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Tapasin's protein interactions in the rainbow trout peptide-loading complex

Short title: Tapasin's protein interactions in rainbow trout.

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Summary

Major histocompatibility complex (MHC) class I receptors play a key role in the immune system by presenting non-self peptides to T cell lymphocytes. In humans, the assembly of the MHC class I with a peptide is mediated by machinery in the endoplasmic reticulum referred as the peptide loading complex (PLC). Although, the identity of the PLC has been widely explored in humans, this complex has not been characterized in fish. Co-immunoprecipitation and mass spectrometry analysis revealed that the protein-protein interactions which exist in the human PLC are conserved in the monocyte/macrophage rainbow trout cell line (RTS11), in particular the interaction of tapasin with the transporter associated with antigen processing (TAP), MHC class I and ERp57. Importantly, a 20 kDa tapasin version that contains an intact C and N terminal domains was found to associate with ERp57 and form a 75 kDa heterodimer. These results suggest a possible novel alternative spliced version of tapasin may regulate the formation of the peptide-loading complex in teleosts.

Keywords: Antigen presentation, peptide loading complex, tapasin, rainbow trout

Introduction

The recognition of intracellular pathogens such as viruses by cytotoxic T cell lymphocytes (CD8+) is mediated through MHC class I receptors on the cell surface of all nucleated cells where they present either self or foreign peptides (Blum et al., 2013). The MHC class I receptor is composed of three essential components: the polymorphic heavy chain, β2 microglobulin and a peptide of either self or foreign origin. Lack of any of these three, results in poor stability and degradation of the MHC class I receptor (Pamer and Cresswell, 1998; Wearsch and Cresswell, 2008). Peptides to be loaded into the MHC class I receptor are mainly generated by proteasomal cleavage of native proteins. These peptides are then delivered from the cytosol into the endoplasmic reticulum (ER) by transporter associated with antigen processing (TAP; Hulpke and Tampe, 2013). Once the peptides reach the ER they are destined to bind to the MHC class I, which needs to achieve optimal folding in the presence of a changing repertoire of peptides (Peaper and Cresswell, 2008).

In humans, the lectin chaperone calnexin associates with the MH class I heavy chain during its early folding and recruits the thiol-disulfide oxidoreductase, ERp57. Subsequently, upon assembly with β2 microglobulin, the MHC class I heavy chain is incorporated into the peptide-loading complex (PLC) containing TAP, the lectin chaperone calreticulin and a disulphide linked conjugate of tapasin and ERp57 (Lindquist et al., 2001; Peaper et al., 2005, Rizvi and Raghavan, 2010). Tapasin is a keystone component in the PLC that forms a structural bridge between TAP and the MHC class I (Sadasivan et al., 1996; Momburg and Tan, 2002). This protein contains an

immunoglobulin constant domain, a transmembrane domain and a cytoplasmic domain with ER retrieval sequence (Ortmann et al., 1997). One of its first proposed functions was to stabilize TAP and increase peptide flow to the ER (Lehner et al., 1998; Raghuraman et al., 2002), however subsequent studies showed that a soluble tapasin version which does not interact with TAP can still facilitate the recruitment of the MHC class I and peptide binding (Lehner et al., 1998). Other studies focused on the role of tapasin as a "peptide editor" (Howarth et al., 2004; Chapman and Williams, 2010), which optimizes the selection of peptides that are loaded on the MHC class I. Through a cell free system approach, it was demonstrated that recombinant tapasin-ERp57 conjugates, but not recombinant tapasin alone, can effectively recruit the MHC class I and support binding and the selection of high affinity peptides (Wearsch and Cresswell, 2007).

The first teleost major histocompatibility gene fragments were isolated from carp (Hashimoto et al., 1990) and genomic linkage studies revealed a surprising genetic architecture in which the class I and II major histocompatibility genes do not form the complex seen in other vertebrates, and therefore are referred as MH (major histocompatibility) genes (Stet et al., 2003; Phillips et al., 2003). The teleost genes involved in antigen processing, delivery and selection such as TAP, the proteasome induced subunits LMP2 and LMP7 and tapasin have been characterized and are usually linked to the MH class I genes in teleost fishes (Takami et al., 1997; Hansen et al., 1999; Landis et al., 2006). Promoter analysis of MH class I and tapasin genes revealed the presence of a putative interferon regulatory factor binding site and a interferon-γ binding site that supported their transcriptional induction by interferons during salmonid

viral infection (Hansen et al., 1999: Jørgensen et al., 2006). In addition, the ER chaperones: calreticulin, ERp57 and calnexin, which are involved in the formation of the PLC, have been identified and studied in several teleost fishes (Kales et al., 2004; Fuller et al., 2004; Sever et al., 2013a; Sever et al., 2014b). To date, no evidence has been published describing a possible PLC in a non-mammalian vertebrate mainly due to the lack of antibodies. This report shows, for the first time, the unique and conserved protein interactions that may constitute the PLC in teleost fish, as well as the possible unique mechanisms that regulate the formation of this PLC.

Material and methods

Fish Cell Cultures

A semi-adherent monocyte/macrophage RTS11 cell line originally established from rainbow trout spleen (Ganassin and Bols, 1998), was maintained in Leibovitz's L-15 medium (ThermoFisher Scientific, Nepean, ON) with 20% fetal bovine serum and 150 U/mL of penicillin and 150 mg/mL streptomycin (ThermoFisher, Nepean, ON) in 75 cm^2 culture flasks. Two adherent rainbow trout cell lines: RTmt from the testis and RTovarian fluid from the ovarian fluid (Vo NTK and Bols NC, unpublished) were grown in room temperature in L-15 with 10% FBS. Long-term leukocyte cultures that were rich in dendritic cells and macrophages were grown from caudal fin organ explants from rainbow trout in L-15 with 30% FBS (Vo NTK and Bols NC, unpublished). All rainbow trout cell cultures were maintained at 20°C. The fathead minnow-derived adherent epithelial EPC cell line (Winton et al., 2010) was maintained in L-15 medium with 10%

FBS in 75 cm^2 culture flasks at 26°C. All adherent cell lines were sub-cultured on a weekly basis by trypsin (Lonza, Allendale, NJ).

VHSV IVa propagation and Infection of RTS11 cells for immunoprecipitation

A North American group viral hemorrhagic septicemia virus IVa isolate (VHSV IVa) obtained from Pacific herring (Kocan et al., 1997) was routinely propagated on EPC cultures in L-15 medium with 2% FBS at 14°C as described (Sever et al., 2014a). Briefly, once the confluent cell monolayer was completely destroyed, the virus-containing conditioned media was spun at 4500 X g for 5 minutes at 4°C. The supernatants were filtered through 0.2-µm membranes (Pall Corporation, Mississauga, ON) and stored in aliquots at -80°C for later use. Viral titers were determined by $TCID_{50}$/mL assays as previously described (Pham et al., 2013). For cell infection, approximately, 1 X 10^8 RTS11 cells were challenged with 1 X 10^8 $TCID_{50}$/mL of VHSV IVa in L-15 media supplemented with 2% FBS. In the control (non-infected) cultures, the virus was absent. Both cultures were incubated for 7 days at 14°C. Cells were then collected and centrifuged at 500 X g for 4 minutes at 4°C. Subsequently, cell pellets were incubated in 5 mL of ice-cold PBS containing 10 mM of methyl methanethiosulfonate (MMTS: ThermoFisher, Nepean, ON) for 3 minutes followed by quick centrifugation at 500 X g.

