Herein we report that, despite the similarity of Rac2 to Rac1 (92% amino acid identity), macrophages derived from Rac2−/− mice, which continue to express Rac1, display a marked defect in αβ2/αβ3 and αβ1 integrin-directed migration measured on vitronectin and fibronectin fragments (FN-H296), respectively. In contrast, mouse embryo fibroblasts derived from the Rac2 knockout mice utilize Rac1 for migration via αβ2/αβ3 and αβ1 integrin. The genetic reconstitution of bone marrow-derived macrophages (BMMø) with Rac2 restores the integrin-dependent migration of Rac2-deficient macrophages on vitronectin (VN) and FN-H296. The levels of GTP-Rac2 generated upon specific integrin engagement in wild type macrophages parallels the phenotypic defect observed in Rac2-deficient macrophages; i.e. FN-H296, αβ2 > VN, αβ2/αβ3, αβ1, > FN-C1H271, > FN-H296, > intact FN. In a COS7 cell system, the expression of Syk kinase alone is insufficient to convert the αβ1 migration response to Rac2 dependence. Therefore, we present the first evidence that the αβ1 receptor in blood cells has evolved a Syk-Rac2 signaling axis to transmit signals required for integrin-directed migration suggesting that Syk kinase in part encodes myeloid Rac2 specificity in vivo.

Cell migration is an essential process during development and wound healing. During cell migration coordination between membrane traffic, cell substrate adhesion, and actin polymerization and reorganization is required for protrusion of the leading edge. Actin cytoskeletal reorganization is regulated by Rho family GTPases and, with a contribution from the endocytic cycle, serves to extend the forward edge of mammalian cells (1, 2). The Rho family GTPases have been recognized as the regulators of signal transduction pathways that mediate distinct actin cytoskeleton changes required for cell migration (3, 4). The Rho family of small GTPases (including Rac, Cdc42, and RhoA) comprise a complex group of at least 15 proteins critically involved as molecular switches in a large number of biochemical events in many cell types (5–7). Rac activation results from a combination of reduced association with GDP dissociation inhibitors and/or enhanced exchange of GDP to GTP promoted by guanine nucleotide exchange factors. Activated GTP-bound Rac proteins then transduce signals to downstream effector proteins. Finally, through association with GTPase-activating proteins, the GTP-bound small GTPase returns to an inactive GDP-bound form by hydrolysis of the bound GTP. Importantly, the events by which receptors orchestrate the activation of Rac are likely dependent upon the upstream activation of distinct protein-tyrosine kinases and the recruitment of specific adapter proteins to specific subcellular local to transmit specific signals.

One feature of the Rac proteins is their capacity to regulate the architecture of the actin cytoskeleton (3, 8–11). Dynamic rearrangement of the actin cytoskeleton is key for morphological changes observed under conditions of adhesion to the extracellular matrix and for cell migration. The structure and function of normal tissue is determined by reciprocal dialogue that is mediated in part through interactions with extracellular matrix, ECM. The ECM is a complex network of proteins and glycoproteins that provide both architectural support of the cells and contextual information to define the proper response to a given stimulus (12) at least in part via the regulation of Rac and other Rho GTPases.

Members of the Rac sub-class are comprised of Rac1, Rac2, and Rac3. Rac1 and Rac2 share 92% sequence identity and differ primarily in the C-terminal 10 residues, whereas Rac1, but not Rac2, contains a polybasic region. Rac1 and Rac3 share 77% identity and Rac2 and Rac3 share 83% identity (13, 14). Despite the high degree of sequence conservation the Rac2 knockout mice display a number of hematopoietic defects (15–19) mostly in the context of blood cell-specific receptor function or hematopoietic-specific effector mechanisms; e.g. fMLP-induced migration and NADPH oxidase activity, chemokine-induced chemotaxis response, FcεRI-induced mast cell degranulation, but also in kinase pathway-activated cell survival. These studies clearly identify a role of Rac2 in blood cell-specific signaling. However, the mechanisms that underlie Rac2-specific signals in hematopoietic cells remain unclear. We speculate a Rac2-specific receptor found in blood and nonblood cells to gain insight into the process, which encodes Rac2 specificity in the hematopoietic cell compartment. Although several reports suggest an importance for Rac in cell motility, the role of Rac2 in macrophage-specific integrin-dependent haptotaxis has not.

*This work was supported by National Institutes of Health Grants CA81403 and CA94233 (to D. L. D.) and by the Riley Research Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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This paper is available on line at http://www.jbc.org

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www.jbc.org

Published, JBC Papers in Press, August 12, 2003, DOI 10.1074/jbc.M306491200

The abbreviations used are: ECM, extracellular matrix; BMMø, bone marrow-derived macrophages; MEFs, mouse embryo fibroblasts; VN, vitronectin; FN, fibronectin; fMLP, formylmethionylleucylphenylalanine; mAb, monoclonal antibody; M-CSF, macrophage/colony-stimulating factor; IMDM, Iscove’s modified Dulbecco’s medium; WT, wild type; KO, knockout; PBS, phosphate-buffered saline; PE, phycoerythrin; FACS, fluorescence-activated cell sorting; EGFP, enhanced green fluorescent protein; GTP-γS, guanosine 5'-3-[O-thiotriphosphate; ITAM, immunoreceptor tyrosine-based activation motif.
been extensively evaluated. Our current data provide evidence that the integrins, αβ1 and αβ2αδδ9, expressed in macrophages display a requirement for the Rac2 not seen in fibroblasts. Moreover, using a reductionist approach in COS7 cells, we generate evidence that the presence of the nonreceptor protein-tyrosine kinase Syk is sufficient to convert nonhematopoietic cells to Rac2 dependence in the context of an αβ1 migration. Hence, we conclude that blood cells evolved an integrin-specific signalosome, which involves the nonreceptor protein-tyrosine kinase, Syk, in a signal transduction cascade that is selective for Rac2 and required for myeloid haptotaxis.

