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A HYDROPHOBIC REGION OF RICIN A CHAIN WHICH MAY HAVE A ROLE IN MEMBRANE TRANSLOCATION CAN FUNCTION AS AN EFFICIENT NONCLEAVED SIGNAL PEPTIDE

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Summary. Ricin A chain is a polypeptide of 267 amino acids containing a hydrophobic region near its carboxyl-terminus (residues 245-256) which has been implicated in the membrane translocation step necessary for this catalytically active toxin to reach its intracellular substrate. DNA fusions were constructed that encoded hybrid proteins consisting of carboxyl-terminal residues 233-267 or residues 238-267 of ricin A chain preceding mouse dihydrofolate reductase. When *in vitro* transcripts prepared from these constructs were translated in cell-free systems, the ricin A chain-derived sequences functioned as efficient signal peptides which directed dihydrofolate reductase into microsomes or into proteoliposomes containing microsomal membrane components.

Ricin A chain (RTA) kills mammalian cells by catalytically inactivating cellular protein synthesis (1). To do so, RTA must reach the cytosol where its ribosomal RNA substrate is located (2). RTA, as part of the heterodimeric ricin holotoxin, enters cells by endocytosis. After intracellular transport, RTA is believed to translocate into the cytosol from the lumen of the endoplasmic reticulum (ER) (3). The mechanism of the membrane translocation step is unclear at present, but a stretch of hydrophobic amino acids near the C-terminus of RTA has been implicated as playing a role since point mutations in this stretch significantly reduce the cytotoxicity of ricin but are without effect on the catalytic activity of RTA (4). The hydrophobic region might facilitate RTA translocation by interacting with existing ER membrane protein translocators, such as that involved in signal sequence-mediated transport across or insertion into the membrane of nascent secretory or transmembrane proteins (5).

Examination of the amino acid sequence of RTA in the vicinity of the hydrophobic region indicates some similarity to a typical N-terminal signal peptide (6). That is, the hydrophobic region is flanked on the N-terminal side by a region containing charged residues, and on the C-terminal side by a region dominated by charged and polar amino acids. In the present study, we addressed the question of whether a C-terminal peptide derived from RTA could function as a signal peptide

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when placed at the N-terminus of an appropriate passenger protein. The data presented clearly show that it can function in this way, establishing a functional co-translational interaction in this fusion protein context with ER membrane translocon components such as Sec61p (5).

MATERIALS AND METHODS

Hybrid construction and DNA manipulations. Dihydrofolate reductase (DHFR) preceded by RTA residues 233-267 (pRA-DHFR 1) or RTA residues 238-267 (pRA-DHFR 2) were constructed in pGEM3Z (Promega) by amplification of the coding region of DHFR from the vector pDS5/3 (7) and RTA from pGEMRA (8). Control pDHFR DNA was created by amplification of the DHFR sequence and insertion into pGEM3ZF(-) (Promega). Hybrid DNA was fully sequenced to establish clone integrity. Plasmid DNA was prepared by alkaline lysis (9) and linearised by digestion with *Hin*dIII prior to transcription. Truncated constructs were prepared by cleavage at base position 291 with *Afl*II.

In vitro transcription/translation. Linearised hybrid DNA was transcribed using T7 polymerase under standard conditions (8). Transcripts encoding preprolactin and prepro-alpha-factor were prepared as described earlier (10). Transcripts were stored at -70°C prior to *in vitro* translation. Translation was performed in cell-free systems derived from wheatgerm (11) and rabbit reticulocyte lysate (12) in the presence of ^{35}S-methionine at 30°C for 30 and 60 minutes respectively.

In vitro translocation assay. Puromycin and potassium chloride-washed microsomes (PKRMs), reconstituted membranes and proteoliposomes were prepared as described elsewhere (13). Rough microsomes were kindly donated by Dr. S. High (University of Manchester, UK). *In vitro* import of translation product was carried out as described (13). Proteinase K digestion of samples was performed at a concentration of 0.5mg/ml for 40 minutes on ice, in the presence or absence of 1% NonidetP40. Protease was inactivated by the addition of phenylmethylsulfonyl fluoride to 2mM prior to SDS-PAGE. Samples were analysed on 15% polyacrylamide gels under reducing conditions. Gels were dried and subjected to autoradiography.

