The targeting information of the mitochondrial outer membrane isoform of cytochrome b_5 is contained within the carboxyl-terminal region

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Abstract Two isoforms of mammalian cytochrome b_5, which have homologous cytosolic amino-terminal catalytic domains, are located one on endoplasmic reticulum (ER b_5) the other on mitochondrial outer membranes (OM b_5). A cDNA coding for the previously unknown carboxyl-terminal domain of OM b_5 was cloned and a chimera between the catalytic domain of ER b_5 and the carboxyl-terminal region of OM b_5 was expressed in cultured mammlian cells. The chimera localized to mitochondria, indicating that the carboxyl-terminal 43 amino acids of OM b_5 contain sufficient information to target the catalytic domain of ER b_5 to the mitochondrial outer membrane.

Key words: Endoplasmic Reticulum; Cytochrome b_5; Membrane biogenesis; Membrane protein; Mitochondrial outer membrane; Protein targeting

1. Introduction

During the past few years there has been increasing interest in the mechanism of targeting of a class of membrane proteins characterized by an N-terminal cytosolic domain and a short C-terminal anchoring domain. These proteins, which have recently been called 'tail-anchored' [1], lack a signal sequence and are inserted into membranes post-translationally. Current evidence favours a transmembrane topology for their membrane anchor [1,2]. Members of this class of proteins are involved in fundamental processes within the cell, such as vesicular traffic (t-snares and v-snares), regulation of apoptosis (bcl-2), tyrosine dephosphorylation (PTB-1B), electron transport (cyt b_5) [1,3] and references therein).

Although it was previously thought that tail-anchored proteins could insert non-specifically into any phospholipid bilayer, it is now known that many of them have restricted and specific subcellular distributions, and it is thought that unknown targeting mechanisms are responsible for their localization (see [3] and [4] for reviews). As a starting point for thinking about the targeting mechanisms of tail-anchored proteins, two general models can be considered. In the first one, the cytosolic active domain, by interacting permanently or transiently with specifically located protein partners, determines, in addition to the function of the protein, also its localization. In this scenario, the C-terminal anchoring domain would have a non-specific role in the integration of the protein into the phospholipid bilayer. In the second model, information for targeting would be distinct from the structural features required for function, and could be located within the C-terminal anchoring domain.

A system which could allow the distinction between these 2 mechanisms is offered by the 2 homologous isoforms of cytochrome (cyt) b_5. Mammalian tissues express an endoplasmic reticulum (ER)-bound cyt b_5 (ER b_5) and an mitochondrial outer membrane isoform (called outer membrane cyt b by the group that discovered it [5], and which we will refer to as OM b_5), which are the products of 2 different genes [6]. ER b_5 consists of an N-terminal, heme-binding, cytosolic domain, which can be detached by trypsin from the C-terminal anchoring domain. The latter includes: (i) a polar hinge region, which connects the anchor to the cytosolic domain; (ii) the membrane anchor itself; and (iii) the polar residues located at the extreme C-terminus. As far as OM b_5 is concerned, until the present, only the primary structure of a tryptic fragment, corresponding to the cytoslic, heme-containing, domain had been determined [6]. The sequence of this domain is 60% identical to the corresponding region of ER b_5. Since OM b_5 has a higher apparent M_r than the sequenced tryptic fragment and behaves like an integral membrane protein [7], it was anticipated that it would have a membrane-anchoring domain, however, it was not known whether this domain was at the C- or N-terminus of the protein.

In a previous study, by using monospecific anti-peptide antibodies, we demonstrated that the 2 cyt b_5 isoforms have non-overlapping subcellular distributions and are each located exclusively, or nearly exclusively, on a single target membrane [7]. Homologous proteins with different subcellular localizations are good models for the study of intracellular targeting, because the regions which are less similar between them are good candidates for targeting signals, and because it is possible to construct chimerae which are likely to retain the native structure of the wild-type parent proteins. We report here the finding that the anchoring domain of OM b_5 is C-terminal and that the information contained therein is sufficient to target the cytosolic domain of ER b_5 to the mitochondrial outer membrane.

