The EMBO Journal (2007) 26, 505–515 | © 2007 European Molecular Biology Organization | All Rights Reserved 0261-4189/07
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THE EMBO JOURNAL

PSGL-1 and mTOR regulate translation of ROCK-1 and physiological functions of macrophages

Richard Fox[1,*], Thomas Q Nhan[1], G Lynn Law[2], David R Morris[2], W Conrad Liles[1,3] and Stephen M Schwartz[1,*]

[1]Department of Pathology, University of Washington, Seattle, WA, USA, [2]Department of Biochemistry, University of Washington, Seattle, WA, USA and [3]Department of Medicine, University of Toronto/University Health Network, Toronto General Hospital, Toronto, Ontario, Canada

Rho-associated kinases (ROCKs) are critical molecules involved in the physiological functions of macrophages, such as chemotaxis and phagocytosis. We demonstrate that macrophage adherence promotes rapid changes in physiological functions that depend on translational upregulation of preformed ROCK-1 mRNA, but not ROCK-2 mRNA. Before adherence, both ROCK mRNAs were present in the cytoplasm of macrophages, whereas ROCK proteins were undetectable. Macrophage adherence promoted signaling through P-selectin glycoprotein ligand-1 (PSGL-1)/Akt/mTOR that resulted in synthesis of ROCK-1, but not ROCK-2. Following synthesis, ROCK-1 was catalytically active. In addition, there was a rapamycin/sirolimus-sensitive enhanced loading of ribosomes on preformed ROCK-1 mRNAs. Inhibition of mTOR by rapamycin abolished ROCK-1 synthesis in macrophages resulting in an inhibition of chemotaxis and phagocytosis. Macrophages from PSGL-1-deficient mice recapitulated pharmacological inhibitor studies. These results indicate that receptor-mediated regulation at the level of translation is a component of a rapid set of mechanisms required to direct the macrophage phenotype upon adherence and suggest a mechanism for the immunosuppressive and anti-inflammatory effects of rapamycin/sirolimus.

The EMBO Journal (2007) **26,** 505–515.
doi:10.1038/sj.emboj.7601522
Subject Categories: immunology; proteins
Keywords: mTOR; rapamycin–sirolimus; ROCK-1; translation

Introduction

Macrophages exist in environments ranging from the air–liquid interface of the alveolus, to cells floating free in peritoneal or joint fluid, to cells adherent to a wide variety of extracellular matrices in different tissues. Profound phenotypic changes occur as macrophages transit from one environment to another, especially in areas of inflammation. These changes are presumably under control of signaling mechanisms initiated by adherence to matrix and to other cells (Silverstein, 1979; Gordon, 1998; Greenberg and Grinstein, 2002; Takumi *et al*, 2005; Xu *et al*, 2005). Candidate molecules that may signal such changes include matrix proteins, integrins, selectins and other intercellular adhesion molecules (Alon and Feigelson, 2002; Alon *et al*, 2003; Shamri *et al*, 2005). Signaling pathways initiated by these diverse receptors converge, in part, on the serine/threonine kinases, Rho-associated kinase-1 (ROCK-1) and Rho-associated kinase-2 (ROCK-2).

In addition to their role at the trailing edge of cells undergoing directed migration (Somlyo *et al*, 2000; Worthylake *et al*, 2001), Rho-associated kinases (ROCKs) are important for multiple physiological functions of leukocytes. ROCKs play a critical role in the regulation of actin polymerization and cytoskeletal rearrangement, and complement receptor-mediated phagocytic cup formation and chemotaxis (Maekawa *et al*, 1999; Somlyo *et al*, 2000; Ashida *et al*, 2001; Worthylake *et al*, 2001; Hauert *et al*, 2002; Olazabal *et al*, 2002; Chodniewicz and Zhelev, 2003). β_2-Integrins signal to the small GTPase Rho, the canonical upstream activator of ROCKs, to facilitate cytoskeletal changes involved in integrin segregation and clustering, thereby promoting firm adherence to substrates (Laudanna *et al*, 1996, 1997). Pharmacological evidence from the ROCK-selective inhibitors, fasudil and Y27632, support these properties of ROCKs (Miyata *et al*, 2000; Cohen, 2002; Kandabashi *et al*, 2002; Horton *et al*, 2005).

ROCK proteins have been described as constitutively expressed proteins (Nishimura *et al*, 1996) with their enzymatic activity controlled by the small GTPases, Rho-A, -B, -C and -E (Riento and Ridley, 2003). Until recently, ROCKs have been viewed as functionally redundant. Yoneda *et al* (2005) demonstrated that ROCK-1 has higher specific activity than ROCK-2 and that ROCK-1 was essential for focal adhesions and stress fiber formation (Yoneda *et al*, 2005). In addition, although closely related in sequence (nearly 65% at the amino-acid level) (Nakagawa *et al*, 1996) and predicted structure, the mechanisms of regulation of these two proteins differ. Rho-A, Rho-B and Rho-C function as activators of both ROCK-1 and ROCK-2, whereas Rho-E negatively regulates ROCK-1 (Riento and Ridley, 2003). Conversely, ROCK-2 is not regulated by Rho-E (Riento *et al*, 2005a, b; Riento and Ridley, 2006). p160-ROCK-1 is cleaved by caspase-3 generating the constitutively active p130-ROCK-1, suggestive of cell death and promoting membrane blebbing (Coleman *et al*, 2001; Sebbagh *et al*, 2001). Unlike ROCK-1, ROCK-2 activity is enhanced by phosphatidylinositol binding (Yoneda *et al*, 2005). Granzyme B-dependent as opposed to caspase-dependent proteolysis of ROCK-2 generates p130-ROCK-2 that promotes membrane blebbing during cell death (Sebbagh *et al*, 2005). Finally, ROCK-1 activity fails to compensate for ROCK-2 function in ROCK-2-deficient

*Corresponding authors. R Fox, Department of Pathology, University of Washington, 815 Mercer Street, Seattle, WA 98109, USA.
Tel.: +1 206 390 2997; Fax: +1 206 897 1540;
E-mail: richardfox.fox@gmail.com or SM Schwartz, Department of Pathology, University of Washington, 815 Mercer Street, Room 421, Seattle, WA 98109-4714, USA. Tel.: +1 206 543 0258;
Fax: +1 206 897 1540; E-mail: steves@u.washington.edu

Received: 13 March 2006; accepted: 30 November 2006

mice (Thumkeo *et al*, 2003). These data suggest that these proteins are not completely redundant and, presumably, function differently under varying conditions and in different cell types.

During investigation of ROCK function in primary murine peritoneal macrophages, we observed that ROCK-1, but not ROCK-2, undergoes profound changes in translation during cell adherence. Because the mTOR kinase signaling complex (mammalian target of rapamycin) selectively controls translation, we were interested in a potential regulatory role of mTOR in ROCK-1 synthesis. Furthermore, P-selectin glycoprotein ligand-1 (PSGL-1) has been shown to function as a receptor that signals to mTOR in monocytes (Mahoney *et al*, 2001). Therefore, PSGL-$1^{-/-}$ mice became a candidate model to test the hypothesis that mTOR-mediated translation of ROCK proteins is a pivotal control point in the activation of macrophages.

We report here that macrophage adherence promotes Akt/mTOR-dependent signaling, promoting the translation of preformed ROCK-1 mRNAs. Furthermore, we demonstrate that PSGL-1 functions as a signaling receptor initiating mTOR-dependent translation of ROCK-1. These signaling events result in rapid and broad changes in macrophage phenotype and function. These observations indicate a previously unknown mechanism that may account, in part, for the immunoregulatory and anti-inflammatory effects of rapamycin/sirolimus, a potent immunosuppressive agent that inhibits mTOR activity.