Immunoprecipitation

RTS11 and primary fin cultures (1 X 10^8 cells) were collected and incubated for 2 minutes with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4; pH 7.4) containing 10 mM of MMTS (ThermoFisher Scientific, Nepean, ON). Subsequently, cells were centrifuged at 500 X g for 3 minutes and lysed with 1% digitonin (Sigma Aldrich, St. Louis, MO) containing 10 mM MMTS, 50 mM of Tris-HCl, 150 mM of NaCl supplemented with 1X protease inhibitors (Roche, Mannheim, Germany). Cells were rotated at 4°C for 30 minutes followed by centrifugation at 10,000 rpm for 30 minutes to remove cellular debris. Immunoprecipitation was performed using either 1:50 affinity purified tapasin antibody (Sever et al., 2013a); 1:50 pre-immune tapasin rabbit serum; 1:200, anti-MH polyclonal antibody (Kawano et al., 2010) or 1:200 of anti TAP1 antibody (SAB2102370: Sigma Aldrich, St. Louis, MO). Antibodies were rotated with lysates overnight at 4°C, followed by the addition of 50 µL of protein A agarose beads (Sigma Aldrich, St. Louis, MO) for 1 h. The beads were spun down by centrifugation at 3000 rpm and subsequently washed three times with 500 µL of 0.1% of digitonin lysis buffer. Primary elution for tapasin pull downs was performed by adding 100µM tapasin peptide in 0.1% of digitonin lysis buffer, which corresponds to the last 21 aa of trout tapasin's C terminal domain. Trout tapasin's protein sequence was obtained from the GeneBank accession number (NP_001118025.1).

Primary elution was followed by a second boiling elution in 50 µL of 2X SDS sample buffer. Boiled elutions were separated on a 10% SDS page gel, transferred to a nitrocellulose membrane and probed with either anti MH class I (1:400) antibody or (1:200) anti-tapasin antibody for one hour. Detection was performed using anti-rabbit

IgG VeriBlot (HRP) diluted with 1:800 of skim milk and clarity™ (Roche, Mannheim, Germany) according to manufacturer's instructions.

Tapasin peptide elutions were separated on a 10% SDS page gel and stained with Coomassie blue (0.1% Coomassie blue, 40% methanol, 10% acetic acid, 50% Milli-Q water) for 1 hour followed by addition of destaining solution (5% methanol, 7% acetic acid, 88% Milli-Q water) overnight at 4°C. Proteins with relative molecular weight of 75 kDa, 55 kDa and 25 kDa were excised and stored at 4°C in 1% acetic acid in Milli-Q water, until ready for digestion.

Immunoprecipitations for tapasin and MH class I were repeated 4 times (see supplementary figures 1 and 2 for examples). IPs for tapasin and ERP57 were repeated 3 times. The IP for the tapasin and TAP interaction was only performed once so this result might be preliminary, but TAP peptides were detected in small quantities in the mass spectrometry of tapasin bands, corroborating this interaction.

Western blotting

For the detection of the interaction between ERp57 and tapasin, non-stimulated $2x10^6$ RTS11 cells were collected and incubated for 2 minutes with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4; pH 7.4) containing 10 mM of MMTS (ThermoFisher Scientific, Nepean, ON). Subsequently, cells were lysed for 30 min with 1% digitonin (Sigma Aldrich, St. Louis, MO) containing 10 mM of MMTS, 50 mM of Tris-HCl, 150 mM of NaCl supplemented with 1X protease inhibitors (Roche, Mannheim, Germany). Supernatants were collected after centrifugation at 13,000 rpm for 20 min.

Protein samples were prepared under reducing conditions with β-mercaptoethanol or non-reducing conditions, without β-mercaptoethanol, and loaded onto a 10% SDS-page gel and probed with 1:1000 anti-ERp57 antibody (Sever et al., 2013b) or 1:200 anti-tapasin antibody (Sever et al., 2013a). Detection was performed using anti-rabbit IgG VeriBlot (HRP) diluted with 1:800 of skim milk and clarity™ (Roche, Mannheim, Germany) according to manufacturer's instructions. For detection of tapasin protein in RTtestis and RTovarian fluid, 2 X 10^6 cells were lysed in 1% NP-40 lysis buffer containing 150 mM NaCl and 50 mM Tris [pH 8.0] supplemented with 1X of protease inhibitor cocktail (Roche, Mannheim, Germany). Detection was performed as previously described (Sever et al., 2014a).

Mass spectrometry and protein identification

In gel tryptic gel digestion and mass spectrometry analysis (MS/MS) were performed by SPARC BioCenter, mass spectrometry facility (Hospital for Sick Children, Toronto, Canada). Briefly, excised gel fragments containing the protein bands were reduced with 10 mM of DTT for 30 min at 56°C and alkylated with 100 mM iodoacetamide for 15 min at room temperature in the dark. Supernatants were then discarded, followed by addition of 50 µL of solution containing 1:1 of 50% acetonitrile and 25 mM ammonium bicarbonate. In-gel tryptic digestion was performed by adding 13 ng/µL trypsin (Sigma Aldrich, St. Louis, MO) for 3 hours at 37°C. Peptides were loaded onto a 50 cm x 75 µm ID column with RSLC 2 µm C18 packing material (EASY-Spray, Thermo-Fisher, Odense, Denmark) with an integrated emitter. The peptides were eluted into a LTQ-

Velos Pro Elite hybrid mass spectrometer (Thermo-Fisher, San Jose, CA) using an Easy-Spray nLC 1000 chromatography system (Thermo-Fisher, Odense Denmark) with a 2 hr gradient from 0% to 35% acetonitrile in 0.1% formic acid.

Data processing parameters

Tandem mass spectra were extracted by XCalibur version 2.2. All MS/MS samples were analyzed using Sequest (Thermo Fisher Scientif c, San Jose, CA, USA; version 1.4.0.288) and X! Tandem (The GPM, thegpm.org; CYCLONE; version 2010.12.01.1). The software was set up to search the Oncorhynchus mykiss database. Scaffold (version Scaffold 4.2.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identif cations.

Results

Tapasin associates with the MH class I under normal and viral induced conditions

Tapasin's transcript expression and the 48 kDa isoform levels were previously shown to upregulate in RTS11 cells during viral hemorrhagic septicemia virus (VHSV) infection which highlighted its possible conserved function in trout (Sever et al., 2014a). In order to investigate if trout tapasin can bind the MHC class I heavy chain, tapasin protein was pulled down from RTS11 cell lysates in uninfected conditions and after seven days of VHSV infection. As seen in Fig.1A, the MH class I heavy chain band was detected in tapasin immunoprecipitates obtained from control and infected cells. This association was identified between tapasin and the upper MH class I band, previously shown to be

the mature glycosylated form, in gill tissue (Pham et al., 2013) and in RTS11 (Sever et al., 2014a). The reciprocal interaction of tapasin with the MH class I was further supported by detection of tapasin protein band in MH class I pull downs both in control and infected cells (Fig.1B). Importantly, in tapasin pull downs, a 20 kDa band was detected by tapasin antibody in addition to the predicted 48 kDa tapasin protein. This 20 kDa band appeared both in control and VHSV infected cells when the tapasin antibody was employed. The 20 kDa band was not seen in the MHC class I pulldowns, although its levels were relatively low compared with 48 kDa tapasin protein, so there may simply be not sufficient quantities present for detection in these lanes.

