Differential Regulation of Phosphoinositide Metabolism by \( \alpha_5\beta_3 \) and \( \alpha_5\beta_5 \) Integrins upon Smooth Muscle Cell Migration*

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Smooth muscle cell migration is a key step of atherosclerosis and angiogenesis. We demonstrate that \( \alpha_5\beta_3 \) and \( \alpha_5\beta_5 \) integrins synergistically regulate smooth muscle cell migration onto vitronectin. Using an original haptotactic cell migration assay, we measured a strong stimulation of phosphoinositide metabolism in migrating vascular smooth muscle cells. Phosphatidic acid production and phosphoinositide 3-kinase IA activation were triggered only upon \( \alpha_5\beta_3 \) engagement. Blockade of \( \alpha_5\beta_3 \) engagement or phospholipase C activity resulted in a strong inhibition of smooth muscle cell spreading on vitronectin. By contrast, blockade of \( \alpha_5\beta_5 \) reinforced elongation and polarization of cell shape. Moreover, Pyk2-associated tyrosine kinase and phosphoinositide 4-kinase activities measured in Pyk2 immunoprecipitates were stimulated upon cell migration. Blockade of either \( \alpha_5\beta_3 \) or \( \alpha_5\beta_5 \) function, as well as inhibition of phospholipase C activity, decreased both Pyk2-associated activities. We demonstrated that the Pyk2-associated phosphoinositide 4-kinase corresponded to the \( \beta \) isoform. Our data point to the metabolism of phosphoinositides as a regulatory pathway for the differential roles played by \( \alpha_5\beta_3 \) and \( \alpha_5\beta_5 \) upon cell migration and identify the Pyk2-associated phosphoinositide 4-kinase \( \beta \) as a common target for both integrins.

Integrins \( \alpha_5\beta_3 \) and \( \alpha_5\beta_5 \) have been involved in cell adhesion and migration of different cell types. Despite their ability to bind the same ligand, vitronectin, \( \alpha_5\beta_3 \) and \( \alpha_5\beta_5 \) integrins represent a good example of functional differences generated by different combinations of the integrin \( \alpha \) and \( \beta \) subunits. Indeed, previous data showing that blocking antibodies against \( \alpha_5\beta_3 \) affect both cell spreading and migration but that those directed to \( \alpha_5\beta_5 \) inhibit only cell migration argue in favor of specific events regulated by \( \alpha_5\beta_3 \) or \( \alpha_5\beta_5 \) upon cell motility (1). Little is known on the specific signal transduction pathways triggered by \( \alpha_5\beta_3 \) or \( \alpha_5\beta_5 \) engagement. However, some differences have been shown, such as a more diffuse distribution of \( \alpha_5\beta_3 \) compared with \( \alpha_5\beta_5 \), which is most frequently found in focal adhesion contacts (2, 3). Clustering of \( \alpha_5\beta_5 \) induces a weaker colocalization of actin, \( \alpha \)-actinin, and tensin than \( \alpha_5\beta_3 \) (4). Engagement of \( \alpha_5\beta_3 \) is able by itself to trigger protein kinase C or FAK1 activation and cell migration, whereas \( \alpha_5\beta_5 \) needs the concomitant activation of growth factors or cytokines receptors for this signaling pathway (4, 5).

The importance of phosphoinositides in chemotactic signaling was pointed out by the ability of receptors to chemotactic factors to directly bind and regulate the key enzymes phospholipase C (PLC) and phosphoinositide 3-kinase (PI3K) (6). Studies with mutant receptors have demonstrated that one of these two pathways might be predominant, depending on the cell status (7). The PLC activity and the PI3K activity products have common targets involved in cell migration, such as specific isoforms of protein kinase C, calcium mobilization through either internal stocks or calcium channels, and PIP 5-kinase activity allowing PIP2 formation and interaction with actin-associated proteins. Integrins have also been shown to be able to activate PLC-γ1 and PI3K IA (p85α/p110α) through FAK regulation (8–10). Moreover, integrins may regulate PIP2 production either through activation of the rac-dependent PIP 5-kinase (11) or, for some integrins, through activation of the tetraspan family-associated PI4K (12). Upon cell migration, these signaling pathways contribute to an adapted response of the cell to its environment through cytoskeleton reorganization and mobilization of membrane receptors. Depending on the nature of the matrix protein and its state (intact or hydrolyzed), integrins may regulate either formation or disorganization of focal adhesions (13, 14). PI3K was shown to regulate focal adhesion formation through modulation of integrin affinity (15) but also through disorganization of focal complexes (16). Because cell migration requires both focal adhesion formation at the front and focal adhesion disorganization at the rear, an important question to resolve is the nature of integrins involved at each step, and the specific enzymes activated.

Vascular smooth muscle cell (VSMC) migration is a key process in atherosclerosis, restenosis, and angiogenesis. Because VSMCs express both vitronectin receptors, \( \alpha_5\beta_3 \) and \( \alpha_5\beta_5 \) (17), we focused our studies on phosphoinositide-dependent signaling pathways upon VSMC migration, downstream of

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and anti-human PI4K CA). Anti-Pyk2 polyclonal antibodies (#600 for immunoprecipitation; P1F6) were purchased from Chemicon International, Inc. (Temecula, CA). Anti-p85 and anti-human PI4K α5 integrins were both found to control a PI4K activity associated with the FAK homolog Pyk2.

EXPERIMENTAL PROCEDURES

Cell Culture—FIG VSMCs were prepared from thoracic aorta of 6-week-old pigs using the explant technique (18). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum. For all experiments, VSMCs were used at passages 2–5.

