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Maintenance of the *Salmonella*-containing vacuole in the juxtanuclear area: A role for intermediate filaments

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ABSTRACT

Until recently, intermediate filaments (IF) were thought to be only involved in resistance to physical stress and mechanical integrity of cells and tissues. Recent data indicate that IF play a much more important role in cellular physiology including organelle structure and positioning within the cell. Here, we show that *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) induces in epithelial cells and macrophages the formation of an aggresome-like structure with a dramatic remodelling of cytoplasmic IF (vimentin and cytokeratin) networks and the adaptor proteins 14-3-3 which are recruited around intracellular *S. typhimurium* microcolonies. These rearrangements are not necessary for bacterial replication. Depletion of vimentin and cytokeratin by siRNA indicates that IF remodelling is required to maintain *Salmonella* microcolonies in the juxtanuclear area.

1. Introduction

As part of their intracellular lifestyle, many pathogens survive and replicate within membrane-bound vacuoles that interact extensively with the host cell. *Salmonella enterica* serovar Typhimurium (*Salmonella typhimurium*) is a facultative intracellular pathogen responsible of gastroenteritis in humans and a fatal typhoid-like affliction in susceptible mice [1–3]. Internalization into epithelial cells is directed by the type III secretion system encoded by the *Salmonella* pathogenicity island 1 (SPI-1 T3SS) that allows translocation of effector proteins from the bacterial cytosol, through the plasma membrane, to the host cell cytosol [4–6]. These translocated effectors cause actin polymerization, membrane ruffling, and uptake of bacteria into a membrane-bound compartment known as the *Salmonella*-containing vacuole (SCV) [5]. It is well established that in epithelial cells, maturation of the SCV is dependent on a series of selective interactions with the endocytic pathway. After internalization, the SCVs interact with the early endocytic pathway and acquire EEA1 and the transferrin receptor [7,8]. These proteins are rapidly recycled from the maturing SCV and, simultaneously, the SCVs are diverted from the normal endocytic pathway and undergo a specific biogenesis process that involves sequential acquisition of Rab5 and Rab7, the lysosomal glycoproteins (Lgps) lamp1, lamp2 and CD63, the vacuolar ATPase and the lysobisphosphatidic acid (LBPA). The mannose 6-phosphate receptor and lysosomal hydrolases are essentially excluded from SCVs, indicating that they avoid fusion with mature lysosomes [9–13]. Bacterial replication starts when SCVs have reached a juxtanuclear location after a lag phase of 4 h post internalization. Intracellular replication of *S. typhimurium* requires the expression of the type III secretion system encoded by the *Salmonella* pathogenicity island-2 (SPI-2 T3SS), which translocates effector proteins into and across the vacuolar membrane [14]. SPI-2 T3SS effectors contribute to numerous intracellular events, including maturation and integrity of the vacuole, formation of vacuolar membrane extensions called Sifs, bacterial replication, and the systemic spread of bacteria [15–20]. Extensive remodelling of the actin cytoskeleton and microtubules also occurs during the course of intracellular replication of *S. typhimurium* and both networks are necessary for bacterial replication, Sif formation and maintenance or dynamics of the vacuolar membrane [19,21–25].

Dynamic interactions between the three cytoskeletal systems, microfilaments (MF), microtubules (MT) and intermediate filaments (IF) regulate to a great extent the structural organization of

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the cytoplasm of animal cells. Although IF constitute one of the three major cytoskeletal systems of eukaryotic cells, they are the least studied of the networks. This can be explained in part by the complexity of this network which includes about 50 different proteins classified into six distinct groups of IF, whose expression patterns are usually cell-type specific. IF build two distinct systems, one in the nucleus and one in the cytoplasm. Lamin A/C belongs to type V IF and constitutes the nuclear lamina and nuclear matrix. Cytoplasmic IF, that are clearly not required for the survival of individual cells, are considered to be the major contributors to the mechanical integrity of cells and tissues. This has been particularly well studied in epithelial cells in which cytokeratins (type I and II IF) and vimentin (type III IF) constitute the cytoplasmic IF networks. Recent data demonstrate that IF are highly dynamic and motile structures [26–28] and are reorganized during numerous cellular events such as mitosis and apoptosis. They seem to be involved in positioning organelles as well as contributing to their shape and function, targeting proteins to their proper subcellular compartments, trafficking of lipid droplets within cells and formation of autophagic vacuoles [29,30]. Reorganization and dynamics of IF are thought to be mediated by phosphorylation [31].

In view of the ability of *S. typhimurium* to remodel MF and MT during intracellular replication and their importance in *S. typhimurium* pathogenesis, we investigated whether IF are also affected during *S. typhimurium* infection of HeLa cells.

