Various pathogenic bacteria such as *Shigella* deliver effector proteins into mammalian cells via the type III secretion system. The delivered *Shigella* effectors have been shown to variously affect host functions required for efficient bacterial internalization into the cells. In the present study, we investigated the IpaH proteins for their ability to be secreted via the type III secretion system and their fate in mammalian cells. Upon incubation in a medium containing Congo red, the bacteria secrete IpaH into the medium, but secretion of IpaH occurs later than that of IpaB. Immunofluorescence microscopy indicated that IpaH<sub>9.8</sub> is secreted from intracellular bacteria and transported into the nucleus. On microinjection of the protein, intracellular IpaH<sub>9.8</sub> is accumulated at one place around the nucleus and transported into the nucleus. This movement seems to be dependent on the microtubule network, since nuclear accumulation of IpaH<sub>9.8</sub> is inhibited in cells treated with microtubule-destabilizing agents. In nuclear import assay, IpaH<sub>9.8</sub> was efficiently transported into the nucleus, which was completely blocked by treatment with wheat germ agglutinin. The nuclear transport of IpaH<sub>9.8</sub> does not depend on host cytosolic factors but is partially dependent on ATP/GTP, suggesting that, like β-catenin, IpaH<sub>9.8</sub> secreted from intracellular *Shigella* can be transported into the nucleus.

Various Gram-negative pathogenic bacteria possess a type III secretion system through which they deliver a set of proteins into the environment as well as into the target host cells required for infection (1, 2). Although the mechanisms underlying the secretion of these proteins remain to be elucidated, the secreted proteins seem to be variously utilized for the secretory process by affecting the target host functions. For example, *Salmonella* species deliver an array of effectors such as SipA, SipB, SipC, SopB/SigD, SopE, SptP, and SphH1 into the host cells during infection (3–5). These molecules modulate the host cellular signaling pathways and rearrange the cytoskeleton required for invasion of host cells. Enteropathogenic

### Shigella Protein IpaH<sub>9.8</sub> Is Secreted from Bacteria within Mammalian Cells and Transported to the Nucleus*

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**Takahito Toyotome‡, Toshihiko Suzuki‡, Asaomi Kuwae‡, Takashi Nonaka‡, Hiroyuki Fukuda‡, Shinobu Imajoh-Ohmiš, Toshihiko Toyoufukuš, Masatsugu Horiš, and Chihiro Sasakawa‡**

*From the ‡Division of Bacterial Infection, Department of Microbiology and Immunology and the §Division of Cell Biology and Biochemistry, Department of Basic Medical Sciences, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108-8639 and the Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan*
determined by monitoring the gene expression using the lacZ transcriptional fusion system (22). Furthermore, it has recently been reported that when S. flexneri 2a (2457T) infected mouse macrophages (J774) or human monocyte-derived macrophages, IpaH2a facilitated escape from the vacuole (23).

In the present study, we attempted to determine whether the IpaH proteins can be secreted from S. flexneri via the type III secretion system, and we further investigated the fate of the secreted IpaH protein, IpaH_{2a}, in the host cells. Our results indicated that IpaH_{2a}, IpaH_{4.5}, and IpaH_{4.5} can be secreted through the type III secretion system but that secretion occurred at a later stage. Importantly, the secretion from S. flexneri seemed to be stimulated primarily within the host cell cytoplasm, where the secreted IpaH_{2a} can be efficiently transported into the host cell nucleus.

