When bugs meet cells

Conference: Frontiers of cellular microbiology and cell biology

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special emphasis on cytoskeleton plasticity and signaling. Here we highlight several of the fundamental questions of cell biology that were addressed by cellular microbiologists.

Lipidic microdomains in microbial host cell invasion and endocytosis

It is now clear from the work of several laboratories during the last decade, that the organization of the plasma membrane is more complex than the fluid mosaic model proposed by Singer and Nicholson 30 years ago (reviewed by Simons and Toomre, 2000). Lipid rafts, consisting mainly of dynamic assemblies of cholesterol and sphingolipids, form in the exoplasmic leaflet of the bilayer, conferring an intrinsic heterogeneity to the plasma membrane. In turn, according to their affinities for different lipids, proteins can also be heterogeneously distributed in various microdomains. In particular, molecules that are used as receptors for the binding of pathogens may associate with membrane microdomains, causing a preferential association of pathogens with these domains.

Jean Pieters (Basel, Switzerland) showed that cholesterol accumulates at the site of *Mycobacteria* entry into macrophages. Depleting cell cholesterol specifically inhibited mycobacterial uptake, suggesting that the preferential association of the parasite with cholesterol is important for its entry. To understand further how this association takes place, Isabelle Maridonneau-Parini (Toulouse, France) studied the phagocytosis of *Mycobacterium kansasii* by human neutrophils. She showed that antibodies directed against the complement receptor type 3 (CR3), as well as antibodies directed against several GPI-anchored proteins, strongly inhibit phagocytosis of the mycobacteria. In contrast, although phagocytosis of yeast extracts (zymosan) or *M. kansasii* coated with a specific antiserum (opsonized particles) involved CR3, it involved neither cholesterol nor GPI-anchored proteins. CR3 is known to associate with several GPI-anchored

Introduction

Early research into microbiological pathogenesis attempted to determine which microbial properties make micro-organisms pathogenic. Over time, however, a growing number of research projects began to focus on the intimate relationships between microbes and their hosts and, more precisely, on the consequences of infection for the cell biology of the host. This approach has brought together two traditional disciplines—microbiology and cell biology. Signs of the recent successes of this merger include a new journal—*Cellular Microbiology*—and several meetings on the subject, including the Euresco/EMBO workshop held in Giens, France (October 7–12, 2000) entitled 'Frontiers of cellular microbiology and cell biology'. Progress in understanding a number of cellular processes was discussed at this meeting, with

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proteins in neutrophils, and *N*-acetyl-D-glucosamine, which disrupts this interaction, also strongly diminished phagocytosis of the mycobacteria. Maridonneau-Parini proposed that CR3, rather than any specific GPI-anchored protein, functions as a *M. kansasii* receptor, and that CR3 needs to associate with lipid rafts to mediate phagocytosis. When CR3 is not associated with a GPI-anchored protein, it remains outside these domains and mediates phagocytosis of zymosan and of opsonized particles, but not of *M. kansasii*.

Considerable progress has been made in cell biology to identify the different pathways of endocytosis and to characterize their molecular basis. The best characterized pathway is via clathrin-coated pits, and several of the molecular interactions involved in the concentration of membrane proteins into clathrin-coated pits and in the invagination of the membrane into clathrin-coated vesicles have been identified. Much less is known about the machineries involved in the clathrin-independent pathways of entry, mostly due to the lack of specific markers to characterize them. One example of these alternative pathways is caveolae, bottle-shaped invaginations of the plasma membrane coated with caveolin, which can undergo internalization. Highly purified caveolae can be obtained from rat lung endothelial cells, following perfusion with cationic colloidal silica particles, a technique developed by Jan Schnitzer (San Diego, CA). This protocol has allowed the differentiation of caveolae from other membrane subdomains whose flotation characteristics on sucrose gradients are similar and can therefore not be distinguished by standard biochemical methods. A first insight into understanding the molecular regulation of endocytosis via caveolae may come from the observation that endothelin-1 stimulates the budding of caveolae, in which the endothelin receptor-B is localized.

