Protein Kinase C α Associates with Phospholipase D_1 and Enhances Basal Phospholipase D Activity in a Protein Phosphorylation-Independent Manner in Human Melanoma Cells

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It is well known that phospholipase D plays a crucial part in the signal transduction of many types of cells, and is activated by protein kinase C α when cells are stimulated. To elucidate the role of phospholipase D in melanoma, the expression of phospholipase D_1 and protein kinase C α in primary and metastatic lesions of acral lentiginous melanoma and superficial spreading melanoma was investigated using immunohistologic techniques. In addition, the mechanism of regulation of phospholipase D_1 by protein kinase C α was examined in a human melanoma cell line HM3KO using an adenovirus-mediated gene transfer technique. Both phospholipase D_1 and protein kinase C α were strongly expressed in primary and metastatic lesions of superficial spreading melanoma. Conversely, in acral lentiginous melanoma lesions, the expression of these two proteins increased dramatically with tumor progression; the expression of both phospholipase D_1 and protein kinase C α was almost negative in the radial growth phase of primary acral lentiginous melanoma lesions, and increased synchronously in a progression-related manner in advanced acral lentiginous melanoma lesions, including vertical growth phase and metastatic lesions. Immunoprecipitation study showed that phospholipase D_1 and protein kinase C α are associated physiologically in resting melanoma cells. Further immunoprecipitation study using HM3KO cells after adenovirus-mediated simultaneous overexpression of phospholipase D_1 and protein kinase C α, or phospholipase D_1 and the kinase-negative mutant of protein kinase C α revealed that both protein kinase C α and the kinase-negative mutant of protein kinase C α are associated with phospholipase D_1 in melanoma cells in the absence of an external signal. Overexpression of protein kinase C α or the kinase-negative mutant of protein kinase C α in melanoma cells by the adenovirus vectors resulted in the enhancement of basal phospholipase D activity in a viral concentration-dependent manner. Furthermore, enhanced basal phospholipase D activity increased the *in vitro* invasive potential of HM3KO cells. These results suggest that upregulation of phospholipase D_1 and protein kinase C α plays a part in the progression of acral lentiginous melanoma from the radial growth phase to the vertical growth phase. The present results also suggest that protein kinase C α associates with phospholipase D_1 and enhances basal phospholipase D activity in a protein phosphorylation-independent manner in melanoma cells, which contributes to the cell's high invasive potential. ***Key words: invasion/melanoma/phospholipase D/phosphorylation/protein kinase. J Invest Dermatol 121:69–76, 2003***

Phospholipase D (PLD) is an enzyme that hydrolyzes phosphatidylcholine to generate a lipid mediator, phosphatidic acid. Phosphatidic acid and its dephosphorylated product diacylglycerol are thought to be important second messengers, and it has been recognized that PLD plays a crucial part in the signal transduction of many types of cells (Exton, 1999; Frohman *et al*, 1999; Liscovitch *et al*, 2000). PLD is activated by a variety of agonists through the heterotrimeric guanosine triphosphate-binding regulatory protein-coupled or tyrosine kinase type receptors, and its activation has been implicated in a broad range of physiologic responses, including proliferation, differentiation, secretion, and immune responses. In mammals, two major PLD genes have been identified, PLD1 and PLD2, both of which are expressed in many cell types (Meier *et al*, 1999). Of the PLD isoforms, the regulation mechanisms of PLD1 have been studied most extensively, and several activators of PLD1, including protein kinase C (PKC), small guanosine triphosphatases such as the adenosine diphosphate-ribosylation factor and Rho families, have been identified.

PKC is a multifunctional protein-serine/threonine kinase that plays an important part in the intracellular signaling of various

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Abbreviations: ALM, acral lentiginous melanoma; EMEM, Eagle's minimal essential medium; KN-PKCα, kinase-negative mutant of PKCα; PFU, plaque-forming unit; PKC, protein kinase C; PLD, phospholipase D; SSM, superficial spreading melanoma.

biologically active substances, and is generally regarded as the major intracellular receptor for tumor-promoting phorbol esters (Nishizuka, 1995). PKC comprises a family of related enzymes that are differentially expressed in a variety of tissues and cell types, and more than 10 PKC isoforms including α, βI, βII, γ, δ, ε, η, θ, ξ, λ, and μ have been identified in mammalian tissues. The members of the PKC family appear to have individual properties and show differential distribution in tissues and cells. Therefore, it has been speculated that each member of the family plays a distinct role in the processing and modulation of a variety of physiologic and pathologic responses to external signals.

The importance of PKC in the regulation of PLD1 has been implicated in studies showing that the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate stimulates PLD1 activity, and that PKC inhibitors suppress the activation of PLD1 in intact cells (Kiss, 1996; Exton, 1999; Frohman *et al*, 1999). Furthermore, of the PKC isoforms, the PKC α isoform (PKCα) has been shown to activate PLD1 when cells are stimulated (Eldar *et al*, 1993; Balboa *et al*, 1994; Mukherjee *et al*, 1996; Hammond *et al*, 1997). For this reason, the role of PKCα-mediated PLD activation in cells has generated great interest in recent years.

