Evidence That the Transmembrane Biogenesis of Aquaporin 1 Is Cotranslational in Intact Mammalian Cells

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Most polytopic membrane proteins are believed to integrate into the membrane of the endoplasmic reticulum (ER) cotranslationally. However, recent studies with *Xenopus* oocytes and dog pancreatic microsomes have suggested that this is not the case for human aquaporin 1 (AQP1). These experiments indicate that membrane-spanning segments (MSSs) 2 and 4 of AQP1 do not integrate into the membrane cotranslationally so that this protein initially adopts a four MSS topology. A later maturation event involving a 180-degree rotation of MSS 3 from an N_{lum}/C_{cyt} to an N_{cyt}/C_{lum} orientation and the concomitant integration of MSSs 2 and 4 into the membrane results in the final six MSS topology. Here we examine the biogenesis of AQP1 in the human embryonic kidney cell line HEK-293T. To do this, we constructed an expression vector for a fusion protein consisting of the enhanced green fluorescent protein followed by an insertion site for AQP1 sequences and a C-terminal glycosylation tag. We then transiently transfected HEK-293T cells with this vector containing the AQP1 sequence truncated after each MSS. Glycosylation of the C-terminal tag was used to monitor its location relative to the ER lumen and consequently the membrane integration and orientation of successive MSSs. In contrast to previous studies our results indicate that AQP1 integrates into the ER membrane cotranslationally in intact HEK-293T cells.

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The abbreviations used are: ER, endoplasmic reticulum; MSS, membrane-spanning segment; AQP, aquaporin; EGFP, enhanced green fluorescent protein; PNGase F, peptide:N-glycosidase F; PK, proteinase K; HEK, human embryonic kidney cells.
The final AQP1 topology is then attributed to a 180-degree rotation of MSS 3 to an Nα,gC-tres orientation, which pulls MSSs 2 and 4 into the membrane and translocates the extramembrane loops on either end of MSS 3 to the opposite sides of the bilayer.

In the present studies, we have examined the biogenesis of AQP1 in HEK-293T cells. In contrast to the earlier results discussed above and the proposal of Lu et al. (10), our results indicate that AQP1 integrates into the ER membrane cotranslationally in these intact mammalian cells.

MATERIALS AND METHODS

Vector Construction—The mammalian expression vector, pEGFP-β, used in our experiments was constructed from pEGFP-C3 (CLONTECH) by ligating the sequence for the 177 C-terminal amino acids of the β-subunit of the rabbit gastric H,K-ATPase (a glycosylation tag) between the HindIII and EcoRI sites of pEGFP-C3. The HindIII-EcoRI fragment of the β-subunit was taken from the vector M0 (16), a generous gift from Dr. George Sachs. Sequences coding for AQP1 truncation mutants were synthesized by the polymerase chain reaction (PCR) and ligated between the BglII and HindIII sites of pEGFP-β using standard methods. The human AQP1 template was the plasmid pXβg-ev1 (17), a generous gift from Dr. Peter Ager. Each AQP1 truncation mutant began at the AQP1 start codon. Six of the seven truncation mutants studied here ended at the C terminus of the six MSSs of AQP1 (Lys-36, Ser-66, Thr-120, Ala-155, Ala-183, or Asp-228) as identified in recent crystallographic studies (14, 15) and the seventh ended at Leu-164 close to the N terminus of MSS 5. The PCR primers were designed in such a way that each of the final constructs coded for a fusion protein consisting of the enhanced green fluorescent protein (EGFP) followed by an AQP1 truncation mutant and the H,K-ATPase β-subunit fragment; expression of these fusion proteins was driven by the cytomegalovirus promoter of the original pEGFP-C3 plasmid (Fig. 1). The resultant expression vectors are referred to as K36S, S66S, T120S, A155S, L164S, and D228S, where the subscript refers to the number of AQP1 MSSs included in the AQP1 truncation mutant.

