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TIMP1 induces CD44 expression and the activation and nuclear translocation of SHP1 during the late centrocyte/post-germinal center B cell differentiation

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Abstract

Tissue inhibitor of metalloproteinase-1 (TIMP1) is a survival factor of germinal center (GC) B cells, and its over-expression is correlated with aggressive B cell lymphomas and classical Hodgkin lymphomas. We previously demonstrated that TIMP1 down-regulates B-cell receptor and BCL6, and activates interleukins-6,-10 (ILs)/signal transducer and activator of transcription-3 (STAT3) signaling in GC B cells. The activation of ILs/STAT3 signaling can amplify CD44 function, and vice versa, and induce protein-tyrosine phosphatase SHP1 activity by a negative feedback mechanism. Here, we show that TIMP1 up-regulates cell surface CD44 (standard and variants 3 and 7–10) and induces the activity and nuclear localization of SHP1 in an Epstein Barr virus (EBV)-negative Burkitt lymphoma cell line, the neoplastic counterpart of GC centroblasts. These results suggest that TIMP1 functions as a differentiating and survival factor of GC B cells by modulating CD44 and SHP1 in the late centrocyte/post-GC stage, regardless of EBV infection.

Keywords

TIMP1; CD44; SHP1; Hodgkin lymphoma

1. Introduction

Tissue inhibitor of metalloproteinase-1 (TIMP1) is a survival factor with multiple functions involving cell growth and differentiation [1], as well as matrix metalloproteinase (MMP) inhibition [2]. TIMP1 expression is highly correlated with a subset of aggressive diffuse large B cell lymphomas [3,4], classical Hodgkin lymphomas [5,6], and a variety of carcinomas with poor clinical outcomes [7,8]. Microarray analysis of genes induced by TIMP1 revealed that CD44 and suppressor of cytokine signaling-3 (SOCS3) were over-

expressed and accompanied by the activation of interleukins (ILs)-6 or -10/signal transducer and activator of transcription-3 (STAT3) signaling pathways [9].

CD44, a highly variable cell surface glycoprotein, is involved in lymphocyte adhesion and homing and tumor progression and metastasis. The structural and functional diversity of CD44 is generated by alternative splicing of messenger RNA (mRNA) and variations in glycosylation [10,11]. Although CD44 is highly expressed on primitive hematopoietic cells, the level of CD44 expression is known to change according to the specific stage of B cell development [12]. Intriguingly, CD44 is also tightly regulated by MMPs and TIMP1. CD44 can modulate the secretion and activation of MMP2 and anchor the active form of MMP9 on breast cancer cell surfaces, leading to extracellular matrix degradation of the tumor microenvironment [13,14]. In addition, CD44 can be cleaved by membrane type 1 (MT1)-MMP, which is inhibited by TIMP1. The cleavage of CD44 plays a critical role in cell migration [15,16]. CD44 is known to amplify the IL6/STAT3 signaling pathway in myeloma cells, and vice versa [17]. The activation of STAT3, in turn, up-regulates its negative signaling regulators, such as SOCS3 or Src homology-2 domain-containing protein-tyrosine phosphatase (SHP1), forming a negative feedback loop [18].

Generally, SHP1 acts as a negative regulator of B-cell receptor (BCR) signaling [19]. SHP1 is uniformly expressed both in normal B cells from the mantle and marginal zones, as well as in plasma cells and in their malignant counterparts. However, it is down-regulated in normal germinal center (GC) B lymphocytes and Burkitt lymphoma (BL) cells [20]. These data suggest that SHP1 expression is not only tightly regulated during B cell differentiation, but also reflects histogenesis of malignant lymphomas [21,22]. Furthermore, the induction of SHP1 expression leads to hematopoietic cell differentiation and adhesion in HL60, K562, and BL cell lines [23,24]. However, although the expression of SHP1 can be induced by some hematopoietic differentiating inducers, including sodium butyrate, dimethyl sulfoxide (DMSO), phorbol 12-myristate 13-acetate (PMA), and polyclonal anti-Ig M antibody, the mechanism by which SHP1 is expressed and activated remains poorly understood [20,23,24].

To understand how TIMP1 acts on GC B cell differentiation, the expression of CD44 and SHP1, the putative downstream molecules of TIMP1, were analyzed in an Epstein-Barr virus (EBV)-negative BL cell line with enhanced expression of TIMP1. Here, we demonstrate that TIMP1 up-regulates cell surface CD44 expression (standard and variants 3, and 7–10 isoforms), and induces the activity and nuclear translocation of SHP1 in the stage of late centrocyte/post-GC differentiation.