2. Materials and methods

2.1. General

Recombinant DNA techniques were carried out by standard procedures [8,9]. DNA sequencing was carried out by the dideoxy chain

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Abbreviations: cyt, cytochrome; ER, endoplasmic reticulum; ER b_5, endoplasmic reticulum isoform of cytochrome b_5; NER-COM, chimera between N-terminal domain of ER b_5 and C-terminal domain of OM b_5; OM b_5, outer mitochondrial membrane isoform of cytochrome b_5; PDI, protein disulphide isomerase.

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termination method on inserts cloned in pGEM 3 (Promega, Madison, WI), using the Taq Track kit from Promega.

2.2. Cloning of cDNA for OM b_5 by anchored polymerase chain reaction (PCR)

A 200 bp cDNA fragment coding for a portion of the cytosolic domain of OM b_5 was amplified from rat liver cDNA. The latter was obtained by reverse transcription of 5 μg total rat liver RNA, prepared by the method of [10], using random hexanucleotides as primers. To amplify this cDNA, 2 oligonucleotides were designed from the known primary structure of the cytosolic domain of OM b_5 [6]: 5′ oligonucleotide: 5′ *CGGGATCC*AACACTGCTGAGG-AGACCTGGATGGTG-ATCCA 3′; 3′ oligonucleotide: 5′*GCGGTACC*G-TCATTGGGGAC-ATCCCCAATGTAGTACTGCTT 3′. The 5′ and 3′ oligonucleotides each contained an extra-sequence (in italics) with a *Bam*HI and *Kpn*I site, respectively. The amino acid sequences used for the construction of these oligonucleotides are overlined with dashed arrows in Fig. 1A. The cDNA (deriving from 1/4 of the total) was amplified for 30 cycles (94°C, 1 min; 58°C, 1.5 min; 72°C, 1 min – 10 min in the final cycle) in a standard buffer containing 1.5 mM $MgCl_2$. 1/10 of the reaction mix was reamplified under the same conditions. The amplified DNA was purified, cut with *Kpn*I and *Bam*HI, and subcloned into pGEM 3. The sequence of 2 separate clones were in agreement with each other and predicted the expected amino acid sequence previously determined by [6].

To obtain a clone specifying the previously unkown C-terminal portion of OM b_5, the RACE-PCR protocol [11] was applied, using a sequence internal to the previous clone as 5′ primer, and $dT_{(17)}$ extended by an adaptor sequence containing restriction sites, as well as the adaptor sequence alone, as 3′ primer. The sequence of the 5′ primer (overlined by solid arrow in Fig. 1A) was: GAGTCTACGATAT-CACCCG. Rat liver cDNA was obtained from total RNA as described above but using the $dT_{(17)}$-adaptor instead of random hexanucleotides as primer. 1/10 of the cDNA was amplified with the primers described above for 30 cycles (94°C, 1 min; 55°C, 2 min; 72°C, 1 min – 10 min in the final cycle). After purification, the amplified DNA was cut within sites present in the adaptor (*Sal*I) and in the known part of the amplified fragment (*Hin*dIII, at position 91 in Fig. 1A), and subcloned into pGEM 3. 3 positive clones, selected by colony hybridization with the 200 bp probe, were sequenced.

2.3. Construction of 'NER-COM' chimera

A chimeric cDNA coding for the first 91 residues of ER b_5 joined to residues 93–134 of OM b_5 (Fig. 1A and B) was constructed taking advantage of a unique *Pvu*I site present in ER b_5 cDNA at the junction between codons 87 and 88.