Results

ROCK-1 protein accumulates and is active following adherence of peritoneal macrophages

Equal numbers of primary peritoneal macrophages were allowed to adhere to plastic tissue culture dishes in the presence of 10% serum for a defined time or lysed directly following harvest. Immunoblot analysis was performed for both ROCK-1 and ROCK-2. ROCK-1 protein was undetectable in lysates of non-adherent peritoneal macrophages (Figure 1A). Following adherence, ROCK-1 protein rapidly accumulated and was detectable (Figure 1A). Specifically, ROCK-1 was present within 10 min of adherence and remained elevated 120 min later. ROCK-2 protein was undetectable by immunoblot of the same lysates at any time (data not shown). Previously, macrophage adherence was shown to promote caspase activity in living cells (Nhan *et al*, 2005; Nhan *et al*, 2006). In addition to p160-ROCK-1, accumulation of a caspase-mediated 30 kDa cleavage fragment of ROCK-1 was detected following 30 min of adherence (Figure 1A).

ROCK-1 phosphorylates several substrates that play pivotal roles in cytoskeletal rearrangement and chemotaxis, including myosin light chain kinase phosphatase (MYPT-1), (Hartshorne and Hirano, 1999; Somlyo and Somlyo, 2003). In agreement with the time course observed for ROCK-1 protein accumulation, immunoblot analysis of lysates of peritoneal macrophages showed that MYPT-1 was phosphorylated at T850 within 10 min of adherence. Phosphorylation of MYPT-1 was present

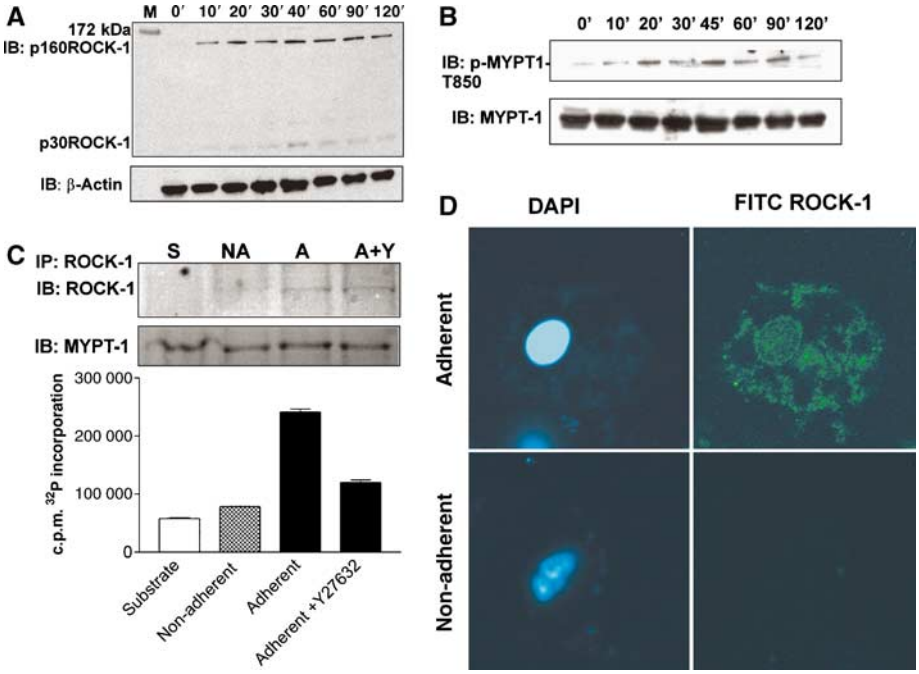

Figure 1 ROCK-1 protein accumulates in an adherence-dependent manner in murine macrophages. (**A**) Immunoblot analysis for ROCK-1 in macrophage lysates before and after adherence for the specified period (β-actin served as a loading control). Lane M is a marker at 172 kDa. (**B**) Immunoblot analysis of phosphorylated MYPT-1 T850 in macrophage lysates before and after adherence for the specified period (total MYPT-1 served as a loading control). (**C**) *In vitro* kinase assay of whole-cell lysates of peritoneal macrophages immunoprecipitated for ROCK-1 and incubated with [γ-^{32}P]ATP and rMYPT-1 as substrate and in the presence or absence of 10 μM Y27632. c.p.m. were determined by scintillation counter with the average of three experiments in the composite graph with error bars representing s.e.m. Immunoprecipitated ROCK-1 was analyzed by immunoblot for ROCK-1: S = substrate alone, NA = non-adherent, A = adherent and A + Y = adherent + 10 μM Y27632. Immunoblot for total MYPT-1 served as a loading control for substrate addition to the reaction. (**D**) Confocal immunofluorescence microscopy performed on 30-min adherent or cytospun macrophages. Cellular staining for ROCK-1 protein used the G6 antibody with DAPI nuclear counterstain. Panel 1: DAPI; panel 2: FITC-labeled ROCK-1.

through 120 min of adherence; however, the pattern of phosphorylation varied slightly over time (Figure 1B).

To confirm that phosphorylation of MYPT-1 was ROCK-specific, the ROCK-selective inhibitor, Y27632, was used during immunoprecipitation *in vitro* kinase assays. ROCK-1 was immunoprecipitated from equal amounts of total protein from lysates of either non-adherent or adherent macrophages. Immunoprecipitated ROCK-1 was incubated with [γ-^{32}P]ATP and substrate—recombinant GST-MYPT-1 (rMYPT-1)—in the presence or absence of 10 μM Y27632. Substrate alone showed background incorporation of ^{32}P. ^{32}P incorporation by ROCK-1 immunoprecipitated from non-adherent macrophages was similar to the substrate-alone (SA) negative control (Figure 1C). ROCK-1 immunoprecipitated from cells adherent for 30 min showed an approximate four-fold increase in ^{32}P incorporation (Figure 1C). ROCK-1 immunoprecipitated from adherent macrophages and treated with 10 μM Y27632 showed a marked decrease in ^{32}P incorporation (Figure 1C). Immunoblot for ROCK-1 protein of ROCK-1 immunoprecipitates showed an absence of ROCK-1 in the SA group and the non-adherent group (NA) (Figure 1C), whereas ROCK-1 was present in the adherent group (A) and the adherent group plus Y27632 (A+Y) (Figure 1C). Immunoblot for total MYPT-1 showed equal loading of substrate for each condition (Figure 1C). A reciprocal experiment was performed in which phosphorylation of immobilized rMYPT-1 was used as a substrate for whole-cell lysates, either treated with PBS or 10 μM Y27632, and had similar results as Figure 1C (data not shown).

Immunofluorescence confocal microscopy studies were performed to confirm the results of immunoblot experiments (Figure 1A). ROCK-1 protein was not detected in non-adherent macrophages, whereas ROCK-1 protein was detected in the cytoplasm of adherent peritoneal macrophages (Figure 1D). Control experiments using an irrelevant IgG confirmed the specificity of the ROCK-1 antibody (data not shown). ROCK-2 protein was undetectable by immunofluorescent confocal microscopy at any time examined (data not shown).

Additional experiments performed in human monocyte-derived macrophages showed results similar to the murine peritoneal macrophages (Supplementary Figure 1).

