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Serine 756 of β_2 integrin controls Rap1 activity during inside-out activation of $\alpha_M\beta_2$

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SYNOPSIS

During $\alpha_M\beta_2$ -mediated phagocytosis, the small GTPase Rap1 activates the β_2 integrin by binding to a region between residues 732 – 761. Using COS-7 cells transfected with $\alpha_M\beta_2$, we show that $\alpha_M\beta_2$ activation by the phorbol ester, PMA, involves serine residue 756 of β_2 . This residue is critical for the local positioning of talin, and biochemically interacts with Rap1. Using the calmodulin antagonist W7, we found Rap1 recruitment and inside-out activation of $\alpha_M\beta_2$ to be affected. We also report a role for calcium/calmodulin kinase II (CamKII) in the activation of Rap1 during integrin activation. These results demonstrate a distinct, physiological role for the serine residue 756 of β_2 integrin, in conjunction with the actions of talin and Rap1, during $\alpha_M\beta_2$ activation in macrophages.

INTRODUCTION

Phagocytosis is the physiological process by which cells internalize 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 [1]. Phagocytic receptors can bind their targets directly or indirectly through opsonins [2], and can exist as constitutively active or inducible receptors prior to binding to their opsonised targets. An example of an inducible phagocytic receptor is the integrin $\alpha_M\beta_2$ aka complement receptor 3, CR3, Mac-1, CD11b/CD18. This is expressed on the cells of the monocyte/macrophage lineage and polymorphonuclear neutrophils, but also on Natural Killer (NK) cells, B- and T-lymphocytes [3]. Phagocytosis through $\alpha_M\beta_2$ is a multistep process that sequentially involves receptor-mediated particle recognition via interactions between active integrins and their C3bi ligand, actin-driven uptake, and phagosome closure and maturation [4].

Integrins are heterodimeric, cell surface receptors made up of single α and β chains. Integrin chains are divided into a large extracellular ligand-binding domain, a single pass transmembrane domain and a short cytoplasmic tail, and they are bi-directionally regulated. 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 [5]. However, the ligand-binding activity of many integrins is

regulated by “inside-out” signalling events which affect integrin conformation [6,7]. The cytoskeletal protein, talin, and the small GTP-binding protein, Rap1, are involved in “inside-out” signalling to many integrins [6,7]. In addition, RIAM (Rap1-GTP-interacting adaptor molecule) is reported to be involved in regulation of $\alpha_{IIb}\beta_3$ activity [8-10].

Previously, we established that both talin and Rap1, but not RIAM, are essential regulators of “inside-out” activation of integrin $\alpha_M\beta_2$ during phagocytosis in macrophages [11,12]. Furthermore, Rap1 activation of $\alpha_M\beta_2$ integrin is dependent on binding of talin to the cytoplasmic tail of β_2 [11,12]. Activation of $\alpha_M\beta_2$ by Rap1 was dependent upon residues 732 to 761 of the β_2 integrin chain, of which residues W747 and F754 control the binding of the talin head domain to the β_2 tail [12]. This is essential for optimal inside-out activation of β_2 integrins in transfected cells [11,13,14]. Also in this region, the triple-threonine residues 758–760, which regulate the stable recruitment of active RhoA, actin polymerization and phagocytosis in response to $\alpha_M\beta_2$ ligation [15], are dispensable for talin head domain binding and V12Rap1 activation [11].

Fagerholm et al [16] demonstrated that, in human T cells, serine residue 756 of β_2 cytoplasmic tail peptide was phosphorylated after phorbol ester stimulation. This effect was suppressed by the calmodulin antagonist, W7, suggesting a role for calmodulin in Ser-756 phosphorylation. Incidentally, W7 also inhibits β_1 -mediated adhesion of monocytic cell line THP-1 to VCAM-1 and fibronectin in a dose-dependent manner [13]. Calmodulin (CaM) is a ubiquitous, calcium-binding protein that binds and regulates different protein targets, and affects different cellular functions. CaM is composed of 148 conserved amino acids and several common CaM-binding motifs have been identified in CaM-binding proteins, including calcium/CaM-dependent protein kinases [18].

In this study we show that the serine residue S756 is responsible for the recruitment and activation of the Rap1/talin complex to $\alpha_M\beta_2$ integrin. This leads to activation of the integrin and uptake of C3bi-opsonised RBC. We suggest this process is mediated by calcium/calmodulin dependent protein kinase II (CamKII), either by Rap1 activation, Rap1 recruitment to $\alpha_M\beta_2$, or by a combination of both processes.