2. Materials and methods

2.1. Cell culture

EBV-free JD38 BL cells were used to induce TIMP1 expression by LXSJ retroviral transduction, as described by Guedez et al. [25]. All cell lines designated JD38 (parent), LXSJ JD38 (empty vector control), and TIMP1 JD38 (clones 20 and 24) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin G, 100 µg/ml streptomycin sulfate, and L-glutamine (Gibco, Gaithersburg, MD, USA). All cells were incubated in 5% CO₂ with 95% humidity at 37 °C.

2.2. Immunohistochemistry

Immunohistochemical staining was carried out using a ChemMate EnVision Detection Kit (Dako, Carpinteria, CA, USA). Tissue sections from reactive palatine tonsils were deparaffinized and dehydrated. The sections were submerged in 1-mM EDTA buffer (pH 8.0), heated in a microwave oven or pressure cooker for 10 min, and then incubated with

0.3% H₂O₂/methanol for 20 min. Next, the slides were incubated with anti-TIMP1 (Labvision-Neomarkers, Fremont, CA, USA), anti-CD44 (Santa Cruz Biotech., Santa Cruz, CA, USA), anti-SHP1 (Santa Cruz Biotech.), anti-BCL6 (Dako), and anti-MUM1 (Santa Cruz Biotech.) antibodies at room temperature (RT) for 1 h. After incubation with secondary antibody at RT for 30 min, the sections were developed with 3,3'-diaminobenzidine (DAB) and counterstained with hematoxylin.

2.3. Western blot analysis

Cells were cultured (2×10^6 cells/ml) for 24 h in serum-free RPMI media and then lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 5 mM EDTA) containing a cocktail of proteinase inhibitors. A range of 20–40 µg of protein was electrophoresed in a 4–20% (wt/vol) sodium dodecyl sulfate/polyacrylamide gel (SDS-PAGE). Proteins were electroblotted onto polyvinylidene difluoride membranes (PVDFs). After blocking with Tris-buffered saline (TBS) containing 3% nonfat dry milk, the membranes were washed and blotted with primary antibodies (1:200 to 1:1000 dilutions). The primary antibodies used in this study included anti-BCL6 (Dako), anti-CD44 (Santa Cruz Biotech.), anti-SHP1 (monoclonal, BD Transduction Laboratories, San Jose, CA, USA; polyclonal, Santa Cruz Biotech.), and anti-MUM1 (Santa Cruz Biotech.) antibodies. The membranes were washed in phosphatebuffered saline (PBS) containing 0.05% Tween 20, then developed using a horseradish peroxidase-conjugated secondary antibody (Pierce, Rockville, MD, USA) and a chemiluminescence kit (DuPont NEN, Boston, MA, USA).

2.4. Flow cytometry

Cells (4×10^5) were washed twice in PBS-containing 1% bovine serum albumin (BSA) and then incubated with fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-CD44 diluted at 1:100 (1 µg) (Santa Cruz Biotech.), and FITC-conjugated mouse IgG₁ (Dako). The cells incubated for 1 h at 4 °C in the dark and then rinsed twice with PBS-containing 1% BSA. To determine the percentage of positive cells, immunofluorescence-labeled cells were analyzed in an FACSCAN (Becton–Dickinson, San Jose, CA, USA) with CellQuest software (Becton–Dickinson). Fluorescence intensity was also calculated. After data acquisition, fluorescent calibrated beads were used to standardize fluorescence intensity and expressed as molecule equivalent of surface fluorochrome (MESF) units.

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

CD44-specific RT-PCR was performed using a Gene-Amp Gold RNA PCR Reagent kit (PE Biosystems, Foster city, CA, USA) essentially as previously described [26]. Briefly, total cellular RNA was prepared using 1×10^7 cells and isolated using an RNA Easy extraction kit (Qiagen, Valencia, CA, USA). Total RNA was reversetranscribed using oligo dT primers at 42 °C for 10 min. The primers used in the first and exon-specific amplifications were as follows:

P1 (sense, exon 5) 5'-AAGACATCTACCCCAGCAAC-3';

P2 (common antisense, exons 17–18) 5'-CCAAGATGATCAGCCATTCTGG-3';

V2 (sense, exon 7) 5'-GATGAGCACTAGTGCTACAG-3';

V3 (sense, exon 8) 5'-ACGTCTTCAAATACCATCTC-3';

