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Novel Pathogenic Mechanism of Microbial Metalloproteinases: Liberation of Membrane-Anchored Molecules in Biologically Active Form Exemplified by Studies with the Human Interleukin-6 Receptor

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Certain membrane-anchored proteins, including several cytokines and cytokine receptors, can be released into cell supernatants through the action of endogenous membrane-bound metalloproteinases. The shed molecules are then able to fulfill various biological functions; for example, soluble interleukin-6 receptor (sIL-6R) can bind to bystander cells, rendering these cells sensitive to the action of IL-6. Using IL-6R as a model substrate, we report that the metalloproteinase from *Serratia marcescens* mimics the action of the endogenous shedding proteinase. Treatment of human monocytes with the bacterial protease led to a rapid release of sIL-6R into the supernatant. This effect was inhibitable with TAPI [*N*-{D,L-[2-(hydroxyaminocarbonyl)methyl]-4-methylpentanoyl}L-3-(2′ naphthyl)-alanyl-L-alanine, 2-aminoethyl amide], a specific inhibitor of the membrane-bound intrinsic metalloproteinase, but not with other conventional proteinase inhibitors. sIL-6R-liberating activity was also detected in culture supernatants of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Listeria monocytogenes*, organisms that are known to produce metalloproteinases. sIL-6R released through the action of *S. marcescens* metalloproteinase retained biological activity and rendered IL-6-unresponsive human hepatoma cells sensitive to stimulation with IL-6. This was shown by Northern (RNA) blot detection of haptoglobin mRNA and by quantitative measurements of de novo-synthesized haptoglobin in cell supernatants. Analysis of immunoprecipitated, radiolabeled sIL-6R revealed that the bacterial protease cleaved IL-6R at a site distinct from that utilized by the endogenous protease. These studies show that membrane-anchored proteins can be released in active form through cleavage at multiple sites, and they uncover a novel mechanism via which microbial proteases possibly provoke long-range biological effects in the host organism.

Bacterial pathogens secrete a wide array of proteases, many of which have been extensively characterized at functional and structural levels (9). Although microbial proteases are now standard tools in biochemistry and cell biology, there is a discrepant paucity of information regarding the biological events that they may provoke in a physiological setting. Most studies deal with the problem of local substrate cleavage and, hence, the destruction of host molecules. For example, one of the best-studied bacterial proteases is the elastase of *Pseudomonas aeruginosa*. This protease degrades immunoglobulin G and complement components, possibly providing the bacterium with survival advantages in the hostile host environment (9, 10, 14, 25).

Substrate inactivation is, however, but one of several mechanisms by which proteases can elicit biological effects. An emerging novel mechanism is the release of membrane-anchored proteins in a functionally active state. This shedding process has been discovered to be characteristic of many mediators, including several cytokines and their receptors (6, 15, 21). The shed proteins can assume a variety of functions. For example, soluble receptors may act as competitive inhibitors of the cognate ligands. Alternatively, soluble cytokine receptors may also act as agonists. For example, the soluble interleukin-6 receptor (sIL-6R) binds by specific interactions to bystander cells, rendering these cells sensitive to the action of IL-6 (21). An analogous transsignalling phenomenon has been documented for the soluble lipopolysaccharide (LPS) receptor (soluble CD14). Such transsignalling processes may play important roles in the pathogenesis of systemic inflammatory reactions (2).

It is known that endogenous, membrane-bound metalloproteinases are responsible for the physiological shedding of tumor necrosis factor alpha and its receptors, IL-6R, and the Fas ligand (4, 12, 18). Shedding occurs through proteolytic cleavage of the membrane anchors at specific sites close to the membrane surface. The endogenous metalloproteinases are inhibitable by hydroxamic acid compounds and to a lesser degree by conventional inhibitors of metalloproteinases, such as 1,10-phenanthroline (8, 16, 17). The events leading to activation of the intrinsic metalloproteinase are not well known; phorbol esters are conventionally used as activators, but physiological stimuli other than LPS have not been discovered. The shedding protease has not yet been isolated (15, 21).

Pathogenic microorganisms often derange homeostasis in the host by deregulating physiological processes. Several pathogens, including *Staphylococcus aureus*, *P. aeruginosa*, *Serratia marcescens*, *Listeria monocytogenes*, and *Bacillus subtilis*, elaborate metalloproteinases, and it occurred to us that these microbial enzymes might mimic the action of the endogenous shedding protease. To test this hypothesis, we utilized *S. marcescens* metalloproteinase (SMP), which is available in a highly purified form. The crystal structure of the enzyme has been elucidated (1), and the proteinase is structurally related to the alkaline protease of *P. aeruginosa* (11).

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The model system employed in our study involved the shedding of IL-6R from primary human monocytes or from transfected COS-7 cells. sIL-6R could be detected immunologically by enzyme-linked immunosorbent assay (ELISA) and could be biochemically characterized by immunoprecipitation with monospecific antibodies. Further, the biological activity of sIL-6R could be assessed in a well-established experimental system. We will show that SMP releases IL-6R from cell membranes in an active form, rendering sIL-6R available for trans-signalling events. IL-6R shedding was also detected in cells treated with culture supernatants from *S. marcescens*, *S. aureus*, *P. aeruginosa*, and *L. monocytogenes*, organisms that are known to produce metalloproteinases. These studies uncover a novel pathway via which microbial proteases may provoke long-range reactions in the host organism.