MATERIALS AND METHODS

Antibodies and Reagents—Rac2 Ab was a gift from Drs. G. Bokoch and U. Knaus (The Scripps Research Institute, La Jolla, CA). Mouse mAb against Rac1 and a PAN-Rac antibody, which reacts with Rac1 and Rac2 (R56220), were purchased from Upstate Biotechnology (Lake Placid, NY) and BD Transduction Laboratories (Lexington, KY), respectively. The recombinant p21-activated kinase p21 binding domain (PAK-1 PBD, for the Rac2 pull-down assay) conjugated to agarose was purchased from BD Pharmingen (San Diego, CA). Monoclonal antibodies were used to quantitate murine integrin expression in different cell lineages using flow cytometry and to block migration via specific integrins. M-CSF was bought from Peprotech (Rocky Hill, NJ). Human fibronectin (FN) and recombinant human FN peptides, H296 and CH271 were obtained from Collaborative Biomedical (Bedford, MA) or a gift from Takara Shuzo (Osuka, Japan), respectively. Vitronectin was purified in our laboratory as described previously (20, 21). All other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Animals—Rac2-deficient (−/−) mice and their normal littermate in a C57BL6/J genetic background have been described by Roberts et al. (15). C57BL6/J mice were used for wild type controls in these experiments. The Rac2 knockout mice used in this study had been backcrossed into C57BL6/J mice for >12 generations. Mice were housed in microisolation units under specific pathogen-free conditions and were fed autoclaved food and acidified water ad libitum. Mice used in these experiments were 8–12 weeks of age.

Isolation of Bone Marrow-derived Macrophages and Mouse Embryo Fibroblasts—Bone marrow-derived macrophages (BMMs) were generated by flushing the bone marrow cavity with culture media (IMDM, Invitrogen, Rockville, MD). Following centrifugation at 1500 rpm, reseparated cells were washed twice with PBS-0.1% BSA and subsequently incubated with PE-conjugated secondary Abs were purchased from BD Pharmingen (San Diego, CA). Monoclonal antibodies were used to quantitate murine integrin expression in different cell lineages using flow cytometry and to block migration via specific integrins. M-CSF was bought from Peprotech (Rocky Hill, NJ). Human fibronectin (FN) and recombinant human FN peptides, H296 and CH271 were obtained from Collaborative Biomedical (Bedford, MA) or a gift from Takara Shuzo (Osuka, Japan), respectively. Vitronectin was purified in our laboratory as described previously (20, 21). All other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated.

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Analysis of Integrin and Mac1 Receptor Expression—Cells (macrophages or MEFs) isolated from wild type (WT) or knockout (KO) mice were collected, washed, and resuspended in incubation buffer (PBS contained 0.1% BSA) at 2 × 10⁶ cells/sample. Following incubation with Fcγ receptor-blocking Ab (CD16/32), samples were washed once with PBS-0.1% BSA and incubated with one of the following mAbs (1 μg/ml) for 30 min on ice. The antibodies were either PE-conjugated anti-MAC1 or monoclonal antibodies against the murine integrin subunits: α4 (CD49d), α5 (CD49e), α6 (CD11α), α7 (CD11b), α9 (CD49b), α10 (CD49g), β1 (CD29), or β2 (CD61) or the respective isotype control. After incubation, cells were washed twice with PBS-0.1% BSA and subsequently incubated with secondary antibody (PE-conjugated) and then incubated for 30 min on ice followed by adjustment to volume of 250 μl in PBS. After washing the cells twice with PBS-0.1% BSA, flow cytometric analyses were performed using FACSScan (BD Biosciences, San Diego, CA).

Cell Migration and Adhesion Assays—Cell migration assays were performed on polycarbonate membranes using Transwell migration chambers (diameter 6.5 mm, pore size 8 μm; Costar Corp., Cambridge, MA). The underside of the membrane to which cells migrate was coated with 20 μg/ml of either fibronectin, vitronectin, collagen type I, or fragments of fibronectin H296 (binds αvβ3) or CH271 (binds α5β1) (Fig. 1) (22). Surfaces were subsequently blocked with heat-denatured BSA. Transwells were placed into the lower chamber containing 600 μl of serum-free medium. Cells (8- to 10-day cultures of bone marrow-derived macrophages or embryo fibroblasts) were scrapped and washed in serum-free medium. 2 × 10⁵ cells in 100 μl of media/Transwell were added to the top of the migration chamber (uncoated side) and allowed to migrate to the coated side of the chamber for 4 h at 37 °C. Haptotaxis was quantitated as described previously (25–27). The haptotaxis response of each cell type was further confirmed by demonstrating the complete abrogation of migration by coating both sides of the membrane with vitronectin, H296, CH271, or FN. To confirm the specificity of the integrin migration and to functionally validate the anti-integrin antibodies used in our FACS analysis, we preincubated wild type macrophages with antibody against the α1 or α5 integrin subunit (1 μg/ml). These cells were then examined for capacity to migrate toward vitronectin or H296 peptide in haptotaxis assay (see Fig. 3D). Cell adhesion assays were also performed. Briefly, flat bottom 96-well polystyrene plates (BD Biosciences) were coated with 20 μg/ml of either fibronectin, vitronectin, or fragments of fibronectin (H296 or CH271) in PBS for 1 h at 37 °C. Wells were washed once with PBS, incubated with 20 μg/ml BSA for 1 h at 37 °C for blocking nonspecific sites, and again washed twice with PBS. To examine cell adhesion to the coated surface, 1 × 10⁵ cells were added to each well and incubated at 37 °C for 4 h. At the end of the incubation, medium and unbound cells were removed with a washing vapor wash with PBS. Adherent cells were fixed with 3.5% formaldehyde and stained with 0.1% crystal violet. The stain was eluted with 10% acetic acid, and absorbance was determined at 600 nm with a microplate reader (Spectramax 250, Molecular Device) (28).

