

Protein translocation across the endoplasmic reticulum membrane in cold-adapted organisms

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Summary

Secretory proteins enter the secretory pathway by translocation across the membrane of the endoplasmic reticulum (ER) via a channel formed primarily by the Sec61 protein. Protein translocation is highly temperature dependent in mesophilic organisms. We asked whether the protein translocation machinery of organisms from extremely cold habitats was adapted to function at low temperature and found that post-translational protein import into ER-derived microsomes from Antarctic yeast at low temperature was indeed more efficient than into mesophilic yeast microsomes. Analysis of the amino-acid sequences of the core component of the protein translocation channel, Sec61p, from Antarctic yeast species did not reveal amino-acid changes potentially adaptive for function in the cold, because the sequences were too divergent. We therefore analyzed Sec61 α (vertebrate Sec61p) sequences and protein translocation into the ER of Antarctic and Arctic fishes and compared them to Sec61 α and protein translocation into the ER of temperate-water fishes and mammals. Overall, Sec61 α is highly conserved amongst these divergent taxa; a number of amino-acid changes specific to fishes are evident throughout the

protein, and, in addition, changes specific to cold-water fishes cluster in the luminal loop between transmembrane domains 7 and 8 of Sec61 α , which is known to be important for protein translocation across the ER membrane. Secretory proteins translocated more efficiently into fish microsomes than into mammalian microsomes at 10°C and 0°C. The efficiency of protein translocation at 0°C was highest for microsomes from a cold-water fish. Despite substantial differences in ER membrane lipid composition, ER membrane fluidity was identical in Antarctic fishes, mesophilic fishes and warm-blooded vertebrates, suggesting that membrane fluidity, although typically important for the function of the transmembrane proteins, is not limiting for protein translocation across the ER membrane in the cold. Collectively, our data suggest that the limited amino-acid changes in Sec61 α from fishes may be functionally significant and represent adaptive changes that enhance channel function in the cold.

Key words: Sec61 channel, ER lipid composition, Secretion, Antarctic fish, Antarctic yeast

Introduction

Protein secretion is an essential process in prokaryotes and eukaryotes (Matlack et al., 1998). Protein synthesis takes place in the cytoplasm, therefore secretion requires one protein translocation step, directly across the plasma membrane in prokaryotes, and across the ER membrane in eukaryotes (Johnson and van Waes, 1999; Matlack et al., 1998). The protein translocation channel in the ER membrane is composed of three subunits: Sec61 α , Sec61 β and Sec61 γ (Sec61p, Sbh1p, and Sss1p in yeast) (Johnson and van Waes, 1999). These proteins form heterotrimers, several of which assemble in the ER membrane in response to the presence of a functional signal sequence to form the protein translocation channel for secretory proteins (Matlack et al., 1998). The principal channel subunit, Sec61 α , contains 10 transmembrane domains, which line the channel (Johnson and van Waes, 1999; Matlack et al., 1998). It is currently unknown how the channel opens and closes. One possibility is a conformational change in Sec61 α , but no high-resolution structure of Sec61 α is available, and mobile regions in the protein have not yet been identified. In

addition, the Sec61 channel in the ER membrane mediates export of misfolded proteins from the ER to the cytosol for degradation (Römisch, 1999). The mechanism of channel opening from the luminal side for export is also not understood.

Protein translocation across biological membranes is dependent on temperature and membrane lipid composition (Baker et al., 1988; Leheny and Theg, 1994; Nilsson et al., 2001). In mesophilic organisms this process is strongly inhibited by low temperatures, yet 80% of life on earth exists at temperatures below 4°C, suggesting that the protein translocation machinery of these organisms has been adapted to permit function in the cold (Baker et al., 1988). Protein translocation channel subunits from psychrophiles have not been characterized so far. In soluble cold-adapted enzymes, the observed amino-acid changes in many cases lead to increased flexibility of hinge regions, which allow the enzymes to undergo conformational changes necessary for activity at low temperatures (Gianese et al., 2001; Russell, 2000).

Transmembrane proteins intimately interact with the lipids

of the membrane into which they are integrated, and their function is often dependent on membrane lipid composition (Fyfe et al., 2001). Nilsson and coworkers showed recently that increasing the concentration of cholesterol in dog pancreas microsomes, which putatively increases membrane rigidity, strongly inhibits translocation of proteins into the microsomal lumen (Nilsson et al., 2001). Membrane lipid composition is known to vary with the body temperature of organisms (Hazel, 1995; Logue et al., 2000). Membranes from cold-adapted animals usually contain higher proportions of unsaturated fatty acids, resulting in increased membrane fluidity to offset the rigidifying effects of the cold (Hazel, 1995). Thus membranes of organisms from different thermal environments may have similar fluidities when measured at their respective body temperatures, as seen in brain synaptic vesicles from an Antarctic fish, temperate fishes and mammals (Logue et al., 2000). Cold-adaptation of the ER translocation machinery may therefore also be a function of the physical structure of the lipid bilayer in which it is integrated.

