Reports

Stop-Transfer Regions Do Not Halt Translocation of Proteins into Chloroplasts

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Protein targeting in eukaryotic cells is determined by several topogenic signals. Among these are stop-transfer regions, which halt translocation of proteins across the endoplasmic reticulum membrane. Two different stop-transfer regions were incorporated into precursors for a chloroplast protein, the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. Both chimeric proteins were imported into chloroplasts and did not accumulate in the envelope membranes. Thus, the stop-transfer signals did not function during chloroplast protein import. These observations support the hypothesis that the mechanism for translocation of proteins across the chloroplast envelope is significantly different from that for translocation across the endoplasmic reticulum membrane.

PROTEINS SYNTHESIZED ON CYTOplasmic ribosomes in eukaryotic cells must be correctly targeted to their proper intracellular location. For many proteins, this requires that one or more membranes be crossed. Details of the process by which proteins cross this hydrophobic environment are not well understood. For translocation across the endoplasmic reticulum (ER), which occurs co-translationally, two distinctly different models have been proposed. In one, proteins are inserted directly into and traverse the lipid bilayer (*1*). In the other, proteins are thought to be transported through a hydrophilic (protein) pore (*2–5*). For translocation of proteins into mitochondria and chloroplasts, which occurs posttranslationally, it has been proposed that precursor proteins initially interact with a receptor on the outer membrane (*1, 6*). The proteins are then unfolded (*7*) and threaded through the two lipid bilayer membranes at special regions where the two membranes are held in close proximity (*8*).

We reasoned that if import into chloroplasts involves unfolding and threading proteins through the bilayer, it should be possible to halt protein translocation by incorporating hydrophobic stop-transfer (ST) regions (*9, 10*) into precursor proteins. ST regions from mouse immunoglobin M (IgM) and vesicular stomatitus virus glycoprotein (VSV G protein) have been shown to be necessary and sufficient to halt translocation of proteins across the ER, thereby causing the translocated proteins to become anchored in the ER membrane (*11, 12*). We have created chimeric precursor proteins containing these ST regions fused to the precursor to the small subunit (SS) of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), a nuclear-encoded chloroplast protein. These chimeric precursor proteins were characterized with respect to efficiency of import and intraorganellar location.

Chimeric precursor molecules (Fig. 1) were produced by in vitro transcription and translation. Radioactive precursors were incubated with isolated pea chloroplasts as described (*13*) to determine if they could be imported into chloroplasts. TPSSVSV and TPSSμM3 were imported into chloroplasts and were processed to appropriate sizes. A small amount of unprocessed precursor was sensitive to protease and presumably was bound to the outside of the chloroplasts (Fig. 2). The processed molecules were protected from protease treatment, indicating they were inside the chloroplasts (Fig. 2). Time course studies showed that TPSSVSV and TPSSμM3 were imported at a rate 10 to 20% that of TPSS (*14*).

To determine whether the chimeric precursors were being imported via the same mechanism as TPSS, we first determined if import was dependent upon a transit peptide, the NH_2-terminal extension which directs transport of precursors into chloroplasts (*6*). This control was necessary because it has been reported that one of the ST regions used here (IgM) can act as a signal sequence and initiate translocation across microsomal membranes (*15*). Two proteins, SSVSV and SSμM3, which lack transit peptides, were constructed (Fig. 1). These proteins were not imported into chloroplasts (*14*). These results indicated that the transit peptide, rather than the hydrophobic ST region, was necessary for import. Second, we determined that the import of both chimeric precursors was inhibited by nigericin and could be restored by addition of adenosine triphosphate (ATP) (*14*). The confirmation that the import of the chimeric proteins depended on both a transit peptide and ATP supported the conclusion that they were imported by the same mechanism utilized for TPSS.