The synthetic cDNA for ER b_5, [12], a gift of Dr. Stephen Sligar, University of Illinois, was recloned into the *Pst*I–*Eco*RI sites of pGEM 3. A ~300 bp fragment, coding for the first 87 residues of ER b_5 was excised with *Hin*dIII (within the polylinker, upstream of the cloning site) and *Pvu*I (at the junction between codons 87 and 88), and purified. A fragment of the cDNA for OM b_5, coding for residues 93–134 was amplified with a 5′ primer comprising the desired junction between the 2 clones, and containing the *Pvu*I site of ER b_5 (5′*AT* CGATCGTTCTAAAGATGGTGACAAGGACCCT 3′: bases in italics are extrasequence; underlined bases contain ER b_5 sequence) and a 3′ primer extending from 5 to 20 nts downstream to the stop codon (5′ *GGAATTC*CGACACTTCAACGTGGC 3′ – bases in italics are extra-sequence containing an *Eco*RI site). The amplified fragment was purified, digested with *Pvu*I and *Eco*RI, and ligated together with the *Hin*dIII–*Pvu*I fragment deriving from ER b_5 into pGEM 3 cut with *Hin*dIII and *Eco*RI. The absence of errors due to amplification was controlled by sequencing.

2.4. Transfection of CV1 cells

The cDNAs coding for ER b_5 and for the NER-COM chimera were subloned into the *Hin*dIII and *Eco*RI site of the mammalian expression vector pCB6 [13], modified to contain an *Eco*RI site within the polylinker.

Cells, plated on 1.7 × 1.7 cm glass coverslips, were transfected by the calcium phosphate method [14], but the glycerol shock was omitted. Uptake of DNA was increased by post-incubation of the cells with 0.1 mM chloroquine in complete medium for 3 h. 24 h after transfection, cells were fixed with 4% paraformaldehyde in 0.120 M sodium phosphate buffer, pH 7.4, at 37°C for 30 min.

2.5. Antibodies and immunofluorescence

Coverslips containing the paraformaldehyde-fixed cells were washed in HS (0.5 M NaCl, 20 mM sodium phosphate buffer pH 7.4) and preincubated for 1 h on a drop of blocking buffer (= HS containing 0.2% gelatin and 0.6% Triton X-100). The coverslips were then incubated for 2 h with primary antibody diluted in blocking buffer, washed briefly 5 times with HS, incubated for 1 h with fluorescent secondary antibodies diluted in blocking buffer, washed again 5 times with HS and once with PBS, mounted in PBS containing 70% glycerol and 0.1% phenylenediamine, and observed under a Zeiss Axioplan microscope equipped for epifluorescence. In some experiments, the signal was amplified by use of biotinylated secondary antibodies followed by streptavidin-Texas red (Amersham, Buckinghamshire, UK). In this case, 2 blocking steps were carried out with the reagents of the blocking kit from Vector Laboratories (Burlingame, CA).

Primary antibodies used were: (i) a polyclonal antibody raised in rabbits against a bacterially expressed fusion protein of rabbit ER b_5. The cDNA for rabbit ER b_5 [15], a gift of Dr. A. Steggles (Northeastern Ohio Universities College of Medicine), cut with *Fsp*I and *Hin*dII was cloned into the filled *Bam*HI site of pET3a (Novagen, Madison, WI). The resulting construct coded for a fusion protein consisting of the first 11 residues of the gene 10 protein of phage T7, followed by residues 3–134 of rabbit ER b_5. The bacterially expressed fusion protein was enriched in a low speed pellet after lysis of bacteria with lysozyme and DNase treatment of the lysate. This crude preparation of fusion protein was used as immunogen. The resulting anstiserum was affinity-purified, using a nitrocellulose strip containing the SDS-PAGE-purified fusion protein as affinity matrix [16]. The affinity purified antibodies were used at a dilution corresponding to 1: 125 of the original antiserum; (ii) a monclonal antibody against bovine protein disulfide isomerase (PDI) from *Stress*Gen (Victoria, BC, Canada), used at 1:500 dilution. Secondary antibodies were from Jackson Immunoresearch (West Grove, PA) or from Amersham (Buckinghamshire, UK).

Mitochondria were stained with Mitotracker CMX Rose from Molecular Probes (Eugene, OR). Cells were incubated with the dye dissolved in the culture medium at a concentration of 800 nM for 15 min in the CO_2 incubator, and then fixed with paraformaldehyde and processed for immunofluorescence as described above.

