ARTICLE
Journal of Cellular Biochemistry 111:999–1009 (2010)

Journal of Cellular Biochemistry

Rap1 Controls Activation of the $\alpha_M\beta_2$ Integrin in a Talin-Dependent Manner

Jenson Lim,[1*] Aurélien G. Dupuy,[1] David R. Critchley,[2] and Emmanuelle Caron[1]

[1] *Centre for Molecular Microbiology and Infection, Division of Cell and Molecular Biology, Imperial College London, London, UK*

[2] *Department of Biochemistry, University of Leicester, Leicester, UK*

ABSTRACT

The small GTPase Rap1 and the cytoskeletal protein talin regulate binding of C3bi-opsonised red blood cells (RBC) to integrin $\alpha_M\beta_2$ in phagocytic cells, although the mechanism has not been investigated. Using COS-7 cells transfected with $\alpha_M\beta_2$, we show that Rap1 acts on the β_2 and not the α_M chain, and that residues 732–761 of the β_2 subunit are essential for Rap1-induced RBC binding. Activation of $\alpha_M\beta_2$ by Rap1 was dependent on W747 and F754 in the β_2 tails, which are required for talin head binding, suggesting a link between Rap1 and talin in this process. Using talin1 knock-out cells or siRNA-mediated talin1 knockdown in the THP-1 monocytic cell line, we show that Rap1 acts upstream of talin but surprisingly, RIAM knockdown had little effect on integrin-mediated RBC binding or cell spreading. Interestingly, Rap1 and talin influence each other's localisation at phagocytic cups, and co-immunoprecipitation experiments suggest that they interact together. These results show that Rap1-mediated activation of $\alpha_M\beta_2$ in macrophages shares both common and distinct features from Rap1 activation of $\alpha_{IIb}\beta_3$ expressed in CHO cells. J. Cell. Biochem. 111: 999–1009, 2010.

KEY WORDS: PHAGOCYTOSIS; CR3; MACROPHAGE; TALIN; RAP1; RIAM

Phagocytosis is the physiological process by which cells internalise particles larger than 0.5 μm in diameter (e.g. inorganic material, cell debris, microorganisms, necrotic and apoptotic cells) and it plays an essential role in development, homeostasis and immune defences [Desjardins et al., 2005]. Phagocytic receptors can bind their targets directly or indirectly through opsonins [Underhill and Ozinsky, 2002], and can exist as constitutively active or inducible receptors prior to binding to their opsonised targets. One of the best-characterised inducible phagocytic receptors is the integrin $\alpha_M\beta_2$ aka complement receptor 3, CR3, Mac-1, CD11b/CD18. Integrin $\alpha_M\beta_2$ is expressed predominantly on leukocytes, particularly on cells of the monocyte/macrophage lineage and polymorphonuclear neutrophils, but also on natural killer (NK) cells, B- and T-lymphocytes and endothelial cells [Wagner et al., 2001]. Phagocytosis through $\alpha_M\beta_2$ is a multistep process [Griffin et al., 1975] that sequentially involves receptor-mediated particle recognition via interactions between active integrins and their C3bi ligand, actin-driven uptake, and phagosome closure and maturation.

Integrins are heterodimeric, surface expressed receptors made up of single α and β chains. Topologically, integrin chains are divided into three regions, a large extracellular ligand-binding domain, a single pass transmembrane domain and a short cytoplasmic tail. Integrin function is bi-directionally regulated. Thus, ligation of the extracellular domain of integrins can trigger 'outside-in' signalling pathways that induce changes in cell adhesion, motility and phagocytosis, and promote cell survival and cell cycle progression [Hynes, 2002]. However, the ligand binding activity of many integrins are activated by 'inside-out' signalling [reviewed by Caron, 2003; Shattil et al., 2010] which is proposed to induce global conformational rearrangements in the integrin heterodimers. This includes unclasping of the α and β integrin tails, separation of the transmembrane helices [Lau et al., 2009] and/or the shortening of the trans-membrane domain [Armulik et al., 1999; Stefansson et al., 2004] and these are thought to trigger a large conformational change in the extracellular domains from the 'bent' to the 'extended' form which is associated with integrin activation [Takagi et al., 2002; Nishida et al., 2006]. Recent studies clearly establish that binding of the N-terminal FERM (band 4.1, ezrin, radixin and moesin) F3 domain of the cytoskeletal protein talin to both a conserved NPXΦ motif [reviewed by Liu et al., 2000; Lim et al., 2007] and a membrane proximal helical region of β integrin tails

Grant sponsor: Wellcome Trust; Grant number: 068556/Z/02/Z; Grant sponsor: Biotechnology and Biological Sciences Research Council (BBSRC); Grant number: 28/C18637.

*Correspondence to: Dr. Jenson Lim, Nanotherics Ltd, Guy Hilton Research Centre, Thornburrow Drive, Stoke-on-Trent ST4 7QB, UK. E-mail: jensons_blog@yahoo.co.uk

Received 25 February 2010; Accepted 16 July 2010 • DOI 10.1002/jcb.22788 • © 2010 Wiley-Liss, Inc.
Published online 27 July 2010 in Wiley Online Library (wileyonlinelibrary.com).

[Wegener et al., 2007; Anthis et al., 2009] plays a key role in this process. The small GTP-binding protein Rap1 is also involved in 'inside-out' signalling to many integrins [reviewed by Caron, 2003; Shattil et al., 2010] and recent studies have defined a pathway in which Rap1 activates $\alpha_{IIb}\beta_3$ via the protein RIAM (Rap1-GTP-interacting adaptor molecule) [Han et al., 2006; Watanabe et al., 2008], which binds to talin and Rap1 in order to activate integrins [Lee et al., 2009]. RIAM binding to talin is thought to relieve the intramolecular interactions which keep talin in an inactive state [Goksoy et al., 2008; Goult et al., 2009], thereby leading to integrin activation.

The role of 'inside-out' signalling in $\alpha_M\beta_2$-mediated mammalian phagocytosis is less well researched, and most studies have focussed on β_1 and β_3 integrins [Liu et al., 2000; Abram and Lowell, 2009; Shattil, 2009]. However, we have firmly established that both talin and Rap1 are also essential regulators of 'inside-out' activation of integrin $\alpha_M\beta_2$. Talin is essential for $\alpha_M\beta_2$-mediated phagocytosis in macrophages, and interaction of the N-terminal talin head with the β_2 integrin cytoplasmic domain is necessary and sufficient for 'inside-out' integrin activation, and optimal binding of C3bi-opsonised particles to macrophages, and to $\alpha_M\beta_2$-expressing COS-7 cells [Lim et al., 2007]. Rap1 is activated by a variety of agonists and mediators known to promote $\alpha_M\beta_2$ binding and phagocytic activity, including phorbol esters, lipopolysaccharide (LPS), tumour necrosis factor-α (TNF-α) and platelet activating factor (PAF) [Caron et al., 2000]. Unlike the talin head, which is able to rescue particle binding but not uptake when expressed in talin-deficient cells [Lim et al., 2007], expression of active Rap1 in macrophages is sufficient to induce maximal binding and subsequent engulfment of particles [Caron et al., 2000]. However, it has not been established how Rap1 and/or talin regulate $\alpha_M\beta_2$-mediated binding and phagocytosis of complement-opsonised ligands. Here, we show that Rap1 acts upstream of talin to regulate $\alpha_M\beta_2$ ligand binding activity and macrophage spreading. Rap1 activity controls the recruitment of talin to sites of particle binding, although the effects were independent of RIAM.

MATERIALS AND METHODS

REAGENTS

Sheep red blood cells (RBC) were purchased from TCS Biosciences Ltd, while 4-hydroxytamoxifen, rhodamine-phalloidin, gelatin veronal buffer, protein G-agarose and C5-deficient serum were obtained from Sigma.

The antibodies used in this study were rat anti-mouse α_M (clone 5c6; Serotec), mouse anti-human α_M (clone ICRF44; BD-Pharmingen), mouse anti-human β_2 (clone 6.7; BD-Pharmingen); mouse anti-GFP (clones 7.1 and 13.1; Roche); mouse (clone 9E10; Santa Cruz) and rabbit anti-myc (clone 71D10; Cell Signalling); rat anti-HA (clone 3F10; Roche); rabbit anti-actin (Sigma) and rabbit IgM anti-RBC antibodies (Cedarlane Laboratories). Anti-RIAM antibody was kindly provided by Theresia Stradal (University of Pennsylvania, Philadelphia). Conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (immunofluorescence) or GE Healthcare (Western blotting).

DNA CONSTRUCTS

Eukaryotic expression vectors (pRK5) encoding human wild type (wt) and mutant α_M and β_2 were previously described [Caron and Hall, 1998; Wiedemann et al., 2006; Lim et al., 2007]. pEGFP-RIAM (GFP-RIAM), pRKGFP-Talin (GFPTFL) and pRKGFPTalinHead (GFPTH) were kindly provided by Theresia Stradal (University of Pennsylvania, Philadelphia), Kazue Matsumoto (National Institute of Health, MA) and Neil Bate (Leicester University, UK), respectively.

