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Preparation of Protein Transduction Domain-Fused Peptidyl Prolyl *cis/trans* Isomerase Pin1

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The phenotypes of mice lacking peptidyl prolyl *cis/trans* isomerase Pin1 ($Pin1^{-/-}$) indicated that deficient Pin1 might be related to a variety of diseases. We created TAT-Pin1, a fusion protein of human immunodeficiency virus 1 *trans*-activator of transcription factor with Pin1. Treatment of HeLa cells with TAT-Pin1 increased the ratio of the S phase. Moreover, TAT-Pin1 restored the proliferating function of $Pin1^{-/-}$ mouse embryonic fibroblasts which cannot restart proliferation after G0 arrest. These results indicate that TAT-Pin1 is useful in studying the functions of Pin1 and can be developed as a macromolecular drug for diseases related to Pin1 loss.

Key words: protein transduction domain human immunodeficiency virus-1 *trans*-actuvatir if transcription factor (TAT); peptidyl prolyl *cis/trans* isomerase Pin1; cell cycle

A peptidyl prolyl *cis/trans* isomerase (PPIase) Pin1 specifically binds phosphorylated Ser/Thr-Pro protein motifs and catalyzes *cis/trans* isomerization of the peptide bond.[1] When Pin1 was considered an essential PPIase regulating mitosis,[2] we created a *pin1*-deleted ($Pin1^{-/-}$) mice and found that Pin1 is not essential, but is implicated in saving the cell cycle from G0 arrest by using a mouse embryonic fibroblast (MEF).[3] The $Pin1^{-/-}$ mice showed phenotypes similar to *cyclin D1*-deficient mice, which suggests that Pin1 is involved in the G1/S progression by maintaining cyclin D1 levels constant.[4] Various transcription factors and regulators have been identified as substrates of Pin1. It is involved in controlling the functions of p53, a tumor-suppressor against DNA damage at the checkpoint, to respond to genotoxic stress smoothly.[5,6] Analysis of *Pin1*-deficient mice has also provided evidence that Pin1 is involved in a variety of diseases, indicating cancer and Alzheimer's disease.[1,7] Recently it was reported that Pin1 regulates parathyroid hormone mRNA stability and determines the response of the parathyroid to chronic hypocalcemia and chronic kidney disease.[8] These results suggest that an increased Pin1 level might cure or protect from a variety of diseases.

In order to increase the Pin1 level, we thought to utilize membrane-permeable Pin1. There are several methods of delivering proteins into cells. The most widely used approach is to link several small regions of protein transduction domain (PTD). PTDs are small cationic peptides such as human immunodeficiency virus-1 *trans*-activator of transcription protein (TAT),[9] *Drosophila melanogaster* antenna-peptide protein,[10] herpes simplex virus protein VP22,[11] and synthetic Lys/Arg rich peptides.[12,13] Although the exact mechanism of transduction is still unknown, The PTD fusion approach can be used to introduce a target protein into cells. The TAT peptide, consisting of the amino acid sequence GRKKRRQRRRPPQ, is less toxic to cells than other basic membrane-interacting agents, and a TAT-protein has been successfully delivered to the brain when injected into mice.[14]

In this study, we found that TAT-Pin1 can be a macromolecular drug to recover Pin1 loss. The TAT-Pin1 we created maintained the PPIase activity of native Pin1 and was successfully delivered into cells.

Materials and Methods

Construction of recombinant plasmid. TAT-fused Pin1 was designed by adding the TAT protein sequence GRKKRRQRRRPPQ to the N-terminal (TAT-Pin1), the C-terminal (Pin1-TAT), and both termini (TAT-Pin1-TAT) of glutathione *S*-transferase (GST) fusion Pin1 (Fig. 1). An oligonucleotide encoding TAT PTD was ligated to the 5′ end and/or the 3′ end of the Pin1 coding sequence by PCR to construct *tat-pin1*, *pin1-tat*, and *tat-pin1-tat*, respectively. The sequences were ligated into pGEX-4T-1 (GE Healthcare, UK) plasmid at the *Bam*HI and *Xho*I sites. The integrity of the construct was confirmed by DNA sequencing. *Escherichia coli* BL21 (DE3) cells transformed with the various expression vector plasmids, were cultured, followed by treatment with isopropyl β-D-thio-galactopyranoside (IPTG) to induce protein production. The TAT-proteins were analyzed by 12.5% SDS-poly-acrylamide gel electrophoresis (SDS–PAGE), and then stained with Coomassie Brilliant Blue (CBB) R-250. The purified protein preparations were stored at $-80\,^{\circ}C$.

