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TIMP-1 Regulates Cell Proliferation by Interacting With the Ninth Zinc Finger Domain of PLZF

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Abstract The tissue inhibitors of metalloproteinases (TIMPs) are multifunctional proteins that specifically inhibit matrix metalloproteinases (MMPs) and regulate extracellular matrix (ECM) turnover and tissue remodeling. This is directed by forming tightly bound inhibitory complexes with MMPs. Recent years have revealed important differences of various biological activities between TIMP families but molecular mechanisms are not clear. To define the molecular mechanisms of TIMP-1-dependent biological processes, we used TIMP-1 as bait in a yeast two-hybrid screen, along with a human ovary cDNA library. Further characterization revealed the ninth zinc finger domain as an interacting domain of the promyelocytic leukemia zinc finger protein (PLZF). Interaction of PLZF with TIMP-1 in mammalian cells was also confirmed by co-immunoprecipitation and with in vitro binding assays. We investigated whether TIMP-1-mediated anti-apoptotic activity could promote the growth of ovarian cancer in an experimental model system. TIMP-1 treatment was found to be more effective at increasing ovarian cancer growth when compared with PLZF in parallel experiments. Subsequently, the efficacy of a combined treatment with TIMP-1 and PLZF was investigated. In the presence of both of these proteins, TIMP-1 significantly reduced apoptosis induced by PLZF in cervical carcinoma cells. These combined results indicate that TIMP-1 functions as an anti-activator of the transcriptional repressive activity of PLZF. J. Cell. Biochem. 101: 57–67, 2007.

Key words: matrix metalloproteinases; PLZF; apoptosis; caspase-3; small interfering RNA; transcriptional anti-activator

INTRODUCTION

The tissue inhibitors of metalloproteinases (TIMPs) play a pivotal role in the cellular homeostasis of the extracellular matrix (ECM) by regulating the activities of matrix metalloproteinases (MMPs) including cell proliferation, differentiation, apoptosis, tumor angiogenesis, tumorigenesis, and metastasis [Juliano and Haskill, 1993; Whitelock et al., 1996; Amour et al., 2000; Visse and Nagase, 2003]. TIMPs form a family of at least four members, TIMP-1, -2, -3, and -4. They are expressed by a variety of cell types, most tissues, and body fluids [Lambert et al., 2004]. However, recent studies have indicated that the biological role of TIMPs is to interact with cell surface proteins and modulate intracellular downstream signaling pathways independent of its MMP-inhibitory activity [Fernandez et al., 2003; Qi et al., 2003]. During the past 10 years, another study has shown evidence that TIMPs acts as a regulator of apoptosis. Anti-apoptotic activity of TIMP-1 and pro-apoptotic activity of TIMP-3 have been well reported, whereas both pro-apoptotic and anti-apoptotic activities of TIMP-2 and TIMP-4 were reported [Alexander et al., 1996; Baker et al., 1998; Guedez et al.,

Abbreviations used: TIMPs, tissue inhibitors of metalloproteinases; PLZF, promyelocytic leukemia zinc finger protein; BTB, broad complex, tramtrack, bric-a-brac; ECM, extracellular matrix; MTT, 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl-^{2}H-tetrazolium bromide; ONPG, *O*-nitrophenyl β-D-galactopyranoside; HDAC1, histone deacetylases.

Seung Bae Rho and Bo Mee Chung contributed equally to this work.

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1998a; Guedez et al., 1998b; Barasch et al., 1999; Li et al., 1999; Jiang et al., 2001; Tummalapalli et al., 2001; Bond et al., 2002; Yoshiji et al., 2002; Ahonen et al., 2003; Liu et al., 2005]. Based on this, TIMPs could appear as a new type of inhibitors in cancer therapy. TIMP-3 promotes extrinsic apoptosis in a number of cancer cells by inhibiting the shedding of tumor necrosis factor-α receptors [Bond et al., 2002]. In contrast, TIMP-1 inhibits intrinsic apoptosis and angiogenesis in various cell types, and may also be capable of regulating tumor growth, positively acting as a growth or cell survival pathway factor for cancer cells [Alexander et al., 1996; Guedez et al., 1998a; Guedez et al., 1998b; Li et al., 1999; Yoshiji et al., 2002; Liu et al., 2003].

To begin characterizing the regulating mechanism of the TIMP-1 biological phenomena, we used a yeast two-hybrid system to screen a human ovary cDNA library for novel TIMP-1 interacting partners. We identified human promyelocytic leukemia zinc finger (PLZF), well known as a transcriptional repressor protein, to be a TIMP-1-interacting partner. Deletion mapping studies revealed the ninth zinc finger domain as a PLZF interacting domain. We also investigated whether TIMP-1-mediated anti-apoptotic activity could promote the growth of ovarian cancer in an experimental model system. TIMP-1 treatment, due to its ability to interfere with PLZF activity, was found to be more effective at increasing ovarian cancer growth when compared with PLZF in parallel experiments. The combined treatments of TIMP-1 and PLZF were investigated in order to display the efficacy of this study. In the presence of both proteins, TIMP-1 significantly reduced the apoptosis induced by PLZF in cervical carcinoma cells.