The $\beta_2\Delta762$ deletion was generated by polymerase chain reaction using wt β_2 as a template, the sense primer 5′-GGGGGGTCTAGAATGCTGGGCCTGCGCCCCCCACTG-3′, which incorporates an internal *Xba*I site, and the antisense primer 5′-GGGGGGAAGCTTCTACATGACCGTCGTGGTGGCGCT-3′, which incorporates an internal *Hin*dIII site. Plasmid products were transformed into One Shot TOP10 chemically competent *Escherichia coli* (Invitrogen) and checked by DNA sequencing (MWG). DNA was later prepared using the QIAGEN Endofree maxi-prep kit.

CELL CULTURE AND TRANSFECTION

Cells from the simian kidney fibroblast COS-7 cell line (American Type Culture Collection number CRL-1651) were maintained and seeded as previously described [Caron and Hall, 1998]. Mouse embryo fibroblasts carrying a floxed talin1 allele as well as a tamoxifen-inducible Cre-recombinase allele (kindly provided by Susan J. Monkley, University of Leicester) were maintained in DMEM medium (Invitrogen) supplemented with 10% heat-inactivated foetal bovine serum (PAA Laboratories). Human monocyte-like THP-1 cells (ATCC number TIB-202) were maintained in RPMI 1640 medium, supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES (Sigma) and 1.0 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol and 10% (v/v) heat inactivated foetal bovine serum. To differentiate THP-1 cells into adherent macrophage-like cells [Stefansson et al., 2004], 50 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma Chemical) was added to the medium for 48 h. COS-7 cells were transfected using the DEAE-dextran method [Caron and Hall, 1998], talin conditional knock-out cells using SuperFect (QIAGEN, West Sussex, UK) [Lim et al., 2007] with 4-hydroxytamoxifen added immediately after transfection when needed. THP-1 cells were transfected by nucleofection (Kit V, AMAXA). All cells were left to express constructs for 48 h before phagocytic challenge.

For siRNA experiments, 1×10^6 undifferentiated THP-1 monocytes were nucleofected (AMAXA) with medium alone or with 80 nM siRNA (pools of four siRNA directed against human talin1, accession number NM_006289, human RIAM, accession number NM_019043 or siLuciferase siRNA pool, Dharmacon) and incubated for 48 h, before any further assays were performed.

PHAGOCYTIC CHALLENGE

IgG- and C3bi-opsonised RBC (later referred to as IgG- and C3bi-RBC, respectively) were prepared and used as previously described [Caron and Hall, 1998; Wiedemann et al., 2006], using 0.1 μl (0.5 μl for macrophages) fresh RBC per 13 mm glass coverslip. To elicit 'inside-out' signalling, macrophages and $\alpha_M\beta_2$-expressing COS-7 cells were pretreated with 150 ng/ml PMA (Sigma) in HEPES-buffered, serum-free DMEM for 15 min at 37°C as described

previously [Caron et al., 2000]. After challenge with C3bi-RBC for 30 min at 37°C, cells were washed with PBS to remove unbound RBC and fixed in cold, 4% paraformaldehyde for 10 min at 4°C.

FLOW CYTOMETRY

Transfected COS-7 cells were prepared as previously described [Lim et al., 2007], and analysed for the relative fluorescence of gated cells, using a FACSCalibur analyser (Becton Dickinson).

IMMUNOFLUORESCENCE AND SCORING

Cells were either stained for surface β_2 or permeabilised with 0.2% Triton X-100 and incubated with the appropriate primary and secondary antibodies. β_2-expressing cells were distinguished from attached RBC using different fluorochrome-conjugated secondary antibodies, for example a Cy2-conjugated donkey anti-mouse antibody for the β_2 and a Rhodamine Red X-conjugated donkey anti-rabbit antibody to stain opsonised RBC. Coverslips were finally mounted in Mowiol (Calbiochem) containing *p*-phenylene diamine (Sigma) as antifading reagent and analysed by microscopy using an epifluorescence microscope (BX50, Olympus). Cells co-expressing surface β_2 and the different constructs were scored for attached RBC. The association index is defined as the number of RBC bound to 100 phagocytes. For spreading experiments, 100 macrophages were observed and those above 20 μm in their longer axis were considered spread.

The enrichment in GFP- or myc-tagged proteins at sites of RBC binding was scored by confocal microscopy (LSM510, Zeiss). For these experiments, a minimum of 20 transfected cells per condition were analysed for a discrete local enrichment in marker signal at bound RBC. Nascent phagosomes were scored as positive when at least a quarter of the underlying/surrounding area showed significant enrichment, compared to the neighbouring areas.

IMMUNOPRECIPITATION

Serum-starved transfected COS-7 cells were lysed on ice in lysis buffer (1% NP-40, 50 mM Tris pH 7.6, 150 mM NaCl, 1 mM PMSF, 1 mM DTT and protease inhibitor cocktail (Roche Applied Science, East Sussex, UK)). Lysates were incubated for 2 h at 4°C with the appropriate antibodies and Protein G-Agarose, followed by three washes in cold lysis buffer, before analysis by SDS–PAGE and Western blotting. Mouse anti-GFP or anti-myc antibodies (diluted 1:1,000) were added for 1 h, followed by goat anti-mouse-HRP. Detection was carried out using the Enhanced ChemiLuminescence detection kit (ECL, Amersham).

RESULTS

BINDING OF C3bi-OPSONISED RBC TO $\alpha_M\beta_2$ INTEGRIN EXPRESSED IN COS-7 CELLS IS REGULATED BY Rap1

We have previously shown that Rap1 regulates the binding of C3bi-opsonised particles to the integrin $\alpha_M\beta_2$ (aka CR3) in mouse macrophages [Caron et al., 2000]. To characterise the mechanism involved, we turned to COS-7 cells, which do not contain any endogenous α_M or β_2 integrin chains and offer a robust system for the study of $\alpha_M\beta_2$ function in phagocytosis [Caron and Hall, 1998; May et al., 2000; Wiedemann et al., 2006]. COS-7 cells expressing either the α_M or the integrin β_2 subunit alone bound RBC poorly or not at all as reported previously [Fig. 1A; Wiedemann et al., 2006]. By contrast, co-expression of the wild type (wt) α_M and β_2 integrin chains enabled COS-7 cells to bind C3bi-RBC efficiently, and binding was increased twofold by PMA (phorbol 12-myristate 13-acetate), a known activator of β_2-dependent functions in mouse macrophages [Wiedemann et al., 2006]. This is not due to increased $\alpha_M\beta_2$ expression, as the levels of surface expressed $\alpha_M\beta_2$ on transfected COS-7 cells did not vary as measured by flow cytometry (data not shown). Importantly, basal and PMA-induced binding of C3bi-opsonised RBC was sensitive to Rap1 activity (Fig. 1A,B), and cells co-transfected with $\alpha_M\beta_2$ and dominant negative Rap1 (N17) showed decreased RBC binding and were no longer responsive to PMA. That this effect is due to down-regulation of Rap1 activity was confirmed by the decreased RBC binding observed in cells expressing RapGAP, a Rap-specific GTPase activating protein which binds to and activates Rap1's intrinsic GTPase activity, leading to the

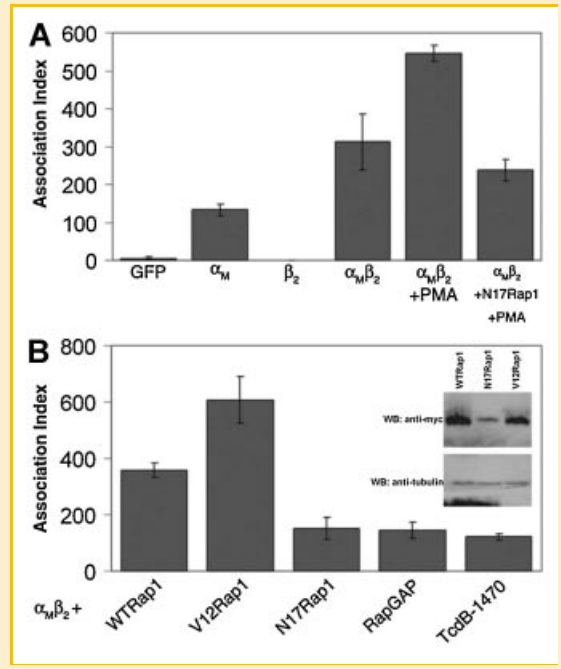

Fig. 1. $\alpha_M\beta_2$-transfected COS-7 cells are sensitive to Rap1-mediated inside-out signalling. A,B: COS-7 cells were co-transfected with $\alpha_M\beta_2$ integrin, Rap1 and RapGAP constructs as shown. Where necessary, cells were pretreated with PMA (A, 150 ng/ml for 15 min) or *Clostridium difficile* toxin B-1470 (B, 1 pg/ml for 2.5 h), challenged with C3bi-RBC, processed for immunofluorescence and scored for RBC association, as described in the Materials and Methods Section. Association indices are given as absolute values. Only cells that were successfully transfected with the plasmids were counted for RBC binding. Results are expressed as the mean ± SD of at least three independent experiments. B, inset: Expression levels of Rap1 constructs. Lysates of COS-7 cells co-expressing either WT-, N17- or V12Rap1 were separated by SDS–PAGE and analysed by Western blotting, using the indicated antibodies. Results are representative of three independent experiments. N17Rap1 is less well expressed than V12Rap1 or WTRap1 (B, inset), and increasing the levels of N17Rap1 expression is toxic to cells (data not shown).

termination of the signalling event (Fig. 1B), Binding was also inhibited by the toxin TcdB-1470 from *Clostridium difficile* (Fig. 1B) which blocks activation of protein kinase C (PKC) by PMA [Schmidt et al., 1998] and Rap1 activity [Chaves-Olarte et al., 1999; Caron et al., 2000]. It is known that PKC acts upstream of Rap1 in $\alpha_{IIb}\beta_3$ activation [Han et al., 2006]. Conversely, expression of constitutively active (V12)Rap1 resulted in increased binding. None of these treatments affected the steady-state levels of surface expressed $\alpha_M\beta_2$ integrins, as analysed by flow cytometry (data not shown), indicating that the $\alpha_M\beta_2$ integrin is sensitive to both PKC and Rap1 regulation in COS-7 cells.

