Membrane Localization and Topology of Leukotriene C₄ Synthase*

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Leukotriene C₄ (LTC₄) synthase conjugates LTA₄ with GSH to form LTC₄. Determining the site of LTC₄ synthesis and the topology of LTC₄ synthase may uncover an unappreciated intracellular role for LTC₄, as well as how LTC₄ is transferred to its export carrier, the multidrug resistance protein-1. We have determined the membrane localization of LTC₄ synthase by immunoelectron microscopy. In contrast to the closely related five-lipoxigenase-activating protein, LTC₄ synthase is distributed in the outer nuclear membrane and peripheral endoplasmic reticulum but is excluded from the inner nuclear membrane. We have combined immunofluorescence with differential membrane permeabilization to determine the topology of LTC₄ synthase. The active site of LTC₄ synthase is localized in the lumen of the nuclear envelope and endoplasmic reticulum. These results indicate that the synthesis of LTB₄ and LTC₄ occurs in different subcellular locations and suggests that LTC₄ must be returned to the cytoplasmic side of the membrane for export by multidrug resistance protein-1. The differential localization of two very similar integral membrane proteins suggests that mechanisms other than size-dependent exclusion regulate their passage to the inner nuclear membrane.

Leukotrienes (LTs) are proinflammatory products of arachidonic acid (AA) metabolism. LTC₄, the parent sulfidopeptide LT, is synthesized on the luminal face of the ER membrane and exported by MRP. In addition, the differential localization of two integral membrane proteins to the inner nuclear membrane supports the possibility that the intracellular synthesis of LTB₄ and LTC₄ may be differentially compartmentalized and that LTC₄ synthase is localized in the lumen of the nuclear envelope and ER. These results support the possibility that the intracellular synthesis of LTB₄ and LTC₄ may be differentially compartmentalized and that LTC₄ synthase is synthesized on the luminal face of the ER membrane from where it must be transported to the cytoplasm for eventual export by MRP.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—LTC₄ synthase cDNA was cloned by reverse transcriptase-PCR. mRNA was prepared from KG-1 cells with Triazol (Invitrogen), and first strand cDNA synthesis was performed using...
Localization and Topology of LTC4 Synthase

Localization of LTC4 Synthase—We initially analyzed the subcellular localization of endogenous LTC4 synthase in RBL-2H3 cells. These cells were chosen because they express high levels of endogenous LTC4 synthase and generate LTC4 after stimulation with IgE or ionophore. The cells were permeabilized with 0.1% Triton X-100 and analyzed by conventional immunofluorescence. As shown in Fig. 2A, the enzyme was detected with a perinuclear staining pattern that extended out toward the peripheral ER, a distribution typical of intrinsic ER proteins. We next compared the distribution of LTC4 synthase-Myc-His expressed in CHO cells by conventional (Fig. 2B) and confocal microscopy (Fig. 2, C and E) with that of the inner nuclear membrane protein lamin A/C (Fig. 2D). Transfected cells were fixed, permeabilized with Triton X-100, and then probed with both anti-Myc monoclonal antibody and anti-lamin A/C polyclonal antibody (Fig. 2, B–E), followed by rhodamine-conjugated donkey anti-mouse IgG and fluorescein-conjugated goat anti-rabbit secondary antibody. When the localization of LTC4 synthase was determined by immunofluorescent microscopy, the highest intensity of rhodamine staining was detected at the nuclear rim (Fig. 2B); however, the Myc epitope was detected with a distribution consistent with that of the peripheral ER and with a pattern matching that of the endogenous protein. To further analyze the nuclear envelope, we performed confocal microscopy. In this case, a clear nuclear rim was observed (Fig. 2C), but additional staining was observed extending outwards with a pattern consistent with an ER distribution. Lamin A/C was also detected with a clear green ring around the nucleus (Fig. 2D). Digital overlay indicated a yellow rim around the nucleus and indicates co-localization of LTC4 synthase and lamin A/C at this level (Fig. 2E). However, as
discussed below, these findings cannot discern whether LTC₄ synthase is present in both the inner and outer nuclear membrane.

