Identification of a Domain That Mediates Association of Platelet-activating Factor Acetylhydrolase with High Density Lipoprotein

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The plasma form of platelet-activating factor (PAF) acetylhydrolase (PAF-AH), also known as lipoprotein-associated phospholipase A₂ (Lp-PLA₂) inactivates potent lipid messengers such as PAF and modified phospholipids generated in settings of oxidant stress. In humans, PAF-AH circulates in blood in fully active form and associates with high and low density lipoproteins (HDL and LDL). Several studies suggest that the location of PAF-AH affects both the catalytic efficiency and the function of the enzyme in vivo. The distribution of PAF-AH among lipoproteins varies widely among mammals. Here, we report that mouse and human PAF-AHs associate with human HDL particles of different density. We made use of this observation in the development of a binding assay to identify domains required for association of human PAF-AH with human HDL. Sequence comparisons among species combined with domain-swapping and site-directed mutagenesis studies led us to the identification of C-terminal residues necessary for the association of human PAF-AH with human HDL. Interestingly, the region identified is not conserved among PAF-AHs, suggesting that PAF-AH interacts with HDL particles in a manner that is unique to each species. These findings contribute to our understanding of the mechanisms responsible for association of human PAF-AH with HDL and may facilitate future studies aimed at precisely determining the function of PAF-AH in each lipoprotein particle.

The platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine, PAF) acetylhydrolase (PAF-AH) activity expressed in mammalian plasma is a phospholipase A₂ secreted by cells of the hematopoietic system, primarily macrophages (1). This enzyme catalyzes the hydrolysis of short and/or oxidized acyl groups present in biologically active lipids such as PAF, oxidatively fragmented glycerophospholipids, esterified F₂-isoprostanes, and phospholipid hydroperoxides (2–5). We and others (6) have proposed that the most likely function of this enzymatic activity is to provide a safety mechanism to limit the levels of pro-inflammatory mediators, the accumulation of which can have undesirable consequences. In human plasma, two-thirds of the PAF-AH activity are found associated with LDL and one-third circulates as a complex with HDL. This distribution profile varies among species, possibly because of differences among PAF-AH orthologs, combined with a wide diversity of lipoprotein levels and composition. Our previous studies suggested that intrinsic properties of PAF-AH played important roles as determinants of the location of the enzyme in vivo (7). These findings led to the identification of PAF-AH domains essential for the human enzyme to associate with LDL (7). A number of clinical studies from various laboratories indicate that altered location of PAF-AH correlates with human diseases such as coronary artery disease (8), hypercholesterolemia (9), paroxysmal atrial fibrillation (10), and chronic kidney disease (11). These observations suggest that the distribution of PAF-AH in lipoproteins may define its physio-pathological function in humans. We previously reported that PAF-AH can migrate among lipoproteins (12), found that the location of the enzyme impacts its catalytic activity (13), and presented evidence supporting a model wherein HDL may act as a transport system to distribute PAF-AH among LDL particles (14). These combined observations suggest that the lipoprotein environment regulates the function of PAF-AH, and they underscore the need to precisely characterize the nature of these interactions. Here, we report that individual species display unique lipoprotein distribution profiles and seem to utilize distinct PAF-AH domains to associate with the particles. In addition, we report the identification of key C-terminal residues required for association of human PAF-AH with human HDL. These studies are likely to facilitate the development of much needed in vivo model systems that faithfully recapitulate the unique lipoprotein distribution of human plasma PAF-AH and that can potentially be utilized to assess the role of the lipoprotein environment on the function of this enzyme.

EXPERIMENTAL PROCEDURES

Materials—[3H-acetyl]PAF was purchased from Amersham Biosciences (Piscataway, NJ) and unlabeled PAF was from Avanti Polar Lipids (Alabaster, AL). Pfu was from Stratagene (La Jolla, CA), and dNTPs were purchased from Fermentas Inc. (Hanover, MD). Pefabloc was from Calbiochem. Secondary anti-
Molecular Basis for PAF Acetylhydrolase Association with HDL

Expression and Purification of Mutant and Chimeric Proteins—Site-directed mutagenesis was performed by a two-step amplification protocol using Pfu as the polymerase, as previously described (16). A FLAG tag was inserted at the N-terminal end to facilitate immunoblot detection and purification. The products were cloned into a pUC cloning vector under the control of the tryptophan promoter, as described (16). Plasmid DNA was purified using a plasmid miniprep purification kit (Qiagen Inc, Valencia, CA).

