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Prointerleukin-18 Is Activated by Meprin β *in Vitro* and *in Vivo* in Intestinal Inflammation*

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Sanjita Banerjee and Judith S. Bond[1]

From the Department of Biochemistry and Molecular Biology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

Interleukin-18 (IL-18), a pro-inflammatory cytokine, is a key factor in inflammatory bowel disease (IBD). Caspase-1 activates this cytokine, but other proteases are likely involved in maturation. Because meprin metalloproteinases have been implicated in IBD, the interaction of these proteases with proIL-18 was studied. The results demonstrate that the meprin β subunit of meprins A and B cleaves proIL-18 into a smaller 17-kDa product. The cleavage is at the Asn^{51}–Asp^{52} bond, a site C-terminal to caspase-1 cleavage. The cleavage occurred *in vitro* with a K_m of 1.3 μM and in Madin-Darby canine kidney cells transfected with meprin β when proIL-18 was added to the culture medium. The product of meprin B cleavage of proIL-18 activated NF-κB in EL-4 cells, indicating that it was biologically active. To determine the physiological significance of the interactions of meprins with proIL-18, an experimental model of IBD was produced by administering dextran sulfate sodium (DSS) to wild-type and meprin β knock-out (βKO) mice, and the serum levels of active IL-18 were determined. DSS-treated meprin βKO mice had lower levels of the active cytokine in the serum compared with wild-type mice. Furthermore, in meprin αKO mice, which express meprin β but not α, active IL-18 was elevated in the serum of DSS-treated mice compared with wild-type mice, indicating that the meprin isoforms have opposing effects on the IL-18 levels *in vivo*. This study identifies proIL-18 as a biologically important substrate for meprin β and implicates meprins in the modulation of inflammation.

Interleukin-18 (IL-18)[2] is a pro-inflammatory cytokine that is similar in structure to IL-1β, and both cytokines play important roles in innate host defense and inflammatory processes (1). These two cytokines are synthesized as inactive proforms that lack signal sequences and are expressed in a variety of cells, including monocytes, macrophages, and epithelial cells. Caspase-1 has been identified as one of the proteases able to generate biologically active mature forms of IL-18 and IL-1β in response to a signal such as endotoxin (1, 2). The relationship between cytokine activation and exit from the cytosol is unresolved, and the cytokines in their proforms can be released from the cell in the absence of proteolytically functional caspase-1 (3, 4).

Meprins, tissue-specific metalloproteinases, are highly expressed in intestinal and kidney epithelial cells and in the epidermis (5, 6). They are normally found extracellularly at the apical membrane of these cells or secreted into the lumen of these tissues (7, 8). Meprins are also expressed in leukocytes of the lamina propria of human inflammatory sites and in mouse mesenteric lymph nodes during intestinal inflammation, and they are found at high concentrations in human urine in women with urinary tract infections (5, 7, 9). The localization of meprins at the interface with the external environment, in leukocytes at inflammatory sites, and in response to bacterial infections implicates them in host defense. Furthermore, the observation that leukocytes from mice lacking the meprin β gene have a diminished ability to disseminate through the extracellular matrix indicates that meprins affect leukocyte infiltration (9). *In vitro* studies have demonstrated that meprins are capable of cleaving extracellular matrix proteins such as laminin, collagens IV and V, fibrinogen, and cytokines such as osteopontin, bradykinin, and MCP-1 (monocyte chemotactic protein-1) (10–12). Thus, meprins are capable of cleaving a variety of substrates that are intimately involved in the host response to injury.

Meprins are composed of two multidomain subunits (α and β) that are encoded on different chromosomes (α on human chromosome 6 and mouse chromosome 17; β on chromosome 18 in humans and mice) and share ~40% amino acid identity. Despite the fact that the subunits are evolutionarily related, they have different substrate specificities and different structural properties. For example, the meprin β subunit has a preference for cleavage of peptide bonds flanked by negatively charged amino acids, whereas the meprin α subunit cleaves at bonds flanked by small or hydrophobic amino acids (11). Both subunits contain signal sequences that direct the proteins to the secretory pathway, prosequences that keep the enzymes inactive, proteolytic domains of the "astacin family of metalloproteinases," and other C-terminal non-catalytic domains (13). The subunits form homo- or heteromeric dimers that are linked by disulfide bonds (14). During biosynthesis, the meprin α subunit is proteolytically cleaved near the C terminus and thus loses its epidermal growth factor-like, transmembrane, and cytoplasmic domains. Thus, dimers of meprin α are

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[1] To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Pennsylvania State University College of Medicine, 500 University Drive, H171, Hershey, PA 17033. Tel.: 717-531-8586; Fax: 717-531-7072; E-mail: jbond@psu.edu.

[2] The abbreviations used are: IL-18, interleukin-18; IBD, inflammatory bowel disease; DSS, dextran sulfate sodium; MS/MS, tandem mass spectrometry; MDCK, Madin-Darby canine kidney; KO, knock-out; WT, wild-type; PR-3, proteinase-3.

secreted into the extracellular space. The meprin β subunit retains its epidermal growth factor, transmembrane, and cytoplasmic domains and moves to the plasma membrane as a type 1 membrane-bound protein (15). As a consequence, homo-oligomers of meprin α (called homomeric meprin A) are secreted into the extracellular space; heterodimers of meprins α and β (heteromeric meprin A) form tetramers and are membrane-bound; and homo-oligomers of meprin β form membrane-bound dimers (meprin B) (5, 16).