In this study, we try to elucidate some of the complex structure-function relationships of the protein translocation channel in the ER membrane and its lipid environment by comparing protein translocation efficiencies into the ER, Sec61 protein primary structures and ER lipid compositions of select organisms from extremely cold habitats and their mesophilic counterparts. Specifically, we examined post-translational protein translocation into the ER of Antarctic and mesophilic yeasts over a range of temperatures to establish that psychrophilic yeasts are indeed more efficient at translocation in the cold. Although several factors required for protein translocation across the ER membrane may be cold sensitive, we focused our subsequent analysis on the pore-forming component of the protein translocation channel, Sec61p or Sec61 α , because channel opening is essential for protein entry into the secretory pathway and has recently been shown to involve a conformational change in the channel (Beckmann et al., 2001). We obtained and sequenced *SEC61* cDNAs from Antarctic yeast in order to identify amino-acid changes potentially responsible for improved protein translocation channel function at low temperature. In order to better distinguish between changes due to phylogenetic divergence and those due to cold adaptation, we further sequenced *SEC61* cDNAs from Antarctic and Arctic fishes. Fish have substantially longer generation times than yeast and are therefore expected to have a lower degree of sequence divergence from mesophilic *SEC61*, and thus fewer changes that are not related to function. To examine Sec61 channel function in vertebrates, we post-translationally translocated a truncated, ribosome-bound, secretory protein into the ER of an Antarctic fish, a temperate fish, and a mammal over a temperature range. In addition, we assessed the potential influence of lipid bilayer properties on protein translocation across the ER membrane by comparative analyses of lipid composition and membrane order of liver microsomes from vertebrates spanning a wide range of body temperatures, from Antarctic fish to mammals.

Materials and Methods

RNA and cDNA preparation, sequencing and alignments

RNA from yeast was isolated using TRIzol (Invitrogen) and bead

beating, and mRNA from 200-400 μ g total RNA using the MicroPoly(A)Pure kit (Ambion). RNA from fish liver was purified using the Ultraspec RNA isolation system (Biotecx). cDNA was synthesised and *SEC61* sequences amplified using the Marathon cDNA Amplification Kit (Clontech), or the SuperscriptII Preamplification System plus 5'RACE and 3'RACE kits (Invitrogen). Amplified *SEC61* products were directly sequenced, or cloned and sequenced. Sequences were aligned using Multalin (Corpet, 1988) with the following parameters: Symbol comparison table: blosum62; gap weight:12; gap length weight: 2.

Preparation of liver microsomes, lipid analysis, and fluidity measurements

Liver microsomes from Antarctic *Dissostichus mawsoni* (caught and held at -1.6°C), trout (acclimated to 10°C), carp (25°C) and rat were prepared as described previously (Logue et al., 1998). Membrane fluidity was determined by measurements of fluorescence anisotropy using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a probe (Logue et al., 2000). Total lipid extraction and fatty acid analysis were performed using established methods (Logue et al., 1998). Microsomes for translocation experiments: dog pancreas microsomes were a gift from Stephen High (University of Manchester). Carp microsomes were prepared from livers of animals acclimated to 17°C .

Yeast strains and microsomes

The *Saccharomyces cerevisiae* wild-type strain used in this study was RSY255 (Stirling et al., 1992). Antarctic yeasts *Cryptococcus adeliensis* (CBS8351), *C. antarcticus* (CBS7687), and mesophilic *C. laurentii* (CBS942) were obtained from the CBS culture collection, Utrecht, The Netherlands (Garancis et al., 1970; Scorzetti et al., 2000; Vishniac and Kurtzman, 1992). Microsomes were prepared from cells grown to OD₆₀₀ of 3.0-5.0 in YPD at 15°C (Antarctic species) or 24°C (*C. laurentii*, *S. cerevisiae*); under these conditions, the generation times were 10 hours for *C. antarcticus* and *C. adeliensis*, 2 hours for *C. laurentii* and 1.7 hours for *S. cerevisiae*. Cells were lysed by liquid nitrogen lysis, and microsomes isolated by differential centrifugation as described elsewhere (Lyman and Schekman, 1995).

Electron microscopy

C. antarcticus, *C. adeliensis* grown at 15°C and *C. laurentii* grown at 24°C to OD₆₀₀ of 4.0 in YPD were fixed in 0.1 M cacodylate and processed for standard electron microscopy.

Translocation assays

Into yeast microsomes: in vitro translated, radiolabelled mutant alpha-factor precursor (p Δ gp ϕ ; 4 μ l=10⁶ cpm per 20 μ l reaction) was translocated into yeast microsomes in the presence of ATP and an ATP-regenerating system at the indicated temperatures for 15 minutes. Microsomal protein in the reactions (2-5 μ g) was limiting for translocation under these conditions. Reactions were terminated by addition of an equal volume 20% trichloro-acetic acid, protein samples analyzed by SDS-PAGE on 18% acrylamide/4 M urea gels, and individual bands were quantified with a phosphorimager (Packard). Translocation was defined as a percentage of signal-cleaved alpha-factor precursor. Protease-protection of the signal cleaved form was at least 90%.

Into vertebrate microsomes: plasmid pSPB4 was linearized within the preprolactin coding sequence using *Pvu*II and a truncated mRNA encoding the N-terminal 86 amino acids of preprolactin transcribed using SP6 polymerase (Siegel and Walter, 1988). PPL86 was synthesized in reticulocyte lysate (Promega) at 25°C for 15 minutes in the presence of [³⁵S]-methionine. Translations were transferred to ice and the translation terminated by adding cycloheximide to a final

concentration of 250 μM . Limiting amounts of membranes (2 μg protein) from *D. mawsoni*, *C. carpio* or *C. familiaris* were added to 15 μl in vitro translations, and reactions were incubated for 10 minutes at the indicated temperatures (targeting). Then puromycin was added to 1 mM final concentration, and incubation continued for 10 minutes at the indicated temperatures (translocation). Membranes were sedimented (4 minutes, 4°C, 16,000 g), taken up in SDS sample buffer and radiolabelled proteins resolved by SDS PAGE and analyzed using a phosphorimager (Perkin Elmer, Cambridge, UK). Translocation was assessed by quantifying signal-cleaved PL56. For protease protection, sedimented membranes were resuspended in 0.1 mg/ml Proteinase K and incubated on ice for 20 minutes. The signal-cleaved form was, on average, 85% protease-protected in these experiments with no significant differences between species. The signal-sequence-containing PPL86 associated with the membranes was >80% protease sensitive.

