Coronin-1 Function Is Required for Phagosome Formation

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Coronin-1 is an actin-associated protein whose function in actin dynamics has remained obscure. All coronin proteins have a variable N-terminal domain, followed by WD repeats and a C-terminal coiled-coil dimerization domain. Transfection of coronin-1-GFP into RAW 264.7 cells revealed that coronin rapidly and transiently associates with the phagosome. To determine if coronin is involved in mammalian phagocytosis we used a dominant-negative approach by expressing only the central WD domains. However, this caused cell rounding and dissociation from the substratum, hampering analysis of their phenotype. We therefore developed TAT-fusion constructs of coronin-1 WD domains to acutely introduce the recombinant protein fragment into live cells. We show that although TAT-WD has no effect on binding of opsonized RBCs to RAW 264.7 cells, receptor clustering or several downstream signaling events, lamellipodial extensions, and actin accumulation at the base of the bound particle were diminished. Furthermore, Arp3 accumulation at the phagosome was impaired after TAT-WD treatment. Interestingly, whereas coronin-1 also accumulates at the sites of actin remodeling associated with *Salmonella* invasion, TAT-WD had no effect on this process. Together, our data demonstrates that coronin-1 is required for an early step in phagosome formation, consistent with a role in actin polymerization.

INTRODUCTION

Phagocytosis is a vital component of the host defense against infection. Invading microorganisms, often coated by soluble host opsonins such as complement C3 or immunoglobulins, are recognized by receptors on the surface of leukocytes. This leads to clustering of the opsonin receptors adjacent to the surface of the phagocytic particle, followed by their tyrosine phosphorylation. Phosphorylation of tyrosine residues within the immunoreceptor tyrosine activation motif (ITAM) by nonreceptor kinases of the *src* family provides docking sites for SH2-containing molecules, including the tyrosine kinase Syk (Greenberg *et al.*, 1994). These early signaling events ultimately lead to local remodeling of the submembranous actin cytoskeleton (Greenberg *et al.*, 1990) and the recruitment of a complex comprised of the Fyb/*src*-like adaptor protein (SLAP), SLP-76, Nck, vasodilator-stimulated phosphoprotein (VASP), and Wiskott-Aldrich syndrome protein (WASP; Coppelino *et al.*, 2001) that may function to synchronize the localization of key mediators of actin remodeling, such as profilin and Arp2/3. The Arp2/3 complex is necessary for particle ingestion via both Fcγ receptor (FcγR; Booth *et al.*, 2002)- and CR3-mediated phagocytosis (May *et al.*, 2000), suggesting that de novo nucleation of actin structures is required for phagosome formation.

Another actin-associated protein that has been implicated in phagocytosis in *Dictyostelium* is the WD-domain protein coronin. Coronin was first identified as a soluble protein from *D. discoideum* that bound to actin-myosin complexes (deHostos *et al.*, 1991). Importantly, loss of the coronin gene product results in cells with impaired chemotaxis and

phagocytosis (deHostos *et al.*, 1993). Coronins are conserved from yeast to man, with at least six isoforms being expressed in mammals (deHostos, 1999) but little is known about the specific roles of the mammalian forms or their functional homology to the *Dictyostelium* form. Of the mammalian forms, coronin-5 and coronin-6 are mainly neural, and only coronin-1 (originally called p57) has a predominantly hemopoietic expression pattern. The sequence of *Dictyostelium* coronin predicts a 49 kDa protein containing five WD-40 repeats similar to the ones found in the β subunit of heterotrimeric G proteins, and a C-terminal coiled coil domain implicated in dimerization. *Dictyostelium* ingest nutrients from the environment by macropinocytosis and phagocytosis. It is noteworthy that coronin null mutants perform phagocytosis at only 1/3 the rate of wild-type cells (Maniak *et al.*, 1995). GFP-tagged versions of coronin are capable of rescuing the null phenotype, indicating that the GFP moiety has no deleterious effects on its function and can be used safely to monitor the distribution of the protein in situ.

Coronin not only colocalizes extensively with actin-rich structures, but has also been shown to bind actin in vitro (deHostos *et al.*, 1991; Goode *et al.*, 1999; Mishima and Nishida, 1999). Nevertheless, the actin-binding domains of the protein have not been fully defined. In the yeast Crn1p, actin binding has been mapped to the N-terminal half of the protein (Goode *et al.*, 1999). In contrast, *Xenopus* coronin cosediments with actin but this was impaired if either end of coronin was truncated and abolished if only the middle of the protein containing the WD repeats was present (Mishima and Nishida, 1999). For mammalian coronin-1, two regions were identified as having actin-binding capacity. The strongest actin binding was identified in the N-terminal 34 amino acids, while the second and third WD domains also had weak actin-binding capacity (Oku *et al.*, 2003).

The role of coronin in actin assembly remains unclear. In yeast, the coronin homolog Crn1p enhances barbed-end as-

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sembly, apparently by reducing the lag phase of polymerization (Goode *et al.*, 1999). In contrast, *Dictyostelium* coronin associates with the entire length of actin filaments and it has been suggested to speed up depolymerization (Gerisch *et al.*, 1995). Interestingly, recent studies in yeast have also shown a physical association of coronin with the Arp2/3 complex (Humphries *et al.*, 2002), supporting earlier evidence of an association between coronin and the Arp2/3 complex in mammalian neutrophils (Machesky and Hall, 1997).

In this study we set out to examine the role of coronin in the phagocytic process of macrophages. We demonstrate that coronin-1 transiently accumulates at the nascent phagosome in a temporal sequence similar to that of actin. Moreover, by introducing the WD domains of coronin-1 into macrophages we observed significant changes in their adhesion properties and phagocytic potential, with impaired accumulation of Arp3 and actin at the phagocytic cup. These results provide the first evidence for a requisite function of coronin-1 in macrophage phagocytosis.

