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Dedifferentiation of adenocarcinomas by activation of phosphatidylinositol 3-kinase

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ABSTRACT **Signet ring cell carcinoma is a malignant type of poorly differentiated adenocarcinomas in stomach, which is characterized by the occasional presence of signet ring-like cancer cells. We found that expression of constitutively active phosphatidylinositol 3-kinase (PI 3-kinase) in well differentiated adenocarcinoma cell lines induced the loss of cell–cell contact and some of the cells changed their shapes to signet ring cell-like, characterized by appearance of mucus droplets in the cytoplasm with well developed endplasmic reticulum and Golgi complexes. The active PI 3-kinase-expressing cells formed poorly differentiated tumors in nude mice, which were clearly different from those of the original cell lines. The PI 3-kinase activities detected in anti-phosphotyrosine immunoprecipitates were higher in several signet ring cell carcinoma-derived cell lines than in other adenocarcinoma cell lines. In addition, PI 3-kinase was found to be associated with a 200-kDa protein phosphorylated in tyrosine in 4 of 6 signet ring cells but not in other cell lines, suggesting that PI 3-kinase is possibly activated in these cells by binding to the 200-kDa protein. The 200-kDa protein–PI 3-kinase complex was exclusively fractionated in the membrane fractions. The specific activity of the PI 3-kinase immunoprecipitated with anti-phosphotyrosine antibody was ≈3-fold higher than that with anti-PI 3-kinase antibody. These results suggest that PI 3-kinase in signet ring cell carcinoma is recruited to the membrane and activated by the binding to the 200-kDa protein.**

Phosphatidylinositol 3-kinase (PI 3-kinase) is the enzyme that catalyzes the phosphorylation of the D-3 position of phosphatidylinositol (PI) and its derivatives. PI 3-kinase can be regulated by various mechanisms including G proteins and tyrosine kinases (1–3). PI 3-kinase, which is mainly activated by tyrosine kinases, consists of two subunits: a catalytic 110-kDa subunit (p110) and a regulatory 85-kDa subunit (p85) (4). P85 is an adapter molecule harboring an SH3 domain and two SH2 domains. This enzyme uses PI 4,5-diphosphate as a substrate *in vivo* to produce PI 3,4,5-triphosphate and triggers many cell responses including signal transduction to the nucleus (5), cytoskeletal rearrangement (6, 7), and vesicle transport (8–10). Recent studies suggest that PI 3-kinase may be involved in tumor formation in animals. A transforming retrovirus that causes hemangiosarcoma in chickens carries activated PI 3-kinase as an oncogene (11), and a mutant p85 was found in a mouse irradiated by UV can transform fibroblasts *in vitro* (12). In addition, an elegant study that used PI 3-kinase fused to an estrogen receptor suggests that prolonged expression of the activated PI 3-kinase can contribute to cellular changes that are characteristic of cellular transformation (13). In addition, it has been reported that PI 3-kinase may contribute to the mortality (14) and invasiveness of transformed cells, probably through cytoskeletal rearrangements downstream of integrin signaling (15–17). However, no direct evidence has been reported for the involvement of PI 3-kinase in the development of human tumors.

Dedifferentiated carcinoma often brings the worst prognosis in patients because of its aggressive and infiltrative nature with desmoplastic reaction, making surgical removal difficult (18). The cells of the carcinoma lack the ability to maintain cell–cell contact and therefore diffusely infiltrate the stroma, resulting in increased invasion and metastasis. High incidence of stomach tumors is seen in Japan. Each year, approximately 50,000 people are killed by stomach tumors, most of which are dedifferentiated. Signet ring cell carcinoma, one of the typical dedifferentiated carcinomas, occasionally contains signet ring-shaped cancer cells (signet ring cells) (see Fig. 4*a*). Signet ring cells exhibit round shapes with eccentric nuclei and have abundant mucus granules in the cytoplasm. Despite serious implications for human health, the molecular mechanism regulating the dedifferentiation of tumors is not well known. In this paper, we report expression of constitutively active PI 3-kinase (19, 20) in human adenocarcinoma cell lines by an adenovirus-mediated Cre-loxP recombination system (21). We found that high PI 3-kinase activity can convert differentiated cells with polarity to a less differentiated and more malignant stage similar to signet ring cell carcinoma and that PI 3-kinase is indeed activated in native signet ring cell carcinomas.

MATERIALS AND METHODS

Cell Culture. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS. Seventeen human gastric or colon carcinoma cell lines were used in this study. KATOIII (JCRB0611), NUGC-4 (TKG0049), HSC-39 (22), HSC-45 (22), HSC-58 (22), and HSC-60 (22) were established from signet ring cell carcinomas of the stomach; MKN45 (JCRB0254), AZ-521 (TKG0185), GOTO (JCRB0612), SCH (JCRB0251), and SH10-TC (TKG0412) were cell lines from well or moderately differentiated adenocarcinoma of stomach; HCC2998 (23), COLO 205 (TKG0457), CoLo-TC (TKG0404), and DLD-1 (TKG0379) were differentiated colon cancer cell lines. Cells with TGK and JCRB numbers were obtained from Cancer Cell Repository, Institute of Development, Aging and Cancer, Tohoku University, and Health Science Research Resources Bank, respectively.

