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Probing for Actinase Activity of Protealysin

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Abstract—The ability of protealysin, a thermolysin-like metallopeptidase from *Serratia proteamaculans* 94, to cleave actin and matrix metalloprotease MMP2 is reported. In globular actin, protealysin and *S. proteamaculans* 94 cell extracts are shown to hydrolyze the Gly42–Val43 peptide bond within the DNase-binding loop and the Gly63–Ile64 and Thr66–Ile67 peptide bonds within the nucleotide cleft of the molecule. At enzyme/substrate mass ratio of 1 : 50 and below, a 36 kDa-fragment produced by the cleavage between Gly42 and Val43 was virtually resistant to further breakdown. Judging from the results of zymography, protealysin transforms proMMP2 into a 66 kDa polypeptide characteristic of mature MMP2, indicating that protealysin can activate MMP2. Upon incubation of *S. proteamaculans* 94 with human larynx carcinoma Hep-2 cells intracellular bacteria were detected in about 10% of Hep-2 cells, this being the first evidence for invasion of eukaryotic cells with bacteria of this species. Thus, *S. proteamaculans* 94 turned out to be one more bacterial strain in which synthesis of actin-specific metalloprotease is coupled with bacterial invasion. These results are consistent with the idea of the actinase activity of bacterial metalloproteases being a factor that may promote bacterial invasion of eukaryotic cells.

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Key words: actin, metalloprotease, *Serratia*, bacterial invasion, ECP32, grimelysin

Zinc-containing metalloproteases are common in all living organisms. In eukaryotic tissues, metalloproteases play a key role in proliferation, differentiation, cell migration, cell–cell interactions, and other normal processes as well as in pathogenesis [1-4]. In bacteria, zinc-containing metalloproteases are involved in sporulation, regulation of cell envelope composition, and specific gene expression [5, 6]. Many toxins and bacterial virulence factors are metalloproteases [5, 7]. Specifically, various bacteria produce extracellular proteases that act as virulence factors activating or inhibiting matrix metalloproteases [1, 8, 9]. It has also been shown that metalloproteases of pathogenic bacteria can interact with proteins of the cell surface and cytoskeleton [7, 10]. Studying the role of these interactions is important to reveal both mechanisms of bacterial virulence/invasiveness and the ligand–receptor relationships and their involvement in susceptibility of eukaryotic cells to infection.

Bacterial zinc-containing metalloproteases are used in studies on protein structure and function by the limited proteolysis method. This method is employed to detect local conformational changes in proteins and protein complexes, to identify domains, and to isolate protein fragments [11]. The efficiency of the limited proteolysis is higher when more specific enzymes are employed. Earlier we discovered, isolated, and partially characterized a novel enzyme, bacterial metalloproteinase ECP32, with a high specificity toward actin [12-14]. Actin is cleaved with protease ECP32 at a single site between Gly42 and Val43 within the DNase-binding loop of subdomain 2; this site is not attacked by any other known protease, and its cleavage results in reversible loss of actin polymerization [15, 16]. Therefore, protease ECP32 has been an efficient tool for studying actin structure/function relationship and mechanisms of actin polymerization [15-23].

Furthermore, we have shown that nonpathogenic bacteria synthesizing protease ECP32 can penetrate into eukaryotic cells and produce actin cytoskeleton rearrangements [24]. The correlation between the actin-hydrolyzing and invasive activities was also found in furazolidon-induced *Shigella flexneri* mutants [25], and,

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according to our preliminary data, in *Serratia grimesii* producing metalloprotease grimelysin that seems to be identical to ECP32 [26].

Recently a new metalloprotease of *Serratia proteamaculans*, protealysin, has been identified [27]. This enzyme is similar to ECP32/grimelysin in amino acid sequence and some properties of its active form [26, 27]. At the present time, protealysin is one of the best characterized thermolysin-like proteases [28, 29]. Moreover, the *S. proteamaculans* genome is completely sequenced (GenBank ID CP000826). Therefore, use of these bacteria and protealysin is promising for studies on mechanisms of invasion associated with actin cytoskeleton rearrangements. This work is aimed to elucidate whether protealysin cleaves actin, and whether *S. proteamaculans* 94, which naturally produces protealysin, can penetrate into eukaryotic cells.