RESULTS AND DISCUSSION

Fusions were constructed between DNA encoding RTA residues 233-267 and mouse DHFR (RA-DHFR 1) and RTA residues 238-267 and DHFR (RA-DHFR 2) (Fig. 1). Each sequence was mutagenised to make the first codon that for methionine. While the RTA-derived sequences exhibited the general features of typical N-terminal signal peptides, no consensus site for signal peptidase was present, implying that if these sequences could function as signal peptides they were likely to remain uncleaved during segregation of the fusion proteins. The longer RTA sequence contained a putative N-glycosylation site at Asn236, but this site was not glycosylated *in vitro* in

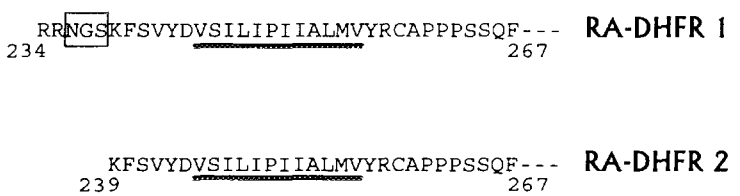

Figure 1. Fusion proteins used in this study. The RTA carboxyl-terminal peptides MetArg234-Phe267 and MetLys239-Phe267 were fused to the amino-terminus of native mouse dihydrofolate reductase to create RA-DHFR 1 and RA-DHFR 2,respectively. The N-glycosylation site is boxed and the hydrophobic region underlined.

the presence of microsomes (see below), probably because of its proximity to the N-terminus of the protein.

RA-DHFR 1 transcripts translated in reticulocyte lysates in the absence (Fig. 2, lanel) or the presence (Fig. 2, lane 3) of dog pancreas microsomes produced two polypeptide products; full length RA-DHFR and a faster moving product resulting from initiation at Met255 within the hydrophobic stretch of the RTA-derived sequence (Fig. 1). The same full length and truncated products were seen in the wheatgerm system (see Fig. 4). Protease treatment of the products formed in the absence of microsomes converted both to a single faster moving band (Fig. 2, lane 2). This band represents DHFR from which the N-terminal peptides derived from RTA had been digested. The DHFR moiety itself was resistant to protease under the conditions employed here (Fig. 2, lanes 12 to 16). Protease treatment of the products synthesised in the presence of microsomes showed that the full length RA-DHFR 1 was protected from degradation whereas the truncated product was not (Fig. 2, lane 4). Since the truncated product lacked most of the hydrophobic region of the putative RTA-derived "signal peptide", this served as a useful control showing that the complete "signal peptide" was necessary for membrane translocation. Protection in the presence of microsomes in the case of RA-DHFR 1 was due to segregation into the vesicles, since protease treatment in the presence of detergent did result in degradation to the size of mature DHFR (Fig. 2, lane 5). Identical results were obtained in the case of RA-DHFR 2 (Fig. 2, lanes 6 to 10). Preprolactin, which was employed as a control, behaved predictably in the assays used (Fig. 2, lanes 17 to 21).

The experiments described above were complicated somewhat by the well established resistance of full length DHFR to protease digestion. We therefore prepared another set of fusions between the RTA sequences and a truncated version of DHFR lacking 90 C-terminal residues. *In vitro* translation of truncated transcripts gave product of the expected size in both the absence (Fig. 3, lanes 1 and 7) and presence (Fig. 3, lanes 3 and 9) of microsomes. In this case, however, product formed in the absence of microsomes was completely sensitive to protease (Fig. 3, lanes 2 and 8), whereas that formed in the presence of microsomes was protected (Fig. 3, lanes 4 and 10) unless detergent was added to disrupt the vesicles (Fig. 3, lanes 6 and 12). Following translation, an

