Restoration of β₁A Integrins is Required for Lysophosphatidic Acid-induced Migration of β₁-null Mouse Fibroblastic Cells

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Cells lacking the β₁ integrin subunit or expressing β₁A with certain cytoplasmic mutations have poor directed cell migration to platelet-derived growth factor or epidermal growth factor, ligands of receptor tyrosine kinases (Sakai, T., Zhang, Q., Fässler, R., and Mosher, D. F. (1998) J. Cell Biol. 141, 527–538). We investigated the effect of expression of β₁A integrins on lysophosphatidic acid (LPA)-induced migration of fibroblastic cells derived from β₁-null mouse embryonic stem cells. These cells expressed edg-2, a G-protein-linked receptor for LPA, as well as the related edg-1 receptor. Cells expressing wild type β₁A demonstrated enhanced cell migration across filters coated with gelatin or adhesive proteins in response to LPA, whereas β₁-deficient cells lacked LPA-induced cell migratory ability. Checkerboard analyses indicated that LPA causes both chemotaxis and chemokinesis of β₁-replete cells. Cells expressing β₁A with mutations of prolines or tyrosines in conserved cytoplasmic NPXY motifs, threonine in the inter-motif sequence, or a critical aspartic acid in the extracellular domain had low migratory responses to LPA. These findings indicate that active β₁A integrin is required for cell migration induced by LPA and that the cytoplasmic domain of ligated β₁A interacts with pathways that are common to both receptor tyrosine kinase and G-protein-linked receptor signaling.

Directed cell migration in a concentration gradient (chemotaxis) is important for a large number of physiological and pathological processes, including development, immunity, wound healing, and cancer metastasis (1–4). Chemotaxis involves the sensing of the gradient of chemoattractant, reorganization of the actin cytoskeleton, and subsequent movement toward the chemoattractant. Cell movement requires the fine control of cellular association with and release from the extracellular matrix (5, 6). Integrins are transmembrane heterodimeric cell adhesion receptors that mediate organization of focal contacts, actin-containing cytoskeleton, and extracellular matrix and may also contribute to cell migration by participating in signal transduction cascades (7–14). It has been suggested that integrin function involves interaction with adhesive ligands (“outside-in” signaling) and cellular control of binding avidity (“inside-out” signaling) (9, 15, 16). In addition, cell migration may be regulated in part by the cycling of integrins between cytoplasmic compartments and the cell surface (17, 18).

Lysophosphatidic acid (LPA)₁ is a product of activated platelets and cells and has diverse actions on cells (19). LPA is the serum enhancement factor of fibronectin matrix assembly; enhancement of assembly closely correlates with LPA-induced actin stress fiber formation and cell contraction (20–22). LPA is a mitogen for a number of cells, including endothelial cells (23, 24). LPA induces in vitro invasion across host cell monolayers by several types of tumor cells (25, 26). LPA also stimulates random, nondirectional migration (chemokinesis) of Rat1 fibroblasts (27). Recently, the specific LPA receptor, ventricular zone gene-1 (vzg-1) (28), also known as endothelial differentiation gene-2 (edg-2) (29), has been identified in mouse and human. Related proteins, Edg-1 (30, 31) and Edg-3 (32, 33), have been identified as receptors for sphingosine 1-phosphate. LPA signaling is mediated by the Ras-ERK pathway via Ras activation and through the small GTP-binding protein Rho, leading to activation of mitogen-activated protein kinase (19, 29, 34). Expression of a dominant negative Ras mutant inhibits migration of NIH(M17) cells in response to LPA as well as to other chemotactants such as platelet-derived growth factor (PDGF) (35).

We recently expressed a set of β₁A integrin subunits with point mutations of the cytoplasmic domain in fibroblasts derived from β₁-null stem cells and showed that cells lacking the β₁ integrin subunit or expressing β₁A with certain cytoplasmic mutations had impaired ability to migrate toward PDGF or epidermal growth factor (EGF), ligands of receptor tyrosine kinases (36). This system allows the effects of wild type and mutated β₁ on cell migration to be analyzed without the confounding presence of endogenous β₁ subunits. In the present study, we demonstrate that restoration of β₁ integrins is stringently required for LPA-induced cell migration of β₁-null fibroblasts.