MATERIALS AND METHODS

The following reagents were used in this study: Tris, ATP (disodium salt), sodium azide, thermolysin, Triton X-100, reagents for electrophoresis, fluorescein isothiocyanate (FITC), rhodamine-phalloidin, and gelatin were purchased from Sigma (USA). Peptone and yeast extract were from Difco (USA).

Bacteria and cell line. *Serratia proteamaculans* 94 [27] was grown in Luria broth (LB medium) at 30°C, with aeration. Human larynx carcinoma Hep-2 cells (Russian Cell Culture Collection, Institute of Cytology, St. Petersburg) were grown on glass coverslips in culture dishes in Dulbecco's modified Eagle's medium (DMEM) (Biolot, Russia) containing 10% fetal calf serum (Sigma) in a 37°C incubator supplemented with 5% CO_2 for the time required to form a monolayer (usually about 48 h).

Protein preparations. Protealysin was purified from *E. coli* BL21(DE3) (pSIT20) lysate by sequential chromatography steps on an Econo-Pac High Q anion-exchange column (Bio-Rad, USA), Phenyl Sepharose CL-4B column (Amersham Biosciences, USA), and gel filtration on a Superdex 75 HR 10/30 column (Amersham Biosciences) as described previously [27]. Homogeneity of the samples was proved by SDS-PAGE [30]. Protealysin was stored in 25 mM Tris-HCl buffer, pH 8.0, at 4°C.

Rabbit skeletal muscle actin was isolated by a standard procedure of Spudich and Watt [31]. Globular actin (G-actin) was stored in buffer G (0.2 mM ATP, 0.1 mM $CaCl_2$, 5 mM Tris-HCl, pH 7.5, 0.02% NaN_3) on ice during a week or as 0.2-ml aliquots (0.5-1.0 mg/ml) frozen at –20°C for a single use.

Limited proteolysis assay. Actin (0.5 mg/ml in buffer G) was incubated with protealysin (0.3 mg/ml in 25 mM Tris-HCl, pH 8.0) at various enzyme/protein mass ratios at 22°C. At different time points, the digestion was stopped by addition of an equal volume of the electrophoresis sample buffer containing 4% SDS, 125 mM Tris-HCl, pH 6.8, followed by a 3-min boiling. The digestion products were analyzed by SDS-PAGE [30]. The actinase activity was defined by the appearance of specific actin fragments.

To determine the ability of bacterial extracts to cleave actin, bacteria grown in LB medium were harvested by centrifugation at 9600*g* for 10 min. The pellets were resuspended in buffer G, concentration of bacteria was determined by optical density at 600 nm, and the bacteria were lysed by five cycles of freezing and thawing. The lysates (bacterial extracts) were clarified by centrifugation at 9600*g* for 10 min. Actin in buffer G (0.5 mg/ml) was mixed with an equal volume of the clarified lysates and incubated for 2 h at room temperature. The reaction was stopped by addition of the SDS-buffer, and the products were analyzed by SDS-PAGE as described above.

Thermolysin (2.6 mg/ml in 25 mM Tris-HCl, pH 8.0) was added to G-actin (0.5 mg/ml in buffer G) at different enzyme/protein mass ratios at 22°C. The digestion was stopped with 1 mM *o*-phenanthroline.

To determine susceptibility to protealysin of matrix metalloprotease MMP2, zymography with gelatin was performed [32]. Fibroblast-conditioned medium containing MMP2 was incubated with protealysin for 2 h at room temperature. The samples were then incubated with electrophoresis sample solution (62.5 mM Tris-HCl, pH 6.8, 0.1% SDS) for 30 min and analyzed by SDS-PAGE differing from a regular SDS-PAGE by the presence of 0.3% gelatin in the resolving gel. After electrophoresis, the gel was washed twice with 2.5% Triton X-100 to remove SDS and incubated in 5 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.2-7.4, for 18 h at 37°C to renature the proteases and carry out proteolysis. Finally, the gel was fixed in 25% isopropanol with 10% acetic acid for 30 min and stained with Coomassie brilliant blue G-250.

