The Sec61 complex is located in both the ER and the ER-Golgi intermediate compartment

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SUMMARY

The heteromeric Sec61 complex is composed of α, β and γ subunits and forms the core of the mammalian ER translocon. Oligomers of the Sec61 complex form a transmembrane channel where proteins are translocated across and integrated into the ER membrane. We have studied the subcellular localisation of the Sec61 complex using both wild-type COS1 cells and cells transfected with GFP-tagged Sec61α. By double labelling immunofluorescence microscopy the GFP-tagged Sec61α was found in both the ER and the ER-Golgi intermediate compartment (ERGIC) but not in the trans-Golgi network. Immunofluorescence studies of endogenous Sec61β and Sec61γ showed that these proteins are also located in both the ER and the ERGIC. Using the alternative strategy of subcellular fractionation, we have shown that wild-type Sec61α, β and γ, and GFP-tagged Sec61α, are all present in both the ER and the ERGIC/Golgi fractions of the gradient. The presence of the Sec61 subunits in a post-ER compartment suggests that these proteins can escape the ER and be recycled back, despite the fact that none of them contain any known membrane protein retrieval signals such as cytosolic di-lysine or di-arginine motifs. We also found that another translocon component, the glycoprotein TRAM, was present in post-ER compartments as demonstrated by subcellular fractionation. Our data indicate that the core components of the mammalian ER translocon are not permanently resident in the ER, but rather that they are maintained in the ER by a specific retrieval mechanism.

Key words: Endoplasmic reticulum, Translocation, GFP, ERGIC, ER retrieval

INTRODUCTION

The Sec61 complex is a core component of the mammalian translocon, forming a channel across the endoplasmic reticulum (ER) membrane through which newly synthesised secretory proteins are translocated and integrated into the ER membrane. We have studied the subcellular localisation of the Sec61 complex using both wild-type COS1 cells and cells transfected with GFP-tagged Sec61α. By double labelling immunofluorescence microscopy the GFP-tagged Sec61α was found in both the ER and the ER-Golgi intermediate compartment (ERGIC) but not in the trans-Golgi network. Immunofluorescence studies of endogenous Sec61β and Sec61γ showed that these proteins are also located in both the ER and the ERGIC. Using the alternative strategy of subcellular fractionation, we have shown that wild-type Sec61α, β and γ, and GFP-tagged Sec61α, are all present in both the ER and the ERGIC/Golgi fractions of the gradient. The presence of the Sec61 subunits in a post-ER compartment suggests that these proteins can escape the ER and be recycled back, despite the fact that none of them contain any known membrane protein retrieval signals such as cytosolic di-lysine or di-arginine motifs. We also found that another translocon component, the glycoprotein TRAM, was present in post-ER compartments as demonstrated by subcellular fractionation. Our data indicate that the core components of the mammalian ER translocon are not permanently resident in the ER, but rather that they are maintained in the ER by a specific retrieval mechanism.

Key words: Endoplasmic reticulum, Translocation, GFP, ERGIC, ER retrieval
the KDEL sequence at the C terminus of ER lumenal proteins of mammalian cells (Pelham, 1990). Proteins with a KDEL retrieval motif bind to the KDEL receptor (ERD2) and are then recycled back to the ER (Lewis and Pelham, 1992a). The KDEL receptor is mainly localised in the first post-ER compartment, the ERGIC (ER to Golgi intermediate compartment) together with the cis-Golgi (Griffiths et al., 1994). As a consequence of its retrieval function, the KDEL receptor has also been localised to the ER, albeit to a lesser extent than to the ERGIC or cis-Golgi (Griffiths et al., 1994). A number of single-spanning membrane proteins have a carboxy-terminal di-lysine or an amino-terminal di-arginine retrieval motif (reviewed by Teasdale and Jackson, 1996), and these are found in type I and type II membrane proteins, respectively. COP1 subunits have been shown to bind directly to proteins bearing the cytosolic di-lysine motif and are believed to function in the retrieval of the proteins to the ER (Cosson and Letourneur, 1994). Very little is known about the ER retention and retrieval of multiple spanning membrane proteins.

