Ubiquicidin, a novel murine microbicidal protein present in the cytosolic fraction of macrophages

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Abstract: Previously we have identified and characterized three murine microbicidal proteins purified from the granule fraction of cells from the murine macrophage cell line RAW264.7. During these studies evidence was obtained for the presence of an additional antimicrobial protein in the cytosolic fraction of RAW264.7 cells that had been activated with interferon-γ (IFN-γ). In this study we have purified this protein, designated ubiquicidin, to apparent homogeneity and demonstrated that it is a cationic, small (M, 6654) protein. Ubiquicidin displayed marked antimicrobial activity against Listeria monocytogenes and Salmonella typhimurium. Using a gel overlay procedure evidence was obtained that the protein also displays activity against Escherichia coli, Staphylococcus aureus, and an avirulent strain of Yersinia enterocolitica. Amino-terminal amino acid sequencing and mass spectrometry analysis of purified ubiquicidin indicated that it is most likely identical to the ribosomal protein S30. This protein is produced by posttranslational processing of the Fau protein, a 133-amino-acid fusion protein consisting of S30 linked to an unusual peptide with significant homology to ubiquitin. The fau gene has been reported to be expressed in a variety of tissues in humans and various animal species. The presence of ubiquicidin in the cytosol of macrophages may serve to restrict the intracellular growth of microorganisms. In addition, because macrophage disintegration will likely lead to release of ubiquicidin into the extracellular environment, it may contribute to host defense after macrophage death. J. Leukoc. Biol. 66: 423-428; 1999.

Key Words: monocytes/macrophages · antibacterial activity · antimicrobial peptides

INTRODUCTION

A main function of macrophages in the host defense against infection is the phagocytosis of nonopsonized or opsonized microorganisms, and the subsequent growth restriction or killing of ingested microorganisms. The antimicrobial activity of macrophages is mediated by oxidative and non-oxidative mechanisms. The oxidative antimicrobial mechanisms, which include the action of reactive oxygen intermediates (and in rodents nitrogen intermediates), have been the subject of numerous studies [reviewed in refs. 1, 2]. Non-oxidative mechanisms include vacuolar acidification, nutrient deprivation, and the action of antimicrobial polypeptides. These peptides have been extensively studied in neutrophils [1, 3] and are stored in the azurophilic and specific granules of these cells. On phagocytosis of microorganisms by neutrophils, these granule-associated polypeptides are transferred to the phagolysosome where they can kill ingested microorganisms. In addition to granule-associated antimicrobial polypeptides, neutrophils also contain a cytosolic antimicrobial protein called calprotectin [4, 5].

Macrophages also contain granule-associated antimicrobial polypeptides, although these are less abundant than in neutrophils [reviewed in refs. 6, 7]. These polypeptides include lysozyme [8], present in monocytes and macrophages from various species, and defensins that are present only in rabbit alveolar macrophages [9, 10]. In addition, at an early stage of the differentiation of mononuclear phagocytes, their cytoplasmic granules contain the antimicrobial polypeptides cathepsin G, elastase, proteinase 3, and azurocidin, collectively also known as serprocidins [reviewed in refs. 6, 7, 11]. The capacity to synthesize these polypeptides is strongly reduced on differentiation of promonocytes into monocytes and subsequently macrophages. In addition to lysozyme, defensins, and serprocidins, murine macrophages also contain additional antimicrobial proteins that we have designated murine microbicidal proteins (MUMP) [12]. Three MUMPs were purified from interferon-γ (IFN-γ)-activated cells from the murine macrophage cell line RAW264.7, and characterized as histone-related granule-associated proteins (MUMP-1, -2, and -3). Evidence was obtained for the presence of an additional antimicrobial protein in the cytosol fraction of IFN-γ-activated RAW264.7 cells. The aim of this study was to purify and characterize this additional protein that we have designated ubiquicidin.