Cells were fed three times weekly and passaged by treatment with 0.25% trypsin and 0.02% EDTA before confluence. Cultures were maintained in a humidified incubator with 5% CO2 at 37 °C.

Stable transfectants of HEK 293 cells expressing α5β3 or α5β1 integrins were obtained from Dr. J. W. Smith (The Barnham Institute, La Jolla, CA) and cultured in DMEM supplemented with 10% fetal calf serum and 0.25% trypsin and 0.02% EDTA before confluence. Cultures were maintained in a humidified incubator with 5% CO2 at 37 °C.

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12-well Evergreen plates (untreated; Polylabo, Strasbourg, France) were first coated for 1 min with 17 μg/ml poly-L-lysine (P-Lys) (Sigma) in PBS. Under these conditions, coating efficiency for P-Lys was determined previously to be 0.2 μg/PE. After PE drying at room temperature, nonspecific sites were blocked with PBS containing 0.5% fatty acid-free BSA (w/v) for 2 h at room temperature. Before seeding, PE filters were washed twice with PBS and once with DMEM-0.5% BSA and incubated for an additional 5 min at 37 °C in the last wash.

VSMCs grown near confluence were detached with trypsin-EDTA, washed, resuspended in DMEM containing 0.5% BSA (Albunax; Life Technologies, Inc.), and finally plated into wells containing the P-Lys-coated PE filters (300,000 cells/well) in a final volume of 1 ml. Cell adhesion was allowed for 24 h at 37 °C. Efficiency of seeding was checked by counting the remaining nonadherent VSMCs with a cell counter (Coulter) and was determined to be 80%.

(ii) Cell Migration—Bottom PE filters with adherent cells were washed twice in DMEM-0.5% BSA and transferred into new 12-well plates containing DMEM-0.5% BSA. Migration was initiated with an overlay of the middle and top PE filters coated with human vitronectin from Life Technologies, Inc. (1 h with 3 μg/ml vitronectin in PBS followed by BSA blocking). The three PE filters were maintained in tight contact with each other by applying a monolayer of glass beads (3-mm diameter) (Fig. 1). For inhibition studies, antibodies, synthetic peptides, and small molecule antagonists were preincubated at 37 °C for 1 h before their overlay at appropriate concentrations established for porcine VSMCs. In some assays, 20 μM LY-294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) from Biomol Research Laboratories (Plymouth Meeting, PA) or 50 or 100 nM wortmannin from Sigma, or 5 μM LY-294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) from Biomol Research Laboratories (Plymouth Meeting, PA) or 50 or 100 nM wortmannin from Sigma, or U-73122 from Novabiochem (France Biochem, Meudon, France) was preincubated for 1 h with VSMCs to block PI3K IA/PI4K β or PLC activities, respectively. When inhibitors were dissolved in Me 2SO, the final amount of Me 2SO did not exceed 0.6% (v/v). For phosphoinositide labeling, cells were incubated for 4 h with 0.4 mCi/ml [32P]phosphate (Amersham Pharmacia Biotech) before overlay. Migration was allowed in the 32P-containing medium for 24 h at 37 °C in a humidified cell culture incubator. Under our experimental conditions, VSMCs were not proliferative, as determined by cell cycle distribution analysis performed by flow cytometry after staining with a propidium iodide solution (data not shown).

At the end of the incubation period, all PE filters were rinsed twice in PBS, fixed in a Karnovsky solution for 5 min at 4 °C, and dried on tissue paper. Lipids were extracted following the modified procedure of Bligh and Dyer (20) on the same PE filters used for cell migration evaluation. Control experiments were performed and showed that cell fixation before lipid extraction did not modify lipid quantification, nor was the lipid extraction detrimental to the subsequent evaluation of cell migration (data not shown). The PE filters were removed from the lipid extraction tube after the one-phase solvent extraction step and dried on tissue paper. Then, PE filters were stained in crystal violet solution for 5 min. After six washes under running water, the crystal violet staining of PE filters was dissolved by adding 500 μl (w/v) 2% deoxycholate, 0.15 M NaCl, or shaking for 2 min. Absorbance was measured at 595 nm on 200 μl in 96-well plates using a HTS 7000 Bio Assay Reader (PerkinElmer Life Sciences). Cell migration was quantified by comparison of the absorbance from the middle + top PE filters (cells that migrated) to the absorbance from total cells that adhered on P-Lys (the sum of absorbances from the bottom, middle, and top PE filters). Cells that migrated on the top PE filter represented ~30% of...
total cells that migrated (middle + top PE filters). Each test group was performed in at least triplicate wells.

**Lipid Analysis**—After extraction, lipids were separated by TLC on Silica Gel G plates (Merck, Darmstadt, Germany) previously impregnated with 1% (w/v) potassium oxalate or 3% (w/v) oxalic acid in appropriate solvents: (i) to separate PtdIns-4,5-P₂ and PtdIns-4,5-P₃, the elution solvent was chloroform, acetone, methanol, acetic acid, and water (80:30:26:24:16, v/v) as described by Julles et al. (21); (ii) for PtdOH quantification, lipids were resolved by chloroform, methanol, 10 N hydrochloric acid (87:33.05:5, v/v) according to Cohen et al. (22); (iii) to separate phosphatidylinositol 3,4'-bisphosphate, lipid extracts were developed twice in chloroform, methanol, 0.1 N ammonium hydroxide, 40:15:5, v/v, followed by the procedure established by Pignataro and Ascoli (23); and (iv) to separate PtdIns-3-P and PtdIns-4-P, the procedure of Walsh et al. (24) was used, and lipids on trans-1,2-diaminocyclohexane-N,N',N''-tetraacetic acid-impregnated silica gel plates were separated by methanol, chloroform, pyridine, water, and 88% formic acid (75:60:45:7.5:3, v/v) supplemented with 12 g of boric acid. The plates were run up to the top for 3 h. In some experiments, the spot of PtdIns obtained with the elution solvent chloroform/methanol/3 N ammonium hydroxide (90:70:20, v/v) was scraped off, solubilized, and further run with the solvent described by Walsh et al. (24).

