Complement Factor H Is a Serum-binding Protein for Adrenomedullin, and the Resulting Complex Modulates the Bioactivities of Both Partners*

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Adrenomedullin (AM) is an important regulatory peptide involved in both physiological and pathological states. We have previously demonstrated the existence of a specific AM-binding protein (AMBP-1) in human plasma. In the present study, we developed a nonradioactive ligand blotting assay, which, together with high pressure liquid chromatography/SDS-polyacrylamide gel electrophoresis purification techniques, allowed us to isolate AMBP-1 to homogeneity. The purified protein was identified as human complement factor H. We show that AM/factor H interaction interferes with the established methodology for quantification of circulating AM. Our data suggest that this routine procedure does not take into account the AM bound to its binding protein. In addition, we show that factor H affects AM in vitro functions. It enhances AM-mediated induction of cAMP in fibroblasts, augments the AM-mediated growth of a cancer cell line, and suppresses the bactericidal capability of AM on Escherichia coli. Reciprocally, AM influences the complement regulatory function of factor H by enhancing the cleavage of C3b via factor I. In summary, we report on a potentially new regulatory mechanism of AM biology, the influence of factor H on radioimmunoassay quantification of AM, and the possible involvement of AM as a regulator of the complement cascade.

Human adrenomedullin (AM) is a 52-amino acid peptide originally isolated from a human pheochromocytoma and identified as a molecule capable of elevating rat platelet cAMP (1). AM belongs to the calcitonin gene-related peptide superfamily based on its slight homology with calcitonin gene-related peptide (CGRP) and amylin (1). The human mRNA is 1.6 kilobases long and encodes for a predicted 185-amino acid precursor from which two amidated peptides are generated: AM and a second peptide denoted as proadrenomedullin N-terminal 20 peptide (PAMP) (2). The expression of AM has been demonstrated in many tissues and biological fluids such as plasma (3), cerebrospinal fluid (4), sweat (5), amniotic fluid (6), urine (7), and milk (8). AM has been implicated in the modulation of numerous physiological functions, which include cardiovascular tone, central brain activity, bronchodilation, renal function, hormone secretion, cell growth, differentiation, and immune response (9).

Recently, we have demonstrated that plasma proteins from several species can specifically bind AM (10). The existence of these binding proteins was established using a radioligand blotting technique based on a method originally described for the detection of insulin-like growth factor-binding proteins (11). Most of the species analyzed, including humans, had an AM-binding protein (AMBP) with a Mr of 120,000 under non-reducing conditions. Interestingly, the plasma from ruminant species (calf, goat, and sheep) had an additional band of Mr 140,000. Whether these proteins are different or represent two glycosylation patterns from the same protein remains to be determined. An analysis of plasma from calves undergoing an acute phase response to a parasitic infection revealed a decrease in the expression of AMBP as compared with uninfected calves (10), whereas AM levels increased (12). The presence of a protein that specifically binds AM and the regulation of its expression in pathological situations may have a critical impact on AM physiology.

In this study, we isolate and characterize the human AMBP (AMBP-1) from plasma as complement factor H. We also describe how factor H may interfere with the quantification of AM by conventional radioimmunoassay (RIA) and how both binding partner proteins may modulate their respective biological activities.

**EXPERIMENTAL PROCEDURES**

**Labeling of Adrenomedullin**—Nonradioactive labeling was accomplished by conjugation of synthetic AM-(1-52) (Peninsula Laboratories, Belmont, CA) with fluorescein-5-EX succinimidyl ester (Molecular Probes, Inc., Eugene, OR). Briefly, 100 µg of AM were dissolved in 1 ml of 50 mM sodium bicarbonate, pH 8.5, and the succinimidyl ester was added to a final molar concentration ratio of 10:1 (linker:AM). The mixture was incubated with slow agitation for 1 h at room temperature, and the reaction was terminated by the addition of 0.1 M ethanolamine, pH 8.5, followed by another incubation of 1 h. Labeled AM was mixed...
with an equal volume of 0.1% alkali-treated casein (ATC) in Tris-buffered saline (TBS), pH 7.4, and extracted using reverse phase Sep-Pak C18 cartridges (Waters, Milford, MA) with 80% acidic isopropyl alcohol as the elution buffer. The extract was lyophilized and reconstituted in 1 ml of TBS containing 0.1% ATC, 0.1% Tween 20, and 0.05% Triton X-100. 1% NaFluorescein- labeled AM was stored at 4 °C for as long as 3 months without significant loss of activity.

