Myeloid-related Protein (MRP) 8 and MRP14, Calcium-binding Proteins of the S100 Family, Are Secreted by Activated Monocytes via a Novel, Tubulin-dependent Pathway*

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Myeloid-related protein-8 (MRP8; S100A8) and MRP14 (S100A9) are two calcium-binding proteins of the S100 family (1–3), which has grown to be one of the largest subfamilies of EF-hand proteins (4). Members of this family are defined by their homologies to two calcium-binding proteins highly enriched in nervous tissue, S100α and S100β. S100 proteins are characterized by the presence of two calcium-binding sites of the EF-hand-type, the N-terminal of which differs from the conserved EF motive by two additional amino acids. They have a relatively small molecular mass of around 10 kDa and, in contrast to other calcium-binding proteins such as calmodulin, show tissue-specific expression patterns. S100 proteins play a role in cell differentiation, cell cycle progression, regulation of kinase activities, and cytoskeletal-membrane interactions (4, 5). In addition, extracellular functions have been reported for distinct S100 proteins; S100β, the prototypic member of this family, can be found as an extracellular protein inducing neutrope extension (5, 6). S100L (S100A2) is chemotactic for eosinophils (7), whereas psoriasin (S100A7) exhibits chemotactic activity for neutrophils and CD4+ lymphocytes (8).

MRP8 and MRP14 are expressed at high concentrations by granulocytes and during early differentiation stages of monocytes but are absent in lymphocytes and mature tissue macrophages (9–12). Down-regulation of MRP8 and MRP14 expression in monocytes involves a calcium-mediated suppressor mechanism (13). Phagocytes express MRP8 and MRP14 under multiple inflammatory conditions, e.g. during rheumatoid arthritis, allograft rejections, or inflammatory bowel diseases (9, 14, 15). Inflammatory disorders as chronic bronchitis, cystic fibrosis, or rheumatoid arthritis are associated with elevated serum levels of MRP8 and MRP14 (16, 17). The close correlation between serum levels and disease activity led to the assumption that MRP8 and MRP14 are released into the extracellular medium (13). They therefore function as biomarkers in inflammatory disorders. Since the pathway of S100 protein release has not yet been elucidated in recent years several studies reported extracellular functions of MRP8 and MRP14, including antimicrobial, cytokostatic, or chemotactic activities (18–20), thus favoring an active mechanism of secretion. However, neither MRP8 and MRP14 nor any other S100 protein has a signal sequence for an active cellular secretion. However, it is not known whether all nonclassically secreted proteins use the same mechanisms of release.

Since the pathway of S100 protein release has not yet been examined (4), we intended to elucidate the molecular mechanisms associated with the release of MRP8 and MRP14 by human monocytes. Here we prove evidence that MRP8 and MRP14 are secreted after activation of protein kinase C via a novel pathway requiring an intact microtubule network.

MATERIALS AND METHODS

Cells and Cell Culture—Human peripheral blood leukocytes were obtained from buffy coats which arise during preparation of packed red blood cells. Leukocytes were isolated by Ficoll-Hypaque gradient centrifugation. Human monocytes were enriched by adherence to plastic dishes. Monocytes were used at a concentration of 10^6 cells per ml. Human peripheral blood mononuclear cells were cultivated in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% antibiotics, 1% L-glutamine, 0.1% heparin, and 2% interleukin 2 at 37 °C in 5% CO2. The cells were harvested at the indicated times and analyzed to determine the proportion of monocytes.