Expression Purification of Mutant and Chimeric Proteins—We generated various truncated forms of PAF-AH that started at Leu-41 and Ile-42 for mouse and human PAF-AH, respectively, and expressed the recombinant proteins in the Escherichia coli strain BL-21. Protein extracts were obtained as previously described (16). Where indicated, the supernatants were purified using anti-FLAG affinity beads, following the instructions provided by the manufacturer (Sigma). We determined enzymatic activity and protein content of the mutant preparations recovered after purification and assessed the level of expression by Western analyses using a monoclonal anti-FLAG (M2) antibody (Sigma), as described (3). Unless otherwise stated, the mutant proteins expressed significant levels of PAF-AH activity and mass, suggesting that the folding of the recombinant proteins was comparable to that of wild-type PAF-AH.

HDL Binding Assay—To test binding to HDL we incubated a source of PAF-AH with pHDL (range: 4–29 mg) for 30–120 min at 37 °C, in a total volume of 500 μl. The amount of detergent was normalized and kept at a level that did not affect the integrity of HDL particles as judged by their ability to bind wild-type PAF-AH. The mixtures then were adjusted to a volume of 10 ml and a density of 1.3 g/ml with solid KBr. The solutions were layered with 0.9% NaCl and centrifuged at 50,000 rpm in a VTi 50 Beckman rotor, for 3 h at 4 °C. The gradients were fractionated, and individual fractions were assayed for PAF-AH activity, as described (15). Where indicated, we utilized “modified” KBr density gradients designed to improve resolution in the heavy density region. These gradients were identical to those described above except that they were generated by layering 20 ml of a 1.3 g/ml KBr solution containing pHDL and PAF-AH with 0.9% NaCl.

RESULTS

The Association of Plasma PAF-AH with Lipoproteins and the Total Levels of Enzymatic Activity Vary among Species—Previous studies reported differences in the distribution of PAF-AH activity in human compared with mouse or rat plasma, but to our knowledge no comparative studies among species have been presented to date. We investigated the distribution of PAF-AH activity in freshly isolated plasma from six different species and found vast differences in the distribution pattern among lipoproteins (Fig. 1). PAF-AH associated with both LDL and HDL in plasma from bovine, canine, and human sources (Fig. 1, A–C). However, the distribution between lipoprotein particles varied among the three species; notably, humans were the only species in which most of the circulating PAF-AH associated with LDL. In rat plasma, PAF-AH associated exclusively with HDL particles, in agreement with a previous report (Fig.
Molecular Basis for PAF Acetylhydrolase Association with HDL

TABLE 1
Expression levels of PAF-AH activity in freshly isolated plasma samples from various species

| Plasma source | PAF-AH activity |
|---------------|-----------------|
|               | umol/min/ml     | umol/min/mg |
| Bovine        | 47.7 ± 0.6      | 0.33 ± 0.01 |
| Human         | 73.2 ± 0.8      | 1.23 ± 0.23 |
| Dog           | 61.4 ± 2.0      | 2.32 ± 0.15 |
| Rat           | 183.1 ± 3.1     | 3.89 ± 0.13 |
| Guinea pig    | 234.1 ± 9.5     | 10.78 ± 0.88 |
| Mouse         | 630.0 ± 5.2     | 15.72 ± 0.26 |

1D) (17). Initial studies revealed that the densities of HDL particles with which PAF-AH associated were higher in guinea pig and mouse compared with other species (not shown), so it was necessary to adapt the standard fractionation protocol to improve resolution in the heavy density region. We adjusted the density of the gradients as described under “Experimental Procedures” and found almost complete association of guinea pig and mouse PAF-AH with HDL particles (Fig. 1, E and F), as previously reported (18, 19). Our studies also provided a quantitative comparison of the total amount of plasma PAF-AH activity expressed in six species tested. Interestingly, species in which PAF-AH associated with both HDL and LDL expressed lower total levels of activity compared with those in which the location of the enzyme was limited to HDL particles (Table 1).