MATERIALS AND METHODS

Chemicals. Staurosporin, SMP, phorbol myristate acetate (PMA), pepstatin A, phenylmethylsulfonyl fluoride, and Nonidet P-40 were obtained from Sigma (Taufkirchen, Germany). *N*-Ethylmaleimide, Pefabloc [4-(2-aminoethyl)benzenesulfonyl fluoride, hydrochloride], 1,10-phenanthroline, aprotinin, and leupeptin were from Boehringer (Mannheim, Germany). Protein A-Sepharose CL-4B was obtained from Pharmacia (Freiburg, Germany). Pansorbin was purchased from Calbiochem (La Jolla, Calif.). Tran^{35}S-label (44 TBq/mmol) was obtained from ICN (Meckenheim, Germany). Dulbecco's modified Eagle's medium (DMEM) with Glutamax, minimum essential medium, and penicillin-streptomycin were from Gibco (Eggenstein, Germany). Fetal calf serum was from Seromed (Berlin, Germany). The novel metalloprotease inhibitor TAPI [*N*-{D,L-[2-(hydroxyaminocarbonyl)methyl]-4-methylpentanoyl}L-3-(2′ naphthyl)-alanyl-L-alanine, 2-aminoethyl amide] was prepared at Immunex Corp., Seattle, Wash. (17). The preparation of the polyclonal monospecific antiserum against human IL-6R (huIL-6R) was described previously (24). Monoclonal antibodies against huIL-6R, MT18, and PM-1 were obtained from K. Yasukawa (Tosoh Corp., Tokyo, Japan). The generation of the expression vector pCDM8-huIL-6R was described previously (20). Soluble huIL-6R was expressed as described previously (13). Goat polyclonal serum anti-human haptoglobin and rabbit polyclonal serum anti-human haptoglobin were purchased from Sigma (Deisenhofen, Germany). Human haptoglobin standard was purchased from The Binding Site (Birmingham, United Kingdom), and alkaline phosphatase-conjugated donkey polyclonal serum anti-rabbit immunoglobulin G was purchased from Pierce (BA Oud Beijerland, The Netherlands).

Monocyte isolation and treatment. Monocytes were isolated from citrated buffy coats, which were kindly provided by the Blood Transfusion Center of the University of Mainz (Mainz, Germany). Monocytes were isolated as described previously (5). Monocyte cell suspensions regularly contained 70 to 90% monocytes as determined by flow cytometry. They were adjusted with minimum essential medium to densities of 1.5×10^6 cells/ml, and 1-ml aliquots of cell suspensions were applied per well to 24-well cell culture plates (Nunc AS, Roskilde, Denmark). After a 1-h incubation of cells at 37°C in 5% CO_2, the plates were washed twice with Hanks balanced salt solution. The cells were then treated with 0.01 to 100 μg of SMP per ml. At the times noted in the figure legends, the media were removed and centrifuged for 5 min at $13,000 \times g$.

Quantification of sIL-6R. The quantification of sIL-6R in cell supernatants was undertaken by following the instructions supplied by the manufacturers of the ELISA kits. The sIL-6R ELISA was supplied by Laboserv Diagnostica (Giessen, Germany).

Cell culture. COS-7 cells and HepG2-IL-6 cells (13) were grown in DMEM supplemented with 10% fetal calf serum, penicillin (60 mg/liter), and streptomycin (100 mg/liter) at 5% CO_2 and 37°C in a water-saturated atmosphere. HepG2-IL-6 cells were cultured in 96-well tissue cell culture plates. Confluent monolayers were washed with phosphate-buffered saline (PBS) and starved for 1 h in fetal calf serum-free DMEM. Triple sets of cells were stimulated with supernatants from transiently transfected and stimulated COS-7 cells or with soluble receptor (sIL-6R) for 24 h in 200 μl of fetal calf serum-free DMEM.

Transient transfection and metabolic labeling of COS-7 cells. Transient transfections of COS-7 cells were performed by electroporation (Gene Pulser; Bio-Rad). Confluent growing cells (50 to 70%) were trypsinized, resuspended in 1 ml of DMEM containing 20 μg of the appropriate DNA, pulsed (960 F; 240 V; distance of electrodes, 4 mm) and further grown for 1 to 2 days. Cells were metabolically labeled for 2 h with 50 μCi of [^{35}S]cysteine–methionine per ml in cysteine-methionine-free minimum essential medium. Cells were chased in DMEM containing cysteine-methionine for 1 h.

Immunoprecipitation of IL-6R. Supernatants of transiently transfected and metabolically labeled COS-7 cells were harvested and supplemented with 0.3% sodium dodecyl sulfate (SDS)–1% Nonidet P-40. Supernatants were pretreated with Pansorbin and subsequently incubated with monoclonal antibodies against IL-6R for 1 h at 4°C. Three antibodies of defined specificity, designated PM-1, MT18, and 6.2, were employed. Figure 1 shows a structural model of IL-6R consisting of three extracellular fibronectin III domains and then a tether region, transmembrane domain, and intracellular domain. The epitopes recognized by the antibodies have been mapped to the positions depicted in the figure (24, 28). Immune complexes were precipitated with protein A-Sepharose and separated on SDS–12.5% polyacrylamide gels. After being subjected to intensifying fluorography with sodium salicylate, the gels were dried and exposed to X-ray film.