PPIase assay. The PPIase assay was performed by protease coupled assay as described previously.[15] The total volume of the assay was 1,500 µl, and the sample buffer consisted of 35 mM HEPES, pH 7.8. Stock solutions of chromogenic substrate, Suc-AAEPF-*para*-nitroanilide (pNA) (Bachem, Switzerland), was prepared in 0.47 M LiCl/

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Abbreviations: CBB, Coomassie Brilliant Blue; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; FCS, fetal calf serum; GST, glutathione *S*-transferase; IPTG, isopropyl β-D-thio-galactopyranoside; MEF, mouse embryonic fibroblast; PBS, phosphate-buffered saline; PI, propidium iodide; pNA, *para*-nitroanilide; PPIase, peptidyl prolyl *cis/trans* isomerase; PTD, protein transduction domain; SDS–PAGE, SDS-poly-acrylamide gel electrophoresis; TAT, human immunodeficiency virus-1 *trans*-activator of transcription factor; TFE, trifluoroethanol

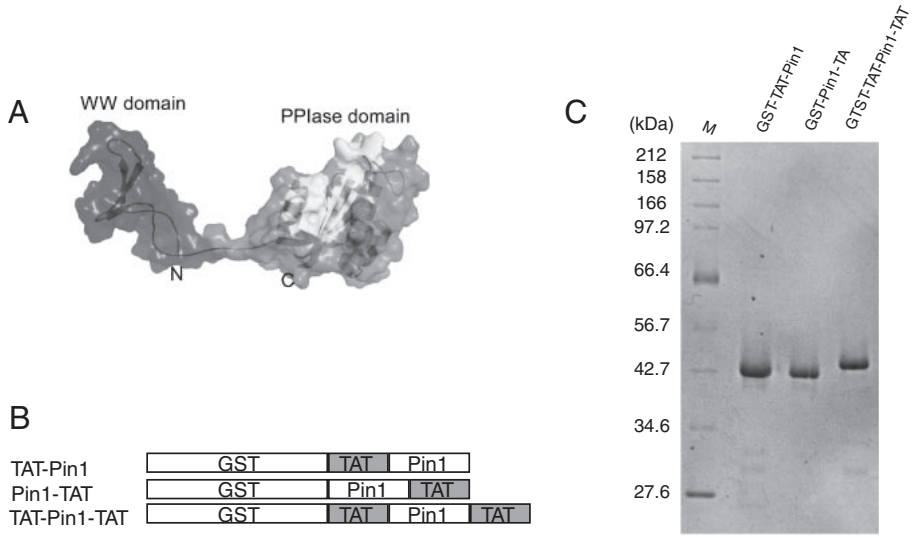

Fig. 1. TAT-Pin1 Structure.

A, Transparent molecular surface and ribbon diagram of the NMR structure of Pin1 (PDB ID 1 nmv). The N-terminal WW domain and the C-terminal PPIase domain are colored in blue and red respectively. The phosphorylated Ser/Thr-Pro binding site is colored in yellow. The N-terminal and C-terminal ends are labeled N and C respectively. B, Schematic structures of the recombinant proteins. The expression vectors were constructed as described in "Materials and Methods." GST indicates the glutathione *S*-transferase domain. TAT indicates the GRKKRRQRRRPPQ sequence. C, Purified GST-tagged fusion proteins were analyzed by SDS–PAGE and stained with CBB. Lane M, molecular weight marker; lane 1, GST-TAT-Pin1; lane 2, GST-Pin1-TAT; lane 3, GST-TAT-Pin1-TAT.

trifluoroethanol (TFE) at a concentration to 30 mg/ml, and chymotrypsin was dissolved in 35 mM HEPES, pH 7.8 at a concentration to 100 mg/ml. After incubation of the sample buffer with chymotrypsin (0.2 mg/ml) at 4 °C, the reaction was started by injection of the substrate (0.04 mg/ml). After the initial phase, at which all pre-existing *trans*-peptides were cleaved, Pin1 was added to the sample buffer. The reaction was recorded with an Agilent 8453 UV-Vis spectrophotometer (Agilent, USA), and the signal difference between 390 and 510 nm was used to calculate the first-order rate constants. One kat of PPIase activity was defined as the amount of Pin1s releasing 1 mol of p-NA per s. The PPIase activity was measured 3 times for each Pin1.

