Identification of a Region in the Vitamin D-binding Protein that Mediates Its C5a Chemotactic Cofactor Function*

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The vitamin D-binding protein (DBP), also known as group-specific component or Gc-globulin, is a multifunctional plasma protein that can significantly enhance the leukocyte chemotactic activity to C5a and C5a des-Arg. DBP is a member of the albumin gene family and has a triple domain modular structure with extensive disulfide bonding that is characteristic of this protein family. The goal of this study was to identify a region in DBP that mediates the chemotactic cofactor function for C5a. Full-length and truncated versions of DBP (Gc-2 allele) were expressed in Escherichia coli using a glutathione S-transferase fusion protein expression system. The structure of the expressed proteins was confirmed by SDS-PAGE and immunoblotting, whereas protein function was verified by quantitating the binding of [3H]vitamin D. Dibutyryl cAMP-differentiated HL-60 cells were utilized to test purified natural DBP and recombinant expressed DBP (reDBP) for their ability to enhance chemotaxis and intracellular Ca2+ influx to C5a. Natural and full-length reDBP (458 amino acid residues) as well as truncated reDBPs that contained the N-terminal domain I (domains I and II, residues 1–378; domain I, residues 1–191) significantly enhanced both cell movement and intracellular Ca2+ concentrations in response to C5a. Progressive truncation of DBP domain I localized the chemotactic enhancing region between residues 126–175. Overlapping peptides corresponding to this region were synthesized, and results indicate that a 20-amino-acid sequence (residues 130–149, 5′-EAFRKKDPEKEYANQFWMWEYST-3′) in domain I of DBP is essential for its C5a chemotactic cofactor function.

Vitamin D-binding protein (DBP) is a multifunctional and highly polymorphic plasma protein synthesized primarily in the liver (1, 2). DBP is expressed as a single polypeptide chain with a molecular mass of ~56 kDa and circulates in plasma at 6–7 μM (1, 2). Due to its extensive polymorphisms, DBP initially was named the group-specific component of serum, later shortened to Gc-globulin (3). DBP is a member of the albumin (ALB), α-fetoprotein (AFP), and α-albumin/afamin (AFM) gene family and thus has the characteristic multiple disulfide-bonded, triple domain modular structure (1). Besides functioning as a circulating vitamin D transport protein, it has been demonstrated that plasma DBP effectively scavenges G-actin released at sites of necrotic cell death and prevents polymerization of actin in the circulation (1, 2). Distinct binding regions within the 458-amino-acid sequence of DBP have been identified: a vitamin D sterol binding segment in the N-terminal domain (amino acids 35–49) and a G-actin binding region in the C-terminal domain (amino acids 373–403) (4, 5). More recent work on the crystal structure of DBP (bound to either vitamin D3 or actin) has confirmed the vitamin D sterol binding site but has demonstrated that actin interacts with distinct amino acid sequences in all three DBP domains (6–9).

Complement C5a is a 74-amino-acid peptide generated by limited proteolytic cleavage of C5 during complement activation (10). C5a is a very potent chemotactic factor for all leukocytes as well as several other cell types, and the peptide has several other proinflammatory functions as well (10). C5a exerts these activities by binding to its high affinity receptor (C5aR or CD88) on the plasma membrane of target cells (11). Several groups have demonstrated that purified DBP can significantly enhance the neutrophil chemotactic activity (i.e. chemotactic activity) of C5a and its stable breakdown product C5a des-Arg (12–17). In addition to neutrophils, DBP can also augment the C5a chemotactic activity for monocytes and fibroblasts (18, 19). The chemotactic enhancing properties of DBP appear to be restricted to C5a/C5a des-Arg since this protein cannot enhance the chemotactic activity of formylated peptides, IL-8, leukotriene B4, or platelet-activating factor (12, 13, 16, 18). The mechanisms by which DBP acts as a chemotactic cofactor for C5a are not known. The goal of this study was to determine whether a cochemotactic region could be located within DBP. The results demonstrate for the first time that DBP enhances both C5a-mediated chemotaxis and Ca2+ influx in differentiated HL-60 cells. In addition, a 20-amino-acid sequence within the N-terminal domain I of DBP (residues 130–149) was found to possess the C5a chemotactic cofactor activity.