Rap1-DEPENDENT REGULATION OF $\alpha_M\beta_2$ ACTIVITY IS MEDIATED BY THE CYTOPLASMIC TAIL OF THE β_2 SUBUNIT

To ascertain which integrin chains (α_M, β_2 or both) Rap1 acts upon, we co-transfected COS-7 cells with N17- or V12Rap1, and combinations of wild type and mutant integrins devoid of their cytoplasmic tails. Cells were then scored for their ability to bind C3bi-RBC. When expressed alone, the α_M integrin chain conferred low-level binding activity, which was essentially insensitive to N17- or V12Rap1 co-expression (Fig. 2). Cells expressing a mutant $\alpha_M\beta_2$ heterodimer containing a α_M ($\alpha_M\Delta1136$) cytoplasmic tail truncation and a wild type β_2 bound similar numbers of C3bi-RBC compared to wild type $\alpha_M\beta_2$ (compare Fig. 2 and Fig. 1A). Importantly, like the wild-type integrin, the binding activity of the $\alpha_M\Delta1136\beta_2$ integrin was decreased by co-expression of N17Rap1 and increased by V12Rap1 (P values of 0.005 and 0.03, respectively). In contrast, cells expressing wild type α_M and a β_2 mutant lacking the cytoplasmic tail ($\beta_2\Delta724$) showed enhanced binding, that was unresponsive to either V12- or N17Rap1 (Fig. 2). We conclude that Rap1 acts on the β_2 chain to regulate $\alpha_M\beta_2$ binding activity.

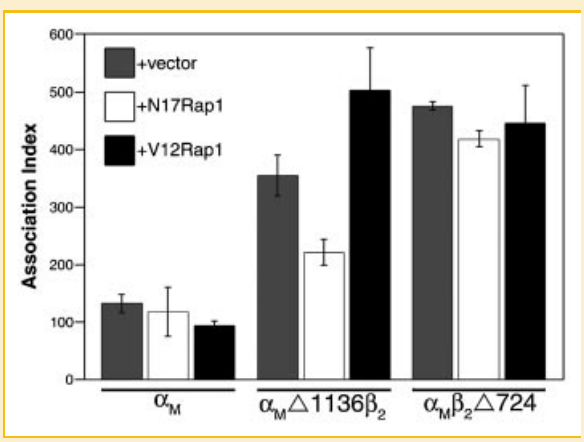

Fig. 2. Rap1-mediated regulation of $\alpha_M\beta_2$ binding function requires the β_2 cytoplasmic tail. COS-7 cells were co-transfected with constructs encoding integrin subunits (wild-type or cytoplasmic tail deletions) and N17 or V12Rap1 as indicated, challenged with C3bi-RBC, processed for immunofluorescence and scored for RBC association (association index), as described in the Materials and Methods Section. Results are expressed as the mean ± SD of at least three independent experiments.

Rap1 ACTIVATION OF $\alpha_M\beta_2$ INTEGRIN IS DEPENDENT ON BINDING OF TALIN TO THE CYTOPLASMIC TAIL OF β_2

To identify the region on the β_2 tail upon which Rap1 acts, we co-transfected COS-7 cells with β_2 cytoplasmic tail truncation mutants (Fig. 3A) along with wild type α_M, and tested their ability to bind C3bi-RBC. The most extensive β_2 deletions up to residue 724 and 732 showed increased binding, while shorter deletions showed decreased binding relative to wild type $\alpha_M\beta_2$ (Fig. 3B). However, all but two ($\Delta762$ and $\Delta767$) of the β_2 deletion mutants failed to show increased RBC binding upon co-expression of V12Rap1 (Fig. 3C). We conclude that activation of $\alpha_M\beta_2$ by Rap1 depends upon residues 732–761 of the β_2 integrin chain.

Interestingly, this region of the β_2 subunit harbours several residues known to regulate $\alpha_M\beta_2$ function. In particular, we and others have shown that residues W747 and F754 control the binding of the talin head to the β_2 tail, which is essential for optimal inside-out activation of β_2 integrins in transfected cells [Sebzda et al., 2002; Takagi et al., 2002; Lim et al., 2007]. In addition, threonine residues 758–760 (Fig. 3A, underlined) regulate the stable recruitment of active RhoA, actin polymerisation and phagocytosis in response to $\alpha_M\beta_2$ ligation [Wiedemann et al., 2006]. To test whether the Rap1-dependent activation of C3bi-RBC binding involves the talin head, we over-expressed wild type α_M and β_2 point mutants that cannot bind talin head (β_2W747A and β_2F754A) [Lim et al., 2007] and V12Rap1. Figure 3D shows that β_2 mutants that are unable to bind talin head cannot be activated by V12Rap1. In contrast, a β_2F766A point mutant and a β_2AAA triple mutant (where threonines 758–760 are substituted to alanines) still bind the talin head [Lim et al., 2007] and are still activated by V12Rap1. These results strongly suggest that talin head binding to β_2 is critical for Rap1-induced activation of the $\alpha_M\beta_2$ integrin.

To confirm this hypothesis, we used conditional talin1 knock-out mouse embryonic fibroblasts (MEFs) carrying a floxed talin1 allele and a tamoxifen-inducible Cre recombinase allele. Tamoxifen-treated Talin1 Flox/+ cells retain one talin1 allele, whilst Flox/− MEFs are rendered talin1-deficient [Jiang et al., 2003]. When transfected with $\alpha_M\beta_2$, in the absence of tamoxifen, the two MEF cell lines bound similar levels of C3bi-RBC (375 ± 113 vs. 300 ± 36, respectively, $P = 0.76$), and co-transfection of V12Rap1 increased RBC binding as expected (Fig. 4A). In contrast, tamoxifen-induced deletion of the floxed talin1 allele in $\alpha_M\beta_2$-expressing Flox/− MEFs strongly impaired RBC binding (50% of control) but had little effect on binding to $\alpha_M\beta_2$-expressing Flox/+ cells (Fig. 4A). Importantly, addition of tamoxifen to Flox/− cells markedly reduced the ability of V12Rap1 to up-regulate RBC binding (Fig. 4A). The residual binding probably reflects the fact that the cells express talin2 which can compensate for loss of talin1 [Zhang et al., 2008].

To verify these results in phagocytic cells expressing endogenous $\alpha_M\beta_2$ integrin, we turned to the human monocytic cell line, THP-1. Consistent with our previously published data obtained in mouse macrophages [Lim et al., 2007], siRNA-induced knock-down of talin1 in PMA-differentiated THP-1 cells (Fig. 4B), markedly decreased RBC binding ($AI = 91 \pm 7.8$), compared to a non-targeting siRNA (Luciferase, Luc; $AI = 191 \pm 9.3$, $P = 0.001$). Unsurprisingly, co-transfection of V12Rap1 doubled the number of RBC bound to

