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SIGNAL PEPTIDE PEPTIDASE (SPP) ASSEMBLES WITH SUBSTRATES AND MISFOLDED MEMBRANE PROTEINS INTO DISTINCT OLIGOMERIC COMPLEXES

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Abbreviations used: SPP, signal peptide peptidase; ER, endoplasmic reticulum; pPrl, preprolactin; HCMV, human cytomegalovirus; BN-PAGE, blue native PAGE; HEK, human embryonic kidney; SP^{Prl}, signal peptide of preprolactin; SPF^{Prl}, signal peptide fragment of preprolactin; Ii, MHC class II associated invariant chain; RAMP4, ribosome-associated membrane protein 4; DDM, n-dodecyl β -D-maltoside; ERAD, ER-associated degradation;

ABSTRACT

Signal peptide peptidase (SPP) is an aspartyl intramembrane cleaving protease, which processes a subset of signal peptides, and is linked to the quality control of endoplasmic reticulum (ER) membrane proteins. We analysed SPP interactions with signal peptides and other membrane proteins by co-immunoprecipitation assays. We found that SPP specifically and tightly interacts with a large range of newly synthesized membrane proteins, including signal peptides, preproteins and misfolded membrane proteins, but not with all co-expressed type II membrane proteins. Signal peptides are trapped by the catalytically inactive SPP mutant SPP^{D/A}. Preproteins and misfolded membrane proteins interact with both, SPP and the SPP^{D/A} mutant, and are not substrates for SPP-mediated intramembrane proteolysis. Proteins interacting with SPP are found in distinct complexes of different sizes. A signal peptide is mainly trapped in a 200 kDa SPP complex, while a preprotein is predominantly found in a 600 kDa SPP complex. A misfolded membrane protein is detected in 200 kDa, 400 kDa and 600 kDa SPP complexes. We conclude that SPP does not only process signal peptides but also collects preproteins and misfolded membrane proteins that are destined for disposal.

INTRODUCTION

Signal peptide peptidase (SPP) is an intramembrane cleaving protease of the GxGD-type aspartyl protease-family [1]. These proteases are polytopic membrane proteins with their active sites located in two adjacent transmembrane domains. They promote peptide bond hydrolysis of transmembrane proteins in the plane of cellular membranes [2].

SPP is located in the endoplasmic reticulum (ER) membrane, where it functions in the processing of signal sequence-derived signal peptides [3]. Signal sequences are N-terminal extensions of nascent secretory and membrane proteins, and mediate protein targeting to the ER membrane and insertion into the membrane [4]. They typically have a tripartite structure consisting of an N-terminal region, a central hydrophobic h-region, and a C-terminal region including the cleavage site for signal peptidase [5]. Following insertion of the nascent polypeptide, signal peptidase usually cleaves off the signal sequence from the parent preprotein [6]. SPP processes some of the resulting signal peptides within their membrane-spanning h-region and thereby generates fragments that are liberated from the ER membrane [3, 7]. Some of them act as bioactive peptides, for example those derived from the signal sequences of MHC class I molecules, the hepatitis C virus polyprotein, the HIV-1 Env glycoprotein or preprolactin (pPrl) [8, 9]. In these cases, SPP-mediated processing affects diverse pathways like immune surveillance, virus maturation, or cellular signalling. All SPP substrates identified thus far are signal peptides [10, 11]. They span the ER membrane in a type II topology, exposing the N-terminus to the cytosol and the C-terminus to the exoplasm [12]. Therefore, it is assumed that SPP processes only type II oriented peptides, whereas type I oriented peptides are processed by other intramembrane proteases [11]. Signal peptides need to be released from their parent preproteins by signal peptidase cleavage to become SPP substrates, a process called ectodomain shedding [10, 13].

Besides processing signal peptides, SPP may also contribute to protein quality control at the ER membrane. Such a function was proposed from the identification of SPP as a cross-linking partner of misfolded variants of the polytopic membrane protein opsin [14]. Furthermore, SPP is implicated in the US2-dependent dislocation of MHC class I heavy chains from the ER membrane. US2 is a human cytomegalovirus (HCMV) encoded protein and forms a complex with SPP. Both proteins are required for the dislocation of full-length MHC class I heavy chains to the cytosol [15].

SPP can be isolated as a monomer and as an SDS-stable dimer [16, 17]. For proteolytic processing, SPP does not require additional cofactors since purified recombinant SPP is able to process a substrate [18]. This is in contrast to presenilin-dependent intramembrane processing. The aspartyl protease presenilin is the catalytic core unit of the gamma-secretase complex consisting of at least four subunits. All components are essential for complex assembly, substrate selection and protease function [19].

Considering the processing of signal peptides and the proposed role of SPP in the dislocation of other membrane proteins [14, 15], the question arises how SPP selects its substrates for intramembrane processing or ER membrane dislocation and degradation. In the present study, we characterized SPP interactions with potential substrates and other proteins bearing a type II oriented transmembrane domain by co-immunoprecipitation. We found that SPP specifically interacts with a large range of newly synthesised membrane proteins including a signal peptide, a preprotein and a misfolded polytopic membrane protein. Using BN-PAGE, we identified distinct SPP complexes and discovered that a substrate and a preprotein as well as a misfolded membrane protein assemble with SPP complexes of different sizes, respectively.

EXPERIMENTAL

Plasmids

SPP-myc and SPP-D265A-myc were generated by PCR using the primers 5'CCC GGG TCA TTT CTC TTT CTT CAA GTC CTC TTC AGA AAT GAG CTT TTG CTC CTC CAG CCC CTT CGA TGC and 5'-ATT TAG GTG ACA CTA TAG with pSV-Sport1-SPP and pSV-Sport1-SPP-D265A [17] as template. Thereby, a myc tag is introduced N-terminal of the KKEK ER retention signal. PCR-products were cloned into pRK5rs. pRK5rs and pRK5rs-pPrI have been described previously [20]. Starting with pRK5rs-pPrI, pRK5rs-pPrI^{PP29} was generated by site directed-mutagenesis using the primers 5' CTA CTC TTG TGC CAG GGT CCG CCT TCC ACC CCC GTC TGT CCC and 5' GGG ACA GAC GGG GGT GGA AGG CGG ACC CTG GCA CAA GAG TAG. C-terminal HA-tagged forms of pPrI and pPrI^{PP29} were generated by PCR on pRK5rs-pPrI and pRK5rs-pPrI^{PP29} using primers 5' CG CTC GAG TTA AGC GTA ATC TGG AAC ATC GTA TGG GTA GCA GTT GTT GTT GTA GAT GAT TCT GC and 5' CGA TTG AAT TCC CCG GGG ATC CTC TAG AGT CGA CCC AGC TTG and cloned into pRK5rs. pcDNA3.1-RAMP4op is derived from pGEM4Z-RAMP4/3'UTR/op [21] by PCR using the primers 5'-GAA TTC TCC ACC ATG GTC GCC AAG CAG AGG and 5'- GAA TTC AAG CTT CAG CCC GTC TTG TTG G. pSV51-Ii is described in [22]. pRK5rs-OP91H* was generated by PCR on pZEO-OP91H+(cys56) (compare [14]) using the primers 5'-TAA TAC GAC TCA CTA TAG GG and 5'- TAG AAG GCA CAG TCG AGG and cloning into pRK5rs.

Antibodies

Anti-myc and anti-opsin antibodies were prepared from 9E10 or R2-15 hybridoma cell supernatants, respectively, using standard procedures. SPP-specific rabbit antibodies (C18) recognize an epitope in amino acids 353-377 of human SPP [23]. Anti-HA and anti- α -tubulin antibodies were purchased from Covance and Sigma ImmunoChemicals, respectively. Anti-pPrI, anti-SP^{PrI} [24], anti-invariant chain, and anti-RAMP4 antibodies [22] have been described previously. Anti-mouse IgG and anti-rabbit IgG antibodies conjugated with horseradish peroxidase were obtained from Sigma.

Cell culture and transient transfection

Human embryonic kidney (HEK) 293 cells (ECACC, 85120602) were grown in Dulbecco's modified Eagle's medium/Hams F12 (Invitrogen) supplemented with 10% foetal calf serum and 2 mM L-glutamine. Cells were regularly controlled for the absence of mycoplasma using standard PCR. 3×10^5 cells were seeded in 6-well slots, used for calcium-phosphate transfection as described in [20], and harvested 44 h later.

