Permissive effect of ceramide on growth factor-induced cell proliferation

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Addition of bacterial sphingomyelinase to quiescent Swiss 3T3 cells effectively potentiated the platelet-derived growth factor (PDGF)-stimulated cell proliferation, though the enzyme by itself had little effect on the cell proliferation. Such potentiation of the cell growth could also be observed by the addition of ceramide, a product of the sphingomyelinase-catalysed reaction. In contrast, phosphocholine, another product of the enzyme reaction, had no synergistic effect on the action of PDGF. Treatment of the cells with sphingomyelinase or ceramide

increased the cellular activity of mitogen-activated protein kinases (MAP kinases), which have been implicated in the regulation of cell proliferation. However, the synergistic effect of sphingomyelinase on the PDGF-induced cell growth could still be observed even when the cellular MAP kinase activity was fully activated by the growth factor alone. These results indicate that a ceramide-mediated cellular event(s) other than the MAP kinase activation is potentially involved in the regulation of cell growth.

INTRODUCTION

Membrane lipids are now recognized as not only the structural constituent of cellular membranes but also the precursors of the second messengers generated following stimulation of cell-surface receptors. Increased metabolism of phospholipids induced by receptor-mediated activation of phosphoinositide-specific phospholipase C, phospholipase A₂ (PLA₂), phospholipase D and phosphatidylinositol 3-kinase has been recognized as a key event in the signal transduction cascade leading to the functional changes of cells [1]. Sphingomyelin hydrolysis is another potential signalling pathway mediating receptor-induced cellular events [2,3]. Activation of membrane receptors by agonists, such as tumour necrosis factor α [4,5], interleukin-1 [6,7], interferon γ [4], and nerve growth factor [8], stimulates cellular sphingomyelinase activity leading to the breakdown of sphingomyelin to phosphocholine and ceramide. Membrane-permeable short-chain ceramide induces the same biological actions as these agonists [4,8,9]. Furthermore, treatment of intact cells with bacterial sphingomyelinase mimics the effects of agents that stimulate endogenous sphingomyelinase [10,11]. Sphingomyelinase activities have also been described in serum and urine [12]. These humoral enzymes are expected to play some roles in cell regulation by hydrolysing sphingomyelin located on the outer leaflet of the membrane.

Most studies on the sphingomyelin pathway have focused on its role in regulation of cell differentiation. In one report, on the other hand, sphingomyelinase is shown to be an activator of cell growth [13]. In the present paper, we show that sphingomyelinase and its metabolite ceramide have little effect by themselves but effectively potentiate the growth factor-stimulated proliferation of Swiss 3T3 cells. Because little is known about the early intracellular events induced by sphingomyelinase, we have also examined the effect of sphingomyelinase on the intracellular activity of mitogen-activated protein kinase (MAP kinase), which is recognized to be a component common to signalling pathways

leading to cell proliferation and differentiation [14]. The results suggest that the potentiation of growth factor-stimulated proliferation is mediated by ceramide-induced cellular event(s) other than the MAP kinase activation.

MATERIALS AND METHODS

Materials

[¹⁴C]Serine, [¹⁴C]choline, [³H]thymidine, [³²P]P_i and [γ -³²P]ATP were purchased from DuPont-NEN. Recombinant human platelet-derived growth factor (PDGF)-BB and the monoclonal antibody against a peptide corresponding to a 21-amino-acid sequence near the C-terminus of MAP kinase were from Toyobo and Zymed respectively. Sphingomyelinase (*Streptomyces* sp.), ceramide (bovine brain), myelin basic protein (MBP), and fatty acid-free BSA were from Sigma. When the effects of ceramide on intact cells were examined, the lipid was dissolved in 2% decane/98% ethanol just before use, and an aliquot of the solution was added directly to the cell culture.

Cell culture

Swiss 3T3 fibroblasts were maintained in 100-mm-diam. Corning tissue-culture dishes under a humidified atmosphere containing 5% CO₂. The cells were subcultured in a culture medium (Dulbecco's modified Eagle's medium supplemented with 100 units/ml of penicillin, 100 μ g/ml of streptomycin, and 0.6 mg/ml of glutamine) containing 5% (v/v) fetal-calf serum (FCS). The subconfluent cells were arrested in G₀ phase by incubation in the same medium containing 0.2% FCS for 48 h before use.