Nonradioactive Ligand Blotting Assay—Proteins from human plasma (2 μl) were electrophoretically fractionated on 3–8% Tris acetate gels (Novex, San Diego, CA) under nonreducing conditions and transferred to a 0.2-μm nitrocellulose membrane. The membrane was washed with PBS containing 1% Tween 20 and incubated with 50 μl fluorescein-labeled AM for 16 h at 4 °C in blocking buffer containing 0.1% Tween 20. Binding was detected with a primary rabbit anti-fluorescein IgG (1:1000; Molecular Probes, Inc.), a secondary antibody coupled to alkaline phosphatase (1:2000; Dako, Carpinteria, CA), and nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (Roche Molecular Biochemicals) as the color-substrate solution. For competition studies with unlabeled peptides, the membrane was preincubated with 5 μM unlabeled ligands at 4 °C for 6 h. Then labeled AM was added, and the membrane was incubated for 16 h at 4 °C. AM fragments, PAMP, and CGRP were purchased from Phoenix Pharmaceuticals (Belmont, CA).

Reverse-phase HPLC—Preparative reverse phase HPLC was performed on a Delta Pak C18 300-Å column (30 × 300 μm; Waters, Tokyo, Japan) and the “System Gold” modular system (Beckman Instruments Inc., Fullerton, CA). 2.5 ml of human plasma were mixed with an equal volume of 10% acetonitrile with 0.2% trifluoroacetic acid, processed through a 0.2-μm filter, and loaded onto the column. After 5 min with 0.1% trifluoroacetic acid in 5% acetonitrile, the column was eluted with a linear gradient of acetonitrile containing 0.75% trifluoroacetic acid from 5 to 60% at a flow rate of 12 ml/min over 60 min. Each fraction (12 ml) was collected, freeze-dried, and dissolved in 0.3 ml of TBS, 0.1% Tween 20. Fractions were tested for the presence of AMB-1 using the nonradioactive ligand blotting technique.

Amino Acid Analysis—Amino acid analysis was performed by The Protein/DNA Technology Center at the Rockefeller University, New York. HPLC (NovaPak C18 30-μm column) with the Waters FicoTag Work station and a two-pump gradient system (model 510) equipped with a model 490 UV multiwavelength detector were used as previously described (13).

Edman Degradation—The N-terminal sequence analysis was performed by the Biotechnology Resource Laboratory, Protein Sequencing and Peptide Synthesis Facility (Medical University of South Carolina, Charleston, SC). The sample was subjected to automated Edman degradation using a PE Biosystems Procise 494 Protein Sequencer and a PE Biosystems eLC Microblotter 173, using standard cycles and reagents (14, 15).

Mass Spectrometry—After fractionation by SDS-polyacrylamide gel electrophoresis under reducing conditions (5% β-mercaptoethanol), the gel was stained with Coomassie Blue, and the AMB-1 band was excised from the gel, digested, and subjected to extraction and fractionation as previously described (16). One-tenth of the extracted protein digest was analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry on a PerSeptive Voyager-DE STR (PE Biosystems) MALDI-TOF mass spectrometer (ThermoQuest; Finnigan MAT Division, Piscataway, NJ). The instrument was operated in reflector mode with the operating parameters as follows: sheath gas flow, 5 l/min with 0.1% trifluoroacetic acid in 5% acetonitrile, the column was washed with 1% Nonidet P-40, blocked with 0.1% ATC in TBS, and transferred to a 0.2-μm filter, and loaded onto the column. After 5 min, the column was preincubated with 5 μM unlabeled ligands at 4 °C for 6 h. Then added to the mix. After a 30-min incubation, the sample was centrifuged, and the pellet was extensively washed with cAMP PBS, 0.1% Triton X-100. The final pellet was resuspended in 100 μl of LDS sample buffer (Novex) and boiled before the Western blot analysis.