Western Blotting and Analysis— SDS-PAGE was carried out using 4–20% Tris/glycine Ready Gels (Bio-Rad), and proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) by semidry blotting (20 V for 30 min) using a Trans-Blot SD Cell (Bio-Rad) and a transfer buffer containing 50 mM Tris-HCl (pH 7.4), 10% methanol, and 20% methanol. Immunoblotting was carried out in 100 mM Tris-HCl (pH 7.4) containing 0.9% NaCl, 4% skim milk powder (Giant Foods), and 0.04% Tween 20. (Tween 20 was omitted during incubation with the primary antibody.) The primary and secondary antibodies were a rabbit anti-GFP polyclonal (Molecular Probes) used at dilution of 1:10,000. Incubation with the primary antibody was done overnight at 4 °C. Detection was carried out using the ECL kit (Amersham Biosciences) and X-Omat AR film (Kodak). In preliminary experiments (not shown) we found that the signal from the anti-GFP antibody in Western blots was greatly increased if an SDS-PAGE sample buffer containing only 0.5% SDS was used, and samples were not boiled before electrophoresis. Accordingly all sample conditions were used in our experiments. We suspect that this increase in signal occurs because the anti-GFP antibody is more effective at recognizing the non-denatured form of EGFP, but we have not explored this phenomenon further. Quantitation of Western blots was done using a Molecular Dynamics computing densitometer. Quantitative results shown are means ± S.E. for three or more independent experiments.

RESULTS AND DISCUSSION

Fig. 2A shows a schematic representation of the transmembrane topology of AQP1 as determined from crystallographic studies (14, 15); the locations of the end points of the seven truncation mutants used in our experiments are also indicated (see “Materials and Methods”). Fig. 2B shows the initial four MSS transmembrane topology of AQP1 previously deduced from experiments carried out with truncation mutants expressed in Xenopus oocytes and in the presence of dog pancreatic microsomes (10, 11). As already mentioned, in these

[Fig. 1. Schematic representation of the expression cassette of the EGFP vector. The expression of a fusion protein consisting of EGFP, an AQP1 truncation mutant and the C-terminal 177 amino acids of the β-subunit of the rabbit gastric H,K-ATPase (a glycosylation tag) is driven by the cytomegalovirus (CMV) promoter (see “Materials and Methods”). The locations of the BglII and HindIII restriction sites used to ligate the AQP1 truncation mutants into the vector are indicated.]
experiments a reporter peptide (derived from bovine prolactin) was fused to the C terminus of the AQP1 truncation mutants, and its location inside or outside the ER lumen was determined by proteinase K (PK) accessibility. Briefly stated, in the experiments with Xenopus oocytes it was found that when AQP1 was truncated at residue Val-52, 67% of the reporter peptides were inaccessible to PK, indicating that MSS 1 was integrated into the membrane in an N_{cyt}/C_{lum} orientation. However, when AQP1 was truncated at Pro-77 or Arg-93, ~80% of the reporter peptides were still inaccessible to PK, suggesting that MSS 2 was not membrane-integrated. Furthermore, in truncations at Thr-120, Leu-139, and Pro-169, ~90% of the reporter peptides were accessible to PK, demonstrating that MSS 3 was integrated into the membrane, but in an N_{lum}/C_{cyt} orientation, and that MSS 4 like MSS 2 was not membrane-integrated. Experiments with AQP1 truncations in the loop between MSSs 5 and 6 and after MSS 6 showed that these MSSs were in their correct (final) orientations in the oocyte membranes. Hence the proposal (10) that AQP1 initially integrates into the ER membrane with the topology shown in Fig. 2B. Similar conclusions were reached from the experiments with dog pancreatic microsomes.

In their experiments, Lu et al. (10) also examined the protease accessibility of a c-Myc epitope inserted into AQP1 truncation mutants at Thr-120 as a way of monitoring the orientation of MSS 3. In membranes from Xenopus oocytes these authors found that this epitope was more protected from digestion (indicating that MSS 3 had assumed its final N_{cyt}/C_{lum} orientation) as more C-terminal AQP1 MSSs were included in their truncation mutants; more specifically, after 2 h of expression, ~48% of the c-Myc epitope was protected in mutants truncated after MSS 3, MSS 4, and MSS 5, and MSS 6, respectively. They also found evidence that protection of this epitope increased with time after the synthesis of full-length AQP1, reaching a maximum of 78% protected sites within 5 h of synthesis. Smaller effects were seen in dog pancreatic microsomes (23% protection 12 h after synthesis). On the basis of these results and the authors’ conclusions regarding the initial (cotranslational) topology of AQP1 (Fig. 2B) they proposed that AQP1 undergoes a maturation step after the synthesis of MSSs 4–6 in which MSS 3 rotates 180 degrees from an N_{lum}/C_{cyt} to an N_{cyt}/C_{lum} orientation. This reorientation pulls MSSs 2 and 4 into the membrane and translocates the extramembrane loops on either end of MSS 3 to the opposite sides of the bilayer.