Determination of actin cleavage site. Actin fragments resulting from the proteolysis with protealysin were separated by SDS-PAGE, blotted to PVDF membrane (Millipore, USA), and subjected to N-terminal Edman degradation using an ABI Procise 491 sequencer (Proteome Factory AG, Germany).

Invasion assay. Bacteria were grown in LB medium at 30°C with aeration until actinase activity of their extracts could be determined (usually 48-49 h). Thirty minutes before the experiment, FITC (50 μg/ml of the bacterial suspension) was added to the bacterial culture to visualize bacteria. Bacteria were pelleted at 9600*g* for 10 min; the pellets were resuspended in DMEM and added to Hep-2 cells in a fresh portion of DMEM. Hep-2 cells and bacteria were co-cultivated at 37°C in 5% CO_2 for 3 h.

Confocal microscopy. To visualize actin cytoskeleton, the cells were washed with PBS, fixed with 3.7% formaldehyde for 10 min, incubated with 0.1% Triton X-

100 for 5 min, and stained with rhodamine-phalloidin for 15 min. The samples were examined under a Leica TCS SL confocal scanning microscope using argon (488 nm) and helium-neon (543 nm) laser systems.

Electron microscopy. Cells on cover slips were fixed with 2.5% glutaraldehyde in PBS for 40 min followed by post-fixation with 2% OsO_4 in PBS for 30 min. The fixed cells were dehydrated in ethanol of ascending concentration and embedded in Epon-Araldite (Fluka, Germany). Ultrathin sections were counterstained with lead citrate and 2% uranyl acetate in 50% ethanol and examined with a JEM-100U electron microscope at 80 kV.

RESULTS

Cleavage of actin with protealysin. In the actin molecule, ECP32 cleaves only one peptide bond between Gly42 and Val43 producing two fragments of 36 and 5 kDa, which are resistant to further proteolysis [15]. Lysates of the bacteria synthesizing ECP32/grimelysin also hydrolyzed actin between Gly42 and Val43 [12, 26]. Similar proteolysis was observed upon interaction of actin with protealysin (Fig. 1). Protealysin completely cleaved actin already in 30 min of the incubation as evidenced by transformation of actin into the 36 kDa fragment. (The small 5 kDa fragment is not seen on the gels; for its visualization, a special staining of the sample with fluorescent dye is required [16].) At the protealysin/actin mass ratio of 1 : 50, further cleavage of the 36 kDa fragment was not observed even on 4-h incubation (Fig. 1a). At the higher protealysin concentration (enzyme/actin mass ratio of 1 : 10), the 36 kDa fragment was digested to yield a 33 kDa fragment (Fig. 1b).

To determine the cleavage sites, protealysin was added to actin at the ratios of 1 : 5 and 1 : 50. Actin fragments were separated by SDS-PAGE, transferred to PVDF membrane, and their N-terminal sequences were analyzed. The N-terminal sequence of the 36 kDa-fragment was determined to be Val-Met-Val-Gly-Met-Gln, which corresponds to the cleavage between Gly42 and Val43. This site is identical to the actin cleavage site for ECP32/grimelysin.

In the sample containing the 33 kDa-fragment, sequencing revealed the presence of two fragments, which were not separated by electrophoresis. The N-terminal sequences of these fragments were determined to be Leu-Lys-Tyr-Pro-Ile-Glu and Ile-Leu-Thr-Leu-Lys-Tyr. These sequences correspond to hydrolysis of the bonds Thr66–Leu67 and Gly63–Ile64.

