP1 subunit and two mapping to TAP2. Based on the TAP dimer model in Fig 5.1 the binding sites are distant and a single gpUS6 molecule is not able to bind to all sites simultaneously. The formation of gpUS6 oligomers is consistent with a model in which gpUS6 complexes bridge distant contact sites on the TMDs of TAP1 and TAP2. A similar oligomerization pattern was found for the gpUS6 related HCMV glycoprotein US3 (for sequence relatedness see (Benz and Hengel, 2000)), which prevents MHC class I molecules from leaving the ER (Misaghi et al., 2004). The authors propose a model in which interacting gpUS3 molecules enhance the ability to retain MHC class I molecules in the ER. Oligomerization of gpUS3 was described not to involve disulfide bonds (Misaghi et al.,

2004), whereas here gpUS6 clusters were found to be sensitive to reducing agents. The active lumenal domain of gpUS6 comprises eight cysteins and thus is likely to form a complex intra- as well as intermolecular network of disulfide bridges. Indeed, expression of this active soluble domain in *E. coli* yielded functional gpUS6 monomers and dimers (Kyritsis et al., 2001). Based on these findings one may envisage gpUS6 mutants selectively lacking the interaction with either TAP1 or TAP2. By studying such gpUS6 mutants, segregation of the molecular inhibition upon TAP subunits will be possible and will complement the findings based on human/rat intrachain chimeras.

5.1.5 ICP47 interaction with TAP

In contrast to gpUS6 the HSV-1 encoded ICP47 is a small soluble protein localized in the cytosol, i.e. it inhibits TAP from the cytosolic side of the ER membrane. The inhibitory mechanism of ICP47 is different from that of gpUS6. The first 35 residues of ICP47 were shown to be sufficient for effective TAP inactivation (Galocha et al., 1997). In early investigations ICP47 was found to block peptide binding to TAP (Ahn et al., 1996; Tomazin et al., 1996) and because high peptide concentration inhibited ICP47 interaction with TAP, it was concluded that the ICP47 binding site overlaps with the peptide binding site, i.e. ICP47 was suggested to be a high affinity competitor of the peptide binding site (Tomazin et al., 1996). However, a binding domain for ICP47 on TAP was not defined, therefore, the question whether ICP47 is a true competitor or rather an allosteric regulator, remained to be answered. The established species-restriction of ICP47 for its substrate, inhibiting human but not mouse and rat TAP (Ahn et al., 1996; Jugovic et al., 1998), allowed the employment of mixed human and rat TAP transporters and intrachain human/rat TAP chimeras at hand for analysis of ICP47 binding to TAP. Unlike gpUS6, ICP47 binding to TAP is absolutely dependent on interaction with the TAP2 subunit. Only when a human TAP2 subunit was part of the interchain chimeric TAP complex, ICP47 binding was detectable. Still, when applying the TAP2 human/rat intrachain chimeras a dependency on human TAP1 sequences was observed. This is in agreement with a previous finding: Coupling of the UV photoactivable phenylalanine analogue Tpa (4´- (trifluoromethyl-diazirinyl)-phenylalanine) to an ^{125}I-labeled ICP47-peptide (aa 1-35) resulted in labeling of both TAP1 and TAP2 with equal efficiency, pointing at ICP47 interaction with both TAP subunits (Galocha et al., 1997). The contribution of the ICP47 contact sites on TAP for functional inhibition revealed an absolute dependency on ICP47 binding to both subunits. The interaction observed with the rT1/hT2 complex did not mediate measurable TAP inhibition.