The interaction of tapasin with the glycosylated form of the MH class I was also detected in long-term fin leukocyte-like cultures, that contain many cells with characteristics of dendritic cells (TK Vo, L. Sever, B. Dixon. and NC Bols, unpublished) suggesting that this interaction might be conserved in antigen presenting cells *in vivo* (Fig. 2).

TAP association with tapasin and MH class I under normal and viral infected conditions

In mammals, tapasin is known to associate with TAP through its transmembrane domain, which facilitates a structural bridge between TAP and the MHC class I (Wearsch and Cresswell, 2008). In order to determine if the tapasin:TAP interaction is conserved in fish, pull downs of MH class I and tapasin were probed with a human anti TAP1 antibody which recognizes a 50 aa peptide, sharing 61% identity with the trout TAP1 protein (GeneBank accession number: AAD53033).

In RTS11 cell lysates TAP1 was recognized as a 75 kDa protein and its association with tapasin and MH class I was identified both in control and VHSV IVa infected cells as seen in Fig.3. Although this experiment was only performed once, these results were further confirmed by mass spectrometry and protein identification, which revealed peptides derived from trout TAP1 and TAP2 in trout tapasin pull downs (Table 1).

Tapasin protein expression in trout cell lines derived from reproductive tissues

Trout tapasin protein is mainly detected as a 48 kDa protein which corresponds with its predicted molecular weight (Sever et al., 2013a). Interestingly, an additional 20 kDa band was detected by tapasin antibody in most trout cell lines and tissues, and the protein level of this 20 kDa band didn't change in response to poly I:C stimulation and viral infection in contrast to the upregulation of tapasin 48 kDa protein (Sever et al., 2013a). When tapasin protein levels were detected in cell lines derived from trout reproductive organs such as testis (RTmt) and ovarian fluid (RTovarian fluid), only the 20 kDa band was observed (Fig. 4). This result further supports a possible 20 kDa version for trout tapasin protein which is expressed differentially in trout cell lines and yet contains an intact C terminal domain, which contains the epitope recognized by our anti-trout tapasin antibody.

The possible identity of the 20 kDa band as a tapasin isoform was supported by mass spectrometry and protein identification which showed that peptides derived from both the N and the C terminal domains of tapasin were present in tapasin's 20 kDa pull downs (Table I). No peptides from the immunoglobulin domains were identified in the 20

kDa band mass spectrographs, although band from these domains were found in mass spectrograph analyses of the larger band.

Tapasin association with ERp57

To elucidate the possible interaction of ERp57 with tapasin, RTS11 cells were pretreated prior to the detergent extraction with methyl methanethiosulfonate (MMTS), which protects native disulfide bonds during detergent extraction (Dick et al., 2002; Peaper et al., 2005). Under reducing conditions, only monomers of tapasin and ERp57 were detected by their respective antibodies (Fig 5). Under non-reducing conditions, a 110 kDa band was detected by both tapasin and ERp57 antibodies, in addition to ERp57 and tapasin monomers. The size of this band and its presence in both tapasin and ERp57 blots suggest a conserved heterodimer of ERp57 and tapasin (Fig. 5), similar to the one seen in mammals which is maintained via disulfide bonds (Lindquist et al. 2001; Peaper et al., 2005). These results were further confirmed by mass spectrometry, which identified peptides derived from trout ERp57 in tapasin pulldowns (Table 1).

Interestingly, an additional 75 kDa band was detected by tapasin and ERp57 antibodies under non-reducing conditions. Based on the detection of a 20 kDa tapasin protein in trout cell lines and tissues (Fig.4; Sever et al., 2013a), it suggested that this shorter version of tapasin could possibly also interact with ERp57 to form a 75 kDa disulphide linked heterodimer. Amplification of the full open reading frame of trout tapasin from RTS11 and spleen tissue has shown multiple smaller transcripts that could encode a

truncated mRNA, although to date the specific transcript encoding this variant has not been sequenced (data not shown).

Discussion

In this work, the potential protein-protein interactions involved in the PLC of rainbow trout were examined in the rainbow trout macrophage cell line RTS11. Although, indirect evidence suggests that antigen presentation does occur in teleost fish (Vallejo et al., 1991; Vallejo et al., 1992; Nakanishi et al., 2002), the molecular mechanisms involved in peptide loading are still unknown. The RTS11 cell line was used as a model since the trout proteins such as calreticulin (Kales et al., 2007), ERp57 (Sever et al., 2013b), and calnexin (Sever et al., 2014b) were previously studied in this cell line. In addition, both MH class I and class II proteins are abundantly expressed in RTS11 (Kawano et al., 2010) and thus it should possess a functional endogenous pathway PLC.

Interaction of tapasin with the MH class I and TAP

The specific interaction of MHC class I heavy chain and tapasin is not fully understood due to their low affinity (Wearsch and Cresswell, 2008). Nevertheless, mutagenesis studies have shown that there are at least two sites of interaction of tapasin with the MHC class I heavy chain, one of which involves the N terminus and the other involving the membrane proximal immunoglobulin domain of tapasin (Lewis et al., 1996; Yu et al., 1999). The first 50 amino acids of tapasin's N terminal domain were shown to be important for binding to MHC class I, ERp57 and calreticulin (Bangia et al., 1999),

whereas the C-terminal transmembrane domain of tapasin is involved in tapasin's interaction with TAP (Leonhardt et al., 2005). In this study, the association of trout tapasin with the trout MH class I heavy chain has been identified, which provides the first evidence for the conserved role of tapasin as an MH class I binding protein in teleost fishes. Additionally, trout tapasin was also shown to interact with TAP1 and TAP2, confirming tapasin's conserved role as a bridge between TAP and MH Class I receptors.

Although tapasin's leucine motif, known to be involved with TAP binding in mammals (Tan et al., 2002), it is absent from the trout sequence but there are three leucine residues in the transmembrane domain of the trout sequence that could putatively interact with TAP (Landis et al., 2006). In addition, the charged lysine residue within the transmembrane region of tapasin that has previously been shown to be involved in binding of TAP (Petersen et al., 2005) is conserved in both the zebrafish and salmonid sequences, which suggests that the interaction of trout tapasin with TAP could be mediated through tapasin's transmembrane domain in a fashion similar to mammals. These results suggest the structure of a putative peptide-loading complex in teleost, that includes three main constitutents: TAP, tapasin and MH class I.

