Phagocytosis and the actin cytoskeleton

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Summary

The process of engulfing a foreign particle – phagocytosis – is of fundamental importance for a wide diversity of organisms. From simple unicellular organisms that use phagocytosis to obtain their next meal, to complex metazoans in which phagocytic cells represent an essential branch of the immune system, evolution has armed cells with a fantastic repertoire of molecules that serve to bring about this complex event. Regardless of the organism or specific molecules concerned, however, all phagocytic processes are driven by a finely controlled rearrangement of the actin cytoskeleton. A variety of signals can converge to locally reorganise the actin cytoskeleton at a phagosome, and there are significant similarities and differences between different organisms and between different engulfment processes within the same organism. Recent advances have demonstrated the complexity of phagocytic signalling, such as the involvement of phosphoinostide lipids and multicomponent signalling complexes in transducing signals from phagocytic receptors to the cytoskeleton. Similarly, a wide diversity of 'effector molecules' are now implicated in actin-remodelling downstream of these receptors.

Key words: Phagocytosis, Complement, Actin, Cytoskeleton, Fc-receptor

Introduction

The internalisation of large particles by cells was first documented over a century ago by Elie Metchnikoff (Metchnikoff, 1893), in his observations of amoeboid cells moving within a transparent starfish larva towards an inserted rose thorn. Today, phagocytosis (a phrase first coined by Metchnikoff) is defined as the cellular engulfment of large particles, usually those over 0.5 μm in diameter. This is a functional definition and as such encompasses a variety of distinct processes involving different signalling pathways (Aderem and Underhill, 1999; Kwiatkowska and Sobota, 1999; Sanchez-Mejorada and Rosales, 1998b).

Phagocytosis has been known to be an actin-dependent process since 1977, when Kaplan showed that cytochalasin B, a toxin that blocks actin polymerisation, inhibits uptake of IgG-coated erythrocytes by mouse macrophages (Kaplan, 1977). Although originally proposed to be a unique feature of Fc-receptor-mediated phagocytosis, remodelling of the actin cytoskeleton is now known to be required for phagocytic uptake through other receptors too. Here, we consider the recent progress in our understanding of the link between signalling and actin remodelling during phagocytosis.

Fc-receptor-mediated phagocytosis

In mammals, binding of immunoglobulins (Igs) to foreign particles leads to the prompt clearance of those particles from the organism. Igs act as opsonins, molecules that render the particle they coat more susceptible to engulfment by phagocytic cells. The conserved Fc domain of the Igs are recognised by Fc receptors present on professional phagocytes, such as neutrophils and macrophages, and the opsonised particle is rapidly internalised. This internalisation is characterised by the dramatic, actin-dependent extension of the plasma membrane around the particle (Fig. 1) and is followed by secondary activity, such as the production of superoxide and the release of inflammatory cytokines from the phagocyte (Roitt, 1994).

Fc receptors and their activation

The major Ig opsonin is IgG, which binds to corresponding Fcγ receptors (FcγRs), although IgA and IgE also have cognate Fc receptors (FcαR and FcεR, respectively) that are involved in phagocytosis (van Egmond et al., 1999; Yokota et al., 1992).

A range of FcγRs exist (Sanchez-Mejorada and Rosales, 1998b). FcγRI (Indik et al., 1994a), FcγRIIA (Tuijnman et al., 1992) and FcγRIIIA (Park et al., 1993) can all support phagocytosis. FcγRIIB (an isoform of FcγRIIA) negatively regulates phagocytosis (Hunter et al., 1998), unless its cytoplasmic tail is mutated to resemble that of FcγRIIA, when it becomes phagocytic (Indik et al., 1994b). FcγRIIIB is able to initiate calcium signalling and actin polymerisation, but its role in phagocytosis remains unclear (Kimberly et al., 1990; Salmon et al., 1991; Chuang et al., 2000).

The interaction between FcγR and IgG triggers rapid phosphorylation of specific tyrosine residues in the receptor within motifs termed ITAMs (for immunoreceptor tyrosine-based activation motifs; Isakov, 1997). FcγRIIA contains an ITAM within its cytoplasmic domain, whereas the relevant ITAMs for FcγRI and FcγRIIIA are contained within a separate molecule, the dimeric γ subunit, which associates with the receptor (Sanchez-Mejorada and Rosales, 1998b). The way in which binding of ligand to the extracellular domain of the receptor leads to conformational change in its cytoplasmic domain is not yet known, but the recently published structures of FcγRIII (Sondermann et al., 2000) and FcεRI (Garman et al., 2000) are likely to improve our understanding of these early signalling steps.

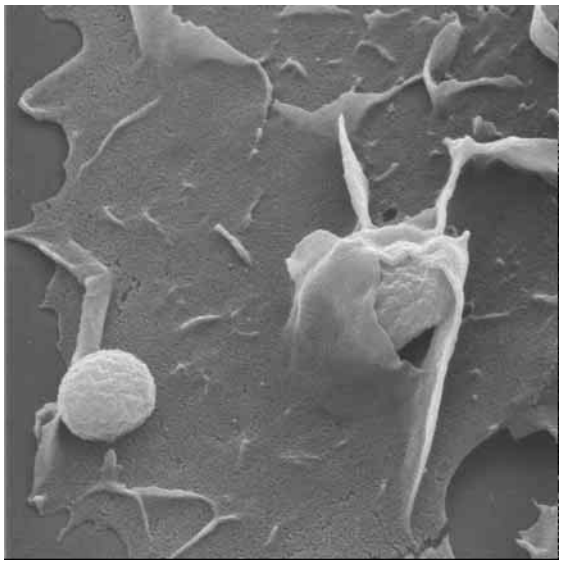

Fig. 1. The dramatic nature of FcR-mediated phagocytosis. The scanning electron micrograph shows an IgE-opsonised zymosan particle being engulfed by an RBL-2H3 cell, an $Fc_{\varepsilon}R$-mediated event. Note the dramatic protrusion of membrane around the particle. Image kindly provided by Philippe Montcourrier and Philippe Chavrier.

The Src family

The initial ITAM phosphorylation is carried out by tyrosine kinases of the Src family (Ghazizadeh et al., 1994). These are normally maintained in an inactive state by an interaction between a phosphorylated tyrosine residue within their tail and their SH2 domain (Erpel and Courtneidge, 1995). Activation is generally thought to be achieved by dephosphorylation, which in leukocytes might be carried out by the cell surface phosphatase CD45 (Adamczewski et al., 1995), although protein-protein interactions might also activate Src (Erpel and Courtneidge, 1995).

Professional phagocytes contain five members of the Src family – Src, Hck, Fgr, Lyn and Fyn. Of these, Hck, Fgr and Lyn are the predominant family members, although their relative contributions to FcγR-mediated phagocytosis remain unclear. $Hck^{-/-}$ (Fitzer-Attas et al., 2000) or $Src^{-/-}$ (Hunter et al., 1993) macrophages show no defect in phagocytosis, Lyn knockouts show a mild defect (Fitzer-Attas et al., 2000), whereas $Hck^{-/-}$ $Fgr^{-/-}$ $Lyn^{-/-}$ triple knockouts show a more severe delay in phagocytosis (Fitzer-Attas et al., 2000). This is not a clear case of redundancy, however, since Fgr can apparently negatively regulate phagocytosis (Gresham et al., 2000), and cells do not express all five kinases simultaneously (Katagiri et al., 1991).

Although both neutrophils and macrophages are professional phagocytes, comparisons between them can be misleading. For example, both cell types express FcγRI and FcγRIIA, but FcγRIIIA is restricted to macrophages, and FcγRIIIB is present only on neutrophils (Sanchez-Mejorada and Rosales, 1998b). And although FcγRs in both cell types are tyrosine phosphorylated upon clustering, this phosphorylation is essential for phagocytosis only in macrophages (Greenberg et al., 1993) and apparently not in neutrophils (Dusi et al., 1994). Moreover, some of the downstream signals elicited during phagocytosis differ between the two cell types (see below).

