

# An Essential Role for Talin during $\alpha_M\beta_2$ -mediated Phagocytosis

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The cytoskeletal, actin-binding protein talin has been previously implicated in phagocytosis in *Dictyostelium discoideum* and mammalian phagocytes. However, its mechanism of action during internalization is not understood. Our data confirm that endogenous talin can occasionally be found at phagosomes forming around IgG- and C3bi-opsonized red blood cells in macrophages. Remarkably, talin knockdown specifically abrogates uptake through complement receptor 3 (CR3, CD11b/CD18,  $\alpha_M\beta_2$  integrin) and not through the Fc  $\gamma$  receptor. We show that talin physically interacts with CR3/ $\alpha_M\beta_2$  and that this interaction involves the talin head domain and residues W747 and F754 in the  $\beta_2$  integrin cytoplasmic domain. The CR3/ $\alpha_M\beta_2$ -talin head interaction controls not only talin recruitment to forming phagosomes but also CR3/ $\alpha_M\beta_2$  binding activity, both in macrophages and transfected fibroblasts. However, the talin head domain alone cannot support phagocytosis. Our results establish for the first time at least two distinct roles for talin during CR3/ $\alpha_M\beta_2$ -mediated phagocytosis, most noticeably activation of the CR3/ $\alpha_M\beta_2$  receptor and phagocytic uptake.

## INTRODUCTION

Phagocytosis is an essential physiological function, common to most eukaryotic cell types. From serving a feeding role in amoebae, phagocytosis is observed in Metazoa as a homeostatic process that ensures the removal of microorganisms and apoptotic cells (Desjardins *et al.*, 2005). Classically, phagocytosis is a multistep process that sequentially involves receptor-mediated particle recognition, actin-driven uptake, phagosome maturation and particle clearance. Numerous phagocytic receptors exist that can bind their target directly or indirectly through opsonins (Underhill and Ozinsky, 2002). Receptors for phagocytosis can show constitutive or inducible binding activities, as illustrated for the two best-characterized phagocytic receptors: the Fc  $\gamma$  receptor (Fc $\gamma$ R) for complexed IgG and complement receptor 3 (CR3, CD11b/CD18,  $\alpha_M\beta_2$  integrin), respectively (Bianco *et al.*, 1975). Ligand-bound receptors classically zipper around the phagocytic prey and induce intracellular signaling cascades that lead to the activation and recruitment of signaling and adaptor molecules at sites of particle binding. These locally assembled signaling complexes reorganize the actin cytoskeleton and regulate membrane dynamics underneath bound particles through the activation of Rho- and Arf-family GTP-binding proteins, respectively (Cougoule *et al.*, 2004). According to the zipper model, phagocytosis of bound particles

requires continual ligation of phagocytic receptors around the whole phagocytic object, at least for spherical particles (Griffin *et al.*, 1975; Champion and Mitragotri, 2006).

Several cytoskeletal proteins have been shown to be recruited to phagocytic cups, although their role is not always defined. Talin, a cytoskeletal protein of 2541 amino acids and 270 kDa has been repeatedly implicated in phagocytosis. Immunofluorescence studies of phagocytosing macrophages have shown that talin accumulates transiently around IgG-opsonized red blood cells, unopsonized zymosan, and *Leishmania* amastigotes. It also colocalizes with F-actin during the early stages of uptake (Greenberg *et al.*, 1990; Allen and Aderem, 1995, 1996; Love *et al.*, 1998). These data suggest a general role for talin in phagocytosis, because each type of particle ligates different phagocytic receptors. This hypothesis is supported by recent data using *Dictyostelium discoideum* talin-null mutants, which showed a slower rate of uptake than wild-type (wt) cells for both heat-killed yeast particles and latex beads (Niewohner *et al.*, 1997; Gebbie *et al.*, 2004). Nevertheless, the exact role of talin in mammalian phagocytosis remains elusive.

There are two talin genes in mammals (Monkley *et al.*, 2001)—talin-1 and talin-2, which are 74% identical at the protein level—and apparently only one gene in *Drosophila* and *Caenorhabditis elegans*. The talin molecule is composed of two main regions: the N-terminal head region (ca. 50 kDa) contains a FERM (band 4.1, ezrin, radixin, moesin) domain, which binds to the cytoplasmic domain of  $\beta$ -integrin subunits and layilin, a C-type lectin, whereas the large rod domain harbors F-actin- and vinculin-binding sites (Critchley, 2005). Studies in a variety of cell systems and organisms suggest that talin can play distinct cellular roles in different contexts. Indeed, it has been shown to provide a physical link between integrin receptors and the cytoskeleton (Giannone *et al.*, 2003), to regulate the conformation of transmembrane

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receptors (Tadokoro *et al.*, 2003), and to support the assembly of signaling complexes (Calderwood and Ginsberg, 2003; Nayal *et al.*, 2004; Tanentzapf *et al.*, 2006).

Herein, we confirm that talin is transiently recruited to different types of particles during phagocytosis, specifically after ligation of the  $\alpha_M\beta_2$  integrin and the Fc $\gamma$ R in mammalian macrophages. We show that talin is essential for  $\alpha_M\beta_2$ - but not Fc $\gamma$ R-mediated phagocytosis. Furthermore, we show that talin interaction with the  $\beta_2$  integrin cytoplasmic domain of  $\alpha_M\beta_2$  is required for optimal binding of C3bi-opsonized particles and that it has a dramatic albeit secondary influence on phagocytic uptake. Our results therefore establish talin as an essential regulator of integrin-dependent engulfment in mammalian phagocytes.

## MATERIALS AND METHODS

### Reagents

Sheep red blood cells (RBCs) were purchased from TCS Biosciences (Buckingham, Buckinghamshire, United Kingdom). EZ-Link-Sulfo-NHS-Biotin and streptavidin conjugated to horseradish peroxidase (HRP) were purchased from Pierce Chemical (Rockford, IL). Rhodamine-phalloidin, gelatin veronal buffer, protein G-agarose, and C5-deficient serum were from Sigma Chemical (Poole, Dorset, United Kingdom).

The antibodies used in this study were mouse anti-talin (clone 8d4; Sigma Chemical), rat anti- $\alpha_M$  (clone 5c6; Serotec, Oxford, United Kingdom), mouse anti-human  $\alpha_M$  (ICRF44; BD Biosciences Pharmingen, San Diego, CA), mouse anti-human  $\beta_2$  (clone 6.7; BD Biosciences Pharmingen), mouse anti-green fluorescent protein (GFP) (clone JL-8; Clontech, Mountain View, CA), mouse anti-tubulin (clone tub2.1; Sigma Chemical), and rabbit IgM anti-sheep RBC antibodies (Cedarlane Laboratories, Hornsby, Ontario, Canada). Conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA) (immunofluorescence) or GE Healthcare (Little Chalfont, Buckinghamshire, United Kingdom) (Western blotting).

### DNA Constructs

Eukaryotic expression vectors (pRK5) encoding human wt and mutant  $\alpha_M$  and  $\beta_2$  were described previously (Caron and Hall, 1998; Wiedemann *et al.*, 2006). pCRE-Pac, pRKGFP-Talin, and pRKGFP-TalinHead (GFP-tagged talin head; GFP<sup>TH</sup>) were kindly provided by Takeshi Yagi (National Institute for Physiological Sciences, Aichi, Japan), Kazuo Matsumoto (National Institutes of Health, Bethesda, MD) and Neil Bate (Leicester University, Leicester, United Kingdom), respectively.

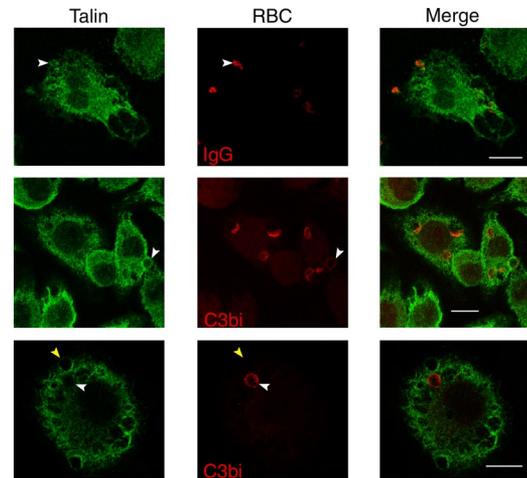
To generate the  $\beta_2$  W747A and F754A mutants, mutations were introduced into pRK5- $\beta_2$  by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), by using the following combinations of primers (mutation underlined): W747A, 5'-CTCAAGTCCCAGGCGAACAATGATAATCCC-3' and 5'-GGGATTATCATTGTCGCGCTGGGACTTGAG-3'; and F754A, 5'-AATGATAATCCCCTTGCAAGAGCGCCACCACG-3' and 5'-CGTGGTGGCGCTCTTGGCAAGGGGATTATCATT-3'.