Bacteria synthesizing ECP32/grimelysin exhibited the specific proteolytic activity at the post-logarithmic

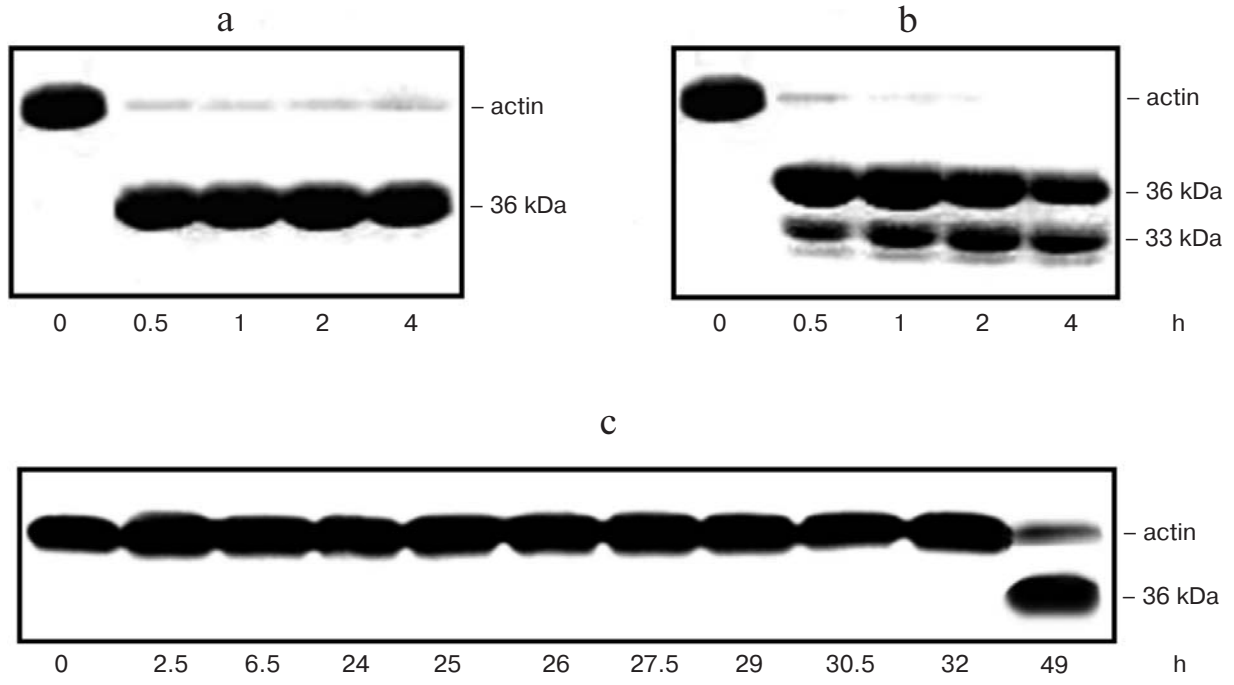

Fig. 1. Actinase activity of protealysin (a, b) and bacterial extracts from *S. proteamaculans* 94 (c). To determine kinetics of actin cleavage, protealysin (0.3 mg/ml in 25 mM Tris-HCl, pH 8.0) was added to G-actin (0.5 mg/ml in buffer G) at mass ratios of 1 : 50 (a) and 1 : 10 (b). After incubation at the time intervals indicated at 22°C, the reaction was stopped by addition of an equal volume of electrophoresis sample buffer containing 4% SDS, 125 mM Tris-HCl. To reveal actinase activity in extracts of *S. proteamaculans* 94, the extracts were obtained as described in "Materials and Methods" and incubated with actin for 2 h at 22°C. Numbers indicate the time of cultivation of the bacteria whose extracts were tested for actinase activity shown on the corresponding lane.