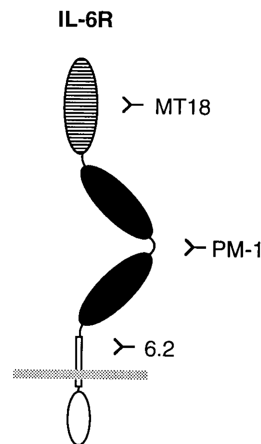

FIG. 1. Recognition sites of the IL-6 antibodies MT18, PM-1, and 6.2. The structure of IL-6R is schematically depicted (for details, see references 19 and 28).

Northern (RNA) blot analysis. RNA was prepared with RNeasy (Qiagen, Hilden, Germany). Northern blotting was carried out as previously described (22). Nylon membranes (GeneScreen Plus; NEN) were hybridized with a 0.9-kb *Hin*fII fragment of human haptoglobin cDNA labeled by random priming (7).

Haptoglobin ELISA. An ELISA was performed essentially as described previously (23). Briefly, 96-well microtiter plates were coated with 100 μl of goat polyclonal serum anti-human haptoglobin (1:1,000 dilution in 50 mM sodium carbonate, pH 9.4) per well for 1 h at room temperature. Blocking was done with 200 μl of 3% bovine serum albumin in PBS for 2 h. Cell culture medium (50 μl) or standard haptoglobin (0.1 to 100 ng/ml) diluted in blocking buffer was added to each well and incubated for 1 h at room temperature. Dilutions of cell culture media were chosen in order to obtain absorbance values in the linear range of the standard curve. After being washed four times with PBS, plates were incubated with 50 μl of rabbit polyclonal serum anti-human haptoglobin (1:1,000 dilution in blocking buffer) per well for 30 min at 37°C. Plates were washed, and a final incubation was done with 50 μl of alkaline phosphatase-conjugated donkey polyclonal serum anti-rabbit immunoglobulin G per well at 37°C (1:1,000 dilution in blocking buffer) for 30 min. Secondary antibody binding was detected by the addition of 50 μl of alkaline phosphatase substrate (0.5 mg of *p*-nitrophenylphosphate per ml, 0.9 M diethanolamine [pH 9.6], 1 mM $MgCl_2$). After 10 min at 37°C, the enzyme reaction was stopped and the A_{405} was read with a microplate reader (Immunoreader NJ 2000; InterMed).

RESULTS

SMP releases huIL-6R into supernatants. Human monocytes were treated with SMP at the concentrations given in the legend to Fig. 2 for 40 min at 37°C, and sIL-6R in cell supernatants was assayed by ELISA. sIL-6R concentrations measured in the buffer controls were 130 ± 10 pg/ml. Substantial liberation of sIL-6R from monocytes occurred at a concentration of 1 μg of SMP per ml, and peak concentrations were observed at 5 to 10 μg of SMP per ml. At the very high concentration of 100 μg of SMP per ml, the amounts of immunologically detectable sIL-6R were reduced, probably because of extensive cleavage and destruction of the soluble receptor. The kinetics of released sIL-6R induced by 10 μg of SMP per ml are shown in Fig. 3. Release was very rapid and approached maximum levels after 10 min.

Monocytes were treated with 10 μg of SMP per ml in the presence of 1 mM *N*-ethylmaleimide, an inhibitor of cysteine proteinases; 3 mM Pefabloc, an inhibitor of serine proteases; 1 μg of pepstatin per ml, an inhibitor of carboxyl proteases; 3

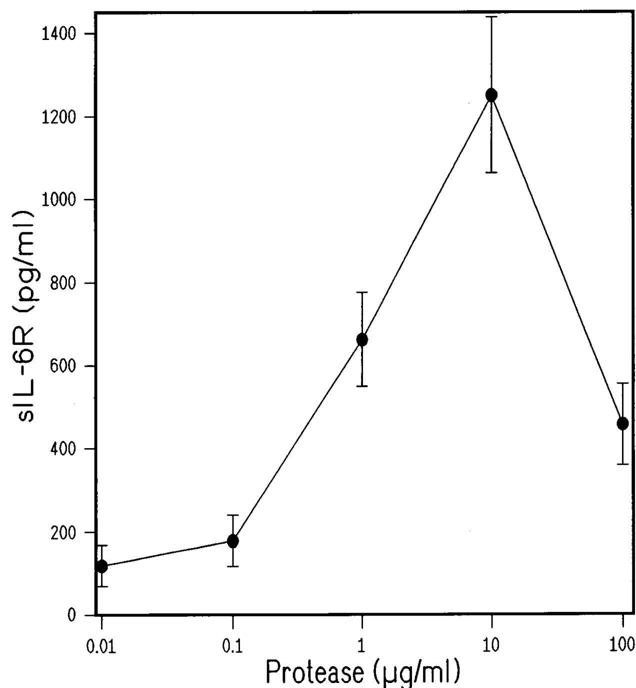

FIG. 2. Generation of soluble huIL-6R from monocytes induced by SMP. Monocytic cells (1.5×10^6) were treated with 0.01, 0.1, 1, 10, or 100 μg of SMP per ml for 40 min. sIL-6R concentrations in the supernatants of stimulated cells were determined by ELISA ($n = 3$). Error bars indicate standard deviations.

