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Structure–function studies on inhibitory activity of *Bungarus multicinctus* protease inhibitor-like protein on matrix metalloprotease-2, and invasion and migration of human neuroblastoma SK-N-SH cells

Wen-Min Chou [a,b], Wen-Hsin Liu [a,b], Ku-Chung Chen [a,b], Long-Sen Chang [a,b,*]

[a] *Institute of Biomedical Sciences, National Sun Yat–Sen University, Kaohsiung 804, Taiwan*
[b] *National Sun Yat–Sen University–Kaohsiung Medical University Joint Research Center, National Sun Yat–Sen University, Kaohsiung 804, Taiwan*

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ABSTRACT

In view of the findings that several Kunitz-type protein inhibitors suppress tumor invasion and metastasis, the aim of the present study is to explore whether *Bungarus multicinctus* protease inhibitor-like protein-2 (PILP-2) and PILP-3 exhibit anti-tumor activity. Although approximately 28% of amino acid substitutions occurred between PILP-2 and PILP-3, molecular modeling suggested that PILP-2 and PILP-3 shared similar folded structures. Unlike PILP-2, PILP-3 showed a notable activity in abolishing migration and invasion of human neuroblastoma SK-N-SH cells. The ability of PILP-3 to inhibit matrix metalloprotease-2 (MMP-2) activity was higher than that of PILP-2. Pull-down assay revealed protein–protein interaction between PILP-3 and MMP-2. In contrast to mutation on N-terminal region, replacement of amino acids at C-terminus attenuated notably the ability of PILP-3 to inhibit cell invasion, cell migration and MMP-2 activity as well as the binding capability of PILP-3 with MMP-2. Molecular docking showed that N-terminal region of PILP-2 and PILP-3 fitted into the cleft around the active site of MMP-2 catalytic domain. In contrast to that of PILP-2, C-terminal region of PILP-3 was suggested to be in close contact with catalytic domain of MMP-2. Collectively, our data indicate that PILP-3 is a MMP-2 inhibitor and shows an activity in inhibiting migration and invasion of neuroblastoma, and suggest that intact C-terminus is crucial to the activities of PILP-3.

1. Introduction

Snake venoms are complex mixtures of pharmacologically active polypeptide toxins that are believed to have evolved to alter functionally the physiological activities along with predator-prey interaction (Kordis and Gubensek, 2000; Zupunski et al., 2003; Lynch, 2007). In addition to enzymes and toxins, snake venom also contains serine protease inhibitors. Several Kunitz/bovine pancreatic trypsin inhibitors (BPTI) from the venom of Viperidae and Elapidae snakes have been isolated and sequenced (Ritonja et al., 1983; Shafqat et al., 1990; Chang et al., 2001; Cheng et al., 2005). These snake venom Kunitz-type protease inhibitors have been demonstrated to specifically inhibit the proteolytic activity of trypsin or chymotrypsin. Nevertheless, their physiological roles in the regulatory mechanisms that influence the proteases in coagulation, fibrinolysis and inflammation have been considered. Snake venom protease inhibitors are structurally homologous to BPTI, dendrotoxins and B chains of β-bungarotoxin (Chang et al., 2001; Chen et al., 2001). However, dendrotoxins and B chains of β-bungarotoxin are unable to inhibit protease activity. Three protease inhibitor-like protein (PILP) genes cloned from *Bungarus multicinctus* genome have recently

* Corresponding author. Fax: +886 7 5250197.
E-mail address: lschang@mail.nsysu.edu.tw (L.-S. Chang).

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been found to be evolutionarily related to B chain genes of β-bungarotoxin (Chang et al., 2008). The deduced protein sequences of PILPs (PILP-1, PILP-2 and PILP-3) are homologous with those of Kunitz-type protease inhibitors and B chains of β-bungarotoxins. Efforts had been made to purify PILPs from *B. multicinctus* venom, but it failed. Nevertheless, recombinant PILPs have been successfully prepared (Chang et al., 2008). In contrast to recombinant PILP-2 and PILP-3, recombinant PILP-1 has an inhibitory activity toward trypsin (Chang et al., 2008).

A number of proteases such as urokinase-type plasminogen activator and matrix metalloproteinases (MMPs) on the plasma membrane play important roles in matrix degradation, cell migration and cell invasion (Ellis and Murphy, 2001). Several Kunitz-type protease inhibitors including bikunin, HGF activator inhibitor-2 and tissue factor pathway inhibitor-2 are found to suppress tumor invasion and metastasis (Kobayashi et al., 2003; Suzuki et al., 2003; Sierko et al., 2007; Tung et al., 2009). In view of the fact that the physiological targets of PILP-2 and PILP-3 remain elusive, the present study aims to assess anti-tumor activity of PILP-2 and PILP-3. Our data showed that PILP-2 and PLIP-3 exerted different potencies on inhibiting cell migration, cell invasion and MMP-2 activity. Structure–function analyses revealed that C-terminal region of PILP-3 was crucial to exerting these biological activities.