The introduction of the TAP2 chimeras in the binding assay rendered different recognition patterns by ICP47, implying manifold and hierarchic interactions of ICP47 with TAP. TAP2 chimeras with a human N-terminal TMD were recognized by ICP47 only if combined with human TAP1, whereas the interaction was lost with rat TAP1 (see Table 5.3 for summary of ICP47 interaction with TAP2 chimeras). This provides evidence for an interaction of ICP47 with TAP1, which is dependent on an initial contact with TAP2 and explains why no binding of ICP47 to the combination hTAP1/rTAP2 was observed. In addition, the finding proposes further binding domains on TAP2 conferring the co-precipitation seen with the combination rTAP1/hTAP2. These sites were included in the chimera r4hT2, which was precipitated by ICP47 not only as dimer with human TAP1 but also with rat TAP1. However, when the human part of r4hT2 was shortened from the N-terminus, leaving only the TMS8-9 of the TMD and the NBD human, the interaction with ICP47 was lost completely, also when combined with human TAP1. Therefore, the domain important for initiating the ICP47-TAP interaction could be restricted to the N-terminus (aa 1-363) of TAP2. Furthermore, the findings map the domain conferring the TAP1 independent precipitation of the TAP complex by ICP47 downstream of aa 178 on TAP2. In conclusion, ICP47 differs between three independent domains on TAP2, an N-terminal TMD domain (aa 1-177; domain I) sufficient for ICP47 recognition in combination with human TAP1and a central TMD domain (aa 178-363; domain II), and a C-terminal domain (aa 364-703; domain III), both required for formation of the C-terminal ICP47 binding site on TAP2. Interestingly, this binding site co-localizes to some extent with the peptide binding domain, which was determined to reside between aa 301-389 and 414-433 of TAP2 (Nijenhuis and Hammerling, 1996). Furthermore, the number of sites enabling interaction raises the supposition that several ICP47 molecules could be involved in inhibition of one TAP complex. In fact, this has been suggested earlier by D. Johnson and co-workers. They found that ICP47 binding to TAP reached a plateau at an ICP47 concentration of 1µM, while the constant TAP concentration was 0,3µM (Tomazin et al., 1996). In addition, the authors describing the inhibition of TAP by the first 35 aa of ICP47 suggested that these residues are taking part in the actual inhibition of the peptide binding, whereas the remaining residues of ICP47 (aa 36-87) rather confer stability for the ICP47-TAP interaction by binding to sites not involved in the inhibitory mechanism (Galocha et al., 1997). This model fits well with our findings, where ICP47 interaction with domains II and III could be engaged in the inhibitory effect and interaction with domain I would be stabilizing the interaction. Still, for an assertive statement, it is necessary to measure the contribution of

each ICP47 contact site on TAP2 for the blocking of peptide binding and transport. Only then it would be possible to discuss the high affinity competitor model proposed for ICP47. This however, is under current investigation.

TABLE 5.3

Interaction of ICP47 with human/rat TAP2 chimeras

TAP2	human parts			ICP47 binding	
	sequence	TMSs	cytosolic loops*	hT1	rT1
hT2	1-703	1-9	1-4	+	+
rT2	–	–	–	–	–
h4rT2	1-177	1-4	1-2	+	–
h7rT2	1-363	1-7	1-4	+	–
r4hT2	178-703	5-9	3-4	+	+
r7hT2	364-703	8-9	–	–	–

* predicted cytosolic loops are given the numbers 1-4, starting with number 1 for the first loop from the N-terminus of TAP2

5.2 Comparing conformational requirements on TAP for gpUS6 and ICP47 recognition

The TAP dependent translocation of peptides across the ER membrane involves multiple sequential and coordinated steps of events comprising ATP hydrolysis by the NBDs that convert the released energy in rearrangement of the TMSs leading to opening and closing of the transmembrane pore. The Walker A domains found on the NBDs of both TAP1 and TAP2 are responsible for ATP binding and hydrolysis. Mutations in the Walker A domain have revealed non-synchronous action by the NBDs which translates into distinct tasks during the transport cycle (van Endert et al., 2002). It has been proposed that in the cycle of events, TAP2 is always one step ahead, starting the translocation cycle by binding ATP, thereby inducing a high affinity state for peptide binding (van Endert et al., 2002). Also, it was shown that ATPase activity by TAP2 is absolutely required for peptide translocation, whereas mutation of the TAP1 Walker A only led to reduced ability to transport substrate (Karttunen et al., 2001; Saveanu et al., 2001). Therefore, it is reasonable to assume that the TAP2 NBD is the operator for the actual substrate transport and the TAP1 NBD is resetting the cycle. Acting on separate sides of the ER membrane, gpUS6 and ICP47 were employed as conformational sensors for TAP dimers during the transport cycle.

