A misassembled transmembrane domain of a polytopic protein associates with signal peptide peptidase

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The endoplasmic reticulum (ER) exerts a quality control over newly synthesized proteins and a variety of components have been implicated in the specific recognition of aberrant or misfolded polypeptides. We have exploited a site-specific cross-linking approach to search for novel ER components that may specifically recognize the misassembled transmembrane domains present in truncated polytopic proteins. We find that a single probe located in the transmembrane domain of a truncated opsin fragment is cross-linked to several ER proteins. These components are distinct from subunits of the Sec61 complex and represent a ‘post-trans-

locon’ environment. In this study, we identify one of these post-translocon cross-linking partners as the signal peptide peptidase (SPP). We find that the interaction of truncated opsin chains with SPP is mediated by its second transmembrane domain, and propose that this interaction may contribute to the recognition of misassembled transmembrane domains during membrane protein quality control at the ER.

Key words: endoplasmic reticulum, membrane protein, opsin, quality control, signal peptide peptidase (SPP).

INTRODUCTION

The endoplasmic reticulum (ER) is a major site of protein synthesis in eukaryotes, providing secretory and membrane proteins for the compartments of the secretory pathway, the plasma membrane and the extra-cellular milieu. The ER possesses a translocation machinery and a complex set of molecular chaperones and folding factors to facilitate the synthesis of these proteins [1]. In addition, the newly synthesized proteins are audited by a process known as quality control where correctly folded proteins and fully assembled complexes are distinguished from terminally misfolded species and unassembled protein subunits [2]. Such misfolded/unassembled polypeptides are normally prevented from exiting the ER and eventually subjected to ER-associated degradation (ERAD) [3]. This process requires the retrotranslocation of the misfolded proteins from the ER lumen to the cytoplasm via a pathway that involves a potentially novel channel for ER export [4,5] and the cytoplasmic AAA ATPase p97 [6], and ends with delivery to the proteasome for degradation [7]. Whilst our understanding of the ER components that enforce quality control over soluble proteins is extensive [2,8], less is known about the recognition of aberrant membrane proteins [4,5,9–11].

In this study we used a well-characterized seven transmembrane (TM) domain protein, bovine opsin [12,13], as a model aberrant membrane protein. Opsin is a G protein-coupled receptor and, when correctly folded and assembled, the wild-type protein is transported to the cell surface in the mammalian retina where, after it is covalently attached to 11-*cis*-retinal, it forms the photoreceptor rhodopsin [14]. In humans, numerous opsin mutations have been discovered [15] that result in trafficking defects such as ER retention and degradation of the misfolded protein, leading to eventual blindness. Clinically relevant mutations occur throughout the opsin gene, some leading to truncations of the opsin protein, whilst others result in single amino acid substi-

tutions [15]. Opsin is also well suited to *in vitro* studies [16,17] providing a suitable vehicle for analysing components that may mediate ER quality control [see 18].

In many cases, studies of membrane proteins have focused on mutations in hydrophilic regions located in the ER lumen or the cytosol, and have identified similar components to those recognizing misfolded secretory proteins in the ER lumen [19,20], or defined a role for cytosolic chaperones in the ER quality control process [20,21]. In other examples, a key feature of membrane protein quality control is the recognition of misfolded or misassembled TM domains [11,22]. In order to identify ER components capable of recognizing such features, we have utilized a site-specific cross-linking approach to identify proteins associated with the TM domains of truncated opsin chains.

We identify the presenilin-related intramembrane-cleaving aspartic protease, signal peptide peptidase (SPP) [23], as a major cross-linking partner of unassembled opsin fragments and show that the interaction is mediated by the second of opsin’s seven TM domains (OPTM2). The association of opsin with SPP is prevented by the SPP inhibitor (Z-LL)₂-ketone [24], suggesting a functional association. We speculate that SPP may contribute to the quality control of misassembled polytopic membrane proteins. This may involve the recognition and delivery of misfolded proteins to other facilitators of quality control, or cleavage within their hydrophobic TM regions.

EXPERIMENTAL

Reagents

The cross-linking reagent bismaleimido-hexane (BMH) was purchased from Pierce and Warriner (Chester, U.K.). Restriction endonucleases, endoglycosidase H (EndoH), peptide:*N*-glycosidase F (PNGaseF) and m⁷G(5')ppp(5')G cap analogue were from New

Abbreviations used: BMH, bismaleimido-hexane; EndoH, endoglycosidase H; ER, endoplasmic reticulum; ERAD, ER-associated degradation; HA, haemagglutinin; OPΔCHO, bovine opsin lacking sites for *N*-linked glycosylation; OP[cys56], bovine opsin with a single cysteine at residue 56; PNGaseF, peptide:*N*-glycosidase F; SPP, signal peptide peptidase; TM, transmembrane.