Signalling post-Src

Following its phosphorylation, the ITAM motif acts as a docking site for SYK (Ghazizadeh et al., 1995; Agarwal et al., 1993), a tyrosine kinase related to ZAP-70 (which fulfils a similar role in signalling from the T-cell receptor; Chan et al., 1992). Docking of SYK leads to its phosphorylation (Greenberg et al., 1994) and activation (Darby et al., 1994), although note that SYK is also capable of auto-activation (Latour et al., 1996). SYK is clearly a critical component for FcγR signalling (but not complement-receptor-mediated phagocytosis, see below; Kiefer et al., 1998), since macrophages lacking SYK cannot internalise IgG-opsonised particles (Crowley et al., 1997; Kiefer et al., 1998; Matsuda et al., 1996).

The point at which SYK acts remains controversial. Some authors report it to be required for formation of the actin filament 'cup' that assembles beneath the bound particle during FcγR- (Cox et al., 1996a; Greenberg et al., 1996) or FcεR-mediated (Oliver et al., 1994) phagocytosis, whereas others describe normal actin rearrangement but a subsequent failure to internalise particles in cells lacking SYK (Crowley et al., 1997). Since a variety of non-haematopoietic cell lines (which express little or no SYK) can be induced to carry out FcγR-mediated phagocytosis upon expression of appropriate receptors (described below), either a SYK-independent phagocytosis mechanism, or a SYK-like molecule, must exist in these cells. Nevertheless, co-expression of SYK with FcγRs in non-haematopoietic cells increases their phagocytic efficiency (Indik et al., 1995), which emphasises its central role in this process.

Interestingly, macrophages lacking SYK show reduced phosphorylation of the FcγR tail (or associated γ subunit) in response to ligand binding (Kiefer et al., 1998); this suggests that SYK cooperates with the Src kinases to phosphorylate the receptor and thus initiate signalling. In addition, SYK can autoactivate (Latour et al., 1996), which may account for the residual actin-cup formation seen in the $Hck^{-/-}$ $Fgr^{-/-}$ $Lyn^{-/-}$ macrophages (Fitzer-Attas et al., 2000; Fig. 2).

In addition to SYK and Src-family kinases, FcγR stimulation activates casein kinase II (Suzuki, 1991; Yamada et al., 1989). The role of this enzyme in phagocytic signal transduction remains unclear, although inhibition of casein kinase II prevents recruitment of several cytoskeletal components to the nascent phagosome (Yamada et al., 1989).

Complement-receptor-mediated phagocytosis

Complement-receptor-mediated phagocytosis is morphologically distinct from that mediated by FcRs, although both processes require actin polymerisation. Complement-opsonised particles 'sink' into the phagocyte; there is minimal membrane disturbance, and internalisation does not usually lead to an inflammatory response or oxidative burst.

Evolutionarily much older than adaptive immunity, the complement system is present even in simple organisms such as sea urchins (Smith et al., 1999), and yet still represents a major branch of the innate immune system in higher organisms, including humans (Ravetch and Clynes, 1998). In

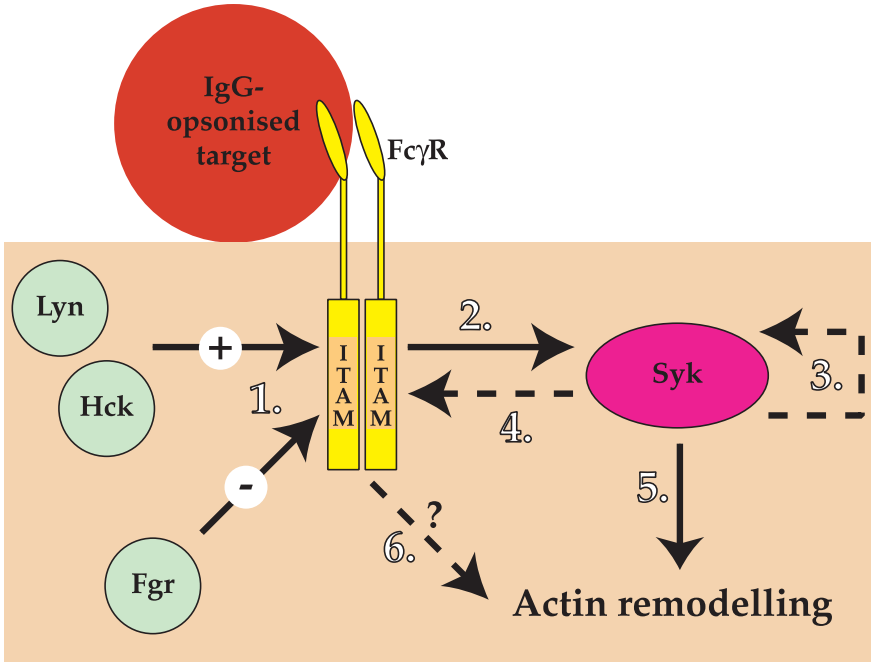

Fig. 2. The early stages of FcR signal transduction. Solid arrows represent the major signal direction; dotted lines represent pathways that can occur to a lesser extent. (1) The initial tyrosine phosphorylation event is carried out by members of the Src family. Hck and Lyn phosphorylate the receptor (or associated γ subunit). In contrast, Fgr negatively regulates phagocytosis by activating the phosphatase SHP-1 (Gresham et al., 2000). (2) SYK tyrosine kinase is then recruited to the phosphorylated ITAM, where it is activated (Darby et al., 1994). (3) SYK is also capable of autoactivation (Latour et al., 1996), which may account for the background phagocytosis observed in $Hck^{-/-}$ $Lyn^{-/-}$ $Fgr^{-/-}$ cells (Fitzer-Attas et al., 2000). (4) SYK can also phosphorylate the receptor (Kiefer et al., 1998), again contributing towards redundancy within the signal cascade. (5) Active SYK then transmits a downstream signal leading to actin polymerisation and particle internalisation. (6) SYK-knockout macrophages are unable to internalise IgG-opsonised particles but remain able to assemble F-actin at phagosomes (Crowley et al., 1997). Thus, it appears that there is a SYK-independent signal that stimulates actin remodelling, as well as a SYK-dependent one.

higher vertebrates the complement system is composed of ~30 proteins, which can be activated by exposure to microbial macromolecules (such as carbohydrates) or by binding to antibodies (primarily IgM or IgG) on the microorganism surface.

C3b, a molecule produced following complement activation, contains an exposed, highly reactive thioester group that can covalently bind to hydroxyl or amino groups on the microbial surface. This deposited C3b acts as an opsonin, being recognised by complement receptor 1 (CR1, also known as CD35), or (following further modification by plasma Factors H and I, which convert it to iC3b) by complement receptor 3 (CR3, also known as Mac-1, CD11b/CD18 or $\alpha_M\beta_2$ integrin) or complement receptor 4 (CR4, also known as CD11c/CD18 or $\alpha_x\beta_2$).

The complement receptors

CR1, CR3 and CR4 are expressed on macrophages and neutrophils and are all implicated in phagocytosis (Brown, 1991). CR1, a single-pass transmembrane protein, primarily functions in particle adherence rather than internalisation (Brown, 1991; Fallman et al., 1993). CR3 and CR4 are both integrin heterodimers and have the same β chain but different α chains. To date, CR3 has been the most widely studied.

CR3 is capable of binding to several ligands through different recognition sites (Diamond et al., 1993). Binding to iC3b leads to particle phagocytosis, but the receptor can also mediate cell spreading and chemotaxis through its interaction with intercellular adhesion molecules ICAM-1 (CD54; Diamond et al., 1990), ICAM-2 (CD102; Xie et al., 1995), ICAM-4 (LW; Bailly et al., 1995) and platelet glycoprotein Ibα (Simon et al., 2000), or matrix molecules such as fibrin and fibrinogen (Wright et al., 1988), collagen (Walzog et al., 1995) or heparan sulphates (Diamond et al., 1995). In addition, CR3 can mediate non-opsonic phagocytosis of particles by binding to molecules such as β-glucan (Thornton et al., 1996). The cell is able to distinguish these various ligands to produce differing downstream effects, which suggests that either the receptor or secondary signalling elements are affected differently by different ligands.