MATERIALS AND METHODS

Reagents and Antibodies

All restriction enzymes were from New England Biolabs (Beverly, MA). Dulbecco's modified Eagle's medium, fetal bovine serum (FBS), phosphate-buffered saline (PBS), trypsin-EDTA, penicillin/streptomycin, and HEPES-buffered RPMI were purchased from Wisent (St-Bruno, Quebec, Canada). Paraformaldehyde was from Canemco (St-Laurent, Quebec, Canada). FuGene 6 was from Roche Molecular Biochemicals (Indianapolis, IN). The electroporation system used was from Amaxa (Gaithersburg, MD). Mammalian and bacterial protease inhibitor cocktails, human IgG, C5-deficient serum, and phorbol myristate acetate (PMA) were obtained from Sigma-Aldrich (St. Louis, MO). RAW 264.7 cells were purchased from the American Type Culture Collection (Manassas, VA). BL21(DE3)LysS cells were from Novagen (Madison, WI). *Salmonella enterica* serovar typhimurium strain WT SL1344 and the invasion-defective mutant *AinvA* SL1344, both expressing dsRed, were kind gifts of Dr. John Brumell (Hospital for Sick Children, Toronto). Ampicillin-resistant plasmid pIZ1590 expressing dsRed under the rpsM promoter, was from Francisco Ramos-Morales (Universitat de Sevilla, Spain). Human coronin-1 cDNA and mouse anti-coronin-1 (p57) antibody were a gift from Dr. Satoshi Toyoshima (National Institute of Health Science, Japan). The pTAT, pTAT- β Gal, and β -galactosidase (non-TAT) plasmids were kindly provided by Dr. S. F. Dowdy (University of California at San Diego, CA). FcyRIIA-GFP was from Dr. Alan D. Schreiber (University of Pennsylvania, Philadelphia, PA). PAK-PBD-YFP was kindly provided by Dr. H. Bourne (University of California, San Francisco, CA). MonoRFP-N1 plasmid was a kind gift of Dr. R. E. Campbell (Campbell *et al.*, 2002). Arp3-GFP was kindly provided by Dr. T. Bretschneider (Max-Planck-Institut für Biochemie, Germany). Polystyrene beads were from Bangs Laboratories (Fishers, IN). Coronin-1 or control nontargeting siRNA duplexes were purchased from Dharmacon (Lafayette, CO). Rabbit and mouse anti-actin antibodies were from Sigma-Aldrich, mouse anti-His antibody was from Amersham (Baie d'Urfe, Quebec, Canada), rabbit anti-*Salmonella* antibody was from BD Biosciences (Franklin Lakes, NJ), mouse anti-phosphotyrosine antibody was from Upstate Biotechnology (Waltham, MA). Cy3- and Cy5-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). Alexa 488-conjugated donkey anti-mouse secondary antibody and Rhodamine-phalloidin were from Molecular Probes (Eugene, OR). Sheep red blood cells (RBCs) and rabbit anti-sheep RBC antibody were obtained from Cappel/ICN (Irvine, CA). Rabbit anti-sheep RBC IgM was from Accurate Chemical and Scientific Corp. (Westbury, NY). Fluorescent Mounting Medium was from DAKO (Carpinteria, CA).

DNA Constructs and Protein Purification

To generate the construct WT-cor-GFP, coronin-1 (GenBank Accession no. D4449) was subcloned into the pEGFP-N1 vector (Clontech, Palo Alto, CA). Full-length coronin-1 cDNA was PCR-amplified using PFU DNA polymerase (Stratagene, La Jolla, CA) with oligomers 5'-GCTGCGGCTCGAGGGCAGCA-GAATGAGCCGGCAG-3' and 5'-GCTGCGGCGATCCGTCTTGGCCTG-GACTGTCTCTC-3'. This PCR product was digested with *XhoI* and *Bam*HI and ligated into the vector pEGFP-N1 (Clontech, Palo Alto, CA). To generate the construct CorRFP, full-length coronin-1 cDNA was digested from the clone WT-cor-GFP using *XhoI* and *Bam*HI and ligated into the vector monoRFP-N1. To generate the clone WD-cor-GFP containing amino acids 65–306, and lacking both N- and C-termini of coronin-1, the five WD repeats were PCR-amplified, as above, using oligomers 5'-CGCGAAGCTTAGATCTAT-GGGCAAGACTGGACGTGTGGAC-3' and 5'-CGCGCGGCCCGGATCCG-

TATAGTCAGGAAAGGGGCTC-3'. This PCR product was digested with *Hind*III and *Bam*HI, and ligated into pEGFP-N1. To generate the clone TAT-WD, the WD-cor-GFP construct was digested with *XhoI* and *Not*I, subcloned into pCDNA3.1+ (Invitrogen, Burlington, Ontario, Canada), and then digested with *XhoI* and *Eco*RI and resubcloned into the pTAT vector containing His6, TAT, and HA tags (all N-terminal). To generate the construct containing the coiled coil (amino acids 410–461) of coronin-1 linked to GFP (CC-GFP) PCR amplification using oligomers 5'-CGGAATTCATGCTGGACAC-CGGGCGCAGGAG-3' and 5'-CGGGATCCGTCTTGGCCTGGACTGTCTC-CTC-3' was performed. This PCR product was digested with *Bam*HI and *Eco*RI and ligated into pEGFP-N1. To make the clone containing the carboxyl terminal 155 residues of coronin-1 (amino acids 307–461) linked to GFP (CT-GFP), PCR amplification was performed using oligomers 5'-CGGAATTCATGCTCTCCATGTTTCAGTTCCAAG-3' and 5'-CGGGATCCGTCTTGGCCTGGACTGTCTCTC-3'. This PCR product was cloned into pEGFP-N1 with the same method as the CC-GFP construct. The fidelities of all constructs were confirmed by DNA sequence analysis. To purify proteins, TAT-WD, TAT- β Gal, and β -galactosidase (non-TAT) plasmids were transformed into BL21(DE3)LysS competent cells and grown in Terrific Broth (Life Technologies, Burlington, Ontario, Canada) overnight. Protein expression was induced for 4 h with 0.5 mM IPTG at 37°C. His6-tagged fusion proteins were lysed by French Press in buffer (8 M urea, 100 mM NaCl, 20 mM HEPES) and centrifuged at 12,000 $\times$ g for 30 min. The supernatant was added to a Ni-NTA matrix column to immobilize the proteins and an AKTA fast-performance liquid chromatography with Frac-950 (Amersham Pharmacia, Baie d'Urfe, Quebec, Canada) was used to purify the proteins. The eluted proteins were desalted with 1 $\times$ PBS using P10 matrix columns (Amersham Biosciences, Piscataway, NJ), aliquoted, and stored at -80°C. All proteins prepared for this study showed essentially only one band on 10% Coomassie-stained SDS-polyacrylamide gel (see Figure 3a).

Cell Culture and Transfections with DNA or siRNA

For cell assays, RAW 264.7 cells were trypsinized, split onto 18-mm round coverslips and grown to 50% confluence in DMEM containing 5% FBS and penicillin/streptomycin, at 37°C in 5% CO₂. DNA transfections were performed using FuGene 6 and 0.25 μ g DNA per 18-mm coverslip, according to the manufacturer's instructions, and assayed after 16 h. Arp3GFP and CorRFP transfections were performed using the Amaxa Nucleofection system. Briefly, 4 $\times$ 10⁶ RAW 264.7 cells were washed in PBS and resuspended in 100 μ l solution V (Amaxa), 0.5 μ g of each of Arp3GFP and CorRFP DNA was added, and cells were electroporated using program U-12. RNA interference against coronin-1 was performed on RAW cells using the sense sequence from coronin-1 5'-UGACAGCUCUAUCCGGUAAUUU, or nontargeting control siRNAs (Dharmacon). RAW 264.7 cells were plated at 20% confluence on 18-mm coverslips and 200 nM coronin or nontargeting control siRNA duplex was transfected using 2 μ l oligofectamine per 20-mm well, with coverslips for phagocytosis assays. Assays were done after 3 or 4 d.

Transduction with TAT- β -Gal or WD-TAT Protein

For all TAT protein assays, RAW 264.7 cells grown on 18-mm coverslips were first washed in PBS, treated with TAT- β Gal or TAT-WD protein at a final concentration of 200 nM, diluted in either HEPES-buffered RPMI, or PBS containing 1 mM calcium chloride, 1 mM magnesium chloride, and 10 mM glucose, and then incubated at 37°C for 1 h, without CO₂.

