Localization of Microsomal Triglyceride Transfer Protein in the Golgi

POSSIBLE ROLE IN THE ASSEMBLY OF CHYLOMICRONS*

Although a critical role of microsomal transfer protein (MTP) has been recognized in the assembly of nascent apolipoprotein B (apoB)-containing lipoproteins, it remains unclear where and how MTP transfers lipids in the secretory pathway during the maturation process of apoB lipidation. The aims of this study were to determine whether MTP functions in the secretory pathway as well as in the endoplasmic reticulum and whether its large 97-kDa subunit interacts with the small 58-kDa protein disulfide isomerase (PDI) subunit and apoB, particularly in the Golgi apparatus. Using a high resolution immunogold approach combined with specific polyclonal antibodies, the large and small subunits of MTP were observed over the rough endoplasmic reticulum and the Golgi. Double immunocytochemical detection unraveled the colocalization of MTP and PDI as well as MTP and apoB in these same subcellular compartments. To confirm the spatial contact of these proteins, Golgi fractions were isolated, homogenized, and incubated with an anti-MTP large subunit antibody. Immunoprecipitates were applied on SDS-PAGE and then transferred on to nitrocellulose. Immunoblotting the membrane with PDI and apoB antibodies confirmed the colocalization of these proteins with MTP. Furthermore, MTP activity assay disclosed a substantial triglyceride transfer in the Golgi fractions. The occurrence of membrane-associated apoB in the Golgi, coupled with its interaction with active MTP, suggests an important role for the Golgi in the biogenesis of apoB-containing lipoproteins.

Lipids constitute the most calorically dense dietary nutrients. They must undergo emulsification within the intestinal lumen, cell membrane permeation, intracellular esterification, and incorporation into chylomicrons before reaching the circulatory system (for reviews, see Refs. 1–4). Despite significant progress, our understanding of the complex biosynthetic process involved in the formation and secretion of triglyceride-rich lipoprotein particles remains rather fragmentary. In particular, we know little about the sequential multistep assembly of apolipoproteins and lipids or the topology of the proteins in intracellular organelles implicated in lipoprotein production.

The study of naturally occurring mutations and genetic variations in humans has greatly contributed to the identification of the proteins essential to the synthetic pathway and to the delineation of key metabolic mechanisms (5–11). Inherited disorders of apolipoprotein B (apoB)1 and microsomal transfer protein (MTP) deficiency have provided a unique source for delineating, at least partially, the role of these specific proteins as well as elucidating the intracellular mechanisms that result in lipid absorption and transport (5–11). The addition of core lipid to the nascent lipoprotein particle is thought to occur in conjunction with the translation and translocation of apoB in the ER (12–15). During this process, apoB remains tightly bound to the ER membrane, where it is folded. The initial complement of lipid is then added to form a nascent, small, dense lipoprotein particle. In the second step, maturation of the particle occurs by the addition of the neutral lipid core (16, 17). Based upon the known lipid transfer activity of MTP, its localization in the lumen of the ER, and the observation that apoB is degraded intracellularly and not secreted in the absence of MTP, it has been proposed that MTP shuttles lipids from the ER membrane to the growing apoB chain in the ER, allowing the protein to translocate completely into the lumen (18–20). However, very little is known about where and how the addition of bulk lipids to the nascent particle takes place.

Several models have been proposed for the formation of VLDL: (a) complete assembly of VLDL in the ER (21–23); (b) association of apoB with membranes until it reaches the Golgi apparatus, whereupon lipid is added to the particle (24, 25); and (c) a sequential addition of lipid to apoB during its passage from the ER to the Golgi for secretion (26–28). If partial assembly takes place in the Golgi and additional core lipids and phospholipids are added in the pre-Golgi and Golgi as has been suggested (25, 29), one would anticipate the obligatory presence of MTP in these compartments.