The interactions of meprin isoforms with cytokines have not been studied systematically. It has been demonstrated, however, that meprin B is capable of activating IL-1β *in vitro* (17). Because of the structural similarity between IL-1β and IL-18 and the importance of these cytokines in inflammatory processes, we chose to determine whether the latter is also a substrate for meprin β in the heteromeric isoform of meprin A and homomeric meprin B. Furthermore, studies aimed to determine whether meprins generate biologically active forms of the cytokine using a cell-based functional assay that tested the ability of meprin β-processed IL-18 to activate NF-κB. In addition, we asked whether mice lacking the meprin β gene produce altered levels of active IL-18 in an experimental model of inflammatory bowel disease (IBD) induced by dextran sulfate sodium (DSS). Several groups have reported high levels of IL-18 in patients with active IBD, and this observation is mirrored in various mouse models of experimental IBD, where intestinal damage can be attenuated by blocking IL-18 (18–21). The results indicate that the meprin β subunit of heteromeric meprin A and homomeric meprin B is able to generate a physiologically active form of IL-18 and that this subunit affects the levels of active IL-18 *in vivo* in a mouse model of IBD.

EXPERIMENTAL PROCEDURES

Expression and Purification of ProIL-18—Full-length mouse IL-18 containing an N-terminal histidine tag with a thrombin cleavage site in the linker region was constructed in a bacterial expression system. ProIL-18 obtained as pCR3.1::IL-18 (a gift from Camille Locht, Institute Pasteur de Lille) was cloned into pET30::IL-18 using NcoI-EcoRI restriction enzyme sites. The induced protein showed anomalous mobility and, upon further purification, gave unsatisfactory yield. Therefore, proIL-18 was subcloned into pET28a. NcoI of pET30::IL-18 was mutated to an NdeI site by site-directed mutagenesis using primers 5′-CGA CGA CGA CGA CAA CAT ATG GCT GCC ATG TCA G-3′ (sense) and 5′-C TGA CAT GGC AGC CAT ATG TTG TCG TCG TCG TCG-3′ (antisense) to generate pET30::IL-18-NdeI. ProIL-18 was excised and inserted into pET28a using NdeI-EcoRI to generate pET28a::IL-18 (Fig. 1*A*).

Recombinant murine proIL-18 with an N-terminal histidine tag (His_6-proIL-18) was transformed into the *Escherichia coli* BL21(DE3)-RIL Codon Plus strain and purified from bacterial extracts, 4 h after induction with 1 mM isopropyl β-D-thiogalactopyranoside, using nickel-nitrilotriacetic acid matrix (Qiagen Inc.) under native purification conditions (Fig. 1*B*). Fractions containing His_6-proIL-18 were pooled and treated with thrombin-agarose using a RECOMT Thrombin CleanCleave kit (Sigma) to remove the N-terminal linker region. Uncut His_6-proIL-18, as well as the histidine tag, was removed by subjecting

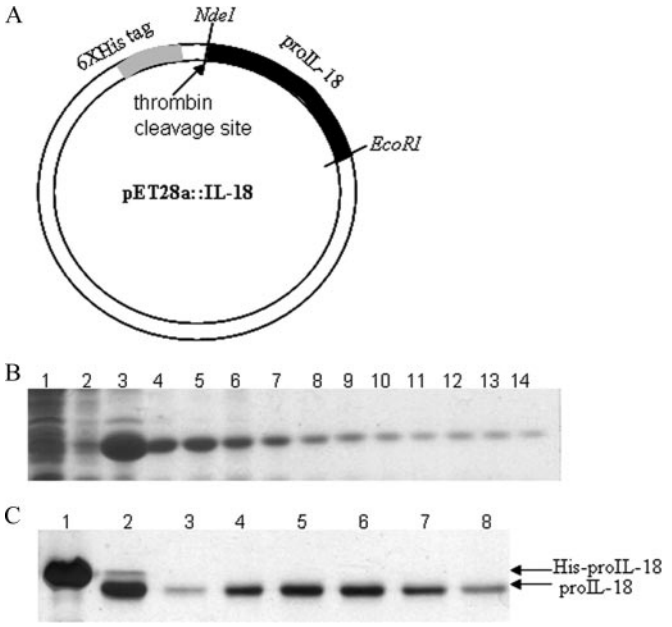

FIGURE 1. **Expression and purification of proIL-18.** *A*, vector map of pET28a::IL-18. ProIL-18 was cloned at the NdeI-EcoRI sites. The N-terminal 6-histidine tag and thrombin cleavage site are indicated. *B*, Coomassie Blue staining of Ni^{2+} purification of His_6-proIL-18 after isopropyl β-D-thiogalactopyranoside induction. *Lane 1*, sample added to Ni^{2+} column; *lane 2*, flow-through fraction; *lanes 3–14*, eluate fractions. *C*, silver staining of purified proIL-18 before and after thrombin cleavage. *Lane 1*, His_6-proIL-18; *lane 2*, thrombin-cleaved proIL-18; *lanes 3–8*, eluate fractions after Ni^{2+} purification.

the protein to another round of Ni^{2+} purification (Fig. 1*C*). Protein samples after each treatment were subjected to 12% SDS-PAGE and visualized by Coomassie Blue or silver staining. The protein concentrations were determined using a Micro-BCA kit (Pierce).