Glutathione S-transferase (GST) fusions of the wt and mutant cytoplasmic tails (GST- $\beta_2$ cyt,  $\beta_2$ cytW747A,  $\beta_2$ cytF754A, and  $\beta_2$ cytF766A, respectively) were made by polymerase chain reaction (PCR) from the corresponding pRK5- $\beta_2$  constructs, by using the following primers: 5'-GGGGGGGATCCCAAGGCTCTGATC-CAC-3' and 5'-GGGGGGAATTCCTAACTCTCAGCAGCCTTGGGGTTCAT-3' for  $\beta_2$ cytF766A; 5'-GGGGGGGAAATTCCTACTAACTCTCAGCAAACTT-3' for the other GST fusions (restriction sites underlined). Amplified fragments were digested as appropriate, cloned into the pGEX-4T2 expression vector (GE Healthcare), and transformed into *Escherichia coli* BL21.

GFP fusions of the wt and mutant cytoplasmic tails (GFP- $\beta_2$ cyt and  $\beta_2$ cytF754A, respectively) were made by PCR from the corresponding pRK5 constructs, by using as primers 5'-GGGGGGCTCGAGCTAAGGCTCTGATC-CAC-3' and 5'-GGGGGGGAAATTCCTACTAACTCTCAGCAAACTT-3' (restriction sites underlined). Amplified fragments were digested and cloned into the pEGFP-C1 expression vector (Clontech). All products were transformed into One Shot TOP10 chemically competent *E. coli* (Invitrogen, Carlsbad, CA) and checked by DNA sequencing (MWG Biotech, High Point, NC). DNA was later prepared using the QIAGEN maxi-prep kit (QIAGEN, Valencia, CA) (note: Endofree kits were used for macrophage transfections).

### Cell Culture and Transfection

Cells from the murine macrophage J774.A1 and simian kidney fibroblast COS-7 cell (nos. TIB-67 and CRL-1651, respectively; American Type Culture Collection, Manassas, VA) were maintained and seeded as described previously (Caron and Hall, 1998). RAW 264.7 (ATCC no. TIB-7) and talin conditional knockout mouse embryo fibroblasts (Critchley, 2005) were maintained in DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal bovine



**Figure 1.** Endogenous talin localizes to sites of RBC binding in mouse macrophages. J774.A1 mouse macrophages were challenged with IgG-RBC (top row) for 5 min or with C3bi-RBCs (middle and bottom rows) for 30 min at 37°C, processed for immunofluorescence, and analyzed by confocal microscopy as described in *Materials and Methods*. Talin localizes occasionally around particles (white arrowheads), but it can also be seen enriched at seemingly empty vacuoles (yellow arrowhead). Bar, 10  $\mu$ m.

serum (PAA Laboratories, Coelbe, Germany). COS-7 cells were transfected using the DEAE-dextran method (Caron and Hall, 1998), talin conditional knockout cells were transfected using SuperFect (QIAGEN). RAW 264.7 cells were transfected by nucleofection (program D-32; Amaxa Biosystems, Gaithersburg, MD) and left to express constructs for 24 h before phagocytic challenge.

J774 macrophages ( $3 \times 10^5$ ) were transfected with 200 nM small-interfering RNA (siRNA) (pool of 4 siRNAs directed against talin-1, accession no. NM\_011602, or siCONTROL NonTarget siRNA pool; Dharmacon RNA Technologies, Lafayette, CO) or mock transfected by using Lipofectamine (Invitrogen) and assayed 48 h later as recommended by the manufacturer.

### Flow Cytometry

Macrophages or transfected COS-7 cells were washed in 0.5% bovine serum albumin, 0.02% sodium azide, and phosphate-buffered saline (PBS) and stained to detect surface  $\beta_2$  by using a combination of mouse anti- $\beta_2$  antibodies and Cy2-conjugated goat anti-mouse antibodies. The relative fluorescence of gated cells was analyzed using a FACSCalibur analyzer (BD Biosciences, San Jose, CA).

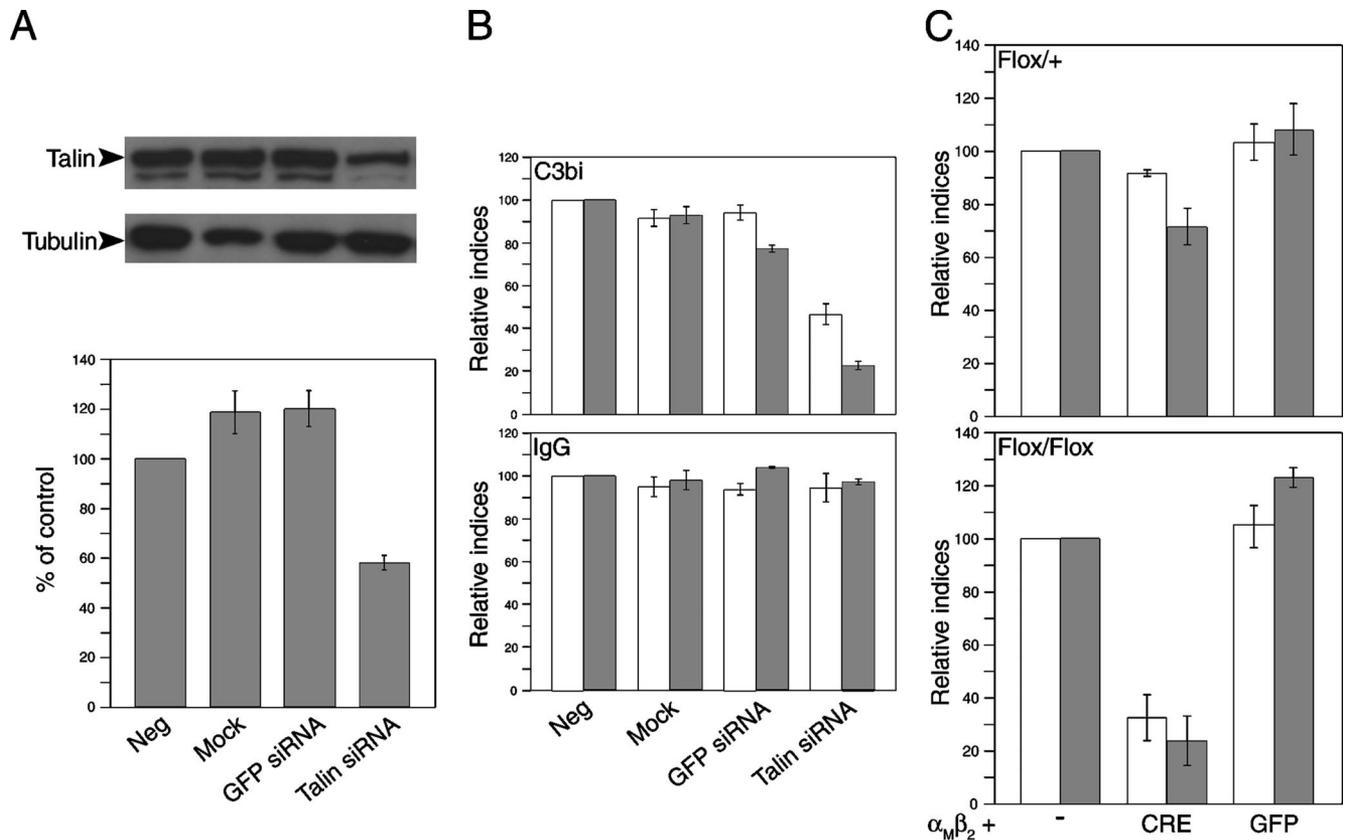
### Phagocytic Challenge

IgG-opsonized and C3bi-opsonized RBCs (later referred to as IgG- and C3bi-RBCs, respectively) were prepared and used as described previously (Caron and Hall, 1998; Wiedemann *et al.*, 2006) by using 0.1  $\mu$ l (0.5  $\mu$ l for macrophages) of fresh RBCs per 13-mm coverslip. For efficient binding and phagocytosis of C3bi-opsonized RBCs, macrophages require preactivation (Wright and Jong, 1986; Caron *et al.*, 2000), i.e., pretreatment with 150 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma Chemical) in HEPES-buffered, serum-free DMEM for 15 min at 37°C. Cells were then challenged with C3bi-RBCs for 30 min at 37°C, washed with PBS to remove unbound RBCs, and fixed in cold 4% paraformaldehyde for 10 min at 4°C.