The phagocytosis of iC3b-opsonised particles by CR3 proceeds efficiently only if the phagocyte is first activated – for example, by inflammatory cytokines or by attachment to the extracellular matrix (Brown, 1986; Pommier et al., 1983). Activation triggers a conformational change in the complement receptor (Oxvig et al., 1999), possibly through phosphorylation of the β subunit (Chatila et al., 1989). This triggers clustering of the receptor (Detmers et al., 1987), a precondition for particle binding, and allows transduction of the phagocytic signal and consequently particle engulfment (Allen and Aderem, 1996a).

Receptor phosphorylation

The α-chains of CR3 and CR4 are constitutively phosphorylated, whereas the β-chain becomes serine phosphorylated following activation (e.g. by the PKC activator PMA; Buyon et al., 1990; Chatila et al., 1989). This phosphorylation appears to depend on PKC activity, which is essential for internalisation (but not binding) of both iC3b- and β-glucan-opsonised particles (Allen and Aderem, 1996b; Roubey et al., 1991). In contrast, tyrosine phosphorylation of CR3 is apparently not required for phagocytosis (Allen and Aderem, 1996b). Caron et al. have recently reported a role for the small GTPase Rap1 in activating CR3 (Caron et al., 2000), and it will be interesting to see how this relates to the phosphorylation state of the receptor.

Early signalling from complement receptors is complicated by the large number of lateral ('cis') interactions between the

receptors and other membrane proteins (Petty and Todd, 1993). Notably, CR3 has been shown to associate with FcγRIII (Zhou et al., 1993), and the two phagocytic receptors cooperate in regulating the respiratory burst (Zhou and Brown, 1994). Receptor activation leads rapidly to the association of the cytoplasmic domain of the receptor with cytoskeletal proteins, such as α-actinin (Pavalko and LaRoche, 1993), but as yet there is no evidence for a direct interaction between CR3 and downstream signalling proteins.

Other phagocytic receptors

It is becoming apparent that a growing number of cell-surface receptors can mediate phagocytic uptake of particles. These include non-complement-receptor integrins such as $\alpha_5\beta_1$ and $\alpha_v\beta_3$ (which mediate uptake of particles coated with fibronectin; Blystone et al., 1994), lectins such as the mannose receptor (Stahl and Ezekowitz, 1998), the lipopolysaccharide (LPS) receptor CD14 (Devitt et al., 1998) and the diverse scavenger receptor group (Platt et al., 1998). Internalisation by these receptors appears, at least in some cases, to be morphologically dynamic, as in the case of Fc receptors but in contrast to uptake through CR3. Membrane is extended around the attached particle, and there is transient ruffling in surrounding areas of the cell (Parnaik et al., 2000). However, uptake does not trigger inflammation (Meagher et al., 1992), and might actively suppress it (Fadok et al., 1998; Voll et al., 1997).

Recently, there has been a resurgence of interest in these receptors in an attempt to understand the removal of apoptotic cells (Fadok and Henson, 1998; Platt et al., 1998). In metazoans, development is accompanied by massive apoptosis (e.g. during limb formation), and these 'corpses' are engulfed both by professional phagocytes and by neighbouring cells that act as 'non-professional' phagocytes (Franc et al., 1999; Rabinovitch, 1995; Savill, 1998). The recent report of a receptor for phosphatidylserine (Fadok et al., 2000), a membrane phospholipid exposed externally on apoptotic cells, is likely to stimulate rapid progress in this field. Additionally, *Caenorhabditis elegans* appears to use a common mechanism to engulf apoptotic and necrotic cell corpses (Chung et al., 2000) – whether this is also true for 'higher' organisms remains to be seen.

In common with FcR- and complement-receptor-mediated phagocytosis, phagocytosis mediated by this diverse group of receptors is also actin dependent (Platt et al., 1998), and many of the downstream components are the same as those lying downstream of CR3 or FcγRs. Nevertheless, the events immediately following receptor-ligand interaction remain largely unknown.

Some of these receptors might act only to tether particles, and then utilise accessory receptors to deliver the phagocytic signal (Savill, 1998). This would explain the ability of some receptors (such as CD14) to induce inflammatory responses when binding to one ligand (LPS) but not another (apoptotic cells; Devitt et al., 1998). Some of these receptors can signal through tyrosine kinases, and uptake is, at least in some cases, phosphorylation dependent (Roubey et al., 1991). In this regard it is particularly intriguing that the newly cloned *ced-1* gene from *C. elegans* (which encodes a transmembrane receptor that is essential for the uptake of apoptotic cells) contains an intracellular YXXL motif (Zhou et al., 2001). This sequence is also found in the ITAMs of mammalian FcγRs, where it mediates interactions with downstream signalling elements.

Professionals, amateurs and model organisms

To tease apart early signalling events during phagocytosis, many groups have made use of non-phagocytic cell lines. When transfected with appropriate receptors, a wide variety of cell lines become competent for phagocytosis (Indik et al., 1991; Tuijnman et al., 1992). Moreover, phagosomes in these cells subsequently fuse with endosomes and acidify in a similar manner to that in which phagosomes acidify in professional phagocytes (Downey et al., 1999), which suggests that the basic machinery of phagocytosis is present in most cells (Aderem and Underhill, 1999). This idea is supported by the observation that, in vivo, many apoptotic cells are phagocytosed by neighbouring cells, such as fibroblasts (Fadok and Henson, 1998).

The phagocytic process has also been extensively investigated in 'simpler' model organisms, such as *C. elegans* and *Dictyostelium discoideum*. Mutation screens in such organisms have identified several genes required for uptake of apoptotic bodies during development of *C. elegans*, as described above, or the non-opsonic uptake of bacteria (feeding of *Dictyostelium*), many of which appear to have homologues in higher organisms.

Downstream pathways

A large range of molecules are implicated in phagocytic signalling. Most of these were initially identified as acting downstream of Fc-receptors, but many now appear to be involved in signalling from other phagocytic receptors as well.

Calcium

One of the first reported signals to be observed in response to FcγR activation is an increase in cytosolic Ca^{2+} concentration (Young et al., 1984). In neutrophils, this calcium pulse has been reported to be required for FcγR-mediated phagocytosis by one group (Kobayashi et al., 1995) but not by another (Della Bianca et al., 1990). In contrast, CR3-mediated phagocytosis in neutrophils appears to be independent of changes in Ca^{2+} concentration (Della Bianca et al., 1990; Lew et al., 1985). Following the internalisation of IgG-opsonised particles by neutrophils, calcium appears to trigger actin depolymerisation at phagosomes (Bengtsson et al., 1993), a step that may be necessary for phagosome-lysosome fusion (Jaconi et al., 1990). In macrophages, neither phagocytosis (Greenberg et al., 1991) nor phagosome-lysosome fusion (Zimmerli et al., 1996) appears to be Ca^{2+} dependent, which may reflect the involvement of different phagocytic receptors (Edberg et al., 1995).

How might Ca^{2+} control actin depolymerisation during phagocytosis? One possible model is that a local rise in Ca^{2+} concentration activates gelsolin. Gelsolin caps the barbed (fast-growing) end of actin filaments, preventing filament elongation, and can also sever filaments in a Ca^{2+}-dependent manner (Harris and Weeds, 1984; Yin et al., 1981). Gelsolin localises to nascent phagosomes in macrophages (Yin et al., 1981), whilst neutrophils from gelsolin knockout ($Gsn^{-/-}$) mice

have a profound defect in FcγR-mediated phagocytosis (Serrander et al., 2000). However, the calcium-dependent depolymerisation of actin filaments from around particles after internalisation is normal in Gsn$^{-/-}$ neutrophils (Serrander et al., 2000), which suggests that Ca^{2+} plays a role wider than simply activating gelsolin.