Extraction of Plasma and AM Radioimmunoassay—Extraction was performed using reverse-phase Sep-Pak C18 cartridges (Waters) as previously reported (18, 19). Briefly, cartridges were activated with 80% methanol and washed with 0.9% NaCl. Plasma samples were mixed with an equal volume of a 0.1% acetonitrile-containing 0.1% acetic acid solution in water to wash the column. After elution with 1 ml of TBS, pH 7.4. Samples were applied to the columns, and, after washing twice with 0.9% NaCl, AM was eluted with 80% isopropyl alcohol containing 125 μM HCl. Extracts were freeze-dried to remove the organic solvent. Concentrations of AM in the extracts were measured by radioimmunoassay as previously described (19).

cAMP Assay—Rat-2 fibroblasts were grown in RPMI 1640 containing 10% fetal bovine serum (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Life Technologies) to Rat-2 cells. AM was secreted into 24-well plates at 2 × 104 cells/well and incubated 48 h at 37 °C in 5% CO2. Before the assay, the cells were incubated in TIS medium (RPMI 1640 plus 10 μg/ml transferrin, 10 μg/ml insulin, and 50 mM sodium selenite) for 15 min. Then cells were treated for 5 min with AM (Behem, King of Prussia, PA) and/or factor H (Sigma) in 250 μl of TIS medium containing 1% BSA, 1 mg/ml bacitracin, and 100 μM isobutylmethylxanthine. The reaction was terminated by adding an equal volume of ice-cold PBS. cAMP was measured using the Biotrac cAMP RIA (Amersham Pharmacia Biotech).

Receptor Binding Assay—Binding of rat 125I-AM (Pharma- ceuticals) to Rat-2 cells was determined as previously reported (20). In brief, Rat-2 cells were plated out at 2 × 104 cells/well in poly-n-lysine-coated 24-well plates (Becton Dickinson, Bedford, MA). Confluent cells were washed twice with ice-cold binding buffer (200 mM HEPES, pH 7.4, 5 mM MgCl2, 10 μM NaCl, 1 mM EDTA, 1 mM KCl, 1 mM glutathione, 0.25 mg/ml bacitracin, 0.3% bovine serum albumin) containing 100 pM rat 125I-AM. After incubation, cells were washed twice with ice-cold binding buffer and then dissolved in 1 ml NaOH for counting. Nonspecific binding was measured by incubating the cells with a 1000-fold excess of unlabeled AM.

Proliferation Assay—The breast cancer cell line T-47D (ATCC, Manassas, VA) was maintained in Dulbecco’s modified Eagle’s medium (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies). The MTT Proliferation Assay (Promega, Madison, WI) was carried out in serum-free conditions as previously reported (21). Briefly, cells were seeded in 96-well plates at 1–2 × 104 cells/well, and appropriate concentrations of the indicated compounds were added. After 5 days of incubation at 37 °C and 5% CO2 in a humid incubator, the MTT colorimetric assay was performed following the instructions from the manufacturer. The plate was read at a wavelength of 540 nm. Eight independent wells per treatment were averaged.

Antimicrobial Activity Assay—The antimicrobial activity was measured using Escherichia coli (ATCC 35218, Gaithersburg, MD) and a radial diffusion assay as previously described (22). Briefly, bacteria were incorporated into a thin underlay gel that contained 1% agarose, 0.1% tryptone, pH 7.2, and 0.1% glucose. A spot of tryptone broth buffer (20 μl) were added to each of a series of wells cut into the agar. Test substances were added and allowed to diffuse for 3 h at 37 °C. After polymerization, small wells of 10 μl of capacity were carved in the agar. Test substances were added and allowed to diffuse for 3 h at 37 °C. The diameters of the inhibition halos were measured to the nearest 0.1 mm and, after subtracting the diameter of the well, were expressed in inhibition units (10 units = 1 mm). We
estimated the minimal inhibitory concentration (MIC) by performing a linear regression and determining the x intercepts.

Cofactor Activity of Factor H—C3b (28 pmol) was incubated with factor I (0.16 pmol) and factor H (0.16 pmol) in the presence or absence of AM and related peptides for 24 h at 37 °C in a final volume of 50 μl of PBS. Samples were analyzed by SDS-polyacrylamide gel electrophoresis using 4–12% Tris-Bis gels (Novex) under reducing conditions and Coomassie Blue staining. C3b and factor I were purchased from Advanced Research Technologies (San Diego, CA).

