Localization and Stoichiometry of Hook-Associated Proteins within Salmonella typhimurium Flagella

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A bacterial flagellum consists of three distinct parts connected in series: a basal body in the membrane, a short curved rod called the hook, and a long helical filament (see for review reference 21). Previous studies have shown that the filament and the hook are each made by regular assembly of a single kind of protein subunit (flagellin and hook protein, respectively) and that both of these structures can be reconstructed in vitro from purified subunits (2, 19). However, little is known as to how the flagellum grows in vivo, except that the filament elongates by subunit addition at the distal end (4, 13).

Salmonella typhimurium mutants defective in the structural genes for flagellin (H1 and H2) or in certain other genes (flaL, flaU, flaV, and flaW) have hooks but not filaments (11). By study of such mutants, Homma et al. have found three proteins (HAP1, HAP2, and HAP3) associated with the hook (11). HAP1 (Mr = 59,000 [59K]), HAP2 (48K or 53K, depending on the strain), and HAP3 (31K) were found to be the products of the flaW, flaV, and flaU genes, respectively (10). Immuno-electron microscopic analysis has led to a model whereby HAPs form a layered structure at the distal end of the hooks from filamentless mutants in the order: hook-HAP1-HAP3-HAP2 (7). In wild-type flagella, HAP1 has been localized at the hook-filament junction, but the other HAPs have not yet been localized (7).

Recently, several lines of indirect evidence have pointed to the interesting possibility that HAP2 is localized at the tip of the filament, rather than at the hook-filament junction. Ikeda et al. (14) have found that the tip of an intact filament is unable to serve as a nucleation center for the assembly of exogenous flagellin and that the tip often has a “capped” appearance, quite different from that of a filament reconstituted in vitro. A similar blockage of the addition of exogenous flagellin has been found with the hook of filamentless mutants, except those defective in flaV, the gene for HAP2 (17, 18). Furthermore, Homma et al. have observed that unassembled flagellin leaks out from growing flaV mutant cells (6) but that the flagellin stops leaking and forms filaments if the mutant cells are incubated in medium containing HAP2 (9). The same phenomenon was observed with flaV mutants on which short flagellar filaments had been grown by the addition of exogenous flagellin (9). These findings suggest that, in wild-type flagella, HAP2 is attached to the flagellin tip, somehow working to trap and assemble endogenous flagellin and to prevent the addition of exogenous flagellin.

To provide direct evidence for the above hypothesis, we have investigated the localization of HAPs in wild-type flagella, using antibodies specific to each HAP species together with a second antibody conjugated with colloidal gold. We found that HAP2 is indeed located at the tip, whereas both HAP1 and HAP3 are at the hook-filament junction, in agreement with a model previously presented (7). Furthermore, gel electrophoresis of the hook-filament complex isolated from a short-flagellum mutant has led to the estimate that a single flagellum contains 10 to 20 HAP2 molecules, as well as 10 to 20 of HAP1 and 10 to 40 of HAP3. These findings indicate that filament growth in vivo is a complex process that involves flagellin-HAP2 interaction in addition to flagellin-flagellin interaction.

MATERIALS AND METHODS

**Bacterial strains.** Two strains were used: SJW806, a strain which produces normal flagella with an e,n,x antigenicity (H2-monophasic) (15), and SJW1254, a short-flagellum, nonmotile strain derived from SJW806 by a spontaneous mutation in the H2 gene (15).

**Isolation of wild-type flagella.** Strain SJW806 was cultured in 1% nutrient broth (Difco Laboratories, Detroit, Mich.). Cells harvested in late log phase were suspended in phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.0) and deflagellated by rigorous pipetting. After the cell bodies were removed by centrifugation at 12,000 × g for 10 min, the detached flagella in the supernatant were sedimented by centrifugation at 100,000 × g for 40 min and resuspended in PBS.