Individual lanes containing commercial standards of PtdOH, PtdIns, PtdIns-4-P, or PtdIns-4,5-P₂ were stained with iodine vapors or with zinizarin spraying. The radioactive spots were visualized and quantitated by a PhosphorImager 445 SI (Molecular Dynamics) after a 2-day exposure. In some experiments, whole lipid extracts were deacylated by methylamine treatment, separated by high pressure liquid chromatography, and quantified by on line radioactivity detection (25).

**Immunoprecipitation of p85, Pyk2, and PI4K β**—For p85 immunoprecipitates, 1.4–2 × 10⁶ VSMCs were lysed with 1.5 ml of RIPA buffer (50 mM HEPES, pH 7.4, 10 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 15 µg/ml benzamidine, 1 µg/ml pepstatin, and 1 µg/ml leupeptin). For Pyk2 immunoprecipitates, 1.4–9 × 10⁶ VSMCs were lysed in 20 mM Tris HCl, pH 7.4, 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 2 µg/ml leupeptin. For PI4K β immunoprecipitates, 500 µl of cold lysis buffer (50 mM HEPES, pH 7.5, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM dithiothreitol, 0.2% Triton X-100, 500 µM Na₃VO₄, 200 µM 4-(2-aminophenyl)benzenesulfonyl fluoride, 10 µM NaF, and 1 µM leupeptin, pepstatin, and aprotinin) was added on 1.4 × 10⁶ VSMCs. Lysates were clarified by a 10-min centrifugation at 8000 × g at 4 °C. 3 volumes of equilibration buffer (20 mM HEPES, pH 7.5, 2.5 mM MgCl₂, 0.1 mM EDTA, 1% Triton X-100, and dithiothreitol and inhibitors as indicated above) was added on PI4K β immunoprecipitates. Lysates were pre-cleared with protein A-Sepharose (Amersham Pharmacia Biotech) and then incubated overnight with anti-p85 antibodies (0.5 µg/ml), anti-Pyk2 antibodies (2.5 µg/ml), or anti-PI4K β antibodies (1 µg/ml). After capture by protein A-Sepharose beads and three washes, one-half of the immune-complexes were tested for in vitro PI kinase activity, and the other half was analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with relevant antibodies.

**In Vitro PI Kinase Assay**—p85 and Pyk2 immunoprecipitates were suspended in PI kinase activity buffer (0.5 mM EDTA, 100 mM NaCl, and 50 mM Tris-HCl, pH 7.4, plus 50 µM ATP and 10 mM MgCl₂) and incubated with PtdIns (30 µg/ml) phosphorylated serine (60 µg) vesicles and 15 µCi of [γ-³²P]ATP for 30 min at 37 °C with shaking, according to Whitman et al. (26). Protein A beads associated with PI4K β were incubated in a 50-µl final volume containing 20 mM HEPES, pH 7.5 and 0.3% Triton X-100 plus 50 µM ATP and 10 mM MgCl₂ and in the presence of 0.2 mg/ml sonicated PtdIns and 30 µCi of [γ-³²P]ATP for 20 min at 37 °C with shaking (27). Reaction products were separated by TLC, visualized by autoradiography, and quantified on a PhosphorImager (Molecular Dynamics).

**In Vitro Tyrosine Kinase Assay**—Equal amounts of lysates from migrating or nonmigrating VSMCs (1.4 × 10⁶ cells) were subjected to immunoprecipitations with antibodies against Pyk2. Immunoprecipitates were washed three times with lysis buffer and once with kinase buffer (50 mM Tris-HCl, pH 7.5, 5 mM MnCl₂, and 5 mM MgCl₂). One-half of the immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-Pyk2 antibodies, whereas the other half were incubated with 40 µl of kinase buffer supplemented with 20 µg of poly(Glu-Tyr) (4:1) and 40 µM ATP including 15 µCi of [γ-³²P]ATP for 15 min at room temperature. The reaction was stopped by the addition of SDS sample buffer, sample was boiled for 2 min, and products were resolved by 7.5% SDS-polyacrylamide gel electrophoresis.