In order to investigate the possible involvement of PLD activation in tumor progression in melanoma, we have examined the expression of PLD1 and PKCα in primary and metastatic lesions of acral lentiginous melanoma (ALM) and superficial spreading melanoma (SSM) using immunohistologic techniques, and showed that the expression of these two proteins increases synchronously with tumor progression in ALM. Using an adenovirus-mediated gene transfer technique, we demonstrated that basal PLD activity in melanoma cells can be enhanced by PKCα through protein–protein interaction in the absence of cell stimulation. Furthermore, we demonstrated that the elevated basal PLD activity enhances the invasive potential of melanoma cells.

MATERIALS AND METHODS

Patients and tissue sections Twenty-one primary melanoma lesions were obtained from 12 male and nine female patients with an average age of 70 y (range: 47–88 y); four had stage I; eight stage II; five stage III; and four stage IV melanoma. All procedures involving human subjects were approved by the Review Boards of Kobe University Graduate School of Medicine and Kumamoto University School of Medicine, and all subjects provided written informed consent prior to entrance in the study. Tumor staging was based on the histopathologic TNM classification system (Beahrs *et al*, 1992). The lesions were ALM in 14 and SSM in seven patients. The mean thickness of the 21 primary lesions was 3.6 mm (range: 0.5–8.0 mm). Lesions were less than 1.5 mm thick in four patients, between 1.5 and 4.0 mm in eight, and more than 4.0 mm in nine. Four metastatic melanoma lesions were obtained from four male patients with an average age of 70.3 y (range: 63–78 y). Two lesions were obtained from each ALM and SSM case. Primary and metastatic melanoma lesions were obtained from patients who underwent surgery in the Department of Dermatology at Kumamoto University School of Medicine, Kumamoto, Japan. Tissue samples were processed within 15 min of their surgical removal. Each tumor sample was divided into two parts: (1) one half was fixed in 10% neutral buffered formaldehyde, processed, and paraffin embedded for routine histopathology, (2) the other half was snap frozen in liquid nitrogen and stored at −80°C.

Antibodies and immunohistochemical reagents The monoclonal antibody to PKCα and goat anti-sera to PLD1 were purchased from Santa Cruz Biotechnology (Santa Cruz, California). The DAKO EnVision+TM, peroxidase, mouse, and rabbit were purchased from DAKO Corporation (Carpinteria, California).

Immunohistochemistry Indirect immunoperoxidase staining of frozen tissue sections with antibodies was performed utilizing the DAKO EnVision+TM, peroxidase, mouse, and rabbit system following the manufacturer's instructions. Briefly, 4 μm thick cryostat sections were air-dried and fixed in absolute acetone for 5 min, and treated with 0.03% hydrogen peroxide to block endogenous peroxidase activity. The tissue sections were incubated with primary antibody for 45 min at room temperature and then incubated with EnVision+TM, peroxidase, mouse, and rabbit for 45 min at room temperature. 3,3′-diaminobenzidine tetrahydrochloride was used as the chromogen. Culture supernatants from the murine myeloma cell line P3-X63-Ag8.653 replaced primary antibodies to control for nonspecific staining. All sections were counterstained with Giemsa solution. The intensity of staining in each section was estimated independently by two observers and graded as: −, when no staining was detectable; +/−, weak; +, clearly positive; and ++, strongly positive.

Cell culture A human melanoma cell line HM3KO was established from a lymph node metastasis of a patient with nodular melanoma as described previously (Sasase *et al*, 1989). Melanoma cell lines of WM115, WM239A, and WM1205Lu were kindly provided by Dr Meenhard Herlyn (The Wistar Institute, Philadelphia, Pennsylvania). All melanoma cell lines were cultured in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal calf serum. Normal human melanocytes from neonatal Caucasian foreskins were obtained from Kurabo (Osaka, Japan) and were maintained in Medium 154 supplemented with 0.5% fetal bovine serum, 0.4% bovine pituitary extract, 3 ng recombinant human basic fibroblast growth factor per mL, 0.5 μM hydrocortisone, 5 μg bovine insulin per mL, 5 μg human transferrin per mL, and 3 μg heparin per mL.

Adenovirus vectors The full length rat PLD1 cDNA (Park *et al*, 1997) was cloned into the pCMV5 vector with a FLAG-epitope tag to make the amino-terminal FLAG-epitope-tagged PLD1 (FLAG-PLD1). The recombinant adenovirus vectors of rabbit PKCα (Ohno *et al*, 1987), rabbit PKCα kinase-negative mutant in which Lys 368 in the adenosine triphosphate-binding site is replaced by Arg (KN-PKCα) (Ohno *et al*, 1990), and FLAG-PLD1 were prepared as described (Oka *et al*, 2002). The adenovirus vectors containing PKCα, KN-PKCα, and FLAG-PLD1 were designated AxPKCα, AxKN-PKCα, and AxPLD1, respectively. The adenovirus carrying the β-galactosidase gene (LacZ) from *Escherichia coli* (Kanegae *et al*, 1995), which was kindly provided by Dr Izumu Saito (Tokyo University, Japan), was designated AxLacZ and used as a control virus.