MATERIALS AND METHODS

Culture and subcellular fractionation of cells from the murine macrophage cell line RAW264.7

Culture, subcellular fractionation, and extraction of RAW264.7 cells was performed essentially as described previously [12]. Briefly, RAW264.7 cells

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obtained from the American Type Culture Collection (ATCC, Rockville, MD) were cultured in Dulbecco's minimal Eagle's medium (Flow Laboratories, Irvine, Scotland) that was supplemented with L-glutamine (2 mM), penicillin G (100 U/mL), streptomycin (100 μg/mL), D-glucose (3.5 mM/mL), and 10% (vol/vol) heat-inactivated newborn calf serum (Flow Laboratories) in 1-L spinner bottles. At a density of 1 to 2 × 10⁶/mL, the cells were activated by culture for 20 h in the presence of recombinant IFN-γ (200 U/mL; kindly provided by R. H. van der Meide, Department of Virology, TNO, Rijswijk, The Netherlands). The activation of these cells was assessed by measuring the release of NO₂⁻; IFN-γ-treated cells released 13.9 nmol of NO₂⁻ per 10⁶ cells (n = 16), whereas control-treated cells released no detectable NO₂⁻ (<0.2 nmol/10⁶ cells). The cells were harvested, subjected to subcellular fractionation after disruption by nitrogen cavitation in the presence of protease inhibitors, and cytosol and granule fractions were obtained [12]. These fractions were extracted with 5% (vol/vol) acetic acid, insoluble material was removed by centrifugation for 20 min at 27,000 g, and the cleared extracts were concentrated by vacuum centrifugation (SpeedVac; Savant Instruments Inc., Hicksville, NY) [12].

Purification and characterization of antimicrobial polypeptides from the cytosol fraction of IFN-γ-activated RAW264.7 cells

Cytosol extract obtained from 1.2 × 10⁹ cells was concentrated to 7 mL by vacuum centrifugation (final protein concentration 12.6 mg/mL). The concentrated extract was fractionated in aliquots derived from 5 × 10⁶ cells by gel filtration at a flow rate of 7.6 mL/h on a column of Bio-Gel P60 (2.5 × 88 cm; Bio-Rad Laboratories, Richmond, CA) that was equilibrated in 5% acetic acid. Fractions of 2.54 mL were collected and assayed for protein content, lysozyme, and antimicrobial activity. Selected fractions were further purified by reverse-phase high-performance liquid chromatography (RP-HPLC) on a C18 column (4.6 × 250 mm; Vydac, The Separations Group, Hesperia, CA) using linear water-acetonitrile gradients that contained 0.13% heptfluorobutyric acid as ion-pairing agent.

Characterization of ubiquicidin

The purity of the antimicrobial polypeptides was determined with tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [13]. The amino-terminal amino acid sequence of ubiquicidin was determined by automated Edman degradation using a pulsed-liquid protein sequencer 477A equipped with an on-line PTH amino acid analyzer 120 A (Applied Biosystems, Foster City, CA). Sequence data were analyzed using the GeneWorks program (Intelligenetics, Mountain View, CA). The molecular mass of ubiquicidin was determined using laser desorption mass spectrometry (Lasermat, Finnigan MAT Ltd., Hemel Hempstead, UK).

Antimicrobial assays

The antimicrobial activity of the cellular and subcellular extracts, and of the fractions obtained after chromatography, was investigated with the following target microorganisms: Listeria monocytogenes EGD, Salmonella typhimurium 14028S (a generous gift from E. A. Groissmann and F. Helfron, Scripps Clinic Research Institute, La Jolla, CA), Staphylococcus aureus 502A, Escherichia coli ML-35p (kindly provided by R. I. Lehrer, Department of Medicine, UCLA School of Medicine, Los Angeles, CA) [14], and an avirulent strain of Yersinia enterocolitica (pYVe+; kindly provided by G. Cornelis, Microbial Pathogenesis Unit, Universite Catholique de Louvain, Belgium) [15]. The antimicrobial activity of the crude cellular extracts and the fractions was determined using mid-logarithmic bacteria as targets in a gel overlay assay and a radial diffusion assay as previously described [12, 16]. In these assays bacteria were dispersed in an underlay agar that consisted of 9 mM sodium phosphate buffer, 1 mM citrate, and 1% (vol/vol) tryptic soy broth (TSB; Difco Laboratories) pH 6.5.