EXPERIMENTAL

Reagents

Sheep red blood cells (RBC) were purchased from TCS Biosciences Ltd. Phorbol 12-myristate 13-acetate (PMA), Gelatin Veronal Buffer, Protein G-Agarose, Guanosine 5'-[γ -thio]triphosphate, Guanosine 5'-diphosphate and C5-deficient serum were obtained from Sigma. Calmodulin antagonist, W7 and calcium/calmodulin-dependent protein kinase II inhibitor, KN62 were obtained from Calbiochem. Rhodamin-phalloidin was obtained from Invitrogen.

The antibodies used in this study were rat anti-mouse α_M (clone 5c6; Serotec), mouse anti-human β_2 (clone 6.7; BD-Pharmingen); mouse anti-GFP (clones 7.1 and 13.1; Roche); mouse anti-myc (clone 9E10; Santa Cruz) and rabbit anti-myc (clone 71D10; Cell Signalling); rat anti-HA (clone 3F10; Roche); mouse anti-Rap1 (clone 3; BD Transduction Laboratories) and rabbit IgM anti-RBC antibodies (Cedarlane Laboratories). Conjugated secondary antibodies were from Invitrogen, 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 [11,15,19]. pRKGF-Talin (GFPTFL) and pRKGFPTalinHead

(GFPTH) were kindly provided by Kazue Matsumoto (National Institute of Health, MA) and Neil Bate (Leicester University, UK) respectively.

To generate the β_2 S756M and S756D mutants, mutations were introduced into pRK5- β_2 by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), by using the following combinations of primers (mutation underlined): S756D, 5'-AATCCCCCTTTTCAAGGACGCCACCACGACGGTC-3' and 5'-GACCGTCGTGGTGGCGTCCTTGAAAAGGGGATT-3'; S756M, 5'-AATCCCCCTTTTCAAGATGGCCACCACGACGGTC-3' and 5'-GACCGTCGTGGTGGCCATCTTGAAAAGGGGATT-3'. 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 murine macrophage J774A.1 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 [19]. COS-7 cells were transfected using the DEAE-dextran method [19], or by nucleofection (program A-024; Amaxa Biosystems, Gaithersburg, MD) and left to express constructs for 48, or 24 hours respectively, before phagocytic challenge (DEAE-Dextran) or immunoprecipitations (Amaza).

Phagocytic challenge

IgG- and C3bi-opsonised RBC (later referred to as IgG- and C3bi-RBC, respectively) were prepared and used as previously described [15,19], using 0.1 μ l (0.5 μ l for macrophages) fresh RBC per 13mm glass coverslip. Where needed, cells were pre-treated with inhibitors as follows: W7, 1-100 μ M for 15 mins or KN62, 25 μ M for 20 mins. To elicit "inside-out" signalling, macrophages and $\alpha_M\beta_2$ -expressing COS-7 cells were pre-treated with 150ng/ml PMA (Sigma) in HEPES-buffered, serum-free DMEM for 15 min at 37°C as described previously [20]. 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 [11], 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, e.g. a Cy2-conjugated donkey anti-mouse antibody for the β_2 and a Rhodamine Red X-conjugated donkey anti-rabbit antibody to stain opsonized RBC. Coverslips were finally mounted in Mowiol (Calbiochem) containing p-phenylene diamine (Sigma) as antifading reagent and analyzed 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. Measurement of spreading was performed using ImageJ software (National Institutes of Health). The spreading index is defined as the average surface area of 50 macrophages.

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 analyzed 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.

Significance was tested using Student's *t* test. $P < 0.05$ was considered significant and is indicated by a single asterisk. Insignificant results is indicated by a double asterisk.

Immunoprecipitation

Serum-starved transfected COS-7 cells were lysed on ice in lysis buffer (1% NP-40, 50mM Tris pH7.6, 150mM NaCl, 1mM PMSF, 1mM DTT and protease inhibitor cocktail (Roche Applied Science, East Sussex, UK)). Lysates were incubated for 2 hours 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 1:1000) were added for 1 hour, followed by goat anti-mouse-HRP (horseradish peroxidase). Detection was carried out using the Enhanced ChemiLuminescence detection kit (ECL, Amersham).

Rap1 pull-down assay

Rap1 pull-downs were conducted as previously described [21] using 20µg GST-RalGDS-RBD and 2×10^7 J774A.1 cells per condition. Cells were kept in HEPES-supplemented SFM before treatment with KN62 (25µM for 20 min). Samples were run on 15% acrylamide SDS-PAGE gels, blotted and probed with a monoclonal mouse anti-Rap1 antibody, followed by goat anti-mouse-HRP. Detection was performed using the Enhanced ChemiLuminescence detection kit (ECL, Amersham). Intensities of bands were determined by densitometric analysis by using the ImageJ software and related to the levels of GST fusions.