Quantitative immunoblotting

Equal amounts of *D. mawsoni* liver or dog pancreas microsomes were resolved on 12.5% SDS gels, transferred to nitrocellulose and Sec61 α detected using anti-dog Sec61 α antibodies (S. High) at 1:1000 dilution, followed by [¹²⁵I]-Protein A (Amersham) and quantitation using a phosphorimager.

Results

Post-translational protein translocation into the ER of Antarctic yeast at low temperature

We compared post-translational translocation efficiency into the ER in the Antarctic yeasts, *Cryptococcus antarcticus* and *Cryptococcus adeliensis*, and the mesophilic species, *Cryptococcus laurentii*, and *Saccharomyces cerevisiae* at 0°C, 10°C and 20°C. In contrast to vertebrates, yeast can import proteins into the ER post-translationally, and thus the effect of temperature on translocation can be determined independently of protein translation, which is itself affected by temperature. Since we measured ratios of protein translocation at different temperatures for each species, our conclusions are also independent of the concentration of protein translocation channels in the respective membranes and of the absolute amount of ER in the individual preparations.

Fig. 1 shows protein translocation into microsomes from *S. cerevisiae* and *C. adeliensis* grown at 24°C and 15°C, respectively. The upper panel shows a phosphorimager scan of an SDS-gel of cytosolic precursor (p $\Delta\text{gp}\alpha\text{f}$) and translocated, signal-cleaved $\Delta\text{gp}\alpha\text{f}$; the lower panel shows the percentage of translocation at different temperatures normalized to 20°C (Fig. 1). Translocation into *S. cerevisiae* microsomes is most efficient at 20°C; translocation at 10°C is approximately 1.8-fold lower, and translocation at 0°C is 3.1-fold lower than at 10°C, suggesting that the activity of the Sec61 channel in *S. cerevisiae* is optimal at its growth temperature in the wild. There is no further increase in translocation into *S. cerevisiae* ER at higher temperatures (data not shown) (Baker et al., 1988). The relative protein import into *C. laurentii* microsomes was similar to import into *S. cerevisiae* microsomes at the same temperatures (data not shown). In contrast, protein translocation into microsomes derived from the Antarctic *Cryptococcus* species, *C. antarcticus* and *C. adeliensis*, was approximately equal at 20°C and 10°C, and decreased by only a factor of 2 between 10°C and 0°C (Fig. 1; *C. antarcticus* data not shown). Microsomes prepared from Antarctic yeast grown

at 6°C showed no further improvement of translocation at low temperature. The absolute amount of translocation per μg of microsomal protein varied between microsome preparations, but the ratios of translocation at different temperatures remained identical.

Electron micrographs were taken, and a representative micrograph for each *Cryptococcus* species is shown in Fig. 2. Compared with its congeners, *C. antarcticus* has unusually large mitochondria, which can occupy a substantial fraction of the cell volume (Fig. 2, top). By contrast, the mesophilic *C. laurentii* and the psychrotolerant *C. adeliensis* display no substantial morphological differences (Fig. 2). On the basis of qualitative assessment of images taken from large numbers of cells at low magnification, all three species contain comparable amounts of ER cisternae. Taken together our results indicate that Antarctic yeast are more efficient at protein translocation across the ER membrane at low temperature than their mesophilic counterparts.

Primary structure of Sec61p in Antarctic yeast

Given the relatively high translocation efficiency at low temperature through the Sec61 channel into the ER of Antarctic yeast, we asked whether Sec61p had acquired amino-acid changes that may be adaptive to function in the cold. We obtained partial sequences of *SEC61* from *C. antarcticus* and *C. adeliensis* and compared them with the *SEC61* sequence

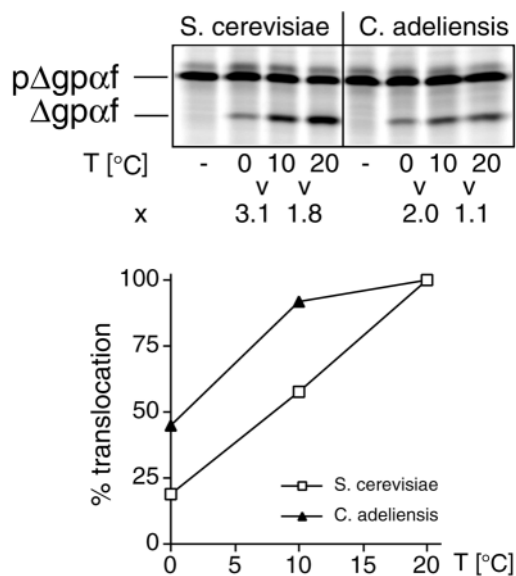


Fig. 1. Protein translocation into the ER of the Antarctic yeast *C. adeliensis* and the mesophilic yeast *S. cerevisiae*. Upper: limiting amounts of microsomal protein were incubated with excess radiolabelled secretory precursor (p $\Delta\text{gp}\alpha\text{f}$) for 0 (lanes 1, 5) or 15 minutes (all other lanes) at the indicated temperatures and samples analyzed by SDS-PAGE. Signal-cleavage ($\Delta\text{gp}\alpha\text{f}$) was used as a measure of translocation and was quantified using a phosphorimager. Signal-cleaved $\Delta\text{gp}\alpha\text{f}$ was >90% protease protected. Samples were taken in duplicate, and the experiment repeated twice. The difference between translocation at different temperatures is indicated (x). Lower: translocation at 20°C for each species was set to 100% to illustrate the relative decrease in translocation efficiency with decreasing temperature.

