

# Active and passive displacement of transmembrane domains both occur during opsin biogenesis at the Sec61 translocon

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## Summary

We used a site-specific crosslinking approach to study the membrane integration of the polytopic protein opsin at the endoplasmic reticulum. We show that transmembrane domain 1 occupies two distinct Sec61-based environments during its integration. However, transmembrane domains 2 and 3 exit the Sec61 translocon more rapidly in a process that suggests a displacement model for their integration where the biosynthesis of one transmembrane domain would facilitate the exit of another. In order to investigate this hypothesis further, we studied the integration of the first and third transmembrane domains of opsin in the absence of any additional C-terminal transmembrane domains. In the case of transmembrane domain 1, we found

that its lateral exit from the translocon is clearly dependent upon the synthesis of subsequent transmembrane domains. By contrast, the lateral exit of the third transmembrane domain occurred independently of any such requirement. Thus, even within a single polypeptide chain, distinct transmembrane domains display different requirements for their integration through the endoplasmic reticulum translocon, and the displacement of one transmembrane domain by another is not a global requirement for membrane integration.

Key words: Endoplasmic reticulum, Integration, Translocation, Protein biosynthesis

## Introduction

The endoplasmic reticulum (ER) is a major site of membrane protein synthesis in eukaryotic cells and insertion typically occurs through the co-translational integration of the polypeptide at the ER translocon (Alder and Johnson, 2004; Lecomte et al., 2003). This ER translocon provides a carefully gated aqueous pore that spans the lipid bilayer and allows the regulated transport of hydrophilic polypeptide across the ER membrane and into the lumen. In the case of membrane proteins, the ER translocon can arrest hydrophobic transmembrane domains (TMs) during translocation and facilitate their lateral exit from the aqueous pore, resulting in membrane integration (Alder and Johnson, 2004; Lecomte et al., 2003). The core of the ER translocon is the Sec61 complex, a heterotrimer comprised of  $\alpha$ ,  $\beta$  and  $\gamma$  subunits, and it is the Sec61 complex that directly mediates TM integration (Alder and Johnson, 2004; Lecomte et al., 2003; Van den Berg et al., 2004). Initial low-resolution studies, together with biophysical measurements, suggested that about four copies of the Sec61 complex combined to form a 'doughnut-like' structure with a large, central cavity through which protein translocation might occur (Alder and Johnson, 2004; Beckmann et al., 2001; Dobberstein and Sinning, 2004; Menetret et al., 2000). However, the only high-resolution structure of an archaeal Sec61-like complex currently available suggests that protein translocation occurs through a narrow pore located at the centre of a single Sec61 heterotrimer (Van den Berg et al., 2004). Although this crystal structure represents an inactive or empty version of the Sec61 complex, it suggests that a ring of

hydrophobic residues within the putative Sec61 channel could maintain the permeability barrier offered by the ER membrane during polypeptide translocation (Van den Berg et al., 2004). This view is also supported by an analysis of the residues in the related SecY protein that are found in close proximity to a translocating polypeptide using a targeted crosslinking approach (Cannon et al., 2005), although the number of copies of the Sec61 heterotrimer that represents an active translocon remains contentious (Mitra et al., 2005).

There have been a number of studies of membrane protein integration occurring through the Sec61 complex using both simple, single-spanning, membrane proteins and more complex polytopic precursors (Alder and Johnson, 2004; Booth and High, 2004; Lecomte et al., 2003; Sadlish and Skach, 2004). Of particular interest is the process by which hydrophobic TMs exit the Sec61 translocon in the lateral plane and enter the lipid bilayer (Alder and Johnson, 2004; Lecomte et al., 2003; Van den Berg et al., 2004). In the case of polytopic membrane proteins, multiple TMs must be integrated by the Sec61 translocon and subsequently assembled into a correctly folded polypeptide. Opsin has seven TMs, and it has been generally assumed that the individual TMs are sequentially integrated into the ER membrane as they emerge from the ribosome and engage the Sec61 complex (Laird and High, 1997; McCormick et al., 2003; Meacock et al., 2002). Such an ordered insertion of opsin TMs is also supported by *in vivo* studies of an archaeal equivalent, bacterioopsin (Dale et al., 2000), and by a detailed analysis of aquaporin-4 biogenesis (Sadlish et al., 2005).

One of the key issues that remains to be answered, particularly in the context of polytopic proteins, is the driving force for the lateral exit of TMs during their biogenesis (von Heijne, 2005). In the simplest case, an individual TM may 'partition' from the ER translocon into the lipid bilayer on the basis of its intrinsic hydrophobicity (Hessa et al., 2005). However, even the presence of two TMs in a polypeptide can result in far more complex models for TM exit (Heinrich and Rapoport, 2003; Sauri et al., 2005). The simplest model describing the integration of polytopic proteins suggests that each TM rapidly exits the Sec61 complex and enters the lipid bilayer shortly after its synthesis by 'sequential insertion' (Alder and Johnson, 2004; High and Laird, 1997; Lecomte et al., 2003; Sadlish et al., 2005; Sadlish and Skach, 2004). However, for some polytopic membrane proteins, it appears that pairs of TMs must associate for their stable integration at the ER [Alder and Johnson (Alder and Johnson, 2004) and references therein]. There are a number of possible scenarios to explain such observations. In at least one case, a 'fully integrated' TM appears to return to the Sec61 complex in order to facilitate the integration of a second TM (Heinrich and Rapoport, 2003). Alternatively, it has been shown that a TM can be fully translocated into the ER lumen before its subsequent integration during a posttranslational 'maturation' event (Lu et al., 2000). A third possibility for the stabilisation of one TM by another is the orchestrated assembly of multiple TM domains within the ER translocon (Borel and Simon, 1996). This model suggests that the relevant TMs are only released into the lipid bilayer after some level of packing and assembly has occurred within the translocon (Alder and Johnson, 2004; Borel and Simon, 1996; High and Laird, 1997; Lecomte et al., 2003). It is striking that the high-resolution structure of the archaeal Sec61 complex appears completely at odds with this latter model, suggesting that the core of the Sec61 complex would be too small to accommodate more than a single  $\alpha$ -helix (Van den Berg et al., 2004). The assembly of TM domains at a different location of the Sec61 complex (Sadlish et al., 2005) or the concerted action of two Sec61 complexes during membrane protein integration would offer potential solutions to this apparent problem (Dobberstein and Sinning, 2004; Mitra et al., 2005).

In order to address directly the issue of TM exit during polytopic membrane protein biogenesis, we have extended our previous studies of opsin integration (Laird and High, 1997; Meacock et al., 2002) to investigate this question specifically. In this study, we have carried out a detailed analysis of the biosynthesis of TM1-TM3 of opsin using a site-specific crosslinking approach. We find that during membrane integration, opsin TM1 encounters two distinct environments provided by the Sec61 complex, which we define as phase I and phase II on the basis of the precise crosslinking patterns observed (Sadlish et al., 2005). By contrast, opsin TM2 and TM3 only display a transient, phase-I-like, association with the Sec61 complex. When the requirements for TM exit from the Sec61 complex were analysed, we found that the lateral exit of TM1 from the Sec61 complex is promoted by the synthesis of the subsequent TMs within the opsin polypeptide. However, the release of TM3 from the Sec61 complex occurs independently of any additional TM synthesis. Thus, even within a single polypeptide chain, different TMs are

differentially processed and released by the Sec61 translocon during integration at the ER membrane.

