A crucial role for Lyn in TIMP-1 erythroid cell survival signalling pathway

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Article info

Article history:
Received 28 December 2012
Revised 19 March 2013
Accepted 27 March 2013
Available online 11 April 2013

Edited by Angel Nebreda

Keywords:
TIMP-1
Erythroleukemic cell
Cell survival
Lyn

Abstract

TIMP-1, a well-known MMP inhibitor, displays other biological activities such as cell survival, proliferation and differentiation in hematopoietic cells. In this report, we investigated the role of the Src-related kinase Lyn in TIMP-1 induced UT-7 erythroleukemic cell survival. We showed that (i) tyrosine 507 of Lyn was dephosphorylated and Lyn kinase activity enhanced by TIMP-1, (ii) Lyn silencing suppressed TIMP-1 anti-apoptotic activity and (iii) Lyn was activated upstream the JAK2/PI 3-kinase/Akt pathway. Our data suggest a novel role for Lyn in erythroid cell survival.

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1. Introduction

In bone marrow, survival, proliferation and differentiation of hematopoietic progenitor cells are supported by cytokines and growth factors but also depend on some microenvironmental factors among which the matrix metalloproteinases (MMPs) and their endogenous inhibitors, the Tissue Inhibitors of Metalloproteinases (TIMPs) [1,2,3,4]. Besides their MMP inhibitory activity, TIMPs and particularly TIMP-1 display MMP-independent cytokine-like activities and are involved in cell growth, angiogenesis, apoptosis, and migration [4]. In bone marrow TIMP-1 participates to hematopoiesis as an autocrine or a paracrine factor and regulates hematopoietic stem cell cycle dynamics [4,5]. We previously reported that human erythroleukemic UT-7 cells secrete TIMP-1 which promotes erythroid differentiation and cell survival via the JAK2/PI-3 kinase/Akt pathway [6,7]. The identification of intracellular pathways activated by TIMP-1 leads us to hypothesize that TIMP-1 may induce a signal through a membrane receptor. Such receptor has been partially characterized with varying molecular characteristics and binding partners according to cell type [8]. In human breast epithelial cells, TIMP-1 interacts with the tetraspanin receptor CD63 and integrin β1 leading to the activation of cell survival and apoptosis inhibition [9]. In human osteosarcoma cells, TIMP-1 interacts with αvβ3 integrins and confers resistance to TNF-α-induced apoptosis [10]. In UT-7 cells, we demonstrated that TIMP-1 binding to a membrane proMMP-9/CD44 complex was a prerequisite for TIMP-1 survival signalling [11]. CD44 is a cell surface transmembrane glycoprotein, widely expressed in hematopoietic cells. CD44 ligation induces intracellular signalling through the association of its intracellular domain with receptor-associated tyrosine kinases such as Src-related kinases [12,13]. Some studies have suggested the existence of a complex between CD44 and Lyn impacting on cell migration via Akt phosphorylation [14].

Nine members compose the human Src kinase family and their activation depends on tyrosine 527 dephosphorylation followed by tyrosine 416 autophosphorylation [15]. Activated kinase phosphorylates tyrosine residues of cytoplasmic substrates among which the PI 3-kinase [16]. Numerous studies underscored the biological importance of the Src kinase Lyn for correct erythroid development with particular attention to erythropoietin-induced terminal differentiation [17]. Lyn is one of the key TK that regulates erythropoietin (Epo)-induced differentiation as well as survival and interacts with a large number of growth factors [18]. In this report, we have hypothesized that Lyn could transduce TIMP-1 survival signalling downstream the proMMP-9/CD44 complex receptor. We showed that Lyn is activated by TIMP-1 and regulates
JAK2/PI 3-kinase/Akt pathway in erythroleukemic UT-7 cell survival.

