Pharmacological Activation of Guanine Nucleotide Exchange Factors for the Small GTPase Rap1 Recruits High-Affinity β_1 Integrins as Coreceptors for Parvovirus B19: Improved *Ex Vivo* Gene Transfer to Human Erythroid Progenitor Cells

Kirsten A.K. Weigel-Van Aken

Abstract

Parvovirus B19 has potential as a gene therapy vector because of its restricted tropism for human erythroid progenitor cells in the bone marrow. B19 binds to the cell surface through P antigen and we identified activated β_1 integrins as coreceptors for internalization. Because differentiation with phorbol ester induces β_1 integrin coreceptor activity, but cell differentiation is not desirable in gene transfer to human progenitor cells and one of the downstream effectors of phorbol esters is the small GTPase Rap1, the role of Rap1 in the recruitment of β_1 integrins on hematopoietic cells was examined. Expression of a constitutively active Rap1 (63E) was sufficient to recruit β_1 integrin coreceptors in erythroleukemic K562 cells by inducing high-affinity integrin conformation. A crucial role of actin polymerization in Rap1-mediated β_1 integrin recruitment was documented by complete inhibition of the 63E Rap1 effect with low-dose cytochalasin D and by the ability of a constitutively active mutant of the actin cytoskeleton regulator Rac1 to sensitize K562 cells to the pharmacological activation of endogenous Rap1, using the Rap1 exchange factor-specific 8-pCPT-2'-O-Me-cAMP [8-(4-chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate]. Interestingly, in primary human erythroid progenitor cells, 8-pCPT-2'-O-Me-cAMP was sufficient to significantly increase B19-mediated gene transfer, suggesting that these cells possess the cytoskeleton organization capacity required for efficient recruitment of β_1 integrins by brief pharmacological stimulation of Rap1 GTP loading. Because 8-pCPT-2'-O-Me-cAMP has been implicated in enhanced homing of progenitor cells, these results identify a novel tool with which to optimize *ex vivo* B19-mediated gene transfer and potentially improve homing of transduced cells by Rap1- β_1 integrin activation with 8-pCPT-2'-O-Me-cAMP.

Introduction

VIRAL VECTORS have been extremely efficient in introducing therapeutic genes into human cells, as exemplified by retroviral gene transfer into hematopoietic stem cells that cured X-linked severe combined immunodeficiency (SCID) in all patients treated (Hacein-Bey-Abina *et al.*, 2002). However, the occurrence of insertional mutagenesis after retroviral integration into the human genome (Hacein-Bey-Abina *et al.*, 2008) poses a threat to the overall success of gene therapy and has fueled the search for alternative vectors. In addition, alternative target cells, such as committed progenitors, and cell type-selective, targeting vectors are being

explored in order to further lower the risk of adverse side effects. Adeno-associated virus 2 (AAV2) vectors have entered clinical trials and demonstrated therapeutic efficacy (Hauswirth *et al.*, 2008; Maguire *et al.*, 2008). Although recombinant AAV2 vectors also integrate randomly, albeit with markedly lower frequency, wild-type AAV2 has been shown to integrate site-specifically and AAV2 has not been linked to any human disease (Srivastava, 2005).

Parvovirus B19 is unique among potential gene therapy vectors because it has evolved to be restricted in its replication to one cell type, erythroid progenitor cells in the human bone marrow. B19 was shown to bind to erythroid cells through the blood group P antigen or globoside (Brown *et al.*,

1993). We demonstrated that P antigen is not sufficient for parvovirus B19 infection (Weigel-Kelley *et al.*, 2001) and subsequently identified functionally activated $\alpha_5\beta_1$ integrins as coreceptors for parvovirus B19 entry into human cells (Weigel-Kelley *et al.*, 2003). In addition, the Ku80 subunit of the DNA double-strand break repair protein Ku, which also functions as an adhesion receptor for fibronectin (Monferran *et al.*, 2004), was reported to provide coreceptor activity for B19 (Munakata *et al.*, 2005).

To exploit the erythroid specificity of parvovirus B19 for therapeutic purposes, we have generated recombinant parvovirus B19 vectors that encapsidate single-stranded or self-complementary AAV2 genomes into B19 capsids (Ponnazhagan *et al.*, 1998; Weigel-Kelley *et al.*, 2001). Because the functional activity of β_1 integrin coreceptors on erythroid cells is rather low and functional recruitment of β_1 integrins by induction of cell differentiation (Weigel-Kelley *et al.*, 2003) is not desirable during gene transfer to hematopoietic progenitor cells, alternative means of integrin activation were explored.

Despite a substantial body of knowledge on the regulation of the adhesive functions of integrins, our understanding of the mechanisms that "recruit" integrins as coreceptors to promote entry of viruses is still sparse. One of the hallmarks of integrin regulation is their activation through signaling pathways from within the cell ("inside-out" activation) that emanate from growth factor and cytokine receptor stimulation and ultimately lead to an increase in integrin affinity, avidity, or both (Katagiri *et al.*, 2000; Sebzda *et al.*, 2002). Rap1, a GTPase of the Ras superfamily, has been implicated in the "inside-out" activation of multiple integrins, including β_1 , β_2 , and β_3 integrins (Bos *et al.*, 2003; Shimonaka *et al.*, 2003; Bos, 2005), and has been shown to be activated by phorbol esters (Maridonneau-Parini and de Gunzburg, 1992; M'Rabet *et al.*, 1998; Bertoni *et al.*, 2002; Caron, 2003; Katagiri *et al.*, 2003; Bivona *et al.*, 2004; Dustin *et al.*, 2004; Bos, 2005). Guanine nucleotide exchange factors (GEFs) swap GDP for GTP, enabling a conformational change and activation of Rap1. Whereas some GEFs regulate both Ras and Rap proteins, C3G and Epac are specific for Rap proteins (Quilliam *et al.*, 2002). A cAMP analog, 8-pCPT-2'-O-Me-cAMP [8-(4-chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate], which specifically activates Epac and selectively increases the GTP-loading of Rap1, has been developed (Enserink *et al.*, 2002).

In an attempt to characterize the mechanisms leading to the functional recruitment of β_1 integrins as coreceptors for parvovirus B19, the current study identifies Rap1 as a central regulator for β_1 integrin coreceptor activity and documents the efficient β_1 integrin coreceptor recruitment on human erythroid progenitor cells by brief exposure to 8-pCPT-2'-O-Me-cAMP.

Materials and Methods

Cells, viruses, antibodies, and reagents

K562 and NIH3T3 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA) and cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% newborn calf serum and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO). Human bone marrow-derived CD36⁺ erythroid progenitor cells were purchased from AllCells (Emeryville, CA). Cells

were cultured overnight in serum-free medium containing stem cell factor (SCF, 50 ng/ml), interleukin (IL)-3 (10 ng/ml), IL-6 (10 ng/ml), and erythropoietin (Epo, 2 U/ml) before transduction with recombinant B19 vectors. The HA-63E Rap1 and GST-RalGDS RBD-expressing plasmids were kindly provided by L. Quilliam (Indiana University, Indianapolis, IN) and the Q61L Rac1-expressing plasmid was purchased from Upstate Biotechnologies/Millipore (Billerica, MA). Recombinant parvovirus B19-*Luc* and B19-*EGFP* vector stocks containing the firefly luciferase and enhanced green fluorescent protein transgenes, respectively, were generated as described previously and viral titers were determined using slot blots and infectious assays (Ponnazhagan *et al.*, 1998; Weigel-Kelley *et al.*, 2003). Activating and inhibitory monoclonal antibodies against β integrin chains were purchased from Chemicon (Temecula, CA). Trypsin (0.05%)–EDTA (0.02%), phorbol 12-myristate 13-acetate (PMA), and cytochalasin D were purchased from Sigma-Aldrich, and 8-pCPT-2'-O-Me-cAMP was purchased from Tocris Bioscience (Ellisville, MO).

