Apolipoprotein B Binding to Microsomal Triglyceride Transfer Protein Decreases with Increases in Length and Lipidation: Implications in Lipoprotein Biosynthesis†

M. Mahmood Hussain,*,‡ Ahmed Bakillah,‡,§ and Haris Jamil||

Departments of Pathology and Biochemistry, Allegheny University of the Health Sciences, Philadelphia, Pennsylvania 19129, and Bristol-Myers Squibb, Pharmaceutical Research Institute, Princeton, New Jersey 08543

Received June 11, 1997; Revised Manuscript Received August 13, 1997⊗

ABSTRACT: Microsomal triglyceride transfer protein (MTP), a heterodimer of 97 kDa and protein disulfide isomerase, is required for the assembly of apolipoprotein B (apoB)-containing triglyceride-rich lipoproteins. These proteins have been shown to interact with each other during early stages of lipoprotein biosynthesis. Our studies indicated that binding between apoB and heterodimeric MTP was of high affinity (K_d 10−30 nM) due to ionic interactions. In contrast to MTP, protein disulfide isomerase alone interacted very poorly with lipoproteins, indicating the importance of the heterodimer in these bindings. Preincubation of lipoproteins with detergents enhanced their interaction with MTP. Native VLDL bound poorly to MTP, but its preincubation with Tween-20 resulted in significantly increased binding to MTP. Furthermore, binding of LDL was enhanced by preincubation with taurocholate, indicating that partial delipidation of apoB-containing lipoproteins results in increased binding to MTP. Subsequently, attempts were made to study interactions between C-terminally truncated apoB polypeptides and MTP. Binding of all the polypeptides to MTP was enhanced in the presence of taurocholate. Comparisons revealed that the binding of different apoB polypeptides to MTP was in the order of apoB18 > apoB28 > apoB42 > apoB100. These studies indicated that optimum interactions occur between apoB18 and MTP, and that the increase in apoB length beyond apoB18 has a negative effect on these interactions. Since apoB18 does not assemble triglyceride-rich lipoproteins, these studies suggest that apoB may interact with MTP before its lipidation. It is proposed that steps in lipoprotein biosynthesis may be dictated by the sequential display of different functional domains on the apoB polypeptide.

Apolipoprotein B (apoB)[1] is an essential structural protein required for the assembly of triglyceride-rich lipoproteins. It is synthesized as a single polypeptide of 4536 (apoB100) or 2152 (apoB48) amino acids by the liver and intestine, respectively. Unlike other secretory proteins, which are co-translationally inserted into the lumen of endoplasmic reticulum (ER), apoB is co-translationally integrated, at least transiently, into the ER membranes in a transmembrane orientation [for reviews, see Innerarity et al. (1996), Hussain et al. (1996), Davis (1991), Sparks and Sparks (1994), Yao and McLeod (1994); Gibbons (1990), Vance and Vance (1990), and Dixon and Ginsberg (1993)]. Translocation of apoB across the ER membrane is inefficient and probably determines lipoprotein production (Bonnardel & Davis, 1995; Sakata et al., 1993). It is thought that apoB can be lipidated even before the completion of peptide synthesis. The cotranslational lipidation of apoB is supported by the observations that incompletely synthesized apoB polypeptides are secreted as lipoprotein particles by cells incubated with puromycin, which stops protein synthesis by releasing peptides from ribosomes (Spring et al., 1992; Boren et al., 1992). Lipidation of apoB results in the release of apoB from the ER membrane and in the formation of primordial lipoproteins.

The early lipidation of nascent apoB polypeptides requires the microsomal triglyceride transfer protein (MTP) [for reviews, see Wetterau et al. (1997) and Gregg and Wetterau (1994)]. The purified MTP activity consists of two subunits of 58 and 97 kDa (Wetterau & Zilversmit, 1985). The 97 kDa subunit is essential for lipid transfer activity and is defective in abetalipoproteinemia patients who lack apoB-containing lipoproteins in their plasma (Gregg & Wetterau, 1994; Wetterau et al., 1992; Sharp et al., 1993). The 58 kDa protein disulfide isomerase (PDI) is required to keep the larger subunit in solution and to retain it in the ER (Ricci et al., 1995; Wetterau et al., 1991). A direct correlation between MTP activity and lipoprotein assembly has been obtained in vitro by co-expressing apoB and MTP in cells that do not secrete lipoproteins (Patel & Grundy, 1996; Wang et al., 1996; Gretch et al., 1996; Leiper et al., 1994; Gordon et al., 1994). Expression of apoB cDNAs in most studies resulted in the intracellular synthesis of apoB polypeptides, but no lipoprotein secretion. In contrast, cotransfection of apoB with MTP resulted in increased secretion of apoB polypeptides (Patel & Grundy, 1996; Wang et al., 1996; Gretch et al., 1996; Leiper et al., 1994; Gordon et al., 1994). It has been suggested that MTP most likely assists in the increased translocation of nascent apoB from the ER

† Supported by grants from the National Institutes of Health (DK-46900 and HL-22633) and American Heart Association, National Center and Southeastern Pennsylvania Affiliate.

* Address correspondence to this author. Phone: 215-991-8497. Fax: 215-843-8849. E-mail: Hussain@auhs.edu.

‡ Allegheny University of the Health Sciences.

§ Visiting scientist from Université Chouaib Doukkali, Faculté des Sciences, Laboratoire de Biochimie Appliquee, El Jadida, Morocco.

|| Bristol-Myers Squibb, Pharmaceutical Research Institute.

⊗ Abstract published in *Advance ACS Abstracts,* October 1, 1997.

[1] Abbreviations: ApoB, apolipoprotein B; ER, endoplasmic reticulum; HDL, high-density lipoproteins; LDL, low-density lipoproteins; MTP, microsomal triglyceride transfer protein; PBS, phosphate-buffered saline; PBS-Tween, PBS containing 0.05% Tween-20; PDI, protein disulfide isomerase; VLDL, very low density lipoproteins.