In order to ensure the minimum leakage of proteins from the ER some proteins possess a combination of both retention and retrieval signals. Hence, Sec12p, a yeast protein involved in the formation of ER transport vesicles, has a transmembrane domain that acts as a retention signal and an N-terminal cytoplasmic domain which is involved in retrieval (Sato et al., 1996). Tang et al. (1997) later showed that the transmembrane domain was responsible for the formation of oligomers, and that this was the molecular basis for preventing the exit of Sec12p from the ER. Clearly proteins which lack any retention/retrieval information in their sequence can be retained in the ER if they are in a stable complex with other proteins that do contain such information (Zhen et al., 1995).

The ER-Golgi intermediate compartment, or ERGIC, (also known as the intermediate compartment or ‘vesicular tubular structures’), is the first recycling compartment of the mammalian secretory pathway. It was originally identified as the compartment where Semliki Forest virus-infected cells accumulated envelope glycoproteins when intracellular transport was arrested by incubation at 15°C (Saraste and Kuismanen, 1984). It is usually defined by the presence of resident marker proteins such as human ERGIC53 (Schweizer et al., 1988), its rat homologue p58 (Saraste and Svensson, 1991) and the KDEL receptor (Tang et al., 1993). These proteins continuously cycle between the ER the ERGIC and the cis-Golgi. Whilst the role of the KDEL receptor (ERD2) has been established for some time (see above), until recently the role of ERGIC53/p58 was less clear. These proteins were shown to have lumenal ‘lectin’ like domains that recognise high mannose oligosaccharides, and hence it was suggested that they may be involved in the sorting or recycling of glycoproteins during export from the ER (Fiedler and Simons, 1994; Itin et al., 1996). Recently, Nichols et al. (1998) found that mutations in the human ERGIC53 protein cause a combined deficiency of the coagulation factors V and VIII. Thus, ERGIC53 appears to be responsible for the sorting and efficient secretion of a very specific subset of newly synthesised glycoproteins including coagulation factors V and VIII (Nichols et al., 1998) and cathepsin C (Vollenweider et al., 1998). To date, the main function that has been attributed to the ERGIC is that it acts as the site for the retrieval of ER resident proteins that have escaped the ER (Bannykh et al., 1998). In other systems, particularly the yeast Saccharomyces cerevisiae, it has been suggested that certain ER proteins, for example some ER chaperones and components of the membrane translocation machinery, are efficiently sorted away from cargo destined for export at the ER. These components are therefore largely excluded from the transport vesicles leaving the ER (reviewed by Aridor and Balch, 1996).

We have studied the localisation of the Sec61 complex in mammalian cells using two approaches, namely fluorescence microscopy and subcellular fractionation. The wild-type Sec61α protein was tagged with the green fluorescent protein (GFP) in order to facilitate its visualisation in both fixed and live cells, and to establish its subcellular location. The subcellular location of the endogenous Sec61β and Sec61γ subunits were also studied by indirect immunofluorescence microscopy, and in all cases double labelling studies were carried out. Taken together with the results from the subcellular fractionation studies we find that a proportion of all three subunits of the Sec61 complex are found beyond the ER of mammalian cells and are clearly present in the ERGIC. We suggest that the Sec61 complex is transported between the ER and the ERGIC, and that its ER location is dependent upon a recycling mechanism.

MATERIALS AND METHODS

Materials

COS1 African green monkey kidney SV40 transformed cell line (ECACC no. 88031701) was obtained from the European collection of animal cell cultures.

The Sec61α canine cDNA was a gift from T. Rapoport (Harvard Medical School, Boston, USA). Restriction enzymes used for recombinant DNA procedures were obtained from New England Biolabs (Hitchin, UK). 35S [S]methionine was from Amersham (Lewes, UK). PCR was carried out using QuikChangeTM site-directed mutagenesis kit (Stratagene, Cambridge, UK). 35S [S]methionine was from NEN-Dupont (Stevenage, UK). Bismaleimido-hexahexylamine (BMH) cross-linking reagent was from Pierce and Warriner (Chester, UK). Rabbit reticulocyte lysate and the amino acid mixture minus methionine were from Promega. The 7-methylguanosine 5′ monophosphate was from Ambion (Austin, Texas). Protein A and G Sepharose came from Zymed (San Francisco, CA). Nycodenz was purchased from Nycomed Pharma (Oslo, Norway). Digitonin was obtained from Calbiochem (Nottingham, UK). Unless otherwise stated all other reagents were purchased from Sigma (Poole, UK) and BDH-Merck (Lutterworth, UK).

