Focal adhesion kinase (p125^{FAK}) and paxillin are substrates for sphingomyelinase-induced tyrosine phosphorylation in Swiss 3T3 fibroblasts

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We examined the effect of sphingomyelinase on tyrosine phosphorylation of intracellular proteins in mouse Swiss 3T3 fibroblasts. Incubation of the cells with bacterial sphingomyelinase resulted in the elevation of tyrosine phosphorylation of multiple cellular proteins of 190, 130, 120, 97 and 70 kDa within minutes. The 120 and 70 kDa tyrosine-phosphorylated peptides were identified as p125 focal adhesion kinase (p125^{FAK}) and paxillin respectively by the use of specific antibodies against the proteins. Tyrosine kinase activity associated with anti-p125^{FAK} immunoprecipitate was stimulated by incubation of cells with sphingomyelinase. Cytochalasin D, which selectively disrupts the network

of actin filaments, inhibited sphingomyelinase-induced tyrosine phosphorylation of p125^{FAK} and elevation of tyrosine kinase activity in the anti-p125^{FAK} immunoprecipitates. Sphingomyelinase-induced phosphorylation of p125^{FAK} was not inhibited by wortmannin, an inhibitor of phosphatidylinositol 3-kinase. This was in sharp contrast with a wortmannin-sensitive phosphorylation of p125^{FAK} observed in platelet-derived growth factor (PDGF)-stimulated cells. Thus hydrolysis of sphingomyelin is considered to regulate the tyrosine kinase cascade including p125^{FAK} and paxillin by a mechanism distinct from PDGF.

INTRODUCTION

Metabolism of membrane phospholipids has been implicated as a key event in the signal transduction cascade regulating cellular proliferation and differentiation [1,2]. Recently the existence of a 'sphingomyelin cycle' was demonstrated in human myeloid HL-60 cells. The cellular sphingomyelin level is decreased transiently and the ceramide level is elevated concomitantly in response to inducers of monocytic cell differentiation such as vitamin D₃, tumour necrosis factor α and interferon γ . The addition of either bacterial sphingomyelinase or the cell-permeable analogues of ceramide results in monocytic differentiation of HL-60 cells. These observations suggest that the sphingomyelin cycle is a potential signalling pathway leading to cell differentiation [3,4]. Cellular sphingomyelinase has also been proposed as a regulatory enzyme of various cellular events such as apoptosis [5], prostaglandin E₂ production [6] and protein transport [7]. Humoral sphingomyelinase activities have been described in serum and urine [8,9]. Sphingomyelinase activity in serum decreases with maturation, and sphingomyelin is accumulated in the aging vessel walls. Thus some circulating sphingomyelinase could hydrolyse sphingomyelin on the surface of cellular elements in blood or on the vascular endothelial surface. These humoral sphingomyelinases are thought to play some roles in cell regulation. Olivera et al. [10] reported that bacterial sphingomyelinase and ceramide added to quiescent Swiss 3T3 cells stimulated DNA synthesis. We have found that ceramide can effectively potentiate the mitogenic action of growth factors such as platelet-derived growth factor (PDGF) and insulin [11]. These findings indicate that ceramide, which has been shown to inhibit the growth of yeast and mammalian cells [5,12], has a potency in stimulating cell proliferation.

Tyrosine phosphorylation of intracellular proteins is one of the key events involved in transmission of proliferative, developmental and oncogenic signals from membrane to nucleus [13–15]. A cytosolic protein tyrosine kinase, p125^{FAK}, has recently

been identified as a major substrate of tyrosine phosphorylation in growth factor-stimulated cells [16,17]. Tyrosine phosphorylation of p125^{FAK} is also observed after integrin stimulation and oncogenic transformation of cells [18]. Hence it follows that p125^{FAK} represents a point of convergence in the actions of growth factors, integrins and oncogenes [19]. In Swiss 3T3 cells, neuropeptides such as bombesin and vasopressin, which act as potent growth factors by stimulating G-protein-linked receptors that do not possess intrinsic tyrosine kinase activity, increase tyrosine phosphorylation of several cellular proteins, including p125^{FAK} [16,17]. Thus p125^{FAK} is a candidate for a tyrosine kinase that catalyses neuropeptide-induced tyrosine phosphorylation of cellular proteins, although the precise mechanism by which p125^{FAK} is tyrosine-phosphorylated and activated in intact cells is not well understood. A recent study with p125^{FAK}-deficient mice demonstrated that p125^{FAK} is involved in the turnover of focal adhesion contacts during cell migration [20].