Assay of DNA synthesis

3T3 cells were seeded into Corning 24-well tissue-culture plates at a density of 1.5×10^4 cells/well. The subconfluent culture (about 6×10^4 cells/well) was arrested and rinsed twice with the culture medium supplemented with 20 μ g/ml of BSA and 5 μ g/ml

Abbreviations used: MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein; PDGF, platelet-derived growth factor; FCS, fetal calf serum; EGF, epidermal growth factor.

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of transferrin. The cells were incubated for 1 h with or without various growth factors and then for 42 h with the further addition of [^3H]thymidine (2 μM , 1.6 $\mu\text{Ci}/\text{ml}$). After the medium was removed by aspiration, the cells were rinsed twice with 1 ml of PBS and then twice with 10% trichloroacetic acid. The insoluble material was dried and dissolved in 2% (w/v) NaHCO_3 and 0.1% SDS in 0.1 M NaOH in order that the radioactivities incorporated could be determined.

Measurement of sphingolipid metabolism

Metabolites of cellular sphingomyelin after sphingomyelinase treatment were examined by the method described by Olivera et al. [13]. Cells were seeded into 100-mm-diam. Corning tissue-culture dishes at a density of 1×10^5 cells/dish and grown for 2 days. Subconfluent cells were incubated for 24 h in the culture medium containing 0.2% FCS and for a further 24 h with the addition of [^{14}C]serine (0.1 $\mu\text{Ci}/\text{ml}$). The cell monolayers were washed with Hepes-buffered solution consisting of 10 mM Hepes/NaOH (pH 7.4), 130 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgCl_2 , 1.5 mM CaCl_2 , 5 mM glucose and 0.2% BSA. The cells were treated with 300 m-units/ml of sphingomyelinase in 5 ml of the same solution at 37 °C. The incubation was terminated by placing the dishes on ice and quickly aspirating the medium. The cells were scraped from the dish into 0.4 ml of 1% HClO_4 and then mixed with 0.9 ml of $\text{CHCl}_3/\text{methanol}$ (1:2, v/v). After being vigorously stirred, the mixture was added to 0.5 ml each of CHCl_3 and 1% HClO_4 . The organic phase was washed twice with a CHCl_3 -saturated solution containing 0.5 M NaCl and 1% HClO_4 . The lipids were saponified in 0.1 ml of 0.1 M methanolic KOH at 37 °C for 1 h to eliminate ester-containing phospholipids [13,15]. The samples were evaporated to dryness and dissolved in 20 μl of $\text{CHCl}_3/\text{methanol}$ (1:1, v/v) to be spotted on a silica gel plate (Merck, Silica Gel 60). Sphingolipids were resolved by sequential one-dimensional TLC using $\text{CHCl}_3/\text{benzene}/\text{ethanol}$ (80:40:75, by vol.) followed by $\text{CHCl}_3/\text{methanol}/25\%$ ammonia (65:25:5, by vol.) as solvents [13,16]. The radioactivities in the spots corresponding to ceramide and sphingomyelin were determined with a Fuji BAS2000 Bioimaging Analyser.

Measurement of MAP kinase activities

Quiescent 3T3 cell monolayers in 100-mm-diam. Corning culture dishes were rinsed twice with the Hepes-buffered solution described above. The cells were incubated at 37 °C for 10 min in 5 ml of the same buffer and then with the addition of growth factors. After the incubation for various lengths of time, the cells were rinsed twice with 5 ml of ice-cold PBS and once with 5 ml of the ice-cold extraction buffer containing 50 mM β -glycerophosphate (pH 7.3), 1.5 mM EGTA, 0.1 mM Na_3VO_4 , 1 mM dithiothreitol, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin, 2 $\mu\text{g}/\text{ml}$ pepstatin and 1 mM benzamidine. The cells were scraped into a total volume of ~ 0.5 ml of extraction buffer, sonicated for 10–30 s with a Branson Sonifier at half-maximal intensity, and then centrifuged at 100 000 g for 20 min at 2 °C. The supernatant was frozen at -70 °C and stored until required for use. No significant alteration of MAP kinase activity was noted after one freeze–thaw cycle. The cytosolic extract was diluted to 0.4 mg/ml, and an aliquot (25 μl) was mixed with 25 μl of the assay buffer containing 50 mM β -glycerophosphate (pH 7.3), 1.5 mM EGTA, 0.1 mM Na_3VO_4 , 1 mM dithiothreitol, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin, 2 $\mu\text{g}/\text{ml}$ pepstatin A, 1 mM benzamidine, 20 μM calmidazolium, 0.67 mg/ml MBP, 20 mM MgCl_2 , and 0.2 mM [$\gamma\text{-}^{32}\text{P}$]ATP (12 $\mu\text{Ci}/\text{ml}$). The mixture was incubated at 37 °C for 10 min before the aliquot (40 μl) was