5.2.1 ATP binding to TAP2 is a prerequisite for gpUS6 recognition of TAP

Influence of ATP binding and hydrolysis on gpUS6 recognition of TAP was analyzed using TAP subunits mutated in the Walker A domain. Whereas mutation in the TAP1 Walker A domain has an inducing effect on gpUS6 binding to TAP, mutations in the TAP2 Walker A domain blocked gpUS6 ability to bind TAP. This type of result was seen also with a soluble gpUS6 mutant, excluding the possibility that gpUS6 interacts with cytosolic or transmembrane parts of TAP. The findings prove conformational changes in the TMD of TAP upon ATP binding to TAP2 needed for gpUS6 interaction. Therefore, the identified gpUS6 binding domains on TAP (C-terminal parts of TAP1 and TAP2 TMD in addition to the N-terminal part of the TAP2 TMD) are formed only following a power stroke induced by ATPase activity by the TAP2 Walker A domain, implying not only *cis*- (TAP2 TMD) but also *trans*- (TAP1 TMD) acting conformational changes. Moreover, the results fit well with the previous observation, that gpUS6 does not inhibit peptide binding to TAP (Hengel et al., 1997), as peptide binding was showed to be dependent on ATP binding to TAP2 (Karttunen et al., 2001; Saveanu et al., 2001). Previous studies have demonstrated that gpUS6 binding to

TAP prevents ATP binding to TAP1 (Hewitt et al., 2001; Kyritsis et al., 2001), which was shown here to be a result of gpUS6 binding to TAP1. Altogether this suggests that gpUS6-mediated stabilization of ER exposed sequences in the TAP TMD accounts for inhibition of further conformational rearrangements required for ATP binding to the NBD of TAP1. This would readily explain why there was an induced gpUS6 binding to the TAP1 Walker A mutant: After ATP binding and hydrolysis by TAP2 proceeding of the transport cycle is dependent on ATP binding to TAP1. If this step does not take place because of a defective TAP1 Walker A motif, the TAP dimers will be trapped at this stage of the cycle, supposably a conformation favorable for gpUS6 binding.

5.2.2 ICP47 dependency on TAP conformation is insensitive to ATP binding and hydrolysis

Similar binding analysis as discussed above was performed with ICP47 and the TAP Walker A mutants. Based on biochemical data it has been reported that ICP47 has no influence on ATP binding to TAP1 nor to TAP2 (Ahn et al., 1996; Tomazin et al., 1996). Here the inverted situation was analyzed using genetic means, i.e. which consequences the complete loss of ATPase activity by TAP1 and TAP2 on ICP47 interaction would have. In clear contrast to gpUS6, ICP47 interaction with all Walker A mutants was maintained, indicating that ICP47 binding to TAP is truly independent of ATP induced conformational changes.

5.2.3 gpUS6 and ICP47 interaction to TAP is mutually exclusive

For closer comparison of gpUS6 versus ICP47 receptive conformations of TAP a system was used in which both TAP inhibitors could be expressed simultaneously in the same cell. The expression of each inhibitor was staggered by using on the one hand stable cell tranfectants and on the other hand rVV. Remarkably, the experiments revealed that gpUS6 and ICP47 binding to TAP is mutually exclusive. Irrespective of which one, gpUS6 or ICP47, the inhibitor expressed first occupied all available TAP molecules in a conformation unrecognizable by the later expressed inhibitor. This again circumstantiates that gpUS6 and ICP47 indeed discriminate between different conformational steps in the TAP cycle for binding and inhibition, excluding the possibility that ICP47 binding to TAP is independent on TAP conformation. Furthermore, it indicates that ICP47 recognizes a different TAP domain than the peptide, since gpUS6 does not inhibit peptide binding to TAP, whereas ICP47 binding to TAP is inhibited by gpUS6. It would be interesting to investigate whether non-

hydrolyzable ATP analogues would be able to evoke conformations equally unfavorable for ICP47 as the gpUS6 induced conformation. This would shed light on the question concerning the state of the nucleotide at TAP2 during gpUS6 interaction, i.e. is ATP hydrolysis required or is ATP binding sufficient for induction of the gpUS6 receptive conformation. Clearly the findings also demonstrate that cytosolic TAP conformations have direct consequences for the TMD adopted conformation and vice versa.