As an internal control in the antimicrobial assays, rabbit defensin NP-1 (a generous gift from R. I. Lehrer) was used.

Other methods

Protein was measured using the bichinchonic acid method (Pierce, Rockford, IL) and lysozyme activity using a radial diffusion assay [17]. Production of NO was assessed by determining the level of NO₂⁻ in the supernatant of the cells through the use of the Griess reagent [18].

RESULTS

Antimicrobial activity of cellular and subcellular extracts from activated murine RAW264.7 cells

A cetic acid extracts were prepared from unfraccionated IFN-γ-activated RAW264.7 cells (whole cells) and from the cytosol and granule fraction of the cells that were obtained after subcellular fractionation. The presence of antibacterial activity in the extracts was analyzed with a gel overlay procedure using S. typhimurium as target micro-organism (Fig. 1). With all extracts derived from RAW264.7 cells bands of clearing were obtained that we previously designated zones α and β [12]. Compared to whole-cell and granule extracts, these zones were less marked in the extracts from the cytosol fractions. A third zone of clearing (zone γ) was observed in all extracts and was previously identified as lysozyme [12]. A fourth zone of antibacterial activity with a higher electrophoretic mobility, zone δ, was observed in the whole-cell and cytosol extract of IFN-γ-activated RAW264.7 cells. When L. monocytogenes, E. coli, S. aureus, or an avirulent strain of Y. enterocolitica were used as target, zones α, β, γ, and δ were also observed, with the exception that zone γ was not observed with S. aureus (data not shown).

Purification of antimicrobial polypeptides from the cytosol of IFN-γ-activated RAW264.7 cells

The molecules present in an acetic acid extract obtained from IFN-γ-activated RAW264.7 cells causing the zone of antimicrobial activity designated zone δ were further purified. The extract from the cytosol fraction was fractionated on a Bio-Gel P-60 column and the fractions were tested for activity against S. typhimurium and L. monocytogenes with the use of radial
contained a substantially purified protein with an estimated first peak of antibacterial activity (fractions 29 and 30). PAGE analysis of the active fractions (containing antimicrobial activity) were obtained (RP-HPLC using a Vydac C18 column, and two major peaks the fractions were pooled, subjected to further purification by multiple proteins in these fractions (data not shown). Therefore purification. SDS-PAGE analysis demonstrated the presence of assay, these fractions were selected for further analysis and content by bichinchonic assay (open circles) and lysozyme activity (filled circles).

**DISCUSSION**

The results from this study demonstrate that activated mouse macrophages contain a small (M, 6,654) cationic antimicrobial protein. This protein, that we have designated ubiquicidin, was purified from the cytosolic fraction of IFN-γ-activated cells of the mouse macrophage cell line RAW 264.7. Purified ubiquicidin was found to be equally active against L. monocytogenes as one of the most potent defensins, rabbit NP-1.

These results demonstrate that ubiquicidin is identical or highly homologous to S30, a protein that was purified from the small ribosomal subunit fraction of rat liver and shown to be present in various human and murine tissues [19–21]. This conclusion is based on (1) the full identity between the amino-terminal 18-amino-acid sequence of ubiquicidin with mouse, human, and rat S30, and (2) the similar relative molecular masses of these proteins (ubiquicidin, 6,654; S30, 6,643). To our knowledge, antimicrobial activity of ribosomal proteins has not been previously described. Mouse, rat, and human ribosomal protein S30 show full identity; it is a 59-amino-acid cationic protein (pl 12.67) containing 21 basic residues (7 arginine, 13 lysine, and 1 histidine) and only 1 acidic residue [19, 21]. Based on the homology of ubiquicidin with a ribosomal protein and the observation that many antimicrobial peptides are cationic [22], it is possible that ubiquicidin has biological roles that are distinct from its putative role in host defense against infection.