Development and Characterization of a PAF-AH/HDL Binding Assay—To characterize the nature of the interaction between PAF-AH and HDL particles, we developed a binding assay that consisted of incubating either the purified recombinant enzyme, or solubilized extracts expressing various mutant and chimeric PAF-AH constructs, with Pefabloc-treated human HDL particles that lacked enzymatic activity (p-hHDL). To assess the extent of binding to p-hHDL, we subjected the mixtures to ultracentrifugation, fractionation, and activity determinations. We found that under the conditions described under “Experimental Procedures” the human wild-type enzyme, supplied in either purified form or as a solubilized bacterial extract, associated with particles of density identical to that of natural PAF-AH-containing human HDL particles (compare Fig. 1C and supplemental Fig. S1A). Optimal binding was observed after incubation for 1 h at 37 °C (supplemental Fig. S1B). To ensure that the amount of p-hHDL supplemented to each assay was not the factor limiting the extent of binding, we varied the amount of p-hHDL using a fixed level of PAF-AH and found comparable results when p-hHDL ranged between 9 and 29 mg (supplemental Fig. S1C). Additional studies showed that p-hHDL levels could be further reduced to 4 mg without affecting the extent of binding (not shown). We also found that a fixed amount of p-hHDL (9 mg) supported binding of PAF-AH over a relatively wide range of enzyme concentrations (supplemental Fig. S1D). These combined results identified experimental conditions that resulted in optimal binding of PAF-AH to exogenous human HDL. In addition, the studies demonstrated that solubilized bacterial extracts behaved in a manner equivalent to that of purified enzyme sources. Subjecting various types of PAF-AH-containing samples to the binding assay in the absence of exogenous p-hHDL resulted in complete loss of enzymatic activity (not shown).

Mouse and Human PAF-AHs Associate with Human HDL Particles of Different Density—Our next goal was to investigate whether association of PAF-AH with HDL was defined by the enzyme, the lipoprotein particles, or both. To address this issue, we compared the ability of PAF-AH with lipoproteins was not affected by this technical adjustment as judged by the similar behavior of the human plasma enzyme in the two types of gradients (compare Figs. 1C and 2B). We next found that mouse and human PAF-AHs associated with Pefabloc-treated murine HDL (p-mHDL) particles of the same density (i.e. fractions 5–9, Fig. 2C). In contrast, we observed that human PAF-AH associated with lighter p-hHDL particles compared with mouse PAF-AH (Fig. 2D). These results suggested that the mechanisms that govern association of PAF-AH with HDL vary among species and include contributions from both the enzyme and the lipoprotein particles. In addition, these results provided the basis for the next series of experiments.

The C-terminal End of Human PAF-AH Mediates Binding to Human HDL—we next focused our studies on the identification of domain(s) responsible for the association of human PAF-AH with p-hHDL. The observation that the mouse and human enzymes associated with p-hHDL particles of different density (Fig. 2D) provided us with a tool to search for discrete protein domains that contributed to the interaction. Our strategy consisted of replacing regions within human PAF-AH with corresponding sequences derived from the mouse ortholog, and then testing binding of the chimeric constructs to p-hHDL (Fig. 3A, Ref. 16). We found that replacement of residues 339–441 in the human protein with the corresponding murine sequences (construct V) resulted in a chimeric protein whose behavior differed from that of wild-type PAF-AH (compare Fig. 3, B and G). In contrast, the remaining chimeric constructs (constructs I–IV) displayed binding to p-hHDL similar to that of the wild-type human enzyme (Fig. 3, C–F). To further investigate the role of individual PAF-AH domains in binding to p-hHDL, we utilized a complementary approach. We generated a second set of chimeric constructs in which we increased the contribution of sequences derived from human PAF-AH, as we proportionately decreased representation of the mouse protein (Fig. 4A). We next tested the ability of these constructs to associate with p-hHDL and found evidence confirming a requirement for the human C terminus in binding to p-hHDL (Fig. 4, B–F). These studies supported and refined our previous findings as they revealed that human PAF-AH associated with p-hHDL through a domain comprised of amino acids 340 and 415.

