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Processing of HEBP1 by Cathepsin D Gives Rise to F2L, the Agonist of Formyl Peptide Receptor 3

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The peptide F2L was previously characterized as a high-affinity natural agonist for the human formyl peptide receptor (FPR) 3. F2L is an acetylated 21-aa peptide corresponding with the N terminus of the intracellular heme-binding protein 1 (HEBP1). In the current work, we have investigated which proteases were able to generate the F2L peptide from its precursor HEBP1. Structure-function analysis of F2L identified three amino acids, G3, N7, and S8, as the most important for interaction of the peptide with FPR3. We expressed a C-terminally His-tagged form of human HEBP1 in yeast and purified it to homogeneity. The purified protein was used as substrate to identify proteases generating bioactive peptides for FPR3-expressing cells. A conditioned medium from human monocyte-derived macrophages was able to generate bioactivity from HEBP1, and this activity was inhibited by peptatin A. Cathepsin D was characterized as the protease responsible for HEBP1 processing, and the bioactive product was identified as F2L. We have therefore determined how F2L, the specific agonist of FPR3, is generated from the intracellular protein HEBP1, although it is unknown in which compartment the processing by cathepsin D occurs in vivo. The Journal of Immunology, 2011, 187: 1475–1485.

Inflammation is a critical response of the organism to pathogens, damaged cells, or inflammatory stimuli. However, uncontrolled inflammation can lead to diseases such as atherosclerosis (1), arthritis (2), and cancer (3). Therefore, inflammatory processes need to be well coordinated and regulated. An optimal immune response depends in particular on specific leukocyte subsets recruited to the sites of inflammation. Neutrophils, macrophages, and dendritic cells (DCs) are important cellular mediators of innate immune defenses (4). Numerous molecules are able to elicit chemotactic responses, and they can be classified into different chemical and structural families, among which are chemokines (5), complement factors C3a and C5a (6), leukotrienes, and formylated peptides. These latest factors, such as the prototypic fMLF, are among the first identified and most potent chemoattractants for phagocytic leukocytes (7–9).

These various families of chemotactic factors activate G protein-coupled seven transmembrane domains receptors expressed not only by leukocytes but also by a variety of other cell types. A receptor for formylated peptides was first described in 1976 (10), and the cDNA encoding the human formyl peptide receptor (FPR, now called FPR1) was cloned in 1990 (11, 12). Two closely related human receptors, FPR-like 1 (FPRL1/FPR2) and FPR-like 2 (FPRL2/FPR3), were subsequently cloned by low-stringency hybridization, using the FPR1 cDNA as a probe (13–15). The three genes are clustered on human chromosome 19q13.3 and encode receptors sharing a high level of sequence identity (13, 14). N-formylated peptides are derived either from bacterial proteins or from endogenous mitochondrial proteins. FPR1, and presumably the other two members of the family, are therefore believed to be involved in host defense mechanisms against invading pathogens and also in the sensing of internal danger signals resulting from cellular dysfunction. The prototypic fMLF displays high affinity for FPR1 (10) and low affinity for FPR2 (15). In addition to formylated peptides, a number of structurally diverse agonists of FPR1 and/or FPR2 have been described during recent years (16). In contrast, FPR3 does not respond to fMLF (17) and presents a distinctive expression pattern among leukocyte populations. FPR3 is expressed in monocytes, myeloid and plasmacytoid DCs, some tissue-specific macrophage subpopulations (particularly in lung, skin, and colon) and eosinophils, but not in neutrophils (18).

In contrast to FPR1 and FPR2, a very few ligands have been identified for FPR3. Humanin, a neuroprotective peptide in models of Alzheimer’s disease, was shown to bind FPR3 and FPR2 with high affinity (19, 20). The most specific ligand described for FPR3 is, however, the endogenous peptide F2L (21), an acetylated 21-aa peptide derived from heme-binding protein 1 (HEBP1). F2L was isolated from porcine spleen extracts on the basis of its bioactivity. The prototypic formyl peptide receptor-like 2 (FPRL2/FPR3) displays high affinity for FPR2 (15). In addition to formylated peptides, a number of structurally diverse agonists of FPR1 and/or FPR2 have been described during recent years (16). In contrast, FPR3 does not respond to fMLF (17) and presents a distinctive expression pattern among leukocyte populations. FPR3 is expressed in monocytes, myeloid and plasmacytoid DCs, some tissue-specific macrophage subpopulations (particularly in lung, skin, and colon) and eosinophils, but not in neutrophils (18).

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Abbreviations used in this article: CTS D, cathepsin D; DC, dendritic cell; FPR, formyl peptide receptor; FPRL1/FPR2, formyl peptide receptor-like 1; FPRL2/FPR3, formyl peptide receptor-like 2; HEBP1, heme-binding protein 1; siRNA, small interfering RNA.

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on FPR3-expressing leukocytes, F2L promotes calcium mobilization and chemotaxis (18, 21).

How and in which circumstances F2L is generated in the organism is, however, still unknown. HEBP1 is an intracellular tetrapyrole-binding protein of 22 kDa. Initially purified from mouse liver, HEBP1 is expressed in many tissues. Knockdown of mouse HEBP1 by antisense oligonucleotides induces a reduction of the cell heme content, suggesting that HEBP1 may be involved in heme regulation, biosynthesis, or transport (22, 23). However, no additional data have reinforced this hypothesis, and the biological function of HEBP1 remains therefore poorly defined. The three-dimensional structure of murine p22HBP was determined by nuclear magnetic resonance and consists of a 9-stranded distorted β-barrel flanked by two long α-helices (24, 25). Located outside the globular structure of HEBP1, residues 1–17 are disordered, whereas residues 18–23 form a β-strand. This part of the protein is not found in bacterial homologues of the SOUL/HEBP family. It is therefore conceivable that cleavage of HEBP1 after the leucine 21 would release the F2L ligand while keeping the heme-binding domain functional (24).