PLD assay PLD activity was assayed by monitoring *in vivo* transphosphatidylation activity (Kiss, 1996). Cells in six-well culture dishes were infected with various doses (0–25 plaque-forming units (PFU)/cell) of adenovirus. One day after infection, cells were incubated with 1-[1-^{14}C]palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine (54.0 mCi per mmol, Amersham Pharmacia Biotech, Tokyo, Japan) (0.25 mCi/1 × 10^7 cells) for 16 h at 37°C in serum-free EMEM, and washed three times with phosphate-buffered saline. Then, cells were incubated (in a total volume of 1 mL) in the presence of 1% ethanol in serum-free EMEM for 30 min at 37°C. During the incubation, the reaction proceeded linearly. After incubation, the medium was removed by aspiration, and ice-cold methanol (400 μL) was added to each well. The cell debris was scraped into an Eppendorf tube (1.5 mL) and kept on ice, and a solution of $CHCl_3$/HCl (1:0.006, vol/vol) (400 μL) was added to each tube. Phase separation, lipid extraction, and thin layer chromatography analysis were carried out as described previously (Cockcroft, 1992). Radioactivity was quantitated using a Bioimaging analyzer (BAS, 2000; Fuji Film, Tokyo, Japan), and the PLD activity was expressed as the percentage of radioactivity in phosphatidylethanol relative to the total lipid radioactivity found in a given lane. Data are expressed as the mean ± SEM (n = 3).

Immunoprecipitation and immunoblot analysis Cells plated out into 10 cm tissue culture dishes were treated as for the PLD assay described above except that they were incubated in the absence of the radioactive lipid. In the case of immunoprecipitation of PKCα and KN-PKCα, cells were then washed three times with phosphate-buffered saline and lyzed in 20 mM Tris–HCl (pH 7.5) containing 1 mM ethylenediamine tetraacetic acid, 1 mM ethyleneglycol-bis-(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid, 10 mM 2-mercaptoethanol, 1% Triton X-100, 150 mM NaCl, 10 mM NaF, 1 mM sodium orthovanadate, and 50 μg phenylmethylsulfonyl fluoride per mL. The cell lysate was incubated for 3 h at 0 to 4°C with antibodies against PKCα (Santa Cruz Biotechnology). Then, protein A-Sepharose (Pharmacia Biotech, Uppsala, Sweden) (30 μL) was added to the mixture and incubated for 1 h at 0 to 4°C with constant mixing. In the case of the immunoprecipitation of FLAG-PLD1, cells were washed three times with phosphate-buffered saline and lyzed as above. The cell lysate was then incubated with anti-FLAG M2 affinity gel (30 μL) (Sigma, St Louis, Missouri) for 3 h with constant mixing. The immunoprecipitates were boiled in sodium dodecyl sulfate sample buffer, and proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred on to an Immobilon P membrane (Millipore, Bedford, Massachusetts). Immunoblot analysis was

carried out using either the anti-PKCα or anti-FLAG-epitope tag (Sigma) antibody as the primary antibody. An alkaline phosphatase-conjugated anti-mouse antibody (Promega, Madison, Wisconsin) was employed as the secondary antibody. The color reaction was carried out using 5-bromo-4-chloro-3-indoyl-phosphate and nitroblue tetrazolium as substrates as described (Konishi *et al*, 1997). Where indicated, the lysates from the melanoma cells and melanocytes were directly subjected to immunoblot analysis. Data shown are representative of three independent experiments.

***In vitro* invasion assay** Melanoma cell invasion was measured using a membrane invasion culture system (Albini *et al*, 1987) with a minor modification. Briefly, polycarbonate filters with a pore size of 8 μm were coated with basement membrane matrigel (Becton Dickinson Labware, Lincoln Park, New Jersey). The coated filters were placed in Boyden chambers, in the upper compartment of which 1×10^5 HM3KO cells were suspended in serum-free EMEM. In the lower compartment, fibronectin (25 μg per mL) (Calbiochem, Darmstadt, Germany) diluted with serum-free EMEM was added as a chemoattractant. After 72 h incubation, the filters were fixed in methanol and stained with Giemsa solution. The number of cells that had migrated to the lower surface of the filters was counted manually. Each assay was performed in triplicate.

Statistical analysis Student's t test was used for determining the statistical difference of *in vitro* invasive potential. Probability values less than 5% were considered significant.

RESULTS

Synchronous expression of PLD1 and PKCα in ALM and SSM lesions Expression of PLD1 and PKCα was examined in 21 cases of primary melanoma (ALM: 14 cases; SSM: seven cases), and four cases of metastatic melanoma (ALM origin: two cases; SSM origin: two cases). Expression of PLD1 and PKCα was almost negative in the radial growth phase of ALM lesions (**Fig 1*A*,*D***; **Tables I and III**), whereas expression of these proteins dramatically increased during the tumor progression of ALM lesions (**Fig 1*B*,*C*,*E*,*F***; **Tables I and III**). When the tumor thickness was less than 1.5 mm, both PLD1 and PKCα were negative in all cases. Expression of PLD1 and PKCα increased synchronously as the tumor thickness increased, and all specimens of primary ALM in which the tumor thickness was greater than 4 mm and of metastatic ALM lesions showed strong expression of the two proteins. In contrast, expression of PLD1 and PKCα was constantly strong in all stages of SSM lesions (**Tables II and III**). Immunoblot analysis of each protein was performed to compare the level of expression of PLD1 and PKCα in melanoma cells and normal melanocytes (**Fig 2**). Expression levels of both PLD1 and PKCα were higher in HM3KO, WM115, WM239A, and WM1205Lu melanoma cells than in normal melanocytes derived from three different donors.