Retrovirual Reconstitution of Wild Type Rac2 into Rac2-deficient (Rac2−/−) Macrophages—Mice were injected intraperitoneally with 50 mg/kg 5-fluorouracil. After 48 h, mice were sacrificed and bone marrow was isolated. For transduction, nontissue cultured plastic 6-well plates were coated with CH296 fibronectin peptide as described (29). The MIEG-3 retroviral vector has been described before (30). Briefly, the MIEG-3 vector contains internal ribosomal entry site sequences, which are located downstream of a FLAG epitope-tagged Rac2 DNA (MIEG3-FR2), which drives the expression of EGFP in cells that express Rac2. The viral titers of MIEG-3 or MIEG-3FR2 viral supernatant were 2.5 × 10⁸ and 1.4 × 10⁹ colony forming units/ml, respectively. These retroviral supernatants were used to infect wild type and Rac2-deficient macrophage progenitors. 24-h bone marrow cultures (2 × 10⁵ cells/well) were plated in the presence of freshly thawed retrovirus supernatants supplemented with 100 ng/ml stem cell factor, 100 ng/ml megakaryocyte growth and development factor, 100 ng/ml granulocyte-CSF, and 50 ng/ml M-CSF (18). Following the incubation at 37 °C for 4 h, supernatants along with nonadhering cells were removed; cells were washed, resuspended in fresh conditioned medium (virus free) containing all the above-mentioned supplements and 24-h retrovirus supernatant was removed, cells were washed and resuspended in freshly thawed retrovirus supernatant supplemented with all cytokines and plated in the same well. After 4 h of infection, supernatants were aspirated; cells were washed and then plated in virus-free conditioned...
medium. Cells were incubated for 36–48 h at 37 °C. Following the incubation, supernatant was removed and adhering cells were harvested (16, 31). Green fluorescent protein-positive cells were isolated using a fluorescence-activated cell sorter (FACStar Plus, BD Biosciences) under sterile conditions. The transfection efficiency was 30–70%. EGFP-positive cells were washed and resuspended in IMDM for migration assay, and cell lysates were prepared for Western blot analysis for Rac2.

**Immunoblot Analysis of Rac1 and Rac2 Expression**—Lysates of mouse bone marrow macrophages and embryo fibroblasts were prepared, and protein was estimated with Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) using BSA as a standard. Protein lysates were resolved by SDS-PAGE. Blots were probed with mouse mAb for Rac1 or rabbit polyclonal Ab for Rac2 (32) and developed with an ECL method (Amersham Biosciences, UK). We also employed the PAN-Rac antibody (BD Transduction Laboratories, Lexington, KY) against both Rac1 and Rac2 pull-down assays. Using purified recombinant Rac1 and Rac2, we have observed that this antibody detects both GTPases with 20% greater sensitivity for Rac2 in Western blots (data not shown).

**Assay for Integrin-induced Rac2 Activation**—Macrophages (BMMs) (from wild type animals) were obtained by culturing bone marrow for 10 days in the medium defined above. They were stimulated in 10 cm of nonnecrosis culture-coated Petri dishes with 20 μg/ml filrobentin, vitronectin, or fragments of fibronectin (H296 (α5β1) or CH271 (α1β1) in PBS for 1 h at 37 °C. Cells were scraped and washed in serum-free medium (5 × 10^6 cells in 2 ml of serum-free media) and then plated onto vitronectin, fibronectin, and different fragments of fibronectins (H296 and CH271, 20 μg/ml) coated in 1-cm Petri dishes for 10 min. Following adhesion, cells were chilled with Hank’s balanced salt solution at 4 °C. Cell lysates were prepared in 25 ml HEPES, pH 7.5, 150 mM NaCl, 1% Tris/glycerol, 10 mM MgCl2, 1 mM EDTA, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aproptin, 25 mM sodium fluoride, and 1 mM sodium orthovanadate. For in vitro guanine nucleotide binding-positive control, cell lysate was incubated for 15 min at 30 °C in the presence of 10 μM GTPS and 100 μM GTP-βS. GTP loading was stopped by addition of MgCl2 to 60 mM. Binding reaction was initiated by adding 10 μl of Pak-1-agarose (GST fusion protein, corresponding to the p21 binding CRIB domain, PBD, residues 67–150, of human Pak-1, expressed in E. coli and bound to glutathione agarose) to each sample and incubated for 45 min at 4 °C and processed as described (18).