Because little is known about the signal transduction pathway activated by increased hydrolysis of membrane sphingomyelin, we examined here protein tyrosine phosphorylation in sphingomyelinase-treated Swiss 3T3 cells. We observed that sphingomyelinase and ceramide stimulated tyrosine phosphorylation of several proteins, including p125^{FAK} and paxillin. We also found that tyrosine kinase activity associated with p125^{FAK} was increased after cell treatment with sphingomyelinase or ceramide. Our results suggest that p125^{FAK} and paxillin might participate in the signal transduction pathway initiated by sphingomyelin hydrolysis.

MATERIALS AND METHODS

Materials

[γ -³²P]ATP and ¹²⁵I-labelled Protein A were purchased from DuPont NEN. Monoclonal antibody against p125^{FAK} was from

Abbreviations used: LPA, lysophosphatidic acid; p125^{FAK}, p125 focal adhesion kinase; PDGF, platelet-derived growth factor; PI, phosphatidylinositol. ‡ To whom correspondence should be addressed.

UBI. Monoclonal mouse anti-paxillin and anti-phosphotyrosine (PY20) antibodies were from Zymed. Rabbit polyclonal anti-phosphotyrosine antibody was from Chemicon. Anti-mouse IgG agarose beads were purchased from American Quarlex. Sphingomyelinase (*Streptomyces aureus*), ceramide (bovine brain) and lysophosphatidic acid (oleoyl) were from Sigma. Raytide was obtained from Oncogene Science.

Cell culture

Swiss mouse 3T3 fibroblasts were grown in 100 mm Corning tissue culture dishes under a humid atmosphere containing 5% CO₂. The culture medium used was Dulbecco's modified Eagle's medium supplemented with 100 i.u./ml penicillin, 100 µg/ml streptomycin, 0.6 mg/ml glutamine and 5% (v/v) fetal calf serum. The cells were seeded into 100 mm Corning tissue culture dishes at a density of 10⁵ cells per dish and grown in the medium for 4–6 days. Confluent cultures of the cells (approx. 2.4 × 10⁶ cells per dish) were used for each experiment.

Immunoprecipitation

The cell monolayers were rinsed with Krebs–Ringer Hepes buffer, pH 7.4 (KRH), and warmed to 37 °C. The composition of KRH was 10 mM Hepes, 130 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgCl₂, 1.5 mM CaCl₂, 5 mM glucose and 0.2% fatty acid-free BSA. The cells were treated with sphingomyelinase or ceramide in 5 ml of KRH at 37 °C. The incubation was terminated by placing the dishes on ice and quickly aspirating the medium. After being rinsed twice with ice-cold Tris-buffered saline, the cells were lysed on ice with 800 µl of lysis buffer [10 mM Tris/HCl, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 µM Na₃VO₄, 1% (w/v) Triton X-100, 1% (w/v) BSA, and 1 mM PMSF, pH 7.4]. The lysates were centrifuged at 10000 g for 20 min at 4 °C and precleared by incubation with anti-mouse IgG agarose beads at 0 °C for 1 h. After brief centrifugation, the supernatants were transferred to a new tube, and incubated at 0 °C for 1 h with anti-phosphotyrosine (PY20), anti-p125^{FAK} or anti-paxillin antibodies and then for 1 h with the further addition of 5 mg of anti-mouse IgG agarose beads. The immunocomplex was subjected to sequential washing with lysis buffer followed by lysis buffer containing no BSA. Each washing was repeated twice.

Western blotting

Immunoprecipitates prepared as described above were heated at 100 °C for 5 min in 40 µl of sample buffer containing 1% SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.02% (w/v) Bromophenol Blue, and 62.5 mM Tris, pH 6.8. Solubilized peptides were then subjected to SDS/PAGE [7.5% (w/v) gel]. The gels were electroblotted on to Immobilon membranes (Millipore) by using a semi-dry electroblotting system. The membranes were blocked for 1 h with 5% BSA in Tris-buffered saline (TBS; 20 mM Tris/HCl, pH 8.0, containing 100 mM NaCl) and incubated for 1 h with 1 µg/ml polyclonal rabbit anti-phosphotyrosine antibody in TBS supplemented with 5% BSA and 0.1% (w/v) NaN₃. The membranes were washed four times (5 min each) with TBS containing 0.1% Nonidet P40 (TBSN) and incubated for 1 h with ¹²⁵I-rabbit anti-mouse IgG (0.2 µCi/ml) in TBS supplemented with 5% BSA and 0.1% NaN₃. The membranes were washed three times with TBSN and once with TBS, and subjected to analysis for associated radioactivity with a Fuji BAS2000 Bioimaging Analyser.