Phospholipases

Substantial evidence supports the involvement of phospholipases in phagocytic signalling (Lennartz, 1999), but the precise stage at which they act remains unclear. Inhibition of phospholipase A (PLA) blocks FcγR-mediated phagocytosis in macrophages (Lennartz et al., 1997) but not neutrophils (Della Bianca et al., 1990). Phospholipase Cγ (PLCγ) is activated downstream of FcγRs (Azzoni et al., 1992; Liao et al., 1992) and localises to phagocytic cups during the uptake of IgG-opsonised particles (Botelho et al., 2000). Inhibiting PLCγ blocks phagocytosis in *Dictyostelium* (Seastone et al., 1999), and FcγR-mediated phagocytosis in macrophages (Botelho et al., 2000). Phospholipase D (PLD) is also implicated in phagocytic signalling, given that it is activated at an early stage during complement-receptor-mediated phagocytosis (Fallman et al., 1992; Serrander et al., 1996).

PKC and friends

Serine/threonine kinases of the PKC family are activated by the phospholipase product diacylglycerol (DAG), by Ca^{2+} and by pharmacological agents such as phorbol esters. PKCα localises to macrophage phagosomes during FcγR-, CR3- and mannose-receptor-mediated phagocytosis (Allen and Aderem, 1996b; Allen and Aderem, 1995; Larsen et al., 2000). Complement-receptor-mediated phagocytosis, both of iC3b- and β-glucan-opsonised particles, appears to require PKC activity (Allen and Aderem, 1996b; Roubey et al., 1991). In contrast, conflicting results have been obtained for its involvement in FcγR-mediated phagocytosis (Allen and Aderem, 1996b; Greenberg et al., 1993; Larsen et al., 2000; Zheleznyak and Brown, 1992).

In addition to the PKCα isoform, PKCβ (Dekker et al., 2000), PKCγ (Melendez et al., 1999), PKCδ (Brumell et al., 1999) and PKCε (Larsen et al., 2000) have all been shown to localise to the phagosome membrane during FcγR-mediated phagocytosis. The isoforms recruited may depend on the differentiation state of the cells and/or the exact FcγR involved (Melendez et al., 1999), different isoforms controlling different aspects of phagocytosis (Larsen et al., 2000).

PKC has a range of downstream targets that are implicated in phagocytosis. For example, plekstrin, the major PKC-phosphorylation target in platelets, is expressed in macrophages and recruited to the phagosome membrane during FcγR-mediated phagocytosis (Brumell et al., 1999), although its role there is unknown. More is known about MARCKS and MacMARCKS, two other PKC targets implicated in phagocytosis. MARCKS (myristoylated alanine-rich C kinase substrate) and the closely related MacMARCKS (Li and Aderem, 1992) are actin-filament crosslinking proteins that can also link actin filaments to the membrane (Hartwig et al., 1992).

MARCKS localises to phagosomes (Allen and Aderem, 1996b; Allen and Aderem, 1995) and becomes phosphorylated during zymosan phagocytosis (Allen and Aderem, 1995). However, macrophages derived from MARCKS$^{-/-}$ mice show normal rates of FcγR- and CR3-mediated phagocytosis and only a minor reduction in the uptake of zymosan particles (Carballo et al., 1999). MacMARCKS also localises to phagosomes (Underhill et al., 1998; Zhu et al., 1995). Mutations in MacMARCKS were reported to block zymosan phagocytosis (a mannose-receptor-mediated event) by one group (Zhu et al., 1995) but MacMARCKS$^{-/-}$ macrophages do not show phagocytic defects (Underhill et al., 1998) – a discrepancy that might be attributable to the use of different cell lines (Underhill et al., 1998).

Phosphoinositide kinases

Phosphoinositol lipids are major regulators of actin remodelling during several cellular phenomena (Martin, 1998). To date, the major phosphoinositide kinase implicated in phagocytosis is phosphoinositide 3-kinase (PI3-K). Unlike most signalling proteins implicated in phagocytosis, PI3-K does not seem to accumulate at nascent phagosomes (Strzelecka et al., 1997), perhaps because it is displaced from its binding partners (such as FcγRIIA; Chacko et al., 1996) by binding to phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3), one of its products (Kwiatkowska and Sobota, 1999; Rameh et al., 1995). PI3-K is, however, activated by FcγRs (Ninomiya et al., 1994), associates with FcγRIIA in platelets (Chacko et al., 1996) and is required for FcγR-mediated phagocytosis by macrophages (Araki et al., 1996; Cox et al., 1999; Ninomiya et al., 1994). In addition, Cox et al. have recently shown PI3-K to be required for CR3-mediated phagocytosis (Cox et al., 2001), although PI3-K is not required for phagocytosis in *Dictyostelium* (Seastone et al., 1999).

PI3-K appears not to regulate the initial actin polymerisation during phagocytosis, which proceeds normally when PI3-K is inhibited, but rather to control closure of the phagosome (Araki et al., 1996; Cox et al., 1999). The dependence on PI3-K is reduced for smaller particles (Cox et al., 1999); PI3-K might therefore have a role in the regulation of membrane availability. Intriguingly, although not required for actin polymerisation, PI3-K is nevertheless able to induce local actin reorganisation and subsequent particle uptake if it is artificially activated beneath bound beads (Lowry et al., 1998a).

This wealth of data, combined with the observation that initial extension of the plasma membrane around particles during phagocytosis is independent of actin polymerisation (Lowry et al., 1998b), leads to the following ‘four step’ model of internalisation (Fig. 3): (1) particles are bound by surface receptors; (2) this triggers an actin-independent membrane protrusion around the particle, which involves as-yet-unidentified molecules; (3) an actin-dependent (but PI3-K independent) enhancement of the phagocytic cup takes place; and (4) fusion of the membrane leading to particle internalisation requires actin remodelling and possibly vesicle trafficking steps, which are driven by the PI3-K product PtdIns(3,4,5)P_3.

Recent data has additionally implicated another phosphoinositide kinase, phosphatidylinositol phosphate 4-phosphate 5-kinase Iα (PIP5KIα), in phagocytic signalling. PIP5KIα regulates the level of PtdIns(4,5)P_2, a molecule that plays a key role in regulating signalling and cytoskeletal proteins. PtdIns(4,5)P_2 accumulates at the phagocytic cup early