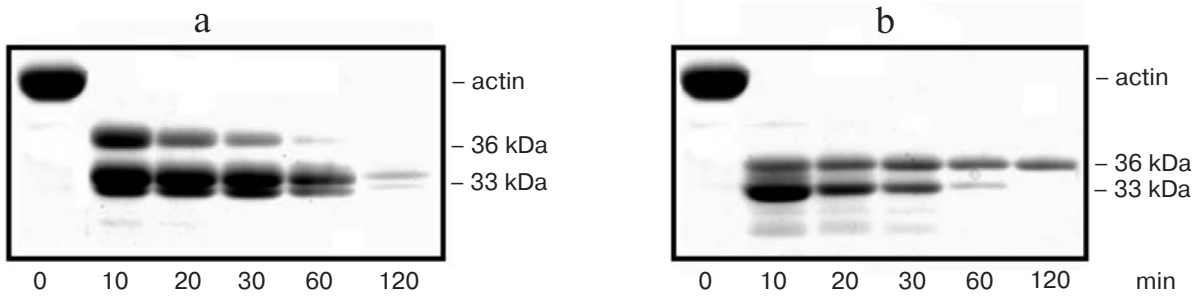

Fig. 2. Proteolysis of actin with thermolysin. Thermolysin (2.6 mg/ml in 25 mM Tris-HCl, pH 8.0) was added to G-actin (0.5 mg/ml in buffer G) at mass ratios of 1 : 50 (a) and 1 : 10 (b). After incubation at the time intervals indicated at 22°C, the reaction was stopped by addition of 1 mM *o*-phenanthroline.

growth stage [12, 26]. We obtained similar data for *S. proteamaculans* 94. In the assay used, the lysates cleaved actin only when the bacteria were cultivated for 49 h (Fig. 1c).

Protealysin is a member of the peptidase family M4 whose characteristic enzyme is thermolysin [33]. The results of actin proteolysis with thermolysin are shown in Fig. 2. Similarly to protealysin, thermolysin cleaved actin yielding both the 36- and 33-kDa fragments. However, these fragments were intensively digested even at the thermolysin/actin mass ratio of 1 : 50. At the ratio of 1 : 10, rapid unlimited actin degradation was observed.

MMP2 as a substrate for protealysin. Some bacteria are known to secret proteases, which can act as virulence factors while activating or inhibiting matrix metalloproteases [8, 9]. Therefore, we examined susceptibility of matrix metalloprotease MMP2 to protealysin using zymography with gelatin. The method is based on the ability of MMP2 to renature after SDS-PAGE, which results in the cleavage of gelatin in the area of the gel where MMP2 is present. Matrix metalloprotease MMP2, whose inactive form (proMMP2) has molecular mass of 72 kDa [34], is contained in fibroblast-conditioned medium [35] (Fig. 3). Upon incubation of the conditioned medium with protealysin, intensity of the band corresponding to proMMP2 was diminished,

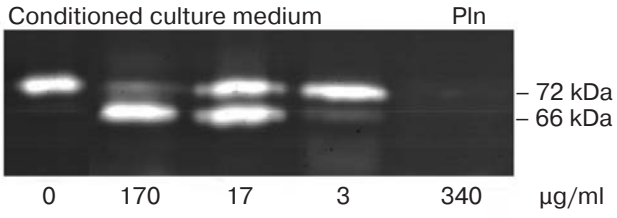

Fig. 3. Susceptibility of matrix metalloprotease MMP2 to protealysin. Conditioned culture medium containing MMP2 was incubated with protealysin at the protealysin concentrations indicated for 2 h at room temperature. Pln, protealysin incubated under the same conditions. Other conditions and details are described in "Materials and Methods".

whereas the 66 kDa fragment consistent with molecular mass of activated MMP2 [34] appeared. Efficiency of this transformation depended on protealysin concentration (Fig. 3). As shown by the last lane in Fig. 3, protealysin after the electrophoresis run did not digest gelatin. These data indicate that protealysin can activate MMP2.

Invasive capacity of *S. proteamaculans* 94. We have previously shown that bacteria displaying ECP32-like actinase activity are also able to invade eukaryotic cells [24, 25]. Therefore, the next part of the work was aimed to reveal capacity to invasion of *S. proteamaculans* 94 which naturally produce protealysin.

Figure 4 (see color insert) represents the results of experiments in which human larynx carcinoma Hep-2 cells were incubated with bacteria *S. proteamaculans* 94 grown for 49 h. Within 3 h of incubation, the cell monolayer had partially loosened, Hep-2 cells changed their shape, numerous actin-containing protrusions appeared on the cell surface, and bacteria were revealed in the cytoplasm of about 10% of the cells.