Human peripheral blood mononuclear cells were stimulated with a panel of compounds and lectins which are known to activate protein kinase C (21). Compounds were applied at the following concentrations: 0.3 μM 4a-phorbol 12-13-acetate; 0.6 μM PMA; 5 μM ionomycin; 0.1 μM phorbol 12-myristate 13-acetate; 1 μM tumor necrosis factor-α; 10 μM PMA; and 0.1 μM 4a-phorbol 12-13-acetate. LPS was used at a concentration of 10 μg/ml. Cells were stimulated for 15 min and 60 min, respectively. For the determination of protein kinase C activity, the cells were incubated with 10 μM bisindolylmaleimide I (BIM) and 5 μM BIM at 37 °C for 30 min. The cells were then stimulated for 15 min and 60 min, respectively. The activity of protein kinase C was determined as described previously (22). The cells were incubated with 10 μM bisindolylmaleimide I (BIM) and 5 μM BIM at 37 °C for 30 min. The cells were then stimulated for 15 min and 60 min, respectively. The activity of protein kinase C was determined as described previously (22).
blood cell concentrates. Monocytes were isolated by Ficoll-Paque and Percoll (Pharmacia, Freiburg, FRG) density gradient centrifugation or by leukopheresis of individual donors using a cell separator CS 3000 (Becton Dickinson, CA). Vimentin intermediate filaments were isolated from mouse liver (Dianova, Hamburg, FRG), actin filaments by FITC-labeled phallolidin (Sigma, Deisenhofen, FRG) and microtubuli by mAb TUB 2.1 against β-tubulin (Sigma). mAb H21 directed against S100 protein p11 (S100A10) (24) was kindly provided by Dr. V. Gerke, University of Münster. For controls, monoclonal mouse IgG, (Dianova) and polyclonal rabbit IgG (Pharmacia) of irrelevant specificity were employed. Affinity-purified goat-anti-mouse or goat-anti-rabbit secondary antibodies conjugated with either Cy3, Texas Red, or FITC were obtained from Dianova. Protein G Sepharose 4 Fast Flow was purchased from Pharmacia.

Stimulation of MR8/ MR14 Secretion—For stimulation of monocytes lipopolysaccharide (1 μg/ml, Sigma), granulocyte-macrophage colony-stimulating factor (GM-CSF, 100 units/ml; Sigma), IL-1β (10 units/ml), IL-4 (5 units/ml), IL-6 (100 units/ml; all from Calbiochem, Bad Soden, FRG), interferon-γ (50 units/ml; Boehringer Mannheim, Mannheim, FRG), 4β-phorbol-12-myristate-13-acetate (PMA, 1–100 nM), 4α-phorbol 9-myristate-9-acetate (4α-PMA, 1–100 nM), 12-deoxyphorbol 13-phenoxyacetate 20-acetate (dPPA, 1–100 nM), pertussis toxin (100 ng/ml), cholera toxin (100 ng/ml), forskolin (100 nM), and dibutyryl cAMP (50 μM, all from Sigma) were employed.

Inhibitory effects on PMA-induced MR8/MR14 secretion were analyzed by concomitant application of 10 nM PMA and either staurosporine (0.2–50 nM), H7 (0.1–30 μM), HA1004 (0.1–200 μM, all from Calbiochem), cycloheximide (10 μg/ml), carbonyl cyanide chlorphenylhydrazone (CCCP, 1 μM), dinitrophenol (1 mM), monensin (10 μM), and cytochalasin B (5 μg/ml, all from Sigma). Cell viability was assayed by trypan blue exclusion staining, by propidium iodide labeling with subsequent flow cytometry, or by determination of lactate dehydrogenase activity in the medium at the end of exposure periods as described earlier (22). Viability was found to be higher than 95% in all experiments. Lactate dehydrogenase activity in the medium differed maximally up to 20% between controls and the various treatment procedures.

Enzyme-linked Immunosorbent Assay (ELISA) for MR8/MR14, Tumor Necrosis Factor-a (TNF-α), and IL-1β—The MR8/MR14 content in the supernatants of cultured monocytes was quantified by a sandwich ELISA as described earlier (16, 22). The ELISA was calibrated with recombinant MR14 in concentrations ranging from 1 to 1000 ng/ml. Sensitivity was less than 2.5 ng/ml. The sensitivity was calculated from the concentration yielding a 50% response to the mean of four parallel determinations.

For detection of secreted IL-1β and TNF-α, Biotrak sandwich ELISA systems were obtained from Amersham-Buchler (Braunschweig, FRG) and employed according to the manufacturer’s instructions. The sensitivities were less than 5 and 1 pg/ml for TNF-α and IL-1β, respectively.

