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C-Terminal Sequences Can Inhibit the Insertion of Membrane Proteins into the Endoplasmic Reticulum of *Saccharomyces cerevisiae*

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We have constructed three gene fusions that encode portions of a membrane protein, arginine permease, fused to a reporter domain, the cytoplasmic enzyme histidinol dehydrogenase (HD), located at the C-terminal end. These fusion proteins contain at least one of the internal signal sequences of arginine permease. When the fusion proteins were expressed in *Saccharomyces cerevisiae* and inserted into the endoplasmic reticulum (ER), two of the fusion proteins placed HD on the luminal side of the ER membrane, but only when a piece of DNA encoding a spacer protein segment was inserted into the fusion joint. The third fusion protein, with or without the spacer included, placed HD on the cytoplasmic side of the membrane. These results suggest that (i) sequences C-terminal to the internal signal sequence can inhibit membrane insertion and (ii) HD requires a preceding spacer segment to be translocated across the ER membrane.

Topological studies of membrane proteins have been aided by the use of reporter enzymes fused to various positions along membrane proteins. The intracellular location of a reporter enzyme can easily be determined in vivo if its enzymatic activity depends on the cellular compartment in which the enzyme is located. Using this approach, topological studies were initiated with *Escherichia coli* by using the periplasmic and cytoplasmic enzymes alkaline phosphatase and β-galactosidase, respectively, as reporter enzymes (6, 12). These studies revealed that the information necessary for determining the topology of the membrane protein appears not to require the protein in its entirety but that N-terminal fragments become integrated in the proper orientation. The disposition of a particular segment in the intact membrane protein can therefore be inferred from the disposition of a reporter enzyme that has been fused to the C terminus of an N-terminal fragment of the membrane protein truncated at the point of interest. One general exception was found when the placement of a reporter enzyme next to a hydrophilic segment resulted in the deletion of positively charged residues that were required to stably localize that segment on the cytoplasmic side of the *E. coli* inner membrane (2).

Gene fusion methodology has recently been applied to test structural predictions of the topology of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, a membrane protein with seven transmembrane domains, in the yeast *Saccharomyces cerevisiae* (13). This analysis involved the use of the cytoplasmic enzyme histidinol dehydrogenase (HD), encoded by the *HIS4* gene, and was based on the earlier observation that HD enzymatic activity was absent in cells containing HD localized to the lumen of the endoplasmic reticulum (ER) (3). The inactivation of HD in the ER lumen may be caused by one or more of the following: aberrant folding, glycosylation, oxidation, selective degradation, or being sequestered away from available substrate by the ER membrane.

HD is located at the carboxy-terminal end of a multifunctional cytoplasmic enzyme involved in the biosynthesis of histidine (4). When HD is expressed in the cytoplasm of *his4* mutant cells, it allows these cells to grow on histidinol, a metabolic precursor to histidine. Since the enzyme is inactivated when it is expressed after a signal sequence which directs it to the lumen of the ER, the positioning of HD next to an extracytoplasmic membrane protein segment results in the failure of *his4* mutant cells to grow on agar plates that contain histidinol but lack histidine. Cells grow, however, on agar plates containing histidine. Here, we report initial studies of the membrane integration of the multispanning yeast membrane protein, arginine permease. Surprisingly, we found during these studies that the integration of transmembrane domains was influenced by amino acid sequences located C terminal to the arginine permease segment of two chimeric proteins.

MATERIALS AND METHODS

Reagents, strains, and media. Histidinol was obtained from Sigma (St. Louis, Mo.) and used at a concentration of 10 mM on agar plates. For testing the growth of cells in the presence of 10 mM histidinol, agar plates contained SD medium (20 g of 0.67% yeast-nitrogen base without amino acids–2% agar–2% glucose). Supplements included 0.1 mg each of leucine, adenine, tryptophan, and uracil per ml. Trasylol was obtained from Mobay Corp., New York, N.Y. [^{35}S]methionine was obtained from ICN Pharmaceuticals Inc., Irvine, Calif. *S. cerevisiae* strains JC1-3C (*MAT***a** *ade5-1 trp-1 his4-401 ura3-52 leu2-1 HOL1-1*) and FC2-12B (*MAT*α *trp1-1 leu2-1 ura3-52 his4-401 HOL1-1 can1-1*) were obtained from Christine Guthrie (University of California, San Francisco, Calif.). Strain CSa42 (*MAT***a** *sec61-3 his4-401 ura3-52 HOL1-1 trp1-1*) was obtained from Colin Stirling and Randy Schekman (University of California, Berkeley, Calif.).

SD-CAS medium (SD medium plus Casamino Acids [0.7%] and 1 mg each of tryptophan and adenine per ml) was

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used for the growth of cells containing plasmids marked with *URA3*. The Casamino Acids supplement contains leucine and histidine, but the uracil concentration is too low for normal cell growth of *ura3* mutants. We took advantage of the low uracil concentration in SD-CAS to detect cells lacking plasmids marked with *URA3* which grew to very small colonies. These small colonies were then tested for normal growth on SD-CAS agar plates plus uracil (0.1 mg/ml).