Generation of Cell Lines Bearing the Constitutively Active PI 3-Kinase Gene. HCC2998 and MKN45 were transfected

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Abbreviations: PI, phosphatidlyinositol; MAP, mitogen-activating protein; PKB, protein kinase B; PAS, periodic acid/Schiff reagent.

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with the expression vectors carrying the mutant PI 3-kinase genes. MKN45 is often referred to as a poorly differentiated type carcinoma, however, this cell line actually contains highly differentiated cells, as evidenced by the *in vivo* tumor formation experiment in this study. The neomycin-resistant colonies were subcloned, and the expression of the proteins was examined after infection with AxCANCre, an adenovirus coding for the Cre recombinase. HCC2998 cell clones capable of expressing the BD110, the BD110X, and the BD110E proteins were randomly selected and designated as HCC2998/BD110, HCC2998/BD110X, and HCC2998/BD110E, respectively. MKN45 cells capable of expressing pBD110 were named as MKN45/BD110. AxCANLacZ is an adenovirus coding for LacZ instead of Cre (21).

Analysis of Phospholipid. For the lipid analysis, HCC2998/BD110 cells were infected with AxCANCre. After incubation for 1, 2, or 3 days, medium was replaced with the phosphate-free minimal essential medium containing [^{32}P]orthophosphate (1 mCi/ml; 1 Ci = 37 GBq) and 25 mM Hepes·NaOH (pH 7.4). After 4 hr, the reaction was stopped with MeOH/1 N HCl (1:1), and the lipid was extracted with chloroform. After a deacylation reaction, the resulting water-soluble components were analyzed by anion exchange chromatography with Partisfere SAX5 column (Whatman) (24).

Periodic Acid/Schiff Reagent (PAS) Staining. The cultured cells on a glass slide were reacted with PAS after periodic treatment.

Immunostaining of the Cells. The cultured cells were fixed in 4% formaldehyde solution and embedded in paraffin. The immunocytochemical staining was performed on paraffin sections with anti-CA15–3 (DAKO) as a primary antibody, with biotin-conjugated anti-mouse IgG (DAKO), and with peroxidase-conjugated streptavidin. Peroxidase activity was visualized with diaminobenzidine solution and counterstaining was performed with hematoxylin.

Electron Microscopy. The cultured cells were washed with PBS and fixed in a cacodylate buffer containing 2% paraformaldehyde and 0.5% glutaraldehyde and post-fixed in an osmium tetroxide solution. After dehydration and embedding in Epon, 80 nm ultrathin sections were stained with lead citrate and uranium acetate and observed with an electron microscope (JEM1200EX, JEOL).

Soft Agar Colony Formation Assay. BD110-expressing or unexpressing HCC2998/BD110 or MKN45/BD110 were suspended at 300 cells in 4.5 ml of RPMI containing 10% FBS (GIBCO) and 0.4% low-melting-temperature agarose (FMC). The cell suspension was laid on top of 5 ml of 0.72% agarose-containing medium in 60-mm tissue culture dishes and incubated in a CO_2 incubator for 2 weeks to form colonies. The BD110-expressing cells were used 40–60 days after the AxCANCre infection. Colonies >0.5 mm in diameter were scored in two experiments, each with duplicate dishes.

Histological Studies. For transplantation of the signet ring-like cells to nude mice, the pBD110-expressing and the original cells were implanted subcutaneously into the backs of nude mice (5×10^6 cells per mouse, 3 mice per cell line). The mice were pretreated with anti-asialo GM1 serum (WAKO, 20 ml:0.3 ml PBS per mouse) for 3days (25). Tumors formed between days 14 and 28 were fixed in 4% formalin solution. The paraffin sections were stained with hematoxylin and eosin. These animal experiments were performed in accordance with the guidelines in National Cancer Center Research Institute, Japan.

Antibodies. Monoclonal anti-p85 antibodies Ab6 and CA3 were described previously (26). Anti-phospho-p38 mitogen-activated protein (MAP) kinase (Thr-180/Tyr-182) antibody and anti-phospho-PKB (Ser4–73) were from New England Biolabs. Anti-p38 MAP kinase antibody (N-20) and anti-PKB antibody (C-20) were from Santa Cruz Biotechnology.