Activation of Recombinant Meprins—Secreted forms of recombinant histidine-tagged mouse meprin α (homomeric meprin A), rat meprin β (meprin B) and rat meprin $\alpha\beta$ (heteromeric meprin A) were purified from stably transfected HEK-293 cells overexpressing the respective proteins as described previously (22, 23). Meprin α and β subunits are secreted as disulfide-linked dimers. One molar unit of homomeric meprin A and meprin B was an α/α and β/β dimer, respectively; 1 molar unit of heteromeric meprin A was a $\beta\alpha/\alpha\beta$ tetramer. Meprin isoforms (1–4 μM) were activated by limited proteolysis by trypsin (20:1 molar ratio) at 37 °C for 1 h and passed through a Sephadex G-25 column (Sigma) to remove trypsin. Activities of meprin α and β subunits were assayed using their respective fluorogenic peptide substrates, BK^+ (a bradykinin analog; 2-aminobenzoyl-RPPGFSPFRK-(dinitrophenyl)-G-OH) and OCK^+ (an orcokinin and cholecystokinin analog; 2-aminobenzoic acid-MGWMDEIDK-2,4-dinitrophenyl-SG-OH), as described previously. Actinonin (3.5 μM) was used to inhibit the activities of both subunits (22).

Cleavage of ProIL-18 by Meprins—ProIL-18 (2.2 μM) was incubated with homomeric meprin A (0.11 μM) and heteromeric meprin A (0.11 μM) and meprin B (0.11 μM or 2 nM) in 20 mM Tris-Cl and 50 mM NaCl (pH 7.5) in a total reaction volume of 50 μl for up to 12 h at 37 °C. The reaction was stopped by addition of EDTA (5 μM), and the samples were boiled in SDS-PAGE loading buffer containing β-mercaptoethanol. The reac-

tion mixtures were subjected to 15% SDS-PAGE under reducing conditions, and proteins were visualized by silver staining or transferred onto nitrocellulose membranes. The membranes were probed with rabbit anti-mouse IL-18 polyclonal antibody (Rockland Immunochemicals, Inc.) followed by horseradish peroxidase-conjugated donkey anti-rabbit IgG (GE Healthcare), and the signal was detected by chemiluminescence using SuperSignal Dura substrate (Pierce). The detection limit for the anti-IL-18 antibody was 50 ng. For meprin B inhibition studies, reaction mixtures were incubated in the presence or absence of actinonin (35 μM) at 37 °C.

Identification of the ProIL-18 Site of Cleavage—ProIL-18 (2.2 μM) incubated with meprin B (0.11 μM) for 30 min was subjected to 15% SDS-PAGE under reducing conditions, and proteins were detected by silver staining. The cleavage product was excised and prepared for mass spectrometry. After destaining, the sample was alkylated with 55 mM iodoacetamide and in gel-digested with mass spectrometry-grade trypsin gold (Sigma). The resultant peptides were extracted with 50% acetonitrile and 0.1% trifluoroacetic acid and suspended in 0.5% trifluoroacetic acid. The sample was added to a C18 nanoflow column and then subjected to tandem mass spectrometry (MS/MS). The peaks obtained were searched against the theoretical peaks generated by proIL-18 trypsin digestion using FindPept (ExPASy), and the peak list was submitted for data base searching using the MASCOT MS/MS Ions Search computer program (Matrix Sciences) with parameters set for semi-trypsin digestion. The identified peak was further subjected to collision-induced dissociation followed by MS/MS for sequence analysis.

Kinetic Measurements—The rate of product formation of proIL-18 by cleavage by meprin isoforms was determined using quantitative Western analysis. ProIL-18 (0.5–8 μM) was incubated with 0.1 μM meprin B or heteromeric meprin A at 37 °C in total volume of 8 μl in 20 mM Tris-Cl and 50 mM NaCl (pH 7.5) for varying times (0–5 min). Samples of the incubated mixtures were subjected to SDS-PAGE, and product formation was quantified using Quantity One software (Bio-Rad). The rates of product formation were plotted against substrate concentration using GraphPad Prism to calculate K_m and V_{max}. The catalytic constant (k_{cat}) and specificity constant (k_{cat}/K_m) were further calculated.

Meprin β Transfection and IL-18 Interaction in Madin-Darby Canine Kidney (MDCK) Cells—MDCK cells were grown in minimum essential medium with Earle's salts supplemented with HEPES (0.049 g/liter) and 10% (v/v) fetal bovine serum. Full-length rat meprin β cDNA was transfected into MDCK cells grown to 30% confluence in a 24-well plate using Lipofectamine 2000 (Invitrogen). Meprin β was activated by limited trypsin proteolysis 36 h after transfection. MDCK cells were washed twice with serum-free medium; 10 μl of trypsin (1 mg/ml in 50 mM Tris-Cl (pH 7.5)) was added to 1 ml of serum-free medium and incubated at 37 °C for 30 min. The medium was removed, cells were washed with serum-free medium, and 10 μl of soybean trypsin inhibitor (2 mg/ml in water) was added in 1 ml of serum-free medium and incubated for 30 min at 37 °C. MDCK cells were washed again before addition of proIL-18 (1 μg in 1 ml of serum-free minimum essential medium) and incubated at 37 °C for 22 h. Controls were subjected to the same treatment without trypsin. The culture media were collected, subjected to 15% SDS-PAGE, transferred to nitrocellulose, and probed with anti-IL-18 polyclonal antibody. The cells were washed with phosphate-buffered saline and sonicated, and proteins were separated on 7.5% polyacrylamide gels and probed with anti-meprin β antibody.