S0006-2960(97)01395-0 CCC: $14.00

membrane (Gretch et al., 1996). Furthermore, MTP-assisted increased translocation and secretion of nascent apoB polypeptides required the presence of the N-terminal 17% of apoB. Why does MTP require the N-terminal 17% of apoB to assist the secretion of nascent apoB polypeptides? Recently, evidence has been presented for a transient interaction between apoB and MTP using coimmunoprecipitation techniques (Patel & Grundy, 1996; Wu et al., 1996). Very little is known concerning the biochemical, biophysical, or molecular nature of these interactions. Furthermore, factors that help to dissociate MTP from nascent lipoproteins prior to their secretion are not known. To characterize these interactions, we have developed in vitro assays and have shown that the N-terminal 18% of apoB has the optimum capacity to bind with MTP and that the interactions between apoB and MTP are affected by the length and lipidation of apoB polypeptide.

MATERIALS AND METHODS

Materials. All the assays were performed with the purified heterodimeric MTP complex (Wetterau et al., 1990, Jamil et al., 1995,1996). The partially purified PDI (Wetterau et al., 1990) used was devoid of 97 kDa subunit as determined by Western blot analysis. The purified 97 kDa subunit was not used because it could not be isolated without denaturing the protein (Ricci et al., 1995; Wetterau et al., 1991). Furthermore, the denatured, purified subunit had no lipid transfer activity (Wetterau et al., 1991). Antibodies used for ELISA have been described (Hussain et al., 1995b).

Cells. McA-RH7777 cells stably transfected with different C-terminally truncated forms of apoB have been described (Wang et al., 1994; Hussain et al., 1995; Yao et al., 1991). HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in minimal essential medium containing 10% fetal bovine serum (Biofluids, Maryland, MD) and 1% antibiotic−antimycotic (Life Technologies).

Binding of Radiolabeled MTP or PDI to Lipoproteins. MTP or PDI (200 μg) was iodinated (Hussain et al., 1995a) with 0.2 mCi of ^{125}I (NEN) using 1 mg of Iodogen (Pierce), and dialyzed extensively against 0.01 M Na_2HPO_4, 1.76 mM KH_2PO_4, 0.137 M NaCl, 2.68 mM KCl, and 0.001% thimerosal buffer, pH 7.4 (PBS). The specific activities of the radiolabeled proteins ranged from 800 to 1000 cpm/ng and contained less than 4% free iodine. To study the binding of ^{125}I-MTP or ^{125}I-PDI to lipoproteins, microtiter wells were coated (100 μL, 2 h, 37 °C) with different amounts of human LDL, VLDL, or HDL in PBS. After washing 3 times with PBS, wells were incubated (1 h, 37 °C) with PBS containing 0.05% Tween-20 (PBS-Tween). The amount of apoB or apoA-I immobilized was determined by ELISA (Hussain et al., 1995b). In separate experiments, lipoprotein-coated wells pretreated with PBS−Tween were incubated with the indicated amounts of ^{125}I-MTP in PBS−Tween, washed, and counted in a γ-counter to determine the amount of bound MTP. Human plasma LDL was iodinated by iodomonochloride procedure (Hussain et al., 1991; Goldstein et al., 1983). To study the binding of ^{125}I-LDL, microtiter wells were coated with heterodimeric MTP or PDI. Wells were then incubated with PBS−Tween followed by various concentrations of ^{125}I-LDL as described above. Nonspecific binding was determined in the presence of a 30−50-fold excess of unlabeled LDL. The nonspecific binding was generally less than 25% of the total binding.

Binding of Lipoproteins to Immobilized MTP. ELISA plates were coated with different indicated amounts of MTP in 100 μL of PBS in triplicate wells by incubating at 37 °C for 2 h. Wells were washed with PBS, and incubated with either PBS−Tween or albumin (1 mg/100 μL) for 1 h at 37 °C to block all the binding sites in the wells. Wells were then incubated with indicated amounts of LDL diluted in PBS−Tween for 2 h at 37 °C. In different experiments, detergents or salt was added during these incubations. Microtiter wells were washed 3 times with PBS−Tween. Bound apoB was quantitated by a sandwich ELISA (Hussain et al., 1995b). In parallel, a standard curve for the apoB was generated by coating wells with a monoclonal antibody, 1D1, and incubating with different concentrations of LDL (0−14 ng/well) as described before (Hussain et al., 1995b).

Binding of Different Truncated ApoB Polypeptides to MTP. For experiments, 80% confluent monolayers of McA-RH7777 cells transfected with different C-terminally truncated apoB polypeptides were incubated with serum-free medium containing 0.2% BSA for 48 h. The conditioned medium was concentrated and used to determine the amount of apoB present and to study the binding of apoB polypeptides to the immobilized MTP. The apoB polypeptides that interacted with MTP were quantitated by ELISA.

Other Analyses. Protein was determined using the Coomassie Plus reagent (Pierce Chemical Co., Rockford, IL) with BSA as a standard. Radioactivity was counted in a γ-counter (Packard, Meriden, CT). Optical density in ELISA plates was measured using a Dynatech MRX microplate reader (Dynatech Labs, Chantilly, VA). The data were plotted as mean ± standard deviations, and the binding isotherms were analyzed using Prism2 (Graphpad, San Diego, CA). The molecular masses used for LDL, MTP, and PDI subunit were 512, 146, and 55 kDa, respectively. For VLDL, we assumed that apoB100 constituted one-third of the total proteins.

RESULTS

Interactions between ApoB100 and MTP. To investigate whether heterodimeric MTP would interact with plasma lipoproteins, different amounts of human plasma lipoproteins were immobilized to plates, and the amount of ^{125}I-MTP bound was determined (Figure 1A). The maximum binding of MTP to immobilized LDL and VLDL was significantly higher than that of binding to HDL. The interactions between MTP and lipoproteins were then studied in more detail to obtain kinetic parameters (Figure 1, panels B−D). In addition, we studied in parallel the binding of PDI to different lipoproteins. The binding of radiolabeled heterodimeric MTP to the LDL and VLDL was significantly higher than the binding of PDI (Figure 1, panels B and C). The maximum binding of both MTP and PDI to HDL was very low (Figure 1D). The MTP binding to LDL and VLDL exhibited saturation kinetics, whereas the binding of PDI was linear under the experimental conditions (Figure 1B,C). The curves for the binding of MTP to LDL and VLDL were subjected to nonlinear regression analysis using an equation [$y = ax/(b + x)$; a is B_{max}, b is K_d]. Different kinetic parameters are summarized in Table 1. MTP bound to LDL and VLDL with a high affinity (K_d) in the range of 17−26 nM. At B_{max}, one molecule of heterodimeric MTP was found