MATERIALS AND METHODS

Reagents—Recombinant C5a was purchased from Sigma. Purified human DBP was obtained from Biodesigen International (Kennebunkport, ME). Full-length human DBP cDNA (Gc-2 allele), clone number CSODM004YP02, was purchased from Invitrogen. DNA restriction and modification enzymes were purchased from New England Biolabs (Beverley, MA). Oligonucleotides were synthesized by Invitrogen. Anti-human DBP was purchased from Diasorin, (Stillwater, MN). pGEX-4T-2 expression vector was purchased from Amerham Biosciences. Dibutyryl-cAMP (Bt2cAMP), Me2SO, and protease inhibitor mixture were purchased from Calbiochem. Recombinant expressed DBP, GST, glutathione S-transferase, HBSS, Hank’s buffered saline solution; BSA, bovine serum albumin.

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1 The abbreviations used are: DBP, vitamin D-binding protein; reDBP, recombinant expressed DBP; GST, glutathione S-transferase; HBSS, Hank’s buffered saline solution; BSA, bovine serum albumin.
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RESULTS

DBP Enhances C5a-induced Chemotaxis and Ca2+ Influx in Differentiated HL-60 Cells—Previous published work in our laboratory has demonstrated that neutrophils co-incubated with DBP display significant increased movement to suboptimal concentrations (10–100 pM) of C5a, i.e. the cochemotactic effect (12, 23, 25). However, neutrophils are short-lived, terminally differentiated cells and cannot be genetically manipulated. A cell culture model for neutrophils is the promyelocytic cell line HL-60 that can be induced to differentiate into neutrophil-like cells using agents such as methylSO or Bt2cAMP. Differentiation of HL-60 cells for 48 h using 250 μM Bt2cAMP induced expression of the C5a receptor and both permitted chemotaxis to C5a alone and significantly enhanced movement to C5a in the presence of purified natural DBP (Fig. 1A). These results are consistent with our previous reports using neutrophils and indicate that differentiated HL-60 cells will serve as a good cell culture model to investigate the cochemotactic function of DBP.

Intracellular calcium mobilization is a rapid and well-characterized event in response to C5a binding to its receptor. Fig. 1B shows that cells pretreated with DBP for 30 min display a significantly enhanced intracellular calcium influx in response to C5a. In contrast, untreated cells that had DBP and C5a added simultaneously did not show augmented calcium influx (data not shown), supporting previous studies showing that DBP needs to bind to the cell surface for at least 15 min before enhanced chemotaxis is observed (25). Nevertheless, the results presented in Fig. 1 show that DBP can enhance C5a-induced chemotaxis and calcium mobilization in differentiated HL-60 cells and indicate that this cell line will serve as a good cell culture model to investigate the cochemotactic function of DBP.

Analysis of E. coli-expressed DBP by SDS-PAGE and Western Blotting—The commercially available (Invitrogen) 1374-nucleotide DBP cDNA, Gc2 allele, was expressed in E. coli BL-21 in the plasmid vector pGEX-4T-2 fused to GST. Upon isopropyl-1-thio-b-D-galactopyranoside induction, the GST-DBP fusion protein was expressed, as judged by SDS-PAGE (Fig. 2A). SDS-PAGE of the elution protein revealed an ~80-kDa band corresponding to the molecular mass of full-length DBP fused to GST. The bands at ~65, 55, and 45 kDa correspond to the molecular mass of GST with truncated forms of DBP. All lanes have a small amount of a 30-kDa band. This probably represents a degradation product of GST or the unfinished product of the protein synthesis. Thrombin cleavage of the purified fusion protein resulted in separation of GST from DBP. The SDS-PAGE and immunoblotting after cleavage and separation of GST revealed a single protein band of 56 kDa for full-length DBP, 40 kDa for domains II and III, and 20 kDa for domain I or domain II (Fig. 2B).

Functional Characterization of rDBP—The capacity of reDBP to bind 25(OH)-vitamin D3 was measured to determine whether the expressed proteins could functionally bind a physiological ligand. Competitive binding assays of reDBP with a fixed amount of [3H]25(OH)-D3, and various amounts of unlabelled 25(OH)-D3 demonstrated that all reDBPs could displace the radiolabel in a similar dose-dependent manner (Fig. 3), indicating that the vitamin D sterol binding site of the reDBPs is similar to the purified, natural DBP. In addition, full-length reDBP-bound G-actin in a 1:1 molar complex as detected by
non-denaturing PAGE (data not shown). The ability of reDBPs to enhance C5a-mediated chemotaxis and Ca\(^{2+}\) flux in differentiated HL-60 cells was determined next. Fig. 4 demonstrates that reDBPs containing the N-terminal domain I have the capacity to enhance chemotaxis (Fig. 4A) and intracellular Ca\(^{2+}\) flux (Fig. 4B) in response to a C5a stimulus. Cells treated with DBP alone showed no response (data not shown). In addition, undifferentiated HL-60 cells, which express very little C5a receptor, did not react to C5a or C5a plus DBP (data not shown).