In the current study, we investigated the potential pathways leading to the generation of F2L in the organism. According to the role of macrophages in cleaning up cellular debris and the resolution of inflammatory processes, we searched for the generation of F2L when recombinant HEBP1 was submitted to the action of macrophage proteases or conditioned media. The use of specific protease inhibitors and purified proteases demonstrated that the lysosomal aspartyl endopeptidase cathepsin D (CTS D) is able to process HEBP1 and generate F2L. This observation suggests that F2L might be generated after tissue damage and macrophage recruitment, favoring the recruitment of additional monocyte/macrophages and DCs, which would contribute to tissue repair and the control of the inflammatory process.

Materials and Methods

Plasmid construction and yeast transformation

The cDNA corresponding to human HEBP1 was amplified by PCR from pCDNA3–HEBP1 using the primer pair oeq313 and oeq312 and was introduced in the pCSC2 Saccharomyces cerevisiae expression vector (26) by homologous recombination. The resulting plasmid, pCSC2–HEBP1 (pCSC294), allows the production of recombinant HEBP1 displaying a C-terminal tag of six histidines. The resulting plasmid, pCSC2–HEBP1 (pCSC294), was transformed into yeast strain BY4709 strain (S. cerevisiae MATa his3Δ1 leu2Δ2 trp1Δ901 ura3Δ100), which was transformed using the lithium acetate procedure (27). Transformants were selected on YNB plates containing 20 mg/ml glucose.

HEBP1 production and purification

Strain BY4709 expressing HEBP1 was cultured on YNB containing 20 mg/ml glucose in a 13-l batch bioreactor (Biolafitte) to an OD660nm of 1.69. Cells were harvested by centrifugation and the pellet washed twice in water. Cells were resuspended in buffer A (300 mM NaCl, 50 mM NaH2PO4) with EDTA-free Complete Protease Inhibitor Cocktail (Roche) and lysed with a French press. The lysate was centrifuged at 12,000 rpm ( Sorvall Rcsb, SS-34 rotor) for 30 min at 4˚C. The supernatant was purified on an Ni-NTA Sepharose column (Qiagen), eluted by a step gradient of 0.90, and 150 mM imidazole in buffer A. The serine protease inhibitor PMSF (1 mM; Sigma) was added to the collected fractions (4 ml/fraction). A sample of the fractions was loaded on a 12% polyacrylamide gel, followed by Western blotting using a rabbit polyclonal Ab (Phoenix). Fractions of interest were analyzed on a 12% polyacrylamide gel, followed by HEBP1 immunodetection on Western blots, and purity was checked by Coomassie blue staining.

Truncated synthetic peptides and alanine scanning

Acetylated F2L (Ac-MLGMIKNSLFGSVETWPWQVL) and alanine variants were synthesized locally by using the solid-phase Fmoc strategy. Monoisotopic masses and sequences of all peptides were verified by mass spectrometry. Because of their hydrophobicity, peptides were dissolved in DMSO at 1 mM, and 25-fold intermediate dilutions were made in 50% CH3CN, followed by further dilution in assay buffer to working concentrations. All peptides were assayed from 0.1 to 3000 nM (2 points per log) in the aequorin-based assay on FPR3-expressing cells, and the EC50 was determined. The results are presented as the ratio between the EC50 of the peptide and the EC50 of native F2L.

Aequorin-based luminescence assay of intracellular calcium release

Calcium release was measured by an aequorin-based bioluminescence assay, as previously described (28, 29). In brief, CHO-K1 cells coexpressing apoaequorin, CTS D, and FPR3 or control GPCRs were collected from culture dishes, pelleted, and centrifuged, and resuspended at 5 × 106 cells/ml in DMEM/Ham’s F12 containing 0.1% BSA (aequorin buffer). The cell suspension was supplemented with 5 μM coelenterazine h (Promega, Madison, WI) and incubated under shaking for 3 h 30 min at room temperature in the dark, then diluted 5-fold in aequorin buffer. Fifty microliters of cell suspension was injected onto 50 μl of agonist-containing medium in 96-well plates, and light emission was recorded for 40 s in a Centro LB 960 luminometer (Berthold Technologies). ATP (20 μM; Sigma) was used as standard to normalize the data.

Binding assays

For the analysis of truncated F2L peptides, a F2L variant containing an additional C-terminal tyrosine was used as tracer after labeling with 125I using the iodogen method (the sp. act. was 900 Ci/mmol), as described previously (16). Afterwards, we obtained a custom-made fluorescent F2L derivative, containing S66-carboxyi fluorescein linked to an additional C-terminal lysine (F2L–FAM), from JPT Peptide Technologies (Berlin, Germany). Two hundred thousand FPR3-expressing CHO-K1 cells or parental CHO-K1 cells in 100 μl binding buffer (DMEM-F12, containing 0.5% BSA and 0.1% NaCl) were incubated in siliconized 1.5-ml microcentrifuge tubes (Sigma) with increasing concentrations of F2L–FAM for 1 h at room temperature in the dark. The cells were then washed with 2 volumes of binding buffer, pelleted, resuspended in 250 μl binding buffer, and analyzed by FACS (FACScan; Becton Dickinson). FACS data were analyzed with the WinMDI software. Nonspecific binding was determined in the presence of 10 nM unlabeled F2L. For competition binding assays, cells were incubated with 10 nM F2L–FAM and increasing concentrations of HEBP1, unlabeled F2L, or variants thereof. The binding data were analyzed with the GraphPad Prism software.