In our experiments we have also employed a strategy using truncation mutants to examine the biogenesis of AQP1 in intact mammalian HEK-293T cells. We have used the 177 C-terminal amino acids of the β-subunit of the rabbit gastric H,K-ATPase as our reporter peptide. This sequence represents a portion of the extracytosolic tail of the β-subunit and includes five consensus sites for N-linked glycosylation (16). When this reporter is translocated into the interior of the ER it acquires ~14 kDa of apparent molecular mass due to glycosylation (20), an increase that is easily detected by SDS-PAGE electrophoresis. The use of this glycosylation tag in membrane topology determinations is now well established (16, 20–22). We have also included EGFP at the N terminus of our constructs for ease of detection on Western blots (Fig. 1).

The results of our studies are presented in Fig. 3. In each of the panels in Fig. 3A we show a typical experiment where the membrane fraction from HEK-293T cells, transiently transfected with the truncation mutant indicated, was treated with (+) or without (−) PNGase F. All procedures are described under “Materials and Methods.” B, pulse-chase study of the truncation mutant T120o. HEK-293T cells were transiently transfected with T120, as described under “Materials and Methods.” Membranes were then harvested from cells incubated with 10 μM cycloheximide (added to culture medium) for 3 h (+0) or from cells incubated with 10 μM cycloheximide for 3 h and then washed and incubated in culture medium for an additional 1 h (+1). A Western blot from a typical experiment is shown. C, quantitation of the glycosylation of transiently transfected AQP1 truncation mutants. Results from membrane fractions from the truncation mutants indicated are shown. The density of the glycosylated band was calculated as a percentage of the total expressed recombinant protein (glycosylated band plus unglycosylated band). All results represent the averages ± S.E. from three or more independent determinations.
then isolating the membrane fraction by flotation on a sucrose gradient (see “Materials and Methods”). Membrane fractions treated with or without PNGase F were separated by SDS-PAGE and probed by Western blotting to determine the extent of glycosylation of the β-subunit and thus its location inside or outside the ER lumen. Thus, for example, for membranes from cells transfected with the plasmid K36, we observed two bands in the Western blot of untreated membranes (–), a dense upper band of ~56 kDa and a much weaker lower band of ~44 kDa.

After treatment with PNGase F, the upper band disappeared, and all of the immunoreactivity was found in the lower band, confirming that the difference in apparent molecular masses of the two bands was due to glycosylation of the recombinant protein. Quantitation of the two bands observed without PNGase F treatment (Fig. 3D) shows that ~90% of the AQP1 proteins truncated at Lys-36 were glycosylated, and thus that MSS 1 in these constructs was predominantly integrated into HEK-293T cell membranes in an Nlum/Ccyt orientation. Thus MSS 1 has strong signal anchor activity. On the other hand, ~50% of AQP1 proteins truncated at Ser-66 were glycosylated indicating that MSS 2 has somewhat weak stop transfer activity (Fig. 3, A and D). Extending the AQP1 sequence to Arg-93, the N-terminal end of MSS 3, had little effect on this result (data not shown). However, when AQP1 was truncated at Thr-120, most of the recombinant proteins (~85%) were glycosylated (Fig. 3, A and D), demonstrating that when the first three MSSs of AQP1 are expressed together they are found to be predominantly integrated into HEK-293T cell membranes in their correct (final) orientations. These results are in marked contrast to those described above from oocytes and microsomes.

Our data also suggest that MSS 2 is integrated into the membrane more effectively in the presence of MSS 3 than in its absence. One possible explanation for this result is that in the 50% of the cases where MSS 2 does not integrate into the membrane (Fig. 3D), MSS 3 is inserted in an Nlum/Ccyt (reverse) orientation and then undergoes a 180-degree rotation as suggested by Lu et al. (10). To test this possibility we carried out the pulse-chase type experiment shown in Fig. 3B. Here HEK-293T cells transiently transfected with T120, were incubated with cycloheximide for 3 h to deplete the cellular content of recombinant protein. Cycloheximide was then removed, and membranes were harvested 1 h later to study the glycosylation of newly synthesized protein. In these experiments we found that 90.0 ± 3.2% (n = 3) of the recombinant proteins observed 1 h after cycloheximide removal were glycosylated. Thus this experiment provides no evidence for the presence of a transient pool of proteins with MSS 3 inserted in an Nlum/Ccyt orientation. We also note that the proposal of Lu et al. (10) was in fact that the reorientation of MSS 3 occurred only after the synthesis of MSSs 4–6 (see above).