NF-κB Activation in EL-4 Cells by IL-18—IL-18 bioactivity was tested as a function of NF-κB activation in EL-4 cells. EL-4 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) horse serum. The cells were washed with phosphate-buffered saline and suspended in serum-free medium to a concentration of 1 million cells/ml. ProIL-18 (2.2 μM) and meprin B (0.11 μM) was preincubated for 10 min at 37 °C, and 200 ng of meprin B-treated proIL-18 was added to 1 ml of EL-4 cells and incubated for 30 min. Control incubation mixtures contained meprin B alone or proIL-18 alone at equivalent amounts of respective proteins. Cell lysates were prepared, and total protein was quantified using a Micro-BCA kit. Total protein (50 μg) was used for the NF-κB activation assay using a p65 TransAM kit (Active Motif), and absorbance was determined at 450 nm. Nuclear extracts of Jurkat cells served as the positive controls for the NF-κB assay.

Induction of IBD and Measurement of Serum IL-18 by Enzyme-linked Immunosorbent Assay—IBD was induced in 8–9-week-old male meprin α knock-out (αKO) mice (on a C57BL/6×129 mixed background), meprin βKO mice (on a congenic C57BL/6 background), and their corresponding wild-type (WT) mice by administering 3.5% DSS in their drinking water for 4 days and normal water on the 5th day (9). Control groups received normal drinking water over the 5-day study period. Mice ($n = 5$) from all of the different groups were killed by inhalation of isoflurane followed by cervical dislocation, and blood was collected by cardiac puncture into an EDTA-coated Microvette (Sarstedt). The blood sample was centrifuged at 10,000 rpm for 10 min at 4 °C to obtain serum. The serum was tested for active IL-18 levels using a murine IL-18 enzyme-linked immunosorbent assay system (MBL International Corp.). The antibodies in this assay recognize only the active form of IL-18. The detection limit of this assay is 25 pg/ml.

Statistical Analysis—The unpaired two-tailed t test was used for all statistical analysis. p values <0.05 were considered significant.

RESULTS

Interaction of Meprins with ProIL-18—ProIL-18 was incubated with purified preparations of activated recombinant meprins, and the products were separated by SDS-PAGE (Fig. 2, *A* and *B*). Both heteromeric meprin A and meprin B cleaved proIL-18 (24 kDa) and generated a 17-kDa fragment (Fig. 2, *A* and *B*, *lanes 4–6* and *7–9*, respectively). Little or no decrease in the amount of proIL-18 or the appearance IL-18 fragments was detected when proIL-18 was incubated with homomeric meprin A (Fig. 2, *A* and *B*, *lanes 1–3*; and in repeated experiments not shown). In addition, repeated experiments showed meprin B to be capable of generating greater amounts of the 17-kDa cleaved product than hetero-