PLD1 is physiologically associated with PKCα in melanoma cells As the results obtained from the immunohistochemical examination indicated that PLD1 and PKCα are synchronously expressed in a manner related to ALM tumor progression, and that both proteins are strongly expressed in SSM lesions, we hypothesized that PLD1 makes a complex with PKCα in melanoma cells. To test this hypothesis, the association between endogenous PLD1 and PKCα was examined in resting cultured melanoma cells. As shown in **Fig 3**, endogenous PKCα was clearly coimmunoprecipitated with endogenous PLD1 from HM3KO cells. Endogenous PLD1 from WM115, WM239A, and WM1205Lu melanoma cells was also associated with PKCα (data not shown). These results suggest that PLD1 is physiologically associated with PKCα in melanoma cells. In contrast, such association was not detected in normal melanocytes (**Fig 3**). To investigate the mechanism and significance of PLD1–PKCα association in melanoma cells in more detail, FLAG epitope-tagged PLD1 and PKCα were simultaneously overexpressed using adenovirus vectors in HM3KO cells. Molecules associated with PLD1 were analyzed by immunoprecipitation of PLD1 using anti-FLAG M2 affinity gel. **Figure 4** shows that PKCα is constitutively associated with FLAG-PLD1 in the absence of cell stimulation, and that this association increased concomitant with the level of expression of PKCα.

Overexpression of PKCα induces the enhancement of basal PLD activity in melanoma cells To investigate the functional significance of the association of PLD1 and PKCα in melanoma cells, basal PLD activity in HM3KO cells overexpressing PKCα was examined. Basal PLD activity was strongly potentiated by the infection of AxPKCα in a concentration-dependent manner (**Fig 5**). Infection of HM3KO cells with the control adenovirus AxLacZ did not alter basal PLD activity even at the maximal PFU used in this study (data not shown).

Kinase-negative mutant of PKCα (KN-PKCα) induces the enhancement of basal PLD activity and associates with PLD1 in melanoma cells To examine whether the enhancement of the basal PLD activity by PKCα requires the protein phosphorylation function of PKCα, KN-PKCα was introduced into HM3KO cells using an adenovirus vector, and the basal PLD activity was measured. As shown in **Fig 6**, basal PLD activity was enhanced by the infection of AxKN-PKCα in a concentration-dependent manner. The basal PLD activity at each PFU of AxKN-PKCα was approximately the same as in the case of AxPKCα at the corresponding PFU. Association of KN-PKCα with PLD1 was investigated by immunoprecipitation study after coinfection with AxPLD1 and AxKN-PKCα (**Fig 7**). Similar to wild-type PKCα, KN-PKCα associates with FLAG-PLD1 in the absence of cell stimulation, and this association increased concomitant with the expression of KN-PKCα.

Elevated basal PLD activity enhances the invasive potential of melanoma cells To investigate the functional significance of elevated basal PLD activity in melanoma cells, the effect of increased basal PLD activity on the invasive potential of HM3KO cells was examined using an *in vitro* invasion assay. As shown in **Fig 8**, the results showed that HM3KO cells with elevated basal PLD activity had significantly enhanced invasive potential compared with control cells infected with only AxLacZ.

DISCUSSION

In this study we examined the expression of PLD1 and its activator PKCα in human melanoma tissues, and found that the expression of both PLD1 and PKCα increases synchronously and in a manner related to the tumor progression of ALM lesions. In contrast, expression of these proteins is constantly strong in SSM lesions irrespective of tumor stage. We also showed that PLD1 is physiologically associated with PKCα in melanoma cells, and that PKCα associating with PLD1 increases basal PLD activity by a protein phosphorylation-independent mechanism. As it has generally been thought that PLD is activated only upon stimulation of cells, our finding that PLD activity in cells can be enhanced by PKCα in the absence of an external signal is novel. Furthermore, we demonstrated that enhanced PLD activity induces high invasive potential in melanoma cells. Collectively, it is assumed that PLD1 is associated with PKCα in melanoma cells, resulting in the enhancement of basal PLD activity and contributing to the cells' high invasive potential.

The marked difference in the expression of PKCα and PLD1 between ALM and SSM during tumor progression may reflect the potential role of these enzymes in the progression of the two types of melanoma. The significantly higher expression of PLD1 and PKCα in the vertical growth phase than in the radial growth phase of ALM suggests that these proteins play an important part in the progression of the disease in ALM. Conversely, constant strong expression of both PLD1 and PKCα in all stages of SSM

