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ORIGINAL ARTICLE

Evidence for a protective role of the STAT5 transcription factor against oxidative stress in human leukemic pre-B cells

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STAT5 transcription factors are involved in normal B lymphocyte development and in leukemogenesis. We show that the inhibition of STAT5A expression or activity in the NALM6, 697 and Reh leukemic pre-B cell lines, results in a higher spontaneous apoptosis and an increased FAS-induced cell death. However, the molecular mechanisms underlying the altered pre-B cell survival are unclear. We used a proteomic approach to identify proteins that are differentially regulated in cells expressing (NALM6Δ5A) or not a dominant negative form of STAT5A. Among the 14 proteins identified, six were involved in the control of the oxidative stress like glutathione (GSH) synthetase and DJ-1. Accordingly, we showed increased levels of reactive oxygen species (ROS) in NALM6Δ5A cells and suppression of the increased sensitivity to Fas-mediated apoptosis by the GSH tripeptide. Similar results were observed when NALM6 cells were treated with TAT-STAT5Δ5A fusion proteins or STAT5A shRNA. In addition, the 697 and Reh pre-B cells were found to share number of molecular changes observed in NALM6Δ5A cells including ROS generation, following inhibition of STAT5 expression or function. Our results point out to a hitherto undescribed link between STAT5 and oxidative stress and provide new insights into STAT5 functions and their roles in leukemogenesis.

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Keywords: STAT5; leukemic pre-B cells; apoptosis; oxidative stress; proteomics

INTRODUCTION

The signal transducer and activator of transcription factors STAT5A and STAT5B are two closely related STAT family members that play a major role in cytokine and growth factor signaling.[1] Number of studies have demonstrated that STAT5 plays a crucial role in hematopoiesis. In particular, the knockout mice models (*Stat5a/b*$^{\Delta N/\Delta N}$ and *Stat5a/b*$^{null/null}$) have pointed up the important role of STAT5 in hematopoietic cell development.[2,3] These mice have multiple hematopoietic defects, which affect the proliferation and/or survival of both myeloid and lymphoid lineages, including B-cell development.[4] In the *Stat5a/b*$^{null/null}$ mice, the precursor B-cell compartment is substantially reduced presumably due to an inability of progenitor B cells to proliferate in response to interleukin-7 (IL-7). However, differentiation towards mature B cells seems to be unaffected.[5,6] STAT5 is also involved in the regulation of IL-7-dependent germline transcription, histone acetylation and DNA recombination of distal VH gene segments, which are the hallmark of B lymphocyte development.[7] Accordingly, a constitutively active form of STAT5B in *IL-7R*$^{-/-}$ mice, is sufficient to restore B-cell development.[8] However, the instructive role for STAT5 and IL-7-R signaling in early B-cell development has been controversial in a mouse model of conditional mutagenesis of STAT5.[9] This model showed that the main role of IL-7-mediated activation of STAT5 is to promote pro-B-cell survival via Mcl1 and to prevent premature rearrangements of the immunoglobulin-κ light-chain locus (*Ig*κ) by binding to the *Ig*κ intronic enhancer.[9] The role of STAT5 in B-cell development may not be restricted to IL-7 signaling. It is also involved in thymic stromal lymphopoietin signaling[10] and possibly in B-cell receptor (BCR) transduction signal.[11] Moreover, Bruton's tyrosine kinase, which is activated upon pre-BCR and BCR ligation, has been shown to interact with and to activate STAT5 *in vitro*.[12] STAT5 also functionally interacts with the phosphatidylinositol-3′ kinase/Akt pathway, which is implicated in the signaling cascades of some major receptors expressed by pre-B as well as immature and mature B cells.[13,14] Finally, STAT5 has been involved in the regulation of memory B-cell differentiation.[15]