EXPERIMENTAL PROCEDURES

Cells—The GD25 and GD10 fibroblast lines were established after differentiation from β₁-null stem cells and immortalization with SV40 large T antigen (37, 38). β₁A with mutations of the cytoplasmic domain were constructed from pBSβ₁A encoding full-length mouse β₁A integrin subunit and expressed in GD25 cells as described (36). A point mutation in the extracellular domain of β₁A (D130A) was introduced as follows. cDNA for β₁A was excised with XbaI and AccI6I from pBSβ₁A (38) and cloned into pGEM-7ZI. The mutant was generated by oligonucleotide-primed DNA synthesis using a mutagensis kit (Pharmacia Biotech, Uppsala, Sweden). The region spanning the HindIII and ClaI sites was

₁ The abbreviations used are: LPA, lysophosphatidic acid; edg, endothelial differentiation gene; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; vzg-1, ventricular zone gene-1; BSA, bovine serum albumin; RT-PCR, reverse transcription-polymerase chain reaction.
analyzed by DNA sequence analysis, and the correctly mutagenized HindIII-ClaI fragment was isolated and ligated into HindIII-ClaI-digested pGEM-7Zf containing the original XhoI-BgII fragment of βA. The XhoI-ClaI fragment was then excised and ligated into XhoI-ClaI-digested pBSβA, yielding cDNA encoding full-length βA polypeptide containing the point mutation. The plasmid was linearized with XhoI and transfected into GD25 cells by electroporation. Clones stably expressing mutant βA were obtained with the selection by puromycin and analyzed for expression of βA by flow cytometry (36). The population of higher expressing cells in several clones was selected by fluorescence-detected cell sorting and expanded. By flow cytometry, the cells selected for flow sorting expressed mutant cell-surface βA at comparable levels with cells expressing wild type βA.

Wild type βA was expressed in GD10 cells by transfecting with pBSβA using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions.

Cell Migration—Cell migration assays were performed in modified Boyden chambers containing Nucleopore polycarbonate membranes (8-μm pore size; Costar Corp., Cambridge, MA) as described previously (36). The filters were soaked overnight in a 10 μg/ml solution of vitronectin, fibronectin, or laminin-1 or 100 μg/ml gelatin, briefly rinsed with phosphate-buffered saline containing 0.2% (w/v) bovine serum albumin (BSA), air-dried, and then placed in the chamber. PDGF from porcine platelets (R&D systems, Minneapolis, MN) or 1-oleoyl-LPA (Avanti Polar Lipids, Birmingham, AL) in Dulbecco’s modified Eagle medium containing 0.2% fatty acid-free BSA was added to the lower (and/or upper) compartment of the chambers. Cells suspended in Dulbecco’s modified Eagle medium containing 0.2% fatty acid-free BSA were introduced into the upper compartment. The chambers were then incubated for 6 h at 37 °C. The filters were fixed and stained, and the cells that had migrated to the lower surface were counted at ×40 magnification. In each experiment, two areas from each of two wells were counted. Values are the mean ± S.D. of cells per 0.16-mm² field.

RNA Isolation and RT-PCR—Total RNA was isolated using total RNA isolation system (Promega Corp., Madison, WI). RT-PCR was performed on Perkin-Elmer 480 thermal cycler (Perkin-Elmer Corp.) using the Access RT-PCR system (Promega Corp.) as described elsewhere (36). Controls for RT-PCR were studied by omitting the avian myeloblastosis virus reverse transcriptase.

Immunoprecipitation and Immunoblotting—Immunoprecipitation analysis was performed as described previously elsewhere (39), with a slight modification. Briefly, cells grown to 80–90% confluence were starved in medium without serum for 16 h and then stimulated with slight modification. Briefly, cells grown to 80–90% confluence were selected for study expressing mutant cell-surface βA, and analyzed for expression of βA by flow cytometry (36). The population of higher expressing cells in several clones was selected by fluorescence-detected cell sorting and expanded. By flow cytometry, the cells selected for flow sorting expressed mutant cell-surface βA at comparable levels with cells expressing wild type βA.

