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Involvement of Phosphoinositide 3-Kinase in Regulation of Adhesive Activity of Highly Metastatic Hepatoma Cells

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We have established hepatoma clones from benzopyrene-treated liver cells, one of which (G-5) shows extensive metastasis to the lung when injected subcutaneously into mice [Tanigaki, Y. *et al.* (1995) *Invasion Metastasis* 15, 70-80]. In the present study, we performed *in vitro* assays suitable for examination of the adhesive and invasive properties of the highly metastatic cells. G-5 cells efficiently entered the pores of fibronectin-coated filters. Treatment of the cells with an inhibitor of phosphoinositide 3-kinase (PI 3-kinase), wortmannin, significantly impaired the invasive activity. A structurally unrelated inhibitor, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) also prevented invasion. Both inhibitors suppressed cell adhesion to fibronectin-coated dishes. G-5 cells were next transfected with a mutant regulatory subunit (Δp85) of PI 3-kinase, which was expected to impair the function of PI 3-kinase. The transfectants showed suppressed adhesion to the dishes and did not efficiently migrate into the filters. The lower adhesive ability of the transfected cells was not further affected by inhibitors of PI 3-kinase. Thus, PI 3-kinase activity contributes significantly to the adhesive and invasive properties of G-5 cells.

Key words: hepatoma cells, integrin, lung metastasis, tumor invasion, wortmannin.

Progression of tumor cells to the invasive phenotype results in metastasis, a lethal event critically influencing the mortality of patients with malignancy. Tumor invasion is a multistep process involving attachment of cells to extracellular matrix *via* cell-surface receptors (adhesion), their migration to nearby vessels (motility), and the proteolytic degradation of tissue barriers. Although many environmental factors and cell-surface proteins have been shown to be involved in these steps, relatively little is known regarding the signal transduction pathways responsible for their regulation.

Previously, we established two tumor cell sublines from benzopyrene-treated murine liver cells (*1*). When injected subcutaneously into mice, one subline (G-5) was highly metastatic to the lung, while the other (G-1) showed poor metastatic activity. The poorly metastatic G-1 cells possess the ability to form colonies in the lung as effectively as G-5 cells when injected intravenously. Therefore, their different metastatic potencies were predicted to be largely attributable to different properties in the early stages of the metastatic process.

In the present study, we performed *in vitro* assays to examine the adhesive and motile properties of cells. G-5 cells were characterized by their extensive invasion into the pores of fibronectin-coated filters. Experiments using pharmacological tools and a mutant protein suggested that phosphoinositide 3-kinase (PI 3-kinase) enhances the invasive activity of G-5 cells by changing their adhesive properties.

MATERIALS AND METHODS

Cells and Reagents—Two clones of mouse benzopyrene-induced hepatoma cells, G-1 and G-5, were established from a day 16 BALB/c mouse embryo as described previously (*1*). The cells were cultured in Eagle's minimal essential medium (MEM, Nissui, Tokyo) supplemented with 1 mM glutamine and 10% fetal bovine serum (Cell Culture Laboratories, OH) at 37°C in a 5% CO_2 atmosphere.

Wortmannin was a gift from Kyowa Hakko Kogyo (Tokyo). LY294002 was purchased from Funakoshi (Tokyo). Human fibronectin was purified from 10 mM EDTA- or citrate phosphate dextrose-supplemented blood plasma as described previously (*2*). Δp85 cDNA was a gift from Dr. M. Kasuga, Kobe University, Kobe. The reagents and vectors for transfection were prepared as described previously (*3*). [γ-^{32}P]ATP and [^{32}P]orthophosphate were from ICN (CA) and DuPont-NEN (MA), respectively.

Polyclonal antibody against the regulatory subunit (p85) of PI 3-kinase and rat monoclonal antibodies against mouse LFA-1 and VLA-4 were from UBI (NY) and Seikagaku Kogyo (Tokyo), respectively. Rat monoclonal antibodies

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against mouse CD49e and CD18, and hamster monoclonal antibodies against CD51 and CD61 were from Phamingen (CA). A hamster monoclonal antibody against rat CD29, which is cross-reactive with mouse CD29 (*4*) and partially blocks its fibronectin binding, was a gift from Drs. K. Okumura (Juntendo University, Tokyo) and M. Miyasaka (Osaka University, Osaka).

