Protein Fusions of BPI with CETP Retain Functions Inherent to Each

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ABSTRACT: Cholesteryl ester transfer protein (CETP), bactericidal/permeability inducing protein (BPI), and lipopolysaccharide binding protein (LBP) are members of the lipid transfer/lipopolysaccharide binding protein (LT/LBP) family of proteins that share a common secondary/tertiary structure. Despite this commonality of structure, very different patterns of lipid binding and protein−protein interactions are observed among the family members. BPI was previously shown to retain aspects of its own function when part of it was fused with LBP to form a chimeric protein. We have extended those observations to CETP. Some aspects of cholesteryl ester transfer function can be maintained in a chimeric protein even when over 40% of the sequence is from BPI. Further replacement of an additional 60 amino acids resulted in a complete loss of CETP function even though the chimera was able to retain some BPI-like properties. These artificial fusions retain BPI functions such as lipopolysaccharide (LPS) binding and protein−protein interactions that are not observed with native CETP. BPI−CETP chimeras are inhibited by LPS but cannot be inhibited by small molecule CETP inhibitors as effectively as native CETP. These results localize the site of LPS binding in BPI to a region no larger than the amino terminal 155 amino acids. This region can participate in some protein−protein interactions similar to intact BPI. Chimeras containing the amino terminus of CETP and the carboxy terminus of BPI did not retain any observable CETP function. These results further confirm the modular nature of the LT/LBP family of proteins but also highlight the discrete nature of their individual functions.

The lipid transfer/lipopolysaccharide binding protein (LT/LBP) family includes the lipid transfer proteins cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP) and lipopolysaccharide binding proteins such as bactericidal/permeability inducing protein (BPI), lipopolysaccharide binding protein (LBP), as well as multiple other less characterized proteins (*1*, *2*). LBP and BPI are both involved in innate immunity and help to coordinate the body's response to bacterial endotoxins by binding to them and signaling through various pathways. CETP is involved in triglyceride and cholesteryl ester transfer, while PLTP is involved in phospholipid transfer among lipoprotein particles.

Members of this protein family are highly hydrophobic and typically 450−500 amino acids long. The family is characterized by relatively poor primary sequence homology but strongly conserved secondary/tertiary structure. The crystal structure of BPI has been determined (*3*) and can be used to model the other members of the family (*4*). These structures include extensive regions of β-sheet and lesser amounts of α-helix. These proteins are split into similar halves by a pseudo-2-fold axis of symmetry, and these discrete domains can often be assigned specific functions. For example, the amino half of BPI has been shown to bind LPS and other lipids, while the C-terminal half is involved in cellular interactions (*5*). This modularity of function is clearly seen by examining artificial fusions that were constructed with parts of both BPI and LBP. These chimeras retained many properties of the original proteins (*6*, *7*).

The importance of many members of this family in human disease makes understanding their structure and function of particular interest. Fragments of BPI have been tested for preventing endotoxic shock (*8*). Inhibitors of CETP are now in clinical development for raising high-density lipoprotein cholesterol (HDL-C) and preventing cardiovascular disease (*9*, *10*).

The extent of natural variation in CETP amino acid sequence shows that the protein can be active with many individual sequence changes (*11*, *12*) although certain residues and regions are critical for function and significant truncations from either end are not tolerated (*13*, *14*). To determine whether CETP is like BPI and LBP with respect to functional domains and, if so, to map those domains, artificial fusions of CETP with BPI have been constructed and assayed for activity.

METHODS AND MATERIALS

Expression Constructs. Full-length human BPI cDNA was PCR amplified from bone marrow mRNA using primer [#1] 5′GCCAGCTACCGGTGTCAACCCTGGCGTCGTGGTCAGG3′, and primer [#2] 5′GTACAGGCGGCCGCTCATTTATAGACAAC3′. Full-length human CETP cDNA was PCR amplified from liver mRNA using primer [#3] 5′GACACCGGTTGCTCCAAAGGCACCTCGCACGAG3′, and primer [#4] 5′TCGATCGCGGCCGCCTAGCTCAAGCTCTGGAGGAAATCC3′. Chimera 1, chimera 2, and chimera 3 contain N-terminal BPI sequence fused to C-terminal CETP sequence. Chimera 1 was generated by fusing BPI