**Experimental Procedures**

**Bacterial Strains, Eukaryotic Cell Lines, and Growth Conditions**—Bacterial strains and plasmids used in this study are listed in Table I. All S. flexneri-derived strains were grown routinely in brain heart infusion broth (Difco) at 37°C. The strains used in this study are listed in Table I. The plasmids used were constructed as follows. The IpaH genes were ligated into the N terminus, into the pMEsf-neo vector. Plasmids pHis-IpaH_{2a} which has a FLAG tag and linker (MDYKDDDDKVDGIDKLDIEF), were cloned into pQE30 (Qiagen). Resultant plasmids were digested with EcoRI/HindIII fragment of the IpaH_{2a} gene cloned into pGEX-6P-1. Plasmid pFLAG-IpaH_{2a} was constructed by cloning the FLAG-IpaH_{2a} gene, which has a FLAG tag and linker (MDYKDDDDKVDGIDKLDIEF) fused to the N terminus, into the pMEsf-neo vector. Plasmids pHis-IpaH_{2a} (x: 9.8, 7.8, or 4.5) were used as follows. The IpaH genes were cloned into pQE30 (Qiagen). Resultant plasmids were digested with EcoRI and HindIII, followed by ligation of the EcoRI-HindIII fragment containing the His-tagged IpaH_{2a} gene into pTB101. Expression and Purification of Recombinant Proteins—Recombinant IpaH proteins were purified as follows. E. coli cells carrying pGST-IpaH_{2a} (x: 9.8, 7.8, or 4.5) were cultivated in L broth supplemented with ampicillin (50 μg ml^{-1}) for 3 h at 37°C. Expression was induced by the addition of 1 mg ml^{-1} isopropyl-1-thio-β-D-galactopyranoside and incubation for 2 h at 37°C. Bacteria were disrupted by sonication using an ultrasonic disruptor UD-200 (TOMY) for 1 min, 4 times with incubation on ice. Purification of the GST fusion proteins with glutathione-Sepharose 4B (Amersham Pharmacia Biotech) and cleavage of the GST proteins with thrombin were performed according to the manufacturer's protocol. Recombinant β-catenin was purified as follows. E. coli cells carrying pGST-β-catenin were cultivated in L broth supplemented with ampicillin (50 μg ml^{-1}) for 3 h at 37°C. Expression was induced by the addition of 1 mg ml^{-1} isopropyl-1-thio-β-D-galactopyranoside and incubation for 2 h at 37°C. Bacteria were disrupted by sonication. Purification of the GST fusion protein with glutathione-Sepharose 4B and cleavage of the GST protein with PreScission Protease (Amersham Pharmacia Biotech) were performed according to the manufacturer's protocol.

**Antibodies**—Polyclonal rabbit anti-IpaH antibody was raised against recombinant IpaH_{2a} protein. Purified IpaH antibody reacted with the IpaH gene products encoded on the large virulence plasmid (pMYSH6000) in S. flexneri 2a (YS6000). Anti-IpaB antibody was purchased from Sigma. Cy5-labeled anti-rabbit IgG was purchased from Amersham Pharmacia Biotech. Detection of Ipa Proteins in the Whole-cell Lysates and Culture Supernatants—Shigella cells cultivated in 5 ml of brain heart infusion broth at 37°C for 4 h were washed in ice-cold phosphate-buffered saline (PBS) and resuspended in 2 ml of PBS. After incubation at 37°C for 5 min, 6 ml of 1% Congo red (CR) was added to the bacterial suspension, followed by incubation for 10 min at 37°C. After centrifugation, the supernatant was passed though a 0.45-μm pore size filter, and the proteins in the resultant supernatant and bacterial pellet were precipitated with trichloroacetic acid. Samples were separated by SDS-PAGE and immunoblotted with appropriate antibodies.

**Collection of Ipa proteins at each time point was performed as follows. Shigella cells were cultivated in 2 ml of tryptone soy broth at 37°C for 3 h. After the addition of 1 μl of 1% CR to the bacterial culture, the bacterial cells were incubated at 37°C for 0, 30, 60, 120, 180, or 240 min. Each sample was centrifuged, and the proteins in the resultant supernatant and bacterial pellet were precipitated with trichloroacetic acid. Each sample was separated by SDS-PAGE and immunoblotted with appropriate antibodies.**

**Table I**

| Strain or plasmid | Characteristics | Reference or source |
|-------------------|-----------------|---------------------|
| S. flexneri YSH6000 | Serotype 2a wild type | 27 |
| S. flexneri S325  | YSH6000 mxa:A/Th5 | 28 |
| E. coli MC1061    | Employed for DNA technology and protein preparation | 29 |
| Plasmids         |                  |                     |
| pTB101           | lacZ and trimethoprim resistance gene inserted into pKK223-3 | Amersham Pharmacia Biotech |
| pGEX-2T          | GST fusion protein expression vector | Amersham Pharmacia Biotech |
| pGEX-6P-1        | GST fusion protein expression vector | Amersham Pharmacia Biotech |
| pMEsf-neo        | Mammalian expression vector | Gift from Y. Horiguchi |
| pIpaH_{2a}       | IpaH_{2a} gene cloned into pTB101 | This study |
| pHis-IpaH_{2a}   | His-IpaH_{2a} gene cloned into pTB101 | This study |
| pGST-IpaH_{9.8}  | IpaH_{9.8} gene cloned into pGEX-2T | This study |
| pGST-β-catenin   | β-catenin gene cloned into pGEX-6P-1 | This study |
| pFLAG-IpaH_{2a}  | FLAG-IpaH_{2a} gene cloned into pMEsf-neo | This study |