Statistical Analysis—The MTT assay values and the MIC values were analyzed by the Student’s t test. cAMP values were analyzed with a one-way analysis of variance and Tukey’s test. p < 0.05 was considered significant.

RESULTS

Development of a Novel Nonradioactive Ligand Blotting Assay for AMBP Detection—Using the radioligand blotting technique originally described by Hossenlopp et al. (11), we have previously demonstrated that human plasma contains at least one adrenomedullin-binding protein (AMBP-1) with Mr 120,000 under nonreducing conditions (10). In the present study, we have developed a nonradioactive ligand blotting assay. Our initial approach included the labeling of AM with three different reporters: biotin, fluorescein, or dinitrophenyl. With all of these tracers, we were able to detect AMBP-1 in human plasma (data not shown). We discarded the use of the biotinylated AM reagent due to the possible interference with avidin- or biotin-like proteins present in plasma; this problem has already been described in the development of a nonradioactive ligand blotting assay for insulin-like growth factor-binding proteins (IGFBPs) using biotinylated insulin-like growth factor I (23). The procedure using fluorescein-labeled AM gave a better signal/noise ratio than the dinitrophenyl tag and was used for the evaluation of AMBP-1 expression. Fig. 1A compares the radioligand blotting using 125I-AM or fluorescein-labeled AM. One band with Mr 120,000 was visualized in both systems; however, the nonradioactive technique resulted in sharper band definition. To demonstrate the specificity of the assay, fluorescein-labeled AM was incubated with 5 μM AM, the gene-related peptide PAMP, and the structurally related peptide CGRP (Fig. 1B). Only AM was able to displace the nonradioactive tracer. When the competition was carried out with different fragments of AM, only the intact AM molecule reduced the binding (Fig. 1C).

Isolation and Characterization of AMBP-1—Human plasma (2.5 ml) was fractionated by reverse phase HPLC (Fig. 2A). Each fraction was tested for the presence of AMBP-1 using the nonradioactive ligand blotting technique. AMBP-1 was revealed in fractions 48–51 (Fig. 2C). Glycoprotein staining of AMBP-1 on SDS-polyacrylamide gels revealed that AMBP-1 was glycosylated (Fig. 3A). Isoelectric focusing showed a pl 6 for the protein (Fig. 3B). For the identification of purified AMBP-1, we used three different techniques: total amino acid analysis, Edman degradation, and mass spectrometry. Table I shows the amino acid composition of AMBP-1. A data base search revealed complement factor H as the protein with the highest degree of similarity to this composition profile. The amino acid composition of factor H is also shown in Table I. The percentage of methionine was lower than expected; however, the recovery of methionine in the case of bovine serum albumin, used as a control protein, was approximately half of the expected value. The analysis of the N-terminal amino acid sequence of AMBP-1 yielded a mix of at least two main N-terminal sequences; the data base search gave us again factor H as the protein with highest homology. The sequence of the 15 amino acids analyzed was identical to the N-terminal sequence of factor H with the exception of the threonine in position 12. Furthermore, a new search with the amino acids obtained from the segment peptide that did not correspond to the N terminus of factor H showed a 65% homology with an internal sequence of factor H (residues 578–592), suggesting that this secondary sequence could correspond to factor H fragmentation. Finally, the peptide masses obtained by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry after trypsin digestion of AMBP-1 corresponded unequivocally to the tryptic digestion of factor H, with the detected peptides covering 21.8% of the total factor H sequence. MS/MS applied to the ion with a molecular mass of 1396.6 Da yielded the sequence, confirmed by both the b and the y series, of 10 out of the 12 amino acids from the fragment 737–748 in the factor H molecule.

To further confirm that AMBP-1 was in fact factor H, we analyzed the ability of anti-factor H antibody to recognize AMBP-1 by Western blot detection. AMBP-1 was immunoreactive for this antibody, giving a band of Mr 120,000 under nonreducing conditions, an identical size to that of commercially available human factor H (Fig. 3C). Moreover, with nonradioactive ligand blotting using fluorescein-labeled AM, we demonstrated that factor H binds to AM (Fig. 3D). Under reducing conditions, AMBP-1 had a Mr of 150,000 (Fig. 3A), which corresponds to the Mr reported for factor H in such conditions (24). The reduction of the disulfur bonds prevented
the binding of AMBP-1 to the fluorescein-labeled AM (Fig. 3E).