2. Materials and methods

2.1. Materials

γ-ATP was from Amersham Health (Velizy, France). Human recombinant TIMP-1 was purchased from Calbiochem (VWR, Fontenay-sous-Bois, France). Purified recombinant human erythropoietin (Epo) (specific activity of 5000 U/mL) and PP1 were from Biomol International (Tebu-Bio, Le Perray-en-Yvelines, France). Fetal Calf Serum (FCS) was from BioWest (Nuaillé, France). Annexin-V-FLUOS International (Tebu-Bio, Le Perray-en-Yvelines, France). Fetal Calf Serum (FCS) was from Biowest (Nuaillé, France). Annexin-V-FLUOS International (Tebu-Bio, Le Perray-en-Yvelines, France). Anti-phospho-Lyn (Tyr 507), -Lyn, -phospho-Akt (Thr 308). Anti-p85 antibody was from Upstate Biotechnology Inc (Millipore, Cell Signalling Technology (Ozyme, St Quentin en Yvelines, France)). Anti-p85 antibody was from Upstate Biotechnology Inc (Millipore, Cell Signalling Technology (Ozyme, Meylan, France)). Anti-phospho-Lyn (Tyr 507), -Lyn, -phospho-Akt (Thr 308), -Akt, -phospho-JAK2, JAK2 antibodies (1:1000) were from Cell Signalling Technology (Ozyme, St Quentin en Yvelines, France). Anti-p85 antibody was from Upstate Biotechnology Inc (Millipore, Molsheim, France). α-Minimum essential medium (α-MEM) and Opti-MEM were from Invitrogen (Fisher Bioblock Scientific, Illkirch, France). α-Minimum essential medium (α-MEM) and Opti-MEM were from Invitrogen (Fisher Bioblock Scientific, Illkirch, France). SiRNA Lyn were from Santa Cruz Biotechnology (Tebu-Bio, Le Perray-en-Yvelines, France). All other reagents were purchased from Sigma–Aldrich (St Quentin Fallavier, France).

2.2. Cell culture and stimulation

Epo-dependent UT-7 erythroid cells were cultured in α-MEM containing 10% (v/v) FCS, 2 mM L-glutamine and 0.5 U/mL Epo. To study signal transduction, cells were serum- and Epo-starved by incubation for 16 h in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 0.2% (v/v) denatured bovine serum albumin (BSA), 2 mM L-glutamine and 20 µg/mL human holotransferrin. Cells were then incubated in IMDM and stimulated with 5 ng/mL TIMP-1 for 5 min. In some experiments, cells were incubated for 30 min or 16 h with 50 µM PP1 or 50 µM AG490 respectively before stimulation.

2.3. Western blot

Following stimulation, the reaction was stopped by adding ice-cold phosphate-buffered saline (PBS) containing 50 µM Na2VO4. Cells were then washed with PBS containing 50 µM Na2VO4 (170 g, 10 min, 4°C) and solubilized in 85 µL Laemmli sample buffer (62.5 mM Tris, 2% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) β-mercaptoethanol and 1 mM Na3VO4). Whole cell extracts were then analysed by SDS–PAGE followed by autoradiography. Proteins were transferred to a nitrocellulose membrane that was blocked with Tris-buffered Saline Tween (TBST) containing 5% (w/v) non-fat dry milk for 2 h at room temperature. The blot was then incubated with primary antibodies in blocking solution overnight at 4°C. After 5 washes with TBST, the blot was incubated with horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were revealed with an ECL plus chemiluminescence kit from Amersham Biosciences by using a ChemiDoc-XRS imaging station from Biorad or exposed to Kodak X-Omat film. When necessary, the blot was stripped by incubation for 30 min at 55°C in 62.5 mM Tris–HCl pH 6.7 containing 100 mM β-mercaptoethanol and 2% (w/v) SDS, then washed 5 times with TBST and treated as described above. Western blot presented are representative of at least three separate experiments. The specific signal of β-actin was used to ensure equal loading.

2.4. Immunoprecipitation

After stimulation, cells were washed twice with PBS containing 50 µM Na2VO4 and lysed for 15 min at 4°C in 250 µL lysis buffer (10 mM Tris–HCl pH 7.4, 1% (v/v) Brij 98, 150 mM NaCl, 5 mM EDTA, 10% (v/v) glycerol, 1 mM Na3VO4, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 1 µM pepstatin, 10 µg/mL E64 and 1 mM phenylmethylsulfonylfluoride (PMSF)). Insoluble material was then removed by centrifugation (15800 g, 20 min, 4°C) and supernatants were immunoprecipitated with specific antibodies as previously described [11]. Samples were analysed by SDS–PAGE and Western blotting.

2.5. Cell transfection

UT-7 cells in exponential phase of growth were washed twice with Opti-MEM and suspended at a final concentration of 1 × 107 cells/mL in the same medium. Cells were then electroporated using a Biorad Gene Pulser (280 V, 250 µF) in presence of various concentrations of siRNA Lyn or snRNA and incubated in α-MEM containing 10% (v/v) FCS, 1% (v/v) L-glutamine 200 mM and 0.5 U/mL Epo for 24 h.