ROCK mRNA levels do not change in macrophages following adherence

To determine if ROCK-1 protein accumulation was controlled at the level of transcription, quantitative real-time RT–PCR (Q–PCR) was performed on RNA from murine peritoneal macrophages harvested either directly following isolation from the animal, or after adherence in the presence of 10% serum to tissue culture plates for 15, 60 or 120 min. No significant change in ROCK-1 or ROCK-2 mRNA levels was observed at any time following adherence ($P>0.1$) (Figure 2, light gray and dark gray bars, respectively). In contrast, by 60 min of adherence, levels of macrophage colony-stimulating factor (M-CSF) mRNA increased five-fold and were eight-fold higher by 120 min of adherence (Figure 2, black bars). Transcript abundance of RhoA reached a maximum increase of 7.5-fold at 60 min and decreased to near baseline levels at 120 min (Figure 2, striped bars). Transcript abundance of tumor necrosis factor-α (TNF-α) steadily increased over time to four-fold (Figure 2, hatched bars). The absence of a detectable increase in ROCK-1 mRNA levels implied that appearance of protein was due to either enhanced translation or protein stability.

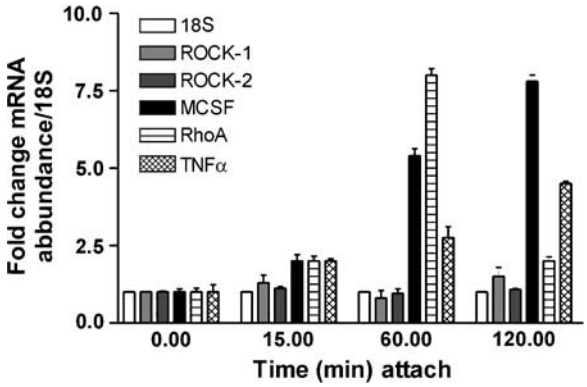

Figure 2 ROCK-1 transcript abundance does not change before or after adherence. Quantitative PCR analysis was performed for 18S, ROCK-1, ROCK-2, RhoA, TNF-α and M-CSF (labeled in the graph) transcript abundance before and after adherence. Quantitative PCR analysis was performed within the linear range of amplification as determined by limited dilution and amplification of 18S ribosomal RNA. Error bars represent s.e.m. for $n=3$ for each transcript and condition.

Transcription of ROCK-1 mRNA is not required for synthesis of ROCK-1 in macrophages following adherence

To determine if adherence affected general protein synthesis, metabolic labeling of proteins was performed in non-adherent and adherent murine peritoneal macrophages in the presence or absence of transcription and translation inhibitors. Incorporation of ^{35}S into protein was measured in either non-adherent or adherent macrophages pretreated as described. Following 1 h of adherence, incorporation of [^{35}S]methionine increased two-fold (Figure 3A, open bars). Protein synthesis was blocked by the translational inhibitor cycloheximide (CHX) (Figure 3A, light gray bars), whereas the transcription inhibitor actinomycin-D (act-D) (Figure 3A, hatched bars) blocked approximately 60% of total protein synthesis both before and after adherence. Rapamycin/sirolimus, an mTOR-specific inhibitor, blocked approximately 50% of total protein synthesis before adherence, but only modestly reduced synthesis over the 60 min of adherence (Figure 3A, black bars). These, data suggest that adherence has a direct effect on total protein synthesis, and both transcription and translation are important components.

To determine if accumulation of ROCK-1 protein was controlled at the level of protein synthesis, experiments were performed using continuous radiolabeling of murine peritoneal macrophages followed by immunoprecipitation of ^{35}S-labeled ROCK-1. Before adherence, ^{35}S-labeled ROCK-1 protein was undetectable (Figure 3B). After 15 min of adherence, the ^{35}S-labeled protein product was detected by immunoprecipitation and autoradiography (Figure 3B). CHX strongly depressed ROCK-1 synthesis, whereas actinomycin-D had no effect on ROCK-1 protein synthesis (Figure 3B), indicating that *de novo* transcription of the ROCK-1 gene is not required for synthesis of ROCK-1 protein.

Similar to the results with CHX (Figure 3B), rapamycin/sirolimus depressed ROCK-1 synthesis, in comparison to its

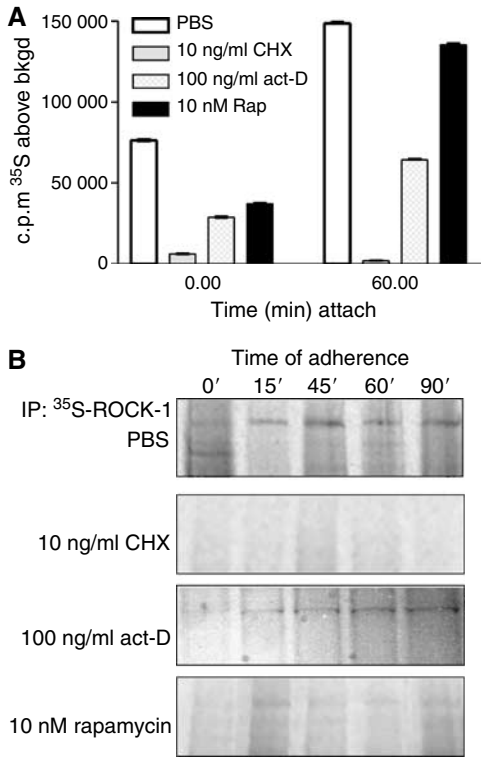

Figure 3 Inhibition of translation, but not transcription, results in ablation of immunoprecipitable ROCK-1. (**A**) Total protein synthesis determined by TCA precipitation of [^{35}S]methionine-labeled proteins with composite data from three independent experiments and error bars representing s.e.m. Treatment conditions were 30 min pretreatment with either PBS (open bars), CHX 10 ng/ml (light gray bars), act-D 100 ng/ml (hatched bars) or rapamycin/sirolimus 10 nM (black bars), then either directly lysed or allowed to adhere for 60 min ($n=4$). (**B**) Radioimmunoprecipitation of ROCK-1 from macrophage lysates pretreated with PBS vehicle, act-D 100 ng/ml, CHX 10 ng/ml or rapamycin/sirolimus 10 nM, then either directly lysed or allowed to adhere for the indicated period. Equal TCA-precipitated c.p.m. of [^{35}S]methionine-labeled proteins from peritoneal macrophage cell lysates were immunoprecipitated with ROCK-1 G-6 antibodies and separated by SDS–PAGE before autoradiography ($n=3$).