spotted on to Whatman P-81 phosphocellulose filter paper which had been soaked in 17.5 mM H_3PO_4 . The filter paper was washed several times with 175 mM H_3PO_4 and counted for the trapped radioactivity. The levels of phosphate incorporation measured in the absence of substrate were subtracted from values obtained in the presence of substrate to correct for non-specific phosphorylation.

Renatured kinase assay in MBP-containing gel

The cytosolic extract prepared as described above was subjected to electrophoresis on an SDS/polyacrylamide gel (7.5%) containing 0.5 mg/ml MBP. The gel was incubated once in 50 mM Tris/HCl (pH 8.0) containing 20% 2-propanol for 15 min, twice in 50 mM Tris/HCl (pH 8.0) containing 5 mM 2-mercaptoethanol each for 30 min, and then twice in 50 mM Tris/HCl (pH 8.0)/5 mM 2-mercaptoethanol/6 M guanidine for 30 min each. The separated polypeptides were renatured by washing the gel for 16 h with four changes of 50 mM Tris/HCl (pH 8.0)/5 mM 2-mercaptoethanol/0.04% Nonidet P-40. The gel was incubated for 30 min in 40 mM Hepes (pH 8.0) containing 0.1 mM EGTA and 20 mM MgCl_2 , and for a further 1 h with the addition of 25 μM [$\gamma\text{-}^{32}\text{P}$]ATP (10 $\mu\text{Ci}/\text{ml}$). The gel was washed repeatedly with 5% (w/v) trichloroacetic acid containing 1% sodium pyrophosphate before being dried and analysed for the radioactivities using a Fuji BAS2000 Bioimaging Analyser.

RESULTS AND DISCUSSION

Sphingomyelinase-stimulated proliferation of quiescent fibroblasts

Proliferation of quiescent Swiss 3T3 fibroblasts, as measured by [^3H]thymidine incorporation, was stimulated by the addition of PDGF. The action of the growth factor was observed to be significant at 1 ng/ml and maximal at 30 ng/ml (Figure 1a) under the present conditions. Bacterial sphingomyelinase itself had little effect on the cell growth, even at a concentration as high

Figure 1 Synergistic stimulation of DNA synthesis by PDGF and sphingomyelinase in Swiss 3T3 cells

[^3H]Thymidine incorporation was measured in quiescent Swiss 3T3 cells as described in the Materials and methods section. (a) The cells were stimulated with increasing concentrations of PDGF in the presence (●) or absence (○) of 300 m-units/ml of sphingomyelinase. (b) The cells were stimulated with increasing concentrations of sphingomyelinase in the presence (●) or absence (○) of 30 ng/ml of PDGF. Each value represents the mean of duplicate determinations. Similar results were obtained in five additional experiments.

Figure 2 Breakdown of sphingolipid observed in the sphingomyelinase-treated Swiss 3T3 cells

Swiss 3T3 cells were labelled with [^{14}C]serine for 24 h before being treated with 300 m-units/ml of sphingomyelinase. After the treatment for the indicated times, radioactivities in the fractions of sphingomyelin (●) and ceramide (○) were determined as described in the Materials and methods section. Total (sphingomyelin plus ceramide fractions) radioactivities (□) were also shown in the Figure. A similar result was obtained in a replicate experiment.

as 3 units/ml (Figure 1b). However, the PDGF-stimulated proliferation was markedly increased by the simultaneous addition of the enzyme. The action of the enzyme was observed at levels as low as 100 m-units/ml and did not reach its maximum at 3 units/ml (Figure 1b). The synergistic activation of cell growth by sphingomyelinase could be observed even when the maximum concentration of PDGF (30 ng/ml) was used (Figure 1a). A similar effect of sphingomyelinase could be observed when cell growth was stimulated with insulin or lysophosphatidate instead of PDGF (results not shown). The action of sphingomyelinase was destroyed by boiling at 100 °C for 10 min (results not shown).