Proteolytic processing of HEBP1 in conditioned medium from monocyte-derived macrophages

Monocytes were isolated from venous blood of healthy donors by immunomagnetic bead cell sorting (MACS) according to the manufacturer’s specifications. These procedures received authorization from the Ethics Committee of the Free University of Brussels Medical Faculty. After Ficoll density gradient, monocytes were purified by positive selection using CD14 microbeads (Miltenyi Biotec). Macrophages were differentiated from monocytes in the presence of 50 ng/ml recombinant human M-CSF (R&D Systems) for 6 to 8 d. The purity of the cell prepreparations was evaluated to 95% or more by flow cytometry (CD206* CD14+, or CD68* for permeabilized cells). Macrophage monolayers were incubated in a proteolysis buffer (25 mM sodium acetate pH 3.6, 100 mM NaCl) at 37˚C in a humidified atmosphere of 5% CO2 during 24 h. The medium was then collected and incubated with HEBP1 with or without 10 μg/ml pepstatin A (Sigma) for different periods of time at 37˚C. The medium was then adjusted to pH 7 by 10 mM sodium bicarbonate pH 8, and samples were engaged in the aequorin-based assay.

Inhibition of CTS D expression

CTS D expression was inhibited in human monocyte-derived macrophages by transfection of specific small interfering RNAs (siRNAs; A, HSS102578; B, HSS175648; C, HSS175649; Invitrogen), using their respective scrambled oligonucleotides as controls. Oligonucleotides (0.1, 1, and 10 nM) were transfected using the INTERFERin reagent (Polyplus-transfection) at days 6 and 7 of macrophage differentiation from monocytes, and the purity of fully differentiated macrophages at day 7 was evaluated by flow cytometry (CD68* CD206+ cells). Transfection efficiency was monitored with 100 nM FITC-labeled oligonucleotide (BLOCK-it Fluorescent Oligo; Invitrogen). CTS D levels were evaluated 48 h after the second transfection by Western blotting using a mouse anti-
CTS D mAb (clone BC011, IgG2a, 0.1 μg/ml; Calbiochem), a rabbit polyclonal Ab against GAPDH as control (1/2000, Cell Signaling), and an Odyssey infrared imaging system analysis.

**Proteolysis of HEBP1 by CTS D**

Native CTS D from human spleen was purchased from Calbiochem. HEBP1 was incubated in proteolysis buffer with CTS D at 37˚C with or without pepstatin A. The medium was adjusted to pH 7 by sodium bicarbonate pH 8, and samples were tested for activity in the aequorin-based assay. For mass spectrometry analysis, the samples were vacuum-dried and resuspended in 10 μl 0.1% trifluoroacetic acid mixed with matrix mix (2 mg/ml 2,5-dihydroxybenzoic acid, 10 mg/ml α-cyano-4-hydroxycinnamic acid, 2 mM fucose). Mass spectrometry analysis was performed on a Q-TOF Ultima Global mass spectrometer equipped with a MALDI source. In parallel, samples were vacuum-dried, boiled for 10 min in 1× Novex loading buffer (Invitrogen), and loaded on 10–20% polyacrylamide gradient gels in Tricine buffer (Invitrogen). Gels were blotted onto 0.2-μm pore-size polyvinyl difluoride membranes (Millipore), and HEBP1 fragments were immunodetected with a rabbit polyclonal Ab directed at the N terminus of the protein (Phoenix Pharmaceutical) or stained using an improved Blum’s silver staining protocol modified for high-sensitivity protein identification (30).

**Preparation of mouse neutrophils**

Fresh blood samples were collected from posterior vena cava of 8–wk-old C57BL/6 mice. Neutrophils were recovered on a Ficoll density gradient (Lymphoprep; Axis-Shield), and erythrocytes were lysed by ammonium chloride. The cell population was consistently composed of >90% neutrophils, as determined by flow cytometry analysis (CD11b+ Gr1+ population).

**Chemotaxis assays**

Leukocytes were isolated as described earlier and incubated for 30 min in RPMI 1640 containing 5% decomplemented FBS before running the chemotaxis assays. HEBP1 (100 nM) was incubated for 30 min at 37˚C with 60 nM CTS D in saline buffer (25 mM acetate buffer, 150 mM NaCl, pH 3.6). The medium was neutralized with 1 M sodium bicarbonate pH 8 before testing. Chemotaxis was assayed by modification of the Boyden micropore filter technique, as previously described (31). Briefly, the assay was conducted in 48-well microchemotaxis chambers (Neuroprobe), using 5-μm (human monocytes and monocyte-derived macrophages) or 3-μm (mouse neutrophil) pore-size Nuclepore track-etched polycarbonate membranes (Whatman). The cell suspension (10,000 macrophages/well and 50,000 monocytes or neutrophils/well) was loaded in the upper chamber and the chemotacticant solution or vehicle in the lower chamber. Positive control of cell migration was 10 nM synthetic F2L. Migration was conducted for an adequate period of time (1 h for monocytes, 1 h 30 min for macrophages, and 30 min for neutrophils) at 37˚C in humidified air containing 5% CO2. After membrane removal, nonmigrated cells were scraped off its upper side. The filters were fixed in methanol and stained with Hoechst for 2 min. Micrographs of the lower surface of the filters were taken, and the number of cells was counted with ImageJ software (version 1.36b). Results are expressed as a chemotactic index (ratio of migrated cells in the presence versus the absence of chemotacticant). Chemotaxis was distinguished from chemokinesis by a checkerboard test, in which the chemoattractant was added to the upper chamber with the cells or to both chambers.

