Cloning and Characterization of HB₂, a Candidate High Density Lipoprotein Receptor

SEQUENCE HOMOLOGY WITH MEMBERS OF THE IMMUNOGLOBULIN SUPERFAMILY OF MEMBRANE PROTEINS

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The protection against coronary artery disease attributed to high density lipoprotein (HDL) may be associated with several functions, including its central role in reverse cholesterol transport, possible antioxidant and antithrombotic properties and others not yet identified which may depend on specific interactions between HDL and cell receptors. Several HDL-binding proteins have been identified including two candidate liver HDL receptors, HB₁ and HB₂ recently purified in this laboratory. We now report the cloning, sequencing, and some properties of HB₂, the most abundant of the pair. It shows significant homology with the adhesion molecules ALCAM and BEN of the immunoglobulin superfamily and the cDNA, when transfected into HepG2 or COS cells, caused specific HDL₃ binding to increase by 80–100%. Further, ligand blotting of glycoproteins isolated from phorbol 12-myristate 13-acetate-treated THP-1 cells or from transfected HepG2 and Chinese hamster ovary cells also provided evidence of increased binding of HDL₃ to HB₂. Differentiation of THP-1 cells into macrophages resulted in a striking increase in HB₂ mRNA which was attenuated if cells were cholesterol-loaded by incubation with acetylated low density lipoprotein. If the interaction between HDL and HB₂ reduces the adhesion-induced inflammatory cellular events that characterize arterial wall injury, thereby achieving the protection associated with higher plasma levels of HDL, these findings may provide a clue to one mitigating effect of HDL in heart disease.

The incidence of premature artery disease is lowered in the presence of high levels of circulating high density lipoprotein (HDL) suggesting that HDL may protect individuals against atherosclerosis (1). Part of this protection may be attributed to the participation of HDL in the reverse cholesterol transport pathway, a mechanism which reduces the accumulation of cholesterol in the arterial wall and the narrowing which subsequently occurs (2).

More recent evidence would indicate that HDL influences many other biological events in a manner consistent with a protective function, suggesting that the antiatherogenic role of HDL is likely to be complex. HDL may have antioxidant and antithrombotic properties (3–5), it may act as a signal transducing agent and as a secretagogue (6), and it may possibly influence the production of some cell adhesion molecules such as VCAM and ICAM (7).

Despite these observations, the mechanism(s) responsible for the HDL-induced physiological effects remain enigmatic. The fact that HDL binds to a variety of cells with varying degrees of specificity has invited speculation about the existence of an HDL receptor. Our laboratory (8) and others (9, 10) have purified liver cellular HDL-binding proteins, thereby strengthening the evidence for the existence of HDL receptor(s) that may have important functions as determinants of HDL metabolism. To date, only SR-B₁, a member of the scavenger receptor class of membrane proteins that binds LDL, modified LDL and HDL, has been shown to elicit a physiological response, viz. the transfer of cholesteryl ester into cells, particularly in steroioidogenic tissues (11).

We have identified two liver plasma membrane proteins named HB₁ and HB₂ (HDL-binding proteins 1 and 2) which are candidate HDL receptors (8, 12). Although present in low abundance, HB₂ was purified in sufficient quantities to provide amino acid sequence data. This paper describes the subsequent cloning of HB₂, its complete amino acid sequence, and evidence for increased binding of HDL to HB₂ in cells overexpressing HB₂.

MATERIALS AND METHODS

Cell Lines and Tissue Culture—HepG2, COS, CHO, and THP-1 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained under standard tissue culture conditions using Dulbecco’s modified Eagle’s medium for HepG2 and COS, α-minimal essential medium for CHO cells, and RPMI 1640 medium for THP-1 cells, plus 10% fetal bovine serum. THP-1 cells were differentiated into adherent macrophages by treatment with 200 ng phorbol 12-myristate 13-acetate (PMA) for 72 h.

Isolation and Labeling of HDL—Human HDL₃ (d 1.21–1.21 g/ml) was obtained from plasma as described previously and contained apoAI and apoAII but no apoE (8). HDL₃ was radiolabeled using IODOBEADS (Pierce) to specific activities ranging from 150–210 cpm/ng of protein. HDL₃ was also labeled with [³H]cholesteryl oleoyl ether (13). Brieﬂy, HDL₃ was incubated with donor particles containing egg yolk phosphatidycholine, [³H]cholesteryl oleoyl ether, and lipoprotein-deﬁcient serum (d > 1.21 g/ml) as a source of cholesteryl ester (CE) transfer protein for 6 h at 37 °C, and the HDL₃ was then separated from donor particles by ultracentrifugation at 1.06 g/ml. HDL₃ was labeled with [¹⁴C]CE by incubating a d > 1.12 g/ml (dialyzed) fraction of human
plasma (lecithin:cholesterol acyltransferase source) with [14C]-cholesterol (Amersham). The CE-labeled HDL was isolated by ultracentrifugation at d 1.21 g/ml and dialyzed.