Stimulation of the sphingomyelin pathway has been reported, in several cell lines, to inhibit their growth [10,17–19], except in one report where sphingomyelinase treatment instead stimulates the proliferation of fibroblasts [13]. We have confirmed the latter finding, although we could not observe the stimulatory action of the enzyme alone. Instead, we showed that sphingomyelinase effectively potentiated the actions of growth factors. The difference could not be explained. However, it is fascinating to consider that the potentiating action of sphingomyelinase is so marked, especially when the higher concentrations of the enzyme were used (Figure 1b), that the enzyme treatment could make prominent the effect of the residuary trace amounts of growth factors in the culture medium.

Effects of the products of sphingomyelinase on the cell proliferation

The cells, which had been labelled with [^{14}C]serine, were treated with sphingomyelinase to be analysed for sphingolipid metabolism. The radioactivity in the sphingomyelin fraction was decreased, while that in the ceramide fraction was increased, by the treatment with sphingomyelinase. The changes were detected within 1 min and had almost reached an equilibrium after 5 min (Figure 2). Total radioactivity (sphingomyelin plus ceramide fractions) was increased slightly. Degradation of sphingomyelin

Figure 3 Effects of phosphocholine and ceramide on DNA synthesis in Swiss 3T3 cells

[^3H]Thymidine incorporation was measured in quiescent Swiss 3T3 cells as described in the Materials and methods section. (a) The cells were stimulated with increasing concentrations of phosphocholine in the presence (●) or absence (○) of 30 ng/ml of PDGF. (b) The cells were stimulated with increasing concentrations of ceramide in the presence (●) or absence (○) of 30 ng/ml of PDGF. Each value represents the mean of duplicate determinations. Similar results were obtained in a replicate experiment.

could also be observed when cellular lipids were labelled with [^{14}C]choline or [^{32}P]P_i (results not shown). Decrease in radioactivity after 5 min of exposure to sphingomyelinase was by about 40% regardless of the different labelling method. No significant change has been found in lipids other than sphingolipids, indicating that the main metabolites of sphingomyelin in 3T3 cells were ceramide and phosphocholine.

We next examined the direct effects of ceramide and phosphocholine on the cell growth (Figure 3). In the previous studies on the biological actions of ceramide, short-chain analogues of ceramide, C-2 ceramide and C-6 ceramide, have been used because of the very low solubility of the natural form [9,20]. Here we examined the effect of the natural form of ceramide by virtue of the use of an appropriate solvent (2% decane/98% ethanol) developed by Hirabayashi and colleagues [21]. The natural form of ceramide (from bovine brain) had no effect by itself but effectively potentiated the action of the maximum concentration of PDGF (Figure 3b). Short-chain analogues also potentiated the action of PDGF, although higher concentrations were necessary (results not shown). The effect of natural ceramide reached a maximum at a concentration of approx. 3 μM and was decreased at higher concentrations (Figure 3b). Treatment of the quiescent cells with 1 mM phosphocholine stimulated cell proliferation by itself (Figure 3a). Cell proliferation observed in the presence of PDGF was also increased by 1 mM phosphocholine. However, no synergistic interaction could be observed between PDGF and phosphocholine (Figure 3a). These results indicated that the treatment of quiescent 3T3 cells with sphingomyelinase produced ceramide, which in turn could potentiate the actions of growth factors.