MATERIALS AND METHODS

Yeast Two-Hybrid Analysis

For bait construction with human TIMP-1, cDNA encoding full-length human TIMP-1 was sub-cloned into the *Eco*RI and *Xho*I restriction sites of the pGilda. The resulting plasmid pGilda-TIMP-1 was introduced into yeast strain EGY48 [*MATa*, *his3*, *trp1*, *ura3-52*, *leu2::pLeu2-LexAop6*/pSH18-34 (*LexAop-lacZ* reporter)] by a modified lithium acetate method [Ito et al., 1983]. The cDNAs encoding B42 fusion proteins were introduced into the competent yeast cells that already contained pGilda-TIMP-1 and the tryptophan prototrophy (plasmid marker) transformants were selected for on a synthetic medium (Ura^-, His^-, Trp^-) containing 2% glucose. We tested their interactions with pGilda-TIMP-1 on a medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside as described [Rho et al., 1996]. Then, β-galactosidase activity was measured by adding 140 μl of 4 mg/ml *O*-nitrophenyl β-D-galactopyranoside (ONPG) [Rho et al., 1996, 2004]. The β-galactosidase activity was calculated using the formula units $= [1{,}000 \times (A_{420} - 1.75 \times A_{550})]/(\text{time} \times \text{volume} \times A_{600})$.

Subcloning of Deletion Mutants of PLZF

Three deletion mutants of PLZF were isolated by PCR using the following primers (PLZF-(BTB)-F1, 5′-CGGGAATTCATGGATCTGACA-AAAATG-3′; PLZF(BTB)-R1, 5′-ATTCTCGA-GTCACTTCAGGCACTGTTC-3′; PLZF-(RD2)-F2, 5′-ATTGAATTCACCAAGGCTGCAGTGG-AC-3′; PLZF(RD2)-R2, 5′-ATTCTCGAGTTAG-CCAGCCTCAGCTGG-3′). PLZF(ZF)-F3, 5′-CGGGAATTCAGCTACATCTGCAGTGAG-3′; PLZF(ZF)-R3, 5′-ATTCTCGAGTCACACATA-GCACAGGTA-3′). PCR products were cloned into the *Eco*RI and *Xho*I restriction sites of the pGilda and sequenced. Each constructed plasmid was introduced into yeast EGY48 expressing either TIMP-1 or PLZF hybrid protein.

Co-Immunoprecipitation Assays

cDNA encoding human TIMP-1 was isolated by PCR using a specific template and was then cloned into pEGFPC1 (Clontech) or pcDNA4/HisMax (Invitrogen) and digested with *Bgl*II and *Eco*RI (pEGFPC1-TIMP-1) or *Eco*RI and *Xho*I (pcDNA4/HisMax-TIMP-1). The human PLZF cDNA was then ligated into pcDNA4/HisMax (Invitrogen) using *Eco*RI and *Xho*I (pcDNA4/HisMax-PLZF) or *Bgl*II and *Eco*RI (pEGFPC1-PLZF). For co-immunoprecipitation, 293 cells were co-transfected with cDNA constructs of pEGFPC1-TIMP-1 and pcDNA4/HisMax-PLZF or pEGFPC1-PLZF and pcDNA4/HisMax-TIMP-1 using FuGENE6 (Roche Applied Science, Basel, Switzerland). As a negative control, pEGFPC1-TIMP-1, PLZF, and an empty vector pcDNA4/HisMax were also co-transfected. Two days after transfection, cells were harvested by trypsinization and centrifugation. Cell pellets were washed in PBS, re-suspended in cell lysis solution (50 mM Tris, pH 7.2, 150 mM NaCl, 1% Triton X-100, 1 μg/ml

leupeptin, 1 μg/ml pepstatin, 2 μg/ml aprotinin, 200 μg/ml PMSF). Lysates were incubated with anti-His, anti-PLZF (Santa cruz), anti-TIMP-1 (Sigma) antibody, and then precipitated with protein A-agarose. The precipitated proteins were resolved by SDS–PAGE, and immunoblotted with anti-GFP (Santa cruz), anti-PLZF, anti-TIMP-1 antibody. An ECL system (Amersham) was used for detection.

MTT Assays

Relative rate of cell proliferation were quantified by MTT assays. Briefly, HeLa cervical cancer cells were grown in DMEM medium containing 10% FBS. Cells were seeded at a density of 3.5×10^3 cells per well in 96-well plates. Three days after transfection, fresh medium containing 10% FBS, and 20 μl of3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl-^{2}H-tetrazolium bromide (MTT) solution (Sigma, 5 mg/ml) was added to each well. Each well was then incubated for an additional 4 h at 37°C. The amounts of MTT-formazan generated were determined as absorbance using a microculture plate reader at 540 nm. Each sample was assayed in triplicate and experiments were repeated three times.

Apoptosis Assays

HeLa cervical cancer cells were plated onto 4-chamber slides and then transfected with TIMP-1, PLZF, and TIMP-1 plus PLZF cDNAs for 24 h using Effectene as described above. Cells were incubated with fluorescein isothiocyanate (FITC)-labeled Annexin V and PI for 15 min according to the supplier's protocol (Boehringer Mannheim, Mannhein, Germany) and then analyzed on a FACS Vantage (Becton Dickinson, San Jose). For evaluation of nuclear morphology, cells were fixed in methanol and stained with 2 μg/ml of DAPI (Boehringer Mannheim) at 37°C for 15 min, washed twice with PBS, and examined with the fluorescence microscope.