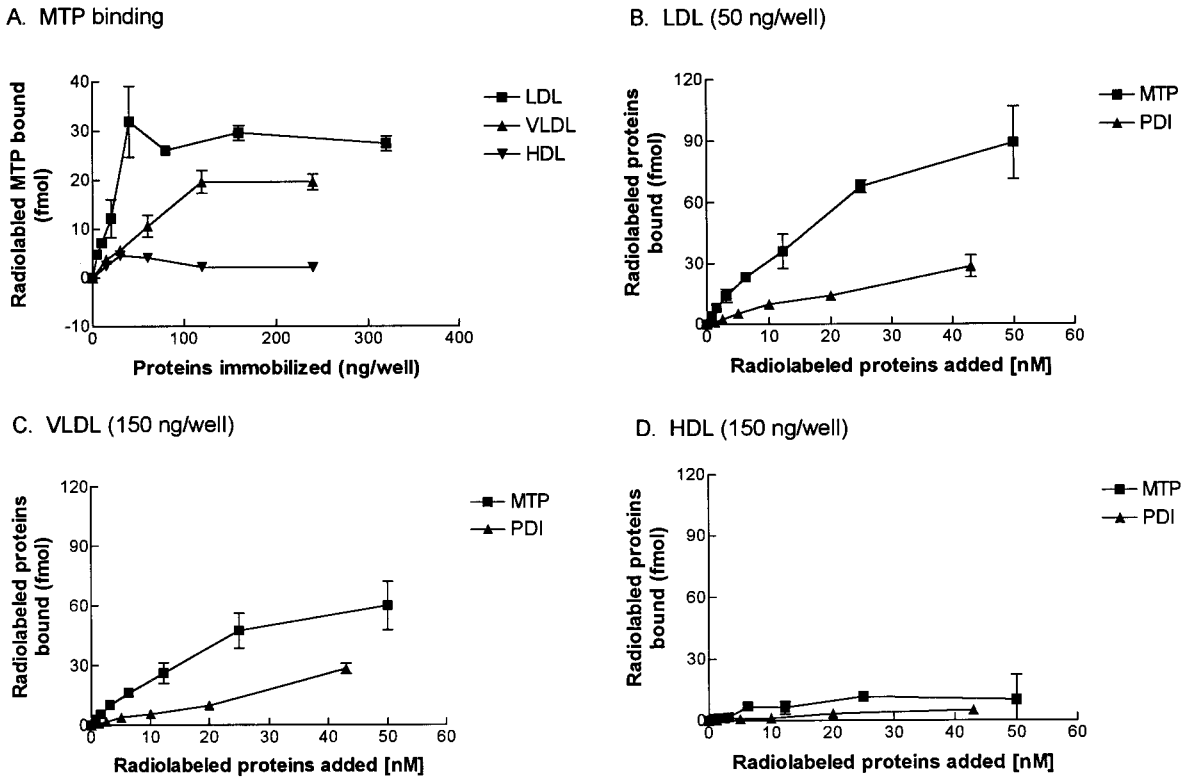

FIGURE 1: Binding of MTP to immobilized human plasma lipoproteins. Panel A: Triplicate microtiter wells were incubated (2 h, 37 °C) with 100 μL of the different indicated amounts of LDL, VLDL, or HDL in PBS, and unoccupied binding sites were blocked with PBS−Tween (1 h, 37 °C). Wells were washed and incubated with 100 μL of ^{125}I-MTP (1 μg/mL, 6.8 nM) in PBS−Tween for 2 h at 37 °C, and the amount of MTP bound was quantitated. The binding of radiolabeled MTP to noncoated wells was performed in parallel. This nonspecific binding constituted less than 10% of the total binding and was subtracted. Mean values for binding and standard deviations are plotted as line graphs and error bars. Panels B−D: Microtiter wells were coated (2h, 37°C) with LDL (50 ng), VLDL (150 ng) or HDL (150 ng), as described above. Wells were then incubated with PBS−Tween for 2 h at 37 °C. The indicated amounts of radiolabeled MTP (specific activity, 829 cpm/ng) or PDI (specific activity, 996 cpm/ng) were added to wells in triplicate and incubated for 2 h at 37 °C. Binding was plotted as line graphs. Error bars represent standard deviations.

Table 1: Kinetic Parameters of ApoB/MTP Interactions

proteins or lipoproteins					
soluble	immobilized	B_{max} (fmol)	K_d (nM)	r^2	data from
^{125}I-MTP	LDL	101.20 ± 8.44[a]	22.90 ± 3.57	0.9943	1B
^{125}I-MTP	VLDL	64.50 ± 4.78	19.52 ± 2.85	0.9944	1C
^{125}I-LDL	MTP	96.02 ± 55.66	86.81 ± 108.4	0.7344	2B
LDL	MTP	13.77 ± 0.57	13.72 ± 2.07	0.9861	3B
VLDL	MTP	6.22 ± 1.62	120.4 ± 69.24	0.8834	4A
VLDL* [b]	MTP	7.52 ± 0.76	16.16 ± 6.76	0.9108	4A

[a] Mean ± standard error. [b] VLDL*, VLDL preincubated with PBS−Tween for 2 h at 37 °C.

to bind to one molecule of the immobilized LDL (Figure 1B,C). These studies indicated that MTP binds to apoB-containing lipoproteins with high affinity. In contrast to heterodimeric MTP, the PDI interacts poorly with these lipoproteins.

Next, MTP was immobilized on microtiter wells and used to study the binding of plasma lipoproteins. Immobilized MTP retained 14−20% of the triglyceride transfer activity. The radiolabeled LDL bound significantly to MTP-coated wells but not to PDI-coated or noncoated wells (Figure 2A). The specific binding of LDL to MTP was saturable (Figure 2B). Nonlinear regression analysis revealed that the binding of radiolabeled LDL to immobilized MTP exhibited 4-fold lower affinity than the binding of radiolabeled MTP to immobilized LDL (Table 1).