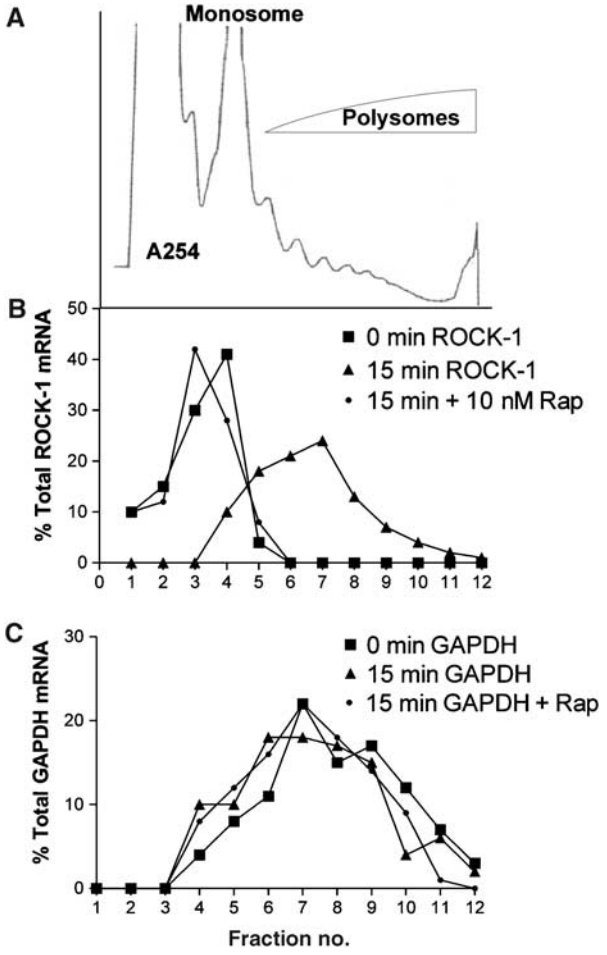

Figure 4 Translation state analysis before and after adherence demonstrates increased ribosome loading of ROCK-1 messages. Macrophages were harvested as described and either lysed immediately and polysome preparations generated or allowed to attach for 15 min and lysed for polysome preparation. (**A**) A254 profile of a representative sucrose gradient of polysome fractions. The *Y*-axis depicts the intensity of the A254 trace with the *X*-axis depicting fractions eluted off the gradient. (**B**) Q–PCR using ABI (Applied Biosystems, Foster City, CA) Assays on Demand primer probe sets analyzed polysome fractions for ROCK-1 transcript abundance; $n=3$ for each primer probe set under each condition. Total mRNA abundance was normalized to UT2 as described. ROCK-1 messages before adherence (squares), for 15 min after adherence (triangles) or 15 min after adherence in the presence of 10 nM rapamycin (circles). (**C**) Q–PCR using ABI assays on Demand primer probe sets analyzed polysome fractions for GAPDH messages before adherence (squares), for 15 min after adherence (triangles) or 15 min after adherence in the presence of 10 nM rapamycin (circles); $n=3$ under each condition.

ability to only partially inhibit global translation (Figure 3B). Together, these data show that ROCK-1 mRNA was present, but not translated in non-adherent macrophages, and then was translated upon contact with a substrate. As ROCK-1 synthesis in adherent cells was blocked by rapamycin/sirolimus, these data suggest that translation of ROCK-1 mRNA in macrophages is controlled in part by mTOR.

Ribosome loading on ROCK-1 mRNA increases following adherence

Because increased ribosome loading correlates directly with the efficiency of protein synthesis (Mathews, 1996; Ruan *et al*, 1997), we determined polysome loading profiles for ROCK-1 mRNAs before or after adherence. A254 absorbance traces of sucrose gradients showed separation of polyribosomes by density gradient centrifugation and fractionation (Figure 4A). In non-adherent macrophages, ROCK-1 mRNA was sedimented in fractions of the gradient corresponding with ribonucleoprotein (RNP) particles and monosomes (large peak, Figure 4A), consistent with the lack of translation of this transcript (Figure 4B, squares). Following 15 min of adherence, ROCK-1 mRNA shifted to higher-order fractions, corresponding to polysome-loaded mRNAs, indicating active translation of ROCK-1 messages (Figure 4B, triangles). Rapamycin treatment of macrophages prevented ribosome loading of ROCK-1 mRNAs (Figure 4B, circles). In contrast, GAPDH mRNA remained associated with large polysomes, both before (Figure 4C, squares) and after adherence (Figure 4C, triangles). These data suggest that, upon adherence, ROCK-1 mRNA in macrophages becomes loaded onto polysomes, thereby allowing the rapid accumulation of

ROCK-1 protein observed within 10 min of adherence (Figures 1A, C, 3B and 4B).

Adherence-dependent ROCK-1 accumulation in macrophages is PSGL-1-, Akt- and mTOR-dependent

Immunoblot analysis of ROCK-1 protein in macrophage lysates before and after adherence and in the presence of 10 nM rapamycin/sirolimus showed total ablation of ROCK-1 protein at all time points following adherence (Figure 5A), which is in agreement with the previous results showing that no ROCK-1 protein could be immunoprecipitated from rapamycin/sirolimus-treated macrophages (Figures 3B and 4B, circles). Therefore, inhibition of mTOR was sufficient to block the accumulation of ROCK-1 protein.

Immunoblot analysis for phosphorylation of Akt at S473 was not detectable in lysates of non-adherent macrophages (Figure 5A). Following adherence, phosphorylated Akt S473 was detectable (Figure 5A and B). Immunoblot analysis of phosphorylated Akt S473 in lysates of macrophages treated with rapamycin/sirolimus showed a decrease in phosphorylated Akt S473 compared to phosphate-buffered saline (PBS) controls (Figure 5A). This observation is not totally surprising, as others have reported a rapamycin-sensitive effect on Akt phosphorylation (Sarbassov *et al*, 2005; Shaw and Cantley, 2006; Vega *et al*, 2006).

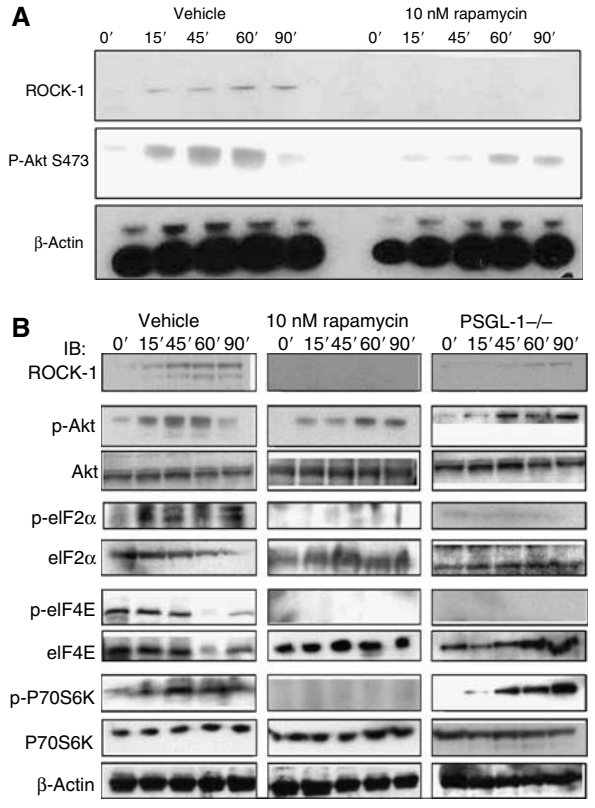

Figure 5 PSGL-1-dependent Akt phosphorylation is necessary for ROCK-1 synthesis whereas rapamycin/sirolimus inhibits ROCK-1 synthesis. (**A**) Immunoblot analysis of peritoneal macrophages pretreated with vehicle or 10 nM rapamycin/sirolimus for 30 min, then allowed to adhere for the indicated time. Immunoblot analysis of peritoneal macrophage lysates was performed for p-Akt-S473 and ROCK-1 (β-actin served as a loading control). This figure is representative of a minimum of three independent experiments per panel. (**B**) Immunoblot analysis was performed on lysates of peritoneal macrophages from wild-type mice in the presence or absence of 10 nM rapamycin and PSGL-$1^{-/-}$ mice adherent for the indicated time. Immunoblot analyses were performed for ROCK-1, ROCK-2 (no data), p-Akt S473, total Akt, p-eIF2α, total eIF2α, p-eIF4e, total eIF4E, p-P70s6K, total p70S6K and β-actin. This figure is a composite figure of representative immunoblots of three replicate experiments; each phosphorylated protein and total protein immunoblot were performed on the same blot demonstrating the total amount of protein available for phosphorylation. However, the same blots were not used for all antibodies.