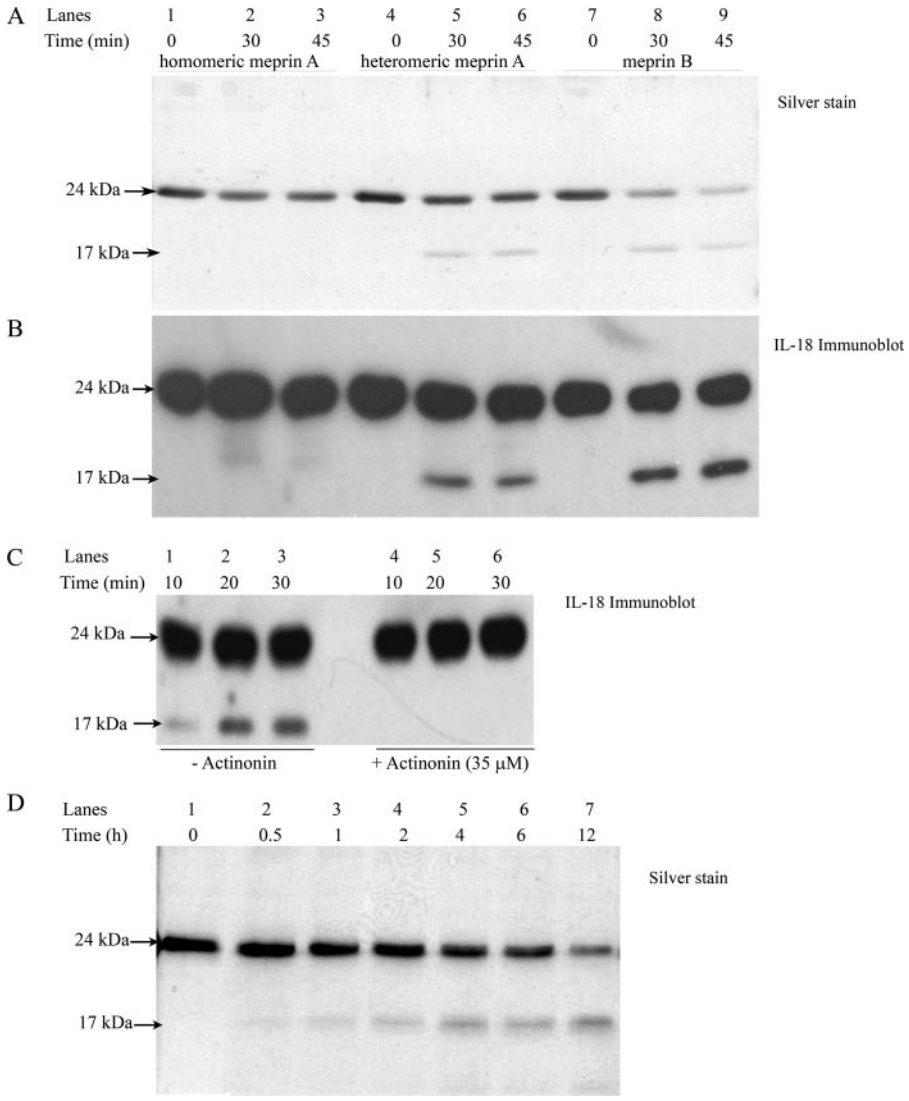

FIGURE 2. **Cleavage of proIL-18 by meprin isoforms.** *A* and *B*, proIL-18 (2.2 μM) was incubated with meprin isoforms (0.11 μM) as described under "Experimental Procedures." The mixtures were subjected to SDS-PAGE, and the substrates and products were visualized by silver staining (*A*) or by immunoblotting using a polyclonal antibody to IL-18 (*B*). Meprin isoforms were homomeric meprin A (*lanes 1–3*), heteromeric meprin A (*lanes 4–6*), and meprin B (*lanes 7–9*). *C*, the hydrolysis of proIL-18 by meprin B was inhibited when actinonin (35 μM) was included in the incubation mixture. *D*, proIL-18 (2.2 μM) was incubated with 2 nM meprin B for up to 12 h, and the proteins were visualized by silver staining.

TABLE 1

Kinetic constant determination of proIL-18 cleavage

ProIL-18 (0.5–8.0 μM) was incubated with meprin B or heteromeric meprin A (0.1 μM) in 20 mM Tris-Cl and 50 mM NaCl (pH 7.5) for varying times (0–5 min). Kinetic parameters were calculated by quantitative Western analysis by directly fitting the rate of product formation against substrate concentration to the Michaelis-Menten equation. Meprin B had a significantly lower K_m and k_{cat} (*, $p = 0.004$) and a higher k_{cat}/K_m (**, $p = 0.05$) compared with heteromeric meprin A ($n = 3$).

	K_m	k_{cat}	k_{cat}/K_m
	$M \times 10^{-6}$	s^{-1}	$(M^{-1} s^{-1}) \times 10^6$
Meprin B (meprin β/β)	1.31 ± 0.001*	6.79 ± 0.55*	5.19 ± 0.47**
Heteromeric meprin A (meprin βα/αβ)	5.49 ± 0.27	16.44 ± 0.24	3.0 ± 0.19

meric meprin A under the same reaction conditions (Fig. 2, *A* and *B*, compare *lanes 5–6* and *8–9*). At meprin B concentrations of 0.11 μM or higher and during prolonged incubations, meprin B was capable of degrading the 17-kDa product (Fig. 2*A*, *lane 9*). However, at lower meprin B concentrations (*e.g.* 2 nM), the 17-kDa product accumulated over a 12-h time period (Fig. 2*D*). Actinonin, an inhibitor of meprins A and B, clearly inhibited the cleavage of IL-18 (Fig. 2*C*, *lanes 4–6*).

Biochemical Characterization of ProIL-18 Cleavage by Meprins—The rate of IL-18 product formation (generation of the 17-kDa fragment) over time was determined by quantitative Western blotting and densitometric analysis. The kinetic constants K_m and k_{cat} and the specificity constant k_{cat}/K_m for the reactions were calculated by directly plotting the rates of product formation against substrate concentration using the Michaelis-Menten equation by nonlinear regression analysis (Table 1). Meprin B had a significantly lower K_m (~5-fold) and a slightly greater k_{cat}/K_m for hydrolysis of proIL-18 compared with heteromeric meprin A, indicating greater affinity and efficiency.

Identification of the Site of Cleavage of ProIL-18 by Meprin B—To identify the site of proIL-18 cleavage, the products of proIL-18 cleaved by meprin B were separated by electrophoresis as described under "Experimental Procedures." A peak corresponding to the C terminus of IL-18 was found in the spectrum, indicating that the cleavage removed an N-terminal fragment from the protein. Comparison of the peaks generated against the theoretical peaks predicted for limited trypsin digestion of proIL-18 revealed a 1119-Da fragment, which could result from a semi-trypsin digestion (*i.e.* a trypsin cleavage on the C terminus and a non-trypsin cleavage on the N terminus). The amino acid sequence of the fragment was identified by further subjecting it to collision-induced dissociation followed by MS/MS. From the set of y ions thus generated, the sequence was deduced to be (N)DQVLFVDKR(Q) (Fig. 3). The identified cleavage is consistent with previously established substrate preferences for meprin B, *i.e.* an acidic residue in the P_1' position (11). The meprin B cleavage site was distinct from caspase-1, caspase-3, and proteinase-3 (PR-3) cleavage sites, as shown in Fig. 3. Caspase-1 and PR-3, which cleave N-terminally to the meprin β cleavage site, activate proIL-18, whereas caspase-3, which cleaves C-terminally to the meprin β cleavage site, is known to inactivate IL-18 (1). Therefore, it was important to determine how meprin B affects IL-18 activity.