Finally, we carried out binding assays of fluorescein-labeled AM to immobilized factor H. Fig. 3F shows the competitive displacement with unlabeled AM that could not be achieved with either CGRP or PAMP.

**Interference of Factor H with the AM Radioimmunoassay**—We previously reported that the C18 reverse-phase separation technique used to prepare plasma for AM RIA analysis effectively eliminates AMBP-1 from the extract (10). In the present work, we confirmed this observation by analyzing the presence of factor H in plasma before and after the C18 extraction. When plasma is processed through the C18 column, factor H is obtained in the unbound portion of the sample and not in the fraction used for AM quantification (Fig. 4A). Based on this observation, we tested the possibility that a certain amount of AM may pass through the column bound to factor H. For that purpose, 1 ml of human plasma was processed through the column, and both the bound and unbound extracts were recovered. We immunoprecipitated AM from the extracts and determined its presence by Western blotting. AM was detected in both the unbound and the bound fractions (Fig. 4B), suggesting that the traditional procedure used for peptide purification does not recover the total amount of AM present in plasma. Western blot after immunoprecipitation in the absence of extract did not yield any band, excluding the possibility that AM comes from the rabbit anti-AM serum (data not shown).

Disruption of AM/factor H interaction demonstrated a second source of AM in the plasma that was not routinely accounted for by traditional C18 purification procedures prior to RIA determination. A ligand blotting was performed with purified AMBP-1. After incubation with fluorescein-labeled AM, we tested several conditions for the dissociation of the binding between AM and factor H. For that purpose, the membrane was cut in strips and washed six times for 10 min each under the different conditions. Finally, the strips were equilibrated again in the assay buffer and the ligand blotting was developed (Fig. 5A). Extreme conditions such as acidic pH and high salt concentration did not dissociate the interaction of factor H with AM. However, the incubation of the blot with labeled AM in those conditions effectively avoided the interaction (data not shown). One of the treatments that disrupted the binding was basic pH; however, further experiments indicated that this

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**Fig. 3. Biochemical characterization of AMBP-1 as human factor H.** A, Coomassie Blue and glycoprotein staining (GelCode Glycoprotein Staining Kit; Pierce) after SDS-polyacrylamide gel electrophoresis. Lane 1, horseradish peroxidase (5 μg), a glycosylated protein used as positive control. Lane 2, soybean trypsin inhibitor (5 μg), a nonglycosylated protein used as negative control. Lane 3, AMBP-1 (2 μg). B, samples were run in a pH 3–10 isoelectric focusing gel (Novex, San Diego, CA) and stained with Coomassie Blue. Lane 1, isoelectric focusing markers. Lane 2, AMBP-1 (4 μg). C, Western blot with an anti-factor H antibody. Lane 1, AMBP-1 (100 ng). Lane 2, AMBP-1 (200 ng). Lane 3, commercially available human factor H (50 ng). Lane 4, human plasma (0.2 μl). D, nonradioactive ligand blotting of 1 μg of AMBP-1 (lane 1) and factor H (lane 2). E, ligand blotting of AMBP-1 (250 ng) run under unreduced (lane 1) or reduced conditions (lane 2). F, binding of fluorescein-labeled AM (50 nm) to a 96-well plate coated with factor H (5 ng/well) was competed with increasing concentrations of unlabeled AM (○). Neither PAMP (*) nor CGRP (□) affected the binding. The results of one of two independent experiments are shown. Values represent mean and S.D. of three determinations. B/B₀ represents the ratio of signals generated in the presence/absence of unlabeled peptide.

