The ligation of available α6β1 integrin in adherent LOX melanoma cells by laminin G peptides and integrin stimulatory antibodies induced cell invasiveness, independent of adhesion activity of integrins that were prebound to extracellular matrix (Nakahara, H., Nomizu, M., Akiyama, S. K., Yamada, Y., Yeh, Y., and Chen, W.-T. (1996) J. Biol. Chem. 271, 27221–27224). Here, we show that this induced invasion involves an increase in tyrosine phosphorylation of a 190-kDa GTPase-activating protein for Rho family members (p190 RhoGAP; p190) and membrane-protrusive activities at invadopodia. This tyrosine phosphorylation does not occur when the adherent cells are treated with non-activating antibody against β1 integrin, control laminin peptides, or tyrosine kinase inhibitors genistein and herbimycin A. Although p190 and F-actin co-distribute in all cell cortex extensions, tyrosine-phosphorylated proteins including p190 appear to associate with F-actin specifically in invadopodia. In addition, the localized matrix degradation and membrane-protrusive activities were blocked by treatment of LOX cells with tyrosine kinase inhibitors as well as microinjection of antibodies directed against p190 but not by non-perturbing antibodies or control buffers. We suggest that activation of the α6β1 integrin signaling regulates the tyrosine phosphorylation state of p190 which in turn connects downstream signaling pathways through Rho family GTPases to actin cytoskeleton in invadopodia, thus promoting membrane-protrusive and degradative activities necessary for cell invasion.

Integrin receptors play important roles in organizing the actin-containing cytoskeleton and in signal transduction from the extracellular matrix (ECM) upon cell adhesion to immobilized ECM components (1–3). The assembly of integrin adhesion complexes requires both ECM and Rho/Rac family signaling proteins (4–6). However, integrin’s role in cell invasiveness remains to be determined. Invasive cells express a specialized surface structure for invasion, termed invadopodia, which exhibit dynamic motility, adhesion to, and degradation of the ECM (7). Invadopodia show the following biochemical properties: localization of oncogenic variants of pp60 c-Jun (8), dramatic association of cytoskeleton talin-β1 integrin with ECM (9), the elevated tyrosine phosphorylation state of a subset of proteins (10), and the localization of ECM-degrading proteases (11) including gelatinase A (12), seprase (13), and membrane type-1 metalloproteinase (14).

Activated Src may associate with the state of tyrosine phosphorylation of a major RasGAP-associated protein p190 (p190 RhoGAP (p190) (15–17) or p190B (5), which in turn creates a binding site for the SH2 domains of other signaling molecules to promote actin cytoskeletal motility. p190 contains a GTPase domain at its amino terminus and a GTPase-activating protein (GAP) domain at its carboxyl terminus. It forms a stable association with the p120 RasGAP that down-regulates Rho and Rac activity (16, 18, 19). Specifically, pp60 c-Jun regulates the simultaneous rearrangement of actin cytoskeleton and p190 following epidermal growth factor stimulation (17). Microinjection of the p190 into Swiss 3T3 cells blocks Rho-mediated changes in membrane ruffling (20) and the decrease in fibronectin adhesion (21). These findings suggest a role for p190 in connecting actin motility to downstream signaling pathways mediated through different Rho family GTPases.

Recently, the laminin α1 chain carboxyl-terminal globular domain (G domain amino acid residues 2111–3060) has been shown to have adhesion activities involving integrins (22–24). We showed that laminin G peptide AG-10 acts on the α6β1 integrin signaling of invasion by stimulating localized ECM degradation at invadopodia that are distinct from their direct effects on cell adhesion on immobilized ECM (25). Here we have examined tyrosine phosphorylation of signaling molecules in β1 integrin-ligation using this recently developed model of melanoma invasion that can be induced by laminin G peptides and anti-β1 integrin antibodies (25). Soluble peptides and antibodies specific for β1 integrins were added to melanoma cells that had already adhered to immobilized fibronectin and gelatin films and the state of protein tyrosine phosphorylation and membrane-protrusive activities at invadopodia were assessed.