Cell culture. MEFs were produced and cultured as previously described.[3] HeLa and MEF cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal calf serum (FCS) and 50 units/ml of penicillin and 50 µg/ml of streptomycin. For the transduction of TAT-Pin1 into MEFs, cells were plated at 0.5×10^5 cells/well in 24-well plates (Becton Dickinson) in DMEM containing 10% FCS for 1 d. When they were 60% confluent, the culture medium was replaced with fresh medium containing 0.1% FCS, and the cells were cultured for an additional 72 h to synchronize them in the S phase. After synchronization of the cells, 0.5 µM of TAT-Pin1 was added to the medium, and the numbers of cells were counted at day 1 (24 h), day 3 (72 h), day 4 (96 h), day 5.5 (132 h) and day 7 (168 h).

Analysis of the transduction of the fusion protein into the cells by fluorescence microscopy. For transduction of TAT fusion Pin1 into HeLa cells, the cells were plated at 1.0×10^4 cells/well in an 8-well Glass Slide of the Lab-Tek II Chamber Slide™ System (Nalge Nunc, USA). After culture for 18 h at 37 °C, the medium were replaced with fresh medium containing 10% FCS, and the cells were exposed to 0.5 µM TAT fusion proteins for 4 h. They were rinsed with phosphate-buffered saline (PBS) twice, fixed in 4% w/v paraformaldehyde in PBS for 20 min, treated with 0.2% v/v Triton X-100 in PBS for 10 min, blocked in HISTO-FINE blocking reagent II (Nichirei, Japan) for 15 min, and incubated for 3 h with a rabbit antibody to GST (Bethyl, USA) at a dilution of 1:400. After washing with PBS 3 times, the slides were incubated with Alexa Flour 594-labeled goat anti-rabbit antibody (Invitrogen, Japan) at a dilution of 1:500 for 1 h at 37 °C. After another wash with PBS, the cells were incubated in 1 µg/ml of 4′,6-diamidino-2-phenylindole (DAPI) in PBS at room temperature for 3 min, washed with PBS and water 3 times each, treated with 50% ethanol and 50% methanol, and dried. They were mounted in Aqueous Mounting Medium Permafluor (Beckman Coulter, Japan) and observed under a fluorescence microscope, Biozero 8000 (Keyence, Japan).

Flow cytometry analysis. Cell-cycle assay was done with a flow cytometer, as described previously.[16] HeLa (2.0×10^5) cells in a 60 mm dish were cultured overnight. They were treated with 0.5 µM purified TAT fusion proteins. After 48 h, they were washed with PBS, trypsinized, and washed with fresh medium. They were immobilized with 700 µl of −20 °C methanol for 1 h, washed with saline buffer 3 times, treated with saline buffer containing 0.1% NP-40 and 20 ng/ml of propidium iodide (Dojin, Japan) for 1 min, washed with saline buffer 3 times, and stained with 50 ng/ml of propidium iodide and 100 µg/ml of RNase. Cell-cycle analysis was done using a flow cytometer (FACS caliber, Becton Dickinson) and the ModFit LT program (Becton Dickinson).

Examination of the delivery of TAT-Pin1 to mouse organs. Our study was approved by the Tohoku University Animal Use and Care Committee. All investigations were conducted according to the principles of the Declaration of Helsinki.

TAT-Pin1 (100 µg) was injected into the mouse tail vein, and 1 h later histological sections were of each organ was prepared and expression of TAT-Pin1 was detected with rabbit anti-GST antibody.

Results and Discussion

Preparation of TAT-fused Pin1

Pin1 consists of two domains, an N-terminal WW domain and a C-terminal PPIase domain, as shown in Fig. 1A. Recently, it was reported that the TAT-WW domain of Pin1 was created,[17] but TAT-Pin1 has never been reported. It is necessary to introduce whole Pin1 molecules into cells to increase Pin1 PPIase activity in them. Therefore, we established an expression and purification system for TAT-fusion-Pin1, including TAT-Pin1, Pin1-TAT, and TAT-Pin1-TAT, which retain PPIase activity. The TAT-tag was fused with GST-Pin1 (Fig. 1B) due to expression level and protein stability, but the effects of GST on PPIase activity and permeability can be ignored, as described below. TAT-Pin1, Pin1-TAT, and TAT-Pin1-TAT were expressed in *E. coli*