2.6. Lyn kinase activity

Anti-Lyn immunoprecipitates were incubated for 15 min at 30°C in 50 µL kinase buffer (25 mM Hepes pH 7.4, 2 mM MnCl2, 10 mM MgCl2, 0.5 mM Na3VO4 and 25 mM ATP) containing 5 µCi [γ-32P] ATP and 5 µg rabbit denatured enolase as substrate. The reaction was stopped by adding Laemmli buffer and samples were analysed by SDS–PAGE followed by autoradiography.

2.7. PI 3-kinase activity

Anti-p85 immunoprecipitates were washed thrice with lysis buffer then twice with kinase buffer (25 mM Hepes pH 7.4, 100 mM NaCl, 5 mM MgCl2 and 200 µM adenosine). The reaction was initiated by adding 70 µL phosphatidylinositol (PI) and phosphatidylycerine (2 mg/mL), 10 µCi [γ-32P] ATP and 25 µM ATP and stopped by adding 100 µL 1 M HCl after incubation for 15 min at 30°C. Phospholipids were extracted and analysed by thin layer chromatography (TLC) as previously described [7].

2.8. Quantification and statistical analyses

The quantification of bands from Western blots was performed by densitometry and subsequent analysis using Quantity One software (Biorad). Mean values of triplicate experiments were given with standard deviation (S.D.). The statistical significance of the differences was calculated using student’s t test.

3. Results and discussion

3.1. Lyn is crucial for TIMP-1 survival activity

We hypothesized that cytoplasmic TK other than JAK2 may play critical roles in TIMP-1 erythroid cell survival signalling and we particularly focused on Lyn, one member of the Src kinases predominantly expressed in myeloid cells. The two protein isoforms are produced in UT-7 cells (Fig. S1). The contribution of Lyn in TIMP-1-induced UT-7 cell survival was first evaluated by chemical inhibition of Lyn with PP1, a potent and selective inhibitor of Src kinases (Fig. 1A and B). Flow cytometry analyses (Annexin V/PI) show that TIMP-1 has significant survival activity in UT-7 cells in a manner similar to Epo (82 ± 5% and 86 ± 2% viable cells respectively), as we previously described [7]. PP1 (50 µM) alone is not cytotoxic for UT-7 cells (41 ± 3% viable cells) whereas it
significantly decreases cell viability when incubated together with TIMP-1 (36 ± 7% viable cells compared to 82 ± 9%).

To confirm these data, Lyn expression was silenced by short-interfering RNA (siRNA). The efficiency of the siRNA targeting Lyn was tested and compared to silencer negative control RNA (sncRNA) (Fig. 1C). Lyn expression is markedly reduced in 100 and 150 nM siRNA Lyn-transfected cells. Flow cytometry analyses of Annexin V/PI-labelled cells show that siRNA cell transfection does not alter cell viability since the viable cell ratio remains unchanged in sncRNA- or Lyn siRNA-transfected cells as compared to that in non-transfected cells (Fig. 1D). TIMP-1 survival activity is abolished in siRNA Lyn-transfected cells (51.65 ± 4.82 as compared to 86.71 ± 3.51). These results confirm that Lyn is required for TIMP-1 survival effect in erythroleukemic UT-7 cells.

Numerous studies reported the importance of Lyn in signal transduction during hematopoiesis even though the majority of them focused on erythroid differentiation. Indeed, Lyn has been described as playing a key role in Epo-induced maturation of normal and leukemic erythroid cells [18,19]. The role of Lyn is not restricted to in vitro studies since Lyn−/− mice display extramedullary stress erythropoiesis and develop anemia [20]. Over the last few years, Src kinases have also been reported as key regulators in apoptosis prevention and furthermore v-src expression has been described to rescue several cell types from apoptosis [21]. In addition, Lyn silencing induces apoptosis in primary and drug-resistant leukemia cells [22]. All these data document that Lyn is necessary to erythroid cell survival besides erythroid differentiation.

3.2. Lyn is activated by TIMP-1

Lyn activation was first examined by Lyn tyrosine 507 phosphorylation status (Fig. 2A). Western blot with anti-phospho-Lyn antibodies detects p-Tyr507-Lyn (the inactivated form) in untreated cells and dephosphorylated Lyn (the activated form) in TIMP-1-treated cells. Tyrosine 507 dephosphorylation is followed by tyrosine 416 autophosphorylation leading to kinase activation. Lyn kinase activity was thus tested by its ability to phosphorylate enolase as exogenous substrate in vitro. As shown in Fig. 2B, the level of phosphorylated enolase is significantly increased in TIMP-1-stimulated cells and abolished by 50 μM PP1. These results show that Lyn is activated in response to TIMP-1.