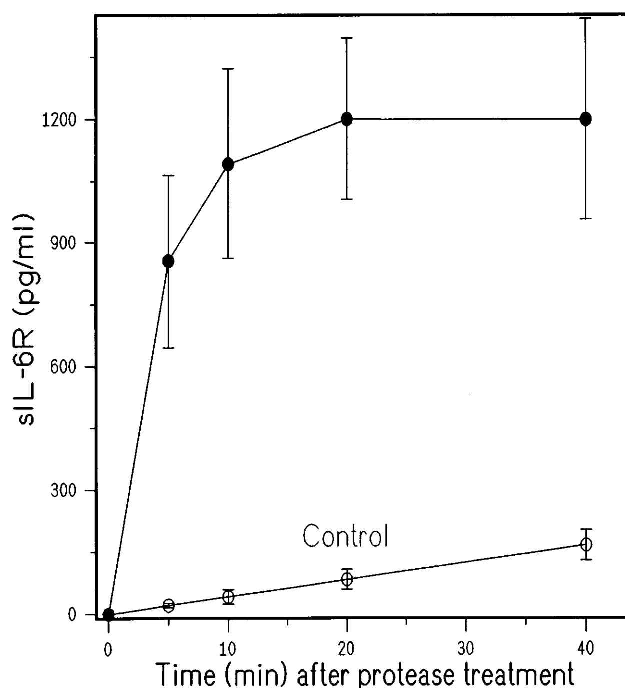

FIG. 3. Time dependency of sIL-6R generation. Monocytic cells (1.5×10^6) were treated with 10 μg of SMP per ml, and sIL-6 concentrations were measured in the supernatants at various time points. Cells incubated with supernatants of buffer alone (control) were assayed in parallel. Error bars indicate standard deviations ($n = 3$).

mM 1,10-phenanthroline, a conventional inhibitor of metalloproteinases; or 300 μM TAPI, a novel, specific inhibitor of the endogenous mammalian shedding metalloproteinase (17). As shown in Fig. 4, complete inhibition of sIL-6R liberation was observed in the presence of TAPI. Under the conditions noted in the legend to Fig. 4, 1,10-phenanthroline inhibited shedding of IL-6R by 50% whereas other inhibitors were completely ineffective.

sIL-6R liberated by SMP is biologically active in transsignalling. The biological activity of shed IL-6R was assessed by a well-defined experimental system. This system consisted of HepG2 cells that, after being stably transfected with a huIL-6 cDNA, constitutively express and secrete IL-6. These cells, termed HepG2-IL-6 cells, do not express IL-6R on the cell surface and, therefore, in the absence of extraneous sIL-6R, do not respond to IL-6 stimulation. These cells can be used to measure the biological activity of sIL-6R (13). As a source for sIL-6R, transfected COS-7 cells that expressed membrane-bound IL-6R were employed. In the absence of a shedding stimulus, supernatants from these cells do not contain significant amounts of sIL-6R. Accordingly, addition of the control supernatants did not provoke synthesis of haptoglobin mRNA in the HepG2 cells (Fig. 5A, control lane). When the COS-7 cells were stimulated with PMA, sIL-6R was shed into the supernatant and transsignalling activity became evident through the appearance of haptoglobin mRNA in the bystander HepG2-IL-6 cells (Fig. 5A). A positive Northern hybridization signal was similarly observed when supernatants of SMP-treated COS-7 cells were tested, and haptoglobin mRNA synthesis was virtually absent when TAPI was present during protease treatment. The far right lane in Fig. 5A (lane + sIL-6R) depicts the positive control experiment wherein recombinant sIL-6R was added to the HepG2-IL-6 cells. Incubation of HepG2-IL-6 cells with SMP alone did not induce haptoglobin mRNA synthesis (not shown). The synthesis of haptoglobin induced by the transsignalling process was revealed by ELISA measurements of haptoglobin in supernatants of HepG2 cells, and the results of two experiments are

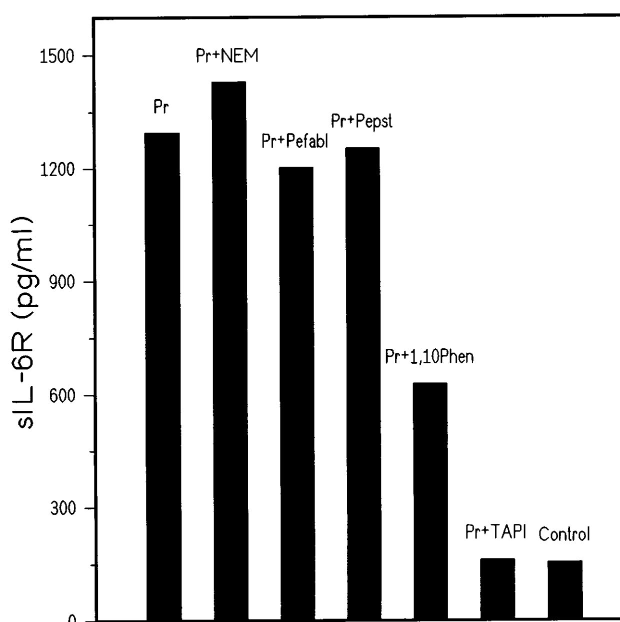

FIG. 4. Effect of protease inhibitors on sIL-6R generation. Monocytic cells (1.5×10^6) were treated with 10 μg of SMP (Pr) per ml alone or in the presence of 1 mM *N*-ethylmaleimide (NEM), 3 mM Pefabloc (Pefabl), 1 μg of pepstatin (Pepst) per ml, 3 mM 1,10-phenanthroline (1,10Phen), or 300 μM TAPI. sIL-6 was assayed by ELISA in supernatants of untreated (control) and treated cells.