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amino acid residues 1−155 (PCR primers [#1] and [#5] 5′AGCTACAATTGGATCAGCCACCCGAC3′) with CETP amino acids 165−476 (PCR primers [#6] 5′CGATCCAATTGTTCACAAATTTCATCTCC3′ and [# 4]) through an engineered Mfe I restriction site. Chimera 2 consists of BPI residues 1−193 (PCR primers [#1] and [#7] 5′AGCTACAGAGTCTGGAAATAAGG3′) and CETP residues 203−476 (PCR primers [#4] and [#8] 5′CGATCCAGACTCTGGCCAGCATCCTTTCAG3′) linked through an AlwN I restriction site. Chimera 3 is comprised of BPI residues 1−253 (PCR primers [#1] and [#9] 5′AGCTAGCATGCGGTCATGGGCA3′) linked with CETP residues 261−476 (PCR primers [#4] and [#10] 5′CGATCGCATGCTGTACTTCTGG3′) via an Sph I site. Chimera 4 and chimera 5 consist of N-terminal CETP sequence fused with C-terminal BPI sequence. Chimera 4 contains CETP residues 1−167 (PCR primers [#3] and [#11] 5′AGCTACAATTGCTTGATCCACCCAGG3′) and BPI residues 159−456 (PCR primers [#12] 5′AGCTACAATTGTTCCACAAAAAAATTGAG3′ and [#2]) joined through an Mfe I site. Chimera 5 contains CETP residues 1−200 (PCR primers #3 and [#13] 5′AGCTACAGAGTCTGGACAAAATCGGCCATG3′), and BPI residues 193−456 (PCR primers [#2] and [#14] 5′-AGCTACAGACTCTGCCAGTAATGACC-3′) joined through an AlwN I site. All constructs were cloned into a modified pSecTag2/Hygro (Invitrogen) vector.

Full-length human lipopolysaccharide-binding protein (LBP) cDNA was PCR amplified (primers [#15] 5′AGCTAACCGGTGCCAACCCCGGCTTGGTCGCC3′ and [#16] 5′GTACAGCGGCCGCTCAAACTCTCATGTATTGGAC3′) from HepG2 mRNA and subsequently expressed/purified from HEK293S cells as described for the chimeras.

pSecTag2/Hygro-based vectors (Invitrogen) were used to express all proteins, native and chimeric, in HEK293S cells. After transient transfection, media was collected and proteins were purified via His-tag affinity resin as described previously (*11*). After purification, the size of each protein was determined by Coomassie staining on SDS gels (polyacrylamide gradient 8−12%). All proteins, including those without any CETP sequence, were tested in an in vitro fluorescence-based cholesteryl ester transfer assay.

Fluorescence Assays. Bodipy-cholesterol ester substrate for CETP assays (*11*) was generated using 7 mg of phosphatidyl choline, 0.75 mg of triolein (Avanti Polar Lipids), and 3 mg of bodipy-CE (Molecular Probes) by drying and vacuum desiccating at 60 °C. Lipids were solubilized at 65 °C in phosphate-buffered saline (PBS) by sonication (at 25% of full power setting) with a microtip Misonix Ultrasonic processor for 2 min under a stream of nitrogen. The preparation was cooled to 45 °C and 5 mg of apolipoprotein A-1 (Biodesign) was added. The preparation was resonicated (at 25% of full power) for 20 min at 45 °C, pausing after each minute to cool the probe. The sonicate was spun for 30 min at 3000*g*, adjusted to 1.12 g/mL using sodium bromide, and layered below a solution of 1.10 g/mL sodium bromide. After spinning of the sample for 48 h at 100000*g*, the unincorporated protein and small dense particles were discarded. The remainder was collected and dialyzed in PBS/0.02% (w/v) azide.