β -Galactosidase Histochemical Assay

After treatment with TAT- β Gal or β -galactosidase protein, RAW 264.7 cells were washed three times with 1 $\times$ PBS, fixed for 5 min at 4°C in 1 ml of fixing solution (2% formaldehyde, 0.2% glutaraldehyde in 1 $\times$ PBS), and washed in PBS. Cells were then incubated for 24 h at 37°C in histochemical staining solution (3 mM potassium ferricyanide, 3 mM ferrocyanide, 10% dimethyl sulfoxide, 2 mM MgCl₂, 1 mg/ml X-gal, made up in 1 $\times$ PBS). Samples were examined using an Axiovert 200M light microscope (Carl Zeiss, Thornwood, NY) with a Microcolor RGB filter (see Figure 3, b and c).

Phalloidin Staining

RAW 264.7 cells grown on 18-mm coverslips were treated with either TAT- β Gal (see Figure 3, e and g) or TAT-WD (see Figure 3, f and h) for 1 h, fixed in 4% paraformaldehyde/PBS at 4°C for 1 h, and permeabilized for 15 min using 0.1% Triton X-100 containing 100 mM glycine. To label F-actin, rhodamine-phalloidin was diluted to 0.4 U/ml in PBS and incubated with cells for 30 min. Coverslips were washed in PBS and mounted using DAKO Fluorescent Mounting Medium. Images were taken on a DM-IRE2 fluorescence microscope (Leica, Deerfield, IL) using a Cy3 filter cube and processed using Open Lab v 3.1.7 software (Improvision, Lexington, MA).

Western Analysis of Triton X-100-soluble Proteins and siRNA Lysates

After treatment of RAW 264.7 cells with TAT-WD, total Triton X-100-soluble proteins were collected as follows; after three washes in ice-cold PBS, samples

were incubated with ice-cold extraction buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 0.5% Triton X-100) and agitated on ice for 5 min. Triton-soluble supernatant was recovered, diluted in 4× Laemmli loading dye and passed five times through a 27-gauge needle to break up chromosomal DNA. Samples were subjected to 10% SDS-PAGE, and Western blot was probed for coronin-1 (p57 antibody, 1:1000), rabbit anti-actin (1:1000), or mouse anti-His tag (1:3000; see Figure 3d). For siRNA knockdown of coronin-1, after 72 h siRNA treatment, RAW cells were washed three times in ice-cold PBS, solubilized in RIPA buffer (1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, pH 7.2), scraped, and incubated on ice for 30 min. After passing three times through a 27-gauge needle, equal amounts of samples were loaded on 10% SDS-PAGE gels. Western blots were probed for coronin-1 using a p57 monoclonal antibody (1:1000) or mouse anti-actin (1:1000) as a loading control.

Scanning Electron Microscopy

RAW 264.7 cells were grown on 18-mm round coverslips, treated with TAT-βGal or TAT-WD protein, and then IgG-opsonized RBCs were allowed to bind for 10 min at 4°C in HEPES-buffered RPMI. Unbound RBCs were washed away with PBS. Samples were fixed in universal fixative (1% glutaraldehyde, 4% formaldehyde in 0.1 M phosphate buffer) at room temperature, washed twice in PBS, once in distilled water, and fixed in 2% osmium tetroxide for 1 h. Samples were dehydrated in an ascending series of ethanol solutions from 50 to 100%, before coating with gold-palladium. Samples were then examined, and images were acquired on an XL-30 scanning electron microscope (FEI Company, Hillsboro, OR).

Fc Receptor-mediated Phagocytosis of RBCs

Generally, sheep RBCs were opsonized with rabbit anti-sheep RBC IgG (50:1) by rotating at 37°C for 1 h and washed three times in PBS. Alternatively, polystyrene beads were opsonized with human IgG. Opsonized RBCs were allowed to attach to RAW 264.7 cells for 5 min, in ice-cold HEPES-buffered RPMI. Unbound RBCs were washed away with ice-cold PBS. RAW 264.7 cells were warmed to 37°C and allowed to undergo phagocytosis for 5 or 10 min. In some cases, RBCs that were not internalized were lysed by exposure to distilled water for 15 s. After phagocytosis, RAW 264.7 cells were fixed in 4% paraformaldehyde at 4°C for 1 h.

For Fc Receptor-mediated binding index assays (see Figures 4c and 5, a and b), RAW 264.7 cells were treated with either TAT-βGal or TAT-WD protein, CC-GFP, CTerm-GFP plasmid, coronin siRNA or control siRNA, and opsonized RBCs were allowed to bind at 4°C for 5 min before unbound cells were washed away with ice-cold PBS. RAW 264.7 cells were then fixed in 4% paraformaldehyde at 4°C for 1 h. The binding index was calculated using a phase contrast microscope to count the average number of remaining RBCs bound per RAW 264.7 cell. The index data are means ± SE of three experiments, with at least (30) cells counted in each case.

For Fc Receptor-mediated Phagocytic Index assays (see Figure 4d), RAW 264.7 cells were treated with either TAT-βGal, TAT-WD protein, or untreated (control), CC-GFP plasmid, CT-GFP plasmid, coronin siRNA, or control siRNA and allowed to undergo phagocytosis of IgG-opsonized RBCs for 10 min at 37°C. RBCs that were not internalized were lysed by a 15-s exposure to distilled water. RAW 264.7 cells were fixed in 4% paraformaldehyde for 1 h and the Phagocytic Index was calculated using a phase contrast microscope to count the average number of internalized RBCs per RAW 264.7 cell. The index data are means ± SE of three experiments, with at least (30) cells counted in each case. Asterisk indicates $p < 0.05$.

For live phagocytosis assays, RBCs were opsonized (as above), stained with Cy3 donkey anti-rabbit antibody (1:1000) at room temperature for 1 h and washed three times in PBS before adding to RAW 264.7 cells (transfected with WT-cor-GFP or WD-cor-GFP) in ice-cold HEPES-buffered RPMI. RAW 264.7 cells were allowed to warm to 37°C for phagocytosis to occur and live images were captured at 10-s intervals (Figure 1). Alternatively, phagocytosis of RBCs was allowed for 10 min at 37°C (Figure 2e). RBCs that were not internalized were lysed by hypotonic shock, and samples were analyzed by using an LSM 510 laser scanning confocal microscope (Zeiss).

Complement Receptor-mediated Phagocytosis of RBCs

For C3-receptor-mediated phagocytic index measurements, RBCs were opsonized with IgM as follows: 100 μl of stock RBCs were washed once in PBS and 40 μl rabbit anti-sheep RBC IgM was added, incubated on a rotator at room temperature for 1 h, and subsequently washed two times in PBS. Fifty microliters of C5-deficient serum was added and incubated at 37°C for 20 min. RAW 264.7 cells were serum-starved for 2 h in DMEM at 37°C in 5% CO₂, activated by 100 nM PMA for 20 min in DMEM, and treated with either TAT-WD or TAT-βGal protein. Opsonized RBCs were washed in PBS three times and overlaid on RAW 264.7 cells, and phagocytosis was allowed to occur for 60 min at 37°C. External RBCs were then lysed using hypotonic shock, and RAW 264.7 cells were fixed in 4% paraformaldehyde for 1 h. The Phagocytic Index, defined as the number of phagosomes/macrophage, was calculated by using a phase contrast microscope (see Figure 4e) to count the average number of internalized RBCs per macrophage.