Immunoprecipitaion of PI 3-Kinase. Cells were lysed in a buffer containing 20 mM Tris·HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, and 1% Nonidet P-40, and PI 3-kinase or phosphotyrosine-containing proteins were immunoprecipitated with anti-p85 antibodies, AB6 or CA3, or with anti-phosphotyrosine antibody, PY20, bound to protein A-Sepharose. The immunocomplexes were washed three times with RIPA buffer containing 10 mM Tris·HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton-X 100, 0.1% SDS, and 1% sodium deoxycholate.

Fractionation of the Cells. Cells were exposed to a buffer containing 10 mM Tris (pH 7.5) and 10 mM NaCl. After homogenizing with a Dounce homogenizer, the nuclear fraction was removed by centrifuging at $600 \times g$ for 5 min. The supernatant was further centrifuged at $100{,}000 \times g$ for 45 min to separate membrane and cytosolic fractions.

RESULTS

Two human adenocarcinoma cell lines, HCC2998 from colon and MKN45 from stomach, were transfected with a pCALNLw vector carrying the active PI 3-kinase gene (BD110) or pCALNLw vectors carrying truncated derivatives of BD110 lacking enzyme activity, BD110X or BD110E (Fig. 1*a*), to establish cell lines. The genes consist of a CAG promoter, a neomycin-resistance gene with loxP sequences at each end, and the PI 3-kinase genes (Fig. 1*b*). The PI 3-kinase genes were kept silent during the selection for stable transformants. The established cell lines maintained the characteristics of the parental adenocarcinoma cell lines, exhibiting tight cell–cell adhesion and polarity (Fig. 1*e*), and exhibited the differentiated-type histology when transplanted into nude mice (Fig. 3*c*). After infection with the adenovirus coding for a recombinase, Cre, the neomycin-resistant gene between the two loxP sequences was excised, bringing the PI 3-kinase genes under control of the CAG promoter (Fig. 1*b*). After this recombination, the BD110 protein (pBD110) and its derivatives were expressed >5 times more abundantly than the endogenous p110 in the cells and maintained at a high level over 30 days (Fig. 1*c*). Immunostaining analysis using an anti-myc antibody revealed that ≈97% of the cells infected with the Cre adenovirus were expressing pBD110 (data not shown). A control adenovirus coding for the lacZ gene did not induce recombination. The levels of PI 3,4-diphosphate and PI 3,4,5-triphosphate were significantly elevated after expression of pBD110 (Fig. 1*d*), suggesting that expression of pBD110 elevated the PI 3-kinase activity in these cells. No such effect was seen after induction of pBD110X or pBD110E (data not shown). To test whether the downstream molecules of PI 3-kinase also were activated after expression of pBD110, we analyzed phosphorylation of p38 MAP kinase (27, 28) and PKB (29–31). As shown in Fig. 1*f*, enhancement of phosphorylation of p38 MAP kinase was observed in HCC2998/BD110 and MKN45/BD110 cells after expression of pBD110. Another downstream molecule, PKB, also was activated (Fig. 1*f*), but MAP kinase was not (data not shown). Expression of wild-type PI 3-kinase did not show any effect, suggesting that simple overexpression of this protein was not sufficient to induce a biological effect (data not shown).

Expression of pBD110 resulted in a dramatic morphological change, in which cells were round and lacked interaction with other cells; some of the cells even grew in suspension, suggesting loss of anchorage dependence in these cells. In spite of these drastic changes in morphology, the growth of the cells was not affected (data not shown). After a few days, large cells containing huge vacuoles in the cytoplasm with eccentric nuclei, which are typical features of signet ring cells, were occasionally observed (Fig. 1*e*, compare with Fig. 4*a*). PAS–alcian blue staining revealed the presence of a mucous substance in the vacuole (Fig. 2*b*), which was barely seen in