PSGL-1 has been shown to activate mTOR signaling in monocytes (Mahoney *et al*, 2001); therefore, we utilized PSGL-$1^{-/-}$ mice to dissect the mTOR pathway following adherence. To test this model, we performed immunoblot analysis for ROCK-1, ROCK-2, phosphorylated eIF2α, eIF4E and p70S6K in macrophage lysates of wild-type mice, wild-type mice treated with 10 nM rapamycin/sirolimus or PSGL-$1^{-/-}$ mice. Macrophages were allowed to adhere in the various treatment groups for the indicated time and then lysates were generated. Following adherence, lysates of wild-type mice showed the anticipated patterns of phosphorylation with enhanced phosphorylation of eIF2α, eIF4E and p70S6K (Figure 5B, vehicle). Treatment with 10 nM rapamycin inhibited the phosphorylation of eIF2α, eIF4E and p70S6K (Figure 5B, 10 nM rapamycin). Macrophages from PSGL-$1^{-/-}$ mice showed the anticipated lack of phosphorylation of eIF2α and eIF4E (Figure 5B, PSGL-$1^{-/-}$). Surprisingly, macrophages from PSGL-$1^{-/-}$ mice showed an accumulation of phosphorylated p70S6K following 45 min of adherence. The phosphorylation of p70S6K accompanied a modest accumulation of ROCK-1, but not ROCK-2, suggesting alternative activation of the mTOR pathway may occur. Alternatively, a receptor other than PSGL-1 may be capable of activating mTOR. However, the second alternative seems unlikely, given the delayed activation of p70S6K and modest accumulation in ROCK-1 following adherence.

Macrophage function is impaired by antagonism of ROCK-1 and mTOR

To understand the physiological role of mTOR-mediated regulation of ROCK-1 in macrophages, we assessed if PSGL-$1^{-/-}$ macrophages were capable of *in vivo* recruitment to the peritoneum. Recruitment of macrophages to the peritoneum following thioglycollate injection was about 1/10 that of wild-type controls (Figure 6A, gray bars). Macrophages from PSGL-$1^{-/-}$ mice not injected with thioglycollate had macrophages in the peritoneum, similar to injected animals, suggesting that the majority of cells harvested by lavage were a resident population (Figure 6A, black bar).

Pharmacological blockade of ROCK-1 synthesis by rapamycin/sirolimus or ROCK-1 function by Y27632 resulted in a decrease in chemotaxis of peritoneal macrophages from wild-type animals *in vitro*. Control macrophages were capable of chemotaxis toward a gradient of monocyte chemoattractant protein 1 (MCP-1) (Figure 6B, gray bars) with a maximal response at 50 ng/ml MCP-1. Pretreatment of peritoneal macrophages with 10 μM Y27632 strongly attenuated chemotaxis (Figure 6C, gray bars). In the presence of 10 nM rapamycin/sirolimus, chemotaxis was greatly reduced (Figure 6C, black bars). An inhibitory antibody against β2 integrins (2E6) had a modest effect on chemotaxis (Figure 6C, dark gray bars), suggesting that firm adherence is not required to generate a chemotactic response. These observations are consistent with a publication in which rapamycin/sirolimus

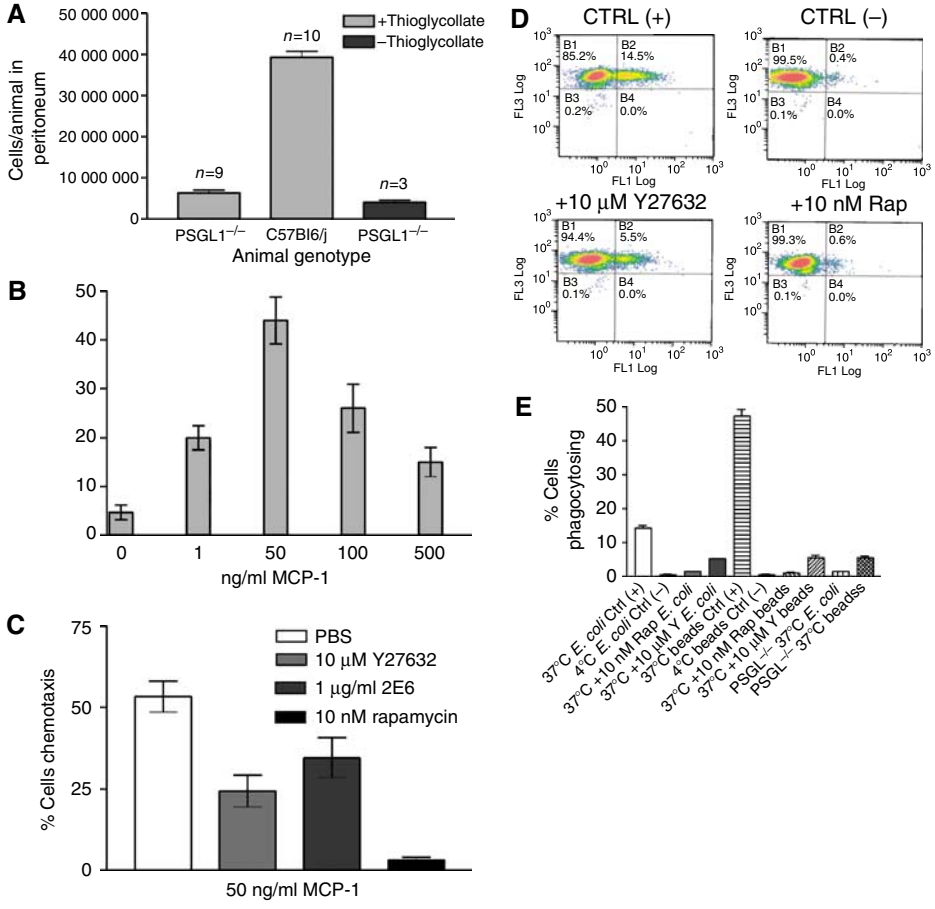

Figure 6 PSGL$^{-/-}$ mice are defective in *in vivo* macrophage recruitment and phagocytosis and recapitulate the effects of rapamycin/sirolimus and Y27632. (**A**) *In vivo* recruitment of macrophages to the peritoneal cavity was measured by counting the number of cells in lavage fluid from PSGL$^{-/-}$ mice or wild-type (CB57Bl6/j) mice. Mice were treated for 5 days by intraperitoneal injection of thioglycollate (light gray bars) or intraperitoneal injection of sterile saline (black bars); n = number of animals per group with error bars representing s.e.m. (**B**) Chemotaxis of peritoneal macrophages toward a gradient of MCP-1 (indicated in the figure). A maximal dose of MCP-1 was determined to be 50 ng/ml. (**C**) Chemotaxis was measured in cells pretreated for 30 min in vehicle alone (open bars), the ROCK-selective inhibitor Y27632 (10 μM) (light gray bars), the b2 integrin inhibitory antibody 2E6 (dark gray bars) or the mTOR inhibitor rapamycin/sirolimus (10 nM) (black bars). The relative fluorescence of each sample was calculated using an internal standard (nontreated cells placed into the bottom well to determine the percentage of cells migrating into the lower chamber). Error bars represent s.e.m. ($n = 3$). (**D**) Representative flow cytometry analysis (FACS) of phagocytic uptake of *E. coli* by peritoneal macrophages at 37°C (ctrl +) and 4°C (ctrl −), in the presence of 10 μM Y27632 or 10 nM rapamycin/sirolimus. Before the phagocytosis assays, peritoneal macrophages were allowed to adhere to tissue culture plate in the presence of 10% FBS/RPMI-1640 for 10 min to stimulate ROCK-1 synthesis before incubation with the respective pharmacological agent for 30 min. (**E**) Graphical display of three independent (FACS) experiments of phagocytic uptake of either FITC-labeled *E. coli* or FITC-conjugated opsonized beads by peritoneal macrophages expressed as the percent of cells that are FITC positive and PI positive. Displayed on the *X*-axis of the graph are the conditions and genotypes of peritoneal macrophages.

inhibited neutrophil chemotaxis (Gomez-Cambronero, 2003). Although inhibition of ROCK-1 had less effect on chemotaxis than did inhibition of mTOR, these data suggest that mTOR-mediated translational control is an important component of directed motility of macrophages.