Metabolic labelling and immunoprecipitation

Cells were depleted and metabolically labelled essentially as described in [20] and in the figure legends. They were lysed in Triton X-100-containing lysis buffer (50 mM HEPES, pH 7.5; 150 mM NaCl; 1% Triton X-100 (v/v); 10% (v/v) glycerol; 1.5 mM MgCl₂; 1 mM EGTA, pH 8; 1 mM PMSF; Complete protease inhibitor cocktail [Roche]) or in digitonin-containing lysis buffer (1% digitonin; 20 mM Tris/Cl, pH 7.5; 1.5 mM MgCl₂; 1 mM EGTA; 50 mM NaCl, 10% (v/v) glycerol; and protease inhibitors as described above). Non-solubilised material was removed by centrifugation (16000 x g, 10 min, 4 °C). When indicated, 1/10 of the lysate was directly mixed with SDS-sample buffer (for Tris/Tricine gels: 4% SDS; 75 mM Tris/Cl, pH 6.8; 12% (v/v) glycerol; 0.01% Serva Blue G; 50 mM DTT; for Tris/Glycine gels: 2% SDS; 50 mM Tris/Cl, pH 6.8; 10% (v/v) glycerol; 0.01% bromphenolblue; 50 mM DTT). For immunoprecipitation, the cell lysate was diluted in HNTG low salt buffer (20 mM HEPES, pH 7.5; 150 mM NaCl; 0.1% (v/v) Triton X-100; 10% (v/v) glycerol) or in TNDG buffer (20 mM Tris/Cl, pH 7.5; 50 mM NaCl; 0.1% digitonin; 10% glycerol;). Antibodies and either Protein A-sepharose or Protein G-sepharose beads (GE Healthcare) were added for 3 h at 4°C. Beads were washed five times with HNTG high salt buffer (HNTG buffer with 500 mM NaCl instead of 150 mM) or with TNDG buffer. Samples were heated at 65 °C for 10 min and separated by Tris/Tricine or Tris/Glycine SDS-PAGE according to Schägger and Laemmli, respectively [25, 26]. Dried gels were analysed by PhosphorImaging (Fuji BAS 1500).

Western blotting

Cells were lysed and proteins separated as described above. Then, proteins were transferred onto membranes in a semi-dry blotting unit. Blocking and first antibody incubation was done in 0.25 % gelatin in TBS-T (50 mM Tris/Cl, pH 7.5; 150 mM NaCl; 5 mM EDTA, pH 8; 0.05% (v/v) Triton X-100), washing and secondary antibody incubation was done in TBS-T. For immunodetection, BM chemiluminescence blotting substrate (POD, Roche Applied Science) was used. In case of re-probing the membranes with anti-tubulin antibodies, the membranes were stripped with 62.5 mM Tris/Cl, pH 6.7; 2% SDS and freshly added 0.5% (v/v) β -mercaptoethanol for 30 min at 50 °C.

Blue Native (BN)-PAGE

Cells were lysed in BN-lysis buffer (see above digitonin-containing lysis buffer but containing 1% of detergents as indicated in the figure legends). Non-solubilised material was removed by centrifugation at 16000 x g for 10 min at 4 °C. 1/40 volume of BN-sample buffer (500 mM 6-aminohexanoic acid; 100 mM BisTris, pH 7; 5% Coomassie G250) was added to the lysate before subjection to BN-PAGE [27]. Samples were loaded onto 5 to 9%, 6 to 13%, or 6 to 16% gradient gels (with 67 mM 6-aminohexanoic acid and 50 mM BisTris, pH 7) and run at 4 °C for 1 h at 15 mA with cathode buffer B (50 mM Tricine, pH 7; 15 mM BisTris; 0.02% Coomassie G250). Then, the cathode buffer was exchanged for cathode buffer B/10 (buffer B but only 0.002% Coomassie G250) and gels were run at 100 V (anode buffer: 50 mM BisTris, pH 7). As molecular marker, the high molecular weight kit for native electrophoresis (GE Healthcare) was used. For Western blotting, BN-gels were soaked in Tris/Glycine-SDS-electrophoresis buffer [26], then in BN-transfer buffer (25 mM Tris; 150 mM glycine; 0.02% SDS; 20% (v/v) methanol) and proteins were transferred via semi-dry blotting onto PVDF membranes (Millipore). Membranes were destained with methanol and immunodetection was performed as described above.

2D-BN/SDS-PAGE

Lanes from the BN-gel were cut, incubated in SDS-sample buffer for 30 min, and placed into a SDS-PAGE. The gel run and the following Western blotting were performed according to standard protocols as described above.

Affinity purification of SPP complexes and mass spectrometry

To purify SPP^{D/A}-myc containing complexes, anti-myc antibodies were coupled to CnBr-Sepharose beads (GE Healthcare) according to the manufacturer's instructions. Cells transfected with pRK5rs-SPP^{D/A}-myc and pRK5rs-Prl or mock-transfected cells were lysed in BN-lysis buffer containing 1% digitonin. Beads were equilibrated in the same buffer, mixed with the lysates, and incubated for 16 h at 4°C. Non-bound material was removed by washing the beads four times with BN-lysis buffer, and bound material was eluted by competition with synthetic myc-peptide (Sigma) for 2 h at 4°C. Fractions of each step were mixed with BN-sample buffer and subjected to BN-PAGE. The gel was silver stained [28]. For LC/MSMS analysis, the band containing the 200 kDa complex was cut out from the gel together with a reference region from a lysate of mock-transfected cells.

Densitometric analyses of protein bands

Densitometric analysis was done with ImageJ. Each signal, represented by a defined area, was corrected for background by subtracting a signal from an identical area within the same lane. For statistical analyses, relative amounts were calculated, *i.e.* signals from each time point were adjusted to 100%. Relative amounts are given in percent as a mean of three independent experiments with standard deviation.

RESULTS

The SPP substrate SP^{Prl} tightly interacts with SPP^{D/A}

To characterize the interaction of SPP with potential substrates and with other integral membrane proteins, we employed co-immunoprecipitation of metabolically labelled proteins. As model substrate we used the preprolactin (pPrl) derived signal peptide, SP^{Prl} (Fig. 1B), which previously was shown to be processed by SPP in an *in vitro* translation/translocation system [3, 7]. To generate SP^{Prl} in cells, we overexpressed pPrl. Additionally, we expressed myc-tagged SPP or the catalytically inactive SPP mutant SPP-D265A, here termed SPP^{D/A}, which is known to lack proteolytic activity (Fig. 1A, [1, 29]).

HEK293 cells transiently expressing pPrl alone or together with either SPP or SPP^{D/A} were metabolically labelled and then lysed either directly or chased for 1 hour. To detect SP^{Prl} by immunoprecipitation, we used an antibody recognizing an epitope in the n-region of the pPrl signal sequence (anti-SP^{Prl}), which had been characterised previously [24]. Aliquots of total lysates (Fig. 1C, lanes 1-8) and the immunoprecipitated proteins (lanes 9-16) were separated by Tris/Tricine-SDS-PAGE, which allows a high resolution of small proteins. Finally, labelled proteins were visualised by autoradiography. When pPrl alone was expressed, the 3 kDa SP^{Prl} and its smaller fragment, SP^{F^{Prl}}, were detected after pulse labelling in the immunoprecipitate (lane 10) but not any more after 1 h chase (lane 14). This indicates that SP^{Prl} also in cells is processed and that the resulting fragment SP^{F^{Prl}} has a rather short half-life. After co-expression with SPP, no SP^{Prl} and only small amounts of SP^{F^{Prl}} were found (lanes 11 and 15), suggesting that overexpressed SPP increases the rate of processing and leads to a rapid degradation of SP^{F^{Prl}}. When SPP^{D/A} was co-expressed with pPrl, SP^{Prl} accumulated (lanes 4 and 12) and was stabilized over time (lanes 8 and 16). Thus, SPP^{D/A} can efficiently prevent SP^{Prl} processing and degradation. The anti-SP^{Prl} antibody did not efficiently immunoprecipitate SP^{Prl} under these native conditions (lanes 12 and 16 compared to 4 and 8). This may indicate that under these conditions SP^{Prl} is not accessible for the antibody.

To see whether SP^{Prl} interacts with SPP^{D/A}, we performed co-immunoprecipitation using the anti-myc antibody. This antibody recognizes myc-tagged SPP and SPP^{D/A}, which are detected in the SDS-gel as monomers and dimers (Fig. 1C, lanes 17-24). Similar amounts of SPP and SPP^{D/A} were immunoprecipitated (lanes 19, 20, 23, 24) and could be detected after Western Blotting (Fig. S1), demonstrating similar expression levels of both proteins. SP^{Prl} was efficiently co-immunoprecipitated with SPP^{D/A} (lanes 20, 24 and Fig. S2).