**Table 1**

Amino acid composition of AMBP-1 and factor H

| Amino Acid | AMBP-1 | Factor H |
|------------|--------|----------|
| Asx        | 12.7   | 9.4      |
| Glx        | 13.2   | 12.2     |
| Ser        | 8.1    | 8.2      |
| Gly        | 8.3    | 8.5      |
| His        | 2.6    | 2.5      |
| Arg        | 4.4    | 4.9      |
| Thr        | 6.4    | 6.6      |
| Ala        | 3.4    | 3.3      |
| Pro        | 7.8    | 8.0      |
| Tyr        | 5.8    | 6.1      |
| Val        | 6.4    | 5.5      |
| Met        | 0.9    | 1.7      |
| Ile        | 5.8    | 5.9      |
| Leu        | 5.0    | 5.0      |
| Phe        | 2.8    | 2.7      |
| Lys        | 6.4    | 7.0      |

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a Since tryptophan and cysteine are destroyed by the hydrolysis of the protein in 6 M HCl, the calculated composition of factor H corresponds to the 18 amino acids recovered.

b The combined values for asparagine and aspartic acid, and glutamine and glutamic acid are expressed as Asx and Glx, respectively.

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**Fig. 4. Western blot for factor H and AM after C18 extraction.** A, 1 ml of human plasma was processed through a Sep Pak C18 cartridge, and both the bound and unbound fractions were analyzed for factor H presence by Western blot. Lane 1, commercially available human factor H (10 ng). Lane 2, whole human serum (0.5 μl). Lane 3, unbound fraction (1 μl). Lane 4, bound fraction (1 μl). B, the same was done for the detection of AM (immunoprecipitating AM before the Western blot analysis). Lane 1, synthetic AM (1 ng). Lane 2, fraction immunoprecipitated with normal rabbit serum (30 μl). Lane 3, fraction immunoprecipitated with rabbit anti-AM antibody (30 μl).
Effect could in fact correspond to an artifact, since this extreme pH alters the structure of the fluorescein and doing so negates its affinity for the anti-fluorescein antibody (data not shown). Using the binding assay in a 96-well plate, we further analyzed the dissociating ability of the chaotropic agent sodium thiocyanate (NaSCN). After incubation of solid-phased factor H (50 ng) with fluorescein-labeled AM (50 nm) and prior to the development of the assay, wells were incubated during different periods of time with PBS, 3 M NaSCN, pH 7.4. Values represent the mean and S.D. of six determinations. $B/B_0$ represents the percentage of total binding.

Plasma samples of three healthy donors were quantified by RIA following either the method previously described (18, 19) or the following modification. 1 ml of plasma was preincubated with an equal volume of 6 M NaSCN in PBS, 0.1% ATC, 0.1% Triton X-100, pH 7.4, for 10 min at room temperature. After that incubation, plasma was extracted through the C18 cartridge and quantified. The detected levels with the new protocol were 2-fold higher than those obtained with the standard technique (mean and S.D. values of the three donors were $23.0 \pm 4.8 \text{ versus } 54.3 \pm 8.6 \mu g/ml$). The same results were obtained when a longer preincubation with the chaotropic agent (16 h at 4°C) was used. Using the new protocol with NaSCN, recovery of unlabeled AM (200 pg) added to human plasma was $93.9 \pm 18.7\%$ ($n = 3$), whereas recovery of $^{125$I-AM was $82.7\% \pm 4.4\%$ ($n = 6$). The dilution curve in the RIA obtained from a fourth plasma sample was parallel to that of the synthetic human AM used in the standard curve and to the curve generated with the same plasma extracted under the traditional conditions (Fig. 6). The parallel curves confirm that the increase in AM immunoreactivity generated by NaSCN treatment was not artifactual.

Effect of Factor H in AM-mediated cAMP Induction—AM was initially identified as a peptide capable of elevating cAMP (1). Recently, it has been reported that Rat-2 fibroblast cells express a specific AM receptor coupled to adenylate cyclase that produces a dose-dependent increase in cAMP upon exposure to AM (20). Using this cell line as a model, we studied the effect that factor H could have on AM-mediated cAMP response. Treatment of Rat-2 fibroblasts with 100 nm AM produced a 2-fold increase in cAMP (Fig. 7A). When the same concentration of AM was combined with increasing concentrations of factor H (50, 100 and 200 nm), we observed a significant and dose-dependent augmentation in cAMP production. The highest factor H concentration (200 nm) used alone had no effect on cAMP levels, confirming that the observed increase was in all cases due to the presence of AM (Fig. 7A). On the other hand, the presence of factor H did not apparently modify the kinetics of the binding between rat AM and its receptor. Neither the association rate nor the competitive binding of human AM was altered by the presence of factor H (Fig. 7, B and C).