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RESULTS

Expression of vgz-1/edg-2 and edg-1—GD25 and GD10 cells were differentiated from βA-null embryonic stem cells and are of uncertain lineage. We used RT-PCR to check the expression of vgz-1/edg-2 and edg-1, which have been identified as receptors for LPA and sphingosine 1-phosphate, respectively (28–31). GD25 and GD10 cells lacking βA, and GD25 and GD10 cells expressing wild type βA, were found to express vgz-1/edg-2, a LPA-specific receptor (Fig. 1). The related edg-1 receptor was also expressed by these cells (Fig. 1).

Migration through Gelatin-coated Filters—The effects of βA integrin on LPA-induced cell migration through gelatin-coated filters were analyzed. GD25 cells lacking βA migrated very little when LPA (500 nm) was added to the lower compartment (Fig. 2A and Table I). GD25 cells expressing wild type βA migrated 10-fold more than nontransfected GD25 cells in response to LPA. GD25 cells and GD10 cells expressing βA migrated equally well in response to PDGF (Table I). GD10 cells and GD10 cells expressing wild type βA behaved similarly to the GD25 counterparts (Table I). Thus, βA-null fibroblasts demonstrate a profound defect in migration through gelatin-coated filters in response to LPA that is not because of an intrinsic inability to migrate through the gelatin-coated pores.

Cells expressing mutant βA were tested for an ability to migrate in response to LPA. The mutations in the cytoplasmic domain fell into two groups, active (D759A; Y795F; Y783,785F) and inactive (T788P; P781,793A), based upon the reactivity of expressing cells with anti-βA (9EG7) antibody, antibodies dependent on amino acid residues that are involved in interaction with internal proteins, and reactivity with the 9EG7 antibody, antibodies dependent on amino acid residues that are involved in interaction with internal proteins, and reactivity with the 9EG7 antibody, antibodies dependent on amino acid residues that are involved in interaction with internal proteins, and reactivity with the 9EG7 antibody, antibodies dependent on amino acid residues that are involved in interaction with internal proteins.

Migration through Filters Coated with Adhesive Proteins—Expression of βA in GD25 cells enhances haptotactic migration through filters coated with vitronectin, fibronectin, or laminin-1 or 100 μg/ml solution of vitronectin, fibronectin, or laminin-1 or 100 μg/ml gelatin, briefly rinsed with phosphate-buffered saline containing 0.2% (w/v) bovine serum albumin (BSA), air-dried, and then placed in the chamber. PDGF from porcine platelets (R&D systems, Minneapolis, MN) or 1-oleoyl-LPA (Avanti Polar Lipids, Birmingham, AL) in Dulbecco’s modified Eagle medium containing 0.2% fatty acid-free BSA was added to the lower (and/or upper) compartment of the chambers. Cells suspended in Dulbecco’s modified Eagle medium containing 0.2% fatty acid-free BSA were introduced into the upper compartment. The chambers were then incubated for 6 h at 37 °C. The filters were fixed and stained, and the cells that had migrated to the lower surface were counted at ×40 magnification. In each experiment, two areas from each of two wells were counted. Values are the mean ± S.D. of cells per 0.16-mm² field.

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Immunoprecipitation and Immunoblotting—Immunoprecipitation analysis was performed as described previously elsewhere (39), with a slight modification. Briefly, cells grown to 80–90% confluence were starved in medium without serum for 16 h and then stimulated with agonists for 5 min or left unstimulated. The cells were then lysed on ice in buffer containing 1% (v/v) Triton X-100, 150 mM NaCl, 5 mM EDTA, 100 mM sodium fluoride, 1 mM sodium orthovanadate, 0.5 mM sodium molybdate, 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 0.1 μg/ml pepstatin A, 0.4 μg/ml pefabloc SC, and 20 mM Tris-HCl, pH 7.4 and centrifuged for 15 min at 12,000 × g. The samples were then subjected to SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes.