Plasmid constructions. The plasmids pA189HD, pA255HD, and pA559HD contain fragments of the *CAN1* gene (10) from DNA nucleotide −95 to +566, +677, and +1679, respectively. The numbers, 189, 255, and 559, in the plasmid names refer to the number of amino acids of arginine permease contained in each fusion protein. These DNA fragments were fused in frame to a fragment (nucleotides +97 to +2649) of the *HIS4* gene (4). An *Xho*I restriction site was preserved at each fusion joint. The plasmids pA189HD, pA255HD, and pA559HD also contain the 2μm origin of replication and the *URA3* gene. Upstream of the *CAN1-HIS4* gene fusion, the *ADH1* promoter sequence (1) was introduced into pA189HD and pA255HD for high levels of expression. High levels of expression of pA559HD were lethal to cells. Therefore, the *GAL1* promoter sequence (11) was introduced into pA559HD. Low levels of expression of pA559HD were found in cells grown in the presence of glucose. However, cell death occurred when cells were placed in the presence of galactose. The amino acid sequences of the fusion joints are SVVGSSLS (A189HD), AGVTRSSLS (A255HD), and GDVDSSLS (A559HD), in which SSLS begins the HD fragment sequence. The plasmids pA189invHD, pA255invHD, and pA559invHD are identical to pA189HD, pA255HD, and pA559HD, respectively, except for the inclusion of a fragment of the *SUC2* gene (nucleotides +789 to +1193) encoding a fragment of cytoplasmic invertase (15). This fragment was inserted at the *Xho*I restriction site between the *CAN1* and *HIS4* fragments of pA189HD, pA255HD, and pA559HD. The amino acid sequences of the fusion joints are SVVGSSSFNG (A189 invHD), AGVTRSRSFNG (A255invHD), and GDVDSRSF NG (A559invHD), in which SFNG begins the invertase fragment sequence. The specific details of the plasmid constructions are available on request.

Preparation of anti-HD antibodies. A 2.2-kb *Eco*RI restriction fragment from plasmid pA255invHD was inserted into pGEX-1 (14). The new plasmid, named pNG110, was transformed into *E. coli*. The encoded fusion protein between glutathione *S*-transferase and the A255invHD fragment was induced in cells at an optical density at 600 nm of 0.6 for 1 h with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Cells were broken by sonication, mixed with sample buffer, boiled for 5 min, and then analyzed by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The strongly staining protein band at an M_r of 60,000 was excised, frozen in liquid nitrogen, and ground to a powder. The powder was diluted in water to 1 mg of protein per ml and frozen. This material was used by Cal Tag Animal Pharm Services (Healdsburg, Calif.) to prepare anti-HD serum from rabbits. Approximately, 3 to 5 mg of the fusion protein was obtained from a 100-ml cell culture, and 0.2 mg of protein was used per injection. Note that the DNA fragment fused to the glutathione *S*-transferase gene also contained the invertase fragment and a short fragment from the arginine permease gene. The resulting antiserum may therefore contain antibodies directed against these determinants in addition to antibodies to HD. These additional determinants are not present in A189HD which is recognized by the antiserum, indicating that anti-HD antibodies were raised.

Immunoprecipitation with anti-HD. Yeast cells were grown at 30°C to an optical density at 600 nm of 1 in SD-CAS (5 ml) which was supplemented appropriately. Cells (1 ml) were resuspended in SD medium (1 ml) and supplemented appropriately (0.67% yeast-nitrogen base without amino acids–2% glucose–0.1 mg each of uracil, histidine, leucine, tryptophan, and adenine per ml), and then they were incubated with shaking for 1 h at 30°C. An aliquot of [^{35}S]methionine (50 μCi) was added, and the incubation was continued for 5 min. In some experiments, cells were chased for 1 h with unlabeled methionine at a concentration of 1 mg/ml. Strain CSa42 (*sec61*^{ts}) cells were pulse-labeled at the permissive temperature, 22°C. Cells were suspended in 0.2 ml of 10% trichloroacetic acid and broken by vortex mixing with glass beads. The suspension was centrifuged for 1 min at 10,000 rpm in an Eppendorf centrifuge. The pellet was resuspended in 20 μl of SDS-PAGE sample buffer and boiled for 5 min. The resulting mixture was diluted with 0.7 ml of phosphate-buffered saline PBS–1% Triton X-100, and protease inhibitors (0.1 mM PMSF [phenylmethylsulfonyl fluoride]–1% Trasylol–4 μg each of antipain, leupeptin, chymostatin, and pepstatin per ml) were added. This material was centrifuged at 10,000 rpm in an Eppendorf tube, and 0.5 ml of the supernatant was incubated overnight on ice with 5 μl of anti-HD serum. Protein A-Sepharose (20 μl of a 1:1 suspension in PBS–1% Triton X-100) was added, and the incubation was continued with shaking for 45 min. The material was centrifuged at 10,000 rpm for 1 min, and 20 μl of sample buffer was added to the pellet. After being boiled for 5 min, the sample was analyzed by SDS-PAGE.