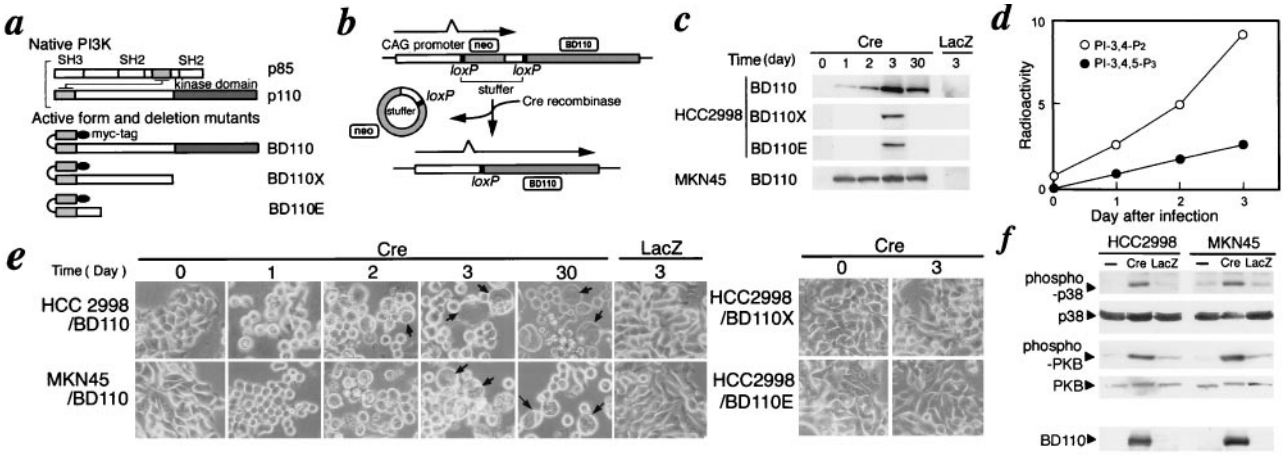

FIG. 1. Formation of signet ring-like cells after expression of the constitutively active PI 3-kinase. (*a*) Structure of the mutant PI 3-kinases used in establishment the stable transformants. The active PI 3-kinase, BD110, has the binding site for p110 in p85 at the amino-terminal end of p110 as well as the myc tag. The truncated versions of BD110, BD110X, and BD110E lack the kinase domain at the carboxyl-terminal portion of BD110 and almost all of the p110 sequence except the p85 binding domain, respectively. (*b*) Scheme of induction of the PI 3-kinases by the Cre–loxP system. A recombinase, Cre, cleaves out the sequence between the two loxP sequences. (*c*) Expression of PI 3-kinases after infection of the cells with an adenovirus bearing the Cre recombinase. The HCC2998 and MKN45 cells were infected with AxCANCre or AxCANLacZ. Expression of the PI 3-kinases was analyzed by Western blotting with anti-myc monoclonal antibody, 9E10. The numbers show the incubation time after infection with the viruses. (*d*) Elevation of levels of 3′-phosphorylated polyphosphoinositides after induction of pBD110. HCC2998/BD110 cells were infected with AxCANCre. After indicated periods of incubation, the cells were labeled with [^{32}P]orthophosphate for 4 hr, and the lipid was extracted. After deacylation reaction, the resulting water-soluble components were analyzed by a strong anion column chromatography. The radioactivities in the PI 3,4-diphosphate and PI 3,4,5-triphosphate fractions were plotted on the graph. The numbers were normalized by the total radioactivity in the lipid fraction. A representative of two experiments with two cell lines is shown in the figure. (*e*) Morphological change of HCC2998 and MKN45 cells expressing the PI 3-kinase mutants. Cells bearing PI 3-kinase genes, HCC2998/BD110, HCC2998/BD110X, HCC2998/BD110E, and MKN45/BD110 cells were infected with AxCANCre (Cre) or with AxCANLacZ (LacZ) and photographed at the indicated time. The cells expressing BD110 changed their shapes, becoming round and separated. Large signet ring-like cells appeared among the round cells after 2–3 days (indicated by arrows). No such changes were observed in the control cells. (*f*) Phosphorylation of p38 MAP kinase and PKB after expression of pBD110. HCC2998/BD110 and MKN45/BD110 cells were incubated for 3 days after infection with AxCANCre (Cre) or AxCANLacZ (LacZ). The cells were harvested, and Western blotting was performed with anti-phospho-p38 antibody (phospho-p38) and anti-phospho-PKB antibody (phospho-PKB), respectively. Total p38 MAP kinase and PKB were detected by using anti-p38 antibody (p38) and anti-PKB antibody (PKB). Expression of the myc-pBD110 was detected by anti-myc antibody (*Bottom*). (−), mock-infected cells.

mock-infected cells (Fig. 2*a*). In addition, DF3 (CA15-3), a mucous tumor-associated antigen generally secreted from the apical borders of secretory epithelial cells, was detected in the vacuoles as well as in the plasma membrane (Fig. 2*c*). An ultrastructural examination of the signet ring-like cells revealed marked dilation of the Golgi elements with increase in their numbers and irregular elongation of the microvilli above the Golgi elements, suggesting that the secretion system of the BD110-expressing cells was not disturbed but rather was activated (Fig. 2*d*). Indeed, the secretion level in culture medium of another tumor-associated antigen, CA19-9, was enhanced 14-fold after the induction of pBD110 (data not shown). Such enhanced function of the secretion system is typically observed in the cells of signet ring cell carcinomas (32). Immunostaining of pBD110 with anti-myc antibody revealed that this protein localized in the plasma membrane and the vacuole membrane, implicating the location of the targets of PI 3-kinase in these cells. Other HCC2998- or MKN45-derived cell lines capable of expressing pBD110 showed the same phenotypes after expression of pBD110 (data not shown). These BD110-expressing cells could be maintained for over 3 months without marked change in the morphology and growth rate. No such effects were observed in the cells expressing pBD110X or pBD110E or after infection with the control adenovirus (Fig. 1*e*). These results suggest that activation of PI 3-kinase can convert differentiated adenocarcinomas to more dedifferentiated ones, such as signet ring carcinoma, *in vitro*.