Signaling of the Phagocytic Pathway in RAW 264.7 Cells

RAW 264.7 cells were split onto 18-mm coverslips and transfected using FuGene 6 and 0.25 μg FcγRIIA-GFP (see Figure 6, a and b), PAK-PBD-YFP (Figure 6, g and h), PLCδ-PH-GFP (Figure 6, i and j), AKT-PH-GFP (Figure 6, k and l), C18-GFP (Figure 6, m and n), both PLCδ-PH-CFP and C18-YFP (Figure 6, o and p), or not transfected (Figure 6, c–f). Alternatively, 4×10^6 RAW 264.7 cells were resuspended in solution V (Amaxa), electroporated with 0.5 μg Arp3GFP (see Figure 7, a–c, g, and h), or coelectroporated with 0.5 μg Arp3GFP and 0.5 μg CorRFP (Figure 7, e–h) using program U-12 (Amaxa). Cells were grown for 16 h and pretreated with TAT-WD or TAT-βgal protein as control. IgG-opsonized RBCs were overlaid, and phagocytosis occurred for 1 min (Figure 6i, insets A and B, and 6j, insets A and B), 3 min (Figure 7A), 5 min (Figures 6, c–p, and 7, b and c), or 10 min (Figure 6, a and b) at 37°C. Unbound RBCs were washed out with PBS and the RAW 264.7 cells were fixed in 4% paraformaldehyde at 4°C for 1 h. Fixed cells were permeabilized for 15 min using 0.1% triton X-100 containing 100 mM glycine and blocked in 5% serum. Mouse anti-phosphotyrosine (Figure 5, c and d) was diluted to 1:200 in 5% serum and incubated with cells for 1 h at room temperature, washed three times in PBS, and incubated with Alexa 488 donkey anti-mouse secondary antibody (1:1000) in 1% serum. Total RBCs were labeled with Cy3 donkey anti-rabbit antibody. To label F-actin, rhodamine-phalloidin (Figure 5, e and f) was diluted to 0.4 U/ml and incubated with cells for 30 min in 1% serum. Coverslips were washed in PBS and mounted using DAKO Fluorescent Mounting Medium. Fixed samples were imaged using an LSM510 laser scanning confocal microscope (Carl Zeiss) and an oil-immersion 100× objective. GFP, Alexa 488, rhodamine, Cy3, CFP, and YFP fluorescence were examined using standard filter sets.

Salmonella and Invasion

For bacterial invasion index analysis (Figure 6, c and d), both wild-type or InvA mutant *Salmonella* expressing dsRED from plasmid pIZ1590 were grown overnight in LB containing ampicillin. After pretreatment of RAW 264.7 cells with TAT-βGal or TAT-WD protein, a final concentration of 5–10 million cells/ml *Salmonella* was added to RAW 264.7 cells in DMEM containing 5% FBS. Invasion proceeded for 30 min at 37°C in 5% CO₂, coverslips were washed three times in PBS, and fixed in 4% paraformaldehyde for 1 h and stained for external *Salmonella* using rabbit anti-*Salmonella* primary antibody (1:500) and Cy5 donkey anti-rabbit secondary antibody (1:1000). Cells were imaged using an LSM510 laser scanning confocal microscope. Internalization index was calculated as the average difference between dsRED bacteria (total) and Cy5-staining bacteria (external) per macrophage.

Alternatively, wild-type or InvA *Salmonella* were overlaid on RAW 264.7 cells transfected 16 h previously with WT-cor-GFP, in DMEM containing 5% FBS. Live images were acquired every 10 s at 37°C over a period of 5 min (Figure 8, a and b), using confocal microscopy.

RESULTS

Coronin-1 Accumulates at Phagosomes

We initially analyzed the presence and distribution of coronin-1 in RAW 264.7 cells, a murine macrophage line. Using monoclonal antibodies we confirmed that these cells express coronin-1 (p57) by Western blotting (unpublished data). We then set out to analyze its distribution during phagocytosis. Unfortunately, this and other available antibodies specific for coronin were not capable of detecting the protein at endogenous levels by immunofluorescence microscopy. As an alternative means of monitoring coronin-1 distribution, we transiently transfected RAW 264.7 cells with full-length coronin-1-GFP. In *Dictyostelium*, coronin-GFP has been shown to be fully functional (Maniak *et al.*, 1995), so we anticipated that the GFP-tagged chimera would faithfully represent the distribution of the endogenous coronin-1 in RAW 264.7. Transfected cells were then incubated with IgG-opsonized sheep RBCs, and the location of coronin was examined. Figure 1 shows a series of images of a representative transfected cell captured after an RBC had bound to the Fc receptors. Coronin-1-GFP was seen to rapidly accumulate at the nascent phagosome and concentrate at the leading edge of the lamellipodia. After only 180 s the coronin was seen to rapidly dissipate from the phagosome. This is consistent with observations of coronin-1 in differentiated HL-60 cells (Itoh *et al.*, 2002), where coronin was found to transiently associate with the phagosome.

Figure 1. Distribution of wild-type human coronin-1-GFP (WT-cor-GFP) during phagocytosis. RAW 264.7 cells expressing WT-cor-GFP were allowed to bind to IgG-opsonized sheep RBCs at 4°C, and unbound RBCs were washed away. Phagocytosis images were collected at 30-s intervals after the temperature warmed to 37°C, using confocal fluorescence microscopy. The time after warming is indicated in seconds at the top right of each panel. Arrowhead indicates a bound RBC. Scale bar, 10 μ m.

Expression of Dominant-Negative Coronin-1 Affects Cell Adhesion and Phagocytosis

To determine the role of coronin-1 in phagocytosis we constructed a putative dominant-negative construct containing only the five WD repeat sequences of coronin-1. This choice was based on previous studies of *Xenopus* coronin that had shown this region of the protein to possess dominant-interfering properties (Mishima and Nishida, 1999). The WD domain was linked to GFP and the cDNA construct was introduced by transfection. We immediately noticed that only a very low number of transfected cells remained after 24 h of expression. More careful analysis revealed that expression of the WD domains led to rounding and, ultimately, detachment of the cells from the substratum. As seen in Figure 2a, RAW 264.7 cells transfected with wild-type coronin-1-GFP spread normally on the substratum, acquiring a fried egg appearance when viewed from the side in a confocal Z-axis reconstruction. Actin is seen at the periphery of the cell membrane, including the surface contacting the substratum (dotted line at bottom). Coronin-1-GFP staining is seen at the plasma membrane but is also present elsewhere in the cell (inset). In contrast, cells expressing WD-GFP underwent a marked rounding and a significant reduction in the surface area in contact with the substratum (Figure 2b). This loss of substratum contact likely accounts for the apparent reduction in transfection efficiency, as a fraction of the transfected cells probably detached from the coverslips before analysis, during the period allowed for expression. In the few cells remaining attached, we noted a clear reduction in their capacity for phagocytosis. Figure 2c shows a RAW 264.7 cell expressing coronin-1-GFP in the process of engulfing several opsonized RBCs. In contrast, cells expressing the WD-cor-GFP construct were typically found with adherent IgG-opsonized RBCs, but there was little evidence of phagocytosis. As an independent approach to determine if IgG-opsonized RBCs were being internalized we used hypo-osmotic lysis to eliminate externally adherent RBCs. Unlike the RBCs that are internalized and thereby acquire osmotic resistance, extracellular adherent RBCs are readily susceptible to osmotic lysis. As shown in Figure 2e, RAW 264.7 cells transfected with WD-cor-GFP (green) were