Cell fractionation and carbonate extraction. A 10-ml culture was grown in SD-CAS medium, supplemented appropriately, to an optical density at 600 nm of 1. Cells were pelleted and resuspended to 10 times the optical density at 600 nm per ml in 0.2 M Tris HCl (pH = 8)–0.05 M EDTA plus protease inhibitors (see above). Cells were then broken by vortex mixing with glass beads. The extract was centrifuged at 10,000 × *g* for 5 min to remove unbroken cells and other large debris and then centrifuged for 1 h at 100,000 × *g*. The supernatant (S_1) was saved, while the pellet was extracted with sodium carbonate as described previously (8). The supernatant (S_1 and S_2) and pellet (P) fractions were analyzed by SDS-PAGE.

RESULTS

Expression of arginine permease/HD fusion proteins. To begin the study of the membrane integration of arginine permease, a multispanning yeast plasma membrane protein, we constructed three gene fusions which encoded differently sized fragments of arginine permease fused to HD. Three different gene fragments encoding the N-terminal 189, 255, and 559 amino acids of arginine permease were fused to HD, yielding constructs which encode the fusion proteins A189HD, A255HD, and A559HD, respectively. Fusion after the N-terminal 189 amino acids placed the HD moiety after a transmembrane segment as predicted from the hydrophobicity profile of the arginine permease primary structure (Fig. 1A). We have previously shown that the segment preceding the fusion joint does, in fact, span the membrane and can function as an internal signal sequence (7). In that study, we used the identical 189-amino-acid fragment of arginine permease fused to galactokinase as a reporter