Tapasin association with ERp57

Interestingly, trout tapasin contains amino acid substitutions that may influence its putative interactions with ERp57. In all teleost tapasin sequences reported to date, the conserved Cys95, known to recruit ERp57 in mammals, is substituted with serine, a feature surprisingly also conserved in birds (Van Hateren et al., 2010). Nevertheless,

another upstream cysteine residue in position 57, which is found only in teleost fishes could possibly replace Cys95 in forming a disulfde bond with ERp57. Both ERp57 and tapasin antibodies detected the conserved heterodimer of tapasin and ERp57 in RTS11 cell lysates. This result indirectly suggests the possible recruitment of ERp57 to a PLC through its interaction with tapasin that might have evolved to optimize the unique folding requirements of the MH class I with a peptide in the fish ER.

Possible 20 kDa version of tapasin in trout

Interestingly, while tapasin protein is detected in both human and mice as a single 48 kDa band, in trout cell lines tapasin is detected as the predicted 48 kDa protein with an additional 20 kDa band. This finding raises the interesting possibility that there is a 20 kDa form of trout tapasin. As the anti-trout tapasin antibody recognizes the C terminal domain of tapasin, the 20 kDa protein contains an intact cytoplasmic tail which could possibly interact with TAP. The 75 KDa complex seen in figure 5 suggests that it can also bind ERp57 through a cysteine residue in its N terminal domain. Thus, the 20kDa trout tapasin version could be an alternative splice variant and might be lacking the middle Ig domains that form the primary interaction with the MH class I. Interestingly, while the 48 kDa isoform is glycosylated, the 20 kDa protein was found not to be subject to N glycosylation (data not shown). As the tapasin N-glycosylation site is located in the distal IgV domain, it further suggests that the IgV domain of tapasin is missing in the 20 kDa protein.

Evidence for tapasin alternative splice variants was originally identified in the human B-lymphoblastoid cell line, .220, which exhibits defective antigen presentation and

reduced MHC class I levels on the cell surface (Greenwood et al., 1994; Grandea et al., 1995). This phenotype was attributed to a genomic point mutation, which results in an alternative spliced tapasin. This human tapasin form lacks exon 2 thus encoding a protein missing a signal peptide and 49 aa of tapasin's N terminal domain and was found to be present in very low levels in .220 cells possibly due to aberrant protein translocation into the ER (Ortmann et al., 1997). This genomic defect was restored upon transfection with a full-length tapasin cDNA sequence into .220 cells (Ortmann et al., 1997), but a normal phenotype was also restored when cells were transfected with a soluble version of tapasin, which lacks its transmembrane and cytoslic domains (Lehner et al., 1998). Another human tapasin cDNA variant that retained introns 4-6 has been described. When the cDNA of this variant was transfected into .220 B cell line, both exon 4 and 6 were successfully excluded, however exon 5 remained intact. This alternative spliced tapasin transcript produced a soluble protein version that lacks tapasin's transmembrane domain and contained eight novel amino acid residues at the C terminal end, due to the incorporation of a new stop codon (Gao et al., 2004).

In contrast to the alternative spliced variants of tapasin produced by genomic mutations, a third variant that lacks exon 3 was described in a human melanoma cell line. This variant encodes the distal immunoglobulin domain, yet also encodes an intact transmembrane domain and a cytoplasmic tail. Transfection of this variant into tapasin deficient cells demonstrated that, in spite the intact binding of tapasin to TAP, MHC class I surface expression was still impaired (Belicha-Villanueva et al., 2010). This result raised the possibility of a possible dominant negative tapasin form that can potentially

regulate antigen presentation in cancer cells. However, a tapasin's version that lacks exon 3 hasn't been described in non-cancerous mammalian cell lines.

In this study, confirmation that the tapasin version seen here is indeed an alternative spliced variant for tapasin is still lacking, as we have not yet identified a cDNA corresponding to this protein. However, when Northern blotting was performed using probes generated against tapasin's C-terminal, middle (immunoglobulin) and N-terminal domains, a minor lower band was detected by the C-terminal and N-terminal probes in addition to the main expressed transcript, whereas no expression of this band was observed with the immunoglobulin domain probe (Sever et al., 2013a). In addition, mass spectrometry followed by protein identification of the 20 kDa band revealed two unique peptides that match the N and C terminal domain of tapasin; but no other peptides were identified from tapasin's immunoglobulin domain. Mass spectrometry of the 48 kDa band in these same experiments did include peptides from the middle of the molecule. Combined with the presence of smaller transcripts by PCR noted above, these results, including the lack of glycosylation of the 20 kDa tapasin form, support a possible trout tapasin spliced transcript, which does not encode the immunoglobulin domains. The fact that the mRNA encoding it is hard to isolate may suggest that it is ephemeral by nature because of the negative regulatory role its product plays.

Another possibility could be the presence of a second gene for tapasin, which encodes a shorter version of the protein. Interestingly, in trout there are two identified genes for tapasin, which reside on different chromosomes: TAPBP.A and TAPBP.B, the latter of which does not appear to encode for a full-length TAPBP due to deletion of exons 5-7. The authors of this study could not find a sequence encoding the 3' end of the protein

(Landis et al., 2006); however, if this gene encodes the 20 kDa protein, seen here, it should encode an in intact C terminal domain, as this domain is recognized by the tapasin antibody, but the published version lacks the exons encoding this region.

What is the function of a possible tapasin version in trout? It is possible that this smaller isoform of tapasin could compete with the full length protein for binding to TAP, yet does not bring MH class I receptor into the complex. It could be further speculated that expression of both tapasin versions allows regulation of antigen presentation at important immune system sites such as the head kidney and spleen during normal conditions, whereas in sites such as testis and ovaries where only the 20 kDa band is expressed, restricted immune responses might be desirable, in a manner similar to human melanoma cells (Belicha-Villanueva et al., 2010) and as a reflection of the immune privileged status of these tissues seen in mammals. Thus, the high expression of this 20 kDa form reduces the amount of antigen presented.

Since trout tapasin is the only MH class I chaperone that was upregulated during viral infection (Sever et al., 2013a), it is possible that during immune responses tapasin plays a critical role in regulating PLC function in trout. When the larger isoform is upregulated by stimuli, it can outcompete the shorter tapasin form in binding to TAP and thus more MH class I could enter the peptide loading complex and exit the ER to the cell surface. Thus, while the trout PLC may be similar in composition to mammalian PLCs during immune responses (Fig. 6), its regulation might be very different due to the concurrent expression of two tapasin protein forms, the 20 kDa version of which reduces immune responses by tying up TAP and ERp57 while excludling MHC class I receptors.

Interestingly, in humans numerous novel alternatively spliced versions of the human tapasin-related protein named TAPBPR have been isolated, one of which, TAPBPR γ encodes a protein which lacks an IgC domain but still contains the transmembrane and cytoplasmic domains. Although, other isoforms of TAPBPR protein were shown to associate with MHC class I, TAPBPR γ did not (Porter et al., 2014). This supports the suggestion that the 20 kDa form of trout tapasin which similarly lacks immunoglobulin domains also lacks the ability to bind to MH class I receptors. Given the evolutionary distance between trout and humans, human TABBPR and tapasin are equally valid homologues of trout tapasin.

Conclusions

In this work, the conserved association of the trout MH class I heavy chain and tapasin was identified, along with the dual interaction of tapasin with TAP and ERp57. These findings suggest trout possess a PLC similar in composition to that of mammals. However, unlike mammals another 20 kDa version tapasin was identified, which is suggested here to play a role in regulating the formation of the PLC in teleosts.