RESULTS

$\alpha_M\beta_2$ activation by PMA is regulated by Rap1 and through residue S756 of β_2

Previously, we showed that in both J774A.1 macrophages and COS-7 cells, treatment with the phorbol ester phorbol myristate acetate (PMA) increased binding of C3bi-coated RBC (C3bi-RBC) via the activity of Rap1, an essential regulator of “inside-out” signalling to integrins [12,20]. We demonstrated that Rap1 acts on the β_2 and not the α_M chain, through β_2 residues 732 to 761 [12]. In Figure 1A, $\alpha_M\beta_2$ -expressing COS-7 cells co-transfected with dominant negative Rap1 (N17) or RapGAP (Rap specific GTPase activating protein which binds and activates Rap1's intrinsic GTPase activity, turning ‘off’ the signalling cascade) were unresponsive to PMA stimulation ($\alpha_M\beta_2$ +N17Rap1 +/-PMA, $p=0.18$; $\alpha_M\beta_2$ +RapGAP +/-PMA, $p=0.21$). COS-7 cells transfected with wild type α_M and β_2 point mutants W747A or F754A, known to be needed for talin head domain binding and activation [11], were also unresponsive (Figure 1B). Interestingly, expression of the triple-threonine mutant of β_2 (to triple-alanines), known to recruit active RhoA needed for “outside-in” signalling [15], resulted in increased C3bi-RBC binding, in response to PMA treatment. However, expression of the phosphorylation-insensitive point mutant β_2 S756M with wt α_M , resulted in a lack of C3bi-RBC binding, after PMA stimulation, although surface expression was not affected. These data suggest serine 756 is required for the regulation of $\alpha_M\beta_2$, possibly through Rap1 (Figure 1B and data not shown).

Serine 756 of β_2 is required for the recruitment of talin

To address the role(s) of serine 756 in the regulation of $\alpha_M\beta_2$, COS-7 cells were transfected with wild-type α_M and β_2 or β_2 S756M, and Rap1 or talin constructs. These cells were challenged with C3bi-RBC and the association indices and recruitment of Rap1 or talin were analysed. When wt β_2 was replaced with β_2 S756M, only the GFP-tagged talin head domain (GFPTH) was able to maintain binding to C3bi-RBC (Figure 2A). Rap1 was previously shown to act upstream of talin, and they influence each other's localisation to phagocytic cups [12]. There is also a correlation between the recruitment of constitutively active Rap1 (V12), full length talin (GFPTFL) or GFPTH to $\alpha_M\beta_2$ -dependent C3bi-RBC binding [12]. We found that S756 was important for the recruitment of full length talin. COS-7 cells transfected with wt α_M and β_2 S756M, and challenged with C3bi-RBC, showed a significant reduction in recruitment of GFPTFL, when compared to cells transfected with wt β_2 (Figure 2B, $p=0.001$). These data suggest serine 756 is involved in the recruitment of full-length talin, prior to talin head domain exposure and integrin activation.

Rap1 interacts with β_2 through serine 756

Since the talin head domain activates $\alpha_M\beta_2$ S756M and Rap1 acts upstream of talin in activating $\alpha_M\beta_2$, we analysed interactions between Rap1 and β_2 S756M, using co-immunoprecipitation. We immunoprecipitated GFPTH, but not GFP from COS-7 cells expressing $\alpha_M\beta_2$ S756M (Figure 3A). To establish if Rap1 interaction with $\alpha_M\beta_2$ is mediated by S756, we used an anti- α_M antibody to immunoprecipitate from COS-7 lysates co-transfected with myc-tagged wt Rap1, wt α_M and phosphorylation-insensitive (S756M) or phospho-mimicking (S756D) mutants of β_2 . Western blot analysis revealed wt Rap1 co-immunoprecipitated with the phospho-mimicking but not the phosphorylation-insensitive point mutant (Figure 3B). We conclude that Rap1 interacts with β_2 through the serine 756 residue.