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**RESULTS**

αβ₃ and αβ₅ Integrins Are Both Involved in VSMC Migration on Vitronectin—Expression of αβ₃ and αβ₅ integrins in cultured human or porcine VSMCs has already been demonstrated (17, 28). Interaction of VSMC αβ₃ and αβ₅ integrins with their natural ligand, vitronectin, was then demonstrated in human atheromatous plaque (29). However, the respective roles of both αβ₃ and αβ₅ receptors in VSMC migration on vitronectin have not yet been elucidated. Under our experimental conditions, serum-deprived porcine VSMCs efficiently migrated on a vitronectin-coated polystyrene. To evaluate the importance of each integrin in our migration system, we used both specific blocking antibodies and RGD analog compounds from Hoffmann-La Roche, the functional characteristics of which are shown in Fig. 2. VSMC migration was significantly detectable as early as 3 h after overlay of vitronectin-coated polystyrene.
aβ3 and aVβ5 Phosphoinositides, and Cell Migration

Table I
Characterization of aβ3 and aβ5 antagonists Ro 64 and Ro 66

| Human | Solid phase assay IC50 | Adhesion assay (HEK 293) IC50 |
|-------|------------------------|-------------------------------|
|       | aβ3 | aβ5 | aβ3 | aβ5 | aβ3 | aβ5 |
| Ro 64 | 0.1 | 0.6 | >100,000 | 84 | 9 | 5500 |
| Ro 66 | 1.3 | 55,000 | >100,000 | 1800 | 232,000 | 597,000 |

Roche products Ro 64 and Ro 66 were characterized by solid phase assays and adhesion assays as described under "Experimental Procedures." For solid phase assays, inhibition of fibrinogen or vitronectin binding to isolated human aβ3, or aβ5 integrins, respectively, was measured by enzyme-linked immunosorbent assay in the presence or absence of Ro 64 or Ro 66 compounds. For adhesion assays, stable transfectants of HEK 293 cells (expressing aβ3 or aβ5) and parental HEK 293 cells (expressing aβ3) were allowed to adhere on vitronectin or fibronectin, respectively, in DMEM-0.5% BSA and in the presence or absence of Ro 64 or Ro 66. Then, adherent HEK cells were fixed and stained with crystal violet, and absorbance was measured for cell adhesion evaluation and IC50 determination.

Table I. Whereas the Hoffmann-La Roche compound Ro 64 blocked both integrins aβ3 and aβ5, Ro 66 was specifically directed against aβ5. Anti-a3a5 activity in both solid phase and adhesion assays showed that the IC50 values for Ro 66 were 10–20 times greater than the IC50 values for Ro 64. Moreover, as shown in Fig. 3A, a maximal inhibition of VSMC migration was obtained at 10 μM Ro 64 and near 700 μM Ro 66. Thus, in VSMC migration inhibitory studies, 30 μM Ro 64 and 650 μM Ro 66 were used to be equally potent on aβ3 blockade. In VSMCs, blockade of aβ3 function by the specific antibody LM609 inhibited cell migration by 20% after 24 h (Fig. 3B). Ro 66 inhibited VSMC migration by 34% (Fig. 3B). Blockade of aβ3 by the specific antibody P1F6 also decreased VSMC migration by ~35% (Fig. 3B). When LM609 + P1F6, P1F6 + Ro 66, or Ro 64 alone was applied on VSMCs, inhibition of cell migration was more efficient than blockade of each separate integrin (Fig. 3B). The peptide GpenGRGDSPCA (penRGD), which blocked both aβ3 and aβ5 integrins, induced also a strong inhibition of VSMC migration, whereas the control peptide GRGESP (RGE) was inefficient (Fig. 3B).

These data demonstrated that both integrins aβ3 and aβ5 are involved in VSMC migration on vitronectin. The effects of the Hoffmann-La Roche antagonists did not result from aβ3 or aβ5 integrin blockade. Indeed, it has been shown that aβ3 is not involved in VSMC adhesion on vitronectin (17), and under our experimental conditions, aβ3 blockade did not affect VSMC migration (data not shown). A synergistic effect was observed on cell migration inhibition when both aβ3 and aβ5 were blocked. However, the LM609 antibody appeared to be less efficient than Ro 66 in blocking porcine aβ3 integrin and induced a much stronger synergistic inhibition in association with P1F6 when human VSMCs were used (data not shown). It has been suggested previously that porcine aβ3 integrin was poorly recognized by the LM609 antibody (28). Thus, in further experiments, we chose Ro 66 as a aβ5 integrin blocker.

Not aβ3 Engagement Regulates Phosphatidic Acid Production and PI3K IA Activity—Little is known about the variations of phosphoinositides in migrating cells. We took advantage of our cell migration system, which allows an easy separation of nonmigrating cells from migrating cells, to measure changes in 32P-labeled phosphoinositides. As shown in Fig. 4B, an increase in the labeling of PtdIns-4-P, PtdIns-4,5-P2, 3-phosphoinositides, and PtdOH was measured after 24 h in migrating VSMCs. These data strongly suggested that the key enzymes of phosphoinositide metabolism, PI4K, PI5-kinase, PI3K, and PLC, were all stimulated upon VSMC migration. Because recovery of migrating cells was not sufficient to study the kinetics of phosphoinositide-dependent signal production, we measured phosphoinositide changes in P-Lys-adherent VSMCs at early times after overlay with vitronectin-coated PE (1–3 h). Interestingly, PtdIns-4-P and PtdOH production was increased (Fig. 4A). Thus, PI4K and PLC might be specific signals correlated with the starting steps of cell migration (<3 h).