Antibodies

The mouse monoclonal antibody against ERGIC53 was provided by H. P. Hauri (University of Basle, Switzerland), that against the KDEL sequence (1D3) was from V. Allan (University of Manchester, UK), and the Myc epitope specific monoclonal antibody was purchased from Invitrogen (Leek, Netherlands). All other antiserum were raised in rabbits and sourced as follows: ERD2 from H. D. Soling (University of Goettingen, Germany), TRAM from B. Dobberstein (University of Goettingen, Germany), TGN46 from V. Ponnambalam (University of Dundee, UK). The antibody specific for the 22/23 kDa signal peptidease subunit was provided by C. Nicchitta (Duke University, USA). The antisera against GFP and calnexin were purchased from Clontech (Palo Alto, CA) and StressGen (Victoria, Canada), respectively. The Sec61α
antibody used for immunoblotting, and the Sec61β antibody, were both gifts from R. Zimmerman (University of Saarland, Hamburg, Germany). The Sec61α antibody used for immunoprecipitation of cross-linking products was raised against an N-terminal peptide (MAIKFLEVIKFP) by Research Genetics Inc. (Huntsville, AL, USA). The Sec61γ antibody was raised against a peptide close to the N terminus of the sequence (EPSRQFVKDISR) by Research Genetics.

** Constructs 

The vector used for the expression of GFP-tagged Sec61α was a modified version of the mammalian expression vector pcDNA3.1(-) (Invitrogen, Paisley, UK) supplemented with 10% FCS and 1 mM L-glutamine. Cells were transfected with Sec61α-GFP, Sec61α-Myc/His or with the pcDNA3.1(-) plasmid alone by lipofection. DNA was mixed with the lipofection reagent Lipofectamine (Invitrogen BRL, Paisley, UK) and OPTI-MEM medium for 30 minutes according to the manufacturer’s recommendations. Cells were incubated at 37°C in OPTI-MEM containing the DNA and lipofectamine for 5 hours after which the medium was replaced with the full medium described above, and the cells grown for a further 48 hours.

For microscopy, 1×10⁵ cells were placed in a 3 cm diameter dish with a glass coverslip, grown for 20 hours and then transfected using 1 μg DNA. This was scaled up accordingly for the preparation of semi-permeabilised cells or for subcellular fractionation experiments.

** Cross-linking analysis of recombinant Sec61α function 

Transfected cells were permeabilised using digitonin at a concentration of 30 μg/ml and truncated preprolactin (PPL) was used as a model secretory protein for translocation (Wilson et al., 1995). For the generation of a truncated RNA encoding the first 86 amino acids of PPL, pGEM4 T7 PPL was linearised with PvuII and transcribed with T7 RNA polymerase (High et al., 1993). PPL RNA was then translated for 15 minutes at 30°C. The translation reaction contained 14 μl rabbit reticulocyte lysate, 0.4 μl of 1 mM amino acids minus methionine, 15 μCi of [L-35S]methionine, 2 μl RNA and 4-8 μl of digitonin treated, semi-permeabilised, COS1 cells (equivalent to 1×10⁵ cells). After 30 minutes 7-methylguanosine 5' monophosphate was added to 4 mM, incubation at 30°C was continued for a further 5 minutes and then cycloheximide was added to a final concentration of 2 mM to prevent any further protein synthesis. The cells were washed and resuspended in 150 μl KHM buffer, and then incubated at 30°C in the presence of 0.33 mM BMH cross-linker (cross-links the -SH groups of cysteine residues). After a 10 minutes incubation 2-mercaptoethanol was added to a final concentration of 7.33 mM to act as a quenching agent. Immunoprecipitation of the crosslinking products was carried out for 16 hours at 4°C using a Triton based buffer with no SDS present (‘native conditions’; see Oliver et al. 1996). The samples were then incubated for 2 hours with 30 μl of Protein A Sepharose or a mixture of Protein A and Protein G Sepharose before recovery of the immunoprecipitates by centrifugation. These were then washed extensively (Oliver et al., 1996) and analysed by SDS-PAGE.