### Immunofluorescence and Scoring

Different staining procedures helped to differentiate internalized from total associated RBCs. Because all RBCs were opsonized with rabbit Ig, cells were incubated with rhodamine red X-conjugated donkey anti-rabbit antibodies, permeabilized with 0.2% Triton X-100, and incubated with Cy2-conjugated donkey anti-rabbit antibodies. In transfection experiments, only cells expressing surface  $\beta_2$  and GFP were scored. Internalized particles, which were red, were easily distinguishable from extracellular RBCs, which were yellow. The association and phagocytic indices, respectively, are defined as the number of RBCs bound to and engulfed by 100 phagocytes. Coverslips were finally mounted in Mowiol (Calbiochem, San Diego, CA) containing *p*-phenylene diamine (Sigma Chemical) as antifading reagent, and they were analyzed by microscopy.

The enrichment in TalinH/Talin/ $\beta_2$  at sites of RBC binding was studied and scored by confocal microscopy (LSM510; Carl Zeiss, Jena, Germany). For each experiment, at least 20 transfected cells per condition were analyzed for



**Figure 2.** Talin is essential for RBC binding and phagocytosis during  $\alpha_M\beta_2$ -mediated uptake, but it is dispensable for IgG-dependent phagocytosis of RBCs. (A and B) J774.A1 macrophages were transfected with pools (200 nM total) of talin- or GFP-specific siRNA as indicated. Forty-eight hours later, they were analyzed for talin expression (A) and binding and phagocytosis of IgG- and C3bi-RBCs (B). (A) Lysates of control, mock-, and siRNA-transfected cells were analyzed by Western blotting for the presence of talin and tubulin (top). Bottom, relative band intensities were determined as described in *Materials and Methods*, with the ratio of talin and tubulin intensities set to 100% for the negative control. (B) Association (open bars) and phagocytosis (closed bars) indices of J774 macrophages challenged with either C3bi- (top) or IgG- (bottom) RBCs. Indices were related to the values obtained from the negative controls (phagocytic indices of  $110.5 \pm 5.7$  and  $131.5 \pm 2.1$  for C3bi- and IgG-RBCs, respectively). (C) Conditional talin knockout mouse embryonic fibroblasts (Flox/+, top; Flox/Flox, bottom) were transfected with plasmids (- for empty vector) as indicated, challenged for 30 min with C3bi-RBCs, processed for immunofluorescence, stained for surface-expressed  $\beta_2$  and RBCs, and the  $\beta_2$ -expressing cells were scored for RBC association (open bars) and phagocytosis (closed bars). Association and phagocytosis indices were related to the  $\alpha_M\beta_2$  control (phagocytic index of  $185.5 \pm 1.4$ ). Results are expressed as the mean  $\pm$  SD of at least three independent experiments.

a discrete local enrichment in marker signal at bound RBCs. Phagosomes were scored as positive when at least a quarter of the underlying/surrounding area showed significant enrichment, compared with the neighboring areas.

### Protein Expression and GST Pull-Down Assay

Protein expression was induced in subcultures of *E. coli* BL21 expressing various  $\beta_2$  cytoplasmic tails ( $\beta_2$ cyt) constructs in pGEX-4T2 with 0.5 mM isopropyl  $\beta$ -D-thiogalactoside for 2 h at 37°C. Cells were harvested by centrifugation, resuspended in 50 mM Tris, pH 8, 40 mM EDTA, 25% sucrose, 100 mM MgCl<sub>2</sub>, 0.2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), and Complete protease inhibitor cocktail (Roche Applied Science, East Sussex, United Kingdom) and sonicated. After clearing, fusion proteins were affinity purified from the soluble fraction on glutathione-Sepharose 4B beads (GE Healthcare) according to the manufacturer's instructions.

J774.A1 or transfected COS-7 cells were lysed on ice in lysis buffer (10% glycerol, 1% NP-40, 50 mM Tris, pH 7.6, 200 mM NaCl, 2.5 mM MgCl<sub>2</sub>, 1 mM PMSF, and Complete protease inhibitor cocktail). Lysates were incubated for 2 h at 4°C with a 50% slurry of glutathione-Sepharose 4B beads coupled to 15  $\mu$ g of GST or GST fusion proteins. Beads were washed three times in cold lysis buffer before analysis by SDS-PAGE and Western blotting. Anti-GFP or anti-talin antibodies (both diluted 1:1000) were added for 1 h each, followed by goat anti-mouse HRP. Detection was carried out using the enhanced chemiluminescence detection kit (GE Healthcare). Intensities of bands were determined by densitometric analysis by using the ImageJ software (National Institutes of Health) and related to the levels of GST or GST fusions.

### Immunoprecipitation

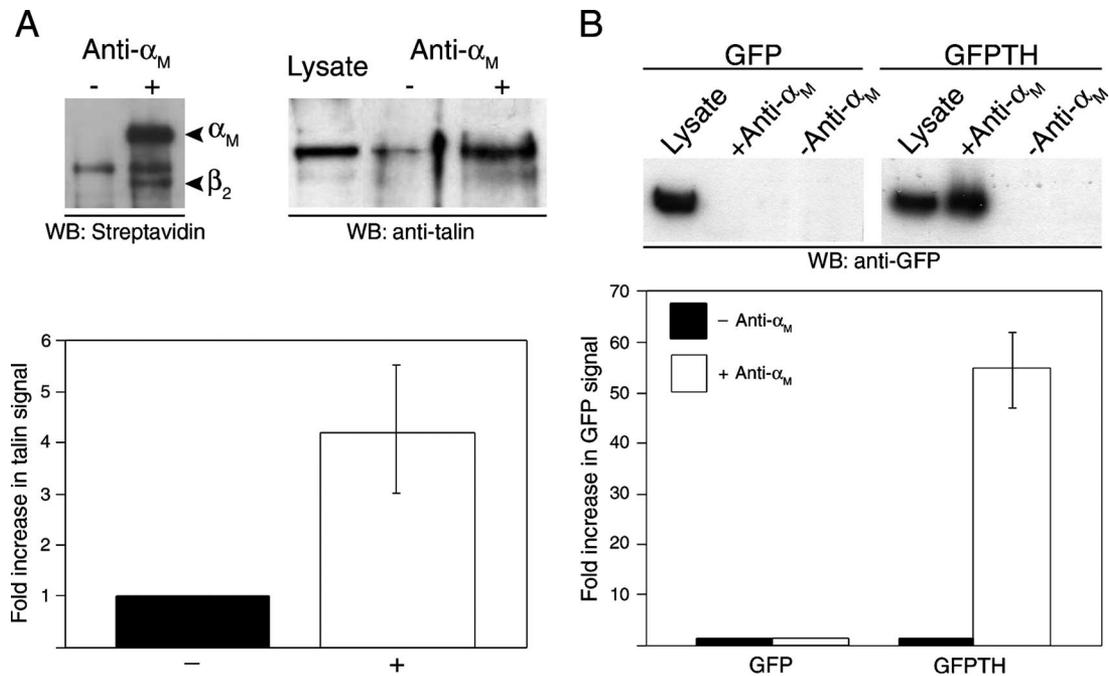
Serum-starved J774.A1 or transfected COS-7 cells were surface biotinylated with 0.5 mg/ml EZ-Link-Sulfo-NHS-biotin for 1 h at 4°C and then lysed on ice

as described above. Lysates were incubated for 2 h at 4°C with anti- $\alpha_M$  antibody and protein G-agarose, followed by three washes in cold lysis buffer. Beads and lysates were analyzed by SDS-PAGE and Western blotting as described above. Immunoprecipitation of  $\alpha_M\beta_2$  was confirmed using streptavidin-HRP. Intensities of bands were determined as described above and related to the levels of talin, GFP, or GFP<sup>TH</sup>.