To evaluate the respective involvement of aβ3 and aβ5 integrins in the regulation of PLC and PI3K activities, we measured PtdOH production and PI3K IA (p85α/p110) activity in the presence or absence of β3 or β5 blockers. As shown in Fig. 5A, Ro 66 inhibited PtdOH production by 26%, whereas the P1F6 antibody had no inhibitory effect. An inhibition similar to that of Ro 66 was obtained with the aβ3-blocking peptide penRGD or the PLC inhibitor U-73122. Measurement of PI3K activity in p85α immunoprecipitates from nonmigrating and migrating VSMCs is shown in Fig. 5B. A strong stimulation of the p85-associated PI3K activity was observed upon cell migration. The aβ3 blocker Ro 66 inhibited lipid kinase activity by 70%. In contrast, blockade of the aβ5 integrin by P1F6 did not significantly modify PI3K activity. Incubation of cells with the PI3K inhibitor LY-294002 inhibited PI3K activity by 55%. It
appropriate solvents as described under after fixation. Phosphoinositides and PtdOH were separated by TLC in the incubation period, lipid extraction was performed on both nonmigrating cells (bottom PE filter) and migrating cells (middle and top PE filters) after fixation. Phosphoinositides and PtdOH were separated by TLC in appropriate solvents as described under “Experimental Procedures” and quantified on a PhosphorImager. A, the variations of PtdOH (.), PtdIns-4-P (△), and PtdIns-4,5-P2 (●) were measured in VSMCs adherent on the bottom PE filter at different times after overlay with vitronectin-coated PE. Values were obtained after subtraction of the control values (overlay with BSA-coated PE filter). Comparison to the starting overlay time: *, *p < 0.05; and **, **p < 0.01 (unpaired Student’s t test). B, 24 h after overlay with vitronectin-coated PE, the increase of each lipid was measured in migrating cells as compared with nonmigrating cells (control). Results are from 1.4 × 10⁶ cells and are the mean ± S.E. of three to six experiments. Comparison to control (nonmigrating cells): *, *p < 0.05; and **, **p < 0.01 (unpaired Student’s t test). In our conditions, phosphoinositide labeling in 1.4 × 10⁶ nonmigrating VSMCs before overlay was as follows: PtdOH, 3738 cpm; PtdIns-3-P, 41836 cpm; phosphatidylinositol 3-phosphate, traces; and PtdIns-4,5-P2, 2023 cpm.

should be noted that application of Ro 64 at 0.1 μM inhibited VSMC migration by 40% but did not modify PtdOH production and PI3K IA activity (data not shown). Even though 0.1 μM Ro 64 could not have sufficiently blocked α5β3 function, its IC50 is 10 times greater than that for α5β3 (Table I). Therefore, these data reinforce the lack of α5β3 involvement in PtdOH production and PI3K IA activity. Altogether, our data demonstrated that α5β3 engagement, but not α5β3 engagement, might act upstream of PLC and PI3K IA.

Evidence for a PI4K Activity Regulated by αvβ3 and αvβ5 Engagement—We then explored phosphoinositide signaling pathways downstream of αvβ3 integrin engagement upon VSMC migration. It was previously demonstrated that both αvβ3 and the FAK homolog Pyk2 are prelaminarily found outside focal adhesions (2, 30). Moreover, Pyk2 bears putative binding sites for PI3K (31). We therefore hypothesized that Pyk2 could be a good candidate to interact with a PI kinase downstream of the α5β3 integrin engagement. We assessed Pyk2 immunoprecipitates from VSMCs preincubated with αvβ3 or αvβ5 blockers. Activity assays performed on Pyk2 immunoprecipitates with poly(Glu-Tyr) or PtdIns as exogenous substrate revealed that: (i) the Pyk2-associated tyrosine kinase was increased upon VSMC migration (Fig. 6A), and (ii) a PI kinase (PI3K and/or PI4K) was associated with Pyk2 and was strongly increased upon VSMC migration (Fig. 6B). Treatment of VSMCs with αvβ3 or αvβ5 blockers decreased both Pyk2-associated tyrosine kinase and PI kinase activities in migrating VSMCs. Moreover, treatment of VSMCs with PI3K or PI4K blockers decreased the phosphorylation of the PYK2 substrate (p85) in migrating VSMCs. These data suggest that PI3K and PI4K are involved in the regulation of Pyk2 activity during VSMC migration.

FIG. 4. Changes of phosphoinositides and PtdOH upon VSMC migration on vitronectin. VSMCs adherent on bottom PE filters were labeled for 4 h with 0.4 μCi/ml [32P]phosphate before overlay. Migration was allowed to proceed for 30 min, 60 min, 3 h, or 24 h at 37°C in DMEM-0.5% BSA-0.4 μCi/ml [32P]phosphate. At the end of the incubation period, lipid extraction was performed on both migrating cells (bottom PE filter) and migrating cells (middle and top PE filters) after fixation. Phosphoinositides and PtdOH were separated by TLC in appropriate solvents as described under “Experimental Procedures” and quantified on a PhosphorImager. A, the variations of PtdOH (.), PtdIns-4-P (△), and PtdIns-4,5-P2 (●) were measured in VSMCs adherent on the bottom PE filter at different times after overlay with vitronectin-coated PE. Values were obtained after subtraction of the control values (overlay with BSA-coated PE filter). Comparison to the starting overlay time: *, *p < 0.05; and **, **p < 0.01 (unpaired Student’s t test). B, 24 h after overlay with vitronectin-coated PE, the increase of each lipid was measured in migrating cells as compared with nonmigrating cells (control). Results are from 1.4 × 10⁶ cells and are the mean ± S.E. of three to six experiments. Comparison to control (nonmigrating cells): *, *p < 0.05; and **, **p < 0.01 (unpaired Student’s t test). In our conditions, phosphoinositide labeling in 1.4 × 10⁶ nonmigrating VSMCs before overlay was as follows: PtdOH, 3738 cpm; PtdIns-3-P, 41836 cpm; phosphatidylinositol 3-phosphate, traces; and PtdIns-4,5-P2, 2023 cpm.