GST-RBD pull-down assay

The GST-RBD pull-down assay has been extensively used to estimate the activities of Ras family proteins, including Rap1, in cellular extracts (Castro *et al.*, 2005). It is based on the preferential binding of a glutathione *S*-transferase (GST)-fused Rap binding domain (RBD) from the downstream effector RalGDS (C-terminal 97 residues) to GTP-bound versus GDP-bound Rap1 proteins. To determine Rap1–GTP levels, control K562 cells and HA-63E Rap1-transfected K562 cells were lysed in lysis/wash buffer (50 mM Tris-HCl, pH 7.4; 10% glycerol; 200 mM NaCl; 200 mM MgCl₂; 1% Triton X-100; 1 mM phenylmethylsulfonyl fluoride [PMSF]; aprotinin [0.05 trypsin inhibitor U/ml]). Lysates were cleared by centrifugation for 10 min at 12,000 rpm and supernatants were transferred to fresh tubes containing 10 μ g of GST-RalGDS RBD. After a 1-hr incubation on a rotor at 4°C, GSH-agarose beads (50% slurry) were added and further incubated. The beads were washed three times with 1 ml of cold lysis/wash buffer, resuspended in 2 $\times$ sodium dodecyl sulphate (SDS)-loading buffer, heated for 3 min at 100°C, and loaded on a SDS–12% polyacrylamide gel. Proteins were transferred to nitrocellulose membrane and Rap1 levels were detected with a monoclonal anti-Rap1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Recombinant parvovirus B19 vector transduction assay

K562 cells were washed twice in serum-free IMDM and incubated with either 10 or 500 nM cytochalasin D for 20 min, or with 100 μ M 8-pCPT-2'-O-Me-cAMP for 15 min at 37°C before infection with recombinant parvovirus B19-*Luc* vector. Cell extracts were assayed for luciferase activity, using a luciferase assay kit (Promega, Madison, WI) 24 hr postinfection. For antibody studies using anti-integrin antibodies, cells were preincubated for 10 min at room temperature with normal human IgG (50 μ g/ml) to block Fc receptors and then with β_1 , β_2 , or β_3 integrin antibodies (50 μ g/ml) for 25 min at room temperature before infection with recombinant parvovirus B19-*Luc* vector. For integrin

cross-linking experiments, cells were preincubated for 10 min at room temperature with normal human IgG (50 μ g/ml) to block Fc receptors followed by incubation with β_1 integrin antibodies (50 μ g/ml) for 25 min at room temperature and cross-linking anti-mouse IgG antibodies (25 μ g/ml) for 25 min at room temperature before infection with recombinant parvovirus B19-*Luc* vectors. NIH3T3 murine fibroblasts were plated in 12-well plates and treated 24 hr after plating with 100 μ M 8-pCPT-2'-O-Me-cAMP for 15 min at 37°C immediately before infection. Human CD36⁺ primary erythroid progenitor cells were cultured overnight either in suspension or on fibronectin-precoated 12-well plates in the presence of SCF (50 ng/ml), IL-3 (10 ng/ml), IL-6 (10 ng/ml), and Epo (2 U/ml). Erythroid progenitor cells were infected with recombinant B19-EGFP vector either in suspension or on fibronectin-coated plates and EGFP expression was detected by fluorescence microscopy and flow cytometry 36 hr post-infection.

Southern blot detection of viral genomes in NIH3T3 cells

Wild-type B19 and wild-type AAV2 virions were incubated with NIH3T3 cells for 2 hr at 37°C to allow virus binding and internalization. Uninternalized viral particles were removed from the cell surface by extensive trypsin treatment. Cells were lysed, nuclear and cytoplasmic compartments were separated, and low molecular weight DNA was isolated and run on an agarose gel. Viral genomes were detected in cytoplasmic and nuclear compartments by Southern hybridization with ³²P-labeled wild-type B19 and wild-type AAV2 DNA probes, respectively.

Flow cytometric and immunoblot detection of integrins

To determine the expression levels of β_1 , β_2 , and β_3 integrins on untransfected and 63E Rap1-transfected K562 cells, unpermeabilized cells were washed with cold phosphate-buffered saline (PBS)–1% bovine serum albumin (BSA) twice; incubated with mouse anti-human β_1 , β_2 , and β_3 integrin antibodies and rabbit anti-mouse fluorescein (FITC)-conjugated secondary antibodies on ice for 30 min; and analyzed by flow cytometry. Cells incubated only with secondary FITC-conjugated antibodies were used as controls. Expression of β_1 integrins in murine NIH3T3 fibroblasts was detected in cell extracts by immunoblot analysis with anti-mouse β_1 integrin antibody. Extracts from $\beta_1^{-/-}$ murine fibroblasts were used as negative control. For the detection of high-affinity conformation β_1 integrins on untransfected and 63E Rap1- and Q61L Rac1-transfected K562 cells, unpermeabilized cells were incubated with a β_1 integrin antibody, HUTS-21, which binds only high-affinity conformation β_1 integrins, and JB1A antibody, which binds all β_1 integrins irrespective of their conformation, and FITC- and phycoerythrin (PE)-conjugated secondary antibodies, respectively, and the percentage of HUTS-21-positive β_1 integrins was determined by flow cytometry.

Statistical analysis

Experiments were performed three times and all measurements shown represent means and SEM. *P* values were calculated by Student *t* test.

Results

Constitutively active Rap1 is sufficient to make the erythroleukemia cell line K562 permissive for parvovirus B19 infection

Integrins on the surface of unstimulated hematopoietic cells are known to display low affinity for their ligands. However, external stimuli can generate intracellular signals that rapidly activate integrins through “inside-out” signaling, inducing a high-affinity and/or avidity state in which integrins bind to adhesion molecules on other cells or extracellular matrix components (Harris *et al.*, 2000; Laudanna *et al.*, 2002). We have previously demonstrated that β_1 integrins expressed on unstimulated K562 cells fail to promote parvovirus B19 entry, even in the presence of high β_1 integrin affinity-stabilizing or β_1 integrin clustering antibodies, and that differentiation induced by phorbol ester (PMA) leads to the functional recruitment of β_1 integrins as parvovirus B19 coreceptors (Weigel-Kelley *et al.*, 2003). Because PMA affects a variety of intracellular signaling pathways and cell differentiation in most instances is not desirable in the context of gene transfer to human hematopoietic progenitor cells, alternative means of β_1 integrin coreceptor activation to enhance parvovirus B19-mediated gene transfer were explored.

Phorbol esters activate the Ras family GTPase Rap1, which plays a central role in controlling integrin function (Arai *et al.*, 2001; Bos *et al.*, 2001; Katagiri *et al.*, 2002; Caron, 2003; Carmona *et al.*, 2008). In Jurkat T cells, for example, PMA induces colocalization of Rap1 with the cytoplasmic domain of β_1 integrins, which is required for Rap1 GTP loading and increased β_1 integrin-mediated cell adhesion (Medeiros *et al.*, 2005). Rap1 modulates integrin activation by affecting its affinity and avidity through several known effectors: regulator of cell adhesion and polarization enriched in lymphoid tissue (RAPL), Rap1-GTP-interacting adapter molecule (RIAM), and protein kinase D (PKD) (Cantor *et al.*, 2008). Interestingly, PKD is a direct substrate of protein kinase C (PKC), a kinase that is activated by PMA (Kucik *et al.*, 1996). Activation of Rap1 and its downstream effectors could therefore be involved in the functional recruitment of β_1 integrins as B19 coreceptors that we had previously observed in PMA-treated K562 cells.