V4 (sense, exon 9) 5'-TCAACCACACCACGGGCCTT-3';

V5 (sense, exon 10) 5'-GTAGACAGAAATGGCACCAC-3';

V6 (sense, exon 11) 5'-CAGGCAACTCCTAGTAGTAC-3';

V7 (sense, exon 12) 5'-CAGCCTCAGCTCATACCAGC-3';
 V8 (sense, exon 13) 5'-TCCAGTCATAGTACAACGCT-3';
 V9 (sense, exon 14) 5'-CAGAGCTTCTCTACATCACA-3';
 V10 (sense, exon 15) 5'-GGTGAAGAAGAGACCCAAA-3';
 β -actin primers (sense) 5'-TGCTATCCAGGCTGTGCTAT-3';
 (antisense) 5'-GATGGAGTTGAAGGTAGTTT-3'.

The cDNA was first amplified using Taq-DNA polymerase in a final volume of 50 μ l. The reaction solution contained 10 mM dNTP, 1 U Taq-DNA polymerase, 10 $\times$ PCR buffer, 25 mM MgCl₂, and 0.2 μ M of each primer. PCR reactions were performed as follows: 2 min at 95 $^{\circ}$ C, followed by 30 cycles of denaturation at 95 $^{\circ}$ C for 30 s, annealing at 56 $^{\circ}$ C for 90 s, and extension at 73 $^{\circ}$ C for 60 s, with a final extension of 5 min at 72 $^{\circ}$ C. Exon-specific amplification was performed in 50 μ l containing 10 mM dNTP, 1 U Taq-DNA polymerase, 10 $\times$ PCR buffer, 25 mM MgCl₂, and 0.2 μ M of each primer. Different sense-variable primers with the same antisense primer were used. The reaction conditions were as follows: 10 min at 95 $^{\circ}$ C, followed by 30 cycles of denaturation at 94 $^{\circ}$ C for 45 s, annealing at 55 $^{\circ}$ C for 1 min, and extension at 72 $^{\circ}$ C for 1 min, with a final extension of 7 min at 72 $^{\circ}$ C. The amplified products were separated on a 1.5% agarose gel and visualized using ethidium bromide staining.

2.6. Protein-tyrosine phosphatase assay

Protein-tyrosine phosphatase activities were assayed following the immunoprecipitation of SHP1 (BD Transduction Laboratories). Phosphotyrosine phosphatase activity of the immunoprecipitates was measured as the amount of phosphate released after the addition of known amounts of phosphotyrosine-containing peptide substrates (Upstate Biotechnology, Lake Placid, NY, USA). After JD38 cells were starved with serum-free media for 24 h, the cells were lysed with RIPA buffer-containing proteinase inhibitors (without orthovanadate). The relative protein concentrations were determined using the BCA assay (Pierce). Equal amounts of protein (250 μ g) were immunoprecipitated with anti-SHP1 antibody at 4 $^{\circ}$ C for 1 h, followed by incubation with anti-mouse agarose beads (Santa Cruz Biotech.) for 1 h. The beads were subsequently washed once with lysis buffer (without orthovanadate) and twice with phosphatase assay buffer. The immunoprecipitate was resuspended in assay buffer and the reaction initiated by the addition of phosphotyrosine peptide. The enzyme reaction was incubated for 20 min at 37 $^{\circ}$ C and then terminated by the addition of Malachite Green solution. Color developed after 15 min, the optical densities were measured at 650 nm. Data were normalized to blank assay values and plotted as relative OD units of phosphate released resulting from the enzyme reaction.

2.7. MALDI-TOF mass spectrometry analysis

Total cell lysate was solubilized in RIPA buffer-containing freshly added protease inhibitors. Cell lysates were pre-cleared with normal mouse IgG and protein A/G plus-agarose (Santa Cruz Biotech.) at 4 $^{\circ}$ C for 30 min. The supernatants were incubated with monoclonal anti-SHP1 antibody (BD Transduction Laboratories) at 4 $^{\circ}$ C for 1 h, followed by protein A/G plus-agarose at 4 $^{\circ}$ C overnight or for 1 h. Immunoprecipitates were washed twice with RIPA buffer and once with PBS, and resolved by 4–10% SDS-PAGE. The gels were stained with Coomassie blue (Pierce). The 70-kDa bands of the gel were excised and trypsin-digested. The in-gel tryptic peptides were extracted and analyzed using an Ettan MALDI-TOF Pro (Amersham Bioscience) mass spectrometer.