Several pathogens use the host cell's endocytic machinery to deliver proteins into eukaryotic cells. Several of these molecules, such as ricin or Shiga toxin, have even been used by cell biologists as markers to follow endocytic routes. Along these lines, Gisou van der Goot (Geneva, Switzerland) presented new results on the sorting of GPI-anchored proteins in CHO and in BHK cells, using the pore forming toxin aerolysin from *Aeromonas hydrophyla* as a marker: indeed, aerolysin specifically binds all GPI-anchored proteins, and can therefore be used as a specific marker for the membrane microdomains with which these proteins preferentially associate. In CHO cells, surface GPI-anchored proteins are transported to the recycling compartment which was identified by labelling with transferrin, while in BHK cells, GPI-anchored proteins were targeted to late endosomes. This work suggests that different mechanisms may operate to sort GPI-anchored proteins/rafts in the endocytic pathway in the two cell types.

Generation of motion through actin polymerization

There are at least two ways in which actin can generate motion. First, it has long been known that actin filaments form tracks on which motor proteins move by interacting sequentially with different actin subunits: this mechanism is central to both muscle contraction and cytokinesis, and its use for vesicle motion has been demonstrated in a few systems (reviewed by DePina and

Langford, 1999). Secondly, it has been shown that sustained actin nucleation induces the growth of actin filaments and pushes forward whatever triggers the nucleation: this was first observed with bacteria moving through cytosol, which have helped to characterize the molecular machinery underlying actin nucleation. Several systems in which motility can be produced through actin polymerization were discussed at the meeting.

Mimicking receptor tyrosine kinase signal transduction. Vaccinia virus can polymerize actin in the cytosol of infected cells, and move at the tip of actin comet tails. Michael Way (Heidelberg, Germany) showed that tyrosine phosphorylation of the integral membrane viral protein A36R is necessary for actin tail formation. Using the Src-family kinase inhibitor PP1 or dominant-negative forms of Src, which reduce A36R phosphorylation, Way showed that Src is involved in tyrosine phosphorylation of A36R and actin tail formation. The adaptor protein Nck, and the Nck downstream partner N-WASP, are also recruited to the site of vaccinia tail formation upon A36R phosphorylation, providing a mechanism for the recruitment of the Arp2/3 complex to the vaccinia tail. Vaccinia virus therefore mimics the receptor tyrosine kinase signal-transduction pathways involved in the control of actin polymerization at the plasma membrane in order to favour its own actin-based motility.

Vesicle movement. Laura Machesky (Birmingham, UK) reported that various stimuli, such as overexpression of the PIP5-kinase, activation by platelet derived growth factor (PDGF) in the presence of pervanadate, or subjection of the cells to an osmotic shock, result in the appearance of vesicles coated with actin comet in murine 3T3 cells. Conversely, actin comets are inhibited by overexpression of truncated forms of Scar (ScarWA) or of N-WASP, (showing the involvement of the Arp2/3 complex in comet formation), cytochalasin D addition, cholesterol depletion (by β -methylcyclodextrin addition) and serum starvation. By testing the vesicles for several markers, Machesky found a significant enrichment of markers from the Golgi apparatus (such as ceramide, cholera toxin B, influenza hemagglutinin A), indicating that these vesicles may originate from this organelle.

Results reported by John Cooper (St Louis, MO) showed that actin comets are also observed in cells overexpressing Arf6Q67L, a constitutively active form of the small G protein Arf6. Under these conditions, fluid-phase endocytosis is enhanced by 50% and the moving particles observed may correspond to pinocytic vesicles. The number of moving particles decreases upon expression of ScarWA, indicating that actin nucleation via Arp2/3 is involved, and by inhibitors of tyrosine phosphorylation. Occasionally, antibodies directed against phosphorylated tyrosine could label the tip of the moving particles, but the phosphorylated protein has not been identified.