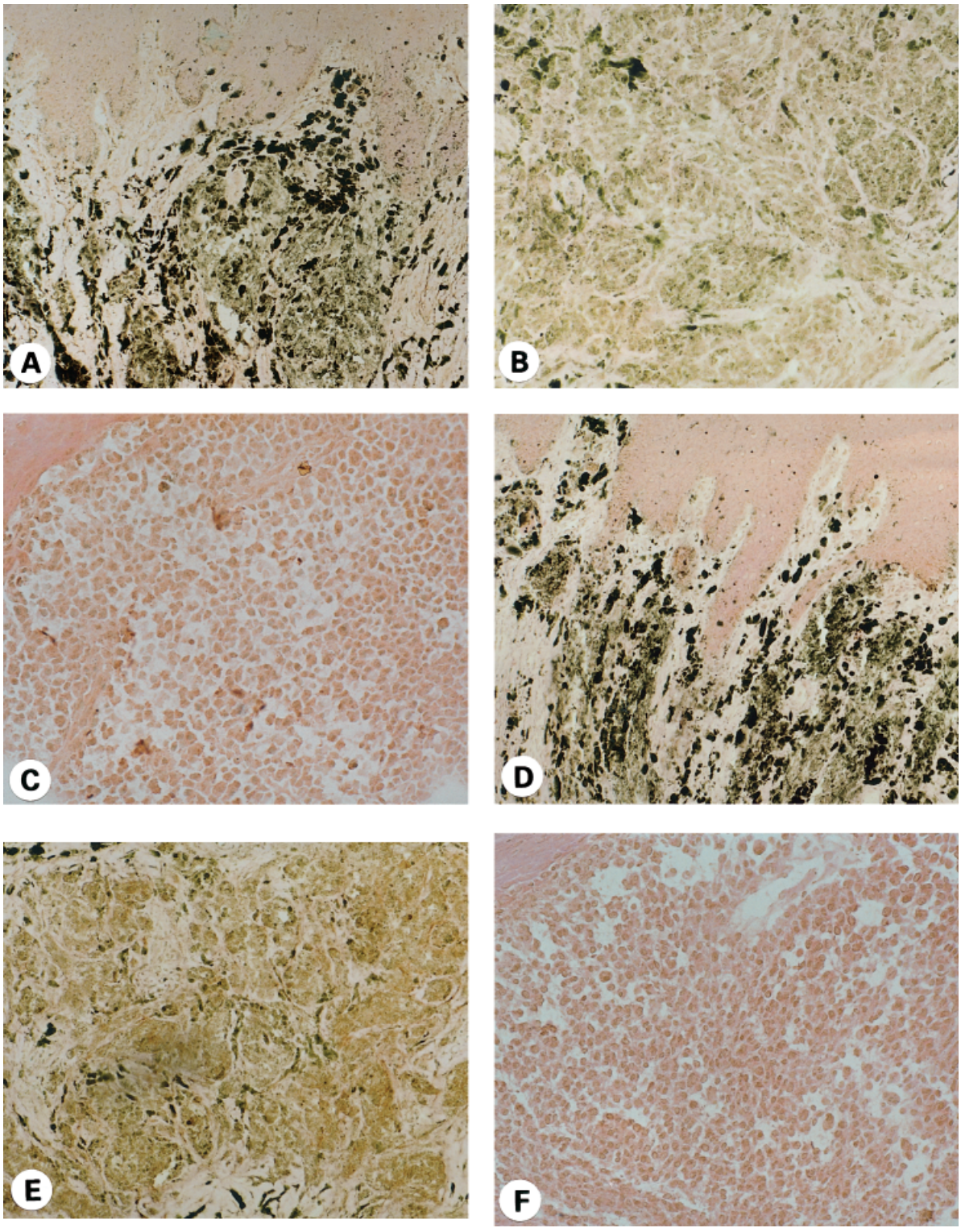

Figure 1. Immunohistochemical staining of ALM lesions with anti-PLD1 antibody (*A–C*) and anti-PKCα monoclonal antibody (*D–F*). Representative examples of staining of the radial growth phase of primary ALM (*A,D*), the vertical growth phase of primary ALM (*B,E*) and metastatic ALM lesions (*C,F*) with anti-PLD1 antibody and anti-PKCα monoclonal antibody are shown. The cytoplasm of melanoma cells in the vertical growth phase and in the metastatic lesions was stained homogeneously with anti-PLD1 antibody and anti-PKCα monoclonal antibody, whereas melanoma cells in the radial growth phase were not stained. Original magnification: (*A,B,D,E*) 100; (*C,F*) 150.

suggests that PLD1 and PKCα are not involved in the tumor progression of SSM, although these enzymes may contribute to the malignant phenotype of SSM. The present data and previous results showing that the expression levels of melanoma associated antigens and integrin αvβ3 are different in ALM and nodular melanoma lesions (Kageshita *et al*, 1991, 2000) indicate that the biologic properties of ALM are distinct from other types of melanoma.

Table I. Relationship between the expression of PKCα and PLD1 and the thickness of primary ALM lesions

		Radial growth phase		Vertical growth phase	
Patient no.	Thickness (mm)	PKCα	PLD1	PKCα	PLD1
1	0.5	−	NT[a]	NT	NT
2	1.0	−	NT	NT	NT
3	1.5	−	−	−	−
4	1.6	+ +	+	+ +	+
5	1.8	−	NT	+ +	NT
6	2.0	−	NT	+	NT
7	3.0	−	−	+ +	+ +
8	3.5	−	−	+	+
9	3.5	NT	NT	+ +	NT
10	3.5	−	−	+ +	+ +
11	5.5	NT	NT	+	NT
12	5.5	NT	NT	+ +	+ +
13	6.0	NT	NT	+/−	+/−
14	8.0	NT	NT	+	+

[a]Not tested.