Kinase assay in vitro

This was performed by the method of Zachary et al. [21]. Briefly, immunoprecipitates prepared as described above were washed twice with kinase buffer (50 mM Hepes, pH 7.5, 0.1 mM EDTA, 75 mM NaCl, 0.03% (v/v) Brij 35), and resuspended in 20 µl of kinase buffer containing 5 µg of Raytide. Reactions were initiated by the addition of 10 µl of ATP mixture {330 µM [γ -³²P]ATP (1.1 µCi/µl) and 33 mM MgCl₂}. After incubation at 30 °C for 20 min, reactions were stopped by adding 30 µl of 330 µM ATP and cooling on ice. Immunoprecipitates were collected by centrifugation, and the supernatants were applied to P-81 ion exchange chromatography paper, which was washed thoroughly before being dried and counted in a scintillation counter.

RESULTS

Effect of sphingomyelinase and ceramide on tyrosine phosphorylation

We first examined protein tyrosine phosphorylation in Swiss 3T3 cells treated with sphingomyelinase. Lysate from the cells was incubated with specific antibody against phosphorylated tyrosine residue, and the immunoprecipitates were analysed by Western blotting with the same antibody. Incubation of quiescent Swiss 3T3 cells with *S. aureus* sphingomyelinase resulted in enhanced tyrosine phosphorylation of multiple proteins of 190, 130, 120, 97 and 70 kDa (Figure 1A). The phosphorylation was detectable within 1 min of the addition of sphingomyelinase, peaked at 5 min, and persisted for at least 20 min (results not shown, but see Figure 2 for p125^{FAK} and paxillin). This time course was similar to those of sphingomyelin hydrolysis and ceramide formation obtained previously in the same cells [11]. Treatment of the cells with sphingomyelinase from *Bacillus cereus* also induced tyrosine phosphorylation of the same set of proteins (results not shown).

To investigate whether the tyrosine phosphorylation induced by sphingomyelinase was the result of sphingomyelin hydrolysis, we next examined the effect of ceramide, one of the products of sphingomyelinase. Ceramide was able to induce the same pattern of tyrosine phosphorylation as sphingomyelinase (Figure 1B).

Figure 1 Sphingomyelinase-induced tyrosine phosphorylation in Swiss 3T3 cells

(A) Quiescent Swiss 3T3 cells were untreated (lane 1) or treated with 1 unit/ml sphingomyelinase for 2.5 min (lane 2) or 5 min (lane 3). (B) The cells were incubated in the absence (lane 1) or presence of 10 µM (lane 2) and 30 µM (lane 3) bovine brain ceramide for 5 min. (C) The cells were untreated (lane 1) or treated with 1 µg/ml of LPA for 2.5 min (lane 2) or 5 min (lane 3). Tyrosine-phosphorylated proteins were then precipitated by anti-phosphotyrosine and analysed by Western blotting using the same antibody. The molecular masses (kDa) of the molecular size standards are indicated.

Figure 2 Sphingomyelinase-induced tyrosine phosphorylation of p125^{FAK} and paxillin in Swiss 3T3 cells: dose response and time course

Quiescent 3T3 cells were treated with various concentrations of sphingomyelinase for 5 min (A and C) or with 1 unit/ml sphingomyelinase for various times (B and D), lysed and immunoprecipitated with anti-p125^{FAK} (FAK; A and B) or anti-paxillin (C and D) before analysis by anti-phosphotyrosine Western blotting. Each result is representative of an experiment performed at least three times.

Phosphatidylcholine-specific phospholipase C, which catalyses the hydrolysis of phosphatidylcholine to diacylglycerol and phosphorylcholine, did not cause tyrosine phosphorylation of p125^{FAK} and paxillin (results not shown). The result indicates that disturbance of lipid bilayer construction is not itself sufficient to cause a sphingomyelinase-triggered cellular event, and also that hydrolytic formation of phosphorylcholine from sphingomyelin may not be involved in the induction of tyrosine phosphorylation.