Actin and endocytosis. The results described above suggest that actin may play a role in the motion of endocytic vesicles. Conflicting results have been reported regarding the role of actin in endocytosis, probably because it may depend on the system used, and because many studies were conducted using drugs which probably have an incomplete effect on actin polymerization and other non-specific effects. New tools, such as

dominant-negative forms of proteins of the endocytic machinery or of proteins linked to the actin cytoskeleton, are now available, and several recent studies point to a molecular interaction between the endocytic machinery and the actin cytoskeleton (reviewed in Qualmann *et al.*, 2000). In this direction, Michael Kessels (Magdeburg, Germany) has identified the mouse homologue of the yeast actin binding protein 1 (abp1p), a molecule involved in cytoskeletal regulation and endocytosis in *Saccharomyces cerevisiae*. Mammalian Abp1 possesses two independent actin binding regions and, upon Rac1 activation, relocates to lamellipodia and regions of cortical actin rearrangement. Abp1 can interact with dynamin via a SH3 domain, is observable at dynamin-rich sites of endocytosis upon stimulation of NIH 3T3 cells with growth factors, and overexpression of its SH3 domain leads to a drastic reduction of transferrin endocytosis, suggesting that Abp1 can functionally link membrane trafficking events to a dynamic actin cytoskeleton.

Other data supporting a role for actin in endocytosis were reported by Folma Buss (Cambridge, UK). Myosin VI is the only protein of the myosin superfamily that moves towards the minus end of actin filaments away from the plasma membrane. Therefore, it is a good candidate for an endocytic motor with a role in the transport of endocytic material into the cell. Immunofluorescence studies have shown that myosin VI is associated with the Golgi complex, with membrane ruffles and with vesicles distributed throughout the cell. This punctate pattern was identified as clathrin-coated pits/vesicles, as it coincides with the localization of clathrin and of the adaptor protein AP-2. Moreover myosin VI is present in a protein complex with AP-2 and clathrin since it can be immunoprecipitated with both, it can be pulled down from whole cytosol *in vitro* with just the α -ear domain of AP-2, and it is present in purified clathrin-coated vesicles. Buss has identified three splice variants affecting the myosin VI tail domain: without insert, with a small insert or with a large insert. The distribution of these forms is tissue specific. In polarized cells, the myosin VI isoform with the large insert is expressed and is concentrated at clathrin-coated vesicles at the apical domain. Overexpression of the myosin VI tail without a functional motor domain as a dominant negative in rat fibroblasts significantly reduces transferrin uptake, suggesting that myosin VI indeed has a function in endocytosis via clathrin-coated vesicles.

Interfering with host cell functions

Secretion of bacterial proteins across the plasma membrane of a host cell. Several Gram negative pathogens have developed complex structures—termed type III secretion systems—designed to translocate specific bacterial effectors into the cytoplasm of target cells (reviewed by Cornelis and Van Gijsemen, 2000). The secretion apparatus consists of about 30 proteins that assemble into a ‘needle’, which extends through the bacterial cell envelope and protrudes as an external spike that can be inserted into the plasma membrane of host cells, forming a pore through which bacterial proteins can be delivered directly to the cellular cytosol. Ariel Blocker (Paris, France), who used *Shigella* as a model, was able to purify the entire needle complex, including the large proximal bulb positioned within the bacterial cytoplasm, and the attached neck domain which traverses both

bacterial membranes. Using high resolution electron microscopy, she obtained a two-dimensional image of the negatively stained needle complex at 17 Å resolution, in which a central 2–3 nm canal could be discerned. Blocker’s work favours a model in which type III secretion apparatuses build a continuous canal between the bacterial cytoplasm and the host cell cytosol.

Type IV secretion systems are also supramolecular complexes that have been described as transporters of DNA from bacteria-to-bacteria, or to eukaryotic cells. In the past few years, additional members of the family have been discovered, and these have alternative functions including transport of multi-subunit proteins across bacterial membranes. Markus Stein (Sienna, Italy) presented data showing that the *cag* system of *Helicobacter pylori*, which encodes a specific type IV secretion engine, is used to deliver a bacterial protein, CagA, into epithelial cells. An as yet unidentified host cell kinase phosphorylates CagA on tyrosine residues, a process which may be part of the mechanism responsible for the actin polymerization observed at the site of contact between *H. pylori* and the host cell.