In order to test whether SP^{Prl} also interacts with an SPP-like (SPPL) protease, pPrl was co-expressed with the ER-localized SPPL3 [30] and its catalytically inactive mutant SPPL3-D271A, here termed SPPL3^{D/A} (Fig. S3). No SP^{Prl} accumulated in presence of SPPL3^{D/A} and no co-immunoprecipitation of SP^{Prl} and SPPL3^{D/A} was detected. This suggests that SPP^{D/A} cannot be functionally replaced by SPPL3^{D/A}. Taken together, we show that SP^{Prl} is processed by SPP in cells and that the catalytically inactive mutant SPP^{D/A} is an efficient dominant-negative mutant that tightly interacts with SP^{Prl}.

pPrl interacts with SPP and is no substrate for SPP-mediated processing

In addition to SP^{Prl}, we observed the specific co-immunoprecipitation of a 25 kDa protein with SPP and SPP^{D/A} (Fig. 1C, right panel and Fig. S2). As this protein could be immunoprecipitated with an anti-prolactin antibody [24] but is about 3 kDa larger than prolactin it must be preprolactin (Fig. 1D). When pPrl alone was expressed, mainly prolactin of about 22 kDa was immunoprecipitated and only a small amount of pPrl accumulated (Fig. 1D, lanes 2 and 8), indicating that pPrl is efficiently inserted into ER membranes where its signal sequence is cleaved off. By contrast, higher amounts of pPrl accumulated in presence of co-expressed SPP or SPP^{D/A} (lanes 5, 6, 11, 12) and were co-immunoprecipitated (lanes 17, 18 and 23, 24). This suggests that overexpression of SPP increases the amount of the preprotein pPrl at the expense of processed prolactin. pPrl interacts with SPP^{D/A} and catalytically active SPP, accumulates and is not processed with time. This strongly indicates that it is not a substrate for SPP. This is consistent with the earlier finding that cleavage by signal peptidase is required for intramembrane proteolysis by SPP [10].

SPP does not interact with the type II oriented membrane proteins Ii or RAMP4

Signal peptides, like their preproteins, span the ER membrane in a type II orientation, exposing the N-terminus towards the cytosolic side of the membrane [12]. In order to investigate whether other type

II oriented membrane proteins that comprise non-cleaved signal sequences also interact with SPP or SPP^{D/A}, we tested the MHC class II associated invariant chain (Ii) and the ribosome-associated membrane protein 4 (RAMP4). Ii is a signal-anchor glycoprotein with an N-terminal transmembrane domain [31], while RAMP4 is a tail-anchored membrane protein, which is inserted in the ER membrane via its C-terminal transmembrane domain [21, 32]. To detect RAMP4 membrane insertion, an opsin-tag encoding a glycosylation site has been appended to its C-terminus (Fig. 2A and [21]).

In transfected cells, Ii was immunoprecipitated mainly as a bi-glycosylated protein of 33 kDa (Fig. 2B, lane 2). Similar amounts of Ii were detected upon co-expression with SPP or SPP^{D/A} but Ii was not co-immunoprecipitated with SPP or SPP^{D/A} (lanes 11 and 12). As no smaller forms of Ii accumulated in the presence of SPP or SPP^{D/A}, this indicates that Ii is neither a SPP substrate nor a binding partner (lanes 5 and 6).

RAMP4op was immunoprecipitated as a 12 kDa glycoprotein as previously characterised (Fig. 2C, lane 2; [21]). In addition, endogenous RAMP4 of 8 kDa was immunoprecipitated by the anti-RAMP4 antibody (compare lanes 1 and 2). The co-expression of SPP and SPP^{D/A} did not affect the amount of RAMP4op nor endogenous RAMP4. No co-immunoprecipitation of RAMP4op with overexpressed SPP or SPP^{D/A} was found (lanes 11 and 12). Together, these data indicate that Ii and RAMP4 are neither SPP-substrates nor interact with co-expressed SPPs. Furthermore, we conclude that not all type II oriented membrane proteins interact with SPP/SPP^{D/A}.

SPP interacts with misfolded opsin

Previously, Crawshaw *et al.* have shown that SPP can be cross-linked to a misassembled transmembrane domain of a truncated mutant of polytopic opsin. This protein-protein interaction could be prevented by addition of an SPP-inhibitor [14]. Here, we characterized this interaction in our cell-based assay using co-immunoprecipitation. We used OP91H*, a truncated opsin mutant that spans the membrane twice (type I and type II oriented transmembrane domains) and can be bi-glycosylated in the N-terminal region (Fig. 3A and B, lanes 2-4, [14]).

OP91H* was immunoprecipitated in its non-, mono-, and bi-glycosylated forms after pulse labelling as previously characterised (Fig. 3B, lane 2; [14]). Similar amounts of OP91H* were immunoprecipitated upon co-expression with SPP or SPP^{D/A}. Additionally, SPP and SPP^{D/A} co-immunoprecipitated with OP91H* (lanes 3 and 4). Employing the anti-myc antibody for immunoprecipitation revealed that a small portion of OP91H* was non-specifically immunoprecipitated in the absence of myc-tagged SPP (lane 6). However, increased amounts OP91H* specifically co-immunoprecipitated with co-expressed SPP and SPP^{D/A} (lanes 7 and 8). Taken together, these data indicate that both SPP and SPP^{D/A} tightly interact with a newly synthesised misfolded membrane protein.

SPP impedes the degradation of misfolded opsin

Truncated and misfolded opsin mutants are retained in the ER membrane where they do not assemble correctly but can aggregate or undergo ER-associated degradation (ERAD) [33, 34]. To test whether co-expression of SPP affects the degradation of OP91H*, we pulse-chase labelled cells expressing either OP91H* alone or together with SPP or SPP^{D/A}.

OP91H* was substantially degraded after 6 h, when expressed alone (Fig. 4A, lanes 1-4 and quantification in B). By contrast, the co-expression of either SPP or SPP^{D/A} resulted in a longer half-life of OP91H* indicating that its degradation was significantly impeded (Fig. 4A, lanes 5-6 and 9-12, and quantifications in B, respectively). Accumulation was in particular found for the bi-glycosylated OP91H* suggesting that SPP-bound OP91H* was still competent for glycosylation. The stabilizing effect of co-expressed SPP/ SPP^{D/A} on OP91H* could also be confirmed under steady state conditions as depicted by the Western blot in Figure 4C. Together, these results show that the misfolded membrane protein is stabilized in presence of high SPP amounts. Since co-expression of both, SPP and SPP^{D/A}, leads to similar observations, we conclude that the stabilisation is independent of the catalytic SPP activity.

SPP is found in distinct high molecular weight complexes

Our co-immunoprecipitation data revealed that SPP interacts with a range of membrane proteins including substrates as well as preproteins and misfolded opsins, which are not SPP substrates. In

order to further characterize the SPP complexes, we analysed them by Blue Native (BN)-PAGE. First, we tested whether SPP, which has been characterized under denaturing conditions as a monomer of 40 kDa and as an SDS-stable dimer of 90 kDa (Fig. 5A and [16]) is detectable under native conditions in higher molecular weight complexes. Mock-transfected cells expressing endogenous SPP and transfected cells expressing SPP-myc were solubilised by three different detergents (1% digitonin, 1% Triton X-100, and 1% n-dodecyl β -D-maltoside [DDM]), and proteins separated by BN-PAGE (Fig. 5B). Using an SPP-specific antibody we detected endogenous (lanes 1, 3, 6) and overexpressed SPP complexes after Western blotting (lanes 2, 4, 6). Both migrated as a major complex of about 200 kDa (C200). Additionally, complexes of about 400 kDa (C400) and 600 kDa (C600) were detected. Endogenous SPP was detected in one further complex of about 250 kDa. All these complexes were detected after solubilisation with the three detergents indicating that they are rather stable.

A signal peptide is trapped in a 200 kDa SPP complex

Since SP^{Prl} accumulates in the presence of SPP^{D/A} and can be identified by Western blotting (Fig. 6A, lane 4), we next used BN-PAGE to identify SP^{Prl}/SPP^{D/A} complexes. Upon Western blotting, the anti-SP^{Prl} antibody recognized its epitope in a 200 kDa complex, when pPrl was co-expressed with SPP^{D/A} (Fig. 6B, lanes 1-4). SPP^{D/A} complexes were identified with the anti-myc antibody, which recognized the above-described complexes C200, C400 and C600 (lane 5). The 200 kDa SP^{Prl} complex co-migrates with the SPP^{D/A}-C200, suggesting that SP^{Prl} is trapped in this SPP complex. SP^{Prl} could also be detected in this complex, when proteins were solubilised in the presence of Triton X-100 or DDM, indicating that the SP^{Prl}/SPP^{D/A}-C200 is stable under different detergent conditions (Fig. S4).

To identify the protein components of SP^{Prl}/SPP^{D/A}-C200, we affinity purified SPP^{D/A} complexes on anti-myc antibodies. Figure 6C shows the purification profile and elution of the three SPP complexes on a silver-stained BN-gel and after Western blotting (lanes 1-8). SP^{Prl} could be identified as a component of purified SPP^{D/A}-C200 (lane 9). Next, mass spectrometry was used to identify polypeptides within the three SPP complexes. In the C200 complex, three different peptide sequences derived from SPP were obtained. Other peptide sequences were non-specifically found in the SPP complexes since they were also found in samples obtained after a mock-purification. Together these data suggest that SPP is the main, possibly the sole, constituent of the C200 complex. We also attempted to identify the protein constituents of the SPP-C400 and C600 complexes. However, no peptide sequences were obtained by mass spectrometric analysis most likely due to the small amounts.

pPrl is found in a 600 kDa SPP complex

In addition to SP^{Prl} that is trapped by SPP^{D/A}, pPrl tightly interacts with SPP^{D/A} and SPP (compare Fig. 1). Therefore, we also identified pPrl/SPP complexes by BN-PAGE. Since only small amounts of pPrl accumulated in cells when pPrl was co-expressed with SPP or SPP^{D/A} (Fig. 7B, lanes 1-4), we generated the pPrl mutant pPrl^{PP29} that is not cleaved by signal peptidase and thus leads to the accumulation of pPrl (Fig. 7A and B, lanes 5-7, [10, 13]).