Consideration was given to the possibility that iodination of LDL in the previous experiments might have affected the interactions between apoB and MTP. Thus, we developed assays to study interactions between native LDL and immobilized MTP (Figure 3). In these assays, native LDL was allowed to interact with immobilized MTP or PDI subunit, and the amount of apoB bound was quantitated by ELISA (Figure 3A). LDL interacted significantly with the immobilized heterodimeric MTP but not with the PDI. We then studied the binding of different concentrations of LDL to the immobilized MTP (Figure 3B). The binding of LDL to the immobilized MTP was saturable and exhibited a rectangular hyperbola. In these assays, 1 molecule of LDL was found to associate with 200 molecules of the immobilized heterodimeric MTP. This is probably because the binding of one large LDL molecule might have covered several immobilized MTP molecules, thus rendering them inaccessible for binding. Native LDL bound to immobilized MTP with 6-fold higher affinity than radiolabeled LDL (Table 1), which suggests that this assay is preferable to the

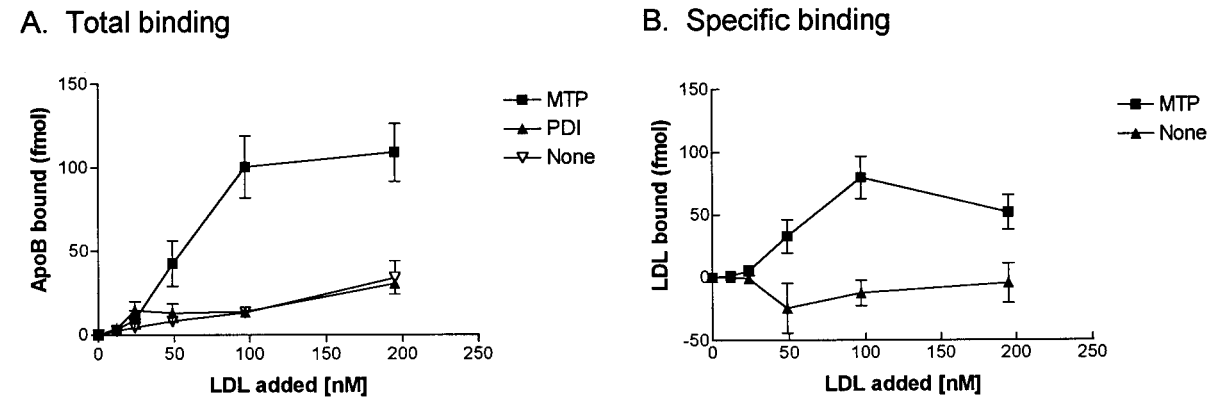

FIGURE 2: Interactions between radiolabeled LDL and immobilized MTP. Panel A: Wells were coated in triplicate with MTP (1 μg/well), PDI (1 μg/well), or no protein (none) as described in Figure 1A and under Materials and Methods. Wells were incubated with the different indicated concentrations of ^{125}I-labeled LDL (specific activity, 200 cpm/ng) for 2 h and washed, and the amount of bound LDL was measured by γ-counting. Panel B: Experiments were performed as described in panel A. The nonspecific binding was subtracted and specific binding was plotted as a line graph. Error bars represent standard deviation.

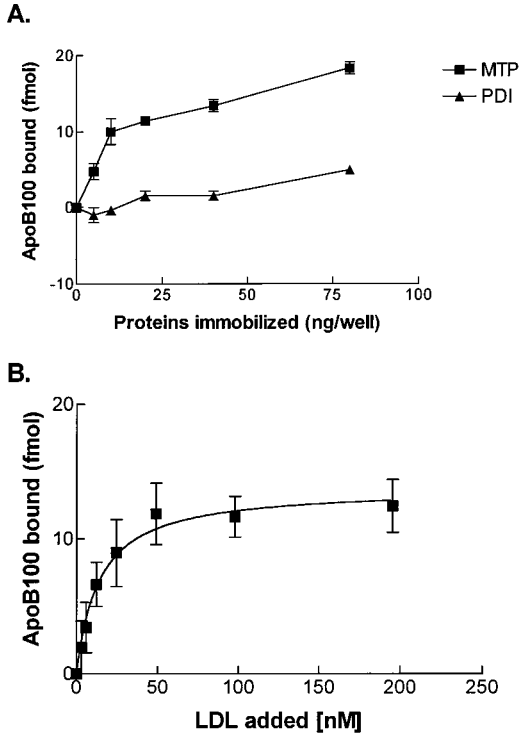

FIGURE 3: Binding of native LDL to immobilized MTP. Panel A: Wells were incubated (2 h, 37 °C) with indicated amounts of heterodimeric MTP or PDI, blocked with PBS−Tween (2 h, 37 °C), washed, and then incubated (2 h, 37 °C) with LDL (20 nM). The amount of apoB bound was measured by ELISA. The values plotted are means ± standard deviations. Panel B: Wells were coated with 1 μg of MTP, washed, blocked with BSA, and incubated (2 h, 37 °C) in triplicate with 100 μL of different concentrations of LDL (0−200 nM). The amount of apoB bound to MTP was measured by ELISA. The binding of LDL was subjected to nonlinear regression analysis using a one-binding site isotherm. The means and standard deviations are plotted as line graphs and error bars, respectively.

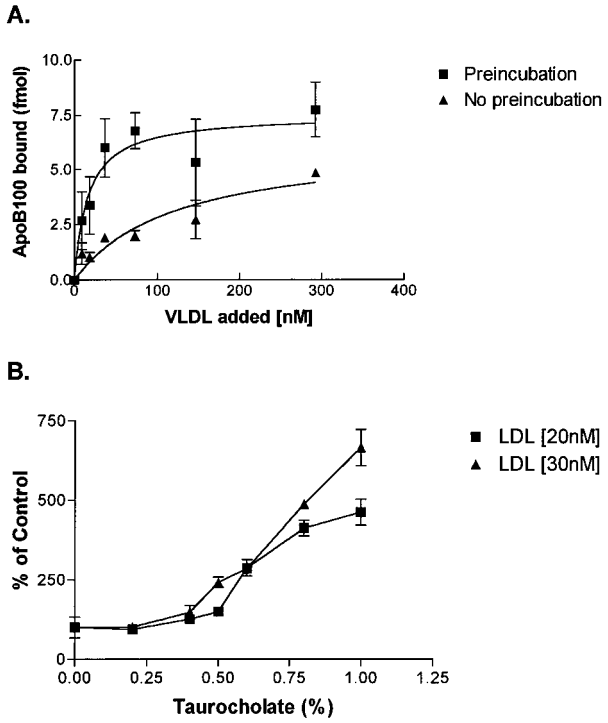

FIGURE 4: Effect of preincubation of plasma lipoproteins with detergents on their interactions with MTP. Panel A: Preincubation of VLDL with Tween-20: Wells were coated with MTP, washed, blocked with PBS−Tween, and incubated (2 h, 37 °C) with different concentrations of VLDL in PBS−Tween. In one set of experiments, VLDL was diluted in PBS-Tween immediately before the addition on the wells (no preincubation). In another set, VLDL was diluted in PBS−Tween, incubated at 37 °C for 2 h, and then added to immobilized MTP (preincubation). Bound apoB was quantitated by ELISA. The graph was analyzed by nonlinear regression analysis. Panel B: Preincubation of LDL with taurocholate: LDL (20 or 30 nM) was preincubated in the absence or presence of different concentrations of taurocholate for 2 h at 4 °C, and incubated (2 h, 37 °C) with immobilized heterodimeric MTP. The amount of LDL bound was measured. ApoB bound in the absence of detergent was used as 100%.

other assays described above. All subsequent experiments were performed using this assay.