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England Biolabs (Herts, U.K.). T7 RNA polymerase, transcription reagents and rabbit reticulocyte lysate were supplied by Promega (Southampton, U.K.). Easytag L-[³⁵S]methionine was purchased from NEN Dupont (Stevenage, U.K.). Reagents for cell culture were obtained from Invitrogen (Paisley, U.K.), whilst all other chemicals were purchased from BDH/Merck (Poole, U.K.) and Sigma (Poole, U.K.). The monoclonal antibody specific for the N-terminus of bovine opsin was a gift from Dr Paul Hargrave (Department of Ophthalmology, University of Florida, U.S.A.) [25].

Opsin-derived constructs

The versions of bovine opsin with a single cysteine at residue 56 (OP[cys56]) and lacking sites for *N*-linked glycosylation (OPΔ-CHO) are as described previously [17]. Templates for the transcription of truncated opsin mRNAs were prepared by PCR [16], forward primers were located 160 bases 5' of the RNA polymerase promoter, whilst reverse primers were designed to generate truncations encoding the N-terminal 91 and 150 amino acids of opsin. No stop codon was present in the mRNAs synthesized, so that the resulting polypeptides remain attached to the ribosome unless released by treatment with puromycin [16,17,26]. Reverse primers incorporating the haemagglutinin (HA) epitope tag at the C-terminus of the resulting polypeptides were also designed to generate opsin-derived chains with a length of 80, 91, 109, 150, 190 and 348 amino acids. A reverse primer was also used to add the peptide sequence PMQNATKYG (one-letter amino-acid symbols) from residue 108 of OPΔCHO, generating the construct OP117 [cys56,asn112] with a novel glycosylation site near its C-terminus. PCR products were purified directly from the reaction mixture using the QIAquick PCR purification kit (Qiagen, Crawley, U.K.).

Transcription, translation and cross-linking

Transcriptions were carried out using T7 RNA polymerase as described by the manufacturer (Promega) and the RNA obtained was purified from the reaction mixture using the RNeasy RNA purification kit (Qiagen) before use in translation reactions. Cultured HT-1080 fibroblasts (ATCC CCL-121, American Type Culture Collection, Rockville, MD, U.S.A.) were semi-permeabilized with the detergent digitonin (Calbiochem, Nottingham, U.K.) as described previously [27] and used to provide a source of ER derived membranes. The RNA was translated in a rabbit reticulocyte lysate system (Promega) for 15 min at 30 °C in the presence of [³⁵S]methionine and semi-permeabilized HT-1080 cells. Subsequently, aurintricarboxylic acid was added (100 μM final concentration) to inhibit translation initiation and 10 min later translation was terminated by the addition of cycloheximide to a final concentration of 2 mM. Where nascent chains were released from the ribosome prior to cross-linking, samples were treated with 2 mM puromycin and 50 mM EDTA for 10 min at 30 °C in place of the cycloheximide treatment. Where appropriate, (Z-LL)₂-ketone [24] was diluted in the rabbit reticulocyte lysate prior to the addition of RNA.

The membrane-associated integration intermediates, or the membrane-associated polypeptides resulting from puromycin/EDTA treatment, were recovered from the translation mix by centrifugation for 10 s at 16 000 *g* and the resulting membrane fraction washed twice by resuspension in KHM buffer (110 mM potassium acetate, 2 mM magnesium acetate, 20 mM Hepes, pH 7.2). The resulting membrane pellet was resuspended in KHM and the cross-linking reagent BMH added to a final concentration of 1 mM. BMH cross-links adjacent proteins via the -SH groups of available cysteines. Samples were incubated at 30 °C for 10 min and the cross-linking reaction quenched by the addition of

Figure 1 Cross-linking partners of OP91[cys56]

A 91-residue-long integration intermediate of opsin with a single cysteine at residue 56 (OP91[cys56]) was synthesized in a rabbit reticulocyte lysate translation system in the presence of semi-permeabilized HT-1080 cells. The membrane-associated radiolabelled polypeptides were isolated and treated with either BMH (lanes 2–4 and 6–8) or a DMSO solvent control (lanes 1 and 5). When the integration intermediate was stabilized by cycloheximide treatment, three major BMH-dependent, cross-linking products were observed (compare lanes 1 and 2). Two of these were shown to be adducts with Sec61α (lane 3, band α) and Sec61β (lane 4, band β) by immunoprecipitation. A third adduct contained the radiolabelled nascent chain and both Sec61α and Sec61β (lanes 3 and 4, ×). Thus, after treatment with cycloheximide (CHX) the ribosome-bound nascent chain is adjacent to components of the ER translocon [17]. Treatment with puromycin (PURO) releases the nascent chain from the ribosome, enabling complete integration and exit from the Sec61 translocon as judged by cross-linking (lanes 7 and 8). BMH treatment of the ribosome-released chains results in a number of novel adducts (lane 6, adducts labelled a–k in order of increasing mobility). All opsin-derived products were immunoprecipitated with a monoclonal antibody [17], and the locations of the *N*-glycosylated (OP91.2CHO) and non-glycosylated (OP91) polypeptides are shown. In the construct cartoons for all figures, cysteine probes are represented by a star, *N*-glycosylated residues are represented by branched structures and hydrophobic transmembrane regions are represented by zig-zag lines.