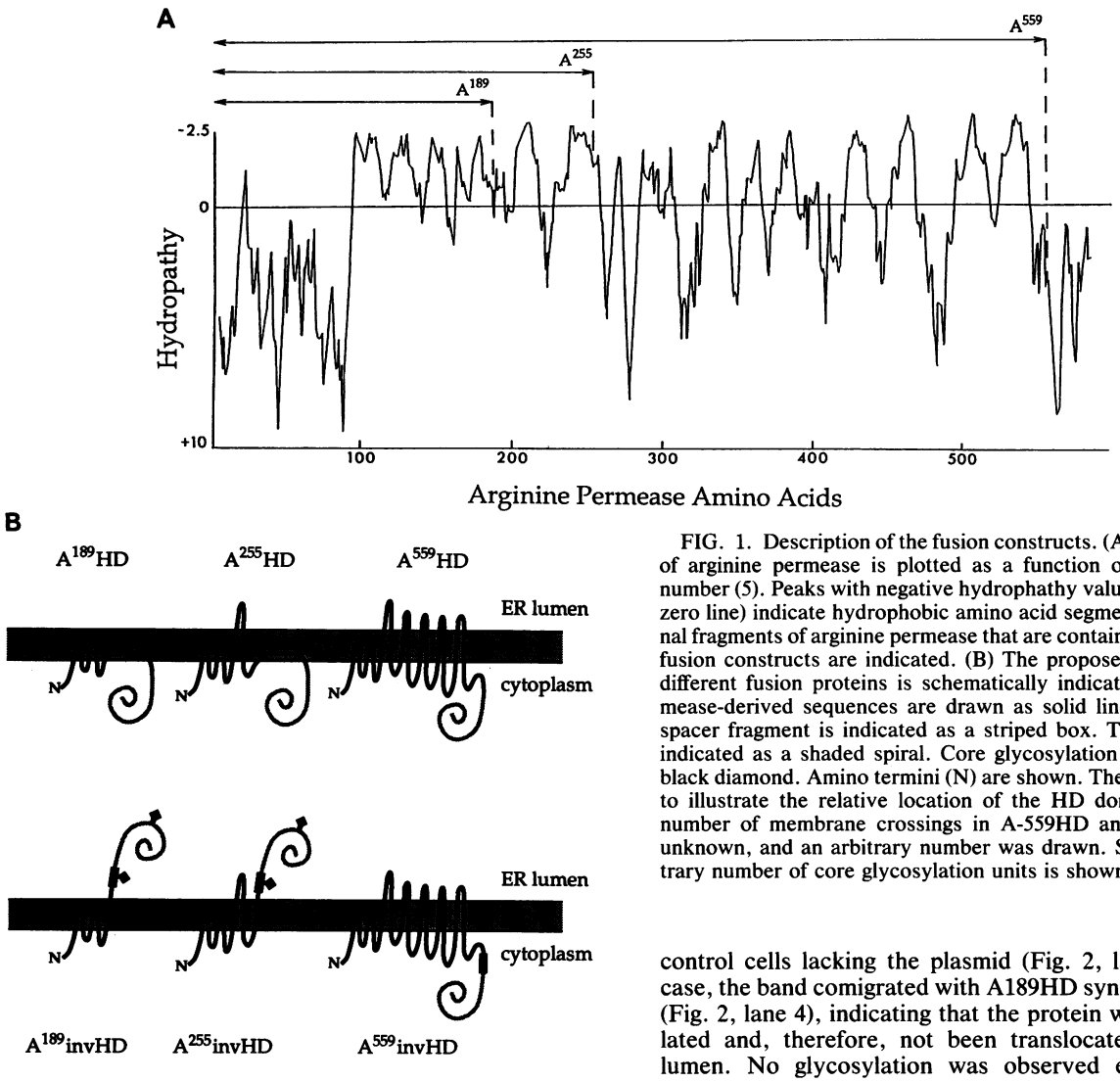

FIG. 1. Description of the fusion constructs. (A) The hydropathy of arginine permease is plotted as a function of the amino acid number (5). Peaks with negative hydrophathy values (i.e., above the zero line) indicate hydrophobic amino acid segments. The N-terminal fragments of arginine permease that are contained in the different fusion constructs are indicated. (B) The proposed topology of the different fusion proteins is schematically indicated. Arginine permease-derived sequences are drawn as solid lines. The invertase spacer fragment is indicated as a striped box. The HD domain is indicated as a shaded spiral. Core glycosylation is indicated by a black diamond. Amino termini (N) are shown. The cartoon attempts to illustrate the relative location of the HD domain; the precise number of membrane crossings in A-559HD and A-559invHD is unknown, and an arbitrary number was drawn. Similarly, an arbitrary number of core glycosylation units is shown.

enzyme and showed that the galactokinase moiety was translocated into the ER lumen in vitro; conversely, a 163-amino-acid fragment of arginine permease fused to galactokinase failed to translocate the galactokinase reporter enzyme. These results suggest that amino acid 189 is located on the extracytoplasmic side of the ER membrane and that amino acid 163 is located on the cytoplasmic side. In striking contrast to these previous results, however, we found that expression of A189HD in *his4* mutant cells (strain JC1-3C) allowed for cell growth on agar plates lacking histidine but containing histidinol. This indicated that the HD domain of A189HD was not localized efficiently to the ER lumen.

To corroborate this finding, we immunoprecipitated the fusion proteins from labeled JC1-3C (*his4*) cells with anti-HD antibodies. As shown below, HD that has been translocated into the lumen of the ER becomes glycosylated, resulting in a considerable molecular weight increase, which is indicative of its intracellular localization. A single immunoreactive band was precipitated from cells bearing the plasmid pA-189HD which were pulse-labeled with [^{35}S]methionine (Fig. 2, lane 2) and from cells that were pulse-labeled and chased for an additional hour in the absence of [^{35}S]methionine (Fig. 2, lane 3), but this band was not precipitated from labeled control cells lacking the plasmid (Fig. 2, lane 1). In each case, the band comigrated with A189HD synthesized in vitro (Fig. 2, lane 4), indicating that the protein was not glycosylated and, therefore, not been translocated into the ER lumen. No glycosylation was observed even during an extended chase period. Similarly, expression of the other two fusion constructs, A255HD and A559HD in JC1-3C (*his4*) cells, allowed the cells to grow on histidinol. Again, immunoprecipitation experiments showed that A255HD could be precipitated from extracts of JC1-3C (*his4*)/pA-255HD cells as a single band (Fig. 3, lane 1), which comigrated with the in vitro translation product (Fig. 3, lane 3). No immunoprecipitation analyses of JC1-3C (*his4*)/pA-559HD cells were performed (see below).

Insertion of a spacer domain. The data presented so far indicate that HD was not translocated across the ER membrane in vivo, even though previous studies have shown that an identical fragment of arginine permease effected the translocation of a different reporter enzyme, galactokinase, to the ER lumen. Since the HD moiety was efficiently translocated into the ER lumen in similar studies (3, 13), we reasoned that it could not be a global property, such as a particularly tight-folding characteristic, which would make HD a poor translocation substrate. Rather, the discrepancy between HD and galactokinase could have been due to local sequence elements of HD which, in the specific context of the fusion proteins, impeded its translocation. We therefore tested directly whether the insertion of a spacer region between the arginine permease fragment and HD would help overcome the ER translocation block of HD. For this

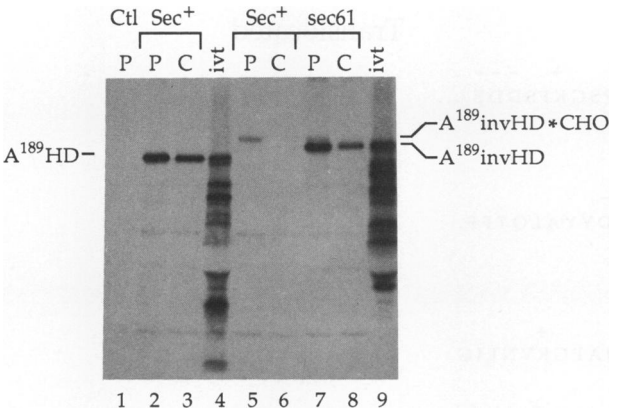

FIG. 2. Immunoprecipitation of A189HD and A189invHD. Strain JC1-3C/pA189HD (lanes 2 and 3), strain JC1-3C/pA189invHD (lanes 5 and 6), strain CSa42 (*sec61*)/pA189invHD (lanes 7 and 8), and, as a control strain, JC1-3C not bearing a plasmid (lane 1) were pulse-labeled (P) with [^{35}S]methionine (lanes 2, 5, 7) and then chased (C) with unlabeled methionine (lanes 3, 6, 8), as described in Materials and Methods. Proteins were precipitated with anti-HD antibodies and displayed by SDS-PAGE. The positions of A189HD, A189invHD, and glycosylated A189invHD (indicated by A^{189}inv HD*CHO) are indicated. As a size reference, genes encoding both of the fusion proteins were translated in vitro as described previously (8) and analyzed on the same gel (lanes 4 and 9). The highest-molecular-weight translation products correspond to full-length A189HD and A189invHD, respectively; the lower-molecular-weight bands are probably early termination products of the in vitro translation system.