After BN-PAGE and Western blotting, the anti-SP^{Prl} antibody recognized its epitope in a 200 kDa complex when wild-type pPrl was co-expressed with SPP^{D/A} (lanes 8-14). However, we could not detect pPrl^{PP29} complexes after BN-PAGE and Western blotting. Most likely the corresponding epitope was not available after blotting from the BN-gel. To circumvent this problem, we separated the complexes in a second dimension by SDS-PAGE, termed 2D-BN/SDS-PAGE (Fig. 7C). When HA-tagged pPrl^{PP29} was co-expressed with SPP^{D/A}, it was detected as a 30 kDa protein after the second dimension SDS-PAGE (lower panel). SPP migrated as 90 kDa dimers (middle panel) indicating an even-numbered stoichiometry of SPP in the complexes. pPrl^{PP29} co-migrates with the large SPP-C600 complex strongly indicating that it is present in this complex. As a control, a similar analysis applying wild-type pPrl showed that mature prolactin is not incorporated into higher molecular weight complexes but migrated in a range below 60 kDa according to the first dimension BN-PAGE (data not shown). We conclude, that SP^{Prl} and its preprotein assemble into SPP complexes of different sizes. While SP^{Prl} is trapped in C200, the preprotein predominantly accumulates in C600.

Misfolded opsin accumulates in three distinct SPP complexes

As misfolded opsin OP91H* also interacts with SPP and SPP^{D/A} (Fig. 3), we next analysed OP91H*/SPP complexes by BN-PAGE. Lysates of cells expressing OP91H* alone or together with

SPP or SPP^{D/A} were analysed by BN-PAGE and Western blotting (Fig. 8A). Upon co-expression with SPP and SPP^{D/A}, OP91H* is found to co-localize with the three SPP complexes C200, C400 and C600 (compare lanes 3 and 4 with 6 and 7). Very low amounts of the OP91H*/SPP complexes were detected in lysates from cells expressing OP91H* alone (lane 2 and compare Fig. S5 for long exposure). This again corroborates the strong stabilizing effect of SPP on OP91H* as found after pulse-chase labelling and immunoprecipitation (compare Fig. 4).

OP91H* has two N-glycosylation sites and is detected as a non-, mono-, and bi-glycosylated form after SDS-PAGE (Fig. 3 and 4). In order to test whether the glycosylation status determines the assembly into one particular SPP complex, we analyzed OP91H*/SPP and OP91H*/SPP^{D/A} complexes by 2D-BN/SDS-PAGE (Fig. 8B-D). When OP91H* was expressed alone, glycosylated and non-glycosylated forms of OP91H* were mainly detected in high molecular weight complexes of about 600 kDa after second dimension SDS-PAGE (Fig. 8B). When co-expressed with SPP or SPP^{D/A}, glycosylated and non-glycosylated OP91H* forms were detected to similar extent in the 200, 400, and 600 kDa complexes (Fig. 8C and D), indicating that the glycosylation status does not determine OP91H* assembly into one particular SPP complex.

DISCUSSION

SPP tightly interacts with a signal peptide and a range of membrane proteins

We have investigated SPP interactions with a set of membrane proteins with type II oriented transmembrane regions. The prolactin (pPrI)-derived signal peptide, SP^{PrI}, is an SPP substrate and tightly interacts with the catalytically inactive SPP mutant SPP^{D/A}. The parent preprotein pPrI as well as the misfolded opsin OP91H* also interact with SPP^{D/A} and furthermore with catalytically active SPP. Since pPrI and OP91H* accumulate in the presence of catalytically active SPP and no smaller processing fragments were detected, these proteins are not substrates for SPP-mediated processing. They bind to SPP but are not processed and are in the following termed SPP client proteins. No interaction of the type II membrane proteins Ii and RAMP4op with SPP was detected. We conclude that SPP interacts with a range of membrane proteins that is not restricted to substrates, but it does not interact with all type II membrane proteins.

The question arises what are the features that distinguish proteins that bind to SPP from those that do not. A preprotein, like prolactin, accumulates because its signal sequence has not been cleaved off by signal peptidase. It remains anchored in the ER membrane via its signal sequence resulting in a type II oriented membrane protein. SPP co-expression enhances the accumulation of the preprotein pPrI and both also tightly interact with each other. The most likely explanation is that SPP is in close proximity to the translocation site in the ER membrane and optimally placed to compete with signal peptidase for binding to the signal sequence in the newly synthesised preprotein. Such an SPP binding may then prevent signal peptidase to cleave. Consistent with this model, SPP is known to interact with the translocon-associated TRAM1 protein [35]. A signal peptide and its parent preprotein are only distinguished by the absence or presence of the luminal ectodomain of the preprotein. Since mature prolactin did not interact with SPP, it must be the signal sequence that mediates the interaction with SPP. Although preceding signal sequence cleavage is required to allow SPP-mediated processing of the signal peptide, as demonstrated previously [10], a luminal ectodomain does not generally prevent an interaction with SPP. Similarly, additional transmembrane domains, like in OP91H* do not prevent an SPP interaction.

In its overall topology Ii resembles a preprotein that spans the membrane with its signal anchor sequence. However, no interaction between SPP and Ii has been detected. Since a luminal domain in principle does not abolish an SPP interaction, there may be intrinsic features in the transmembrane domain required for SPP interaction. RAMP4 and a signal peptide are similar in their overall topology. Both expose their N-terminal domains in the cytosol, span the membrane with their hydrophobic domains and have a small C-terminal domain in the ER lumen. In contrast to a signal peptide, RAMP4 does not interact with SPP. Taken together, a type II topology and a small C-terminal domain in the ER lumen are not sufficient for an interaction with SPP but a large luminal domain, as well as additional transmembrane domains do not prevent an SPP interaction. It appears that not one single and simple feature determines whether a protein interacts with SPP. Since all proteins that interact with SPP have a transmembrane domain, there might be intrinsic features in this domain that are essential for an SPP interaction. Possibly, the structural arrangement of the transmembrane domains determines the accessibility to SPP. Truncated opsin proteins were shown to have a less compact structure and a reduced alpha-helical content compared to the correctly assembled wild type opsin [36]. Thus, misfolded proteins that are not correctly assembled may expose their hydrophobic domains that are in turn accessible to SPP. Similarly, preproteins are usually generated as by-products of an inefficient process, so that one can assume them to remain unassembled and destined for disposal. In contrast, Ii and RAMP4 are known to oligomerise with their physiological interaction partners. Ii rapidly assembles into a homo-oligomeric complex upon membrane insertion [37], while RAMP4 associates with the Sec61 translocon and ribosomes [38]. Consequently, both proteins are presumably not accessible to SPP and therefore do not interact. In conclusion, we propose that the oligomeric state of type II oriented membrane proteins may be one critical determinant for their accessibility to SPP.

SPP assembles with substrates and client proteins into complexes of different sizes

Using BN-PAGE, we identified endogenous and overexpressed SPPs in three distinct high molecular weight complexes of 200, 400, and 600 kDa (C200, C400 and C600). A substrate, i.e. a signal peptide, is trapped in SPP-C200 when SPP^{D/A} was co-expressed, while its preprotein is predominantly

found in SPP-C600. In contrast, a misfolded membrane protein was identified in all three SPP complexes.

Since the substrate is only detected in the SPP-C200 complex, we suggest that this is the catalytically active SPP complex. Besides SPP we did not find any other protein in this complex consistent with the fact, that SPP alone is sufficient to process signal peptides [1, 18]. The 200 kDa complex is probably a dimer of SPP-dimers since we detect dimers after 2D-BN/SDS-PAGE and since one SPP dimer has a molecular weight of about 90 kDa [16]. In contrast, SPP client proteins accumulate predominantly in the large SPP complexes C400 and C600. The presence of these complexes correlates with the finding that the interacting proteins are not substrates. As the amount of these complexes was small after affinity purification, we could not identify their protein constituents by mass spectrometry. However, we found SPP dimers by 2D-BN/SDS-PAGE and Western blotting indicating that this is the minimal SPP-core in the large complexes. The higher mass suggests that either SPP-dimers homo-oligomerise into larger complexes or that SPP-dimers associate with further proteins.