To further characterize the change of cell properties induced by the expression of pBD110, ability of colony formation in soft agar was tested. As shown in Fig. 3*a*, cells expressing BD110 formed significant numbers of colonies (61–108 colonies from 300 cells plated) as compared with parental cells (2–8 tiny colonies from 300 cells plated) in both cell lines. In addition, the BD110-expressing cells formed large colonies with grape-like morphology, indicating invasion of cells into the surrounding agar, whereas parental cells formed round colonies (Fig. 3*b*).

The change of cell characteristics accompanied by pBD110 expression also was investigated *in vivo*. HCC2998/BD110 and MKN45/BD110 cells with or without expression of pBD110 were subcutaneously xenotransplanted into nude mice. All of these cells developed tumors with similar growth rates regardless of expression of pBD110, however, their growth patterns disclosed by histological examination were quite different depending on the expression of pBD110 (Table 1, Fig. 3*c*). In the tumors formed by HCC2998/BD110 and MKN45/BD110 cells without expression of pBD110, most of the cells proliferated in papillary or tubular structures. Occasionally, well defined lumen formation was observed, indicating preservation of cell polarity and the ability to form organized tissue-like structures. On the contrary, the BD110-expressing cells proliferated more diffusely with alveolar patterns and infiltration into the surrounding fibrous tissues. No lumen formation was observed, indicating loss of polarity. These results suggest that activation of PI 3-kinase can induce a dedifferentiation in adenocarcinomas. We could not detect clear metastasis in BD110-expressing cells. However, the mice transplanted with the cells expressing pBD110 lost weight and died within an average of 48 days; this is a life span more than 2 months shorter than observed in those transplanted with the control cells. This result suggests that the BD110-expressing cells were more malignant than the parental cells. It is possible that this could be caused by the production of proinflammatory cytokines, such as tumor necrosis factor α, that cause cachexia.

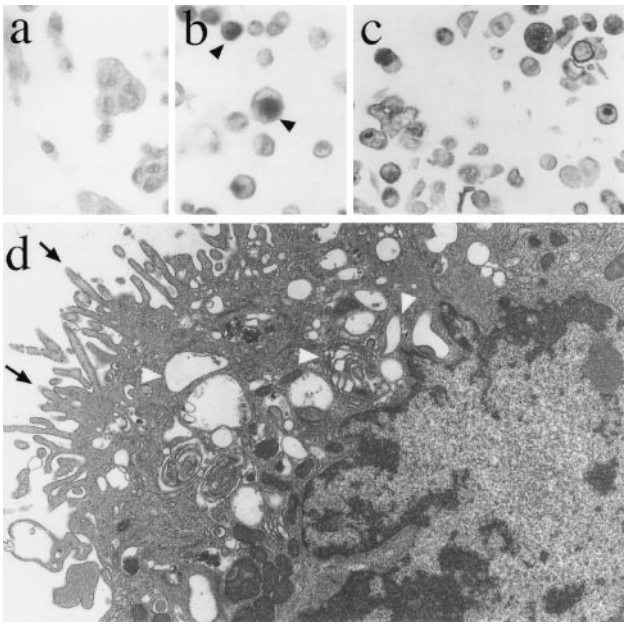

FIG. 2. Characterization of the signet ring-like cells formed by the expression of pBD110 in HCC2998/BD110 cells. (*a* and *b*) PAS staining of mucinous substance in the pBD110-expressing cells (*b*) or control cells (*a*). HCC2998/BD110 cells were cultured on a coverslip for 3 days after infection with AxCANCre, fixed with 4% paraformaldehyde, and PAS stained. Eccentric nuclei and large droplets containing diastase-resistant PAS-positive substance can be seen in the signet ring-like cells (indicated by an arrowheads). (*c*) Intracellular localization of a secretory glycoprotein antigen, CA15–3. Cells prepared as above were embedded in paraffin and the section was stained with anti-CA15–3 antibody. CA15–3 antigen was detected in various sized vacuoles in cytoplasm as well as the entire cell surface. Various cross sections of the cells were observed. (*d*) Electron micrograph of a signet ring-like cell found in the BD110-expressing cells. HCC2998/BD110 cells cultured for 3 days after AxCANCre infection were used to prepare the section. A typical signet ring-like cell was photographed. Dilation of the Golgi apparatus was observed from perinuclear to the submembranous regions (indicated by arrowheads). Irregular elongation of microvilli was localized above the dilated Golgi apparatus (indicated by arrows). (Original magnification, ×4000.)