Establishment of G-5 Subclones Expressing a Mutant Regulatory Subunit of PI 3-Kinase—A plasmid for the expression of a mutant bovine p85a (⊿p85) lacking a binding site for p110 was prepared by placing ⊿p85 cDNA (*5*) in the mammalian expression vector pME18s using standard gene manipulation techniques (*3*). G-5 cells (80% confluency on 100-mm dishes) were transfected with ⊿p85/pME18s (20 μg) using lipofectamine (30 μg). The empty vector (20 μg) was used instead of ⊿p85/pME18s as a control. Transfectants were selected by addition of G418 (final concentration of 200 μg/ml) to the culture medium. Resistant cells were screened by immunoblotting with anti-p85 antibodies and subcloned by the limiting dilution method. Three independent clones of G-5/⊿p85, named A1, B5, and C2, were established together with a clone containing vector only (C1) for the present study. On SDS-PAGE/immunoblotting, the ⊿p85 band appeared just below the p85 band in these clones. The ratios of ⊿p85/endogenous p85 in A1, B5, and C2 clones were 2.7, 1.6, and 2.1, respectively, based on densitometric analysis of the blotting sheet (data not shown). The obtained cells proliferated as monolayers almost normally in MEM containing 1 mM glutamine and 10% fetal bovine serum.

Flow Cytometry—Flow cytometry was used to assess the levels of surface expression of integrin subunits. Cells (1×10^6) were incubated with the first antibody against α or β subunit of integrin for 45 min at 0°C. After two washes with phosphate-buffered saline containing 0.5% BSA, the cells were incubated with FITC-labeled goat $F(ab')_2$ against rabbit, rat, or hamster IgG (Organon Teknika, NC). The samples were fixed with 0.5% paraformaldehyde and analyzed using an Epics Profile II within 3 days.

Cell Adhesion Assay—Cells were cultured for 24 h in serum-free GIT medium (Nippon Seiyaku, Tokyo), containing serum growth factor (SGF), insulin, and transferrin. The cells were detached from culture dishes with 10 mM EDTA, suspended in MEM, then incubated with 0.05 μM wortmannin, 0.1 mM LY294002 or DMSO (vehicle alone) for 10 min at 37°C. After three washes, the cells were suspended in MEM containing 0.02% BSA at a concentration of 4×10^5 cells/ml. Samples of the cell suspension (2 ml) were plated on fibronectin-coated dishes (Nippon Becton Dickinson, Tokyo). At 1 h intervals, the unbound cells were aspirated off and the numbers of attached cells were determined by a colorimetric assay (*6*). The assays were performed three times in triplicate. In some experiments, cells were plated on dishes which had been filled with 0.02% BSA/MEM containing 20 μg of nonimmune mouse IgG or 2-20 μg of monoclonal antibodies against α5β1 integrin (*4*).

Invasion Assay—Aliquots of 100 μl of purified fibronectin (125 μg/ml) were poured over polycarbonate filters with 8.0 μm pores. The filters were dried at room temperature for 15 h, then immersed in serum-free MEM and placed in a Chemotaxicell (Kurabo, Osaka) modified Boyden chamber system. The lower compartment of the chamber was filled with MEM containing 5% mouse serum. Cells (5×10^5 cells/0.2 ml) which had been treated with 0.05 μM wortmannin, 0.1 mM LY294002, or DMSO for 10 min at 37°C as described above, were added to the upper compartment of the chamber and incubated for 0-6 h at 37°C. At timed intervals, the cells remaining on the upper surface of the filters were removed by wiping with cotton swabs. The cells which migrated to the lower surface of the filters were then fixed with 70% ethanol, stained with Giemsa solution, and counted under a microscope. The assays were performed three times in quadruplicate.