Flow Cytometry—Monocytes of culture day 1 were stimulated as described above and processed for flow cytometry. Immunostaining procedures were performed as reported earlier employing mAb 27E10 (9, 23). Surface expression was analyzed employing a FACScan (Becton Dickinson, Heidelberg, FRG) equipped with Lysis II software.

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Flow Cytometry—Monocytes of culture day 1 were harvested, washed, and preincubated in modified Eagle’s medium without methionine (Life Technologies, Inc.) at a density of 5 × 10⁶ cells/ml. Monocytes were then labeled by adding 250 μCi/ml [35S]methionine (Amersham-Buchler) to the same medium for 2 h. Medium was removed and replaced by McCoy’s 5A medium supplemented with 15% fetal calf serum. Cells (2 × 10⁶) were treated for another 4 h with either medium, 10 nM PMA, or 10 nM PMA plus 20 nM staurosporine. Thereafter, supernatants were collected, and cells were washed and lysed in phosphate-buffered saline containing 1% Nonidet P-40 and 1% phenylmethylsulfonyl fluoride (Sigma). Supernatants and lysates were prepared for immunoprecipitation as described earlier (13) using monospecific affinity-purified antisera against MR8 or MR14, mAb 27E10, mAb H21 against p11, or nonspecific isotype-matched antibodies as a control. Samples were separated by 15% SDS-polyacrylamide gel electrophoresis under reducing conditions. The relative amounts of MR8 and MR14 monomers were determined densitometrically by scanning of autoradiography bands using a Fast Scan supplied with Image Quant software (Molecular Dynamics, Sunnyvale, CA).

Immunofluorescence Microscopy—Monocytes cultured for 3 days on fibronectin (Becton Dickinson)-coated Lab-Tec chamber slides (Nunc, Wiesbaden, FRG) were either left untreated or incubated for 4 h with 10 nM PMA. In some experiments 5 μg/ml colchicine, 2 μg/ml nocodazole, 1 μM demecolcine, or 5 μg/ml cytochalasin B was added during the last hour of the incubation period. Cells were washed with phosphate-buffered saline, permeabilized by 10 nM Heps, pH 6.8, 100 μM KC1, 3 μM MgCl2, 200 mM saccharose, 1 mM phenylmethylsulfonyl fluoride (Sigma), and incubated with 0.5% Triton X-100 for 2 min. washed twice in CS buffer for 5 min, subsequently fixed with 3.7% formaldehyde in phosphate-buffered saline for 4 min and methanol for 6 min at −20 °C, and processed for single- or double-labeling immunofluorescence as described earlier (25) using a MR8, a MR14, mAb 27E10, phalloidin–FITC, mAb TUB 2.1 against β-tubulin, and mAb V9 against vimentin as primary antibodies. For double-labeling experiments staining of MR8 or MR14 with a polyclonal rabbit serum was followed by detection of cytoskeletal components employing mAbs from mice. No cross-reactivity or spillover was detected in control experiments after omitting specific antibodies or replacing them by isotype-matched control antibodies of irrelevant specificity. Fluorescence stainings were analyzed on a Zeiss photomicroscope.

Northern Blot Analysis—Total RNA of monocytes was prepared according to the guanidine hydrochloride method (13). Filters were hybridized with cDNA probes specific for MR8 or MR14 which were labeled with [32P] by a random primer method (Multiprime DNA labeling system; Amersham). Membranes were probed with cDNAs described above with stripping the blots in between employing 0.1% SDS at 95 °C. Autoradiographic bands were quantified by densitometrically scanning. Data obtained from MR8 or MR14 mRNA bands were normalized to the corresponding 18 S rRNA bands.

Statistics—The U test according to Mann-Whitney (for values without normal distribution) was performed to determine significant differences in MR8/MR14 secretion. Values of p > 0.05 was considered not to be significant.