Cell Cycle Analysis

HeLa cells co-transfected with TIMP-1 and PLZF expression vectors were fixed with ice-cold 70% ethanol, centrifuged for 5 min at 1,000*g*, and re-suspended in PBS containing 5 mM EDTA and RNase A (50 μg/ml). After incubation for 30 min at 37°C, the cells were stained with propidium iodide (50 μg/ml) and analyzed by flow cytometry with a FACS.

Caspase-3 Activity Assays

Caspase-3 enzymatic activities in cell lysates were determined using actyl-DEVD-7-amino-4-trifluoromethyl coumarin as substrate, according to the manufacturer's protocol (BDPharmingen, San Diego). Activity was measured using a Spectramax 340 microplate reader (Molecular Devices, Sunnyvale) in fluorescence mode using excitation at 400 nm and emission at 505 nm. Enzyme activities were calculated from fluorescence readings using the formula provided by the manufacturer.

Small Interfering RNA (siRNA) Construction

The siRNA oligonucleotide sequence targeting TIMP-1 (AAGATGTATAAAGGGTTCCAA) corresponded to nucleotides 261–281 in the human sequence. siRNA was synthesized by using an siRNA construction kit (Ambion) and was then transfected by oligofectamine (Invitrogen) according to the manufacturer's protocol. Total RNA was isolated using a TRIZOL Reagent (Life Technologies) and reverse transcription (RT) PCR was then performed.

Promoter-Reporter Gene Assays

Constructs, that is, IL-3R-Luc and pCyclinA2-Luc, for promoter-reporter assay were kindly provided by Dr. Inpyo Choi (Korea Research Institute of Bioscience and Biotechnology, Taejon, Republic of Korea) [Han et al., 2003]. HeLa cells plated on 60-mm dishes were transfected with the indicated plasmids using Effectene (Qiagen, Hilden, Germany). After lysis, the cell extracts were incubated with luciferase substrate for 30 min at room temperature. Then, a 5 μl aliquot of each sample was transferred to a luminometer plate, and luciferase activity was measured using Luciferase Assay Systems (Promega, Madison, WI).

Electrophoretic Mobility Shift Assays

Nuclear extracts were prepared from cultured HeLa cells. Electrophoretic mobility shift assay (EMSA) was done with a double-stranded, ^{32}P-radiolabeled oligonucleotide that contained the binding site for human PLZF promoter (5′-TCGAGGTATTCAGTACAGTACCAT-3′). Supershift analysis was done using antibodies against normal IgG, TIMP-2, or TIMP-1. The DNA-protein complexes were resolved on a non-denaturing polyacrylamide

gel, and were vacuumed dried and autoradiographed.

Data and Statistical Analysis

All data values are presented as the mean ± SD or means ± SEM. Statistical comparisons were carried out using the Student's *t*-test. $P < 0.05$ were considered relevant.

RESULTS

Identification of PLZF as a TIMP-1 Binding Protein and Mapping of the Interaction Region

To identify which proteins bind directly to TIMP-1, we screened a human ovary cDNA library, using full-length TIMP-1 cDNA fused to the pGilda DNA-binding domain as bait. Approximately 3.1×10^6 independent transformants were pooled and re-spread on selection media (Ura^-, His^-, Trp^-, Leu^-) containing 2% galactose to induce the expression of cDNA. If a B42-tagged protein interacts with TIMP-1 under these conditions, it will activate the transcription of *LEU2* gene, which allows the host cells to grow on a synthetic medium lacking leucine. Of the 13 colonies that were grown on the selection media, a total of five colonies showed galactose dependency. The plasmids were then isolated from the selected yeast cells and introduced into *E. coli* KC8 to isolate the plasmids carrying pJG4-5-cDNA inserts. The plasmids were then isolated by the plasmid marker *trp* in the *E. coli* host, and the purified plasmids were sequenced. A homology search in GenBank using BLAST revealed that all five plasmids encoded human protein PLZF (GenBank accession number: NM_006006).

To identify the PLZF binding region of TIMP-1, cDNA constructs containing three PLZF deletion mutants were designed as shown in Figure 1A. In the two-hybrid system, full-length human TIMP-1 cDNA accompanied with either