## RESULTS

### Talin Localizes to Sites of Particle Binding during Fc $\gamma$ R- and $\alpha_M\beta_2$ -mediated Phagocytosis in Mouse Macrophages

Talin regulates phagocytosis in *Dictyostelium* (Niewohner *et al.*, 1997) and is recruited to sites of phagocytic uptake in mammalian macrophages (Greenberg *et al.*, 1990). To establish the role of talin during mammalian phagocytosis, we first sought to confirm the recruitment of talin at sites of uptake. We used the monoclonal anti-talin antibody 8d4, which readily stained focal adhesions in normal but not talin-deficient mouse embryonic fibroblasts (data not shown). We challenged J774.A1 mouse macrophages with either C3bi- or IgG-RBCs to promote uptake via  $\alpha_M\beta_2$  or Fc $\gamma$ R, respectively (Figure 1). There were clear examples of talin recruitment to both types of bound RBCs as observed by confocal microscopy. However, their frequency was low with only a maximum of  $24.4 \pm 3.1\%$  talin-positive phago-



**Figure 3.**  $\alpha_M\beta_2$  integrins and talin interact biochemically in phagocytes. Lysates of surface-biotinylated J774.A1 mouse macrophages (A) or COS-7 cells expressing either GFP or GFP-TH (B) were incubated with equal amounts of beads coated with or without an anti- $\alpha_M$  mAb (5c6). Pellet-associated proteins were separated by SDS-PAGE and analyzed by Western blotting by using streptavidin-HRP (A, top left), anti-talin (A, top right), or anti-GFP antibodies (B). Corresponding band intensities were quantified and shown below, with the negative control value (no antibody or GFP) arbitrarily set to 1. Results are expressed as the mean  $\pm$  SD of at least three independent experiments.

somes for IgG-RBCs and  $21.8 \pm 1.8\%$  recruitment for C3bi-RBCs observed 5 min after RBC challenge. By comparison,  $69.3 \pm 2.5\%$  of the phagosomes forming around IgG-RBC were enriched in F-actin (Cougoule *et al.*, 2006) and  $68 \pm 2.2\%$  of phagosomes forming or formed around C3bi-RBCs showed  $\alpha_M\beta_2$  recruitment at the same time points. Altogether, these data are in line with previous observations that talin is transiently recruited to forming phagosomes, and we extend these findings to  $\alpha_M\beta_2$ -mediated phagocytosis. Puzzlingly, talin was also enriched around empty vacuoles (Figure 1, bottom row), suggesting that talin can redistribute to other sites at the plasma membrane during phagocytic challenge.

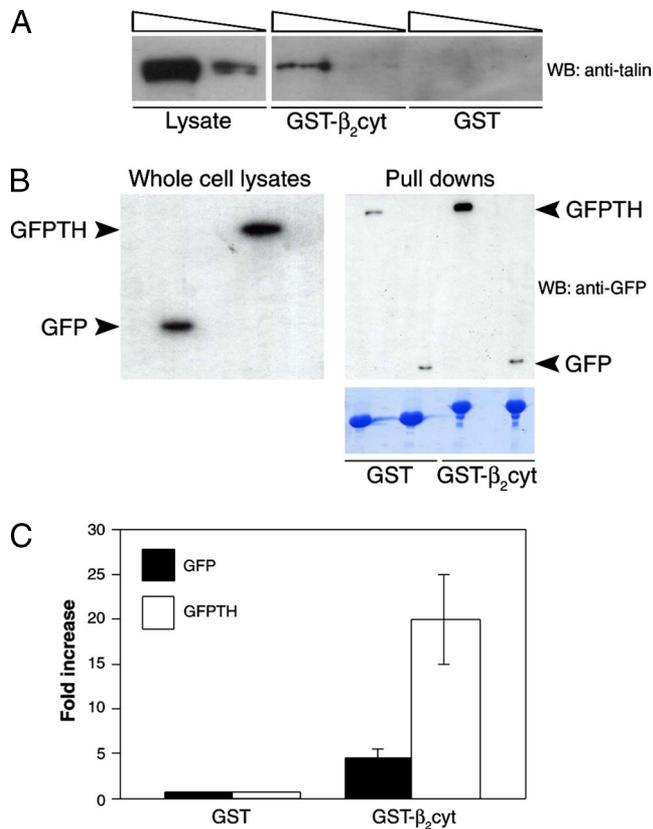
#### Essential Role for Talin in $\alpha_M\beta_2$ -dependent Binding and Phagocytosis

To test the functional role of talin during phagocytosis, we studied the phagocytic properties of talin-deficient cells. J774.A1 macrophages were transfected with control and talin-specific siRNA and analyzed for talin expression by Western blotting (Figure 2A) or challenged with opsonized RBCs and scored for binding and phagocytosis (Figure 2B). We observed a reduction in overall talin expression of around 40% in talin siRNA-knocked down J774.A1 cells but not in control J774.A1 cells (Figure 2A). This significant decrease in talin protein levels was accompanied by a potent inhibition of C3bi-RBC phagocytosis (Figure 2B). By contrast, talin knockdown had no effect on Fc $\gamma$ R-mediated uptake in these conditions, suggesting a preferential involvement of talin in  $\alpha_M\beta_2$ -dependent phagocytosis. Importantly, inhibition of  $\alpha_M\beta_2$ -dependent internalization was accompanied by a parallel decrease in RBC binding, whereas talin siRNA had no effect on binding of IgG-RBCs to Fc $\gamma$ R. These results suggest a specific role for talin in regulating the ability of  $\alpha_M\beta_2$  to bind C3bi-RBCs. To confirm this result, we

made use of conditional talin knockout mouse embryo fibroblast (MEFs) cell lines (Figure 2C). In these cells, one or both copies of the talin-1 gene are flanked by Flox sequences, which can be excised by Cre recombinase (Cre). In heterozygous Flox/+ cells, coexpression of Cre with  $\alpha_M\beta_2$  had little effect on RBC binding, compared with control cells ( $\alpha_M\beta_2$ ; no Cre), although there was a slight decrease in phagocytosis. However, overexpression of Cre and  $\alpha_M\beta_2$  in Flox/Flox cells strongly impaired RBC binding (33% of control) and phagocytosis (24% of control). This effect was specifically due to Cre expression, because the GFP transfection control showed no deficiency in binding or phagocytosis (Figure 2C). These data confirm the siRNA data obtained in macrophages and demonstrate an essential role for talin during  $\alpha_M\beta_2$ -mediated uptake, most likely due to the regulation by talin of the binding activity of this phagocytic receptor.

#### $\alpha_M\beta_2$ and Talin Interact in Macrophages and $\alpha_M\beta_2$ -expressing COS-7 Cells

Because of the role of talin in  $\alpha_M\beta_2$  binding activity and phagocytosis, we next examined whether  $\alpha_M\beta_2$  interacted with talin biochemically. To confirm that the 5c6 anti- $\alpha_M$  antibody can immunoprecipitate  $\alpha_M\beta_2$  (Rosen and Gordon, 1990; van Gisbergen *et al.*, 2005), lysates from surface-biotinylated J774 cells were mixed with the 5c6 antibody, and protein G-agarose and associated proteins were separated by SDS-PAGE. Probing Western blot membranes with streptavidin-HRP revealed the presence of the two bands characteristic of the chains of  $\alpha_M\beta_2$  ( $\alpha_M$ , ca. 160 kDa and  $\beta_2$ , ca. 100 kDa). Endogenous talin was specifically coimmunoprecipitated with  $\alpha_M\beta_2$  (Figure 3A). Because talin head domain interacts with the cytoplasmic tail of various  $\beta$  integrins (Garcia-Alvarez *et al.*, 2003; Calderwood, 2004), we tested whether it would also coimmunoprecipitate with