2. Materials and methods

2.1. Preparation of recombinant PILP-2, PILP-3 and mutated PILP-3

Recombinant PILP-2 and PILP-3 were prepared essentially according to our published procedure (Chang et al., 2008). The cDNA fragments encoding PILP-3 mutants, PILP-3N and PILP-3C, were prepared by PCR methods. PILP-3N and PILP-3C had N-terminal region and C-terminal region of PLIP-2, respectively. The sense and antisense primers for preparing PILP-3N cDNA were 5′-GATATCAAGAACCGTCC ACCGTTTTGTAATCTGCTTCCTGAGCC-3′ and 5′-CTCGAGTT-CAGCACAGGTGCGTTTGCATTCA-3′, respectively. The sense primer (5′-GATATCAGAAAGCGTCATCAGTTTTGTAATGTGCC TCCTGA GCCT-3′) and antisense primer (5′-CTCGAGTTCA GCACAGGTGCGTTGGCAT TCTTCTATGGTCTTAAAATTG-3′) were synthesized for preparing PILP-3C cDNA. The underlines indicated the *Eco*RV and *Xho*I restriction enzyme sites. The PCR-amplified DNA fragments were subcloned into the *Eco*RV and *Xho*I sites of expression vector pET29a (+). Because the antisense primers did not contain a termination codon, the C-terminus of recombinant proteins contained an extra six His residues. The expressed vector was transformed into *E. coli* strain BL21(DE3), and the recombinant PILPs appeared as soluble proteins. Purification of recombinant PILPs on His-Bind resin (Novagene) was carried out essentially according to the manufacturer's instruction. Then recombinant PILPs were further purified by reverse phase HPLC on a SynChropak RP-P column (4.6 mm × 25 cm) equilibrated with 0.1% trifluoroacetic acid and eluted with a linear gradient of 7.5–75% acetonitrile for 50 min. The eluate was monitored at 280 nm.

2.2. Cell culture

Human neuroblastoma SK-N-SH cells obtained from ATCC (Rockville, MD, USA) were routinely cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 2 mM glutamine and penicillin (100 units/ml)/streptomycin (100 μg/ml) in an incubator humidified with 95% air and 5% CO_2.

2.3. In vitro wound-healing assay

A sufficient number of serum-starved SK-N-SH cells in a medium containing 10% FCS were seeded into 12-multiwell plates. After the cells grew to confluence, a scratch wound in the monolayer was made by dragging a pipette tip across the layer. Wounded monolayers were then washed four times with medium to remove cell debris and incubated in medium in the absence or presence of PILPs for 24 h. Cell migration into the wound surface was determined under an inverted microscopy at 24 h.

2.4. Cell migration and invasion assays

SK-N-SH cells were treated with wild-type and mutated PILPs, respectively. Cell migration was assessed using transwell (Millipore) insert using polycarbonate membranes of 8-μm pore size, and invasion assays were performed using transwell precoated with Matrigel (Becton Dickinson Labware, Bedford, MA). In brief, 8×10^3 cells resuspended in a 100-μl serum-free medium were placed in the upper chamber and allowed to migrate or invade for 24 h. The lower part of the transwell unit was filled with 10% FCS medium. The total number of migrated or invaded cells on the lower chamber (which were localized on the bottom surface of polycarbonate filters) were fixed in formaldehyde, stained with Giemsa solution, and counted under a microscopy.

2.5. Gelatin zymography

Cells were incubated in serum-free medium overnight with or without PILPs. Culture medium or cell lysates were mixed with SDS loading buffer (0.125 M Tris–HCl, pH 6.8, 4% SDS and 0.02% bromophenol blue) and incubated for 30 min at 37 °C. Samples were electrophoresed in a 10% polyacrylamide gel containing 0.1% gelatin. The gel was then washed in 2.5% Triton X-100 to remove SDS. The gel was incubated at 37 °C for 48 h in 50 mM Tris–HCl (pH 7.6) containing 150 mM NaCl and 5 mM $CaCl_2$. After staining with Coomassie Blue R-250, gelatinases were identified as clear bands.

2.6. MMP-2 activity measurement

MMP-2 activity was analyzed utilizing a SensoLyte™ 490 MMP-2 assay kit (AnaSpec Inc., Japan) using EDANS/DABCYL FRET peptide as substrate. Experiments were carried out according to the manufacturer's protocol. Purified MMP-2 (0.7 nmol, R&D systems) and different concentrations of PILP-2 were incubated for 30 min at 37 °C, and the mixtures were then added into the substrate