**Membrane Topology of CYP4F3A**—To determine the membrane topology of LTC₄ synthase, we used a strategy based on the ability of SLO to permeabilize the plasma membrane as opposed to intracellular membranes. Triton X-100 will permeabilize all cellular membranes including the ER and both membranes of the nuclear envelope. Thus, antigenic epitopes that are in the cytosol are revealed by treatment with SLO, but not those oriented to the ER lumen or contained within the nucleus; these require Triton X-100 to make them accessible to immunological detection. To establish this method, we employed a protein with a known topology that is a member of a class of proteins in which this approach has been successfully used. The protein chosen was CYP4F3A. CYPs are localized to the ER and possess a single transmembrane domain with the N terminus oriented to the lumen (21). We generated a functional
variant of CYP4F3A that contained an N-terminal FLAG epitope. This allowed detection of the luminal N terminus using anti-FLAG antibody and the cytoplasmic C terminus using affinity-purified anti-CYP4F3A-(410–520) antibody generated in our laboratory. COS cells were transfected with the pCMV5-CYP4F3A plasmid, fixed, and permeabilized with Triton X-100. They were probed simultaneously with fluoresceinated anti-CYP4F3A-(410–520) to detect the cytoplasmic portion of the molecule and anti-FLAG antibody to detect the N terminus. The FLAG epitope was detected with Texas Red-conjugated donkey anti-mouse IgG. When analyzed for fluorescein and rhodamine (Fig. 3, A and B), both the C and N termini showed an identical pattern consistent with ER localization. COS cells were then transfected and selectively permeabilized with SLO and probed with both antibodies. The cells demonstrated a pattern consistent with ER localization when analyzed for flu-
orescein (Fig. 3). However, no fluorescence was detected when they were analyzed for rhodamine (Fig. 3D), indicating that the FLAG epitope was not accessible to antibody. The detection of the cytoplasmic C terminus, but not the N terminus, after treatment with SLO confirms that this approach is effective in determining the topology of protein domains relative to the peripheral ER and nuclear envelope.

**Topology of the C and N Termi of LTC₄ Synthase**—We employed differential permeabilization to determine the topology of the C terminus of LTC₄ synthase. CHO cells that expressed LTC₄ synthase Myc-His were treated with SLO using the conditions established for CYP4F3A. The cells were then simultaneously probed for the Myc epitope and lamin A/C as described above. The same staining pattern was observed for the Myc epitope when cells were permeabilized with SLO (Fig. 2F). However, lamin C was not detected (Fig. 2G). These results indicated that the C terminus of LTC₄ synthase and the C terminus of CYP4F3A shared the same relationship (cytosolic) to the ER and outer nuclear envelope. To determine the topology of the N terminus, a FLAG-LTC₄ synthase vector was created that places a FLAG epitope on the N terminus of LTC₄ synthase. This vector was expressed in CHO cells, and differential permeabilization with SLO was performed. In these experiments (Fig. 2H), the FLAG epitope was detected, indicating its cytoplasmic orientation.

We next determined the orientation of the two hydrophilic loops of the active site. When CHO cells expressing LTC₄ synthase-Myc-His were permeabilized with Triton X-100 and analyzed by immunofluorescent microscopy (Fig. 4, A–C), both loop 1 (Fig. 4A) and loop 2 (Fig. 4B) were detected. In addition, the nuclear protein nucleoredoxin (Fig. 4C) was detected (as was lamin; data not shown). In these experiments, the nonspecific background was secondary to antisera, and the dark red staining nucleus was clearly visible, with nucleoli visible as dark holes. In contrast, neither loop 1 nor loop 2 could be detected following permeabilization with SLO (Fig. 4, D–F). When cells permeabilized with SLO were probed with anti-nucleoredoxin antibody, the background remained unchanged, whereas the nuclei appeared black secondary to a complete lack of staining. These results localize the active site of LTC₄ synthase to the same luminal side of the membrane as the N terminus of CYP4F3A, and the opposite side of the membrane as the N- and C termini of LTC₄ synthase.