To examine additional physiological roles of ROCK-1, phagocytosis experiments were performed using murine peritoneal macrophages from either wild-type mice or PSGL-1$^{-/-}$ mice. Utilizing a flow cytometry-based technique to analyze phagocytosis, macrophages from wild-type mice had functional phagocytic activity of both complement opsonized *Escherichia coli* and antibody opsonized agarose beads. The ROCK-selective inhibitor, Y27632, decreased the phagocytic activity in wild-type macrophages. Macrophages from wild-type mice treated with rapamycin and macrophages from PSGL-1$^{-/-}$ mice had significantly reduced phagocytic activity when compared to controls (Figures 6D and E).

Discussion

The phenotypic transition that occurs when a suspended macrophage becomes an adherent, motile cell occurs rapidly. Peritoneal macrophages, for example, are viable despite being non-adherent. In contrast, adherent macrophages die if they become non-adherent (Hume *et al*, 2002; Xu *et al*, 2005). We propose this transition begins with a non-adherent cell in the circulation, which has the components for the attached phenotype as preformed untranslated mRNAs (Figure 2). These untranslated messages are available for rapid synthesis (Figures 1A, 3B, 4B, and 5). The findings of the current study demonstrate that adherence-dependent signaling events result in upregulation of ROCK-1, but not ROCK-2 protein. The process of ROCK-1 upregulation results from increased ribosome loading of preformed ROCK-1 mRNAs (Figure 4). Following adherence, activation of mTOR by PSGL-1,

functioning as a receptor, results in the rapid accumulation of ROCK-1 protein (Figure 6). Not surprisingly, blockade of ROCK-1 activity by Y27632 or ROCK-1 synthesis by rapamycin/sirolimus significantly reduces the ability of macrophages to adhere (data not shown). However, blocking firm adherence through β2 integrins had no effect on ROCK-1 synthesis (data not shown).

Before the findings reported in this study, ROCK proteins have been viewed in many cells as a constitutively available signaling enzyme regulated primarily through two distinct pathways, the Rho-dependent signaling pathways or proteolytic cleavage by caspase-3 for ROCK-1 or granzyme-B for ROCK-2 (Nishimura *et al*, 1996; Coleman *et al*, 2001; Sebbagh *et al*, 2001; Riento and Ridley, 2003). Our results demonstrate that ROCK-1 protein was undetectable in non-adherent murine peritoneal macrophages (Figures 1, 3 and 6) and non-adherent human monocyte-derived macrophages (Supplementary Figure 1), even though ROCK-1 and ROCK-2 mRNAs were present in the cytoplasm as untranslated mRNAs. Following adherence, synthesis of ROCK-1 (but not ROCK-2) occurred, and the nascent ROCK-1 kinase became enzymatically active, as indicated by phosphorylation of the downstream target MYPT-1 (Figure 1 and Supplementary Figure 1).

Following adherence, macrophages underwent an approximate 50% increase in translation, including an 85–90% increase in the proportion of ROCK-1 mRNA in higher-order polysome fractions (Figure 4B). The translation of ROCK-1 messages was inhibited by rapamycin/sirolimus (Figure 4B). Unfortunately, performing ribosome-loading studies in macrophages from PSGL-$1^{-/-}$ mice would be difficult because of the limited number of cells available. This transition was not simply a general increase in translation, as there was no change in ribosome-loading profiles of GAPDH messages before or after adherence (Figure 4C).

Our data further suggest that translation of ROCK-1 occurred via a PSGL-1/Akt/mTOR-dependent mechanism (Figure 5). Macrophages from PSGL-deficient mice or macrophages from wild-type mice treated with either rapamycin/sirolimus or the ROCK-1 inhibitor Y27632 blocked both chemotaxis and phagocytosis (Figure 6). We suggest that the inhibition of physiological activation of macrophages depends, in part, on mTOR-dependent translation of ROCK-1 and, presumably, other molecules.

Finally, PSGL-1 functions as a receptor involved in initiating ROCK-1 translation. PSGL-1 has been shown to activate Akt signaling to mTOR in the human monocyte cell line U937 (Mahoney *et al*, 2001). This signaling results in the translational upregulation of urokinase plasminogen activator receptor (uPAR). uPAR, in turn, promotes firm adhesion of macrophages *in vivo* and promotes chemotaxis and directed motility during transit from the circulation to tissue sites (Gyetko *et al*, 1994; Chavakis *et al*, 2002; Petty *et al*, 2002; Yoshitake *et al*, 2003). PSGL-$1^{-/-}$ mice are defective in leukocyte recruitment in response to thioglycollate challenge (Dunon *et al*, 1996; Tchernychev *et al*, 2003). PSGL-1 directly inhibits leukocyte rolling by all three selectins (E-selectin, L-selectin and P-selectin) *in vivo* (McEver and Cummings, 1997; Hicks *et al*, 2003). These data suggest that inhibition of monocyte rolling and adherence of macrophages, as studied herein, may utilize similar signaling pathways, which converge on the rapid translational regulation of key effector molecules, such as ROCK-1.

Both uPAR and ROCK-1 are molecules translationally regulated by adherence signaling via mTOR. A third example is CD36 scavenger receptor. Although often called a scavenger receptor, CD36 controls foam cell formation (Shashkin *et al*, 2005). The CD36 scavenger receptor is thought of as being necessary for foam cell formation through the uptake of lipids resulting in the promotion of atherosclerosis. In addition, CD36 has been implicated in chemotaxis toward and clearance of apoptotic cells (Platt *et al*, 1998; Godson *et al*, 2000; Moodley *et al*, 2003; Truman *et al*, 2004). mTOR-dependent translation of CD36 is regulated via structural elements in the 5′ UTR (Griffin *et al*, 2001). Computational studies of the ROCK-1 5′ UTR suggest a similar structural element to that of the CD36 5′ UTR with upstream open reading frames that could block ROCK-1 translation (Morris and Geballe, 2000) (Figure 7A). To date, it is unknown if the polypeptides encoded by these small open reading frames are expressed or play any role *in vivo*.

The observed functions for mTOR suggest a broader pharmacological perspective for the mTOR kinase-specific drugs, such as rapamycin/sirolimus. Rapamycin/sirolimus is most prominently used as an immunosuppressive following renal transplantation (Vincenti *et al*, 1998; Groth *et al*, 1999; Guba *et al*, 2002). The proposed mechanism of action for rapamycin/sirolimus is inhibition of mTOR-mediated synthesis of critical molecules that control the signaling equilibrium between cell survival and death within effector T cells. Cancer therapy is another potential application for rapamycin/sirolimus. Several reports suggest that inhibition of mTOR decreases tumor metastasis by eliciting an anti-angiogenic response and downregulation of VEGF (Guba *et al*, 2002; Bjornsti and Houghton, 2004). We propose that these beneficial effects may depend on blockade of translation of specific proteins required for the pathological process, including blockade of ROCK-1 translation. Given the significant role of ROCK-1 for cell motility, blockade of ROCK-1 synthesis may have a direct effect on inhibiting metastasis, as well as other pathological events, such as transplantation atherosclerosis.

The question remains, if PSGL-1 functions as a receptor to modulate mTOR-dependent translation, what ligand is engaging the receptor both *in vivo* and *in vitro*? PSGL-1 interacts with all three selectins (P-, E- and L-selectin) (Martinez *et al*, 2005) and can homodimerize to form a stable signaling complex (Ramachandran *et al*, 2001). Soluble P-selectin and soluble PSGL-1 have been shown to be at high circulating levels in a multitude of inflammatory and disease pathologies, for example, chronic obstructive pulmonary disease, irritable bowel syndrome and peripheral artery occlusive disease, to name a few (Kappelmayer *et al*, 2004; Yanaba *et al*, 2004; Galkina and Ley, 2006). Blocking antibodies against selectins have been shown to reduce some of these pathologies, as well as deep venous thrombosis. However, circulating ligands do not explain the activation of mTOR following adherence in culture. We speculate that adherence-induced PSGL homodimers are sufficient to initiate signaling resulting in ROCK-1 synthesis. Following ROCK-1 synthesis, β2 integrins signal through the Rho family members to activate ROCK-1, resulting in reorganization of the cytoskeleton followed by clustering of β2 integrins, thus promoting firm adherence in preparation for penetration into the tissue.