Although the role of STAT5 in B lymphopoiesis is unquestionable, the targets of STAT5 in the differentiation, proliferation and survival signaling pathways in human B-cell precursors remain to be clarified. It is now well agreed that STAT5A and STAT5B are significant regulators of cell proliferation and survival through their ability to modulate expression of various genes involved in apoptosis or cell-cycle progression, as well as genes encoding cytokines or growth factors.[16] Involvement of STAT5 in cell proliferation comes from the description of persistent activation found in many types of cancer cells including leukemic B cells.[17–19] Upstream signaling components of the JAK-STAT pathway instead of STAT5 *per se* are now clearly involved in the mechanisms leading to STAT5 deregulation in leukemic B cells.[4] However, the most direct evidence for STAT5 involvement in cellular proliferation and transformation comes from the analysis of the

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STAT5 dominant positive mutants that are capable to relieve cell lines from growth factor dependence and can also induce a fatal myeloproliferative disease or a multilineage leukemia in mice.[20,21] Conversely, we have demonstrated that expression of DN-STAT5, in the human 697 leukemic pre-B cell line leads to an increased spontaneous apoptosis that is massively enhanced upon IL-7 stimulation. These cells also exhibited a higher sensitivity to Fas-mediated cell death. Altogether, these results underline a potential connection of STAT5 with apoptotic pathways.[22] We show that the NALM6 and Reh pre-B cells display similar features following expression of DN-STAT5A or knockdown of this transcription factor. To identify the proteins involved in this connection, we carried out a differential proteomic analysis of NALM6 cells modified or not by the DN-STAT5A. We found that expression of this truncated form of STAT5A modulated the expression of 14 proteins most of which were directly or indirectly involved in cell survival or apoptosis. Number of these proteins was also modulated in 697 and Reh cells following inhibition of STAT5. We also brought evidence for the first time for a link between STAT5 and oxidative stress, observed in all three leukemic pre-B cell lines.

MATERIALS AND METHODS

Pre-B cell lines

NALM6, 697 and Reh pre-B cells were maintained in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal calf serum and 2 mM L-glutamine.[23]

Antibodies and reagents

The following antibodies (Abs) were used: anti-CD95, clone CH11 (Beckman Coulter/Immunotech, Marseille, France), anti-STAT5 (Transduction Laboratories, Lexington, KY, USA), anti-STAT5A (Zymed, San Francisco, CA, USA) anti-phosphotyrosine-STAT5 (New England BioLabs, Beverly, MA, USA), anti-topoisomerase, anti-phospho-Hsp27 (ser87), anti-actin, anti-raf-1, anti-Hsp27, anti-Hsp70 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-prohibitin (PHB), anti-DJ-1, anti-Daxx (Cell Signaling Technology, Beverly, MA, USA), anti-ribonuclease H2 subunit A (RNase H2 subunit A), anti-glutathione synthetase (GSH synthetase) (Abnova, Taipei, Taiwan), anti-replication protein A 32kD subunit (RP-A p32), anti-thioredoxin domain-containing protein 9 (proteins 1–4), anti-Quinolinate Phosphoribosyl Transferase (QPRTase) (Abcam, Cambridge, UK), anti-peroxydase-conjugated Abs specific for rabbit or mouse IgG (Amersham Pharmacia Biotech, Little Chalfont, UK), for goat IgG (Santa Cruz Biotechnology) or for chicken IgG (Abcam) GSH (Sigma-Aldrich, Saint-Quentin Fallavier, France).

Stable transfection of NALM6 cells with a dominant negative form of STAT5A

The pRSV-STAT5AΔ749 corresponding to the COOH-truncated STAT5A or pRSV-neo plasmids[24] were electroporated at 300 V, 975 μF in the NALM6 pre-B cell line[25] (20 μg per 4×10^6 cells). Electroporated cells were expanded for 24 h before G418 selection (1 mg/ml). The NALM6Δ5Acells expressing the DN-STAT5 were used in this study in parallel to the NALM6neo containing the empty vector.

Knockdown STAT5A expression by RNA interference

Green fluorescent protein-shRNA (GFP–shRNA) plasmid kit for STAT5A was used according to the manufacturer's instructions (SABiosciences, Qiagen, Frederick, MD, USA). Four shRNA STAT5A plasmids (1–4) and a shRNA control-plasmid were tested in transient transfection assays with the Amaxa Technology (Lonza, Verviers, Belgium). Transfected cells were expanded for 24 h in medium and the GFP + cells were sorted by flow cytometry. Downregulation of STAT5A protein expression was assessed by western blot. Plasmids 2 and 3 were found to induce the strongest inhibition of STAT5A expression. Results presented here were obtained with plasmid 2.