PMA- and Rap1-mediated regulation of $\alpha_M\beta_2$ activity is sensitive to the calmodulin antagonist W7

Fagerholm et al [16] demonstrated phosphorylation of β_2 S756 from human T cells stimulated with the phorbol ester, phorbol 12,13-dibutyrate (PDBu). This effect was suppressed by the calmodulin antagonist, W7 [16]. To explore a role for calmodulin in $\alpha_M\beta_2$ -mediated C3bi-RBC binding, we used both J774A.1 macrophages and $\alpha_M\beta_2$ -transfected COS-7 cells. J774A.1 cells were pre-treated with W7, stimulated with PMA, challenged with C3bi-RBC and scored for binding and spreading capabilities. Basal, but not PMA-stimulated, binding was significantly decreased at low levels of W7 pre-treatment (25 μ M and under, $p<0.05$) (Figure 4A). However, at 100 μ M W7, only PMA-stimulated J774A.1 binding of C3bi-RBC was significantly decreased (Figure 4A). A similar result was also observed in the $\alpha_M\beta_2$ -transfected COS-7 cells (data not shown). This effect of W7 is not restricted to binding, as cell spreading was also affected. Unlike binding, spreading decreased in a dose-dependent manner, with basal and PMA-stimulated cells, pre-treated with 100 μ M W7 showing significant ($p<0.05$) decrease in spreading (Figure 4B). To dissect the effect of W7 on Rap1-mediated inside-out activation, the $\alpha_M\beta_2$ -transfected COS-7 model was used. Increased C3bi-RBC binding was observed when V12Rap1 was over-expressed in $\alpha_M\beta_2$ -transfected COS-7 [12], which was blocked by 100 μ M W7 (Figure 4C, $\alpha_M\beta_2$ +/-V12Rap1, $p=0.14$). In $\alpha_M\beta_2$ F754A-transfected COS-7 cells (β_2 defective in talin head domain binding), addition of W7 did not completely abolish binding levels (Figure 4C, $p=0.09$). These data suggest the calmodulin antagonist W7 blocks Rap1-mediated inside-out signalling to $\alpha_M\beta_2$. A decrease in

V12Rap1 localisation to sites of C3bi-RBC binding was also seen in W7 pre-treated, $\alpha_M\beta_2$ -expressing COS-7 cells (Figure 4D, $p=0.03$). We conclude that calmodulin may be involved in the recruitment of Rap1 to $\alpha_M\beta_2$ during inside-out signalling.

Rap1 activity is blocked by CamKII inhibitor KN62

Previous studies have demonstrated a role for calcium/calmodulin-dependent protein kinase II (CamKII) during integrin-mediated signalling events [22,23]. We analysed whether CamKII played a role in Rap1-mediated inside-out signalling to $\alpha_M\beta_2$. J774A.1 macrophages pre-treated with a CamKII inhibitor, KN62, displayed a small, but significant decrease in binding to C3bi-RBC (Figure 5A, $p=0.002$). However, PMA was still able to significantly increase binding of C3bi-RBC to KN62-treated J774A.1 macrophages, though not to the same level as the control (Figure 5A, $p=0.02$). This suggests CamKII functions upstream of the PMA target molecule, presumably Rap1. However, in contrast to cells treated with W7, spreading was not significantly affected (Figure 5B). This suggests a specific role for CamKII during inside-out activation of $\alpha_M\beta_2$ in J774A.1 macrophages. To study the effects of KN62 on Rap1 activity, we performed a GST pull-down experiment to detect levels of GTP-bound Rap1 in macrophages [20]. This was achieved using the Rap1-binding domain of RalGDS, a known Rap1 effector [21]. Glutathione-Sepharose beads coated with GST-RalGDS were mixed with lysates from mouse macrophages J774A.1 loaded with the unhydrolysable GTP, GTP γ S or GDP, mimicking active and inactive Rap1, respectively. Western blot analysis (Figure 6A) demonstrated that the GST-RalGDS-coupled Glutathione-Sepharose beads were functional, pulling down more GTP-loaded Rap1 compared to the GDP-bound form. To determine the effect(s) of KN62 on Rap1 activation, GST pull-down was repeated but with KN62-treated J774A.1. Figures 6B and 6C shows that lysates of KN62-treated J774A.1 contained less GTP-bound Rap1, compared to the DMSO control. This suggests the CamKII inhibitor, KN62, inhibits Rap1 activation. We conclude there is a potential role for CamKII in the activation of Rap1 during $\alpha_M\beta_2$ activation.