FIG. 5. α5β3 integrin but not α5β3 integrin engagement regulates PtdOH production and PI3K IA activity. VSMCs adherent on P-Lys-coated PE filters were labeled (A) or were not (B) labeled for 4 h with 0.4 μCi/ml [32P]phosphate and allowed to migrate on vitronectin-coated PE filters for 24 h in DMEM-0.5% BSA. In some assays, before starting migration, VSMCs were preincubated for 1 h at 37°C with integrin blockers (Ro 66, 650 μM as anti-α5β3; P1F6, 10 μg/ml as anti-α5β3; penRGD, 50 μg/ml as anti-αvβ3 integrins), the PLC inhibitor U-73122 (2 μM), or the PI3K inhibitor LY-294002 (20 μM). When Ro 66 was used, control was performed in presence of the vehicle Me2SO. A, at the end of the incubation period, lipid extraction was performed on both nonmigrating cells (NM, bottom filter) and migrating cells (M, middle-top filters; Ro 66, P1F6, penRGD, and LY-294002) or nonmigrating (NM, bottom filter) cells were lysed in RIPA buffer and then incubated with an anti-p85 antibody for immunoprecipitation as described under “Experimental Procedures.” An in vitro PI3K assay was performed on p85 immunoprecipitates. The reaction product PtdIns-3-P was separated by TLC and quantified on a PhosphorImager. Results are from 1.4 × 10⁶ VSMCs and are expressed as the amount of PtdIns-3-P/PtdOH produced in each assay compared with the amount of PtdIns-3-P/PtdOH produced by cells that migrated in the control (M). Results shown are the mean ± S.E. of three experiments. Comparison to control migrating cells: *, *p < 0.05; and **, **p < 0.001 (unpaired Student’s t test). The cell migration percentages (mean ± S.E. of three experiments) and the Western blots of immunoprecipitated p85 showing comparable amounts between assays are displayed at the bottom of the figure.
Fig. 6. Both α,β₁ and α,β₂ integrins regulate Pyk2-associated tyrosine kinase and PI kinase activities. VSMCs adherent on P-Lys-coated PE filters were allowed to migrate on vitronectin-coated PE filters for 24 h in DMEM-0.5% BSA. In some assays, before starting migration, VSMCs were incubated with integrin blockers (Ro 66, 650 μM as an anti-α,β₁; P1F6, 10 μg/ml as an anti-α,β₂), with a PI3K inhibitor (LY-294002, 20 μM) or a PLC inhibitor (U-73122, 2 μM) for 1 h at 37 °C. When LY-294002, U-73122, or Ro 66 was used, control was performed in the presence of the vehicle MeSO₄. Nonmigrating and migrating control cells (NM and M) and migrating cells from each assay were lysed and incubated with an anti-Pyk2 antibody for immunoprecipitation as described under “Experimental Procedures.” A, an in vitro kinase assay with poly(Glu-Tyr) as exogenous substrate was performed on Pyk2 immunoprecipitates from 1.4 x 10⁶ VSMCs as described under “Experimental Procedures.” Results shown are from one of two representative experiments with similar results. Western blots of immunoprecipitated Pyk2 are displayed below. B, an in vitro PI kinase assay was performed on Pyk2 immunoprecipitates from 1.4 x 10⁶ VSMCs. Reaction products were separated by TLC and quantified on a PhosphorImager. Results are expressed as the amount of PtdInsP produced in each assay compared with the amount of PtdInsP produced by cells that migrated in the control assay. Each bar represents the mean ± S.E. of at least three experiments. Comparison to control migrating cells: *p < 0.05 (unpaired Student’s t test). The cell migration percentages (mean ± S.E. of at least three experiments) are shown at the bottom of the figure.

VSMCs (Fig. 6, A and B). Furthermore, pretreatment of intact cells with LY-294002 or the PLC inhibitor U-73122 induced an inhibition of the Pyk2-associated tyrosine and PI kinases (Fig. 6, A and B). These data demonstrate that a Pyk2-dependent signaling pathway is activated upon VSMC migration.

We further attempted to characterize this Pyk2-associated PI kinase from nonmigrating VSMCs (in culture). As shown in Fig. 7A, wortmannin (50 nM; added in the kinase assay) induced a 40% reduction of Pyk2-associated PI kinase activity, whereas the immunoprecipitated PI3K IA activity was reduced by 67%. In the same assays, LY-294002 (20 μM) decreased the Pyk2-associated PI kinase activity by 28 ± 6% (mean ± S.E.; n = 5) and the p85-associated PI3K activity by 60 ± 6% (mean ± S.E.; n = 4) (data not shown). Furthermore, the Pyk2-associated PI kinase was able to phosphorylate PtdIns in the presence of either Ca²⁺ or Mg²⁺ (Fig. 7A), but the activity was decreased by 54% when Ca²⁺ was used instead of Mg²⁺. By contrast, PI3K IA was not active in the presence of Ca²⁺ (Fig. 7A). Thus, a PI kinase different from PI3K IA but still sensitive to wortmannin and LY-294002 might be isolated in Pyk2 immunoprecipitates.