**Immunohistochemistry and tissue microarrays**

Normal tissues obtained from 30 different human organs in the Service d’Anatomie Pathologique of the Erasme Hospital were selected using H&E-stained slides. Corresponding paraffin-embedded blocks were then precisely aligned with the marked slides. Three tissue cores (0.6 mm in diameter) for each sample were punched using a precision instrument (Beecher) and arrayed into a recipient block. Each organ was represented by samples from three to five different patients. These procedures received authorization from the Ethics Committee of the Free University of Brussels and the Erasme Hospital. Sections (5 μm) of formalin-fixed and paraffin-embedded tissue samples or tissue microarrays were subjected to standard immunohistochemical procedures. Primary Abs were a mouse monoclonal anti-HEBP1 [aa 1–21] (clone 729, IgG1b; Euroscreen) and a mouse monoclonal anti-CTS D (clone C5, IgG2b; AbD Serotec). Microwave Ag retrieval was performed twice in citrate buffer, pH 6, for 5 min. Detection was made by the Vectastain Elite ABC kit (Vector Laboratories) using 3,3’-diaminobenzidine tetrahydrochloride (Dako) as the peroxidase substrate. The slides were counterstained with hematoxylin, dehydrated, and mounted in DPX mounting medium (Sigma-Aldrich). Negative controls were conducted by replacing primary Abs with corresponding isotype controls (Vector Laboratories). HEBP1 and CTS D expression were evaluated by two independent observers and assessed by scoring each spot: 0 for no expression; 1 for low expression; and 2 for high expression. Results are presented as the score means for different donors.

**Statistics**

For the results of aequorin-based and chemotaxis assays, statistical significance was determined using the one-way ANOVA test, and p values <0.05 were considered significant.

**Results**

**HEBP1 production and purification**

F2L is to date the only specific ligand of FPR3. However, no information is available yet regarding the situations and mechanisms leading to the generation of F2L in the organism, including the proteolytic enzymes involved in the cleavage of HEBP1. To gain insight into this process, we first expressed and purified full-length recombinant human HEBP1.

The HEBP1 sequence starts with an acetylated methionine and contains no apparent signal peptide. N-terminal acetylation occurs in cytosolic mammalian proteins and yeast proteins but rarely in prokaryotic or archaeal proteins (32, 33). In a subset of these proteins, acetylation is required for some of their biological properties, such as enzymatic activity, stability, DNA binding, protein–protein interaction, or peptide–receptor recognition. Other proteins are acetylated without known functional consequences. In the case of F2L, acetylation is not required for binding to FPR3 or activation of the receptor (21). It might, however, be necessary to stabilize F2L and/or HEBP1 in vivo, for the (essentially unknown) functions of HEBP1, or for the recognition of the protein by the putative pro tease(s) generating F2L (34). For all these reasons, we produced recombinant human HEBP1 in the eukaryotic cell S. cerevisiae, so that it would be correctly folded and acetylated. As F2L is the N-terminal peptide of HEBP1, we placed a poly-histidine tag for purification purposes at the C-terminal end of the protein.

After selection of a yeast clone, the amount of HEBP1 evaluated by Western blotting represented close to 1% of the total protein extract (data not shown). The recombinant protein was purified by immobilized metal ion affinity chromatography and gel filtration (Fig. 1A–C). Size-exclusion chromatography showed that HEBP1 is monomeric. The purity of the purified protein was evaluated on 12% polyacrylamide gels after Coomassie blue staining (Fig. 1C). Using this procedure, we produced and purified around 300 μg HEBP1 per liter of yeast culture. The integrity of purified HEBP1 was evaluated by electrospray mass spectrometry. The major peak (21,943.9 Da) corresponded with acetylated HEBP1 and a smaller peak (21,901.9 Da) with nonacetylated HEBP1 (Fig. 1D, inset), suggesting that most of the purified protein is indeed acetylated.

**Characterization of the binding and functional activities of HEBP1 on FPR3**

The functional activity of purified full-length HEBP1 was evaluated on FPR3-expressing CHO-K1 cells by using an aequorin-based calcium mobilization assay. The activity was expressed as a percentage of the response induced by 10 μM ATP, acting on endogenous P2Y receptors. No significant activity was detected at low nanomolar concentrations of HEBP1 compared with the activity of F2L used as a positive control. A weak activity was, however, identified for 1 μM HEBP1 (Fig. 2A).

We then evaluated the capacity of full-length HEBP1 to bind FPR3-expressing CHO-K1 cells in competition binding assays using a fluorescent F2L analogue as tracer. F2L labeled by carboxyfluorescein at its C terminus (F2L–FAM) was obtained and samples were tested for activity in the aequorin-based assay. F2L labeled by carboxyfluorescein at its C terminus (F2L–FAM) was obtained and samples were tested for activity in the aequorin-based assay. F2L labeled by carboxyfluorescein at its C terminus (F2L–FAM) was obtained and samples were tested for activity in the aequorin-based assay. F2L labeled by carboxyfluorescein at its C terminus (F2L–FAM) was obtained and samples were tested for activity in the aequorin-based assay.
binding was detected on parental CHO-K1 cells (Fig. 2B). In competition binding experiments, unlabeled F2L competed for F2L–FAM binding with an IC50 of 17 nM, whereas HEBP1 did not inhibit F2L–FAM binding (Fig. 2C). We also investigated the potential antagonist activity of HEBP1 in a calcium mobilization assay. FPR3-expressing CHO-K1 cells were stimulated with 50 nM F2L in the presence of various concentrations of HEBP1. Full-length HEBP1 was unable to antagonize the activity of F2L (Fig. 2D), and the weak agonist effect of the protein was observed at high concentration (1 μM). In agreement with this agonist activity, preincubation of cells with high concentrations of HEBP1 for 20 min desensitized the cells, which displayed decreased calcium release in response to 90 nM F2L (Fig. 2E).