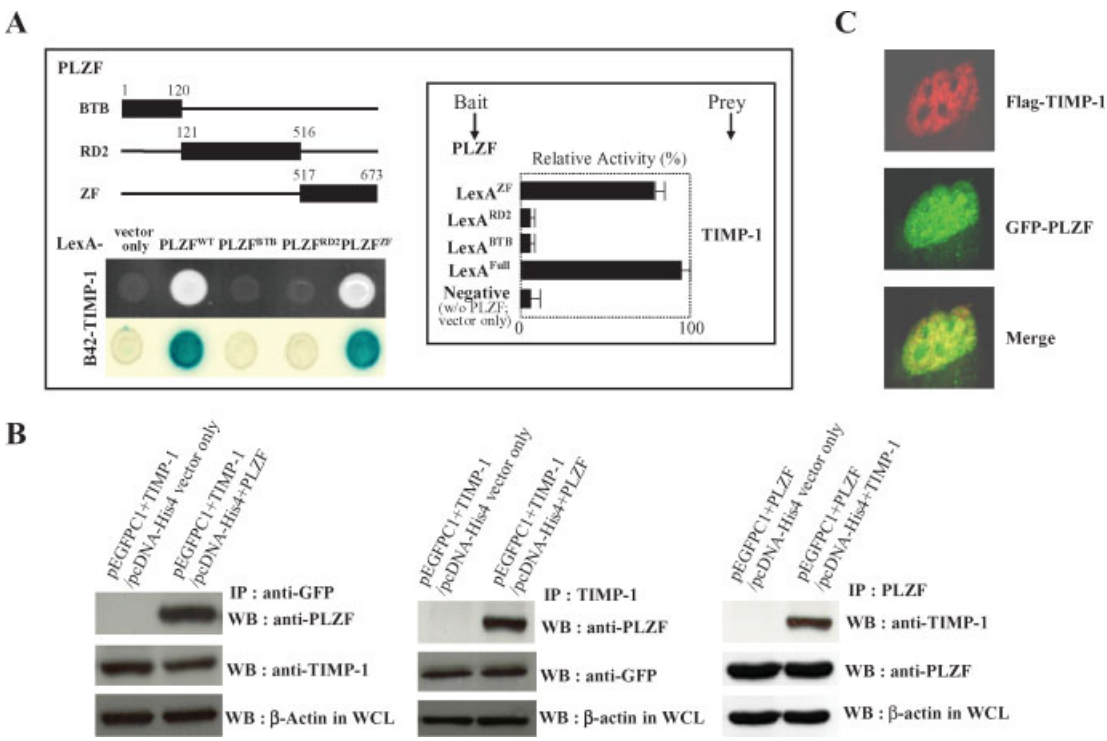

Fig. 1. Interaction analysis between human TIMP-1 and PLZF. **A**: Schematic representation of cDNA constructs for each PLZF deletion mutant (**upper panel** in the left). Testing the interaction of TIMP-1 and PLZF in the yeast two-hybrid system. Positive interactions were revealed by cell growth for 3 days at 30°C on medium lacking leucine (**lower panel** in the left), as well as by the formation of blue colonies on medium containing X-gal. Then, β-galactosidase activity was measured by adding *O*-nitrophenyl β-D-galactopyranoside (ONPG) (right). **B**: Co-immunoprecipitation of PLZF with anti-GFP and anti-TIMP-1. **Lanes 1**: Lysate from pEGFPC1-TIMP-1 and pcDNA4/HisMax (vector only) co-transfectant; **2**: Lysate from pEGFPC1-TIMP-1 and pcDNA4/HisMax-PLZF co-transfectant. Conversely, proteins immunoprecifitated with anti-PLZF antibody were analyzed by Western analysis with anti-TIMP-1 antibody. IP means immunoprecipitation and WB means immunoblotting with indicated antibodies. **C**: Co-localization of TIMP-1 and PLZF in the nucleus. HeLa cells were co-transfected with expression plasmids encoding FLAG-TIMP-1 and GFP-PLZF. The cells were then stained with antibodies to FLAG and Texas Red-conjugated secondary antibodies (red fluorescence). The GFP-PLZF fusion protein was visualized by GFP fluorescence (green). Co-localization of the two types of fluorescence is indicated in the merged image.

a plasmid containing full-length human PLZF cDNA (Full, Fig. 1A) or plasmids containing three truncation mutant forms (BTB, RD2, and ZF, Fig. 1A) of cDNAs were co-transformed into EGY48 yeast cells. Cells containing only the full-length PLZF cDNA construct and ZF-PLZF grew on the Ura, His, Trp, and Leu deficient plates, whereas yeast cells transformed with both deletion mutant colonies (BTB and RD2) failed to grow (Fig. 1A). To confirm this result, we determined the binding activities of these constructs by measuring the relative expression levels of β-galactosidase. As shown in Figure 1A, β-galactosidase assay results confirmed that neither of these mutants (BTB and RD2) bound TIMP-1.

For co-immunoprecipitation, cDNA constructs of TIMP-1 (pEGFPC1-TIMP-1) and PLZF (pcDNA4/HisMax-PLZF), or pEGFPC1-TIMP-1 and pcDNA4/HisMax (vector only) were co-transfected into 293 cells. Subsequently, immunoprecipitation was performed using anti-GFP antibody with lysates from both transfected cells. After immunoprecipitation, the precipitated proteins were immunoblotted by either using anti-TIMP-1 or anti-PLZF antibody. As shown in Figure 1B, pcDNA4/HisMax-PLZF was co-immunoprecipitated with pEGFPC1-TIMP-1 (lane 2 in upper panel), whereas no interaction was observed between pcDNA4/HisMax (vector only) and pEGFPC1-TIMP-1 (lane 1 in upper panel). Immunoblotting using anti-TIMP-1 antibody, confirmed that an equal amount of TIMP-1 was precipitated in both samples (middle panel). Whole cell lysates from both samples contained equivalent proteins when immunoblotted using anti-β-actin antibody (lower panel). Additionally, we also demonstrated the binding specificity of proteins immunoprecipitated with anti-PLZF antibody and analyzed them through Western analysis employing anti-TIMP-1 antibody.