Subsequently, we studied the binding of VLDL to immobilized MTP. Under the conditions described for LDL binding in Figure 3B, VLDL bound with 9-fold lower affinity to the immobilized MTP (Table 1 and Figure 4A, no preincubation), compared with LDL. Furthermore, the affinity between VLDL and MTP was 6-fold lower than that observed in Figure 1C. In Figure 1C, VLDL was immobilized and then incubated with buffer containing Tween-20 to block nonspecific sites. We reasoned that the immobilized VLDL was being partially delipidated during the preincubation. Thus, we determined the effect of detergent treatment of VLDL on its interaction with MTP. To evaluate the effect of detergent pretreatment, VLDL was preincubated with Tween-20 and then assayed for its binding

to immobilized MTP (Figure 4A, preincubation). The pretreatment did not result in the precipitation of apoB as determined by centrifugation. The extent of VLDL delipidation was not determined. The pretreatment with Tween-20 increased the affinity of VLDL to the immobilized MTP by 7.5-fold probably due to partial delipidation. The binding was saturable and was of high affinity similar to that observed in Figure 1C. Furthermore, the affinity was similar to that of plasma LDL (Table 1). These studies showed that the MTP binding site was probably masked in plasma VLDL and preincubation with Tween-20 resulted in the exposure of this site.

In contrast to VLDL, the binding of LDL was not affected by preincubation with Tween-20 (data not shown). Next, we studied the effect of taurocholate on the LDL/MTP interactions. Taurocholate was selected because it had been used for the measurement of microsomal apoB (Wilkinson et al., 1993). Furthermore, it has a high critical micellar concentration, does not induce protein denaturation, and has been used for the solubilization of membrane enzymes (Helenius & Simons, 1975). Preincubation of LDL with different concentrations of sodium taurocholate (1%) at 4 °C increased the LDL binding by 3−6-fold to the immobilized heterodimeric MTP (Figure 4B). Under similar conditions, taurocholate did not increase the immunological detection of LDL in a solubilized mixture (data not shown), suggesting that the detergent neither enhanced interactions between apoB and its polyclonal antibodies nor precipitated apoB. These studies indicate that preincubation of taurocholate increases the binding of LDL to MTP, most likely by partially delipidating apoB and exposing binding epitopes.

Interactions between C-Terminally Truncated ApoB Polypeptides and MTP. Next, we studied the effect of detergents on the interactions of different C-terminally truncated apoB polypeptides that are secreted with different degrees of lipidation or secreted as lipid-poor polypeptides. We used conditioned medium from HepG2 cells and McA-RH7777 cells stably transfected with apoB42, apoB28, and apoB18. HepG2 cells secrete apoB100 as LDL-size particles. ApoB42 is mainly secreted as an HDL-size particle, whereas apoB28 is secreted both as lipid-free and in the lipidated state. On the other hand, apoB18 is secreted as lipid-free polypeptide by the stably transfected cells (White et al., 1992; Wang et al., 1994; Hussain et al., 1995; Yao et al., 1991). The effect of taurocholate on the interactions of different apoB polypeptides to immobilized MTP is shown in Table 2. Incubation with sodium taurocholate increased (2−9-fold) the binding of all the peptides. These studies also indicated that the effect of taurocholate was more pronounced on lipidated polypeptides compared to lipid-free apoB18.

The binding of different C-terminally truncated apoB polypeptides at equimolar concentrations was compared in Table 3. At two different concentrations of the added polypeptides, the amount of apoB18 bound was higher than the other larger apoB polypeptides. Decreasing the length of apoB from apoB18 to apoB15 resulted in no detectable binding. Patel and Grundy (1996) have shown that apoB polypeptides as small as apoB13 coimmunoprecipitate with MTP. Thus, it is quite likely that apoB15 might interact with MTP, albeit with low affinity compared to the other polypeptides studied. When the N-terminus of apoB was extended from apoB18 to apoB28, the binding decreased by 2-fold. A further increase in apoB length from apoB28 to apoB42 resulted in 1.5−2.5-fold decreased binding to MTP (Table 3). The decreased binding of apoB42 was not due to competition with endogenous rat apoB100 and apoB48 because the apoB42-expressing cell line secreted less amounts of rat apoBs than the apoB18-expressing cell line as determined by radiolabeling followed by immunoprecipitation (data not shown). The binding was, however, not affected by an additional increase in apoB length to apoB100. These studies indicated that optimum interactions occurred between the N-terminal 18% of apoB and MTP probably because the MTP binding site is present in this region of apoB and is better exposed.

Table 2: Effect of Sodium Taurocholate on the Interaction of Different ApoB Polypeptides to Immobilized MTP

		apoB bound (fmol)		
apoB length	n^b	−taurocholate	+taurocholate	fold increases
apoB18	6	12.85 ± 1.02[c]	30.15 ± 1.04	2
apoB28	6	3.87 ± 0.66	8.71 ± 0.87	2
apoB100	3	0.19 ± 0.34	1.63 ± 0.30	9

[a] Stably transfected McA-RH7777 cells expressing various lengths of N-terminal apoB fragments and HepG2 cells were incubated in serum-free medium containing 0.2% BSA for 48 h. The conditioned medium was used for determining apoB concentrations by ELISA and to study the binding of these fragments to immobilized MTP. To study the binding of different length apoB polypeptides to immobilized MTP, conditioned media containing 10 nM of various apoB polypeptides were preincubated (2 h, 4 °C) with or without sodium taurocholate (1%) and incubated with immobilized MTP, and the amount of apoB bound was quantitated. [b] Number of wells. [c] Mean ± standard deviations.