Accumulation of PtdIns(3,4,5)P_3—Cells incubated for 24 h at 37°C in MEM containing 0.1% BSA were suspended at a density of 1.7×10^8 cells/ml in labeling medium consisting of 20 mM HEPES/NaOH (pH 7.4), 136 mM NaCl, 4.9 mM KCl, and 5.5 mM glucose. The cells were incubated for 15 min at 37°C with [^{32}P]orthophosphate (100 μCi/ml), then suspended at 5×10^7 cells/ml in 0.1% BSA/MEM. Samples of the suspension (280 μl) were incubated at 37°C for 5 min with or without 0.05 μM wortmannin or 0.1 mM LY294002, then for a further 0-180 min on fibronectin-coated dishes. The cells were collected in glass tubes and 1.55 ml of chloroform/methanol/8% $HClO_4$ (50:100:5) was added. After vigorous mixing, 0.5 ml of chloroform and 0.5 ml of 8% $HClO_4$ were added, followed by centrifugation to separate the organic phase, which was then washed repeatedly with chloroform-saturated 0.5 M NaCl containing 1% $HClO_4$. The dried lipids were dissolved in 20 μl of chloroform/methanol (95:5) to be spotted on TLC plates (Silica gel 60, Merck) impregnated with potassium oxalate. [^{32}P]PtdIns(3,4,5)P_3 was separated from other lipids by developing in chloroform/acetone/methanol/acetic acid/water (80:30:26:24:14) (*7, 8*).

RESULTS

We first examined the cell-surface expression of integrin subunits in two hepatoma clones (see "Introduction" for these clones) by flow cytometry using monospecific antibodies (Table I). Of the integrin α chains examined, only CD49e (α5) was positive in both G-1 and G-5 cells. Likewise, of the β chains only CD29 (β1) was expressed in both sublines. Thus, these molecules were expected to function as adhesion molecules on the cells. In fact, monoclonal antibodies against α5 and β1 significantly inhibited the adhesion of G-5 cells to fibronectin-coated dishes (by 26 and 10% at their concentration of 10 and 2 μg/ml, respectively). Yet, undetermined molecules (including other α chain moieties) might modulate cell adhesion additionally.

As α5β1 integrin functions as a fibronectin receptor, we next performed *in vitro* invasion assay (*9*) using fibronectin-coated filters. As shown in Fig. 1A, highly metastatic G-5 cells extensively moved into the pores of the filters. Only marginal infiltration was observed unless the filters were coated with fibronectin (data not shown). In contrast, G-1 cells with low metastatic activity did not efficiently migrate into the pores even on filters coated with fibronectin (Fig. 1B). Treatment of G-5 cells with 0.05 μM wortmannin, a potent inhibitor of PI 3-kinase (*7, 10*), blocked the invasive activity of the cells (Fig. 1, C and D). The time course of infiltration of G-5 cells into the fibronectin-coated filter is shown in Fig. 1E. The number of invading cells

increased linearly with time. Wortmannin-treated cells did not enter the pores even after 6 h. LY294002, another inhibitor of PI 3-kinase, which is structurally unrelated to wortmannin (*11*), also inhibited the invasive activity of G-5 cells (Table II).

To further elucidate the role of PI 3-kinase, we established stable subclones from G-5 cells by transfection with a dominant negative mutant of PI 3-kinase. For this purpose, Δp85, a mutant regulatory subunit of PI 3-kinase lacking the binding site to the catalytic subunit (*5, 12*), was utilized. Three subclones expressing Δp85, A1, B5, and C2, were established. The invasive activity of these cells was significantly impaired (Fig. 2). Cells transfected with the empty vector (C1) showed higher invasive activity than the Δp85-transfected cells. In these cells, surface expression of both the α5 and β1 chains of integrin as determined by flow cytometry varied only marginally (data not shown).

We next measured the adhesive properties of these hepatoma cells. As shown in Table III, wortmannin at 0.05 μM inhibited the adhesion of G-5 cells to fibronectin-coated dishes. The adhesion of G-1 cells to the dishes was not inhibited by wortmannin. Similar results were obtained when these cells were treated with 100 μM LY294002 (data not shown, but see Fig. 3 for G-5 cells). Surface expression of both the α5 and β1 chains of integrin was unaffected by wortmannin or LY294002 (data not shown). All three clones transfected with Δp85 showed lower

TABLE I. **Surface expression of integrin subunits in G-1 and G-5 cells.**

Molecules	Mean fluorescence shift[a]	
	G-1 cells	G-5 cells
LFA1 (αL)	0.21	0.15
VLA4 (α4)	0.44	0.00
CD49e (α5)	3.02	2.22
CD51 (αV)	0.00	0.00
CD29 (β1)	11.04	4.55
CD18 (β2)	0.36	0.05
CD61 (β3)	0.00	0.00

[a]G-1 and G-5 subclones were analyzed by flow cytometry using monoclonal antibodies specific for the indicated integrin subunits.