To determine the sub-cellular localization interactions of PLZF with TIMP-1, HeLa cells were co-transfected with green fluorescent protein GFP-tagged PLZF and FLAG-tagged TIMP-1. Immunofluorescence analysis, along with the antibodies to FLAG and confocal microscopy, indicated that FLAG-TIMP-1 and GFP-PLZF fluorescence were co-localized in the nuclear granules (Fig. 1C). These findings confirmed that TIMP-1 and PLZF were both present in the same intracellular compartment, that is, in the nucleus, and thus provided evidence that they were capable of interacting with each other. Furthermore, the data also suggests that the PLZF-TIMP-1 interaction may take place in the nucleus.

Effect of TIMP-1 on Inhibition of PLZF-Induced Apoptosis

To investigate the effects of TIMP-1 in human cancer cells, TIMP-1, PLZF, and TIMP-1 plus PLZF were induced in HeLa cervical cancer cells. Transient overexpression of PLZF caused a change in cell morphology (data not shown) and inhibited cell growth (Fig. 2A). To confirm that this reduction in cell number represents apoptosis, fragmented DNA was examined by DAPI staining in HeLa cells overexpressing control vector (Mock), TIMP-1, PLZF, and TIMP-1 plus PLZF. Full-length PLZF-overexpressing cells showed fragmented nuclei, which is characteristic of apoptosis, while transfectants containing Mock or TIMP-1 did not show any signs of DNA fragmentation. Cells with a combination of full-length PLZF and TIMP-1 demonstrated a significantly reduced number of cells containing fragmented DNA (Fig. 2B). Apoptosis was also examined by FACS analysis after double staining Annexin V-FITC and propidium iodide (PI). PLZF-overexpressing cells showed strikingly similar characteristics of apoptotic cells, notably a low forward scatter (FCS) and high side scatter (SSC) profile (Fig. 2C). In the TIMP-1 culture, 92.03% of the cells were viable, while 3.41% were in early apoptosis and the remaining 4.56% were in the late or final stages of apoptosis ($P < 0.01$) (left panel). Initial analysis of HeLa cells induced with PLZF or PLZF plus TIMP-1 during 12 and 24 h showed no significant modifications in relation to the TIMP-1 culture (data not shown). However, in HeLa cells treated with PLZF or PLZF plus TIMP-1 during 72 h, 47.58% (middle panel) and 74.68% (right panel) of the cells were viable, while 36.28% and 15.38% were in early apoptosis, respectively. These combined results suggest that TIMP-1 expression efficiently blocks PLZF-mediated apoptosis. Next, to define the mechanism in determining the functional relationship between TIMP-1 and PLZF-mediated apoptosis, we measured the caspase-3 activity of those transfectants. Although TIMP-1 is involved in cell proliferation, no significant changes in caspase-3 activity were observed in the transfectants of TIMP-1 cDNA alone, when compared

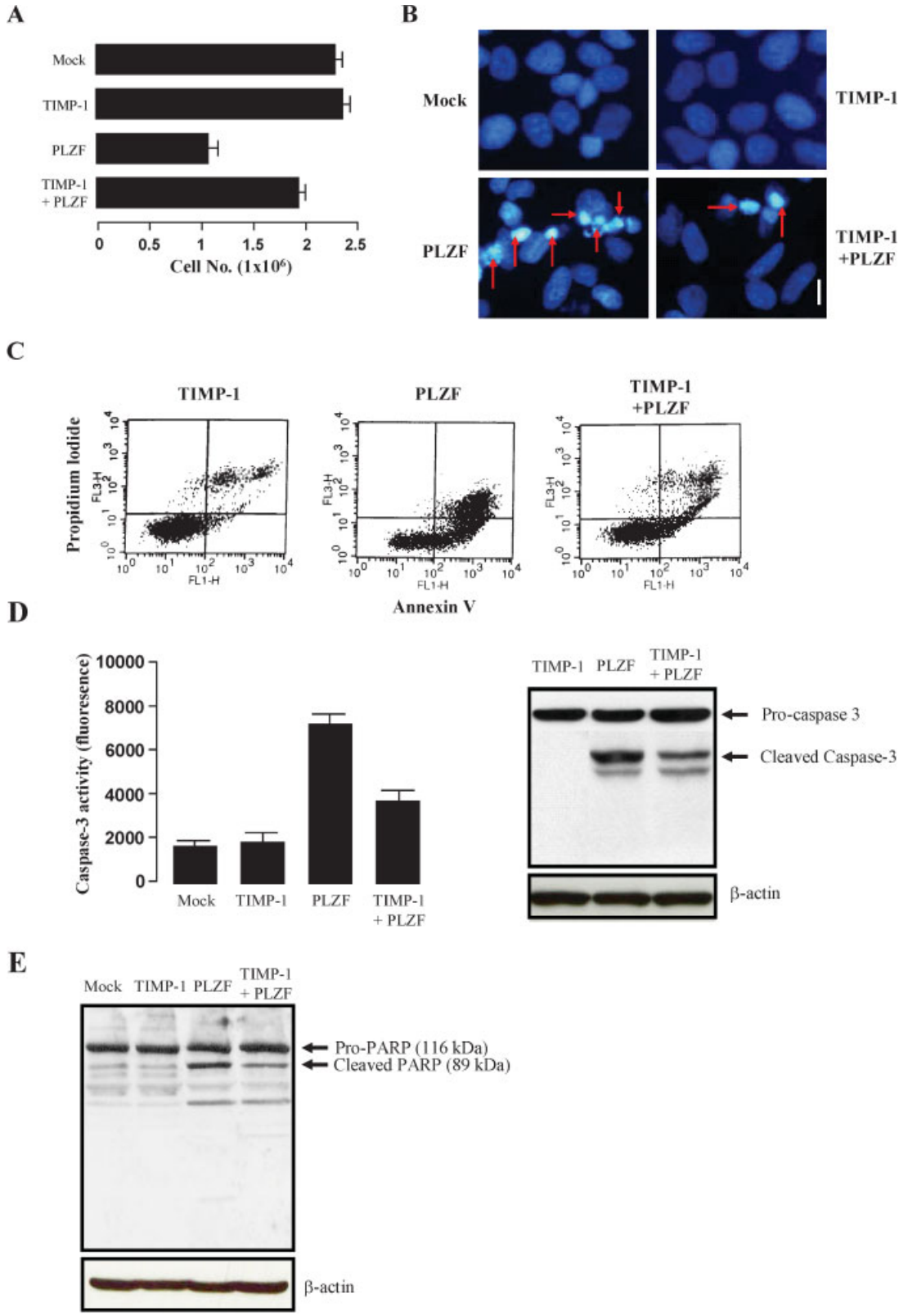
A
Mock
TIMP-1
PLZF
TIMP-1 + PLZF
0 0.5 1 1.5 2 2.5
Cell No. (1x10⁶)
B
Mock
TIMP-1
PLZF
TIMP-1 +PLZF
C
TIMP-1
PLZF
TIMP-1 +PLZF
Propidium Iodide
FL3-H
FL1-H
Annexin V
D
Caspase-3 activity (fluorescence)
10000
8000
6000
4000
2000
0
Mock
TIMP-1
PLZF
TIMP-1 + PLZF
TIMP-1 PLZF TIMP-1 + PLZF
Pro-caspase 3
Cleaved Caspase-3
β-actin
E
Mock TIMP-1 PLZF TIMP-1 + PLZF
Pro-PARP (116 kDa)
Cleaved PARP (89 kDa)
β-actin