Table 3: Binding of Different Length ApoB Polypeptides to Immobilized MTP

apoB polypeptide	apoB added (nM)	apoB bound (fmol)	apoB added (nM)	apoB bound (fmol)
apoB15	3	not detectable	not determined	
apoB18	3	15.61 ± 1.09[b]	50	33.17 ± 0.26
apoB28	3	7.84 ± 0.93	50	15.14 ± 1.30
apoB42	3	2.98 ± 0.21	50	10.61 ± 0.43
apoB100	3	2.25 ± 0.20	not determined	

[a] Conditioned media from stably transfected McA-RH7777 cells expressing apoB15, apoB18, apoB28, or apoB42 and HepG2 cells expressing apoB100 were preincubated with 1% sodium taurocholate for 2 h at 4 °C, and used to study the binding of apoB polypeptides to immobilized MTP. The amount of apoB bound was measured by ELISA. The data are from two independent experiments performed at different times. [b] Mean ± standard deviations.

Nature of Interactions between ApoB and MTP. To test whether the interactions between apoB and MTP were ionic in nature, we determined the effect of different concentrations of salt on the binding of apoB to immobilized MTP. The binding of LDL (Figure 5A) and apoB18 (Figure 5B) to MTP was maximum when no additional salt was added. Increasing salt concentrations progressively decreased the binding of LDL and apoB18 to MTP. The binding was completely inhibited at 0.5 M NaCl, and 50% inhibition was observed at 0.05 M. In contrast, binding of LDL to 1D1 was stimulated at low salt concentrations and was not inhibited at higher concentrations, suggesting that the effect of salt was specific to apoB/MTP interactions. These studies indicated that the interactions between MTP and apoB are

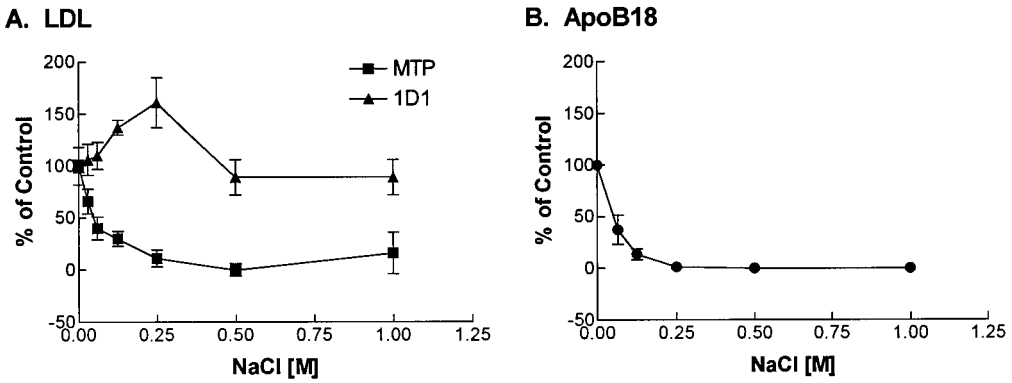

FIGURE 5: Inhibition of apoB/MTP interactions by NaCl. Panel A: Immobilized MTP or 1D1 was incubated (2 h, 37 °C) with LDL in the absence and presence of different concentrations of salt in 0.01 M Na_2HPO_4, 1.76 mM KH_2PO_4, 0.001% thimerosal, and 0.05% Tween-20 buffer, pH 7.4. The amount of LDL bound in the absence of salt was used as 100%. Panel B: Immobilized MTP was incubated with 100 μL of conditioned medium obtained from McA-RH7777 cells stably transfected with human apoB18 cDNA in the presence and absence of the indicated concentrations of NaCl. The amount of apoB18 bound in the absence of added salt was used as 100%.

ionic in nature. It has been shown that coimmunoprecipitated apoB/MTP complexes are resistant to quick salt washes (Patel & Grundy, 1996). To study the effect of salt washes, LDL was allowed to interact with immobilized MTP, and then complexes were washed 3 times with various concentrations of NaCl (0−5 M). These washes had no effect on the apoB/MTP interactions. Subsequently, preformed apoB/MTP complexes were incubated with high salt concentrations for 1 h. This treatment also did not result in the disruption of these complexes, suggesting that salt must be present during interactions to exert its effect and that the apoB/MTP complexes cannot be disrupted by salt washes. These studies indicate that the initial interactions between these proteins are ionic but subsequent interactions may be hydrophobic.

DISCUSSION

Interactions between ApoB and MTP. The interactions between apoB and MTP were of high affinity with K_d values in nanomolar range (Table 1). On the other hand, interactions between apoB and PDI were significantly lower than those observed between heterodimeric MTP and apoB (Figures 1−3). The low binding of apoB to PDI should be interpreted cautiously because partially purified PDI was used in these studies. We could not study the binding of LDL to purified 97 kDa subunit because it could not be isolated without denaturing the protein (Ricci et al., 1995; Wetterau et al., 1991). Furthermore, the denatured, purified subunit had no lipid transfer activity (Wetterau et al. 1991). These studies suggest that the presence of 97 kDa subunit is required for high-affinity interactions with apoB. However, we cannot rule out the possibility that PDI as part of the MTP complex may play an important role in apoB interactions.

High-affinity interactions between plasma lipoproteins and MTP were not anticipated because these interactions were supposed to occur in the ER prior to or during lipoprotein assembly. In our studies, native VLDL interacted poorly with MTP, but preincubation with Tween-20 increased these interactions (Figure 4A), indicating that the MTP interaction site was probably masked in the secreted lipoproteins. LDL interacted with high affinity to MTP (Figure 3B). These interactions could be increased further by taurocholate treatment (Figure 4B). Thus, fully lipidated lipoproteins appear to bind poorly with MTP, and partial delipidation of these lipoproteins exposes MTP binding sites.

Consideration was given to the possibility that MTP interacted with apoB and not with lipids associated with apoB. To test this possibility, interactions between apoB18 and MTP were studied. ApoB18 does not form TG-rich lipoproteins (White et al., 1992; Yao et al., 1991). Since apoB18 showed more binding than apoB present in lipoproteins (Tables 2 and 3), we conclude that interactions between MTP and apoB are protein−protein interactions.