Identification of amino acids involved in F2L binding to FPR3
We investigated the structure–function relationship of F2L using modified peptides derived from the F2L sequence. First, an alanine-scanning experiment was devised, in which each amino acid of F2L was replaced by an alanine (Fig. 2F). In a functional assay, we identified three residues (G3, N7, and S8) as important for FPR3 activation. Indeed, replacement of these residues by alanine increased significantly the EC50 of the peptides by 2- to 9-fold. Concurrent replacement of K6, N7, and S8 by alamines increased the EC50 by almost two logs, confirming the important role of these amino acids (Fig. 2F, 2G). Some individual substitutions (such as L7, I5, and F10) appeared to improve slightly the potency of the peptide, but the combination of these substitutions did not confirm this tendency (Fig. 2G). The apparent importance of glycine at position 3 suggests that flexibility is required in the N-terminal part of the peptide for its interaction with FPR3. Finally, none of the residues in the C-terminal half of F2L, including some large hydrophobic amino acids, appeared to play a critical role.

We further investigated the importance of specific residues of F2L by testing N- and C-terminally truncated F2L variants in binding and functional assays. At the N terminus, removal of the first three amino acids had little impact on the activation parameters (Fig. 2J). In contrast, a clear loss of potency was observed after deletion of M4, very poor activation was obtained when deleting I5, and the peptide starting at K7 was totally inactive. In the binding assay, competition for radioiodinated F2L was significantly impaired by deletion of the first three amino acids, and no competition was detected for shorter peptides (Fig. 2K). These data are consistent with the important role of the structure surrounding G3 in the N terminus of the peptide.

At the C terminus, no differences in activation and binding parameters were observed after deletion of the last three residues. Peptides 1–17 and 1–16 displayed an EC50 twice as high as that of F2L. EC50 values could still be determined for peptides 1–15 and 1–12, but their efficacy was decreased. The activity of peptides 1–10 and 1–9 was still detectable at high concentrations, although EC50 values were not measurable. Peptide 1–6 was totally inactive (Fig. 2H, 2I). As for N-terminal truncations, the binding parameters were affected more rapidly with successive truncations. None of the peptides tested behaved as an antagonist, as binding and activation parameters decreased consistently in parallel. These results, summarized in Supplemental Table I, fit with the presence of a core of residues, necessary for binding and activation of FPR3, in the N-terminal part of the F2L peptide.

Proteolysis of HEBP1 in conditioned medium from monocyte-derived macrophages
To identify conditions allowing proteolysis of HEBP1 and F2L release, we incubated purified HEBP1 in different media potentially containing required proteases. Conditioned media were

**FIGURE 1.** Production and purification of full-length HEBP1. A, A lysate of yeast *S. cerevisiae* expressing HEBP1 C-terminally tagged with six histidines was loaded on a nickel column and eluted by a step gradient of imidazole (dashed line). The black box indicates the fractions containing HEBP1. B, The three fractions were concentrated and separated on a Superdex 75 column (1 ml/min). The third peak corresponds with HEBP1 (black box). C, Samples from different purification steps were loaded on a 12% polyacrylamide gel stained with Coomassie blue. D, Electrospray mass spectrometry profile of purified HEBP1.
prepared from human monocyte-derived macrophages, human neutrophils purified from peripheral blood, and purified mouse spleen cells. Incubation of HEBP1 for 30 min at 37°C in macrophage conditioned medium resulted in the generation of a biological activity on FPR3-expressing CHO-K1 cells using the aequorin-based assay (Fig. 3A). Neither the conditioned medium without HEBP1 nor HEBP1 in the unconditioned medium induced calcium release. Weaker activities were recorded after incubation of HEBP1 in conditioned media from human neutrophils or mouse spleen cells (Fig. 3B).

To identify the class of proteolytic enzymes responsible for HEBP1 processing, we investigated the effect of specific protease inhibitors on the generation of FPR3 agonists in macrophage conditioned medium. The activity was inhibited very efficiently by pepstatin A (1 μg/ml), a potent inhibitor of aspartyl proteases. The vehicle alone (10% ethanol, 1% acetic acid) and other protease inhibitors (pMSF and leupeptin) had no effect (Fig. 3C and data not shown). These data suggest that macrophages release an aspartyl protease able to cleave HEBP1 under acidic conditions.
Proteolysis of HEBP1 by purified CTS D

CTS D, a lysosomal aspartyl protease, was considered as a good candidate for the processing of HEBP1. Indeed, macrophages contain high amounts of CTS D, and the activity of this enzyme is strongly inhibited by pepstatin A. The concentration of CTS D in the medium of macrophages cultured for 24 h in acetate buffer was evaluated to 63.9 ng/ml (n = 3). Incubation of human HEBP1 (250 nM) with purified human CTS D (10 ng/ml) for 30 min at 37˚C generated a strong biological activity for FPR3-expressing CHO-K1 cells (Fig. 4A). The specificity of this activity was demonstrated by the absence of response of parental CHO-K1 cells and cell lines expressing FPR2, ChemR23, or GPR88 as control receptors (Fig. 4B).

We then evaluated the biological activity resulting from the incubation of HEBP1 (250 nM) with increasing concentrations of CTS D and showed, in the aequorin-based assay, a bell-shaped curve with a maximum corresponding with 60 nM CTS D (Fig. 4C). In other experiments, a 15-min incubation of 60 nM CTS D with increasing concentrations of HEBP1 (≤2 μM) resulted in a saturation curve with typical Michaelis–Menten kinetics (Km: 148 nM) (Fig. 4D). Finally, we performed a 24-h time course of the bioactivity generated from HEBP1 (250 nM) by CTS D (60 nM). Activity was observed at early time points (5 min) of hydrolysis with a peak at 30 min, after which it slowly decreased with time. The same samples were loaded on 12% polyacrylamide gels, showing that most of the substrate was cleaved after 5 min, whereas slower degradation was observed afterward (Fig. 4E, 4F).