2.8. Immunofluorescence study and cell fractionation

JD38 cells were incubated with serum-free medium for 24 h and spun onto glass slides at 500 rpm for 5 min using a Cytospin 3 (Shandon, Pittsburgh, PA, USA). The cells were washed for 5 min in PBS and then fixed in 4% paraformaldehyde in PBS for 20 min. After a further 5-min wash in PBS, the cells were incubated in 10% normal goat serum (NGS), and 0.2% Triton X-100 for 30 min, and then incubated overnight at 4 °C with anti-SHP1 monoclonal antibody (BD Transduction Laboratories) diluted 1:250 (0.5 µg/ml) in PBS and 1% NGS. The cells were washed four times for 5 min in PBS, 10% NGS, and 0.2% Triton X-100 before Alexa 488-conjugated goat anti-mouse antibody (Molecular Probes, Eugene, OR, USA) was applied at a 1:200 dilution in PBS and 1% NGS for 1 h. After four additional 5-min washes in PBS, 10% NGS, and 0.2% Triton X-100, the cells were immersed briefly in distilled water and mounted with a Vecta Shield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). Alexa-488 and DAPI fluorescence were viewed at 467 nm using an Axiovert 135 microscope (Zeiss, Thornwood, NY, USA) coupled to a monochrome light source. JD 38 cells (5×10^6) were fractionated using an NE-PER nuclear and cytoplasmic extraction kit (Pierce). The procedure was carried out at 4 °C according to the manufacturer's instructions. The purity and integrity of nuclei were monitored using inverted light microscopy and Western blotting with a nuclear marker (anti-lamin B).

3. Results and discussion

3.1. TIMP1, CD44, SHP1, and MUM1 are expressed in the late centrocyte/post-GC stage

To determine on what stage of GC B-cell differentiation TIMP1 acts, the expression of TIMP1 and its associated proteins, such as CD44 and SHP1, was investigated in reactive lymphoid tissues using immunohistochemistry. TIMP1, CD44, SHP1, and MUM1 were localized in the centrocytes of the GC light zone, interfollicular B cells, and plasma cells. CD44 and SHP1 were also expressed in the pre-germinal center mantle zone cells (Fig. 1a). Most follicular dendritic cells expressed CD44. However, the centroblasts in the dark zone expressed BCL6 (data not shown), but lacked the expression of TIMP1, CD44, SHP1, and MUM1 (Fig. 1a). A panel of phenotypic markers including BCL6, MUM1, and CD138 has been established for the characterization of physiologic GC B-cell development and molecular histogenesis of their malignant counterparts: centroblasts ($BCL6^+/MUM1^-/CD138^-$), late centrocyte/early post-GC B cells ($BCL6^-/MUM1^+/CD138^-$), and post-GC B cells ($BCL6^-/MUM1^+/CD138^+$) [27]. Thus, these results suggest that TIMP1, CD44, and SHP1 play a role in the stage of late centrocyte/post-GC differentiation. They can be used as additional histogenetic markers of late centrocyte/post-GC B cells because these proteins are tightly regulated at various stages of GC B-cell development (Fig. 1b).

3.2. TIMP1 differentiates GC centroblasts into the late centrocyte/post-GC phenotype

To determine whether TIMP1 functions as a differentiation factor on GC centroblasts, the expression of phenotypic markers was analyzed by Western blotting using the EBV-free TIMP1 JD38 cell lines. The EBV-negative BL cell lines were used because BL cells are a malignant counterpart of centroblasts, and EBV is known to drive GC Bcell maturation to the post-GC stage [28,29]. Western blot analyses showed that TIMP1 JD38 cells considerably upregulated CD44, SHP1, and MUM1 expression compared to the control JD38 and LXS cell lines (Fig. 2). In contrast, BCL6 expression is markedly decreased or absent in TIMP1 JD38 cells. This phenotypic pattern ($BCL6^-/CD44^+/SHP1^+/MUM1^+$) suggests that TIMP1 promotes the differentiation process from centroblasts to the centrocytic stage of GC B-cell differentiation.