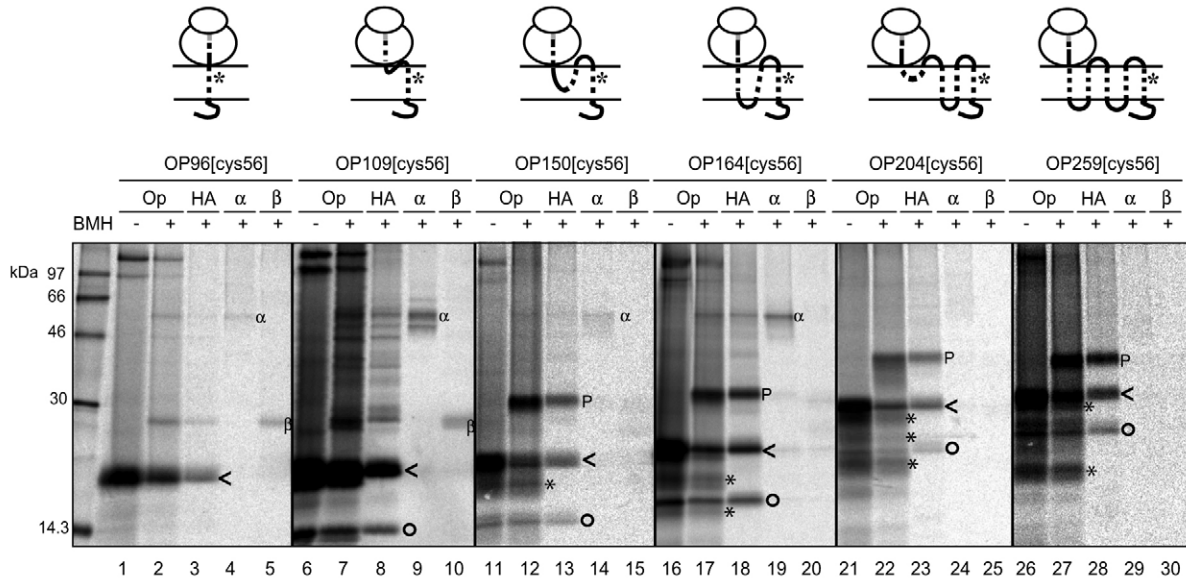
## Results

Our previous studies of opsin biogenesis have concentrated on the environment of the hydrophilic loops (Laird and High, 1997) and the first and second transmembrane domains (TMs) (Meacock et al., 2002). In order to extend these studies, we have now investigated the integration of TM1-TM3 of opsin by using a site-specific crosslinking approach combined with the use of chimeras that lack selected TMs. This N-terminal region of opsin was chosen because *in vivo* expression studies show that it behaves as an independent folding domain (Ridge et al., 1995), and hence its biogenesis and assembly are biologically relevant. Integration intermediates are frequently used to study membrane protein integration, and these are normally generated by the use of precisely truncated mRNAs that lack a stop codon, leading to the resulting polypeptide remaining attached to the ribosome as a peptidyl-tRNA (Gilmore et al., 1991). In the case of ER-targeted proteins, this causes the polypeptide to be trapped at the ER translocon unless the ribosome is released by puromycin treatment (Gilmore et al., 1991).

### A C-terminal epitope tag can be used to authenticate adducts with specific integration intermediates and reveals complexity during opsin TM1 integration

During a previous study of opsin biosynthesis, we found evidence that the population of nascent chains generated using truncated mRNAs can be heterologous, with different chain lengths apparently present in a single translation reaction resulting in complex crosslinking patterns (Meacock et al., 2002). In order to confirm the identity of specific integration intermediates, we introduced a haemagglutinin (HA) epitope tag at the C-terminus of several truncated opsin chains of the same length as those we had previously studied (Meacock et al., 2002). We then carried out a series of BMH (bismaleimido-hexane)-mediated crosslinking reactions from a previously characterised single cysteine probe present at residue 56 of opsin TM1 (Meacock et al., 2002).

Our criteria for authentic crosslinking of nascent opsin chains to components of the ER translocon were that the adduct should be unambiguously immunoprecipitated by antisera specific for both the N-terminus ( $\alpha$ OP) and the C-terminus ( $\alpha$ HA) of the nascent opsin chain in addition to an antiserum recognising the crosslinking partner where available. When opsin chains of between 96 and 259 residues (including the nine residue HA tag) were analysed, it was apparent that there was significant heterogeneity in the non-crosslinked nascent chains resulting from the translation reaction (see Fig. 1). This was especially apparent when nascent chains of 150 residues and longer were analysed where a number of products were recognised by the  $\alpha$ OP but not the  $\alpha$ HA serum (Fig. 1, see products identified by asterisks). These translation products lack a completed C-terminus, consistent with the proposal that they result from ribosome stacking on the truncated mRNA templates (Meacock et al., 2002), and such incomplete integration intermediates have the potential to generate adducts with subunits of the ER translocon. Any aberrant crosslinking products can be distinguished from adducts with authentic



**Fig. 1.** TM1 displays a biphasic association with the Sec61 complex. A schematic of ribosome-bound integration intermediates is presented above each set of products. The dashed line represents the transmembrane domain (TM), the solid line indicates the hydrophilic portion of the nascent chain and the star denotes the cysteine probe within TM1. Each integration intermediate was synthesised in a rabbit reticulocyte lysate translation system in the presence of [ $^{35}$ S]Met/Cys and semi-permeabilised HT1080 cells. Membrane-associated integration intermediates were isolated by centrifugation and crosslinking performed by the addition of the homobifunctional reagent, BMH. Opsin-derived nascent chains were immunoprecipitated using either a monoclonal antibody specific for the N-terminus of the polypeptide (Op), or for the HA epitope on the C-terminus of the integration intermediates (HA). For each chain length, authentic doubly N-glycosylated opsin chains are indicated by an open arrowhead and their non-glycosylated counterparts by an open circle. Shorter opsin chains that lack the C-terminal HA epitope tag are indicated by asterisks. Adducts to Sec61 $\alpha$  and Sec61 $\beta$  were identified by immunoprecipitation with appropriate antisera (denoted  $\alpha$  and  $\beta$ ). P indicates adducts to an unidentified protein of  $\sim 10$  kDa, denoted PAT-10 (lanes 13, 18, 23 and 28). Vertical black lines indicate sets of samples resolved on different SDS-polyacrylamide gels. Positions of molecular size markers are indicated to the left.

integration intermediates by their failure to be immunoprecipitated by the  $\alpha$ HA serum.

A number of discrete crosslinking products that were efficiently immunoprecipitated by both the  $\alpha$ OP and  $\alpha$ HA sera were clearly visible. Amongst these, adducts with the Sec61 $\alpha$  and Sec61 $\beta$  subunits of the ER translocon could be unambiguously detected (Fig. 1, lanes 2-5, 7-10, 12-14 and 17-19, products labelled  $\alpha$  and  $\beta$  respectively). The third major component that was clearly crosslinked to authentic integration intermediates was the previously defined PAT-10, a protein associated with the translocon of  $\sim 10$  kDa (Fig. 1, lanes 13, 18, 23 and 28, product labelled P). PAT-10 is specifically associated with the first TM of the G-protein-coupled receptors opsin and the neurotensin transporter, but remains to be fully characterised in terms of its sequence and biological function (Meacock et al., 2002). As previously reported (Meacock et al., 2002), no crosslinking of opsin TM1 to the TRAM protein was detected (data not shown).

This detailed analysis of the opsin TM1 crosslinking partners revealed a previously unidentified level of complexity in its behaviour during membrane integration. Thus, after strong crosslinking of shorter chains to Sec61 $\beta$ , most notably the OP96 and OP109 chains (Fig. 1, lanes 2, 3, 5, 7, 8 and 10,  $\beta$ ), no definitive Sec61 $\beta$  adducts were seen with any of the longer opsin chains (Fig. 1, lanes 12-15, 17-20, 22-25 and 27-30). By contrast, clear adducts with Sec61 $\alpha$  were seen up to a chain length of 164 residues (Fig. 1, lanes 2-4, 7-9, 12-14 and 17-19,  $\alpha$ ). The variation in the strength of the Sec61 $\alpha$  adduct

primarily reflected differences in the translation efficiencies of different truncated opsin chains used (data not shown). On the basis of these data, we propose that TM1 experiences at least two distinct, Sec61-associated environments during opsin biogenesis, that are adjacent to and distant from the Sec61 $\beta$  subunit. Furthermore, this analysis confirms our previous suggestion that once adjacent to PAT-10, opsin TM1 remains in close proximity to this component until the completion of protein synthesis and/or the release of the polypeptide from the ribosome (Meacock et al., 2002).

#### TM1 relocation is delayed in the absence of subsequent TMs

The distinct alterations in BMH-dependent crosslinking from opsin TM1 that we observe when analysing different integration intermediates may reflect differing consequences of chain extension. Thus, the environment of TM1 may simply alter in response to the length of the polypeptide 'tether' at the C-terminus of TM1 (Sauri et al., 2005), or alternatively, the synthesis of the additional TMs (TM2, TM3, etc.) present in this region may actively displace TM1 from the Sec61 translocon. In order to distinguish between these two possibilities, we replaced the polypeptide at the C-terminus of opsin TM1 with a region of the secretory protein preprolactin that lacked any hydrophobic transmembrane domains (denoted OPTM1PPL, see Table 1), and analysed the environment of opsin TM1 using integration intermediates of this chimera identical in length to the opsin chains previously investigated (cf. Fig. 1).