x: 9,8, 7,8, 4.5

"\[\text{Shigella-secreted Protein, IpaH}\]"
Preparation of Cy3-labeled Proteins and Nuclear Localization Signal (NLS)-conjugated Allophycocyanin—Cy3-labeled IpaH$_{9.8}$ and β-catenin were prepared according to the manufacturer's protocol (FluoroLink-Ab Cy3 labeling kit; Amersham Pharmacia Biotech). Allophycocyanin (APC: Calbiochem) was conjugated with synthetic peptide (CYGGPKKKRRKVE) containing the SV40 large T antigen NLS as described previously (32).

Microinjection—Cy3-labeled IpaH$_{4.5}$ protein (about 1.5 mg ml$^{-1}$) was injected through a glass capillary using a micromanipulator (model 5171, Eppendorf) into the cytoplasm of host cells grown on coverslips. The microscope stage was maintained at 37°C and 5% CO$_2$ during the time of injection and data acquisition. Nocodazole pretreatment was performed as described above.

In Vitro Nuclear Import Assay—HeLa cells were grown on coverslips to ~70% confluence in the absence of antibiotics. Cells were permeabilized in ice-cold transport buffer (20 mM HEPES, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 5 mM sodium acetate, 0.5 mM EGTA, 2 mM dithiothreitol, 1 µg ml$^{-1}$ aprotinin, leupeptin, and pepstatin) containing 40 µg ml$^{-1}$ digitonin (Research Biochemicals Incorporated) for 5 min on ice. After digitonin permeabilization, ATP-depleted samples were pre-treated with transport buffer containing 0.1 units ml$^{-1}$ apyrase (Sigma) and 2% bovine serum albumin for 5 min at 30°C. For wheat germ agglutinin (WGA; Sigma) treatment, permeabilized cells were incubated with 0.5 mg ml$^{-1}$ WGA for 5 min on ice. After washing the cells with cold transport buffer, the coverslips were blotted and inverted over 10 µl of test solution on a Parafilm sheet in a humidified box. The compositions of each test solution are indicated in the respective figure legends. The import reaction was performed for 30 min at 30°C or 4°C. For + ATP conditions, 1 mM ATP, 0.5 mM GTP, 5 mM phosphocreatine (Sigma), 20 units ml$^{-1}$ creatine phosphokinase (Sigma), 2 units ml$^{-1}$ alkaline phosphatase (Sigma) were present during incubation. For + cytosol conditions, 20 ng ml$^{-1}$ rabbit reticulocyte lysate (Promega) was present during the incubation. After rinsing the cells with ice-cold transport buffer, the cells were fixed with 4% paraformaldehyde in PBS.

RESULTS

Structures of the ipaH Gene Family on the 230-kilobase Plasmid of S. flexneri YSH6000—The presence of three ipaH genes, ipaH$_{4.5}$, ipaH$_{7.8}$, ipaH$_{9.8}$, on the large plasmid (pMYSH6000) of S. flexneri 2a YSH6000 was investigated by polymerase chain reaction cloning. The nucleotide sequences of the polymerase chain reaction-amplified ipaH genes indicate that all except ipaH$_{9.8}$ were almost identical to those of pWR100 (22, 24, 25). Our sequence data of ipaH$_{7.8}$ from pMYSH6000 contained one additional nucleotide at position 26 from the 5’ end of ipaH$_{7.8}$ of pWR100, resulting in a 98-nucleotide extension from the 5’ end of the ipaH$_{7.8}$ open reading frame (Fig. 1A). Consequently, the predicted N-terminal IpaH$_{7.8}$ sequence encoded by the ipaH$_{7.8}$ of pMYSH6000 possessed 33 additional amino acids at the N terminus of IpaH$_{9.8}$ on pWR100 (Fig. 1A). Accordingly, the deduced IpaH$_{4.5}$, IpaH$_{7.8}$, and IpaH$_{9.8}$ sequences of pMYSH6000 were composed of four distinctive domains: (i) the N-terminal 60–70-amino acid stretch; (ii) the following 200–355 amino acid region containing 6–9 LRRs; (iii) the intervening sequence bracketed by LRRs and the C-terminal conserved region; and (iv) the C-terminal conserved region (CTR) (Fig. 1B). Although the length of CTRs varied slightly between members of the IpaH family, they showed significant similarity with other cognates such as in the C-terminal regions of SlrP of Salmonella and y4fR of Rhizobium (33, 34).