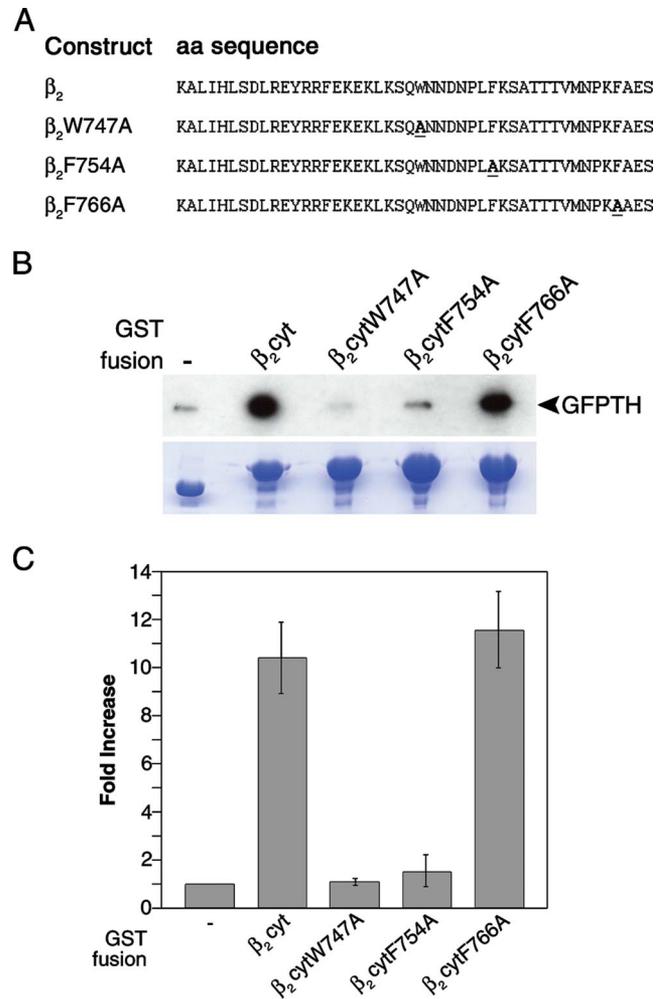
**Figure 4.** Talin interacts through its head domain with the  $\beta_2$  tail in pull-down assays. Cell lysates of J774.A1 mouse macrophages (A) or COS-7 cells expressing either GFP or GFP<sup>PTH</sup> (B and C) were mixed with equal amounts of either GST or GST- $\beta_2$ cyt coupled to glutathione-Sepharose beads. Precipitated proteins were separated by SDS-PAGE and analyzed by Western blotting by using anti-talin (A) and anti-GFP monoclonal antibodies (B). (A) Right-angled triangles indicate the increasing amounts of macrophage lysates (200 or 600  $\mu$ g) introduced to the beads. In B, the levels of GST and GST- $\beta_2$ cyt, as determined by Coomassie staining of the SDS-PAGE gel, are shown at the bottom. (C) Quantification of the band intensities measured in pull-down assays in COS-7 cells (B), with the negative control value (GST/GFP) arbitrarily set to 1. Results are expressed as the mean  $\pm$  SD of at least three independent experiments.

$\alpha_M\beta_2$ . COS-7 cells were transfected with  $\alpha_M\beta_2$  and GFP<sup>PTH</sup> or GFP alone. Using the 5c6 antibody, GFP<sup>PTH</sup> was coimmunoprecipitated with  $\alpha_M\beta_2$ , as shown in Figure 3B.

To confirm these interactions, we set up pull-down assays by using cytoplasmic tails of  $\beta_2$  fused to GST (GST- $\beta_2$ cyt) or GST alone as a control. Endogenous talin could be specifically precipitated from J774 macrophage lysates by using GST- $\beta_2$ cyt but not GST alone (Figure 4A). The same assay was performed using lysates from COS-7 cells expressing similar amounts of GFP or GFP<sup>PTH</sup>. As seen in Figure 4, B and C, GFP<sup>PTH</sup> was again specifically pulled down with GST- $\beta_2$ cyt. We conclude that talin interacts with the cytoplasmic tails of the  $\alpha_M\beta_2$  receptor, most likely through the binding of talin head domain to the  $\beta_2$  integrin.

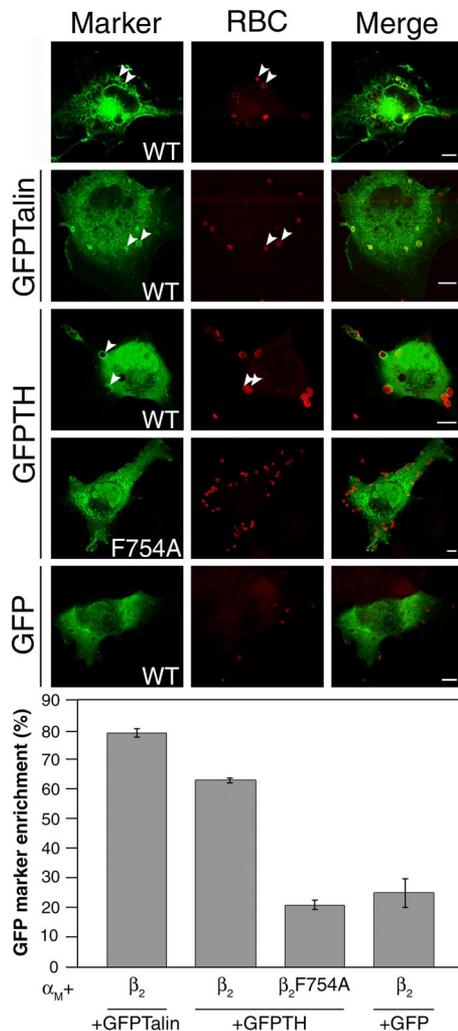
#### Residues W747 and F754 of the $\beta_2$ Cytoplasmic Tail Are Essential for Talin Head Association

Recent work has mapped the residues that control talin head association with the  $\beta_3$  integrin (Tadokoro *et al.*, 2003), spe-



**Figure 5.** Residues W747 and F754 of  $\beta_2$  cytoplasmic tail control talin head binding. (A) Amino acid sequence of the different GST-fused  $\beta_2$  cytoplasmic tails used in this study. Introduced mutations are underlined. (B) Lysates of COS-7 cells transiently transfected with GFP<sup>PTH</sup> were mixed with beads coated with GST, wild-type, or mutant GST- $\beta_2$ cyt as indicated. Proteins were separated by SDS-PAGE and analyzed by Western blotting by using anti-GFP antibodies. The corresponding amounts of GST or GST- $\beta_2$ cyt fusion proteins used are shown, as revealed by Coomassie staining. (C) Band intensities were determined as described in *Materials and Methods* and are related to the values obtained for GST alone (arbitrarily set to 1). Results are expressed as the mean  $\pm$  SD of at least three independent experiments.

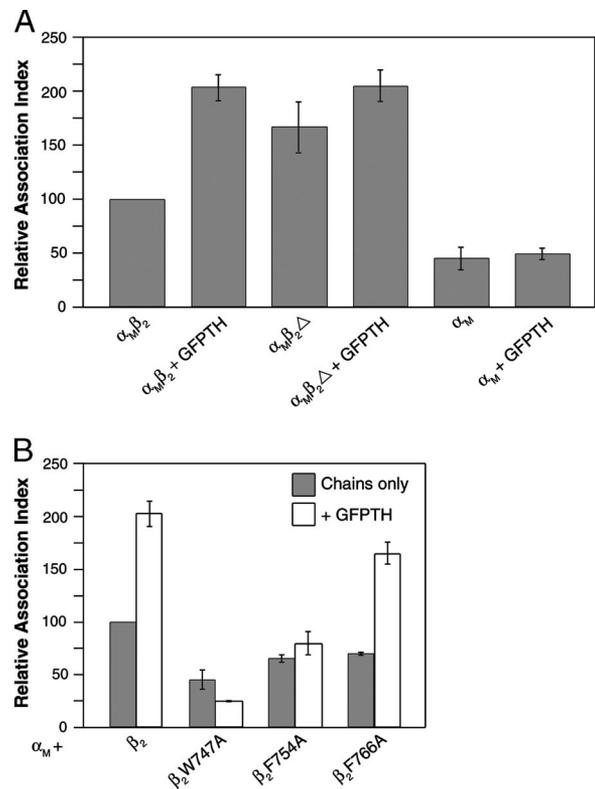
cifically to a NPX $\Phi$  (where  $\Phi$  is an aromatic residue) motif preceded by a single tryptophan seven or eight residues upstream. The amino acid sequence of the human  $\beta_2$  tail (Figure 5A) reveals two NPX $\Phi$  motifs, one motif membrane proximal (residues 751-754) and one motif distal (residues 763-766). We created mutants of  $\beta_2$ cyt harboring single amino acid substitutions in the tryptophan and in the aromatic residues within the two NPX $\Phi$  sequences (Figure 5A). Pull-down assays were performed to determine whether GFP<sup>PTH</sup> could interact with the GST- $\beta_2$ cyt mutants. Alanine substitution of W747 and F754 (membrane proximal NPX $\Phi$ ) but not F766 (distal NPX $\Phi$ ) abolished  $\beta_2$  interaction with talin head in vitro (Figure 5, B and C). These data establish the essential role of  $\beta_2$  integrin cytoplasmic domain residues W747 and F754 in the interaction with talin head.