To investigate a potential role of Rap1 in β_1 integrin recruitment, K562 cells were stably transfected with a constitutively active mutant of Rap1 (63E Rap1). Hemagglutinin (HA)-tagged 63E proteins were detected by Western blot with anti-HA antibodies or anti-Rap1 antibodies, with the latter depicting endogenous and HA-tagged Rap1 proteins as distinct bands because of the slightly slower migration of the HA-tagged proteins (Fig. 1A, top right). Rap1-GTP pull-down assays using the GST-fused Rap binding domain (RBD) from the downstream effector RalGDS were used to determine the levels of GTP-loaded Rap1 in untransfected and 63E Rap1-transfected K562 cells. Incubation of whole cell extracts with 10 μ g of GST-RalGDS RBD and increasing amounts of GSH-agarose beads resulted in the immunoprecipitation of increasing amounts of GTP-bound Rap1 in 63E Rap1-transfected, but not untransfected, K562 cells (Fig. 1B, top). These results document low endogenous Rap1-GTP levels and substantially increased Rap1-GTP levels after stable expression of 63E Rap1. Morphologically, 63E Rap1-transfected K562 cells demonstrated occasionally adherent,

elongated cells (Fig. 1C, bottom, long arrow), and tight cell-cell interactions as demonstrated by flattened plasma membrane shape at sites of cell-cell contact (Fig. 1C, bottom, short arrows). In contrast, untransfected control K562 cells always displayed the rounded morphology of suspension cells and lacked tight cell-cell interactions (Fig. 1C, top, short arrows). To test whether 63E Rap1-expressing K562 cells were permissive for parvovirus B19 infection, untransfected and 63E Rap1-expressing K562 cells were exposed to recombinant B19-*Luc* vector and luciferase activity was determined 24 hr postinfection. As observed previously, untransfected K562 cells were not transduced with B19-*Luc* vector. Expression of the constitutively active Rap1 protein (63E Rap1-K562), however, was sufficient to convert undifferentiated K562 cells into B19-permissive cells as documented by a significant increase in luciferase activity (Fig. 2A). As a control, K562 cells were treated with the cAMP analog 8-pCPT-2'-O-Me-cAMP (8-CPT-2-Me-cAMP), which specifically activates the Rap1 GEF Epac1 and increases Rap1 GTP loading (Erserink *et al.*, 2002), but no effect on B19 permissiveness was observed (Fig. 2A).

Constitutively active Rap1 induces permissiveness for parvovirus B19 infection in K562 cells through functional recruitment of β_1 integrins

Because K562 cells express high levels of β_1 integrins and low levels of β_2 and β_3 integrins, and Rap1 has been demonstrated to be involved in the inside-out activation of all three integrins (Katagiri *et al.*, 2000; Bertoni *et al.*, 2002; Sebzda *et al.*, 2002), the contribution of each of the integrins to the 63E Rap1-mediated effect on B19-*Luc* transduction was investigated. Untransfected and 63E Rap1-transfected K562 cells were preincubated with function-blocking antibodies against β_1 , β_2 , and β_3 integrins before exposure to parvovirus B19-*Luc* vector. No transduction was observed in untransfected K562 cells in the absence or presence of anti-integrin antibodies, confirming our previous results (Weigel-Kelley *et al.*, 2003). K562 cells expressing 63E Rap1, however, were readily transduced with B19-*Luc* vector, and transduction was completely blocked by β_1 integrin function-blocking antibodies (Fig. 2B, 63E Rap1-K562/ β_1 -P4C10). In contrast, no inhibitory effect was observed after preincubation with β_2 and β_3 integrin function-blocking antibodies (Fig. 2B, 63E Rap1-K562/ β_2 and β_3). In fact, function-blocking β_3 integrin antibodies led to an increase in B19 transduction, an effect reminiscent of the β_1/β_3 integrin cross-talk we previously observed in primary human erythroid cells (Weigel-Kelley *et al.*, 2006). Because β_2 integrins are functionally recruited

by constitutively active Rap1 in leukocytes (Katagiri *et al.*, 2000), these data confirm our earlier results that β_2 integrins are not involved in B19 internalization (Weigel-Kelley *et al.*, 2006). These results corroborate that 63E Rap1 induces permissiveness for parvovirus B19 in K562 cells by functionally recruiting β_1 integrins. Interestingly, "outside-in" activation of β_1 integrins on 63E Rap1-expressing K562 cells with high-affinity stabilizing antibodies (Fig. 2B, 63E Rap1-K562/ β_1 -N29) had only a moderate effect on B19-*Luc* transduction, whereas preincubation with low-affinity-stabilizing β_1 integrin antibodies followed by clustering secondary anti-mouse IgG antibodies (Fig. 2B, 63E Rap1-K562/ β_1 -JB+sec.) substantially increased B19-*Luc* transduction. These results suggest that 63E Rap1 exerts its effect on β_1 integrin coreceptor function through both an increase in integrin affinity and avidity, although the latter seems less pronounced because antibody-mediated integrin cross-linking was able to provoke a substantial enhancement of coreceptor activity.

To further investigate the mechanism of 63E Rap1-mediated β_1 integrin recruitment, total levels of β_1 integrins and levels of high-affinity β_1 integrins on the surface of 63E Rap1-K562 cells were measured. As reported in other cell systems, 63E Rap1 expression did not change the total levels of β_1 integrins on the surface of K562 cells as detected by flow cytometry (Fig. 2C). For the detection of high-affinity β_1 integrins, the β_1 integrin antibody HUTS-21, which binds only high-affinity conformation β_1 integrins, was used. As expected, untransfected K562 cells showed little HUTS-21 antibody binding, as these cells express default low-affinity β_1 integrins (Lundell *et al.*, 1996). In contrast, on 63E Rap1-expressing K562 cells up to 33% of β_1 integrins were in a high-affinity conformation (Fig. 2D). The pharmacological activation of endogenous Rap1-GTP, in contrast, led to high-affinity conformations in less than 3% of surface-expressed β_1 integrins, which might have contributed to the lack of B19-mediated transduction in 8-pCPT-2'-O-Me-cAMP-treated cells (Fig. 2A).

De novo actin polymerization is required for Rap1-mediated recruitment of β_1 integrins as coreceptors for parvovirus B19 on K562 cells

The preservation of low-affinity conformations in integrins expressed on circulating blood cells is thought to involve cytoskeletal restraints that prevent the lateral movement of integrins within the plasma membrane. We previously reported a dual effect of the actin polymerization inhibitor cytochalasin D on β_1 integrin coreceptor function in PMA-differentiated K562 cells: at low cytochalasin D concentrations, which

FIG. 1. Expression of constitutively active Rap1 (63E Rap1) in K562 cells. (A) K562 cells were stably transfected with a plasmid encoding a hemagglutinin (HA)-tagged 63E Rap1 protein. Total Rap1 levels were detected by immunoblot with either a Rap1 antibody (*top*) or an HA antibody (*middle*). Phorbol ester (PMA) differentiation of K562 cells slightly increased endogenous Rap1 levels (*top left*) but had no major effect on the expression of transfected HA-63E Rap1 (*middle right*). (B) Levels of active, GTP-loaded Rap1 were determined in K562 and 63E Rap1-expressing K562 cells, using a glutathione S-transferase (GST)-fused Rap binding domain (RBD) from the downstream effector RalGDS that preferentially binds the GTP-bound form of Rap1. GTP-loaded Rap1 was precipitated with increasing amounts of GST-RalGDS RBD in 63E Rap1-transfected K562 cells (*top right*), but not untransfected control K562 cells (*top left*). Total levels of endogenous and HA-tagged 63E Rap1 in whole cell extracts are shown in the bottom panels. (C) Phase-contrast microscopy of untransfected and 63E Rap1-transfected K562 cells demonstrates the typical rounded cell morphology and loose cell-cell interactions (small arrows) in untransfected cells (*top panel*) and elongated cell shapes (long arrow) and tight cell-cell adhesions (short arrows) in 63E Rap1-transfected cells (*bottom panel*). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IP, immunoprecipitation; WCE, whole cell extract.