Experimental Procedures

Materials—The human amelanotic melanoma cell line LOX was obtained and cultured as described (25). Mouse laminin-1 and all lamin G peptides, including AG-10 (NPWHSIYITRFG) and control AG-10-s (scrambled peptide of AG-10, NYITRFPGPWHSI), were prepared as described (23). Rat mAb 13 (against human β1 integrin subunit) was provided by Dr. S. K. Akiyama (NIH, Bethesda, MD). Monoclonal antibody K20 (against human β1 integrin subunit) was purchased from Immunotech (Westbrook, ME); anti-FAK antibody, anti-RasGAP, anti-

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The abbreviations used are: ECM, extracellular matrix; FAK, focal adhesion kinase; GAP, GTPase activating protein; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SH2, Src homology 2 domains; YPP, tyrosine-phosphorylated proteins.
Integrin-mediated Cell Invasion

p120 pp60src, anti-phosphotyrosine mAb 4G10 from Upstate Biotechnology (Lake Placid, NY); anti-p190, anti-cortactin, anti-p190src from Transduction Laboratory (Lexington, KY); and genistein, herbimycin A, and cytochalasin D from Calbiochem Novabiochem (San Diego, CA).

Culture of Cells on ECM Substrata, in Vitro Degradation/Invagination Assay, and Immunofluorescence—Suspended cells (10⁵/ml) were added to fibronectin-fibronectin-coated cross-linked gelatin films on a 15-mm round glass coverslip in a 12-well plate for in vitro degradation/invagination assay as described (25, 26). Cells were allowed to grow on the films for the indicated times, fixed and stained with rhodamine conjugate of fluorescein isothiocyanate (FITC) and antibodies were directed against p190 or other proteins. The cells were further stained with fluorescent conjugate of secondary antibodies. Stained samples were photographed using the Planapo 63/1.4 or 25/1.2 objectives on a Zeiss Photomicroscope III (Carl Zeiss, Inc., Thornwood, NY) under epifluorescence as described (13).

Preparations of Cell Lysate, Immunoprecipitation, and Immunoblotting—After treatment with laminin G peptides or anti-β1 integrin antibodies, cell monolayers were washed with ice-cold PBS and lysed with 0.1 M Tris-HCl, pH 7.5, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 10 mM sodium fluoride, 0.5 mM orthovanadate, 10 mg/ml aprotinin, 5 mg/ml leupeptin) at 1 mg/ml were microinjected into the cytoplasm with Femtotip microinjection system (Transjector 3246/micromanipulator 5171, Eppendorf). Mouse immunoglobulin G (IgG) at 1 mg/ml was used as a control and in some experiments the injected IgG was visualized with fluorescein-labeled goat anti-mouse IgG. In all cases, cells showing a response to the microinjected antibodies contained the mouse IgG inside the cells.

RESULTS

To examine tyrosine phosphorylation of proteins in cells during the induced matrix degradation, we seeded a human melanoma cell line LOX on immobilized fibronectin-cross-linked gelatin films in the presence of serum and allowed the cells to adhere for 1 h. Serum-free media containing soluble peptides or anti-integrin antibodies were then added to cells for an additional 5 h to assess the induced ECM degradation/invagination. As shown in Fig. 1A (lanes marked m13, AG-10), ligation of β1 integrin by stimulatory anti-β1 integrin mAb 13 or laminin G peptide AG-10 resulted in elevated tyrosine phosphorylation of proteins of 190, 120–130, and 60–90 kDa in molecular mass as revealed by immunoblotting using anti-phosphotyrosine mAb 4G10. When lysates obtained from cells treated with a nonactivating anti-β1 integrin mAb K20, non-immune IgG, or control laminin peptide AG-10-s, major bands around 120–130 kDa were detected by enhanced chemiluminescence (ECL, Amersham Corp.). To detect other proteins shown in Fig. 2, the YPP blot was stripped and reprobed with antibodies against individual proteins, followed by horseradish peroxidase-conjugated secondary antibodies.