** Fluorescence microscopy 

Live cells transfected with the GFP-tagged Sec61α construct were transferred to Opti-MEM medium and visualised using an Olympus BX60 fluorescence microscope. Images were captured with a Princeton Instruments Pentamx integrated slow-scan cooled CCD camera. For indirect immunofluorescence studies, wild-type or transfected cells were fixed in methanol at −20°C. The cells were rehydrated after fixing by incubation in PBS with 1% BSA for 15 minutes, followed by incubation with various primary antibodies for 1 hour. The cells were washed and then incubated with the appropriate fluoroscein, rhodamine or Cy3 labelled second antibody for 1 hour. After washing, the slides were mounted with mowiol and DABCO antifade and the cells were observed with a Zeiss axiophot microscope or a Leica TCS NT laser scanning confocal microscope with the appropriate filters.

** Subcellular fractionation 

Subcellular fractionation was carried out on Nycodenz gradients using a modified protocol based on that of Hammond and Helenius (1994). One 225 cm² flask of approximately 80% confluent cells was sufficient for one gradient and the resulting fractions could be used for two immunoblots. Cells were washed twice in homogenisation buffer (10 mM triethanolamine, 10 mM acetic acid, 250 mM sucrose, 1 mM EDTA, and 1 mM DTT) then scraped into 2 ml/flask of homogenisation buffer containing 10 μg/ml each of PMSF, antipain, pepstatin A and leupeptin. Cells were homogenised by passing them five times through 19, 23 and 25-gauge hypodermic needles consecutively and then spun at 1500 g for 5 minutes at 4°C. The postnuclear supernatants were then loaded directly onto preformed Nycodenz gradients as described by Hammond and Helenius (1994) and nine fractions were collected. Equal volumes of the fractions were precipitated using trichloroacetic acid, separated by SDS-PAGE, and then analysed by western blotting using ECL detection (Amersham, UK).

** RESULTS 

** Expression of the GFP-tagged Sec61α and functionality assay 

The transfection efficiency of the GFP-tagged Sec61α in COS1 cells was relatively high and approximately 40% of the cells showed a high intensity GFP dependent signal when visualised by fluorescence microscopy. Metabolic labelling and immunoprecipitation was used to determine the amount of the GFP-tagged Sec61α protein produced by the COS1 cells. We found that it was expressed at approximately 2.5 times the level of the endogenous Sec61α protein present in both the same cells and in cells transfected with the expression vector alone (data not shown). Since only 40% of the cells were actually transfected, the average expression level per GFP-tagged Sec61α expressing cell was probably 4-5 times the level of endogenous Sec61α.

To test whether the GFP-tagged Sec61α was functional we used an established cross-linking assay (Wilson et al., 1995). Transiently transfected cells were semi-permeabilised using digitonin and then added to a cell free translation reaction programmed with mRNA encoding a truncated version of the secretory protein preprolactin (PPL). Since the truncated PPL lacked a stop codon it would be targeted to the ER membrane.
and then become trapped in the ER translocation channel because it remains attached to the ribosome (see High, 1995). Upon the addition of the homobifunctional cross-linking reagent BMH, the ER translocon components adjacent to the translocating PPL chain can be cross-linked to it (cf. Laird and High, 1997) and can be identified by immunoprecipitation. It had already been shown that endogenous Sec61a can be cross-linked to a PPL translocation intermediate (Wilson et al., 1995), and therefore this assay could be used to determine whether the tagged Sec61a was also adjacent to secretory proteins. The ability of tagged Sec61a proteins to cross-link PPL translocation intermediates would indicate that the tagged protein was functional in mediating ER membrane translocation.