2. Results

2.1. S. typhimurium induces redistribution of vimentin, cytokeratins and 14-3-3

The ability of *S. typhimurium* to subvert the actin and microtubule networks is well documented [19,21,22,32,33]. In contrast, the ability of *S. typhimurium* to modulate IF proteins, which constitute the third major component of the cytoskeleton, is not well known. In order to determine if *S. typhimurium* infection affects the IF, HeLa cells were infected for 10 h with wild-type *S. typhimurium*, fixed and stained with antibodies directed against cytoplasmic IF (vimentin and cytokeratins) and nuclear IF (lamin). Microscopic examination of uninfected control cells revealed that while vimentin and cytokeratins form a dense filament network radiating from the nucleus and extending to the plasma membrane, lamin remained confined to the nuclear envelope region. Confocal X/Y sectioning of infected cells revealed a dramatic accumulation of vimentin, CK18 and CK8 around $83 \pm 12\%$, $71 \pm 17\%$, and $86 \pm 6\%$ *S. typhimurium* microcolonies, respectively (Fig. 1A). Three-dimensional reconstructions of serial optical sections revealed that the SCVs were surrounded by a uniform concentration of CK18 and vimentin fibres (Fig. 1B). Analysis of vimentin accumulation over time showed that these rearrangements were first detectable at 7 h post infection which increased both in terms of intensity and frequency thereafter (Fig. 1C). Similar vimentin rearrangements also occurred in *S. typhimurium*-infected murine elicited peritoneal macrophages and in the intestinal epithelial cell line INT407 (data not shown).

IF network dynamics is controlled by the adaptor proteins 14-3-3, which interact directly with vimentin and CK [34]. We investigated if *S. typhimurium* infection also induces redistribution of 14-3-3. In uninfected cells, 14-3-3 was distributed homogeneously throughout the cytoplasm. In contrast, following 10 h *S. typhimurium* infection, extensive accumulation of 14-3-3 was seen around *S. typhimurium* microcolonies (Fig. 1D)

2.2. IF are involved in maintaining the SCVs in the juxtanuclear area

S. typhimurium migrates to the juxtanuclear location after internalization into epithelial cells and starts to replicate in the proximity of the Golgi apparatus at around 4 h post infection [17]. In order to determine if IF proteins play a role in intracellular *S. typhimurium* localisation, we treated cell with either acrylamide, which induces selective collapse and disassembly of CK and vimentin networks [35] (Fig. 2, left panel) or small interfering RNAs (siRNA), which deplete cells from vimentin and/or CK18 (Fig. 2, right panel). While migration of internalized bacteria to juxtanuclear area occurred normally within 3 h post infection (not shown), analysis of treated cells 10 h post *S. typhimurium* infection revealed that acrylamide disruption or siRNA depletion of IF proteins led to SCVs being scattered in the cytoplasm and at cell periphery; $68 \pm 20.1\%$, $82 \pm 14.2\%$; $90 \pm 13\%$ and $88 \pm 13.5\%$ cells contained dispersed SCVs in acrylamide-treated, CK18-, vimentin-, and vimentin and CK18 siRNA-depleted cells, respectively, compared to $22 \pm 5.3\%$ in untreated cells (Fig. 3A). Importantly, disruption or depletion of IF proteins had no adverse effects on the Golgi or caused the release of intracellular bacteria from the SCVs into the cytoplasm, as intracellular bacteria in treated cells remained associated with Lamp1 (Fig. 3B) and Sif formation was not affected (not shown).

We next investigated if IF rearrangements are necessary for intracellular replication of *S. typhimurium*. To this end, HeLa cells were infected with *S. typhimurium* for 10 h and bacteria were enumerated in acrylamide-treated and CK18- or vimentin siRNA-depleted cells by microscopy. No significant inhibition of bacterial replication was observed compared to untreated cells (Fig. 4A). Similar results were obtained in a gentamycin protection assay comparing acrylamide treated cells to untreated cells (not shown).

In a reciprocal study we investigated if intracellular bacterial replication is needed for IF rearrangement. Using the auxotrophic *aroC* and *purD* double *S. typhimurium* mutant which does not duplicate intracellularly [16], we found that remodelling of the IF was not significantly different from that caused by the wild-type strain (Fig. 4B).

2.3. Rearrangement of IF by S. typhimurium is dependent on protein synthesis

In order to determine if IF remodelling requires live bacteria, we compared their distribution after internalization of *S. typhimurium* and opsonized latex beads of similar diameter into HeLa cells transfected with a vector encoding the human Fc gamma receptor IIA (FcγRIIA). This revealed that while the *S. typhimurium* triggered typical IF reorganization, no IF proteins were detected around the internalized IgG-opsonized latex beads up to 10 h after uptake (Fig. 5A).

In order to investigate the specificity of IF remodelling around intracellular *S. Typhimurium*, we examined the ability of *E. coli* expressing invasin to promote IF remodelling. When cells were infected with *E. coli* expressing invasin, no IF remodelling was observed around intracellular bacteria (Fig. 5B).

Finally, we investigated if bacterial protein synthesis is required for IF remodelling. At 5 h post *S. typhimurium* infection, while bacterial replication and IF rearrangement had only just begun, we blocked bacterial protein synthesis by the addition of tetracycline to the medium of infected cells. Following a further 7 h incubation the tetracycline-treated cells were fixed and vimentin rearrangement was examined by confocal microscopy. In the majority ($75.4 \pm 7.3\%$) of tetracycline-treated cells, *S. typhimurium* microcolonies were found in the juxtanuclear area. However, only $26 \pm 10.5\%$ of the *S. typhimurium* microcolonies were surrounded by a vimentin network compared with $83.3 \pm 6\%$ in untreated cells (Fig. 5C). This suggests that bacterial protein synthesis is required to promote or maintain vimentin remodelling. Similar results were obtained for CK18 (not shown).