Sphingosine, a deacylated metabolite of ceramide, is known to potentiate the growth factor-stimulated DNA synthesis in quiescent Swiss 3T3 cells at an appropriate concentration [22]. However, we could not observe any stimulatory effect of sphing-

Figure 4 Effect of sphingomyelinase on MBP kinase activity in Swiss 3T3 cells

Activity of cell extract to phosphorylate MBP was measured as described in the Materials and methods section. Before the preparation of cell extract, (a) quiescent Swiss 3T3 cells were treated with 1 unit/ml of sphingomyelinase for the indicated times, or (b) the cells were treated with increasing concentrations of the enzyme for 3 min. Each value represents the mean of duplicate determinations. Similar results were obtained in three additional experiments.

goline on cell growth under our conditions (results not shown). We have not succeeded in detecting the increased formation of sphingosine in sphingomyelinase-treated Swiss 3T3 cells in agreement with the result obtained by other authors [13]. Thus it is unlikely that the intracellular accumulation of sphingosine or its metabolite is mediating the ceramide-induced cellular events.

Figure 5 Effects of phosphocholine and ceramide on MBP kinase activity in Swiss 3T3 cells

Quiescent Swiss 3T3 cells were treated for 3 min with increasing concentrations of phosphocholine (a) or ceramide (b) before the activities of cell extracts to phosphorylate MBP were measured as described in the Materials and methods section. Each value represents the mean of duplicate determinations. Similar results were obtained in two additional experiments.

Figure 6 Effect of sphingomyelinase on MAP kinase activity in Swiss 3T3 cells

(a) Quiescent Swiss 3T3 cells were incubated for 3 min without (lane 1), or with 1 unit/ml of sphingomyelinase (lane 2), 30 ng/ml of PDGF (lane 3), or both (lane 4) before the preparation of cell extracts, which were analysed for the MAP kinase activities by the renatured kinase assay described in the Materials and methods section. An autoradiogram of the dried gel used for the assay is shown with the positions of molecular-mass markers (left) and the activated forms of Erk 1 and Erk 2 (right) indicated. (b) Quiescent Swiss 3T3 cells were incubated for 3 min without (lanes 1 and 5), or with 1 unit/ml of sphingomyelinase (lane 2), 30 ng/ml of PDGF (lane 3), or both (lane 4) before the preparation of cell extracts. The peptides were then resolved by SDS/PAGE, transferred to polyvinylidene difluoride membrane, and blotted with antibodies against Erk 1 and Erk 2. Similar results were obtained in a repeated experiment.

Stimulation by sphingomyelinase of cellular MAP kinase activities

Treatment with sphingomyelinase activates MAP kinase in HL60 cells, where the sphingomyelin cycle is proposed to function in tumour necrosis factor-induced cell differentiation [23]. Because MAP kinases have been implicated in the regulation of cell differentiation and also proliferation [14], we examined the effects of sphingomyelinase and the natural form of ceramide on the MAP kinase activity in Swiss 3T3 cells. Exposure of quiescent 3T3 cells to sphingomyelinase increased the activity of cellular extract to phosphorylate MBP, which is known to be a good substrate for MAP kinases (Figure 4a). The effect of the enzyme addition to the cells did not reach a maximum level at concentrations as high as 1 unit/ml (Figure 4b). Direct addition of sphingomyelinase to the MBP kinase assay mixture had no effect on the kinase activity (results not shown). Phosphocholine increased the cellular MBP kinase activities (Figure 5a). Ceramide was also found to activate cellular MBP kinase (Figure 5b). However, the concentrations of ceramide required for the activation were much higher than those required for potentiation of the PDGF-stimulated cell growth (see Figure 3b).

In order to characterize the MBP kinase activity in Swiss 3T3 cells, we performed the renatured kinase assay in MBP-containing gel [24,25]. PDGF increased the MBP kinase activities migrating with 43-kDa and 41-kDa polypeptides, which were considered to be Erk-1 and Erk-2 subtypes of MAP kinase respectively (Figure 6a). Treatment of the cells with sphingomyelinase also increased the activities with the same molecular masses (Figure 6a). The same results were obtained when cell lysate was first treated with the antibody against Erk-1 and Erk-2, and then the immunoprecipitate therefrom was examined by the renatured kinase assay (results not shown). It is well known that activated forms of Erk-1 and Erk-2 are phosphorylated on serine and threonine residues and that the phosphorylation causes the shift in electro-