**Heterologous COS7 Migration System**—COS7 cells were plated at 1 × 10^5 cells on a 10-cm tissue culture dish overnight. Cells were transiently transfected with plasmids using the Lipofectamine reagent. Epithelial plasmids were used to express Rac1, Rac2, or the EGFP-tagged Syk protein. After 4 h, medium was changed and cells were further incubated for 48 h. For the migration assay the underside of the polycarbonate membrane of the Transwell was coated with 20 μg/ml of specific and/or noncoated integrin (20). Cells were scraped and washed in serum-free medium (5 × 10^6 cells) and then plated onto vitronectin, fibronectin, and different fragments of fibronectins (H296 and CH271, 20 μg/ml) coated in 1-cm Petri dishes for 10 min. Following adhesion, cells were chilled with Hank’s balanced salt solution at 4 °C. Cell lysates were prepared in 25 ml HEPES, pH 7.5, 150 mM NaCl, 1% Tris/glycerol, 10 mM MgCl2, 1 mM EDTA, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aproptin, 25 mM sodium fluoride, and 1 mM sodium orthovanadate. For in vitro guanine nucleotide binding-positive control, cell lysate was incubated for 15 min at 30 °C in the presence of 10 μM GTPS and 100 μM GTP-βS. GTP loading was stopped by addition of MgCl2 to 60 mM. Binding reaction was initiated by adding 10 μl of Pak-1-agarose (GST fusion protein, corresponding to the p21 binding CRIB domain, PBD, residues 67–150, of human Pak-1, expressed in E. coli and bound to glutathione agarose) to each sample and incubated for 45 min at 4 °C and processed as described (18).

**Fig. 2.** Expression of Rac1 and Rac2 in BMMs and MEFs. Western blot analysis was employed to determine the levels of expression of Rac2 and Rac1 in bone marrow-derived macrophages and mouse embryo fibroblasts. Whole cell lysates were prepared from Rac2 wild type and deficient macrophages or MEFs and probed with Rac1- and Rac2-specific antisera (32). A β-actin Western blot was used as a loading control. Data are representative of 10 experiments performed.

| Protein (ng) | BMMs | MEFs |
|-------------|------|------|
| WT | KO | WT | KO |
| 1 | 9 | 5 | 7 |
| 2 | 12 | 6 | 8 |
| 3 | 15 | 9 | 10 |
| 4 | 18 | 12 | 11 |
| 5 | 21 | 15 | 14 |
| 6 | 24 | 18 | 17 |
| 7 | 27 | 21 | 20 |
| 8 | 30 | 24 | 23 |

**Immunoblot**

*Expression of Rac2 and Rac1 in MEFs and BMMs*—It has previously been shown that Rac2 is the predominant Rac isoform in hematopoietic cells (13). We determined the levels of Rac1 and Rac2 protein in mouse bone marrow-derived macrophages and MEFs from Rac2−/− and +/+ animals by immunoblotting with specific antibodies for Rac1 and Rac2 (Fig. 2). Rac2 was only expressed in BMMs, whereas a similar amount of Rac1 was detected in BMMs and MEFs (Fig. 2). These results confirm the selective expression of Rac2 in murine BMMs. The data are similar to the previously reported expression pattern for Rac1 and Rac2 in murine neutrophils and mast cells (16, 18). The faint band noted in Fig. 2, lane 4 in the Rac−/− macrophage cell lysate probed with anti-Rac2 antibody is due to a minor cross-reactivity between the anti-Rac2 antisera and Rac1 as previously reported (15). Moreover, our results suggest that the loss of Rac2 in −/− macrophages does not result in augmentation in expression of Rac1 in these cells under these culture conditions. This is in contrast to mast cells, where in vitro culture of Rac2-deficient cells is associated with a significant increase in Rac1 expression (19).

**Rac2 Is Required for Macrophage Integrin-directed Cell Migration**—Previous experiments have utilized microinjection and/or transfection of dominant negative mutants of Rac to implicate Rac as an important event in the induction of actin polymerization and integrin-directed movement in macrophages (33). Importantly, the use of dominant negative and activated mutants of Rac do not provide an adequate dissection of the relative importance of Rac1, -2, or -3 isoforms in integrin signaling. To directly and specifically evaluate the role Rac2 plays a role in hematopoietic integrin signaling required for migration, we examined integrin-directed migration via several integrins in a Rac2-deficient genetic model. A careful evaluation of the α5β1, α5β2, and α5β3 expression patterns in macrophages isolated from −/− and +/+ mice as well as comparison of these integrins in MEFs reveals no change in integrin expression pattern between the different genotypes. Expression of integrin subunits; α5, β3, α4, α6, and β1 as determined by flow cytometric analysis revealed that both the percentage of macrophages and MEFs expressing these integrins and the levels of expression are similar in cells isolated from the Rac2−/− and +/+ mice (data not shown).