DISCUSSION

This study examines the role(s) of residue serine 756 of β_2 (β_2 S756) in Rap1-mediated inside-out signalling to $\alpha_M\beta_2$, during phagocytosis. Previously, we showed Rap1 activation of $\alpha_M\beta_2$ was dependent on residues 732 to 761 of the β_2 chain and Rap1 acts upstream of talin, in a RIAM-independent manner [12]. This β_2 region contains two residues known to control talin head domain binding (W747 and F754), one of which (F754) is part of a highly conserved NPxY motif found in cytoplasmic tails of β integrins [24]. These 2 key residues are essential for maximal inside-out activation of the $\alpha_M\beta_2$ integrin in macrophages and $\alpha_M\beta_2$ -transfected COS-7 cells [11]. There is also a triple threonine motif (758–760) that regulates the recruitment of active RhoA to $\alpha_M\beta_2$ during outside-in signalling, actin polymerization, phagocytosis and recruitment of 14-3-3 proteins [15,25]. We found $\alpha_M\beta_2$ S756M-expressing COS-7 cells unresponsive to both PMA stimulation and V12Rap1 activation, but sensitive to activation by the talin head domain. Mutation of β_2 S756 to alanine had no effect on phorbol ester-induced binding to ICAM-1, a known ligand of the integrin [26]. Our results are also in agreement with research on the homologous serine residue 785 in β_1 , which is involved in a variety of integrin-mediated events including adhesion, spreading and migration [27]. The fibroblast cell line (GD25) and the teratocarcinoma cell line (F9) expressing a β_1 mutant containing a dephosphorylated serine residue promoted cell attachment, spreading and migration on laminin [27]. While S756 was essential for Rap1 association with β_2 , it is only partially involved in talin recruitment to β_2 . This suggests other molecule(s) may be involved

in talin recruitment, which in turn will lead to Rap1 promoting talin head domain exposure thereby regulating integrin activation.

β_2 S756 was reported to be phosphorylated after phorbol ester stimulation in T cells and in reconstituted systems using COS-7 and B lymphoblastoid cells expressing mutant β_2 subunits [16,26]. We found that Rap1 interacts with β_2 through the phosphor-mimicking S756D residue, suggesting the involvement of a kinase. As β_2 S756 was found not to be phosphorylated by PKC isoforms in T cells after phorbol ester stimulation [16], other possibilities were considered. The calmodulin antagonist W7 was known to affect actin reorganisation and cell spreading in T cells challenged with phorbol esters [28]. We found that W7 decreases C3bi-RBC binding, by preventing the recruitment of V12Rap1 to sites of particle binding, in both J774A.1 macrophages and $\alpha_M\beta_2$ -expressing COS-7 cells. Spreading is also decreased, suggesting W7 blocks a molecule involved in Rap1's localisation to $\alpha_M\beta_2$ and other integrins. Interestingly, in the monocytic cell line THP-1, W7 inhibits vortex-induced adhesion to vascular cell adhesion molecule-1 (VCAM-1) and fibronectin in a dose-dependent manner [17]. Both adhesion mechanisms are through $\alpha_4\beta_1$ and $\alpha_5\beta_1$, respectively [17]. Recently, it was revealed that calmodulin forms a complex with ezrin/radixin/moesin (ERM) and binds to the cytoplasmic tail of the cell adhesion molecule, L-selectin [29]. With talin also containing a N-terminal FERM (protein 4.1, ezrin, radixin, moesin) domain, and L-selectin clustering involved in inside-out activation of integrins, it is tempting to suggest that these two pathways are linked [29].

A possible candidate for a W7-sensitive serine kinase is calcium/calmodulin-dependent protein kinase II (CamKII). CamKII is a multi-isoform calcium-dependent serine/threonine kinase. CamKII regulates gene expression, membrane trafficking, secretion, cytoskeletal dynamics, apoptosis and the β_3 -mediated phagocytosis of apoptotic tumor cells [30]. CamKII activity is regulated by binding of calcium-calmodulin, autophosphorylation of stimulatory and inhibitory sites, and the action of protein phosphatases [31]. Data which implicates CamKII in various integrin-mediated signalling events are somewhat contradictory, particularly with reference to the use of KN62, a CamKII inhibitor. Although KN62 specifically inhibited calcium/calmodulin protein kinase isoforms [32], and decreased phagocytosis in K562 cells [23], it showed no effect on integrin-mediated T cell-cell adhesion [28]. In addition, KN62 was reported to increase adhesion and spreading in CHO cells [22]. We found KN62 blocked C3bi-RBC binding in PMA-stimulated J774A.1 macrophages, possibly by decreasing Rap1 activity. Interestingly, spreading was unaffected by KN62 suggesting a specific role for CamKII in $\alpha_M\beta_2$ -mediated particle binding and Rap1 activation. Though β_2 integrin controls a variety of leukocyte functions, including phagocytosis, leukocyte transendothelial migration, motility, and the formation of stable immunological synapses, inside-out signalling to β_2 is not clearly understood. Other cytoskeletal proteins such as radixin (part of the FERM family of proteins) or vinculin also activate integrins [33,34]. There is also the possibility of a NPxY-independent activation of β_2 , as inferred by work on the β_5 integrin which regulates phagocytosis of apoptotic cells [35]. The model we propose is that CamKII acts on β_2 at serine 756, enabling $\alpha_M\beta_2$ to recruit Rap1, expose the talin head domain and activate $\alpha_M\beta_2$. CamKII functions upstream of Rap1, possibly regulating a guanine exchange factor (GEF), thereby promoting full activation of Rap1. Further work is needed to fully understand the role of CamKII during $\alpha_M\beta_2$ -mediated phagocytosis.