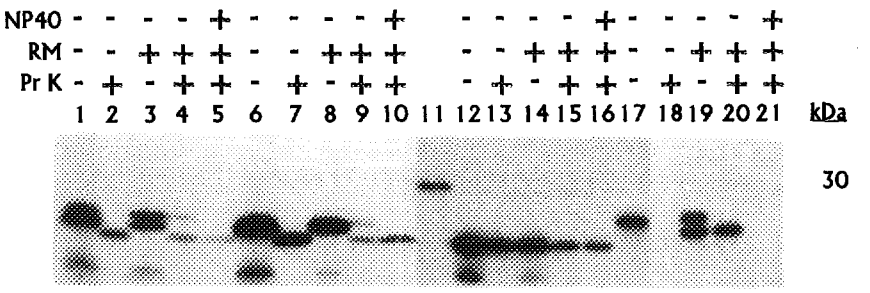

Figure 2. ***In vitro* translocation of RA-DHFR hybrids onto microsomes.** Transcripts encoding RA-DHFR 1 (lanes 1-5), RA-DHFR 2 (lanes 6-10), DHFR (lanes 12-16) and preprolactin (lanes 17-21) were translated in rabbit reticulocyte lysates in the absence or presence of canine pancreas microsomes (RM) and treated with proteinase K (PrK) in the absence or presence of the detergent NonidetP-40 as indicated. Translation products were separated by SDS-PAGE and visualised by autoradiography. Lane 11 represents molecular weight markers whose sizes are indicated.

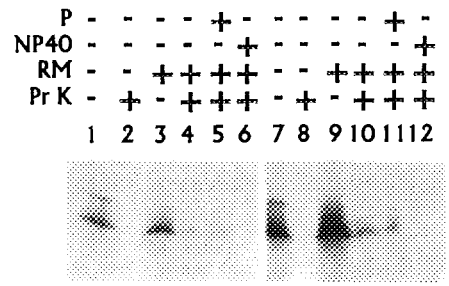

Figure 3. *In vitro* translocation of truncated RA-DHFR hybrids into microsomes. Transcripts encoding C-terminally truncated RA-DHFR 1 (lanes 1-6) or RA-DHFR 2 (lanes 7-12) were translated in rabbit reticulocyte lysates in the absence or presence of canine pancreas microsomes (RM) and treated with proteinase K (PrK) in the absence or presence of NonidetP-40 as indicated. Translations treated with puromycin (P) are also shown. Translation products were separated by SDS-PAGE and visualised by autoradiography.

aliquot was treated with puromycin (2mM) to release the truncated products from the ribosome. No increase in sensitivity to protease was observed (Fig. 3, lanes 5 and 11). The bands were slightly distorted on this gel since they comigrated with unlabelled globin present in the lysates.

RA-DHFR 1 and 2 were also co-translationally segregated into proteoliposomes reconstituted from either total components of detergent-solubilised dog pancreas microsomes followed by detergent removal using Biobeads (13), or from purified signal recognition particle (SRP) receptor, the Sec61p complex and the translocating chain-associating membrane (TRAM) protein and purified phospholipids (13). Transcripts were translated in the wheatgerm system in the absence of added membranes (Fig. 4a, lanes 1 and 5), in the presence of microsomes (Fig. 4a, lanes 2 and 6), in the presence of proteoliposomes reconstituted from total ER membrane components (Fig. 4a, lanes 3 and 7), or in the presence of proteoliposomes reconstituted from purified components (Fig. 4a, lanes 4 and 8). Protease treatment established that the full-length products were segregated into all types of vesicle (Fig. 4b, lanes 1 to 8). The functional integrity of the vesicles was shown using