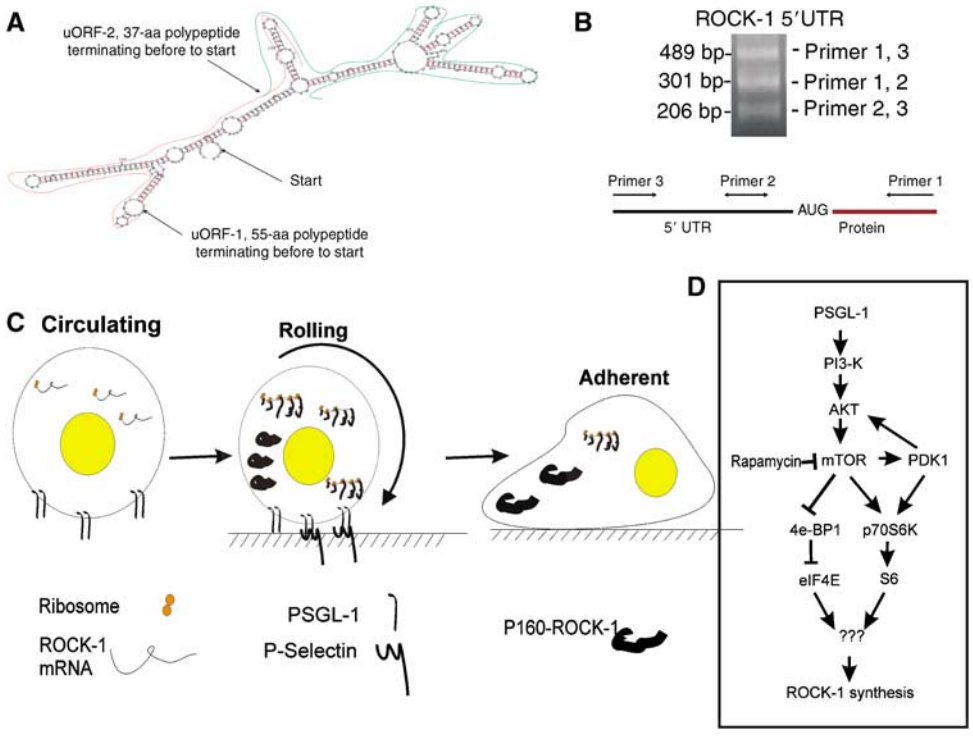

Figure 7 Model of adherence-dependent mTOR-mediated accumulation of ROCK-1 in macrophages. (**A**) Computational structural analysis suggests that the ROCK-1 5′ UTR has two predicted uORFs that end in termination codons before the start of the canonical ROCK-1 message. uORF 1 is depicted in red and uORF 2 is predicted in green. (**B**) 5′ RACE experiments were performed to identify and sequence the 5′UTR depicted in (A). Multiplex PCR shows amplification of products of the correct predicted size based on primer design as noted. (**C**) Before adherence, ROCK-1 protein is undetectable. The ROCK-1 message is stable and available for translation with the predominant ROCK-1 mRNA species (black symbol) as RNP particles or loaded with a single ribosome (red symbol). Following adherence, mTOR activation occurs via a PSGL-1-dependent receptor-mediated mechanism (purple symbol), resulting in increased ribosome loading on the ROCK-1 message and rapid accumulation of the functional protein. Activation of ROCK-1 results in physiological functions of macrophages to include chemotaxis and phagocytosis. (**D**) Schematic of PSGL-1-dependent signaling events with alternative mTOR activation via a putative PDK1-dependent event in PSGL-1-deficient macrophages.

Finally, we propose a model in which adherence-dependent signaling events result in the upregulation of ROCK-1 and other proteins via increased ribosome loading of preformed mRNAs (Figure 7). In this model, before adherence, ROCK-1 protein is undetectable in both peritoneal macrophages and human monocyte-derived macrophages. ROCK-1 mRNA is stable and available for translation. The predominant ROCK-1 mRNA species in non-adherent macrophages are present in RNP particles or loaded on a single ribosome, which could be trapped at the upstream open reading frames (Morris and Geballe, 2000). Following adherence, PSGL-1 signals through Akt and mTOR activation occurs, resulting in ROCK-1 mRNA loading with polysomes and rapid accumulation of ROCK-1 protein. The downstream events of mTOR activation occur through the 4E-BP family of proteins. mTOR regulates the association of eIF4E with the pre-initiation complex (Hershey, 1991), a rate-limiting step in protein synthesis. eIF4E binds to the m7GpppN cap structure located at the 5′ end of eukaryotic messages. The dependence of translation on eIF4E increases with the degree of secondary structure in the 5′ UTR of a transcript, thus providing a potential mechanism for selective translational control (Gingras *et al*, 2001; Raught *et al*, 2001; Gingras *et al*, 2004; Hay and Sonenberg, 2004).

This study demonstrates that adherence-dependent, receptor-mediated, translational control of protein abundance plays a critical role in multiple settings and pathologies where rapid differentiation is necessary for cellular function.

Materials and methods

Animal care protocol

All studies were performed according to animal care protocols approved in advance by the Institutional Animal Care and Use Committee of the University of Washington (2052-04). Dr Bruce Furie provided PSGL-$1^{-/-}$ mice.

Preparation of mouse peritoneal macrophages and human monocyte-derived macrophages

Retired male breeder mice C57BL6/J (Jackson Laboratories, Bar Harbor, ME) were maintained in a modified SPF facility on a chow diet. Peritoneal macrophages were harvested by lavage in 5% fetal bovine serum (FBS)-supplemented RPMI-1640 following thioglycollate injection as described previously (Nhan *et al*, 2003). Human monocytes were prepared from the venous blood of healthy volunteer donors using the Rosette Sep monocyte negative selection kit (StemCell Technologies Inc., Vancouver, Canada) as per the manufacturer's directions, and differentiated into macrophages by 7-day cultivation in Teflon tubes with recombinant human M-CSF as described (Hubel *et al*, 2002).

Adherence assay

Following harvest from animals, peritoneal macrophages were washed once with PBS and 10×10^6 macrophages were plated on 10 cm tissue-culture dishes in 10 ml of RPMI-1640 medium supplemented with 10% FBS for the designated time.

Immunoblot analysis

Cell were lysed using RIPA lysis buffer (0.15 M NaCl, 5 mM EDTA, pH 8, 1% Triton X-100, 10 mM Tris–HCl, pH 7.4) with HALT protease inhibitor (Pierce Chemical, Rockford, IL). Protein concentration was determined using the BCA kit (Pierce Chemical, Rockford, IL). SDS–PAGE electrophoresis was performed to separate lysates by size and then transferred to PVDF membranes (Invitrogen, Carlsbad, CA). Primary antibodies were diluted 1:1000 in 1% BSA:TBS-T. The following primary antibodies were used: ROCK-1 G-6 (Santa Cruz Biotechnology, Santa Cruz, CA), p-MYPT-1 T850, p-MYPT-1 T696 and total MYPT-1 (Upstate Biotech USA, Lake Placid, NY), p-Akt-S473, total Akt, p-eIF2α, total eIF2α, p-eIF4E, total eIF4e, p-p70S6K, total p70S6K (Cell Signaling Technology, Beverly, MA) and β-actin (Novus Biologicals Inc., Littleton, CO, ab6276). Primary antibodies were detected using the appropriate species HRP-conjugated secondary antibody at 1:3000 diluted 1:1000 in 1% BSA:TBS-T and then reacted with Pierce SuperSignal west-pico ECL reagent (Pierce Chemical, Rockford, IL).