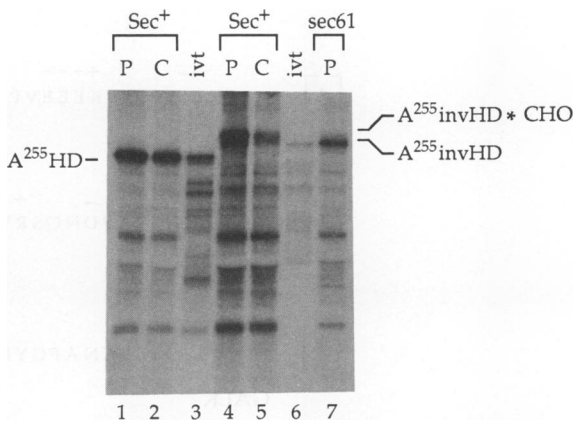

FIG. 3. Immunoprecipitation of A255HD and A255invHD. Strain JC1-3C/pA255HD (lanes 1 and 2), strain JC1-3C/pA255invHD (lanes 4 and 5), and strain CSa42 (*sec61*)/pA255invHD (lane 7) were pulse-labeled (P) with [^{35}S]methionine (lanes 1, 4, 7) and then chased (C) with unlabeled methionine (lanes 2 and 5), as described in Materials and Methods. Proteins were precipitated with anti-HD antibodies and displayed by SDS-PAGE. The positions of A255HD, A255invHD, and glycosylated A255invHD (indicated by A^{255}inv HD*CHO) are indicated. As a size reference, in vitro translation products were analyzed on the same gel (lanes 3 and 6). The highest-molecular-weight translation products correspond to full-length A255HD and A255invHD, respectively; the lower-molecular-weight bands are probably early termination products of the in vitro translation system. About fourfold more material was loaded in lanes 4 and 5.

purpose, we used a gene fragment encoding 134 amino acids of invertase and inserted it between the genes encoding the truncated arginine permease fragments and HD, resulting in plasmids encoding A189invHD, A255invHD, and A559inv HD. As summarized in Fig. 1B, the insertion of the invertase spacer fragment indeed allowed the HD domain to become translocated. JC1-3C (*his4*)/pA189invHD cells and JC1-3C (*his4*)/pA255invHD cells failed to grow on histidinol, indicating that the HD moiety was inactivated in vivo.

Immunoprecipitation experiments confirmed that this inactivation was indeed due to the translocation of HD into the ER lumen. Immunoprecipitates from labeled JC1-3C (*his4*)/pA189invHD cells showed a single band (Fig. 2, lane 5), which was notably larger than the in vitro translation product (Fig. 2, lane 9). The increase in molecular weight is consistent with N-linked glycosylation of either a subset or of all 11 potential glycosylation sites that are present in the HD domain (6 potential glycosylation sites) and the invertase spacer domain (5 potential glycosylation sites). Arginine permease does not contain potential glycosylation sites within the first 255 amino acids. Membrane integration was blocked when A189invHD was expressed in *sec61* mutant cells (strain CSa42), which are defective in protein translocation across the ER membrane (Fig. 2, lane 7) (3). Note that in this case the immunoreactive band was not glycosylated as it comigrated with the in vitro translation product (Fig. 2, compare lanes 7 and 9). Similar results were obtained when cells expressing the second fusion protein [JC1-3C (*his4*)/pA-255invHD cells] were analyzed by immunoprecipitation. The predominant product was glycosylated (Fig. 3, lane 4) as indicated by its slower migration on SDS gels compared with that of the in vitro translation product (Fig. 3, lane 6). As for A189invHD, the HD moiety of A255invHD was not translocated in CSa42 (*sec61*) cells (Fig. 3, lane 7). Furthermore, CSa42 cells containing either pA189invHD or pA255invHD were able to grow at 22°C on agar plates containing histidinol, whereas JCI-3C cells containing either pA189invHD or pA225invHD were unable to grow on agar plates containing histidinol. These results support our conclusion that the HD moieties of A189invHD and A255invHD are translocated into the lumen of the ER in JCI-3C cells.

Stability of fusion proteins in vivo. During these analyses we noted that the amount of glycosylated A189invHD that was immunoprecipitated from pulse-labeled cells was reduced compared with the amount of immunoprecipitated A189HD (Fig. 2, compare lane 5 with lane 2). This raised the possibility that the fusion proteins containing the invertase spacer fragment are unstable in vivo and that the lack of HD activity in these cells could have resulted from HD degradation. Indeed, when cells were subjected to a 1-h chase with an excess of unlabeled methionine after the labeling procedure, glycosylated A189invHD was no longer detectable (Fig. 2, lane 6). This instability was, however, not a property of the fusion protein per se, as A189invHD produced in CSa42 (*sec61*) cells was more stable during the pulse-chase analysis (Fig. 2, lane 8). Similar results were obtained for A255invHD (Fig. 3), although glycosylated A255invHD was more stable as indicated by the pulse-chase analysis (Fig. 3, lane 5). Thus, we can conclude that the relative enzymatic activity of HD indeed reflected the intracellular location of HD and was not simply due to its stability in cells.