Lysophosphatidic acid (LPA) is a biologically active phospholipid known to stimulate tyrosine phosphorylation in Swiss 3T3 cells. As shown in Figure 1(C), LPA induced tyrosine phosphorylation of 130, 120, 97 and 70 kDa proteins, whose mobilities were similar to those phosphorylated on sphingomyelinase treatment. Unlike sphingomyelinase, however, the lipid did not induce tyrosine phosphorylation of the 190 kDa peptide.

Sphingomyelinase stimulates tyrosine phosphorylation of p125^{FAK} and paxillin

Several groups have recently observed that p125^{FAK} and paxillin are tyrosine-phosphorylated in response to mitogens, including LPA, in Swiss 3T3 fibroblasts [22,23]. This prompted us to examine whether p125^{FAK} and paxillin can serve as substrates for sphingomyelinase-induced tyrosine phosphorylation.

Cell lysate was incubated with either anti-p125^{FAK} or anti-paxillin antibodies, and the immunoprecipitates were probed with anti-phosphotyrosine. Tyrosine phosphorylation of p125^{FAK} was increased markedly and rapidly after sphingomyelinase treatment (Figures 2A and 2B). The increase was 4–5-fold after a 5 min exposure of the cells to 1 unit/ml enzyme, and the level persisted for at least 20 min. The lowest effective concentration of sphingomyelinase was approx. 0.03 unit/ml under the conditions. When cell lysate from which p125^{FAK} had been precipitated by anti-p125^{FAK} was subjected to analysis by anti-

Figure 3 Inhibition by cytochalasin D of tyrosine phosphorylation of p125^{FAK}

Quiescent Swiss 3T3 cells were incubated for 2 h in the absence (None) or presence of 2 μ M cytochalasin (CytD) and then treated for the indicated times with 1 unit/ml sphingomyelinase (A), 30 μ M ceramide (B) or 1 μ g/ml LPA (C). The cells were lysed and anti-p125^{FAK} immunoprecipitate was analysed by anti-phosphotyrosine Western blotting. The results shown are representative of two separate experiments.

phosphotyrosine immunoblotting, the 120 kDa phosphorylated peptide disappeared totally and specifically (results not shown).

Paxillin is a focal adhesion-associated protein that co-localizes with p125^{FAK}. Tyrosine phosphorylation of paxillin was also increased by sphingomyelinase treatment as judged by anti-phosphotyrosine immunoblotting analysis of the anti-paxillin immunoprecipitates (Figures 2C and 2D). After sphingomyelinase addition, not only was the intensity of the immunoreactive band enhanced but also characteristic diffusion of the band was observed. Tyrosine phosphorylation of paxillin showed similar kinetics and concentration-dependence to those of p125^{FAK}.

Cytochalasin D inhibits sphingomyelinase-induced tyrosine phosphorylation of p125^{FAK}

Tyrosine phosphorylation of p125^{FAK} and paxillin in response to many growth factors has been shown to depend on the integrity of the actin cytoskeleton. Thus we next examined the effect of cytochalasin D, which disrupts the network of actin microfilaments, on sphingomyelinase-induced phosphorylation of p125^{FAK}.

Treatment of the cells with 2 μ M cytochalasin D for 2 h completely prevented sphingomyelinase-induced tyrosine phosphorylation of p125^{FAK} (Figure 3A). Ceramide-induced phosphorylation of p125^{FAK} was also inhibited by cytochalasin D (Figure 3B). The effect of LPA, which stimulates tyrosine phosphorylation of p125^{FAK} to a level similar to that induced by sphingomyelinase (Figure 3C), was inhibited by the treatment with the agent, in agreement with a previous report [22]. Treatment of the cells with a high concentration of PDGF (30 ng/ml), which is known to disrupt the actin cytoskeleton and to inhibit LPA-stimulated tyrosine phosphorylation of p125^{FAK} [22], inhibited the effects of sphingomyelinase and ceramide (results not shown). These results indicate that sphingomyelinase and ceramide induce tyrosine phosphorylation of p125^{FAK} through a similar mechanism to that of LPA and other growth factors that require the integrity of actin cytoskeleton.