Although the protease protection experiments established that the processed molecules were inside chloroplasts, it was necessary to determine their intrachloroplastic locations. In particular, it was important to determine whether the ST regions caused the chimeric proteins to become embedded in the envelope membrane. Chloroplasts containing imported chimeric proteins and a control sample containing imported SS were lysed and fractions representing various chloroplast compartments were isolated. The major portion (approximately 90%) of the imported molecules were found inside the chloroplasts, in the thylakoid and stromal fractions (Fig. 3). Only 5 to 10% of the imported molecules were in the envelope membrane fraction.

The location of the imported molecules within each fraction was determined by further analysis. The envelope membrane fraction was separated into inner and outer

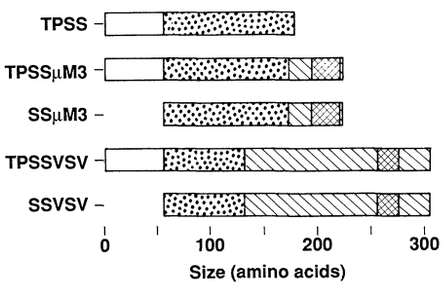

Fig. 1. Precursor proteins containing stop-transfer regions. Constructions were carried out in plasmids that contained an SP6 RNA polymerase promoter upstream of the coding region. TPSS is encoded by pSP81/4 and is a chimeric Rubisco small subunit precursor (*18*). TPSSμM3 is encoded by the plasmid pTPSSμM3. This protein contains the transit peptide and 117 amino acids (aa) of the mature peptide of TPSS, followed by 50 aa of mouse IgM (*19*). SSμM3 is the same as TPSSμM3 but lacks the transit peptide (*19*). TPSSVSV is encoded by the plasmid pTPSSVSV. This protein contains the transit peptide and 77 aa (of a total of 123 aa) of the mature peptide of TPSS (*18*) followed by the COOH-terminal 172 aa of VSV G protein (*20*). SSVSV is the same as TPSSVSV but lacks the transit peptide (*20*). Radiolabeled precursor proteins were produced from purified plasmids by in vitro transcription and translation (*21*). The chimeric molecules are diagrammed; different shading shows the origin of the different regions. Open box, the transit peptide of Rubisco small subunit; dotted box, the mature peptide of Rubisco small subunit; hatched box, soluble portions of the transmembrane proteins; cross-hatched box, the stop-transfer region of each transmembrane protein.

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envelope fractions by centrifugation through a discontinuous sucrose gradient (*16*). Nearly all (greater than 85%) of the imported molecules in the envelope fraction sedimented with the inner membrane (*14*). Treatment of thylakoids with thermolysin resulted in the complete removal of radioactive label. No limit digestion product was found (*14*). Thus, the molecules associated with the thylakoids were only loosely associated with the membrane surface and were not inserted into the membranes.

Stromal contents were analyzed by sedimentation through sucrose gradients to determine whether the imported chimeric molecules had been assembled into Rubisco holoenzyme. Greater than 90% of the imported SSVSV and 60% of imported SSμM3 were found to cosediment with the 18*S* Rubisco holoenzyme (*14*). From these observations we conclude not only that the imported proteins were present in the stroma, but that most of these chimeric proteins had been assembled into Rubisco holoenzyme.

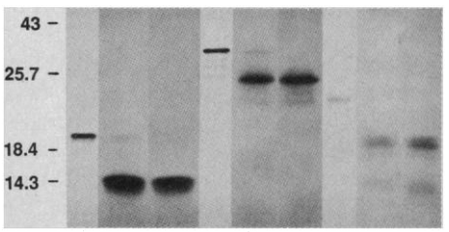

Fig. 2. Import of precursor proteins containing stop-transfer regions. Precursor proteins were incubated with chloroplasts in an in vitro import assay (*18*). Following incubation, chloroplasts were incubated with protease (thermolysin) to remove molecules bound to the outside (lanes 3, 6, and 9). A control sample was treated identically except that it was not treated with protease (lanes 2, 5, and 8). Intact chloroplasts containing imported proteins were isolated by centrifugation through a Percoll cushion, and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography (*13*). Lanes 1 to 3, TPSS; lanes 4 to 6, TPSSVSV; lanes 7 to 9, TPSSμM3. Translation products are shown in lanes 1, 4, and 7; both bound and imported molecules are shown in lanes 2, 5 and 8; imported, protease-protected molecules are shown in lanes 3, 6, and 9. The migration of molecular weight standards are shown; sizes are in kilodaltons.

