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Membrane Localization of the HflA Regulatory Protease of *Escherichia coli* by Immunoelectron Microscopy

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The *hflA* locus of *Escherichia coli* specifies a multisubunit protease that selectively degrades the cII transcriptional activator of phage λ. The regulated turnover of cII is critical for the choice between the lytic and lysogenic pathways of viral development. Previous cell fractionation work has indicated that HflA is associated with the inner membrane fraction. We have sought to demonstrate that the HflA protease is localized in the cell membrane of intact cells. To achieve this goal, we have combined electron microscopy of thin-sectioned *E. coli* cells with antibody tagging by a colloidal gold label. Using antibody to purified HflA protein, we have found preferential membrane labeling for *hflA*$^+$ cells but not for *hflA* mutant cells. We conclude that HflA protease is localized in the cell membrane. The membrane location for HflA protein may serve as a component of a targeting mechanism to limit the action of the regulatory protease to selected cytoplasmic proteins.

The temporal regulation of the lytic and lysogenic pathways for bacteriophage λ has been extensively characterized. This work has led to the conclusion that the relative activity of the λ cII protein is the key variable for the developmental decision between the two pathways: a high intracellular concentration of cII favors a lysogenic response, whereas low cII favors lysis (reviewed in references 9, 10, and 13). The primary mechanism controlling the amount of cII in an infected cell is probably the turnover of this unstable protein (2, 16). Whereas almost all *Escherichia coli* proteins remain stable for 2 h or longer (18), the half-life of intracellular cII in an infected cell is approximately 2 min (16). The rate of degradation of cII is determined by proteins encoded by the phage and by the bacterial host. The λ cIII gene product channels λ toward the lysogenic pathway (17); the cIII protein acts to stabilize cII (2, 16, 20). The products of the bacterial *hflA* and *hflB* loci direct the phage toward the lytic pathway (2–4, 11); the HflA and HflB proteins facilitate the turnover of cII (2, 16). The HflA and HflB proteins are also probably important regulatory proteins for the bacteria. The introduction of an *hflA* mutation results in the appearance of a number of additional proteins in two-dimensional gels (5).

The *hflA* locus has been cloned and shown to encode three cotranscribed polypeptides, designated HflX, HflK, and HflC (1); mutations in the *hflK* and *hflC* genes confer an HflA mutant phenotype (1). The HflK and HflC polypeptides copurify as subunits of a cII-cleaving enzyme termed the HflA protease (6). The initial purification studies for HflA revealed that the protein was tightly associated with the cell membrane (6, 15). After a sucrose gradient fractionation to separate inner and outer membrane, the HflK and HflC polypeptides were found in the inner membrane fraction (15). Since HflA acts as a regulatory protease for a DNA-binding transcriptional activator, this apparent membrane localization was surprising and interesting.

To pursue further the cellular location of the HflA protein, we have used immunoelectron microscopy to probe the site of HflA in intact cells. We have found that the membrane of *hflA*$^+$ cells is labeled specifically by antibody to HflA protein. The membrane labeling is absent in *hflA* mutant bacteria carrying a transposon insertion mutation in the *hflK* gene. We have concluded that the HflA protease is a membrane-localized protein. We discuss below some implications of this finding.