FIG. 2. Constitutively active Rap1 is sufficient to convert parvovirus B19-resistant undifferentiated K562 cells into parvovirus B19-permissive cells. **(A)** Untreated K562 cells, K562 cells treated for 15 min with the Rap1 GEF-specific cAMP analog 8-pCPT-2'-O-Me-cAMP, and 63E Rap1-expressing K562 cells were infected with recombinant parvovirus B19-*Luc* vector and luciferase activity was determined in cell extracts 24 hr postinfection. Experiments were performed three times and all measurements shown represent means and SEM. **(B)** Control and 63E Rap1-expressing K562 cells were incubated with high-affinity-stabilizing (N29) or low-affinity-stabilizing β_1 (P4C10 and JB1A), β_2 , and β_3 integrin antibodies before infection with recombinant parvovirus B19-*Luc* vector. For β_1 integrin cross-linking, incubation with low-affinity-stabilizing antibody (JB1A) was followed by incubation with cross-linking anti-mouse IgG antibodies (JB+sec.) before infection with recombinant parvovirus B19-*Luc* vector. Luciferase activity was determined in cell extracts 24 hr postinfection. Experiments were performed three times and all measurements shown represent means and SEM. **(C)** Expression of constitutively active Rap1 does not affect surface expression of β_1 , β_2 , or β_3 integrin. Cells were washed with cold PBS-1% BSA, incubated with primary anti-integrin antibodies and fluorescein isothiocyanate (FITC)-conjugated secondary anti-mouse IgG antibodies, and analyzed by flow cytometry. **(D)** Expression of constitutively active Rap1 induces high-affinity conformation in β_1 integrins. Untreated K562 cells, K562 cells treated for 15 min with the Rap1 GEF-specific cAMP analog 8-pCPT-2'-O-Me-cAMP, and 63E Rap1-expressing K562 cells were stained with JB1A anti- β_1 integrin antibodies (to determine the total level of β_1 integrins) and high-affinity conformation-specific HUTS-21 β_1 integrin antibodies and FITC- and phycoerythrin (PE)-labeled secondary antibodies, respectively, and analyzed by flow cytometry. The percentage of HUTS-21-positive β_1 integrins present on the cell surface is shown. Experiments were performed three times and all measurements shown represent means and SEM.

FIG. 3. Functional recruitment of β_1 integrin coreceptors by constitutively active Rap1 is dependent on *de novo* actin polymerization. **(A)** Control and 63E Rap1-expressing K562 cells were preincubated with low (10 nM) and high (500 nM) concentrations of the actin filament elongation blocker cytochalasin D (CD) and subsequently infected with recombinant parvovirus B19-*Luc* vector. Luciferase activity was determined in cell extracts 24 hr postinfection. **(B)** Control, 63E Rap1-expressing, and Q61L Rac1-expressing K562 cells were either left untreated or treated with 8-pCPT-2'-O-Me-cAMP for 15 min, stained with JB1A anti- β_1 integrin antibody and high-affinity-specific HUTS-21 β_1 integrin antibody and with FITC- and PE-labeled secondary antibodies and analyzed by flow cytometry. The percentage of HUTS-21-positive β_1 integrin present on the cell surface is shown. **(C)** Control and Q61L Rac1-expressing K562 cells were either left untreated or treated with 8-pCPT-2'-O-Me-cAMP for 15 min and infected with recombinant parvovirus B19-*Luc* vector. Luciferase activity was determined in cell extracts 24 hr postinfection. RLU, relative light units.

presumably only partially disrupt F-actin structures (Kucik *et al.*, 1996), we observed an increase in β_1 integrin-mediated B19 transduction, whereas at higher cytochalasin D concentrations B19 transduction was progressively reduced (Weigel-Kelley *et al.*, 2006).

It was next investigated whether partial disruption of cytoskeletal restraints could further increase B19 transduction in 63E Rap1-expressing K562 cells. To this end, untransfected and 63E Rap1-transfected K562 cells were incubated with either low (10 nM) or high (500 nM) concentrations of cytochalasin D, and subsequently infected with B19-*Luc* vector. Interestingly, low-level transduction with B19-*Luc* vector could be elicited in untransfected K562 cells by low-dose cytochalasin D treatment, supporting our previous observations in PMA-treated K562 cells (Weigel-Kelley *et al.*, 2006). Higher concentrations of cytochalasin D, as expected, abrogated B19 transduction in untreated K562 cells (Fig. 3A). Surprisingly, in 63E Rap1-expressing K562 cells, B19-*Luc* transduction was completely abolished both in low and high cytochalasin D concentrations (Fig. 3A), suggesting that the functional recruitment of β_1 integrins by 63E Rap1 was critically dependent on *de novo* actin polymerization.

To further explore this possibility, a constitutively active mutant of the actin reorganization regulator Rac1 (Q61L Rac1) was transfected into K562 cells. Rac1 has previously been demonstrated to be involved in the internalization of AAV2 (Sanlioglu *et al.*, 2000). Rac1 affects integrin-mediated cell adhesion by assembly of an integrin activation complex containing its downstream effector WAVE2, which binds the actin regulator protein complex Arp 2/3 that induces site-restricted actin polymerization through *de novo* actin nucleation and branching of filaments (Abou-Kheir *et al.*, 2008). Stable expression of Q61L Rac1 alone was sufficient to induce high-affinity conformations in ~17% of surface-expressed β_1 integrins in K562 cells (Fig. 3B). Interestingly, in the presence of Q61L Rac1, K562 cells became responsive to 8-pCPT-2'-O-Me-cAMP treatment, which resulted in an increase in high-affinity β_1 integrins in Q61L Rac1-expressing cells to ~30% of total surface-expressed β_1 integrins, reaching levels close to those observed in 63E Rap1-transfected cells (Fig. 3B). These results strongly suggest a role of the actin polymerization machinery in the functional recruitment of β_1 integrins on K562 cells. To test whether the observed increase in high-affinity β_1 integrins in Q61L Rac1-transfected cells led to coreceptor activity for parvovirus B19, control K562 cells and Q61L Rac1-transfected cells were exposed to B19-*Luc* vector. Indeed, moderate levels of *Luc* transgene expression were observed in Q61L Rac1-K562 cells, which could be further increased by preincubation of the cells for 15 min with 8-pCPT-2'-O-Me-cAMP (Fig. 3C). It is noteworthy that Q61L Rac1 has been demonstrated to activate NF- κ B (Joyce *et al.*, 1999; Singh *et al.*, 2004) and that the recombinant AAV2 construct packaged into the B19-*Luc* vector is known to contain NF- κ B-binding sites within the inverted terminal repeats (ITRs). It thus cannot be excluded at this point that the increase in *Luc* transgene expression observed in Q61L Rac1-expressing K562 cells is partially due to an NF- κ B-mediated increase in transcription of the recombinant AAV2 genome. This is, however, rather unlikely to account for the observed transgene expression, because Q61L Rac1-expressing K562 cells also demonstrated an increase in high-affinity β_1 integrin coreceptors, as detected by HUTS-21 binding, suggesting that

improved viral entry, not an increase in transgene transcription, is likely responsible for the increase in *Luc* expression. The ability of 8-pCPT-2'-O-Me-cAMP to increase B19-*Luc* transduction in Q61L Rac1-K562 cells suggests that in the presence of an active actin cytoskeleton-remodeling machinery brief induction of GTP loading of endogenous Rap1 is sufficient to recruit β_1 integrin coreceptors for B19 in K562 cells. Taken together, these results suggest that the non-permissiveness of erythroleukemic K562 cells is based on the lack of high-affinity β_1 integrins and the inability of these cells to efficiently rearrange their cytoskeleton, which both lead to inefficient recruitment of β_1 integrins as coreceptors for virus internalization.

Pharmacological activation of endogenous Rap1 enhances B19 transduction of murine fibroblasts

The results obtained in K562 cells indicate that a cell's ability to efficiently rearrange its actin cytoskeleton is critical for β_1 integrin coreceptor activity. It was of interest to determine next whether cells that display an adherent phenotype, and presumably reorganize and polymerize actin *de novo* to maintain this phenotype, were susceptible to B19 transduction and amenable to pharmacological activation of the Rap1- β_1 integrin signaling pathway.