**Isolation of short-mutant flagella.** The method used to isolate short-mutant flagella was essentially as described for the isolation of polyhook filaments (19). Briefly, strain SJW1254 was grown in M9 medium (23) and harvested in late log phase. The cell pellet was suspended in the same volume of a buffer solution containing 10 mM Tris hydrochloride (pH 8.0) and 5 mM EDTA. This suspension was blended with a homogenizer, followed by centrifugation at

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FIG. 1. (a) Electron micrographs of the hook portions of wild-type flagella labeled with anti-HAP1 antibody and colloidal gold-conjugated goat anti-rabbit IgG; (b) electron micrographs of the hook portions of wild-type flagella labeled with anti-HAP3 antibody and colloidal gold-conjugated goat anti-rabbit IgG; (c) electron micrographs of the tips of wild-type flagella labeled with anti-HAP2 antibody and colloidal gold-conjugated goat anti-rabbit IgG; (d) high-magnification electron micrograph of the hook-filament junction of a wild-type flagellum. Arrowhead indicates the junction. Bars, 50 nm.
Judged by electron microscopy, the detached flagella were condensed by centrifugation and resuspended to CsCl density-gradient centrifugation. Flagella-rich fractions, as judged by electron microscopy, were dialyzed against PBS.

**Antibody staining.** Rabbit polyclonal antibodies specific to each HAP species were obtained as described before (7). Flagella suspended in 100 μl of PBS containing 1% bovine serum albumin were mixed with 5 μl of anti-HAP antibody in PBS (7) and incubated at 26°C for 1 to 2 h. The mixture was centrifuged at 100,000 × g for 40 min. The pellet was then suspended in PBS containing 1% bovine serum albumin and mixed with pre- and anti-rabbit immunoglobulin G (IgG) conjugated with 5 nm colloidal gold (Jansen Pharmaceutica, Beerse, Belgium). This mixture was incubated at 26°C for 1 to 2 h. After dilution with a large volume of PBS containing 1% bovine serum albumin, the stained flagella were sedimented by centrifugation at 100,000 × g for 40 min and suspended in a small volume of PBS containing bovine serum albumin. This specimen was observed with a JEOL 100C microscope, using 1% uranyl acetate as a negative stain. As control experiments, we incubated flagellar specimens with the gold-conjugated goat antibody, with and without preincubation with preimmune rabbit serum, and observed them in exactly the same way as described above.

**Immunoblotting.** Immunoblotting to identify the bands of flagellin, hook protein, and HAPs separated in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis patterns was carried out by the method of Towbin et al. (26) as modified by Masuda et al. (22).

**Electrophoresis and densitometry.** Polyacrylamide gel electrophoresis in the presence of SDS was carried out by the method of Laemmli (20) as modified by Hubbard and Lazarides (12). Gels stained with Coomassie brilliant blue were analyzed by using a Densitorol densitometer (Toyo Kagaku Sangyo Co., Ltd., Tokyo) with a slit of 0.5 by 5 mm and a wavelength of 565 nm.

**RESULTS**

**Localization of HAPs in flagellar filaments.** Using antibodies specific to each HAP and a second antibody conjugated with colloidal gold, we examined the localization of HAPs in wild-type flagella. The isolated flagellar filament appeared to have been fragmented into several pieces during the isolation procedure, as judged by the observation that only 10 to 20% of the filaments had the hook portion. When this sample was incubated with HAP1- or HAP3-specific antibody and a colloidal gold-conjugated second antibody, gold particles were found associated with the hook-filament junction (Fig. 1a and b). The frequency of occurrence of labeled particles was about 1 in 3 to 10 hook-filament junctions with anti-HAP1 and 1 in 10 to 20 junctions with HAP3. No noticeable association of gold particles with the junction was observed in control samples which had been incubated with the gold-conjugated second antibody alone or preincubated with preimmune rabbit serum. In negatively stained specimens, a short (10 to 20 nm long) region with a smooth surface often appeared between the filament and hook (Fig. 1d). We suppose that this is where HAP1 and HAP3 molecules are located.