The activity of each protein was analyzed with a bodipy-CE assay. The listed amount of each protein (250 ng to 1 μg) was combined with HDL (Biomedical Technologies Inc, 80 μL of a 160-fold dilution in PBS) in a black 96-well plate and then incubated for 5 min at 37 °C. After addition of bodipy-CE (10 μL of a 4-fold dilution in PBS) the plate was read in a Perkin-Elmer Victor plate reader (excitation 485 nm, emission 520 nm), then incubated at 37 °C for 3 h with fluorescence readings taken every 10 min to measure transfer of liposomal bodipy-CE to HDL. Baseline fluorescent values were subtracted from the later time points (*11*). *Escherichia coli* 0111:B4 lipopolysaccharide reagent was purchased from Sigma-Aldrich.

RESULTS

To determine the modularity of CETP and the function of its domains, chimeric proteins containing different extents of N- and C-terminal CETP were replaced with homologous parts of BPI. Using the BPI crystal structure and the CETP homology as a basis (*3*, *4*), junction points were placed at conserved residues near the ends of α-helical regions. As shown in Figure 1, proteins with multiple fusion points and different extents of CETP and BPI were constructed and expressed in HEK293S cells. Chimera 1 contained amino acids 1−155 from BPI and amino acids 165−476 from CETP, chimera 2 contained 1−193 from BPI and 203−476 from CETP, and chimera 3 contained 1−253 from BPI and 261−476 from CETP. These amino acid positions are all based on the mature form of the proteins. Mature BPI is 20 amino acids shorter than mature CETP with 12 amino acids of the difference occurring at the carboxy terminus. As a result, some chimeras were eight to twelve amino acids shorter than native CETP. Constructs with the same fusion points as chimeras 1 and 2 were also made but containing sequence from the reverse proteins, the amino terminus of CETP and the carboxy terminus of BPI (chimeras 4 and 5, respectively). In all cases, the predicted DNA sequence was confirmed prior to protein expression and the appropriate protein size was determined by SDS gel electophoresis. All chimeras tested were secreted with approximately the same efficiency as wild-type CETP (data not shown). Even single amino acid changes in CETP can cause profound defects in secretion in this system (*11*) so the secretion similarity among proteins suggests normal folding. Protein quantities and purities were not sufficient to carry out more detailed structural analyses.

To assess CETP activity, we used an in vitro assay in which the physical environment of fluorescent bodipy-cholesteryl ester molecules can be monitored (*11*). Bodipy-cholesteryl ester is transferred from labeled micelles in which it is present at high concentration and its fluorescence emissions quenched to unlabeled HDL in which it is present at low concentration and its fluorescence becomes unquenched. When unquenched, the transferred bodipy-CE molecule is able to emit fluorescent light upon excitation. In this setting, the transfer of fluorescent bodipy-cholesteryl ester molecules does not occur spontaneously but requires added protein. Transfer of bodipy-CE from the apoAI-containing micelles to unlabeled HDL can be carried out by CETP. Without apoAI present on the micelles, CETP is unable to stimulate transfer. However, any other process that causes the labeled micelles to fuse with the unlabeled HDL can also result in elevated fluorescence.

With wild-type CETP, increased light emission caused by labeled CE transfer has little or no lag time and is linear over the first 100 min (Figure 2). Linearity of the reaction and its lack of lag time are independent of the amount of