Effect of Factor H in AM-induced T-47D Growth—A human breast cancer cell line, T-47D, was used to investigate the effect of AM and factor H interaction on tumor cell growth. We have previously demonstrated that AM can act as an autocrine/paracrine growth factor in several cancer cell lines (25). In the present work, we used the MTT proliferation assay to examine the growth-promoting activity of AM in the presence or absence of factor H. In a serum-free medium, AM had a growth-promoting activity on the cell line T-47D (data not shown). The presence of factor H further induced the proliferation of T-47D in a dose-dependent manner. Factor H in the absence of AM had no effect on growth (Fig. 8).

Modulation by Factor H of the Antimicrobial Activity of AM—A radial diffusion assay was used to characterize the influence of human complement factor H on the antimicrobial activity of AM (Fig. 9). Factor H by itself did not have any antimicrobial effect on E. coli. On the other hand, AM had an intense inhibitory impact on the bacterial growth. When AM and factor H were added together, a significant reduction in the inhibitory effect of AM was observed ($p < 0.001$, $n = 8$), suggesting that factor H is able to hinder the antimicrobial activity of AM. The MIC for AM by itself was $18.4 \pm 1.3 \mu g/ml$, and it became $35.4 \pm 1.1 \mu g/ml$ when factor H (50 $\mu g/ml$) was added.

Modulation by AM of the Cofactor Activity of Factor H—We finally tested whether AM affects the cofactor activity of factor H in the factor I-mediated cleavage of C3b (26, 27). Treatment of C3b with factor H and factor I caused cleavage of C3b (Fig. 10). When AM was added to the reaction, an increase in the cleavage of C3b was observed (Fig. 10A). Note that as the levels...
of AM are increased in the reaction mixture, there is a parallel increase in the split product formation with a reciprocal reduction in the 104-kDa band. AM (10 μM) had no activity in the absence of factor H. Neither CORP nor PAMP (10 μM) had any effect on the cofactor activity (Fig. 10B).

**DISCUSSION**

We demonstrate here that AMBP-1 circulating in human plasma corresponds to complement factor H. The purification of AMBP-1 from plasma was greatly facilitated by a novel nonradioactive assay for the detection of AM binding proteins. This new technique gives us some distinct advantages over our previously described radioactive method (10); its development required a shorter period of time, and the use of the fluorescein-labeled AM had better reproducibility and sharper band formation than the use of 125I-AM. In addition, it simplified handling procedures and extended the half-life of the tracer. Since both methods revealed a protein with the same molecular weight and the binding with the labeled AM could only be totally displaced by the intact unlabeled AM, we conclude that the protein detected with the nonradioactive method corresponds to the protein previously detected with the radioactive ligand (10).

By a combination of HPLC, electrophoretic fractionation, and the nonradioactive detection system, we have been able to isolate AMBP-1 to homogeneity and complete its biochemical identification. Several different analytical techniques led to the unequivocal conclusion that the purified protein corresponds to complement factor H. Factor H is a single chain glycoprotein consisting of 20 subunits called short consensus repeats (28). Factor H binds to C3b, displacing Bb from the C3 convertase. It also acts as a cofactor for the factor I-mediated proteolytic cleavage of the α' chain of C3b. The final result of these activities is the inhibition of the alternative pathway of the complement (24, 26, 27). Additional roles have been identified for factor H; it binds to the integrin Mac-1 (C11b/CD18) enhancing the activation response of human neutrophils (29), is a ligand for L-selectin (30), induces the secretion of interleukin 1/β by...
Factor H is not retained in the C18 matrix, probably because it is too big to penetrate through the particle pores (the pore size is 125 Å based on the manufacturer’s specifications). The way factor H circulates through the column suggests that the AM bound to factor H will not be retained and therefore the extraction protocol would recover only the free AM in plasma. We have confirmed this by demonstrating the presence of a significant amount of AM in the unbound fraction after the extraction. Furthermore, treatment with a chaotropic agent (NaSCN) seems to dissociate, at least partially, the binding between factor H and AM, allowing the detection of higher levels of AM. Plasma levels of AM are elevated in several pathological conditions (39), and although AM seems to act in an autocrine/paracrine manner, a physiological role for circulating AM remains possible (40). Therefore, we believe that determining the total AM concentration in plasma (versus the free AM currently measured) may be important to better understand the role of AM in the physiological and pathological conditions in which it is implicated. In addition, the variations in the levels of AMBP previously observed in infected animals (10) suggest that changes in circulating AM may be also dependent on modifications of its binding protein expression.