RESULTS

Induction of MR8 and MR14 Release by Monocyte-stimulating Agents—To study the regulation of MR8 and MR14 secretion, monocytes were cultured for 1 day and subsequently stimulated with GM-CSF, IL-1β, IL-4, IL-6, interferon-γ, or lipopolysaccharide for 4 h. Thereafter, supernatants were analyzed for MR8/MR14 content by ELISA. As shown in Fig. LA, GM-CSF, IL-1β, and lipopolysaccharide induced significant MR8/MR14 release by human monocytes. MR8 and MR14 have earlier been demonstrated to assemble to noncovalently linked di-, tri-, and tetraheteromeric complexes (26, 27). A sandwich ELISA using a mAb against MR14 does not provide information about the stoichiometric ratio of hetero-meric complexes; therefore, the data presented refer to MR8/14 (MR8/MR14). The exact molecular ratio of secreted MR8 and MR14 was determined by metabolic labeling (see below). To consider nonspecific MR8 and MR14 release due to cell lysis, lactate dehydrogenase activity in the supernatant was determined concomitantly. Specific release was then presented as the ratio of MRP/lactate dehydrogenase (nanograms/unit).

To analyze intracellular signaling pathways resulting in MR8/MR14 release, monocytes were exposed to PMA, Bt2cAMP, cholera toxin, pertussis toxin, or forskolin. ELISA assays revealed a strong induction of MR8/MR14 release by PMA. Modulators of intracellular cAMP had no effect (Fig. 1B). PMA effects on MR8/MR14 release could be inhibited by the protein kinase A release inhibitor H-7 (7 μM, no effect) and by 10 μM cAMP (IC50 = 2 μM), HA-1004 (IC50 = 18 μM), and staurosporine (IC50 = 2 nM). Of these inhibitors resembled their K, regarding protein kinase C, thus confirming involvement of the latter (Fig. 2A). The PMA analogue 4aPMA which does not exhibit intrinsic activity regarding protein kinase C activation (28) had no effect on MR8/MR14 release even at 10-fold higher concentra-
trations (Fig. 2B). dPPA, an agonist of the protein kinase C isotype \( \beta_1 \) (29), the most abundant protein kinase C isoform in monocytes (30), did not affect MRP8/MRP14 release, indicating a protein kinase C subtype-specific pathway of intracellular signaling. PMA- and cytokine-induced release of MRP8/MRP14 was not associated with translocation of the latter to the cell surface as determined by flow cytometry (data not shown).

Identification of MRP8 and MRP14 Subunits in Monocyte Supernatants—To study potential complex assembly of released MRP8/MRP14, supernatants of monocytes exposed to \(^{35}\)Smethionine and stimulated with PMA were analyzed by immunoprecipitation, subsequent SDS-polyacrylamide gel electrophoresis, and autoradiography. Using a-MRP8, a-MRP14, or mAb 27E10 against the complex of MRP8/MRP14, all antibodies precipitated a similar pattern of MRP8 and MRP14, indicating that complexes of both represent the predominant extracellular form (Fig. 3, A–D). Quantification of precipitated MRP8 and MRP14 by densitometrical scanning revealed a ratio of MRP8:MRP14 of 1:3 which reflects the relative methionine content of both proteins (2 in MRP8 and 6 in MRP14). Furthermore, there was no difference between the intracellular and extracellular MRP8:MRP14 ratio, indicating that both are released at similar rates. To exclude nonspecific release of MRP8 and MRP14 into the supernatant, parallel immunoprecipitation experiments were performed for p11, another member of the S100 family expressed by myelomonocytic cells (4). As demonstrated in Fig. 3E, p11 expression was induced by exposure to PMA. In contrast to the strong \(^{35}\)Smethionine incorporation into intracellular p11, no p11 could be detected in the supernatant of PMA-treated monocytes. This observation adds to the evidence that PMA-stimulated cells were viable and that MRP8 and MRP14 were selectively released.

Molecular Mechanisms of MRP8/MRP14 Secretion—In the next set of experiments we analyzed molecular mechanisms involved in PMA-induced MRP8/MRP14 release. To evaluate dependence of PMA-induced MRP8/MRP14 secretion on de novo protein synthesis, monocytes were exposed to cycloheximide. Treatment with this protein synthesis inhibitor did not affect the amount of MRP8 and MRP14 in the supernatant.
Furthermore, double-labeling experiments were performed and showed a completely different pattern (data not shown).