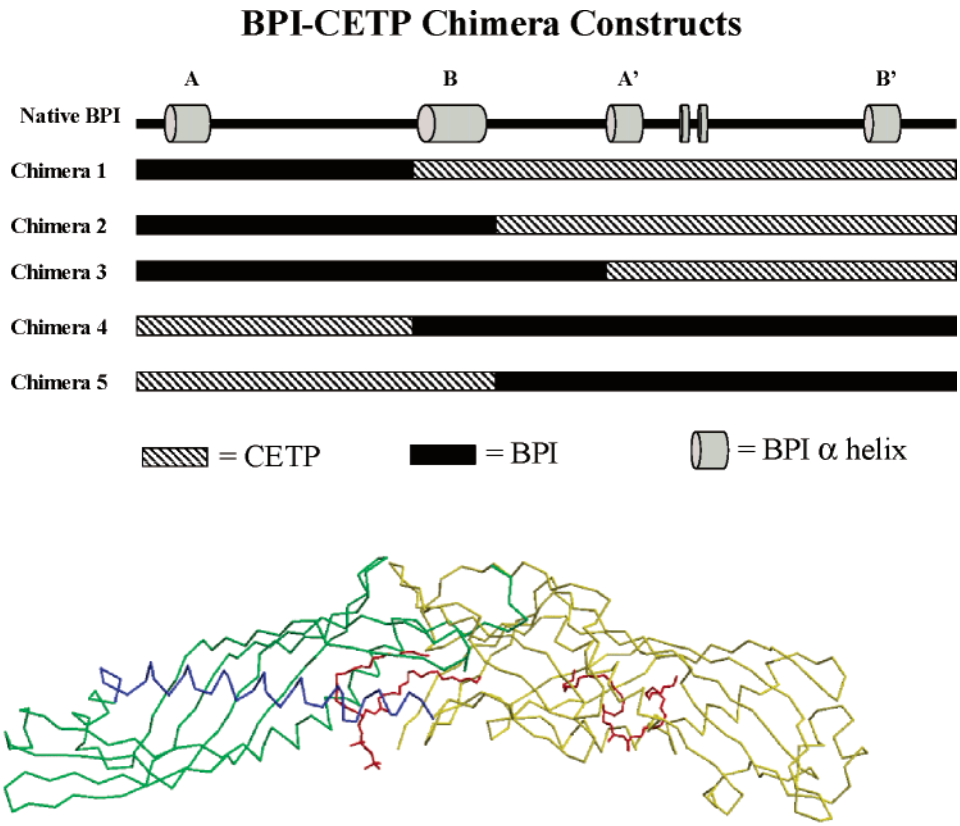

FIGURE 1: (Upper panel) α-Helices from BPI are shown approximately to scale from the secondary structure determined by Beamer et al. (*3*). α-Helices include the long helices A and B in the amino half of the protein and the homologous A′ and B′ helices in the carboxy half. Additionally, there are unnamed, shorter helices in the carboxy half of the protein not found in the amino half. The approximate lengths of CETP sequence (hatched) and BPI sequence (solid) are shown for chimeras 1 through 5. (Lower panel) The mapping of BPI segments onto the inferred structure of CETP based on BPI (*11*). In green, the residues in chimera 1 from BPI are shown. In blue, the additional BPI residues in chimera 2 are shown. The remainder of the sequence in yellow is from CETP for both chimeras 1 and 2. The red molecules correspond to lipids found in the BPI structure (*3*). On the basis of these results, the green segment is sufficient for LPS and BPI interactions, while the yellow is required for apoAI/HDL interactions.

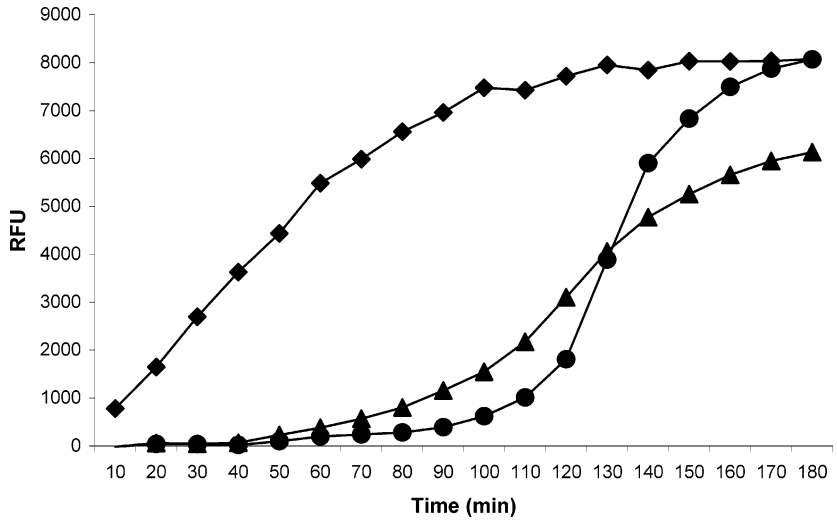

FIGURE 2: Bodipy-cholesteryl ester fluorescence unquenching was measured for 1 μg each of CETP (diamonds), chimera 1 (triangles), and chimera 2 (circles) as a function of time (minutes). Relative fluorescence units (RFU) are shown relative to samples with no added protein at time zero.