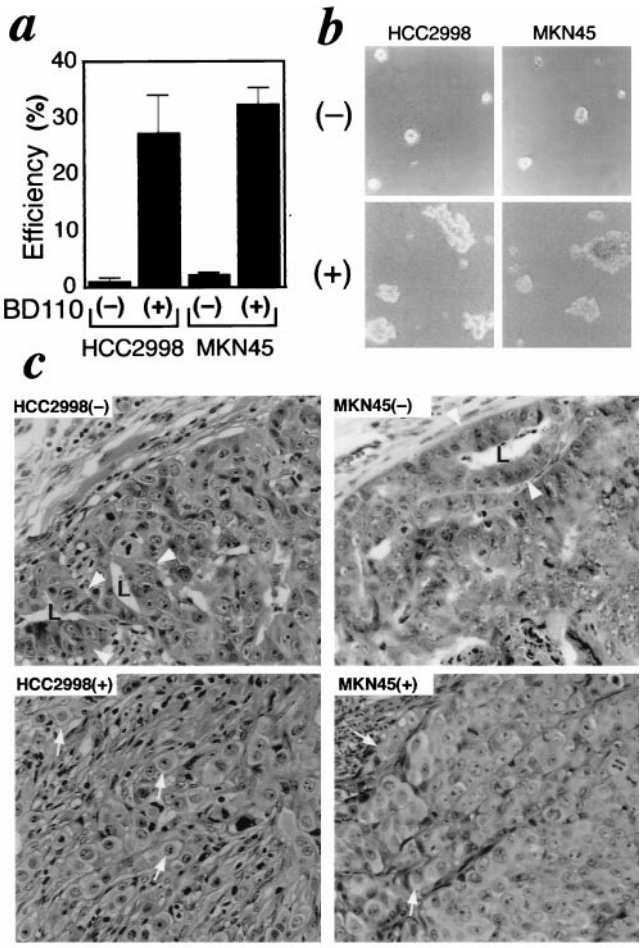

FIG. 3. Conversion of HCC2998 and MKN45 cells to a more malignant, invasive type after expression of constitutively active PI 3-kinase. (*a*) Elevation of colony-forming efficiency in soft agar after expression of constitutively active PI 3-kinase. Three hundred cells of HCC2998/BD110 and MKN45/BD110 before and after induction of BD110 were embedded in a medium containing 0.4% agarose. After two weeks, the numbers of colonies over 0.5 mm in diameter were scored in two independent experiments each with duplicate dishes. (*b*) Representative colonies formed by active PI 3-kinase-expressing cells or their parental cells. (*c*) Constitutively active PI 3-kinase-expressing cells formed malignant, poorly differentiated carcinomas showing high invasiveness in nude mice. HCC2998/BD110 cells or MKN45/BD110 cells were transplanted into subcutaneous tissue of nude mice before or 40 days after the infection with AxCANCre. Cells without pBD110 expression preserved their intestinal cell nature and polarity as shown by the presence of papillary or tubular structures (arrowheads) with lumen (L, *Upper*), in which the presence of mucus was detected by the PAS reaction (data not shown). In contrast, the BD110-expressing cells (shown by arrows) in the tumors lost their polarity and proliferated diffusely in solid or trabecular patterns. Stromal fibroblasts proliferated among the cancer cells. The histological features of the xenotransplants are summarized in Table 1.

These findings urged us to test whether PI 3-kinase was activated in natural dedifferentiated carcinomas, especially in signet ring cell carcinomas. Activation of PI 3-kinase is considered to be mediated by binding to tyrosine phosphorylated proteins including tyrosine kinases or their substrates. We examined PI 3-kinase activities in the immunoprecipitates of anti-phosphotyrosine antibodies from various gastric cell lines including some signet ring carcinoma cells. As shown in Fig. 4*a*, significant levels of PI 3-kinase activity was detected in the immunoprecipitates from signet ring cell carcinoma cell lines KATOIII and NUGC-4. Those immunoprecipitates from other gastric tumor cell lines exhibited much lower activities. Immunoblot analysis using an antibody reactive to all of the p85 family (α p85$^{\text{PAN-UBI}}$) revealed that the major p85s found in these cells were p85α and p85β and that other species of p85 were almost undetectable in the cell lysates and in the anti-phosphotyrosine immunoprecipitates (data not shown). We next investigated the existence of tyrosine phosphorylated molecules binding to PI 3-kinase. We used CA3 mAb, which recognizes p85α and p85β, or AB6, an anti-p85α antibody, as an anti-PI 3-kinase antibody thereafter. When the phosphotyrosine-containing proteins in CA3 immunoprecipitates were examined, a protein with the molecular mass of 200 kDa was detected reproducibly in both KATOIII and NUGC-4 (Fig. 4*b*). The two bands are the same proteins because V8 protease peptide mapping gave identical patterns (data not shown). We therefore tested other signet cell carcinomas for the presence of the 200-kDa protein (p200). p200 was detected in four of six signet ring cell lines but not in seven other carcinoma cell lines, suggesting that PI 3-kinase is indeed activated in some signet ring cell lines by binding to p200 (Fig. 4*b*). Because p200 of HSC39 and HSC58 cells were difficult to handle because of the strong protease activity or the heterogeneous nature of the cell line, NUGC4 and KATOIII cells were mainly used for the further study. Although we could not detect the activation of PI 3-kinase in some signet ring cell lines such as HSC-45 and HSC-60, it is possible that some factors downstream of PI 3-kinase required for the formation of signet ring cell carcinomas may be activated in other ways.

