The *Helicobacter pylori* 19.6-Kilodalton Protein Is an Iron-Containing Protein Resembling Ferritin

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The gastric pathogen *Helicobacter pylori* has been shown to produce a 19.6-kDa protein with apparent binding activity for erythrocytes, human buccal epithelial cells, and laminin. In this report we demonstrate that it is an iron-binding protein, resembling ferritin both structurally and biochemically. Also, because its binding activity for laminin, erythrocytes, and buccal cells was abolished by low concentrations of Tween 20, binding is likely nonspecific.

Helicobacter pylori, a spiral-shaped, gram-negative bacterium, is a major cause of type B gastritis (2, 3, 12) and is thought to be a factor in peptic ulcer formation (16) and in the development of gastric carcinoma (5, 14, 15). This bacterium specifically colonizes the gastric epithelium. The reasons for this tissue tropism are unclear but may in part be due to specific recognition of and subsequent attachment to the host epithelium by the bacterium. Recently, we have reported that *H. pylori* produces a 19.6-kDa protein that binds to laminin and is capable of causing weak hemagglutination of erythrocytes from various species (7). We have further characterized this protein biochemically and with respect to its binding function and found that it appears to be an iron-binding protein resembling ferritin.

Characterization of the 19.6-kDa protein. When the purified 19.6-kDa protein (7) was stored at 4°C for more than 2 days at concentrations of >500 µg/ml (a protein assay [Bio-Rad Laboratories, Mississauga, Ontario, Canada] employing bovine serum albumin as a standard was used throughout this study to quantitate protein), it was noted that a red-brown precipitate formed. This material was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and found to be composed of the 19.6-kDa protein. The pigmented appearance of the precipitated, purified protein led us to examine the preparation for the presence of iron. Purified protein was analyzed for iron content by atomic absorbance spectrophotometry with ferric chloride as a standard and purified horse spleen ferritin (Pharmacia, Baie D'Urfe, Quebec, Canada) as a positive control. With this method, iron represented 11.9% (ratio of weight of Fe/weight of protein ranged from 8.2 to 16.7%) in purified preparations of the protein. The relatively high concentrations of iron associated with the 19.6-kDa protein indicated that this protein might be a bacterioferritin-like molecule.

Careful reexamination of negatively stained 19.6-kDa protein preparations with an electron microscope revealed aggregates of material, as was previously reported. However, occasional ring-like structures (9.1 nm in diameter) could be observed in some of the preparations (Fig. 1A). Examination of unstained preparations showed that these structures contained dark central cores (Fig. 1B) similar to horse spleen ferritin (Fig. 1D) but less electron dense. Bacterioferritins have also been reported to have less dense

cores than mammalian ferritins, probably because of their lower iron content, higher phosphate content, and lower degree of crystallinity (11). These morphological observations confirmed that this protein did indeed form a ferritin-like structure and that the previously reported aggregates were either denatured material or material in which the ring-like appearance was obscured.

To estimate the molecular mass of the structure produced by the 19.6-kDa protein, fast protein liquid, size exclusion chromatography was performed with a Superose 12 HR column (Pharmacia) equilibrated with 20 mM Tris (pH 7.5). This structure eluted as a broad peak with a mean size of 350 kDa or approximately 18 subunits per structure. Ferritins are composed of 24 subunits (19, 20, 24). The reason for this discrepancy may be due to inaccuracies in the estimation of the molecular mass. The broad peak obtained may represent denatured and/or damaged complexes migrating with a smaller molecular mass. This would be in agreement with the observations with the electron microscope and would also result in a low estimate of the structure's mass and resulting number of subunits.

To further confirm that the 19.6-kDa protein formed a ferritin-like complex, the phosphate content of the preparation was determined. By using the method described by Treffry and Harrison (21) employing potassium phosphate as a standard, the phosphate content was found to be 14.2% (ratio of weight of phosphate/weight of protein ranged from 10.8 to 20.9%). This represents a molar ratio of Fe to phosphate of 1.4:1 (range, 1.3:1 to 1.6:1). This ratio is similar to the range of 1.4:1 to 1.9:1 reported previously for bacterioferritins (9, 13, 23).

To further characterize the ferritin-like structure of *H. pylori*, absorption spectra were measured by using the purified 19.6-kDa protein. The absorbance spectra indicated a steep increase in the UV range (Fig. 2). The slope of this curve was steeper than that for horse spleen ferritin and resembled more closely that reported for bacterioferritins (Fig. 2). At concentrations as high as 300 µg/ml, no indication of absorption maxima at 417, 530, and 560 nm was observed. Other bacterioferritins exhibit peaks at these wavelengths because of a low protoheme IX content (4, 10, 24). Therefore, the absence of such absorption peaks may indicate that this protein does not contain protoheme IX and, hence, does not bind heme in any appreciable way. If the ferritin-like complex of *H. pylori* does not bind heme, then it

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FIG. 1. Micrographs of the 19.6-kDa protein stained with 1.0% ammonium molybdate (A), showing its ring-like appearance, and unstained (B), demonstrating its electron-dense core region, and of horse spleen ferritin stained with 1% ammonium molybdate (C) and unstained (D) are shown. Bars represent 50 nm.

would be unlike all other bacterioferritins reported to date. The uniqueness of this ferritin-like structure is further exemplified by its lack of N-terminal homology with other bacterioferritins, whereas there is considerable conservation within other members of the group (1, 7, 8, 10).