Thus far, we have demonstrated that although A189HD and A255HD contain HD fused to the extracytoplasmic segments of two truncated forms of arginine permease, a spacer fragment was required to direct translocation of the HD fragment into the ER lumen. To demonstrate that the invertase fragment did not contain a cryptic signal sequence

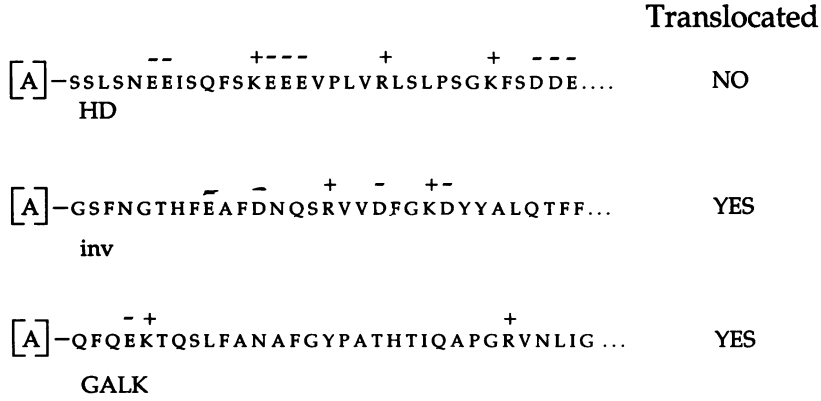

FIG. 4. Comparison of the amino acid sequences fused to the arginine permease fragments. The N-terminal amino acid sequences from HD, the invertase fragment, and the galactokinase domain are shown (see Materials and Methods and reference 7), and charged residues are indicated. The [A]- represents the arginine permease fragments composed of the first 189 and 255 amino acids as used in the fusion protein constructs.

itself, we tested the integration of two additional fusion proteins, A559HD and A559invHD. JC1-3C (*his4*) cells expressing either A559HD or A559invHD grow on histidinol, indicating that HD has not been translocated to the lumen of the ER and that HD is sufficiently stable to synthesize histidine. We infer from these data that the HD moiety has been fused to a cytoplasmic domain of arginine permease and that, therefore, the presence or absence of the invertase spacer did not affect the membrane integration.

The arginine permease fragment used in this fusion construct encoded most of the protein, including all of its putative transmembrane segments. To show that the arginine permease portion of the fusion protein was indeed properly integrated into the membrane and was stably expressed in vivo, we tested directly for arginine permease activity. For this purpose, we introduced pA559HD or pA559invHD into FC2-12B (*can1*) cells. FC2-12B (*can1*) cells are mutant in the gene encoding arginine permease, *CAN1*, and their growth is, therefore, resistant to the arginine analog canavanine. However, FC2-12B (*can1*)/pA559HD and FC2-12B (*can1*)/pA559invHD cell growth was sensitive to canavanine, indicating that the arginine permease fragments in A559HD and A559invHD were indeed integrated correctly into the ER membrane and that the fusion proteins were transported to the plasma membrane to provide functional permease. FC2-12B cells are also *his4* mutant. Therefore, we also tested FC2-12B cells bearing pA559HD or pA559invHD for growth on histidinol. As with JC1-3C cells, FC2-12B cells also grew when pA559HD or pA559invHD were expressed. As expected, FC2-12B cells failed to grow on histidinol when pA189invHD and pA255invHD were expressed but grew when pA189HD and pA255HD were expressed. At the onset of these studies, we learned that expression of A559HD and A559invHD from a strong promoter was lethal to the cells. This restriction required us to express these proteins from a weak promoter and prevented further biochemical analysis.

Clusters of charged amino acids may inhibit HD translocation. As we demonstrated in Fig. 2 and 3, the presence of the invertase fragment in A189invHD and A255invHD played an essential role in the translocation of the HD domain into the lumen of the ER. As reasoned above, one possible role for the invertase fragment could have been to act as a spacer separating the arginine permease fragment from sequences in HD that inhibited its translocation. This would suggest that this inhibiting sequence(s) exerted its effect by being placed in close proximity to the arginine permease fragment. The amino acid sequences immediately following the fusion joint to the arginine permease fragments are shown in Fig. 4. When we compared by visual inspection the HD sequence with the sequence of the invertase fragment or the sequence of galactokinase, we noted a considerable abundance of negatively charged amino acids in HD, most of which were clustered. Such charge clusters were absent in the invertase fragment or in galactokinase, both of which were efficiently translocated when they were fused to the same fragments of arginine permease.