Purification of TAT-STAT5 fusion proteins

The purification of TAT-WT-STAT5A and the TAT-DN-STAT5 was performed as previously described.[26]

Intracellular reactive oxygen species levels

Intracellular reactive oxygen species (ROS) levels were detected by using the fluorescent dye 2′,7′-dichlorodihydro fluorescein diacetate (DCFH-DA; Sigma-Aldrich). Cells (0.5×10^5) were harvested, washed with PBS (phosphate-buffered saline) and suspended in 500 μl PBS. After addition of DCFH-DA (10 μM), the cell suspension was incubated at 37°C for 15 min and then kept on ice until measurement. Fluorescence intensity was immediately measured by flow cytometry.

Analysis of cell death

Cells were incubated as triplicates in flat bottom 24- or 96-well plates (5×10^5 cells/ml). Culture medium was supplemented or not with anti-Fas Ab (CD95, 100 ng/ml) or with the transducible TAT-WT-STAT5A or TAT-DN-STAT5A fusion proteins (100 nM) for 24 or 48 h. Baseline cell death was measured in culture medium. The percentage of living and dead cells was evaluated using the trypan blue dye exclusion assay. In some experiments, cells were pre-incubated for 2 h with 10 mM GSH before addition of anti-Fas Ab.

Subcellular fractionation

Cells were lysed in 10 mM Hepes, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2% NP40, protease inhibitors, 1 mM Na_2VO_4 containing buffer and centrifuged for 5 min at 800 *g*. Supernatants (cytoplasmic fractions) were frozen at −70°C. Pelleted nuclei were resuspended in hypertonic buffer (20 mM Hepes, 0.42 M NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 25% glycerol, protease inhibitor cocktail, 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM vanadate) for 30 min at 4°C. Debris were removed by centrifugation, and nuclear extracts were frozen at −70°C. Protein quantification was performed with the Bradford assay. Protein extracts were used in western blot or proteomic analysis.

Western blot analysis

The cytoplasmic and nuclear extracts obtained as described above were resuspended in Laemmli's buffer. In other experiments, 50 μl/10^6 cells or 30 μl/2×10^5 shRNAGFP + cells were lysed in Laemmli's buffer or in NP40 buffer (1% NP40, 10% glycerol, 0.05 M Tris pH 7.5, 0.15 M NaCl, protease inhibitor cocktail (Roche, Basel, Switzerland), 1 mM PMSF, 1 mM vanadate). After boiling, 25 μl of Laemmli's extracts or 50 μg of NP40 protein extracts or 50 μg of nuclear and 20 μg of cytoplasmic proteins were resolved by SDS–PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and analyzed in western blot using an enhanced chemiluminescence detection system.

Protein sample preparations and proteomic assays

Cytoplasmic (60 μg) or nuclear protein (60 μg) extracts were resuspended in 400 μl 2D sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 0.2% IPG buffer and traces of bromophenol blue). IPG strips (18 cm, linear pH 4–7 gradients; Amersham Biosciences, Buckinghamshire, UK) were rehydrated for 16 h with the various samples. Isoelectric focusing was performed at 20°C with IPGphor (Amersham Biosciences) by stepwise increases in voltage for a total of 80 000 V h *per* IPG strip. Focused IPG strips were first incubated in equilibration buffer containing 6 M urea, 2% SDS, 2% DTT, 30% glycerol and 50 mM Tris–HCl (pH 8.8) for 20 min, and then with 2.5% iodoacetamide for 15 min. After the equilibration steps, the strips were analyzed onto 8–16% (W/V) gradient acrylamide gels. Analytic and preparative gels were silver stained as previously described.[27]

Image analysis and statistics

Stained gels were scanned using an image scanner (Amersham Biosciences) and differential analysis was performed using the ImageMaster 2D Platinium software v5.0 (Amersham Biosciences) for spot detection, quantification, matching and comparative analysis. Statistics were calculated with 24 gels from three different cell cultures corresponding to the cytoplasmic fraction (12 gels from NALM6 neo and 12 gels from NALM6Δ5A) and 24 gels corresponding to the nuclear fraction. The expression level of each spot was determined by its relative volume in the gel and expressed as % volume (%Vol) and are presented as Supplementary Figure S1.