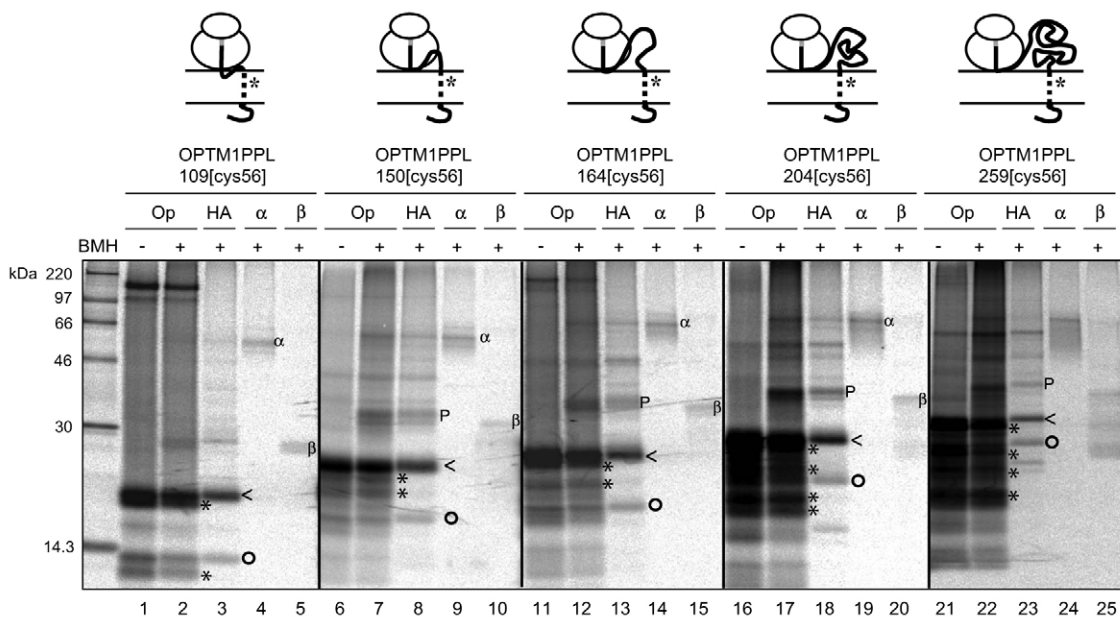
**Table 1. Amino acid sequences of opsin derivatives used in this study**

OP[Cys56]	<u>1</u> MNGTEGPNFYVPPFSNKTGVVRSFFEAPQYYLAEPWQFSMLAAYMFLLIIMLGFPINCLTLY	60
OPTM1PPL[Cys56]	<u>1</u> MNGTEGPNFYVPPFSNKTGVVRSFFEAPQYYLAEPWQFSMLAAYMFLLIIMLGFPINCLTLY	60
OP[Cys115]	<u>1</u> MNGTEGPNFYVPPFSNKTGVVRSFFEAPQYYLAEPWQFSMLAAYMFLLIIMLGFPINFLTLY	60
OPTM1-3PPL[Cys115]	<u>1</u> MNGTEGPNFYVPPFSNKTGVVRSFFEAPQYYLAEPWQFSMLAAYMFLLIIMLGFPINGLTLY	60
	▼	
OP[Cys56]	<u>61</u> VTVQHKKLRTPNLNILLNLAVADLFMVFGGFTTTLTYSLSLHGYPVFGPTGGNLEGGFATLG	120
OPTM1PPL[Cys56]	<u>61</u> VTVQHKKLRTPVGPNGPGNGQVSLRDLFDRAVMVSHYIHDLSSEMFNEFDKRYAQKGF	120
OP[Cys115]	<u>61</u> VTVQHKKLRTPNLNILLNLAVADLFMVFGGFTTTLTYSLSLHGYPVFGPTGGNLEGGFATLG	120
OPTM1-3PPL[Cys115]	<u>61</u> VTVQHKKLRTPNLNILLNLAVADLFMVFGGFTTTLTYSLSLHGYPVFGPTGGNLEGGCFATLG	120
	▲	
OP[Cys56]	<u>121</u> GETALWSLVVLAIERYVVVGKPMNSNFRFGENHAIMGVAFTWVMALAGAAPPLVGSRYIP	180
OPTM1PPL[Cys56]	<u>121</u> ITMALNSGHTSSLPTPEDKEQAQQTTHHEVLSLILGLLRSWNPPLYHLVTEVRGMKGAPD	180
OP[Cys115]	<u>121</u> GETALWSLVVLAIERYVVVGKPMNSNFRFGENHAIMGVAFTWVMALAGAAPPLVGSRYIP	180
OPTM1-3PPL[Cys115]	<u>121</u> GETALWSLVVLAIERYVVVGKPTVPGPNGPGNGQVSLRDLFDRAVMVSHYIHDLSSEMFN	180
OP[Cys56]	<u>181</u> EGMQSGGIDYYTPHEETNNSFVIYMFVVHFIPLIVIFFGYGQLVFTVKEAAAQQQES	240
OPTM1PPL[Cys56]	<u>181</u> AILSRAIEIEEENKRLLEGMEMIFGQVIPGAKETEPYVWSGLPSLQTKDEDARYSAFYN	240
OP[Cys115]	<u>181</u> EGMQSGGIDYYTPHEETNNSFVIYMFVVHFIPLIVIFFGYGQLVFTVKEAAAQQQES	240
OPTM1-3PPL[Cys115]	<u>181</u> EFDKRYAQKGFITMALNSGHTSSLPTPEDKEQAQQTTHHEVLSLILGLLRSWNPPLYHL	240
OP[Cys56]	<u>241</u> ATTQKAEKEVTRMVIIMVIA	260...
OPTM1PPL[Cys56]	<u>241</u> LLHGLRRDSSKIDTYLKLLN	260...
OP[Cys115]	<u>241</u> ATTQKAEKEVTRMVIIMVIA	260...
OPTM1-3PPL[Cys115]	<u>241</u> VTEVRGMKGAPDAILSRAIE	260...

The amino acid sequences of the opsin and opsin-preprolactin chimeras used in this study, including the cysteine probes at residues 56 and 115, are shown. The point of divergence between the opsin and OPTM1PPL sequences is shown by a downward-pointing arrowhead and the point of divergence between opsin and OPTM1-3PPL by an upward-pointing arrowhead. The transmembrane domains are underlined and represent those defined by the crystal structure (Palczewski et al., 2000). For each chain length analysed, the last nine residues of the sequence listed above was replaced by the HA epitope tag, YPYDVPDYA.

When OPTM1PPL[Cys56] integration intermediates were analysed, discrete crosslinking to both Sec61 $\alpha$  and Sec61 $\beta$  was observed from a chain length of 109 residues (Fig. 2, lanes 2-5), and both adducts were clearly maintained with intermediates of 150, 164 and 204 residues (Fig. 2, lanes 7-10, 12-15 and 17-20, see  $\alpha$  and  $\beta$ ). This behaviour is clearly quite distinct from that observed with the comparable opsin

integration intermediates (cf. Fig. 1). Although adducts are also formed with Sec61 $\alpha$  and Sec61 $\beta$  at a chain length of 259 residues, the bulk of these products appear to lack the HA epitope (Fig. 2, cf. lanes 23-25), and hence probably reflect crosslinking to shorter chains present in the translation reaction (Fig. 2, lanes 22 and 23, see asterisks). The crosslinking of opsin TM1 to PAT-10 was also observed with



**Fig. 2.** The exit of TM1 from the ER translocon is facilitated by C-terminal TMs in the opsin nascent chain. A set of integration intermediates containing only opsin TM1 with a single cysteine probe [Cys56] were analysed by crosslinking with BMH as described for Fig. 1. The remainder of the opsin sequence including the C-terminal TMs was replaced with hydrophilic sequence derived from preprolactin. Nascent chain lengths identical to those studied with opsin were investigated, and all symbols are as defined in the legend to Fig. 1.