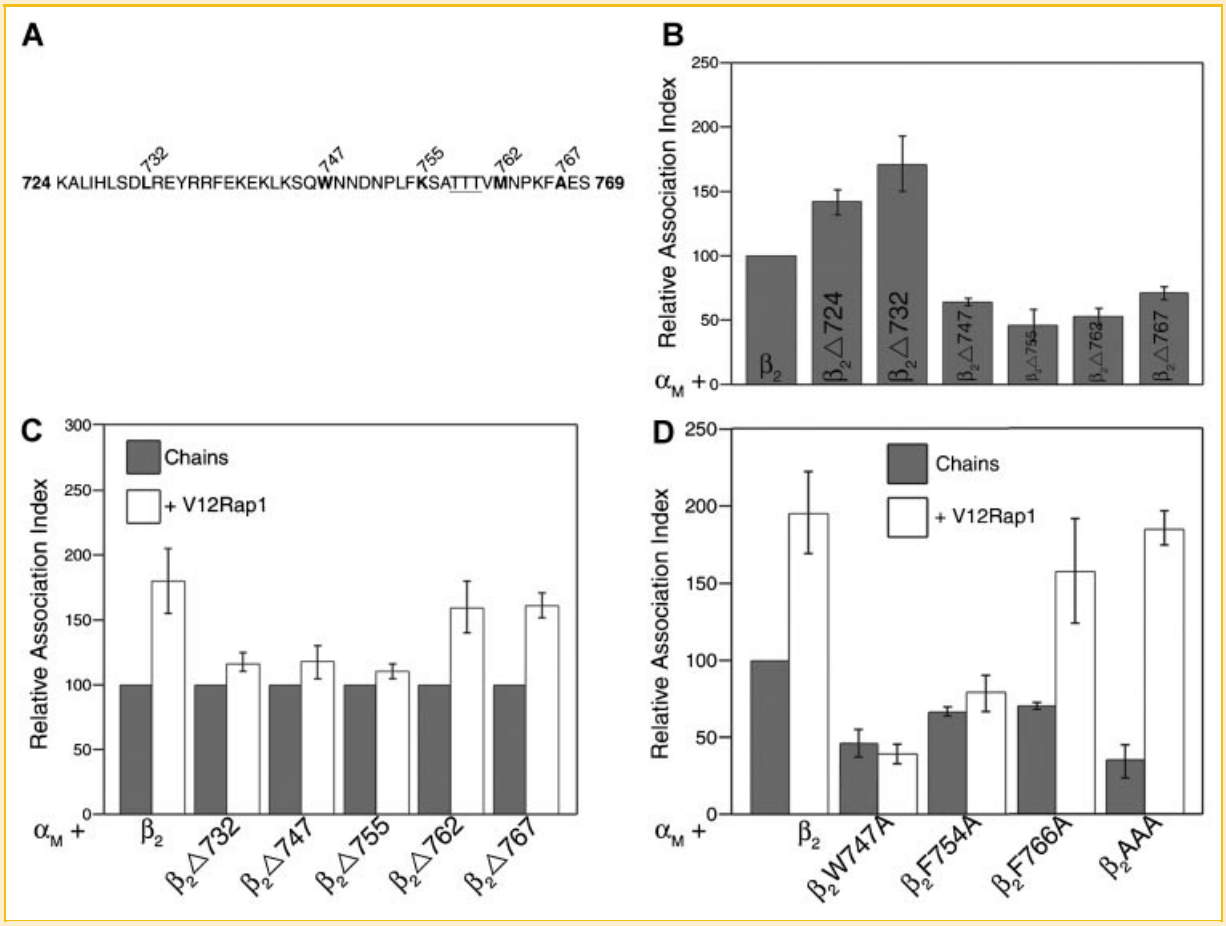

Fig. 3. Identification of the β_2 cytoplasmic domain region necessary for $\alpha_M\beta_2$ activation by Rap1 signalling. A: Amino acid sequence of the cytoplasmic domain of the β_2 integrin. B–D: COS-7 cells were co-transfected with wt α_M, β_2 integrin deletion and point mutants as indicated, in the presence or absence of V12Rap1 (C,D) as shown. Surface expression levels of the mutants were comparable to wild type as assessed by flow cytometry (data not shown). Transfected cells were challenged with C3bi-RBC, processed for immunofluorescence and scored for RBC association, as described in the Materials and Methods Section. Results are expressed relative to the values obtained for wt $\alpha_M\beta_2$ (B,D) or individual β_2 deletion mutants (C) (all arbitrarily set to 100). Results are expressed as the mean ± SD of at least three independent experiments.

THP-1 cells treated with Luc siRNA (AI = 388 ± 15.6, $P = 0.03$) whereas co-transfection of talin1 siRNA dramatically reduced C3bi-RBC binding (AI = 89 ± 11.3, $P = 0.001$ compared to Luc) in response to V12Rap1, similar to the result observed in talin1 knocked-down MEFs. These results provide strong evidence that Rap1-induced inside-out activation of $\alpha_M\beta_2$ requires talin, a dependency that was observed both in reconstituted $\alpha_M\beta_2$-expressing fibroblasts and in human macrophages expressing endogenous $\alpha_M\beta_2$.

Interestingly, the relationship between Rap1, talin and integrins in macrophages is not limited to C3bi-RBC binding, and we observed that talin1 knock-down also affected macrophage spreading, which we showed is controlled by Rap1 [Schmidt et al., 2001]. As shown in Figure 4C, over 70% of control (Luc), siRNA-transfected THP-1 cells were spread and larger than 20 μm in diameter, as expected for PMA-differentiated cells. However, the number of spread cells was dramatically reduced after talin1 knock-down (18.6 ± 4.7% against 70 ± 9.7% for control cells; Fig. 4C). Co-expression of V12Rap1 was unable to rescue spreading in talin1 knock-down THP-1 cells (26.2 ± 3.4% spread cells against 75.7 ± 6.1% for control cells (Luc)), suggesting that talin lies downstream of Rap1 in the activation of the integrin receptors mediating the spreading of THP-1 cells.

Rap1 CONTROLS TALIN RECRUITMENT TO SITES OF PARTICLE BINDING

To confirm that Rap1 acts upstream of talin in inside-out activation of $\alpha_M\beta_2$, we co-transfected $\alpha_M\beta_2$-expressing COS-7 cells with a variety of GFP-tagged talin constructs in the presence of myc-tagged Rap1 alleles. Cells expressing $\alpha_M\beta_2$ and talin (either full-length talin or the isolated head domain) showed an increased level of bound C3bi-RBC binding compared to control ($\alpha_M\beta_2$ + GFP) (Fig. 5). As expected, expression of talin head was not sufficient to induce RBC binding in the absence of $\alpha_M\beta_2$. As another control, we co-transfected $\alpha_M\beta_2$ with a talin full-length point mutant (R358A) that has a reduced capacity to bind β3 integrin [Garcia-Alvarez et al., 2003]. Under these conditions, no increased binding of C3bi-RBC was observed. Importantly, N17Rap1 decreased C3bi-RBC binding to cells co-expressing $\alpha_M\beta_2$ and full-length talin, suggesting that Rap1 lies upstream of talin in this pathway. By contrast, N17Rap1 expression did not decrease binding in cells

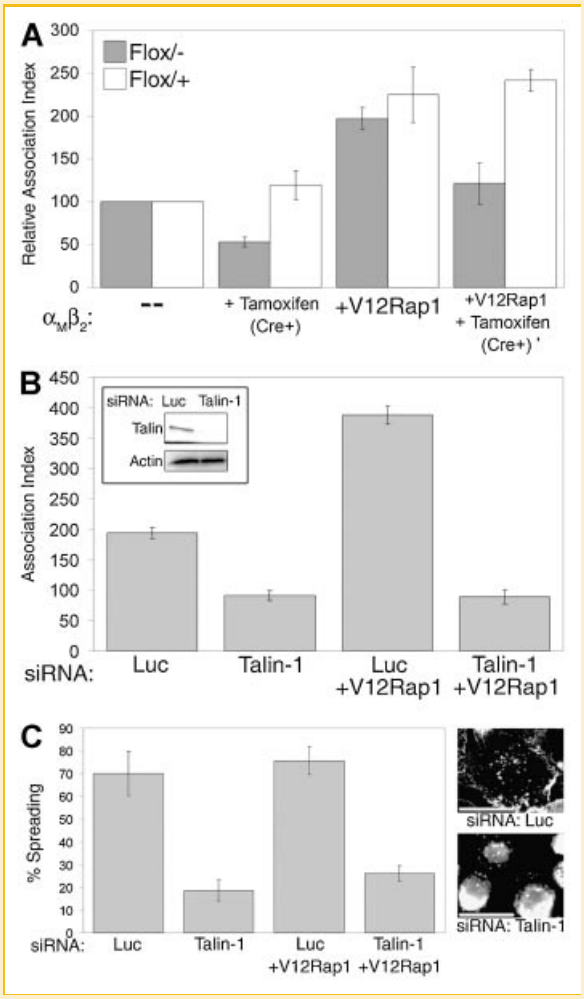

Fig. 4. Control of inside-out signalling by Rap1 requires talin. Conditional talin knock-out MEFs (Flox/+ or Flox/−) (A) or THP-1 cells (B,C) were transfected as indicated (− for empty vector; Luc for Luciferase, non-targeting siRNA), treated with 1 μM 4-hydroxytamoxifen (tamoxifen) when necessary, challenged for 30 min with C3bi-RBC, processed for immunofluorescence and stained for surface expressed β_2 and RBC (A,B) or F-actin (C). Deletion of the talin1 gene did not affect surface expression of $\alpha_M\beta_2$ (data not shown). In (A), association indices were related to the control (−) values obtained respectively in Flox/− and Flox/+ cells transfected with only $\alpha_M\beta_2$; (B) shows the absolute numbers of C3bi-RBC bound, expressed as association indices; (C) gives the percentage of spread cells as defined in the Materials and Methods Section. Right, representative examples. Scale bar, 20 μm. Results are expressed as the mean ± SD of at least three independent experiments.