Structurally, MTP is a heterodimer composed of a unique

* This was supported by research grants from the Canadian Institutes of Health Research (MT-10585) and the Canadian Heart Association, as well as research scholarship awards from the Fonds de la Recherche en Santé du Québec (to E. L. and E. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: apoB, apolipoprotein B; MTP, microsomal transfer protein; ER, endoplasmic reticulum; VLDL, very low density lipoprotein; PDI, protein disulfide isomerase; PBS, phosphate-buffered saline; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
large subunit (97 kDa) and a smaller subunit (58 kDa) (30–33). The latter has been identified as the multifunctional enzyme, protein disulfide isomerase (PDI). The role of PDI in the function of MTP is not entirely clear. The two subunits form a tight complex, and their dissociation eliminates MTP activity. The intracellular location of MTP in the intestine has not been thoroughly elucidated. Subfractionation of crude liver homogenates suggested that MTP is located in hepatocellular microsomes (32, 33). Nevertheless, this finding was not confirmed by immunoelectron microscopy. In the present paper, the following issues are addressed. 1) Can MTP be found in secretory pathway sites other than the ER? 2) Does it occur alone or in close proximity to PDI in these compartments? 3) Does it localize with apoB? 4) If MTP can be detected in the Golgi apparatus, is it functional? 5) Can it facilitate apoB translocation from Golgi membranes? The answers to these questions provided by the experiments described herein help elucidate the mechanisms involved in the assembly and secretion of apoB-containing lipoproteins.

MATERIALS AND METHODS

Intestinal Specimens—Sprague-Dawley rats were used for all experiments. Jeunal specimens were taken at the ligament of Treitz, mounted on nickel grids with a carbon-coated Parlodion film and probed with gold complexes. Segments were examined by light microscopy to select well oriented villus blocks were examined by light microscopy to select well oriented villus tips. Thin sections (60–80 nm) of the different tissue blocks were mounted on nickel grids with a carbon-coated Parlodion film and processed for immunocytochemistry. Immunocytochemical Labeling—Protein A-gold immunocytochemical techniques were employed to detect the presence of MTP, PDI, and apoB in rat intestinal tissue as we have described previously (34, 35). Briefly, the tissue sections were fixed in immediate distilled water, incubated for 5 min on a drop of PBS containing 1% ovalbumin, and transferred subsequently to a drop of the PBS-diluted antibody (see below). After incubation (90 min) at room temperature, the grids were rinsed with PBS to remove unbound antibodies. They were transferred to the PBS-ovalbumin (2 min) and incubated on a drop of protein A-gold (pH 7.2) for 30 min at room temperature. The tissue sections were then thoroughly washed with PBS, rinsed with distilled water, and dried. Sections were stained with uranyl acetate and lead citrate before examination with a Phillips 410 electron microscope. Polyclonal antibodies were routinely used as a control. The protocol allowed for the detection of MTP, PDI, and apoB in the liver as described previously (34, 36). Control experiments were performed to assess the specificity of the results. Excess purified MTP (10-fold) was added to the antibody solution. Incubation with this solution was followed by the protein A-gold complex. Pre-immune rabbit serum (diluted 1:10) was used on tissue sections before incubation with protein A-gold complexes. Inclusions were also performed with the protein A-gold complex alone, omitting the antibody step to test for non-specific adsorption of the protein A-gold complex to tissue sections (36).

Double-labeling Technique—To reveal the existence of MTP-PDI as well as MTP-ApoB complexes within the cellular compartments, the double-labeling technique was applied. The tissue sections were labeled simultaneously for either MTP and PDI or MTP and ApoB. The two-phase labeling technique (36, 37) was applied to avoid any cross-reaction between reagents. The small protein A-gold complex (5 nm) was used for the first labeling protocol, and the larger 10 nm protein A-gold complex was used for the second. This protocol allows for the simultaneous visualization of two antigens (MTP or PDI or MTP and ApoB) in the same tissue section.

ApoB antibodies for the MTP, PDI, and MTP large subunits were kindly provided by John Wettera, Harris Jamil, and one of the authors (C. Shoulders). These antibodies have been characterized and utilized successfully in previous studies (7, 31, 38, 39). The antibody directed against rat apoB was raised in rabbits (35). Confirmation of the specificity of the antibodies was obtained when: 1) MTP large and small subunits as well as apoB from total homogenate were fractionated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with each polyclonal antibody; and 2) cellular lysates were first reacted with the antibodies (directed against large MTP subunit, small MTP subunit, and apoB antibodies) before the immunoprecipitates were subjected to gel electrophoresis, transferred to nitrocellulose membranes, and reacted with the underlying antibodies.