Factor H is present in plasma and has also been detected in extravascular compartments such as the synovial fluid (41, 42). The liver is considered to be the main source of factor H, although it is also synthesized by extrahepatic cells such as mononuclear phagocytes, fibroblasts, endothelial cells, mesangial cells, astrocytes, oligodendrocytes, and neurons (43).

This suggests that the presence of factor H in tissues may affect the autocrine/paracrine actions of AM. We describe here preliminary insights into the effect of factor H on AM activity. An increase in the cAMP induction mediated by AM was observed when Rat-2 fibroblasts were incubated with AM in the presence of factor H. On the other hand, factor H did not affect the binding between AM and its receptor. Factor H was also able to augment the growth-promoting activity of AM on the human breast cancer cell line T-47D. The exact mechanism by which the factor H-AM complex augments AM activity remains to be clarified; however some observations may shed some light on this issue. Factor H is able to bind to cell surfaces through at least three glycosaminoglycan binding sites present in its structure (44–46). It has also been reported that factor H binds to human neutrophils through the integrin Mac-1 (CD11b/CD18) (29), and it is a ligand for L-selectin (30). Hypothetically, the augmentation in cAMP production and cell growth may involve enhanced cell surface attachment, presenting AM in closer proximity to its membrane receptor. Factor H would act as a carrier and a reservoir of AM, which could provide high local levels of AM to stimulate its receptor. In this way, factor H would increase the AM effectiveness without modifying the affinity for its receptor. Other binding proteins enhance the biological activity of their ligands; the case of the well characterized IGFBPs is a good example. IGFBPs can either inhibit or augment the IGF actions (47). How IGFBPs enhance IGF function is not well understood, although it is known that many IGFBPs associate with cell surfaces and that the enhancing activity is probably mediated by this binding (47). A similar example of this phenomenon can be seen with the latent transforming growth factor-β (TGF-β)-binding protein (LTBP), which seems to play an important role in the activation of latent TGF-β, probably through targeting the latent TGF-β complex to the cell surface (48). The presence of an Arg-Gly-Asp (RGD) sequence may account for the cell attachment properties of some IGFBPs (47) as well as the LTBP (49). This sequence is present in several matrix proteins, and it is the essential structure recognized by the integrin superfamily of receptors (50).
Interestingly, factor H also possesses an RGD cell adhesion sequence in its structure (28). Finally, it would also be interesting to determine whether the enhancing effect of factor H on Rat-2 eAMP production and on T-47D growth may be due to a protective effect of factor H on AM degradation.

We have also been able to demonstrate that factor H down-regulates the antimicrobial activity of AM. It has been postulated that AM exerts its bactericidal effect by forming membrane pores, which ultimately cause pathogen lysis (51). Factor H inhibition of AM antimicrobial activity could be mediated by decreasing the concentration of available AM in the microenvironment, thus limiting its access to the pathogen’s outer membrane. Although the physiological relevance of AM’s antimicrobial activity still has to be addressed, the inhibition by factor H of this AM activity is in line with the fact that the binding of factor H by certain microorganisms seems to protect them from complement-mediated host defense (33). It has been suggested that this resistance could be due to the degradation of C3b by the membrane-bound factor H. The inactivation by factor H of the host-produced AM, a molecule with antimicrobial activity, could now be considered as an additional mechanism of microorganism resistance.

Another important aspect of this study is the identification of a role for AM in C3b degradation mediated by factor H/factor I interaction. We have demonstrated that AM accelerates this process. We can speculate that AM may induce conformational changes in the structure of factor H, increasing its affinity for C3b, similarly to what has been reported to occur in the presence of soluble polyanions (52). Since plasma AM is increased during sepsis and after endotoxin challenge (12, 53–56), AM may regulate the antimicrobial activity of AM. We have demonstrated that AM accelerates this interaction. We have shown that AM increases the plasma half-life of AM (50). Ruoslahti, E., and Pierschbacher, M. D. (1987) J. Biol. Chem. 262, 2592–2599

In summary, we present here the interaction between complement factor H and adrenomedullin.
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