**Figure 6.** Recruitment of overexpressed talin and talin head to sites of  $\alpha_M\beta_2$ -dependent RBC binding. Top, COS-7 cells were transfected with wt  $\alpha_M$ , wt or mutant  $\beta_2$ , and GFP constructs as indicated, challenged for 30 min with C3bi-RBCs, and processed for confocal microscopy as described in *Materials and Methods*. Arrows indicate typical anti- $\beta_2$  (top set of pictures) or GFP (bottom four sets) staining patterns. Bar, 10  $\mu$ m. Bottom,  $\beta_2$ -expressing cells were scored for enrichment of GFP at sites of RBC binding. Results are expressed as the mean  $\pm$  SD of at least three independent experiments.

#### The $\beta_2$ Cytoplasmic Tail Controls Talin Recruitment to $\alpha_M\beta_2$ during Phagocytosis

To relate these findings to the regulation of talin recruitment to sites of particle binding, we transfected COS-7 cells with wt  $\alpha_M$  and various (wt or point mutants)  $\beta_2$  integrin constructs. We could detect  $\beta_2$  underneath  $60.29 \pm 0.91\%$  of all C3bi-RBCs bound to cells transfected with wt  $\alpha_M\beta_2$ , as determined by confocal analysis after staining with a monoclonal antibody (mAb) against  $\beta_2$  (Figure 6, top). Similarly, both GFP-tagged full-length talin and talin head were seen to accumulate at sites of particle binding, with 79 and 63% ( $p = 0.179$ , as analyzed by Student's *t* test) of the bound RBCs showing enrichment in GFP signal, respectively. However, when the  $\beta_2$ F754A mutant was cotransfected with  $\alpha_M$  and GFPTH, talin head was only marginally recruited to sites of RBC binding, with localization levels similar to those observed for GFP recruitment to wt  $\alpha_M\beta_2$  (negative control);



**Figure 7.** Activation of  $\alpha_M\beta_2$ -mediated RBC binding is controlled by talin head interaction with the  $\beta_2$  cytoplasmic tails. COS-7 cells were cotransfected with constructs encoding integrin subunits (wild-type or mutant) and GFPTH as indicated, challenged with C3bi-RBCs, processed for immunofluorescence, and scored for RBC association as described in *Materials and Methods*. Results are expressed relative to the values obtained for wt  $\alpha_M\beta_2$  (arbitrarily set to 100). (A) Wild-type integrin subunits and mutant receptors deleted of their cytosolic domain ( $\Delta$ ) were used alone or in combination. (B) Distinct  $\beta_2$  chains (wild type or point mutants) were cotransfected with wild-type  $\alpha_M$  as indicated. Results are expressed as the mean  $\pm$  SD of at least three independent experiments.

Figure 6). These data confirm the *in vitro* binding results and link the ability of talin to bind  $\beta_2$  integrin to its enrichment at sites of RBC binding *in vivo*.

#### Talin Interaction with $\beta_2$ Activates $\alpha_M\beta_2$ Binding Activity

We next examined the role of the  $\beta_2$ /talin interaction in regulating  $\alpha_M\beta_2$  function. For this, COS-7 cells were transfected with wt  $\alpha_M$  alone or in combination with  $\beta_2$  (wt or  $\beta_2\Delta$ , in which the entire cytoplasmic domain of  $\beta_2$  was truncated), and cotransfected with GFPTH (Figure 7A). All  $\alpha_M$  and  $\beta_2$  combinations were surface expressed to similar levels and cotransfection of GFPTH did not affect surface expression of  $\alpha_M\beta_2$ , as determined by flow cytometry (data not shown). After phagocytic challenge,  $\alpha_M$ - or  $\beta_2$ -expressing cells were scored for binding of C3bi-RBCs. Expressing GFPTH with  $\alpha_M\beta_2$  increased RBC association by  $>90\%$ . Coexpression with wt  $\alpha_M$  and truncated  $\beta_2\Delta$  also resulted in a higher binding capacity, which was not further increased with the presence of GFPTH. By contrast, COS-7 cells expressing  $\alpha_M$  alone had minimal RBC association, and this was not further increased by GFPTH. These results indicate that the binding activity of  $\alpha_M\beta_2$  is up-regulated by talin head in a manner that depends on the  $\beta_2$  cytoplasmic tail.

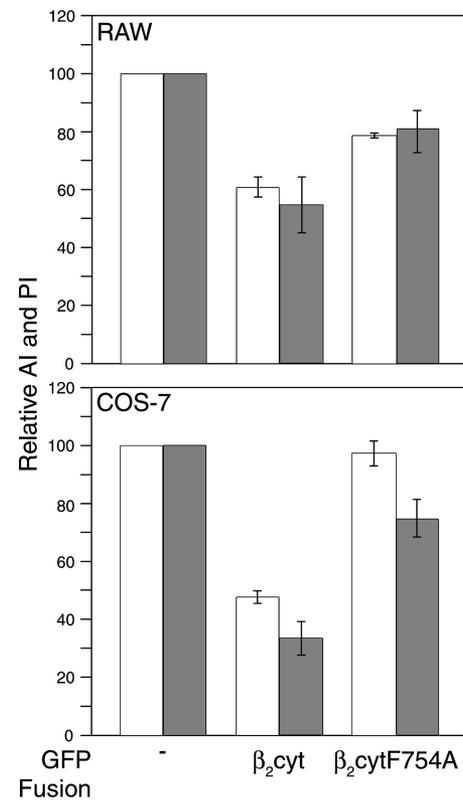
Next, the full-length version of the  $\beta_2$  point mutants described in Figure 5A were cotransfected with wt  $\alpha_M$  and GFP-TH. Combinations of  $\alpha_M$  and  $\beta_2$ W747A, or  $\alpha_M$  and  $\beta_2$ F754A led to wild-type levels of surface expression, as measured by flow cytometry. However, there was a 22% decrease in surface expression of the  $\alpha_M\beta_2$ F766A heterodimer (data not shown), consistent with previously published data (Wiedemann *et al.*, 2006). Expression of the various  $\beta_2$  integrin mutants with wt  $\alpha_M$  integrin led to a decrease in RBC association for all mutants. Importantly, in  $\beta_2$ F766A, decreased RBC binding was correlated to decreased expression (Wiedemann *et al.*, 2006). Moreover, this  $\beta_2$  integrin mutant was sensitive to talin head-induced up-regulation of binding activity. The ability of GFP-TH to regulate RBC association to  $\beta_2$  point mutants was dependent on its ability to bind the  $\beta_2$  tail. Indeed, expression of  $\beta_2$ W747A and  $\beta_2$ F754A mutants with  $\alpha_M$  resulted in reduced basal binding activities and the mutants were refractory to the stimulatory effect of GFP-TH expression on RBC binding (Figure 7B). Therefore, the *in vitro* and *in vivo* data are in agreement and show that residues  $\beta_2$ W747 and F754 control talin head interaction with  $\beta_2$  and the subsequent activation of  $\alpha_M\beta_2$ .

#### Titration of Talin *In Vivo* Decreases $\beta_2$ Function

To independently confirm the importance of talin in  $\alpha_M\beta_2$  function, we transfected macrophages or  $\alpha_M\beta_2$ -expressing COS-7 cells with a GFP-fusion of the wt and F754A  $\beta_2$  tails (GFP- $\beta_2$ cyt and GFP- $\beta_2$ cytF754A, respectively) or with GFP alone. For these experiments, we used RAW264.7 macrophages that are transfectable with DNA constructs, unlike J774.A1 cells. All three overexpressed proteins were expressed in similar amounts, and expression of GFP constructs had no effect on  $\alpha_M\beta_2$  surface expression as measured by flow cytometry (data not shown). In both cell systems, GFP- $\beta_2$ cyt expression decreased the association and phagocytosis of C3bi-RBCs, although the effect was more pronounced in COS-7 cells (Figure 8). This suggested that an important regulator of  $\alpha_M\beta_2$  binding activity was titrated by GFP- $\beta_2$ cyt. The lack of effect of GFP- $\beta_2$ cytF754A on  $\alpha_M\beta_2$ -dependent binding activity strongly suggests that this regulator is talin. None of the overexpressed GFP proteins influenced Fc $\gamma$ R-mediated binding and phagocytosis (data not shown) supporting the results presented in Figure 2, and the idea of a specific role for talin during  $\alpha_M\beta_2$ -mediated uptake.