Taken together, our findings indicate that substrates and client proteins can interact with SPP and are segregated by SPP. Both, SPP substrates as well as its clients, initially may bind to a docking site in SPP. Substrates may then enter the active site and are processed, while other proteins may be excluded from this site due to sterical hindrance by their ectodomains. This model could apply for a signal peptide and its preprotein. As the amino acid sequences of the signal peptide and the signal sequence are identical, it must be the large luminal ectodomain of the preprotein that prevents processing [10, 39]. Similarly, also misfolded polytopic membrane proteins might be sterically hindered to enter the active site as they contain more than one transmembrane domain. The model of distinct docking and active sites has previously been deduced from the finding that a helical peptide inhibitor binds to another SPP-site than a transition-state analogue [40]. Moreover, this concept also implies that a signal peptide, which entered the SPP active site, may be buried inside the protease. In contrast, when a preprotein or a misfolded polytopic membrane protein are bound to SPP, their additional domains, which are the luminal domain and the transmembrane domain, respectively, may stick out of the SPP molecule enabling further interactions and accumulation in larger SPP complexes. In these complexes, SPP itself or additional co-factors could contribute accessory binding sites. Since we identified all SPP complexes under steady-state conditions after Western blotting, we do not know how they are generated. It may be a dynamic process in which initially all SPP interacting proteins bind the SPP-C200 homo-oligomer and then, if not processed, mature into larger complexes. Alternatively, all SPP complexes could pre-exist and recruit preferentially signal peptides or unassembled membrane proteins with type II oriented transmembrane domains.

Also for the SPP-like proteases, SPPL2a and SPPL2b, an interaction with substrates and the corresponding precursors has been shown [41-43]. As observed for SPP, the precursors interact with active SPPLs and accumulate. In contrast to this, no interaction of precursors with presenilin as the catalytic core of the gamma-secretase complex has been observed so far [44, 45]. One explanation is that gamma-secretase is a multi-protein complex that consists of four subunits [19]. The catalytic core subunit is presenilin, an aspartyl intramembrane protease related to SPP, while a further subunit pre-selects peptide substrates and transfers them to the docking site in presenilin [46]. The characterisation of gamma-secretase by BN-PAGE identified heterogeneous complexes ranging in size from 200 to 900 kDa but in contrast to SPP, always included the mentioned subunits [47, 48].

In contrast to gamma-secretase, SPP functions may be characterised by a broad range of clients. Besides processing of signal peptides, SPP may collect disposal-prone membrane proteins. The interaction of SPP with clients that are prone to disposal is consistent with the proposed role of SPP in ER quality control of membrane proteins [14]. In such a scenario, SPP may shield unassembled, exposed transmembrane regions. The SPP complexes we detect are possibly disposal-intermediates that represent part of a platform at which proteins are dislocated from the ER membrane [49]. In fact, SPP-dependent protein dislocation of full-length MHC class I heavy chains from the ER membrane has previously been demonstrated in cells expressing the HCMV-encoded immunoevasin US2 [15]. Furthermore, a recent study demonstrates that SPP co-operates with the ubiquitin E3 ligase TRC8 in the dislocation of MHC class I molecules from the ER membrane [50].

ER-associated degradation includes substrate recognition, targeting to the dislocation-machinery, dislocation from the ER membrane, and degradation of the substrate by the proteasome [51]. Misfolded opsin proteins can be retained in the ER and can undergo ER-associated degradation *via*

the proteasome [34]. We show that misfolded opsin OP91H* is substantially degraded when overexpressed in cells. An interaction of misfolded opsin with endogenous SPP has been demonstrated previously [14]. However, co-expression of SPP impedes OP91H* degradation resulting in accumulation. This apparent discrepancy can be explained by the overexpression situation. It is conceivable that high SPP amounts result in a prolonged interaction with degradation substrates thereby impeding their efficient degradation. We attempted to clarify whether reduced SPP levels in the cell may affect the degradation of misfolded proteins by employing RNA interference. Substantial reduction of SPP did not lead to impeded degradation of OP91H* (Fig. S6). This could mean that SPP is not necessary for the degradation of OP91H or that low SPP amounts in the cell are sufficient for SPP function.

In an alternative model, SPP may act in the collection of disposal-prone membrane proteins by co-aggregating with them. Misfolded membrane proteins that expose hydrophobic domains tend to aggregate in the cell in an uncontrolled manner and can even recruit correctly folded proteins into aggregates, which in turn may lead to cell toxicity [34]. SPP with its ability to tightly bind such membrane proteins and to assemble with them into large oligomeric complexes, possibly aggregates, may contribute to a controlled sequestering of toxic membrane protein species. Large protein aggregates may then be eliminated by autophagic mechanisms.

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FIGURE LEGENDS

Figure 1 The effect of SPP and SPP^{D/A} on SP^{Prl} and pPrl processing and binding.

(A) Schematic representation of signal peptide peptidase (SPP) in the ER membrane with amino acids of the active site motif in one-letter code. The D to A mutation in SPP^{D/A} and position of the myc-tag are indicated. KKEK: ER retrieval motif. Fork-like structures indicate N-linked glycosylation sites. (B) Outline and amino acid sequence of the signal sequence of preprolactin (pPrl) with its N-terminal (n), hydrophobic (h), and C-terminal (c) region. The cleavage site for signal peptidase (SPase) is indicated and the h-region is underlined. (C) *SP^{Prl} is trapped by the dominant-negative mutant SPP^{D/A}.* HEK293 cells transiently expressing pPrl, SPP-myc, or SPP^{D/A}-myc, were metabolically pulse labelled for 30 minutes and chased for 1 hour when indicated. Cells were lysed in presence of 1% Triton X-100. 1/10 of the total lysate was directly analyzed by SDS-PAGE and the rest was used for immunoprecipitation (IP) using either anti-SP^{Prl} antibodies or anti-myc antibodies to identify myc-tagged SPP/SPP^{D/A}. Samples were subjected to Tris/Tricine SDS-PAGE and labelled proteins were visualized by autoradiography; v: empty vector control; SP^{Prl}: signal peptide fragment of SP^{Prl}; Prl: mature prolactin; SPP-myc dimer (di) and monomer (mo) are indicated; Note that the monomer is detected as double-band, representing non-glycosylated and glycosylated SPP (data not shown). (D) *pPrl co-immunoprecipitates with SPP and SPP^{D/A}.* HEK392 cells were transfected and pulse-chase labelled as described in C. To detect pPrl and mature prolactin (Prl) anti-Prl antibodies were used for immunoprecipitation. Anti-myc antibodies were used for the identification of myc tagged SPP/SPP^{D/A} and for co-immunoprecipitation. Proteins were separated by Tris/Glycine SDS-PAGE and visualised as described above.

Figure 2 SPP does not associate with the type II membrane proteins invariant chain and RAMP4op.

(A) Membrane disposition of a signal peptide (SP^{Prl}), invariant chain of MHC class II molecules (Ii), and opsin-tagged RAMP4 (RAMP4op). Fork like structures indicate N-linked glycosylation sites. (B)+(C) Invariant chain (B) and RAMP4op (C) were transiently expressed alone or together with SPP-myc or SPP^{D/A}-myc, pulse labelled for 30 minutes, and proteins immunoprecipitated (IP) with anti-Ii (B), anti-RAMP4 (C), or anti-myc antibodies (B, C). Proteins were separated by Tris/Glycine SDS-PAGE (B) or Tris/Tricine SDS-PAGE (C) and visualised by autoradiography; v: empty vector control; RAMP4: endogenous RAMP4; RAMP4op: non-glycosylated RAMP4op; RAMP4op-g: glycosylated RAMP4op; SPP dimer (di) and monomer (mo) are indicated.

Figure 3 SPP interacts with misfolded opsin.

(A) Membrane disposition of OP91H* in comparison to a signal peptide (SP^{Prl}). Fork like structures indicate N-linked glycosylation sites. (B) *OP91H* co-immunoprecipitates with SPP and SPP^{D/A}.* HEK293 cells transiently expressing OP91H*, SPP-myc or SPP^{D/A}-myc were pulse labelled for 30 minutes, lysed with 1% digitonin and immunoprecipitated (IP) with anti-opsin antibodies (lanes 1-4), or anti-myc antibodies (lanes 5-8). Proteins were separated by Tris/Glycine SDS-PAGE and visualised by autoradiography. v: empty vector control. -1g and -2g indicate mono- and bi-glycosylated opsin forms, respectively. SPP dimer (di) and monomer (mo) are indicated.

Figure 4 SPP impedes the degradation of misfolded opsin.