The observation that most of the chimeric proteins were found inside chloroplasts meant that ST regions had not halted translocation across the chloroplast envelope. The distribution of imported proteins among the membrane fractions most probably resulted from an association of the hydrophobic ST regions with the membranes. The distribution between the envelope and the thylakoid fractions is consistent with such a nonspecific association based on surface areas.

It has been recently reported that a chimeric protein containing an ST region from VSV G protein was imported into mitochondria and located in the inner membrane (*17*). Nguyen and Shore concluded that the ST region had halted translocation of the chimeric protein at the inner membrane. However, they did not eliminate the possibility that association with the inner membrane resulted from insertion into the membrane after import into the matrix. In mitochondria, such an insertion after import has been shown to occur with the Fe/S protein of ubiquinol-cytochrome c reductase (*8*).

The data presented here show that protein translocation across the chloroplast envelope membranes is sufficiently different from translocation across the ER so that an ER ST sequence is not recognized. Several explanations are possible. The chloroplast envelope membrane may not be sufficiently hydrophobic to cause the ST regions to anchor the chimeric proteins. This does not seem likely. A more plausible explanation is that the ST regions in the chimeric proteins were never exposed to the lipid bilayer during translocation. There are several ways this could occur. Protein import could occur via a hypothetical translocator protein complex (*3*). In this case, it would be necessary to postulate that the specificity of the chloroplast translocator complex is sufficiently different that it does not recognize the ER ST sequence. Alternatively, chloroplast protein import may proceed by an entirely different mechanism. Although at present we cannot distinguish between these two possibilities, we suggest that import of proteins into chloroplasts does not involve threading an unfolded protein directly through the lipid bilayer.

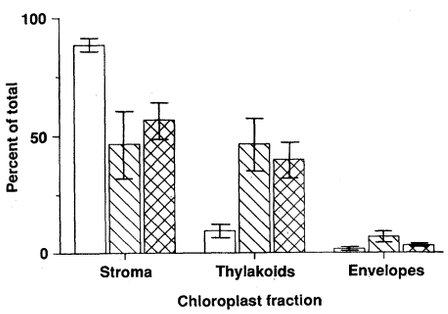

Fig. 3. Localization of stop-transfer proteins-chloroplast fractionation. After incubation of TPSS, TPSSVSV, and TPSSμM3 in a standard import assay, intact chloroplasts were lysed by two freeze-thaw cycles in 0.6*M* sucrose, and fractionated by differential centrifugation (*16*). Thylakoids were recovered by centrifugation at 4500*g* for 15 minutes, and mixed envelope membranes by centrifugation at 45,000*g* for 30 minutes. The supernatant fraction from the final centrifugation represented the stroma. Aliquots of each fraction were analyzed by SDS-PAGE, fluorography, excision of protein bands, and quantitation of radiolabeled protein by liquid scintillation methods (*13*). Quantitation of proteins and calculations of numbers of molecules per chloroplast were as described (*13*) from the number of leucine residues per protein molecule: TPSS, 13; TPSSVSV, 29; and TPSSμM3, 19. Processed molecules, without transit peptides, contained one less leucine molecule than the precursor forms. Results are expressed as the percentage of the total imported protein that was found to be associated with each of the three chloroplast locations shown. Error bars show the standard deviations from the averages of three separate experiments. Open box, TPSS; hatched box, TPSSVSV; cross-hatched box, TPSSμM3.