0.1 vol. of 100 mM 2-mercaptoethanol and incubation on ice for 10 min. As observed previously [16,17], the truncated integration intermediates were correctly membrane inserted, and efficient glycosylation of the asparagine residues at positions 2 and 15 of the N-termini of the various polypeptides was seen (e.g. Figure 1).

Immunoprecipitation and EndoH treatment

Denaturing immunoprecipitations were performed by heating the quenched, cross-linked, samples for 30 min at 37 °C in the presence of 1% SDS. Four volumes of Triton IP buffer (10 mM Tris/HCl, pH 7.6, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100) were then added and the samples were incubated on ice for approx. 30 min, followed by centrifugation at 16 000 *g* for 5 min. Aliquots

of the resulting supernatant were gently agitated overnight at 4 °C with the relevant antisera in the presence of 200 µg/ml PMSF and 1 mM methionine. Protein A–Sepharose, preincubated with 20 % BSA for 30 min and then washed five times with IP buffer, was added and the incubation continued for 2 h. Protein A–Sepharose-bound material was isolated by centrifugation at 16 000 *g* for 1 min, washed four times with IP buffer and then heated to 37 °C for 30 min in SDS/PAGE sample buffer. Where used, EndoH cleavage of high mannose forms of *N*-linked glycans from the cross-linking products was carried out after immunoprecipitation, by adding 500 units of EndoH and the appropriate enzyme buffers (New England Biolabs) to the Protein A–Sepharose beads, and incubating for 30 min at 37 °C prior to the addition of an equal volume of 2 × SDS/PAGE sample buffer.

Sample analysis

Cross-linking products were analysed by SDS/PAGE (8 % or 14 % gels) as indicated and exposed for three days to a phosphorimaging plate for visualization on a Fuji BAS 3000 phosphorimaging system. Quantitative analysis of gels was carried out using AIDA version 2.31 (Raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany).

RESULTS

Truncated opsin chains are cross-linked to several novel ER components via a single probe in TM domain 1

As previously shown, when the first 91 amino acids of bovine opsin, comprising all of TM1 and part of TM2, are synthesized as a ribosome-bound integration intermediate (OP91, see Figure 1), a single cysteine probe present at residue 56 within the first TM domain (OP91[cys56]) generates BMH-dependent cross-linking products with both Sec61α and Sec61β (Figure 1, compare lanes 1–4, see also [17]). Upon release of the nascent polypeptide from the ribosome by treatment with puromycin and EDTA (Figure 1, compare lanes 5–8), cross-linking to Sec61α and Sec61β is lost (Figure 1, lanes 7–8) and several new adducts are seen upon BMH treatment (Figure 1, lane 6, bands a–k). These adducts represent an association with proteins distinct from those present at the site of integration at the ER membrane [16,17]. At least 11 BMH-dependent cross-linking products were consistently observed after puromycin/EDTA treatment of OP91[cys56] chains (Figures 1–3, and results not shown) and we investigated further the nature of these components.

Several of the adducts (a–c, e/f) could only be resolved individually by SDS/PAGE when analysed on low percentage polyacrylamide gels (compare Figure 3A, lane 5 and Figure 3B, lanes 2 and 4). Furthermore, adducts a–c were obscured by two high molecular mass BMH-independent products that were seen after immunoprecipitation with the αOP serum (Figure 1, lanes 1, 2, 5 and 6; Figure 3A, lanes 1, 2, 4 and 5). By resolving the BMH-dependent adducts on an 8 % polyacrylamide gel after immunoprecipitation with the αOP serum, and comparing these to the total membrane associated products (Figure 3B, lanes 1–4), we were able to distinguish adducts a–c individually, and confirm their identity as BMH-dependent cross-linking products (Figure 3B, lanes 1 and 2).