**Protein Sequencing**—HB2, a glycoprotein of M, 100,000 was purified from rat liver plasma membrane as described previously (12). Attempts at direct subcloning of the purified HB2 by the cloning kit (Invitrogen) were unsuccessful. Instead, cDNA was isolated from a cDNA library prepared from rat thymus, fractionated by formaldehyde-agarose gel electrophoresis, and transferred to nylon membranes. These were probed with [32P]-labeled probes (see “Results”), and hybridizing bands were identified by autoradiography or bioimaging. Membranes were stripped and rehybridized with a cDNA probe for rat glyceroldehyde phosphate dehydrogenase. For detection of HB2 mRNA in human tissues, human multiple RNA blots (CLONTECH) were similarly probed as described above, except that the actin probe provided with the blots was used as an internal standard.

**Computer Analysis**—Data base searches with the nucleotide sequence of rat HB2 cDNA were performed with GenBank and EMBL data bases by the FASTA program from the Genetics Computer Group sequence analysis software. Alignments and consensus sequence derivation of peptide sequences were analyzed by GENETYX (version 9.0) from Software Development Co., Ltd. (Tokyo, Japan). Further analyses of the HB2 gene product for common motifs and potential secondary structure were performed using GENETYX.

**RESULTS**

HB2 antiseraum was used to probe membranes from various rat organs as described under “Materials and Methods.” As shown previously (18), the strongest expression of HB2 was present in liver, lung, and intestine. Total RNA from lung and liver were therefore subjected to reverse transcription-PCR as described under “Materials and Methods,” with primers 9511 and 9519.

Under the annealing/elongation conditions described for PCR, a single product of 600 base pairs was amplified from lung, but not from liver. Sequencing of this fragment confirmed the presence of both primers as well as an additional internal peptide of HB2, in the correct reading frame. To obtain a full-length clone, a lung cDNA library was prepared and probed with the 600-base pair fragment. Of 2.5 × 107 plaque-forming units probed, 33 positive clones remained after three rounds of screening. Fifteen of these were further analyzed. As shown in Fig. 1, the cDNA of HB2 encoded a protein of 65 kDa consisting of 18,000 × g for 10 min. Glycoproteins were isolated with wheat germ lectin agarose (Pharmacia) and then applied to SDS-PAGE gels. Ligand blotting was performed as described previously (12).

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**Binding to HDL**—To demonstrate that this transmembrane protein functions as an HDL-binding protein, COS, CHO, or HepG2 cells were transfected or mock-transfected as described under “Materials and Methods.” As shown in Fig. 2, A and B, specific binding of [125I]-labeled HDL was increased (at saturation) approximately 2-fold in transfected HepG2 cells and by 1.8-fold in transfected COS cells compared with mock-transfected cells. This increased binding however was not associated with transfer of cholesteryl esters from HDL to cells because no differences were observed in cholesteryl ester uptake between mock and transfected COS or HepG2 cells (data not shown).

Glycoproteins isolated from mock- or transiently transfected
CHO or HepG2 cells were compared for their HDL binding capacity by ligand blots. As shown in Fig. 2C, untransfected HepG2 cells showed binding of HDL3 to both HB2 and HB1, but the signal for HB2 was much stronger in transfected cells. Similarly the faint signal for HB2 observed in mock-transfected CHO cells was markedly increased when membrane proteins from transfected CHO cells were incubated with HDL3 (Fig. 2D).

Expression of HB2 mRNA in Rat and Human Tissues—Fig. 3A shows Northern blot analyses of HB2 mRNA expression in various rat tissues. Strongest expression was found in the lung, then brain, liver, and kidney. In comparison, to the rat, human mRNA to HB2 (probed with rat HB2 cDNA) was strongest in the brain, prostate, pancreas, small intestine, and liver of human (Fig. 3B), but lower in the lung.

To determine whether blood monocytes or macrophages express HB2, RNA from THP-1 cells or from cells differentiated by treatment with PMA was subjected to Northern blot analysis. As shown in Fig. 4A, HB2 mRNA, hardly detectable in untreated THP-1 cells, was strongly induced when the cells were transformed into macrophages by PMA treatment. After incubating THP-1 cells with 50 or 100 μg/ml acetylated LDL, a dose-dependent reduction in HB2 mRNA expression was observed. No changes were observed in expression of glyceraldehyde phosphate dehydrogenase mRNA with any of the treatments (Fig. 4B). Ligand blots of membrane proteins revealed weak HDL3 binding for THP1 cells, but after differentiation with PMA, strong binding was detected for HB2 as well as an increase in binding to HB1. Binding was also stronger to another protein (approximately 66 kDa) which may represent degraded HB2 or another isoform of HB2.