TABLE II. **Inhibitory effects of PI 3-kinase inhibitors on invasion.**

Treatment	Number of invading cells[a]	
	G-1 cells	G-5 cells
DMSO (control)	2.2±0.5	19.4±9.9
Wortmannin	2.0±1.8	6.3±3.9
LY294002	2.2±1.2	6.7±3.8

[a]G-1 and G-5 cells were examined for their *in vitro* invasion activities after treatment for 5 min with DMSO, 0.05 μM wortmannin, or 0.1 mM LY294002. Numbers of cells/0.6 mm^2 migrating through the pores of fibronectin-coated filters during the 6 h of incubation were counted.

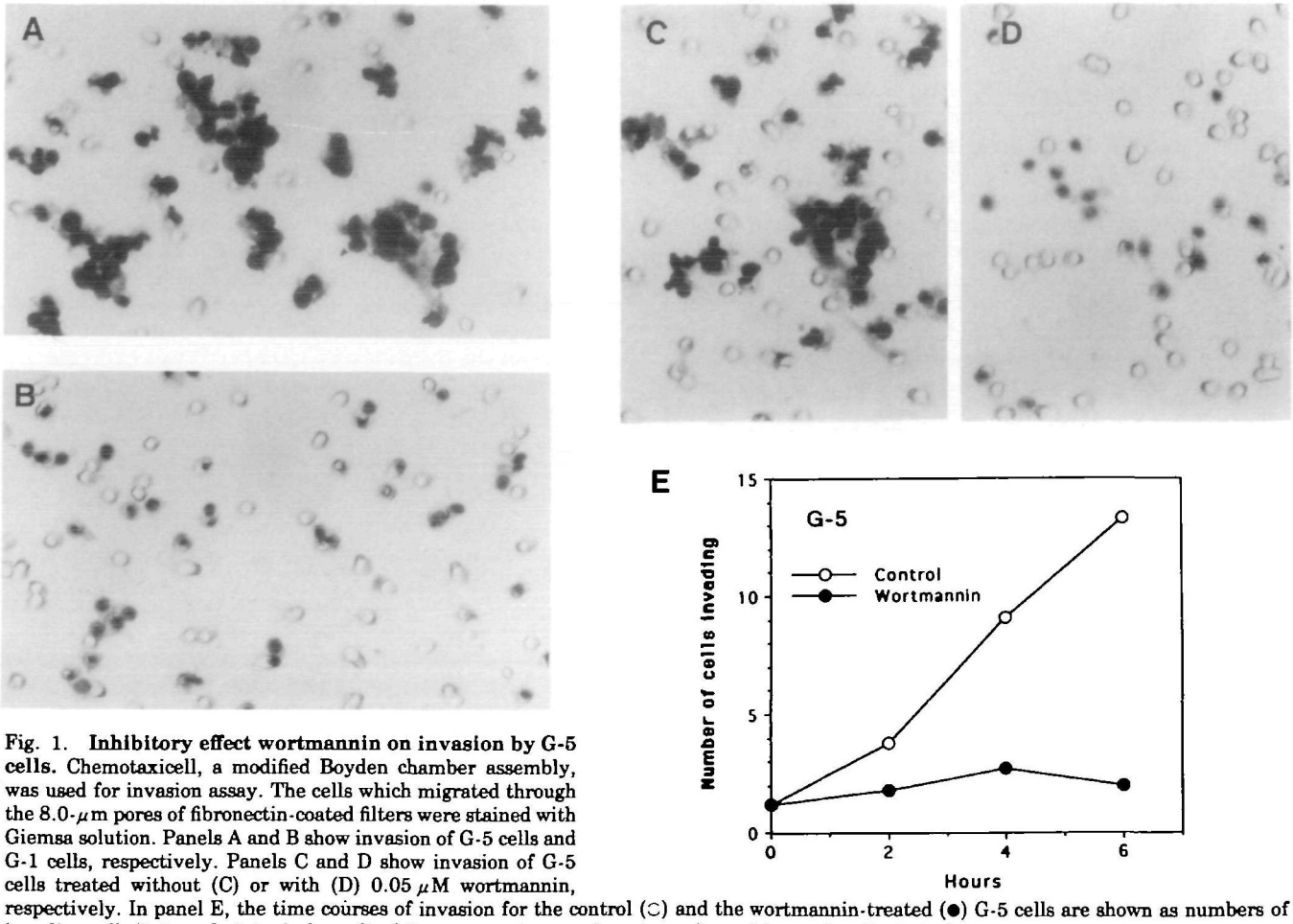

Fig. 1. **Inhibitory effect wortmannin on invasion by G-5 cells.** Chemotaxicell, a modified Boyden chamber assembly, was used for invasion assay. The cells which migrated through the 8.0-μm pores of fibronectin-coated filters were stained with Giemsa solution. Panels A and B show invasion of G-5 cells and G-1 cells, respectively. Panels C and D show invasion of G-5 cells treated without (C) or with (D) 0.05 μM wortmannin, respectively. In panel E, the time courses of invasion for the control (○) and the wortmannin-treated (●) G-5 cells are shown as numbers of invading cells/0.6 mm^2. A typical result of three separate experiments performed in quadruplicate is shown.

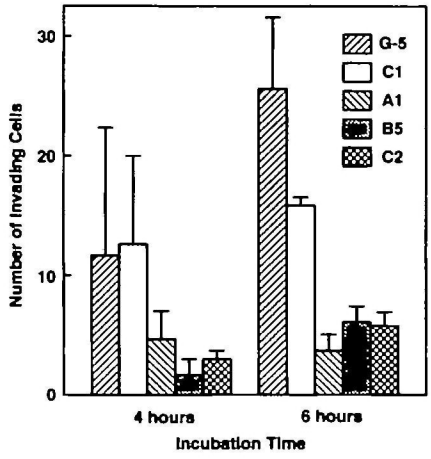

Fig. 2. **Impaired invasion of Δp85-transfected G-5 subclones.** G-5 cells were transfected with Δp85/pME18s (subclones A1, B5, and C2) or the empty vector (subclone C1). The parent and transfected cells were examined for *in vitro* invasion activities as in Fig. 1. Numbers of cells/0.6 mm² migrating through the pores of fibronectin-coated filters during the 4 or 6 h of incubation were determined. A typical result of three separate experiments performed in quadruplicate is shown.