Electron microscopy provided additional evidence for the presence of *S. proteamaculans* 94 bacteria in the cytoplasm of Hep-2 cells (Fig. 5). In the samples prepared on 3 h of infection, the bacteria were observed both as being attached to the Hep-2 cell surface (Fig. 5b) and inside the cells residing in vacuoles (Fig. 5c). In contrast to the control cells in which cortical actin, intermediate filaments, and microtubules are seen well (Fig. 5a), the apical area of the infected cells does not contain these cytoskeleton components (Fig. 5c) probably due to their depolymerization.

Taken together, these data show that *S. proteamaculans* 94 bacteria, whose lysates have the ECP32-like actinase activity, can invade Hep-2 cells.

DISCUSSION

Protealysin, like protease ECP32/grimelysin, belongs to the thermolysin-like proteases [27]; most of

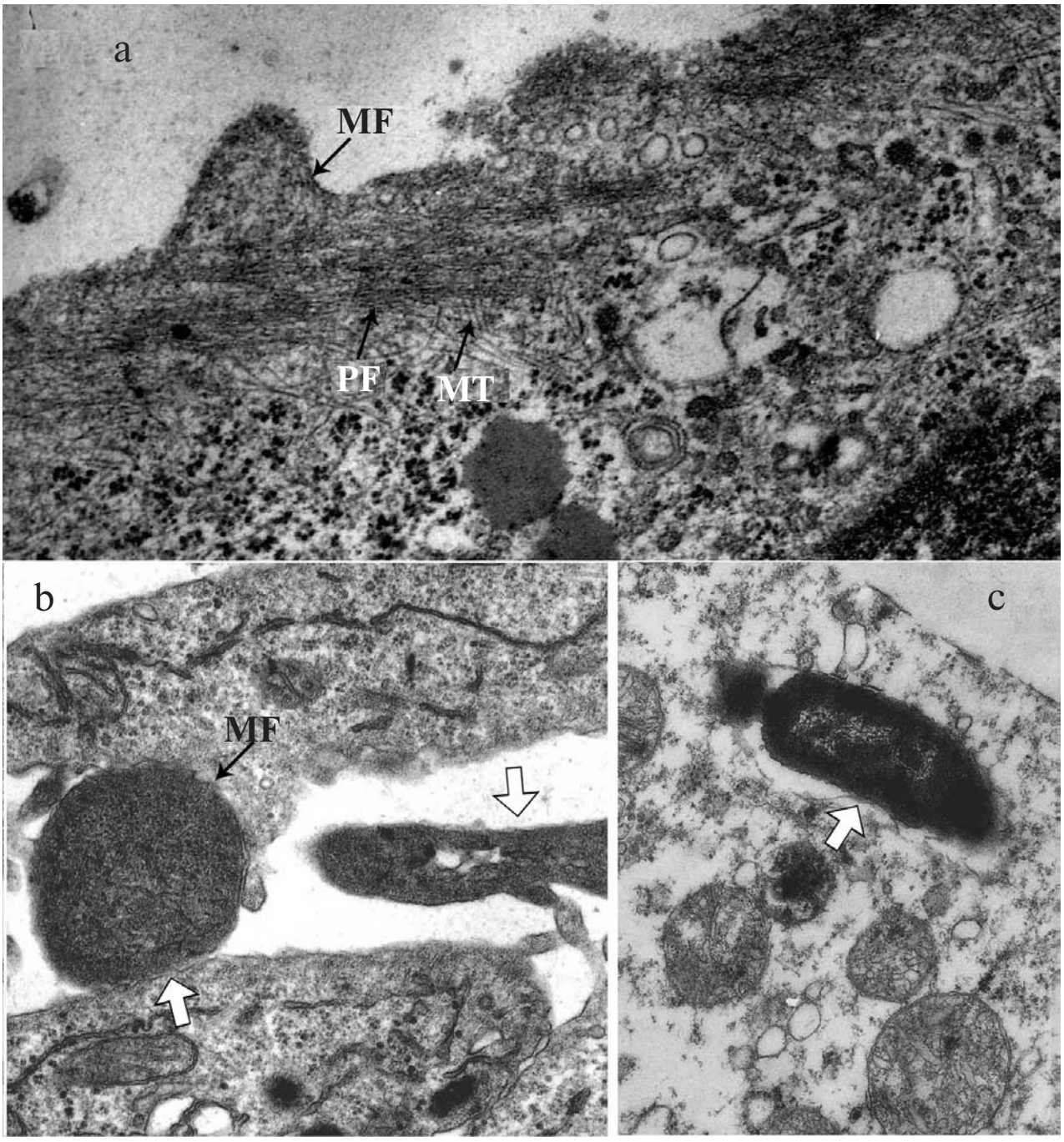

Fig. 5. Different stages of invasion of human larynx carcinoma Hep-2 cells by *S. proteamaculans* 94 bacteria observed under electron microscopy. Hep-2 cells were incubated with bacteria in the same manner as for confocal microscopy: a) non-infected Hep-2 cells; b) a tight contact of a bacterium with Hep-2 cell; c) intracellular localization of bacterium. Labeling: MF, microfilaments; PF, intermediate filaments; MT, microtubules.

these enzymes display broad substrate specificity [36]. However, protease ECP32/grimelysin was found to show quite narrow specificity toward intact proteins. Only actin, histones, melittin, and bacterial heat shock protein DnaK have been found to be substrates for protease ECP32 among more than 30 native proteins tested so far [12-14]. In actin molecule, protease ECP32 hydrolyzes only one peptide bond, between Gly42 and Val43; the produced fragments do not undergo further proteolysis and remain noncovalently associated to each other [15, 16]. Because amino acid sequences of protealysin [27] and ECP32/grimelysin [26] are similar, we suggested that protealysin would cleave actin limitedly. The results of our work have proved this suggestion to be true.