#### Additional Roles for Talin in Integrin Activation and Phagocytosis

To shed more light on the mechanism by which talin regulates  $\alpha_M\beta_2$  binding properties, we studied the impact of conditional talin knockout on  $Mn^{2+}$ -induced activation of  $\alpha_M\beta_2$ .  $Mn^{2+}$  treatment activates integrins from the outside by opening up the folded, inactive extracellular domain, and it converts integrins to their extended, high-affinity conformation (Takagi *et al.*, 2002). In control (Flox/Flox) MEFs transfected with  $\alpha_M\beta_2$ ,  $Mn^{2+}$  treatment led to a twofold increase in RBC association ( $207.63 \pm 12.41\%$ ) but not phagocytosis. Coexpression of Cre recombinase in these cells knocked out the remaining talin allele, decreased RBC association ( $37.02 \pm 9.52\%$ ), and markedly impaired phagocytosis (in agreement with data in Figure 2C). However, both binding ( $p = 0.24$ ) and phagocytosis ( $p = 0.19$ ) remained at low levels when these cells were treated with  $Mn^{2+}$  (Figure 9A). This suggests additional roles for talin, both in activation of integrins for RBC binding and in outside-in signaling. To independently confirm the former hypothesis, we used COS-7 cells coexpressing  $\alpha_M$  and the talin

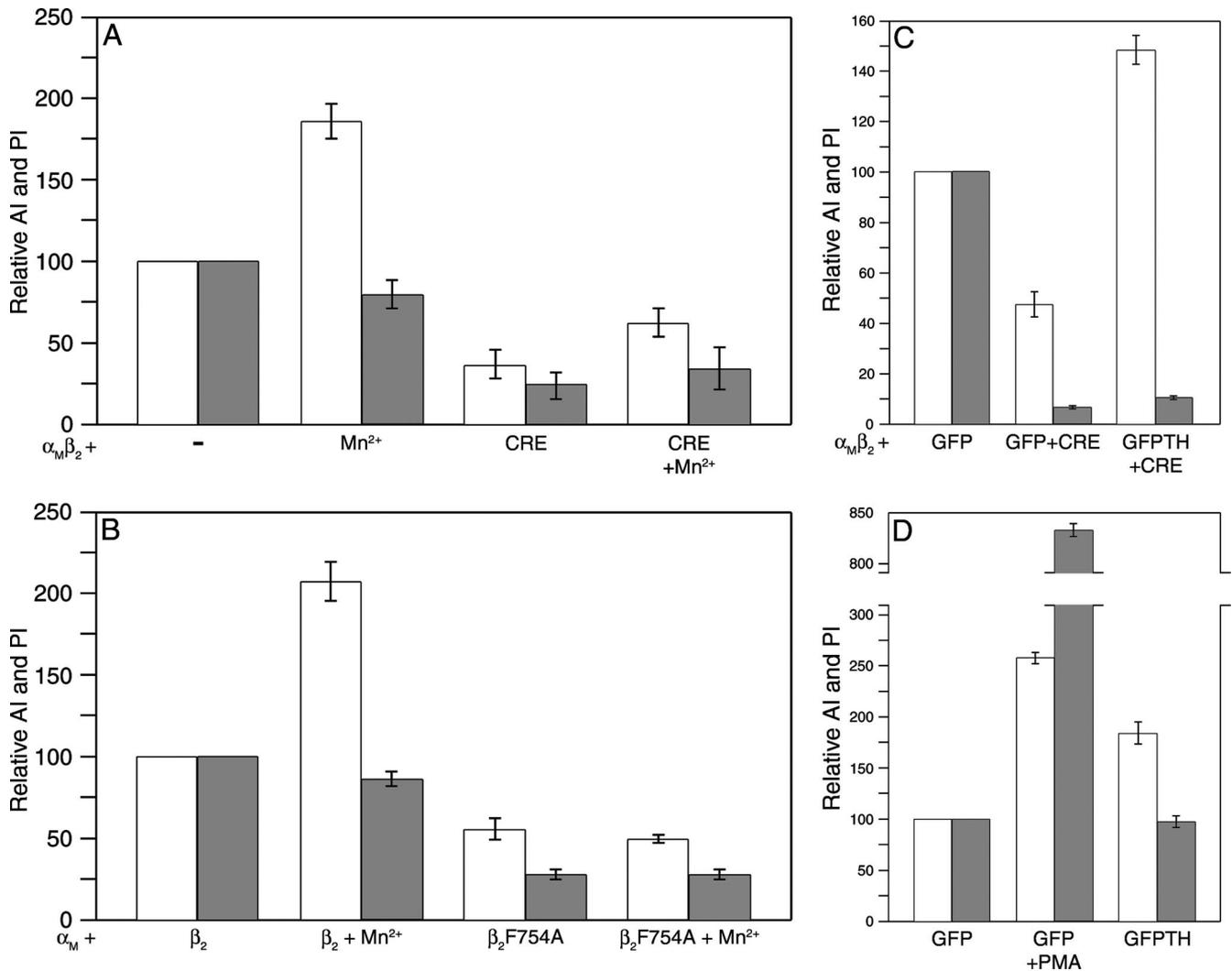


**Figure 8.** Overexpression of GFP- $\beta_2$ cyt leads to decreased binding and phagocytosis in macrophages and  $\alpha_M\beta_2$ -expressing COS-7 cells. RAW 264.7 macrophages (top) or  $\alpha_M\beta_2$ -expressing COS-7 cells (bottom) were transfected with the indicated GFP-fusion proteins, challenged with C3bi-RBCs, and processed and scored as described in *Materials and Methods*. Association (AI; open bars) and phagocytosis (PI; closed bars) indices are shown, with values obtained for negative controls set at 100%. Results are expressed as the mean  $\pm$  SD of at least three independent experiments.

binding-deficient  $\beta_2$  integrin F754A. As shown in Figure 9B,  $Mn^{2+}$  compared with cells expressing wt  $\alpha_M\beta_2$ , was totally unable to induce increased binding, as observed in talin knockout cells.  $Mn^{2+}$  had no effect on phagocytosis, whether in control (wt  $\alpha_M\beta_2$ ) or in  $\alpha_M\beta_2$ F754A-expressing cells. To confirm the role of talin in outside-in signaling from  $\alpha_M\beta_2$ , we investigated whether talin head expression was sufficient to rescue phagocytosis in talin knockout cells. Cotransfection of GFP-TH and Cre recombinase in MEF (Flox/Flox) cells led to an increase in RBC binding but not phagocytosis (Figure 9C), indicating that the whole talin molecule, not just talin head, is required for phagocytosis. Independent confirmation of this hypothesis was obtained in RAW 264.7 macrophages. Talin head expression was almost as efficient at activating RBC binding as PMA. However, talin head was unable to substitute for PMA to induce phagocytosis in macrophages (Figure 9D).

## DISCUSSION

This study examines the role of the cytoskeletal molecule talin in mammalian phagocytosis. We first showed that endogenous talin is recruited to forming phagosomes during Fc $\gamma$ R- and  $\alpha_M\beta_2$ -dependent uptake. This is consistent with previous reports showing that in mammalian phagocytes, talin accumulates at sites of particle binding regardless of the



**Figure 9.** Talin rod domain is required for  $\alpha_M\beta_2$ -mediated uptake. Conditional talin knockout MEFs (Flox/Flox) (A and C) or COS-7 cells (B) were transfected as indicated (– for empty vector), challenged for 30 min with C3bi-RBCs, processed for immunofluorescence, and stained for surface expressed  $\beta_2$  and RBCs. In A and B, cells were pretreated for 20 min at 37°C with 1 mM  $Mn^{2+}$  before phagocytic challenge. Association (open bars) and phagocytosis (closed bars) indices were related to the  $\alpha_M\beta_2$  (A and B) or  $\alpha_M\beta_2$  + GFP (C) controls, which were set at 100%. (D) RAW 264.7 macrophages were transfected with the indicated GFP constructs, pretreated with 150 ng/ml PMA where indicated, challenged with C3bi-RBCs, and processed and scored as described in Figure 2 and *Materials and Methods*. Association (open bars) and phagocytosis (closed bars) indices are shown, with values obtained for negative controls set at 100%. Results are expressed as the mean  $\pm$  SD of at least three independent experiments.

receptor involved in initial particle recognition (Greenberg *et al.*, 1990; Allen and Aderem, 1995, 1996; Allen *et al.*, 2002). The role of talin during phagocytosis seems conserved in *Dictyostelium*, because a GFP-tagged, actin-binding fragment of talin decorates phagosomes (Weber *et al.*, 2002).