3. Results and discussion

To clone the cDNA coding for the C-terminal portion of OM b_5, we first constructed a 200 nt long probe by amplification of reverse-transcribed liver RNA using as primers oligonucleotides designed on the basis of the known amino acid sequence

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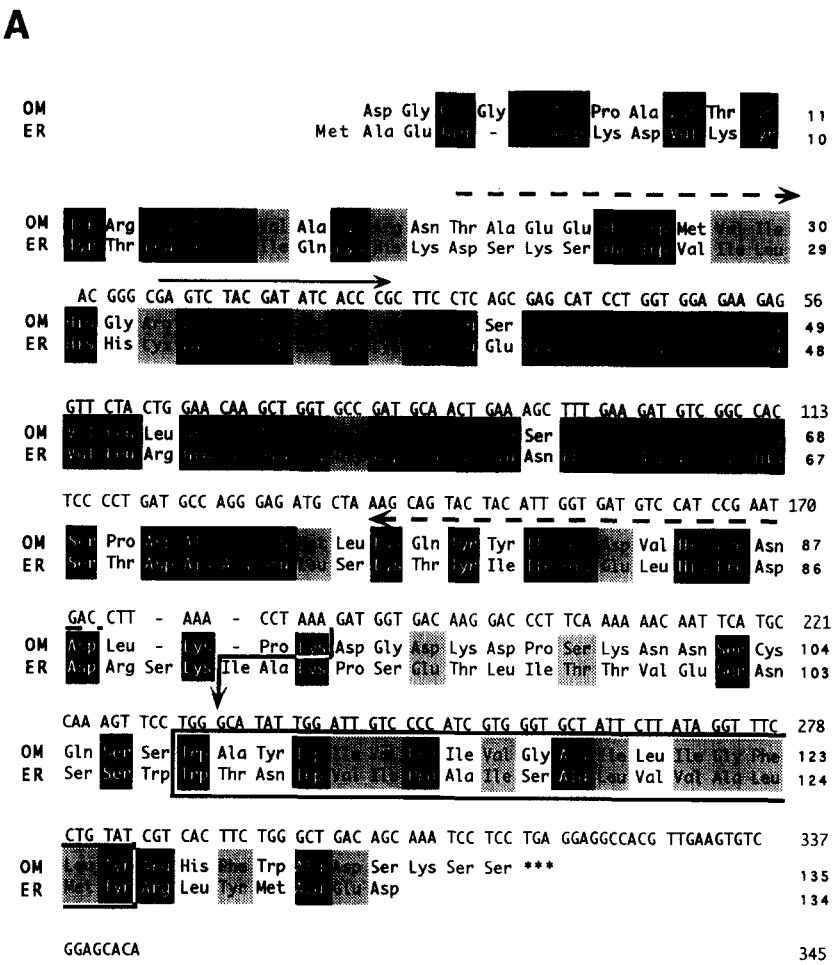
Fig. 1. Primary structures and hydropathy plots of ER b_5 and OM b_5, and construction of NER-COM chimera. (A) Upper line shows cDNA sequence derived from the 2 overlapping clones (200 bp probe and ~800 bp clone obtained by RACE-PCR). The dashed arrows overline the regions used to design by reverse translation the oligonucleotides used for the initial cloning of the 200 bp probe; the continuous arrow indicates the oligonucleotide used for RACE-PCR (see section 2). The deduced amino acid sequence is given on the line marked 'OM' while the line marked 'ER'shows the primary sequence of rat ER b_5 from [17]. The first 31 residues of OM b_5, not encoded in our clones, are from [6] Residues 32–92, deduced from the cDNA, coincide with those determined by [6] on the tryptic fragment of the protein. Alignment of ER b_5 and OM b_5 was done with the Align program [24]. Residues within black or grey boxes are identical or similar, respectively. The vertical arrow passing between residues 92 and 93 of OM b_5 and between residues 90 and 91 of ER b_5 indicates the site of cleavage of trypsin. The open rectangle, close to the C-terminus of the 2 proteins, encloses an uninterrupted stretch of uncharged amino acids in both proteins. (B) Kyte–Doolittle hydropathy plot for OM b_5 and ER b_5 (window setting = 7). The grey and open horizontal bars below the plots indicate the tryptic fragments of OM b_5 and ER b_5 respectively, while the filled black and striped bars correspond to the respective C-terminal anchoring domains. The bar at the bottom of the panel schematizes the structure of NER-COM and shows the amino acid sequence at the junction between the 2 domains in the chimera.