**FIG. 6. Membrane interactions of LTC₄ synthase.** EGFP-LTC₄ synthase (A and D) or EGFP-CYP4F3A (C and F) were expressed in CHO cells and analyzed after permeabilization with 0.1% triton X-100 (TX-100) or after extraction with 1% Triton X-100 and NaCl (Extraction). Alternatively, they were analyzed before and after extraction using antibody to lamin A/C (B and E). In the latter experiments, nonextracted cells were stained with fluoresceinated secondary antibody, and extracted cells were stained with rhodamine-conjugated secondary antibody.

**FIG. 7. EM of LTC₄ synthase.** Immunogold staining of endogenous LTC₄ synthase in the outer nuclear membrane is shown by arrows. EM was performed using antibody to loop 1 at a dilution of 1:100. Nuclei are shown in a and b. n, nucleus; c, cytoplasm; bar, 0.1 μm.

We next used a second, intramolecular approach to confirm these observations. Cells were transfected with LTC₄ synthase Myc-His, fixed, permeabilized with Triton X-100, and then simultaneously probed with anti-Myc and antibodies to either loop 1 or loop 2. As shown in Fig. 5, these antibodies gave the same intracellular distribution when analyzed under fluorescein (loop 1 or 2) or rhodamine (Myc epitope) (Fig. 5, A–D).
When cells were permeabilized with SLO and analyzed simultaneously for loop 1 and for the C-terminal Myc epitope, only the C-terminal Myc epitope was detected (Fig. 5, E and F).

We next determined whether LTC4 synthase possessed physical properties similar to an ER protein. ER or nuclear envelope proteins that are not linked to cytoskeletal or nucleoskeletal structures are completely extracted from membranes with a combination of 1% Triton X-100 and 0.35 mM NaCl (22). As shown in Fig. 6, both LTC4 synthase-EGFP and CYP4F3A-EGFP were both visualized with a characteristic ER pattern (Fig. 6, A and C) and were completely extracted with 1% Triton X-100 and 350 mM NaCl (Fig. 6, D and F). When cells were fixed and probed with antisera to lamin A/C before (Fig. 6B) and after (Fig. 6E) extraction with Triton X-100 and 350 mM NaCl, the protein was detectable. These results indicate that LTC4 synthase does not interact with any structural protein in any cellular compartment in a manner similar to lamin and lamin-associated protein. These experiments characterize a molecule localized to the peripheral ER and the outer nuclear envelope that, based on its size of 17 kDa and relationship to FLAP, would be expected to freely distribute to all contiguous domains of the ER. To determine whether LTC4 synthase could also distribute to the inner nuclear membrane, we identified the ultrastructural localization of endogenous LTC4 synthase in RBL cells. Surprisingly, endogenous LTC4 synthase was distributed throughout the ER and was also localized to the outer nuclear envelope but was excluded from the inner nuclear membrane in any section or cell (Fig. 7). Only very rare gold particles in occasional cells were detected in the inner membrane. These findings are strikingly different from the localization of FLAP, which was shown to be preferentially localized to the inner nuclear membrane.