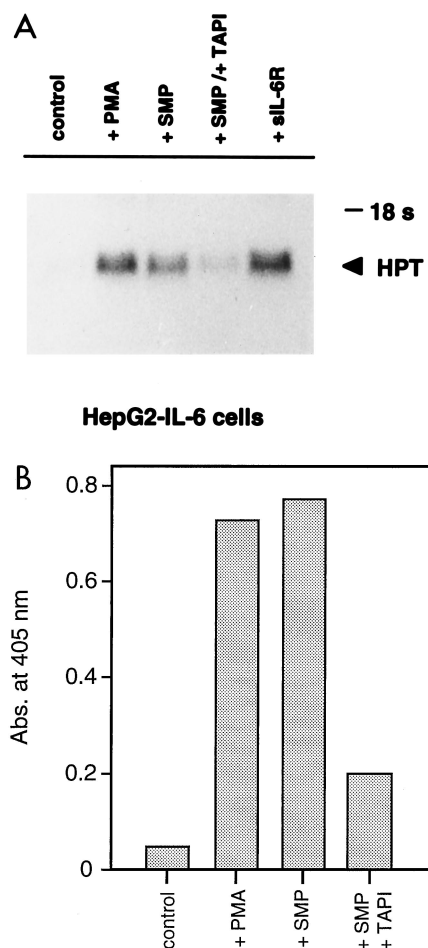

FIG. 5. Biologic activity of released sIL-6R. The biological activity of the released sIL-6R was determined by measuring the induction of synthesis of haptoglobin in HepG2-IL-6 cells. HepG2-IL-6 cells were stimulated for 18 h with supernatants from control COS-7-IL-6R cells or with supernatants of COS-7-IL-6R cells that had been treated with 10^{-7} M PMA, 5 μg of SMP per ml alone, or 5 μg of SMP in the presence of 300 μM TAPI (+SMP/+TAPI). As a positive control, HepG2-IL-6 cells were incubated with 10 ng of sIL6-R. (A) The expression of haptoglobin (HPT) was determined by Northern blot analysis. (B) Supernatants were analyzed by a haptoglobin ELISA. Means of two separate experiments are shown. Abs., absorbance.

depicted in Fig. 5B. Control HepG2-IL-6 cells incubated with supernatants from nonstimulated COS-7 cells secreted no detectable haptoglobin, whereas cells incubated with supernatants of COS-7 cells that had been treated either with PMA or with SMP secreted haptoglobin into the supernatant. Again, transsignalling activity was virtually absent in supernatants of COS-7 cells that had been treated with SMP in the presence of TAPI.

SMP and endogenous metalloproteinase cleave IL-6R at different sites. COS-7 cells transfected with IL-6R were metabolically labeled with [^{35}S]methionine-cysteine. The cells were exposed to PMA, SMP, or SMP plus TAPI. The supernatants were immunoprecipitated with three antibodies designated PM-1, MT18, and 6.2 and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

In controls, no immunoprecipitable IL-6R was recovered in supernatants (Fig. 6, control lanes). IL-6R was found exclusively in cell-bound form (data not shown). PMA stimulation led to almost quantitative shedding of IL-6R (Fig. 6, lanes +PMA), which was precipitated with all three antibodies in the supernatant. Treatment of cells with 5 μg of SMP per ml also led to a virtual disappearance of cellular IL-6R (data not shown). However, it was difficult to detect the protein in supernatants by immunoprecipitation. No immunoprecipitate could be detected with monoclonal antibody 6.2, which recognizes an epitope located very close to the membrane anchor (Fig. 1) (24). With monoclonal antibodies PM-1 and MT18, a faint immunoprecipitate of markedly lower molecular mass than that of sIL-6R shed by PMA stimulation was observed (45 versus 58 kDa). In the presence of TAPI, shedding by SMP was markedly inhibited (Fig. 6, lanes +SMP/+TAPI). From these findings, we conclude that cleavage by SMP produced a smaller sIL-6R molecule than stimulation with PMA, but the shed sIL-6R nevertheless retained transsignalling biological activity.

Shedding of IL-6R occurs through the action of other microbial proteases. To discern whether liberation of sIL-6R was an exclusive property of SMP, a panel of randomly selected bacterial isolates obtained from the diagnostic laboratory of the Institute for Medical Microbiology were cultured in Luria-Bertani medium, the culture supernatants were applied to human monocytes for 60 min, and sIL-6R was then assayed in the cell supernatants. Shedding activity was detected in supernatants of *S. marcescens*, *S. aureus*, *P. aeruginosa*, *B. subtilis*, and *L. monocytogenes*. In all these cases, shedding was at least partially inhibitable (>50%) by 300 μM TAPI or 3 mM 1,10-phenanthroline. This indicated that metalloproteinases, which are known to be produced by these bacteria, were indeed capable of cleaving IL-6R from the cells under the preservation of binding epitopes. In contrast, shedding activity was not detected in supernatants of *Streptococcus pyogenes* A, group B streptococci, *Streptococcus faecalis*, *Staphylococcus epidermidis*, *Escherichia coli*, *Proteus vulgaris*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, or *Aspergillus fumigatus*,