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microsomal and Golgi fractions were immunoprecipitated, subjected to SDS-PAGE electrophoresis, excised from the gel, and counted (45).

**RESULTS**

The first step in our studies was to test the specificity of the polyclonal antibodies that were generously provided by investigators (see “Acknowledgments”) who remarkably advanced the knowledge of MTP. By immunoprecipitating epithelial cell lysates, separating the immunoprecipitates on SDS-PAGE, and Western blotting them separately with large 97-kDa and small 58-kDa antibodies, we identified the expected 97- and 58-kDa subunits corresponding to the MTP and PDI components, respectively (Fig. 1A). The use of polyclonal antibodies directed against the whole MTP complex resulted in the recovery of both MTP and PDI subunits (Fig. 1A). Similar experimental procedures displayed and confirmed the specificity of anti-apoB antibodies (Fig. 1A). In a second step, the specificity of antibodies was verified by fractionating the total homogenate by SDS-PAGE, transferring it to nitrocellulose, and immunoblotting. Once again, we found only the signal corresponding to the targeted protein (Fig. 1B).

We subsequently used immunocytochemical techniques and isolated microsomal and Golgi fractions to reveal the presence of MTP in these subcellular compartments. The purity of microsomal and Golgi fractions was determined by the assay of galactosyltransferase, as a specific marker for Golgi membranes, and glucose-6-phosphatase as a marker for the ER. The results of marker protein assays from a typical fractionation demonstrated the purity of the organelle fractions was assessed by determining the specific activities of UDP-galactose galactosyltransferase and glucose-6-phosphatase. The enrichment of the marker enzymes in each cellular fraction was calculated by dividing the specific activity of the subcellular fraction by that of the homogenate (data are given in parentheses). The data represent the average of n = 2.

| Organelle       | Galactosyltransferase (nmol/mg protein/min) | Glucose-6-phosphatase (nmol/mg protein/min) |
|-----------------|--------------------------------------------|--------------------------------------------|
| Whole homogenate| 0.041 (×1.00)                              | 0.720 (×1.00)                              |
| Microsomal fraction| 0.101 (×2.46)                           | 2.735 (×3.80)                              |
| Golgi fraction  | 3.926 (×95.75)                             | 0.907 (×1.26)                              |

The antibody used in this first series of experiments recognizes the whole 97-kDa-58-kDa MTP protein complex. We therefore attempted to distinguish between the two subunits by employing antibodies specific to the 97-kDa and the 58-kDa polypeptides separately. With the antibody directed against the MTP large subunit, the distribution of the labeling (Fig. 4) over the rough ER and the Golgi apparatus was qualitatively identical to that recorded for the entire MTP complex, as illustrated in Fig. 2. A similar distribution was observed when anti-PDI antibody was utilized (Fig. 5). Again, negligible labeling was noted with control experiments (data not shown).

**Double Immunocytochemical Detection of MTP and PDI as Well as MTP and apoB.—**The interaction of MTP with PDI is required for the production of the soluble, active MTP complex (33), which in turn is crucial for the assembly of apoB-containing lipoproteins (7, 8, 18, 19). In addition to demonstrating their labeling in the secretory pathway, as described above, we assessed their ultrastructural colocalization using double immunocytochemical detection. MTP was revealed by 10-nm protein A-gold, and PDI was shown using 5-nm protein A-gold (Fig. 6). Both particles were present over the rough ER and the Golgi area, many of them in very close association. Only very few gold particles were detected over the nuclei and mitochondria. These data provide evidence for the presence of MTP-PDI complexes not only in rough ER but also in the Golgi apparatus.