Adaptation of freeze-substitution cell preparation to HflA visualization. To visualize HflA in the cell membrane, we needed an approach that would provide morphologically intact and antigenically reactive bacteria. Early experiments with conventional fixation and embedding were not successful. We adapted the freeze-substitution method to provide the needed immunoreactivity and structural preservation. To prepare cells for electron microscopy, bacteria were grown in Luria-Bertani medium with aeration to an optical density at 600 nm of 0.3 to 0.5 at 37°C. The culture was quickly cooled to 0°C by immersion in an ice-water bath, and cells were collected by centrifugation at 4,000 rpm for 15 min at 0°C. The subsequent freeze-substitution procedure was adapted from methods previously described (7, 8, 12, 21). Cell pellets from the centrifugation were resuspended in 1/100 volume of 18% (vol/vol) glycerol at 0°C. Squares of Zig-Zag cigarette paper (0.5 mm^2) were dipped into this suspension and immediately frozen on a polished copper block cooled to −196°C. Samples were stored for further processing at −196°C. The paper squares were then substituted in ethanol containing 3% (vol/vol) glutaraldehyde in the presence of activated molecular sieve beads (8/12 mesh; E. M. Sciences), and were slowly warmed from −196 to −20°C over 72 h by using an aluminum block as described previously (19). The samples were then embedded in LR White resin by successive infiltration of the following mixtures: 100% ethanol for 1 h, ethanol:LR White (2:1) for 1 h, ethanol:LR White (1:2) for 1 h, and two changes of 100% LR White with 0.05% (wt/wt) benzoin methyl ether for 1 h each. Samples were placed in polyethylene flat molds (Fullam) with LR White–0.05% benzoin methyl ether, overlaid with Saran Wrap, and polymerized by UV irradiation (360 to 400 nm) for 72 h at −20°C, followed by UV irradiation for 72 h at 25°C. Thin sections (60 to 70 nm) were taken onto 200-mesh gold grids that were Formvar coated and carbon stabilized.

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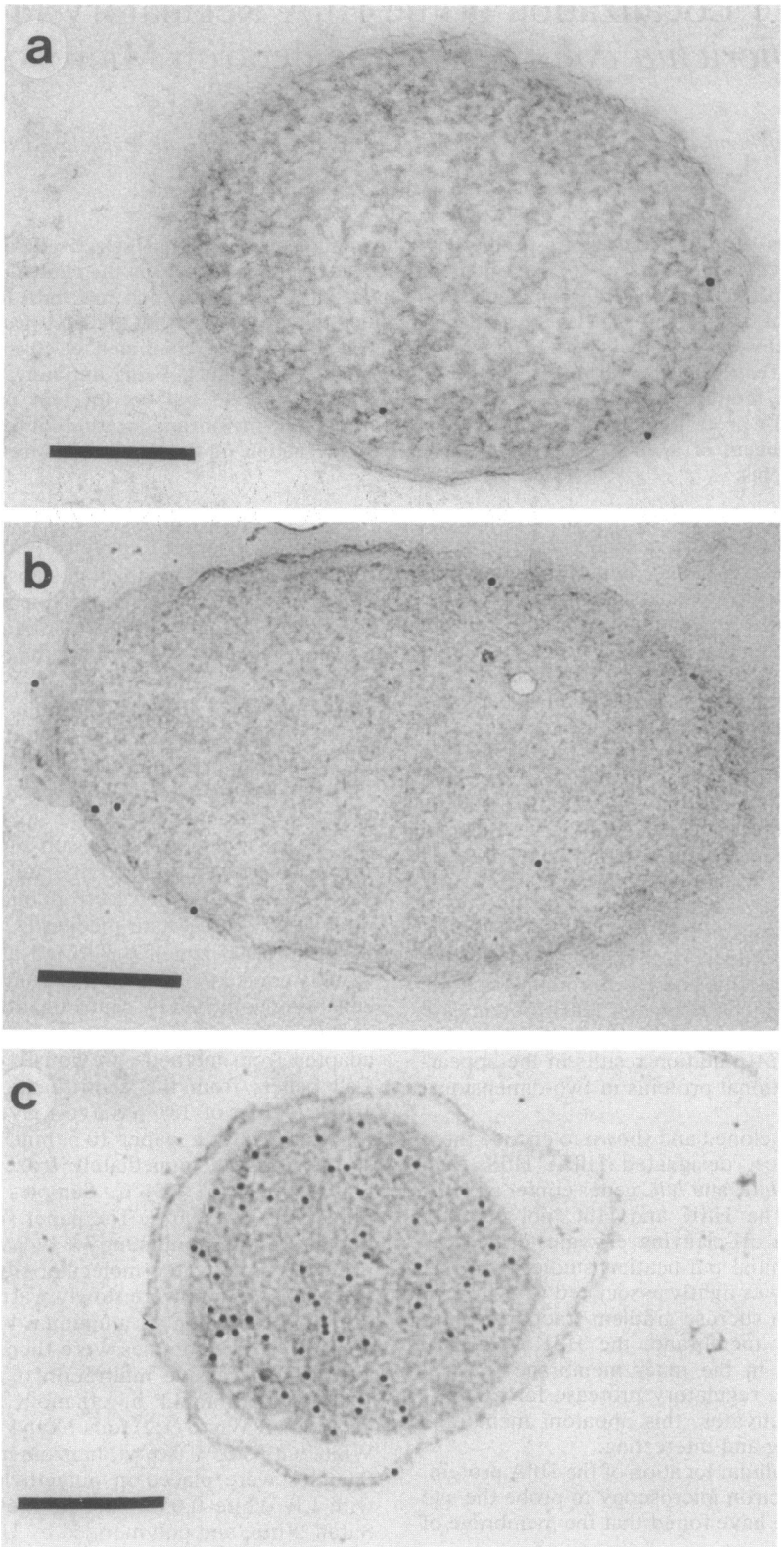
a
b
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FIG. 1. (a and b) Typical sections of *E. coli hflA*$^+$ cells labeled with colloidal gold by anti-HflA antibody. (c) Section of an *hflA*$^+$ cell labeled with anti-cII antibody after synthesis of cII protein from a thermally inducible plasmid. Sectioned cells overproducing cII (or HflA) exhibit a morphology different from that of nonoverproducing cells with smaller size and diminished membrane integrity. The membrane area was calculated by using an image digitizer. The area included was that defined by the inner and outer membrane plus an additional 10 nm (size of gold particle) on either side of the membrane. The cytoplasmic area was taken to be all of the cellular area inside the defined membrane ring. Gold particles were separated into either a membrane or cytoplasmic category based on these definitions. For example, the cell in plate a was counted as two membrane and one cytoplasmic; the cell in plate b was counted as four membrane and two cytoplasmic. Bar, 0.25 μm.