Identification of Specific Residues Involved in PAF-AH/HDL Interaction—Our next goal was to more precisely map the minimal domain necessary to confer binding to p-hHDL. To accomplish this, we cloned the guinea pig and rat PAF-AH...
Molecular Basis for PAF Acetylhydrolase Association with HDL

FIGURE 2. Mouse and human PAF-AHs associate with different types of human HDL particles. A, profile of PAF-AH activity in mouse plasma. The data presented in Fig. 1F are reproduced for comparison with panels B–D. B, profile of PAF-AH activity in human plasma. 1 ml of fresh human plasma was subjected to ultracentrifugation using a modified KBr gradient. C, mouse and human PAF-AHs associate with murine HDL particles of the same density. Purified human or mouse FLAG-tagged PAF-AHs (36 and 67.5 nmol/min, respectively) were incubated with p-mHDL (4.8 mg) and subjected to ultracentrifugation using modified KBr gradients. D, mouse and human PAF-AHs associate with human HDL particles of different density. Solubilized extracts expressing human or mouse FLAG-tagged PAF-AHs (6.9 and 9.8 nmol/min) were incubated with p-HDL (9.5 mg) and subjected to ultracentrifugation in modified KBr gradients. These studies were conducted using modified KBr density gradients to improve resolution in the heavy density region (see “Experimental Procedures”). Panel A illustrates the density of fractions corresponding to all gradients shown in this figure.

cDNAs, expressed and purified the recombinant proteins, and then investigated whether they associated with p-hHDL. We found that the behavior of these proteins differed from that of human PAF-AH and resembled the binding pattern displayed by the murine ortholog (compare supplemental Fig. S2 and Fig. 4B). Next, we aligned the sequences comprised by amino acids 340–415 from the human, mouse, rat, and guinea pig orthologs and searched for residues in the human sequence that were absent in all the rodent orthologs (Fig. 5A). This led to the identification of Arg-347, His-367, Lys-370, Asn-378, Ala-379, Ser-384, and Ile-409 as candidates for further testing (Fig. 5A). We generated human R347K and N378R mutants and found that these mutants displayed normal binding to p-hHDL (Fig. 5, B and C). Next, we replaced the mouse string NKLT comprising residues 366–369 with HMLK, corresponding to amino acids 367–370 in the human protein. Interestingly, the resulting chimeric construct (mHMLK) associated with p-hHDL in a manner similar to that of the human wild-type protein (Fig. 5D). As expected, mHMLK constructs that mimicked the human protein at residues 384 and 409 behaved in a manner comparable to that of the mHMLK mutant (not shown). The role of Ala-379 will be discussed below. These results suggested a key role for the HMLK domain in the interaction of PAF-AH with p-hHDL. To characterize contributions from individual residues within the HMLK domain, we conducted additional experiments (Fig. 6A). We found that mutation of residues Met-368 and Leu-369 prevented binding to p-hHDL (Fig. 6, C and D). In addition, individual replacement of His-367 and Lys-370 with the corresponding mouse residues affected binding to a lesser extent (Fig. 6, B and E). These combined results further establish participation of the HMLK domain in the association of human PAF-AH with p-hHDL. Our results provide a possible explanation to account for the failure of rodent PAF-AH orthologs to associate with p-hHDL.

Naturally Occurring Polymorphic Forms of PAF-AH Display Normal Binding to Human HDL—The human plasma PAF-AH gene displays several polymorphisms and three of them (R92H, I198T, and A379V) result in amino acid changes that have been described in the Caucasian population (20–22). The results depicted in Fig. 4 did not allow us to rule out contributions from these residues to binding to p-hHDL. First, Arg-92 is conserved between the human and mouse orthologs; second, there is a conservative substitution at position 198 of the mouse ortholog (Val-197); third, a valine replaces Ala-379 in the mouse ortholog. To investigate whether the presence of these polymorphisms affected association of human PAF-AH with p-hHDL, we expressed human constructs R92H, I198T, and A379V and then tested the ability of extracts expressing the recombinant proteins to bind to the lipoprotein. We found (Fig. 7) that the three naturally occurring PAF-AH polymorphic forms displayed normal binding to p-hHDL. In addition, introduction of a histidine or a threonine residue at positions 91 and 197, respectively, of the mouse HMLK mutant (see Fig. 5D) did not prevent association with p-hHDL (not shown). These combined data firmly establish that the polymorphic forms of PAF-AH R92H, I198T, and A379V retain the ability to associate with HDL.