As a first step towards characterizing these novel adducts, we used EndoH treatment to establish whether any of the unknown cross-linking partners contained *N*-linked glycans (Figure 2). In order to simplify this analysis, we used a version of opsin that lacked the two asparagine residues normally modified with *N*-linked glycans [17]. After synthesis of OP91ΔCHO[cys56] and

Figure 2 Characterization of the post-translocon OP91[cys56] cross-linking products

OP91ΔCHO[cys56] a derivative of OP91[cys56] lacking the two *N*-glycosylation sites of the wild-type protein, was used in order to more accurately assess the size and glycosylation state of the novel BMH-dependent cross-linking partners, labelled a–k in order of increasing mobility. EndoH treatment increased the mobility of adducts a–c, d and e/f, identifying them as *N*-glycosylated proteins (lanes 2 and 4). Three other prominent novel cross-linking partners were unaffected by treatment with EndoH (lanes 2 and 4, adducts h, i and k) or PNGaseF (results not shown) indicating these components are not *N*-glycosylated. The location of the non-glycosylated OP91 polypeptide (OP91ΔCHO) is indicated.

puromycin/EDTA-treatment, the released nascent chains were treated with BMH or subjected to a control reaction and immunoprecipitated (Figure 2, lanes 1 and 2). The pattern of cross-linking products appeared slightly simpler than that observed with the *N*-glycosylated form of OP91[cys56], suggesting that glycosylation of the OP91 chain was required for its association with a minority of the post-translocon components detected (adducts g and j appear to be absent for OP91ΔCHO, compare Figures 1–4). Half of each sample was treated with EndoH to remove *N*-linked glycans from the BMH-dependent adducts, and the resulting changes in relative mobility were identified by SDS/PAGE (Figure 2, compare lanes 2 and 4). From this analysis it was apparent that the three largest adducts were all EndoH sensitive, suggesting that these novel cross-linking partners are glycoproteins (Figure 2, lane 4, adducts a–d and e/f). By estimating the relative migration of the products after EndoH treatment, and subtracting the contribution of the OP91ΔCHO chain to the adducts, the approximate sizes of the cross-linking partners of the non glycosylated opsin fragment were estimated to be: ~93 kDa (a–c) ~55 kDa (d) and ~38 kDa (e/f) excluding any glycans. The estimated sizes of the three smaller, non-glycosylated, cross-linking partners were: ~27 kDa (h), ~24 kDa (i) and ~8 kDa (k). PNGaseF treatment failed to affect the mobility of the three smaller adducts confirming that they lacked typical *N*-linked glycans of any form (results not shown). It should be noted that these molecular masses are estimations based on the mobility of cross-linked adducts and as such may be prone to significant levels of error.

Figure 3 The SPP cross-links to OP91[cys56]

(A) After puromycin/EDTA treatment of the OP91[cys56] nascent chain, two BMH-dependent adducts were immunoprecipitated by an antiserum to the SPP (lane 7, adducts c and f), but not by a matched pre-immune (P.I.) serum from the same animal (lane 6). In contrast, no cross-linking to SPP was observed when the OP91[cys56] chains were stabilized as ribosome-bound integration intermediates using cycloheximide (lanes 2 and 3). Other labels are as defined in the legend to Figure 1. (B) Adducts a–f were resolved individually by analysing the cross-linking adducts on an 8% polyacrylamide gel.

SPP is present in two of the novel cross-linking products

Only a subset of ER proteins are *N*-glycosylated, and we particularly noted that the EndoH treatment of adduct e/f suggested the presence of two *N*-linked glycans, since an intermediate was apparent between the major forms present before and after EndoH digestion (Figure 2, lanes 2 and 4, adduct e/f). The size of this adduct, and the likelihood that it had two *N*-linked glycans prompted us to test whether adduct e/f may be the recently identified SPP [23]. Given the limitations of size estimation from cross-linking products, SPP was of an appropriate size, had two *N*-linked glycans, and contained five cysteine residues, two within its putative TM domains, that would be capable of BMH-dependent cross-linking to OP91[cys56]. Immunoprecipitation of the OP91[cys56] cross-linking products was carried out with an antiserum specific for SPP, after denaturation with SDS. This analysis showed that in fact two of the BMH-dependent adducts were specifically recognized by the αSPP serum (Figure 3A, compare lanes 6 and 7). SPP had previously been characterized via its role in the cleavage of signal peptides [23,28], and hence the cross-linking of SPP to a fragment of opsin was somewhat unexpected since it does not contain a cleavable N-terminal signal sequence. When OP91[cys56] was treated with BMH as a cycloheximide-stabilized integration intermediate, efficient cross-linking to the α and β subunits of the Sec61 complex was detected (Figure 3A, lane 2; cf. Figure 1, lanes 2–4). However, no cross-linking to SPP was seen under these circumstances (Figure 3A, compare lanes 3 and 7). Thus, it seems that the OP91 polypeptide is only associated with SPP after it has exited the Sec61 translocon.