Type III-dependent Secretion of IpaH—Upon incubation of Shigella in PBS containing 0.003% CR, the bacteria rapidly deliver IpaA, IpaB, IpaC, IpaD, VirA, and IpaG into the medium via the type III secretion system (35). Shigella infectivity has been shown to be dependent on the type III secretion activity (27, 36, 37). To test whether S. flexneri secretes IpaH proteins into the medium via the secretion system, the bacteria were incubated in PBS with or without CR. Although the secretion of IpaB, IpaC, and IpaD could be readily detected on incubation in PBS with CR for 10 min (Fig. 2A, lane 1), IpaH secretion was not detected (Fig. 2A, lane 1). However, after long exposure of the X-ray film, a trace amount of IpaH secretion was detected as a 60-kDa band (data not shown). To confirm the potential ability of IpaH to be secreted via the type III secretion system, we introduced a cloned ipaH$_{9.8}$ plasmid (pipaH$_{9.8}$) into YSH6000 or S325 (a type III-deficient mutant of YSH6000), and IpaH secretion into the medium containing CR was investigated by immunoblotting. As shown in Fig. 2A, secretion of IpaH$_{9.8}$ from YSH6000 but not from S325 was detected as a band of ~60 kDa.

To further investigate the ability of Shigella to secrete IpaH into the conditional medium via the type III secretion system, each IpaH protein was tagged with 6 histidines (His) (see “Experimental Procedures”). YSH6000 overexpressing each of the His-tagged IpaH proteins were examined for their ability to be secreted into the medium when incubated in PBS with or without CR. IpaH$_{4.5}$, IpaH$_{7.8}$, and IpaH$_{9.8}$ were secreted from YSH6000 when incubated in PBS with CR (Fig. 2B), suggesting that at least these IpaH proteins can be secreted through the type III secretion system.

Delayed IpaH Secretion from Shigella in the Presence of...
or pH 4.5 (lanes 1–3). YSH6000 harboring pIpaH 9.8 (lanes 4–6), S325 (lanes 7–9), and S325 harboring pIpaH 9.8 (lanes 10–12). Samples were subjected to SDS-PAGE followed by immunoblotting with antibodies specific for IpaB, IpaC, IpaD, and IpaH. S325 is an mxiA::Tn5 mutant used as a negative control for deficiency in type III secretion. WC, CR+, and CR− denote the whole-cell lysate and supernatant in PBS with and without Congo red, respectively. B, immunoblotting analysis of His-tagged IpaH proteins secreted from and produced by YSH6000 harboring pHis-IpaH4.5 (lanes 1–3), pHis-IpaH7.8 (lanes 4–6), or pHis-IpaH9.8 (lanes 7–9). Samples were subjected to SDS-PAGE followed by immunoblotting with antibodies specific for IpaH. W+ and −, denote the whole-cell lysate and supernatant in PBS with and without Congo red, respectively.