5.3 Integration of gpUS6 and ICP47 into a model of the TAP translocation cycle

The application of gpUS6 and ICP47 in structural and functional analysis of TAP has proven to be an unique approach, conferring clarification at many fronts. We know from earlier studies that ATPase activity of TAP2 is absolutely required for peptide translocation, starting the peptide translocation cycle by enabling peptide binding. This thesis provides evidence for induction of conformational changes upon ATP binding to TAP2, reaching from the cytosolic side to rearrangement of the TMSs and lumenal loops. Peptide binding and ATP hydrolysis at TAP2 are followed by ATP binding to TAP1 and translocation of the peptide. ATP hydrolysis by TAP1 resets the cycle (van Endert et al., 2002). Based on the findings presented so far it is possible to fit gpUS6 and ICP47 binding to this translocation cycle, emphasizing this order of TAP cycle events (Fig 5.2). gpUS6, being dependent on ATP binding to TAP2 and, as found by others, inhibiting ATP binding to TAP1 (Hewitt et al., 2001), binds to TAP at step II, Fig 5.2, in the translocation cycle. gpUS6 binds to TAP at a step in the cycle where rearrangement of the TMSs has already occurred but the completion of the peptide translocation is still a few steps away. In this conformation ATP binding has induced exposure of the binding domains on the lumenal side of TAP. gpUS6, in its oligomerized form, freezes the TAP translocation machine through a net of molecules attaching TAP at many sites. As a consequence ATP binding to TAP1 is not possible anymore, as well as ICP47 binding.

ICP47 binding, on the other hand, is not dependent on ATP induced TAP conformations. ICP47 binds to TAP in a relaxed conformation corresponding to step I in Fig. 5.2. ICP47 interacts initially with the TAP2 subunit but interaction with TAP1 is also observed. This blocks induction of the high affinity state for peptide binding to TAP and, consequently, the gpUS6 binding domains on TAP1 and TAP2 are not formed on the lumenal side.