From the estimated sizes of the cross-linking products, we conclude that adduct e/f represents the glycosylated OP91 chain cross-linked to an SPP monomer (Figure 3A, lane 7, band f). Ad-

duct c is most likely the OP91 polypeptide cross-linked to a homodimer of SPP (Figure 3A, lane 7, band c), consistent with recent work identifying an SDS stable dimer of SPP as the prevalent form in several cell types and tissues [29]. Prolonged heating of the cross-linking products in the presence of SDS did not reduce the proportion of adduct c relative to adduct f (results not shown) and we conclude that in our experiments the SPP dimeric form of SPP is stabilized by intra-subunit BMH-dependent cross-linking between one or more of the multiple cysteines present [29].

When resolved on an 8% polyacrylamide gel (Figure 3B), the two SPP-containing adducts, c and f, were visible after immunoprecipitation (Figure 3B, lane 6, bands c and f) and were now clearly distinct from adducts a, b, d and e after immunoprecipitation with αOP (Figure 3B, cf. lanes 3 and 4, bands a–f) or after analysis of total membrane associated products (Figure 3B, lanes 1 and 2, bands a–f). Two BMH-independent high-molecular-mass products were also clearly separated in the system after immunoprecipitation with the αOP serum (Figure 3B, lanes 3–4, filled circles). Adduct c was not readily visible after immunoprecipitation with the αOP serum (Figure 3B, lanes 2, 4 and 6, adduct c) despite being visible in the total products and also after immunoprecipitation with αSPP serum. This reflects the relatively high background obtained after immunoprecipitation with the αOP serum (Figure 3A, compare lanes 1–6).

An inhibitor of SPP prevents its association with opsin fragments

In order to investigate the authenticity of the association between the OP91 polypeptide and SPP, we exploited a previously characterized inhibitor of SPP activity (Z-LL)₂-ketone [23,28]. (Z-LL)₂-ketone was designed to mimic the signal peptide derived substrates of SPP, and acts as an efficient and specific inhibitor of

Figure 4 (Z-LL)₂-ketone inhibits OP91 cross-linking to SPP

OP91ΔCHO was synthesized in the presence of a serial dilution of (Z-LL)₂-ketone or an equivalent DMSO control. Membrane-associated integration intermediates were then treated with puromycin/EDTA and subjected to BMH-dependent cross-linking. SPP adducts were immunoprecipitated (upper panel) and analysed in parallel with the total reaction products (lower panel). (Z-LL)₂-ketone had no effect upon the translation efficiency (lower panel, OP91ΔCHO) or the formation of adducts d, h, i or k (lower panel) at any of the concentrations tested. In contrast, even the lowest concentration of (Z-LL)₂-ketone caused an almost complete inhibition in the formation of adducts c and f (upper panel).

its protease activity [24,28]. The non-glycosylated form of opsin, OP91ΔCHO[cys56], was synthesized in the presence of semi-intact mammalian cells together with a serial dilution of the SPP inhibitor (Z-LL)₂-ketone, using the solvent DMSO as a control (Figure 4). When the resulting OP91ΔCHO[cys56]–SPP adducts were recovered by immunoprecipitation, it was immediately apparent that the SPP inhibitor caused an almost complete loss of both adducts c and f at the lowest concentration of inhibitor tested (Figure 4, upper panel, cf. lanes 1–12). This effect of (Z-LL)₂-ketone was specific, since when the total reaction products were analysed in parallel, it was clear that the formation of other cross-linking products, such as adduct d, were unaffected by the presence of the inhibitor (Figure 4, lower panel, lanes 1–12, adduct d). The loss of adduct c in the total products was less apparent than in the immunoprecipitated SPP adducts (Figure 4, upper and lower panels, adduct c) since, when OP91ΔCHO is used for crosslinking, adduct c co-migrates with one of two high molecular-mass products consistently seen in the absence of BMH treatment (see Figure 1, lanes 1 and 5; Figure 2, lane 1; Figure 3A, lanes 1 and 4 and Figure 3B, lanes 4 and 6) in addition to adducts a and b (Figure 3B, lanes 2, 4 and 6). The loss of adduct f upon (Z-LL)₂-ketone treatment was clearly apparent in the total reaction

products (Figure 4, lower panel, e/f) suggesting products e and f may represent adducts with different *N*-glycosylated forms of SPP [23]. We therefore concluded that the association between OP91 and SPP that we could detect by cross-linking reflected a genuine interaction that was prevented by an inhibitor in the form of a substrate analogue [28].