Cellular localization of the 19.6-kDa protein. *H. pylori* NCTC 11637 was cultured microaerophilically for 2 days on chocolate agar and harvested in 10 mM Tris–150 mM NaCl

(pH 7.5) (TBS). Cells were collected by centrifugation ($12,000 \times g$ for 15 min at 4°C), washed three times, and broken by three passages through a French pressure cell. Unbroken cells were removed by centrifugation ($3,000 \times g$ for 10 min at 4°C), the membrane fraction was collected ($50,000 \times g$ for 1 h at 4°C), and the supernatant (cytosol fraction) was retained. The membrane pellet was washed three times and suspended to the same volume as the cytosol

FIG. 2. Absorbance spectra of purified 19.6-kDa protein (202 μ g of protein/ml) (A) and horse spleen ferritin (181 μ g of protein/ml) (B) are shown.

fraction. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting were performed as described previously (7). The relative amounts of protein present in each fraction were estimated by the use of a scanning densitometer (Molecular Dynamics, Sunnyvale, Calif.). The 19.6-kDa protein could be detected to some degree in all cellular fractions (Fig. 3). The wash fractions and the membrane fraction accounted for approximately 24 and 20%, respectively, of the total 19.6-kDa protein present. The majority of the protein was localized in the cytosolic fraction (56% of the total). It is likely that the protein detected in the wash fractions originates from cytosolic release due to cell lysis. Further support for the cytosolic localization of this protein was obtained by using sera from *H. pylori*-infected patients. Such sera did not appear to recognize the 19.6-kDa protein, as assessed by Western immunoblot analysis (data not shown). This result would be expected if the protein was located in the cytoplasm and its release into the wash buffer is an in vitro artifact that does not occur in vivo.

Binding properties of the 19.6-kDa protein. In order to examine the affinity of the 19.6-kDa protein for laminin, the 19.6-kDa protein was purified from *H. pylori* NCTC 11637 as described previously (7) and coated onto an enzyme-linked immunoassay microtiter plate (10 μ g/ml in TBS [pH 7.5]; 100 μ l/well) overnight at 4°C. The plate was washed and blocked, and laminin binding was assessed as described previously (22). Addition of Tween 20 (0.1 to 0.003% [vol/vol]) to the binding assay buffer resulted in no detectable binding of laminin to the 19.6-kDa protein. These results led us to conclude that this protein was not the major laminin-

FIG. 3. Immunoblot demonstrating the cellular localization of the 19.6-kDa protein with rabbit anti-19.6-kDa protein (1/1,000 dilution) as the primary antiserum. Lanes: 1, 2, and 3, initial fractions from each of three washes, respectively; 4, cytosolic fraction; 5, membrane fraction. The majority of the 19.6-kDa protein was found in the cytosolic fraction.

FIG. 4. Immunoblot showing the effect of Tween 20 on the binding of the 19.6-kDa protein to human erythrocytes and human buccal cells. The 19.6-kDa protein was detected by using rabbit anti-19.6-kDa protein as the primary antiserum (1/1,000 dilution). Lanes: 1 and 3, 19.6-kDa protein bound to human erythrocytes and human buccal cells; 2 and 4, inhibition of binding of the 19.6-kDa protein to human erythrocytes and human buccal cells, respectively, by 0.025% (vol/vol) Tween 20.

binding protein as had been suggested previously (7, 22) and that the binding activity previously reported for this protein was nonspecific. This was confirmed by reexamination of the erythrocyte- and buccal cell-binding activities of this protein. Binding of the 19.6-kDa protein to either cell type was assessed as described previously (7). The protein bound to both cell types when the incubation was carried out in TBS (Fig. 4). However, addition of 0.025% (vol/vol) Tween 20 to the binding buffer resulted in no detectable binding (Fig. 4).

The evidence presented in this report confirms that the 19.6-kDa protein of *H. pylori* is an iron-binding protein, resembling bacterioferritin morphologically and biochemically. It also appears that the previously reported binding activity of the protein was due to nonspecific hydrophobic interactions. Such low-affinity hydrophobic interactions are not uncommon and have led to considerable confusion in ascertaining the role of various components in the adhesion process (17). *H. pylori* releases a wide variety of components into wash buffers when grown in vitro, and it is likely that a number of these components can bind nonspecifically to various substrates.

This protein does not act as a specific adhesin but may have an important role in vivo nonetheless. Iron availability has been shown to be important to the virulence of many bacteria (6, 18). Because of the low pH in the stomach, the majority of available iron would exist in a soluble, inorganic form. The bacterioferritin of *H. pylori* could have a major role in storing any excess iron that the bacterium sequestered. However, the importance of this ability to store excess iron to the virulence of *H. pylori* is open to question. The iron levels that are present in the particular niche (the stomach) that *H. pylori* occupies are unknown. The occurrence of bacterioferritin would be significant, i.e., in storing the available cytosolic iron for later use and/or avoiding toxicity to the cells, under conditions of constant or intermittent iron excess.

This work was supported by grant 1R01AI29927-01A2 from the U.S. Department of Health and Human Services.

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