Figure 2. Cellular morphology is altered after expression of dominant-negative coronin (WD-cor-GFP) in RAW 264.7 cells. RAW 264.7 cells were transfected with either WT-cor-GFP (a and c) or WD-cor-GFP (b, d, and e), fixed, and stained for F-actin using rhodamine-phalloidin (a and b). Green insets indicate coronin-GFP. Z-stack images of transfected cells (a and b) are projections of 25 confocal slices. A dotted line indicates the position of the substratum. RAW 264.7 cells expressing WT-cor-GFP (c) or WD-cor-GFP (d) were allowed to undergo phagocytosis of IgG-opsonized prelabeled RBCs (red) for 5 min and fixed. Live RAW 264.7 cells expressing WD-cor-GFP and the nontransfected adjacent cells (e) were allowed to undergo phagocytosis of RBCs for 10 min, after which hypo-osmotic lysis removed all except internal RBCs, as seen in nontransfected cell (arrowheads). Bars, 10 μ m.

rarely found to contain internal RBCs, whereas the adjacent, untransfected cells contained internalized IgG-opsonized RBCs (prelabeled with red-conjugated antibodies to facilitate visualization; arrowheads). Unfortunately, the number of cells that remained attached to the coverslips after transfection was too low for us to attempt a systematic quantification of the inhibitory effects of WD-GFP on phagocytosis.

TAT Peptides Permit Transduction of Coronin-1 WD Domains in RAW 264.7 Cells

The finding that expression of the WD domain resulted in detachment and loss of the transfected cells suggested to us that analysis of the role of coronin-1 in phagocytosis would require treatments that would acutely impair coronin function. We therefore set out to implement TAT transduction protocols that permit introduction of proteins into the cytoplasm of live cells within very short time frames (Schwarze *et al.*, 1999). To this end, we engineered a TAT-fusion protein of the WD domain region containing in addition a His6-tag for purification. As a control, we also produced β -galactosidase-TAT (TAT- β Gal). The enzymatic activity of β -galactosidase was used to monitor the ability of the proteins to enter RAW 264.7 cells. The purified preparations of these two fusion proteins are shown in Figure 3a. The equivalent TAT construct of full-length coronin-1 could not be solubilized from bacterial inclusion bodies and could not be used as a control.

To demonstrate that RAW 264.7 cells were efficiently transduced by TAT proteins, we first compared the uptake of TAT- β Gal to β -galactosidase lacking a TAT peptide. Cells were incubated with the respective protein for 1 h and then washed, fixed, and assayed for β -galactosidase activity using X-gal as a substrate. As seen in Figure 3b, little if any β -galactosidase activity was detected in cells incubated with the enzyme lacking a TAT peptide, yet efficient transduction occurred for TAT- β Gal (Figure 3c). To demonstrate that the coronin WD protein could enter cells, RAW 264.7 cells were

Figure 3. TAT-WD protein purification, and transduction into RAW 264.7 cells. (a) Coomassie-stained polyacrylamide gel of purified TAT-βgal (lane 2) and TAT-WD (lane 3) reveals their purities. (b and c) RAW 264.7 cells were assayed for internalization of β-galactosidase after transduction. Cells were incubated for 1 h with β-galactosidase (non-TAT; b) or TAT-βgal (c) added to the medium, and then fixed and assayed histochemically for presence of β-galactosidase. (d) Internalization of TAT-WD was measured by Western blotting of 0.1% Triton X-100-soluble lysates of RAW 264.7 cells transduced with TAT-WD (d, right) or buffer (d, left). Blots were probed for coronin (top), actin (middle), or His tag (bottom). (e–h) Phalloidin staining of actin reveals changes in the adhesion structures after TAT-WD transduction. RAW 264.7 cells were treated with either TAT-βgal (e and g) or TAT-WD (f and h) for 1 h before fixation and staining for F-actin. Fluorescence image slices were taken at the middle (g and h) or basal layer (e and f) of the cells. Bars, 10 μm.

incubated for 1 h in the presence of TAT-WD, washed, and then homogenized in lysis buffer. The lysates were blotted for endogenous coronin-1 (p57), actin as a loading control, and for the His6 epitope on the TAT-WD fusion protein. As can be seen in Figure 3d, His6 was detectable in TAT-WD transduced cells, but not in untransduced controls.

To determine if TAT-WD had the same biological effects on RAW 264.7 cells as overexpression of WD-GFP, we first examined the adhesion of these cells to the substratum. As shown in Figure 3e, RAW 264.7 cells have wide areas of interaction with the substratum, characterized by prominent F-actin rich structures known as focal contacts. At higher focal planes the F-actin appears more homogeneously associated with the membrane (Figure 3g). As seen with WD-GFP transfections, the attachment area of RAW 264.7 cells after TAT-WD transduction is much smaller and the focal

Figure 4. Comparison of RBC binding and phagocytosis by RAW 264.7 cells treated with TAT-WD or TAT-βgal proteins. (a and b) Scanning electron micrographs of RAW 264.7 cells treated with either TAT-βgal (a) or TAT-WD protein (b) for 1 h before RBCs were bound at 4°C for 10 min, washed to remove unbound RBCs, and phagocytosis was initiated at 37°C for 5 min. Bar, 5 μm. Arrowhead indicates phagocytic cup. (c–e) Quantification of binding and phagocytic properties of RAW 264.7 cells after TAT-βgal or TAT-WD transduction. To measure binding index (c), RAW 264.7 cells were treated with either TAT-βgal or TAT-WD protein, IgG-opsonized RBCs were allowed to bind at 4°C for 10 min before unbound cells were washed away, and cells were fixed. The binding index was calculated by counting remaining RBCs bound per RAW 264.7 cell using a phase contrast microscope. To measure Fc receptor-mediated phagocytosis (d) and C3 receptor-mediated phagocytosis (e), RAW 264.7 cells were treated with either TAT-βgal, TAT-WD or untreated (control), and allowed to undergo phagocytosis of opsonized RBCs for either 10 min (Fc receptor) or 60 min (C3 receptor) at 37°C before hypotonic lysis. RAW 264.7 cells were fixed, and the average number of internalized RBCs was counted per RAW 264.7 cell (phagocytic index). Data are means ± SE of three experiments, with at least (30) cells counted in each case; *p < 0.05.

contacts are largely absent (Figure 3f). In contrast, the cortical F-actin associated with the plasma membrane appears unaffected (Figure 3h).