Microinjection—LOX cells were seeded on fibronectin/gelatin films that had been coated on a gridded coverslip (CELLlocate, Eppendorf, Hamburg, Germany). Prior to microinjection, anti-p190 antibodies were dialyzed against PBS for 1 day. Antibody solutions at the concentration of 1 mg/ml were microinjected into the cytoplasm with Femtotip microinjection system (Transjector 3246/micromanipulator 5171). Mouse immunoglobulin G (IgG) at 1 mg/ml was used as a control and in some experiments the injected IgG was visualized with fluorescein-labeled goat anti-mouse IgG. In all cases, cells showing a response to the microinjected antibodies contained the mouse IgG inside the cells.
Integrin-mediated Cell Invasion

The rhodamine conjugate of phalloidin (to stain F-actin, panel B) was detected with a fluorescein conjugate of secondary antibodies (panel A) and the rhodamine conjugate of phalloidin (to stain F-actin, panel B). Both p190 and F-actin were concentrated in membrane protrusions including invadopodia (large arrows) and lamellipodia in the periphery (short arrows) of the cell. Bar, 10 μm.

Fig. 3. F-actin co-distributes with p190 in membrane protrusions. A and B, fluorescent microscopies of F-actin and p190 distribution on AG-10 induced cells. LOX cells were treated with AG-10 peptide (50 μg/ml). Cells were labeled with anti-p190 antibody that was detected with a fluorescein conjugate of secondary antibodies (panel A) and the rhodamine conjugate of phalloidin (to stain F-actin, panel B). Both p190 and F-actin were concentrated in membrane protrusions including invadopodia (large arrows) and lamellipodia in the periphery (short arrows) of the cell. Bar, 10 μm.

Integrin-mediated Cell Invasion

Interestingly, p190 from cells treated with stimulatory integrin antibody m13 (Fig. 1, left two lanes) and p190 (Fig. 3, B) exhibited very low levels of tyrosine phosphorylation (Fig. 2). We conclude that the increased tyrosine phosphorylation of p190 is implicated in invadopodial activities while that of p120^crk (Fig. 2) shows enhanced tyrosine phosphorylation in response to integrin antibody binding to β1 integrins.

To study the connection between p190 tyrosine phosphorylation and actin-driven membrane motility, activated LOX cells that had invaded fibronectin-coated gelatin films were labeled with phalloidin (for F-actin) (Fig. 3, upper panel). When immunoprecipitated from adherent cells treated with non-immune IgG or non-activating β1 integrin antibody K20, p190 exhibited very low levels of tyrosine phosphorylation (Fig. 2, left two lanes). Interestingly, p190 from cells treated with stimulatory β1 integrin mAb m13 shows elevated tyrosine phosphorylation (Fig. 2, right lane). Thus, p190 may be the 190-kDa protein that was seen to increase its tyrosine phosphorylation in response to laminin G peptides and β1 integrin antibody m13 (Fig. 1A). The 120–130- and 60–90-kDa YPPs (Fig. 1A) are major phototyrosine forms of early attachment of cells on the fibronectin-gelatin film (within 6 h). Among these molecules, p120^RasGAP (RasGAP), p120^src substrate (p120^src), cortactin, and pp60^src (Fig. 2) show enhanced tyrosine phosphorylation in cells treated with K20 and m13 antibodies as compared with that treated with non-immune IgG. However, treatment of β1 integrin antibodies had no apparent effect on the tyrosine phosphorylation of FAK and p130^cas (Fig. 2), which already may be highly phosphorylated in cells adherent on ECM as previously shown (29). Therefore, additional β1 ligation may not produce any detectable change in tyrosine phosphorylation of FAK and p130^cas (Fig. 2). We conclude that the increased tyrosine phosphorylation of p190 is implicated in invadopodial activities while that of p120^RasGAP, p120^src substrate, FAK, p130^cas, cortactin, and pp60^src may be due to adhesion and antibody binding to β1 integrins.