Fig. 2.

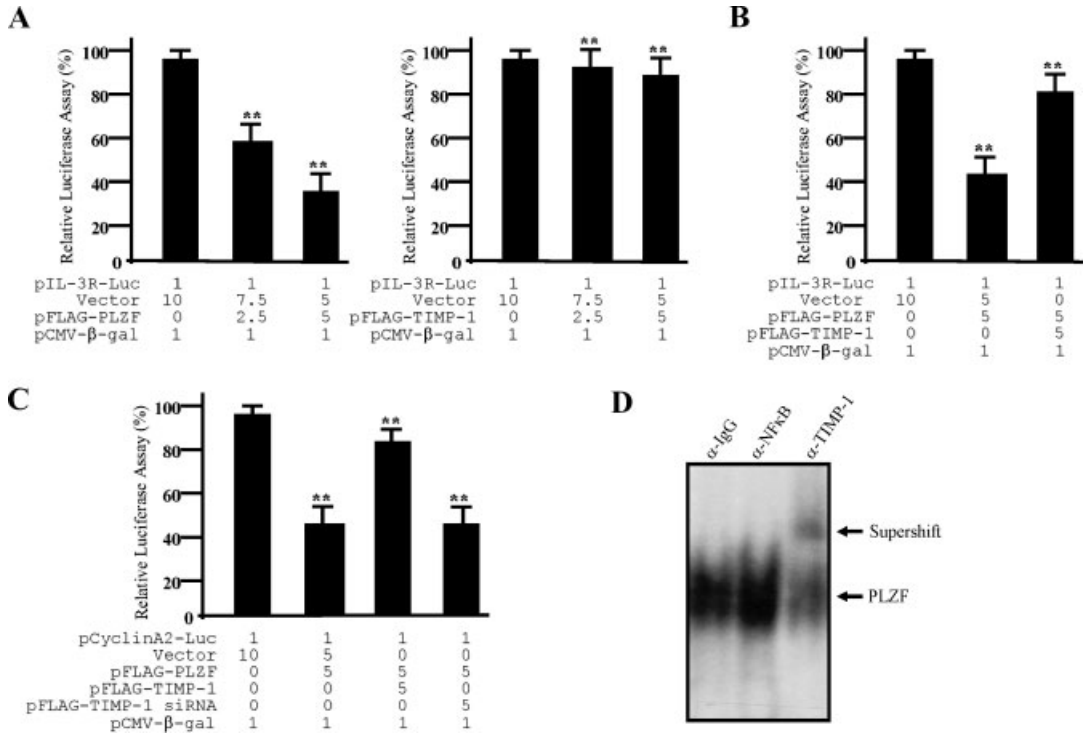

Fig. 3. TIMP-1 rescues a transcriptional activity from PLZF-mediated repression. **A**: Dose-dependent transcriptional activity of PLZF (left) and TIMP-1 (right) on the pIL-3-Luc activity. **B**: Preventing effect of transcriptional activity of PLZF by TIMP-1 on the pIL-3-Luc activity. **C**: Effect as a transcriptional activator of TIMP-1 on the cyclin A2-Luc activity. The indicated amounts (μg) of each plasmid were transiently transfected in HeLa cells. At 48 h after transfection, the cells were assayed for luciferase activity. Data represent mean ± SD from at least three independent experiments. $*P < 0.05$; $**P < 0.01$ compared with control alone. **D**: Nuclear extracts were obtained from cultured HeLa cells and analyzed by EMSA. The anti-IgG, anti-NFκB, and anti-TIMP-1 antibody were used to supershift the DNA–protein complex.