Congo Red During Growth—It has been suggested that induction of ipaH expression in Shigella during growth in medium containing CR is markedly increased as determined by ipaH-lacZ fusion, whereas the expression of ipaBCDA under the same conditions occurred constitutively (22). Therefore, we investigated the kinetics of production within or secretion from YSH6000 of IpaH together with IpaBCD during growth for 4 h in tryptic soy broth containing CR. As shown in Fig. 3A, IpaBCD secretion from YSH6000 was detected as early as 30 min in medium with CR, whereas IpaH secretion was detected after a 2-h growth in medium with CR. Although IpaBCD production in YSH6000 could be detected readily during growth even in the absence of CR, IpaH production in YSH6000 was hardly detected during growth in the presence or absence of CR. In this experiment, none of the IpaH proteins including IpaBCD was secreted from S325 (a type III-deficient mutant of YSH6000), and S325 did not show production of IpaH proteins even with incubation in medium containing CR for 4 h (data not shown). On immunoblotting, two major protein bands were detected with the anti-IpaH antibody. To identify the secreted IpaH proteins corresponding to the 60- and 62-kDa protein bands, each IpaH protein was purified and compared with the sizes of the IpaH proteins secreted from YSH6000. As shown in Fig. 3B, the upper band corresponded to IpaH4.5, whereas the lower band corresponded to IpaH7.8 and IpaH9.8. Also, the identity of the bands was confirmed by peptide mass fingerprinting using matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy. The upper band included IpaH4.5, whereas the lower band included IpaH9.8 (data not shown). Due to unknown technical problems, fingerprinting for IpaH7.8 was not detected in the lower band. These observations suggested that IpaH4.5, IpaH7.8, and IpaH9.8 can be secreted via the type III secretion system, albeit at a late stage, in medium containing CR.

Intracellular Localization of Secreted IpaH—The present results and previous another study (22) led us to speculate that IpaH secretion would take place after bacterial entry into the host cells. To clarify the fate of IpaH secreted from Shigella during infection of epithelial cells, we performed indirect immunofluorescence laser-scanning confocal microscopy of HeLa cells infected with YSH6000 carrying pIpaH4.5. IpaC secreted from bacteria into the epithelial cells was not detected by incubation (data not shown). In contrast, after infection of HeLa cells with YSH6000 carrying pIpaH4.5, internalized bacteria were detected by phase-contrast microscopy and multiplied within the cytoplasm (Fig. 4A, a and c). The internalized bacteria in the cytoplasm as well as the host cellular nucleus were both visualized by staining with TO-PRO3 (e and g). Although IpaH4.5, visualized by the FITC signal was still visible in the cytoplasm, IpaH9.8 was predominantly concentrated within the nucleus (f). Indeed, the FITC signal was mostly colocalized with the TO-PRO3 signal (d). In contrast, no IpaC signal was detected within the nucleus, but it was distributed evenly in the cytoplasm (Fig. 4A, lower panels).
nuclear localization of IpaH was also reproducible when Caco-2 or COS-7 cells were infected with YSH6000 carrying pIpaH 9.8 (data not shown).

To further confirm the fate of intracellular IpaH 9.8, COS-7 cells expressing FLAG-tagged IpaH 9.8 were constructed, and the intracellular distribution of IpaH 9.8 was investigated by immunofluorescence confocal microscopy with anti-FLAG-M5 antibody. As shown in Fig. 4B, FLAG-IpaH 9.8 was localized in the nucleus. Furthermore, Cy3-labeled IpaH 9.8 together with FITC-labeled IgG were injected into COS-7 cells, and the cells were examined after a 30-min incubation. As shown in Fig. 4C, IpaH 9.8 was distributed within the whole cell including the nucleus (left panel), whereas IgG was located only in the cytoplasm (right panel). The distribution was also confirmed by immunohistological method using unlabeled IpaH 9.8 protein (data not shown). These results strongly suggested that IpaH secretion occurred after internalization of Shigella into host cells, in which the secreted IpaH proteins were transported into the nucleus.