CETP over the range of 0.25 to 2 μg per reaction (data not shown). In this range, the slope of the line increases with increasing CETP concentration, but the reaction remains linear (data not shown). Each of the intact proteins and the five chimeras were tested in the same, standard reaction conditions. When added alone, BPI, LBP, chimera 3, chimera 4, and chimera 5 were completely inactive in all conditions tested. Despite having 34−42% of their original CETP sequence replaced with the homologous BPI sequence, chimera 1 and chimera 2 were found to have significant activity when tested at high enough concentration (Figures 2 and 3). Even though substantial activity was observed, there were qualitative differences between the kinetics of the different proteins. Both chimera 1 and chimera 2 can increase

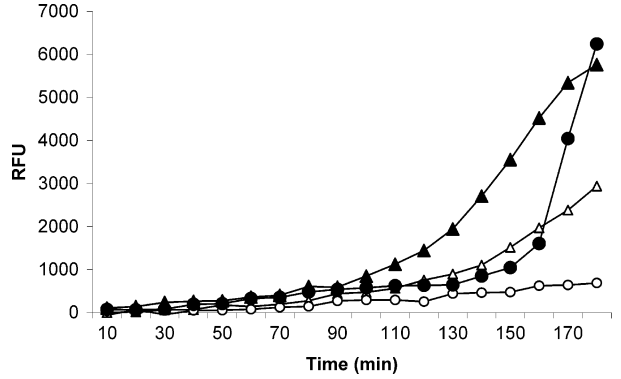

FIGURE 3: Bodipy-cholesteryl ester fluorescence unquenching was measured for chimera 1 (triangle) and chimera 2 (circle). Either 250 ng (small, white symbols) or 500 ng (large, black symbols) of protein was used for each assay.

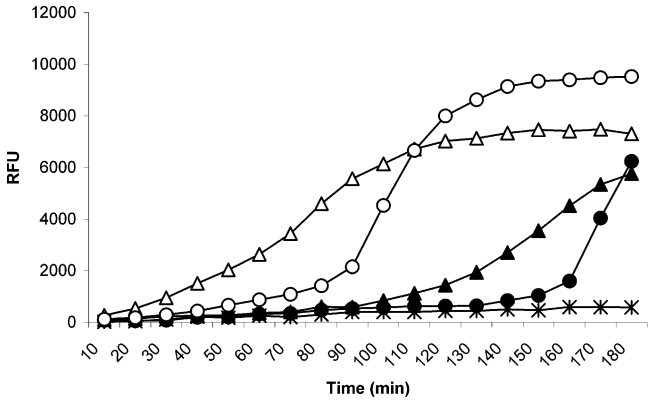

FIGURE 4: Bodipy-cholesteryl ester fluorescence unquenching was measured for 500 ng of chimera 1 (filled triangles) and 500 ng of chimera 2 (filled circles). A total of 1 μg of BPI was also tested either alone (asterisk) or in combination with 500 ng of chimera 1 (white triangles) or 500 ng of chimera 2 (white circles).

fluorescence with a final magnitude equivalent to wild-type CETP but have a significant lag time before any increase occurs. With 1 μg of protein for each chimera, as shown in Figure 2, no significant unquenching occurs until after 70 min, a time at which the same amount of wild-type CETP has completed more than 75% of the final transfer. The CETP reaction is 50% complete after only 45 min, but both chimera 1 and chimera 2 require about 130 min for 50% completion with the same 1 μg of protein. The lag time and final magnitude of unquenching is concentration dependent. With chimera 1 and chimera 2, smaller amounts of protein increase the lag time and the time required for 50% reaction (Figure 3). With 0. 5 μg of chimera 1 or chimera 2, 50% completion is achieved after approximately 150 and 170 min, respectively. With 0.25 μg of chimera 1 or chimera 2, 50% completion is not achieved within 180 min (Figure 3).