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REFERENCES

- 1 Desjardins M., Houde M., and Gagnon E. (2005) Phagocytosis: the convoluted way from nutrition to adaptive immunity. *Immunol. Rev.* **207**, 158-165
- 2 Underhill D. M. and Ozinsky A. (2002) Phagocytosis of microbes: complexity in action. *Annu. Rev. Immunol.* **20**, 825-852
- 3 Wagner C., Hansch G. M., Stegmaier S., Deneffle B., Hug F. and 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
- 4 Griffin Jr. F. M., Griffin J. A., Leider J. E. and Silverstein S. C. (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
- 5 Hynes R. O. (2002) Integrins: bidirectional, allosteric signaling machines. *Cell* **110**, 673-687
- 6 Caron E. (2003) Cellular functions of the Rap1 GTP-binding protein: a pattern emerges. *J. Cell Sci.* **116**, 435-440
- 7 Shattil S. J., Kim C. and Ginsberg M. H. (2010) The final steps of integrin activation: the end game. *Nat. Rev. Mol. Cell. Biol.* **11**, 288-300
- 8 Han J., Lim C. J., Watanabe N., Soriani A., Ratnikov B., Calderwood D. A., Puzon-McLaughlin W., Lafuente E. M., Boussiotis V. A., Shattil S.J. and Ginsberg M. H. (2006) Reconstructing and deconstructing agonist-induced activation of integrin α IIb β 3. *Curr. Biol.* **16**, 1796-1806
- 9 Watanabe N., Bodin L., Pandey M., Krause M., Coughlin S., Boussiotis V. A., Ginsberg M. H. and Shattil S. J. (2008) Mechanisms and consequences of agonist-induced talin recruitment to platelet integrin α IIb β 3. *J. Cell Biol.* **181**, 1211-1222
- 10 Lee H. S., Lim C. J., Puzon-McLaughlin W., Shattil S. J. and Ginsberg M. H. (2009) RIAM activates integrins by linking talin to ras GTPase membrane-targeting sequences. *J. Biol. Chem.* **284**, 5119-5127
- 11 Lim J., Wiedemann A., Tzircotis G., Monkley S. J., Critchley D. R. and Caron E. (2007) An essential role for talin during α M β 2-mediated phagocytosis. *Mol. Biol. Cell* **18**, 976-985
- 12 Lim J., Critchley D. R. and Caron E. (2010) Rap1 controls activation of the α M β 2 integrin in a talin-dependent manner. *J. Cell. Biochem.* **111**, 999-1009
- 13 Sebzda E., Bracke M., Tugal T., Hogg N. and Cantrell D. A. (2002) Rap1A positively regulates T cells via integrin activation rather than inhibiting lymphocyte signaling. *Nat. Immunol.* **3**, 251-258
- 14 Takagi J., Petre B. M., Walz T. and Springer T. A. (2002) Global conformational rearrangements in integrin extracellular domains in outside-in and inside-out signaling. *Cell* **110**, 599-611
- 15 Wiedemann A., Patel J. C., Lim J., Tsun A., van Kooyk Y. and Caron E. (2006) Two distinct cytoplasmic regions of the β 2 integrin chain regulate RhoA function during phagocytosis. *J. Cell Biol.* **172**, 1069-1079