The murine fibroblast cell line NIH3T3 expresses β_1 integrins (Fig. 4A). These cells also allow efficient internalization and nuclear trafficking of wild-type parvovirus B19 particles as determined by Southern blot detection of a majority of wild-type B19 genomes in the nuclear fraction of NIH3T3 cells 2 hr after infection (Fig. 4B). This is in contrast to wild-type AAV2 particles, which have previously been reported to be impaired in intracellular trafficking in NIH3T3 cells (Hansen *et al.*, 2000) and which accumulate in the cytoplasm of NIH3T3 cells with little signal detectable in the nuclei (Fig. 4B). Importantly, when NIH3T3 cells were treated with 8-pCPT-2'-O-Me-cAMP 15 min before infection with recombinant B19 vectors expressing enhanced green fluorescent protein (B19-EGFP), an increase in the number of EGFP-positive cells 24 hr postinfection was observed. These results suggest that, similar to K562 cells transfected with the constitutively active actin reorganization regulator Rac1, the adherent fibroblast cell line NIH3T3 seems to possess an actin cytoskeleton rearrangement capacity that is sufficient to allow brief pharmacological induction of GTP loading of endogenous Rap1 to increase β_1 integrin recruitment as coreceptors for B19.

Pharmacological activation of Rap1 in primary human erythroid progenitor cells enhances parvovirus B19-mediated transduction

As primary human erythroid progenitor cells constitute potential target cells for gene therapy, it was investigated whether pharmacological activation of Rap1 GTP loading could improve parvovirus B19-mediated gene transfer into these cells. As we had described previously, primary human erythroid progenitor cells internalize parvovirus B19 through β_1 integrin-mediated uptake mechanisms (Weigel-Kelley *et al.*, 2006). Using recombinant B19-EGFP vectors, low levels of transgene expression were observed in primary human erythroid progenitor cells, whereas no signal was detected in noninfected control cells (Fig. 5A, left). When the cells were

FIG. 4. Murine NIH3T3 fibroblasts are transduced by parvovirus B19 vectors and transduction efficiency can be increased by pharmacological Rap1 activation. **(A)** Expression of β_1 integrins was determined in cell extracts of $\beta_1^{-/-}$ murine fibroblasts (left lane) and NIH3T3 fibroblasts (right lane) by immunoblot. **(B)** Wild-type (wt) parvovirus B19 enters and traffics to the nucleus efficiently in NIH3T3 cells. NIH3T3 cells were incubated with wild-type AAV2 (left two lanes) and wild-type parvovirus B19 particles (right two lanes) for 2 hr at 37°C to allow viral binding and entry. Uninternalized virions were removed by extensive trypsin treatment, cells were lysed, and cytoplasmic (C) and nuclear (N) fractions were isolated. Viral genomes were detected in cytoplasmic and nuclear fractions by Southern blot hybridization with 32 P-labeled wild-type AAV2- and wild-type B19-specific DNA probes. **(C)** NIH3T3 cells were either left untreated or treated with 8-pCPT-2'-O-Me-cAMP for 15 min, and subsequently infected with recombinant parvovirus B19-EGFP vector. The percentage of EGFP-positive cells was determined by flow cytometry 24 hr postinfection.

pretreated for 15 min with 8-pCPT-2'-O-Me-cAMP, a significant increase in transduction was observed (Fig. 5A, right and Fig. 5B). Taken together, these results indicate that β_1 integrins expressed on primary human erythroid progenitor cells can be functionally recruited as coreceptors for parvovirus B19 vectors by brief pharmacological induction of GTP loading of endogenous Rap1, using the Rap1 GEF-specific activator 8-pCPT-2'-O-Me-cAMP. Interestingly, direct engagement of β_1 integrins on human erythroid progenitor cells with their ligand, immobilized fibronectin, was not beneficial, but instead resulted in a ~3.5- to 4-fold reduction in B19 transduction compared with viral exposure of cells in

suspension culture, as depicted for two different donors in Fig. 5C. However, 8-pCPT-2'-O-Me-cAMP increased B19-mediated transgene expression under both conditions.

Discussion

In previous work we had observed β_1 integrin coreceptor recruitment in nonpermissive K562 cells after treatment with the phorbol ester PMA (Weigel-Kelley *et al.*, 2003), which sparked the hypothesis underlying the current work that the small GTPase Rap1 and its downstream effectors might be involved in the functional recruitment of β_1 integrins as coreceptors for parvovirus B19.

Rap1 modulates integrin activation by affecting affinity and avidity through its effectors RAPL, RIAM, and PKD (Cantor *et al.*, 2008). Among these, PKD is a direct substrate of PKC, a kinase that is activated by PMA, and is involved in PMA-induced, integrin-mediated cell adhesion (Kucik *et al.*, 1996; Medeiros *et al.*, 2005; Stork and Dillon, 2005). RAPL leads to integrin clustering in discrete regions of the plasma membrane (Katagiri *et al.*, 2003; Cantor *et al.*, 2008), and RIAM, which builds a complex with the actin-interacting protein talin, induces high-affinity integrin conformation through direct disruption of the cytoplasmic integrin α - β tail interaction by the talin head domain (Takagi *et al.*, 2001; Vinogradova *et al.*, 2002; Kim *et al.*, 2003; Tadokoro *et al.*, 2003; Campbell and Ginsberg, 2004; Lafuente *et al.*, 2004; Tremuth *et al.*, 2004; Lee *et al.*, 2009).

To investigate a potential role of Rap1 in β_1 integrin coreceptor recruitment, the erythroleukemic cell line K562 was used as a model system because these cells are nonpermissive for B19 infection, despite the expression of globoside as primary virus-binding receptor, efficient virus binding, and expression of β_1 integrin coreceptors (Weigel-Kelley *et al.*, 2001). The current study demonstrates that (1) expression of a constitutively active mutant of the small GTPase Rap1 (63E Rap1) is sufficient to render undifferentiated, B19-resistant K562 cells permissive for B19 infection; (2) this effect is mediated through functional recruitment of β_1 integrin coreceptors, as demonstrated by the presence of high-affinity β_1 integrins on 63E Rap1-K562 cells and complete block of transduction with function-blocking β_1 integrin antibodies; (3) active actin polymerization plays a critical role in β_1 integrin coreceptor recruitment because the 63E Rap1-mediated effect is abolished by inhibition of *de novo* actin polymerization with cytochalasin D. Furthermore, brief pharmacological activation of endogenous Rap1-GTP with 8-pCPT-2'-O-Me-cAMP can induce high-affinity β_1 integrins and B19 transduction only when actin polymerization is enforced by expression of a constitutively active mutant of the actin polymerization regulator Rac1; and (4) in contrast, in primary human erythroid progenitor cells, brief 8-pCPT-2'-O-Me-cAMP treatment is sufficient to significantly increase β_1 integrin coreceptor activity for parvovirus B19 infection.

Surprisingly, firm adhesion of the erythroid progenitor cells to a fibronectin matrix did not support, but rather diminished, B19-mediated transduction. Decreased accessibility of fibronectin-bound β_1 integrins and/or reduced turnover rates of focal complex-associated β_1 integrins could be involved in this phenomenon. In any case, these results suggest that firm ligand engagement of β_1 integrins is not required for its coreceptor activity.

Taken together, the results indicate that high-affinity β_1 integrin conformation and an active actin polymerization machinery, but not necessarily β_1 integrin engagement with its immobilized ligand, are prerequisites for the functional recruitment of β_1 integrins as coreceptors for parvovirus B19. Although we did not directly determine the effect of 63E Rap1 expression on the localization of β_1 integrins within the plasma membrane in this study, an indirect indication that β_1 integrins might be in close proximity to each other on 63E Rap1-expressing cells comes from the substantial increase in coreceptor activity after antibody-mediated β_1 integrin cross-linking, an effect that was not observed in the absence of 63E Rap1.