Double immunocytochemical detection of the large subunit of MTP and apoB was also investigated in the rat jejunal enterocytes (Fig. 7). MTP and apoB were revealed by 10- and 5-nm protein A-gold complexes, respectively. Numerous doublets, formed by large and small gold particles, were found over the Golgi area, suggesting that MTP and apoB are colocalized and associated in the same cellular compartments of the enterocyte. It must be noted that only a certain percentage of the gold particles revealing a large MTP subunit or PDI appeared to be colocalized. This may be due to numerous factors such as the ubiquitous nature of PDI, which fulfills various functions in other locations intrinsic to the ER, and technical limitations stemming from immunogold itself.
Interaction of MTP Large Subunit with PDI and ApoB in the Golgi

The aforementioned ultrastructural studies demonstrated the presence of MTP complex and the colocalization of its two subunits in the Golgi apparatus. Additional studies were carried out to assess its function in this subcellular compartment. Because the association of PDI (58 kDa) with MTP large subunit (97 kDa) is necessary to maintain the catalytically active form of the triacylglycerol transfer protein and to prevent the aggregation of the 97-kDa component, we examined their physical interaction. For this purpose, Golgi fractions were isolated and homogenized in a nondenaturing buffer. Immunoprecipitation was also carried out with an anti-MTP large subunit antibody under nondenaturing conditions. The immunoprecipitates were run onto SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblotting the membrane with anti-PDI antibody confirmed the presence of PDI (Fig. 8A). Thus, the anti-MTP large subunit appeared to precipitate PDI, whereas MTP antibodies recognized the MTP large subunit (Fig. 8B), indicating an interaction between the MTP large subunit and PDI in the Golgi. In a second step, MTP activity was measured after setting up the assay in order to defining the optimal conditions; i.e., incubation time, protein linearity, background transfer in the absence of MTP, and recovery of acceptor vesicles. The assay disclosed substantial triacylglycerol transfer activity in the Golgi fraction compared with that of microsomes (Fig. 9). Together these findings indicate that the MTP complex is present in the Golgi and that it maintains its capacity to transfer triacylglycerol in that organelle. Finally, based on the protein recovery of isolated organelles, intestinal microsomes and Golgi fractions represented 64 and 46%, respectively, of the total cellular MTP activity.

Immunoblotting experiments were performed to confirm the spatial contact of MTP with apoB in the Golgi fractions. Immunoprecipitation was carried out with the antibody directed against the large MTP subunit. The immunoprecipitates were

**Fig. 2.** Immunocytochemical detection of MTP in rat enterocytes. Protein A-gold immunocytochemical technique was applied with the polyclonal antibody directed against the heterodimer MTP/PDI to localize it in absorptive cells of rat jejunum. The labeling by gold particles revealed the antigenic sites of the MTP/PDI heterodimer at the level of the rough ER (RER), Golgi apparatus (G) and the basolateral membrane (blm). Mitochondria (m) and nuclei (N) are devoid of labeling. Magnification, ×40,000.

**Fig. 3.** Assessment of labeling specificity. The tissue section was incubated with antigen-adsorbed antibody against the MTP large subunit followed by the protein A-gold complex. The labeling by gold particles was drastically reduced, with very few particles present over the different cellular compartments. G, Golgi apparatus; RER, rough endoplasmic reticulum; m, mitochondria. Magnification, ×30,000.

**Fig. 4.** Immunocytochemical detection of the MTP large subunit in rat jejunal enterocytes. A specific polyclonal antibody directed against the 97-kDa large subunit of MTP was employed in combination with protein A-gold. Labeling is present over the rough ER (RER) and the Golgi area (G) including small vesicles (v). Mitochondria (m) are devoid of labeling. Magnification: A, ×50,000; B, ×30,000.
run on SDS-PAGE and transferred onto a MTP nitrocellulose membrane. The latter was blotted with anti-apoB antibodies. As shown in Fig. 8C, no apoB was visualized with nonimmune sera, whereas it was clearly detected with anti-apoB antibody. These observations are consistent with the aforementioned ultrastructural findings illustrating the colocalization of MTP with apoB.

Finally, Golgi fractions were exposed to nonimmune serum or anti-apoB antibodies. The immunoprecipitate was run on SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blotted with MTP large subunit antibody. The membrane was stained with 57-kDa PDI subunit against antibody. The immunoprecipitate was run on SDS-PAGE and transferred onto a nitrocellulose membrane. The latter was blotted with anti-apoB antibodies. As shown in Fig. 8C, no apoB was visualized with nonimmune sera, whereas it was clearly detected with anti-apoB antibody. These observations are consistent with the aforementioned ultrastructural findings illustrating the colocalization of MTP with apoB.