For immunoblotting, the blots were probed with the primary antibody, then with a horseradish peroxidase-conjugated secondary antibody (Organon Teknika Corp., Westchester, PA). Immunoreactive bands were developed using the enhanced chemiluminescence (ECL) substrate system (NEN Life Science Products). Rabbit polyclonal antibodies to recognized mouse EGF and PDGF receptor were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody against phosphotyrosine (mAb 4G10) was from Upstate Biotechnology Inc. (Lake Placid, NY). Human recombinant EGF was from Upstate Biotechnology Inc.
vitronectin) or 6-fold (on fibronectin) more than cells lacking wild type
response to LPA of tions (b). LPA-induced migration through laminin-1-coated filters (compare Table I and Fig. 3B). LPA in the concentrations of 50, 150, 500, and 1,500 nM was added in the lower chamber, and the dose dependence was analyzed. Each symbol represents the mean of cell number per 0.16-mm² field. Error bars indicate ± S.D. of quadruplicate determinations.

**TABLE I**

| Chemotactant | β1A-Null | β1A-transfected |
|--------------|----------|-----------------|
|              | GD25     | GD10            |
| None         | <10      | <10             |
| LPA (500 nM) | 17 ± 2   | 39 ± 5          |
| PDGF (30 ng/ml)| 79 ± 18 | 85 ± 9          |

**Fig. 2.** Cell migration through gelatin-coated filters in response to LPA of β1A-deficient GD25 cells and GD25 cells expressing wild type β1A (A) and GD25 cells expressing β1A with mutations (B). LPA (500 nM) was in the lower chamber. Bars represent the means of cell number/0.16-mm² field. Error bars indicate ± S.D. of quadruplicate determinations. GD25, β1A-deficient cells; β1A, GD25, GD25 cells expressing wild type β1A; other cells are designated by mutation(s). In the absence of LPA, less than 10 cells/0.16-mm² moved across filters coated with gelatin.

**Fig. 3.** Effect of LPA or PDGF on cell migration through gelatin-coated filters

Cells and chemotactic agents were added to the upper and lower compartments, respectively. Numbers represent the mean ± S.D. of at least four determinations.

| Chemotactant | β1A-Null | β1A-transfected |
|--------------|----------|-----------------|
|              | GD25     | GD10            | |
| None         | <10      | <10             |
| LPA (500 nM) | 17 ± 2   | 39 ± 5          |
| PDGF (30 ng/ml)| 79 ± 18 | 85 ± 9          |

**Checkerboard Analysis**—Previous studies suggested that LPA causes random, nondirectional migration (chemokinesis) rather than chemotaxis of Rat1 fibroblasts (27). Checkerboard analysis in which different concentrations of chemoattractant were added to the upper and lower chamber of the apparatus was therefore carried out to characterize the effect of LPA on cell migration by derivatives of GD25 cells (Fig. 4). Such an analysis differentiates directed migration across the filter in response to the gradient of chemoattractant (chemotaxis) from increased random motility because of the presence of the chemotactant per se (chemokinesis). Cells expressing wild type β1A, the D759A mutant, or the Y783F mutant displayed directional migration, whereas Y783F,795F cells and nontransfected GD25 cells was too low to classify responses as chemotaxis or chemokinesis.

**Relation to EGF and PDGF Signaling**—Signaling pathways initiated by high concentration of LPA (10–25 μM) are known to “cross-talk” with EGF signaling pathways to cause phosphorylation of the EGF receptors (41, 42). Because GD25 cells are also deficient in migration in response to EGF or PDGF (36), we checked the presence of EGF and PDGF receptors in GD25 cells...
and GD25 cells expressing β1A. Lysates of both cells contained comparable amounts of both receptors when analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting (not shown). As assessed by anti-phosphotyrosine immunoblotting of immunoprecipitated receptors, both receptors in both cells were activated appropriately by concentrations of 3 ng/ml EGF or PDGF but not by 500 nM LPA that caused migration (not shown).