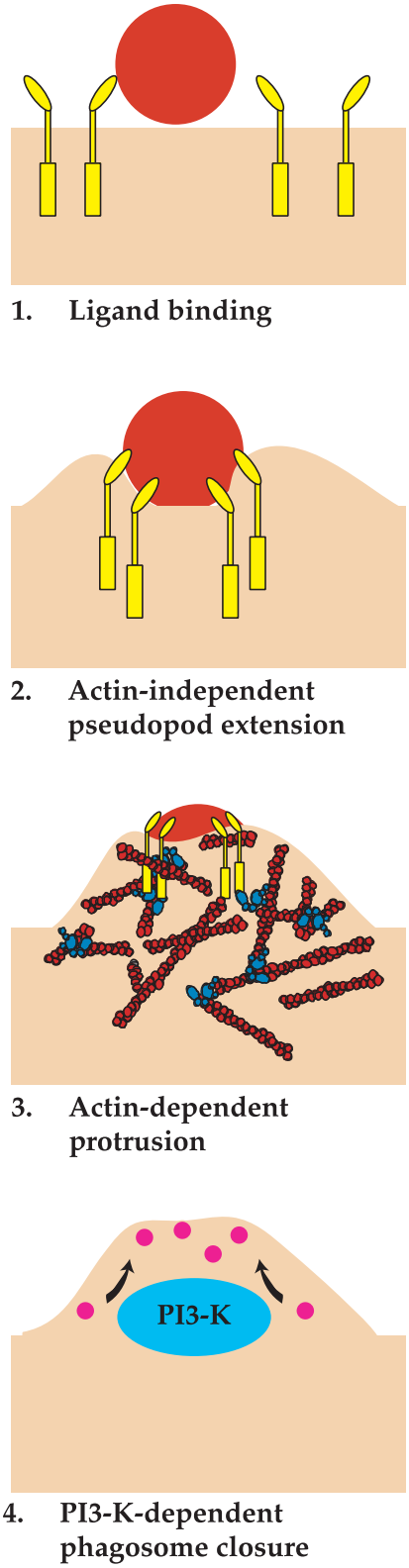

Fig. 3. The four-step model of internalisation. (1) The initial interaction between an Fc-receptor and immunoglobulin upon the particle tethers the target to the membrane. (2) Other receptors are subsequently recruited to the target, leading to zippering as they sequentially engage with ligands on the particle. Zippering drives the initial extension of membrane around the particle in a process that does not require actin polymerisation or signalling from the cytoplasmic domain of the receptor, at least for FcγRIa (Lowry et al., 1998b). (3) Active signalling from the receptor leads to the recruitment of numerous cytoskeletal proteins (Allen and Aderem, 1996b), including the Arp2/3 complex, which nucleates actin filaments beneath the particle (May et al., 2000). The formation of an actin network pushes the plasma membrane further around the target. (4) Once most of the particle is within the cell, PI3-K activity leads to the final engulfment (Araki et al., 1996). How it achieves this is unknown, but possibilities include vesicle delivery (or recycling; Karimi and Lennartz, 1995) and/or a myosin-based contractile activity (Swanson et al., 1999).

during the engulfment process, and reducing its availability inhibits FcγR-mediated phagocytosis (Botelho et al., 2000).

It appears that at least three phosphoinositide products regulate phagocytosis. (1) PtdIns(4,5)P_2 is synthesised early during engulfment and may control actin assembly at the phagosome. (2) The activity of PLC then degrades PtdIns(4,5)P_2, producing DAG, and either the loss of PtdIns(4,5)P_2 or the increase in DAG controls further cytoskeletal changes (Botelho et al., 2000). (3) Simultaneously, PI 3-K converts PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3, triggering phagosome closure.

GTPases

Little is known about the involvement of heterotrimeric G proteins in phagocytosis. In *Dictyostelium*, deletion of the β subunit of heterotrimeric G proteins impairs phagocytosis (Peracino et al., 1998). However, in mammalian systems, inhibition of heterotrimeric G proteins does not inhibit phagocytosis through FcγR (Della Bianca et al., 1993), CR3 (Hiemstra et al., 1992) or non-opsonic receptors (Lad et al., 1986; Rossi et al., 1989), which suggests mammalian cells have made use of alternative signals to replace heterotrimeric G proteins in this process.

In contrast, several monomeric (‘small’) GTPases, including Rho and ARF family members, have been implicated in phagocytosis. The Rho-family GTPases, for example, which regulate processes such as cytoskeletal remodelling, transcriptional activation and superoxide production (Aspenstrom, 1999a), produce distinct changes to the actin cytoskeleton when expressed in fibroblasts (Aspenstrom, 1999b) and have been recently implicated in phagocytic signalling (Chimini and Chavrier, 2000).

Rac1 and Cdc42 are now firmly established as important signalling molecules downstream of FcRs. Inhibition of either protein blocks actin assembly at nascent phagosomes and the subsequent internalisation of IgG-opsonised particles by mouse macrophages (Caron and Hall, 1998; Cox et al., 1997). Similarly, uptake of IgE-opsonised particles (which are phagocytosed by the Fcε-receptor) by rat mast cells is also blocked by inhibition of Rac1 or Cdc42 (Massol et al., 1998). Clustering constitutively active Cdc42 beneath membrane-bound beads triggers actin assembly, leading to finger-like extensions of the plasma membrane around the particles (Castellano et al., 1999). These are reminiscent of the structures that form around IgE-opsonised beads during FcεR-dependent phagocytosis (Massol et al., 1998). Similarly, localised activation of Rac1 also leads to particle engulfment, although in this case there is minimal membrane protrusion (Castellano et al., 2000), which suggests that these two

GTPases control different aspects of phagosome formation. Rac1 and Cdc42 are not required for complement-receptor-mediated phagocytosis (Caron and Hall, 1998). This may reflect the different morphology of actin assembly at CR3-mediated phagosomes as compared with FcγR-mediated phagocytosis (Allen and Aderem, 1996b). Rho appears not to be required for FcγR-mediated phagocytosis (Caron and Hall, 1998; May et al., 2000), although note that this remains controversial (Hackam et al., 1997), whereas it is required for CR3-phagocytosis (Caron and Hall, 1998).

Rac is also important for phagocytosis in other organisms. In *Dictyostelium*, overexpression of RacC leads to a threefold increase in phagocytosis rates (Seastone et al., 1998), whereas the related RacF1, although not essential for phagocytosis, associates transiently with nascent phagosomes (Rivero et al., 1999a). *C. elegans* also has a Rac homologue, which is essential for the uptake of apoptotic cells (Reddien and Horvitz, 2000).

Members of the ADP-ribosylation factor (ARF) family of GTPases that control coat assembly on vesicles (Chavrier and Goud, 1999) and regulate the cytoskeleton (Critchley et al., 1999) have also been implicated in phagocytosis. ARF6 is essential for actin assembly and engulfment during FcγR-mediated phagocytosis (Zhang et al., 1998). It interacts with the Rac-effector POR-1 (D'Souza-Schorey et al., 1997) and appears to lie downstream of Rac1 in macrophages (Zhang et al., 1999). Furthermore, artificially clustered Rac1 induces particle internalisation only if its POR-1-interacting region is intact (Castellano et al., 2000). Hence membrane protrusion during FcγR phagocytosis may rely on a signal pathway leading from the receptor, via Rac and POR-1, to ARF6 (Greenberg, 1999).

Dictyostelium phagocytosis requires RasS, the homologue of mammalian H-Ras (Chubb et al., 2000). However, in vertebrates, neither particle uptake nor FcγR-stimulated gene expression are blocked by a dominant-negative Ras construct (Sanchez-Mejorada and Rosales, 1998a). In mouse cells transfected with human FcγRIIIA, receptor activation results in phosphorylation of proteins thought to inactivate Ras (Zeng et al., 1995), but it is unclear what effect this may have. *Dictyostelium* also requires the GTPase Rap1 for efficient phagocytosis (Seastone et al., 1999). In mammalian macrophages Rap1 is enriched in isolated phagosomes (Pizon et al., 1994) and plays a critical role in activating CR3 prior to ligand binding (Caron et al., 2000) – whether it also functions during the engulfment process is not known.

Clearly the GTPases implicated in phagocytosis must be activated by upstream components, presumably of the guanine-nucleotide-exchange factor (GEF) group, but as yet no such proteins have been implicated in phagocytic signalling. However, it is intriguing that a GEF for Rap1 is activated by DAG (Kawasaki et al., 1998) and might therefore link PLC activity to GTPase activation.

The mechanism of actin assembly

A key challenge in the actin field has been to understand how signals that affect the cytoskeleton are mechanistically linked to the rearrangement of actin filaments (F-actin). Filament remodelling could involve several mechanisms (Pollard et al., 2000): (1) an increase in the filament number through the formation of new filaments (nucleation); (2) an increase in the average filament length by the uncapping of established filaments to allow the addition of new monomers; (3) a reduction in the number or length of filaments by severing and/or of sequestering actin monomers; (4) alterations in the structure of the actin network, without a net change in F-actin content, made by bundling or crosslinking filaments.

Actin nucleation

It has long been predicted that the local reorganisation of F-actin that occurs at a phagosome is due, at least in part, to the recruitment and/or activation of actin-nucleating molecules (Greenberg, 1995). One early candidate for this role was ponticulin, a small protein that links F-actin to the membrane in *Dictyostelium* (Hitt et al., 1994) and nucleates actin filaments there (Luna et al., 1990). However, although ponticulin is present at *Dictyostelium* phagosomes, it is not enriched there (Luna et al., 1990), and cells that lack ponticulin can still phagocytose normally (Hitt et al., 1994).