(A) *Pulse-chase analysis of OP91H*.* HEK293 cells transiently expressing OP91H* in absence or presence of SPP-myc or SPP^{D/A}-myc were pulse labelled for 10 min and chased for the indicated times. Digitonin-lysates were used for immunoprecipitation with the anti-opsin antibody. v: empty vector control. -1g and -2g indicate mono- and bi-glycosylated opsin forms, respectively. SPP dimer (di) and monomer (mo) are indicated. (B) *Quantification of OP91H* accumulation.* Three independent experiments as shown in (A) were used for the quantification of OP91H* accumulation in absence (left panel) or in presence of SPP-myc (middle panel) or SPP^{D/A}-myc (right panel), respectively. The OP91H* amount after the pulse (0 h chase) was set to 100%. The relative mean values of three independent experiments are given and error bars indicate the standard deviation. (C) *Western blotting shows OP91H* accumulation in presence of SPP.* HEK293 cells transiently expressing OP91H* and SPP-myc or SPP^{D/A}-myc as indicated were lysed with 1% digitonin and aliquots separated by Tris/Glycine SDS-PAGE. OP91H* and SPP/SPP^{D/A}-myc proteins were

identified by Western blotting (WB) using anti-opsin (left panel) and anti-myc antibodies (right panel), respectively. Membranes were stripped and re-probed with anti-tubulin antibodies as a loading control (lower panels).

Figure 5 Endogenous and overexpressed SPP is found in distinct high molecular weight complexes after BN-PAGE.

(A) After SDS-PAGE SPP is detected as monomer and SDS-stable dimer. HEK293 cells transiently expressing SPP-myc were lysed with digitonin (Dig). Proteins were separated by SDS-PAGE and SPP monomers (mo) and dimers (di) identified by Western blotting (WB) using anti-myc antibodies. v: empty vector control. (B) Detection of SPP complexes after BN-PAGE. HEK293 cells transiently expressing SPP-myc or transfected with an empty vector (v), were lysed with digitonin (Dig), Triton X-100 (Tx) or n-dodecyl β -D-maltoside (DDM). Total lysates were subjected to blue native (BN)-PAGE (5-9 %). Endogenous and overexpressed SPP complexes of 200 kDa (C200), 400 kDa (C400), and 600 kDa (C600) were then identified by Western blotting (WB) using an SPP-specific antibody.

Figure 6 Characterisation of SP^{Prl}/SPP complexes.

(A) Steady state accumulation of SP^{Prl} in the presence of SPP^{D/A}. HEK293 cells transiently expressing pPrl alone or together with SPP-myc or SPP^{D/A}-myc were lysed in 1% Triton X-100 buffer and proteins separated by Tris/Tricine SDS-PAGE. SP^{Prl} was identified upon SPP^{D/A}-myc co-expression by Western blotting (WB) using the anti-SP^{Prl} antibody. (B) SP^{Prl} is trapped in a 200 kDa SPP-complex. Digitonin-lysates of HEK293 cells expressing pPrl, SPP-myc, or SPP^{D/A}-myc as indicated were separated by BN-PAGE (6-13%). SP^{Prl}-containing complexes were identified by Western blotting (WB) using an anti-SP^{Prl} antibody. SPP^{D/A}-myc complexes were detected with an anti-myc antibody (lane 5). The position of the 200 kDa SP^{Prl}/SPP^{D/A}-C200 complex and higher molecular weight SPP complexes are indicated. (C) Affinity purification of SP^{Prl}/SPP complexes. HEK293 cells co-expressing pPrl and SPP^{D/A}-myc were lysed with 1 % digitonin and SPP^{D/A}-myc complexes were affinity purified on anti-myc beads. Aliquots of the total lysate, flow-through (FT), wash fraction (wash), and eluted fractions (eluate) were characterized by BN-PAGE (5-9 %) and silver staining (lanes 1-7) or by Western blotting (WB) with an anti-myc antibody (lane 8) and an anti-SP^{Prl} antibody (lane 9).

Figure 7 Identification of pPrl/SPP complexes.

(A) Amino acid sequence of the signal sequence of wild-type pPrl and the mutant pPrl^{PP29}, which is not cleaved by signal peptidase (SPase) (both are C-terminally HA-tagged). The V to P mutation (PP29) is underlined. h-regions are indicated with a dotted line. (B) Detection of mature prolactin (Prl), pPrl^{PP29} and SP^{Prl} after SDS-PAGE or BN-PAGE. HEK293 cells transiently expressing pPrl-HA or pPrl^{PP29}-HA alone or together with SPP-myc or SPP^{D/A}-myc were lysed with 1% digitonin and total lysates were subjected to Tris/Glycine SDS-PAGE (lanes 1-7) or to BN-PAGE (6-16%; lanes 8-14). Mature prolactin (Prl-HA) and pPrl^{PP29}-HA were identified after SDS-PAGE by Western blotting (WB) using anti-HA antibodies and SP^{Prl} was identified after BN-PAGE and Western blotting using anti-SP^{Prl} antibodies. v: empty vector control. (C) Identification of pPrl^{PP29} in the 600 kDa SPP complex after 2D-BN/SDS-PAGE. HEK293 cells transiently co-expressing pPrl^{PP29}-HA and SPP^{D/A}-myc were lysed with 1% digitonin and the total lysate was first separated by BN-PAGE (6-13%) and then in the second dimension by SDS-PAGE (12,5%). SPP^{D/A}-myc complexes and pPrl^{PP29}-HA were identified by Western blotting using the anti-myc and the anti-HA antibody, respectively. SPP-dimers (di) and the pPrl^{PP29}/SPP-C600 are indicated. The star indicates a cross-reacting band.

Figure 8 Misfolded opsin accumulates in several SPP complexes.

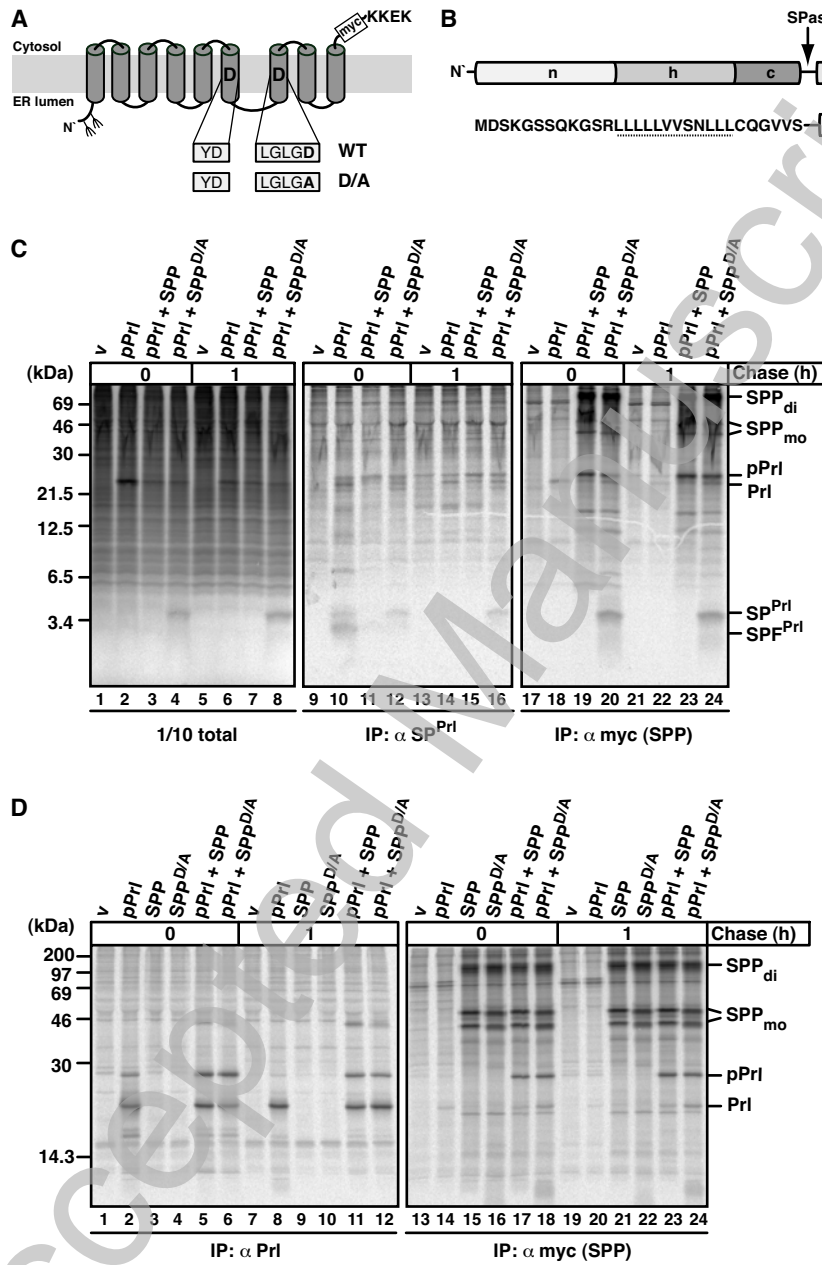
(A) Identification of OP91H*/SPP complexes after BN-PAGE. HEK293 cells transiently expressing OP91H* and SPP-myc or SPP^{D/A}-myc as indicated were lysed with 1% digitonin and aliquots separated by BN-PAGE (5-9%). OP91H* and SPP/SPP^{D/A}-myc complexes were identified by Western blotting (WB) using anti-opsin and anti-myc antibodies, respectively. SPP complexes of 200 kDa (SPP-C200), 400 kDa (SPP-C400), and 600 kDa (SPP-C600) co-migrating with OP91H* are indicated. (B)-(D) Glycosylated and non-glycosylated opsin is found in complexes of the same size. Digitonin-lysates of HEK293 cells transiently expressing OP91H* (B) or co-expressing SPP-myc (C) or SPP^{D/A}-myc (D) were first separated by BN-PAGE (6-13%) and then separated in the second

dimension by SDS-PAGE (15%). Upper panels show Western blots (WB) of the BN-gels using anti-opsin antibodies. Middle panels show Western blots of the upper part of the second dimension SDS-gels using anti-SPP antibodies in B and anti-myc antibodies in C and D. Lower panels show Western blots of the lower part of the second dimension gel using anti-opsin antibodies. SPP dimers (di) and non-, mono- (-1g) and bi- (-2g) glycosylated forms of OP91H* are indicated.