Previous reports from a number of laboratories provide evidence that macrophages express α5β2, α5β3, α5β1, and α5β3 integrins through which they can differentially bind and migrate on the extracellular matrix proteins, vitronectin, and/or fibronectin, respectively. Vitronectin and fibronectin are complex multifunctional glycoproteins present in blood and extracellular matrix. Vitronectin is a 75-kDa matrix protein...
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involved in a diverse array of interactions, including an interaction with plasminogen activator inhibitor 1, suggesting a role in control over matrix degradation (34). Vitronectin contains an RGD sequence (residues 45–47) through which it binds with α5 integrins (34). Similarly, the fibronectin protein is composed of multiple domains each with distinct integrin binding domains and discrete functions (Fig. 1). The peptide sequences represented by FN-H296 and FN-CH271 correspond to the α5β1 and ααβ1 binding sites, respectively, on the full-length fibronectin molecule (FN). Migration of BMMs from Rac2 wild type mice in response to recombinant fibronectin peptide, H296 (35), containing the ααβ1 binding CS-1 sequences (Fig. 1) was significantly higher compared with the full-length FN or VN (Fig. 3A). The capacity of macrophages from Rac2 −/− knockout mouse to migrate on ααβ1- and α5-specific ligands was significantly reduced (p < 0.005) as compared with migration of Rac2 +/+ cells (Fig. 3A). In sharp contrast, we observe that Rac2 is not as important for the migration response on full-length fibronectin, which includes both the ααβ1 and ααβ1 ligands (and the high affinity heparin binding site) or through ααβ1 (CH271) integrin. Although the overall magnitude of macrophage migration on full-length fibronectin and CH271 peptide is considerably reduced compared with migration of VN or H296, it can be accurately and reproducibly quantitated in murine macrophages such that we are confident that the comparison of integrin-specific defects between Rac2 −/− and +/+ mice are valid. Clearly Rac2 appears to function to a greater extent in ααβ1- and ααβ1α5β1-induced migration in macrophages, an observation of some interest to integrin biologists. Hence, we conclude that certain integrins require Rac2 for haptotaxis in macrophages, and others do not. Interestingly, migration via another β1 integrin expressed in BMMs, ααβ1, which binds to an Arg-Gly-Asp-Ser (RGD)-containing sequence within fibronectin (CH271, Fig. 1) (35, 36), was relatively normal in Rac2-deficient macrophages compared with the wild type BMMs. Strikingly, the ααβ1 integrin receptors expressed in

Fig. 3. Rac2 controls αα and ααβ1 migration in macrophages. A, bone marrow-derived macrophages from Rac2 −/− (KO) and +/+ (WT) mice were tested in haptotaxis assay for capacity to migrate on different matrix proteins or fragments of fibronectin corresponding to specific integrin-directed adhesion and migration. VN, vitronectin (via ααβ1/α5β1) FN, fibronectin; H296, via ααβ1; CH271, via ααβ1. Comparison of WT to KO cells on VN protein shows significant difference (p < 0.005); comparison of WT to KO cells on H296 peptide shows significant difference (p < 0.005); comparison of WT to KO cells on CH271 peptide shows significant difference (p < 0.07). Data represent mean ± S.E., representative of 10 experiments performed. B, adhesion of BMMs on VN, FN, H296, and CH271 peptides (n = 4; p > 0.2; comparison of all groups). C, migration of MEFs on VN, FN, H296, CH271 peptides, and collagen (n = 4, p > 0.3; comparison of all groups tested). D, specificity of integrin migration in BMMs. We determined the capacity of monoclonal antibody specific for the αα or α5 integrin subunits to block migration of wild type macrophages on VN or H296 peptide, respectively. Migration of BMMs on VN after preincubation with αα-specific antibody (n = 4, p < 0.02 compared with isotype control) (compare columns 1 to 2). Migration on H296 after preincubation of cells with monoclonal anti-α5-specific antibody, compared with isotype control (compare columns 3 to 4) (p < 0.003). Data are expressed as mean ± S.D. The data are representative of four experiments performed.
MEFs appear to function normally in the absence of Rac2 for migration on the H296 peptide (Fig. 3C). From these data we conclude that macrophages and not fibroblasts (Fig. 3C) require Rac2 for αβ1 and αv integrin-mediated haptotaxis. In addition, macrophages appear not to require Rac2 for αβ1-dependent haptotaxis.

**Specificity of Haptotaxis Response in Macrophages**—To confirm the specific role for the αv and αβ1 integrins in macrophage migration on VN and H296 matrix proteins, respectively, and the specificity within our signaling experiments we used function blocking monoclonal antibodies against the individual integrin subunits, αv and αβ1 (Fig. 3D). Preincubation of BMMs with monoclonal antibody to αv blocks migration on H296 peptide, and preincubation of macrophages with αv antibody blocks migration on vitronectin (Fig. 3D). These data confirm the specificity of H296 and VN for their respective integrins in BMMs. In addition, we have demonstrated that pretreatment of macrophages with H296, CH271, or vitronectin block migration directed by coating the Transwell membranes with monoclonal antibodies αv or αβ1, respectively (data not shown), thus confirming by several methods that our experimental system is evaluating αv and αβ1 integrin signaling and migration in macrophages. Moreover, pretreatment of BMMs with monoclonal antibody against the αv or αβ1 integrin has no effect on migration in response to vitronectin or H296 peptide, respectively (not shown).

**Effect of Rac2 Deficiency on Macrophage and Fibroblast Adhesion**—Surprisingly, adhesion of macrophages and fibroblasts to these integrin-specific ligands is normal in Rac2 null and wild type mice (Fig. 3B). However, the morphology noted for Rac2-deficient macrophages on fibronectin- or vitronectin-coated surfaces is different compared with the Rac2 wild type cells. Rac2-deficient macrophages remain rounded, do not spread, and do not become completely flattened on these specific matrix proteins. In contrast, macrophages from Rac2 wild type mice undergo spreading and acquire a flattened morphology within 1 h of plating on H296 or vitronectin. This correlates with the capacity to migrate on these matrix proteins (Fig. 3A).