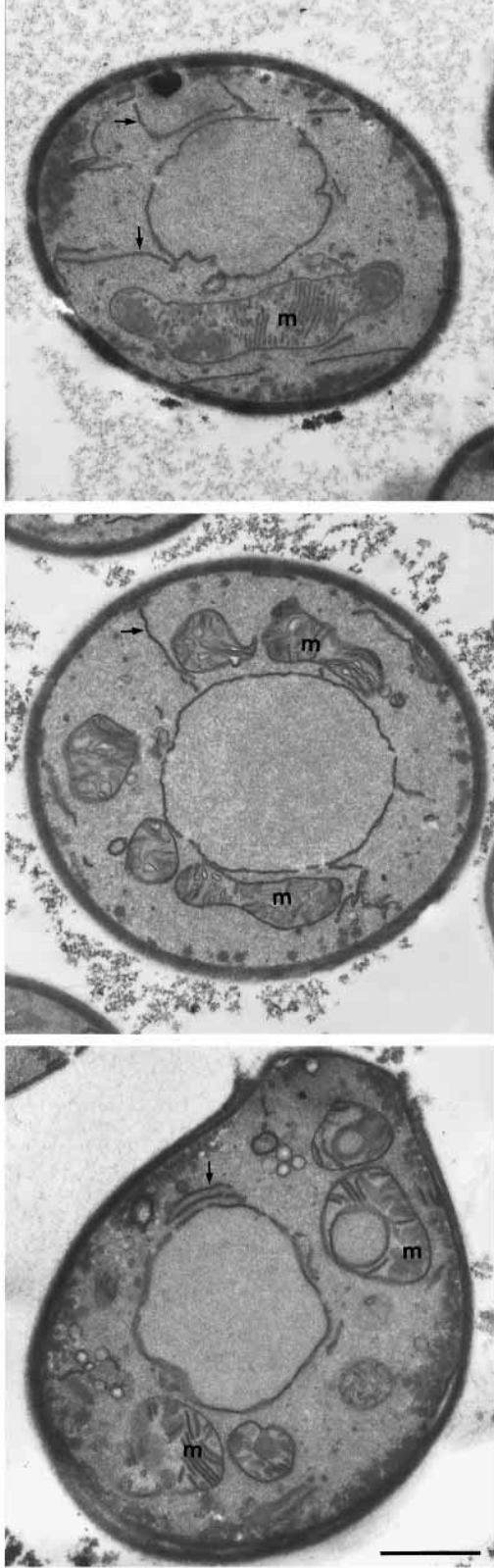


Fig. 2. Electron micrographs of Antarctic and mesophilic *Cryptococcus*. Upper: *Cryptococcus antarcticus*. The large mitochondria were typical for this species; they may provide a respiratory advantage at the extremely low habitat temperature of this organism. Middle: *Cryptococcus adeliensis*. Lower: *Cryptococcus laurentii*. Bar, 1 μ m. Mitochondria (m) and ER (arrows) are indicated.

from *C. laurentii*. Overall, the *SEC61* sequence was relatively divergent between mesophilic and Antarctic *Cryptococcus* species (84% amino acid identity over the 187 amino acid region sequenced); differences appear to have accumulated in the loop between transmembrane domains 7 and 8 (76% identity over 58 amino acids), but it is not immediately obvious which changes are of functional significance.

Comparison of *SEC61* genes from mesophilic and cold-adapted vertebrates

To identify amino-acid changes in Sec61p that are potentially adaptive to function in the cold, we analyzed *SEC61* cDNAs from cold-adapted organisms with substantially longer generation times than yeast. *SEC61* cDNAs from liver of four southern/Antarctic cold-water fish species (*Notothernia angustata*, *Pagothenia borchgrevinki*, *Harpagifer antarcticus*, *Dissostichus mawsoni*) and three northern/Arctic ones (*Hemirhamphus americanus*, *Gadus ogac*, *Boreogadus saida*) were obtained and sequenced, and translated sequences aligned with *SEC61* sequences from the temperate water fishes *Fugu rubripes*, *Oncorhynchus mykiss* (rainbow trout) and *Danio rerio* (zebra fish) and a mammal (*Mus musculus*, mouse) (Fig. 3). The Sec61 α protein sequence and length across these highly divergent vertebrate taxa are remarkably conserved (Fig. 3). Compared to the mouse sequence we observed a number of fish-specific amino-acid changes in Sec61 α : V14A, I15V and K29R occur in the cytoplasmic N-terminus of Sec61 α . S128A and T152I are located in the center of transmembrane domains 3 and 4, respectively. M207T is membrane-proximal in the cytoplasmic loop between transmembrane domains 4 and 5. The I249V exchange is in the center of transmembrane domain 6, and A310T, L316F, S319N, E356D and V363I occur in the ER-luminal loop between transmembrane domains 7 and 8. The last two changes, A430G, and L434M, are located towards the luminal end of transmembrane domain 9. Only a small number of amino-acid changes seem to be specific for cold-water fishes (Fig. 3). These are located at the ER luminal end of transmembrane domain 8 (V363A/G/S) and in the loop region between transmembrane domain 7 and 8 (T327A, S328T and/or S329T, G339A, Y344F or L345F). Although the majority of these changes are conservative substitutions, the cluster of changes occurring in the loop region between transmembrane domains 7 and 8 of Sec61 α may be functionally significant. The functional importance of this region has been demonstrated by the fact that *S. cerevisiae* mutants carrying mutations in this loop of Sec61p are defective in protein translocation across the ER membrane (Stirling et al., 1992; Zhou and Schekman, 1999). Thus the cluster of subtle amino-acid changes in this ER-luminal loop of cold-water fish Sec61 α may represent adaptive changes that improve protein translocation through the Sec61 channel in the cold.