Table 1. Effects of constitutively active PI 3-kinase-expression in the transplanted cells on the properties of tumors formed in nude mice

Cells	BD110 expression	Incidence of tumor formation	Proliferation	Cell polarity
HCCC2998	−	3/3	expansive growth, tubular, partially papillary	+
	+	3/3	invasive growth, alveolar or trabecular	−
MKN45	−	3/3	expansive growth, tubulopapillary	+
	+	3/3	invasive growth, alveolar or trabecular	−

NUGC-4 and KATOIII cells were fractionated into membrane and cytosol, and the localization of p200 was examined. As shown in Fig. 3*c*, p200 was exclusively found in the membrane fractions. Consistent with the localization of p200, PI 3-kinase in these cell lines was almost exclusively distributed in the membrane fraction, whereas that of other gastric tumor cell lines was almost equally distributed in the two fractions. Because PI 3-kinase has been shown to be activated when targeted to the membrane by addition of a myristoylation signal, it is likely that relocalization of PI 3-kinase to the membrane is one of the activation mechanisms (29, 33). P200 may be involved in recruiting PI 3-kinase to the membrane. It has been shown that PI 3-kinase bound to IRS-1, a substrate of the insulin receptor, exhibits higher specific activity than the free PI 3-kinase (34). We tested whether the activity of PI 3-kinase bound to the phosphotyrosine-containing proteins, including p200 was higher than the free enzyme. As shown in Fig. 4*d*, PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates, which contained p200–PI 3-kinase complex, was ≈3-fold higher than that of CA3 immunoprecipitates, which contained a mixture of free and p200-bound PI 3-kinase. Polyclonal anti-p85 antibody gave a similar result (data not shown). To further confirm the activation of PI 3-kinase, we tested whether the features downstream of PI 3-kinase were activated in these cell lines. As shown in Fig. 4*e*, phosphorylation of p38 MAP kinase was clearly elevated compared with two other cell lines, suggesting that PI 3-kinase was indeed activated in these cells. This result and recent studies suggest involvement of p38 in organization of actin cytoskeleton (35, 36). In contrast, no significant activation of PKB was detected, suggesting that activation of PI 3-kinase by binding to p200 did not activate all of the downstream molecules of PI 3-kinase. Taken together, these data suggest that PI 3-kinase is recruited to the membrane and activated by binding to p200 in some dedifferentiated adenocarcinomas and that this activation may contribute to the malignancy of these carcinomas, possibly through the p38 cascade. p200 was shown not to be K-sam or c-met, which had been suggested to be amplified in nondifferentiated gastric tumors (37) (data not shown). We also tested several other candidate proteins that might be phosphorylated on tyrosine, including erbB2 and IRS-1, but none of them corresponded to p200 (data not shown).