Presence of ApoB in the Golgi Membrane—The presence of apoB in the Golgi may be derived from apoB-containing lipoprotein transit from the rough ER or from its close association with the Golgi membrane. To demonstrate that apoB is a resident membrane protein of the Golgi apparatus, membranes were isolated, immunoprecipitated with anti-apoB antisera, applied on SDS-PAGE, and transferred onto a nitrocellulose membrane. Again, we used galactosyltransferase as a marker of Golgi membranes, and the overall enrichment of its specific activity was 133-fold. Anti-apoB antibodies revealed the presence of apoB, suggesting that apoB is associated with Golgi membranes (Fig. 11).
from microsomal and Golgi membranes. In addition, little effect of the MTP inhibitor was observed on the disappearance of apoB from the lumen of the two subcellular compartments. These experiments suggest that MTP protects apoB against co-translational degradation.

**DISCUSSION**

Most of the studies undertaken to delineate the role of MTP in the assembly of apoB-containing lipoproteins have suggested that the ER represents its site of action in view of its involvement in the biogenesis of triglyceride-rich lipoproteins. It was previously unknown whether MTP is found in other subcellular organelles in the small gut. The present investigation examined the intracellular distribution of the large subunit of MTP along with PDI and apoB in the rat small intestine. Our data revealed that, at the electron microscope level, MTP is present particularly in the rough ER and the Golgi apparatus of the absorptive epithelial cells. Double immunocytochemical labeling demonstrated the co-localization of the MTP large subunit with PDI and apoB. The biochemical analyses not only confirmed the ultrastructural studies but also demonstrated the association between the MTP large subunit and PDI in the Golgi, the preservation of its transfer activity, and its interaction with apoB. These observations suggest that this organelle likely represents a site that is active in triglyceride-rich lipoprotein assembly.

The PDI and the MTP large subunit are not covalently linked in the heterodimer, but they form a highly stable protein complex (31). The spatial contact of the large subunit with PDI is necessary to maintain the transfer protein in a nonaggregated and catalytically active form (30–33). The data from the present study documented the presence of the 58- and 97-kDa subunits of MTP in basolateral membranes. Even if our ultrastructural experiments showed that the two subunits seem to be in close physical proximity, we are not able to indicate at this point whether basolateral membranes contain active MTP. Another puzzling question is whether MTP accompanies lipoproteins to this subcellular area. Experiments from our laboratory have shown that freshly isolated lipoproteins from the blood circulation are devoid of MTP (results not shown), suggesting that the latter does not exit the cell. Confirming data were obtained in Caco-2 cells when we were not able to detect any MTP signal by Western blot or by TG transfer activity at the basolateral medium. Currently, it is not clear whether the MTP-PDI complex not only plays a key role in triglyceride-rich lipoprotein assembly but also functions as a chaperone to facilitate the secretion of chyomicrons and VLDL.

Much of our knowledge concerning apoB synthesis and lipoprotein assembly stems from studies using hepatocytes, including human and rat hepatoma cells, McA-RH7777 cells, and primary hepatocytes (13, 18–20, 46). These hepatic models generally produce both apoB-48 and apoB-100. The lipidation of apoB is thought to occur in two phases (16, 17). Initially, the stabilization of apoB necessitates an association with a small quantity of neutral lipids, which protects it from misfolding and degradation. The second step is characterized by the bulk transfer of triacylglycerol to the secretion-competent form of apoB, resulting in the production of mature particles of VLDL. MTP has been shown to transport lipid from the ER membrane to apoB in the lumen of the ER (7, 8, 19, 39, 45). This process may be promoted by a physical interaction between MTP and apoB (46). Our results show that MTP is also located in the Golgi apparatus of rat intestine in its active form and that it interacts with apoB locally, as was demonstrated in the ER by Wu et al. (48) and Patel and Grundy (47).