- 16 Fagerholm S. C., Morrice N., Gahmberg C. G. and Cohen P. (2002) Phosphorylation of the cytoplasmic domain of the integrin CD18 chain by protein kinase C isoforms in leukocytes. *J. Biol. Chem.* **277**, 1728-1738
- 17 Ashida N., Takechi H., Kita T. and Arai H. (2003) Vortex-mediated mechanical stress induces integrin-dependent cell adhesion mediated by inositol 1,4,5-trisphosphate-sensitive Ca^{2+} release in THP-1 cells. *J. Biol. Chem.* **278**, 9327-9331
- 18 Rhoads A. R. and Friedberg F. (1997) Sequence motifs for calmodulin recognition. *FASEB J.* **11**, 331-340
- 19 Caron E. and Hall A. (1998) Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. *Science* **282**, 1717-1721
- 20 Caron E., Self A. J. and Hall A. (2000) The GTPase Rap1 controls functional activation of macrophage integrin $\alpha\text{M}\beta\text{2}$ by LPS and other inflammatory mediators. *Curr. Biol.* **10**, 974-978
- 21 Franke B., Akkerman J. W. and Bos J. L. (1997) Rapid Ca^{2+} -mediated activation of Rap1 in human platelets. *EMBO J.* **16**, 252-259
- 22 Bouvard D., Molla A. and Block M. R. (1998) Calcium/calmodulin-dependent protein kinase II controls $\alpha\text{5}\beta\text{1}$ integrin-mediated inside-out signaling. *J. Cell Sci.* **111**, 657-665
- 23 Blystone S. D., Slater S. E., Williams M. P., Crow M. T. and Brown E. J. (1999) A molecular mechanism of integrin crosstalk: $\alpha\text{v}\beta\text{3}$ suppression of calcium/calmodulin-dependent protein kinase II regulates $\alpha\text{5}\beta\text{1}$ function. *J. Cell Biol.* **145**, 889-897
- 24 Liu S., Calderwood D. A. and Ginsberg M. H. (2000) Integrin cytoplasmic domain-binding proteins. *J. Cell Sci.* **113**, 3563-3571
- 25 Fagerholm S. C., Hilden T. J., Nurmi S. M. and Gahmberg C. G. (2005) Specific integrin α and β chain phosphorylations regulate LFA-1 activation through affinity-dependent and -independent mechanism. *J. Cell Biol.* **171**, 705-715
- 26 Hibbs M. L., Jakes S., Stacker S. A., Wallace R. W. and Springer T. A. (1991) The cytoplasmic domain of the integrin lymphocyte function-associated antigen 1 β subunit: sites required for binding to intercellular adhesion molecule 1 and the phorbol ester-stimulated phosphorylation site. *J. Exp. Med.* **174**, 1227-1238
- 27 Mulrooney J. P., Hong T. and Grabel L. B. (2001) Serine 785 phosphorylation of the β1 cytoplasmic domain modulates β1A -integrin-dependent functions. *J. Cell Sci.* **114**, 2525-2533
- 28 Fagerholm S. C., Prescott A., Cohen P. and Gahmberg C. G. (2001) An essential role for calmodulin in regulating human T cell aggregation. *FEBS Lett.* **491**, 131-136
- 29 Killock D. J., Parsons M., Zarrouk M., Ameer-Beg S. M., Ridley A. J., Haskard D. O., Zvelebil M. and Ivetic A. (2009) In Vitro and in Vivo Characterization of Molecular Interactions between Calmodulin, Ezrin/Radixin/Moesin, and L-selectin. *J. Biol. Chem.* **284**, 8833-8845
- 30 Poggi A., Carosio R., Rubartelli A. and Zocchi M. R. (2002) β3 -mediated engulfment of apoptotic tumor cells by dendritic cells is dependent on CAMKII: inhibition by HIV-1 Tat. *J. Leukoc. Biol.* **71**, 531-537
- 31 Hudmon A. and Schulman H. (2002) Structure-function of the multifunctional Ca^{2+} /calmodulin-dependent protein kinase II. *Biochem. J.* **364**, 593-611
- 32 Hidaka H. and Yokokura H. (1996) Molecular and cellular pharmacology of a calcium/calmodulin-dependent protein kinase II (CaM kinase II) inhibitor, KN-62, and proposal of CaM kinase phosphorylation cascades. *Adv. Pharmacol.* **36**, 193-219
- 33 Tang P., Cao C., Xu M. and Zhang L. (2007) Cytoskeletal protein radixin activates integrin $\alpha\text{M}\beta\text{2}$ by binding to its cytoplasmic tail. *FEBS Lett.* **581**, 1103-1108

- 34 Ohmori T., Kashiwakura Y., Ishiwata A., Madoiwa S., Mimuro J., Honda S., Miyata T. and Sakata Y. (2010) Vinculin activates inside-out signaling of integrin α IIb β 3 in Chinese hamster ovary cells. *Biochem. Biophys. Res. Commun.* **400**, 323-328
- 35 Singh S., D'mello V., van Bergen en Henegouwen P. and Birge R. B. (2007) A NPxY-independent β 5 integrin activation signal regulates phagocytosis of apoptotic cells. *Biochem. Biophys. Res. Commun.* **364**, 540-548
- 36 Lees D. M and Hall A. (2009) Memorial: Emmanuelle Caron 1967 – 2009. *J. Leukoc. Biol.* **85**, 749-750