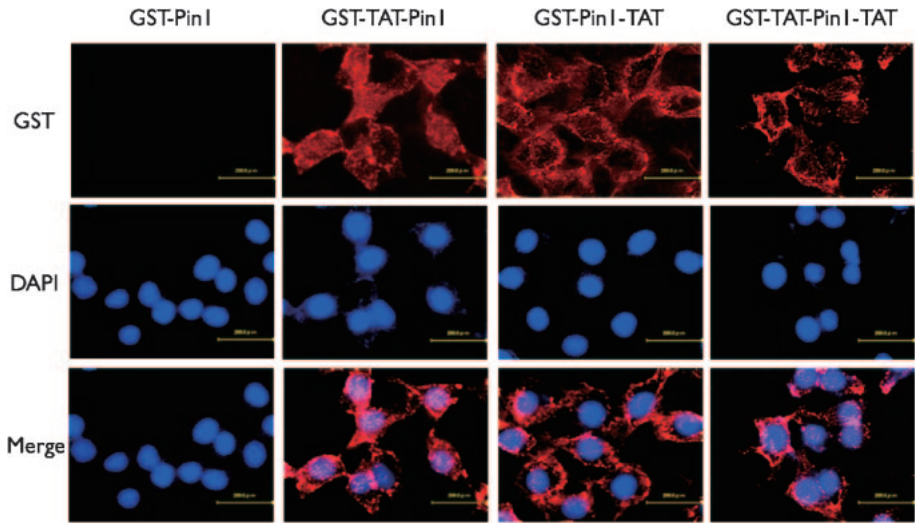

Fig. 2. Transduction of TAT-Pin1 into HeLa Cells.
TAT-Pin1 was detected with anti-GST antibody (red), and the nuclei were stained with DAPI (blue). All scale bars indicate 200 μm.

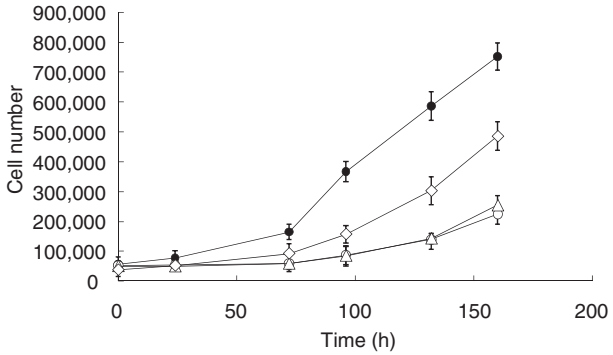

Fig. 3. Effect of TAT-Pin1 on Pin1$^{-/-}$ MEF Proliferation.
The numbers of cells were counted at the indicated time points. MEFs at 60% confluence were cultured in DMEM containing 0.1% FCS for 3 d, then cultured in DMEM containing 10% FCS with or without 0.5 μM TAT-Pin1, and the numbers of cells were counted. ● Pin1$^{+/+}$ MEF; ○ Pin1$^{-/-}$ MEF; △ Pin1$^{-/-}$ MEF with Pin1; ◇ Pin1$^{-/-}$ MEF with TAT-Pin1.

Table 1. PPIase Activities of Various TAT-Pin1s

TAT-Pin1	PPIase activity (kat/mol)
GST-Pin1	$2.5 \pm 0.2 \times 10^{-2}$
GST-TAT-Pin1	$2.4 \pm 0.1 \times 10^{-2}$
GST-Pin1-TAT	$1.5 \pm 0.1 \times 10^{-2}$
GST-TAT-Pin1-TAT	$1.5 \pm 0.2 \times 10^{-2}$

and purified. The purity of these proteins was checked by SDS–PAGE with CBB staining (Fig. 1C). The activities of these proteins are summarized in Table 1. TAT-Pin1 showed a level of PPIase activity similar to that of GST-Pin1. Meanwhile, Pin1-TAT and TAT-Pin1-TAT, which possess the TAT sequence at the C-terminal end of Pin1, showed approximately 40% of the activity of that of GST-Pin1. The X-Ray crystal and NMR structures indicated that the WW domain and the PPIase domain of Pin1 were free to move with respect to each other (Fig. 1A). Therefore, the addition of 13 residues at N-terminal would not affect PPIase activity catalyzed at the C-terminal domain. On the other hand, the TAT sequence at the C-terminal end presumably causes a decrease in