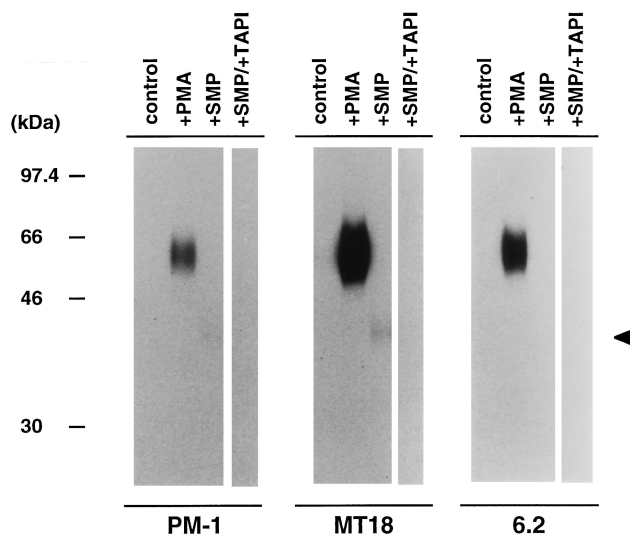

FIG. 6. Immunoprecipitation of sIL-6R released with different monoclonal antibodies. Recognition sites of the IL-6R antibodies MT18, PM-1, and 6.2 are shown. COS-7 cells (2 × 10^6) were transiently transfected with pCDM8-huIL-6R and seeded onto 35-mm-diameter plates. One day after transfection, cells were labeled for 2 h with 50 μCi of Tran^{35}S-label in methionine-cysteine-free medium. Cells were chased in complete medium for 1 h and further incubated for 1 h with 10^{-7} M PMA, 5 μg of SMP alone, or 5 μg of SMP in the presence of 300 μM TAPI. Supernatants of untreated (control) and stimulated cells were immunoprecipitated with different antibodies, as indicated, and analyzed by SDS-PAGE and fluorography. Molecular mass markers are noted at the left. The arrowhead indicates the position of SMP-cleaved sIL-6R.

despite the fact that many of these agents produce other proteinases (9).

DISCUSSION

The results of this investigation led us to conclude that certain microbial proteinases mimic endogenous membrane-bound proteinases in their capacity to liberate mediator molecules from the cell surface. We employed IL-6R as a model because it is particularly well studied at the molecular and functional levels. We believe that our findings will be extrapolative to other mediator molecules, the shedding of which is similarly dependent on the action of endogenous metalloproteinases. Of prime importance is the recognition that, by analogy to the action of the endogenous shedding proteinase, cleavage by microbial metalloproteinases occurs preferentially at sites that are not critical for biological function. Hence, the molecules are released in a biologically active form. In the case of sIL-6R, this was stringently demonstrated by the capacity of sIL-6R to confer IL-6 sensitivity upon HepG2-IL-6 cells that primarily lacked IL-6R. This transsignalling process was documented first by Northern blot analysis of haptoglobin mRNA and second by direct measurements of secreted haptoglobin in the IL-6-stimulated cells. Extrapolated to an in vivo setting, these findings make it easy to envisage how production of metalloproteinases by invading bacteria may provoke short- and long-range effects, including the induction or enhancement of acute-phase reactions and systemic inflammation in the host organism. IL-6R is probably only one of many biologically active molecules that are shed through the action of metalloproteinases. Examples for other molecules include CD14 (LPS receptor), tumor necrosis factor alpha, both types of tumor necrosis factor receptor, and the Fas ligand (4, 12, 18). ELISA measurements have already shown that soluble CD14 is also liberated in an immunologically detectable form from human monocytes through the action of SMP (unpublished observations).

The three-dimensional structure of SMP has been elucidated, and it is known that the protein exhibits homologies with the alkaline protease of *P. aeruginosa* (1). Metalloproteinases are produced by many other bacterial species; the interested reader is referred to a recent compilation for an overview (9). We have not undertaken to test other metalloproteinases for shedding activities, but the positive results obtained with unfractionated culture supernatants from *S. aureus*, *P. aeruginosa*, *L. monocytogenes*, and *B. subtilis*, in addition to those from *S. marcescens*, indicate that the capacity to liberate membrane-bound mediators is widespread. Our inhibition experiments showed that TAPI, a specific inhibitor of the mammalian shedding metalloproteinases, also inhibited shedding of IL-6R by SMP. Inhibition of IL-6R shedding by TAPI was similarly observed when culture supernatants of *S. aureus*, *P. aeruginosa*, and *L. monocytogenes* were assayed (not shown). The experiments with unfractionated supernatants are preliminary. We do not exclude the possibility that other microbial proteinases may also be capable of effecting shedding. We have also not excluded the possibility that shedding activities detected in the supernatants of *S. aureus*, *P. aeruginosa*, and *L. monocytogenes* were due to factors additional to microbial metalloproteinases.