FIGURE LEGENDS

Figure 1. Inside-out signalling to $\alpha_M\beta_2$ is mediated by serine 756 of the β_2 cytoplasmic domain. COS-7 cells were co-transfected with cDNA constructs as indicated, pre-treated PMA (150ng/ml for 15 min) where necessary, challenged with C3bi-RBC, processed for immunofluorescence and scored for RBC association, as described in the Experimental section. Relative Association Index shows the relative number of RBC bound to 100 COS-7 cells (when compared to wt $\alpha_M\beta_2$, arbitrarily set to 100). Results are expressed as the mean \pm S.D. of at least three independent experiments. **, $p \geq 0.05$

Figure 2. Serine 756 of β_2 integrin mediates the recruitment and local activation of talin during $\alpha_M\beta_2$ activation. COS-7 cells were co-transfected with cDNA constructs encoding integrin subunits (wild-type or with β_2 S756M) and V12Rap1, GFP-talin (GFPTFL) or GFP-talin head (GFPTH) as indicated, challenged with C3bi-RBC, processed for immunofluorescence and scored for (A) RBC association or (B) GFP marker recruitment, as described in the Experimental section. Relative Association Index shows the relative number of RBC bound to 100 COS-7 cells (when compared to wt $\alpha_M\beta_2$, arbitrarily set to 100). The % GFP marker recruitment is based on a minimum of 20 transfected COS-7 cells showing local enrichment of marker signal at bound RBC. Results are expressed as the mean \pm S.D. of at least three independent experiments. *, $p < 0.05$

Figure 3. Rap1 interacts with β_2 through serine 756. Lysates of COS-7 cells co-expressing, (A) $\alpha_M\beta_2$ S756 with either GFPTH or GFP; (B) wild-type α_M and β_2 point mutants with myc-WTRap1, were incubated with equal amounts of protein G coated beads coated with anti- α_M (5c6) antibody. After washing with lysis buffer, pellet-associated proteins were separated by SDS-PAGE and analyzed by Western blotting, using the indicated antibodies (equal loading was demonstrated using HRP-conjugated anti-rat antibody which recognises the rat anti-5c6 antibody). Results are representative of three independent experiments.

Figure 4. Rap1 activity and localisation are affected by calmodulin-antagonist W7. Mouse macrophages J774A.1 (A and B) or transfected COS-7 cells (C and D) were pre-treated with W7 for 15 min, challenged with C3bi-RBC, processed for immunofluorescence and scored for RBC association (A and C), spreading (B) or marker recruitment (D), as describe in the Experimental section. Relative Association Index shows the relative number of RBC bound to either 100 untreated J774A.1 (A) or 100 COS-7 cells (when compared to wt $\alpha_M\beta_2$, arbitrarily set to 100) (B). Relative Spreading Index shows the relative surface areas of 50 J774A.1 cells. The %GFP marker recruitment is based on a minimum of 20 transfected COS-7 cells showing local enrichment of marker signal at bound RBC. Results are expressed as the mean \pm S.D. of at least three independent experiments. *, $p < 0.05$; **, $p \geq 0.05$

Figure 5. CamKII inhibitor KN62 blocks PMA-induced binding, but not spreading, in macrophages. Mouse macrophages J774A.1 were pre-treated with KN62 (25 μ M for 20 min), challenged with C3bi-RBC, processed for immunofluorescence and scored for RBC association (**A**) or spreading (**B**), as described in the Experimental section. Relative Association Index shows the relative number of RBC bound to 100 J774A.1 cells (when compared to wt $\alpha_M\beta_2$, arbitrarily set to 100). Relative Spreading Index shows the relative surface areas of 50 J774A.1 cells. Results are expressed as the mean \pm S.D. of at least three independent experiments. *, $p < 0.05$

Figure 6. Rap1 activity is affected by CamKII inhibitor KN62. Lysates from mouse macrophages J774A.1 loaded with GTP γ S or GDP (10 μ M each for 30 min) (**A**) or pre-treated with KN62 (25 μ M for 20 min) (**B** and **C**) were introduced to GST-RalGDS-coupled Glutathione-Sepharose beads. Proteins were separated by SDS-PAGE and analysed by Western blotting, using anti-Rap1 antibody. Corresponding amounts of GST-RalGDS used are shown as revealed by Ponceau S. Lysates were also tested for the levels of Rap1 and actin. (**C**) Band intensities were determined as described in the Experimental section. Results are expressed as the mean \pm S.D. of at least three independent experiments.