The requirement for lipid transfer activity in the assembly of apoB-containing lipoproteins has been underscored by genetic
disorders and various molecular studies. Evidence has been presented for protein-protein interactions between apoB and MTP, which may be crucial for the transfer of lipids to apoB during its translocation or may indicate a chaperone-like activity for MTP in apoB folding, assembly, and secretion. The binding sites involved in these interactions have recently been proposed (49, 50). On the other hand, PDI has been shown to associate with MTP to maintain the MTP complex in a soluble state (30–33). Because PDI contains the "KDEL" ER retention sequence, it might anchor the MTP complex in the ER at the site of apoB translocation (51). Additional studies are required to determine whether most of these events occur in the Golgi apparatus.

The occurrence of membrane-associated apoB in the Golgi, coupled with its interaction with active MTP, suggests an important role for the Golgi in the assembly of apoB-containing lipoproteins. Although direct evidence for such a hypothesis is lacking, earlier reports proposed a pivotal role for the Golgi in the formation of triacylglyceride-rich lipoproteins. Bamberger and Lane (24, 25) reported that 50% of apoB is membrane-associated in both the ER and the Golgi in chicken liver. Using pulse-chase experiments, they concluded that the assembly of triacylglycerol with apolipoproteins occurs in the Golgi. Similarly, Higgins (52) suggested that the trans-Golgi region is the major intracellular site for the assembly of apoB with triacylglycerol and phospholipid. Furthermore, Olofsson et al. (53) emphasized that a substantial portion of the oligosaccharide chains of apoB, along with various posttranslational modifications of both lipid and apolipoprotein moieties, is processed in the Golgi. Accordingly, our data showed that MTP inactivation by BMS-200150 delayed the net removal of newly synthesized apoB from the microsomal and Golgi membranes but not from the corresponding luminal compartments. Similar findings were reported in the liver by Hebbachi and colleagues (54, 55). It seems, therefore, that the association of apoB with lipid components requires active MTP in both the microsomal and Golgi membranes to allow the secretion-competent form of apoB to form chylomicrons in the enterocyte. Taken together, these observations point to the important role of the Golgi in
the assembly of apoB-containing lipoproteins, as suggested by our results herein.

How do our ultrastructural findings and biochemical data fit the two-phase process for apoB core lipiddation? It is possible that nascent apoB-48 HDL particles, originating in the ER, are supplied with neutral lipid droplets at the junction of the rough and smooth ER as proposed by many investigators (for review, see Ref. 4). However, we cannot exclude the possibility that lipid loading is completed in the Golgi compartment. During the continuous maturation process, MTP may shuttle lipids from the Golgi membrane or from locally available lipid droplets to the growing apoB-containing lipoprotein particles. As noted above, a considerable body of literature supports a key role of the Golgi apparatus in triglyceride-rich lipoprotein assembly and phospholipid acquisition (52, 53). On the other hand, we observed the lipid transfer protein activity of MTP in the Golgi as well as its localization to the lumen of Golgi and its association with apoB at this site. One may thus suggest that the Golgi apparatus represents an additional site where MTP enables nascent apoB to attain a secretable conformation by providing sufficient triacylglycerol for the formation of a neutral lipid core. The process may be promoted by a physical interaction between MTP and apoB. This hypothesis is consistent with the recent observations relative to: (a) the heavy labeling of Golgi membrane for apoB following radioactivity pulses (54, 55); (b) Golgi-mediated degradation of apoB (56–58); and (c) the delay of apoB removal from the Golgi membrane following MTP inhibition with BMS-200150 (55). In our studies, the possibility of substantial contamination of Golgi membranes with microsomes was ruled out by the quantification of marker enzymes (59). Moreover, potential major MTP leakage from the ER to the Golgi apparatus was rejected by appropriate immunocytochemical studies. Thus, the Golgi may represent an additional important site for lipoprotein assembly. Nevertheless, many experiments must be carried out to determine the precise function of MTP in the Golgi and the role in this organelle in apoB-containing lipoprotein assembly.

In summary, the present investigation demonstrates the presence of MTP in subcellular compartments associated with the secretory pathway other than the ER. Furthermore, the colocalization of functional MTP and the membrane-associated apoB in the Golgi fraction strongly suggests a role for the Golgi apparatus in the assembly or maturation of intestinal TG-rich lipoproteins.

Acknowledgments—We thank Drs. J. R. Wetterau and H. Jamil for the polyclonal antibodies. We also acknowledge the expert secretarial assistance of Danielle St.-Cyr Huot.

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