**Rac2 Reconstitution of Rac−/− Macrophages Rescues the Integrin-directed Migration Defect**—To confirm a causal role for Rac2 in macrophage integrin-dependent migration, we reconstituted Rac2 expression in Rac2−/− macrophages using retroviral gene transfer (Fig. 4A). Expression of the FLAG-tagged Rac2 protein, which migrates slower on SDS-PAGE due to the FLAG sequence than wild type endogenous Rac2, was confirmed in transfected macrophages (compare lanes 1 and 3 to lanes 5 and 6). The transfer of Rac2 is sufficient to restore normal levels of haptotaxis on vitronectin (αv integrin-mediated adhesion) (Fig. 4A). There was a 4-fold increase in migration observed in Rac2-transduced macrophages above the empty vector control-transduced macrophages. Consistent with the argument for a requirement for Rac2 in macrophage αv integrin migration, we observed that Rac2−/− BMMs express significant levels of Rac1 (Fig. 2).

**The Integrins αv and αβ1 Differentially Activate Rac2 in Macrophages**—To further support the argument that Rac2 functions in αvβ1 and αv integrin signaling in macrophages, we examined the extent to which integrin-specific engagement leads to the activation of Rac2 by quantitating Rac2-GTP levels following adhesion of wild type macrophages to these different ECMs. In these experiments, cell lysates representing equivalent total cell protein were examined for levels of GTP·Rac2 following cellular adhesion to vitronectin, H296, CH271, or full-length fibronectin. We reasoned that, if Rac2 participates in signaling cascade downstream of αv and/or αvβ1, then one might expect that these integrins upon ligand engagement may more robustly activate Rac2 in a wild type macrophage. Conversely, if Rac2 did not participate in αvβ1 signaling, ligands that interact with αvβ1 would not activate Rac2. The results shown in Fig. 5 (A and B) are consistent with this logic in that wild type macrophages upon αv and αvβ1 stimulation preferentially activate Rac2 upon adhesion to VN or H296, respectively. The most dramatic effect is observed with the H296 ligand for αvβ1, representing a response 2-fold greater than VN and 4-fold greater than the CH271 ligand. In contrast engagement of αvβ1 with CH271 or full-length FN does not result in an appreciable activation of Rac2 (Fig. 5). Importantly, these data demonstrate that the magnitude of Rac2 activation is greatest following αvβ1 and αv integrin engagement and correlates with the degree of phenotypic defect observed in Rac2 knockout macrophages as relates to integrin-induced migration on these specific extracellular matrix proteins. Our data are consistent with the model that Rac2 functions in blood-specific αv and αvβ1 receptor complexes to drive migration toward the VN and H296 peptide component of fibronectin.

**Effect of Syk Kinase Expression on Rac2- versus Rac1-dependent αvβ1 Migration and GTPase Activation**—To directly investigate the role played by Syk kinase in Rac2 versus Rac1 signaling, we expressed wild type Syk kinase along with Rac1 or Rac2 in a COS7 cell system. We used this heterologous COS7 cell migration system to evaluate the effects of the hematopoietic-specific nonreceptor protein-tyrosine kinase, Syk, on Rac2-specific migration and to determined the capacity of Syk kinase to preferentially activate Rac2 versus Rac1 downstream of the αvβ1 receptor in the absence of other myeloid proteins. This reductionistic strategy demonstrated that, at least in the context of αvβ1 integrin engagement, that Syk cooperates with Rac2 and not Rac1 to drive haptotaxis on the H296 peptide.
DISCUSSION

The data presented here as well as other published work (15–18) clearly identify a critical role for Rac2 in a number of myeloid-specific phenotypes, including integrin-induced migration (Figs. 2–6), chemotaxis, respiratory burst, and mast cell degranulation. Moreover, it is clear that hematopoietic-specific intra-cyttoplasmic effectors, which include tyrosine kinases, scaffolding/adapter proteins, nucleotide exchange proteins, and phosphatases, likely exist to modulate and orchestrate signals through blood-specific receptors (Fcγ receptors, fMLP receptor, and β2 integrina) (37). It was hypothesized that certain intra-cyttoplasmic effectors may serve to encode a requirement for Rac2 activation by bringing these signals under hematopoietic-specific coordinate control. In this report we focus on the role of the Rac2 GTPase in macrophage integrin-directed migration and the potential upstream signaling elements that may dictate Rac2 specificity in a myeloid cell.

So how is Rac2 specificity encoded in myeloid cells? Recent data suggest that the unique TRQQKRP motif of Rac2 itself may encode a significant component for myeloid-specific function by mediating specific membrane and/or protein-protein, or protein-lipid interactions, and hence the observed delocalization of intracellular Rac2 (38). This report demonstrates that the capacity to rescue certain myeloid-specific phenotypes in Rac2−/− myeloid cells, e.g. NADPH oxidase, requires the TRQQKRP motif. These results are consistent with our model and suggest that the TRQQKRP motif may be necessary but not sufficient to encode some Rac2-specific functions in hematopoietic cells, e.g. integrin-dependent migration, phagocytosis.