We observed several BMH dependent cross-linking products with the PPL translocation intermediate (Fig. 1, lanes 2 and 7). However, when cells transfected with GFP-tagged Sec61α were used, cross-linking to endogenous Sec61α (Fig. 1, lane 4) but not to GFP-tagged Sec61α (Fig. 1, lane 3) was detected. We had previously established that the anti-Sec61α antibody used for this experiment could immunoprecipitate the GFP-tagged Sec61α from metabolically labelled cells and that this product had a molecular weight of around 75 kDa on SDS-PAGE (data not shown). In order to establish whether the C terminus of Sec61α could be modified without affecting function, a much smaller tag consisting of the Myc epitope and six histidine residues (Myc/His) was added in place of the C-terminal GFP tag. In this case, the PPL translocation intermediate could be cross-linked to both endogenous Sec61α (Fig. 1, lane 9) and the Myc/His tagged Sec61α (Fig. 1, lane 8). The cross-linking product with the Myc/His tagged protein was noticeably larger than the wild-type product (the Sec61α-Myc/His construct contains an extra 23 amino acids), and was specifically immunoprecipitated by an anti-Myc antibody which does not recognise the endogenous protein (Fig. 1, lane 8 and data not shown). We therefore conclude that the addition of the 238 amino acid GFP tag to the C terminus of Sec61α specifically inhibits the function of the protein.

The subcellular localisation of GFP-tagged Sec61α visualised by fluorescence microscopy
Fluorescence microscopy was used to determine the subcellular localisation of the GFP-tagged Sec61α following transient transfection of COS1 cells. Our initial studies were carried out using live cells in order to avoid any artefacts that might arise during the fixation process. The cells showed a large amount of perinuclear staining (Fig. 2A) together with a clear reticular pattern that was particularly visible towards the periphery of the cell (Fig. 2B and C). This is the typical pattern of staining obtained with a resident ER protein and indicates that much of the GFP-tagged Sec61α is correctly localised in the ER. No evidence of any labelling at the cell surface in live cells was observed. In order to confirm the localisation of the GFP-tagged Sec61α, double labelling experiments were carried out using fixed cells and a monoclonal anti-'KDEL'
antibody (1D3) recognising several soluble resident ER proteins, particularly protein disulphide isomerase (Vaux et al., 1990). During the course of these experiments both methanol and paraformaldehyde/glutaraldehyde fixation methods were tested. Since both these approaches gave similar results (data not shown), methanol fixation was used for all the studies presented. The reticular network observed with the GFP-tagged Sec61α (Fig. 3A) and using indirect immunofluorescence to visualise the 1D3 reactive proteins (Fig. 3B) were very similar, and when the two images were merged a large degree of co-localisation was observed (Fig. 3C, yellow areas). Hence the reticular network formed by the GFP-tagged Sec61α is largely co-localised with the 1D3 reactive compartment of mammalian cells, ie: the endoplasmic reticulum. We were unable to study the localisation of the endogenous Sec61α protein by indirect immunofluorescence because none of our antibodies were suitable for such studies. We therefore continued to use the GFP-tag to visualise Sec61α throughout this analysis.

Sec61α normally functions as one subunit of a heterotrimer, we therefore compared its subcellular distribution to that of one of the other subunits of the Sec61 complex, Sec61β (Fig. 4). The subcellular distribution of GFP-tagged Sec61α (Fig. 4A) and endogenous Sec61β (Fig. 4B) was very similar with a large degree of co-localisation extending to the periphery of the cell (Fig. 4C). We therefore conclude that the subcellular distribution of GFP-tagged Sec61α is similar to the distribution of endogenous components of the Sec61 complex.

In order to determine whether GFP-tagged Sec61α was localised solely in the ER, we also carried out double labelling immunofluorescence studies using markers for other subcellular compartments of the secretory pathway. The next subcellular location that we analysed was the ERGIC, using antibodies specific for the protein that defines this compartment, namely ERGIC53 (Itin et al., 1995). In contrast to the ER staining pattern described above, ERGIC53 was localised to a discrete perinuclear region of the cell that was visualised as a distinct punctate staining pattern (Fig. 5B). This region partly overlapped with the structures labelled by GFP-tagged Sec61α (Fig. 5A) and specific co-localisation of a sub-population of GFP-tagged Sec61α with ERGIC53 was observed (Fig. 5C). Indeed, few if any of the ERGIC53 staining regions did not overlap with GFP-tagged Sec61α (Fig. 5C). The ERD2 protein (KDEL receptor) is also located primarily in the ERGIC, and partly in the cis-Golgi (Tang et al., 1993) and double labelling studies of ERD2 and GFP-tagged Sec61α gave similar results to those shown for ERGIC53 (data not shown).