SPP association requires the truncated TM2 of OP91

Previous studies of SPP suggest that it binds to, and cleaves, signal peptides oriented with their N-termini in the cytosol [23]. The *N*-glycosylation pattern of OP91 confirmed that it had a fully integrated TM1, with the opposite TM orientation to these previously characterized SPP substrates (see Figure 5 cartoons). In order to clearly establish which region(s) of OP91 was responsible for its association with SPP, we manipulated this opsin fragment to either remove the residual element of TM2 completely and incorporate a C-terminal HA epitope tag (OP80HA), or to replace the last nine residues of the residual TM2 with the HA tag (OP91HA). The original OP91 polypeptide, and its two derivatives, were then analysed by BMH-dependent cross-linking to a single cysteine probe located at the same position (residue 56) in each of their identical TM1 domains. It was immediately apparent that when TM2 was lacking, the truncated opsin chain did not cross-link to SPP (Figure 5, lanes 1–6). In fact OP80HA-[cys56] displayed only a single major BMH-dependent adduct (Figure 5, lane 2, filled circle) that was clearly distant from the SPP adducts and apparently lacked the C-terminal HA epitope present on the nascent chain (Figure 5, lane 4). Analysis of *N*-linked glycosylation using EndoH treatment showed that OP80HA was *N*-glycosylated to a similar degree as OP91HA and OP91 (Figure 5, lanes 19–24) and we conclude that this intermediate inserts in the correct orientation but does not associate with SPP. When the OP91HA derivative was analysed, the incorporation of the HA tag at the C-terminus of the protein caused a large reduction in the relative efficiency of SPP cross-linking and adducts of OP91HA with SPP were barely visible (Figure 5, compare lanes 12 and 18, see also quantification). We therefore conclude that, whilst SPP is cross-linked to OP91 via a probe located in TM1, the principal site of SPP association is with TM2.

SPP can associate with an opsin fragment containing a fully integrated TM2

Given the well-documented association of SPP with signal peptides [30] it seemed likely that the efficient cross-linking of SPP to OP91 reflected its association with the residual TM2 of the OP91 fragment in an equivalent TM topology (see Figure 5 cartoons). However, our previous analysis of OP91 and its derivatives (see Figure 5) left open the formal possibility that none of these opsin fragments possessed a fully membrane-integrated region derived from TM2. In order to address this issue, we generated a version of opsin with complete TM1 and TM2 domains and a C-terminal extension containing a single site for *N*-glycosylation. This polypeptide was efficiently membrane integrated in our *in vitro* system and over half of the polypeptides were *N*-glycosylated, confirming that in the case of these molecules TM2 completely spans the ER membrane with its C-terminus located in the ER lumen (Figure 6, lanes 1 and 2).

Most significantly, OP117 was efficiently cross-linked to SPP and equivalent versions of adducts c and f to those previously seen with OP91 were readily apparent (Figure 6, lanes 3 and 5). In the case of adduct f, a doublet of products was seen (Figure 6, lane 5, bands f and f'). Analysis of *N*-linked glycosylation of these adducts using treatment with EndoH suggest that this is most likely due to the cross-linking of SPP to both the glycosylated and

Figure 5 Opsin TM2 is required for cross-linking to SPP

Radiolabelled integration intermediates of OP80HA, OP91HA and OP91 were treated with puromycin/EDTA and cross-linked to adjacent ER components from cysteine residue 56 using BMH, or subjected to a control reaction with DMSO as indicated (lanes 1–18). The resulting products were immunoprecipitated with antisera specific for the opsin polypeptide (α OP), the HA epitope tag (α HA) or SPP (α SPP). Adducts c, d and f are as previously defined (see legends to Figures 2–4). In the case of OP80HA a BMH-dependent adduct was recognized by the α OP serum but not the α HA serum (compare lanes 2 and 4, filled circle). The relative efficiency of cross-linking to SPP was calculated by quantitative phosphorimaging and is shown in tabular form. Product intensities were measured and used to calculate the proportion of the different opsin nascent chains that were cross-linked to SPP and immunoprecipitated. This was done by determining the amount of each nascent chain cross-linked to SPP and expressing it as a fraction of the total nascent chain present in the reaction. These data were then compared to indicate relative efficiencies, with the SPP adduct containing the highest proportion of nascent chains being arbitrarily defined as having a relative efficiency of 100. The efficiencies shown are averages taken from the results of two separate experiments. In the case of OP80HA[cys56], any SPP adducts formed were below levels of detection (lane 6) and hence no quantification was possible. EndoH treatment showed that all constructs were efficiently *N*-glycosylated indicating integration of the N-terminus of the polypeptide chains into the ER lumen (lanes 19–24).

non-glycosylated versions of the OP117 polypeptide, as adducts f and f' migrate as a single band after EndoH digestion of the *N*-linked glycans (Figure 6, compare lanes 6 and 7). It appears that OP117 generates a distinct pattern of adducts from that seen with OP91, and apart from cross-linking to SPP (adducts c and f), only the adduct denoted k' (Figure 6, lane 3) is of a similar size to that seen with OP91 (Figure 3, lane 5, band k). Other adducts (Figure 6, lane 3, filled circles) may represent distinct components that associate with OP117 but not with OP91. We conclude that SPP associates with short opsin fragments via their second TM region, and that this interaction most likely occurs within the plane of the lipid bilayer.