TABLE III. **Effects of wortmannin on adhesion of G-1 and G-5 cells.**

Treatment	Number of attached cells[a] (Absorbance at 577 nm)	
	G-1 cells	G-5 cells
DMSO (control)	0.095±0.043	0.238±0.028
Wortmannin	0.110±0.036	0.074±0.018

[a]G-1 and G-5 were treated for 5 min with DMSO or 0.05 μM wortmannin before addition to the fibronectin-coated dishes. Cells bound to the dishes after 1 h of incubation were quantified by a colorimetric assay. Absorbance of 0.1 corresponds to 1.2×10^5 cells.

adhesive activity than the parent G-5 cells or the vector control (C1) cells (Fig. 3). The impaired adhesive activities of the Δp85-transfected cells were not further inhibited by LY294002 or wortmannin (data not shown), as was the case of G-1 cells with low adhesive activity (see Table III).

The above results indicated that the PI 3-kinase activity in G-5 cells up-regulated their invasion by changing the adhesive properties of the cells. This conclusion suggested that the PI 3-kinase in G-5 cells is in a more activated state than that in G-1 cells. Hence, we next measured the amounts of PI 3-kinase in these cells. Figure 4A shows the results of immunoblotting analysis using an antibody against the regulatory subunit (p85) of PI 3-kinase. No significant differences in the amount of p85 were observed between these cells. We then measured the PI 3-kinase activity of the anti-p85 immunoprecipitate (Fig. 4B). The ability to phosphorylate PtdIns was not different between G-1 and G-5 cells. The same result was obtained when PtdIns(4,5)P_2 was used as a substrate (data not shown). Thus, the basal activity of PI 3-kinase was not increased in G-5 cells.