In contrast, perturbation of the actin filament system by cytochalasin B had no effect (Fig. 4B). To confirm the latter observations, we performed experiments using immunofluorescence microscopy. In nonstimulated cells, MRP8 and MRP14 show a diffuse staining pattern, whereas treatment with PMA resulted in a filamentous MRP8/MRP14 distribution, which resembled the tubulin network (Fig. 5). Inhibition of tubulin polymerization by nocodazole, colchicine, or demecolcine, however, significantly suppressed release of MRP8/MRP14 into the supernatants (Fig. 4B).

Depolymerization of microtubules by demecolcine (Fig. 6A) resulted in a decrease of PMA-induced TNF-α release. In contrast, IL-1β release was slightly increased by these agents. Thus, MRP8 and MRP14 secretion is different from both the IL-1-like alternative and the classical pathways of secretion, respectively. Brefeldin A and monensin, known to block the vesicular traffic at the ER and Golgi level, significantly inhibited PMA-induced TNF-α release (Fig. 7).

Effects of Brefeldin A, Monensin, and Microtubule-depolymerizing Drugs on Secretion of IL-1β and TNF-α—We then compared the mode of MRP8/MRP14 release with that of IL-1β and that of TNF-α, representatives of an alternative and the classical pathways of secretion, respectively. Brefeldin A and monensin, known to block the vesicular traffic at the ER and Golgi level, significantly inhibited PMA-induced TNF-α secretion, whereas IL-1β release was up-regulated or unaffected, respectively (Fig. 7). Concomitant treatment with microtubule-depolymerizing agents, such as demecolcine and nocodazole, resulted in a decrease of PMA-induced TNF-α release. In contrast, IL-1β release was slightly increased by these agents.
Monocytes cultured for 1 day were incubated for 4 h with 10 nM PMA or medium as control. Activation of protein kinase C led to down-regulation of both MRP8 and MRP14 mRNA. The time course of MRP8 and MRP14 accumulation into the supernatant was closely paralleled by down-regulation of MRP8 and MRP14 mRNA (Fig. 8A). Accordingly, an inverse concentration-response relationship regarding MRP8/MRP14 release and mRNA expression was observed over a range of 1 to 100 nM PMA (Fig. 8B).

**DISCUSSION**

Earlier studies reported different extracellular functions of S100 proteins but did not provide any information on the mechanisms of their release (4, 5, 31). This is quite remarkable since neither MRP8 and MRP14 nor any other member of the S100 family comprise signal sequences that would determine their secretion via the classical ER/Golgi route.

We now demonstrate that MRP8 and MRP14 are released from monocytes during inflammatory activation via a novel secretory pathway. Mechanisms leading to MRP8/MRP14 release were shown to be energy-dependent and to involve protein kinase C activation. Employing metabolic labeling, both MRP8 and MRP14 were demonstrated to be secreted at similar rates and as complexes.

Potential nonspecific release of cytoplasmatic MRP8/MRP14, secondary to toxic effects of PMA, could be excluded at several levels. (i) Cell viability did not change significantly during the experimental procedures. (ii) Concomitant treatment with PMA and several potentially toxic agents inhibited release of MRP8 and MRP14, which is incompatible with a mere nonspecific release due to toxic cell damage. (iii) p11, another member of the S100 family, did not appear in the supernatant, despite intracellular up-regulation after PMA treatment.

The blockade of vesicular traffic through the ER and Golgi did not affect MRP8/MRP14, secondary to toxic effects of PMA, could be excluded at several levels. (i) Cell viability did not change significantly during the experimental procedures. (ii) Concomitant treatment with PMA and several potentially toxic agents inhibited release of MRP8 and MRP14, which is incompatible with a mere nonspecific release due to toxic cell damage. (iii) p11, another member of the S100 family, did not appear in the supernatant, despite intracellular up-regulation after PMA treatment.