Table II. Relationship between the expression of PKCα and PLD1 and the thickness of primary SSM lesions

Patient no.	Thickness (mm)	PKCα	PLD1
1	1.5	+	+
2	1.6	+	+
3	4.5	+ +	+ +
4	4.5	+ +	+ +
5	4.8	+ +	NT[a]
6	5.0	+ +	+ +
7	6.0	+ +	+ +

[a]Not tested.

Table III. Summary of the expression of PKCα and PLD1 in primary and metastatic lesions of ALM and SSM

Tissue	PKCα	PLD1
RGP[a] in ALM	1/9[b]	1/5
VGP in ALM	11/12	7/8
SSM	7/7	7/7
MM from ALM	2/2	2/2
MM from SSM	2/2	2/2

[a]Abbreviations used: RGP, radial growth phase; VGP, vertical growth phase; MM, metastatic melanoma
[b]Results are indicated as number of positive lesions/number of tested lesions.

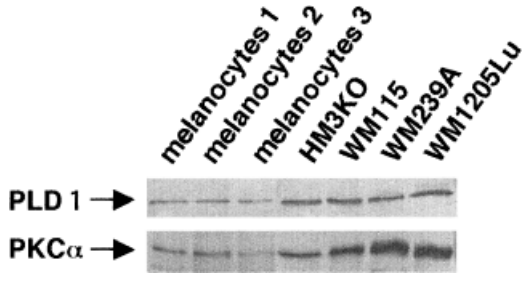

Figure 2. Expression of PLD1 and PKCα in normal human melanocytes and human melanoma cell lines. Equal amounts of total cell lysates of normal human melanocytes derived from three different donors (melanocytes 1–3) and human melanoma cell lines HM3KO, WM115, WM239A, and WM1205Lu were subjected to immunoblot analysis using antibodies against either PLD1 or PKCα.

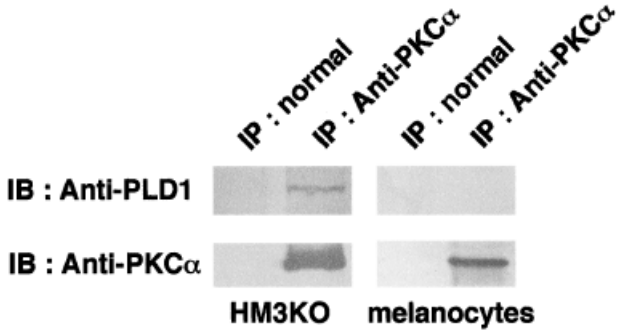

Figure 3. Physiologic association of PLD1 with PKCα in HM3KO cells. HM3KO cells and normal melanocytes were serum-starved for 16 h. Equal amounts of proteins from total cell lysates were immunoprecipitated (IP) with anti-PKCα antibody (Anti-PKCα) followed by immunoblot analysis (IB) using antibodies against either PLD1 (Anti-PLD1) or PKCα (Anti-PKCα). Immunoprecipitation with normal mouse IgG (normal) was carried out as the control experiment.

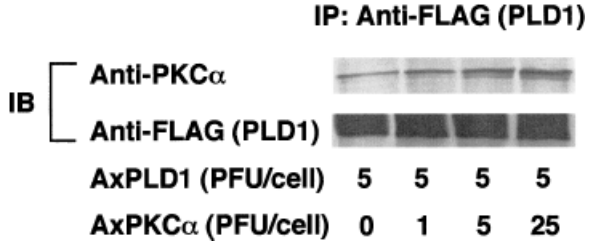

Figure 4. Association of PLD1 with PKCα in HM3KO cells. HM3KO cells were infected with various doses of AxPKCα and AxPLD1 simultaneously, and 24 h after the infection, serum-starved for 16 h. The cell lysate was subjected to immunoblot analysis (IB) using antibodies against PKCα (Anti-PKCα) and FLAG-epitope (Anti-FLAG) after immunoprecipitation (IP) with anti-FLAG M2 affinity gel. Data presented are a typical result of three independent experiments.