In contrast, when HAP2-specific antibody was used as the first antibody, gold particles were found attached exclusively at the filament tip (Fig. 1c). The labeled end was always the one distal to the cell body, as judged by the polarity in the fine structure of the filament (24) and by the shape of the opposite end which was not labeled; this end always had either the hook or a pointed shape characteristic of the proximal end of a filament (3). The frequency of occurrence of the labeled tips was about 1 in 100 to 150 filaments, or, if we consider the filament fragmentation, 1 in 10 to 30 intact distal ends. When a suspension of living cells was used in place of the isolated flagella, gold particles were also found attached to the tips of 2 to 3% of the intact flagella. No such labeling of ends was observed with filaments reconstructed in vitro from purified flagellin, or with control specimens of intact flagella treated with gold-conjugated second antibody alone or preincubated with preimmune serum (data not shown). From these observations, we concluded that HAP2 molecule is localized at the distal end of an intact flagellum. The labeling patterns of the tip or the hook-filament junction, or the extent of the labeling, did not depend noticeably on the growth stage of the cells from which flagella were obtained.

**Quantities of HAPs contained in a single flagellum.** The HAP content in a single flagellum is difficult to estimate with wild-type flagella because the presence of an enormous amount of flagellin interferes with the detection of HAPs in SDS gel electrophoresis pattern. We therefore examined the protein composition of hook-filament complexes isolated from a short-flagellum mutant defective in the structural gene of flagellin (Table 1). With these short-mutant flagella, because the flagellin content is as low as 1/100 to 1/1,000 of the normal amount, we could estimate the amount of HAPs relative to that of hook protein and also the absolute amount of each protein in a single flagellum, since the average number of hook proteins in a flagellum is known.

**Table 1. Quantity of HAPs contained in a single short-mutant flagellum**

| Component | Apparent mol wt (K) | Relative band density* | Estimated content (subunits per flagellum) |
|-----------|---------------------|------------------------|-------------------------------------------|
| Hook protein | 42                  | 1                      | 120-1700*                                 |
| HAP1      | 59                  | 0.12-0.18              | 11-22                                     |
| HAP2      | 52                  | 0.06-0.12              | 6-16                                      |
| HAP3      | 31                  | 0.06-0.17              | 9-39                                      |
| Flagellin | 55                  | 0.12-0.18              | 11-23                                     |

* The band density of each component was normalized against that of the hook protein in four different sets of experiments. The numbers listed show the range of values obtained.

* The number of hook proteins was estimated from the lattice constant of straight polyhook (27) and from the assumption that each hook has a constant length of 50 nm (our own measurement, using the short-mutant flagella labeled with antipolyhook antibody) or 70 nm (5).

Figure 2a shows short-mutant flagella isolated as described in Materials and Methods. As shown previously (15), the flagella appeared to be composed mostly of the hook and a short filament portion which became evident when stained with anti-e,n,x-flagellin antibody (Fig. 2b). When these flagella were mixed with anti-HAP2 antibody, almost all the flagella were clearly stained at the tip (Fig. 2a). Therefore, these short flagella must have normal organization in spite of the great reduction in the flagellin content. Figure 2d shows the SDS-gel electrophoresis pattern of the mutant flagella. Although there are several contaminating protein bands, the bands of flagellin, hook protein, and each HAP are unambiguously identified by reactions with specific antibodies (Fig. 2c). The band density and the relative amount of each protein estimated from its density and apparent molecular weight are listed in Table 1. These values suggest that a single short flagellum contains 10 to 20 HAP1, 10 to 20 HAP2, and 10 to 40 HAP3 molecules.
FIG. 2. Electron micrographs and SDS-polyacrylamide gel electrophoresis pattern of isolated short-mutant flagella. (a) Electron micrograph of isolated short-mutant flagella. (b) Flagella labeled with anti-e,n,x-flagellin antibody. Arrows indicate portions decorated by the antibody. (c) Flagella labeled with anti-HAP2 antibody. Arrows indicate tips of two flagella connected by the antibody. Bars, 100 nm. (d) SDS-polyacrylamide gel electrophoresis pattern of the isolated short-mutant flagella. (e) Detection by immunoblotting of flagellin, hook protein, and HAPs contained in short-mutant flagella: lane 1, band patterns transferred onto a nitrocellulose sheet and stained with amido black; lanes 2 through 6, bands reacted with anti-e,n,x, antipolyhook, anti-HAP1, anti-HAP2, and anti-HAP3 antibodies, respectively; lane 7, bands reacted with preimmune rabbit IgG.
DISCUSSION