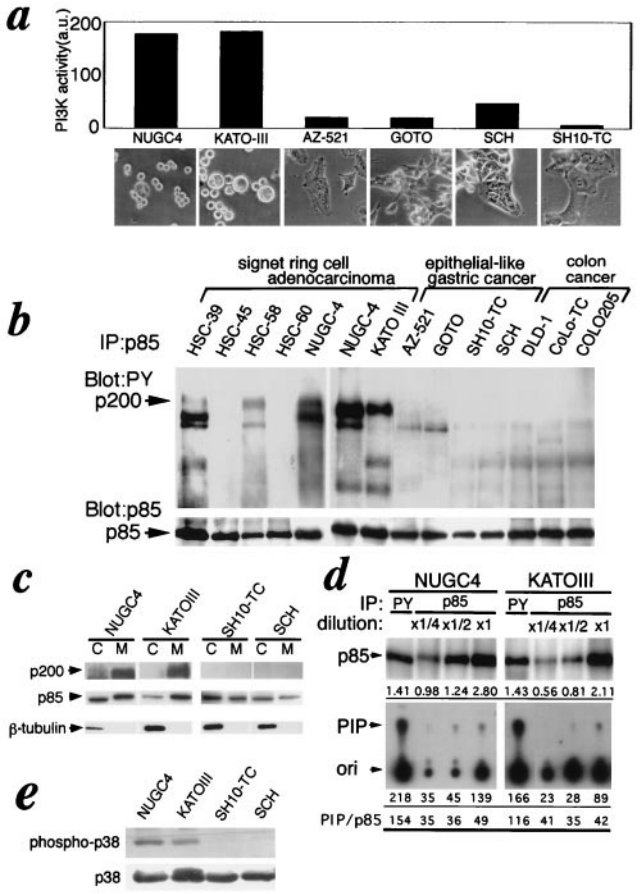

FIG. 4. Activation of PI 3-kinase in the native signet ring cell lines. (*a*) Signet ring cells exhibited relatively high PI 3-kinase activity in the anti-phosphotyrosine immunoprecipitates among gastric cancer cell lines. Phosphotyrosine-containing proteins were immunoprecipitated with anti-phosphotyrosine antibody, PY20, from various gastric tumor cell lines (the morphologies of the cell lines are shown on the abscissa). PI 3-kinase activity in the immunoprecipitates was analyzed with PI as a substrate. The radioactivity in the PI 3-P spots on TLC was quantified with BAS2000 imaging analyzer (Fuji). (*b*) Association of a 200-kDa protein phosphorylated on tyrosine with PI 3-kinase in signet ring cell lines. PI 3-kinase was immunoprecipitated with AB6 from various gastric tumor cell lines. The blots of precipitated proteins were probed for phosphotyrosine-containing proteins (PY20, *Upper*) or p85 α (AB6, *Lower*). (*c*)Membrane localization of PI 3-kinase bound to the 200-kDa protein in the signet ring cells. Cells were fractionated into cytosol (C) and membranes (M). The levels of the 200-kDa protein bound to PI 3-kinase (*Upper*) in anti-p85 immunoprecipitates and of total PI 3-kinase (*Lower*) in total lysates of each fraction were analyzed by using Western blotting probed for phosphotyrosine and p85, respectively. (*d*) Elevation of specific activities of PI 3-kinase in anti-phosphotyrosine immunoprecipitates from signet ring cell lines. PI 3-kinase was immunoprecipitated with PY20 or CA3, which was shown to precipitate both p85α and p85β regardless of binding to the 200-kDa protein from the serial dilutions of the total lysates, and the PI 3-kinase activities in the immunoprecipitates were analyzed (*Lower*). The levels of p85 were analyzed by using Western blotting with CA3 (*Upper*). The numbers under each panel show the relative intensity of the p85 bands (*Upper*) and radioactivities of PI 3-P spots (*Lower*). The numbers under the bottom line show the relative specific activities of PI 3-kinase. (*e*) Activation of p38–MAP kinase in native signet ring cell lines. Phosphorylation of p38–MAP kinase in indicated cell lines was detected by using Western blotting for anti-phospho-p38 antibody (*Upper*). Total p38 was detected by anti-p38 antibody (*Lower*).

DISCUSSION

In this study, we demonstrate that PI 3-kinase is activated in some cell lines established from signet ring cell carcinomas and suggest that this activation may be involved in establishment of the tumors. Expression of constitutively active PI 3-kinase in HCC2998 cells or MKN45 cells did not affect the cell growth or tumorigenicity; however, these cells lost their polarity and cell–cell interactions and developed the secretion system characteristic of signet ring cells. Secretion of mucous substances may provide a good environment for the tumor cells to grow, whereas loss of cell–cell interactions will allow the cells to disseminate widely. Therefore, activation of PI 3-kinase gives the cells an aggressive nature more likely to kill the

animals. In this aspect, the PI 3-kinase gene is not an oncogene, but is a gene that confers on a tumor a more malignant phenotype. Our data suggest that PI 3-kinase in signet ring cells is activated by binding to p200. p200 is not β4 integrin, which has a molecular mass of $\approx$200 kDa and has been suggested to activate PI 3-kinase in collaboration with α6 integrin, by the criteria of mobility in SDS/PAGE and by reactivity to specific antibody (data not shown). However, the β4 integrin was indeed phosphorylated on tyrosine in cell lines such as KATOIII and NUGC-4, implying that the $\alpha 6\beta 4$ integrin could be involved in phosphorylation of p200 (data not shown). Of interest, p200 was found exclusively in the membrane fraction. Because we did not observe autophosphorylation in the immunoprecipitates of various anti-p85 antibodies or of anti-phosphotyrosine antibody under a variety of conditions (data not shown), p200 is unlikely to be a tyrosine kinase but instead a substrate of a tyrosine kinase such as IRS-1, which binds PI 3-kinase to activate it. These phosphotyrosine-containing protein–PI 3-kinase complexes other than those including receptor tyrosine kinases are usually fractionated in the cytosolic fraction. Therefore, membrane localization of p200 is unique as a protein that activates PI 3-kinase, suggesting that characterization of p200 may provide new insights into the role of PI 3-kinase.

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