A useful tool that is often used in studies of the ERGIC (or intermediate compartment) is a 15°C temperature block. This is known to inhibit ER to Golgi transport (Saraste and Kuismanen, 1984), and result in the accumulation of tubulovesicular intermediate compartment structures in peripheral cytoplasm (Saraste and Svensson, 1991). This enables a more precise co-localisation of proteins with this compartment to be made. Following a 2 hour 15°C temperature block we indeed observed a significant increase in the number of ERGIC53 labelled vesicular structures located towards the periphery of the cell (cf. Figs 5B and 6B). We observed a complete co-localisation of the ERGIC53 labelling and the
GFP labelling present in these scattered vesicular structures (Fig. 6C). Scattered vesicular structures labelled with GFP-tagged Sec61α are also clearly visible amongst the reticular ER network (Fig. 6A). In the example shown a complete co-localisation of the ERGIC53 compartment with structures containing GFP-tagged Sec61α was observed after a 15°C temperature block. However, the amount of co-localisation was variable and some cells showed a proportion of ERGIC53 labelled structures that did not co-localise with GFP-tagged Sec61α (data not shown). We believe that this reflects variations in the amount of GFP-tagged Sec61α expressed in individual cells.

In order to determine whether the GFP tagged Sec61α was found in compartments further along the secretory pathway, an immunofluorescence study was carried out using the trans-Golgi network marker TGN46 (Prescott et al., 1997). By standard indirect immunofluorescence microscopy, a substantial portion of TGN46 labelling was distinct from the GFP-Sec61α labelled structures. Nevertheless, some degree of co-localisation was observed, albeit substantially less than that seen with ERGIC53 (data not shown). Since several studies have noted the potential difficulties in distinguishing between the ERGIC and the Golgi (Yang and Storrie, 1998), or the Golgi and the TGN (Banting and Ponnambalam, 1997) using immunofluorescence microscopy, we wanted to determine whether the partially overlapping signals that we observed indicated true co-localisation to the same subcellular structures. We therefore treated cells with brefeldin A, a drug which has been useful in subcellular localisation studies because it redistributes Golgi proteins to the ER whilst those of the TGN ‘collapse’ upon the microtubule organising centre (Banting and Ponnambalam, 1997). Both conventional and confocal immunofluorescence microscopy were used to look at the localisation of GFP tagged Sec61α and TGN46 following Brefeldin A treatment of the cells. Confocal microscopy images of the double labelling results for three different cells are shown in Fig. 7, each image being an average of 18 optical sections (A to C). The collapsed TGN can be seen via immunofluorescence imaging of TGN46 in red, whilst the Sec61α-GFP labelling is in green and any overlapping regions are yellow. Fig. 7A and C show two cells where no co-localisation of GFP-tagged Sec61α and TGN46 was detected. In a few cases a small amount of overlap was still observed following brefeldin A treatment (Fig. 7B). However, this was insignificant when compared to the co-localisation with the ERGIC53.

Taking the results of these microscopy studies together, we conclude that the GFP-tagged Sec61α is localised in the ER and the ERGIC, but that it does not reach the TGN. Furthermore, analysis of the GFP-labelling of live cells showed no detectable presence of the protein in the plasma membrane. On this basis we suggest that GFP-tagged Sec61α can be transported out of the ER to the ERGIC, and perhaps the cis-Golgi (cf. Tang et al., 1993) from where it is most likely recycled back to the ER.

The subcellular localisation of endogenous Sec61β and Sec61γ

It was possible that the presence of GFP-tagged Sec61α in the ERGIC that we observed was a result of either its moderate overexpression or its lack of functionality. In the absence of any suitable antibodies with which we could study the subcellular location of the endogenous Sec61α present in wild-type cells, we investigated the distribution of endogenous Sec61β and Sec61γ, which form a heterotrimeric complex with Sec61α (Gorlich and Rapoport, 1993). Wild-type COS1 cells were stained using antibodies specific for either Sec61β or Sec61γ and ERGIC53, and the subcellular localisation of these proteins was determined by indirect immunofluorescence microscopy. The Sec61β and Sec61γ antibodies both revealed a large reticular network typical of the ER, together with particularly intense staining in the perinuclear region (Fig. 8A and D, respectively). The ERGIC53 antibody also resulted in a distinct punctate staining located in the perinuclear region of the cell (Fig. 8B and E) and this area largely co-localised with Sec61β and Sec61γ (Fig. 8C and F, respectively, yellow

Fig. 5. GFP-tagged Sec61α is present in the ERGIC. The GFP-tagged Sec61α signal (A) and the ERGIC53 detected via monoclonal anti-ERGIC53 and rhodamine conjugated secondary antibody (B) are shown. The image overlay shows that GFP-tagged Sec61α is present in all of the ERGIC53 labelled structures (C).