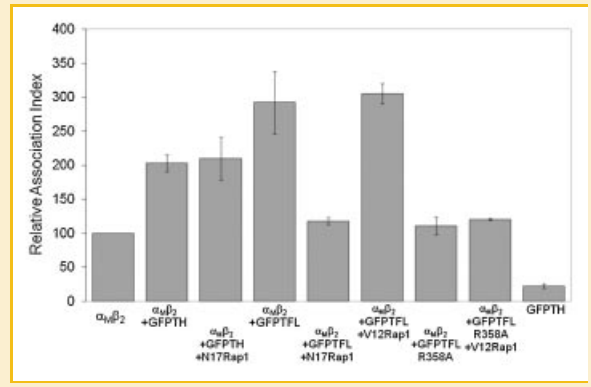

Fig. 5. Rap1 acts upstream of talin head binding to β_2. COS-7 cells were co-transfected with the indicated $\alpha_M\beta_2$ integrin constructs, GFP-talin head (GFPTH), GFP-talin full length (GFPTFL), a talin point mutant unable to bind β integrin chains (GFPTFL-R358A), and/or Rap1 (N17 or V12 versions) as indicated. After challenge with C3bi-RBC, cells were processed for immunofluorescence and scored for RBC association, as described in the Materials and Methods Section. Results are expressed relative to the values obtained for wt $\alpha_M\beta_2$ (arbitrarily set to 100). Results are expressed as the mean ± SD of at least three independent experiments.

expressing the talin head domain. Furthermore, V12Rap1 which is known to up-regulate $\alpha_M\beta_2$ binding activity (see Fig. 1), had no stimulatory effect in cells co-expressing talin R358A (Fig. 5). Together, these data suggest that Rap1 promotes inside-out activation of $\alpha_M\beta_2$ by acting on full-length talin, possibly by exposing the talin head. Interestingly, co-expression of V12Rap1 with full-length talin, did not increase binding above that seen with talin alone, presumably because sufficient talin was activated by endogenous Rap1-GTP to achieve maximal binding.

To see whether Rap1 activity controlled the recruitment of talin to sites of particle binding, we co-expressed $\alpha_M\beta_2$ integrin, GFP-tagged talin fragments and Rap1 constructs in COS-7 cells (Fig. 6A). As expected [Lim et al., 2007], talin and talin head were similarly recruited (78.9 ± 1.6% and 66.4 ± 2.3%, respectively) to RBC associated with phagocytic cups. By contrast, the talin R358A point mutant showed significantly decreased recruitment (44.6 ± 4.6%; $P = 0.04$ when compared to GFP-full-length talin), although it was higher than the GFP controls ($P = 0.02$). This result is in line with the data showing decreased C3bi-RBC association to cells co-transfected with the R358A talin mutant (Fig. 5). Interestingly, recruitment of full-length talin was decreased upon co-expression of RapGAP, a negative regulator of Rap1 activity (47.5 ± 7.7%; $P = 0.03$ compared to GFP control) (Fig. 6A). This correlates with decreased binding of C3bi-RBC in cells co-transfected with GFPTFL and N17Rap1 (Fig. 5). Therefore, Rap1 activity controls both the recruitment of talin to $\alpha_M\beta_2$ and activation of $\alpha_M\beta_2$ RBC binding activity.

Reciprocally, we examined whether Rap1 recruitment to $\alpha_M\beta_2$ was co-dependent on talin localisation. We co-transfected $\alpha_M\beta_2$ or $\alpha_M\beta_2$F754A, an integrin mutant that cannot bind talin head [Takagi et al., 2002; Lim et al., 2007], with tagged versions of N17- or V12Rap1, challenged these cells with C3bi-RBC, and studied the recruitment of the Rap1 constructs to sites of particle binding. As shown in Figure 6B, both N17- and V12Rap1 were recruited to sites of C3bi-RBC binding. However, recruitment was significantly decreased in cells expressing the β_2F754A mutant integrin ($P = 0.01$ and $P = 0.04$, respectively, relative to control). The results strongly suggest that Rap1 recruitment is dependent on talin being able to interact through its head domain with the integrin β_2 chain.

TALIN HEAD CO-IMMUNOPRECIPITATES WITH BOTH N17- AND V12Rap1

Given that Rap1 and talin co-localise at sites of particle binding, we decided to investigate whether the two proteins interact

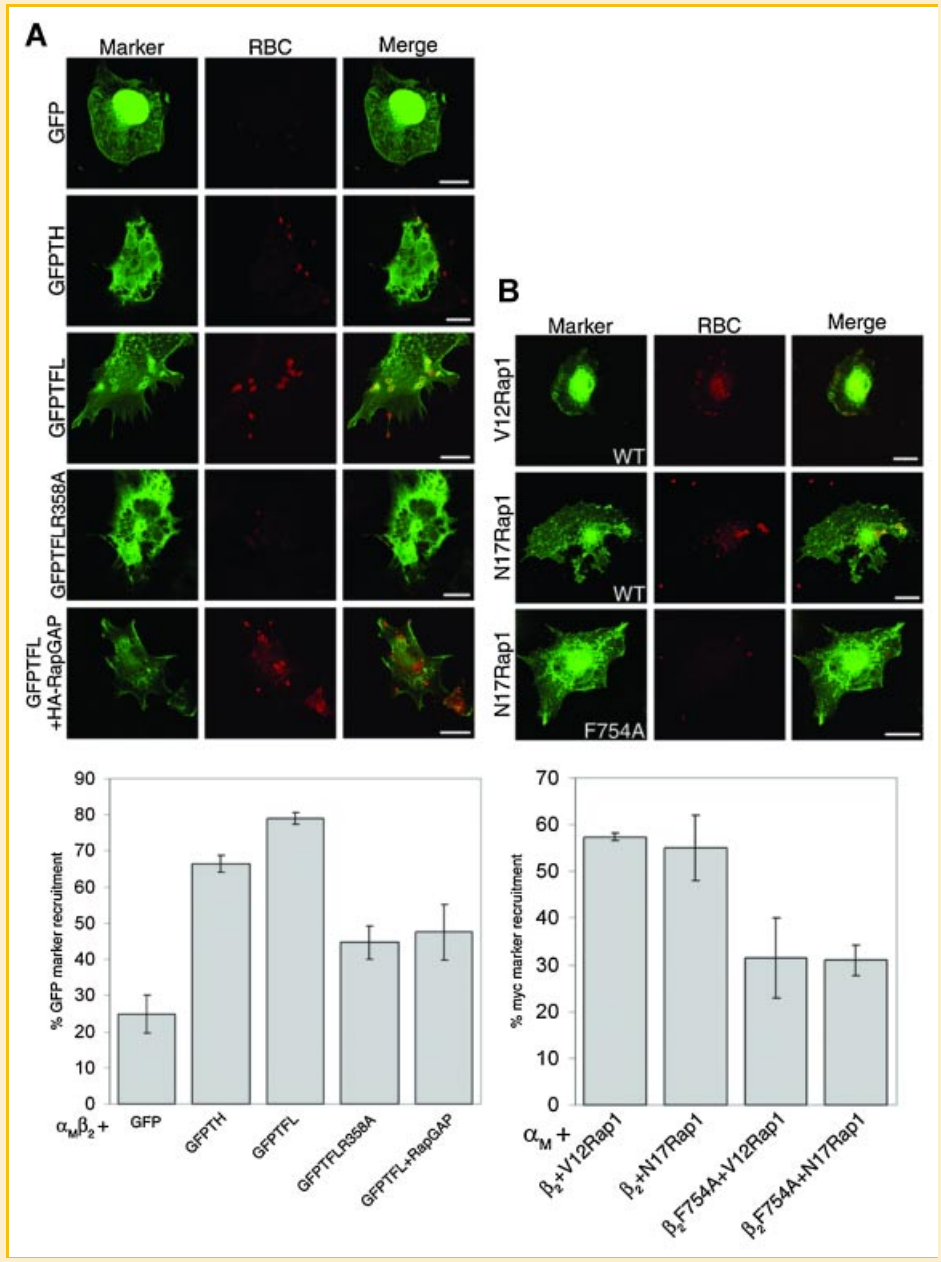

Fig. 6. Recruitment of over-expressed talin to sites of $\alpha_M\beta_2$-dependent RBC binding is dependent on Rap1. COS-7 cells were transfected with wt α_M, wt or mutant β_2, and GFP-, HA- (A) or myc- (B) constructs as indicated, challenged for 30 min with C3bi-RBC, and processed for confocal microscopy as described in the Materials and Methods Section. Top, representative examples. Bottom, GFP- (A), or myc-expressing (B) cells were scored for enrichment of GFP or myc at sites of RBC binding. Results are expressed as the mean ± SD of at least three independent experiments. Scale bar, 10 μm.

biochemically. As shown in Figure 7A, GFP-tagged talin was able to co-immunoprecipitate Rap1 in COS-7 cells. The interaction is specific as another small GTP-binding protein RhoA, did not co-precipitate. Furthermore, there was no co-immunoprecipitation of Rap1 with GFP alone. We conclude that Rap1 interacts either directly or indirectly with talin. To establish whether the interaction is dependent on the activation state of Rap1, we repeated the experiments using lysates from COS-7 cells co-transfected with GFPTH, and either myc-tagged N17- or V12Rap1. Lysates were mixed with the anti-GFP antibody and protein G-agarose, then separated by SDS–PAGE. Western blotting revealed that both N17- and V12Rap1 co-immunoprecipitated with GFPTH, but much less efficiently with GFP alone (Fig. 7B). We conclude that both forms of Rap1 interact with the head domain of talin although it is unclear whether this is a direct or indirect interaction. A direct albeit weak interaction of GTP-bound Rap1 with the talin head has been detected by NMR [Goult et al., 2010].