A
OM
ER
Asp Gly Gly Pro Ala Thr
Met Ala Glu - Lys Asp Lys
11
10
Arg Ala Asn Thr Ala Glu Glu Met
Thr Gln Lys Asp Ser Lys Ser Val
30
29
AC GGG CGA GTC TAC GAT ATC ACC CGC TTC CTC AGC GAG CAT CCT GGT GGA GAA GAG 56
Gly Ser
His Glu
49
48
GTT CTA CTG GAA CAA GCT GGT GCC GAT GCA ACT GAA AGC TTT GAA GAT GTC GGC CAC 113
Leu Ser
Arg Asn
68
67
TCC CCT GAT GCC AGG GAG ATG CTA AAG CAG TAC TAC ATT GGT GAT GTC CAT CCG AAT 170
Pro Leu Gln Tyr Val Asn
Thr Ser Thr Ile Leu Asp
87
86
GAC CTT - AAA - CCT AAA GAT GGT GAC AAG GAC CCT TCA AAA AAC AAT TCA TGC 221
Leu - - Pro Asp Gly Lys Asp Pro Lys Asn Asn Cys
Arg Ser Ile Ala Pro Ser Glu Thr Leu Ile Thr Thr Val Glu Asn
104
103
CAA AGT TCC TGG GCA TAT TGG ATT GTC CCC ATC GTG GGT GCT ATT CTT ATA GGT TTC 278
Gln Ser Ala Tyr Ile Val Gly Leu Leu Pro
Ser Trp Thr Asn Ala Ile Ser Leu Val Val Ala Leu
123
124
CTG TAT CGT CAC TTC TGG GCT GAC AGC AAA TCC TCC TGA GGAGGCCACG TTGAAGTGTC 337
His Trp Ser Lys Ser Ser ***
Leu Met Asp
135
134
GGAGCACA 345
B
Hydrophilicity
5
0
-5
20 40 60 80 100 120
OM
ER
NER-COM
DRSKDGDK

[6]. (Fig. 1A). The nucleotide sequence of the cloned fragment predicted the expected amino acid sequence. Since screening of several cDNA libraries with this probe gave negative results, we turned to a RACE-PCR protocol [11] to obtain the 3′ portion of the cDNA for OM b_5. An oligonucleotide corresponding to a sequence within the probe (overlined with full arrow in Fig. 1A) was used as 5′ primer to amplify liver cDNA together with an oligo(dT) 3′ primer (see section 2). The resulting ~800 nt fragment was cloned and sequenced. The first part of the sequence overlapped with that already determined in the 200 nt probe. The reading frame continued as expected from the amino acid sequence of [6] until the site of cleavage of trypsin at Lys-92 (vertical arrow), to continue with 43 codons followed by a stop codon (Fig. 1A). The additional 43 codons specified a polar linker region (residues 93–107), a hydrophobic region (residues 108–125, enclosed within the rectangle) and 10 residues constituting a short polar region at the extreme C-terminus.

Fig. 1A shows the nucleotide sequence of OM b_5 cDNA determined from the 200 nt probe and the 5′ portion of the 800 nt fragment, as well as the amino acid sequence of the protein – from the work of [6] and deduced from the cDNA sequence of this study – aligned with that of rat ER b_5 [17]. Panel B of the same figure shows the hydropathy plots for the 2 cyt b_5 isoforms. The 2 plots are quite similar, illustrating that the 2 proteins have hydrophobic tails of similar lengths and in similar positions. Although the hydropathy plots of the 2 proteins are similar, examination of Fig. 1A shows that the high degree of sequence similarity present in the N-terminal, trypsin-resistant, heme-binding domains (site of trypsin cleavage indicated by the vertical arrow), is lost in the C-terminal portion of the proteins. This is especially apparent in the linker regions, while the hydrophobic stretch presents a higher percentage of conserved residues.