3.3. TIMP1 up-regulates the expressions of cell surface CD44 standard and its variants

TIMP1 up-regulated the expression of cell surface CD44 and CD44 mRNAs (standard, variant 3, and 7–10 isoforms) as seen by flow cytometry (Fig. 3a) and by exon-specific RT-PCR (Fig. 3b and c), respectively. During GC B cell differentiation, CD44 displays a complex, tightly regulated expression pattern [12]. CD44 is expressed at the resting and early activation stages but is lost upon transition to the centroblast stage. CD44 is up-regulated again at the point of transition from the centroblast to the centrocyte level. This second up-regulation phase appears to be consistent with the phase in which CD44 is induced by TIMP1. During GC B-cell development, CD44 expression may be isoform-specific with different functional significances [11]. Resting lymphocytes express the standard CD44 while activated lymphocytes, lymphoma cells, and epithelial cells preferentially express larger CD44 variants. The CD44 isoforms in our study may be specific in the late centrocyte/post-GC B cell differentiation. CD44v9 is involved in the CD44/IL-6 amplification loop [17], while CD44v10 acts as a homing adhesion molecule of myeloma cells in the bone marrow [30]. CD44v3, decorated with heparan sulfates (HS), serves as a co-receptor for heparin-binding growth factors, such as fibroblast growth factor (FGF)-2 and c- Met [31,32]. CD44 can recruit growth factors, such as MMP9 and MMP7 [13,14,33]. CD44 associates not only with receptor tyrosine kinases, but also with $\alpha 3\beta 1$ integrin or tetraspanins such as CD9 and CO-029 [34]. The antiapoptotic effect of CD44-ligand interaction is associated with phosphatidylinositol-3 kinase (PI3K), the up-regulation and phosphorylation of Akt and BAD phosphorylation, in addition to the activation and up-regulation of BCL-X_L and BCL2 [35].

3.4. TIMP1 induces the activity and nuclear translocation of SHP1

TIMP1 induced the activity and nuclear translocation of SHP1 (Fig. 4). Like CD44, SHP1 is tightly regulated during the GC reaction and up-regulated at the apical light zones of GC, which is the same as the expression pattern of CD44 [21]. Using in vitro tyrosine phosphatase assays, it was shown that TIMP1 up-regulates the activity of SHP1 by 5-fold or higher (Fig. 4a). SHP1 can be upregulated by the activation of ILs-6 or -10/STAT3 signaling pathways. SHP1 associates constitutively with the B cell receptor (BCR) complex to dephosphorylate Ig- α /Ig- β chains in resting B cells [36]. As TIMP1 down-regulates the BCR complex (Fig. 4b), SHP1 was expected to be present exclusively in the cytoplasm. Unexpectedly, SHP1 localized in the nuclei as well as in the cytoplasm of TIMP1 JD38 cells (Fig. 4c); nearly 60% of SHP1 existed in the nuclear fraction of TIMP1 JD38 cells (Fig. 4d). For the first time, it was demonstrated that TIMP1 induced the nuclear translocation of SHP1 in an EBV-free BL cell line, the malignant counterpart of GC centroblasts. However, the nuclear localization of SHP1 was not observed in the GC B cells of the reactive lymphoid follicles (Fig. 1a). Thus, although further studies using another BL cell line transfected by TIMP1 are needed, it is assumed that this translocation of SHP1 is not physiological and occurs only in a subset of the BL cells with a high level of TIMP1 expression. In fact, SHP1 has nucleus localizing signal residues at its carboxy-terminal domain [37]. When cells are stimulated by PMA or cytokines, SHP1 can be translocated to the nucleus or other subcellular components [23]. The activated nuclear SHP1 may dephosphorylate the phosphorylated STAT3 in the nucleus, leading to recycling and therefore, persistent STAT3 activation [38]. However, the precise roles of nuclear SHP1 remain to be further elucidated.

In conclusion, this study shows that TIMP1 promotes the late centrocyte/post-GC differentiation and survival of GC B cells via the up-regulation of CD44 and SHP1 expression. This indicates that TIMP1 might play a role in rescuing the preapoptotic GC B-cells with defective BCR signaling in pathologic states, which is very similar to the effects of EBV on the GC B-cells. Thus, TIMP1, CD44, and SHP1 can be used as new histogenetic

markers for the late centrocyte/post-GC differentiation. In addition, TIMP1 and its putative downstream CD44 and SHP1 may be therapeutically targeted against TIMP1- positive aggressive B cell lymphomas or Hodgkin lymphomas.

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Fig. 1.