Somewhat puzzling was the observation that 8-pCPT-2'-O-Me-cAMP could enhance β_1 integrin coreceptor activity in murine fibroblasts and human erythroid progenitor cells, but not in erythroleukemic K562 cells. It was previously sug-

gested that β_1 integrins on hematopoietic cells derived from patients with chronic myelogenous leukemia, such as the K562 cell line, are functionally uncoupled from the cytoskeleton. Activation of Rac1-dependent pathways was suggested to be involved in the cytoskeleton coupling and functional recruitment of β_1 integrins on these cells (Bhatia *et al.*, 1996). Similarly, in this study a moderate level of β_1 integrin coreceptor activity was observed after expression of an active Rac1 mutant in K562 cells, corroborating a role of Rac1-dependent actin polymerization pathways in the functional recruitment of β_1 integrins.

What could be the role of polymerizing actin in the functional recruitment of β_1 integrins as coreceptors for viral entry?

Actin could be required, for example, to efficiently localize GTP-loaded Rap1 to the plasma membrane, where it can exert its effect on β_1 integrins. In support of this hypothesis,

FIG. 5A. (See page 1675).

an elegant study by Caloca and colleagues in adherent COS cells demonstrated that separate expression of a Rap1 GEF and a Rac1 GEF had a limited effect on β_1 integrin conformation, as detected by HUTS-21 antibody binding. Coexpression of both GEFs, however, led to an increased presence of Rap1-GTP at the plasma membrane and exerted a synergistic effect on β_1 integrin affinity (Caloca *et al.*, 2004). Similarly, in the current study an additive effect on the expression of high-affinity β_1 integrins and B19-mediated transduction was observed in K562 cells upon drug-induced Rap1-GTP loading and concomitant activation of the actin organization machinery by expression of Q61L Rac1. Interestingly, expression of a constitutively active Rap1, 63E Rap1 in the current study and V12 Rap1 in the study by Caloca and colleagues, proved to be sufficient to fully elicit high-

affinity conformations in β_1 integrins (Caloca *et al.*, 2004; and the current study) and β_1 integrin coreceptor function (the current study). This suggests that the constitutively active Rap1 protein exerts additional effects, potentially involving the actin polymerization machinery, that are not sufficiently elicited by brief pharmacological Rap1 GEF activation (the current study) or expression of a Rap1 GEF (Caloca *et al.*, 2004). Indeed, 63E Rap1 has been shown to directly activate Rac1 by binding and targeting a subset of Rac1 GEFs to the plasma membrane, where localized GTP loading of Rac1 and Rac1-dependent actin reorganization occur (Arthur *et al.*, 2004). Rap1 and its effector RIAM have also been suggested to be directly involved in the regulation of actin polymerization, because cellular depletion of Rap1-GTP and RIAM resulted not only in the abolishment of Rap1-mediated cell

FIG. 5. Pharmacological induction of endogenous Rap1 GTP loading significantly increases parvovirus B19 transduction of primary human erythroid progenitor cells. (A) Human erythroid progenitor cells were either left untreated (*left*) or treated with 8-pCPT-2'-O-Me-cAMP for 15 min (*right*), and subsequently infected with recombinant parvovirus B19-EGFP vector. Fluorescence microscopy and phase-contrast micrographs were taken 36 hr postinfection. (B) Human erythroid progenitor cells were either left untreated or treated with 8-pCPT-2'-O-Me-cAMP for 15 min, and infected with recombinant parvovirus B19-EGFP vector. The percentage of EGFP-positive cells was determined 36 hr postinfection by flow cytometry. (C) Human erythroid progenitor cells from two different donors were either left in suspension cultures (-FN) or cultured on fibronectin-coated (+FN) plates overnight. Cells were either left untreated or treated with 8-pCPT-2'-O-Me-cAMP for 15 min, and infected with recombinant parvovirus B19-EGFP vector. The percentage of EGFP-positive cells was determined 36 hr postinfection by flow cytometry. Experiments were performed three times and all measurements shown represent means and SEM. *P* values are calculated by Student *t* test.

adhesion but also in a significant reduction in the cellular content of filamentous (F-), but not monomeric (G-), actin (Lafuente *et al.*, 2004; McLeod *et al.*, 2004).

Actin could also be involved downstream of β_1 integrins, promoting transport of virus-containing endocytic vesicles to the nucleus. Actin polymerization may promote the movement of newly formed endocytic vesicles through the cytoplasm by forming a comet tail. Indeed, endosomes and lysosomes from mammalian cells were demonstrated to recruit the actin nucleation machinery and assemble actin that promoted organelle movement (Taunton *et al.*, 2000) and dynamin-2, known to be involved in the intracellular trafficking of AAV2 (Duan *et al.*, 1999), was shown to regulate actin comet tail formation and comet velocity (Orth *et al.*, 2002). Interestingly, Rap1 is found in exocytic and endocytic vesicles as well as on the plasma membrane (Maridonneau-Parini and de Gunzburg, 1992; Berger *et al.*, 1994; Pizon *et al.*, 1994; Wu *et al.*, 2001) and has been suggested to be involved in intracellular vesicle transport (Bos *et al.*, 2001).

In summary, the current study documents that the cooperative actions of Rap1- and Rac1-dependent pathways lead to the efficient recruitment of β_1 integrins as coreceptors for parvovirus B19 on erythroleukemic K562 cells. Furthermore, pharmacological induction of Rap1 GTP loading by brief treatment with 8-pCPT-2'-O-Me-cAMP results in a significant increase in parvovirus B19-mediated gene transfer into human erythroid progenitor cells *in vitro*. As a similar protocol has been used by another group to increase *in vivo* homing of human endothelial progenitor cells to sites of hindlimb ischemia in a nonobese diabetic/severe combined immunodeficiency mouse model (Carmona *et al.*, 2008), it is conceivable that brief treatment with 8-pCPT-2'-O-Me-cAMP is a valuable tool not only to enhance β_1 integrin function for parvovirus B19-mediated gene transfer but also to promote integrin function for subsequent *in vivo* homing of transduced cells.

Acknowledgments

The author thanks Dr. Lawrence Quilliam (Indiana University, Indianapolis, IN) for the plasmids provided, and Linyuan Chen and Yiwen Xiang for technical assistance. K.W.-V.A. was supported by a New Investigator Research Grant from the Bankhead-Coley Florida Biomedical Research Program of the Florida Department of Health and a UF Shands Cancer Center Institutional Research Grant from the American Cancer Society.

Author Disclosure Statement

The author declares no competing financial interests.