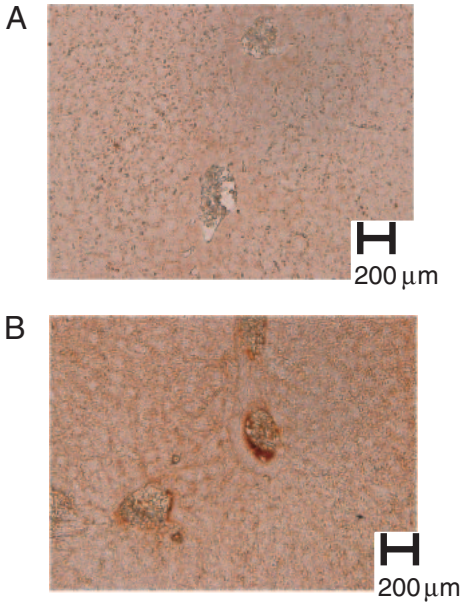

Fig. 4. Delivery of TAT-Pin1.
PBS (A; 25 μl) and 25 μl of 0.5 μM GST-TAT-Pin1 (B) were injected into the mouse tail vein. One h later, the animals were sacrificed and the livers were paraffin-embedded. Deparaffinized tissues were immunostained with anti-GST antibody.

PPIase activity, because the short peptide at the C-terminal can cause steric hindrance to substrate binding or alter the structure of Pin1 not to bind the substrate.

Transduction of TAT fusion proteins into cells

We tested to determine whether the TAT fusion Pin1 can be delivered into mammalian cells *in vitro*. TAT fusion Pin1 was added to a culture medium of HeLa cells, and the cells were incubated for 4 h. They were stained with nucleus-specific marker DAPI and the anti-GST antibody and observed by fluorescence microscopy. As shown in Fig. 2, all the TAT fusion proteins were detected in the cytoplasm. This suggests that the purified TAT fusion Pin1 proteins entered into the cells. Among these fusion proteins, only TAT-Pin1 was detected in the nucleus as well as in the cytoplasm.

Table 2. Cell-Cycle Ratio of HeLa Cells Transduced with TAT-Pin1

Cell cycle	Control (%)	GST-Pin1 (%)	GST-TAT-Pin1 (%)	GST-Pin1-TAT (%)	GST-TAT-Pin1-TAT (%)
G1/G0	52.9 ± 0.9	51.1 ± 1.0	47.1 ± 1.1	47.8 ± 1.6	49.7 ± 1.1
S	34.6 ± 1.0	36.0 ± 1.3	40.7 ± 1.3	41.3 ± 1.8	40.8 ± 1.1
G2/M	12.6 ± 0.3	12.9 ± 0.8	12.1 ± 0.8	11.0 ± 1.0	9.5 ± 0.5

Analysis of the cell cycle after TAT fusion Pin1 treatment

Next we next investigated to determine whether TAT fusion Pin1 functions normally in cells. Pin1 function to make cell-cycle progression from G1 into the S phase.[3,4] HeLa cells were treated with TAT fusion proteins for 2 d, and stained with propidium iodide (PI), and then we analyzed the cell cycle by flow cytometry. Treatment of HeLa cells with TAT fusion Pin1 increased the ratio of cells in the S phase as compared to treatment with GST-Pin1 as control (Table 2). Among the three kinds of TAT-Pin1, TAT-Pin1 consisting of TAT at the N-terminal end promoted the entry of the cells into the S phase.

TAT-Pin1 rescued Pin1$^{-/-}$ MEF to enter the S phase

Pin1$^{-/-}$ MEF showed defect in G1/S transition and slower growth.[3] We analyzed to determine whether TAT-Pin1 can promote S-phase entry and then restore the proliferation rate. Pin1$^{-/-}$ MEFs were synchronized at the G0 phase by serum starvation. After release from starvation, the MEFs were treated with TAT-Pin1, cultured for varying periods of time, and then the cell numbers were counted (Fig. 3). Pin1$^{-/-}$ MEF transduced with TAT-Pin1 restarted proliferation, although the Pin1$^{-/-}$ MEF transduced with TAT-GST or GST-Pin1 did not proliferate.

Examination of TAT-Pin1 delivery in mice

TAT-Pin1 was injected into the vein of the mouse tail and we examined the delivery of TAT-Pin1 to the mouse tissues. The injected TAT-Pin1 may have concentrated in the liver when it was injected from the vein of the tail (Fig. 4). A delivery method should be devised in order to deliver TAT-Pin1 to the other target tissues.

Conclusion

We created TAT-Pin1 that maintined PPIase activity. TAT-Pin1 penetrated into mouse embryonic fibroblasts and functioned in the cells, which suggests that TAT-Pin1 can be useful to recover Pin1 loss. We think that TAT-Pin1 can be developed as a macromolecular drug for diseases related to Pin1 loss, such as cancer, Alzheimer's disease, chronic kidney disease, and so on, in the future.

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