Protealysin cleaves actin within the DNase-binding loop (residues 38-52) that is located at the surface of the molecule, being accessible to many proteases including thermolysin [37]. However, in contrast to thermolysin, protealysin cleaves actin limitedly, and the sites in actin for the cleavages with protealysin and ECP32/grimelysin coincide. Upon the cleavage at this site, two more peptide bonds located in the interdomain (nucleotide-containing) cleft of the actin molecule are rendered accessible for protealysin. These data are consistent with the idea that hydrolysis of the Gly42–Val43 bond results in conformational transition toward a more open nucleotide-containing cleft [17, 21]. Hence, protealysin can be used to study conformational transitions in the nucleotide cleft during actin polymerization and its interactions with actin-binding proteins.

Our data suggest that protealysin performs its biological functions outside the bacterial cell. In favor of this suggestion are the results of confocal and electron microscopy, which have demonstrated that *S. proteamaculans* 94 cells synthesizing protealysin can penetrate into eukaryotic cells. Penetration of bacteria into eukaryotic cells is known to be associated with actin cytoskeleton rearrangements [38-40]. A first step of this process is the interaction of bacterial factor with receptor or actin-binding protein that induces depolymerization of cortical actin at the site of bacterial-cell contact [41]. This is followed by actin polymerization in cell surface protrusions, which rise around the bacterial body and allow its engulfment in a macropinocytic-like process [39, 40]. It is possible that protealysin initiates this process by activating matrix metalloproteases. However, protealysin is not secreted either by recombinant *E. coli* [27] or, according to the preliminary data, by *S. proteamaculans* 94 (I. V. Demidyuk, unpublished results). It is more plausible therefore that the protealysin actinase activity plays the role of a factor promoting invasion. The Gly42–Val43 bond cleaved with protealysin is located in the N-terminal segment of the DNase-binding loop that is intensively involved in the monomer–monomer contacts along actin polymer. Disruption of this bond strongly affects physiological functions of actin [14, 16, 21, 22]; it enhances dynamics of actin polymers, significantly accelerating their depolymerization [21], whereas efficiency of the cleaved actin polymerization is considerably increased by actin-binding proteins [23, 42]. This implies that limited cleavage with protealysin may change actin properties in a direction favorable for invasion and thus promote internalization of the bacteria that synthesize this enzyme.

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