The blockage of vesicular traffic through the ER and Golgi did not affect MRP8 and MRP14 release, thus ruling out involvement of the classical secretory pathway (21). Secretion is dependent on an intact microtubule network since disruption by depolymerizing agents inhibited MRP8 and MRP14 release. The latter observation is in accordance to morphological data demonstrating a clear co-localization of MRP8 and MRP14 with microtubules during the process of secretion.

Properties of MRP8 and MRP14 during secretion resemble in some aspects those of IL-1β, which is supposed to be released via an alternative pathway of secretion (21). IL-1β secretion is not inhibited by monensin or brefeldin A as well (32). Moreover, there is no association of IL-1β with ER, Golgi apparatus, or

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**FIG. 5. Co-localization of MRP8 and MRP14 with microtubules in PMA-stimulated monocytes.** Monocytes were studied by indirect immunofluorescence microscopy either under control conditions (A) or after exposure to 10 nM PMA (B-H). Staining of untreated cells with a-MRP8 (A) revealed a diffuse staining over all the cytoplasm. After stimulation with PMA, a filamentous distribution of MRP8 (B) can be observed, which resembled that of tubulin visualized by mAb TUB 2.1 (D). In contrast, vimentin shows a clearly distinct pattern after PMA stimulation (C). Double labeling using a-MRP8 (polyclonal rabbit antiserum, FITC, E) and mouse mAb TUB 2.1 against tubulin (Cy3, F) revealed an almost identical staining pattern after treatment with PMA. In contrast, double labeling using a-MRP8 (G) and mAb V9 against vimentin (H) resulted in a clearly distinct staining of cytoskeletal structures within the same cell. Similar results were obtained using a-MRP14 or mAb 27E10, which recognizes the MRP8/MRP14 complex (data not shown). Bar, 10 μm.
secretory vesicles, whereas a co-localization with the microtubule network during the secretory process has been reported (33–35). However, secretion of IL-1β is not inhibited by microtubule-depolymerizing drugs (36) (Fig. 7), which is in contrast to our data for MRP8 and MRP14. Furthermore, uncouplers of oxidative phosphorylation increased levels of secreted IL-1β (32), whereas MRP8 and MRP14 release was significantly inhibited by CCCP and dinitrophenol. Thus, IL-1β and MRP8 and MRP14 display clear differences, suggesting that they do not share a common mechanism of release. MRP8 and MRP14 therefore appear to follow neither the classical nor the alternative secretory pathway of the IL-1β-type.

Earlier reports described elevated serum levels of MRP8 and MRP14 during the course of a number of inflammatory diseases (2, 16, 17). Immunohistological data provided indirect evidence that monocytes release MRP8 and MRP14 during endothelial diapedesis at sites of inflammation (11). The complex of MRP8 and MRP14 shows antimicrobial activities, especially against *Candida albicans* (18). The MRP14 subunit seems to be responsible for this antimicrobial effect (37). Furthermore, MRP8-MRP14 complexes exhibit growth-inhibitory activities against murine bone marrow cells, macrophages, and mitogen-stimulated lymphocytes (38), which appears to depend on inhibition of casein kinase II (19). In addition, murine MRP8, but not MRP14, shows chemotactic activity for granulocytes (20). Another recently reported function of MRP8 and MRP14 refers to an antiinflammatory property; systemic application of MRP8/MRP14 mitigated the course of murine experimental arthritis (39). This picture of pleiotropic extracellular activities may reflect different functions of monomeric and complexed MRP8 and MRP14. Such a hypothesis is supported by the observation that MRP8 and MRP14 are differentially

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**FIG. 6.** Effect of demecolcine treatment on intracellular distribution of MRP8 and MRP14. Monocytes were treated with 10 nM PMA and, during the last hour of incubation, concomitantly with 5 μg/ml demecolcine. The filamentous pattern of MRP8 (A, FITC) and tubulin (B, Cy3) was found to be completely disrupted as shown by double-labeling immunofluorescence. The vimentin network (C, Texas Red), however, was still detectable.