CETP on its own does not show any concentration effect on cooperativity or any lag time, suggesting the possibility that the parts of the BPI protein present in chimera 1 and chimera 2 could play a role in these effects. Supporting this, addition of BPI to either chimera 1 or chimera 2 resulted in a shortened lag time and enhanced cooperativity (Figure 4) with 50% completion times shifted earlier to approximately 70 and 100 min with 0.5 μg of protein (compared to 150 and 170 min with the same chimera concentration, Figure 3). The BPI-acceleration effect is specific for chimeras containing the amino terminus of BPI. The reaction rate of native CETP as well as the inactivity of chimera 3, chimera 4, and chimera 5 are unaffected by the addition of exogenous BPI (data not shown).

The localization of the effect to the amino terminus of BPI is further supported by the results observed when chimera 3 is added to either chimera 1 or chimera 2. By itself, chimera 3 has no activity. However, when added to low concentrations of either chimera 1 or chimera 2, the lag time and cooperativity of those proteins are affected in a manner similar to the addition of native BPI (data not shown), suggesting that the sequences responsible for the cooperative behavior are present in the amino terminal half of BPI. The carboxy terminus does not seem to be important for the effect because neither chimera 4 nor chimera 5, both containing C-terminal BPI sequences, had any effect when added to chimera 1 or chimera 2 (data not shown).

Since LBP was previously shown to have very different protein−protein interactions relative to BPI (*15*), it was tested to contrast its effects with those of BPI. LBP had no effect on native CETP activity nor on the initial kinetics of chimera 1 (Figure 5). However, the delayed, sigmoidal increase in fluorescence generally observed with 0.5 μg of chimera 1 (Figure 4) is blocked by LBP, and the reaction does not achieve 50% completion in 180 min. In addition, the stimulation of chimera 1 activity that occurs with addition of BPI (Figures 4 and 5) is also blocked by LBP. With LBP present, the increase in fluorescence continues at a linear rate as if BPI was not added. CETP is unaffected by the addition of either LBP (data not shown) or of both LBP and BPI (Figure 5).

LPS binding has been localized to the amino terminus of both BPI and LBP (*6*). LPS has no effect on native CETP activity but completely inhibits the activity of both chimera 1 and chimera 2 (Figure 6). A small molecule CETP inhibitor was tested with CETP and the chimeric proteins. In Figure 7, the results with one inhibitor, "Pfizer A" (*16*), are shown. At 10 μM "Pfizer A", CETP is greater than 95% inhibited. Chimera 1 and chimera 2 are much less affected with far less than 50% inhibition at 10 μM.

DISCUSSION

Individual members of the LT/LBP family of proteins have diverse functions involving lipid binding and transfer. Some of the proteins appear to be involved in the innate immunity response via binding of bacterial endotoxins like LPS, while others, such as CETP and PLTP, are dedicated to lipid transfer. Solution of the crystal structure of BPI has allowed many inferences to be drawn about how these proteins are folded and how they may carry out their functions, but there are still many unanswered questions about protein−protein and protein−lipid interactions.

Engineered chimeric proteins have been widely used to study protein function and to map protein−protein interactions in a wide variety of systems (*17*). In many systems, activities and interactions are faithfully retained and can be used to identify functional components of the proteins under study. The previous examples of fusions between BPI and LBP suggested that this approach might be fruitful for dissecting some of the functions of CETP as well as extending previous observations with BPI and LBP. This approach complements previous work in which CETP was either truncated or single amino acids were changed. By looking at domain scale alterations, additional information about overall function can be ascertained.