to mock. However, a significant upregulation of caspase-3 activity was observed in cells transfected with PLZF cDNA alone versus Mock transfected cells. Strikingly, TIMP-1 plus PLZF overexpressed cells restored caspase-3 activity almost similarly to Mock and TIMP-1 transfected cells (Fig. 3D). Since apoptosis is most frequently mediated by activated caspases, which leads to PARP binding fragmented DNA, we have attempted to detect caspase activation by Western blot analysis in cancer cells undergoing apoptosis after transfection with TIMP-1, PLZF, or TIMP-1 plus PLZF. As shown in Figure 2E, apoptosis-specific proteolytic cleavage of PARP, to the 85-kDa fragment, were not found on Western analysis in Mock or TIMP-1 overexpressing cells, whereas it was readily detected in the PLZF overexpressed cells. Apoptosis was observed inPLZF plus TIMP-1 overexpressed cells, but due to TIMP-1's cell proliferation ability, apoptosis was not as dramatic as in the case of PLZF overexpressed cells alone. These results suggest that TIMP-1 significantly reduced the apoptosis induced by PLZF in cervical carcinoma cells.

Fig. 2. TIMP-1 restores cell proliferation from PLZF-mediated apoptosis. MTT assay (**A**), DAPI staining (**B**), FACS analysis (**C**), caspase-3 activity assay (**D**), and immunoblot analysis of PARP (**E**) were performed on HeLa cervical cancer cells transfected with TIMP-1 and/or PLZF. The results are representative of three separate experiments. A: The relative rate of cell proliferation was quantified by MTT assays. The amount of MTT-formazan was determined as absorbance at 540 nm. Absorbance of each sample was converted as the relative rate of proliferation. Data are shown as mean ± SEM. B: Cells were stained with DAPI to visualize DNA fragmentations for apoptosis assay. Arrows indicate observed DNA fragmentations. Size bar, 20 μm. C: Cell viability by TIMP-1. Transfected HeLa cells the indicated expression vectors were incubated with FITC-labeled Annexin V and propidium iodide for 15 min and then analyzed by FACS. D: Caspase-3 activity was measured using a Spectramax 340 microplate reader (Molecular Devices) in fluorescence mode using an excitation at 400 nm and an emission at 505 nm according to a manufacturer's protocol (BDPharmingen). Enzyme activity was calculated and indicated as fluorescence according to the formula provided by the manufacturer. Data are shown as mean ± SEM. E: Western blot showing changes in PARP protein expression in whole-cell lysates of TIMP-1, PLZF, and TIMP-1 plus PLZF.

TIMP-1 Rescues a Transcriptional Activity From PLZF-Mediated Repression

To verify the functional relation between the interaction of TIMP-1 and PLZF with respect to transcriptional activity, we performed a luciferase assay with a reporter construct (pIL-3-Luc) containing four copies of the PLZF binding site. We have previously shown that PLZF specifically represses this reporter gene about 2.5-fold. Transcription repression was dose-dependent, which relates to the amount of plasmid but not to TIMP-1 (Fig. 3A). When HeLa cell-expressing PLZF plus TIMP-1 were transiently transfected, transcriptional activity was rescued from PLZF-mediated repression (Fig. 3B). Cyclin A2 expression is essential for cell cycle progression in both normal and cancer cells. TIMP-1 plus PLZF overexpressed cells restored the promoter activity of human cyclin A2, which embraces PLZF-response factors, but this restoration was completely blocked with the treatment of siRNA on TIMP-1 (Fig. 3C). In order to demonstrate the effects of TIMP-1's rescue activity when combined with PLZF, siRNA was employed. Transfection of siRNA restored the PLZF activity interfered by TIMP-1. Herein, TIMP-1 siRNA, which interferes with the production of TIMP-1 mRNA, was used to demonstrate the inhibition of TIMP-1 activity (data not shown).

To identify the components of the PLZF complex, supershift assays were performed with antibodies against different IgG and NFκB proteins (Fig. 3D). In order to verify the specificity of PLZF binding with TIMP-1, IgG, a protein abandoned by the nucleus, and NFκB, a transcriptional factor, were used as controls. The supershift assays indicate that antibodies against only TIMP-1 protein created clear supershift bands and caused a reduction in PLZF DNA binding. Since TIMP-1 is specific to PLZF, the other antibodies did not produce any supershift bands. This data indicate that TIMP-1 is a key regulator of PLZF-mediated transcriptional repression through its effects on reducing PLZF's binding affinity for DNA. In conclusion, these combined results indicate that TIMP-1 functions as an anti-activator of the transcriptional repressive activity of PLZF.