Sphingomyelinase stimulates tyrosine kinase activity in anti-p125^{FAK} immunoprecipitates

We next investigated the effect of sphingomyelinase on the tyrosine kinase activity of p125^{FAK}. Swiss 3T3 cells were treated

Figure 4 Sphingomyelinase-induced increase in tyrosine kinase activity of anti-p125^{FAK} immunoprecipitate

Quiescent cultures of Swiss 3T3 cells were treated with 1 unit/ml sphingomyelinase for various times before cell lysis and immunoprecipitation with anti-p125^{FAK}. The activity of the immune complex in tyrosine-phosphorylating Raytide was measured. Each point is the mean of duplicate determinations.

Table 1 Effect of cytochalasin D on activation by sphingomyelinase and LPA of tyrosine kinase activity associated with p125^{FAK}

Quiescent Swiss 3T3 cells were incubated for 2 h in the absence or presence of 2 μ M cytochalasin D (CytD) and then treated for 5 min with 1 unit/ml sphingomyelinase (SMase) or 1 μ g/ml LPA. After cell lysis and immunoprecipitation with anti-p125^{FAK}, the activity of the immune complex in tyrosine phosphorylation of Raytide was measured. Results are shown as fold increase over the control (no additions) and are the means $\pm$ S.E.M. from three separate experiments.

Activator	CytD added	
	None	2 μ M
None	1	0.81 $\pm$ 0.22
SMase	2.70 $\pm$ 0.18	0.86 $\pm$ 0.11
LPA	2.24 $\pm$ 0.07	1.14 $\pm$ 0.17

with 1 unit/ml sphingomyelinase for various lengths of time before preparation of anti-p125^{FAK} immunoprecipitates. Tyrosine kinase activity associated with the immune complex was assessed by its ability to phosphorylate Raytide, an exogenous peptide substrate for tyrosine kinases. As shown in Figure 4, treatment of intact cells with sphingomyelinase caused time-dependent elevation of tyrosine kinase activity in the anti-p125^{FAK} immunoprecipitate. The activity reached a maximum within 5 min of stimulation by sphingomyelinase, and sustained this level for up to 20 min (Figure 4). When the cells were treated with 30 μ M ceramide for 5 min there was 1.9-fold increase in tyrosine kinase activity in the immunoprecipitates (results not shown). The immune complex prepared with control mouse IgG contained a weak phosphorylating activity towards Raytide, which remained unchanged after sphingomyelinase treatment of the cells (results not shown).

As shown in Table 1, treatment of cells with cytochalasin D

Figure 5 Failure of phosphorylation of p125^{FAK} *in vitro* to stimulate tyrosine kinase activity of anti-p125^{FAK} immunoprecipitate

(A) Quiescent Swiss 3T3 cells were incubated in the absence (lanes 1 and 3) or presence of 1 unit/ml sphingomyelinase (lanes 2 and 4) for 5 min before cell lysis and immunoprecipitation with anti-p125^{FAK}. The immunoprecipitates were then incubated with 0.1 mM ATP and 10 mM MgCl₂ at 30 °C for 20 min before Western blotting analysis for tyrosine-phosphorylated peptides (lanes 3 and 4). Control incubation was performed with 10 mM MgCl₂ in the absence of ATP (lanes 1 and 2). (B) The immunoprecipitate prepared and treated with ATP as described above was washed and determined for activity in phosphorylating Raytide. Each point is the mean of duplicate determinations. Similar results were obtained in a repeated experiment. MW, molecular mass in kDa.

slightly reduced tyrosine kinase activity in anti-p125^{FAK} immunoprecipitate prepared from it. The agent completely blocked sphingomyelinase-induced stimulation of the tyrosine kinase activity. LPA treatment of the cells increased the kinase activity *in vitro* to a level comparable with that induced by sphingomyelinase. The effect of the lipid was also inhibited by cytochalasin D. These results show a good correlation between tyrosine phosphorylation of p125^{FAK} *in vivo* and the activity of p125^{FAK} *in vitro*.

Incubation of the anti-p125^{FAK} immune complex from control cells with 0.1 mM ATP resulted in a marked tyrosine phosphorylation of p125^{FAK} (Figure 5A). Preparation of p125^{FAK}, which had been phosphorylated by cell treatment with sphingomyelinase, could be further tyrosine-phosphorylated by incubation with ATP. No tyrosine-phosphorylated peptide other than p125^{FAK} was observed in the immune complex even after the ATP treatment, regardless of whether or not the cells from which the immune complex was prepared had been treated with sphingomyelinase. Autophosphorylation of p125^{FAK} *in vitro* did not accompany increased tyrosine kinase activity of the immune complex (Figure 5B).