It now appears that the major actin nucleator in cells is the seven-subunit Arp2/3 complex (Machesky and Gould, 1999; Welch, 1999). Previously shown to localise to lamellipodia of crawling cells (Machesky et al., 1997; Welch et al., 1997), the Arp2/3 complex also localises to both FcγR- and CR3-mediated phagosomes, and is required for particle uptake through both receptors (May et al., 2000). Candidate regulators of Arp2/3 behaviour have, until recently, been elusive. However, several proteins that activate the Arp2/3 complex, leading to actin nucleation, have now been identified. In yeast, myosin I proteins bind to the complex, stimulating its activity (Evangelista et al., 2000; Lechler et al., 2000), although mammalian myosins lack the appropriate domain to achieve this interaction (Machesky, 2000). Myosins are involved in phagocytosis (see below), although evidence to date suggests that this is more likely to be in a motor capacity than in regulating the Arp2/3 complex.

The Arp2/3 complex is also activated by members of the WASP family (Machesky and Insall, 1999). WASP is mutated in the hereditary disorder Wiskott-Aldrich Syndrome (WAS; Snapper and Rosen, 1999). WASP (Machesky and Insall, 1998; Yarar et al., 1999), its more widely expressed homologue N-WASP (Rohatgi et al., 1999), the yeast homologue Las17p/Bee1p (Madania et al., 1999; Winter et al., 1999) and the related Scar/WAVE proteins (Machesky and Insall, 1998; Machesky et al., 1999) can all bind to and activate the Arp2/3 complex (Machesky and Insall, 1999).

WASP is recruited to FcγR phagosomes in macrophages (Lorenzi et al., 2000), and macrophages from WAS patients (who express little or no endogenous WASP) exhibit defective FcγR-mediated phagocytosis (Lorenzi et al., 2000). Since WASP interacts with many proteins, however, the complete absence of WASP is likely to lead to multiple effects. This prediction is supported by evidence that apoptosis is increased in macrophages from WAS patients (Rengan et al., 2000).

In other systems, WASP and related proteins provide a link between upstream signals and actin remodelling. For example, Cdc42 and PtdIns(4,5)P_2 can synergise to activate N-WASP, which in turn triggers actin polymerisation via the Arp2/3 complex (Rohatgi et al., 1999). Since FcR-mediated phagocytosis is dependent on Cdc42 (Caron and Hall, 1998; Cox et al., 1997; Massol et al., 1998), there is a potential link

between Cdc42, via WASP or N-WASP (Aspenstrom et al., 1996), and Arp2/3 complex activation (Rohatgi et al., 1999) and thus actin polymerisation. Given that WASP family members can be activated by a wide variety of partners (Snapper and Rosen, 1999), they may provide a central point at which a variety of upstream signalling components can converge.

Actin polymerisation can also be induced by the protein coronin (Goode et al., 1999). First isolated in *Dictyostelium* (de Hostos et al., 1991), coronin binds to F-actin and also, at least in yeast, to microtubules (de Hostos, 1999). Coronin localises to phagosomes in *Dictyostelium* (Maniak et al., 1995), mice (Morrissette et al., 1999) and humans (Grogan et al., 1997). Intriguingly, the human coronin isoform, hcoronin1, also interacts with proteins of the NADPH oxidase complex (Grogan et al., 1997). Coronin could therefore have a dual role in influencing both actin dynamics and the oxidative burst during phagocytosis.

Actin severing and depolymerisation

Several actin-filament-severing proteins are known to be recruited to phagosomes. As already discussed, the actin-severing protein gelsolin localises to phagosomes (Yin et al., 1981) but its role in phagocytosis remains unclear. $Gsn^{-/-}$ neutrophils show a severe defect in FcγR-mediated phagocytosis, and a milder kinetic delay in complement-receptor-mediated phagocytosis (Serrander et al., 2000). However, phagosomal actin reorganisation is normal in these cells (Serrander et al., 2000), which suggests that the absence of gelsolin produces a phenotype more complex than a straightforward defect in actin dynamics.

In *Dictyostelium*, cofilin, a member of the ADF/cofilin family of actin-depolymerising proteins (Bamburg et al., 1999), associates with phagosomes (Aizawa et al., 1997), as does the putative cofilin regulator DAip1 (Konzok et al., 1999). Loss of DAip1 leads to a reduction in phagocytosis in *Dictyostelium* (Konzok et al., 1999), and, in neutrophils, microinjection of anti-cofilin antibodies inhibits phagocytosis (Nagaishi et al., 1999).

Actin bundling and crosslinking

Following polymerisation, the actin filament network can be remodelled by actin-bundling or -crosslinking proteins, several of which are recruited to phagosomes. The Arp2/3 complex, for example, has, in addition to its nucleating behaviour, the ability to crosslink actin filaments into a characteristic ‘dendritic network’ (Mullins et al., 1998), as found in lamellipodia (Svitkina and Borisy, 1999). Although parallels have been drawn between lamellipodial extension and FcγR-mediated phagocytosis (Allen and Aderem, 1996b), the structure of F-actin at phagosomes has yet to be thoroughly elucidated, and so it remains unclear whether the crosslinking behaviour of the Arp2/3 complex is also required at these sites.

Both FcγR- and CR3-mediated phagocytosis are accompanied by the local recruitment of α-actinin (Allen and Aderem, 1996b), a protein that can bundle actin filaments and link them to integrins (Otto, 1994). In this way, α-actinin could link the force generated by the actin network to the bound particle, at least for CR3-mediated phagocytosis (since CR3 is an integrin). However, *Dictyostelium* lacking α-actinin can still engulf bacteria (Rivero et al., 1999b), which suggests that α-actinin is not essential for all phagocytic processes. In contrast, the actin-bundling protein ABP-120 (gelation factor) is required for phagocytosis in this organism (Cox et al., 1996b; Rivero et al., 1996).

The focal adhesion as a model for phagocytosis

Phagocytic receptors can mediate cellular adherence to a substrate, as well as attachment to a particle, provided that the ligand is present over a sufficiently wide area. This can be entirely artificial, as in the case of macrophages spreading on IgG-coated coverslips (Heiple et al., 1990), but other phagocytic receptors, notably CR3, are involved in substrate adherence in vivo – for example, on fibrinogen (Wright et al., 1988). The ability of CR3 to mediate adhesion, together with the appearance of punctate ‘foci’ of cytoskeletal components at complement-receptor-mediated phagosomes (Allen and Aderem, 1996b), suggests that CR3-mediated phagocytosis represents an adapted cell-adhesion mechanism. In this model the foci would then represent modified focal adhesions, the molecular ‘rivets’ that attach cells to the substratum. This is particularly appealing in that CR3-mediated phagocytosis is dependent on the small GTPase Rho (see above; Caron and Hall, 1998), which is also a major regulator of focal adhesion formation in response to growth factors (Ridley and Hall, 1992).

The focal adhesion is a complex assembly of cytoskeletal and signalling proteins, and indeed the term ‘focal adhesion’ may, in fact, encompass a variety of distinct structures, whose components change over time (Zamir et al., 2000). It is clear, however, that several components involved in focal adhesion assembly are also important for phagocytosis. For example, talin, a large protein that binds both cytoskeletal and signalling components at focal adhesions (Critchley, 2000), is enriched at FcγR-mediated (Greenberg et al., 1990) and CR3-mediated (Allen and Aderem, 1996b) phagosomes, and during the uptake of zymosan particles (Allen and Aderem, 1995). In *Dictyostelium*, the loss of talin causes defective phagocytosis (Niewohner et al., 1997). Since talin binds to the β2 subunit of integrins (such as CR3; Sampath et al., 1998) and to F-actin (Hemmings et al., 1996), it could link the complement receptor to the actin cytoskeleton. However, talin is proteolytically cleaved during leukocyte activation, which would normally precede phagocytosis in vivo (Sampath et al., 1998). In this case, the complement receptor may be temporarily released from the cytoskeleton and subsequently reattach through α-actinin (Sampath et al., 1998), which is also enriched at phagosomes (Allen and Aderem, 1996b). Not all CR3 molecules can be attached to α-actinin, however, because the receptor is more widely distributed at the membrane than α-actinin (Allen and Aderem, 1996b). Moreover, the situation is somewhat complicated by the expression of at least two α-actinin isoforms in macrophages, which are recruited to phagosomes to differing extents (Araki et al., 2000).