Interactions between C-Terminally Truncated ApoB Polypeptides and MTP. The N-terminal 18% of apoB has been shown to contain seven of the eight disulfide bonds present in apoB100 (Yang et al., 1990). These disulfide bonds have been suggested to play a role in the early biogenesis of lipoproteins (Shelness & Thornburg, 1996). In addition, this region has been shown to be important in anchoring lipoprotein lipase to endothelial cell surfaces (Sivaram et al., 1994). Furthermore, it is required for MTP to assist in the secretion of apoB polypeptides that can associate with lipids (Gretch et al., 1996). Now we have demonstrated that the 18% N-terminal region of apoB also contains information for the optimum interaction with MTP. Therefore, based on our studies and those of Gretch et al. (1996), it can be speculated that the interactions between apoB and MTP may be necessary for MTP to assist in the translocation of apoB polypeptides from the membrane to the lumen of the ER. If this is true, MTP may well act as a chaperone early in apoB translocation across the ER membrane.

Since apoB18 is secreted as lipid-poor or lipid-free polypeptide, the lipidation of nascent polypeptide may not be necessary for the interactions between apoB and MTP. In fact, lipidation of longer peptides appears to have a negative effect on these interactions (Figure 4 and Table 2). If our data can be extrapolated to in vivo situations, these observations suggest that the MTP binding to apoB may precede lipidation of apoB.

As the length of apoB increased from apoB18 to apoB100, binding between apoB and MTP decreased (Tables 2 and 3). Increase in apoB length beyond apoB18 results in lipidation of apoB polypeptide and secretion of primordial lipoproteins. The decreased interactions could be due to the lipidation of the longer polypeptides. Even though we performed these studies in the presence of detergents, some lipids might not be solubilized from longer apoB polypeptides. Alternatively, the longer polypeptides may contain

regions that exert a negative influence on interactions with MTP.

The physical association between apoB and MTP has been studied in detergent-solubilized HepG2 (Wu et al., 1996) cells and COS cells cotransfected with cDNAs encoding apoB and MTP (Patel & Grundy, 1996). In both these studies, antiserum raised against MTP was used to demonstrate intracellular, physical association between apoB and MTP. Both studies also demonstrated that oleic acid increased interactions between apoB and MTP. In the transfected COS cells, MTP was shown to physically associate with apoB polypeptides ranging from apoB13 to apoB41 (Patel & Grundy, 1996). Since longer apoB polypeptides contain more hydrophobic regions, it was postulated that longer apoB polypeptides would interact more efficiently with MTP. However, in our studies longer apoB polypeptides interacted less efficiently than shorter apoB polypeptides that have fewer hydrophobic regions (Tables 2 and 3). Furthermore, interactions between MTP and apoB were inhibited in the presence of high salt concentrations but not in the presence of detergents such as Tween-20 and taurocholate. In addition, chemical modification of charged amino acid residues in LDL abolished its interaction with MTP (Bakillah et al., manuscript in preparation). Thus, our studies suggest that the initial protein−protein interactions between apoB and MTP are ionic in nature.

ApoB Structure and Lipoprotein Biosynthesis. Our studies were performed in defined biochemical settings different from those observed in the ER. Similarly, the apoB/MTP interactions have been observed in detergent-solubilized cell extracts (Patel & Grundy, 1996; Wu et al., 1996). It is impossible to create conditions in test tubes that are similar to the lumen of the ER. We could generate redox conditions and supplement with some extracts but may never be able to provide all the chaperons, chaperonins, and lipids at lumenal concentrations. Nonetheless, the studies described here and elsewhere (Patel & Grundy, 1996; Wu et al., 1996) may provide some testable directions toward the understanding of lipoprotein biogenesis. Based on our data, in conjunction with the available knowledge, it is speculated that steps in lipoprotein biosynthesis may be dictated by the primary structure of apoB. The N-terminus of apoB contains a signal sequence that is required for the targeting of secretory or membrane proteins to the ER. It is known that the nascent apoB interacts with the ER membrane. The nascent apoB while in the ER membrane may interact with MTP. MTP may then assist in the efficient translocation of the apoB polypeptide from the ER membrane and lipidate it, resulting in the formation of primordial lipoproteins. In the case of apoB48-containing VLDL, sufficient lipidation is believed to occur in a second step different from the synthesis of primordial lipoproteins (Wang et al., 1997; Gordon et al., 1996; Boren et al., 1994). The role of MTP in the second step "core expansion" is still being debated (Wang et al., 1997; Gordon et al., 1996). In light of our observations that poorly lipidated apoB polypeptides bind strongly with MTP, it can be speculated that apoB/MTP interactions may help retain the nascent primordial particles in the ER for optimal lipidation in the second step. Optimal lipidation will then decrease these interactions and provide signals for the exit of these particles for secretion. In the assembly of apoB100-containing lipoproteins, the interactions between apoB and MTP may decrease as the length of nascent apoB polypeptide increases and is lipidated, resulting in the dissociation of MTP from nascent lipoproteins and subsequent exits of these particles from the ER.

In summary, we have demonstrated that the N-terminal 18% of apoB interacts the best with MTP by ionic interactions. The interactions between apoB and MTP are enhanced by detergents, indicating that delipidation of apoB results in enhanced interactions between these proteins. Our studies suggest that apoB/MTP interactions may precede lipidation of the apoB polypeptide. Furthermore, increased lipidation of apoB has a negative effect on these interactions and may help dissociate MTP from nascent lipoproteins. In the future, identification of the MTP binding site(s) within apoB18 will provide important information regarding the role of apoB/MTP interactions in lipoprotein biosynthesis.

ACKNOWLEDGMENT

Helpful discussions with Drs. Ramesh Bhat, Edward Fisher, Julian Marsh, Michael Phillips, and Zemin Yao and the technical assistance of Ms. Jennifer Morello and Shuyun Zhang are gratefully acknowledged.