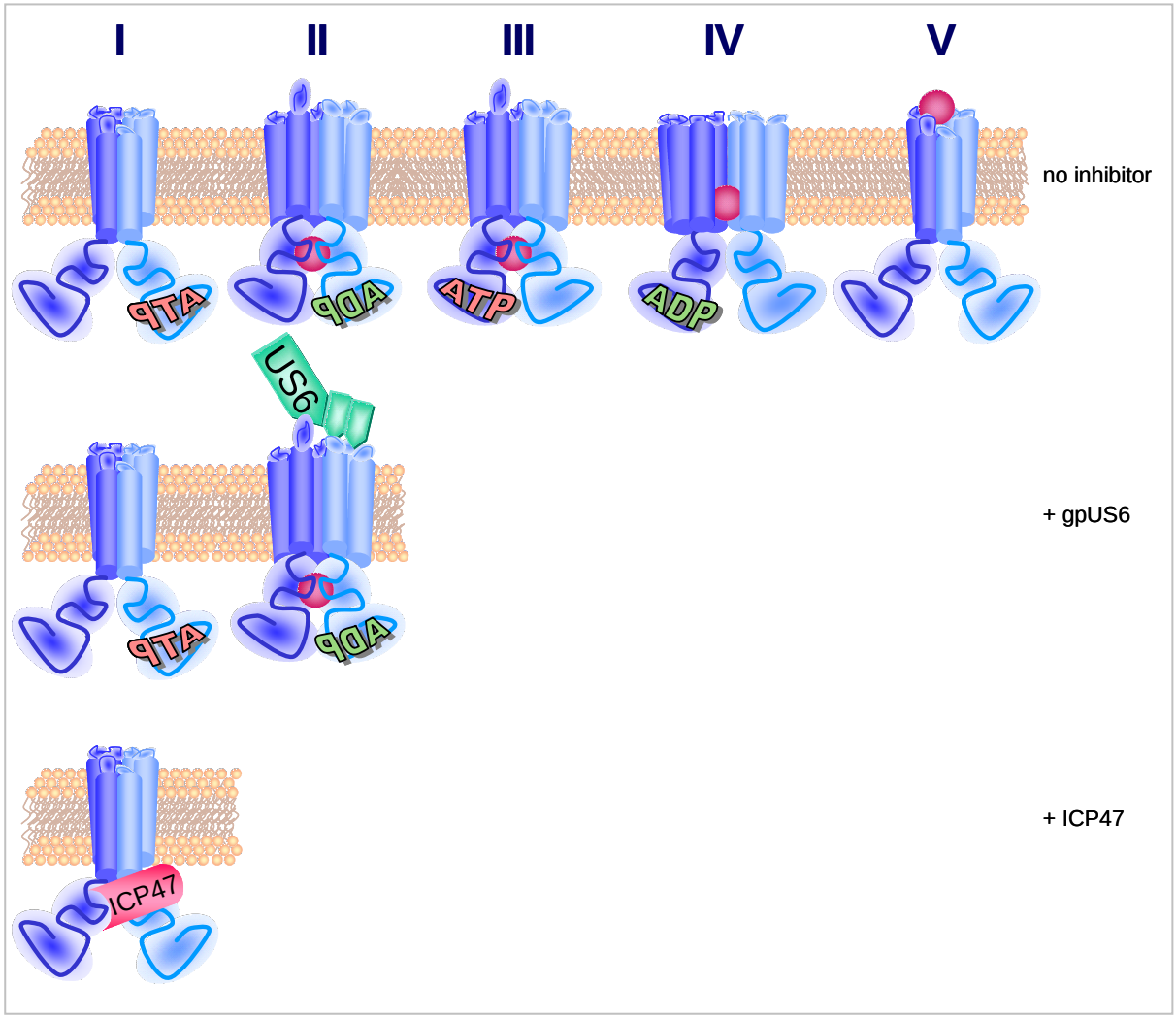

Fig. 5.2 Model of the peptide translocation cycle including gpUS6 and ICP47 binding.

TAP1 and TAP2 are shown in dark and light blue, respectively. The peptide is depicted as a red ball. The peptide translocation cycle starts with TAP in a relaxed conformation and binding of ATP to TAP2 can take place (step I). ATP binding results in a conformation with high substrate affinity (step II). Peptide binding induces ATP hydrolysis at TAP2 and ATP binding to TAP1. ATP binding to TAP1 and release of nucleotide by TAP2 (step III), result in conformational changes and low peptide affinity. The peptide is released. Simultaneously (step IV), ATP is hydrolyzed by TAP1 and a rearrangement of the translocation pore takes place. Release of the nucleotide from TAP1 resets the cycle (steps V and I). gpUS6 binds to TAP at step II after ATP binding to TAP2. ICP47 binds to TAP at step I, independent of ATP binding to TAP.

5.4 Perspectives

Numerous herpesviral proteins have proved to be instrumental for broadening the knowledge of molecular mechanisms of target proteins and signaling pathways in the cell (for examples see (Lilley and Ploegh, 2004; Yin et al., 2003; Zimmermann et al., 2005)). The relatively large genome of herpesviruses allows the seemingly lavish use of gene functions with a highly selective target specificity. In comparison, viruses with smaller genomes have to deal with their limited coding capacity more economically and mostly encode proteins with multiple functions. Therefore, the research of herpesviruses is particularly instructive for the understanding of molecular mechanisms of the cell.

Employing gpUS6 and ICP47 it was possible to characterize the TAP topology, proving the existence of a previously undetected pair of TMSs in TAP1. Furthermore, intermolecular communication between TAP1 and TAP2 leading to structural rearrangements reaching from the cytosolic NBDs to the lumenal part of the TMDs was verified. The findings emphasize the order of events during the peptide transport cycle where TAP2 is always one step ahead. While this study has highlighted a number of issues, further research is necessary. More closely defined gpUS6 and ICP47 interaction domains on TAP are desirable, for which further human/rat TAP chimeras will be constructed. The ICP47 binding site on TAP1 is under current investigation as well as analysis of the functional contribution of ICP47 binding to TAP chimeras.

During the process of writing this thesis the identification of a further herpesviral TAP inhibitor and the existence of another one was published (Koppers-Lalic et al., 2005; Ressing et al., 2005). Putting the new inhibitors of TAP into careful analysis the still unsolved complex mechanism of peptide translocation by TAP will be more comprehensible. Being the only biological inhibitors of an ABC transporter, the herpesviral TAP inhibitors will give new insights for understanding and controlling not only TAP but also other closely related ABC transporters, like the MDR transporter P-gp. P-gp is responsible for transport of drugs out of the cell, causing complications for chemotherapy of cancer patients. Knowledge of inhibitory mechanisms of ABC transporters will be helpful for designing of novel suppressors.