Previous results clearly demonstrate that the C5a cochemotactic function of DBP resides in the N-terminal domain. Therefore, to identify the cochemotactic sequence within this region, a series of truncated versions of domain I were generated. Initially, constructs containing either the N-terminal (amino acids 1–112) or C-terminal half (amino acids 113–191) of domain I were produced. The recombinant protein representing the N-terminal half of domain I-(1–112) was expressed and purified but possessed no chemotactic enhancing activity for C5a (Figs. 5 and 6). Several attempts to express the C-terminal half of domain I-(113–191) in E. coli, however, failed repeatedly (data not shown). The alternative approach of generating C-terminal truncations of domain I was employed next. Full-length domain I-(1–191) was progressively truncated to residues 1–112 since this protein had no chemotactic enhancing
activity. Fig. 5 shows the analysis of domain I truncations by SDS-PAGE (Fig. 5A) and immunoblotting (Fig. 5B). Fig. 6 demonstrates that domain I truncations 1–112 and 1–125 lack enhancing activity, whereas truncations 1–150 and 1–175 possess almost the same level of activity as full-length natural DBP for both chemotaxis (Fig. 6A) and Ca^{2+} flux (Fig. 6B).

The results from Fig. 6 indicate that a region within domain I of DBP, from amino acids 126 to 175, confers the C5a cochemotactic function. Two overlapping peptides within this region of DBP were next synthesized to determine more precisely a cochemotactic sequence. Peptide 1 (residues 130–152, EAFRKDPKEYANQFQMWESTNYG) and peptide 2 (residues 150–172, NYGGAPLSSLVSYTKSYLSMVGS), by themselves or mixed together, could not enhance C5a-mediated chemotaxis (data not shown). Consequently, an alternative approach of using these peptides to block the cochemotactic function of full-length natural DBP by pretreating cells with each peptide was employed. The results from Fig. 7 clearly show that peptide 1-(130–152), but not peptide 2-(150–172), could completely block the cochemotactic function of purified full-length natural DBP (C5a + DBP). In addition, peptide 1-(130–152) also could eliminate the HL-60 cell chemotactic response to complement-activated serum, indicating that this peptide can function to block a potent chemotactic signal in a diverse protein mixture (Fig. 7).

DISCUSSION

This study describes several novel findings. It is the first report of differentiated HL-60 cells displaying increased C5a-mediated chemotaxis to DBP, the first report of a DBP-mediated enhancement of C5a-induced Ca^{2+} influx in any cell type, and the first report to identify a sequence in DBP that mediates the C5a chemotactic cofactor function. Differentiated HL-60 cells are the second myeloid cell line to show enhanced C5a chemotaxis to DBP. Recently, we have demonstrated that undifferentiated U937 cells transfected with the C5a receptor can also be utilized as a cell culture model to investigate the cochemotactic function of DBP (20). Both cell lines will be useful to further elucidate the mechanisms by which DBP augments chemotaxis to C5a. In addition, the demonstration of a DBP-mediated increase in calcium flux to C5a is particularly important because it will permit dissection of intracellular signaling pathways triggered by DBP bound to the cell surface. It is interesting to note that HL-60 cells needed to be pretreated with purified DBP for at least 15 min prior to C5a addition to detect an increased calcium signal (Fig. 1B), perhaps indicating the formation of a DBP signaling complex on the plasma membrane as suggested previously (20). However, the major finding in this report is the identification of a 20-amino-acid sequence in the N-terminal domain of DBP that blocks C5a cochemotaxis to full-length natural DBP.