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19. To make plasmid pTPSSμM3, two fragments were subcloned into Sph I/Eco RI–cut pGM22/3: An Sph I/Fsp I fragment of pSP81/4 (*18*), encoding 117 aa of the mature peptide of Rubisco small subunit, and a Bst NI (blunt-ended with mung bean nuclease)/Eco RI fragment of pG6 (*11*), encoding the 50 aa COOH-terminus of IgM μ fragment, were ligated into Sph I/Eco RI–cut pGM22/3. Plasmid pGM22/3 was constructed by subcloning the Hind III/Sph I fragment of pSP81/4 (*18*), containing the transit peptide of Rubisco small subunit precursor from soybean, into Hind III/Sph I–cut pGEM3. Plasmid pSSμM3, which encodes the protein SSμM3, was made by subcloning the Sph I/Eco RI fragment of pTPSSμM3 into pGEM3.
20. To make pTPSSVSV, the Kpn I/Eco RI fragment of plasmid pSVGL (*12*), encoding the above region of the VSV G protein, was subcloned into Kpn I/Eco RI–cut pSP81/4 (*18*). Plasmid pSSVSV, which encodes the protein SSVSV, was made by subcloning the Sph I/Eco RI fragment of pTPSSVSV into pGEM3.
21. Plasmids were isolated from *Escherichia coli* (HB101) by alkaline lysis (*22*). After precipitation with isopropyl alcohol, plasmid DNA was purified by the procedure of Bywater *et al.* (*23*) with the modifications described (*18*). To produce radiolabeled precursor proteins, plasmids were transcribed with SP6 RNA polymerase to produce messenger RNA, which was translated in a wheat germ–based in vitro translation system (*18*). Mapping across the sites of ligation was used to confirm the identity of the plasmids. Proteins of the correct size were synthesized from the transcripts, thereby confirming that the reading frame remained intact (stop translation codons exist in all other possible reading frames).

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Rat Brain *N*-Methyl-D-Aspartate Receptors Expressed in *Xenopus* Oocytes

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N-methyl-D-aspartate (NMDA) activates a class of excitatory amino acid receptor involved in a variety of plastic and pathological processes in the brain. Quantitative study of the NMDA receptor has been difficult in mammalian neurons, because it usually exists with other excitatory amino acid receptors of overlapping pharmacological specificities. *Xenopus* oocytes injected with messenger RNA isolated from primary cultures of rat brain have now been used to study NMDA receptors. The distinguishing properties of neuronal NMDA receptors have been reproduced in this amphibian cell, including voltage-dependent block by magnesium, block by the NMDA receptor antagonist D-2-amino-5-phosphonovaleric acid, and potentiation by glycine. This preparation should facilitate the quantitative study of the regulation of NMDA receptor activation and serve as a tool for purification of the encoding messenger RNA.

OF THE THREE EXCITATORY AMINO acid (EAA) receptors identified pharmacologically (*1*), the *N*-methyl-D-aspartate (NMDA) receptor has received the most attention. This is due to its potential roles in long-term potentiation (*2*), hypoxic damage (*3*), and epileptic discharges (*4*); to the availability of selective and potent antagonists such as D-2-amino-5-phosphonovaleric acid (D-APV) (*5*), MK-801 (*6*), and 3(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP+) (*7*); and to its large conductance (45 to 50 pS) (*8, 9*), which facilitates single-channel studies. Indeed, patch clamp experiments have revealed a rich complexity in the activation of NMDA receptors that render it well suited to regulation under different physiological conditions. The open NMDA channel appears to be blocked by Mg^{2+} (*8, 10*) and ketamine (*11*) in a voltage-dependent manner, and the frequency of opening of NMDA channels is greatly increased in the presence of low concentrations of glycine (*12*). Single-channel studies (*9*) raise the possibility that the three pharmacologically identified EAA receptors (for NMDA, kainate, and quisqualate) are not independent entities but may instead reflect the use of a single-channel type by the three receptors. The presence of four or more major conductance states for each agonist; preferential activation of different conductance states by the agonists NMDA, quisqualate, and kainate; frequent transitions among the different conductance states; and selective block of only the highest conductance by D-APV and Mg^{2+} favor this hypothesis of receptor sites that share channel properties. Clearly, to investigate the structural and functional relations among the three EAA receptors it would be advantageous to have a preparation that expresses all three receptors but in which receptor expression could be systematically manipulated.