**Intracellular Trafficking of IpaH**—We postulated that the movement of IpaH toward the nucleus may be mediated through interaction with the intracellular trafficking system. Thus, we injected Cy3-labeled IpaH 9.8 into COS-7 cells, and the cells were observed at 5 and 30 min after injection with a microscope equipped with a cooled CCD camera. Although IpaH 9.8-associated fluorescence varied among cells, the IpaH signal, which was initially dispersed within the cytoplasm at 0 min (data not shown), was concentrated around the periphery of the nucleus. In some case, the IpaH-associated fluorescence was accumulated at one place around the nucleus (Fig. 5A). This was not due to an artificial interaction caused by Cy3 modification, since the addition of an excess amount of unlabeled IpaH 9.8 competed with the accumulation of Cy3-labeled signal at one place on the nuclear membrane (without unlabeled protein, 95.5%; with 10-fold excess amount of unlabeled protein, 38.2%; with 100-fold mount of unlabeled protein, 20.0%). When COS-7 cells were pretreated with nocodazole, this phenomenon was not observed (Fig. 5A). Importantly IpaH 9.8 signal coincided with regions of high microtubule density containing γ-tubulin, which indicates a microtubule-organizing center (Fig. 5B). About 80% of the cells showed a similar distribution. Furthermore, HeLa cells treated with various drugs known to affect cytoskeletal dynamics were infected with YSH6000 carrying pIpaH 9.8. At 4 h post-infection, HeLa cells treated with or without cytochalasin D showed an accumulation of IpaH 9.8 within the nucleus as determined by immunofluorescence microscopy (Fig. 6A), suggesting that microfilaments are not in-
volved in the movement. In contrast, in HeLa cells treated with nocodazole or colchicine IpaH₉.₈ were distributed in the cytoplas as well as the nucleus. We examined the structure of microtubules using a mixture of anti-α- and -β-tubulin antibodies. These drugs caused a loss of the normal microtubule structure, and the tubulin that was visible appeared to be aggregated (data not shown). Quantitative assay for the nuclear accumulation of IpaH₉.₈ in HeLa cells treated with the drugs revealed that nuclear accumulation of IpaH was significantly decreased in cells treated with nocodazole and colchicine (Fig. 6B). These results strongly suggested that the microtubule network is involved in the intracellular trafficking of IpaH₉.₈ toward the periphery of the nucleus and transport into nucleus facilitated.

Nuclear Transport of IpaH—Small proteins of less than 40–60 kDa can enter the nucleus passively, albeit in a concentration-dependent manner, whereas some macromolecules larger than 40–60 kDa are actively transported across the nuclear pore complex (38). The active nuclear import of proteins has been shown to be mediated by specific amino acid sequences, which are referred to as NLSs. NLS-mediated nuclear import requires soluble factors such as the importin family (39–42). Although it seemed to have no NLS, IpaH₉.₈ protein was efficiently transported into the nucleus, suggesting that IpaH acts as nuclear transported protein. Therefore, we used a digitonin-permeabilized HeLa cell transport assay and investigated the intracellular fate of exogenously added IpaH using immunofluorescence microscopy, since digitonin permeabilizes the cytoplasmic membrane but not the nuclear membrane (42). In this experiment, we investigated the fate of β-catenin and allopheycocyanin conjugated to SV40 T-antigen NLS peptide (NLS-APC) as a control. Exogenously added β-catenin was rapidly imported into the nucleus in the absence of cell lysate without the addition of ATP/GTP, whereas under these conditions NLS-APC cannot be imported (43). Our results showed that IpaH₉.₈ migrated rapidly into the nucleus in the absence of exogenously added cytosol and ATP/GTP at 30 °C (Fig. 7, panel a). The nuclear transport of IpaH₉.₈ was inhibited as the concentration of cytosol added to the medium was increased (panel m). To investigate the requirement of ATP/GTP for nuclear import, permeabilized cells were pre-treated with apyrase, since apyrase decreases the ATP level (43, 44).
Upon the addition of apyrase, the nuclear transport of IpaH9.8 was inhibited (panel j), although the extent of inhibition was less compared with that for NLS-APC, and the import of IpaH9.8 into the nucleus was sensitive to temperature (panel d) and was inhibited by WGA (panel g), a specific inhibitor of nuclear transport across the nuclear pore complex (45). These results indicated that the nuclear transport of IpaH9.8 is independent of cytosolic factors but dependent on temperature and partly on ATP/GTP. It is thus likely that the nuclear transport of intracellular behavior of IpaH9.8 is similar to that of β-catenin.