Protein identification

Target protein spots were automatically excised by the Ettan spot Picker (Amersham Biosciences). Spots were digested on the Ettan Digester (Amersham Biosciences). Protein identification was performed as previously described.[28]

Matrix assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) analysis and database search based on peptide mass fingerprint spectra

Peptide identification was performed by MALDI-TOF mass spectrometry as previously described.[29]

Statistical analysis

Statistical significance of experiments was determined using Student's *t*-test or Mann–Whitney test as mentioned.

RESULTS

DN-STAT5A increases spontaneous and Fas-induced cell death in pre-B cells

The NALM6 cells were transfected with the DN-STAT5A. According to our previous results,[22] the expression of DN-STAT5A in NALM6Δ5A cells led to an increase in spontaneous cell death and to a massive mortality reaching nearly 70% of the cells after 48 h stimulation with an anti-Fas Ab (Supplementary Figures S2a–c) compared with cells electroporated with the empty vector (NALM6neo). Cell death was related to apoptosis as assessed by annexin V staining (data not shown). Same results were observed with 697 pre-B cells and apoptosis mechanisms were deciphered in our previous paper.[22] To rule out the possibility that the transfection or the overexpression of the non-deleted part of STAT5A was responsible for the increased mortality and sensitivity to Fas-induced cell death of NALM6Δ5Acells, NALM6 cells were treated with the TAT-DN-STAT5A fusion protein or the TAT-WT-STAT5 as control. The use of TAT-fusion proteins is an appealing approach to examine the effects of a protein since they can be transduced rapidly and efficiently (100% of cells in about 20–60 min). TAT-DN-STAT5A transduced in the NALM6 pre-B cells reproduced the effects described with the stably transfected NALM6Δ5A cell line and led to an increase in spontaneous cell death as well as in Fas-induced apoptosis (Supplementary Figure S2d). TAT-fusion proteins had identical effects on 697 pre-B cells (Supplementary Figure S3). Moreover, the knockdown of STAT5A by RNA interference led to the same increase in cell death following Fas stimulation (Supplementary Figures S2e and f).

Proteome modifications induced by the DN-STAT5A

To characterize the mechanisms by which the DN-STAT5A exerts its effects, we resolved NALM6neo and NALM6ΔA proteome by 2D electrophoresis (2DE). Cells were cultured at 8×10^5 cells/ml and harvested after 20 h before protein extraction. Nuclear and cytosolic lysates were obtained from three separate cultures for each cell lines and then analyzed by 2DE in four replicates. The quality of nuclear and cytosolic fractions was first evaluated by western blot with Abs directed against the cytoplasmic Ser/Thr kinase Raf-1 and the nuclear protein topoisomerase1 Scl-70 (Figure 1a). Same extracts were used in 2DE (Figure 1) and in western blot analysis (Figures 3b and c).

A median of 773 (range 684–827) and 774 (range 693–814) spots on gels from cytoplasmic protein extracts and a median of 838 (range 716–917) and 835 (range 724–921) spots on gels from nuclear protein extracts were detected from NALM6neo and NALM6ΔA cells, respectively, within a pI range of 4–7. Representative images of the cytoplasmic and nuclear proteomic profiles obtained in 2DE are presented in Figure 1b. The analysis of the gels showed that eight spots in the cytoplasmic fractions and 10 in the nuclear fractions were differentially expressed between NALM6neo and NALM6Δ5A (Supplementary Figure S1). The proteins were identified with MALDI-TOF MS corresponding to 14 protein entries, because four spots were common to cytoplasmic and nuclear fractions. Some characteristics of the proteins are summarized in Table 1. Seven proteins from the NALM6Δ5A were upregulated as compared with the control NALM6neo while seven others were significantly decreased in NALM6Δ5A cells. The expression of proteins 1, 2, 3 and 4 was upregulated by the DN-STAT5A in both cytoplasmic and nuclear compartments.