DISCUSSION

TIMP-1 is an encoded member of the TIMP family which regulates the activities of the MMPs and also enhances cell proliferation by interacting with a partner concerned with cell signaling. In this present study, we found that PLZF, a component of the transcription factor, to be a novel TIMP-1 binding protein. Here, we have shown that TIMP-1 also effectively inhibits apoptosis induced by exogenous PLZF in human cancer cells. The interaction between TIMP-1 and PLZF could be the first example of a regulation mechanism of PLZF by TIMP-1, which may ultimately provide a role in preventing cell death.

PLZF is a sequence-specific DNA-binding protein containing nine C-terminal C_2H_2 zinc finger motifs and an N-terminal BTB (broad complex, tramtrack, bric-a-brac)/POZ (Pox virus and zinc finger) [Bardwell and Treisman, 1994; Li et al., 1997; Sitterlin et al., 1997; Parrado et al., 2004]. PLZF functions as a transcriptional regulator of cell cycle progression by binding to the promoters of target genes, such as, cyclin A and the interleukin-3 receptor subunit [Ball et al., 1999; Yeyati et al., 1999; Melnick et al., 2000; Kang et al., 2003]. It also interacts with nuclear co-repressor proteins, such as N-CoR, SMRT, and mSin3A, which in turn interacts with histone deacetylases (HDAC1) [Hong et al., 1997; David et al., 1998; Grignani et al., 1998; Guidez et al., 1998c]. PLZF is expressed during early embryogenesis in the axial skeleton, liver, and heart, as a regulator of *Hox* gene expression, and as such, participates as a growth-inhibitory and pro-apoptotic factor in limb budding [Barna et al., 2000].

The regularity of TIMP-1 being located in the nucleus of cells changed in line with that of the proliferating cell nuclear antigen (PCNA). These results convey that the immunoreactive TIMP-1-like protein is localized in the nuclei of human gingival fibroblasts cells and that its content changes according to the cell cycle [Li et al., 1995]. Nuclear TIMP-1's ability of being derived from fetal calf serum (FCS), which was added to the culture medium, brought about the question as to whether TIMP-1 was derived from FCS or not. Since TIMP-1 is known to be a growth factor in serum and to act on a wide range of cells [Hayakawa et al., 1992], it is a possibility that bovine TIMP-1 could enter the cells after internalization with its receptor. As reported previously, TIMP-1 protein was also localized in the nuclei of human fetal lung fibroblasts, human MCF-7 breast carcinoma

cells, and human diploid fibroblast cell line WI-38 cells growing non-simultaneously [Zhao et al., 1998; Ritter et al., 1999]. These observations indicate that the collection of TIMP-1 in the nucleus seems to be widespread, at least among fibroblastic cell types.

In this study, we have found through the use of HeLa cells, PLZF-mediated transcription repression is in fact inhibited by TIMP-1. This result is possible only if TIMP-1 and PLZF are co-localized in the same intracellular region. Identification of TIMP-1 and PLZF co-localization in the nucleus was carried out through confocal microscopy of the HeLa cell, and through EMSA, where it was determined that TIMP-1 resided in the nucleus, allowing it to interact with PLZF. There have been previous research reports of TIMP-1's ability to reside in the nucleus of various cell types as well. The exact mechanism is yet unclear, but further studies are being conducted in order to determine the pathway of TIMP-1's ability to enter the nucleus. What was found is the fact that TIMP-1 is capable of restoring cell proliferation, which was repressed by PLZF-mediated transcription, through their interactions in the nucleus.

TIMPs are complex molecules with both pro- and anti-tumor effects. Thus, these diverse expressions could be due to the multifunctional properties of TIMPs. TIMP-1-inhibited cell death of human breast epithelial cell lines is regulated by Bcl-2 through the constitutive activation of FAK [Li et al., 1999]. A recent study found that TIMP-1 inhibits cell death in breast carcinoma cell T-47D cells via a pathway involving the sequential activations of c-Src, PI3 kinase, and Akt [Lee et al., 2003]. In addition, TIMP-1 is able to exert multiple biological functions including MMP inhibition, tumorigenesis, apoptosis, angiogenesis regulation, and complex formation with signal-related proteins and is also capable of preventing a variety of cellular activities.

As an initial step to define the regulating mechanism of TIMP-1 biological systems, we used a yeast two-hybrid system to screen a human ovary cDNA library for a novel TIMP-1 interacting species. The screening identified human PLZF, a well-known transcriptional regulator, to be a species with the ability to interact with TIMP-1. In the present study, we investigated whether TIMP-1-mediating anti-apoptotic activity can promote the growth of ovarian cancer in an experimental system. TIMP-1 treatment was found to be more effective at increasing ovarian cancer growth than PLZF in our parallel experiments. Subsequently, the efficacy of a combined treatment with TIMP-1 and PLZF was investigated, and TIMP-1 was found to significantly reduce apoptosis induced by PLZF in cervical carcinoma cells. Here, we have shown that the functional relation between the interaction of TIMP-1 and PLZF with respect to transcriptional activity. We performed luciferase assays with a reporter construct (pIL-3-Luc) containing four copies of the PLZF binding site. We have previously shown that PLZF specifically represses this reporter gene about 2.5-fold. The transcription repression of PLZF was found to be dependent on the amount of the plasmid. These results suggest that TIMP-1 transcriptionally activates the transcriptional repressive activity of PLZF. Taken together, these results indicate that TIMP-1-PLZF complex formation induces tumorigenesis in cells by competing with MMP, a signaling molecule known to be critical for cell survival.

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