Three other focal adhesion proteins might also be involved in phagocytosis: vinculin, paxillin and focal adhesion kinase (FAK). Vinculin binds to talin and F-actin, and its activity is regulated by PtdIns(4,5)P_2 in vitro. It is enriched diffusely at FcγR phagosomes, in punctate spots at CR3 phagosomes (Allen and Aderem, 1996b), but not during zymosan uptake (Allen and Aderem, 1995). Paxillin, another focal adhesion

component that has multiple binding partners (including CRKL – see discussion below; Turner, 1998), is strongly phosphorylated during FcγR-mediated phagocytosis (Greenberg et al., 1994) and localises to FcγR-mediated phagosomes in a diffuse pattern (Allen and Aderem, 1996b; Greenberg et al., 1994). It also localises to CR3-mediated phagosomes, forming punctate spots, but is not localised during engulfment of zymosan particles (Allen and Aderem, 1996b). FAK appears to remodel focal adhesions and might relay signals from the extracellular matrix to the cytoplasm (Schlaepfer and Hunter, 1998). However, there are conflicting results as to whether FAK is phosphorylated (and thus presumably activated) following FcγR stimulation (Greenberg et al., 1994; Pan et al., 1999). In addition, monocytes, which lack FAK but express the related molecule FAK2 (also known as Pyk2/CADTK/RAFTK), can still phagocytose in the presence of a dominant negative FAK2 construct (Watson et al., 2001).

Given such an abundance of focal adhesion proteins implicated in phagocytosis, it seems likely that the mechanisms of attachment to a substrate and to a particle are at least partially related. In both cases, mechanical connection and signal transduction go hand-in-hand – a strategy that enables the cell accurately to modify its response to different substrates and/or particles. In addition, this cellular strategy of 'multitasking' proteins raises the possibility that some molecules become enriched at phagosomes not because they perform a function at this site but merely because their binding partner(s) are actively recruited to phagosomes – the molecular equivalent of a free-ride.

The challenge of adaptor proteins and multicomponent complexes

Since many focal adhesion proteins engage in multiple interactions with each other, the focal adhesion represents one type of scaffold complex formed during phagocytosis. Recently, Izadi and colleagues implicated another large signalling complex in phagocytic signalling. This group identified a complex containing the adaptor proteins CBL, NCK and GRB2 in myeloid cells and showed that another protein, CRKL, is recruited to this complex by CBL upon FcγRIIA stimulation (Izadi et al., 1998; Fig. 4). The role of these various components during phagocytosis is unclear, although activated mutants of CBL enhance FcγR-mediated phagocytosis (Sato et al., 1999). In addition, since CRKL can also bind to paxillin (Salgia et al., 1995), this molecule might serve to link the CBL-NCK-GRB2 complex to another complex containing paxillin and other focal adhesion proteins.

The involvement of multiple adaptor proteins raises the complexity of potential downstream interactions to a near-indecipherable point. Until we have a clearer idea of how these individual components regulate each other, any one downstream target is as likely as any other to be a player in the phagocytic signal cascade. However, some candidates are already emerging. For example, PAK1, a downstream effector of Rac and Cdc42 GTPases (Bagrodia and Cerione, 1999) binds to NCK and is involved in remodelling of the actin cytoskeleton through indirect effects on cofilin and myosin (Bagrodia and Cerione, 1999). The interaction of PAK1 with CBL and NCK (Izadi et al., 1998) might result in PAK1 activity downstream of FcγRs, although this has yet to be formally established. Similarly, the adaptors NCK (Rivero-Lezcano et al., 1995) and GRB2 (She et al., 1997) can interact with WASP/N-WASP and, in the case of GRB2, thereby trigger activation of the Arp2/3 complex and thus actin polymerisation (Carlier et al., 2000). NCK might also interact with the small GTPase Rho, through PRK2 (Quilliam et al., 1996). Hence multi-adaptor complexes act to integrate signals from numerous upstream sources by forming a single signalling 'scaffold'.

Adaptor proteins are also involved in phagocytic signalling in other organisms. For example, the *C. elegans* protein CED-6 functions in the phagocytosis of apoptotic bodies (Su et al., 2000), and its human homologue increases apoptotic body engulfment by human macrophages (Smits et al., 1999). Similarly, CED-5 (Ellis et al., 1991), which is homologous to the human adaptor DOCK180 (Wu and Horvitz, 1998), is required for phagocytosis in *C. elegans*. Recently it has been demonstrated that the nematode proteins CED-2 and CED10, which are required for the engulfment of apoptotic cells, are homologues of the human proteins CRKII (an adaptor protein) and RAC1, respectively (Reddien and Horvitz, 2000). Since CED-2 (CRKII) and CED-5 (DOCK180) can interact and apparently control the activity of CED-10 (RAC1), multicomponent complexes are probably also important for phagocytosis in *C. elegans*. This complex has subsequently been found to play a role in the uptake of apoptotic cells by human dendritic cells, a process mediated by the $\alpha_v\beta_5$ integrin (Albert et al., 2000).

Motoring along at the phagosome

Most phagocytosis work has focused on the idea that actin polymerisation/remodelling is the driving force for particle internalisation, but contractile force generated by motor proteins of the myosin superfamily may also play a role.

Recruitment of myosin to phagosomes in macrophages (Stendahl et al., 1980) and neutrophils (Valerius et al., 1981) was first reported twenty years ago. Myosin I is recruited to phagosomes engulfing zymosan particles (Allen and Aderem, 1995) but this process does not require myosin II (de Lanerolle et al., 1993). In contrast, myosin II is important for FcγR-mediated phagocytosis (Mansfield et al., 2000; Swanson et al., 1999), during which myosin IC, myosin V and myosin IXb are also recruited to the phagosome (Swanson et al., 1999). Myosin IC localises to the extreme edge of the protuding membrane that surrounds the particle, which suggests that this isoform has a role in phagosome closure (Swanson et al., 1999). There are, as yet, no data on the involvement or otherwise of myosins in complement-receptor-mediated phagocytosis.

At least one of these myosin isoforms is probably involved in generating contractile force to close the phagosome (Swanson et al., 1999), but in addition myosins such as myosin V have been implicated in vesicle transport (Mermall et al., 1998). The presence of multiple myosin isoforms at the phagosome might therefore reflect an additional role for these proteins in regulating membrane availability. During phagocytosis, vesicles are delivered to the cell surface to maintain membrane availability (Hackam et al., 1998), and recently Bajno and colleagues have demonstrated the polarised insertion of vesicles at the tips of pseudopods as they extend around bound particles (Bajno et al., 2000).

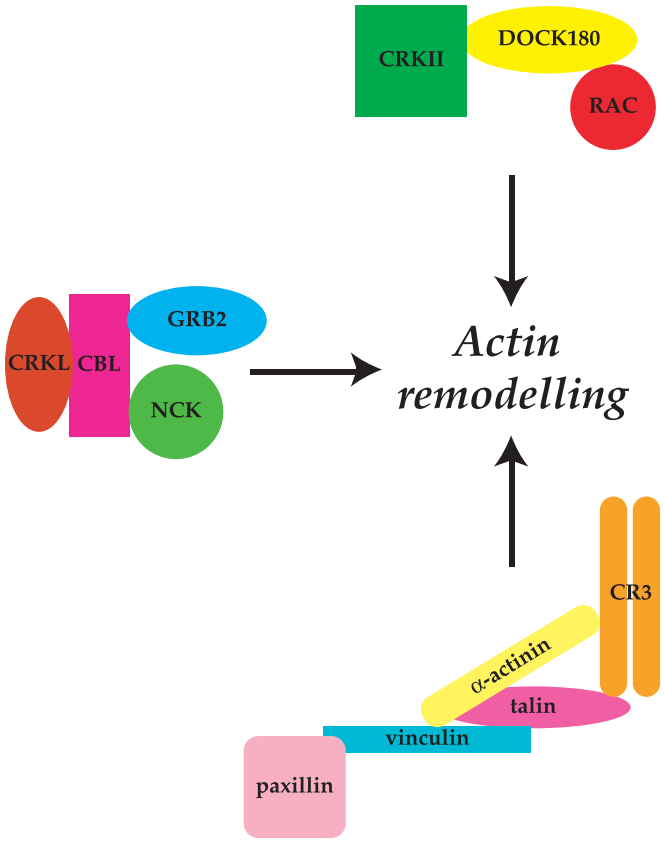

Fig. 4. The involvement of molecular scaffolds in phagocytosis. Three examples of multicomponent signalling complexes formed during phagocytosis are shown (see text for details). All three are able to influence the actin cytoskeleton through at least one component while simultaneously mediating other effects (not shown). Note that, for simplicity, only a small number of components are illustrated, although extremely large complexes are theoretically possible.