Expression of TIMP1, CD44, SHP1, and MUM1 in the light zone identifies the late centrocyte/post-GC stage. (a) Immunohistochemistry of a reactive lymphoid follicle expressing TIMP1, CD44, SHP1, and MUM1 in the light zone (LZ). CD44 and SHP1 are also expressed in the mantle (M) zone cells of lymphoid follicles. CD44 is expressed in centrocytes as well as follicular dendritic cells in the LZ. However, centroblasts in the dark zone (DZ) do not express TIMP1, CD44, SHP1, and MUM1. (b) A model for the physiologic stages of GC B cell development identified by the expression profile of BCL6, TIMP1, CD44, SHP1, MUM1, and CD138 (rectangles). GC B cells (i.e. centroblasts and centrocytes) express BCL6, but not CD138. GC B cells in the light zone (late centrocytes)

and post-GC B cells express TIMP1, CD44, SHP1, and MUM1. The post-GC cells express CD138.

Fig. 2. TIMP1 drives differentiation of JD38 cells to the late centrocyte/post-GC phenotype ($BCL6^{-}/CD44^{+}/SHP1^{+}/MUM1^{+}$). Western blot analysis shows that the expression of TIMP1, CD44, and SHP1 is significantly increased in TIMP1 JD38 cells (clones 20 and 24) compared to the control LXSN and JD38 cells. In contrast, BCL6 expression is markedly decreased or absent in TIMP1 JD38 cells. Relative band intensities were measured by ImageJ image software and shown at the bottom of blots.

Fig. 3. TIMP1 up-regulates the cell surface expression of CD44 (standard, v3, and v7–10 isoforms). (a) Flow cytometry shows that about 92% of TIMP1 JD38 expresses CD44 on their cell surfaces. (b) Schematic representation of CD44 gene and primers for exon-specific nested RT-PCRs. The primers are marked by P1 (sense, exon5), P2 (common antisense, exon 17–18), v2 (sense, exon 7), v3 (sense, exon 8), v4 (sense, exon 9), v5 (sense, exon 10), v6 (sense, exon 11), v7 (sense, exon 12), v8 (sense, exon 13), v9 (sense, exon 14), v10 (sense, exon 15) and β -actin primers. The first reaction was amplified with P1 and P2 primers and the exon-specific amplifications were carried out using a combination of each forward primer derived from exon 7 (v2) to exon 15 (v10) and the common reverse P2 primer. (c)

RT-PCR demonstration of standard CD44 expressing TIMP1 JD38 (clone24) (upper panel). To evaluate CD44 variants, the amplifications were performed using the exon-specific primers in TIMP1 JD38 (clone 24) (lower panel). CD44 v3 and v7–10 were amplified in TIMP1 JD38 cells (clone 24). The amplified products correspond to V3 (277-bp) and V7–10 (680-bp, 540-bp, 432-bp, and 340-bp) of CD44. C and M represent a positive-reaction β -actin control and a 100-bp DNA ladder marker, respectively.

Fig. 4.

TIMP1 induces the activity and nuclear translocation of SHP1. (a) After immunoprecipitation of SHP1, protein-tyrosine phosphatase activities were assayed. Phosphotyrosine phosphatase activity of the immunoprecipitates (IPs) was measured as the amount of phosphate released after the addition of known amounts of phosphotyrosine-containing peptide substrates. The IP from 250 μ g of TIMP1 JD38 (clone24) cell lysate releases 727 ± 44.5 (mean $\pm$ SD) pmol of phosphate (relative OD, 0.16 ± 0.01) after the addition of 500 μ M of phosphotyrosine peptide. In contrast, control LXSN and JD38 releases 126.8 ± 44.5 and 26.7 ± 26.7 pmol at the same conditions, respectively. (b) After pre-clearing of JD38 cell lysates (500 μ g), the supernatants were incubated with anti-SHP1

antibody at 4 °C for 1 h, followed by the addition of protein A/G plus-agarose at 4 °C overnight. After washing, IP was resolved by a 4–10% SDS–PAGE and stained with Coomassie blue for peptide sequencing or silver stained. The ~70-kDa protein bands (arrowheads) seen only in control JD38 cells were analyzed by MALDI-TOF mass spectrometry and identified as a human membrane-bound Ig μ heavy chain. (c) TIMP1 JD38 displays a distinct speckled pattern of nuclear immunofluorescence for SHP1. DAPI was counterstained for the clarification of the nuclear staining (original magnification, 400 $\times$). (d) To verify the nuclear translocation of SHP1, cell fractionation was performed. The purity and integrity of nuclei were monitored using inverted light microscopy and Western blotting with a nuclear marker (anti-lamin B). Nearly 60% of SHP1 was present in the nuclear fraction of TIMP1 JD38. C and N indicate the cytosolic and nuclear fractions, respectively.