DISCUSSION

In recent years, PAF-AH has become known as Lp-PLA₂ because of the fact that the activity circulates in plasma as a complex with LDL and HDL (12, 23). Elevated expression of PAF-AH activity and/or protein has been reported in patients with coronary artery disease by a number of groups, and is thought to be an independent predictor of disease severity in humans (24–33). In addition, PAF-AH protein has been detected in atherosclerotic plaques of humans (34, 35) and in experimental animals (36). These correlative findings led to the proposition that PAF-AH actively contributes to the pathogenesis of vascular disease and that inhibiting its enzymatic activity could be beneficial for the treatment of atherosclerosis and related disorders (37–39). Two plasma PAF-AH inhibitors (SB-22657 and SB-480848) have been reported to attenuate monocyte chemotactic activity and macrophage apoptosis induced...
by oxidized LDL (40, 41) and to reduce atherosclerotic lesion formation in Watanabe heritable hyperlipidemic rabbits (42). SB-480848 (Darapladib) is currently being tested in humans as a potential treatment for atherosclerosis (43). However, the viewpoint that PAF-AH contributes to atherogenesis has been challenged by diverse studies in experimental animals and in human populations. A number of reports indicated that high levels of PAF-AH mass and/or activity may not be significantly associated with disease severity or mortality after adjustment for traditional cardiovascular disease risk factors (44–46). Second, population studies in Asian subjects genetically deficient in PAF-AH pointed at a protective role for the enzyme in vascular disease (see Ref. 20 for review) with the exception of one study (47). Third, overexpression of PAF-AH in experimental models of atherosclerosis led to similar conclusions (18, 48, 49). These combined observations illustrate the prevailing view that the function of PAF-AH in vivo and its contribution to coronary artery disease remain to be rigorously established.

The distribution of PAF-AH activity among lipoprotein particles has been proposed to play a significant role in the pathogenesis of a variety of diseases (10, 50–55). Several groups have reported a high degree of correlation between plasma PAF-AH and total plasma and LDL-cholesterol levels (see Ref. 56 for an excellent recent review on this topic). In addition, patients with primary hypercholesterolemia and combined hyperlipidemia show elevated total plasma- and LDL-associated PAF-AH activity (9, 20, 57). Tsimihodimos et al. (57) have described altered distributions of PAF-AH activity between LDL and HDL as a function of the severity of hypercholesterolemia. In some cases, there is an increase in the level of PAF-AH associated with triglyceride (TG)-rich very low density lipoprotein (VLDL) and intermediate density lipoprotein (IDL, Ref. 20, 57). A common feature shared by dyslipidemic patients is a decrease in the ratio of HDL-associated PAF-AH relative to the total levels of activity in plasma. The correlation between disease and abnormal PAF-AH distribution has led to the hypothesis that differential location of the enzyme in lipoproteins affects its function and/or physiologic role. Treatment with fenofibrates reduced PAF-AH activity associated with small dense LDL and, in some cases, increased the HDL-associated activity (56). We previously reported that HDL- and LDL-associated PAF-AH behave identically in optimized assays designed to measure initial rates of hydrolysis, a finding supported by other studies that used similar assay conditions (13, 58). In contrast, when we and
others (13, 59) performed functional studies under conditions that prevail in vivo, we found that the environment in which PAF-AH exists influenced its catalytic activity. These findings were confirmed using a variety of independent approaches, and they established that PAF-AH is more active against subsaturating concentrations of PAF if it is localized in LDL versus HDL particles. In summary, both clinical and biochemical data suggest that the location of PAF-AH impacts its function, but the mechanisms that define its distribution among lipoproteins are only partially understood.