3.3. Lyn is activated upstream the JAK2/PI 3-kinase/Akt cell survival pathway

Activated Src phosphorylates a number of cytoplasmic substrates among which the PI 3-kinase. As we previously reported, the PI 3-kinase/Akt pathway is activated in the TIMP-1 survival effect [7]. We thus performed PI 3-kinase in vitro assay and our results show a 10-fold increase in the phosphorylated PI (PIP) level by TIMP-1 (Fig. 3A). 50 μM PP1 inhibits this effect by 80%.
confirm that Lyn regulates the PI 3-kinase/Akt pathway. Akt threonine 308 phosphorylation was studied in Lyn-inhibited cells (Fig. 3B). Lyn inhibition by either chemical blockade (Fig. 3B) or protein silencing (Fig. 3C) completely suppresses the TIMP-1 effect on Akt phosphorylation. These results show that Lyn is activated upstream the PI 3-kinase.

As we previously reported, JAK2 is involved in TIMP-1 anti-apoptotic signalling and our data show that both tyrosine kinases Lyn and JAK2 participate to the TIMP-1 survival effect [7]. The involvement of these kinases is in agreement with other papers describing that, in erythroid cells, they are closely related [23].

To elucidate the possible pairwork between JAK2 and Lyn, we studied JAK2 phosphorylation in Lyn-inhibited cells. Western blotting with anti-phospho-JAK2 (tyrosine 1007/1008) antibodies showed that Lyn inhibition by PP1 suppressed TIMP-1-induced JAK2 phosphorylation(Fig. 4A). Secondly, Lyn phosphorylation was studied in JAK2-inhibited cells. Western blotting with anti-phospho-Lyn (tyrosine 507) showed that JAK2 inhibition by AG490 does not impact TIMP-1-induced Lyn dephosphorylation (Fig. 4B). This result indicating that Lyn is activated upstream JAK2 raises the question about the hypothetical association of both kinases to the TIMP-1/proMMP-9/CD44 complex receptor [11]. First, we have hypothesized that JAK2 could interact with CD44. Molecular modeling experiments have demonstrated a possible association between the JAK2 FERM domain and the QKKKLVING CD44 cytoplasmic tail (Fig. S3). However, immunoprecipitation and colocalization experiments by confocal microscopy have failed to support our hypothesis (Fig. S4).
TIMP-1 and/or CD44 [9,24,25]. These transmembrane proteins as LRP-1, integrins or CD63 have been described as binding of CD44/proMMP-9 (Fig. 5). Numerous membrane proteins such components might relay TIMP-1 signalling pathway and that the cytoplasmic domains of CD44 could interact with src kinases [14]. Unfortunately, immunoprecipitation and colocalization experiments by confocal microscopy have not validated our hypothesis (Fig. S5).

Fig. 5. Proposed model for cell survival signalling pathway induced by TIMP-1. TIMP-1 binds to the membrane complex receptor CD44/proMMP-9 leading to Lyn activation by an unknown mechanism. Activated Lyn regulates phosphorylation of JAK2 which could be bound to an unknown transmembrane protein (orange colour). JAK2 activation regulates PI 3-kinase/Akt signalling pathway and promotes cell survival. This model is based on all our results including supplementary data.

Secondly, we have hypothesized that Lyn could interact with CD44. Indeed, Lyn interaction with CD44 leads to Akt phosphorylation in human colon cancer cells [13]. Moreover, it has been demonstrated that a region in the interface of the transmembrane and cytoplasmic domains of CD44 could interact with src kinases [14]. Unfortunately, immunoprecipitation and colocalization experiments by confocal microscopy have not validated our hypothesis (Fig. S5).

These negative results strongly suggest that one or more other components might relay TIMP-1 signalling pathway and that the TIMP-1 receptor is not restricted to a binary complex composed of CD44/proMMP-9 (Fig. 5). Numerous membrane proteins such as LRP-1, integrins or CD63 have been described as binding TIMP-1 and/or CD44 [9,24,25]. These transmembrane proteins could transduce TIMP-1 signalling through Lyn and JAK2 tyrosine kinases with subsequent activation of PI 3-kinase/Akt impacting erythroleukemic UT-7 cell survival.

Acknowledgements

We thank Claude Annie Turlier for her comments and corrections. We thank the Plateforme de Modélisation Moléculaire Multicihellette (P3M) from Reims and the Centre de Calcul de Champagne-Ardenne Roméo for molecular modeling analyses. This work was supported by Region Champagne-Ardenne, the Ligue Nationale contre le Cancer (comité de l’Aube) and the CNRS.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.03.032.

References


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