**DISCUSSION**

Endogenous or overexpressed LTC4 synthase show the same characteristic dense staining in the nuclear envelope extending to the peripheral ER (Fig. 2), which was clearly distinct from that of lamin A/C. The digital overlap with lamin showed a bright yellow rim surrounding the nucleus. Since lamin A/C was restricted to a tight nuclear rim, this suggested that the distribution of LTC4 synthase extended well into the peripheral ER. This distribution was seen for FLAP in peripheral blood human monocytes (7). When examined by EM, FLAP was distributed almost equally to the inner and outer nuclear membrane (7). Furthermore, 5-LO, which was localized in the cytosol of resting monocytes and neutrophils, was associated with the outer and inner nuclear membrane after cell activation. This movement of 5-LO through the nuclear pore has been well characterized (23, 24) and is mediated by a nuclear localization sequence at the C-terminal of the molecule (24, 25). Recently, LTA4 hydrolase has been shown to have the potential to move through nuclear pores and target to the nucleus in RBL cells (26), indicating that LT-B4 would be synthesized in the nuclear compartment. It was not determined whether LTA4 hydrolase was translocated to the cell membrane after activation.

LTC4 synthase and FLAP share 52% identity between amino acids 41 and 97 of FLAP and amino acids 45–101 of LTC4 synthase, which includes the two loops of the active site. Because of this high identity and the small 17-kDa size of these two proteins, it was highly surprising that the distribution of LTC4 synthase between the inner and outer nuclear membrane was distinct from that of FLAP (Fig. 7). This restriction of LTC4 synthase to the outer nuclear membrane combined with the observation that LT-B4 may be made within the nucleus suggests that the synthesis of LT-B4 and LTC4 can be differentially compartmentalized within cells. This points to potential differences in their role in intracellular function and intracellular trafficking. Various studies have suggested a role for LT-B4 in gene transcription as a ligand for peroxisome proliferator-activated receptor γ (27). The exclusion of LTC4 synthase from the inner nuclear membrane suggests that LTC4 is less likely than LT-B4 to play an intracellular role in modulating nuclear function and in transcriptional regulation.

It is generally accepted that after their synthesis and insertion in the ER, integral membrane proteins become localized to the inner nuclear membrane by lateral diffusion through the proteolipid bilayer of the outer nuclear membrane followed by diffusion around the nuclear pore (28, 29). In this model, the proteins are subsequently immobilized in the inner membrane by binding to immobile nucleoskeletal or nucleoplasmic ligands or by multimerization. The main mechanism excluding proteins from entry into the inner membrane is based on size, so that proteins with cytosolic/nucleoplasmic domains greater than 70 kDa fail to localize to the inner nuclear membrane (28). Proteins that do not fall into either category would be potentially free to diffuse between all contiguous membrane domains and be equally represented in the inner and outer nuclear membrane. FLAP fulfills these postulates and fits in the latter group, being equally represented in the inner and outer membrane (7). LTC4 synthase contradicts them, being a small protein essentially the same size as FLAP but being excluded from the inner nuclear membrane. Thus, a different mechanism must exist that allows FLAP free entry into the inner nuclear membrane and excludes LTC4 synthase. This includes the possibility that the primary sequence of FLAP contains a signal that allows it entry to the inner membrane or that LTC4 synthase contains a sequence that signals its exclusion.

The orientation of the N and C termini to the cytoplasm (Fig. 2) and the loops of the active site to the ER lumen (Figs. 4 and 5) support the model of membrane topology shown in Fig 1F. As described above, the release of LTC4 is dependent on its export from cells by the MRP-1 protein, which translocates its substrates from the cytosol to the extracellular space (12–15). LTC4 does not diffuse across membranes and must reenter the cytoplasmic compartment to be accessible to the MRP-1 protein. The mechanism by which this occurs is not known. LTC4 is formed at the luminal face of the ER, where GSH concentrations are 2–3 mM (30). GSH within the ER has been suggested to play a role mostly as a redox buffer controlling the state of sulphydryl bonds (31). Our data suggest that GSH in the ER can serve as an enzymatic substrate in additional reactions. Both prostaglandin H synthases have also been shown to have their active site oriented toward the ER lumen. As for prostaglandin endoperoxides generated by prostaglandin H synthase-1 and -2 (32), how LTC4 moves from the luminal surface to be accessible to intracellular transport and export by the MRP protein remains an open question.

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