To further examine the role of p190 in cell invasion, we microinjected antibodies directed against p190 into LOX cells that were cultured on immobilized fibronectin-cross-linked gelatin films and assessed effects of antibodies on ECM degradation/invasion (Fig. 4). Effects of antibody injection were determined by counting the number of cells among a total of 25 injected cells in a designated area on fibronectin films. Injection of antibodies up to concentrations of 1 μg/ml or PBS into melanoma cells, which had been plated on fibronectin films for 3 h and cultured for another 5 h after injection, did not affect
the number of cells that remained attached 5 h after injection. Microinjection of anti-p190 antibodies into LOX cells blocked their degradative activities (Fig. 4, A and B) whereas that of non-immune IgG did not (Fig. 4, C and D). There was a 10-fold decrease in invasiveness when cells were microinjected with anti-p190 antibodies (p < 0.005) compared with that of cells injected with control non-immune IgG or control saline alone (Fig. 4E). We conclude that p190 exerts its signaling effects on invadopodial activities through β1 integrins.

**DISCUSSION**

The biochemical mechanisms by which cell invasiveness is induced are poorly understood partly because of the difficulty involved in defining signaling molecules of invasion. Recently, we have shown that cell invasiveness can be triggered when α6β1 integrin on ECM-free surfaces of the cell is ligated by soluble laminin G peptides or antibodies (25). Activated α6β1 integrin was shown to induce the recruitment of seprase, a serine-type integral membrane protease, to invadopodia of invading cells, and a subsequent increase of local ECM degradation. In this report, we show that, when α6β1 integrin is ligated by laminin G peptides or antibodies, p190 becomes tyrosine-phosphorylated and associates with F-actin in a specific membrane protrusion, invadopodia. In addition, the stimulated invasive phenotype was blocked by treatment of LOX cells with tyrosine kinase inhibitors and microinjection of antibodies directed against p190. Thus, activation of α6β1 integrin may regulate the monos phosphorylation state of p190 and its association with F-actin organization at invadopodia. The tyrosine-phosphorylated p190 may serve as a binding site for tyrosine kinases containing SH2 and link the Rho pathway to the actin cytoskeleton, thus promoting membrane-protrusive activities necessary for cell invasion.

Following activation of β1 integrins, the tyrosine kinase responsible for p190 phosphorylation remains to be identified. Src family tyrosine kinases are present and active in invadopodia of invasive cells (8, 10). Activated Src may create a binding site for SH2 domains of p190 (15–17) or p190B (5). Thus, c-Src may phosphorylate p190 that in turn may regulate actin polymerization mediated by Rho family GTPases (16–19). Also, p190 associates with membrane-protrusive activities involving Rho-mediated changes in the organization of cytoskeletal actin (20) and the decrease in fibronectin adhesion (21). Microinjection and tyrosine kinase inhibition experiments present in this report thus support the role of tyrosine-phosphorylated p190 in invadopodial activities.

Cell adhesion to ECM promotes tyrosine phosphorylation of many signaling molecules. It has been shown recently that antibody-mediated clustering of α5β1 integrin causes recruitment of p190B and Rho family members to the cell surface (29, 30). Antibody-mediated integrin clustering and adhesion of cells to fibronectin leads to an increase in the tyrosine phosphorylation of Src tyrosine kinases, FAK, p130Cas, talin, paxillin, cortactin, and tensin (2, 6, 29–32). While we show a specific association of p190 tyrosine phosphorylation with invadopodial formation, we have confirmed the observation that antibody activation of integrin promotes tyrosine phosphorylation of pp60src, cortactin, FAK, p120CasGAP, and p120src substrate (Fig. 2). It is possible that tyrosine phosphorylation of these signaling molecules in response to adhesion is a prerequisite for the expression of cell invasiveness. Consistent with the inhibition of invasiveness in cells microinjected with anti-p190 antibody (Fig. 4E), there was a more than 3-fold decrease in invasive potential when cells were microinjected with anti-FAK, cortactin, or pp60src antibodies (data not shown). Thus, we suggest that FAK, cortactin, and pp60src may exert their primary signaling effects on cell invasion through integrin-mediated adhesion on ECM.

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