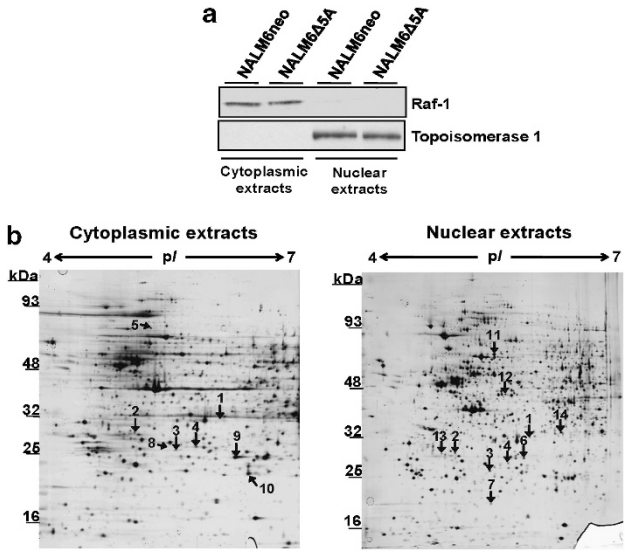

Figure 1. Proteome modifications induced by DN-STAT5A. (**a**) The quality of the nuclear and cytoplasmic extracts was assessed by western blot analysis using Abs directed against the nuclear topoisomerase 1 and the cytoplasmic Raf-1 Ser/Thr kinase. (**b**) Representative silver stained 2D gels of cytoplasmic and nuclear extracts from NALM6Δ5A cells, showing the underexpressed or overexpressed proteins. Proteins were separated on pH 4–7 IPG strips in the first dimension and in 8–16% SDS–PAGE in the second dimension. The 14 proteins significantly (Mann–Whitney test, $P < 0.05$) decreased or increased in NALM6Δ5A cells are indicated numerically and summarized in Table 1.

Pie charts (Figure 2) illustrate the distribution of these proteins according to their biological or enzymatic properties. In all, 42.9% of the proteins play a key role in the oxidative metabolism, 14.3% of the proteins are heat shock proteins (HSP), 14.3% are implicated in DNA repair or recombination, 7.1% in cell proliferation and 21.4% represent other unclassified proteins. These proteins, except the last category are directly or indirectly related to cell survival or apoptosis.

Heat shock protein

In our differential proteomic analysis, we identified the Hsp27 and Hsp70, two members of the HSP family. The downmodulation of their expression in NALM6Δ5A cells were confirmed by western blot experiments (Figure 3a). Like in stable transfectants, Hsp70 and Hsp27 were found to be down-modulated following transduction of TAT-DN-STAT5A in the NALM6 as well as in the 697 and Reh pre-B cells (Supplementary Figure S4a; Figure 4). Charette *et al.* described that phosphorylated dimers of Hsp27 interact with Daxx, a mediator of Fas-induced apoptosis, prevent its interaction with Ask1 and Fas and thereby block Daxx-mediated apoptosis.[30,31] Expression of Hsp27 also prevents the translocation of Daxx from the nucleus to the cytoplasm which is induced upon Fas stimulation.[30,31] Subsequently, we evaluated by