References

- Abou-Kheir, W., Isaac, B., Yamaguchi, H., and Cox, D. (2008). Membrane targeting of WAVE2 is not sufficient for WAVE2-dependent actin polymerization: A role for IRSp53 in mediating the interaction between Rac and WAVE2. *J. Cell Sci.* 121, 379–390.
- Arai, A., Nosaka, Y., Kanda, E., Yamamoto, K., Miyasaka, N., and Miura, O. (2001). Rap1 is activated by erythropoietin or interleukin-3 and is involved in regulation of β_1 integrin-mediated hematopoietic cell adhesion. *J. Biol. Chem.* 276, 10453–10462.
- Arthur, W.T., Quilliam, L.A., and Cooper, J.A. (2004). Rap1 promotes cell spreading by localizing Rac guanine nucleotide exchange factors. *J. Cell Biol.* 167, 111–122.
- Berger, G., Quarck, R., Tenza, D., Levy-Toledano, S., De Gunzburg, J., and Cramer, E.M. (1994). Ultrastructural localization of the small GTP-binding protein Rap1 in human platelets and megakaryocytes. *Br. J. Haematol.* 88, 372–382.
- Bertoni, A., Tadokoro, S., Eto, K., Pampori, N., Parise, L.V., White, G.C., and Shattil, S.J. (2002). Relationships between Rap1b, affinity modulation of integrin $\alpha_{IIb}\beta_3$, and the actin cytoskeleton. *J. Biol. Chem.* 277, 25715–25721.
- Bhatia, R., McCarthy, J.B., and Verfaillie, C.M. (1996). Interferon- α restores normal β_1 integrin-mediated inhibition of hematopoietic progenitor proliferation by the marrow microenvironment in chronic myelogenous leukemia. *Blood* 87, 3883–3891.
- Bivona, T.G., Wiener, H.H., Ahearn, I.M., Silletti, J., Chiu, V.K., and Philips, M.R. (2004). Rap1 up-regulation and activation on plasma membrane regulates T cell adhesion. *J. Cell Biol.* 164, 461–470.
- Bos, J.L. (2005). Linking Rap to cell adhesion. *Curr. Opin. Cell Biol.* 17, 123–128.
- Bos, J.L., De Rooij, J., and Reedquist, K.A. (2001). Rap1 signaling: Adhering to new models. *Nat. Rev. Mol. Cell Biol.* 2, 369–377.
- Bos, J.L., De Bruyn, K., Enserink, J., Kuiperij, B., Rangarajan, S., Rehmann, H., Riedl, J., De Rooij, J., Van Mansfeld, F., and Zwartkruis, F. (2003). The role of Rap1 in integrin-mediated cell adhesion. *Biochem. Soc. Trans.* 31, 83–86.
- Brown, K.E., Anderson, S.M., and Young, N.S. (1993). Erythrocyte P antigen: Cellular receptor for B19 parvovirus. *Science* 262, 114–117.
- Caloca, M.J., Zugaza, J.L., Vicente-Manzanares, M., Sanchez-Madrid, F., and Bustelo, X.R. (2004). F-actin-dependent translocation of the Rap1 GDP/GTP exchange factor RasGRP2. *J. Biol. Chem.* 279, 20435–20446.
- Campbell, I.D., and Ginsberg, M.H. (2004). The talin-tail interaction places integrin activation on FERM ground. *Trends Biochem. Sci.* 29, 429–435.
- Cantor, J.M., Ginsberg, M.H., and Rose, D.M. (2008). Integrin-associated proteins as potential therapeutic targets. *Immunol. Rev.* 223, 236–251.
- Carmona, G., Chavakis, E., Koehl, U., Zeiher, A.M., and Dimmeler, S. (2008). Activation of Epac stimulates integrin-dependent homing of progenitor cells. *Blood* 111, 2640–2646.
- Caron, E. (2003). Cellular functions of the Rap1 GTP-binding protein: A pattern emerges. *J. Cell Sci.* 116, 435–440.
- Castro, A.F., Rebhun, J.F., and Quilliam, L.A. (2005). Measuring Ras-family GTP levels *in vivo*—running hot and cold. *Methods* 37, 190–196.
- Duan, D., Li, Q., Kao, A.W., Yue, Y., Pessin, J.E., and Engelhardt, J.F. (1999). Dynamin is required for recombinant adeno-associated virus type 2 infection. *J. Virol.* 73, 10371–10376.
- Dustin, M.L., Bivona, T.G., and Philips, M.R. (2004). Membranes as messengers in T cell adhesion signaling. *Nat. Immunol.* 5, 363–372.
- Enserink, J.M., Christensen, A.E., De Rooij, J., Van Triest, M., Schwede, F., Genieser, H.G., Doskeland, S.O., Blank, J.L., and Bos, J.L. (2002). A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. *Nat. Cell Biol.* 4, 901–906.
- Hacein-Bey-Abina, S., Le Deist, F., Carlier, F., Bouneaud, C., Hue, C., De Villartay, J.P., Thrasher, A.J., Wulffraat, N., Sor-

- ensen, R., Dupuis-Girod, S., Fischer, A., Davies, E.G., Kuis, W., Leiva, L., and Cavazzana-Calvo, M. (2002). Sustained correction of X-linked severe combined immunodeficiency by *ex vivo* gene therapy. *N. Engl. J. Med.* 346, 1185–1193.
- Hacein-Bey-Abina, S., Garrigue, A., Wang, G.P., Soulier, J., Lim, A., Morillon, E., Clappier, E., Caccavelli, L., Delabesse, E., Beldjord, K., Asnafi, V., Macintyre, E., Dal Cortivo, L., Radford, I., Brousse, N., Sigaux, F., Moshous, D., Hauer, J., Borkhardt, A., Belohradsky, B.H., Wintergerst, U., Velez, M.C., Leiva, L., Sorensen, R., Wulffraat, N., Blanche, S., Bushman, F.D., Fischer, A., and Cavazzana-Calvo, M. (2008). Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J. Clin. Invest.* 118, 3132–3142.
- Hansen, J., Qing, K., Kwon, H.J., Mah, C., and Srivastava, A. (2000). Impaired intracellular trafficking of adeno-associated virus type 2 vectors limits efficient transduction of murine fibroblasts. *J. Virol.* 74, 992–996.
- Harris, E.S., McIntyre, T.M., Prescott, S.M., and Zimmerman, G.A. (2000). The leukocyte integrins. *J. Biol. Chem.* 275, 23409–23412.
- Hauswirth, W.W., Aleman, T.S., Kaushal, S., Cideciyan, A.V., Schwartz, S.B., Wang, L., Conlon, T.J., Boye, S.L., Flotte, T.R., Byrne, B.J., and Jacobson, S.G. (2008). Treatment of Leber congenital amaurosis due to RPE65 mutations by ocular subretinal injection of adeno-associated virus gene vector: Short-term results of a phase I trial. *Hum. Gene Ther.* 19, 979–990.
- Joyce, D., Bouzahzah, B., Fu, M., Albanese, C., D'Amico, M., Steer, J., Klein, J.U., Lee, R.J., Segall, J.E., Westwick, J.K., Der, C.J., and Pestell, R.G. (1999). Integration of Rac-dependent regulation of cyclin D1 transcription through a nuclear factor- κ B-dependent pathway. *J. Biol. Chem.* 274, 25245–25249.
- Katagiri, K., Hattori, M., Minato, N., Irie, S., Takatsu, K., and Kinashi, T. (2000). Rap1 is a potent activation signal for leukocyte function-associated antigen 1 distinct from protein kinase C and phosphatidylinositol-3-OH kinase. *Mol. Cell. Biol.* 20, 1956–1969.
- Katagiri, K., Hattori, M., Minato, N., and Kinashi, T. (2002). Rap1 functions as a key regulator of T-cell and antigen-presenting cell interactions and modulates T-cell responses. *Mol. Cell. Biol.* 22, 1001–1015.
- Katagiri, K., Maeda, A., Shimonaka, M., and Kinashi, T. (2003). RAPL, a Rap1-binding molecule that mediates Rap1-induced adhesion through spatial regulation of LFA-1. *Nat. Immunol.* 4, 741–748.
- Kim, M., Carman, C.V., and Springer, T.A. (2003). Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins. *Science* 301, 1720–1725.
- Kucik, D.F., Dustin, M.L., Miller, J.M., and Brown, E.J. (1996). Adhesion-activating phorbol ester increases the mobility of leukocyte integrin LFA-1 in cultured lymphocytes. *J. Clin. Invest.* 97, 2139–2144.
- Lafuente, E.M., Van Puijenbroek, A.A., Krause, M., Carman, C.V., Freeman, G.J., Berezovskaya, A., Constantine, E., Springer, T.A., Gertler, F.B., and Boussiotis, V.A. (2004). RIAM, an Ena/VASP and Profilin ligand, interacts with Rap1-GTP and mediates Rap1-induced adhesion. *Dev. Cell* 7, 585–595.
- Laudanna, C., Kim, J.Y., Constantin, G., and Butcher, E. (2002). Rapid leukocyte integrin activation by chemokines. *Immunol. Rev.* 186, 37–46.
- Lee, H.S., Lim, C.J., Puzon-McLaughlin, W., Shattil, S.J., and Ginsberg, M.H. (2009). RIAM activates integrins by linking talin to ras GTPase membrane-targeting sequences. *J. Biol. Chem.* 284, 5119–5127.
- Lundell, B.I., McCarthy, J.B., Kovach, N.L., and Verfaillie, C.M. (1996). Activation-dependent $\alpha_5\beta_1$ integrin-mediated adhesion to fibronectin decreases proliferation of chronic myelogenous leukemia progenitors and K-562 cells. *Blood* 87, 2450–2458.
- Maguire, A.M., Simonelli, F., Pierce, E.A., Pugh, E.N., Jr., Mingozzi, F., Bannicelli, J., Banfi, S., Marshall, K.A., Testa, F., Surace, E.M., Rossi, S., Lyubarsky, A., Arruda, V.R., Konkle, B., Stone, E., Sun, J., Jacobs, J., Dell'osso, L., Hertle, R., Ma, J.X., Redmond, T.M., Zhu, X., Hauck, B., Zeleniaia, O., Shindler, K.S., Maguire, M.G., Wright, J.F., Volpe, N.J., McDonnell, J.W., Auricchio, A., High, K.A., and Bennett, J. (2008). Safety and efficacy of gene transfer for Leber's congenital amaurosis. *N. Engl. J. Med.* 358, 2240–2248.
- Maridonneau-Parini, I., and de Gunzburg, J. (1992). Association of rap1 and rap2 proteins with the specific granules of human neutrophils: Translocation to the plasma membrane during cell activation. *J. Biol. Chem.* 267, 6396–6402.
- McLeod, S.J., Shum, A.J., Lee, R.L., Takei, F., and Gold, M.R. (2004). The Rap GTPases regulate integrin-mediated adhesion, cell spreading, actin polymerization, and Pyk2 tyrosine phosphorylation in B lymphocytes. *J. Biol. Chem.* 279, 12009–12019.
- Medeiros, R.B., Dickey, D.M., Chung, H., Quale, A.C., Nagarajan, L.R., Billadeau, D.D., and Shimizu, Y. (2005). Protein kinase D1 and the β_1 integrin cytoplasmic domain control β_1 integrin function via regulation of Rap1 activation. *Immunity* 23, 213–226.
- Monferran, S., Muller, C., Mourey, L., Frit, P., and Salles, B. (2004). The membrane-associated form of the DNA repair protein Ku is involved in cell adhesion to fibronectin. *J. Mol. Biol.* 337, 503–511.
- M'Rabet, L., Coffey, P., Zwartkruis, F., Franke, B., Segal, A.W., Koenderman, L., and Bos, J.L. (1998). Activation of the small GTPase rap1 in human neutrophils. *Blood* 92, 2133–2140.
- Munakata, Y., Saito-Ito, T., Kumura-Ishii, K., Huang, J., Koderia, T., Ishii, T., Hirabayashi, Y., Koyanagi, Y., and Sasaki, T. (2005). Ku80 autoantigen as a cellular coreceptor for human parvovirus B19 infection. *Blood* 106, 3449–3456.
- Orth, J.D., Krueger, E.W., Cao, H., and McNiven, M.A. (2002). The large GTPase dynamin regulates actin comet formation and movement in living cells. *Proc. Natl. Acad. Sci. U.S.A.* 99, 167–172.
- Pizon, V., Desjardins, M., Bucci, C., Parton, R.G., and Zerial, M. (1994). Association of Rap1a and Rap1b proteins with late endocytic/phagocytic compartments and Rap2a with the Golgi complex. *J. Cell Sci.* 107, 1661–1670.
- Ponnazhagan, S., Weigel, K.A., Raikwar, S.P., Mukherjee, P., Yoder, M.C., and Srivastava, A. (1998). Recombinant human parvovirus B19 vectors: Erythroid cell-specific delivery and expression of transduced genes. *J. Virol.* 72, 5224–5230.
- Quilliam, L.A., Rebhun, J.F., and Castro, A.F. (2002). A growing family of guanine nucleotide exchange factors is responsible for activation of Ras-family GTPases. *Prog. Nucleic Acid Res. Mol. Biol.* 71, 391–444.
- Sanlioglu, S., Benson, P.K., Yang, J., Atkinson, E.M., Reynolds, T., and Engelhardt, J.F. (2000). Endocytosis and nuclear trafficking of adeno-associated virus type 2 are controlled by rac1 and phosphatidylinositol-3 kinase activation. *J. Virol.* 74, 9184–9196.
- Sebzda, E., Bracke, M., Tugal, T., Hogg, N., and Cantrell, D.A. (2002). Rap1A positively regulates T cells via integrin activation rather than inhibiting lymphocyte signaling. *Nat. Immunol.* 3, 251–258.
- Shimonaka, M., Katagiri, K., Nakayama, T., Fujita, N., Tsuruo, T., Yoshie, O., and Kinashi, T. (2003). Rap1 translates