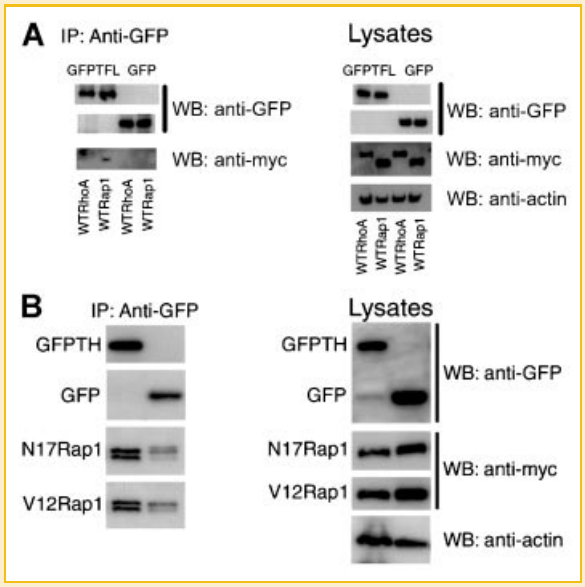

Fig. 7. Rap1 interacts with talin head. Lysates of COS-7 cells co-expressing either GFP-talin full length (GFPTFL), GFP-talin head (GFPTH) or GFP, together with wild-type Rap1 or RhoA were incubated with equal amounts of protein G coated beads coated with either (A) anti-myc or (B) anti-GFP. After washing with lysis buffer, pellet-associated proteins were separated by SDS–PAGE and analysed by Western blotting, using the indicated antibodies. Results are representative of three independent experiments.

THE Rap1-EFFECTOR RIAM DOES NOT REGULATE $\alpha_M\beta_2$ ACTIVATION

Han et al. [2006] have recently proposed that a complex involving Rap1, RIAM and talin regulates $\alpha_{IIb}\beta_3$ activation. As our results link talin and Rap1 to the regulation of inside-out activation of another integrin, $\alpha_M\beta_2$, we decided to test the role of RIAM in our system. THP-1 monocytes, which express endogenous $\alpha_M\beta_2$, were transfected with a RIAM-specific small interfering (si)RNA, analysed for RIAM expression, and scored blind for binding of opsonised RBC. As shown in Figure 8A, we repeatedly observed a 90% reduction in RIAM expression in siRNA treated cells compared to cells transfected with control (Luc) or talin-specific siRNA duplexes. As expected [Lim et al., 2007], talin knock-down led to a potent inhibition of C3bi-RBC binding by THP-1 cells (Fig. 8C). By contrast, RIAM knockdown had no measurable effect on $\alpha_M\beta_2$-mediated RBC binding under these conditions. We conclude that RIAM is not involved in the Rap1-mediated, talin-dependent activation of $\alpha_M\beta_2$. Even more surprisingly, we found that RIAM knockdown had no effects on THP-1 spreading (Fig. 8B), an integrin-dependent function that is strongly impaired in the absence of talin.

To further confirm this finding, we co-expressed GFP-tagged RIAM or GFP with $\alpha_M\beta_2$ integrin in COS-7 cells, and evaluated the effects on C3bi-RBC binding. We found no significant increase in binding between GFP-RIAM and GFP (416.5 ± 23.3 and 378 ± 49.5, respectively; $P = 0.5$) (Fig. 8D), and GFP-RIAM and GFP showed similar low levels of recruitment under bound C3bi-RBC ($34.5 \pm 1.7\%$ and 31.4 ± 10.8, respectively; $P = 0.24$) (Fig. 8E). Therefore, we conclude that the Rap1-effector RIAM is not involved in the regulation of the mammalian phagocytic integrin receptor $\alpha_M\beta_2$.

DISCUSSION

In the present study, we show that COS-7 cells transfected with $\alpha_M\beta_2$ integrin constructs provide an effective model system in which to study the regulation of $\alpha_M\beta_2$. Thus, binding of C3bi-opsonised RBC to $\alpha_M\beta_2$ integrin was dependent on Rap1-mediated inside-out signalling and was potentiated by PKC activation as shown previously for endogenous $\alpha_M\beta_2$ integrin in macrophages [Caron et al., 2000]. Using integrin truncation mutants, we show that Rap1 acts upon the β_2 and not the α_M chain, and that deletion of the β_2 tail results in constitutively active $\alpha_M\beta_2$ that has lost the ability to respond to either dominant negative N17Rap1 or constitutively active V12Rap1. The results are in agreement with data obtained for the $\alpha_L\beta_2$ integrin [Bleijs et al., 2001], and are consistent with the idea that disruption of electrostatic and hydrophobic links between the membrane proximal regions of the α and β cytoplasmic domains results in integrin activation [Vinogradova et al., 2002; Wegener et al., 2007]. Surprisingly, V12Rap1 had been proposed to act on the α_L and not the β_2 chain in BAF B lymphocytic cells expressing wild-type and cytoplasmic tail deletions of the $\alpha_L\beta_2$ (LFA-1) integrin [Tohyama et al., 2003]. The discrepancy could be due to the difference in the cell systems (COS-7 fibroblasts vs. B cells) or the difference in read-outs used. We used a binding assay whereas Tohyama and co-workers performed adhesion assays. In solid phase adhesion assays, defects in 'inside-out' and 'outside-in' signalling cannot easily be distinguished, as by the time the cell has adhered to its extracellular matrix surface, both inside-out and outside-in signalling processes to and from the integrins have taken place.

Further deletion analysis showed that the region spanning residues 732–761 in the β_2 cytoplasmic domain (Fig. 3A) was essential for $\alpha_M\beta_2$ activation by Rap1. Importantly, residues W747 and F754 that are essential for talin binding [Garcia-Alvarez et al., 2003] and activation of $\alpha_M\beta_2$ [Lim et al., 2007] are also required for Rap1-mediated integrin activation. The fact that talin1 knock-out MEFs showed a much reduced response to V12Rap1, indicates that Rap1 acts upstream of talin binding to the cytoplasmic domain of β_2. Similarly, siRNA knockdown of talin1 in THP-1 was accompanied by a 53% decrease in the number of bound C3bi-RBC, as shown previously in RAW264.7 mouse macrophages [Lim et al., 2007], and V12Rap1 did not rescue particle binding in these cells. Moreover, V12Rap1 was unable to increase particle binding to COS-7 cells expressing a talin mutant (R358A) that is unable to bind β tails [Garcia-Alvarez et al., 2003]. Interestingly, the threonine triplet 758–760 in the β2 subunit, which controls the recruitment of RhoA and actin polymerisation downstream of ligated $\alpha_M\beta_2$ [Wiedemann et al., 2006], is dispensable for the activation of $\alpha_M\beta_2$ by Rap1, confirming separate pathways for inside-out (Rap1) and outside-in (RhoA) signalling.

Our results are in broad agreement with those of Han et al. [2006] who showed that activation of $\alpha_{IIb}\beta_3$ expressed in CHO was both Rap1 and talin dependent. However, there are notable differences between the two systems. Thus, a combination of talin