**FIG. 7.** Effects of monensin, brefeldin A, and microtubule-depolymerizing agents on IL-1β, TNF-α, and MRP8/MRP14 secretion. Monocytes were either left untreated (control, □), incubated with solely 10 nM PMA (light gray bar), or concomitantly exposed to PMA and 10 μg/ml monensin ( ●), 0.5 μg/ml brefeldin A (dark gray bar), 2 μg/ml nocodazole ( ▲), or 1 μM demecolcine ( ▼). Amounts of IL-1β, TNF-α, and MRP8/MRP14 secreted into the supernatants were determined by ELISA as described under “Materials and Methods.” Data are related to cytokine or MRP8/MRP14 contents in supernatants of nonstimulated monocytes. Values are presented as means ± S.D. of quadruplicate wells. Data of one out of three independent experiments with essentially similar results are shown.

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**FIG. 8.** Time kinetics and concentration-response relationship of MRP8 and MRP14 secretion and mRNA expression. A, monocytes of culture day 1 were exposed to 10 nM PMA for up to 4 h. Cells and supernatants were harvested after the time intervals indicated and processed for Northern blotting (left, a = 0, b = 10, c = 60, d = 120, and e = 240-min incubation) or ELISA (right), respectively. B, monocytes were treated for 4 h with the PMA concentrations indicated. MRP8/MRP14 release (right) and mRNA expression (left, a = 0, b = 0.1, c = 1, d = 10, and e = 100 nM PMA) were determined in parallel as described in A. In both A and B, a close inverse correlation between secretion and mRNA expression of MRP8 and MRP14 can be observed.
expressed in defined monocyte subpopulations under various inflammatory conditions (15).

Most S100 family proteins appear to play an intracellular role during calcium-dependent signaling (4, 5). They interfere with cell cycle progression, inhibit phosphorylation reactions, or modulate membrane/cytoskeleton interactions. MR8 and MR14 are supposed to be involved in intracellular signaling pathways during calcium-dependent activation of monocytes. They assemble to noncovalently associated complexes (26, 27) that are translocated to membrane structures and intermediate filaments upon elevation of intracellular calcium levels by calcium ionophore A23187 (22). The latter event correlated with cell cycle progression, inhibit phosphorylation reactions, or modulate membrane/cytoskeleton interactions. MR8 and MR14 account for up to 30% of the calcium-binding capacity of EF-hand proteins to distinct stages of inflammatory reactions. Furthermore, secretion into the extracellular space (5).

Supposed that the N-terminal EF-hand binds calcium only at the intracellular calcium levels and up-regulates protooncogene expression (31) and nitric oxide synthetase activity in neuronal cells (45). Thus, activities of distinct S100 proteins are not restricted to either the intra- or extracellular space. Structural properties of S100 proteins support such observations. Their N-terminal EF-hand exhibits a significantly lower affinity to calcium than the C-terminal EF domain. It has therefore been supposed that the N-terminal EF-hand binds calcium only at high calcium concentrations as they predominate in the extracellular environment (5).

Induction of MR8 and MR14 release is associated with down-regulation of de novo synthesis of these proteins at the mRNA level, thus limiting an extracellular function of these proteins to distinct stages of inflammatory reactions. Furthermore, secretion of MR8/MR14 is linked to a marked differentiation step in monocytes, since MR8 and MR14 account for up to 30% of the calcium-binding capacity of EF-hand proteins in these cells, whereas both molecules cannot be found after down-regulation of de novo synthesis in mature macrophages (13, 46).

To date, extracellular functions have been ascribed to five S100 family members. We now for the first time provide evidence that the IL-1 proteins, MRP8 and MRP14, which follow neither the classical data elucidating the mechanism of release of two of these members of the S100 family remains to be elucidated in future studies. We now for the first time provide evidence that these phenomena are independent events.

Other members of the S100 protein family exhibit both intracellular and extracellular functions as well. For example, S100β interferes with calcium-dependent modulation of cytoskeletal structures (41, 42), but also functions as neurite extension factor in the extracellular space (6, 43, 44). S100 proteins to distinct stages of inflammatory reactions. Further-
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