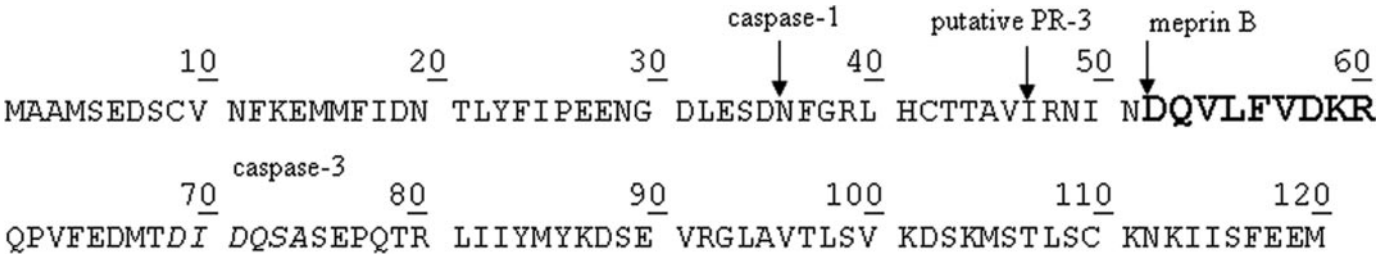

FIGURE 3. **Identification of the proIL-18 site cleaved by meprin B.** The meprin B cleavage site is indicated, and the nine-amino acid sequence identified by MS/MS is shown in *boldface*. Caspase-1 and putative PR-3 sites are also indicated. The region of caspase-3 cleavage is *italicized* (1).

Meprin B and ProIL-18 Interaction in a Cell Culture System—To determine whether proIL-18 could be cleaved in a cell-based system, meprin β was transfected into MDCK cells. After activation of rat meprin B as described under "Experimental Procedures," proIL-18 was added to the cell culture medium. The cell culture medium was collected after 22 h, and the presence of IL-18 was assessed by immunoblotting (Fig. 4). No endogenous IL-18 was detected in the cell culture medium from MDCK cells (*upper panel*, *lanes 1* and *2*). Full-length IL-18 was detected intact after 22 h in the medium from untransfected cells, irrespective of trypsin treatment (*lanes 3* and *4*, respectively). The medium from MDCK cells expressing latent meprin B also contained comparable levels of full-length proIL-18 (*lane 5*). However, MDCK cells expressing active meprin B contained considerably less IL-18 in the culture medium (*lane 6*). The meprin B-generated 17-kDa fragment was not detected, implying that it was either taken up by the cells and/or further degraded by meprin B or other proteases.

Meprin B Cleavage Results in Activation of ProIL-18—To determine whether the meprin B cleavage product of proIL-18 was biologically active, EL-4 cells containing receptors for IL-18 were used. EL-4 cells were treated with proIL-18 alone, meprin B alone, or proIL-18 preincubated with meprin B for 30 min, and the cell lysates were assayed for NF-κB activity (Fig. 5). As IL-18 is capable of enhancing NF-κB activity (24), NF-κB activation reflects, in turn, the levels of active IL-18 present. EL-4 cells treated with proIL-18 alone showed a 1.5-fold increase in NF-κB activity over untreated EL-4 cells. There was a 2.6-fold increase in NF-κB activity in EL-4 cells when proIL-18 preincubated with meprin B was added to the cells ($p < 0.02$). Comparing the two treatments, meprin B-cleaved IL-18 had 1.8-fold higher NF-κB activity than proIL-18. These results indicate that meprin B activated proIL-18.

Meprin βKO Mice Show Decreased Levels of Serum IL-18—To determine whether meprins affect the levels of IL-18 *in vivo*, an experimental model of IBD was induced by administering DSS to mice of different meprin genotypes, and the serum levels of the pro-inflammatory cytokine were measured as described under "Experimental Procedures." Whereas both the WT and meprin βKO mice showed elevated levels of active IL-18 upon DSS treatment, the elevation was less marked in the βKO mice, and the difference between the two genotypes was significant ($p < 0.05$) (Fig. 6*A*). This was consistent with the *in vitro* data that showed that meprin B cleavage was capable of generating active IL-18. In similar experiments with the meprin αKO mice, the elevation in the levels of active IL-18 after DSS-induced colitis was significantly greater than that in the WT mice ($p <$ 0.0015) (Fig. 6*B*). This striking contrast between the two KO groups is a clear indication that the meprin B-mediated activation of proIL-18 is physiologically relevant.