However, our data establish that the functional significance of talin at phagosomes is restricted to specific phagocytic receptors. Despite being recruited in both cases, talin is only required for  $\alpha_M\beta_2$ - not Fc $\gamma$ R-mediated uptake. Interestingly, talin-null *Dictyostelium* cells are unable to phagocytose yeast, although they internalize bacteria normally. This suggests that particle size and/or use of different receptors dictates the requirement for talin during *Dictyostelium* uptake (Niewohner *et al.*, 1997). Our study, which uses one type of phagocytic particle, indicates that preferential receptor use rather than particle size conditions the dependency on talin during phagocytosis. As discussed below, the head domain of talin binds a NPX $\Phi$  motif (where X is I, L, or M

and  $\Phi$ , tyrosine, or phenylalanine) within  $\beta_2$ . This NPX $\Phi$  motif is conserved in most integrin  $\beta$  chains (Calderwood, 2004) and is also present in a family of *Dictyostelium* surface receptors (Cornillon *et al.*, 2006). By contrast, the intracellular domains of the Fc $\gamma$  receptors or dectin-1 (the main receptor for zymosan; Brown *et al.*, 2002), two types of receptors that are associated with talin enrichment at phagocytic cups (Greenberg *et al.*, 1990; Allen and Aderem, 1995), lack this motif. These receptors are thus not predicted to interact with talin (or at least talin head) biochemically.

Why is talin transiently recruited to forming phagosomes and yet dispensable for Fc $\gamma$ R-dependent uptake? Talin could accumulate as a result of local, Fc $\gamma$ R-induced binding of talin to  $\beta_2$ .  $\alpha_M\beta_2$  is enriched on phagosomes containing IgG-coated beads (Gold *et al.*, 1999); it also promotes phagocytosis of RBCs coated with both C3bi and IgG (Ehlenberger and Nussenzweig, 1977) and uptake in cells deficient for Fc $\gamma$ R signaling (Worth *et al.*, 1996). However, as shown in

Figure 2, talin knockdown has no impact on Fc $\gamma$ R-dependent internalization, because in our conditions,  $\alpha_M\beta_2$  plays no functional role during Fc $\gamma$ R-mediated phagocytosis. Alternatively, talin could accumulate underneath IgG-opsonized RBCs independently of the interaction between  $\beta_2$  and talin head region, through an unknown mechanism.

Talin regulates  $\alpha_M\beta_2$ -mediated phagocytosis primarily through its effect on particle binding. As shown using ectopic expression of GFP- $\beta_2$ cyt constructs, talin-1 knockdown and knockout MEFs, talin depletion decreases both binding and phagocytosis of C3bi-RBCs. Down-regulation of talin expression had no detectable effect on cell viability or actin-dependent functions, as shown by normal Fc $\gamma$ R-mediated phagocytosis. It had also no effect on  $\alpha_M\beta_2$  expression (data not shown). These results suggest that the large inhibition of  $\alpha_M\beta_2$  phagocytosis results from a dramatic effect of talin depletion on the ability of  $\alpha_M\beta_2$  to bind RBCs. The zipper model of phagocytosis (Griffin *et al.*, 1975) predicts that receptors have to cluster circumferentially around the entire particle for successful uptake to occur. Suboptimal activation of  $\alpha_M\beta_2$  binding capacity, resulting either from mutations in the  $\beta_2$  tail or talin depletion (Figures 2, B and C, and 8) should therefore have pronounced effects on both binding and phagocytosis.

Interaction of talin head with the cytoplasmic domain of  $\beta_2$  is sufficient to increase binding of C3bi-RBCs in macrophages and transfected COS-7 cells (Figures 7 and 9). Moreover, talin and talin head interact with  $\alpha_M\beta_2$ , as shown by coimmunoprecipitation and GST pull-down assays (Figures 3 and 4). This confirms previous data (Sampath *et al.*, 1998; Kim *et al.*, 2003; Fagerholm *et al.*, 2005). Our results using  $\beta_2$  mutants fit with a model in which talin head interacts with a conserved region of the  $\beta$  integrin cytoplasmic domain consisting of a NPX $\Phi$  motif preceded by a tryptophan residue at position -7/-8 (Garcia-Alvarez *et al.*, 2003). Accordingly, mutation of phenylalanine 754 into alanine in the  $\beta_2$  chain NPXF motif abrogated talin head binding in vitro, prevented redistribution of GFP-tagged talin head to sites of RBC binding, and blocked binding and phagocytosis in transfected COS-7 cells. Conversely, a point mutation in talin (R358A) that reduces talin binding to the  $\beta_3$  integrin in vitro (Garcia-Alvarez *et al.*, 2003) failed to increase RBC binding (Lim, Critchley, and Caron, unpublished data). Our results are in line with similar effects of integrin mutations and talin knockdown on  $\beta_1$ - and  $\beta_3$ -dependent binding abilities (Pfaff *et al.*, 1998; Calderwood *et al.*, 1999; Tadokoro *et al.*, 2003). The general role of talin in activation of RBC binding is further supported by our Mn $^{2+}$  experiments. In  $\alpha_M\beta_2$ -expressing talin knockout MEFs, addition of Mn $^{2+}$ —a strong activator of  $\beta_2$  (Dransfield *et al.*, 1992) and other integrins—had no effect on the binding and phagocytosis of C3bi-opsonized RBC. Similarly, Mn $^{2+}$  treatment had no effect on binding and phagocytosis in COS-7 cells expressing the talin binding deficient integrin  $\alpha_M\beta_2$ F754A. This indicates that talin head binding to the  $\beta_2$  integrin is required for full activation of integrin binding to C3bi-RBCs, both by Mn $^{2+}$  (i.e., from outside the cell) and by inside-out signaling. The mechanism involved remains unclear. Recent in vitro data have shown that, in the presence of Mn $^{2+}$ , ligands bind more stably to unclasped (potentially stabilized by talin) than clasped  $\alpha_V\beta_3$  integrins (Takagi *et al.*, 2002), supporting the notion that talin interaction with the  $\beta$  chain stabilizes ligand binding. However, knockdown of talin-1 had no adverse effect on the binding of reporter antibodies or monovalent ligands to  $\alpha_V\beta_3$ - and  $\alpha_I\beta_2$  in Mn $^{2+}$ -treated cells (Tadokoro *et al.*, 2003; Simonson *et al.*, 2006). Interestingly, our results using C3bi-opsonized RBCs in Mn $^{2+}$ -treated,

talin-deficient cells are in line with Simonson's data, that showed a lack of rescue of CD3- and PMA-induced adhesion or conjugate formation by Mn $^{2+}$  in talin-1 knockdown T-cells. Together, these experiments indicate that talin-1, particularly talin head binding to  $\beta_2$ , is needed for maximal binding of integrins to multivalent ligands (i.e., whole cells or C3bi-opsonized RBCs). Whether this solely involves full integrin activation (transition to the fully extended, open conformation) remains to be seen.

In addition to the role of talin head in promoting integrin activation, our study demonstrates additional roles for talin in  $\beta_2$ -dependent phagocytosis. RBC binding but not phagocytosis was rescued in  $\alpha_M\beta_2$ -expressing, talin-depleted MEFs transfected with talin head. Similarly, talin head expression in RAW 264.7 macrophages increased RBC binding but not uptake. These data strongly suggest that the rod domain of talin also plays a role in integrin-dependent phagocytosis, specifically during uptake. The mechanism involved is unclear, although regulation of F-actin networks (Goldmann *et al.*, 1999), integrin cross-linking (Tremuth *et al.*, 2004; Xing *et al.*, 2001), and regulation of vinculin activation (Chen *et al.*, 2006) are plausible leads for future studies. Interestingly, our data are consistent with results recently obtained in *Drosophila* (Tanentzapf and Brown, 2006).

Regulators of phagocytosis are generally assumed to participate in signaling cascades stemming from occupied receptors. Talin is the first cytoskeletal molecule shown to have dual roles in phagocytosis, i.e., a coordinated effect on receptor activation and phagocytic uptake. The integrin  $\beta_2$  subunit controls other key functions beyond phagocytosis, such as leukocyte transendothelial migration within tissues, motility, and the formation of stable immunological synapses. We anticipate that talin knockdown will have a dramatic negative impact on all  $\beta_2$ -mediated functions, as recently suggested in T-cells (Smith *et al.*, 2005). Exploration of the mechanisms underlying the possible coordinated regulation of inside-out and outside-in integrin signaling by talin will undoubtedly prove fascinating.

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