Our data from intact cells could also be partially reconciled with earlier results if significant amounts of protein with un-integrated MSS 2 were produced in HEK-293T cells but then were rapidly removed from the membrane. (Such proteins might not be as efficiently removed in Xenopus oocytes and not removed at all from microsomes.) However, when we examined the total particulate fraction from the experiments illustrated in Fig. 3B (not shown) we found that 90.2 ± 3.5% (n = 3) of the recombinant proteins observed 1 h after cycloheximide removal were glycosylated. Because this particulate fraction includes proteins removed from the membrane and presumably destined for degradation we conclude that there is no evidence from this result for the preferential removal of proteins with un-integrated MSS 2.

We conclude therefore that MSS 3 is able to assist in the membrane integration of MSS 2 in HEK-293T cells. This effect is consistent with observations from other proteins where a C-terminal MSS with a strong topological signal sequence was able to influence the membrane integration of more N-terminal regions (5, 6). In the case of AQP1, MSS 2 appears to be able to slip within the translocon before the appearance of MSS 3. The effect of MSS 3 may be caused by an interaction with MSS 2, which is sufficient to anchor MSS 2 in the membrane. Alternatively (or additionally), this effect may be a result of the strong Ncyt/Clum (type II) signal anchor activity of MSS 3 that constrains MSS 2 to the membrane simply because it is tethered to MSS 3. In any case, what is important to note is that, in this intact cell system, our results provide strong evidence that MSSs 2 and 3 of AQP1 are integrating into the membrane in their correct (final) orientations cotranslationally.

Analysis of experiments with AQP1 truncated at Ala-155 indicates that MSS 4, like MSS 2, has somewhat weak stop transfer activity (Fig. 3, C and D). However, extending the sequence to Leu-164, which includes the highly charged region DRRRDD (amino acids 158–163), significantly decreases the glycosylation of the recombinant protein, indicating increased membrane integration of MSS 4 (Fig. 3, C and D). This result is consistent with previous observations demonstrating that downstream charged residues can act as powerful topological determinants (3, 23). In this case, presumably the highly charged sequence between Ala-155 and Leu-164 hinders membrane translocation of the nascent chain and thereby anchors MSS 4 in the membrane and the C-terminal glycosylation flag in the cytoplasm. Finally, proteins truncated at Ala-183 are predominantly glycosylated (~90%) whereas those truncated at Asp-228 are virtually all unglycosylated (~95%), indicating that MSSs 5 and 6 are sequentially integrated into the membrane in their correct (final) orientation.

We emphasize that our results argue neither for nor against the existence of the maturation event proposed by Lu et al. (10); however, they do demonstrate quite clearly that this effect is of little relevance to the biogenesis of AQP1 in HEK-293T cells where AQP1 appears to integrate into the ER membrane cotranslationally. What then is the explanation for the differences in the way human AQP1 integrates into these various membrane systems? First, it seems that this protein is intrinsically problematic because of the weak stop transfer activities of MSSs 2 and 4 (Fig. 3). So folding difficulties in Xenopus oocytes are not that unreasonable given the relatively large species differences involved. In this regard, it would be interesting to investigate the membrane biogenesis of Xenopus AQP1 in Xenopus oocytes. Note also that, despite any folding difficulties or complexities, significant quantities of properly folded human AQP1 can clearly be produced in Xenopus oocytes as evidenced by numerous functional studies (24). Although the dog pancreatic microsome system has proven to be a reliable one for the study of many membrane and secretory proteins, it nevertheless is a cell-free system. As such it may lack or be deficient in some intermediaries in the membrane integration apparatus, and these may be particularly important for the processing of problematic proteins such as AQP1. Further studies will be required to identify these putative additional elements.

The experimental system described here for the study of membrane protein topology provides a convenient and reasonable alternative to expression studies carried out with dog pancreatic microsomes and Xenopus oocytes. To our knowledge this is the first use of intact mammalian cells for topology studies involving this now common truncation mutant approach.

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