TAT-WD Inhibits Phagocytosis

We next examined the morphology of control and TAT-WD transduced cells undergoing phagocytosis at the electron microscopic level. As seen in Figure 4a, TAT-βgal transduced RAW 264.7 cells, like untreated cells (unpublished data), bound many IgG-opsonized RBCs and extended lamellipodia that ultimately engulfed the IgG-opsonized RBCs (white arrows). In contrast, cells transduced with TAT-WD (Figure 4b) did not display membrane protrusions, despite the presence of bound IgG-opsonized RBCs. Consistent with this and previous images, quantitative assessment of the number of bound (see *Materials and Methods* for details) IgG-opsonized RBCs revealed that the ability of Fcγ-receptors to interact with their ligand was not affected by treatment of the RAW 264.7 cells with TAT-WD (Figure 4c).

or actin (b, inset). Data are means $\pm$ SE of three experiments, with at least (30) cells counted in each case; * $p < 0.05$.

The absence of distinct lamellipodia (Figure 4b) in RAW 264.7 cells exposed to TAT-WD raised the possibility that, like the WD-GFP construct, the TAT-conjugated WD domain form of coronin-1 inhibited phagocytosis. To measure phagocytosis (Figure 4, d and e), RAW 264.7 cells that had been treated either with TAT- β Gal, TAT-WD, or had been left untreated, were incubated with IgG-opsonized RBCs at 4°C to allow adherence of the target particles, washed, and then warmed to 37°C for 10 min to allow phagocytosis to proceed. The number of internalized RBCs was then counted for at least 30 cells in triplicate by using a phase contrast microscope. Although TAT- β Gal-treated cells ingested RBCs at a rate that was indistinguishable from that of untreated control cells, TAT-WD caused an approximate four-fold reduction in the number of internalized RBCs. To determine if this inhibitory effect was specific to Fc-mediated phagocytosis, we repeated the assay using complement receptor-mediated phagocytosis. In this case RBCs were coated with IgM and incubated with C5-deficient serum to promote the deposition of C3 on the surface of the RBCs. The presence of C3 on the particle surface induces phagocytosis via complement receptors and involves distinct signaling pathways compared with Fc-receptor-mediated phagocytosis (Caron and Hall, 1998; Olazabal *et al.*, 2002). As seen in Figure 4e, complement receptor-mediated phagocytosis was also significantly inhibited by TAT-WD compared with control cells treated with TAT- β Gal.

Because WD repeats are found in a variety of other proteins, it was possible that the dominant-negative effects of TAT-WD were the result of the inhibition of another WD-containing protein. We therefore constructed two additional expression vectors containing GFP-fusions of additional portions of coronin-1 for use as competitive inhibitors of coronin-1 function. Construct CT-GFP consists of GFP fused to the last 155 amino acids of coronin-1, from amino acid 307–461, and therefore not overlapping with the sequence present in the WD domain fragment. A second construct, CC-GFP, was produced that encoded only the last 52 amino acids of coronin-1 (amino acids 410–461), containing the coiled-coil domain implicated in coronin-1 dimerization. Both constructs would be expected to inhibit dimerization of endogenous coronin-1 and this may inhibit its function in a dominant-negative manner (Asano *et al.*, 2001). Like the WD-GFP construct, these plasmids caused cell rounding and likely caused detachment of some cells from the substratum, but sufficient numbers of transfected cells were observed to

Figure 5. Comparison of RBC binding and phagocytosis by RAW 264.7 cells treated with coronin-1 coiled-coil, C-terminus, or siRNA. RAW 264.7 cells were split onto 18-mm glass coverslips, transfected with 0.5 μ g CC-GFP, CT-GFP, or pEGFP-N1 plasmid as control, using FuGene6. RBC binding assays and phagocytosis assays (a and b) were performed as in Figure 4, c and d, except that the phagocytic index was normalized to the control. For siRNA knockdown (b), RAW 264.7 cells were split onto 18-mm glass coverslips, transfected with 200 nM coronin siRNA or control siRNA, using oligofectamine, and assayed for phagocytosis or RBC binding 96 h later. Lysates from RAW 264.7 cells treated with either coronin siRNA or control siRNA were probed on Western blot for either coronin-1 (p57)

permit quantitation of phagocytosis. As shown in Figure 5a, cells expressing either GFP-C-term or GFP-coiled coil were capable of binding to opsonized RBCs normally, but phagocytosis was significantly inhibited. These findings provided independent evidence that coronin-1 function is required for optimal phagocytosis.

As a second proof that the observations made with TAT-WD protein reflected inhibition of coronin-1 function, we used siRNA to deplete coronin-1 from the cells. As shown in the inset of Figure 5b, we were able to routinely obtain $\sim 70\%$ knockdown of coronin-1 protein levels 4 d after transfection with siRNA duplexes. As can be seen in Figure 5b, RBC binding was not affected by coronin-1 depletion, whereas phagocytosis was significantly inhibited ($p < 0.05$), to an extent similar to that seen by TAT-WD treatments, or overexpression of the coiled coil (CC-GFP) or carboxyl terminal (CT-GFP) domains. Together, these results clearly show that coronin-1 function is required for efficient phagocytosis. Given that transfection and protein knockdown by siRNA entail chronic alteration of coronin function that could indirectly affect a host of cellular events, we chose to perform all subsequent studies with TAT-WD, which allows acute inhibition of coronin-1 function.

TAT-WD Inhibits Actin Accumulation at the Nascent Phagosome

The mechanism by which coronin contributes to phagocytosis is not known. We therefore examined the effects of the inhibitory TAT-WD protein on the signaling events that follow binding of the opsonized RBCs to the cell surface. As shown in Figure 6a, control cells transfected with a GFP-labeled Fc γ IIa receptor and transduced with TAT- β Gal, demonstrated clustering and colocalization of the receptors with internalized RBCs. This behavior is identical to what is observed in untreated cells or when antibodies are used to detect the Fc γ receptor. Cells transduced with the TAT-WD protein also showed clear clustering of receptors at the base or adherent RBCs (Figure 6b, arrows), indicating that the failure of particle internalization was not due to lack of receptor clustering. However, the clustered receptors failed to extend into pseudopods and did not surround the particles, as noted in the controls.

Phosphotyrosine accumulation was detected at the nascent phagosomes in RAW 264.7 cells transduced with TAT- β Gal (arrow, Figure 6c), in agreement with earlier observations (Greenberg *et al.*, 1994). Of note, accumulation of

Figure 6. Effects of TAT-WD on Fc receptor signaling pathway of RAW 264.7 cells. RAW 264.7 cells expressing FcγRIIIa-GFP (a and b), PAK-PBD-YFP (g and h), PLCδ-PH-GFP (i and j), AKT-PH-GFP (k and l), C1δ-GFP (m and n), or both PLCδ-PH-CFP and C1δ-YFP (o and p), were treated with TAT-βgal (a, c, e, g, i, k, m, and o) or TAT-WD protein (b, d, f, h, j, l, n, and p), before allowing IgG-opsonized RBCs (a–l) or opsonized polystyrene beads (m–p) to bind for 10 min at 4°C. Unbound particles were washed away, RAW 264.7 cells were warmed to 37°C and phagocytosis continued for 10 min before fixation. Immunofluorescence assays were used to stain for phosphotyrosine (green, c and d) or actin (green, e and f) and total RBCs (red, a–l). Arrows indicate RBCs or polystyrene beads at phagocytic cups or in phagosomes. Panels i and j show 1-min time point (1'A), cup at 1 min (1'B), and 5-min time point (5'). Panels m–p indicate Golgi accumulation of C1δ-YFP (open arrowhead) is separate from phagocytic cup (filled arrowhead). Bars, 10 μm.

phosphotyrosine persisted at the base of bound RBCs in cells transduced with TAT-WD (arrows, Figure 6d), despite the inability of these cells to engulf the particles. These observations indicate that (at least some of) the signaling events downstream from the clustered receptors were occurring.