Remodelling the plasma membrane. The Arp2/3 complex is one of the key players of the actin assembly initiation machinery, and diverse pathogens take advantage of this multimer in order to stimulate actin filament formation (reviewed by Cossart, 2000). Brett Finlay (Vancouver, Canada) showed that enteropathogenic *Escherichia coli* (EPEC) is able to directly recruit the actin nucleation machinery to the site of bacterial attachment at the plasma membrane of host cells, in order to promote the formation of pedestals. Upon contact with the host cell, EPEC secretes the virulence factor Tir (translocated intimin receptor) through a type III secretion system into the cytoplasm of the target cell. Tir is then inserted in the plasma membrane of the host cell and serves as the receptor for the bacterial surface protein intimin, which promotes intimate contact between EPEC and the cell. Tir is phosphorylated at its cytoplasmic region by a yet unknown cellular kinase and recruits a host-cell GTPase of the Chp family, which in turn recruits the Wiskott–Aldrich syndrome protein (WASP). Members of the WASP family of proteins possess a C-terminal highly acidic domain that mediates binding of the Arp2/3 complex. Deletions of the C-terminal domain of WASP inhibited pedestal formation, suggesting that the Arp2/3 complex is indeed involved in the actin polymerization required for pedestal protrusion. Moreover the same group showed the N-terminal part of Tir directly binds α -actinin, which may facilitate actin polymerization much like what happens in focal contacts.

The small GTPases of the Rho family are global regulators of the actin cytoskeleton dynamics and Philippe Sansonetti (Paris, France) described the tightly regulated activation of Cdc42, Rac and Rho during *Shigella flexneri* entry into cells. Incubation of permeabilized cells with IpaC, a bacterial protein secreted by the Mxi-Spa type III secretion system of *Shigella*, promoted the formation of lamellipodia and filopodia at the edges of mouse fibroblasts. Expression of dominant-negative forms of Cdc42 and Rac inhibited the formation of such structures, suggesting that IpaC induces *de novo* actin nucleation through the activity of these two GTPases. Treatment of cells with the C3 toxin of *Clostridium difficile*, which specifically inactivates Rho by ADP-ribosylation, also blocks *Shigella* entry. This result was unexpected as the function of Rho is generally considered to be

What defines the identity of a cellular compartment?

A fundamental property of subcellular compartments of the endocytic and exocytic pathways is their ability to continuously exchange material (membranes or cargo) with one another while maintaining a temporal and morphological stability. Understanding what defines the nature of these compartments and how their integrity is kept remains a key question.

Maria Antonietta de Matteis (Santa Maria Imbaro, Italy) addressed this issue by studying the morphology of the Golgi apparatus and the factors involved in its stability. She demonstrated that upon ARF (ADP ribosylation factor) activation, synthesis of phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate is stimulated in Golgi membranes due to the translocation of PI 4-Kinase β and a yet unidentified PI 5-Kinase; this activity is crucial for the maintenance of the structure of the Golgi apparatus, since expression of a dominant-negative form of the PI 4-K β induces disassembly of the Golgi stacks. An attractive possibility suggested by this work is that the locally produced phosphoinositides serve to recruit spectrin, a molecule known to control membrane organization and shape, by linking phospholipid bilayers to cellular motors as well as to all major filament systems (including actin). Spectrin contributes to the stability of the endoplasmic reticulum-to-Golgi transport by a still uncharacterized mechanism, and could play a functional role in defining the architecture of this complex organelle both spatially and temporally.

The nature of the compartment in which intracellular pathogens reside is also essential for their survival (Mresse *et al.*, 1999). Two examples of how pathogens are able to dictate the composition of the membrane which surrounds them were discussed.

David Holden (London, UK) showed that *Salmonella typhimurium* maintains the integrity of its vacuole (SCV) through the action of the bacterial protein SifA (for *Salmonella* induced filaments). This molecule, previously known to be involved in the formation of tubules enriched in lysosomal glycosylated proteins, was shown by Holden to be linked to the SPI (*Salmonella* pathogenicity island)-2 encoded type III secretion system, because infection of HeLa cells with structural mutants of this secretion apparatus induces loss of Sifs. In macrophages, microscopy studies demonstrated that the absence of this effector leads to vacuole disassembly and bacterial death, since SifA mutant strains are cytosolic and unable to proliferate in this environment. The mechanisms by which SifA maintains the vacuolar membrane are unknown, but in view of the involvement of the rab7 GTPase in the biogenesis of SCVs and its presence on Sifs, it is possible that SifA acts as an activator of this membrane fusion machinery (Figure 2A).