Fig. 6. GFP-tagged Sec61α redistributes with the ERGIC after a 15°C temperature block. The effect of a 15°C temperature block upon the co-localisation of GFP-tagged Sec61α (A) and ERGIC53 labelled structures (B) is shown. The 15°C block causes scattering of some ERGIC53 labeled structures towards the cell periphery, and the image overlay reveals that these scattered structures also contain GFP-tagged Sec61α (C).
When a similar experiment was carried out after a 15°C temperature block the majority of the scattered vesicular structures derived from the ERGIC were also found to contain Sec61β and Sec61γ (cf. Fig. 6, data not shown). We therefore conclude that Sec61β and Sec61γ are resident in both the ER and the ERGIC of wild-type COS1 cells, and perhaps recycle between these two locations.

The localisation of the Sec61 complex using subcellular fractionation

In order to investigate the subcellular distribution of the Sec61 complex by an alternative method, we used subcellular fractionation. Since we were particularly interested in testing our hypothesis that the Sec61 complex is located in both the ER and the ERGIC we used a Nycodenz gradient system developed specifically to separate these compartments (Hammond and Helenius, 1994). The proteins of interest could all be detected by immunoblotting therefore we were able to examine the subcellular distribution of all three subunits of the Sec61 complex using both wild-type (Fig. 9) and transfected cells (data not shown). The method we have used can determine whether or not a particular protein is present in the ER or the ERGIC fractions of the Nycodenz gradient. However, it is particularly important to stress that no estimate of the relative fraction of the protein present in each of these locations can be made. This is because a low speed centrifugation step is used to remove the nuclear material prior to fractionation, and this also results in the loss of a substantial amount of the ER marker calnexin into the pellet. In contrast, very little of the ERGIC/cis-Golgi marker ERD2 is lost (data not shown). Hence, a significant amount of ER material is lost prior to fractionation, presumably as a consequence of the continuity of the ER and the nuclear membranes.

We used calnexin as an ER marker and found that it was restricted to the heaviest fraction of the gradient, i.e: fraction 1, in both wild-type (Fig. 9) and transfected cells (data not shown). This region of the gradient had previously been identified as containing the ER (Hammond and Helenius, 1994). In contrast, our marker for the ERGIC/cis-Golgi, ERD2,
The presence of GFP-tagged Sec61α in mammalian cells we found it to be present in both the ER and the ERGIC. The GFP-tagged protein was shown to be unable to mediate translocation on the basis of its inability to be cross-linked to a secretory protein translocation intermediate. The addition of a 238 amino acid GFP tag to the C terminus of Sec61α, may interfere with its conformation, or with its ability to assemble into a functional translocation complex, both of which would be likely to inhibit its function. Alternatively, the presence of the GFP tag on the cytoplasmic side of the ER membrane may prevent the ribosome binding function of Sec61α (Kalies et al., 1994) and hence block any cotranslational protein translocation. Although in this study the addition of a GFP tag to Sec61α resulted in its loss of function, several studies have shown that both functional and non-functional GFP chimeras can be appropriately localised to their correct subcellular compartments (Cole et al., 1996; Shima et al., 1997). However, in view of the lack of function of GFP-tagged Sec61α we decided not to rely solely on its subcellular localisation to indicate the location of the wild-type Sec61 complex. We therefore studied the localisation of the two endogenous subunits of the Sec61 complex for which suitable antibodies were available, namely Sec61β and Sec61γ. Both these proteins were found to be localised to the ER and the ERGIC by immunofluorescence microscopy studies of wild-type cells. Since Sec61α is found in a heterotrimeric complex with Sec61β and Sec61γ (Gorlich and Rapoport, 1993), these results indicate that the subcellular localisation of GFP-tagged Sec61α faithfully reports the localisation of the wild-type protein.