Altogether, these results demonstrate a fast cleavage of HEBP1 by CTS D, generating an agonist of FPR3, which can be further degraded by the enzyme, although with slower kinetics.
To test whether CTS D is the only (or main) protease contributing to HEBP1 processing in macrophage conditioned medium, we inhibited CTS D expression by transfection of three specific siRNAs during the monocyte-to-macrophage differentiation. The procedure did not affect differentiation, as macrophages appeared fully differentiated 7 d after monocyte selection (Fig. 5A), and transfection efficiency was shown to be near 100% using a fluorescent oligonucleotide as control (Fig. 5B, fluorescence microscopy, 5C, flow cytometry). The three different siRNAs (A, B, and C) significantly reduced the levels of immunoreactive CTS D in monocyte-derived macrophages, as determined by Western blotting (Fig. 5D). In contrast, the corresponding control oligonucleotides (scrambled sequences) did not affect CTS D immunoreactivity. Pretreatment of monocyte-derived macrophages with the three siRNAs prevented the hydrolysis of HEBP1 and the generation of FPR3 agonist in conditioned media prepared from these cells, as evaluated with the aequorin-based assay on FPR3-expressing CHO-K1 cells (Fig. 5E).

Characterization of the peptides resulting from the processing of HEBP1 by CTS D

We analyzed by mass spectrometry the nature of the peptides resulting from the processing of HEBP1 by CTS D to determine which proteolytic product(s) activate FPR3. In the absence of tryp tic cleavage, we identified in the HEBP1 hydrolysate a peptide corresponding with acetylated F2L. Silver staining of polyacrylamide gels showed that several fragments were generated from HEBP1 after 30 min of digestion by CTS D. The molecular weights of these fragments were evaluated to 2.5, 5, 6, 10, and 22 kDa (Fig. 6A). Each band was excised from the gel, trypsinized, and peptides were identified by mass spectrometry. The 2.5- and 6-kDa peptides contained the N terminus of HEBP1, whereas all other fragments did not. Analysis of the peptides identified two main cleavage sites in the protein: one after Leu^21 and the other somewhere between residues 60 and 90 (Fig. 6C). We did not recover enough material to perform sequencing and identify more precisely this second cleavage site. A band around 12 kDa was found for some preparations of HEBP1 in the absence of CTS D. This band did not correspond with a fragment of HEBP1, and we could not identify this yeast contaminant by mass spectrometry. The same samples were loaded on a 10–20% polyacrylamide gel and blotted onto 0.2-μm pore-size polyvinyl difluoride membranes. Immunodetection with an anti-F2L Ab (Euroscreen) identified, among others, a diffuse band at 2.5 kDa (Fig. 6B). Cleavage leading to F2L generation seems to be among the first events during HEBP1 hydrolysis, as short incubations lead to F2L generation (Fig. 6B, left panel) whereas longer incubations generate also larger peptides (Fig. 6B, right panel).

Chemotaxis of monocytes toward HEBP1-derived peptides

F2L is a chemoattractant agent for FPR3-expressing cells, such as monocytes, DCs, and macrophages (18, 21, 29). We evaluated therefore the ability of CTS D-treated HEBP1 to recruit FPR3-expressing leukocytes. In microchemotaxis Boyden’s chambers, processed HEBP1 promoted recruitment of human monocyte-derived macrophages and human monocytes (Fig. 7B, 7C). The chemotaxis index was comparable with that obtained for synthetic F2L (10 nM) used as a positive control. No chemotaxis was observed toward CTS D alone or unprocessed HEBP1. Checkerboard analysis on monocyte-derived macrophages (Fig. 7A) excluded...
The mass of the tryptic fragments detected at 2.5 kDa, the size of F2L. This band was more intense after a short incubation time. The silver-stained bands were analyzed by mass spectrometry after trypsinization. The mass of the tryptic fragments identified are presented in the middle column and the corresponding region of HEBP1 protein sequence in the left column. D, Schematic representation of the cleavage sites in HEBP1 by CTS D (triangles) and of the resulting fragments detected.

**Discussion**

Proteolytic regulation of the biological activity of peptides and proteins acting as ligands of GPCRs is not an uncommon process. Many peptidic GPCR ligands are processed by proteases, resulting in their partial or full inactivation. Almost as frequently, proteolysis is required for the generation of GPCR ligands or results in a significant increase of their affinity and potency for the receptor. We previously identified F2L, a 21-aa peptide, as a high-affinity and specific endogenous ligand for FPR3. F2L corresponds with the N terminus of the heme-binding protein HEBP1, but the mechanisms involved in its production from the precursor protein are unknown. In this work, we investigated how F2L is generated from HEBP1.

Human full-length HEBP1 displays low agonist activity toward FPR3 and does not act as an antagonist. The recent characterization of HEBP1 tridimensional structure has shown that the F2L peptide is located outside the structured domain of the protein. Its proteolytic release might therefore keep intact the function of HEBP1 (24, 25, 36). We determined the structure–function relations of F2L using point mutants and truncated forms of the peptide. It appears that the important part of the peptide lies near its N terminus, the most crucial positions being G3, N7, and S8. However, other low-affinity interaction sites are likely spread over the whole length of F2L, as it was not possible to design much shorter peptides while keeping a reasonable affinity for FPR3. We observed indeed a shift of one log in the EC50 between peptide 1–18 (almost fully active) and peptide 1–15, suggesting a contribution of the WPW motif. F2L is highly conserved among mammalian species, and the human peptide differs from mouse F2L by only one aa in position 6, where lysine is replaced by arginine. Steric hindrance probably prevents the access of full-length HEBP1 to the binding pocket of F2L, limiting greatly its ability to activate FPR3. Additional work will be required to identify peptides with enhanced biological activities or antagonist properties.