SPP preferentially associates with truncated opsin chains

The association of SPP with short fragments of opsin via their second TM region raised the question as to whether there was

any discrimination when opsin TM2 was presented in different contexts. In the case of the T-cell antigen receptor the assembly of the alpha subunit with other subunits of the complex, masks an efficient ER degradation signal present in the single TM domain of the alpha subunit [11]. To investigate whether the synthesis of additional TM domains, C-terminal of opsin TM2, had any effect upon the association of the resulting opsin fragments with SPP, we synthesized a number of opsin constructs of increasing length, all containing the HA epitope tag at the C-terminus. A 109 amino acid fragment of OP[cys56] was therefore synthesized, since this included the entire TM2 domain. When OP109HA[cys56] was analysed for adduct formation and compared with OP150HA[cys56] we found the addition of the entire TM3 domain to the C-terminus of the polypeptide did not cause any significant loss of cross-linking to SPP (Figure 7, compare lanes 4 and 8, bands c and f). Similarly, the addition of the entire TM4 domain in the OP190HA[cys56] polypeptide resulted in similar levels of

Figure 6 SPP can associate with a fully integrated opsin TM2

OP117[cys56,asn112], containing a novel *N*-glycosylation site at residue 112 of OPΔCHO, was synthesized as an integration intermediate and treated with puromycin/EDTA. EndoH treatment confirmed that the C-terminus of the polypeptide was efficiently *N*-glycosylated (compare lanes 1 and 2). Upon BMH treatment, distinct adducts with SPP could be recovered by immunoprecipitation (compare lanes 4 and 5). Adducts f and f' collapse into a single band following treatment with EndoH (compare lanes 6 and 7).

cross-linking to SPP (Figure 7, compare lanes 4, 8 and 12, bands c and f). Only when all seven TM domains were synthesized, in the OP348HA[cys56] polypeptide, were significant differences seen in the efficiency of cross-linking to SPP (Figure 7, lane 16, filled circles). Several adducts were immunoprecipitated by the αSPP serum, but these were not confirmed as adducts with full-length OP348HA chains since they could not be readily seen in the products immunoprecipitated with the αHA serum (Figure 7, lane 14). It is likely that these SPP adducts are cross-linked to incomplete opsin chains that may result from a strong ribosomal pause site present in the mRNA encoding full-length opsin [17]. This possibility is further supported by the observation that several of the OP348HA[cys56] adducts with SPP are smaller than those seen with the purposely truncated chains (Figure 7, compare lanes 4, 8, 12 and 16). The three shorter opsin chains, OP109HA, OP150HA, and OP190HA all show adducts to the presumptive SPP monomer when immunoprecipitated with the αHA serum (Figure 7, compare lanes 2 and 4, 6 and 8, 10 and 12, adduct f). Conversely, adduct c, was not clearly visible after immunoprecipitation with the αHA serum. However, when the cross-linking products of OP109HA[cys56] were analysed by sequential immunoprecipitation using αSPP serum followed by either an αOP or αHA serum, we found adduct c represents a cross-link to a complete OP109HA[cys56] polypeptide (results not shown).

DISCUSSION

The *in vitro* synthesis and cross-linking analysis of OP91[cys56] has provided us with an opportunity to characterize several

unknown ER proteins that are found in association with a short misassembled fragment of a polytopic membrane protein. We have identified SPP as one of the several cross-linking partners of OP91[cys56] and found that it is present in two discrete adducts formed by OP91 after its membrane integration. The estimated sizes of the cross-linked components are ~38 and ~93 kDa, excluding *N*-linked glycans. On this basis, we assume that the smaller adduct (f) represents OP91 cross-linked to an SPP monomer, and this fits well with previous size estimates made using substrate analogues to label SPP [23]. The estimated size of the large adduct (c), and its behaviour upon EndoH treatment, suggest it is probably an SPP dimer cross-linked to one, or more, OP91 polypeptides. A homodimer of SPP has been shown to be the major species in many cells and tissues, and labelling with an active-site probe suggest that the dimer form of SPP may be its functionally active form [29]. Hence, our data are entirely consistent with truncated opsin chains being associated with functionally active SPP. Consistent with a model where SPP contributes to the quality control of aberrant membrane proteins, we find that the OP91 polypeptide is only cross-linked to SPP after it has exited the Sec61 translocon. This presumably represents a stage during the polypeptide's biosynthesis at which it is fully integrated into the lipid bilayer of the ER membrane. We found that the association of OP91 with SPP was completely independent of any requirement for *N*-glycosylation of the nascent chain, consistent with a recognition event occurring within the plane of the membrane. A number of cysteine-containing ER components can be cross-linked to membrane proteins with BMH both during and after integration is completed [16,17,31]. However, immunoprecipitation analysis showed that none of these previously characterized components were present among the BMH-dependent adducts formed by membrane-integrated, ribosome-released, OP91 polypeptides (results not shown). This observation strongly suggested that the novel adducts formed by OP91 after puromycin treatment of the integration intermediate represented the specific association of the OP91 chains with a particular set of ER components.