Post-translational protein translocation into the ER of Antarctic and temperate vertebrates at low temperature

To test the temperature dependence of protein translocation into the ER of cold-water fish, we prepared microsomes from the liver of the Antarctic teleost *D. mawsoni* (habitat

1				TM1				TM2	100	
Mm	MAIKFLEVIK	PFCVILPEIQ	KPERKIQFKE	KVLWTAITLF	IFLVCCQIPL	FGIMSSDSAD	PFYWMRVILA	SNRGTLMELG	ISPIVTSGLI	MQLLAGAKII
Fr	.G.....	.AV.....R.
Dr	.G.....	.AV.....R.
Om	.G.....	.AV.....R.
Sr	.G.....	.AV.....R.
Na	.G.....	.F.....	.AV.....R.
Pb	.G.....	.F.....	.AV.....R.
Ha	.G.....	.AV.....R.
Dm	.G.....	.AV.....R.
Go	.G.....	.AV.....R.
Bs	.G.....	.AV.....R.D.....
101		TM3		TM4					200	
Mm	EVGDTPKDRA	LFNGAQLFG	MIITIGQSIV	YVMTGMYGDP	SEMGAGICLL	ITIQLFVAGL	IVLLLDELLO	KGYGLGSGIS	LFIATNICET	IVWKAFSPTT
FRA.I.M
DrA.I.
OmI.
SrA.I.
NaA.I.
PbA.I.
HaA.I.
DmA.I.
GoA.VI.
BsSA.VI.L
201		TM5		TM6				TM7	300	
Mm	VNTGRGMEFE	GAIIALFHLL	ATRTDKVRAL	REAFYRONLP	NLMNLIATIF	VFAVVIYFQG	FRVDLPIKSA	RYRGQYNTYP	IKLFYTSNIP	IILQSALVSN
FrTSVQ
DrTV
OmTLV
SrTGV
NaTILV
PbTILV
HaTILV
DmTILV
GoTV
BsTV
301			327 328	339			362		400	
Mm	LYVISQMLSA	RFSGNLLVSL	LGTWSDTSSG	GPARAYPVGG	LCYYLSPPES	FGSVLEDPVH	AVVYIVFMLG	SCAFFSKTWI	EVSGSSAKDV	AKQLKEQQMV
FrTF.N.DFI.I
DrTS.F.N.DI
OmTF.N.TFDIAI
SrTF.N.ATADGI
NaTF.N.ATADAI
PbTF.N.ATADAI
HaTF.N.ATADAI
DmTF.N.ATADAI
GoTF.N.TFDSI
BsTFI.N.TFDAI
401		TM9		TM10				476		
Mm	MRGHRETSMV	HELNRVIPTA	AAFGGLCIGA	LSVLADFLGA	IGSGTGILLA	VTIIYQYFEI	FVKEQSEVGS	MGALLF		
FrGM		
DrGM		
Om	.G.....GMM		
SrGM		
NaGMM		
PbGMM		
HaGMM		
DmGMM		
GoGMM		
BsGMMG.F.		

Fig. 3. Amino-acid sequences of Sec61 α from mouse, frog, temperate and cold-water fishes. Species are indicated on the left, Genbank accession number of sequences in brackets: Mm *Mus musculus* (NP058602), Dr *Danio rerio* (AAK40295), Fr *Fugu rubripes* (<http://fugu.hgmp.mrc.ac.uk>), Om *Onchorhynchus mykiss* (AAK29081), Sr Sea Raven/ *Hemipteris americanus* (AY103473), Na *Notothenia angustata* (AY103472), Pb *Pagothenia borchgrevinki* (AY103471), Ha *Harpagifer antarcticus* (AY113840), Dm *Dissostichus mawsoni* (AY113841), Go *Gadus ogac* (AY103475), Bs *Boreogadus saida* (AY103474). Sequence alignments were performed using Multalin. Identical residues are indicated by periods. Transmembrane regions are boxed.

temperature -1.8°C) and the liver of the temperate carp *C. carpio* (acclimation temperature 17°C), and compared post-translational translocation of a ribosome-bound truncated radiolabelled secretory protein consisting of the N-terminal 86 amino acids of preprolactin (PPL86) into the fish microsomes and into dog pancreas microsomes over a temperature range. As shown in Fig. 4A, protein translocation into dog pancreas microsomes was optimal at 25°C and did not increase significantly at higher temperature as shown previously [light grey bars, not shown and (Nicchitta and Blobel, 1989)]. Secretory protein import into dog pancreas microsomes at 0°C was minimal (Fig. 4A). In contrast, protein import into *D. mawsoni* microsomes was efficient at 0°C , and approximately equal at all temperatures tested (black bars, Fig. 4A). Protein import into carp microsomes was more efficient than into dog pancreas microsomes at 0°C , but in contrast to the *D. mawsoni* ER, protein import into the carp ER had an optimum in the range between $10\text{--}20^{\circ}\text{C}$ (Fig. 4A; data not shown). Translocated, signal-cleaved PL56 sedimenting with the membranes was, on average, 85% protease-protected in these experiments, confirming translocation into the ER lumen. In contrast, membrane-associated signal-sequence containing PPL86 was over 80% protease sensitive, suggesting association with the cytoplasmic face of the microsomes. The remaining 15–20% constitute either substrate that was translocated, but not signal-cleaved, or incomplete dissociation of PPL86 from ribosomes. There were, however, no differences in the amounts