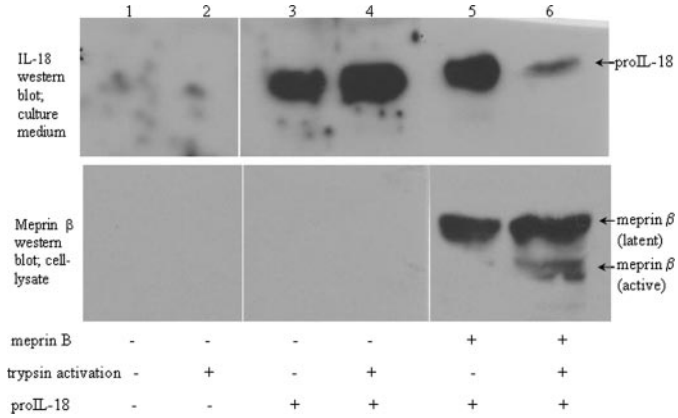

FIGURE 4. **Interaction of proIL-18 with meprin B expressed in MDCK cells.** Full-length meprin β was transfected into MDCK cells, and latent meprin B was activated with trypsin. After removal and inhibition of trypsin, proIL-18 was added to the culture medium and incubated for 22 h; the medium and the cell lysate were then subjected to SDS-PAGE. The *upper panel* is a Western blot of the culture medium using a polyclonal antibody to IL-18; the *lower panel* is a Western blot of the cell lysate fraction using a polyclonal antibody to meprin β. The results are typical of three independent experiments.

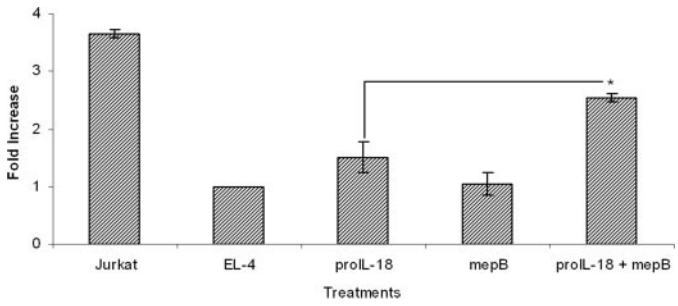

FIGURE 5. **Effect of meprin B on IL-18 activity in EL-4 cells.** NF-κB activation was used to assess IL-18 bioactivity. EL-4 cells were untreated or treated for 30 min with proIL-18 (200 ng/ml) alone, meprin B (*mepB*) alone, or proIL-18 preincubated with meprin B. Cells lysates were assayed for NF-κB activation using a p65 TransAM kit. Jurkat nuclear extract served as a positive control for NF-κB activation. NF-κB activation is shown as -fold increase over unstimulated EL-4 cells ($n = 3$; *, $p < 0.02$). Results are presented as means of three independent experiments.

DISCUSSION

The work described herein identifies proIL-18 as a good substrate for the β subunit of meprin B and heteromeric meprin A and shows that the hydrolysis results in a biologically active form of IL-18. Furthermore, deletion of the meprin β gene results in a

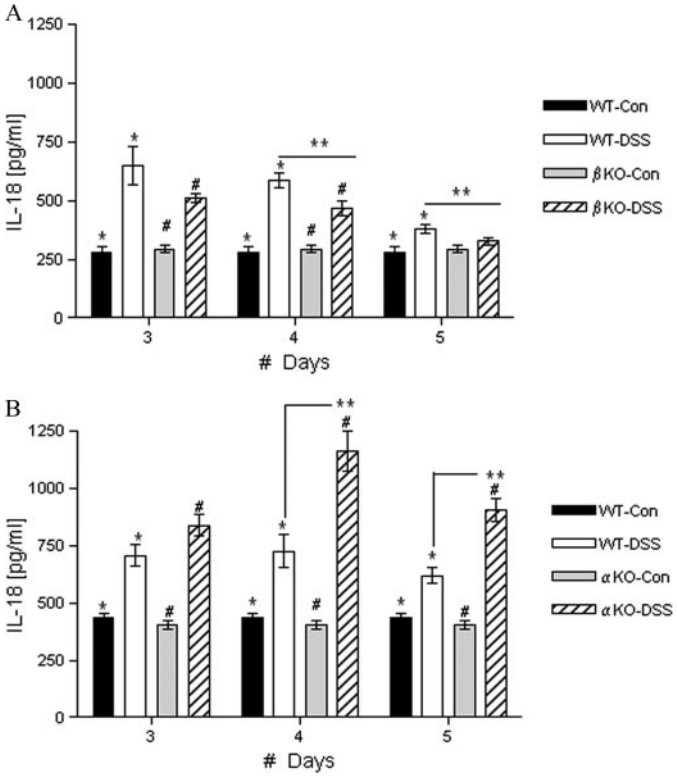

FIGURE 6. **Measurement of active IL-18 levels in the serum of DSS-treated meprin βKO and αKO mice and their corresponding WT littermates.** IBD was induced by administering 3.5% DSS in the drinking water for 4 days and water on the 5th day (9). Controls (*Con*) were given water. Serum was collected on days 3, 4, and 5, and the levels of active IL-18 were measured by enzyme-linked immunosorbent assay. *A*, WT and βKO mice had increased levels of active IL-18 compared with their respective control groups (n = five/group; *, $p < 0.02$, DSS-treated WT *versus* control; #, $p < 0.0002$, DSS-treated βKO *versus* control). Meprin βKO mice treated with DSS showed significantly lower levels of serum IL-18 compared with WT mice given DSS treatment (**, $p < 0.05$). *B*, meprin αKO mice showed significantly elevated levels of IL-18 compared with WT mice after the same DSS treatment (n = seven/group; **, $p < 0.0015$). Both DSS-treated groups showed significant elevation in their serum IL-18 levels compared with the respective control populations (*, $p < 0.0002$, DSS-treated WT *versus* control; #, $p < 5 \times 10^{-11}$, DSS-treated αKO *versus* control).