S30 is the product of posttranslational processing of Fau, a 133-amino-acid fusion protein consisting of a 74-amino-acid polypeptide with significant (38% identity) homology to ubiquitin, and the 59 amino acid S30 polypeptide that is expressed in a variety of tissues [20]. Among the ribosomal proteins this biosynthetic pathway is rather unique because most of the rat ribosomal proteins (more than 60) that have been sequenced to date are unprocessed primary products of their mRNA [23]; the only other exceptions to this rule are two ribosomal proteins that are carboxyl extensions of ubiquitin [24, 25]. Recently, another

**Characterization of ubiquicidin**

Fractions 29 and 30 were pooled and subjected to amino-terminal amino acid sequencing. The sequence of the first 16 amino acids of the purified protein (sequence: KVHGSLARAGKVRGQ), designated ubiquicidin, was identical to that of rat ribosomal protein S30 [19]. Further proof that ubiquicidin is identical to S30 was obtained by analysis using mass spectrometry. This analysis indicated the presence of a single component with a M, of 6,654, whereas the predicted M, of S30 has been reported to be 6,643.

**Comparison of the antimicrobial activity of ubiquicidin and rabbit defensin NP-1**

The antimicrobial activity of purified ubiquicidin against L. monocytogenes was compared to that of rabbit defensin NP-1 using a radial diffusion assay (Fig. 5). On a molar basis, ubiquicidin and NP-1 were equally active.

**Fig. 2.** Gel filtration of cytosol extract from 5 × 10⁷ IFN-γ-activated RAW264.7 cells on Bio-Gel P-60. The fractions were assessed for (A) protein content by bichinchonic assay (open circles) and lysozyme activity (filled circles), and for (B) antibacterial activity against S. typhimurium (open circles) and L. monocytogenes (filled circles).
function of the ubiquitin-like part of the Fau protein was described; a product of a murine T cell hybridoma with nonspecific immunosuppressive activity was found to be virtually identical to the Fau protein [26]. The activity of this product, an isoform of monoclonal nonspecific suppressor factor (MNSF) designated MNSFβ, appeared to reside in the NH₂-terminal ubiquitin-like segment (ubi-L) [27]. IFN-γ caused an increase in the ubi-L/MNSF expression in murine splenocytes by increasing mRNA levels [26, 27]. Therefore the presence of ubiquicidin in the cytosol of activated macrophages as observed in this study may have been the result of IFN-γ-induced Fau/MNSFβ transcription. We have, however, not yet established an effect of macrophage activation on ubiquicidin expression. In a series of experiments the expression of ubiquicidin in IFN-γ-activated RAW264.7 cells was compared to that in nonstimulated cells (data not shown). Although in initial experiments a consistent higher expression of ubiquicidin was found in activated cells, as determined by assessment of the intensity of zone 8 in the gel overlay assay, subsequent experiments yielded more variable results.

Whereas most antimicrobial polypeptides of phagocytes identified to date are stored in cytoplasmic granules and transferred to the phagolysosome where they can kill ingested microorganisms, phagocytes can also secrete antimicrobial compounds into the extracellular environment or store them in the cytosol. Macrophages synthesize and secrete lysozyme constitutively. In addition, IFN-γ-activated mouse macrophages secrete compounds that display static activity against Cryptococcus neoformans [28]; these compounds with Mr of 15,000 and 30,000 were only partially purified and character-

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**Fig. 3.** Purification of ubiquicidin by RP-HPLC. Pooled Bio-Gel P-60 fractions 62–69 were fractionated by RP-HPLC on a Vydac C18 column using 0.13% heptafluorobutyric acid as the ion-pairing agent. Absorbance at 280 nm (A) and antimicrobial activity of collected fractions against L. monocytogenes as assessed using the radial diffusion assay (B) are shown.