In the case of SPP, we were able to investigate further the specificity of its association with OP91 by using a previously characterized inhibitor of SPP, (Z-LL)₂-ketone [24]. When membrane fractions containing puromycin-treated OP91[cys56] chains were preincubated with (Z-LL)₂-ketone at a final concentration of 1.56 μM, almost complete inhibition of cross-linking to SPP was seen. This is entirely consistent with the complete inhibition of SPP reported previously at a 1 μM concentration of the inhibitor [24]. Increasing the levels of (Z-LL)₂-ketone to 50 μM resulted in even lower levels of residual SPP cross-linking without causing any noticeable reduction in the formation of other cross-linking products including the appearance of an adduct with a distinct ER glycoprotein denoted adduct d. Thus, a well characterized SPP inhibitor, the substrate mimetic (Z-LL)₂-ketone [24,28], specifically inhibits the association of membrane-integrated OP91 fragments with SPP. The loss of both adducts c and f upon (Z-LL)₂-ketone treatment confirms that both adducts contain SPP and suggests that both products reflect a biologically relevant interaction. The simplest interpretation of this observation is that the OP91 fragment associates with SPP in the same fashion as its known substrates, *N*-terminal signal peptides.

To investigate further the association of SPP with the OP91 fragment, we manipulated its C-terminal region. We found that the association of SPP with opsin fragments required the presence of all or part of TM2, although our cross-linking probe was located in TM1. We were able to confirm that a fully membrane-integrated TM2 was a substrate for SPP binding, and that SPP can also be cross-linked to opsin fragments via a single cysteine probe

Figure 7 Translation of all the TM domains C-terminal to TM2 reduces efficiency of cross-linking to SPP

Integration intermediates of OP109HA, OP150HA, OP190HA and OP348HA with a probe in the [cys56] position were treated with puromycin/EDTA and subjected to BMH-dependent cross-linking as described previously. BMH-dependent adducts with SPP were recovered by immunoprecipitation (compare lanes 3 and 4, 7 and 8, 11 and 12, 15 and 16) and the relative efficiencies of distinct SPP containing adduct formation compared.

located in TM2 (results not shown). These data all support a model where SPP can specifically associate with hydrophobic regions of polypeptide that span the lipid bilayer of the ER with their N-terminal region in the cytosol and their C-terminus in the ER lumen. To date, a subgroup of N-terminal signal sequences have been shown to meet these criteria [23,30]. We now show that a fragment of the polytopic protein opsin, with two membrane embedded regions, also specifically associates with SPP. In relation to SPP function, it may be that many different TM spanning regions can associate with its substrate binding site, but that only a subset of these can be transferred to the enzyme's active site for proteolytic cleavage [32].

Previous studies of oligomeric protein complexes that assemble in the ER have shown that in many cases when subunits are expressed alone they are recognized by the ER quality control machinery and removed via the ERAD pathway [2]. In some cases, specific features are present within the TM regions of these unassembled 'orphan' subunits that are masked when the complex is partly or fully assembled [11 and references therein]. High resolution structural studies of opsin have shown that TM2 plays an important role in stabilizing the protein and contributes to a number of hydrogen bonds with TM1, TM3, TM4 and TM7 [13]. We therefore investigated whether synthesizing an opsin fragment that included additional TM domains would influence the association of opsin fragments with SPP. We found that when longer fragments were analysed, with up to four integrated TM domains, adduct formation with SPP was essentially unaffected. In contrast, when a version of opsin with all seven TM regions was made, no evidence of cross-linking to the full-length molecule was obtained. Thus, the association of opsin fragments with

SPP may be dependent upon the exposure of specific features that are masked or buried in the full-length correctly folded structure [13].

What is the biological significance of an association between truncated opsin fragments and SPP? One possibility is that SPP contributes to ER quality control by cleaving aberrant polytopic membrane proteins within their TM domains and thereby facilitates their release from the membrane [9,33–35]. Alternatively, SPP may bind such fragments and deliver them to other components of the quality control system operating in the ER [2,3]. At present we are unable to distinguish between such possibilities, and our future work will be aimed at resolving this issue.

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