Sections were cut with a diamond knife on an RMC MT-6000 microtome.

The anti-HflA antibody used here was prepared from rabbit serum obtained after injection with purified HflA protein (6). Purified antibody was prepared by ammonium sulfate precipitation and protein G column chromatography. The immunolabeling procedure was adapted from previous work (7, 8, 19, 22). The goat anti-rabbit monoclonal immunoglobulin G colloidal gold solution (10-nm particle size) was obtained from E. Y. Laboratories. Sections on grids were incubated for 10 min on a drop (20 μl) of 20 mM ammonium chloride and then for 15 min on a bovine serum albumin solution, to saturate nonspecific antibody binding sites (2% bovine serum albumin, fraction V, in PBST [140 mM NaCl, 10 mM $NaPO_4$, 0.05% Tween 20, pH 7.4]). Next, grids were incubated for 2 h on a drop of diluted purified antiserum (50,000 times in PBST). Grids were washed three times in PBST, 5 min each, and then incubated on a drop of 2% bovine serum albumin–PBST again for 15 min. Finally, the sections were incubated for 1 h on a drop of diluted immunoglobulin G-gold (optical density at 520 nm = 0.13 in PBST); washed again three times, 5 min each, in PBST; and then incubated for 10 min on 1% (vol/vol) glutaraldehyde. The grids were then stained for 15 min with 4% uranyl acetate. The grids were observed and photographed with a JEOL 1200CX electron microscope at 80-kV operating voltage. With this technique, no morphological changes were evident even 4 months after polymerization; the efficiency of labeling was also similar for sections collected immediately after polymerization and 4 months later (data not presented).