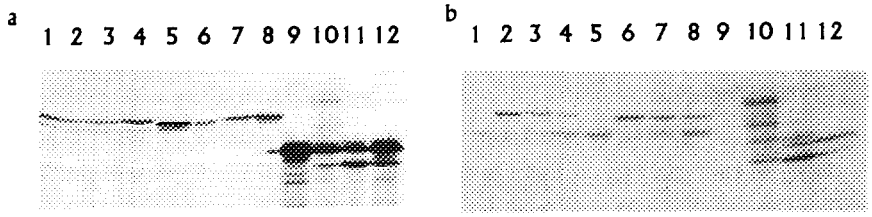

Figure 4. *In vitro* translocation of RA-DHFR hybrids into proteoliposomes. Transcripts encoding RA-DHFR 1 (lanes 1-4), RA-DHFR 2 (lanes 5-8) and yeast prepro-alpha-factor (lanes 9-12) were translated in wheat germ lysates without the addition of membranes (lanes 1, 5 and 9), or in the presence of canine pancreas microsomes (lanes 2, 6 and 10), proteoliposomes reconstituted from total solubilised microsomes (lanes 3, 7 and 11) or proteoliposomes reconstituted from purified components (lanes 4, 8 and 12). Samples were analysed by SDS-PAGE either directly (panel a) or after digestion with proteinase K (panel b), and products were visualised by autoradiography.

transcripts encoding prepro-alpha-factor. Prepro-alpha-factor was core glycosylated on entering microsomes giving product bearing one, two or three oligosaccharide side-chains (Fig. 4a, lane 10) which were protected from protease (Fig. 4b, lane 10). The extent of glycosylation was reduced with proteoliposomes containing total ER membrane components (Fig. 4a and b, lane 11), and was, as expected, absent with proteoliposomes containing a limited number of purified components (Fig. 4a and b, lane 12).

If, during ricin intoxication, RTA translocates into the cytosol from the ER lumen, it is tempting to assume that it utilises one of the protein translocating channels in the ER membrane in the reverse direction. Dissociation of RTA from RTB is thought to occur in the ER lumen (14), possibly facilitated by molecular chaperones and protein disulfide isomerase, and this would expose the RTA C-terminal hydrophobic region which is buried in ricin holotoxin (15). This region of RTA might then insert into the ER membrane and interact with or recruit existing ER translocons to initiate RTA export into the cytosol. Possible translocation channels include that used by nascent secretory or membrane proteins (5), the TAP transporters that deliver cytosolically-derived peptides to MHC class 1 molecules (16), or an as yet uncharacterised transport machinery that exports peptides from the ER (17). We have recently found that cells lacking a functional heterodimeric TAP1-TAP2 peptide transporter remain fully sensitive to ricin, implying that RTA does not utilise this transporter (unpublished data). Since RTA might use the secretory protein translocon in the reverse direction, we decided to investigate whether the C-terminal region of RTA could act as a signal peptide when transposed to the N-terminus of an appropriate passenger protein, in this case DHFR. The data presented here clearly show that it can. Such a finding is not novel in itself since many random peptide sequences have been shown to function as N-terminal signal peptides directing the import of proteins into the ER lumen or into other organelles such as mitochondria (for example, see (18) and (19)). It does, however, show that this RTA sequence can direct nascent proteins into the ER lumen when placed at the N-terminus by productively interacting with components of the ER translocation machinery. Although it has not yet been verified experimentally, it is possible that the same peptide situated at the C-terminus of a protein already present in the ER lumen could also interact with ER membrane components to initiate export from the ER by some unknown mechanism. In keeping with a putative role for this region in RTA membrane translocation, we have found that point mutations in the hydrophobic stretch can significantly reduce ricin cytotoxicity although they do not affect the stability or catalytic activity of RTA itself (4).

Such a mechanism would require the RTA C-terminal sequence to interact with a component of the translocation machinery in the ER membrane in order to gate a channel for toxin export. Relevant to this, a recent report has shown that, in the case of the secretory protein preprolactin, two independent recognition events are required before the protein is allowed to traverse the ER membrane (20). Firstly, the signal sequence of the nascent chain is recognised by SRP in the cytosol. Following interaction of the ribosome-nascent protein-SRP complex with the ER membrane, a second signal sequence recognition event occurs within the membrane which permits the completion of nascent chain insertion into the translocation site. The presence of the Sec61p complex in the membrane is both necessary and sufficient for this second recognition event. Since

the RTA-derived sequence can direct DHFR into proteoliposomes prepared from purified components, the sequence can clearly participate in both SRP and Sec61p recognition steps when it is located at the N-terminus of the hybrid protein. Although at present purely hypothetical, it is possible that the same sequence naturally located at the C-terminus of RTA might productively interact with Sec61p from the lumenal side of the ER membrane, and that this interaction might facilitate the reverse translocation of RTA into the cytosol.

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