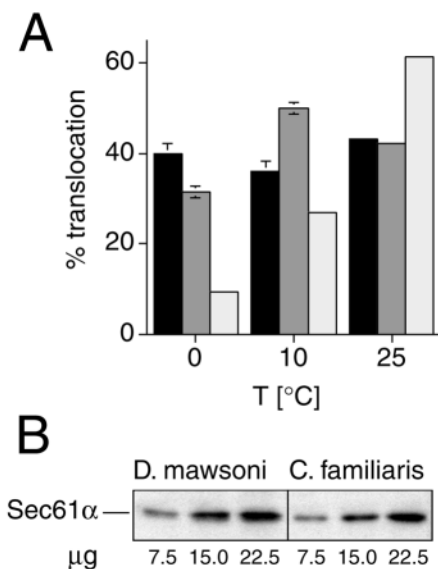


Fig. 4. Protein translocation into ER membranes of the Antarctic fish *D. mawsoni*, carp and dog. (A) Post-translational import of the truncated secretory precursor PPL86 into limiting amounts of microsomes from *D. mawsoni* (black), carp (grey) and dog (light grey) was assayed as described in the Materials and Methods section at the indicated temperatures. Samples were taken in duplicate and the experiment repeated twice. Translocation was defined as the percentage of signal-cleaved PL56 associated with microsomes. (B) Quantitative immunoblot of Sec61 α . The indicated amounts of microsomal protein were resolved on a 12.5% gel, and Sec61 α was detected with polyclonal antibodies against the dog protein and [^{125}I]-Protein A.

of protease-resistant, membrane-associated PPL86 between species.

To investigate whether increased protein import at low temperature into *D. mawsoni* microsomes at low temperature was related to the number of protein translocation channels, we analyzed the amount of Sec61 α in *D. mawsoni* and dog microsomes by quantitative immunoblotting. We found that our membrane preparations contained comparable amounts of Sec61 α (Fig. 4B). The comparatively high protein import efficiency of *D. mawsoni* membranes at low temperature must therefore be due to improved channel function.

Lipid composition of ER membranes in the Antarctic teleost *Dissostichus mawsoni*

The identical length and similar amino-acid composition of the transmembrane domains of Sec61 α from all vertebrates examined (Fig. 3, boxed) suggests that the thickness of the ER membranes of cold-water fishes is similar to those of mesophilic organisms. We therefore compared the lipid compositions of liver microsomes from Antarctic *D. mawsoni*, temperate-water fishes trout and carp and a mammal, the rat. Total lipid fatty acid compositions of liver microsomes from these vertebrate species are shown in Table 1. As generally found in such comparative studies, the cold-adapted or cold-acclimated species had a higher proportion of unsaturated fatty acids in their membranes than temperate or warm-bodied species (Fig. 5A). Microsomes from the Antarctic fish in particular contained an increased percentage of monounsaturated fatty acids (MUFA), compared to trout, carp and rat with increasingly higher body temperatures (Table 1).

Despite the substantial differences in membrane fatty acid composition, the membrane order of liver microsomes derived from these vertebrates measured by DPH anisotropy at a given temperature (21°C) were near identical for *D. mawsoni*, trout, carp and rat (Fig. 5B). Our results indicate that no adaptation has taken place to increase the fluidity of ER membranes from species with low habitat temperatures.

Our data suggest that the protein translocation channel in the ER of Antarctic fish has to assemble and function in membranes that have an increased unsaturated fatty acid content, but are substantially more rigid than mammalian ERs at the body temperature of the fish. We conclude that

Table 1. Liver ER membrane total lipid fatty acid composition [mass%]

Fatty acids	<i>D. mawsoni</i>	Trout	Carp	Rat
16:0	13.0	22.2	28.1	24.3
16:1n-7	6.8	1.6	2.1	1.7
18:0	5.6	7.5	9.0	18.1
18:1n-9	16.7	10.7	11.7	8.3
18:1n-7	8.3	2.0	1.8	3.3
18:2n-6	1.2	3.9	1.9	17.5
20:4n-6	2.8	3.7	3.3	16.1
20:5n-3	13.3	5.9	5.1	1.7
22:6n-3	28.2	36.5	29.2	6.8
% Unsat	78.7	68.9	61.4	57.0
% MUFA	33.1	15.6	19.7	13.3
% PUFA	45.5	53.3	41.7	43.7

MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

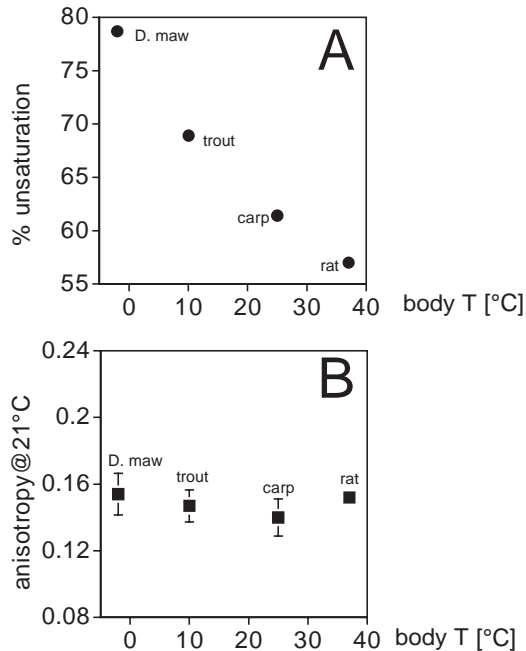


Fig. 5. Lipid composition and fluidity of liver microsomes from four vertebrate species. (A) Correlation between unsaturated fatty acid content and body temperature. (B) 1,6-diphenyl-1,3,5-hexatriene (DPH) anisotropy at 21°C for each species (mean \pm s.d., three separate preparations). Note that the relationship between DPH anisotropy values for different species does not change significantly when measurements are done over a temperature range between 2 and 40°C (Logue et al., 2000).

membrane fluidity is not limiting for protein translocation through the Sec61 channel in the cold.