To investigate whether targeting information is present in the heme-binding and/or in the anchoring domain of cyt b_5 isoforms, we constructed a chimera between the the N-terminal domain of ER b_5 and the C-terminal domain of OM b_5 (NER-COM). The 2 domains were joined at the site of trypsin cleavage, as shown in Fig. 1A and B. The wt ER b_5 and NER-COM were expressed in transiently transfected CV1 cells, and 24 h after transfection their localization was analyzed by immunofluorescence, using an antibody against rabbit ER b_5. The relatively short time allowed for expression of the exogenous DNA was chosen in order to avoid artifacts due to heavy overexpression. The results of these experiments are shown in Figs. 2 and 3. At the dilutions of antibody used, endogenous cyt b_5 of CV1 cells was not detectable (see cells marked by asterisks in Figs. 2 and 3). As can be seen from Figs. 2a and 3a, ER b_5 showed a reticular staining pattern spread out

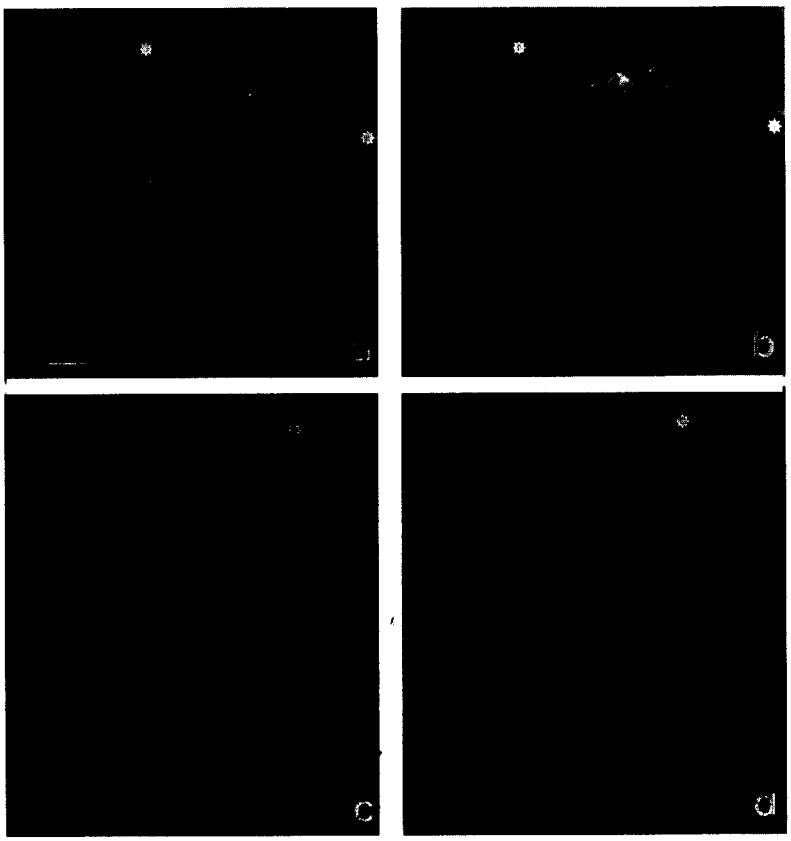


Fig. 2. Comparison of localization of ER b_5 and NER-COM with that of mitochondria in transfected cells. CV1 cells transiently transfected with ER b_5 (panels a and b) or with NER-COM (panels c and d), were incubated with Mitotracker CMX Rose, then fixed, permeabilized and stained for cytochrome b_5, using FITC-labeled secondary antibodies. Panels a and b correspond to the same field of cells transfected with ER b_5, observed for cyt b_5 (fluorescein filter – panel a) and for mitochondria (rhodamine filter – panel b). Panels c and d correspond to the same field of cells transfected with NER-COM, viewed for cyt b_5 (panel c) or for mitochondria (panel d). NER-COM and mitochondria precisely colocalize. Asterisks in all panels correspond to the positions of non-transfected cells, which give a positive stain for mitochondria but do not stain with anti-ER b_5 antibodies. Bar in panel a = 10 μm.