OPTM1PPL[Cys56] chains of 150 residues or more, although the levels of PAT-10 adduct were generally reduced with this precursor (cf. Figs 1 and 2, see adduct P; data not shown). As for the authentic opsin chain (Meacock et al., 2002), if integration intermediates of OPTM1PPL were released from the ribosome before BMH treatment, no adducts with Sec61 $\alpha$ , Sec61 $\beta$  or PAT-10 were observed consistent with the exit of the TM from the ER translocon (data not shown).

Taking the data presented in Figs 1 and 2 together, we conclude that the presence of the TM domains at the C-terminal side of opsin TM1 directly influences its exit from the Sec61 complex.

### TM2 is transiently associated with the ER translocon

The alteration in the behaviour of opsin TM1 when studied in the context of OPTM1PPL raises the question of the relative location of the other TMs that are present in opsin integration intermediates that possess multiple TMs. We therefore analysed the environment of opsin TM2 using a single cysteine probe located at residue 89, a site previously shown to generate distinct adducts with both Sec61 $\alpha$  and Sec61 $\beta$  (Meacock et al., 2002). When an OP140 integration intermediate was analysed, authentic adducts with Sec61 $\alpha$  and Sec61 $\beta$  were seen (Fig. 3, lanes 2-5) and crosslinking to Sec61 $\alpha$  was maintained with OP164 (Fig. 3, lanes 7-10), although adduct formation with Sec61 $\beta$  was significantly diminished. By a chain length of 204 residues, no adducts with any Sec61 subunit were detected (Fig. 3, lanes 13-15) and TM2 had presumably fully exited the ER translocon.

### TM3 has engaged the ER translocon in the OP164 intermediate

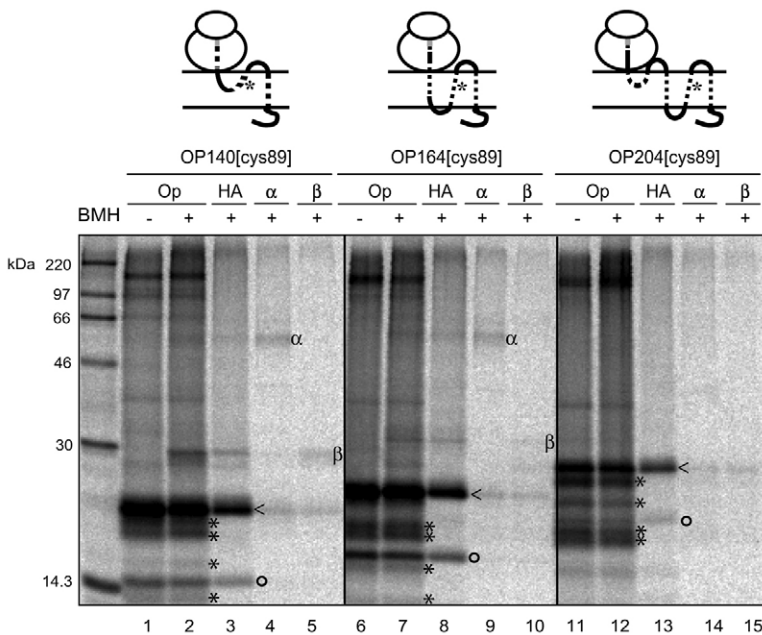
Having identified OP164 as a point at which both TM1 and

TM2 could be crosslinked to the Sec61 $\alpha$  subunit (see Figs 1 and 3), we therefore investigated the environment of TM3 at this chain length. Since we had not previously examined TM3 by crosslinking (Meacock et al., 2002), we first investigated the effect of probe location upon crosslinking efficiency. The high resolution structure of bovine rhodopsin indicates that TM3 is rather long, comprising residues 107-139 (Palczewski et al., 2000), and we tested three cysteines (residues 115, 124 and 132) spread out across this region. We adopted this strategy, since our previous work suggested that small changes in probe location did not influence the ability of the nascent chain to be crosslinked to subunits of the Sec61 complex (Meacock et al., 2002). The proximity of the nascent chain to subunits of the Sec61 complex was then established by BMH-dependent crosslinking using OP164 integration intermediates with a C-terminal HA epitope tag. OP164[Cys115] showed discrete adducts with both Sec61 $\alpha$  and Sec61 $\beta$  (Fig. 4, lanes 2-5) whereas OP164[Cys124] showed significant crosslinking to only Sec61 $\beta$  (Fig. 4, lanes 7-10), and OP164[Cys132] was not crosslinked to either subunit of the Sec61 translocon (Fig. 4, lanes 12-15). These data are entirely consistent with previous studies, and suggest that the regions of opsin TM3 containing Cys115 and Cys124 are adjacent to subunits of the Sec61 complex. By contrast, the region containing Cys132 is probably located close to the ribosomal exit tunnel and is not sufficiently exposed to form adducts with the ER translocon (Heinrich and Rapoport, 2003; Meacock et al., 2002). On the basis of this analysis, we conclude that at a chain length of 164 residues, opsin TM3 has begun to engage the ER translocon. No adducts of OP164 with the TRAM protein could be detected (data not shown) suggesting that like TM1, TM3 does not come into close proximity with this component (Meacock et al., 2002).

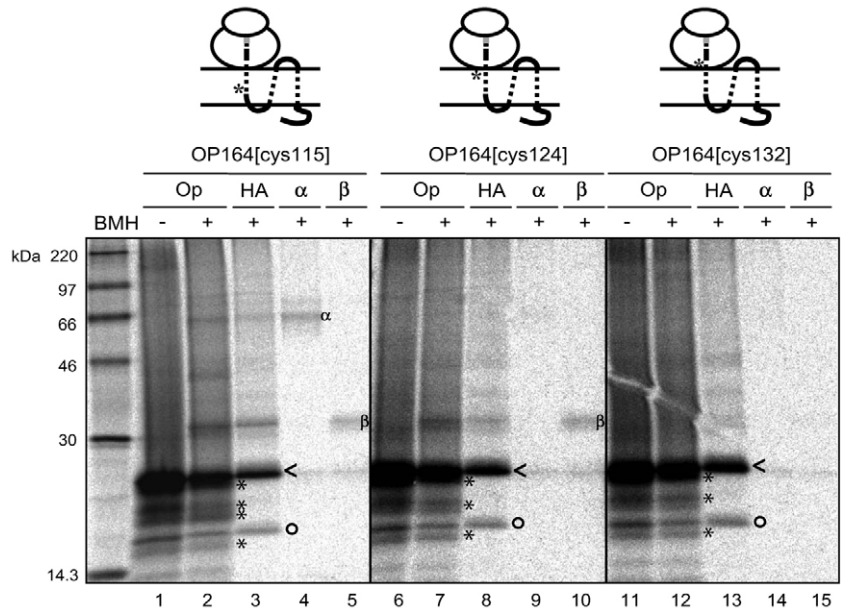
### TM3 vacates the ER translocon upon chain extension

Having established that a cysteine probe located at residue 115 generated discrete adducts with two subunits of the Sec61 complex when present in the OP164 integration intermediate, the environment of this probe at different chain lengths was investigated. When an OP150[Cys115] intermediate was analysed, clear adducts with Sec61 $\beta$  (Fig. 5A, lanes 2, 3 and 5,  $\beta$ ) and a putative ribosomal protein of ~21 kDa (Fig. 5A, lanes 2 and 3, R) were observed. This is consistent with our previous suggestion that the cytosolic domain of Sec61 $\beta$  is close to the exit site of the large ribosomal subunit (Laird and High, 1997). The crosslinking of OP164[Cys115] was as previously described (cf. Fig. 5A, lanes 7-10 with Fig. 4, lanes 2-5) and the crosslinking of the slightly longer OP174[Cys115] intermediate was very similar (Fig. 5A, lanes 12-15). By contrast, when OP204[Cys115] and OP259[Cys115] were analysed, no authentic adducts with Sec61 subunits that contained the C-terminal HA epitope tag were observed (Fig. 5A, cf. lanes 17-21 and 23-27).

The extension of opsin from 164 residues to 204 residues has two consequences, firstly the chain is 40 amino acids longer and secondly TM4



**Fig. 3.** TM2 exit from the Sec61 complex. BMH-dependent crosslinking was performed using opsin integration intermediates of 140, 164 and 204 residues containing a single cysteine probe at position 89 within TM2 (Meacock et al., 2002). All symbols are as previously defined.