We, therefore, tested the simple notion that clusters of charged amino acids, when they are placed closely behind the arginine permease fragment, might inhibit the membrane translocation of the following domain. To this end, we synthesized an oligonucleotide encoding two charge clusters in tandem (Fig. 5A), each resembling a cluster in HD, and inserted the oligonucleotide between the arginine permease fragment and the invertase spacer of A189invHD. The oligonucleotide could have been inserted in one of two orientations; however, only one orientation, denoted linker A, would have provided a charge cluster (Fig. 5A). The opposite orientation, linker B, did not encode a charge cluster (Fig. 5A). As shown in Fig. 5B, JC1-3C (*his4*) cells containing pA189invHD with linker A inserted grew on agar plates containing histidinol, indicating that the translocation of HD was inhibited (Fig. 5B, right). Conversely, the insertion of linker B into pA189invHD did not inhibit the translocation of HD, indicated by the lack of colony formation (Fig. 5B, left). To substantiate these results, we labeled these cells with [^{35}S]methionine as described above and immunoprecipitated A189invHD containing either linker A or linker B (Fig. 6). Note that the glycosylation of A189invHD was unaffected by the insertion of linker B (compare Fig. 6, lane 1, with Fig. 2, lane 5). In contrast, insertion of linker A resulted in the accumulation of unglycosylated A189invHD (Fig. 6, lane 2), which comigrated with unglycosylated A189invHD produced in CSa42 (*sec61*) cells (Fig. 6, lane 3).

Intracellular localization of fusion proteins. The failure to localize HD to the extracytoplasmic side of the ER membrane in A189HD, A255HD, and A189invHD with linker A

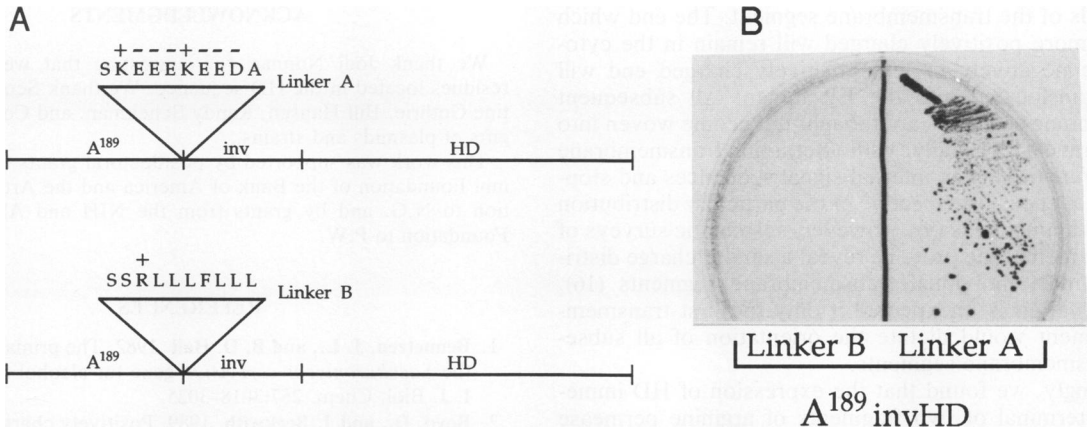

FIG. 5. Linker insertion into pA189invHD. (A) An oligonucleotide linker was synthesized and inserted into the *Xho*I site of A189invHD. The encoded protein sequences for the insertions in either direction (Linker A and Linker B) are shown, and charged residues are indicated. Sequence analysis confirmed the orientation and that the linker was introduced as a single copy. The sequence of the oligonucleotide linker A was 5′-TCGAAAGAAGAAGAAAAAGAAGAAGACGCG-3′. The sequence of oligonucleotide linker B was 5′-TCGACGCGTCTTCT TCTTTTTCTTCTTCTT-3′. (B) JC1-3C cells contained pA189invHD with either linker A or linker B inserted. The agar plate contained SD medium with appropriate supplements and histidinol in place of histidine (see Materials and Methods).

inserted could have resulted from either a failure to target these proteins to the ER membrane or a failure to translocate the HD moiety across the membrane after targeting and partial membrane insertion of the fusion proteins. To distinguish between these two possibilities, we examined the subcellular location of A189HD and A189invHD. Strains bearing pA189HD and pA189invHD (see legend to Fig. 7) were pulse-labeled with [^{35}S]methionine. After cell disruption, the extract was fractionated into a cytoplasmic supernatant fraction, S_1 (Fig. 7, lanes 1 and 4), and a membrane pellet fraction. The pellet fraction was resuspended in a sodium carbonate buffer and fractionated by centrifugation, yielding a supernatant fraction containing proteins that were peripherally bound to the membranes, S_2 (Fig. 7, lanes 2 and 5), and a pellet fraction containing integral membrane proteins, P (Fig. 7, lanes 3 and 6). Note that both A189HD and A189invHD were recovered almost exclusively in the pellet fraction. Some A189HD was also recovered in S_1 and S_2 (Fig. 7, lanes 1 and 2), indicating that a small amount of A189HD was not integrated or more loosely attached to membranes than was A189invHD. Taken together these data suggest, however, that both fusion proteins were integrated into membranes in vivo.