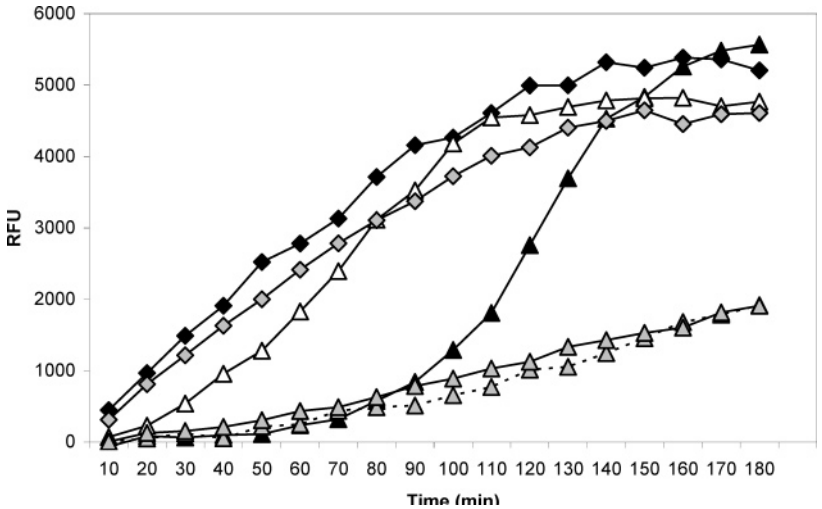

FIGURE 5: Bodipy-cholesteryl ester fluorescence unquenching was measured for 500 ng of CETP (diamonds) and chimera 1 (triangles) as described in Figure 2. Some samples had no other proteins added (solid symbols, solid lines). Alternatively, 1 μg of BPI (white filled symbols, solid lines for chimera 1 only) or 1 μg of LBP (gray symbols, hatched lines for chimera 1 only) or both BPI and LBP (gray symbols, solid lines) was added.

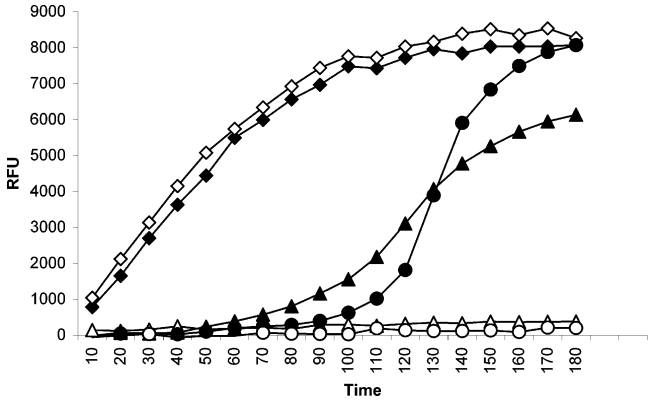

FIGURE 6: Bodipy-cholesteryl ester fluorescence unquenching was measured for 500 ng each of CETP (diamonds), chimera 1 (triangles), and chimera 2 (circles) as described in Figure 2. In addition, 1 μg of LPS was added (open symbols). For samples with no LPS, symbols are solid black.

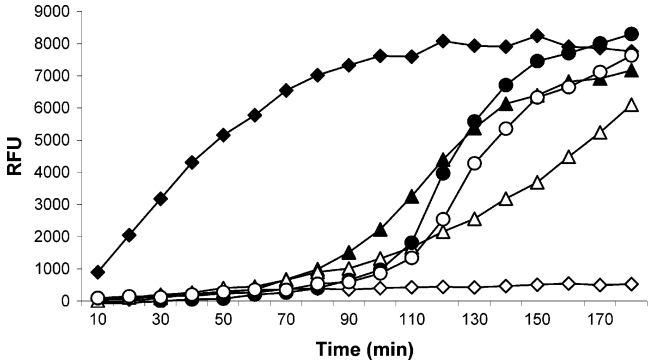

FIGURE 7: Bodipy-cholesteryl ester fluorescence unquenching was measured for 500 ng of CETP (diamonds), chimera 1 (triangles), and chimera 2 (circles) as described in Figure 2. In addition, 10 μM of the CETP inhibitor "Pfizer A" (*16*) was added to each (white symbols). For samples with no added inhibitor, symbols are solid black.