**Fig. 4.** A scanning analysis reveals that TM3 is adjacent to ER translocon components in the OP164 intermediate. Membrane associated OP164 integration intermediates with a single cysteine probe at residues 115, 124 or 132 within TM3 were treated with BMH and the resulting adducts analysed following immunoprecipitation as previously described. Schematic representations of the integration intermediates with the relative location of the cysteine probe (indicated by an asterisk) are presented above the gels and all symbols are as previously stated.

(Palczewski et al., 2000) is fully synthesised and predicted to be largely outside the ribosome (see Fig. 5A, model integration intermediates). In order to establish whether TM4 was now in close proximity to the Sec61 translocon, we analysed the crosslinking pattern of the OP204 chain using a single cysteine probe located at residue 154 near the start of TM4. BMH-dependent crosslinking of OP204[Cys154] showed a discrete adduct with Sec61 $\beta$  that was immunoprecipitated by  $\alpha$ OP and  $\alpha$ HA sera (Fig. 5B, lanes 2, 3 and 6,  $\beta$ ). We therefore conclude that at a chain length of 204 residues, a point at which TM3 appears to have left the ER translocon, opsin TM4 has begun to engage the Sec61 complex.

#### TM3 relocation is independent of subsequent TMs

As with our analysis of TM1 relocation relative to the Sec61 complex, we investigated whether the loss of Sec61 crosslinking to the Cys115 probe in TM3 was dependent upon the synthesis of subsequent TMs, consistent with the displacement of one TM from the ER translocon by the entry of another as it is synthesised. We therefore replaced the polypeptide on the C-terminal side of opsin TM3 with a region of the secretory protein preprolactin (denoted OPTM1-3PPL) and analysed the environment of opsin TM3 using integration intermediates of 164 and 204 residues. The crosslinking pattern of the OPTM1-3PPL164[Cys115] integration intermediate was almost identical to that of the OP164[Cys115] intermediate (cf. Fig. 6, lanes 1-5 with Fig. 5A, lanes 6-10). This is consistent with the fact that the amino acid sequences are virtually identical for these two shorter integration intermediates (see Table 1).

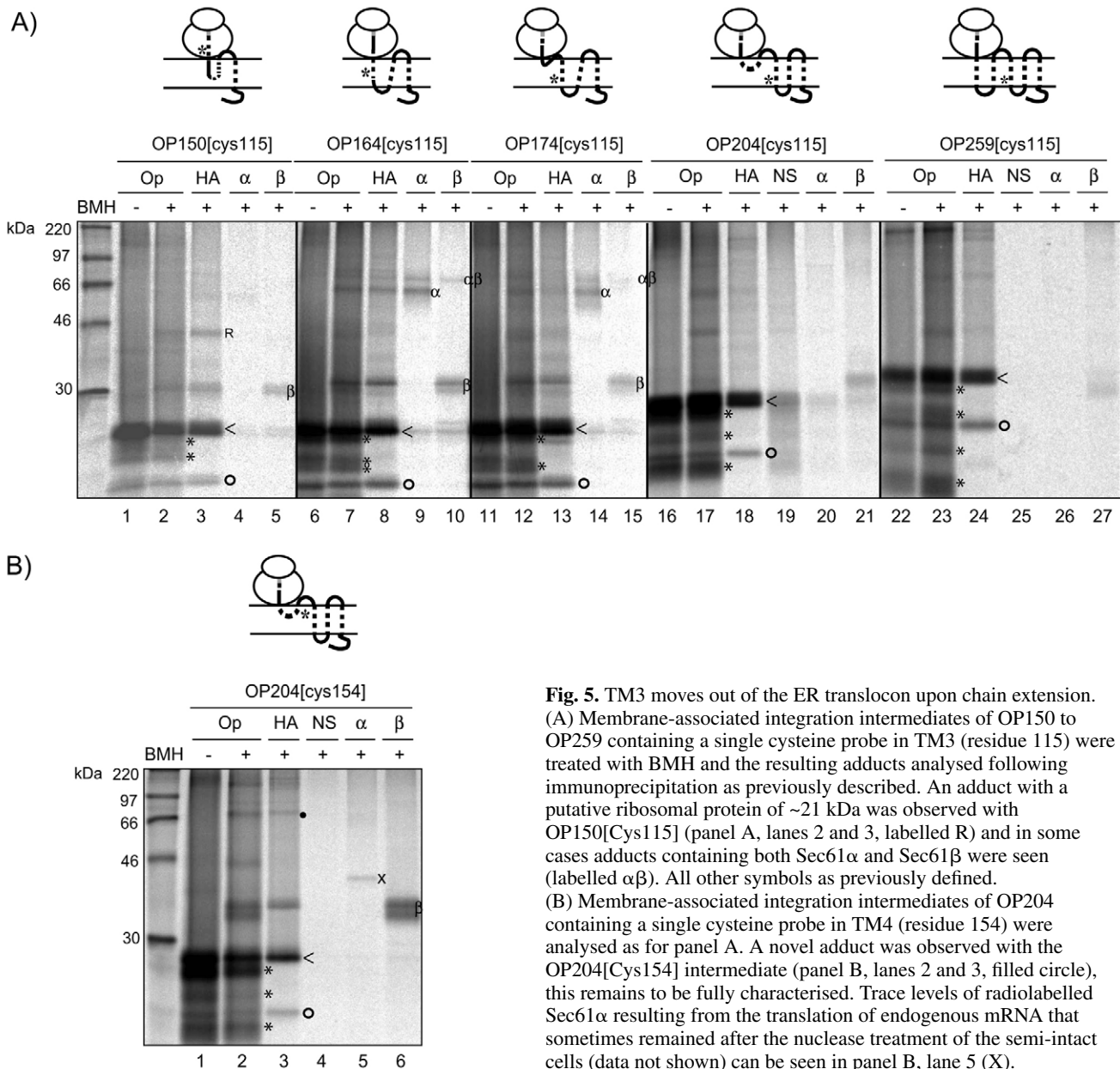
When the chain length of the OPTM1-3PPL intermediate was extended by 40 residues to mimic that analysed with the opsin nascent chain, OPTM1-3PPL204[Cys115] was found to show no authentic crosslinking to either subunit of the Sec61 complex and no candidate adducts bearing a C-terminal HA epitope tag were found (Fig. 6, cf. lanes 8-10). Thus, in the case of opsin TM3, its exit from the Sec61 translocon can occur upon a chain extension of 40 residues independently of

whether or not the additional polypeptide includes opsin TM4 (cf. Table 1).

#### Opsin is associated with a single copy of the Sec61 complex during its integration

One of the assumptions implicit in a site-specific crosslinking analysis is that the environment of the nascent chain as a whole is independent of probe location. Thus, the environment of an OP164 intermediate should be the same whether a cysteine probe is in TM1 or TM3, allowing the data from these different probe locations to be used to generate a composite model. In order to specifically test this assumption, and fully characterise the composition of the ER translocon at which the nascent opsin chain integrates, we carried out a crosslinking experiment using selected opsin integration intermediates each containing two cysteine probes, one located in TM1 and the other in TM3. When OP164[Cys56,115] was subjected to BMH mediated crosslinking, the pattern of adducts reflected the results seen with the two single cysteine probes OP164[Cys56] and OP164[Cys115] (cf. Fig. 1, lanes 17-20, Fig. 4, lanes 2-5, Fig. 5, lanes 7-10 and Fig. 7, lanes 2-5). Thus, adducts with Sec61 $\alpha$ , Sec61 $\beta$  and PAT-10 were all clearly visible and recognised by both  $\alpha$ OP and  $\alpha$ HA sera (Fig. 7, lanes 2-5). Furthermore, a significant proportion of chains were present in a novel adduct of 42 kDa that reflects the crosslinking of a single opsin chain to both PAT-10 (from Cys56) and Sec61 $\beta$  (from Cys115) (Fig. 7, lane 5, adduct  $\beta$ +P). Hence, the bulk of the nascent chains appear to occupy the same environment irrespective of the location of the cysteine probes used to report their nearest neighbours.