Although, many protein-protein interactions were identified in this report, their exact contributions to peptide loading and MH class I assembly are far from being understood. Future work will elucidate the contribution of such interactions to antigen presentation, the timing of events and their effects on T cell activation. Further characterization of the role and source of the 20 kDa tapasin will be pursued.

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Disclosures

The authors have no potential conflict of interest

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Figures

Fig. 1: Detection of the interaction between MH class I heavy chain and tapasin by co-immunoprecipitation. RTS11 cells were infected with VHSV IVa for seven days followed by extraction in 1% digitonin lysis buffer. Immunoprecipitations were performed in cell lysates obtained from control and VHSV IVa infected cells with either tapasin antibody (tapasin), TAP antibody (TAP), MH class I antibody (MHI) or pre immune serum (PI). Blots were probed with either the MH class I antibody (A) or with tapasin antibody (B). The position of MH class I glycosylated band (MHI glyc) is represented in (A) with an

arrowhead on the right margin. The position of tapasin 48 kDa band (Tapasin) and a 20 kDa protein (Band) are represented with an arrowhead on the right margin (B).

Fig.2: Detection of tapasin and MH class I association in long-term fin leukocyte-like cells. Fin leukocyte-like cells was extracted with 1% digitonin lysis buffer followed by immunoprecipitation with pre immune serum (PI), ERp57 antibody, tapasin serum (Tapasin1), affinity purified tapasin antibody (Tapasin2) and MH class I heavy chain antibody (MHI). Elutions were obtained using a synthetic tapasin peptide, followed by a second boiling elution. Immunoprecipitates were separated on 10% SDS- PAGE gel and blotted with MH class I heavy chain antibody. The MH class I heavy chain bands are shown in the cell lysate lane (lysate). The position of MH class I glycosylated band (MHI glyc) is indicated with an arrowhead on the right margin.

Fig.3: Detection of the interaction between tapasin and TAP in RTS11 cells by co-immunoprecipitation. RTS11 cells were infected with VHSV-IVa for seven days followed by extraction with 1% digitonin lysis buffer. Immunoprecipitation was performed with either tapasin antibody (tapasin), MH class I antibody (MHI) or pre immune serum (PI) and blots were probed with TAP antibody.

Fig.4: Western blot analysis of tapasin protein in different trout cell lines demonstrating the detection of two distinct bands in rainbow trout cell lines: the predicted 48 kDa and an additional 20 kDa band in cell lines derived from the testis (RTmt) and ovarian fluid (RTovarian fluid). The position of the molecular mass markers are shown by arrowheads in the right margin

Fig.5: Western blot analysis of RTS11 pretreated with MMTS under reducing conditions with β mercaptoethanol (+) and without β mercaptoethanol (-). Blots were detected with either ERp57 or with tapasin antibody. Arrows indicate the putative ERp57-tapasin conjugate (110 kDa), the free ERp57-tapasin proteins and the 75 kDa band detected by both antibodies.

Fig.6: A model of the possible components of the MH class I peptide loading complex in rainbow trout. (A) During infection, tapasin protein is predominantly expressed compared to its 20 kDa version and the PLC includes tapasin conjugated to ERp57 via disulfide bond, TAP and MH class I. (B) During non-stimulating conditions, the protein levels of tapasin are relatively low which facilitates increased binding of tapasin 20 kDa version to ERp57 and TAP, reducing interactions of these molecules with MH class I.

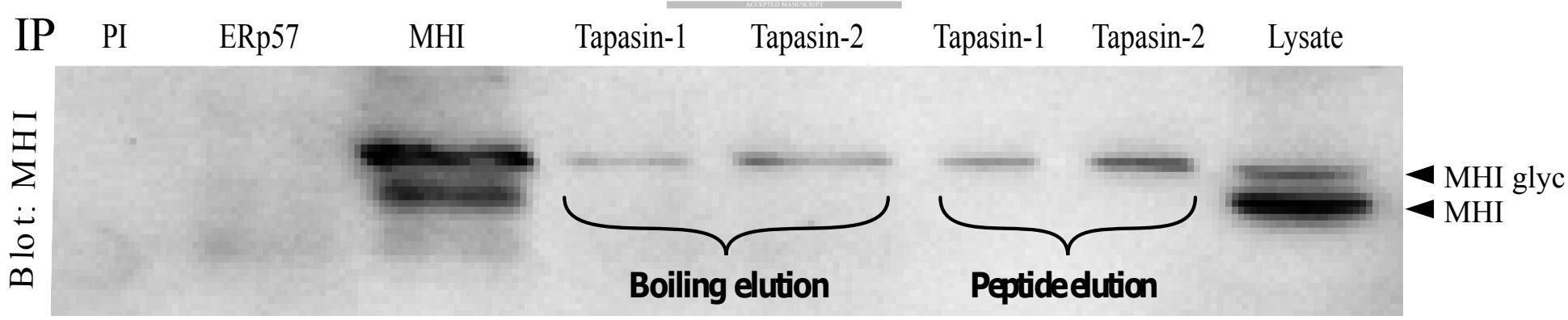
Supplementary Fig 1: A non-cropped version of figure 2.

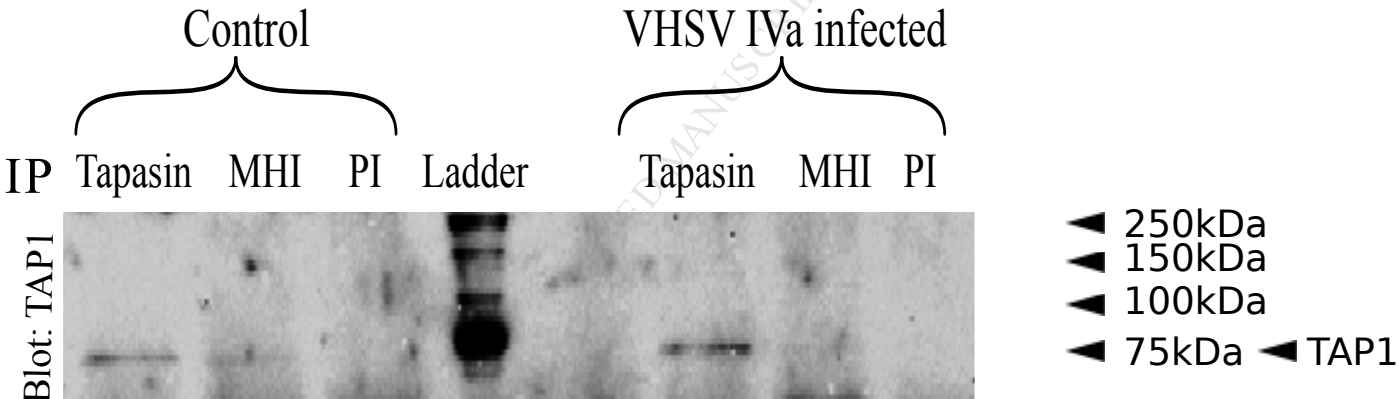
Supplementary Fig 2: A second experiment corroborating the association of the large form of tapasin and MHI shown in Fig1B. In this case, the stimulation to enhance antigen presentation was performed with Poly I:C.