Table 1. Differentially expressed proteins between NALM6neo and NALM6Δ5A

Spot N	*Swiss-Prot Acc. N*	*Protein name*	*Name abbreviation*	*Fraction*[a]	*Aldente score*[b]	*Sequence coverage*	*Matched peptide number*	*Theoritical Mr(Da)/pI*
NALM6 Δ5A upregulated proteins								
1	Q15274	Nicotinate-nucleotide pyrophosphorylase[carboxylating]	QPRTase	C/N	27,88	25%	8	30825/5.80
2	O75792	Ribonuclease H2 subunit A	RNase H2 subunit A	C/N	133,32	37%	12	33395/5.14
3	P35232	Prohibitin	PHB	C/N	120,57	72%	16	29800/5.60
4	O95865	Dimethylarginine dimethylaminohydrolase	DDAH2	C/N	27,20	47%	11	29645/5.66
5	Q13409	Cytoplasmic dynein 1 intermediate chain 2	DH IC-2	C	43,52	42%	16	71465/5.08
6	P15927	Replication protein A 32 kDa subunit	RP-A p32	N	21,94	49%	11	29255/5.75
7	P07741	Adenine phophoribosyltransférase	APRT	N	32,23	76%	10	19485/5.80
NALM6 Δ5A downregulated proteins								
8	O14530	Thioredoxin domain-containing protein 9	Proteins 1–4	C	14,34	40%	6	26535/5.61
9	P04792	Heat shock protein β-1	Hsp27	C	103,06	53%	12	22780/5.97
10	Q99497	Protein DJ-1	DJ-1	C	68,16	72%	11	19691/6.33
11	P08107	Heat shock 70 kDa protein 1	Hsp70	N	54,89	20%	11	70040/5.47
12	P48637	Glutathione synthetase	GSH synthetase	N	110,15	27%	12	52385/5.67
13	P08758	Annexin A5	Annexin A5	N	49,83	45%	12	35805/4.94
14	P37837	Transaldolase	Transaldolase	N	118,00	41%	18	37540/6.35

[a]C denotes cytoplasmic; N denotes nuclear. [b]The score is calculated as described through the Aldente search engine at the following address: http://www.expasy.org/tools/aldente. Acceptance criteria for protein identification were a score above the Aldente identity threshold (set at 95% confidence level) for peptide mass fingerprints.

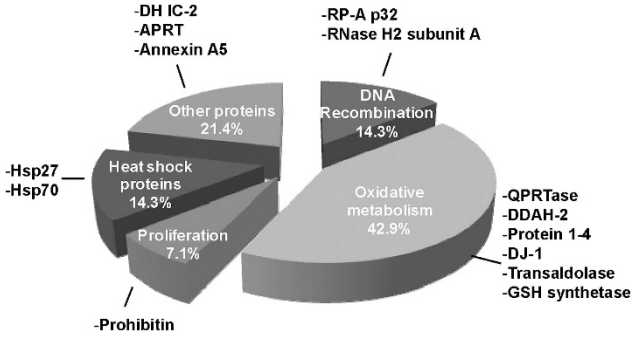

Figure 2. Pie charts showing the distribution of identified proteins according to their biological activities.

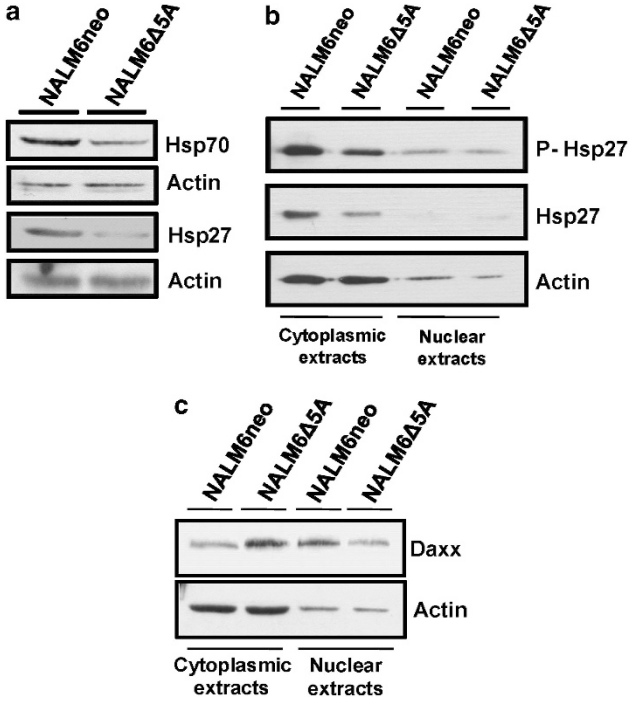

Figure 3. HSP27, HSP70 and Daxx expression in Nalm6Δ5A cells. (**a**) NP40 cell extracts were resolved on SDS–PAGE and immunobloted with Abs directed to Hsp70 and Hsp27. (**b**) Cytoplasmic and nuclear protein extracts prepared from NALM6neo and NALM6Δ5A cells were separated on SDS–PAGE and immunoblots were performed with the anti-phospho-Hsp27 (upper panel) and anti-Hsp27 Abs (lower panel). (**c**) Cytoplasmic and nuclear protein extracts were separated on SDS–PAGE and immunobloted with an anti-Daxx Ab. Results are representative of at least three independent experiments.