Discussion

Protein translocation across biological membranes is strongly temperature dependent. Here, we have tried to increase our understanding of the structure-function relationships of the protein translocation channel in the ER membrane and its lipid environment by comparing protein translocation efficiencies into the ER, Sec61 protein primary structures and ER lipid compositions of select organisms from extremely cold habitats and their mesophilic counterparts.

We found an increase in unsaturated fatty acids in the ER membrane of Antarctic fish, as expected, but surprisingly no changes in fluidity compared to microsomes from fishes with more temperate habitats or a warm-blooded mammal (Fig. 5B). This finding is in sharp contrast to the large membrane fluidity differences in brain synaptic vesicles from the same four species measured with the same technique (Logue et al., 2000). Antarctic fish brain membranes are more fluid at any given temperature than rat brain membranes; as a result fluidity of fish brain membranes at -1.8°C (body temperature of the fish) is comparable to that of rat brain membranes at 37°C (body temperature of the rat), a phenomenon known as homeoviscous adaptation (Logue et al., 2000). Not all membranes of organisms with low habitat temperatures show homeoviscous adaptation (Cossins et al., 1978); thus the lack of it in *D. mawsoni* liver microsomes, although unexpected, is not unique.

A reasonable inference from our data would be that increased membrane rigidity at low temperature is not a primary obstacle to Sec61 channel assembly and function. Our observation is in good agreement with that of Leheny and Theg, who found that protein import into chloroplasts at low temperature was not limited by membrane fluidity, but rather by ATP supply (Leheny and Theg, 1994). The identical length and conserved protein sequence of the 10 transmembrane domains of Sec61 α (Fig. 3) suggest that the protein-lipid interactions between Sec61 α and the ER membrane lipids may be similar in cold-adapted fishes and mammals regardless of the membrane lipid composition. This notion is supported by recent work from Mitra and colleagues (personal communication), who have shown that transmembrane proteins affect the structure of their local environment within the lipid bilayer, which in turn suggests that there is no optimal match between proteins and lipids in biological membranes, but that proteins may generate lipid microdomains that support their individual structure and function.

The lowest habitat temperatures experienced by the fishes whose *SEC61* genes we sequenced (temperate species: *D. rerio* 20°C ; *O. mykiss* 4°C ; New Zealand species: *B. variegatus*, *N. angustata* 4°C ; North Atlantic species: *H. americanus* 1°C ; Antarctic species: *H. antarcticus* -1.0°C , *D. mawsoni*, *P. borchgrevinki*, *P. devriesii* -1.8°C ; Arctic species: *B. saida*, *A. glacialis*, *G. ogac* -1.8°C) are significantly lower than the body temperature of the mouse (*M. musculus* 37°C). The fish-specific amino-acid changes that we observed in Sec61 α (Fig. 3, Fig. 6A) in positions which except for A310, L316 and S319 are completely conserved in mammals, may therefore all reflect adaptations to lower temperature, particularly in light of the fact that protein translocation into the ER of a temperate fish had a much lower temperature optimum than translocation into dog ER (Fig. 4A, dark grey bars versus light grey bars). An alternative explanation is that these changes are not adaptive but inherited from the common ancestor to these fish taxa. These fishes are phylogenetically highly divergent and belong to three different superorders and therefore do not share a recent common ancestor (Cheng, 1998). The superorder Ostariophysi (to which zebrafish belong) and the order Salmoniformes (to which trout belong) arose both in the early cretaceous (140 million years ago), and the DNA sequences of Antarctic and Arctic Sec61 α are equally (about 85%) identical to both trout and zebrafish. *Fugu rubripes* is a tropical fish that belongs to the same superorder as the Antarctic *D. mawsoni* (Acanthopterygii) and evolved after the Antarctic and Arctic species that we examined. Analysis of the *Fugu* Sec61 α amino-acid sequence revealed that *Fugu* does not have the amino-acid substitutions that we identified as cold-specific at positions 327, 328 and 339, and, like mouse and zebrafish, contains a large hydrophobic amino acid (F) at position 362 (Fig. 3). Thus the common amino acid substitutions among the unrelated cold-water fishes (Fig. 3, Fig. 6A) are likely to have occurred independently as an adaptation to the chilling of their respective habitats over geologic times.

The amino-acid changes that we observed specifically in teleosts with low habitat temperatures are subtle and cluster in the loop between transmembrane domains 7 and 8 of Sec61 α (Fig. 3, Fig. 6A). Note that trout can live at habitat temperatures similar to those of the New Zealand notothenioids (lowest winter temperature 4°C), which may explain why trout