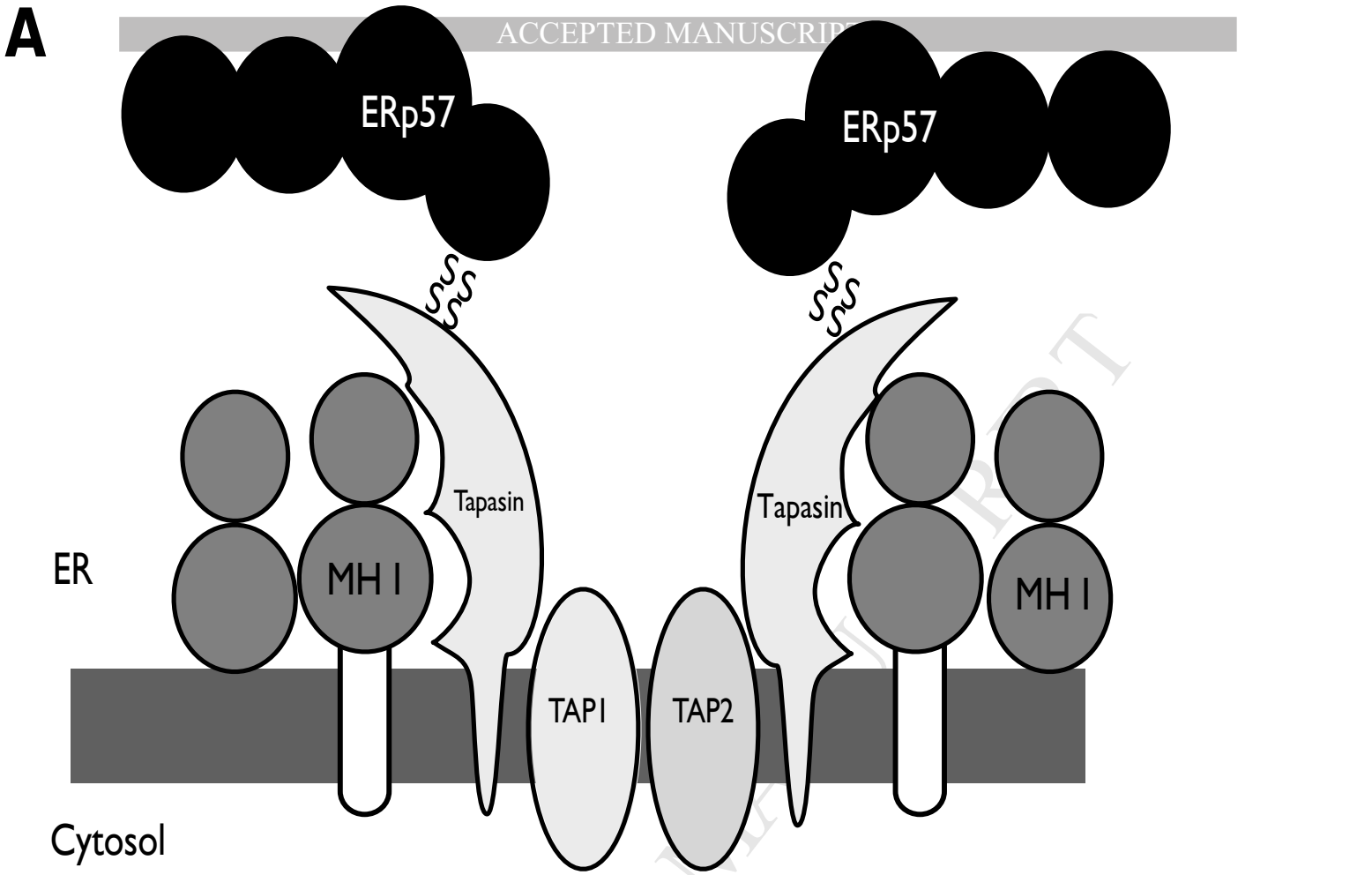
Supplementary Fig 3: A repeat of figure 1A

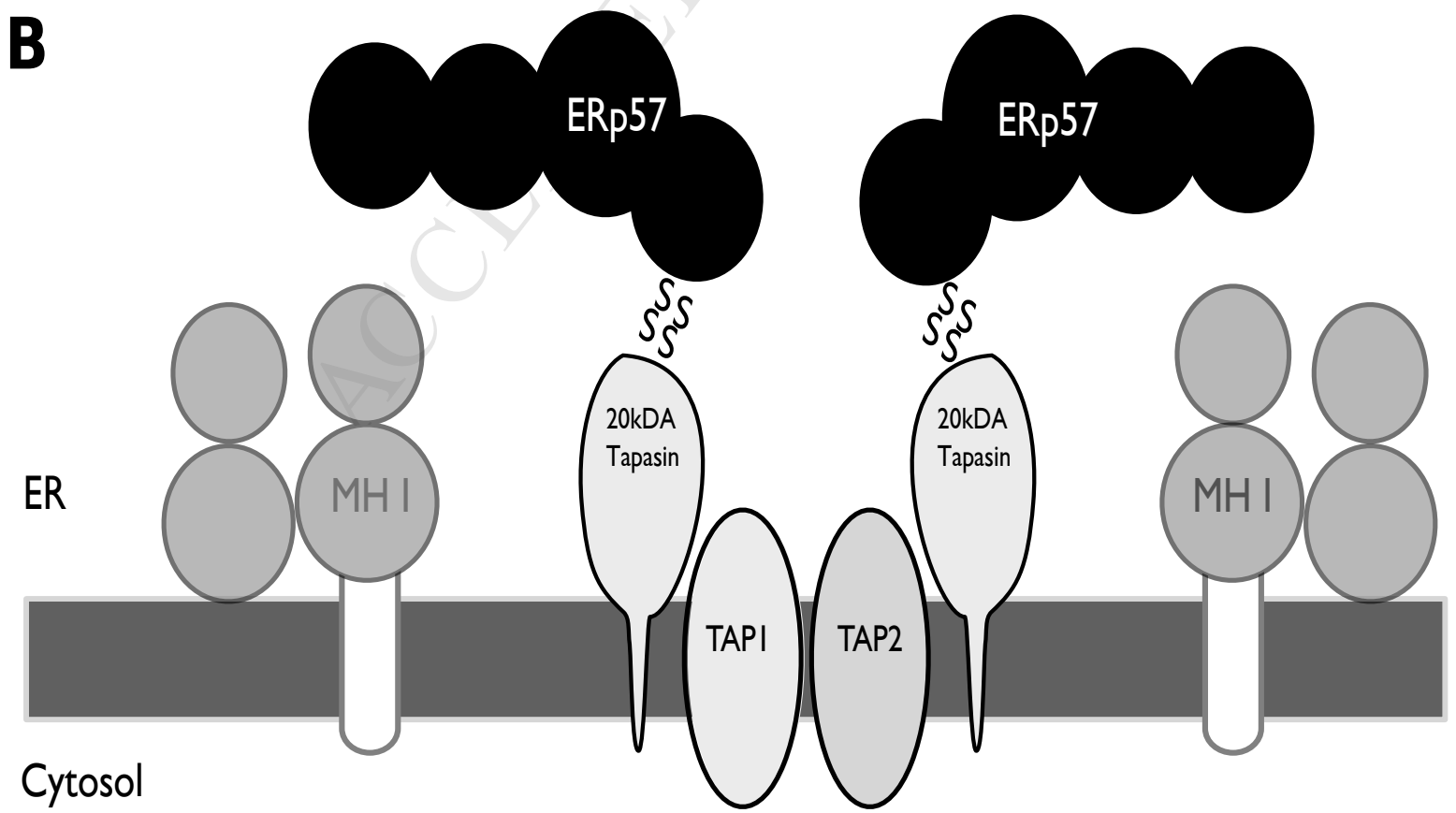
Table 1: Uniquely identified proteins in anti-trout tapasin immunoprecipitation experiments**.** Following IP, molecular weight bands of 75 kDa, 55 kDa and 20 kDa were excised and analyzed by mass spectrometry followed by protein identification using Oncorhynchus mykiss database. Coverage refers to the percentage of the protein sequence that is covered by identified peptides. The number of unique peptides refers to the number of detected distinct peptides that originate from a specific protein