To determine which proteases or protease families are able to cleave HEBP1 and release active FPR3 ligands, human HEBP1 was incubated with a panel of conditioned media. Indeed, no consensus sequence for candidate proteases could be identified in HEBP1 using bioinformatics programs. We selected as a starting point cells known to contain high amounts of proteases, including macrophages. Macrophages are phagocytic cells, which differentiate from circulating monocytes, and are located at strategic sites in the organism. They are involved in innate immune defense and the initiation of adaptive immunity. Upon activation, macrophages release a large set of proteases, including plasminogen activator, collagenases, elastase-like enzymes, and other hydrolases, such as lysozyme and β-glucuronidase. They also secrete high amounts of lysosomal enzymes independently of external stimuli. The activity of these hydrolases is generally low at the neutral pH of extracellular fluids, but macrophages are able to acidify the pericellular space by the activity of their plasma membrane proton pumps (V-ATPase) and secretion of lactic acid. This acidification is possibly a prerequisite for degradative processes, and the concentration

![FIGURE 6. HEBP1 processing by CTS D generates F2L.](http://www.jimmunol.org/)

**FIGURE 6.** HEBP1 processing by CTS D generates F2L. A and B, HEBP1 (6 μg) was processed by 200 ng CTS D for 5 or 30 min at 37˚C and loaded on a 10–20% polyacrylamide gradient gel before silver staining (A) or immunoblot detection with an anti-F2L Ab (B). A diffuse band was detected at 2.5 kDa, the size of F2L. This band was more intense after a short incubation time. C, The silver-stained bands were analyzed by mass spectrometry after trypsinization. The mass of the tryptic fragments identified are presented in the middle column and the corresponding region of HEBP1 protein sequence in the left column. D, Schematic representation of the cleavage sites in HEBP1 by CTS D (triangles) and of the resulting fragments detected.

**Distribution of HEBP1 and CTS D in human organs**

Expression of the mouse ortholog of HEBP1, p22HBP, was previously described in various tissues by Northern blot analysis. High expression was found in liver, kidney, and spleen. To evaluate more precisely the expression of human HEBP1, we characterized a new Ab directed against the N terminus of HEBP1 and used this Ab on tissue microarrays. For each tissue spot, we assessed an immunohistological score from 0 (no detectable expression) to 2 (high expression) and loaded on a 10–20% polyacrylamide gradient gel before silver staining (V-ATPase) and secretion of lactic acid. This acidification is possibly a prerequisite for degradative processes, and the concentration

and lymph nodes, but also in pancreatic islets and proximal kidney tubules. Lower expression was seen in seminal vesicles and Leydig cells of the testis. No expression of HEBP1 was observed in breast, prostate, intestinal segments, ovary, uterus, skin, esophagus, and thymus. Using the same approach, we evaluated the distribution of CTS D. In agreement with published data, CTS D was highly expressed in most tissues, particularly in specialized cells such as macrophages. We observed coexpression of high levels of CTS D and HEBP1 in liver, kidney, and spleen, consistent with a potential role of CTS D in HEBP1 processing in vivo.
of released enzymes is often sufficient for inducing lytic damage to other cells (37). Therefore, in the microenvironment of macrophages, the pH may be lower than in serum or extracellular fluids, resulting in the activation of released lysosomal enzymes.

The testing of conditioned media allowed us to identify CTS D as a candidate for HEBP1 processing and F2L generation, as the activity on FPR3 was totally inhibited by pepstatin A, an aspartyl protease inhibitor, as well as by transfection of siRNAs targeting CTS D transcripts. Moreover, CTS D is described as an endopeptidase cleaving mainly after hydrophobic amino acids, particularly leucine and phenylalanine. This is consistent with the VL/SK motif as the cleavage site for F2L generation. CTS D is a lysosomal aspartic endopeptidase that plays an essential role in cell homeostasis by degrading aging proteins in the lysosomal compartment and recycling their components. This enzyme is also involved in the activation or inhibition of various hormones, growth factors, and enzymes, participates in Ag processing, and is involved in the regulation of programmed cell death (reviewed in Refs. 38–40). Deregulated CTS D activity (altered expression, increased secretion, or unbalance between protease and endogenous inhibitors) is a factor contributing to various diseases characterized by a chronic inflammatory state, such as cancer, asthma, atherosclerosis, Alzheimer’s disease, periodontitis, rheumatoid arthritis, inflammatory bowel disease, osteoarthritis, and pulmonary fibrosis (reviewed in Ref. 41).

In our hands, human purified CTS D cleaved, in less than 5 min, HEBP1 at two main sites, leading to the generation of N-terminal peptides. One of these peptides was identified by mass spectrometry as F2L, the endogenous ligand of FPR3. At this point, we cannot exclude the possibility that other proteases might generate F2L or other HEBP1-derived peptides active on FPR3. A peptide corresponding with the first 50 aa of HEBP1 was indeed identified in extracts from porcine spleen (21), although displaying lower potency than F2L on FPR3. F2L and other related peptides might, however, act together in leukocyte recruitment.