As shown in Fig. 7B (left panel), using a TLC solvent allowing resolution of PtdIns-3-P and PtdIns-4-P, we detected the presence of both PI3K and PI4K activities associated with Pyk2. Left panel, spots of PtdInsP produced by Pyk2- or p85-associated kinase activity as described above (A) were scraped, re-extracted, and separated by TLC to separate PtdIns-3-P from PtdIns-4-P as described under “Experimental Procedures” and quantified on a PhosphorImager. Right panel, VSMCs adherent on P-Lys-coated PE filters were allowed to migrate on vitronectin-coated PE filters for 24 h in DMEM-0.5% BSA in the presence or absence of the anti-α,β₁ antibody P1F6. Lysate of migrating VSMCs (1.4 x 10⁶ cells) were immunoprecipitated with anti-Pyk2, and an in vitro PI kinase assay was performed as described in A in the presence or absence of wortmannin (50 nM). As a control, in the same experiment, we have performed a PI3K assay in immunoprecipitates from migrating cells (3 x 10⁸ VSMCs), as described in A. Reaction products were separated by TLC and quantified on a PhosphorImager. This figure is representative of two independent experiments with similar results.
ated PI4K activity (data not shown). Altogether, these results showed that Pyk2-associated PI4K activity was regulated by both αβ3 and αβ3 integrins.

The PI4K β Isoform Is Associated with Pyk2 and Activated upon β3/β3 Integrin Engagement—Wortmannin/LY-294002 sensitivity and sustained activity in the presence of calcium are two features of the PI4K type III family (32). Two distinct forms of type III PI4K, a 110-kDa β-form and a 230-kDa α-form, have been identified in mammalian cells (33). The yeast homolog of PI4K β, Pik1, and Pyk2 have both been involved in vesicular trafficking (34, 35). We thus hypothesized that PI4K β might be the PI4K isoform associated with Pyk2 in VSMCs. We performed kinase assays of PI4K β or Pyk2 immunoprecipitates followed by re-immunoprecipitation with antibodies to Pyk2 or PI4K β, respectively. As shown in Fig. 8A, phosphoproteins of 105 and 110 kDa were detected in Pyk2 and PI4K β re-immunoprecipitates, respectively, whereas no immunoprecipitation of phosphoproteins was observed with nonimmune control. These data demonstrated that PI4K β and Pyk2 can be isolated as complexes (Fig. 8A).

To check whether PI4K β was the Pyk2-associated PI kinase regulated by αβ3 and αβ3 integrins, immunoprecipitates of PI4K β followed by an in vitro lipid kinase assay were realized on VSMCs that migrated on vitronectin. An increase of PI4K β activity was measured in migrating VSMCs compared with nonmigrating VSMCs. Accordingly, when αβ3 or αβ3 inhibitor was used, its activity in migrating cells was inhibited by 70% and 55%, respectively (Fig. 8B).

αβ3 and αβ3 Integrins Are Differentially Involved in VSMC Spreading—Differences in the signaling pathways of αβ3 and αβ3 integrins strongly suggested that these two receptors were differentially involved upon VSMC migration. We have thus explored their role in cell spreading, a key step of cell migration. Treatment of vitronectin-adherent VSMCs with the αβ3 antagonist Ro 66 induced a complete inhibition of cell spreading (Fig. 9). By contrast, the αβ3 blocking antibody, P1F6, reinforced VSMC elongation and polarization (Fig. 9). We then applied LY-294002 or U-73122 on cells just before adhesion. Whereas LY-294002 did not significantly change VSMC spreading, U-73122 (a PLC inhibitor) treatment resulted in a total inhibition of spreading (data not shown). These results are in favor of the regulation of VSMC spreading by a αβ3-dependent PLC activity. However, pretreatment of VSMCs with U-73122 or LY-294002 induced an inhibition of the migration (Figs. 5 and 6), suggesting that LY-294002-sensitive PI kinases activities (PI3K IA and PI4K β) are required for VSMC migration.

DISCUSSION

The aim of this study was to explore phosphoinositide-dependent signaling pathways regulated by αβ3 and αβ3 integrins in VSMC migration onto vitronectin. We set up an original migration system allowing comparative measurement of phosphoinositides between nonmigrating and migrating cells. Moreover, we characterized new RGD analogs able to block αβ3 or αβ3 and αβ3 functions. Here we show that αβ3 and αβ3 integrins are both involved in VSMC migration on vitronectin in vitro. This work is the first demonstration that endogenous vitronectin receptors transduce distinct phosphoinositide-dependent signals upon cell migration. Indeed, αβ3 integrin regulates PtdOH production and PI3K IA activity in migrating VSMCs and promotes cell spreading, whereas αβ3 is not involved in this signal transduction pathway and regulates other steps of cytoskeleton reorganization. Moreover, both αβ3 and αβ3 integrins regulate Pyk2-associated tyrosine kinase and PI4K β activities. PLC and PI3K are major signaling enzymes involved in cell migration. PLCγ1 and PI3K IA isoforms are activated down-
stream of integrin engagement through their interaction with the focal adhesion protein, FAK (8–10). Addition of α5β3 or α3β5 inhibitors revealed that only α3β5 integrin is implicated in the regulation of PtdOH production and PI3K IA activity. Moreover, α5β3 integrin-vitronectin interaction is clearly required for VSMC spreading and seems to primarily involve PLC activation. Indeed, under our experimental conditions, PI3K inhibitors did not significantly modify VSMC spreading. The positive regulation of cell spreading by PLC activation can be explained by the regulation ofPIP2/profilin association, formation of actin nucleation sites by diacylglycerols, and protein kinase C activation (6). Interestingly, our data suggested that PLC activation could be an early signal of VSMC migration.