The previously noted modular nature of BPI and LBP was further confirmed with these studies and extended to CETP. The N-terminal LPS binding domain of BPI was transferred to two chimeric proteins that contained mostly CETP sequence. Even with only 155 amino acids of BPI sequence, the functional consequences of LPS binding could be observed. CETP's function within the chimeras could be inhibited by binding of LPS to the BPI-specific sequences. Previous work with chimeras and truncations of BPI (*6*, *7*) had shown the LPS binding domain to be in the amino terminal half of the protein, and homology considerations led to the suggestion that a minimum of five lysine residues (positions 42, 48, 92, 95, and 98) were involved in LPS interactions (*18*), although the complete binding site probably includes additional amino acids. Our findings further corroborate these suggestions with these amino acids present in both chimera 1 and chimera 2. In our chimeras, it is not clear whether the LPS binding inhibited CETP function by direct blocking of a functional domain or indirectly via long-range conformational effects. That this effect was independent of CETP sequence was shown by the lack of effect of LPS on native CETP function.

The altered kinetics of the chimeras relative to CETP raised the possibility that the increase in fluorescence might be caused by something other than CETP's standard transfer reaction. Since the mechanism by which even wild-type CETP is able to transfer lipids is poorly understood, eliminating this possibility is a particular challenge. For example, fusion of labeled micelles with unlabeled HDL could cause a decrease in quenching of bodipy-CE even if CETP was not active. Similar experiments with native PTLP and fluorescent LPS had shown that particle fusion was not an issue in that system (*19*). In our system, fluorescence unquenching is specific as CETP and apoAI are both required for the reaction (*11*). Furthermore, none of the proteins or chimeras without CETP C-terminal sequence is able to induce the unquenching on its own. Thus, while we cannot be assured that the standard lipid transfer reaction occurs and causes the observed unquenching, some aspect of CETP function must be involved. This could be the standard lipid transfer reaction or, alternatively, some other process.

It may be easiest to think of the fluorescence unquenching as a simple bodipy-CE transfer accomplished in the standard manner, but we cannot rule out the possibility that the chimeras are unable to transfer lipid as wild-type CETP does but rely on a particle fusion process instead. This fusion activity would have to arise from a combination of protein−

protein interactions between the BPI parts of the chimeras and CETP−HDL interactions from the CETP parts of the fusions. For this to happen, there must be enough of the specific apoAI-binding interface remaining for the chimera to bind HDL. With that interaction established, the remainder of the chimera with its BPI sequence is free to interact with other molecules. At high enough concentration and with enough time, the different BPI-containing proteins could potentially interact both nonspecifically with the unlabeled micelles and with each other. Since BPI has been shown to induce micellar fusion and LBP has been shown to inhibit the process (*15*), our results are consistent with the possibility that the C-terminal two-thirds of CETP is able to bind the apoAI-containing particles and the N-terminal BPI sequences interact with each other, whether on intact BPI or another chimera. These interacting proteins then promote fusion of the different types of labeled and unlabeled micelles. Chimera 3, chimera 4, chimera 5, and BPI would be unable to accomplish even the fusion-mediated transfer of CE on their own because they would lack sufficient CETP-specific sequences to bind HDL and initiate the process.

Additionally, CETP inhibitors such as torcetrapib have been shown to inhibit CE transfer by increasing the binding affinity of CETP for the HDL particle (*10*). Native CETP can be inhibited in this fashion but the chimeras are relatively unaffected. This resistance to inhibition could result from either a lack of inhibitor binding to the chimeras, a lack of importance of the standard lipid transfer reaction, or insufficient apoAI−CETP interface for stabilization to occur. Thus, while we are unable to attribute true CE transfer activity to the chimeras, it is clear that some aspects of CETP function must remain. The fact that some inhibition could be observed in chimera 1 and 2 indicates that some parts of the inhibitor-binding site must be localized to the central or C-terminal regions of CETP.

The LT/LBP family of proteins has attracted much interest because of its central role in multiple processes, especially innate immunity and lipid homeostasis. However, their mechanisms of action and details of their interactions with other proteins and lipids have proved difficult because of the hydrophobic nature of the ligands and their complex interactions. By understanding the modular nature of the proteins and how the individual parts of each protein may function, progress in understanding the overall function may be possible. The results presented here provide additional insight into how each of the family members studied—BPI, CETP, and LBP—may function. The apparently mutually exclusive interactions with individual small molecule ligands suggest that the pathways do not overlap, but each may still provide information about function.

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