In this context, Rac2 specificity may require other lineage-specific proteins in the intracellular milieu. Finally, our data and the data of Tao et al. (38) support the argument that more than one factor will encode and regulate Rac2 specificity in blood cells for different receptor-ligand-induced actions. In the case of αβ1 specificity we suggest that the signaling molecules downstream of the αβ1 integrin can use either Rac1 or Rac3 polybasic regions (KKRRRK or KKPGKK, respectively) to drive fibroblast migration on H296 peptide, whereas the macrophage αβ2 signalsome cannot. These data suggest that some factor other than the integrin receptor itself and/or the Rac structure determines specificity for Rac2 through this integrin within the macrophage.

If factors other than Rac2 GTPase structure contribute to the selectivity for Rac2 in myeloid cells, as has been suggested, then these components are likely downstream of the integrin cytoplasmic domain and upstream of the Rac2-guanine nucleotide exchange factors complex. So what is known about GTPase assembly into the signalsome in response to αβ2 and αα integrin stimulation? It is clear that the upstream activation of tyrosine kinases and phosphatases leads to the assembly of an upstream signalsome composed of adapter proteins that serves to activate small GTPases. Our results provide the first direct evidence that Rac2 is required for integrin-directed haptotaxis and that the hematopoietic-specific nonreceptor protein-tyrosine kinase, Syk, mediates Rac2 specificity in the context of αβ integrin receptor signal transduction. The implications of this work in macrophage signaling and small GTPase specificity are discussed below.

To directly explore the components responsible for Rac2 specificity in myeloid signaling, we employed a reductionistic strategy using COST7 cells transiently transfected with different myeloid-specific proteins together with Rac2 versus Rac1. Our results provide the first evidence that Syk kinase contributes to Rac2 specificity in αβ integrin receptor signaling (Fig. 6, A–D). Our data suggest that Syk is necessary and sufficient to encode αβ1-generated activation of Rac2 and regulate Rac2-dependent migration via this integrin. In contrast, other hematopoietic components, i.e. Vav1, Pyk2, etc., appear not to be required for Rac2 activation or Rac2-mediated migration. Other investigations have examined the role of specific kinases and adapter proteins downstream of the integrin subunits in the propagation of signals required for hematopoietic cell migration. Sun et al. (39) have demonstrated that the adhesive and migratory property of bone marrow-derived macrophages via integrins clearly depends upon myeloid-specific Src family, hck and fgr. They have shown that integrin-dependent signal transduction is impaired and macrophage motility is reduced in cells isolated from hck−/−, fgr−/− double-knockout mouse. In 1994, Clark et al. (40) observed the activation and tyrosine phosphorylation of Syk tyrosine kinase following the cell attachment. The interaction between blood-specific Src family kinases and Syk is somewhat complex and poorly understood. There is evidence that Src family kinases participate in integrin signaling via the downstream activation of Syk kinase (39). Moreover, other laboratories have reported evidence that Syk kinase participates in integrin-specific signaling (41). Woodside et al. (42) have determined that the β3...
FIG. 6. The αβ1 integrin utilization of Rac2 is specified by Syk kinase. A, integrin-dependent migration of COS7 cells transfected with EGFP-Syk plus Rac2 or Rac1. COS7 cells were transfected with pEGFP-Syk, pRK5-Rac1, or pMEP-Rac2. Following transfection, migration response on H296 peptide was quantitated as described under "Materials and Methods." Bars represent S.D. of the mean. B, Western blot analysis of Rac1 and Rac2 expression in COS7 cells. C, flow cytometric analysis to determine the levels of EGFP-Syk expression in different transfected populations of COS7 cells shown in panel A. D, a recombinant PAK-CRIB domain binding assay (pull-down assay) was performed to determine levels of Rac1-GTP, Rac2-GTP in cell lysates of transfected COS7 cells shown in panels A–C. The upper panel shows the levels of GTP-Rac1 detected in COS7 cells cotransfected with EGFP-Syk and Rac1 under conditions of H296 stimulation of αβ1 using the anti-FLAG antisera to quantitate Rac1-GTP. The middle panel is the determination of GTP-Rac2 under similar H296 stimulation conditions using an anti-HA antibody. The lower panel is the detection of levels of Rac1-GTP and Rac2-GTP under conditions of no stimulation (NS) or H296 stimulation for 15 min using an antibody that recognizes both isoforms of Rac to a similar extent in Western blot analysis.
and β1 integrin cytoplasmic domains constitutively associate with the N-terminal Syk-SH2 domain in a nonphosphotyrosine-dependent manner. Other investigators have confirmed a physiological significance for the direct interaction of Syk with the β1 cytoplasmic domain (43). Interestingly, the tyrosine phosphorylation sites in the β2 and β1 subunits (Tyr-747 and Tyr-759) represent closely spaced NXXY motifs, cognate binding sites for the Shc adapter protein (44). The phosphorylation of these sites promotes Shc binding and abrogates the association of Syk with the integrin, suggesting a dynamic interaction between the cytoplasmic domain of these integrins and Syk kinase. Other evidence provides a link between Syk and Rac in T cell signal transduction pathways leading to the activation of JNK kinase (45). Recent evidence suggests a role for Syk kinase in integrin-dependent signaling and migration in neutrophils (46). Other reports have emerged that define a role for Syk and its downstream target, Pyk2, α-tubulin, and Vav1 in integrin-dependent adhesion and migration responses (47). In contrast, Syk is not required for signaling through G-protein-coupled receptors (48) and does not appear to be required for chemotaxis in response to a number of chemokines, suggesting distinct Syk-dependent and -independent pathways may exist for different cell surface receptors in myeloid cells. Information generated in Syk −/− neutrophils provides evidence that Cbl, Vav, and Pyk2 are downstream targets for Syk under conditions of integrin stimulation (46). The link between Syk and Rac2 is particularly interesting in the context of results generated in Syk −/− mice where lymphocytes are defective in tissue entry and distribution in vivo. Our results are consistent with the literature and provide the first direct evidence that Syk encodes Rac2 specificity.