Interestingly, the actin accumulation normally observed in nascent phagosomes appeared to be impaired after TAT-WD transduction. In control cells (Figure 6e) F-actin accumulated at the base of bound particles and transiently

encircled the nascent phagosome, before dissipating. In contrast, no significant enrichment of F-actin was seen at the base of bound RBCs in cells transduced with TAT-WD (Figure 6f).

The polymerization of actin at the phagosomal cup is driven by small GTPases of the Rho family, notably Rac and Cdc42 in the case of FcγR-mediated phagocytosis (Caron and Hall, 1998). To pinpoint the cause of the impairment in F-actin polymerization in TAT-WD-treated cells, we ana-

lyzed the activation of Rac and Cdc42 during phagocytosis. To this end, RAW 264.7 cells were initially transfected with a YFP-tagged chimera of the p21-binding domain (PBD) of PAK, an effector of Rac and Cdc42 (Srinivasan *et al.*, 2003). The PAK-PBD-YFP chimera binds only to the active, GTP-bound form of Rac and Cdc42, and not to their inactive, GDP-associated counterparts. The transfected cells were next treated with the TAT-tagged constructs and analyzed by confocal microscopy. Remarkably, the PAK-PBD-YFP chimera accumulated at nascent phagosomes of cells transduced with either TAT- β Gal (Figure 6g) or TAT-WD (Figure 6h).

Phosphoinositide metabolism plays a central role in signaling and coordinating actin remodelling during phagocytosis. It was of interest to define whether the impairment of phagocytosis caused by inhibition of coronin-1 function was accompanied by changes in phosphoinositide content or dynamics. To this end we used a variety of GFP-coupled probes that reflect the distribution of phosphatidylinositides and their metabolites within the cell. As shown previously (Botelho *et al.*, 2000), PIP₂ detected with the PH domain of phospholipase C δ conjugated to GFP, transiently accumulated at the early stages of nascent phagosome (Figure 6i, inset 1'A, enlarged area shown in 1'B) and was rapidly depleted thereafter (Figure 6i, arrow). After treatment with TAT-WD, PIP₂ is similarly accumulated at the base of the phagosomal cup within 1 min of RBC binding (Figure 6j, insets A and B) but unlike the control case this accumulation persisted for more than 5 min (Figure 6j, arrows) and the secondary depletion was never observed. PIP₃ is produced as a downstream product of PIP₂ through the action of PI-3 Kinase and it can be specifically detected by the PH domain of Akt fused to GFP. As shown in Figure 6k, in otherwise untreated cells PIP₃ transiently accumulated around forming phagosomes and PIP₃ is also seen at the base of RBCs bound to cells treated with TAT-WD (Figure 6l). Thus, the coronin-1 fragment did not noticeably impair phosphatidylinositol 3-kinase, and this mechanism cannot account for the abnormal persistence of PIP₂ in the phagocytic cup.

Diacylglycerol (DAG) is predominantly found in association with the Golgi complex in quiescent cells (Figure 6, m-p, arrowheads). During activation by phagocytic particles DAG is also found transiently at the nascent phagosome, where it is likely generated by phospholipase C-mediated hydrolysis of PIP₂ (Botelho *et al.*, 2000; Figure 6m, arrows). In TAT-WD treated cells, no appearance of DAG was observed at the sites of aborted phagocytosis. This was shown more clearly by cotransfection of cells with PLC δ -PH-CFP, to detect PIP₂, and C1 δ -YFP, which labels DAG. In TAT- β Gal-treated control cells (Figure 6o) DAG can be seen around a particle that had been internalized (arrow) from which the PIP₂ signal had been lost. Only PIP₂ was detectable in the cups aborted by exposure to TAT-WD (Figure 6p).

Arp3-GFP Accumulation Is Blocked by TAT-WD

The observation that actin accumulation was impaired despite the presence of active Rac/Cdc42 and accumulations of PIP₂ and PIP₃ at the phagosome suggested that a signaling event downstream from the Rho-family GTPases was inhibited. Given the existing evidence that coronins can associate with components of the Arp2/3 complex (Machesky and Hall, 1997; Humphries *et al.*, 2002), we set out to determine if TAT-WD affected Arp2/3 complex recruitment to sites of phagocytosis. To monitor the Arp2/3 complex, RAW 264.7 cells were transiently transfected with Arp3-GFP, then transduced with either TAT- β Gal or TAT-WD, and finally incubated with opsonized sheep RBCs. As shown in Figure 7, a, c, and g, significant accumulation of Arp3-GFP was ob-

Figure 7. Effects of TAT-WD on Arp3 accumulation at the phagocytic cup. RAW264.7 cells expressing Arp3GFP alone (a–d) or together with CorRFP (e–h) were treated with TAT- β Gal (a, c, e, and g) or TAT-WD protein (b, d, f, and h) for 1 h before the addition of human IgG-opsonized polystyrene beads. After 10 min of binding in ice-cold buffer, unbound beads were washed away, and cells were warmed to 37°C to allow phagocytosis to proceed for 3 min (a) or 5 min (b–h) before fixation. Arrows indicate bound particles. Differential interference contrast (d) shows particles bound to TAT-WD-treated cell. Bars, 10 μ m.

served at the base of adherent RBCs in cells transduced with TAT- β Gal, reported earlier in untreated phagocytes (May *et al.*, 2000). Importantly, Arp3 recruitment to the phagocytic cup was completely blocked after transduction with TAT-WD (Figure 7b). This suggests that the inhibition of actin polymerization by TAT-WD may be due to the lack of actin nucleating and branching activity provided by Arp2/3. When cells were transfected with full-length coronin-1 conjugated to monomeric red fluorescent protein (Campbell *et*

al., 2002), and Arp3 conjugated to GFP, we could see clear colocalization of these proteins in cells treated with TAT- β gal (Figure 7, e and g). In contrast, in cells treated with TAT-WD, there was accumulation of coronin-1 to the base of the bound particle (Figure 7f, arrows), but no accumulation of Arp3 was detected (Figure 7h, arrows).