Figure 5 shows the cellular accumulation of PtdIns(3,4,5)-P_3, a product of PI 3-kinase. When G-5 cells were labeled with $^{32}P_i$ and then placed on fibronectin-coated dishes, gradual accumulation of [^{32}P]PtdIns(3,4,5)P_3 was observed (Fig. 5B). Treatment of the cells with 0.05 μM wortmannin

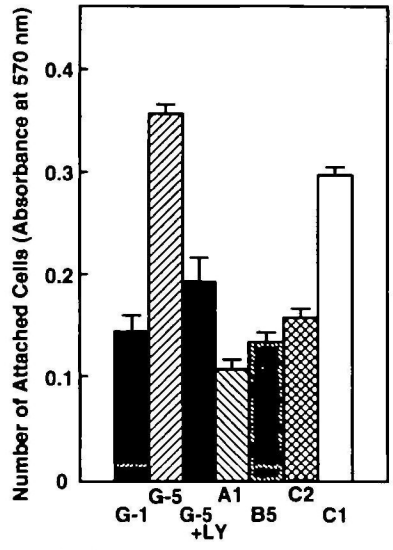

Fig. 3. **Decreased adhesion of Δp85-transfected G-5 subclones.** Adhesion of G-1, G-5, or G-5-derived cells to fibronectin-coated dishes was determined. The effects of 0.1 mM LY294002 (LY) on the G-5 cells were also examined. Cells bound to the dishes after 1 h of incubation were quantified by staining with crystal violet followed by solubilization and determination of the optical density at 577 nm on an automated ELISA reader. An optical density of 0.1 corresponds to 1.2×10^5 cells. A typical result of three separate experiments performed in triplicate is shown.

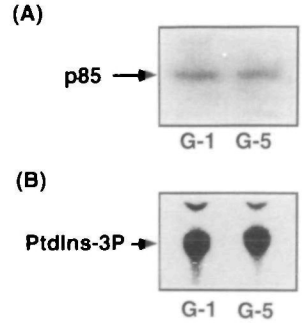

Fig. 4. **Amounts and activities of PI 3-kinase in G-1 and G-5 cells.** G-1 and G-5 cells were solubilized with Nonidet P-40 and sonicated, and the extracts were treated with anti-p85 antibody. In panel A, the immunoprecipitates were resolved by 10% SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, then immunoblotted with anti-p85 antibody. The position of p85 is indicated by an arrow. In panel B, the PI 3-kinase activity of the precipitate was determined using [γ-^{32}P]ATP and PtdIns as substrates. The autoradiogram of the TLC plate used for separation of lipid product is shown with the position of PtdIns(3)P indicated by an arrow.

completely abolished this accumulation. LY294002 also completely inhibited [^{32}P]PtdIns(3,4,5)P_3 accumulation (data not shown). In contrast, no accumulation of the PI 3-kinase product was detected in G-1 cells, regardless of whether the cells were treated with wortmannin or not (Fig. 5A).

DISCUSSION

In the present study, we showed that highly metastatic G-5 hepatoma cells migrate into the pores of fibronectin-coated

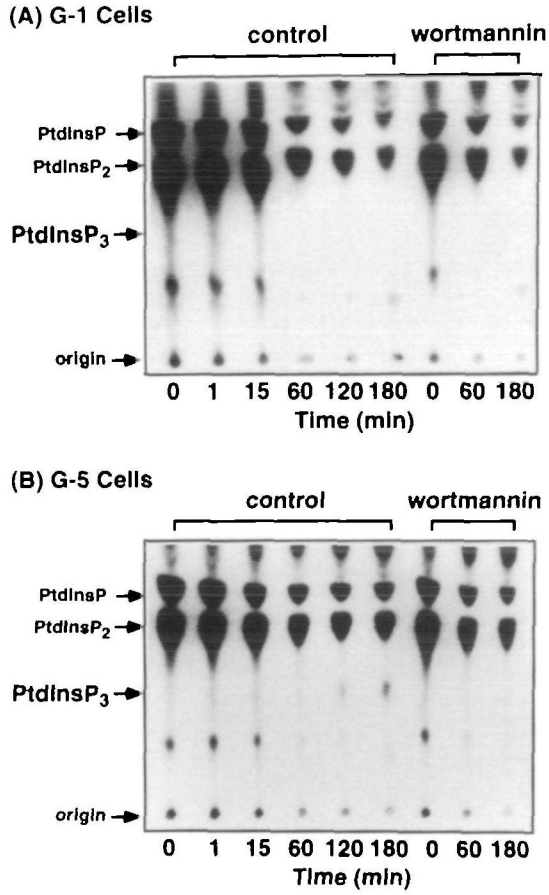

Fig. 5. **Accumulation of PtdIns(3,4,5)P_3 in G-1 and G-5 cells.** G-1 (panel A) and G-5 (panel B) cells, labeled with [^{32}P]orthophosphate, were incubated with (right) or without (left) 0.05 μM wortmannin before addition to the fibronectin-coated dishes. At the indicated times, phospholipids were extracted and separated on an oxalate-impregnated silica gel plate. An autoradiogram of the plate is shown with the positions of the spots corresponding to PtdInsP, PtdInsP_2, and PtdInsP_3 indicated by arrows.