**DISCUSSION**

We previously found that β1A with an intact cytoplasmic tail, including intact NPXY motifs, is important for optimal chemotaxis of GD25 fibroblasts through filters coated with vitronectin, fibronectin, or laminin-1 in response to PDGF or EGF (36). The purposes of the present studies were to learn if GD25 cells or its derivatives migrate in response to LPA and then to compare and contrast LPA-induced migration to that induced by PDGF or EGF. The fact that the extracellular D130A mutation ablated ability of β1A to support migration through filters coated with fibronectin or vitronectin indicates that β1 integrins must be able to interact with extracellular ligands. However, which integrins are responsible for migration on the various coated filters is open to question, with the exception of laminin-1, to which adhesion of β1A-expressing GD25 cells is blocked by anti-α6β1 antibody (36).

GD25 and GD10 cells lacking β1A expressed the vzg-1/edg-2 LPA receptor and also the related edg-1 receptor. The generality of the findings for better defined cell types remains to be elucidated. Bovine heart or aortic endothelial cells expressing edg-1 respond to LPA with enhanced cell migration.3 In contrast, human MG63 osteosarcoma cells expressing vzg-1/edg-2 respond to LPA with inhibited migration.2, 4 Thus, the germ cell-derived lines should be considered as models of cellular behavior rather than of any one cell type.

At least two functions of β1 integrins may account for the need for β1 integrins in migration of the germ cell-derived cells: binding of β1 integrins by insolubilized ligands to allow haptotaxis and synergism between integrin-initiated and LPA-initiated signaling pathways to drive chemotaxis and chemokinesis. Although high concentrations of LPA (10–25 μM) have been shown to induce tyrosine phosphorylation of EGF receptors in a variety of cell types (41, 42), we could not show such cross-talk for EGF or PDGF receptors of GD25 cells with a concentration of LPA (500 nM) that induced optimal chemotaxis, suggesting that the initial steps in LPA-induced signaling are

3 T. Panetti, O. Peyruchaud, and D. F. Mosher, manuscript in preparation.
4 M. K. Magnusson and D. F. Mosher, submitted for publication.
independent of tyrosine kinase receptors. LPA (19) and ligated β1 integrins (43) both signal by multiple pathways. Our results indicate that the β1A integrin must not only be active but have intact NPYX motifs in the cytoplasmic domain to support LPA-induced migration. The latter requirement differentiates the effect of LPA on migration from the effect of LPA on fibronectin matrix assembly, which is up-regulated by LPA in GD25 cells expressing β1A with conservative Tyr to Phe substitutions (36). We previously hypothesized that conversion of both tyrosines between phosphorylated and dephosphorylated states was critical for directed movement, based upon the fact that the Y783F and Y795F mutations caused loss of migration ability to PDGF or EGF (36). One scenario is that the conversion between phosphorylated and dephosphorylated states is important for cycling of integrins to facilitate migration in response to a variety of agonist-receptor systems. Phosphorylated integrins may initiate a pathway leading to changes in F-actin containing cytoskeleton and thus to generation of the cellular polarity required for directional movement. In addition, NPYX motifs may regulate cycling of β1 integrins. Upon phosphorylation of the NPYX motifs, the integrin may lose its affinity for both extracellular ligand and cytoplasmic components of the focal contacts and exit the focal contact. Dephosphorylation of the motifs would allow the integrin to participate in a new round of ligation and focal contact formation. The phosphorylation-dephosphorylation cycle may also allow polarization of receptors for chemotactic agents. An alternative scenario is that the enzymatic cascade initiated by ligation of integrins and phosphorylation of β1A is linked with a common pathway initiated by ligated LPA receptor or tyrosine kinase receptors. Expression of a dominant negative Ras inhibits migration in response to both LPA and PDGF but not to soluble fibronectin (35). Ligated LPA receptors (19), ligated β1 integrins (43), and ligated EGF or PDGF receptors (41, 42) all can work through Ras. Thus, downstream targets of Ras are the most likely common pathway.

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