Figure 7 Failure of sphingomyelinase to potentiate PDGF-stimulated MBP kinase activity

Activity of cell extract to phosphorylate MBP was measured as described in the Materials and methods section. Before the preparation of cell extract, (a) quiescent Swiss 3T3 cells were treated with increasing concentrations of PDGF in the presence (●) or absence (○) of 300 m-units/ml of sphingomyelinase, or (b) the cells were treated with the increasing concentrations of sphingomyelinase in the presence (●) or absence (○) of 30 ng/ml of PDGF. Each value represents the mean of duplicate determinations. Similar results were obtained in a repeated experiment.

phoretic mobility of the isoenzymes. Immunoblotting analysis of cellular peptides with the antibody against Erk-1 and Erk-2 showed that the mobility shift did occur after stimulation of cells with sphingomyelinase (Figure 6b). We next estimated the cellular MAP kinase activity by measuring the activity of cytosolic extracts to phosphorylate a synthetic peptide with a sequence based on the threonine phosphorylation site of the epidermal growth factor (EGF) receptor (Amersham). The activity to phosphorylate this highly selective substrate for MAP kinases was again enhanced by 7-fold in the cells treated with 1 unit/ml sphingomyelinase for 3 min. These results indicated that sphingomyelinase could stimulate the cellular MAP kinase (Erk-1 and Erk-2) activity like many growth factors in Swiss 3T3 cells.

Failure of sphingomyelinase to potentiate the PDGF-stimulated MAP kinase activity

We have shown that sphingomyelinase activated MAP kinases at concentrations similar to those required for potentiation of cell growth (Figures 1b and 4b). Thus we next examined whether the activation of MAP kinase could be the underlying mechanism of the synergistic activation of cell growth by sphingomyelinase. PDGF induced a concentration-dependent increase of cellular MBP kinase activity (Figure 7a). The maximum effect of the growth factor was observed at a concentration of 10 ng/ml. Simultaneous addition of 300 m-units/ml of sphingomyelinase did not potentiate the PDGF-stimulated MBP kinase activity (Figure 7a). We have shown that sphingomyelinase increased cell growth, which was stimulated by the maximum concentration of PDGF (Figure 1b). The enzyme, however, did not affect the PDGF-stimulated activity of MBP kinase under the same conditions (Figure 7b). Synergism between PDGF and sphingomyelinase could not be observed even when MAP kinase activity was estimated by other methods: the renatured kinase assay in an MBP-containing gel (Figure 6a), immunoblot analysis of Erk-

1 and Erk-2 (Figure 6b), and measurement of the activity to phosphorylate the EGF-receptor-related substrate peptide (results not shown). Thus, it is unlikely that activation of Erk-1 and Erk-2 is the mechanism by which sphingomyelinase potentiates the growth factor-induced cell growth. It has been shown that treatment of HepG2 cells with sphingomyelinase activated the 54-kDa kinases (stress-activated protein kinases; SAPKs) which can phosphorylate the EGF receptor peptide substrate [26]. Thus p54 MAP kinase is another candidate that mediates the synergistic action of sphingomyelinase. This possibility is unlikely because we could not observe synergism between PDGF and ceramide in stimulating the activity of cell lysate to phosphorylate the EGF-receptor-related peptide (results not shown). Inability of cell-permeable ceramide to activate this MAP kinase has also been reported in HepG2 cells [27].

MAP kinase has been recognized as a key enzyme in the regulation of cell growth. Our finding that sphingomyelinase effectively potentiated cell growth under the condition where MAP kinase was fully activated by PDGF indicated the significance of the cooperative pathway(s). Growth factors, including pharmacological agents, are known to induce cell growth by stimulating several independent pathways. Specific combinations of growth factors induce synergistic activation of cell growth, although the underlying mechanisms have not been fully understood [28]. Further study on the sphingomyelinase-induced intracellular events is expected to give valuable information on the regulation of cell growth and also on the synergism of growth factors. We have shown here that ceramide, one of the main products of sphingomyelinase, has the ability to potentiate the PDGF-stimulated cell growth. Occurrence of ceramide-activated protein kinase [7,29,30] and protein phosphatase [31,32] has been described. Possible involvement of these enzymes has yet to be examined.

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