Failure of sphingomyelinase to activate phosphatidylinositol 3-kinase

Phosphatidylinositol (PI) 3-kinase catalyses the phosphorylation of inositol phospholipids at the 3-position of the inositol ring. Stimulation of several growth factor receptors with tyrosine kinase activity is known to increase cellular activity of the lipid kinase. A specific association of p125^{FAK} with the regulatory subunit of PI 3-kinase (p85) has been reported in NIH 3T3 cells. We therefore examined the functional correlation between p125^{FAK} and PI 3-kinase.

Figure 6 Functional correlation between phosphatidylinositol 3-kinase and tyrosine phosphorylation of p125^{FAK}

(A) Quiescent Swiss 3T3 cells were incubated for 2 h in the absence (lanes 1–3) or presence (lanes 4–6) of 2 μ M cytochalasin D (CytD). The cells were then incubated without (lanes 1 and 4) or with 1 unit/ml sphingomyelinase for 5 min (lanes 2 and 5) or 6 ng/ml PDGF for 10 min (lanes 3 and 6) before cell lysis and anti-phosphotyrosine immunoprecipitation. The phosphatidylinositol 3-kinase activity of the immune complex was measured. (B) Quiescent Swiss 3T3 cells were incubated for 10 min without (lanes 1–3) or with (lanes 4–6) 0.5 μ M wortmannin. The cells were then incubated in the absence (lanes 1 and 4) or presence of 1 unit/ml sphingomyelinase for 5 min (lanes 2 and 5) or 6 ng/ml PDGF for 10 min (lanes 3 and 6) before cell lysis and immunoprecipitation with anti-p125^{FAK}. The immune complex was analysed by Western blotting analysis using anti-phosphotyrosine. The result shown is representative of two repeated experiments.

PDGF stimulated PI 3-kinase activity in Swiss 3T3 cells, as described previously (Figure 6A). The effect of the growth factor was not inhibited at all by cell treatment with cytochalasin D. No activation of PI 3-kinase occurred after stimulation by sphingomyelinase. PDGF at a concentration of 6 ng/ml increased tyrosine phosphorylation of p125^{FAK} to a similar level to that attained by sphingomyelinase (Figure 6B). Wortmannin, a potent inhibitor of PI 3-kinase [24,25], totally inhibited PDGF-induced phosphorylation of p125^{FAK}. In contrast, sphingomyelinase-induced phosphorylation of the tyrosine kinase was still observed after the treatment with wortmannin.

We attempted to detect tyrosine phosphorylation of phospholipase C γ and GTPase activating protein, which are known to be tyrosine-phosphorylated in response to PDGF in Swiss 3T3 cells [26]. However, neither served as a substrate for sphingomyelinase-induced tyrosine phosphorylation in Swiss 3T3 cells (results not shown).

DISCUSSION

Treatment of cells with sphingomyelinase brings about profound effects on their functions. However, little is known about the direct cellular events following hydrolysis of sphingomyelin. We and other groups have reported that two isoforms of mitogen-activated protein kinase, ERK-1 and ERK-2, are tyrosine-phosphorylated and activated in sphingomyelinase-treated cells [11,27]. Here we observed that two additional proteins, paxillin and p125^{FAK}, were tyrosine-phosphorylated by the treatment (Figure 2). Paxillin and p125^{FAK} are known to be phosphorylated after stimulation of fibroblasts with mitogens including PDGF, bombesin, vasopressin and LPA, and thus have been regarded as possible components through which the mitogens stimulated cell growth [26,28]. We observed previously that incubation of quiescent Swiss 3T3 cells with sphingomyelinase did not stimulate cell growth by itself but effectively potentiated the action of growth factors such as insulin and PDGF [11]. Thus our present results indicate that tyrosine phosphorylation of these proteins cannot itself trigger cell growth.