through the cytoplasm and coinciding with the distribution of the ER marker PDI (compare Fig. 3a with Fig. 3b). Its distribution was completely different from that of mitochondria, which appearsed as distinct, elongated organelles, revealed with a fluorescent mitochondrial stain (compare Fig. 2a and 2b). In sharp contrast, when its level of expression was not too high, NER-COM had a staining pattern precisely matching that of mitochondria (compare Fig. 2c and 2d) and not at all superimposable on that of PDI (Fig. 3c and d). When levels of expression were higher, some NER-COM was also present on the ER (not shown), suggesting that binding of the chimera to mitochondria was saturable and that excess protein could associate with the ER. At yet higher levels of expression, especially in cells observed at later times after transfection, both ER b_5 and NER-COM were concentrated in large bodies (diameters up to 10 μm), probably corresponding to autophagic vacuoles, which accumulated around the nucleus (not shown).

Our results indicate the presence of a targeting signal for OM b_5 in the C-terminal anchoring domain of the protein, since this region is sufficient to relocate the cytosolic functional domain of ER b_5 to the outer mitochondrial membrane. We do not yet know whether the targeting information is contained within the membrane anchor itself or within the hydrophilic flanking sequences, but we favour the latter possibility, since (i) the hydrophobic stretch appears more conserved between the 2 isoforms than the polar flanking regions; (ii) changes in the sequence of the membrane anchor of ER b_5 do not alter its subcellular localization, as judged by immunofluorescence of transfected cells (our unpublished results).

Previous work had suggested a function in targeting for the C-terminal domain of ER cyt b_5 [18] and of the ER form of aldehyde dehydrogenase (msALDH). [19]. For ER cyt b_5 it was shown that in the absence of the 10 C-terminal amino acids, the protein failed to bind to the ER [18]. In the case of msALDH, deletion of residues from either of the regions flanking the anchor did not interfere with localization to the ER, while the double deletion of key residues from both these regions resulted in the protein remaining cytosolic [19]. In both these studies, the deletions caused the proteins to become soluble. Thus, the implicated sequences might be involved in the stabilization of the proteins in the membrane, rather than in their targeting. In contrast, the present work, which shows that the anchoring domain of OM b_5 targets the cytosolic domain of ER b_5 to an alternative membrane (the outer mitochondrial membrane), clearly demonstrates a role in targeting for the C-terminal region.