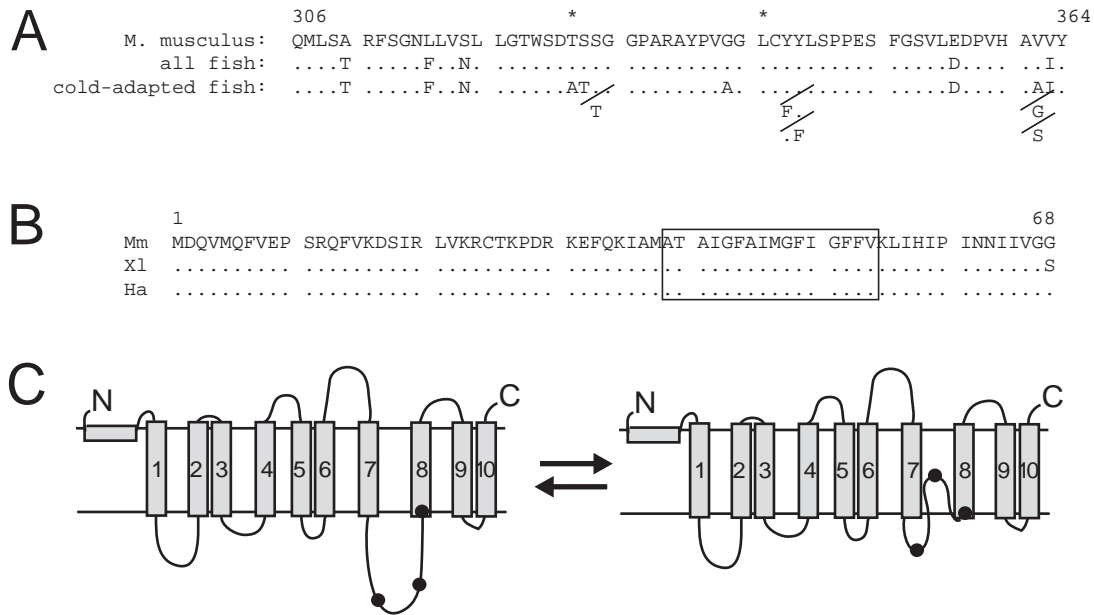


Fig. 6. Sequence changes in the loop between transmembrane domains 7 and 8 of Sec61 α may improve protein translocation across the ER membrane at low temperature. (A) Sequences of the loop regions from mouse, all fishes and cold-water fishes. Positions of mutations in yeast Sec61p defective in translocation are indicated by asterisks. (B) Sec61 γ sequences from *M. musculus*, *X. laevis* and *H. antarticus* (AY258259). The transmembrane region is boxed. (C) Adaptations to the cold may indicate that hinge regions (black circles) in the loop are required for a conformational change during protein translocation into the ER.

Sec61 α contains all but one of the amino-acid changes specific to cold-water fishes in the loop between transmembrane domains 7 and 8 (Fig. 3). These changes are present in fishes with habitat temperatures both above and below 0°C, suggesting that there are no specific adaptations in Sec61 α for function at extremely low temperatures (Fig. 3, Fig. 6A). The majority of these amino-acid positions (S328, G339, Y344, L345, and V363) are absolutely conserved in Sec61 α from warm-blooded vertebrates (mouse, rat, dog and human; data not shown).

Only two subunits of the Sec61 channel, Sec61 α and Sec61 γ , are essential for protein translocation across the ER membrane (Finke et al., 1996; Kalies et al., 1998). We found that Sec61 γ is completely conserved from the Antarctic fish *Harpagifer antarcticus* to mouse at the amino-acid level (Fig. 6B) and hence cannot contribute to improved protein translocation at low temperature. Targeting of nascent chain/ribosome complexes to the ER membrane is mediated by a signal recognition particle (SRP) and its receptor at the ER membrane (Johnson and van Waes, 1999). When we separated the targeting and translocation reactions in vitro, we found that targeting was not limiting for translocation into dog or fish microsomes at low temperature, confirming previous data from Nicchitta and Blobel (Nicchitta and Blobel, 1989), who had addressed this question for translocation into dog pancreas microsomes. The ER luminal Hsp70 BiP may contribute to efficient protein translocation into the ER, although its role in import into mammalian ER is controversial (Gething, 1999; Johnson and van Waes, 1999). In *S. cerevisiae* and in mammalian cells, BiP is inducible by a variety of stress factors (Gething, 1999). BiP recognizes hydrophobic patches of folding intermediates and prevents their aggregation (Rudiger et al., 1997). Hydrophobic interactions, however, are relatively

weak at low temperature (Russell, 2000). Furthermore, liver cells of the Antarctic notothenoid *T. bernacchii* do not express stress-inducible Hsp70s (Hofmann et al., 2000). Unfortunately, the available antibodies against mammalian BiP do not crossreact with the fish protein (K. R. and J.-C. Röper, unpublished). It is therefore unclear whether Antarctic fishes express BiP at all, but even if they do, owing to the specificity of BiP for hydrophobic interactions, the contribution of BiP to protein translocation into the ER and protein folding at low temperature is likely to be limited. Taken together, these considerations further support our notion that the amino-acid changes in Sec61 α from cold-water fishes are responsible for the improved protein translocation into their ER at 0°C (Fig. 4A). At higher temperatures, these changes may be detrimental to channel stability, which may explain why both carp and *D. mawsoni* membranes translocate less efficiently than dog membranes at 25°C.

In soluble proteins, increased activity at low temperature often requires increased flexibility of hinge regions to allow the protein to undergo the conformational changes necessary for its function (Gianese et al., 2001; Russell, 2000). As a result, adaptive amino-acid changes are often observed in these hinge regions. Adaptation to the low temperature of transmembrane channels has not been studied before. Our observations suggest that, as in soluble proteins, only a few key positions need to be changed to improve function at low temperature (Fig. 3 and Fig. 6A). We know from yeast genetics that the loop between transmembrane domains 7 and 8 of Sec61 α is functionally important; mutations in positions 327 and 341 (Fig. 6A, asterisks) lead to defects in protein transport through the channel and to cold-sensitive function of the channel (Stirling et al., 1992; Zhou and Schekman, 1999). Recent structural data suggest that the Sec61 channel undergoes a conformational

change during translocation (Beckmann et al., 2001). It is possible that the amino acids changed in cold-adapted Sec61 α denote hinge regions and that the changes allow the adapted protein to undergo conformational changes more easily. If so, our data would indicate that the loop between transmembrane domain 7 and 8 may need to move during protein translocation into the ER (Fig. 6C).

In summary, our data suggest that membrane fluidity may not be limiting for protein translocation across the ER membrane at and below 0°C and that a conformational change involving the loop between transmembrane domains 7 and 8 of Sec61 α and key positions throughout the protein may be pivotal for protein translocation across the ER membrane.

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