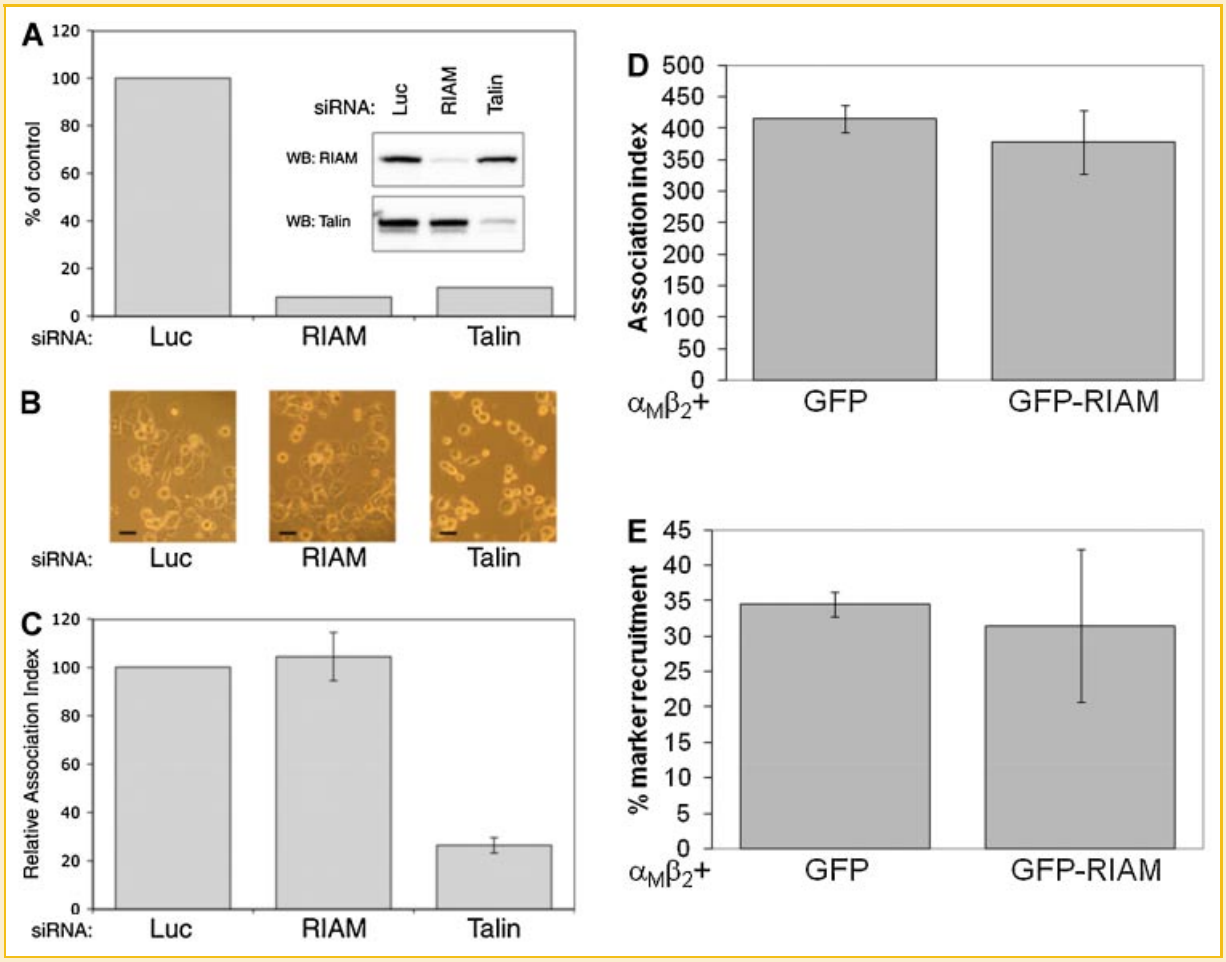

Fig. 8. RIAM is not involved in the regulation of $\alpha_M\beta_2$ binding ability. THP-1 or COS-7 were transfected with pools (80 nM total) of siRNAs directed against luciferase (Luc), RIAM or talin (THP-1) or with GFP-RIAM or GFP (COS-7). Forty-eight hours later, cell lysates were analysed for talin or RIAM expression (A) and binding of C3bi-RBC (B,D,E). A: Lysates of control and siRNA-transfected cells were analysed by Western blotting for the presence of talin, RIAM and tubulin (inset). Relative band intensities were determined as described in the Materials and Methods Section, with the ratio of talin/RIAM and tubulin intensities set to 100% for the negative control (Luc, Luciferase non-targeting siRNA). B,C: THP-1 monocytic cells were transfected with the indicated siRNA, differentiated into macrophages using PMA, challenged with C3bi-RBC, processed for immunofluorescence and stained for RBC. B: representative images of THP-1 prior to challenge with C3bi-RBC. Scale bar, 20 μm. C: Quantitation of RBC binding, with results expressed relative to the values obtained for Luc siRNA control (arbitrarily set to 100). D,E: COS-7 cells were transfected with DNA constructs as indicated, challenged for 30 min with C3bi-RBC, and processed for confocal microscopy as described in the Materials and Methods Section. Cells were scored for C3bi-RBC binding (D) or enrichment of GFP/GFP-RIAM at sites of RBC binding (E). Results are expressed as the mean ± SD of at least three independent experiments.

over-expression and PMA treatment was required to activate $\alpha_{IIb}\beta_3$ in CHO cells in order to achieve the same levels of activation found in platelets [Han et al., 2006]. By contrast, over-expression of talin alone in COS-7 cells is sufficient to induce maximum binding of C3bi-RBC to $\alpha_M\beta_2$. COS-7 cells have been shown to express abundant levels of protein kinase C-alpha [PKCα; Price et al., 2002], and this may explain why over-expression of talin alone is sufficient to induce maximal $\alpha_M\beta_2$ activation in these cells. Interestingly, N17Rap1 blocked both basal and talin-induced particle binding to COS-7 cells suggesting that there is sufficient basal Rap1-GTP in these cells to activate both endogenous and over-expressed talin.

Talin is believed to exist in an inactive auto-inhibited cytoplasmic form where the integrin-binding site in the head domain is masked by an intramolecular interaction between the head and rod domains [Goksoy et al., 2008; Goult et al., 2009]. Compelling evidence has been presented to show that the Rap1 plays a key role in both the recruitment of talin to the membrane and in $\alpha_{IIb}\beta_3$ activation [Han et al., 2006; Watanabe et al., 2008; Lee et al., 2009]. Our data are entirely consistent with these findings, and we found that in COS-7 cells expressing $\alpha_M\beta_2$, Rap1 controls both the recruitment of talin to $\alpha_M\beta_2$ and activation of $\alpha_M\beta_2$ RBC binding activity. The studies on $\alpha_{IIb}\beta_3$ activation in CHO cells also clearly show that the recruitment of talin to membrane-localised Rap1 is mediated by RIAM (Rap1-interacting adaptor molecule), which binds tightly to talin [Han et al., 2006; Watanabe et al., 2008; Lee et al., 2009]. Whether RIAM also activates talin is yet to be resolved, and several other mechanisms have been proposed including phosphatidylinositol (4,5)-bisphosphate binding which relieves the intramolecular interaction between the talin head and rod domain, at least in vitro [Goksoy et al., 2008]. However, we found no

discernable role for RIAM in activation of $\alpha_M\beta_2$ in human monocytes. Thus, macrophages depleted of RIAM still bound C3bi-RBC as efficiently as control cells, and RIAM knockdown had no effects on macrophage spreading. Moreover, over-expression of RIAM in $\alpha_M\beta_2$-transfected COS-7 cells had no effect on the number of bound C3bi-RBC, and RIAM was not recruited to sites of C3bi-RBC binding. We suggest that RIAM, which is part of the MRL (Mig-10/RIAM/Lpd) adaptor molecule family [Lafuente et al., 2004], is specific to megakaryocyte-platelet inside-out signalling, and is not the critical downstream Rap1 effector that controls talin-dependent activation of $\alpha_M\beta_2$. Other Rap1 effectors that are possible candidates in macrophage inside-out signalling are RAPL, Krit-1/CCM1 and several others [reviewed by Bos, 2005; Glading et al., 2007].

In conclusion, our data are consistent with a model in which activated membrane-bound Rap1-GTP drives recruitment of talin to the plasma membrane and its subsequent activation, but in a manner that is independent of the Rap1-effector RIAM. The F3 FERM domain in the talin head is then able to interact with the β_2 cytoplasmic domain and activate the $\alpha_M\beta_2$ integrin heterodimer, promoting particle binding and phagocytosis. β_2 integrins are important in a variety of leukocyte functions including phagocytosis, leukocyte transendothelial migration, motility and the formation of stable immunological synapses. All these events are regulated by Rap1 and involve talin [Shimonaka et al., 2003; Dustin et al., 2004; McLeod et al., 2004; Smith et al., 2005]. Rap1 was also recently demonstrated to be involved in regulation of another phagocytic receptor, the Fcγ receptor and activation of Rap1 by the exchange factor C3G was shown to be an essential step during the process of Fcγ receptor-mediated phagocytosis [Chung et al., 2008]. Finally, it is interesting to note that CD44, a transmembrane adhesion molecule implicated in the phagocytosis of large particles, also interacts with FERM-domain containing proteins [Mangeat et al., 1999] and has been linked with inside-out activation of $\alpha_M\beta_2$ [Vachon et al., 2007].

ACKNOWLEDGMENTS

We apologise for not including all relevant references due to limits placed by the journal. We would like to thank Dr Theresia Stradal, Dr Kazue Matsumoto and Dr Neil Bate for their kind gifts of plasmid constructs, as described in this manuscript. Sadly, Emmanuelle Caron passed away in the prime of her career on 8 July 2009 after a short illness [Lees and Hall, 2009]. We dedicate this manuscript to her memory as a well-respected mentor and a very good friend. Her energy at work and her great sense of humour are greatly missed.

REFERENCES

Abram CL, Lowell CA. 2009. The ins and outs of leukocyte integrin signalling. Annu Rev Immunol 27:339–362.

Anthis NJ, Wegener KL, Ye F, Kim C, Goult BT, Lowe ED, Vakonakis I, Bate N, Critchley DR, Ginsberg MH, Campbell ID. 2009. The structure of an integrin/talin complex reveals the basis of inside-out signal transduction. EMBO J 28:3623–3632.

Armulik A, Nilsson I, von Heijne G, Johansson S. 1999. Determination of the border between the transmembrane and cytoplasmic domains of human integrin subunits. J Biol Chem 274:37030–37034.

Bleijs DA, van Duijnhoven GC, van Vliet SJ, Thijssen JP, Figdor CG, van Kooyk Y. 2001. A single amino acid in the cytoplasmic domain of the beta 2 integrin lymphocyte function-associated antigen-1 regulates avidity-dependent inside-out signaling. J Biol Chem 276:10338–10346.

Bos JL. 2005. Linking Rap to cell adhesion. Curr Opin Cell Biol 17:123–128.

Caron E, Hall A. 1998. Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. Science 282:1717–1721.

Caron E, Self AJ, Hall A. 2000. The GTPase Rap1 controls functional activation of macrophage integrin alphaMbeta2 by LPS and other inflammatory mediators. Curr Biol 10:974–978.

Caron E. 2003. Cellular functions of the Rap1 GTP-binding protein: A pattern emerges. J Cell Sci 116:435–440.

Chaves-Olarte E, Low P, Freer E, Norlin T, Weidmann M, von Eichel-Streiber C, Thelestam M. 1999. A novel cytotoxin from *Clostridium difficile* serogroup F is a functional hybrid between two other large clostridial cytotoxins. J Biol Chem 274:11046–11052.

Chung J, Serezani CH, Huang SK, Stern JN, Keskin DB, Jagirdar R, Brock TG, Aronoff DM, Peters-Golden M. 2008. Rap1 activation is required for Fc gamma receptor-dependent phagocytosis. J Immunol 181:5501–5509.