In our attempts to biochemically characterize the sIL-6R shed through the action of SMP, we encountered difficulties. Following metabolic labeling of IL-6R with [^{35}S]methionine-cysteine, immunoprecipitation experiments utilizing three well-characterized antibodies did show the disappearance of IL-6R from the cells. However, the shed IL-6R molecules were difficult to detect in the supernatants. Figure 6 clearly indicates that the faint ~45-kDa band recognized by the monoclonal antibodies MT18 and PM-1 cannot represent all sIL-6R molecules generated by the action of SMP. One possibility is that the bacterial protease nicks sIL-6R at multiple sites. The nicked molecule may remain intact and functionally active in the absence of SDS but may fragment in the presence of detergent to become undetectable after SDS-PAGE. That SMP cleaves IL-6R at one or several sites distinct from that utilized by the endogenous metalloproteinase was apparent from the fact that sIL-6R was not recognized by antibody 6.2 (24), which precipitated sIL-6R generated by the endogenous shedding protease. That SMP potentially causes extensive fragmentation and destruction of IL-6R was indicated from the finding that a very high protease concentration of 100 μg/ml led to a significantly lower level of recovery of sIL-6R in ELISA assays than a concentration of 10 μg/ml.

It is understandable that a truncated version of sIL-6R exhibits biological activity, since it is known that the NH_2-terminal fibronectin III domain and the tether domain of the extracellular portion of huIL-6R are dispensable for biological function (27, 28). Since the two COOH-terminal fibronectin III domains are essential for ligand binding and signal initiation (27), they must be left intact by the bacterial metalloprotease. It can be concluded that cleavage must occur between the end of the third fibronectin III domain (S-320) and the recognition epitope of antibody 6.2 (E-324 to T-336).

sIL-6R is N glycosylated at position N-350. Deletion of 5 amino acid residues (A-349 to S-353), including the glycosylation site N-350, led to the formation of a sIL-6R protein of 45 to 48 kDa (19). This is the size of the faint protein band detected by immunoprecipitation with the monoclonal antibodies MT18 and PM-1. We therefore speculate that the NH_2-terminal portion of the SMP-shed sIL-6R protein, which was detected by immunoprecipitation with the monoclonal antibodies MT18 and PM-1, is still intact. Irrespective of residual uncertainties, it is apparent that sIL-6R shed through the action of SMP is as active in transsignalling as sIL-6R generated by the endogenous metalloproteinase.

To summarize, this study provides the first evidence that bacterial proteases, in particular metalloproteinases, can liberate membrane-anchored mediators in biologically active form from cells of the host microorganism. The choice of SMP was admittedly not the best one from an etiologic viewpoint, since *S. marcescens* is not a widespread human pathogen. Our choice was dictated by the availability of this and not other metalloproteinases in a highly purified form. The novel concept emerging from this work will of course require further investigation; at present, it is our bias that the concept will prove to be correct and have biological relevance.

The agonistic sIL-6R generated by SMP is only one example of a metalloproteinase with possible pathophysiological relevance to bacterial infection. Cleavage of cell surface recognition molecules or receptors for growth factors and cytokines by the action of bacterial proteases can be expected to perturb cellular communication and interactions. A further obvious consequence that may ensue from receptor cleavage would be refractoriness of the cells to stimulation with the respective cytokine. We have not been able to directly demonstrate such loss of responsiveness, because our present assay system requires prolonged incubation of the cells with IL-6, during which time de novo synthesis of the receptor will occur. Nevertheless, our findings indicate that by analogy to secondary effects that can be evoked by bacterial cytolysins (3) and phospholipases (26), microbial proteases should now be considered

agents that may derange homeostasis in the host in a multifaceted fashion.

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P.V. and I.W. contributed equally to this work.