DBP contains 14 disulfide bonds and, therefore, presents a challenge for expressing the protein in a functional form. Swamy et al. (21) previously reported expression of functional recombinant DBP in E. coli using GST as a fusion partner. Using their approach, we generated a series of functional truncated proteins that localized the C5a cochemotactic function to...
a 50-amino-acid region (residues 126–175) in the C-terminal portion of domain I. Two overlapping peptides covering this region were synthesized and clearly demonstrated that the cochemotactic function was confined to the 20-amino-acid sequence 5'-EAFRKPDEYAQFMVDTST-3' (residues 130–149). This sequence is identical among the three major allelic forms of human DBP (Gc-1F, Gc-1S, Gc-2) and correlates with the fact that there is no difference in the C5a cochemotactic function between these DBP isomers (16). BLAST search of this sequence produced no other match, besides DBP, in any eukaryote, indicating that this region is unique to DBP and that it probably does not function by mimicking a similar sequence in a signaling molecule. In addition, alignment of this human peptide with the corresponding sequences in rat (26), mouse (27), and rabbit DBP (28), the only other mammalian sequences currently described, show a very high degree of amino acid similarity; 78% of the residues are identical, and the remainder are conservative or homologous substitutions. Thus, it is reasonable to speculate that this region in domain I of DBP functions as the cochemotactic sequence in all mammals.

Several recent reports have described the crystal structure of DBP, either unligated or bound to G-actin or vitamin D (6–9). Analysis of the structure has revealed that the protein is comprised of a series of α-helices, much like albumin (29), but in contrast to albumin, DBP folds into a hook-like structure that serves as the G-actin binding site (30). The cochemotactic sequence in DBP (residues 130–149) is located partly in α-helix number 7 (residues 125–134) but mostly in α-helix number 8 (residues 136–150) in domain I (6–9). Three-dimensional analysis of DBP crystal structure using the NIH NCBI software program Cn3D (version 4.1) indicates that this region is not accessible to interact with cells. This structural analysis correlates well with our recent functional studies that demonstrated that ligation of DBP with G-actin, 25-OH vitamin D₃, or both did not alter the C5a cochemotactic activity of DBP. Therefore, this cochemotactic peptide is a distinct functional sequence and constitutes the forth such region (the others are G-actin, vitamin D, and the polysaccharide structure of DBP-MAF (macrophage-activating factor) to be defined in the pleiotropic DBP.

The binding of DBP to cells is required for the protein to mediate its numerous functions: a chemotactic cofactor for C5a, a macrophage or osteoclast-activating factor, clearance of DBP-actin complexes by the liver, and delivery of vitamin D sterols and free fatty acids to cells (1, 2). The cell surface DBP binding site is only partially characterized, but this essential link in DBP physiology is poorly understood. DBP appears to bind either unligated or bound to G-actin or vitamin D (6–9). Analysis of the structure has revealed that the protein is comprised of a series of α-helices, much like albumin (29), but in contrast to albumin, DBP folds into a hook-like structure that serves as the G-actin binding site (30). This structural analysis correlates well with our recent functional studies that demonstrated that ligation of DBP with G-actin, 25-OH vitamin D₃, or both did not alter the C5a cochemotactic activity of DBP. Therefore, this cochemotactic peptide is a distinct functional sequence and constitutes the forth such region (the others are G-actin, vitamin D, and the polysaccharide structure of DBP-MAF (macrophage-activating factor) to be defined in the pleiotropic DBP.

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gion in DBP or whether the sequence identified (residues 130–149) performs both functions. DBP has been shown to bind to chondroitin sulfate proteoglycans, and this protein has several glycosaminoglycan consensus binding sequences (K/R-rich regions) in domains II and III. In particular, an 11-amino-acid sequence (403–413) in domain III (5′-KKKLAERLKAK-3′) is a very basic region that may be a putative glycosaminoglycan binding site. However, the cell binding site on DBP remains to be identified. The results also do not preclude the possibility that there are other sequences in DBP that may be involved in its cochemotactic function. The cochemotactic sequence 5′-EAFRKDPKEYANQFMWEYST-3′ (residues 130–149) can effectively block the capacity of full-length natural DBP to enhance chemotaxis to C5a, but the sequence alone cannot augment chemotaxis, possibly suggesting that other regions of DBP may be involved in this function. Nevertheless, the results reported herein provide strong evidence that amino acids 130–149 play an essential role in the C5a chemotactic cofactor function of DBP and, furthermore, could signify that compounds derived from this sequence may have therapeutic potential to limit C5a-mediated tissue injury.

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