REFERENCES

Bonnardel, J. A., & Davis, R. A. (1995) *J. Biol. Chem. 270*, 28892.
Boren, J., Graham, L., Wettesten, M., Scott, J., White, A., & Olofsson, S. (1992) *J. Biol. Chem. 267*, 9858.
Boren, J., Rustaeus, S., & Olofsson, S. O. (1994) *J. Biol. Chem. 269*, 25879.
Davis, R. A. (1991) in *Biochemistry of lipids, lipoproteins and membranes* (Vance, D. E., & Vance, J., Eds.) pp 403−426, Elsevier Science Publishers, New York.
Dixon, J. L., & Ginsberg, H. N. (1993) *J. Lipid Res. 34*, 167.
Gibbons, G. F. (1990) *Biochem. J. 268*, 1.
Goldstein, J. L., Basu, S. K., & Brown, M. S. (1983) *Methods Enzymol. 98*, 241.
Gordon, D. A., Jamil, H., Sharp, D., Mullaney, D., Yao, Z., Gregg, R. E., & Wetterau, J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 7628.
Gordon, D. A., Jamil, H., Gregg, R. E., Olofsson, S. O., & Borén, J. (1996) *J. Biol. Chem. 271*, 33047.
Gregg, R. E., & Wetterau, J. R. (1994) *Curr. Opin. Lipidol. 5*, 81.
Gretch, D. G., Sturley, S. L., Wang, L., Lipton, B. A., Dunning, A., Grunwald, K. A. A., Wetterau, J. R., Yao, Z., Talmud, P., & Attie, A. D. (1996) *J. Biol. Chem. 271*, 8682.
Helenius, A., & Simons, K. (1975) *Biochim. Biophys. Acta 415*, 29.
Hussain, M. M., Maxfield, F. R., Mas-Oliva, J., Tabas, I., Zhongsheng, J., Innerarity, T. L., & Mahley, R. W. (1991) *J. Biol. Chem. 266*, 13936.
Hussain, M. M., Kancha, R. K., & Tulenko, T. N. (1995a) *Biochemistry 34*, 16074.
Hussain, M. M., Zhao, Y., Kancha, R. K., Blackhart, B. D., & Yao, Z. (1995b) *Arterioscler. Thromb. Vasc. Biol. 15*, 485.
Hussain, M. M., Kancha, R. K., Zhou, Z., Luchoomun, J., Zu, H., & Bakillah, A. (1996) *Biochim. Biophys. Acta 1300*, 151.
Innerarity, T. L., Boren, J., Yamanaka, S., & Olofsson, S. (1996) *J. Biol. Chem. 271*, 2353.
Jamil, H., Dickson, J. K., Jr., Chu, C.-H., Lago, M. W., Rinehart, J. K., Biller, S.A., Gregg, R. E., & Wetterau, J. R. (1995) *J. Biol. Chem. 270*, 6549.
Jamil, H., Gordon, D. A., Eustice, D. C., Brooks, C. M., Dickson, J. K., Jr., Chen, Y., Ricci, B., Chu, C.-H., Harrity, T. W., Ciosek, C. P. J., Biller, S. A., Gregg, R.E., & Wetterau, J. R. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 11991.
Leiper, J. M., Bayliss, J. D., Pease, R. J., Brett, D. J., Scott, J., & Shoulders, C.C. (1994) *J. Biol. Chem. 269*, 21951.
Patel, S. B., & Grundy, S. M. (1996) *J. Biol. Chem. 271*, 18686.
Ricci, B., Sharp, D., Orourke, E., Kienzle, B., Blinderman, L., Gordon, D., Smithmonroy, C., Robinson, G., Gregg, R. E., Rader, D. J., & Wetterau, J. R. (1995) *J. Biol. Chem. 270*, 14281.

Sakata, N., Wu, X. J., Dixon, J. L., & Ginsberg, H. N. (1993) *J. Biol. Chem. 268*, 22967.
Sharp, D., Blinderman, L., Combs, K. A., Kienzle, B., Ricci, B., Wager-Smith, K., Gil, C. M., Turck, C. W., Bouma, M.-E., Rader, D. J., Aggerbeck, L. P., Gregg, R. E., Gordon, D. A., & Wetterau, J. R. (1993) *Nature 365*, 65.
Shelness, G. S., & Thornburg, J. T. (1996) *J. Lipid Res. 37*, 408.
Sivaram, P., Choi, S. Y., Curtiss, L. K., & Goldberg, I. J. (1994) *J. Biol. Chem. 269*, 9409.
Sparks, J. D., & Sparks, C. E. (1994) *Biochim. Biophys. Acta 1215*, 9.
Spring, D. J., Chen-Liu, L. W., Chatterton, J. E., Elovson, J., & Schumaker, V. N. (1992) *J. Biol. Chem. 267*, 14389.
Vance, J. E., & Vance, D. E. (1990) *Annu. Rev. Nutr. 10*, 337.
Wang, H., Yao, Z., & Fisher, E. A. (1994) *J. Biol. Chem. 269*, 18514.
Wang, S., McLeod, R. S., Gordon, D. A., & Yao, Z. (1996) *J. Biol. Chem. 271*, 14124.
Wang, Y., McLeod, R. S., & Yao, Z. (1997) *J. Biol. Chem. 272*, 12272.
Wetterau, J. R., & Zilversmit, D. B. (1985) *Chem. Phys. Lipids 38*, 205.
Wetterau, J. R., Combs, K. A., Spinner, S. N., & Joiner, B. J. (1990) *J. Biol. Chem. 265*, 9800.
Wetterau, J. R., Combs, K. A., McLean, L. R., Spinner, S. N., & Aggerbeck, L. P. (1991) *Biochemistry 30*, 9728.
Wetterau, J. R., Aggerbeck, L. P., Bouma, M.-E., Eisenberg, C., Munck, A., Hermier, M., Schmitz, J., Gay, G., Rader, D. J., & Gregg, R. E. (1992) *Science 258*, 999.
Wetterau, J. R., Lin, M. C. M., & Jamil, H. (1997) *Biochim. Biophys. Acta 1345*, 136.
White, A. L., Graham, D. L., LeGros, J., Pease, R. J., & Scott, J. (1992) *J. Biol. Chem. 267*, 15657.
Wilkinson, J., Higgins, J. A., Groot, P., Gherardi, E., & Bowyer, D. (1993) *J. Lipid Res. 34*, 815.
Wu, X. J., Zhou, M. Y., Huang, L. S., Wetterau, J., & Ginsberg, H. N. (1996) *J. Biol. Chem. 271*, 10277.
Yang, C. Y., Kim, T. W., Weng, S. A., Lee, B. R., Yang, M. L., & Gotto, A. M., Jr. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5523.
Yao, Z., & McLeod, R. S. (1994) *Biochim. Biophys. Acta 1212*, 152.
Yao, Z., Blackhart, B. D., Linton, M. F., Taylor, S. M., Young, S. G., & McCarthy, B.J. (1991) *J. Biol. Chem. 266*, 3300.

BI971395A