We have shown that HAP2 is localized at the flagellar tip whereas HAP1 and HAP3 are located at the hook-flagament junction. The frequency of occurrence of label at the tip and the hook-flagament junction was not very high with wild-type flagella, but this is probably due to the low efficiency in labeling long flagella with colloidal gold-conjugated antibody, where the concentration of tips and hook-flagament junctions was very low relative to that of flagellin. This possibility is supported by the observation that almost all the short flagella of the mutant were labeled by antibodies specific to HAP2 as well as to HAP1 and HAP3 (data not shown). We have previously demonstrated that intact flagellar filaments generally are unable to serve as the nucleation center for the in vitro assembly of flagellin and that more than 90% of the filaments have blunt tips, quite different from the fishtailike tips of the filaments reconstituted in vitro. Moreover, about 50% of the tips appeared to be capped by thin structures (14). The present location of HAP2, together with these previous findings, strongly suggests that the tips of intact flagella of wild-type S. typhimurium are all capped by HAP2 molecules. The recent finding of Homma et al. indicates that capping by HAP2 is necessary for the filament to grow (9). Filaments, then, must grow by addition of new flagellin monomers between the HAP2 cap and the tip of the flagellin polymer. In other words, the HAP2 cap must be pushed up as new flagellin molecules are incorporated into the filament.

We estimate that 10 to 20 HAP2 molecules are present at the filament tip of a short-flagella mutant. Previously we reported that this mutant excretes into the culture medium a large amount of monomeric flagellin and a small amount of proteins that have a strong inhibitory activity on the in vitro polymerization of flagellin (15, 16). We have recently found that HAP2 is the protein responsible for this activity and that HAP2 is present in the medium in about 10 times the amount present on the filament tips (T. Ikeda and R. Kamiya, manuscript in preparation). A recent study by Homma and lino has shown that the excretion of HAP2 occurs also in wild-type cells (8). These findings raise the possibility that the HAP2 cap is a dynamic structure whose subunits are gradually replaced by new ones during the filament growth. Alternatively, it is possible that HAP2 is a stable structure but that it is synthesized in an amount 10 times greater than is necessary to make the cap, and the excess is excreted unassembled. Determination of whether the HAP2 protein in the cap is being turned over during filament assembly must await further studies.

HAP1 and HAP3 have been localized at the hook-flagament junction. Although their fine location was not determined in the present study, they may well occur in the order of hook-HAP1-HAP3, as suggested by previous studies with filamentless mutants (7). Gel electrophoresis of short-mutant flagella has led to an estimate that 10 to 20 HAP1 and 10 to 40 HAP3 molecules are present in a single flagellin. These values agree with those previously estimated in filamentless mutants (11). As the hook and the filament have similar helical arrays of subunits comprising 1-, 5-, 6-, and 11-start helices (1, 24, 25, 27), it is likely that HAP1 and HAP3 are also arranged on such helical lattices. If this is actually the case, HAP1 and HAP3 molecules must have two to four and two to eight turns of the one-start helix, respectively; if we assume the pitch of the one-start helix is similar to that in the hook (2.3 nm) or the filament (2.6 nm), the distance spanned by HAP1 and HAP3 must be 5 to 10 nm and 5 to 20 nm, respectively. Actually, a transition region 10 to 20 nm long has often been observed between the hook and filament in negatively stained flagellar specimens (Fig. 1d). These proteins may function as an adapter between the hook and filament. However, the reason why an adapter is necessary to conjoin the hook and filament is not clear, because it would seem that these two structures could geometrically fit quite well with each other directly (27).

The hook resembles the flagellar filament in subunit packing and the manner of subunit assembly in vitro (19). Hence it may be that these two structures grow in a similar way in vivo. If so, there must be a molecule that is for the hook what HAP2 is for the filament. However, none of HAP1–3 appears to be such a protein, since mutants lacking all these HAPs have hooks of normal length. There may be a related protein yet to be found, or the growth process of the hook may be different from that of the filament. The fact that the hook has a defined length whereas the filament has arbitrary length appears to favor the idea that the growth mechanisms for these two structures are different.

Together with previous studies using filamentless mutants, this study has established that the bacterial flagellum is an elaborate structure comprising basal body, hook, HAP1, HAP3, filament, and HAP2 connected in series. How these distinct parts are constructed and joined together should be a fascinating future problem.

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