Another strategy to create a compartment favourable to proliferation was illustrated with *Mycobacteria*, which sequester proteins from the host cell onto their phagosome. Previous work from several groups has clearly demonstrated that *Mycobacteria* inhibit the maturation of their compartment and replicate in an early endosomal-related phagosome. Jean Pieters (Basel, Switzerland) showed that TACO (also known as coronin-1) was present only in phagosomes containing live microorganisms. TACO is released from phagosomes containing dead *Mycobacteria* which then fuse with lysosomes, suggesting that TACO

Fig. 1. Bacterial signals and cell responses during *Shigella* entry in epithelial cells. IpaC is translocated into the cell cytosol and induces actin polymerization by activating Cdc42 (1). Rho activation is required for the organization of the entry structure; it allows the recruitment of cytoskeletal proteins such as ezrin. Rho activation also leads to Src recruitment, which in turn downregulates Rho activity through a negative feedback loop (2). Once translocated into the cell cytosol the bacterial protein IpaA binds to vinculin, and the vinculin-IpaA complex allows the formation of an adhesion-like structure at the site of bacterial contact with the cell membrane but is also involved in the transformation of filopodial extensions into leaflet structures probably through its F-actin-depolymerizing activity (3). A concerted action of Ipa proteins leads to *Shigella* internalization into a large cellular vacuole (4). (Figure courtesy of G. Tran Van Nhieu, Paris, France)

in determining the formation of stress fibres and focal adhesions. However, Rho inhibition does not prevent actin filament polymerization in the vicinity of the bacterium interacting with the cell membrane, but inhibits filament elongation and bundling. Rho seems to be required during invasion for the recruitment to the site of bacterial entry of two key players: Src (involved in the recruitment of cortactin to sites of actin polymerization) and ezrin (which plays an essential role in the Rho induced extension of filopodia) (Figure 1).

Escaping phagocytosis. *Yersinia* is a bacterial parasite that avoids internalization into professional phagocytes, as shown by Guy Cornelis (Basel, Switzerland). Of the six proteins secreted through its type III secretion system by *Y. enterocolitica* into the cytosol of macrophages, four (YopE, YopH, YopO and YopT) have antiphagocytic activity, blocking also the internalization of latex beads in these cells. Secretion of each one of these four proteins is necessary for complete inhibition of phagocytosis, as mutant bacteria of any of these Yop proteins are phagocytosed by cultured J774 and PU5.8 macrophages more efficiently than are wild-type bacteria. YopE, YopT and YopO act on small GTPases involved in the control of cytoskeletal dynamics, while YopH disturbs focal adhesions.

Fig. 2. Intracellular trafficking of *Salmonella typhimurium* (A) and *Mycobacterium bovis* (B) in target cells. (A) Wild-type *Salmonella* (left branch) translocate a bacterial protein, SifA, into the *Salmonella*-containing vacuole, which contributes to the maintenance of the integrity of the compartment in which the pathogens multiply. Mutant bacteria that do not secrete SifA (right branch) cannot sustain the formation of a vacuolar membrane and are released into the cellular cytoplasm where they are degraded. (B) Live *Mycobacterium* (left branch) infect macrophages and retain a cellular protein, TACO, on the vacuole. This protein inhibits the maturation of the *Mycobacterium*-containing compartments, which remain in an early endosomal stage that favours parasite replication. Phagosomes containing dead bacteria (right branch) do not retain TACO and mature into lysosomes, where the microorganisms are degraded.

can block the fusogenic capabilities of the bacteria-containing compartment (Figure 2B). However the ability to escape fusion with lysosomes may not only rely upon a coat protein. Using an *in vitro* reconstitution of phagosome/lysosome fusion Isabelle Marinnodeau-Parini (Toulouse, France) showed that cytosol of cells infected with *Mycobacteria* inhibited fusion, suggesting that a cytosolic factor was involved.

Conclusions

In this report we have illustrated the diversity of the areas in which cell biology and cellular microbiology overlap. We wish we could have given account of more talks, especially those studies which are less in the current mainstream of research and therefore difficult to accommodate in a global report, but were often fascinating and gave rise to fruitful discussions. Most, if not all, participants agreed that this new conference was a great success and, as a second meeting is already planned for 2002, we hope it will become a regular event attended by both cellular microbiologists and 'pure' cell biologists.

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