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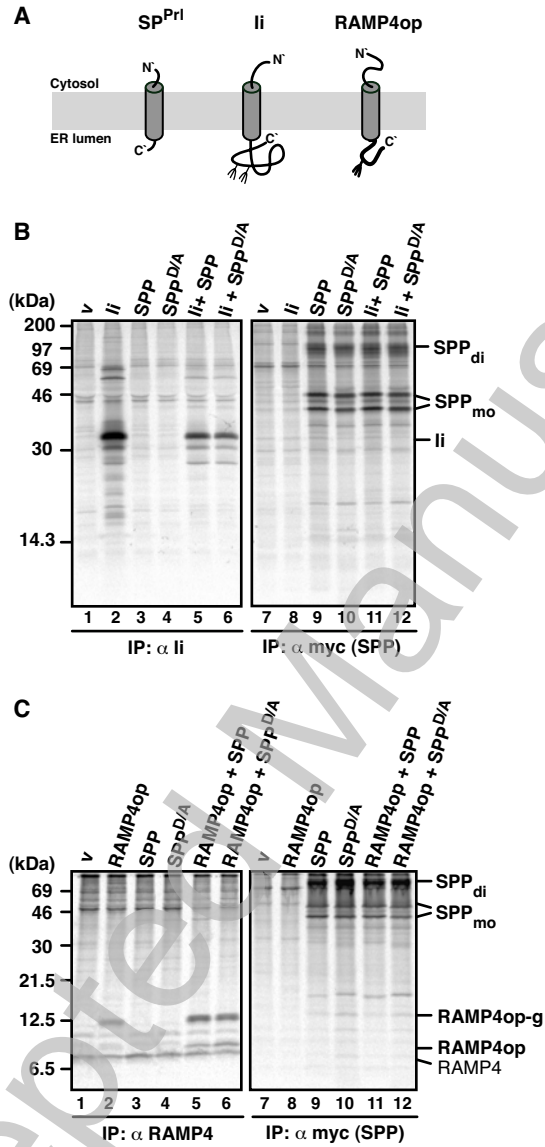
Figure 1, Schrul et al.



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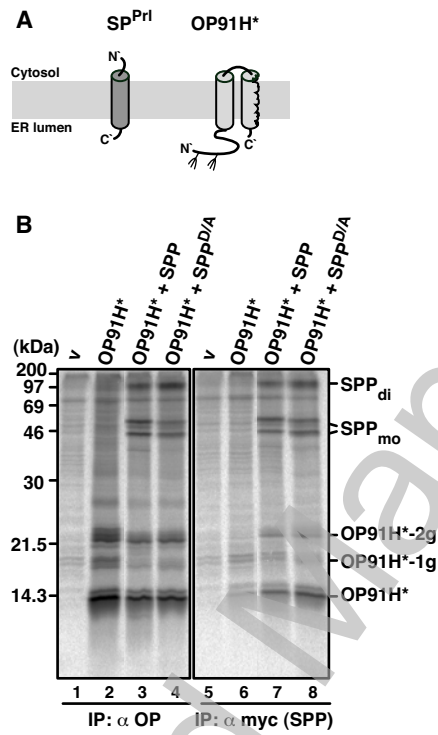
Figure 2, Schrul et al.



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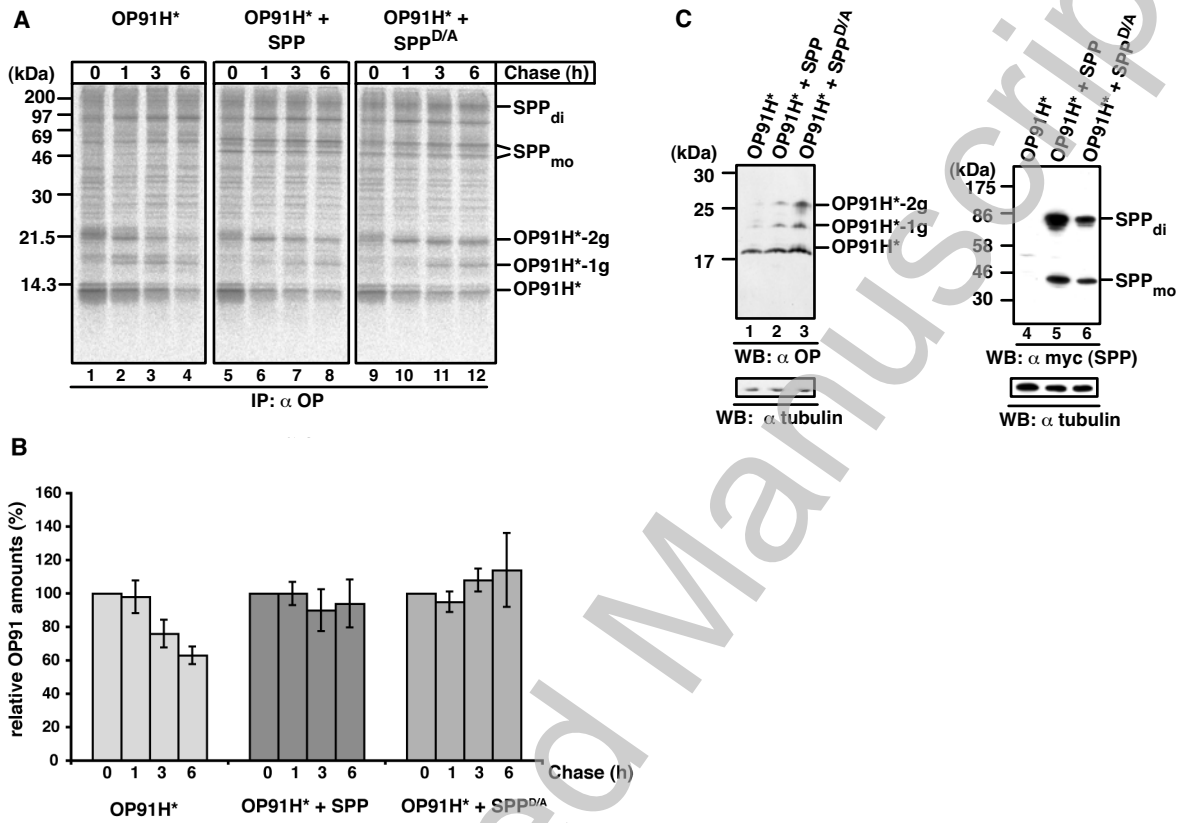
Figure 3, Schrul et al.



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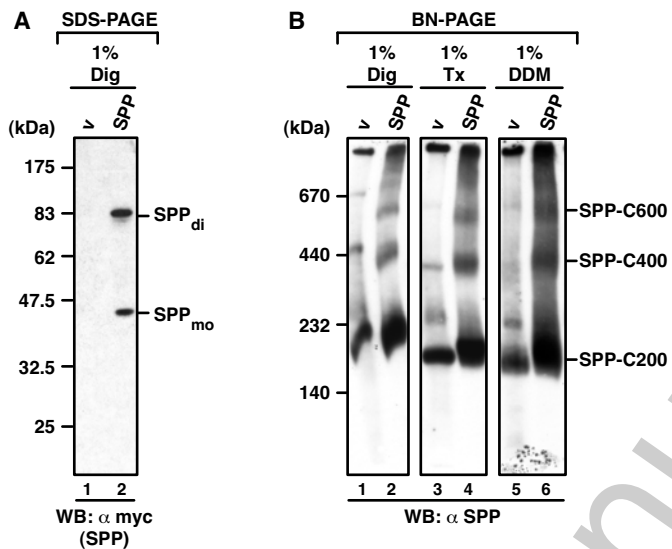
Figure 4, Schrul et al.