In previous work, we searched for features involved in the association of PAF-AH with LDL and identified key domains required for binding to the lipoprotein. In addition, we found evidence for participation of the C-terminal end of apoB100 in interaction (7). The molecular basis for PAF-AH association with HDL has received relatively limited attention (60). This is surprising because of the fact that PAF-AH associates exclusively with HDL in a number of mammalian species commonly used to assess its function in physiological and pathological settings (17, 19, 49, 61, 62). Interestingly, the total activity in the plasma of these animals is at least 3-fold higher than the expression levels observed in species in which PAF-AH associates with both HDL and LDL, including humans (Fig. 1 and Table 1). This suggests the existence of species–specific regulatory mechanisms that control both location and total PAF-AH expression levels. Interestingly, the total activity in the plasma of these animals is at least 3-fold higher than the expression levels observed in species in which PAF-AH associates with both HDL and LDL, including humans (Fig. 1 and Table 1). This suggests the existence of species–specific regulatory mechanisms that control both location and total PAF-AH expression levels. To understand the relationship between function and location of PAF-AH, it is first essential to characterize these processes individually. In this study, we identified species-specific differences in the natural distribution of PAF-AH among lipoproteins and developed assay systems that, combined with homology comparisons, allowed us to identify a domain required for human PAF-AH to associate with HDL. Results from these analyses combined with studies using chimeric constructs resulted in the identification of a string of amino acids (His-367 to Lys-370) unique to human PAF-AH and that is necessary for binding to HDL. Our studies demonstrated a prominent role for Met-368 and Leu-369 and a more moderate contribution of His-367 and Lys-370 to association with HDL. Our data also show that Met-368 and Leu-369 are necessary but not sufficient for binding to HDL, as the guinea pig enzyme, which harbors both of these residues, did not bind to the lipoprotein. These results suggest that His-367 and Lys-370 directly participate in association with HDL or that the residues contribute to the formation of a binding pocket that optimizes interaction of Met-368 and Leu-369 with the lipoprotein. To address whether the string His-367 to Lys-370 was necessary and sufficient for binding to hHDL, we expressed FLAG-tagged C-terminal domains of human plasma PAF-AH and then assessed binding to HDL. Unfortunately, the recombinant peptides were not expressed at the levels necessary for biochemical studies. Thus, it is possible that additional regions conserved between mouse and human PAF-AHs are required for interaction with hHDL.

To investigate whether the binding domain we identified is utilized by additional proteins to interact with HDL, we searched for the presence of the HMLK string in several proteins known to bind HDL, including lecithin cholesterol acyltransferase (LCAT), cholesteryl ester transfer protein, paraoxonase 1, phospholipid transfer protein (PLTP), and several apolipoproteins. We found that only LCAT and PLTP harbored domains with weak homology to the HMLK string and that could potentially participate in the association between these proteins and HDL. Further studies are required to elucidate the
contribution of these domains to the association of LCAT and PLTP with HDL.

We previously reported that binding to human LDL required the presence of Tyr-205, Trp-115, and Leu-116 (7). When we introduced these residues into the mouse enzyme (which does not associate with human LDL and does not harbor the HMLK domain), the mutant protein acquired the ability to associate with LDL (7). In addition, the canine protein, which lacks the HMLK string, associated normally with LDL (7). Thus, our studies suggest that the HMLK domain is not required for association of PAF-AH with LDL. Our work also revealed that residues required for binding to LDL are not necessary for association with HDL. While introduction of Trp-115 and Leu-116 into the mouse ortholog conferred binding to LDL (see above and Ref. 7), these changes did not confer the resulting mutant protein the ability to bind to human HDL (data not shown). These combined observations provide evidence to support participation of distinct domains in binding of PAF-AH to human HDL and LDL.

Finally, the studies presented here revealed differences in the density of HDL particles with which mouse and human PAF-AHs associate. The mouse enzyme seems to have higher affinity for HDL particles of relatively high density, regardless of their source. In contrast, human PAF-AH showed a preference for lighter human HDL particles. While the physiological meaning of these differences remains to be established, our results suggest that the contribution of discrete domains in PAF-AH combined with lipoprotein features that are unique to each species are key components determining the distribution of serum PAF-AH in vivo.

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Molecular Basis for PAF Acetylhydrolase Association with HDL

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