western blot the level of phosphorylated Hsp27 and Hsp27 expression in the cytoplasmic versus nuclear fractions of control and DN-STAT5 expressing cells. As shown in Figure 3b, the level of phosphorylated Hsp27 and Hsp27 itself is clearly lower in the cytoplasmic fraction of NALM6Δ5Acells than in NALM6neo cells. Then, we investigated the distribution of Daxx through cytoplasmic and nuclear fractions. We found that the level of Daxx was higher in the cytoplasmic fraction of NALM6Δ5A than in the NALM6neo cells, while the opposite is found in the nuclear fractions (Figure 3c). Taken together, these findings suggest that changes in the levels of phosphorylated Hsp27 as well as Hsp27 protein could modulate the susceptibility of NALM6Δ5A cells to undergo apoptosis.

Proteins involved in the oxidative metabolism

Among the six proteins identified in differential proteomics and involved in the oxidative metabolism regulation, two of them were upregulated in NALM6Δ5A cells (QRPTase and DDAH2) and

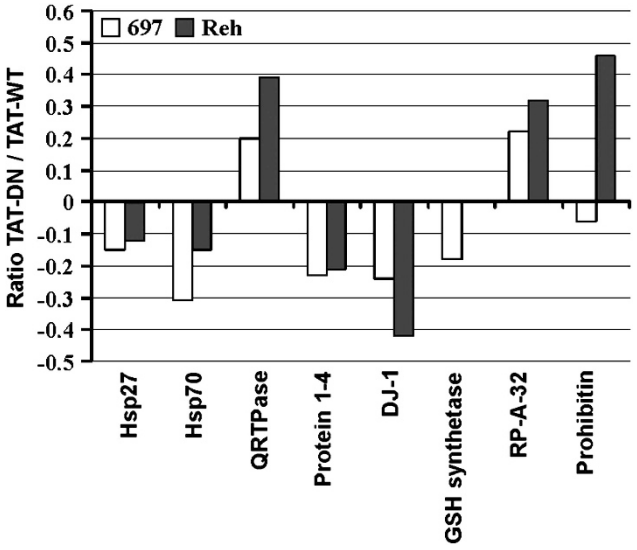

Figure 4. Changes in proteins involved in the oxidative metabolism. 697 and Reh cells were transduced with TAT-WT-STAT5A or TAT-DN-STAT5 proteins (100 nM) for 24 h. NP40 cell extracts were resolved on SDS–PAGE and nitrocellulose membranes were immunobloted with specific Abs for the different proteins and actin. Results were scanned and each protein expression was reported to actin. The value obtained with TAT-DN-STAT5A was divided by the TAT-WT-STAT5A value to objectivize upregulation or downregulation of each protein. Data are presented as (1 − ratio). One out of two representative experiment of each cell line is shown.