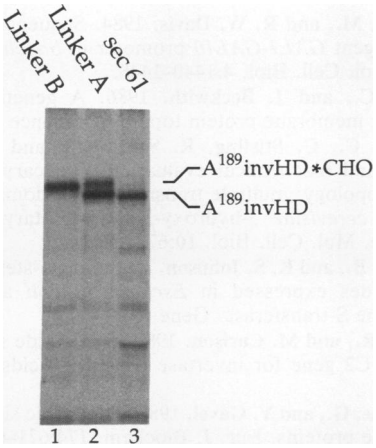

FIG. 6. Immunoprecipitation with anti-HD. JC1-3C cells containing pA189invHD with either linker A (lane 2) or linker B (lane 1) inserted and CSa42 (*sec61*) cells containing pA189invHD without a linker insertion (lane 3) were radiolabeled. Proteins were immunoprecipitated with anti-HD serum as described in the legend to Fig. 2 and in Materials and Methods. A189invHD and glycosylated A189invHD (indicated by A^{189}invHD*CHO) are indicated.

DISCUSSION

For eukaryotic cells, models evolved according to which individual transmembrane segments of multispanning membrane proteins are integrated sequentially by a mechanism in which the most N-terminal membrane-spanning region determines the orientation of the entire protein in the membrane (9). The orientation of the first transmembrane segment in the membrane is correlated with the distribution of charged amino acids flanking either side of the hydrophobic

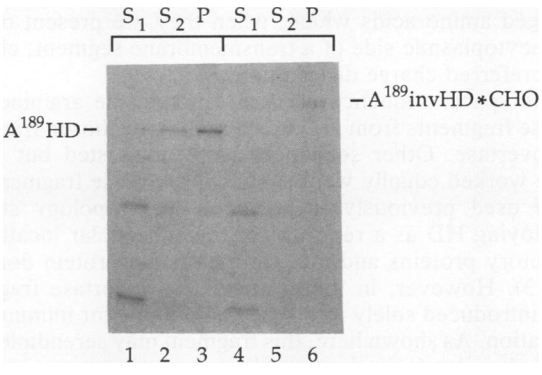

FIG. 7. Immunoprecipitation of A189HD and A189invHD after cell fractionation. JC1-3C/pA189HD cells (lanes 1 to 3) and JC1-3C/pA189invHD cells (lanes 4 to 6) were fractionated as described in Materials and Methods. S_1 corresponds to the supernatant fraction after membrane pelleting. S_2 and P correspond to the supernatant fraction and the pellet fraction from a subsequent carbonate extraction, respectively. The positions of A189HD and glycosylated A189invHD (indicated by A^{189}invHD*CHO) are indicated.

amino acids of the transmembrane segment. The end which is overall more positively charged will remain in the cytoplasm; the negatively or less positively charged end will become translocated into the ER lumen. All subsequent transmembrane segments are thought to become woven into the membrane accordingly, with alternating transmembrane segments functioning as internal signal sequences and stop-transfer sequences, irrespective of the particular distribution of charged amino acids (9). However, systematic surveys of eukaryotic membrane proteins reveal a similar charge distribution in most individual transmembrane segments (16). This observation is unexpected if only the first transmembrane segment would dictate the orientation of all subsequent transmembrane segments.

Surprisingly, we found that the expression of HD immediately C terminal of two fragments of arginine permease impaired its translocation into the ER lumen. Most importantly, HD is not inherently translocation incompetent but can be efficiently moved across the ER membrane when it is placed further from the internal signal sequence(s) in arginine permease. This was accomplished by the insertion of the invertase spacer fragment between arginine permease and HD. These results were surprising for two reasons. First, the sequences in HD that immediately follow the arginine permease fragments contain clusters of negatively charged amino acids, which, when they are present on the extracytoplasmic side of a transmembrane segment, do not change the preferred charge distribution.

Second, these results are not predicted by the current models describing the integration of multispanning membrane proteins in eukaryotic cells. In particular, it was surprising to find that sequences C terminal to internal signal sequences influenced the topology of at least the latter portion of the membrane protein as monitored by the HD reporter domain. These data do not necessarily contradict models invoking a sequential insertion of individual transmembrane segments but add an additional level of complexity. In particular, it appears that an internal signal sequence can be selectively inactivated by the amino acid sequence in the C-terminal proximity. A possibly similar phenomenon has been seen in studies of *E. coli* which showed that a C-terminal sequence of 38-amino-acid residues could inhibit the insertion of a recombinate form of leader peptidase (17). However, in contrast to the results presented here, the 38-residue segment contained a high density of positively charged amino acids which, when they are present on the extracytoplasmic side of a transmembrane segment, change the preferred charge distribution.

The spacer sequence used to separate the arginine permease fragments from HD was a randomly chosen fragment of invertase. Other sequences were not tested but might have worked equally well. A similar invertase fragment has been used previously in secretion and topology studies employing HD as a reporter for the subcellular location of secretory proteins and integral membrane protein domains (3, 13). However, in those studies, an invertase fragment was introduced solely to provide an epitope for immunoprecipitation. As shown here, this fragment may serendipitously have played a dual role. In addition to serving as an epitope for immunological identification of the fusion proteins, the invertase fragment may have functioned also as a spacer sequence required to separate the translocation inhibitory sequences in HD from the preceding signal sequences.

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