In the context of the OP164 integration intermediate, we had already clearly established that both Cys56 and Cys115 can generate discrete adducts with Sec61 $\alpha$  (Fig. 1, lane 19 and Fig. 5A, lane 9). Hence, if the nascent OP164 chain is adjacent to multiple copies of the Sec61 $\alpha$  subunit, one would expect a proportion of the nascent chains to crosslink to two copies of Sec61 $\alpha$ , one from Cys56 and one from Cys115, analogous to the ability of these two probes to be



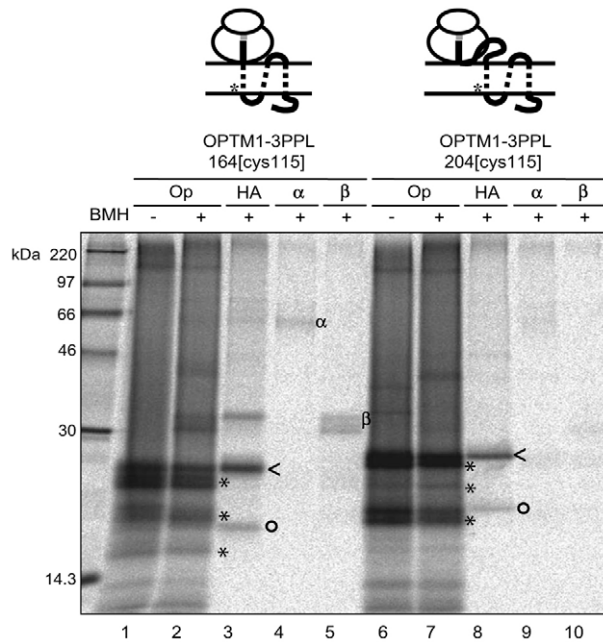
**Fig. 5.** TM3 moves out of the ER translocon upon chain extension. (A) Membrane-associated integration intermediates of OP150 to OP259 containing a single cysteine probe in TM3 (residue 115) were treated with BMH and the resulting adducts analysed following immunoprecipitation as previously described. An adduct with a putative ribosomal protein of ~21 kDa was observed with OP150[Cys115] (panel A, lanes 2 and 3, labelled R) and in some cases adducts containing both Sec61 $\alpha$  and Sec61 $\beta$  were seen (labelled  $\alpha\beta$ ). All other symbols as previously defined. (B) Membrane-associated integration intermediates of OP204 containing a single cysteine probe in TM4 (residue 154) were analysed as for panel A. A novel adduct was observed with the OP204[Cys154] intermediate (panel B, lanes 2 and 3, filled circle), this remains to be fully characterised. Trace levels of radiolabelled Sec61 $\alpha$  resulting from the translation of endogenous mRNA that sometimes remained after the nuclease treatment of the semi-intact cells (data not shown) can be seen in panel B, lane 5 (X).

simultaneously crosslinked to PAT-10 and Sec61 $\beta$  respectively. Although the apparent mobilities of crosslinking products can be variable, a doubly N-glycosylated OP164 chain crosslinked to two copies of Sec61 $\alpha$  would have a mobility of ~100 kDa. In fact, no evidence of any such larger Sec61 $\alpha$ -containing adduct is observed (Fig. 7, lane 4). An adduct of OP164[Cys56, 115] containing both Sec61 $\alpha$  and Sec61 $\beta$  is seen (Fig. 7, lanes 2-5, see adduct  $\alpha\beta$ ), but this product can also be observed with the OP164[Cys115] intermediate (Fig. 5A, lanes 7-10) and reflects the fact that the multiple cysteine residues present in Sec61 $\alpha$  enable it to be crosslinked to both the nascent chain and Sec61 $\beta$  via BMH (Meacock et al., 2002). As previously observed with the single cysteine probes, when the chain length was extended to OP204, discrete adducts with subunits of the Sec61 complex were no longer detectable, and only adducts with

PAT-10, presumably from Cys56 in TM1, remain (cf. Fig. 1, lanes 22-25, Fig. 5A, lanes 17-21 and Fig. 7, lanes 7-10). On the basis of these double probe studies, we can draw two conclusions: firstly, the TM regions of the opsin chain are adjacent to only a single copy of the Sec61 $\alpha$  subunit at this stage of its biosynthesis; and secondly, the PAT-10 protein is in close proximity to the functional Sec61 complex, since a single opsin chain of 164 residues can be crosslinked to both PAT-10 and Sec61 $\beta$  at the same time.

### Discussion

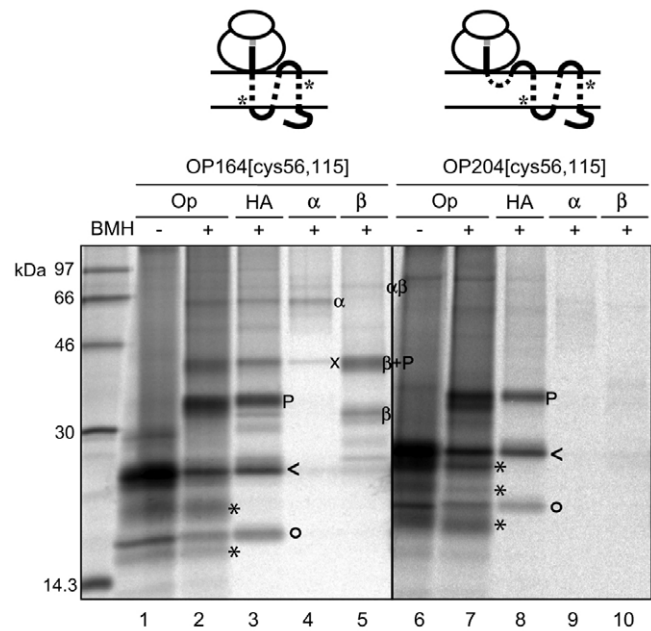
We have used site-specific crosslinking to characterise the molecular environment of TM1 and TM3 of opsin at different stages of its biosynthesis in order to investigate how the TM domains of a polytopic membrane protein are integrated into the ER membrane.



**Fig. 6.** Opsin TM3 exit from the ER translocon is independent of subsequent TM domains. OPTM1-3PPL was constructed by replacing the region of opsin located on the C-terminal side of TM3 with hydrophilic sequence derived from preprolactin. Membrane associated integration intermediates of 164 and 204 residues with a single cysteine probe located in TM3 (residue 115, cf. Fig. 5A) were then subjected to BMH-dependent crosslinking and the resulting adducts recovered by immunoprecipitation and analysed by SDS-PAGE. All symbols are as previously defined.

By using a C-terminal HA epitope tag, we are able to identify adducts with defined integration intermediates, and thereby simplify the analysis of longer polypeptides that can generate multiple adducts (Meacock et al., 2002). On the basis of several previous studies, we propose that the loss of crosslinking to Sec61 $\alpha$  and Sec61 $\beta$  that we observe probably reflects the entry of a TM into a primarily phospholipid-mediated environment at, or beyond, the periphery of the ER translocon (Heinrich and Rapoport, 2003; Martoglio et al., 1995; McCormick et al., 2003; Meacock et al., 2002; Mothes et al., 1997; Sauri et al., 2005). In the case of opsin TM1, an association with PAT-10 is also maintained throughout the process of membrane integration, whereas for TM2 and TM3 no association with known ER proteins was detected after the loss of crosslinking to subunits of the Sec61 complex.

The use of single cysteine probes in combination with thiol-specific homobifunctional crosslinking reagents has provided insight into several aspects of protein biosynthesis at the ER (Heinrich and Rapoport, 2003; Meacock et al., 2002; Oliver et al., 1997). However, this approach can only identify adjacent proteins that contain a suitable cysteine residue. Hence, an alternative explanation for the loss of adduct formation from a particular probe within a nascent chain is that a component is still adjacent, but that its cysteine can no longer be crosslinked (Laird and High, 1997). However, although photocrosslinking approaches display far less specific requirements for adduct formation (Martoglio and Dobberstein, 1996), studies using this approach strongly support the view that a loss of



**Fig. 7.** Double probe analysis of the OP164 and OP204 intermediates. Integration intermediates containing two cysteine probes, the first in TM1 (residue 56) and the second in TM3 (residue 115), were analysed by the BMH-dependent crosslinking of two previously characterised chain lengths (OP164 and OP204). Adducts were identified by immunoprecipitation, and for OP164 a novel crosslinking product corresponding to the opsin nascent chains simultaneously crosslinked to both Sec61 $\beta$  and PAT-10 was observed (lane 5,  $\beta$ +P). All other symbols are as previously defined.

crosslinking equates to the lateral exit of a TM domain from the ER translocon and into the lipid bilayer (Heinrich and Rapoport, 2003; Martoglio et al., 1995; McCormick et al., 2003; Meacock et al., 2002; Mothes et al., 1997; Sauri et al., 2005), and that this conclusion is valid for different types of crosslinking strategies (Heinrich and Rapoport, 2003).