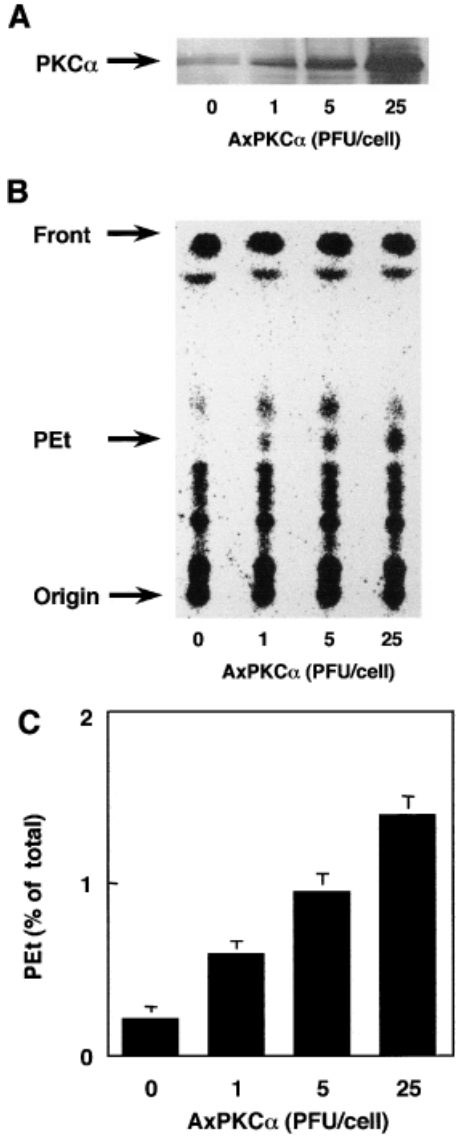

Figure 5. Enhancement of basal PLD activity by the overexpression of PKCα in HM3KO cells. HM3KO cells were infected with various doses of AxPKCα, and 24 h after the infection, serum starved for 16 h, and then incubated in serum-free EMEM containing 1% ethanol for 30 min after which PLD activity was assayed. (*A*) The expression of PKCα was examined by immunoblotting of cell lysates 2 d after infection. (*B*) Representative thin layer chromatography analysis. (*C*) PLD activity was expressed as the percentage of radioactivity in phosphatidylethanol relative to the total lipid radioactivity found in a given lane. Data are expressed as the mean ± SEM. (n = 3).

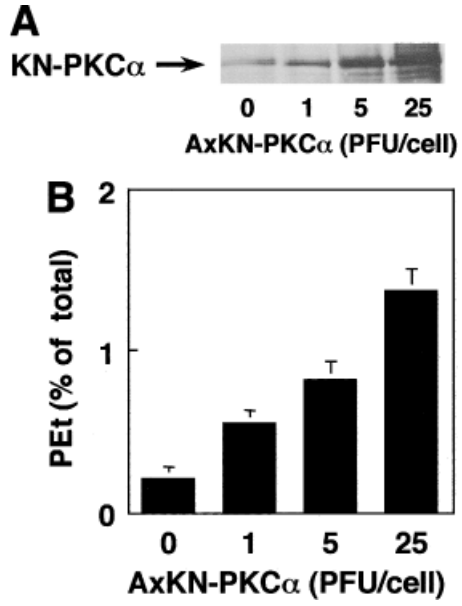

Figure 6. Enhancement of basal PLD activity by the overexpression of KN-PKCα in HM3KO cells. HM3KO cells were infected with various doses of AxKN-PKCα, and 24 h after the infection, serum starved for 16 h, and then incubated in serum-free EMEM containing 1% ethanol for 30 min after which PLD activity was assayed. (*A*) The expression of KN-PKCα was examined by immunoblotting of cell lysates 2 d after infection. (*B*) PLD activity was expressed as the percentage of radioactivity in phosphatidylethanol relative to the total lipid radioactivity found in a given lane. Data are expressed as the mean ± SEM. (n = 3).

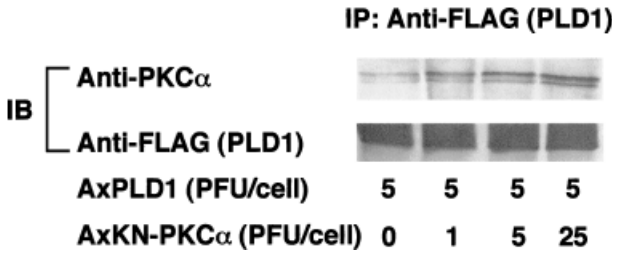

Figure 7. Association of PLD1 with KN-PKCα in HM3KO cells. HM3KO cells were infected with various doses of AxKN-PKCα and AxPLD1 simultaneously, and 24 h after the infection, serum starved for 16 h. The cell lysate was immunoprecipitated (IP) with anti-FLAG M2 affinity gel and then subjected to immunoblot analysis (IB) using antibodies against PKCα (Anti-PKCα) and FLAG-epitope (Anti-FLAG). Data presented are a typical result of three independent experiments.

We have shown that the PLD1 protein level in melanoma cells is higher than that in normal melanocytes. It has been shown that several types of cancer cells express elevated PLD protein levels compared with normal cells (Carnero *et al*, 1994; Uchida *et al*, 1997, 1999; Noh *et al*, 2000; Zhao *et al*, 2000). Also, it has been shown that PLD cooperates with epidermal growth factor receptor to transform rat fibroblasts (Lu *et al*, 2000). These results strongly suggest that PLD is involved in the malignant transformation of cells.

We were not able to detect the association between PLD1 and PKCα in normal melanocytes. We cannot exclude the possibility, however, that the lack of association between PLD1 and PKCα in normal melanocytes is due to the low levels of endogenous expression of both PLD1 and PKCα in these cells.