**DISCUSSION**

In the present study, we investigated the secretion of IpaH proteins (IpaH4.5, IpaH7.8, and IpaH9.8) encoded by the large plasmid (pMYSH6000) of *S. flexneri* 2a (YSH6000) via the type III secretion system. Our results indicated that IpaH4.5, IpaH7.8, and IpaH9.8 were secreted via the type III secretion system when bacteria were incubated in PBS containing CR. These findings agree with those of other studies (21), indicating that IpaH4.5, IpaH7.8, and IpaH9.8 can be secreted from *S. flexneri* 5 (M90T) into the medium under conditions of inactivation of the *ipaBCDA* genes (21). We found that the secretion of IpaH proteins from YSH6000 during growth in the medium stimulated the type III secretion system at a late stage such as after 2 h, whereas the secretion of the IpaBCD under the stimulated conditions occurred as early as after 30 min (see Fig. 3A). The delayed secretion phenotype displayed by IpaH proteins from *Shigella* in activation of the type III secretion system may be reflected by the differences in the transcriptional control system from that required for *ipaBCDA* genes, as reported by Demers et al. (22). Indeed, they showed that the levels of transcription of *ipa* genes, but not *ipaBCDA* genes, were markedly increased during growth in the presence of CR or in the ΔipaBCDA mutant, conditions that enhanced type III secretion (22). Although the mechanism underlying the delayed IpaH expression remains to be elucidated, the delayed IpaH secretion suggested that IpaH plays different roles from IpaBCD in *Shigella* infection.

The assignment of the open reading frame for the *ipaH* gene of YSH6000 in the present study was different from that reported previously for the M90T strain (24), since an additional nucleotide was found at position 26 from the 5′ end of *ipaH* of M90T (see Fig. 1A). The discrepancy might have been due to an error in sequencing of M90T *ipaH*, since Buchrieser et al. (21) very recently reported that the N-terminal amino acid sequence of the secreted IpaH9.8 from M90T was consistent with that of our predicted IpaH9.8 sequence of YSH6000. Therefore, the extended N-terminal 33-amino acid residues of IpaH9.8 also showed similarities with those of IpaH4.5 and IpaH6.8 (see Fig. 1C). Although the precise roles of the N-terminal sequences of IpaH4.5, IpaH7.8, and IpaH9.8 are still to be investigated, the N-terminal sequence may contain amino acid sequences required for secretion via the type III secretion system.

Our observations suggested that the secretion of IpaH from *Shigella* occurs after invasion of epithelial cells. When YSH6000 carrying pIpaH9.8 (a cloned *ipaH* plasmid) was infected into HeLa cells pretreated with cytochalasin D, IpaH9.8 was not detected in the cells by indirect immunofluorescence staining with anti-IpaH antibody. In contrast, when HeLa cells were incubated with the bacteria for 4 h without treatment, *Shigella* were internalized into the HeLa cells, and secreted IpaH was detected within the nucleus, although a small amount of IpaH was present in the cytoplasm. Since the IpaC secreted from the intracellular *Shigella* at 4 h post-infection was observed only within the cytoplasm and not in the nucleus, we concluded that IpaH secretion would be stimulated after bacterial entry into the host cytoplasm and that IpaH9.8 can be transported into the nucleus. Similarly, YopM of *Yersinia pestis* was previously reported to be transported into the nucleus (46). YopM also possesses 15 repeats of LRR, showing similarity with the N-terminal portion of IpaH, but lacks the CTR, which exists in all IpaH family proteins including other homologous proteins such as SlrP of *Salmonella typhimurium* or y4R of *Rhizobium* (see Fig. 1A). Although whether SlrP and y4R would also be translocated into the host nucleus remains to be elucidated, based on the primary structural features and nuclear transport behavior of IpaH9.8 and YopM, the N-terminal portion of IpaH proteins containing the LRR is probably involved in nuclear transport.

Since IpaH9.8 secreted from intracellular *Shigella* at 4 h post-infection appeared to be accumulated in the nucleus, we further investigated the fate of IpaH9.8 including intracellular trafficking by different approaches. In COS-7 transfectants expressing FLAG-tagged IpaH9.8, the IpaH signal was mostly detected in the nucleus. Furthermore, the microinjection of Cy3-labeled IpaH9.8 together with FITC-IgG into COS-7 cells revealed that although the FITC fluorescence signal was detected only in the cytoplasm at 30 min after injection, Cy3 fluorescence was detected in both the cytoplasm and nucleus, implying that IpaH9.8 acts as a mammalian nuclear transport protein. In these experiments, we noted that in some cells the cytoplasmic Cy3 signal, which was associated with IpaH9.8, was concentrated around the microtubule-organizing center. However, the accumulation was not predominant in cells pretreated with nocodazole, a microtubule-destabilizing agent, suggesting that intracellular IpaH9.8 is accumulated in the vicinity of the nuclear surface through association with the microtubule network (see Fig. 8). This was also suggested by investigation of the fate of IpaH9.8 secreted from intracellular *Shigella*, in which accumulation of the secreted IpaH9.8 within the nucleus was almost completely blocked in cells pretreated with nocodazole or colchicine. Although the precise mechanism underlying the concentration of IpaH9.8 remain to be investi-

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2 T. Toyotome, T. Suzuki, A. Kuwae, T. Nonaka, H. Fukuda, S. Imajoh-Ohmi, T. Toyofuku, M. Hori, and C. Sasakawa, unpublished results.