The presence of GFP-tagged Sec61α further along the secretory pathway was also investigated and we found that little, if any, of the protein reached the trans-Golgi network, and none was visible at the plasma membrane of live cells. A second approach was used to determine the subcellular localisation of the Sec61 complex, namely subcellular
fractionation on Nycodenz gradients. Using this method GFP-tagged Sec61α, endogenous Sec61α, Sec61β and Sec61γ were all found to be present in the heavy fraction containing the ER and the lighter fractions where both the ERGIC and the Golgi were located. Since microscopy studies showed that GFP-tagged Sec61α was localised to the ERGIC but not the TGN, we conclude that the presence of the three Sec61 subunits in the lighter fractions is primarily due to their localisation in the ERGIC and perhaps the cis-Golgi.

Our results show that all three components of the Sec61 complex are localised both in the ER and in post ER compartments, particularly the ERGIC. This raises the question of why the Sec61 complex is present in the ERGIC. We believe that the most likely explanation is that the Sec61 complex escapes from the ER to the ERGIC from where it is recycled back to the ER. In contrast, studies in *Saccharomyces cerevisiae* have shown that the yeast Sec61α homologue, Sec61p, is excluded from transport vesicles carrying cargo away from the ER (Barlowe et al., 1994). However, no equivalent of the ERGIC has been reported in *Saccharomyces cerevisiae*.

Our data indicate that the subunits of the Sec61 complex, and the TRAM protein, are located in the ER by virtue of a retention/retrieval mechanism akin to that which recycles membrane proteins bearing a di-lysine motif (Jackson et al., 1993). Although calnexin does have a di-lysine-like retrieval motif (Teasdale and Jackson, 1996), we could not detect the protein in compartments beyond the ER during subcellular fractionation experiments. This result is in agreement with previous studies where calnexin was found to be excluded from ER derived transport vesicles (Rowe et al., 1996). The authors suggested that calnexin has a very low escape rate from the ER, as had previously been suggested by studies of VSV-G protein biosynthesis (Hammond and Helenius, 1994). The fact that calnexin effectively behaves as an ER resident protein may also reflect the fact that it appears to form a complex with the ER luminal chaperone Erp57 which also contains a consensus ER retrieval signal (Oliver et al., 1997; Elliott et al., 1997).

In contrast to calnexin, it would appear that the Sec61 complex has a relatively high escape rate from the ER. The nature of any retrieval signal that could result in the recycling of the Sec61 complex to the ER is presently unclear, and there are no known ER retrieval motifs present in the sequences of any of the components of the Sec61 complex. Thus, recycling between the ER and the ERGIC must either result from unidentified retrieval signals, or via an association with as yet unidentified proteins that do contain known retrieval information. Interestingly, the TRAM protein does have a di-lysine motif at its cytoplasmic carboxy terminus, together with a di-arginine motif at its cytoplasmic amino terminus, both of which are potential ER retrieval motifs (Teasdale and Jackson, 1996). Whether or not they do act as retrieval signals remains to be established. However, such a role would be consistent with our evidence that a proportion of the TRAM protein is located in a post-ER compartment. It is tempting to speculate that an association between the Sec61 complex and the TRAM protein might be the basis for the retrieval of the Sec61 complex to the ER. However, there is currently no evidence for any direct interaction between the TRAM protein and the Sec61 complex.

It may be that the Sec61 complex present in the ERGIC performs a specific function. Hence, misfolded MHC class I molecules were observed to accumulate in the ERGIC (Raposo et al., 1995). Furthermore, the Sec61 complex is believed to participate in the retrograde translocation of misfolded MHC class I proteins prior to their degradation in the cytosol (Wiertz et al., 1996). Thus it may be that such Sec61 dependent retrograde transport takes place at the ERGIC of mammalian cells. This would enable the spatial separation of two functions performed by the same protein complex, and thus allow both forward and retrograde transport to occur at the same time but in different locations.

Our results clearly show the presence of all three subunits of the Sec61 complex, and the TRAM protein, in post ER compartments. We therefore conclude that none of these proteins are permanent residents of the ER (cf. Jackson et al., 1993). Whilst we favour the idea that this reflects specific recycling of these components via a retrieval pathway, the precise biological basis for their subcellular distribution remains to be established.

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