decrease in the serum levels of active IL-18 observed in response to intestinal injury, indicating the functional significance of this reaction *in vivo*. IL-18 is a marker for the severity of IBD in patients, and the cytokine contributes to the cascade of reactions that increase the pathological consequences of the chronic inflammation. Thus, meprins must be considered along with caspase-1 and PR-3 as important factors in the generation of active forms of this cytokine.

ProIL-18 is one of the best meprin B substrates identified to date (11, 23). Previously, gastrin 17, a regulatory molecule of the gastrointestinal tract, was found to be the best meprin B substrate, with a K_m of 1.0 μM and a specificity constant of 10.6×10^6 M^{-1} s^{-1} (22). The kinetic values for proIL-18 found herein (1.3 μM and 5.2×10^6 M^{-1} s^{-}) are comparable to those for gastrin and some of the best peptide substrates found for other metalloproteinases. For example, the specificity constant of TACE (tumor necrosis factor-α-converting enzyme) for a synthetic substrate corresponding to its physiological substrate, tumor necrosis factor-α, is 0.17×10^6 M^{-1} s^{-1} (25). An excellent fluorescent substrate ((7-methoxycoumarin-4-yl)acetyl-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(2,4-dinitrophenol)-NH_2) for stromelysin has a k_{cat}/K_m of 0.22×10^6 M^{-1} s^{-1} (26). In addition, the kinetic constants of meprin B for proIL-18 are similar to those of caspase-1. For meprin B, the estimated K_m for proIL-18 is 1.3 μM; for caspase-1, the K_m is 0.6 μM. The catalytic efficiency of caspase-1 is 2–3-fold greater than that of meprin (1.4×10^7 compared with 0.52×10^7 M^{-1} s^{-1}), a modest difference (27).

Homology modeling of the protease domain of the meprin β subunit showed multiple basic residues in the active site that can potentially form salt bridges with acidic residues in the substrates (11). In conformity with its preference for acidic residues in the P_1' position, meprin B cleaves proIL-18 between Asn^{51} and Glu^{52}, thereby activating the cytokine. It is somewhat unusual for a metalloproteinase to cleave peptide bonds at negatively charged residues; however, there are notable exceptions to this generality. Thus, meprin B shares the ability to hydrolyze peptide bonds at acidic residues with metalloproteinases such as aggrecanases, BMP-1, and stromelysin (28–30). Caspases, intracellular cysteine proteases, have stringent requirements for a four-amino acid peptide sequence (*XXX*D), and as mentioned above, these proteases can activate (caspase-1) or inactivate (caspase-3) IL-18. Thus, several peptide bonds in the N-terminal region of proIL-18 appear to be accessible to proteases, but the specificity of the proteases determines whether activation or inactivation results from hydrolysis.

Meprins, abundant proteases of intestinal epithelial cells and mesenteric leukocytes, are likely responsible for activating a portion of the proIL-18 secreted at intestinal sites as a result of tissue damage in IBD (31, 32). On the basis of the serum values of IL-18 in WT and βKO mice determined in the experimental model of IBD, we suggest that the β subunit of meprin isoforms contributes to ~20% of the active IL-18. There is evidence that proIL-18 is actively released from live (epithelial and leukocytic) cells and passively from dead cells, thereby exposing the procytokine to extracellular proteases. PR-3, a membrane-bound protease of the azurophil granules of polymorphonuclear neutrophils, has been shown to activate proIL-18 at the plasma membrane via a caspase-1-independent pathway (33). Clearly, in meprin βKO mice, there is a decrease in the serum levels of active IL-18 after tissue damage. Meprins may be responsible for this decrease directly or may be involved in the homeostasis of cytokine-producing cells, thereby indirectly contributing to the observed phenotype. In meprin αKO mice, only meprin β is expressed, and therefore, meprin B will be the only isoform of meprins found. Our data show that there are similar levels of expression of meprin β in WT and meprin αKO mice,[3] and when both meprin α and β subunits are expressed, most of the β subunit is found as heteromeric meprin A (16). The observation that the meprin αKO mice have higher levels of serum IL-18 compared with the WT mice implies that meprin B is more efficient than heteromeric meprin A in producing active IL-18. The *in vitro* studies indicate that meprin B has a 5-fold greater affinity for proIL-18 compared with heteromeric meprin A (1 *versus* 5 μM), and perhaps this influences the observed outcome.

There is increasing evidence that matrix metalloproteinases are intimately involved in processing cytokines *in vivo* in the

[3] S. Banerjee and J. S. Bond, unpublished data.

immune system (34). This work provides new evidence that meprins can influence the concentration of IL-18, a key cytokine in the inflammatory response, and provides insight into the molecular mechanism that effects activation of IL-18.

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