TAT-WD Does Not Inhibit *Salmonella* Invasion

To determine if coronin-1 was required for all actin-dependent processes, we examined the effect of TAT-WD on invasion by *Salmonella enterica* serovar typhimurium. *Salmonella* species invade mammalian cells by inducing the formation of large, actin-rich membrane lamella that ultimately engulf the bacteria into large vacuoles. In the case of RAW 264.7 cells, internalization could in principle be due either to invasion driven by the bacteria or to phagocytosis initiated by the macrophage. To control for the latter, we utilized a mutant strain of *Salmonella* lacking InvA, a component of *Salmonella* pathogenicity island 1 that is essential for invasion. These bacteria can only enter RAW 264.7 cells by phagocytosis. As shown in Figure 8, a and b, coronin-GFP accumulates at the sites of bacterial internalization for both wild-type and InvA strains (arrows). However, TAT-WD had essentially no effect on the internalization of wild-type *Salmonella* compared with TAT- β Gal (Figure 8c), whereas the internalization of InvA mutants by phagocytosis was significantly reduced (Figure 8d). Together, these results suggest that while phagocytosis requires a function of coronin-1 inhibited by TAT-WD, *Salmonella* invasion occurs independently of this function.

DISCUSSION

In this study we have shown that coronin-1 accumulates rapidly and transiently at the forming phagosome. The appearance of coronin-1 coincided with the accumulation of actin, consistent with the fact that coronin-1 is an actin-binding protein. Coronin-1 (also known as Taco) had previously been shown to accumulate around phagosomes formed during the ingestion of mycobacteria (Ferrari *et al.*, 1999; Schuller *et al.*, 2001). Persistent accumulation of coronin-1 around phagosomes containing mycobacteria was originally speculated to prevent phagosome maturation and mycobacterial killing (Ferrari *et al.*, 1999). On the other hand, biochemical studies have shown that coronin-1 is only associated with the early stages of mycobacterial phagocytosis and is not associated with late phagosomes whose maturation have been arrested (Schuller *et al.*, 2001).

Overexpression of wild-type coronin-1 (with or without an attached GFP tag) did not appear to significantly alter the morphology or properties of RAW 264.7 cells. Conversely, expression of the central WD domain repeat region dramatically altered the morphology of the cells by reducing the number of focal contacts, actin-based attachment structures responsible for adhesion of the cells to the substratum. This is consistent with previous studies suggesting that the WD domain dominantly interferes with coronin-1 function (Mishima and Nishida, 1999). WD domains are predicted to act as multimolecular scaffolding domains by bringing together interacting proteins on a single surface (Usacheva *et al.*, 2003). The isolated WD domains are likely to interfere with normal cell function by scavenging interacting proteins away from the endogenous, full-length coronin. The studies presented here suggest that the Arp2/3 complex may be among those proteins that interact with coronin-1. Coronin-1 recruitment to the site of receptor engagement was not blocked by the WD domain, suggesting that other portions

Figure 8. Effect of TAT-WD on invasion and phagocytosis of *Salmonella*. RAW 264.7 cells were transfected with coronin-GFP and treated with wild-type *Salmonella* (a) or invasion-defective *Salmonella* (InvA; b). Accumulation of GFP at the sites of invasion is shown. Bacteria were localized by detection of the red fluorescence of endogenously expressed dsRed (unpublished data) and their location indicated with arrows. Live confocal images were taken every 10 s. Bar, 10 μ m. Internalized *Salmonella* particles, whether taken up by phagocytosis or invasion, were quantified as follows: RAW 264.7 cells were pretreated with TAT- β Gal or TAT-WD and then overlaid with wild-type *Salmonella* (c), or invasion-defective *Salmonella* InvA (d). Data are means $\pm$ SE of three experiments; * p < 0.05 with at least 30 cells counted in each.

of the protein are responsible for its recruitment to sites of actin remodeling.

Interestingly, the subcortical F-actin that lines the plasma membrane persisted seemingly unaffected in cells treated with the inhibitory construct, suggesting that the dominant-negative effect of the WD repeats did not impact all actin-based processes. This is consistent with observations from *Dictyostelium*, where coronin appears to contribute to some but not all actin-based functions. For example, cell division in *Dictyostelium* involves two processes: actomyosin-based constriction at the midplane and actin-mediated migration of the cells away from each other. Coronin is normally colocalized with actin at the distal leading edges of dividing cells where it is required for traction-mediated division, but not at the actinomyosin cleavage furrow (deHostos *et al.*, 1993). Interestingly, Arp2/3 is also recruited to the leading edges of the cell in this process but not associated with the cleavage furrow, reinforcing the relationship between this actin regulator and coronin (Insall *et al.*, 2001).

The WD domains of coronin-1 were able to inhibit two forms of phagocytosis, immunoglobulin-mediated uptake

via the Fc γ receptor and complement-mediated uptake via the CR3 receptor, yet these have been shown to signal actin accumulation via distinct pathways. CR3 receptor-mediated phagocytosis occurs via a Rho-dependent process that causes bound particles to be drawn into the cell, whereas the Fc γ receptor activates Cdc42 and Rac to produce large lamellipodia that engulf the particle (Caron and Hall, 1998). The ability of TAT-WD to inhibit both forms of phagocytosis suggests that the function mediated by coronin-1 must reside downstream of the small GTPase step. Both CR3 and Fc γ receptors require the activity of the Arp2/3 complex to support phagocytosis (May *et al.*, 2000).

The role of Arp2/3 in invasion of cells by *Salmonella* is less clearly understood. One report has suggested that the Arp2/3 complex is required for *Salmonella* invasion in polarized MDCK cells (Criss and Casanova, 2003). However, this was demonstrated using overexpression of the W and A domains of Scar1, which causes unregulated actin polymerization. Although this may inhibit Arp2/3 by preventing its recruitment to the site of invasion, it could also lead to depletion of other actin effectors and potentially titrate available G-actin itself, leading to inhibition of invasion by indirect means. In contrast, there is evidence to suggest that *Salmonella* may express effectors that could circumvent the need for Arp2/3 in this process. Among the bacterial effector proteins that participate in invasion are the four Sips (*Salmonella* invasion proteins), of which SipC appears able to bundle actin and promote nucleation in vitro (Hayward and Koronakis, 1999). This protein is injected into the eukaryotic cell and associates with the membrane near the site of infection and its activity is strongly enhanced by SipA (McGhie *et al.*, 2001). SipA also stabilizes actin filaments by arresting cellular mechanisms of actin turnover. SipA binding to actin blocks the binding of ADF and cofilin and prevents the severing activity of gelsolin (McGhie *et al.*, 2001). Based on these and other structural considerations, Hayward and Koronakis (2002) have speculated that SipC and its activator SipA may function like the Arp2/3 complex and its activator WASp, respectively, and may promote actin assembly independently of cellular mechanisms. Our failure to inhibit *Salmonella* invasion after transduction with TAT-WD would be consistent with the notion that although TAT-WD may inhibit Arp2/3 function, bacterial effectors may be able to provide the nucleation activity normally triggered by this complex.

Taken together with previous studies linking coronin and Arp2/3, our data suggest that coronin-1 may be important for the recruitment of the Arp2/3 complex to the sites of dynamic actin remodeling and that the WD domain of coronin-1 acts in a dominant-negative manner by preventing such accumulation. This would suggest that coronin-1 plays a positive role in actin polymerization, in contrast to previous results from yeast that suggested coronin acted as an inhibitor of Arp2/3 function to prevent branching of actin filaments (Humphries *et al.*, 2002). Future studies will be needed to identify coronin-1 interaction partners and determine precisely how it regulates actin dynamics and Arp2/3 function.

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