DISCUSSION

Cloning and sequencing of HB2, one of a pair of HDL-binding proteins previously identified and purified in this laboratory (12), have revealed its identity with a subclass of the immunoglobulin superfamily, ALCAM and BEN. Amino acid sequences obtained from peptide sequencing analysis of rat HB2 protein are underlined.

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DISCUSSION

Cloning and sequencing of HB2, one of a pair of HDL-binding proteins previously identified and purified in this laboratory (12), have revealed its identity with a subclass of the immunoglobulin superfamily of proteins with adhesion type properties. To confirm that HB2, performed a role as an HDL-binding protein, HepG2, CHO, and COS cells were transiently transfected with HB2 cDNA. Compared with control (mock-transfected) cells, HepG2 and COS cells (transfected) demonstrated an 80–100% increase in HDL binding, establishing that HB2, together with other candidate HDL receptors (11, 21), may play...
an important role in HDL metabolism in the body. The level of HDL binding activity resulting from transfection with HB2 cDNA was intermediate between that of an HDL-binding protein found in endothelial cells when expressed in COS cells (21) and that of SR-B1 (11), another candidate HDL receptor. It is not surprising that untransfected cells will demonstrate variable levels of specific HDL binding in view of the presence on most cells of “specific” but low affinity HDL binding sites recently identified in this laboratory (22). This phenomenon is a characteristic of the interaction between HDL and cells and the most likely explanation for the relatively high level of specific binding observed in the mock-transfected cells in this study.

Additional evidence that HB2 is involved in the cellular recognition of HDL came from ligand blotting studies which confirmed that this membrane protein, when overexpressed in

HepG2, CHO, and THP-1 cells, bound HDL3. Untransfected cells, apparently expressing low levels of HB2 compared with HB2 (Fig. 2C) showed a marked increase in HB2 that was active in binding HDL3, when transfected with HB2 cDNA. Similarly, ligand blotting revealed a significant increase in HDL binding to HB2 present in transiently transfected CHO cells, compared with controls. The up-regulation of HB2 mRNA that followed transformation of THP-1 cells into “macrophages” following PMA treatment was also associated with an increase in expression of HB2 which was active in binding HDL3 as seen in Fig. 4C.

These experiments have not yet provided definitive information about the clinical relevance of HB2, but they do support a functional role for HB2 that involves interaction with HDL. Expression of HB2 mRNA, barely detectable in blood monocytes, is up-regulated when THP-1 cells undergo differentiation
metabolism. Our present studies however have demonstrated that expression of HB2 is up-regulated in cells (such as blood monocytes and eosinophils) recognized by HDL or by apoAI. At least eight potential interactions suggested above. In the human, organs that play important roles in lipoprotein transport such as the liver and intestines suggested above. In the human, organs that play important roles in lipoprotein transport such as the liver and intestines (6). In fact, the tissue expression of HB2 mRNA. The structural features of HB2 are also consistent with a “receptor” role for this membrane protein. As shown in the working model (Fig. 5) based on available structural information on this group of membrane proteins of the IgG superfamily, HB2 is characterized by a 32-amino acid cytoplasmic domain, a 24-amino acid hydrophobic transmembrane domain, and approximately 500 residues of an extracellular domain terminating in the NH2 terminus at positions 95, 167, 265, 306, 361, 457, 480, and 499. It is likely that most sites are glycosylated since the protein is mainly expressed in the higher M form, although in HepG2 cells the presence of a faster migrating HB2 isoform was found by Western blotting.2 The function of glycosylation in HDL binding is unclear, although previous ligand blot studies (12) indicate that glycosylation is not essential for HDL binding. The carbohydrate however may contribute to other essential functions such as intracellular transport and influence the capacity of HB2 (in some tissues) to reach sites for HDL binding. Some provisional protein kinase C phosphorylation sites (residues 8, 73, 74, 209, and 421) were found, but since all are extracellular, their potential function as determinants of signaling via protein kinase C is weakened. CAMP- and cGMP-dependent protein kinase phosphorylation sites were also found. Possibly, sites which are inactive when HB2 is membrane-bound are activated if the protein is internalized. Further experiments are planned to investigate the interrelationships between structure and function, as related to HDL binding, and cellular localization of HB2.

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