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REFERENCES

1. **Baumann, U.** 1994. Crystal structure of the 50 kDa metallo protease from *Serratia marcescens*. J. Mol. Biol. **242:**244–251.
2. **Bazil, V.** 1995. Physiological enzymatic cleavage of leukocyte membrane molecules. Immunol. Today **16:**135–140.
3. **Bhakdi, S., F. Grimminger, N. Suttorp, D. Walmrath, and W. Seeger.** 1994. Proteinaceous bacterial toxins and pathogenesis of sepsis syndrome and septic shock: the unknown connection. Med. Microbiol. Immunol. **183:**119–144.
4. **Crowe, P. D., B. N. Walter, K. M. Mohler, C. Otten-Evans, R. A. Black, and C. F. Ware.** 1995. A metalloprotease inhibitor blocks shedding of the 80 kD TNF receptor processing in T lymphocytes. J. Exp. Med. **181:**1205–1210.
5. **Denholm, E. M., and F. M. Wolber.** 1991. A simple method for the purification of human peripheral blood monocytes. A substitute for Sepracell-MN. J. Immunol. Methods **144:**247–251.
6. **Ehlers, M. R., and J. F. Riordan.** 1991. Membrane proteins with soluble counterparts: role of proteolysis in the release of transmembrane proteins. Biochemistry **30:**10065–10074.
7. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
8. **Gearing, A. J. H., P. Beckett, M. Christodoulou, M. Churchill, J. Clements, A. H. Davidson, A. H. Drummond, W. A. Galloway, R. Gilbert, J. L. Gordon, T. M. Leber, M. Mangan, K. Miller, P. Nayee, K. Owen, S. Patei, W. Thomas, G. Wells, L. M. Wood, and K. Woolley.** 1994. Processing of tumor necrosis factor-α alpha precursor by metalloproteinases. Nature (London) **370:**555–557.
9. **Häse, C. C., and R. A. Finkelstein.** 1993. Bacterial extracellular zinc-containing metalloproteases. Microbiol. Rev. **57:**823–837.
10. **Iglewski, B. H., and T. I. Nicas.** 1985. Extracellular factors in the virulence of *Pseudomonas aeruginosa*, p. 257–266. *In* J. A. Roth (ed.), Virulence mechanism of bacterial pathogens. Springer, Berlin.
11. **Katsuya, Y., M. Sato, Y. Katsube, Y. Matsuura, and K. Tomoda.** 1992. Small-angle x-ray scattering study of metal ion-induced conformational changes in Serratia protease. J. Biol. Chem. **267:**12668–12672.
12. **Kayagaki, N., A. Kawasaki, T. Ebata, H. Ohmoto, S. Ikeda, S. Inoue, K. Yoshino, K. Okumura, and H. Yagita.** 1995. Metalloproteinase-mediated release of human Fas ligand. J. Exp. Med. **182:**1777–1783.
13. **Mackiewicz, A., H. Schooltink, P. C. Heinrich, and S. Rose-John.** 1992. Complex of soluble human IL-6-receptor/IL-6 up-regulates expression of acute-phase proteins. J. Immunol. **149:**2021–2027.
14. **Maeda, H., T. Akaike, Y. Sakata, and K. Maruo.** 1993. Role of bradykinin in microbial infection: enhancement of septicemia by microbial proteases and kinin. Agents Actions Suppl. **42:**159–165.
15. **Massagué, J., and A. Pandiella.** 1993. Membrane-anchored growth factors. Annu. Rev. Biochem. **62:**515–541.
16. **McGeehan, G. M., J. D. Becherer, R. C. Blast, Jr., C. M. Boyer, B. Champion, K. M. Conolly, J. G. Conway, P. Furdon, S. Karp, S. Kidao, A. B. McElroy, J. Nichols, K. M. Pryzwansky, F. Schoenen, L. Sekut, A. Truesdale, M. Verghese, J. Warner, and J. P. Ways.** 1994. Regulation of tumor necrosis factor-α processing by a metalloproteinase inhibitor. Nature (London) **370:**558–561.
17. **Mohler, K. M., P. R. Sleath, J. N. Fitzner, D. P. Cerretti, M. Alderson, S. S. Kerwar, D. S. Torrance, C. Otten-Evans, T. Greenstreet, K. Weerawarna, S. R. Kronheim, M. Petersen, M. Gerhart, C. J. Kozlosky, C. J. March, and R. A. Black.** 1994. Protection against a lethal dose of endotoxin by an inhibitor of tumour necrosis factor processing. Nature (London) **370:**218–220.
18. **Müllberg, J., F. H. Durie, C. Otten-Evans, M. R. Alderson, S. Rose-John, D. Cosman, R. A. Black, and K. M. Mohler.** 1995. A metalloprotease inhibitor blocks shedding of the IL-6 receptor and the p60 TNF receptor. J. Immunol. **155:**5198–5205.
19. **Müllberg, J., W. Oberthur, F. Lottspeich, E. Mehl, E. Dittrich, L. Graeve, P. C. Heinrich, and S. Rose-John.** 1994. The soluble human IL-6 receptor. Mutational characterization of the proteolytic cleavage site. J. Immunol. **152:**4958–4968.
20. **Müllberg, J., H. Schooltink, T. Stoyan, M. Günther, L. Graeve, G. Buse, A. Mackiewicz, P. C. Heinrich, and S. Rose-John.** 1993. The soluble interleukin-6 receptor is generated by shedding. Eur. J. Immunol. **23:**473–480.
21. **Rose-John, S., and P. C. Heinrich.** 1994. Soluble receptors for cytokines and growth factors: generation and biological function. Biochem. J. **300:**281–290.
22. **Rose-John, S., G. Rincke, and F. Marks.** 1987. The induction of ornithine decarboxylase by the tumor promoter TPA is controlled at the post-transcriptional level in murine Swiss 3T3 fibroblasts. Biochem. Biophys. Res. Commun. **147:**219–225.
23. **Salvati, A. L., A. Lahm, G. Paonessa, G. Ciliberto, and C. Toniatti.** 1995. Interleukin-6 (IL-6) antagonism by soluble IL-6 receptor alpha mutated in the predicted gp130-binding interface. J. Biol. Chem. **270:**12242–12249.
24. **Stoyan, T., U. Michaelis, H. Schooltink, M. Van Dam, R. Rudolph, P. C. Heinrich, and S. Rose-John.** 1993. Recombinant soluble human interleukin-6 receptor. Expression in *Escherichia coli*, renaturation and purification. Eur. J. Biochem. **216:**239–245.
25. **Tamura, Y., S. Suzuki, and T. Sawada.** 1992. Role of elastase as a virulence factor in experimental *Pseudomonas aeruginosa* infection in mice. Microb. Pathog. **12:**237–244.
26. **Titball, R. W.** 1993. Bacterial phospholipase C. Microbiol. Rev. **57:**347–366.
27. **Vollmer, P., M. Peters, M. Ehlers, H. Yagame, T. Matsuba, M. Kondo, K. Yasukawa, K.-H. Meyer zum Büschenfelde, and S. Rose-John.** Yeast expression of the cytokine receptor domain of the soluble interleukin-6-receptor. Submitted for publication.
28. **Yawata, H., K. Yasukawa, S. Natsuka, M. Murakami, K. Yamasaki, M. Hibi, T. Taga, and T. Kishimoto.** 1993. Structure-function analysis of human IL-6 receptor: dissociation of amino acid residues required for IL-6-binding and for IL-6 signal transduction through gp130. EMBO J. **12:**1705–1712.

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