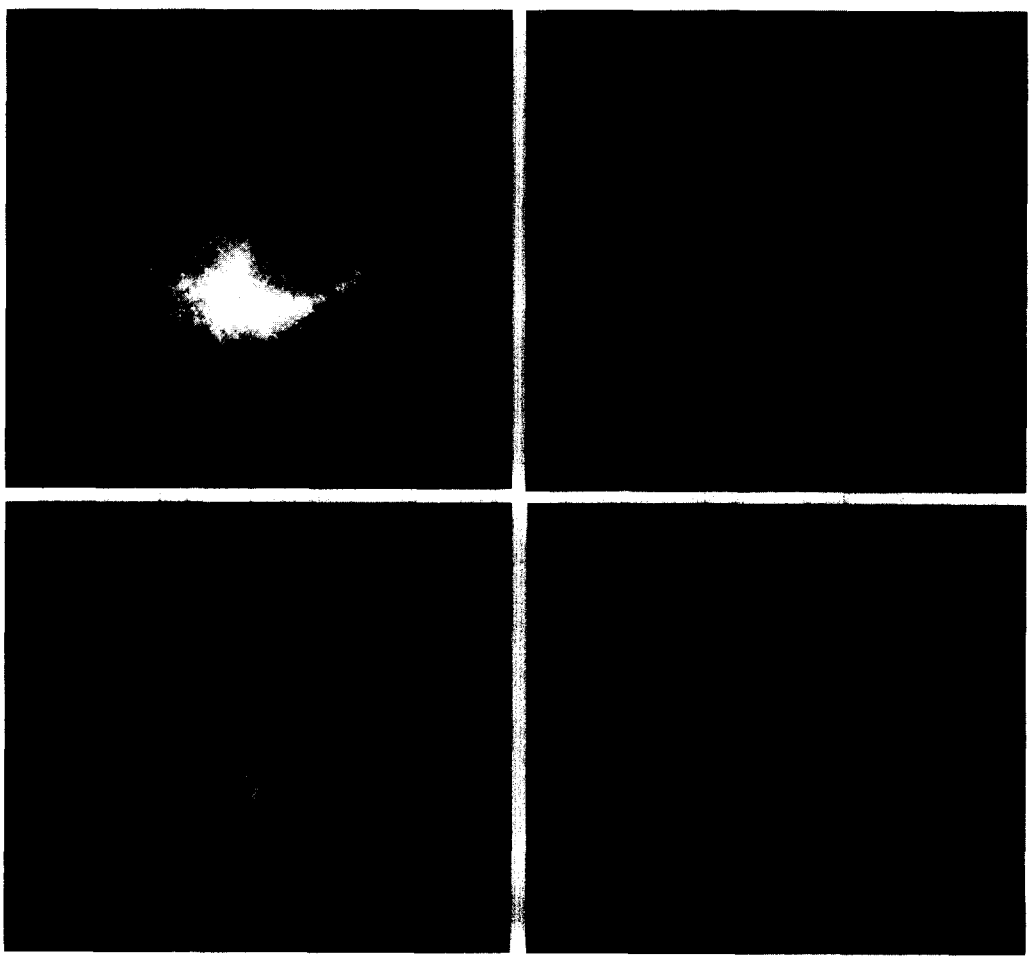


Fig. 3. Comparison of localization of ER b_5 and NER-COM with that of PDI in transfected cells. CV1 cells, transiently transfected with ER b_5 (panels a and b) or NER-COM (panels c and d), were doubly immunostained for cyt b_5 (panels a and c) or PDI (panels b and d). Panels a and b show the same field of cells transfected with ER b_5, while panels c and d show the same field of cells transfected with NER-COM. ER b_5 colocalizes with PDI, while NER-COM has a different pattern. Asterisks and bar are as in Fig. 2.

On the membrane of the ER, cyt b_5 interacts with a number of protein partners, including its electron acceptors, involved in various aspects of lipid and drug metabolism, and its electron donors, i.e. NADH-cyt b_5 reductase and NADPH-cyt *P*-450 reductase. In all of these interactions, the same negatively charged surface area surrounding the exposed heme edge of cyt b_5 appears to be involved, with formation of complementary charge pairs between carboxylate groups of that region and appropriately spaced amino groups at the surface of the various acceptors or donors (see [4] for a review). The present work shows that these protein–protein interactions do not play a dominant role in cyt b_5 targeting, since it can be relocated to a different membrane by a protein region distinct from the cytosolic heme-binding domain. Moreover, our results implicate the C-terminal region of OM b_5 in targeting to the mitochondrial outer membrane rather than an N-terminal amphipathic helix-forming sequence, as found in matrix-directed precursors [20]. It is of interest that a C-terminal mitochondrial outer membrane targeting signal has been reported also for monoamine oxidase B [21]. This protein appears however to be at least partially translocated into the intermembrane space [22]. Although the C-terminal anchoring domain of OM b_5 does not bear any resemblance to known, N-terminal, mitochondrial targeting sequences, it is possible that it interacts with components of the mitochondrial import machinery, as has been shown for other proteins directed to the outer mitochondrial membrane [20,23]. If this turns out to be the case, OM b_5 will be a useful tool to define novel features required for recognition by outer mitochondrial membrane import receptors. As far as the insertion of tail-anchored proteins into the ER is concerned, recent evidence from cell-free studies on the v-snare synaptobrevin indicates a requirement for a trypsin-sensitive component of the ER, distinct from the components of the well-characterized translocation machinery for secretory proteins [1]. Although such a protease-sensitive component has not been shown for the insertion of ER b_5 [2], it is tempting to speculate that common proteins underlie the insertion of all ER-directed tail-anchored proteins.

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