CTS D is produced by almost all cells but is expressed at particularly high levels by macrophages in some tissues, such as liver, spleen, and kidney. In contrast, our immunohistochemical screening of tissues has shown that HEBP1 is not expressed ubiquitously but rather specifically in liver, spleen, lymph nodes, as

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**FIGURE 7.** Leukocyte chemotaxis toward HEBP1 processed by CTS D. A. A checkerboard chemotaxis assay was conducted on human monocyte-derived macrophages. The product of HEBP1 hydrolysis by CTS D was added to the upper and/or lower chambers. Cells were added to the upper chamber and allowed to migrate for 90 min at 37°C. The number of cells having migrated completely through the filter is indicated. B–D. Chemotaxis of human monocytes (B), monocyte-derived macrophages (C), and mouse neutrophils (D) in response to 1 μM (human cells) or 10 μM (mouse cells) HEBP1 processed by 60 nM CTS D for 30 min at 37°C. Synthetic F2L at 10 nM (human cells) or 400 nM (mouse cells) was used as positive control of migration. The displayed responses are representative of three experiments, and results are expressed as the ratio of migrated cells in the presence versus the absence of chemoattractant. **p < 0.01, ***p < 0.001.

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**FIGURE 8.** HEBP1 immunodetection on tissue microarrays. A. Overview of a tissue microarray section. A rectangle of 15 cores corresponds with one organ; each column corresponds with one donor, and the three lines correspond with core replicates. B. Immunohistochemical staining was scored 0 for no expression (left panel), 1 for low expression or dispersed cells (middle panel, arrows), and 2 for high expression of HEBP1 (right panel). Original magnification ×200. Arrows indicate stained cells.
well as in kidney and pancreas, where CTS D is also highly expressed. The correlated expression of HEBP1 and CTS D in several tissues supports the physiological relevance of HEBP1 processing by CTS D.

HEBP1 appears as a good substrate for CTS D, but we cannot exclude a more complex pathway in vivo leading to the production of FPR3 ligands, with the contribution of other proteases acting in parallel or in cascade. A similar situation prevails for other bioactive peptides such as hemorphins (42), a family of small oligopeptides (4-mers to 10-mers) displaying high affinity for atypical peptides such as hemorphins (42), a family of small oligopeptides (4-mers to 10-mers) displaying high affinity for atypical μ opioid receptors. The mechanism of their generation is still not well understood, but the first step is likely the hydrolysis of hemoglobin β-chain by CTS D. Then, other hemorphin variants are produced by other proteases, including DPP-IV (43) and ACE (44). In this system, macrophages cultured in an acidic environment are able to proteolyze hemoglobin and generate hemorphins (45).

In this study, we showed that the F2L peptide, derived from the processing of nanomolar concentrations of HEBP1, is active on FPR3 and able to induce the recruitment of FPR3-expressing monocytes, monocyte-derived macrophages, as well as mouse neutrophils. It is, however, still unclear how cytosolic HEBP1 can give rise to an extracellular chemoattractant factor. HEBP1 is indeed clearly devoid of signal peptide, and all available data point toward an intracellular localization. This raises the question of how HEBP1 or F2L are secreted or otherwise released from cells.

First, HEBP1 might be processed inside the cell and generate F2L, in a process such as apoptosis. Apoptosis is traditionally characterized by the maintenance of organelle integrity and condensation and fragmentation of DNA, ending in the breaking up of the cell into apoptotic bodies. It can occur through numerous pathways, all converging to the activation of the caspase family of proteases. Recent studies have described a significant role for CTS D in the apoptosis of inflammatory cells such as neutrophils. The cleavage of activated neutrophils, essentially by macrophages, is a crucial step in the resolution of inflammation. In this context, CTS D was proposed as a key initiator of apoptosis in neutrophils, through the activation of caspase-8 (39, 46). In this model, loss of lysosomal integrity and release of CTS D in the cytosol were described as primary events in the induction of apoptosis (47). The generation of F2L might therefore take place in apoptotic cells, but the peptide would still require to be secreted to recruit macrophages, thereby contributing to the resolution of inflammation.

HEBP1 might also be released from the cell through a non-conventional secretory pathway or as a result of necrosis. Several distinct nonconventional secretory mechanisms have been described (reviewed in Ref. 48). IL-1α is myristoylated, then translocates to the cell membrane and is released by calpain-induced proteolysis. IL-1β is generated from its precursor by the IL-converting enzyme, a member of the caspase family. It then enters the endosomal compartment via the ABCA1 transporter, before being sorted and exported by secretory vesicles. In the case of F2L, if full-length HEBP1 was released from cells, proteolysis should occur in the extracellular compartment in the presence of extracellular CTS D. A similar situation would prevail if HEBP1 is released when cells undergo necrosis. Necrotic death is associated with the release of the cellular content in the extracellular space. Dying cells and surrounding live cells release a set of inflammatory mediators, which recruit phagocytes (such as neutrophils and macrophages) that clear the cell debris. Under physiological conditions, CTS D is sorted to the lysosomes and found intracellularly. However, in some physiological and pathological conditions, CTS D is secreted by various cell types. Indeed, procathepsin D was found in human, bovine, and rat milk (49), serum, sweat (50), and urine. It is also secreted at high levels in tumors (reviewed in Refs. 51, 52) and atherosclerotic lesions (53). In these pathologies, F2L could participate in the inflammatory response by recruiting leukocytes expressing FPR3, such as macrophages, DCs, and eosinophils.

In conclusion, in this work we identified a protease able to process HEBP1 and to generate biologically active F2L peptide. F2L is the only endogenous ligand specific for FPR3 and chemoattractic for FPR3-expressing leukocyte populations. Further investigations will be required to determine in which physiological and pathological conditions F2L is produced by CTS D. One such condition is possibly apoptosis, in which F2L could contribute to the resolution of inflammation. Alternatively, F2L might be generated extracellularly by CTS D in pathological conditions such as cancer or atherosclerosis. In this context, understanding the mechanisms leading to HEBP1/F2L secretion or release from the cells will be important.

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Disclosures
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