Sphingomyelinase hydrolyses cell surface sphingomyelin, which produces ceramide and phosphorylcholine. We showed that the same set of proteins were tyrosine-phosphorylated in sphingomyelinase-treated and ceramide-treated cells (Figure 1). The kinetics of sphingomyelinase-induced tyrosine phosphorylation (Figure 2B) was similar to that of ceramide formation observed previously in the cells [11]. Ceramide (a natural form) caused tyrosine phosphorylation of p125^{FAK}, although the effect was less pronounced than that induced by sphingomyelinase (Figure 3). A similar finding was obtained by Seufferlein and Rozengurt [29], who demonstrated that a cell-permeable analogue of ceramide and sphingosine induced an increase in the phosphotyrosine content of p125^{FAK}. Phosphorylcholine, another product of sphingomyelinase reaction, had no effect on tyrosine phosphorylation of p125^{FAK} and paxillin (results not shown). We cannot completely rule out the possibility that any action of sphingomyelinase other than phospholipase C activity plays a role in inducing tyrosine phosphorylation of intracellular proteins. However, the above findings suggest that the effect of the enzyme was mediated through hydrolysis of sphingomyelin and accumulation of ceramide or its metabolites in the cells.

It is known that p125^{FAK} is localized in focal adhesion contacts from which actin stress fibres emanate. Treatment of Swiss 3T3 cells with cytochalasin D leads to a complete disruption of the actin cytoskeleton and abolishes tyrosine phosphorylation of p125^{FAK} stimulated by bombesin and LPA [22,30]. Cytochalasin D inhibited sphingomyelinase-induced and ceramide-induced tyrosine phosphorylation of p125^{FAK} (Figure 3). PDGF at high concentration (30 ng/ml), which was previously reported to inhibit LPA-induced tyrosine phosphorylation of p125^{FAK} by disrupting the actin cytoskeleton [22], also prevented the sphingomyelinase-induced change (results not shown). Thus LPA, sphingomyelinase and ceramide seem to regulate p125^{FAK} by a common mechanism dependent on the integrity of the actin cytoskeleton.

In the present study we found for the first time that wortmannin, a potent inhibitor of PI 3-kinase [24,25], effectively inhibited PDGF-induced activation of p125^{FAK} (Figure 6). Wortmannin did not inhibit the effect of sphingomyelinase, which does not activate PI 3-kinase in spite of its profound effect on tyrosine phosphorylation in the cell. Although it remains to be determined whether the effect of wortmannin is the result of inhibition of PI 3-kinase, our results demonstrate that PDGF and sphingomyelinase regulate tyrosine phosphorylation of p125^{FAK} by different mechanisms.

We found that tyrosine kinase activity associated with a specific antibody against p125^{FAK} was increased by cell incubation with sphingomyelinase. The amount of tyrosine residue in p125^{FAK} that was phosphorylated in intact cells correlated well with the tyrosine kinase activity of anti-p125^{FAK} immunocomplex from the cells (Figures 2B and 4). Treatment of Swiss 3T3 cells with cytochalasin D, which blocked tyrosine phosphorylation of p125^{FAK}, completely inhibited the stimulation of the activity of tyrosine kinase in the immunocomplex *in vitro* (Table 1). These results suggest that sphingomyelinase increased the specific activity of p125^{FAK}. An alternative explanation for the elevated tyrosine kinase activity in the anti-p125^{FAK} immunocomplex is the co-precipitation of Src family tyrosine kinases. Four tyrosine residues within p125^{FAK}, Tyr-397, Tyr-407, Tyr-576 and Tyr-577, are known to be phosphorylated in mouse BALB/3T3 cells in an adhesion-dependent manner [31]. Tyr-397 has been proved to be a major site for autophosphorylation. It has also been shown that the autophosphorylated form of p125^{FAK} is able to form stable complexes with protein kinases such as Fyn and Src [32]. Although we could not detect tyrosine-phosphorylated proteins

except p125^{FAK} in the immunoprecipitates even after incubation of the preparation with ATP (Figure 5A), this possibility cannot be excluded at present.

In summary, the present study shows that sphingomyelinase can regulate the tyrosine kinase cascade including p125^{FAK} and paxillin in Swiss 3T3 cells, through the hydrolysis of sphingomyelin and the accumulation of ceramide. The activation of p125^{FAK} is dependent on the integrity of the actin microfilaments, as are many growth factors, but the mechanism of activation is found to be distinct from PDGF. Further study of the sphingomyelinase-specific mechanism will provide information on the physiological role of the 'sphingomyelin cycle'.

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