In vitro kinase assay

Immunoprecipitation P81 filter-binding kinase assay: lysates were generated as described and subjected to immunoprecipitation with the ROCK-1 G6 antibody. Immunoprecipitates were incubated with immobilized rMYPT-1 in kinase buffer (100 mM NaCl, 20 mM HEPES, 5 mM $MgCl_2$, 45 ml water, pH 7.2, 5 mM $MnCl_2$). At time 0, ATP mix was added to the sample (200 μCi/ml [^{32}P]ATP, 0.15 mM cold ATP and 30 mM $MgCl_2$). Samples were incubated for 30 min, bound to P81 filter paper and washed five times in 0.5–10% phosphoric acid, followed by a final wash in 100% acetone. Filter papers were dried and counting was performed on a Coulter scintillation counter (Beckman Coulter Inc., Fullerton, CA).

Quantitative real-time RT–PCR

RNA was collected using the micro-midi RNA spin column kit (Invitrogen, Carlsbad, CA). RNA was quantified and cDNA was prepared using the SuperScript II RT kit (Invitrogen, Carlsbad, CA). The samples were examined for ROCK-1 (mm00485745), ROCK-2, RhoA, TNF-α, GAPDH and M-CSF (mm00432688) transcript abundance using the Applied Biosystems (Foster City, CA) assay on Demand protocol. 18S ribosomal primers from Applied Biosystems were used to normalize data. Experiments were performed in triplicate samples.

Radioimmunoprecipitation

Peritoneal macrophages were harvested from animals in RPMI-1640 without methionine (met) and cysteine (cys) (Mediatech, Herndon, VA). The cells were starved of met and cys for 2 h, then continuously labeled with 1 mCi *trans* [^{35}S]methionine for 30 min, supplemented with 100 μM cys. One hundred minutes into the starvation protocol, cells were treated with either PBS, CHX (10 ng/ml) (Calbiochem, San Diego, CA), actinomycin-D (100 ng/ml) (Calbiochem, San Diego, CA) or rapamycin/sirolimus (10 nM final concentration) (Cell Signaling Technology, Beverly, MA). At time 0, cells were plated in cysteine-supplemented RPMI-1640 on 10 cm tissue culture-treated plastic dishes for the indicated time. Cells were washed twice with 37°C PBS and lysed with TNN buffer (50 mM Tris–HCl, pH 7.4; 100 mM NaCl; 5 mM EDTA; 0.5% Nonidet P-40) and snap frozen. Equal radioactive counts per minute (c.p.m.) of lysates were loaded on a slurry of protein-G Sepharose (Pierce Chemical, Rockford, IL), which had been precleared using the ROCK-1 G-6 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Following immunoprecipitation, the beads were washed three times in TNN buffer and then boiled in SDS-loading buffer. Next, the samples were separated on a 4–20% Tris–glycine SDS gel. The gels were fixed and silver-stained using the Silverquest® staining kit (Invitrogen, Carlsbad, CA) and imaged on film for 5 days.

Total protein synthesis

Total protein synthesis was determined by trichloroacetic acid (TCA) precipitation of metabolically labeled protein preparations as described above. Total cell lysates of 10^6 cell equivalents were added to 50% TCA to yield a final TCA concentration of 10%. Samples were incubated at −20°C for 10 min and then centrifuged at 14 000 *g* for 5 min at 4°C. The pellets were washed with 500 μl of 90% acetone and centrifuged at 14 000 *g* for 5 min at 4°C. Precipitated proteins were resuspended in urea/thiourea buffer (7 M urea, 2 M thiourea, 2% CHAPS, 10 mM DTT, 2% ampholytes, pH 3–10) and then sonicated and loaded onto a scintillation counter.

Polysome analysis

Macrophages were harvested as described and polysomes extracted with modified M-Per lysis buffer (Pierce Chemical, Rockford, IL), supplemented with 10 mM KCl, 15 mM $MgCl_2$, RNAsin® 40 U/μl, 100 μg/ml CHX and 1% DOC. Polysomes were subjected to ultracentrifugation at 39 000 r.p.m. in an SW41 rotor for 1.5 h through 7–47% low-salt sucrose gradients (10 mM HEPES, pH 7.0, 15 mM $MgCl_2$, 10 mM KCl, 0.5 mg/ml heparin, 100 μg/ml CHX) prepared as described (Ruan *et al*, 1997). The gradients were fractionated with a syringe fractionator (Brandel, Gaithersburg, MD) into 1 ml fractions. Fractions were then supplemented with *E. coli* RNA as carrier and UT2 RNA as an internal control, as described (MacKay *et al*, 2004). RNA was isolated using the micro-midi RNA spin column kit (Invitrogen, Carlsbad, CA) and analyzed by quantitative RT–PCR across the fractions as described above.

Mouse peritoneal macrophage chemotaxis

Peritoneal macrophages were harvested as described above and loaded with 0.5 μg/ml calcein-AM (Molecular Probes, Eugene, OR) in phenol red-free RPMI with 5% FBS. Fluorescently labeled macrophages were washed twice with PBS and incubated with one of the two treatment media for 45 min (negative control medium consisted of phenol red-free RPMI; treatment medium consisted of phenol red-free RPMI with 10 μM Y27632 (Calbiochem, San Diego, CA) or 10 nM rapamycin/sirolimus final concentration). After incubation with treatment media, the macrophages were washed twice with PBS resuspended in phenol red-free RPMI with 0.1% BSA, and plated in droplets of 29 μl on top of a 5 μm filter (Neuroprobe, Gaithersburg, MD). The filter on top of a 96-well plate was layered with serially diluted MCP-1 concentrations (Peprotech, Rocky Hill, NJ). Fluorescently labeled macrophages were allowed to chemotax into the wells containing MCP-1 for 2.5 h. At the end of chemotaxis assay, cell droplets were wiped from the top of the filter and the concentration of cells was assessed within each well by measuring fluorescence with a fluorescence plate reader (PerSeptive Biosystems, Framingham, MA) (Frevert *et al*, 1998; Liles *et al*, 2001).

Mouse peritoneal macrophage phagocytosis

Peritoneal macrophages were harvested as described and separated into three groups. Macrophages were allowed to adhere for 10 min, washed from the plate and then treated with the ROCK-selective inhibitor, Y27632, at 10 μM final concentration, pretreated with rapamycin at 10 nM final concentration or PBS vehicle for 30 min. Cells were then fed either opsonized *E. coli* or 2 μM beads coated with protein G and opsonized with AlexaFluor® 488 donkey anti-goat IgG antibodies (Molecular Probes, Eugene, OR) for 30 min. Cells were then assayed using the Phagotest® Kit (Orpegen Pharma, Heidelberg, Germany) as per the manufacturer's directions, then analyzed on a FACScan LM500n flow cytometer (Becton Dickinson, San Jose, CA).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

We thank members of the Schwartz laboratory: Sean Kelly, Dara Kimmel, Jonathan Sargent and Undui Redner for their technical support. We also thank Drs Kelly Smith, Bill Parks and Sirrka Lissa Hostikka for their helpful discussion and critical reading of the paper. This work was supported by NIH grants P01 HL03174, HL07312, CA89807 and HL62945.

Conflcit of interest

The authors have no conflict of interest to declare.

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