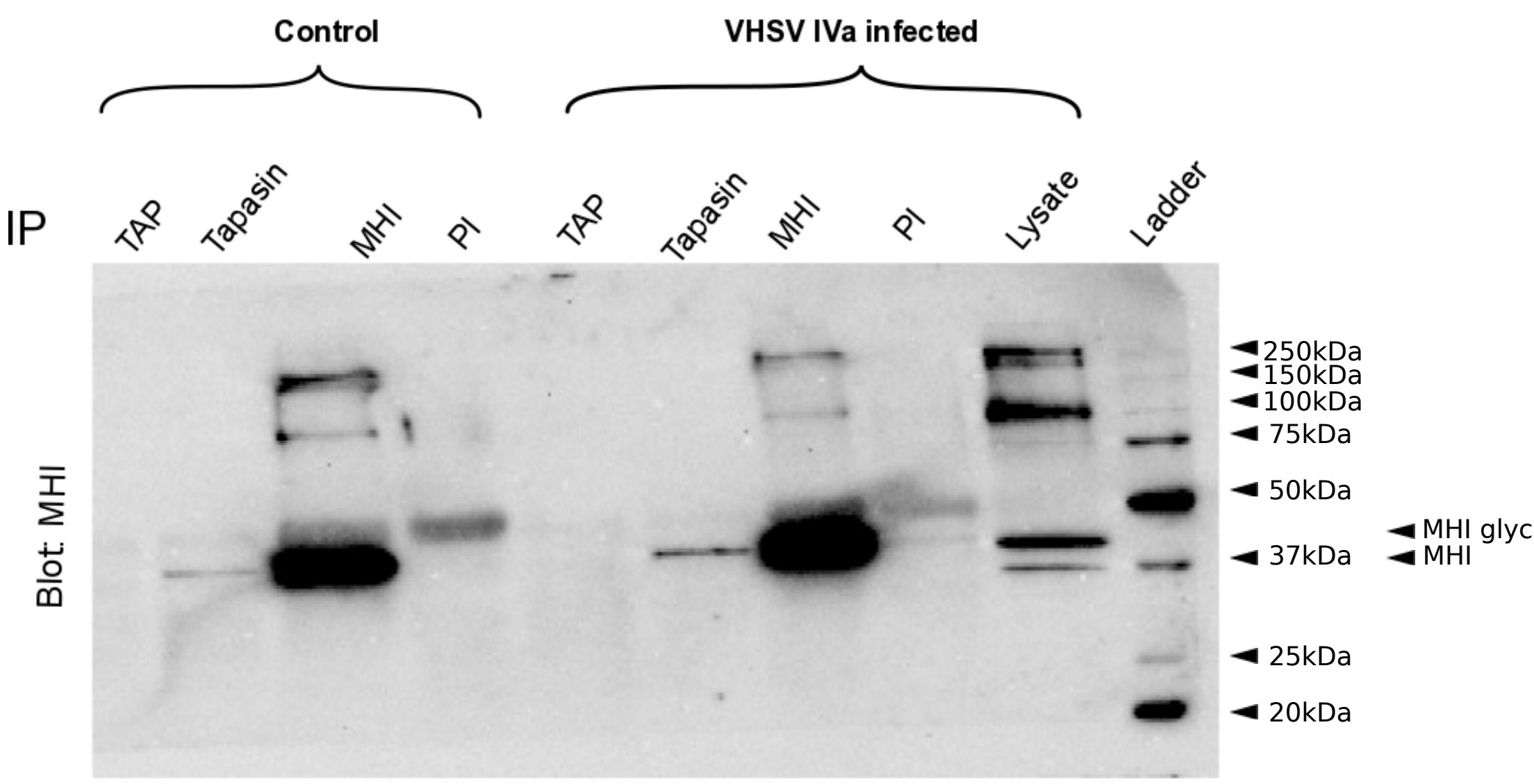
Protein name	Molecular weight	Uniprot accession#	Unique peptides#	%coverage
ERp57	55kDa	L7Z8X5	11	24
TAP1	75kDa	Q00NV7	10	20
TAP2	75kDa	NQ9PT30	2	14
Calreticulin	55kDa	Q6UD79	3	7
Tapasin	20kDa	Q3SAU8	2	7
Tapasin	55kDa	Q3SAU8	14	13