In the case of opsin TM1, we find that this TM exits what we define as a phase I Sec61-based environment fairly quickly, as evidenced by a loss of adducts with Sec61 $\beta$  (cf. Fig. 8, phase I). We propose that this phase I location reflects TM1 occupying the core of the Sec61 complex (Van den Berg et al., 2004), because this pattern of adducts appears at an early stage of the membrane insertion process. The suggestion that proximity to Sec61 $\beta$  reflects an early stage of the integration process is underpinned by our earlier crosslinking studies (Laird and High, 1997), and by recent observations showing that small molecule inhibitors of vascular cell adhesion molecule 1 translocation lead to the specific accumulation of the precursor in proximity to the Sec61 $\beta$  subunit (Besemer et al., 2005; Garrison et al., 2005). An alternative possibility to the relocation of a TM domain during chain extension and accompanying membrane protein integration (cf. Fig. 8), is that the Sec61 translocon undergoes substantial conformational changes, and it is these rearrangements that lead to an alteration in the crosslinking partners of a particular TM within the nascent opsin chain (Van den Berg et al., 2004). In either case, the environment of the TM domain is altered in response to the synthesis of additional polypeptide chain.



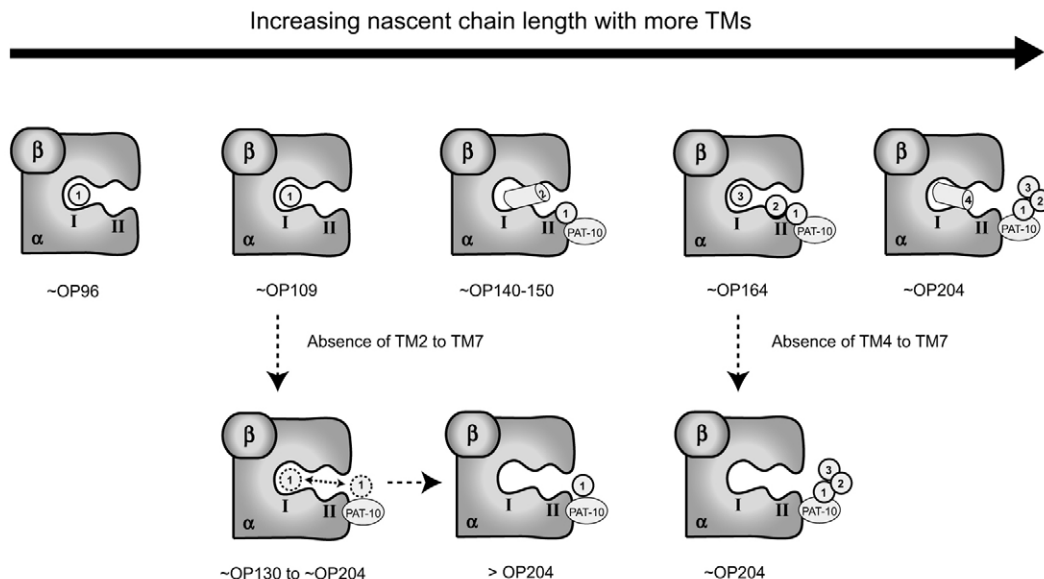
By the time that the nascent opsin chain has reached a length of 150 residues, adducts of TM1 with Sec61 $\alpha$  and PAT-10 are readily detected, but no crosslinking to Sec61 $\beta$  is seen. On this basis we conclude that this second phase of Sec61 $\alpha$  adduct formation represents a distinct association with the Sec61 complex (Sadlish et al., 2005), and have built a model to describe these different environments (see Fig. 8). This phase II environment in the Sec61 translocon, characterised by adducts with Sec61 $\alpha$  but not Sec61 $\beta$ , is maintained with the OP164 intermediate and, in the case of TM1, adducts with PAT-10 are also clearly maintained (Fig. 8, phase II). The phase II association of OPTM1 with the Sec61 complex is lost upon further chain extension to 204 residues.

Having carefully defined the behaviour of opsin TM1 in the context of the normal polypeptide chain, we examined its behaviour when the polypeptide at the C-terminus of TM1 was replaced by a hydrophilic region lacking any additional TM domains. This study very clearly showed that the movement of TM1 out of the phase I Sec61 environment was significantly delayed for OPTM1PPL chains and only completed at chain lengths of greater than 204 residues (Fig. 8). Thus, the exit of TM1 from the Sec61 translocon is promoted by the synthesis of the additional TM regions that are normally present in the polypeptide. The simplest explanation for this observation is that these additional TMs act to displace TM1 from the core of the Sec61 complex, freeing up the entry point into the ER translocon in order to accommodate the next TM in a polytopic protein (Sadlish et al., 2005). In the absence of any additional TMs, opsin TM1 displays a unique crosslinking pattern that shows adducts with both Sec61 $\beta$  and PAT-10. Our favoured interpretation of this behaviour is that opsin TM1 is in a

dynamic equilibrium such that it can reside in either the phase I or the phase II environment until significant additional chain extension (see Fig. 8).

The displacement model outlined above predicts that the relevant subsequent TM would occupy the Sec61 environment that had been vacated by the ousted TM, and for opsin TM1 our crosslinking analysis supports this view. Thus, we found that TM2 engages the Sec61 complex at OP140, remains in proximity at OP164 and has fully exited by a chain length of 204 residues (Fig. 8). Previous studies using opsin point mutants, and opsin fragments, indicate that non-covalent interactions between TM1 and TM2 contribute to the stability of the full-length protein and show that a fragment containing TM1 and TM2 is the minimum requirement to obtain stable membrane integration *in vivo* (Bosch et al., 2003; Heymann and Subramaniam, 1997). Taken together with our own data, we conclude that the authentic membrane integration of opsin TM1 and TM2 is a co-operative process where the two TMs mutually stabilise each other during and/or after integration into the lipid bilayer. This is consistent with studies of *E. coli* leader peptidase and a plant viral protein which suggest that the integration of two TMs at the ER translocon may be a concerted process (Heinrich and Rapoport, 2003; Sauri et al., 2005).

When the environment of TM3 was investigated, we found that it begins to engage the Sec61 machinery in the OP150 intermediate, and is fully associated with the Sec61 complex at chain lengths of 164 and 174 residues with a phase-I-like pattern of adducts. This is consistent with TM3 displacing TM2 from the ER translocon, and suggests that TM3 occupies the phase I environment after TM2 has left (see Fig. 8). Further



**Fig. 8.** Model for the integration of opsin TMs1-4. A schematic version of the view from the cytosolic face of the ER membrane showing the presumptive 'open' conformation of the Sec61 translocon is presented, based on the structure of the archaeal complex (Van den Berg et al., 2004). Only the  $\alpha$  and  $\beta$  subunits of the complex are represented because these are the components that can be crosslinked to various opsin integration intermediates, whereas the PAT-10 protein is as previously described (Meacock et al., 2002). Phase I crosslinking to Sec61 is equivalent to the point where adducts with Sec61 $\alpha$  and Sec61 $\beta$  are seen. Phase II crosslinking is displayed by TM1 and represents specific adducts with Sec61 $\alpha$  that appear after initial exit from the Sec61 complex. The relative location of each TM (numbered circle) is indicated at different stages of the integration process as deduced from the crosslinking patterns of specific integration intermediates. The fates of TM1 and TM3 when synthesised in the absence of the respective C-terminal TMs of the wild-type opsin chain are also illustrated.

extension to OP204 resulted in the loss of authentic adducts with Sec61 $\alpha$  and Sec61 $\beta$  consistent with the complete exit of TM3 from the Sec61 translocon at this point (Fig. 8).