We have demonstrated that high PLD basal activity enhances the *in vitro* invasive potential of melanoma cells. Several reports have indicated that PLD is involved in the process of tumor cell metastasis, including invasion. It has been shown that exogenously added PLD (Imamura *et al*, 1993) and PLD-derived phosphatidic acid (Wakelam *et al*, 1997) have an invasion-inducing activity in cancer cells. It has been demonstrated that activation of PLD induces the secretion of metalloproteinase-9, which is important for cancer cells in the metastatic spread, from fibrosarcoma cells (Williger *et al*, 1999) and breast cancer cells (Wakelam *et al*, 1997). It has been reported that the expression of CD44, which participates in cell adhesion and migration, and is important for the metastatic ability of human melanoma cells (Birch *et al*, 1991; Ahrens *et al*, 2001), is regulated by PLD pathways in murine carcinoma cells (Ladeda *et al*, 1998). In addition, it has

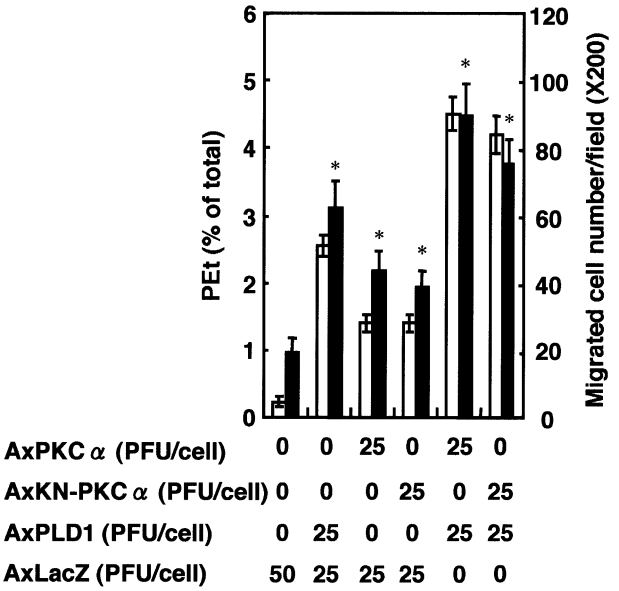

Figure 8. Analysis of the involvement of PLD activity in the invasion of HM3KO cells. HM3KO cells were infected with either AxPLD1, AxPKCα, AxKN-PKCα, or AxLacZ. Two days after the infection, PLD activity (open bar) and invasion activity (solid bar) were assayed. Data are expressed as the mean ± SEM (n = 3). *Differs from control cells (AxPKCα = 0, AxKN-PKCα = 0, AxPLD1 = 0, AxLacZ = 50) by Student's t test.

been suggested that PKCα increases the hematogenous metastasis of human (Dennis *et al*, 1998) and murine (La Porta and Comolli, 1997) melanoma cells. These reports, including present data, suggest that PLD and PKCα play an important part in tumor cell invasion and metastasis. It has been shown that PLD1 and PKCα are involved in the tumor progression of cells in other ways. PLD is involved in the acquisition of multidrug resistance by cancer cells (Fiucci *et al*, 2000). Also, it has been reported that activation of PKCα in NIH3T3 fibroblasts induces the transcription of vascular endothelial growth factor, which is generally believed to play an important part in the angiogenesis of solid tumors (Finkenzeller *et al*, 1992).

We have shown that overexpression of PKCα results in the enhancement of basal PLD activity. Recently, however, it has been reported that basal PLD activity is regulated by the expression level of PLD1 rather than its regulators such as PKCα, Rac1, and adenosine diphosphate-ribosylation factor 6, and that overexpression of PKCα had little effect on basal PLD activity in the rat basophilic leukemic cell line RBL-2H3 (Powner *et al*, 2002). The differential effect of PKCα on basal PLD activity may stem from the different cell systems used. It is attractive to assume that the upregulation of basal PLD activity by its association with PKCα is a function of the malignant nature of melanoma cells. Further studies are required to investigate this possibility.

Concerning the mechanism of agonist-induced, PKCα-mediated PLD activation, conflicting data show phosphorylation-dependent (Lopez *et al*, 1995; Colley *et al*, 1997; Min *et al*, 1998; Morash *et al*, 1998; Houle and Bourgoin, 1999) and phosphorylation-independent (Conricode *et al*, 1992; Singer *et al*, 1996; Hammond *et al*, 1997) activation of PLD by PKCα. As the present results indicate that basal PLD activity is enhanced by both wild-type PKCα and KN-PKCα to similar levels when the expression level of each protein was the same, we concluded that enhancement of basal PLD activity by PKCα is induced in a phosphorylation-independent manner. It is also possible, however, that PKCα and KN-PKCα enhance basal PLD activity by sequestering an inhibitor of PLD, as several inhibitors of PLD have been identified in cells (Exton, 1999).

Recently, the possible involvement of other PKC isoforms in the regulation of PLD1 has been reported (Hornia *et al*, 1999; Kiss *et al*, 1999). In addition, it has also been shown that PLD2 is regulated by various PKC isoforms (Colley *et al*, 1997; Siddiqi *et al*, 2000; Slaaby *et al*, 2000; Han *et al*, 2002). As it has been suggested that PKC plays an important part in differentiation (Gruber *et al*, 1992; Oka *et al*, 1993; Park *et al*, 1993), proliferation (Brooks *et al*, 1993; Arita *et al*, 1994; Oka *et al*, 1995; La Porta *et al*, 2000), and transformation (Linnenbach *et al*, 1988; Yamanishi *et al*, 1991; Oka *et al*, 1996) in pigment cells, studies on the physiologic relevance of the diverse effects of PKC isoforms in the regulation of PLD1 and PLD2 will contribute to an understanding of the biologic properties of pigment cells. Further studies, including identification of the downstream molecular targets of PLD activation, will provide important insights into the pathophysiologic role of PLD in pigment cells.

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