The *Xenopus* oocyte translation system, originally developed by Gurdon and colleagues (*13*) to study messenger RNA (mRNA) that encodes soluble proteins, has been used to translate mRNA encoding neurotransmitter receptors. We (*14*) and others (*15*) have shown that mRNA from rat or chick brain, when injected into oocytes, readily induces responses to kainate and quisqualate, but heretofore NMDA responses have not been observed. We now report that mRNA extracted from adult rat brain or primary cultures of fetal rat brain (*16*) encodes NMDA receptors when injected into oocytes. Our results were mainly from 19 separate experiments in which six independent mRNA preparations were used. An abstract of some of this work has been published (*17*).

Oocytes injected with mRNA from rat brain were voltage-clamped with two microelectrodes, and drugs were applied by perfusion in medium similar to that used to culture the oocytes. At a holding potential of −60 mV, 100 μ*M* NMDA evoked a smooth, readily reversible, nondesensitizing inward current that ranged from 5 to 220 nA (mean ± SEM, 41 ± 5 nA, $n = 73$ cells). In six mRNA preparations that encoded NMDA receptors, 73 of 94 oocytes tested responded to NMDA, whereas uninjected or water-injected oocytes ($n = 12$), or oocytes injected with eight mRNA preparations that encoded only kainate receptors ($n = 36$), did not respond (<5 nA) to NMDA at up to 300 μ*M* concentration. In seven cells, increasing concentrations of NMDA were sequentially applied to construct a concentration response curve; the curve indicated a single component response with a half-maximally effective concentration (EC_{50}) of 38 ± 8 μ*M* (mean ± SEM). The selective NMDA receptor antagonist D-APV (10 μ*M*) reduced the amplitude of the ionic current evoked by 100 μ*M* NMDA by 83 ± 1% ($n = 6$ cells) (Fig. 1A).

Current-voltage curves of NMDA responses constructed in the presence of 1 m*M* Mg^{2+} showed a marked nonlinear region between −80 and −30 mV, similar to results obtained with neuronal NMDA receptors (*8, 10*) (Fig. 1B). As expected, the nonlinear behavior was nearly eliminated when Mg^{2+} was omitted from the perfusion

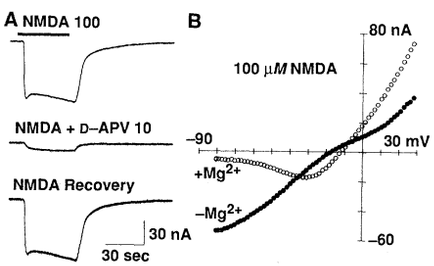

Fig. 1. Block of NMDA-evoked current by D-APV and Mg^{2+}. (**A**) An oocyte injected with approximately 50 ng of mRNA 48 hours before was voltage-clamped with two microelectrodes to a holding potential of −60 mV and superfused continuously with modified Barth's solution (*16*) ($MgSO_4$ replaced by Na_2SO_4 and supplemented with 0.5 m*M* $CaCl_2$ plus 3 μ*M* glycine). During the periods indicated by the solid bar in (A), the superfusion solution was changed to include 100 μ*M* NMDA with or without previous exposure to 10 μ*M* D-APV, as indicated. The input resistance of this cell, measured at its resting potential (−70 mV), was 1.2 Mohm. (**B**) In a different cell the ionic current produced by 100 μ*M* NMDA was plotted as a function of voltage in the absence (●) and presence (○) of 1 m*M* Mg^{2+}. The perfusion solution also contained 3 μ*M* glycine. Current-voltage curves were constructed by ramping the voltage slowly (55 mV per second) from −80 to +30 mV in the presence and absence of 100 μ*M* NMDA. Under these conditions the capacitive current associated with the ramp was small. Leakage and capacitive currents at each voltage were then subtracted from the membrane currents measured in the presence of NMDA. The resting potential and input resistance of this cell were −50 mV and 0.6 Mohm, respectively.

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