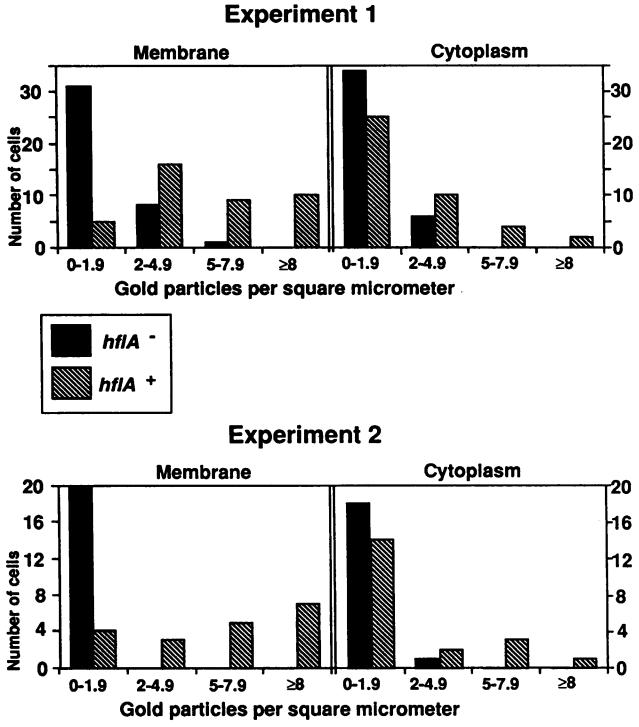

FIG. 2. Immunolabeling data for *hflA*$^+$ and *hflA* mutant bacteria from photographs of sectioned cells. Units are in gold particles per square micrometer. Experiments 1 and 2 represent two different freeze-substitution and immunolabeling experiments following the procedure described in the text. For the data in experiment 1, 40 roughly equally sized and well-preserved bacteria were analyzed for the *hflA*$^+$ strain and for the *hflA* mutant strain; 20 bacteria were analyzed for each strain in experiment 2. The counts for membrane and cytoplasmic label were placed into one of the four categories on the bottom of the bar graphs.

Membrane localization of HflA protein. To verify the specificity of the gold labeling protocol, we compared wild-type *hflA*$^+$ bacteria with a mutant that failed to make the HflK and HflC polypeptides because of a polar insertion mutation in the *hflK* gene (strain X9368 *hflA*::Tn*5* described by Banuett et al. [2]). By gel electrophoresis and immunoblotting, we demonstrated that the *hflA* mutant strain failed to produce proteins reactive to the HflA antibody (data not shown). Using electron microscopy of sectioned cells, we found that gold label was preferentially associated with the membrane of *hflA*$^+$ cells (Fig. 1a and b); almost no gold label was found for *hflA* mutant cells. In contrast, gold label was preferentially found in the cytoplasm with antibody to the cII transcriptional regulator, in separate experiments in which cII protein was produced from an overproducing plasmid construction (Fig. 1c).

To provide a quantitative assessment of the labeling distribution provided by anti-HflA antibody, we counted the gold particles for the membrane and cytoplasm of sectioned cells. The negatives of electron micrographs were displayed at 97,000 times final magnification on an image digitizer tablet. Cells on the negatives were divided into regions of cell membrane and cytoplasm. Areas for each region were measured by using a scale factor accounting for the magnification, and gold particles in each region were counted. The gold labeling data are shown in Fig. 2 for two separate experiments repeating the entire protocol from cell growth through electron microscopy. The data are plotted as a bar graph to show the number of bacteria in four labeling categories; this procedure allows a direct visualization of the labeling pattern from very low (<2 gold particles per μm^2) to very high (≥8 gold particles per μm^2). In both sets of experiments, the *hflA* mutant cells exhibit little or no gold label for either cytoplasm or membrane. In contrast, the *hflA*$^+$ cells exhibit a preferential gold labeling of the membrane; most of the cells have substantial labeling, with a clearly discernible enhancement in the membrane region. For example, over 80% of the *hflA*$^+$ cells fall into the higher labeling categories for the membrane (>2); in contrast, approximately 70% of the cells exhibit very low labeling in the cytoplasm (<2). Antibodies do not generally label the membrane. In similar, separate experiments with antibodies to cII protein, DNA gyrase, and UmuD, most of the labeling

was cytoplasmic (data not shown). On the basis of the data shown in Fig. 2 and previous cell fractionation experiments (6, 15), we conclude that the HflA protein is localized in the cell membrane.

Implications of membrane location of HflA. As noted above, the membrane location of HflA is surprising because the defined target protein for the proteolytic activity of HflA is the cII transcriptional activator, which would be expected to function in the cytoplasm and which fractionates as a soluble protein (14). Possibly HflA has both a membrane receptor regulatory domain and a proteolytic domain. Alternatively (or in addition), the membrane localization may prevent HflA from attacking a cytoplasmic protein unless that protein is specifically targeted to the site of the membrane protease. The targeting mechanism might involve a covalent modification or a chaperone protein to conduct the victim protein to the protease. The HflB protein is a possible candidate for a targeting role.

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