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Fig. 8. Model for the fate of intracellular IpaH9.8. Details are given under “Discussion.” The arrows indicate the fate of IpaH9.8. The gray balls indicate the IpaH proteins. The red ellipses indicate *Shigella*. The blue lines indicate microtubules. The orange ellipses indicate the nuclear pore complex (NPC).
ported into the nucleus (52, 53). Accordingly, IpaH9.8 with an the SV40 large T-antigen, a 94-kDa protein, possesses transported across the nuclear pore complex (38). For example, 60 kDa, if they possess an NLS, are actively identified.

Although the putative motor proteins have not yet been initiating subsequent transport of the genome into the nucleus (47). The cap-

bule-organizing center. Trafficking along the microtubules in important for the condensation of IpaH around the microtu-

trafficking system mediated by microtubule networks would be

fashion. Upon the addition of apyrase, which causes a decrease the large plasmid of

concluded that IpaH9.8 could be transported into the nucleus, infection. Interestingly, with mutation of the

be dependent on transport factors such as importin 

with that of allophycocyanin-conjugated SV40-T-antigen NLS

WGA acting as a nuclear pore plug, suggesting that the nuclear transport was sensitive to temperature and was inhibited by

inhibited. Although the nuclear transport of IpaH 9.8 partially

requires ATP, this requirement for IpaH 9.8 was relatively less

cytosol and without a supply of ATP/GTP, IpaH9.8 was still

gated, as indicated for YopM of

containing CR, we speculate that rapidly motile intracellular

direct evidence has yet been obtained, we speculate that the

independently of host-soluble factors (see Fig. 8). Although no

direct evidence has yet been obtained, we speculate that the

other IpaH proteins may also be imported into the host cell nucleus, since they showed similarities with the LRR motifs of IpaH9.8 and YopM (22, 25).

How intracellular Shigella can sense the host cytoplasmic environment and trigger the secretion of IpaH is still unknown. However, since IpaH secretion would occur mainly after bacte-

rial entry into the host cells, and activation of the type III secretion system would be stimulated by bacterial contact with the host cells or growth under conditions such as in media containing CR, we speculate that rapidly motile intracellular Shigella within the cytoplasm, allowing direct contact with the inner surface of the cytoplasmic membrane, might be triggered to activate the type III secretion system as proposed in Fig. 8.

Finally, it is worth contemplating the significance of nuclear transport of IpaH9.8 including other IpaH proteins in Shigella infection. Interestingly, with mutation of the ipaH7.8 gene of the large plasmid of S. flexneri 2457T or both the ipaH7.8 and ipaH4.5 genes, although the bacterial invasion of epithelial cells had no effect, the mutants induced an exaggerated Sereny response in guinea pig eyes (54), suggesting that ipaH7.8 plays a role in modulating the inflammatory response elicited by infection (23). Although it is unclear whether the roles of each of the IpaH proteins in Shigella infection are similar to each other, the enhanced inflammatory reaction in the Sereny test suggests that the nuclear-transported IpaH proteins participate in modulating gene expression involved in the production of inflammatory mediators such as interleukin-8. If the nuclear transport of IpaH is important for Shigella infection of the human colon, it is necessary to identify the targeting genes or factors of IpaH9.8 protein.

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Shigella Protein IpaH\textsubscript{9.8} Is Secreted from Bacteria within Mammalian Cells and Transported to the Nucleus

Takahito Toyotome, Toshihiko Suzuki, Asaomi Kuwae, Takashi Nonaka, Hiroyuki Fukuda, Shinobu Imajoh-Ohmi, Toshihiko Toyofuku, Masatsugu Hori and Chihiro Sasakawa

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