filters. This invasive activity was suppressed by treatments that inhibited PI 3-kinase activity. Thus, the lipid kinase activity in the hepatoma cells is critical for their high invasive activity. As inhibitors of PI 3-kinase suppressed adhesion, the decreased invasion was at least in part due to the decreased adhesive activity of the cells. The specificity of this action was supported by the observation that the G-5 cells transfected with a dominant negative mutant of PI 3-kinase showed reduced adhesion and by our preliminary result suggesting that the G-1 cells with a constitutively active form of p110 subunit are as adhesive to fibronectin as G-5 cells (Hazeki *et al.*, unpublished data). Furthermore, wortmannin did not inhibit the reduced adherence of G-1 cells or Δp85-transfected G-5 cells.

While this manuscript was in preparation, two groups reported that PI 3-kinase is essential for integrin-mediated invasion of carcinoma cell lines (*13, 14*). In these studies, wortmannin effectively prevented cell motility with little effect on the integrin-mediated adhesion of the cells. In contrast, the action of wortmannin on G-5 hepatoma cells was accompanied by suppressed adhesion as described above. Thus, G-5 cells are considered to possess a unique mechanism regulating the ligand-binding activity of cell-surface integrin. Cytoplasmic domains of integrin are known to modulate their activity, suggesting the existence of a regulatory mechanism by intracellular signals (*15*). One study demonstrated that expression of a constitutively activated R-ras induces integrin activation and enhanced adhesive activity of cells (*16*). The present findings may represent another example of "inside-out" signaling.

We showed that there was a gradual accumulation of [^{32}P]PtdIns(3,4,5)P_3, a product of PI 3-kinase, in G-5 cells placed on fibronectin-coated dishes. Cross-linkage of $\alpha 6\beta 4$ integrin, a laminin receptor, was reported to stimulate PI 3-kinase activity of cells (*14*). Thus, one possible explanation for our results is that ligand binding to $\alpha 5\beta 1$ integrin, *i.e.*, attachment of cells to fibronectin-coated dishes, also triggers the activation of PI 3-kinase. However, the accumulation of [^{32}P]PtdIns(3,4,5)P_3 in G-5 cells was observed only after 2 h of incubation and was more prominent after 3 h, although the adhesion reached a plateau within 1 h. Furthermore, G-1 cells attached to the dish, although less efficiently than G-5 cells, without any production of [^{32}P]-PtdIns(3,4,5)P_3. Thus, the integrin-mediated activation of PI 3-kinase itself cannot be the sole factor responsible for the marked accumulation of PtdIns(3,4,5)P_3 observed in G-5 cells.

The most likely interpretation of the relevant data is that the PI 3-kinase activity in G-5 cells is in an activated state for some as yet undefined reason. If so, it is not surprising that the inhibition of PI 3-kinase suppressed cell adhesion to the level of G-1 cells. It is important to determine the regulatory mechanism of PI 3-kinase in G-5 cells. Many stimuli which activate PI 3-kinase are known to recruit the lipid kinase activity to the immune complex recognized by anti-phosphotyrosine. In a preliminary experiment, however, the activity of this complex was not different between G-1 and G-5 cells (data not shown). We reported previously that the activity of the p85/p110β subtype of PI 3-kinase is increased by activation of heterotrimeric GTP-binding proteins (*17*). This effect is not accompanied by increased lipid kinase activity in the anti-phosphotyrosine immune complex (O. Hazeki, unpublished data). The small GTP-binding protein Ras is known to stimulate PI 3-kinase activities (*18, 19*). Such mechanisms are likely to be in operation in G-5 cells.

In summary, we showed that PI 3-kinase contributes significantly to the adhesive and invasive properties of G-5 cells. Although the mechanism whereby PI 3-kinase-mediated cell motility is enhanced in these cells remains to be clarified, this property is considered to be the basis of the high metastatic activity of these hepatoma cells.

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