- chemokine signals to integrin activation, cell polarization, and motility across vascular endothelium under flow. *J. Cell Biol.* 161, 417–427.
- Singh, A., Karnoub, A.E., Palmby, T.R., Lengyel, E., Sondek, J., and Der, C.J. (2004). Rac1b, a tumor associated, constitutively active Rac1 splice variant, promotes cellular transformation. *Oncogene* 23, 9369–9380.
- Srivastava, A. (2005). Hematopoietic stem cell transduction by recombinant adeno-associated virus vectors: Problems and solutions. *Hum. Gene Ther.* 16, 792–798.
- Stork, P.J., and Dillon, T.J. (2005). Multiple roles of Rap1 in hematopoietic cells: Complementary versus antagonistic functions. *Blood* 106, 2952–2961.
- Tadokoro, S., Shattil, S.J., Eto, K., Tai, V., Liddington, R.C., De Pereda, J.M., Ginsberg, M.H., and Calderwood, D.A. (2003). Talin binding to integrin β tails: A final common step in integrin activation. *Science* 302, 103–106.
- Takagi, J., Erickson, H.P., and Springer, T.A. (2001). C-terminal opening mimics “inside-out” activation of integrin $\alpha_5\beta_1$. *Nat. Struct. Biol.* 8, 412–416.
- Taunton, J., Rowning, B.A., Coughlin, M.L., Wu, M., Moon, R.T., Mitchison, T.J., and Larabell, C.A. (2000). Actin-dependent propulsion of endosomes and lysosomes by recruitment of N-WASP. *J. Cell Biol.* 148, 519–530.
- Tremuth, L., Kreis, S., Melchior, C., Hoebeke, J., Ronde, P., Plancon, S., Takeda, K., and Kieffer, N. (2004). A fluorescence cell biology approach to map the second integrin-binding site of talin to a 130-amino acid sequence within the rod domain. *J. Biol. Chem.* 279, 22258–22266.
- Vinogradova, O., Velyvis, A., Velyviene, A., Hu, B., Haas, T., Plow, E., and Qin, J. (2002). A structural mechanism of integrin $\alpha_{IIb}\beta_3$ “inside-out” activation as regulated by its cytoplasmic face. *Cell* 110, 587–597.
- Weigel-Kelley, K.A., Yoder, M.C., and Srivastava, A. (2001). Recombinant human parvovirus B19 vectors: Erythrocyte P antigen is necessary but not sufficient for successful transduction of human hematopoietic cells. *J. Virol.* 75, 4110–4116.
- Weigel-Kelley, K.A., Yoder, M.C., and Srivastava, A. (2003). $\alpha_5\beta_1$ integrin as a cellular coreceptor for human parvovirus B19: Requirement of functional activation of β_1 integrin for viral entry. *Blood* 102, 3927–3933.
- Weigel-Kelley, K.A., Yoder, M.C., Chen, L., and Srivastava, A. (2006). Role of integrin cross-regulation in parvovirus B19 targeting. *Hum. Gene Ther.* 17, 909–920.
- Wu, C., Lai, C.F., and Mobley, W.C. (2001). Nerve growth factor activates persistent Rap1 signaling in endosomes. *J. Neurosci.* 21, 5406–5416.

Address correspondence to:

Dr. Kirsten A.K. Weigel-Van Aken

Division of Cellular and Molecular Therapy

Departments of Pediatrics, Molecular Genetics, and Microbiology

Powell Gene Therapy Center

University of Florida College of Medicine

Cancer and Genetics Research Complex

1376 Mowry Road, Room 204

Gainesville, FL 32610-3610

E-mail: weigel@ufl.edu

Received for publication July 14, 2009;

accepted after revision August 21, 2009.

Published online: November 13, 2009.