The Syk protein is composed of tandem N- and C-terminal SH2 domains connected by an intervening region termed the I-A domain followed by a linker region and catalytic domain (49, 50). Considerable work has defined a number of important regions for the Syk/ZAP70 family of kinases in immunoreceptor signaling (49). Much less is known regarding the role of Syk and the structural domains of Syk in integrin receptor signal relay. It is interesting to postulate from our results that the Syk Y342/346 region, a region implicated in the activation of Vav and PLC-γ1 downstream of β1 and β2 integrins, is somehow involved in mediating Rac2 activation. In this regard there is some recent evidence that PLC-γ1 is required for actin polymerization and migration through a downstream effect of the phosphatidylinositol 3-kinase cascade, an effect that is linked to the PLC-γ1 pleckstrin homology domain (51). Other evidence suggests that Syk kinase is phosphorylated within a linker region located between its two tandem SH2 domains and the catalytic domain. This region of Syk that includes tyrosines at positions 317, 342, and 346 is involved in the interaction of Syk with downstream effectors of signal transduction, including Cbl adapter protein, Vav, and PLC-γ, respectively. Deckert et al. (52) reported that the Syk Tyr-342 directly binds Vav and catalyzes the phosphorylation of Vav required for nuclear factor of activated T cells activation. Results generated in our laboratory demonstrate that the catalytically dead Syk kinase and Syk mutated at position Y342/346 is defective in the induction of Rac2 activation and Rac2-specific migration in COS7 cells (data not shown). In contrast the Y317F mutation in Syk has no effect on the capacity of Syk to activate Rac2 and drive migration (not shown). In our COS7 cell Fcγ/RITAM phagocytosis system (53), the regions of Syk required for Rac2 activation and phagocytosis are Tyr-317, Tyr-342, and Tyr-346 (data not shown) suggesting as has been reported an important contribution for receptor context for how the Syk pathway may function. Hong et al. (54) recently reported that in one of the Syk linker region mutants, Y317F, the Cbl binding site serves a negative regulatory role in ITAM signaling (54). In our studies Cbl serves both a positive and negative role in myeloid signaling with the capacity to bind several important effectors of signaling, e.g. the p85 subunit of phosphatidylinositol 3-kinase, Crkl, Grb2, and Shc (55, 56). The tyrosine residues in the linker region of Syk, Tyr-342 and Tyr-346, bind to Vav, and phospholipase Cγ1 and phospholipase Cγ2 lipid kinases and are involved in the activation of Ca2+ signaling and PKC (54). It is possible that Syk functions in a similar manner during integrin receptor engagement in myeloid cells. Results generated in our reductionistic system are consistent with our model, which predicts that Syk is immediately upstream of Rac2 in myeloid integrin signaling.

In summary, our data generated in the Rac2 knockout mouse model provide direct evidence that Rac2 is a critical component of myeloid αβ7, αβ8, and αβ4-directed signalsome distinct from the fibroblast αβ7, αβ8, or αβ4-directed signaling complex. This observation is further supported by our results obtained in the COS? cell system where the transfer of one macrophage-specific gene, Syk kinase, can impart Rac2 specificity in the context of integrin αβ7, αβ8, and Rac2 activation (Fig. 6). Our conclusions are supported by several observations: 1) In the Rac2 −/− mouse model the greatest defect in integrin function is noted in αβ7, αβ8, and αβ4/αβ8 migration; 2) The magnitude of Rac2 activation is greatest in macrophage adhesion to H296 and VN; 3) In the heterologous COS 7 cell system Syk activates αβ7, migration in a Rac2-dependent manner and preferentially stimulates the conversion of GDP-Rac2 to its GTP-bound state. Importantly, these comparative observations serve to solidify our model that a Syk-Rac2 axis exists in myeloid cells. This could prove to be one component of αβ7 signaling specificity in myeloid cells where myeloid cells respond in a more robust manner to the H296 region of fibronectin (VLA-4) with an amplified migratory response as compared with lesser response via the αβ7 integrin. This is of possible physiological relevance to a myeloid cell, an issue that remains to be tested. Hence this novel signaling axis may serve as a therapeutic target to control macrophage integrin-directed phenotypes and/or diseases where the VLA-4 pathway is more problematic.

Acknowledgments—We thank Drs. Gary Bokoch and U. Knaus, Scripps Clinic Research Foundation for providing the anti-Rac2 antibody, and thank David A. Williams for providing Rac2 knockout mice and helpful discussion. Dr. Robert Geahlen provided the Syk constructs.

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Rac2 Specificity in Macrophage Integrin Signaling: POTENTIAL ROLE FOR Syk KINASE
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J. Biol. Chem. 2003, 278:41661-41669.
doi: 10.1074/jbc.M306491200 originally published online August 12, 2003

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