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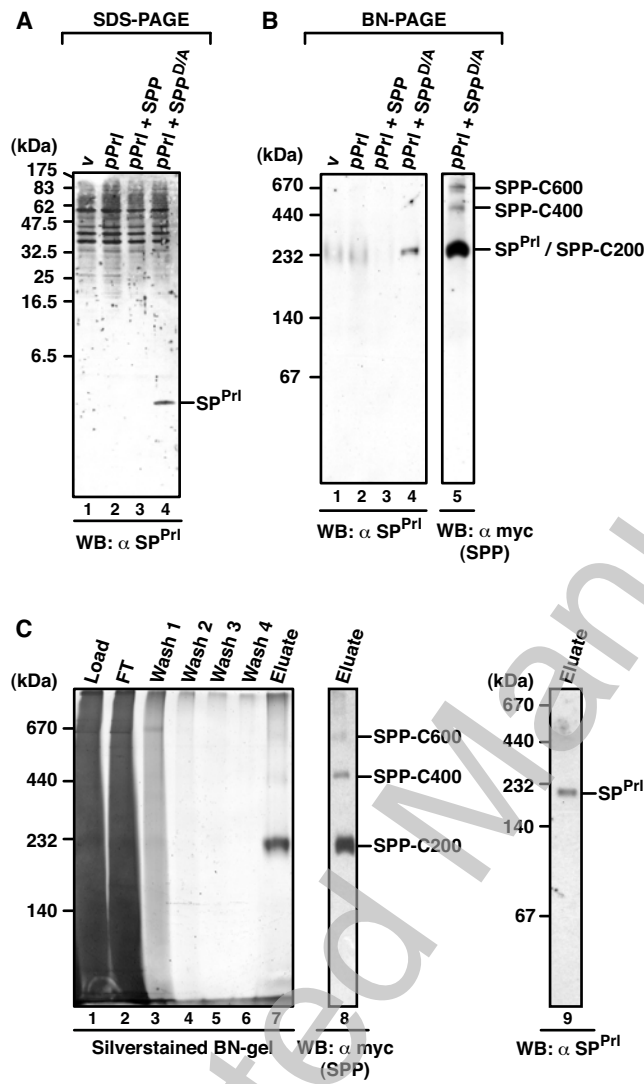
Figure 5, Schrul et al.



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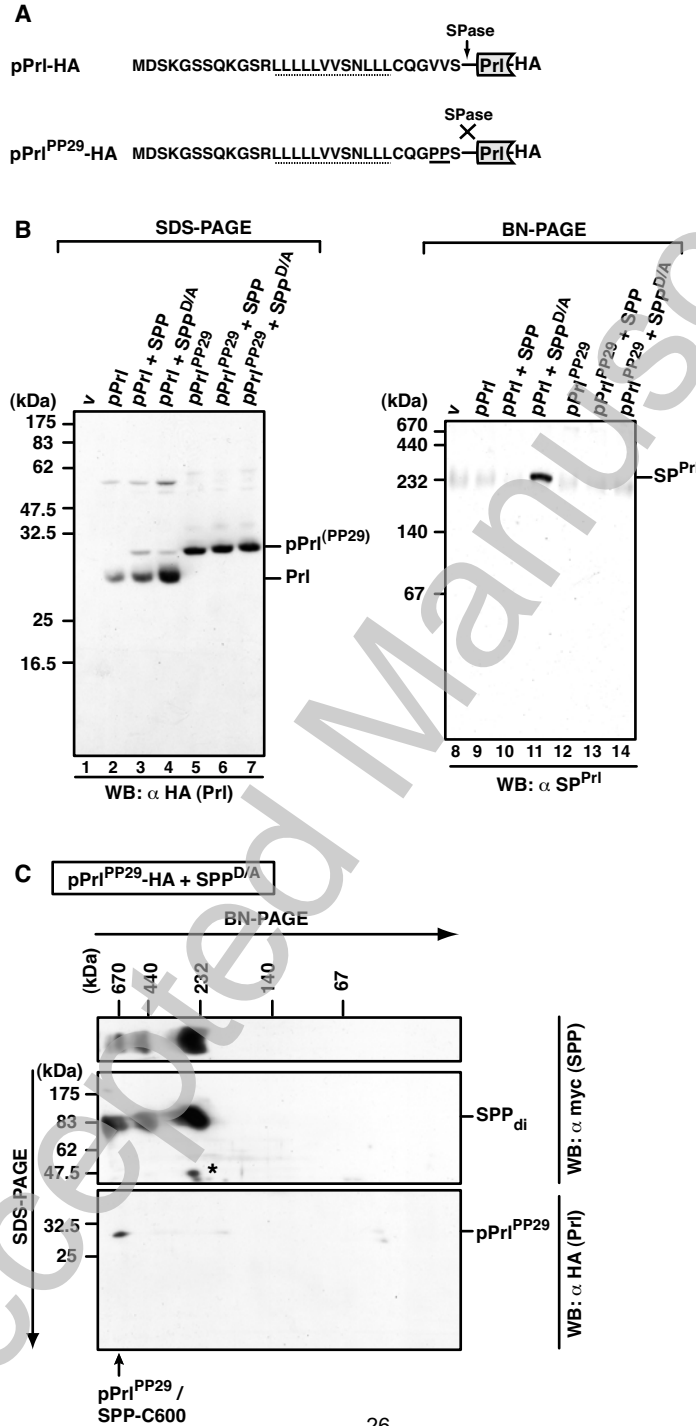
Figure 6, Schrul et al.



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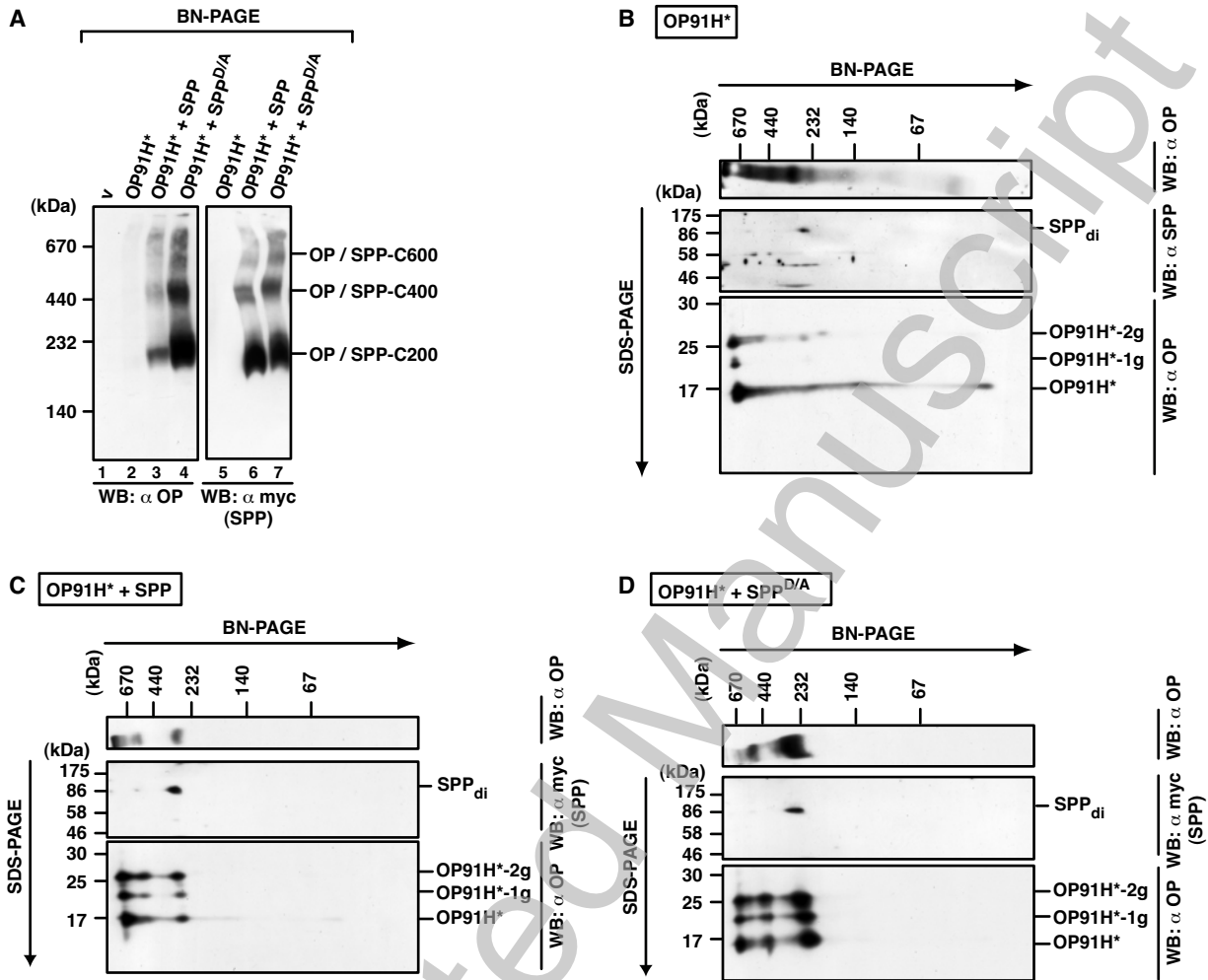
Figure 7, Schrul et al.



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Figure 8, Schrul et al.



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