Desjardins M, Houde M, Gagnon E. 2005. Phagocytosis: The convoluted way from nutrition to adaptive immunity. Immunol Rev 207:158–165.

Dustin ML, Bivona TG, Philips MR. 2004. Membranes as messengers in T cell adhesion signaling. Nat Immunol 5:363–372.

Garcia-Alvarez B, de Pereda JM, Calderwood DA, Ulmer TS, Critchley D, Campbell ID, Ginsberg MH, Liddington RC. 2003. Structural determinants of integrin recognition by talin. Mol Cell 11:49–58.

Glading A, Han J, Stockton RA, Ginsberg MH. 2007. KRIT-1/CCM1 is a Rap1 effector that regulates endothelial cell cell junctions. J Cell Biol 179:247–254.

Goksoy E, Ma YQ, Wang X, Kong X, Perera D, Plow EF, Qin J. 2008. Structural basis for the autoinhibition of talin in regulating integrin activation. Mol Cell 31:124–133.

Goult BT, Bouaouina M, Elliott PR, Bate N, Patel B, Gingras AR, Grossmann JG, Roberts GC, Calderwood DA, Critchley DR, Barsukov IL. 2010. Structure of a double ubiquitin-like domain in the talin head: A role in integrin activation. EMBO J 29:1069–1080.

Goult BT, Bate N, Anthis NJ, Wegener KL, Gingras AR, Patel B, Barsukov IL, Campbell ID, Roberts GC, Critchley DR. 2009. The structure of an interdomain complex that regulates talin activity. J Biol Chem 284:15097–15106.

Griffin FM, Jr., Griffin JA, Leider JE, Silverstein SC. 1975. Studies on the mechanism of phagocytosis. I. Requirements for circumferential attachment of particle-bound ligands to specific receptors on the macrophage plasma membrane. J Exp Med 142:1263–1282.

Han J, Lim CJ, Watanabe N, Soriani A, Ratnikov B, Calderwood DA, Puzon-McLaughlin W, Lafuente EM, Boussiotis VA, Shattil SJ, Ginsberg MH. 2006. Reconstructing and deconstructing agonist-induced activation of integrin alphaIIbbeta3. Curr Biol 16:1796–1806.

Hynes RO. 2002. Integrins: Bidirectional, allosteric signaling machines. Cell 110:673–687.

Jiang G, Giannone G, Critchley DR, Fukumoto E, Sheetz MP. 2003. Two-piconewton slip bond between fibronectin and the cytoskeleton depends on talin. Nature 424:334–337.

Lafuente EM, van Puijenbroek AA, Krause M, Carman CV, Freeman GJ, Berezovskaya A, Constantine E, Springer TA, Gertler FB, Boussiotis VA. 2004. RIAM, an Ena/VASP and Profilin ligand, interacts with Rap1-GTP and mediates Rap1-induced adhesion. Dev Cell 7:585–595.

Lau TL, Kim C, Ginsberg MH, Ulmer TS. 2009. The structure of the integrin alphaIIbbeta3 transmembrane complex explains integrin transmembrane signalling. EMBO J 28:1351–1361.

Lee HS, Lim CJ, Puzon-McLaughlin W, Shattil SJ, Ginsberg MH. 2009. RIAM activates integrins by linking talin to ras GTPase membrane-targeting sequences. J Biol Chem 284:5119–5127.

Lees DM, Hall A. 2009. Memorial: Emmanuelle Caron 1967–2009. J Leukoc Biol 85:749–750.

Lim J, Wiedemann A, Tzircotis G, Monkley SJ, Critchley DR, Caron E. 2007. An essential role for talin during alphaMbeta2-mediated phagocytosis. Mol Biol Cell 18:976–985.

Liu S, Calderwood DA, Ginsberg MH. 2000. Integrin cytoplasmic domain-binding proteins. J Cell Sci 113:3563–3571.

Mangeat P, Roy C, Martin M. 1999. ERM proteins in cell adhesion and membrane dynamics. Trends Cell Biol 9:187–192.

May RC, Caron E, Hall A, Machesky LM. 2000. Involvement of the Arp2/3 complex in phagocytosis mediated by FcgammaR or CR3. Nat Cell Biol 2:246–248.

McLeod SJ, Shum AJ, Lee RL, Takei F, Gold MR. 2004. The Rap GTPases regulate integrin-mediated adhesion, cell spreading, actin polymerization, and Pyk2 tyrosine phosphorylation in B lymphocytes. J Biol Chem 279: 12009–12019.

Nishida N, Xie C, Shimaoka M, Cheng Y, Walz T, Springer TA. 2006. Activation of leukocyte beta2 integrins by conversion from bent to extended conformations. Immunity 25:583–594.

Price MO, McPhail MC, Lambeth JD, Han CH, Knaus UG, Dinauer MC. 2002. Creation of a genetic system for analysis of the phagocyte respiratory burst: High-level reconstitution of the NADPH oxidase in a nonhematopoietic system. Blood 99:2653–2661.

Schmidt A, Caron E, Hall A. 2001. Lipopolysaccharide-induced activation of beta2-integrin function in macrophages requires Irak kinase activity, p38 mitogen-activated protein kinase, and the Rap1 GTPase. Mol Cell Biol 21: 438–448.

Schmidt M, Voss M, Thiel M, Bauer B, Grannass A, Tapp E, Cool RH, de Gunzburg J, von Eichel-Streiber C, Jakobs KH. 1998. Specific inhibition of phorbol ester-stimulated phospholipase D by Clostridium sordellii lethal toxin and Clostridium difficile toxin B-1470 in HEK-293 cells. J Biol Chem 273:7413–7422.

Sebzda E, Bracke M, Tugal T, Hogg N, Cantrell DA. 2002. Rap1A positively regulates T cells via integrin activation rather than inhibiting lymphocyte signaling. Nat Immunol 3:251–258.

Shattil SJ. 2009. The beta3 integrin cytoplasmic tail: Protein scaffold and control freak. J Thromb Haemost 1:210–213.

Shattil SJ, Kim C, Ginsberg MH. 2010. The final steps of integrin activation: The end game. Nat Rev Mol Cell Biol 11:288–300.

Shimonaka M, Katagiri K, Nakayama T, Fujita N, Tsuruo T, Yoshie O, Kinashi T. 2003. Rap1 translates chemokine signals to integrin activation, cell polarization, and motility across vascular endothelium under flow. J Cell Biol 161:417–427.

Smith A, Carrasco YA, Stanley P, Kieffer N, Batista FD, Hogg N. 2005. A talin-dependent LFA-1 focal zone is formed by rapidly migrating T lymphocytes. J Cell Biol 170:141–151.

Stefansson A, Armulik A, Nilsson I, von Heijne G, Johansson S. 2004. Determination of N- and C-terminal borders of the transmembrane domain of integrin subunits. J Biol Chem 279:21200–21205.

Takagi J, Petre BM, Walz T, Springer TA. 2002. Global conformational rearrangements in integrin extracellular domains in outside-in and inside-out signaling. Cell 110:599–611.

Tohyama Y, Katagiri K, Pardi R, Lu C, Springer TA, Kinashi T. 2003. The critical cytoplasmic regions of the alphaL/beta2 integrin in Rap1-induced adhesion and migration. Mol Biol Cell 14:2570–2582.

Underhill DM, Ozinsky A. 2002. Phagocytosis of microbes: Complexity in action. Annu Rev Immunol 20:825–852.

Vachon E, Martin R, Kwok V, Cherepanov V, Chow CW, Doerschuk CM, Plumb J, Grinstein S, Downey GP. 2007. CD44-mediated phagocytosis induces inside-out activation of complement receptor-3 in murine macrophages. Blood 110:4492–4502.

Vinogradova O, Velyvis A, Velyviene A, Hu B, Haas T, Plow E, Qin J. 2002. A structural mechanism of integrin alpha(IIb)beta(3) ''inside-out'' activation as regulated by its cytoplasmic face. Cell 110:587–597.

Wagner C, Hansch GM, Stegmaier S, Denefleh B, Hug F, Schoels M. 2001. The complement receptor 3, CR3 (CD11b/CD18), on T lymphocytes: Activation-dependent up-regulation and regulatory function. Eur J Immunol 31:1173–1180.

Watanabe N, Bodin L, Pandey M, Krause M, Coughlin S, Boussiotis VA, Ginsberg MH, Shattil SJ. 2008. Mechanisms and consequences of agonist-induced talin recruitment to platelet integrin alphaIIbbeta3. J Cell Biol 181:1211–1222.

Wegener KL, Partridge AW, Han J, Pickford AR, Liddington RC, Ginsberg MH, Campbell ID. 2007. Structural basis of integrin activation by talin. Cell 128: 171–182.

Wiedemann A, Patel JC, Lim J, Tsun A, van Kooyk Y, Caron E. 2006. Two distinct cytoplasmic regions of the beta2 integrin chain regulate RhoA function during phagocytosis. J Cell Biol 172:1069–1079.

Zhang X, Jiang G, Cai Y, Monkley SJ, Critchley DR, Sheetz MP. 2008. Talin depletion reveals independence of initial cell spreading from integrin activation and traction. Nat Cell Biol 10:1062–1068.