When the environment of TM4 was analysed in the OP204 intermediate (Cys154), we found it had started to engage the Sec61 translocon as evidenced by crosslinking to Sec61 $\beta$ . Thus, in the OP204 integration intermediate, TM4 appears to engage the phase I location of the Sec61 complex that has been vacated by TM3 (Fig. 8). However, in marked contrast to the behaviour of TM1, when the lateral exit of opsin TM3 was investigated we found that the substitution of the polypeptide on the C-terminal side of opsin TM3 with hydrophilic polypeptide had no effect upon its exit from the phase I Sec61 environment (Fig. 8). Thus, although our data suggest that TM1 is displaced from the Sec61 translocon by TM2, we find that TM3 is rapidly released independently of any requirement for TM4 synthesis. On this basis, we believe that a 'one-in, one-out' model can best describe the membrane integration of opsin TM1-TM3 through the Sec61 complex, but conclude that the driving force for the lateral exit of TM1 and TM3 is distinct (Fig. 8).

One factor that can clearly influence the behaviour of the TM domains within a polytopic protein is the length of the hydrophilic loops that connect the TMs together (Sauri et al., 2005). However, in the case of opsin all of the extramembranous loops are relatively short including those linking TM1 to TM2 and TM3 to TM4, suggesting that this is unlikely to be the basis of the differences in the integration of TM1 and TM3 that we observe. An alternative basis for this difference is that the ability of opsin TM1-TM3 to behave as an independent folding domain (Ridge et al., 1995) may provide the driving force for TM3 exit by favouring its assembly with TM1-TM2 over its residency in the Sec61 translocon. In the case of TM1, no other TMs are immediately available for such assembly and it may be that PAT-10 acts to stabilise TM1 during the assembly of opsin TMs (Meacock et al., 2002).

We were able to exploit double cysteine probes to establish that a single nascent opsin chain (OP164) is adjacent to both Sec61 $\beta$  and PAT-10 at the same stage of membrane integration. Thus, although the identity of PAT-10 remains to be established (Meacock et al., 2002), we conclude that it is in close proximity to the functional Sec61 complex, and hence probably involved in integration and/or translocation or a closely associated process (see Alder and Johnson, 2004). The issue of how many Sec61 channels constitute an active ER translocon remains contentious. Hence, although the native ER translocon contains four copies of the Sec61 heterotrimer (Menetret et al., 2005), high resolution structural studies of the Sec61 complex, and functional studies of the related SecY translocon, suggest that a single Sec61 heterotrimer acts as the protein-conducting channel (Cannon et al., 2005; Menetret et al., 2005; Van den Berg et al., 2004). By contrast, the most recent reconstruction based on an active SecY translocon indicates that two copies of the Sec61 heterotrimer represent an active translocon (Mitra et al., 2005). Our double-probe studies could only detect a single copy of the Sec61 $\alpha$  subunit adjacent to an OP164 intermediate with cysteines located at two positions, each known to independently generate adducts with Sec61 $\alpha$ . This result supports the idea that a single Sec61 complex is the active ER translocon, and we also conclude that both the phase

I and phase II Sec61-based environments defined in this study are located within a single copy of the Sec61 heterotrimer (Sadlish et al., 2005).

This study has confirmed that the biogenesis of polytopic membrane proteins is remarkably complex, with different TMs having quite distinct requirements for their integration into the ER membrane. Precisely why different TMs display such variable behaviour remains to be fully established, and the intrinsic biophysical properties of a TM such as its hydrophobicity will clearly be an important factor (Bowie, 2005; Hessa et al., 2005). However, other features including the relative location of one TM with respect to the others in the chain (Meacock et al., 2002), cooperation between TMs (Heinrich and Rapoport, 2003; Sadlish et al., 2005; Sauri et al., 2005) and the interaction of distinct TMs with different accessory proteins, including TRAM and PAT-10 (Meacock et al., 2002; Ridge et al., 1995; Sadlish and Skach, 2004), may also influence the integration process.

## Materials and Methods

The crosslinking reagent, bismaleimidoethane (BMH), was purchased from Perbio Science (Chester, UK). T7 RNA polymerase, SP6 RNA polymerase, transcription reagents and nuclease-treated rabbit reticulocyte lysate were obtained from Promega (Southampton, UK). The cap analogue m7G(5')ppp(5')G was purchased from NEB (Hitchin, UK) and Easytag L-[<sup>35</sup>S]methionine/cysteine was from PerkinElmer Life Sciences (Stevenage, UK). All reagents for cell culture were obtained from Invitrogen (Paisley, UK). All other chemicals and reagents were purchased from Merck (Poole, UK) and Sigma Chemical (Poole, UK). All sera used are rabbit polyclonal unless otherwise specified. Antisera specific for the Sec61 $\alpha$  and Sec61 $\beta$  subunits were gifts from Richard Zimmerman (University of Saarland, Homburg, Germany) and Bernhard Dobberstein (ZMBH, Heidelberg, Germany) respectively. The mouse monoclonal antibody specific for the N-terminus of bovine opsin (Adamus et al., 1991) was a gift from Paul Hargrave (University of Florida, FL).

## Opsin constructs

Single or double cysteine residues were introduced into a cysteine-null version of bovine opsin (Meacock et al., 2002) using the QuikChange site-directed mutagenesis kit (Stratagene, Cambridge, UK). OPTM1PPL constructs were obtained by combining residues 1-70 of bovine opsin and residues 31-229 of preprolactin (i.e. from the first residue after the point of preprolactin signal sequence cleavage), followed by a cysteine substitution at residue 56. OPTM3PPL constructs were obtained by combining residues 1-142 of bovine opsin and 31-229 of preprolactin, followed by a cysteine substitution at residue 115. Both opsin/preprolactin constructs were cloned into a pSPUTK vector downstream of an SP6 RNA polymerase promoter. PCR was used to generate truncated sections of the opsin coding region that could be used as DNA templates for in vitro transcription. For opsin, the forward primer was located 160 bases 5' of the T7 RNA polymerase promoter of the pGEM3z vector. For the opsin/preprolactin chimeras, the forward primer was located 147 bases 5' of the SP6 RNA polymerase promoter of the pSPUTK vector. The reverse primers were designed to generate truncated nascent chains of 96, 109, 140, 150, 164, 174, 204 and 259 residues, including a nine residue C-terminal HA tag. The PCR reaction mixture was treated with *DpnI* (200 U/ml) for 2 hours at 37°C to remove the methylated parental DNA template. The PCR products were then purified using a QIAquick PCR purification kit (Qiagen, Crawley, UK).

## In vitro transcription and translation

Transcriptions were performed with T7 or SP6 RNA polymerase as described (NEB) and the RNA obtained was purified with an RNeasy Mini Kit (Qiagen). Cultured HT1080 fibroblasts (European Collection of Cell Cultures, Salisbury, UK) were permeabilised with digitonin as previously described (Wilson et al., 1995) and used as a source of ER-derived membranes for all experiments. Translations were performed in a rabbit reticulocyte lysate system for 15 minutes at 30°C in the presence of semi-permeabilised HT1080 cells and [<sup>35</sup>S]methionine/cysteine mix (0.82  $\mu$ Ci/ $\mu$ l final) as described by the manufacturer (Promega). Further initiation of translation was then inhibited by the addition of aurintricarboxylic acid to 0.1 mM for 10 minutes at 30°C and translation was then terminated by the addition of cycloheximide to 2 mM, which stabilised the formation of ribosome-bound integration intermediates. Samples were placed on ice for 5 minutes before the membrane fraction was recovered by centrifugation at 16,000 g for 30 seconds. The resulting pellet was washed twice in KHM buffer (110 mM potassium acetate, 20 mM HEPES, 2 mM magnesium acetate, pH 7.2) and resuspended in KHM buffer before crosslinking reactions.

### Crosslinking and immunoprecipitation

The crosslinking reagent, BMH, was prepared in dimethylsulphoxide (DMSO) as a 20 mM stock solution. For crosslinking, the resuspended membrane fraction was incubated with BMH to a final concentration of 1 mM for 10 minutes at 30°C, whereas for the solvent control DMSO alone was added. 2-mercaptoethanol was added to a final concentration of 5 mM to quench the crosslinking reaction, RNase A (250 µg/ml) was added, and samples incubated at 37°C for 5 minutes to remove any tRNA still attached to the nascent chains. Denaturing immunoprecipitation was then performed in the presence of SDS as previously described (Meacock et al., 2002).

### Sample analysis

All samples were solubilised in SDS-PAGE sample buffer at 37°C for 30 minutes and the radiolabelled products then resolved on 14% polyacrylamide Tris-glycine gels. The gels were then fixed, dried and exposed to a phosphorimage plate for visualisation using a Fuji BAS1800 phosphorimager.

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