**Fig. 4.** SDS-PAGE analysis of RP-HPLC fractions. Approximately 1 µg of selected fractions was subjected to Tricine SDS-PAGE and stained for protein with Coomassie blue. Fractions are indicated by their number and molecular mass markers by M.

**Fig. 5.** Antimicrobial activity of ubiquicidin and rabbit defensin NP-1 against L. monocytogenes. Antimicrobial activity of ubiquicidin (open circles) was compared to that of NP-1 (filled circles) using the radial diffusion assay. Five microliters of serially diluted ubiquicidin or NP-1 in 0.01% acetic acid was added to 3-mm-diameter wells in the underlay gel. The nanomolar concentrations of the peptides are indicated along the abscissa. Results are expressed as mean ± SD of three experiments.
ized and it is therefore not clear whether they are related to ubiquicidin. Recently it was shown that many of the proteins secreted by lipopolysaccharide-stimulated cells of a mouse macrophage cell line are ribosomal proteins [29]. Ubiquicidin was isolated from the cytosol of IFN-γ-activated cells from a murine macrophage cell line. Although we have not detected ubiquicidin activity in the granule fraction using gel overlay assays, we cannot exclude the possibility that some of the antimicrobial activity present in the cytosolic fraction is derived from granules that have been disrupted during cell fractionation. Currently it is not clear whether macrophages secrete ubiquicidin. The cytosolic fraction of human neutrophils also contains an antimicrobial compound, the calprotectin complex [4, 5]. Based on its relative molecular mass and amino-terminal amino acid sequence, it is unlikely that ubiquicidin is the murine equivalent of one of the components of the human calprotectin complex.

Cytoplasmic antimicrobial polypeptides may play a role in host defense against micro-organisms on their release into the extracellular environment after cell disintegration. Such polypeptides may, however, also play a role in inhibiting cytoplasmic growth of intracellular parasites. Members of at least three genera of bacteria have been shown to be able to grow in host cell cytosol (L. monocytogenes, Shigella, and Rickettsiae; [reviewed in ref. 30]). Entry of these bacteria into the cytosol is associated with hemolytic activity of the bacteria, probably resulting in lysis of the vacuolar membrane [30]. Vacular lysis in Listeria-infected cells is mediated in large part by hemolysins such as listeriolysin O [31]. The mechanisms normally limiting cytosolic growth of bacteria are not well understood; although limitation of key nutrients may be a mechanism involved in restriction of cytoplasmic growth, this has not yet been demonstrated [32, 33]. Outgrowth of Listeria is markedly decreased when cell-mediated immunity has developed, probably as a result of macrophage activation by IFN-γ [34, 35]. IFN-γ does not affect the initial phase of killing of L. monocytogenes that occurs shortly after phagocytosis [35, 36]. However, IFN-γ may affect subsequent intracellular replication of L. monocytogenes by another mechanism, i.e. by preventing its escape from the phagolysosome into the cytosol [37]. Our studies imply that in addition to this mechanism, increased levels of ubiquicidin in macrophages and possibly other types of cell, e.g., parenchymal liver cells, may contribute to the limitation of intracytosolic growth and subsequent spread of L. monocytogenes.

In summary, our results indicate that IFN-γ-activated mouse macrophages contain a cytosolic antimicrobial protein that is identical to the ribosomal protein S30. The homology of the precursor element of this protein to ubiquitin and the fact that it is probably widely expressed motivated us to name this protein ubiquicidin (Latin “ubique,” everywhere). Expression of ubiquicidin may enable the activated macrophage to restrict the cytoplasmic growth of intracellular pathogens such as L. monocytogenes once cell-mediated immunity has developed. It may also endow the macrophage with the ability to contribute to host defense against microorganisms as a result of release of ubiquicidin that may occur after disintegration of dead macrophages.

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