Depending on the cellular model, α5β5 was shown to be inside or outside focal adhesions or both. In vitronectin-adherent VSMCs, we found α5β5 distributed mainly in diffuse spots in the cytoplasm, and α5β3 in focal adhesion (data not shown). It has recently been demonstrated that integrins can transduce signals even when they are outside focal adhesion (12, 36). VSMCs express both endogenous FAK and its homolog, Pyk2, and Pyk2 was found localized outside the focal adhesions (30, 37). In contrast with FAK, but similar to α5β5, Pyk2 associates poorly with talin (30). Previous studies have demonstrated β1, β5, or β3 integrin-dependent activation of Pyk2 (38–40). Pyk2-associated tyrosine kinase activity is a good reflection of Pyk2 activation (41), and we found that it was up-regulated upon VSMC migration and by α5β5 engagement. This result was in favor of activation of Pyk2-dependent signaling pathways upon VSMC migration. Indeed, a Pyk2-associated PI4K activity was regulated by α5β5 engagement upon VSMC migration. PI4K was characterized by its wortmannin/LY-294002 sensitivity and its sustained activity in the presence of calcium, two features of the type III PI4K (32). Thus, it is unlikely that this PI4K is the type II PI4K described in association with the tetratranspan family and α5β3 integrin (12). In our assays, the Pyk2-associated tyrosine and PI4K activities were also regulated by α5β5 engagement. Previous reports have demonstrated that Pyk2 was regulated by intracellular calcium, protein kinase C, and PI3K activity (31, 42). We measured a strong inhibition of the Pyk2-associated PI4K activity after the addition of LY-294002 or U-73122. This suggests that PLC activity downstream of α5β3 engagement might be involved in the regulation of the Pyk2-associated PI4K activity. The effect of LY-294002 may be due to the direct inhibition of the Pyk2-associated PI4K and/or indirect inhibition of the α5β3-dependant PI3K IA activity.

We identified the Pyk2-associated PI4K as the PI4K β isoform. As Pyk2, PI4K β is mostly cytosolic, with a significant fraction associated with the Golgi (43). PI4K β bears a proline-rich domain at the N terminus that might promote the interaction of this enzyme with SH3 domains (44). However, Pyk2 does not contain SH2 or SH3 domains, and association of PI4K with Pyk2 might be indirect. The modes of regulation of PI4Ks remain elusive, but recent data demonstrated that a type II phosphatidylinositol 4-kinase was activated through tyrosine phosphorylation by pp60c-src (45). We have shown that PI4K β could be tyrosine-phosphorylated in a kinase assay performed on Pyk2 immunoprecipitates (data not shown). This tyrosine phosphorylation might be the result of the action of Pyk2 or pp60c-src. Indeed, pp60c-src has been shown to interact with Pyk2 through its SH2 domain (46). pp60c-src has an SH3 domain and might mediate the interaction between Pyk2 and PI4K β. Interestingly, it has been shown that PI4K β is recruited by the small GTPase ADP-ribosylation factor, ARF, on the Golgi complex (47). On the other hand, Pyk2 has been involved in the regulation of vesicular transport through its interaction with Rap, a GTPase-activating protein, acting on the small GTPase family ARF (35). It remains to be determined whether PI4K β is present in a complex with Pyk2, pp60c-src, Rap, and ARF. PI4K β phosphorylates only PtdIns to form PtdIns-4-P (43). The synthesized PtdIns-4-P might be a precur- sor for PtdIns-4,5-P_2 or phosphatidylinositol 3',4'-bisphosphate to promote membrane budding or allow recruitment of other factors necessary for membrane trafficking.

We have found both PI4K and PI3K activities associated with Pyk2 from cultured VSMCs. However, only the PI4K activity was detected in Pyk2 immunoprecipitates from migrating VSMCs. The PI3K IA isoform has been shown to associate with Pyk2 in angiostatin II-stimulated VSMCs (48), in thrombin-stimulated platelets (49), and in macrophage colony-stimulating factor-stimulated macrophages (50). Under our conditions, the lack of PI3K activity in Pyk2 immunoprecipitates might be due to either a dissociation of this enzyme from the Pyk2 complex upon cell migration or to a PI3K activity below the level of detection. Thus, we cannot definitely rule out the association of Pyk2 and a PI3K upon VSMC migration. Whereas α5β3 has already been involved in vivo and in vitro in VSMC migration, recent reports based on animal models of neointima formation (51) and expression of vitronectin receptors in smooth muscle cells from human atheromatous plaque (29) have also suggested the potential role of α5β3 integrin in VSMC migration. Our data clearly demonstrate that α5β3 plays a major role in porcine VSMC migration on vitronectin in vitro, as well as in human VSMCs (data not shown). Moreover, both α5 integrins synergistically regulate this VSMC migration. Our data underline the differential involvement of both integrins in VSMC spreading, as has been shown previously for carcinoma cell spreading (52). Clearly, α5β3 promoted VSMC spreading, whereas α5β3 regulated VSMC elongation and polarization. The latter effect may be the result of cytoskeleton reorganization and/or a redistribution of cell adhesion receptors. Thus, we speculate that upon VSMC migration onto vitronectin, cell spreading is a step regulated by α5β3 engagement through phospholipase C activation, which subsequently triggers phosphoinositide 3-kinase IA activity and Pyk2 activity. Conversely, α5β3 engagement seems to be involved in dy-namic changes of cytoskeleton and cell polarization, an effect likely to be due in part to its influence on the Pyk2-dependent signaling pathway. It would be important to define to what extent Pyk2/PI4K β complex is involved in these processes.

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Differential Regulation of Phosphoinositide Metabolism by $\alpha\beta_3$ and $\alpha\beta_5$ Integrins upon Smooth Muscle Cell Migration
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