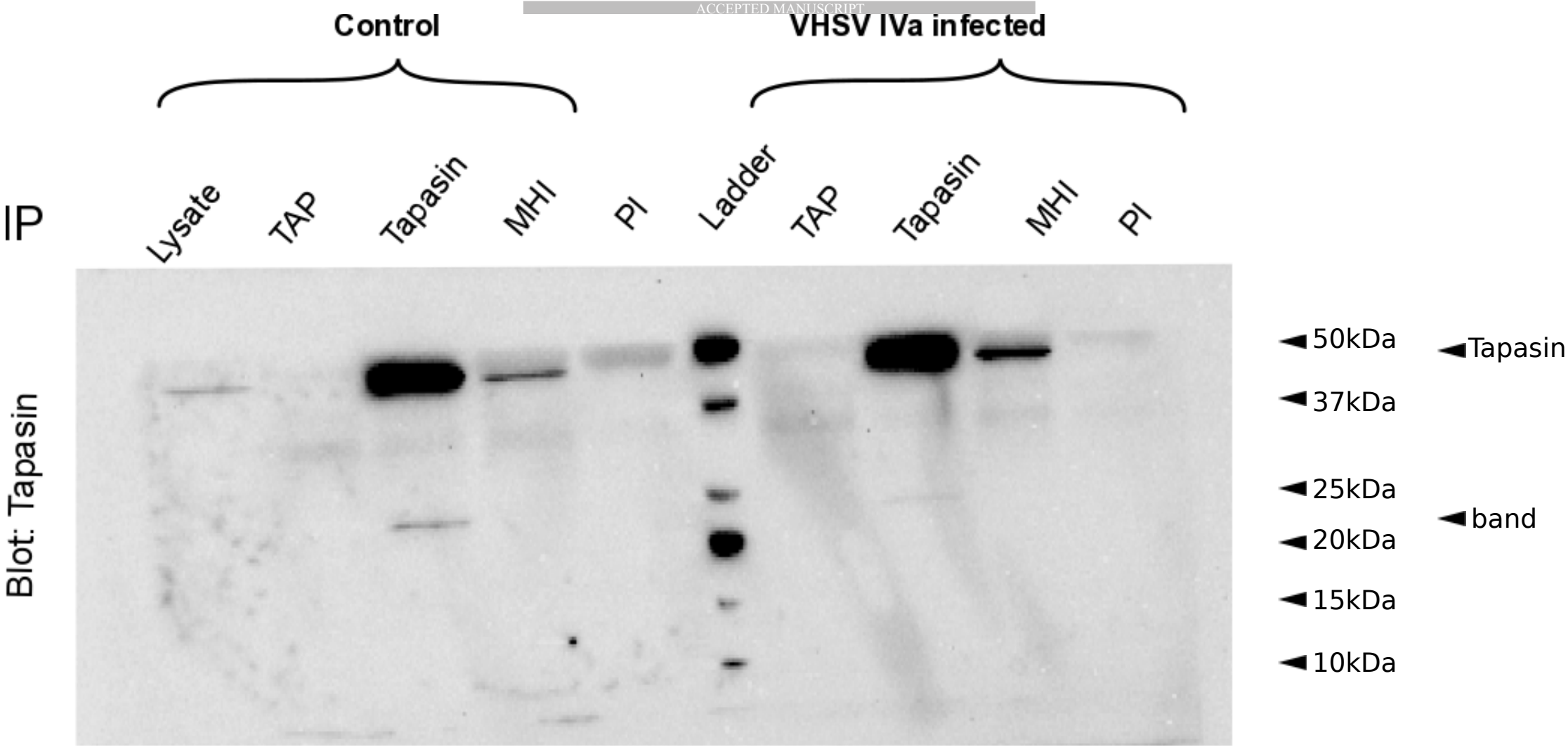


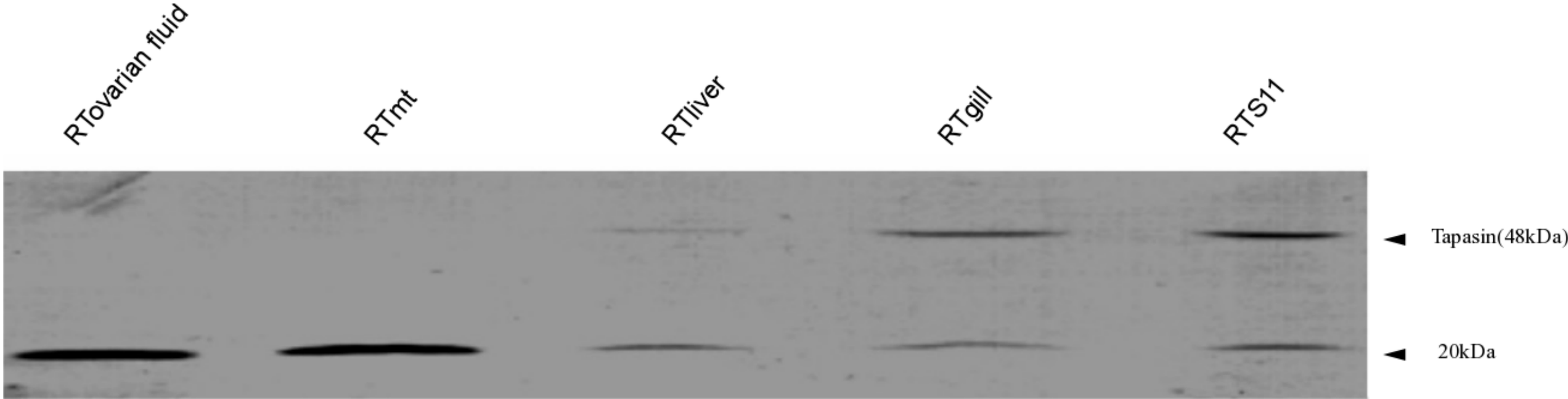


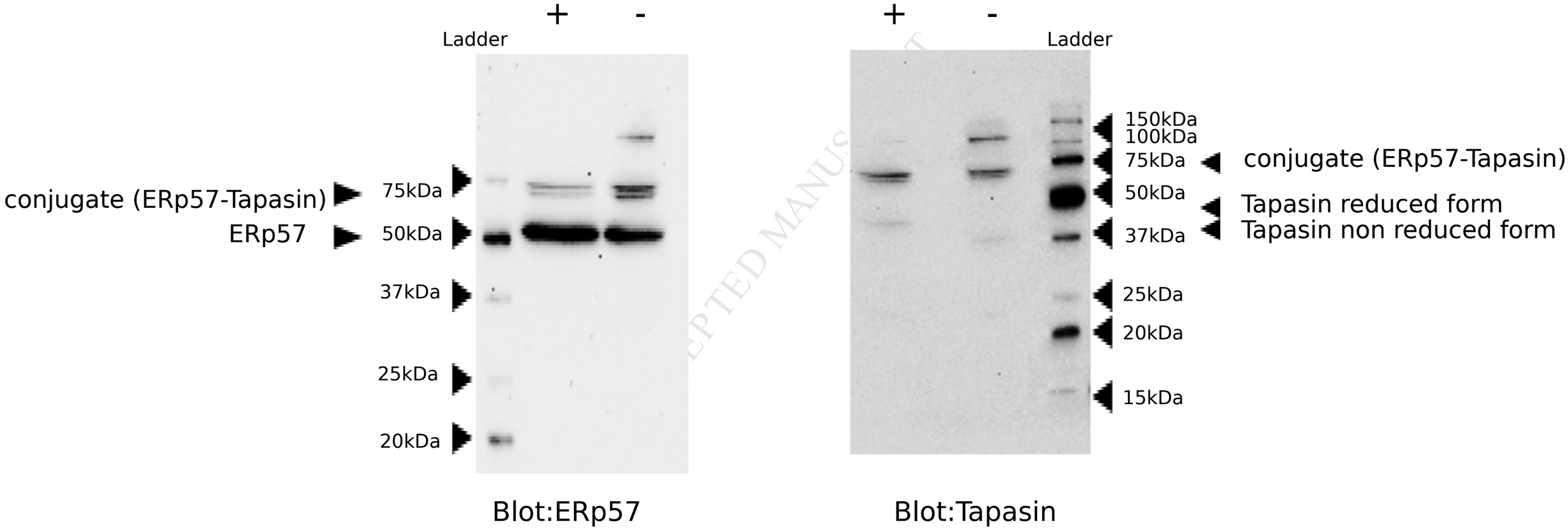





Control
VHSV IVa infected
IP
TAP
Tapasin
MHI
PI
TAP
Tapasin
MHI
PI
Lysate
Ladder
Blot: MHI
250kDa
150kDa
100kDa
75kDa
50kDa
37kDa
25kDa
20kDa
MHI glyc
MHI







Highlights

1. Trout tapasin is present in two isoforms

2. Both trout tapasin isoforms can interact with other peptide loading complex proteins

3. The smaller isoform may regulate antigen presentation by excluding MHC receptors in a dominant negative fashion