In *Dictyostelium*, myosin IB, myosin IC and myosin IK have been reported to be required for efficient phagocytosis (Jung et al., 1996; Schwarz et al., 2000), although it should be noted that Peterson et al. did not find a requirement for myosin IC in phagocytosis (Peterson et al., 1995). Myosin II also localises to *Dictyostelium* phagosomes (Rezabek et al., 1997), whereas cells lacking myosin VII show a significant and specific defect in phagocytosis (Titus, 1999). Given the diversity of structure and function within the myosin superfamily, it is probable that several independent features of phagocytosis (e.g. contractile force and vesicle delivery) are reliant on myosins.

Completing the phagocytic event

During phagocytosis there is a necessity not just to extend membrane to surround the particle (or, in the case of CR3, drag the particle into the cell), but also to 'finish off' – to close the membrane again and form a sealed vacuole containing the particle. PI3-K is important for this process, since inhibition of this enzyme does not perturb actin remodelling at the phagosome but prevents completion of phagocytosis (Araki et al., 1996). This might reflect a role for PI3-K in regulating the delivery of vesicles to the phagosome and thereby providing additional membrane to close the phagosome (Cox et al., 1999).

Recently, other, more surprising, molecules have been implicated in phagosome closure. A large-scale antibody screen of FcγR phagosomes from murine macrophages revealed that a novel amphiphysin isoform, amphiphysin IIm, is enriched at these sites (Morrissette et al., 1999) and regulates membrane extension around the phagosome (Gold et al., 2000). In addition, a binding partner of amphiphysin, the GTPase dynamin 2, is also enriched on phagosomes and is required for membrane extension (Gold et al., 1999). The dynamin-amphiphysin complex is thought to regulate 'pinching off' of clathrin-coated vesicles from the plasma membrane (Schmid et al., 1998); hence its presence at phagosomes (which are not clathrin coated) is a surprise. Gold et al. present evidence that these proteins may act downstream of PI3-K; they propose a model in which PI3-K acts on amphiphysin-dynamin to regulate vesicle delivery to the phagosome and thereby provides material for membrane extension around the particle (Gold et al., 1999). Additionally, the newly discovered role for dynamin-2 as a signal transduction enzyme, relaying transcriptional and apoptotic stimuli (Fish et al., 2000), may prove relevant for phagocytosis.

Maturation of the phagosome

Following particle internalisation the phagosome continues to 'mature', the associated proteins changing over a number of hours (Tjelle et al., 2000). The long-standing belief is that actin filaments that surround newly internalised phagosomes rapidly depolymerise, which leaves the phagosome membrane available to fuse with endosomes and lysosomes. However, this view has been challenged by data showing continued enrichment of actin on phagosomes (Desjardins et al., 1994). Moreover, recent work has shown that purified phagosomes can induce actin polymerisation at their surface (Defacque et al., 2000), a process that requires membrane proteins of the ezrin/radixin/moesin (ERM) family that link actin filaments to membranes (Algrain et al., 1993).

Interestingly, fully internalised phagosomes show enrichment of annexin I (Kaufman et al., 1996), annexin II (Desjardins et al., 1994) and annexin VI (Desjardins et al., 1994), and α-actinin (Desjardins et al., 1994). All four proteins can interact with the actin cytoskeleton (Diakonova et al., 1997; Hosoya et al., 1992). It is therefore possible that, even after internalisation, the phagosome membrane retains a collection of proteins that enable it to continue to interact with the actin cytoskeleton.

What is the purpose of such an interaction? Recent work has demonstrated a possible role for actin polymerisation in driving vesicle movement within cells. In vitro, vesicles can propel themselves using actin polymerisation as the driving force after the addition of PtdIns(4,5)P_2 (Ma et al., 1998). Similarly, increasing PtdIns(4,5)P_2 synthesis in vivo causes the appearance of motile vesicles whose motion is driven by the assembly of an actin 'comet-tail' (Rozelle et al., 2000). Since endocytic vesicles also move by using such an actin tail (Merrifield et al., 1999), it is possible that phagosomes too, once internalised, might use actin polymerisation to drive their movement through the cytoplasm.

Concluding remarks

The mass of data concerning phagocytosis is now quite awesome, and yet we remain some way from a complete model of this important cellular process. This is a measure of its enormous complexity and demonstrates how seemingly distinct pathways might in reality be closely interwoven. Nevertheless, significant advances have been made in identifying molecules that are potentially involved in phagocytic uptake. The task now is to discover how these relate to each other.

An overriding feature of the phagocytic process is redundancy. This undoubtedly makes the job of the researcher more difficult – it is always easier to spot an 'all or nothing' difference – but redundancy should not come as a surprise. After all, phagocytosis is a central process for the survival of an organism, and it is only to be expected that such a process will have several 'fail-safe' mechanisms.

So where next on the phagocytosis trail? One major area of discovery is likely to be the rather eclectic group of 'other receptors' – those involved in the uptake of apoptotic bodies, or in non-opsonic uptake of foreign particles. Long the Cinderellas of phagocytosis, it is likely that they will take centre stage as the cloning of receptor genes (such as the recently identified Phg1 in *Dictyostelium*; Cornillon et al., 2000; or CED-1 in *C. elegans*; Zhou et al., 2001) renders this diverse group amenable for study. Since so many signal components have been identified downstream of FcRs, it should prove possible to repeat these experiments for other receptors and identify similarities and differences. Indeed, comparisons of different phagocytic systems might allow us to identify core components that underpin internalisation. Conversely, much interest will also focus on the differences – why are only some phagocytic events inflammatory, and why does the morphology of internalisation vary so significantly between receptors, when the objective is the same in each case?

More specifically, many components of phagocytic signalling have clearly discernible but indirect downstream targets. For example, Rho influences actin nucleation at CR3 phagosomes through the Arp2/3 complex, but there is still no candidate for a molecule that might link Rho to the complex. At the other extreme, PI3-kinase could influence several downstream targets, but it is unclear what role is performed by each of these.

Two important concepts are key to a clearer understanding of the phagocytic process. Firstly, there is a growing awareness of the 'late' stages of internalisation and the events that follow. Novel biochemical approaches are identifying components of the phagocytic cup that would not previously have been predicted to have a role here (Gold et al., 2000; Morrissette et al., 1999). These hint at the unexpected involvement of vesicle trafficking to and from the phagosome while it is forming. Additionally, a continued role for the cytoskeleton in regulating phagosome behaviour after internalisation is becoming apparent (Defacque et al., 2000). Secondly, cell biologists now recognise that many signalling events occur in the context of signalling scaffolds – multicomponent aggregates of signalling proteins that can be involved in several pathways simultaneously. The recent identification of such a scaffold complex in myeloid cells (Izadi et al., 1998) should focus efforts on the characterisation of such complexes in phagocytic signalling.

Phagocytosis is a complex phenomenon, but, as with other complex cellular behaviours, we should not shy away from its intricacies. Already massive headway has been made, and even the toughest nut can be cracked with sufficient effort. It is worth remembering that the most complex problems are usually those with the most significant impact. To paraphrase Einstein, it's not about being smart, it's about staying with problems longer.

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