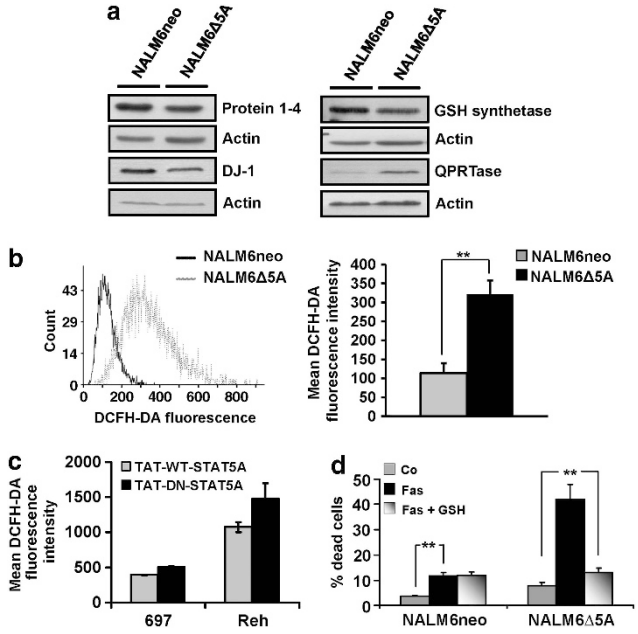

Figure 5. Changes in proteins involved in the oxidative metabolism. (**a**) NP40 cell extracts were resolved on SDS–PAGE and nitrocellulose membranes were immunobloted with specific Abs directed against proteins 1–4, DJ-1, GSH synthetase and QPRTase. (**b**) NALM6neo and NALM6Δ5A cells were incubated with DCFH-DA (5 μM) for 15 min and the generation of intracellular ROS was monitored by flow cytometry. One representative experiment is shown in the left panel. The mean of five independent experiments are shown in the right panel (**$P<0.01$ Student's *t*-test). (**c**) Generation of intracellular ROS levels was assessed by flow cytometry in the 697 and Reh pre-B cells treated with TAT-WT-STAT5A or TAT-DN-STAT5 proteins. (**d**) GSH inhibits the increase of Fas-mediated apoptosis in NALM6Δ5A cells. Cells were pre-incubated or not with 10 mM GSH and stimulated or not with anti-Fas Ab (0.1 μg/ml) for 24 h. Cell death was evaluated by trypan blue dye exclusion. Data are the mean of three independent experiments. **$P<0.01$ Student's *t*-test.

four others were downregulated (Proteins 1–4, DJ-1, GSH synthetase and transaldolase). Proteomic results were confirmed by western blot except for DDAH2 and transaldolase for which there were no available Abs (Figure 5a). To be more convincing that these changes were specifically induced by DN-STAT5A and were not restricted to NALM6 cells the 697 and Reh pre-B cells were treated with the TAT-fusion proteins. Results presented in Figure 4 show an upregulation of QRPTase and down modulation of Proteins 1–4, DJ-1 and GSH synthetase in 697 and/or Reh cells. Interestingly, proteins 1–4, DJ-1 and GSH synthetase participate in the oxidative stress response against ROS-induced cell death.[32,33] In this context, we measured the basal oxidative metabolism of NALM6neo and NALM6Δ5A cells by flow cytometry using the DCFH-DA dye. As shown in Figure 5b, the mean fluorescence intensity was higher in NALM6Δ5A cells than in NALM6neo cells, suggesting a stronger basal oxidative metabolism in the former cells. Similar results were obtained following transient transduction of TAT-DN-STAT5A in 697 and Reh cells (Figure 5c). In order to evaluate the role of ROS in the increased Fas-induced cell death, cells were incubated with the GSH tripeptide prior to stimulation with anti-Fas antibody. As shown in Figure 5d, GSH prevented the hypersensitivity of NALM6Δ5A cells to Fas stimulation as well as Fas-induced ROS generation (Supplementary Figure 4b). The protective effect of GSH on Fas-mediated cell death was confirmed following the knockdown of STAT5A by means of shRNA in NALM6 cells (Supplementary Figure S5a). ShRNA activity was validated by its effects on downregulation of GSH and DJ-1 protein expression following knockdown of STAT5A in NALM6 cells (Supplementary Figure S5b).

Other proteins

Among the proteins identified in 2DE, RP-A p32 and RNAse H2 subunit A were upregulated in NALM6Δ5A cells. These proteins are involved in the process of DNA repair, replication and recombination.[34,35] Their increased expression has been confirmed by western blot (Figure 6a). Moreover, the transduction of TAT-DN-STAT5A proteins led to an upregulation in the level of RP-A p32 in the three pre-B cell lines while the level of RNAse H2 subunit A was only modified in NALM6 cells (Figure 4 and Supplementary Figure S4a).

Prohibitin is one of the upregulated proteins following DN-STAT5A expression. This result was confirmed by western blot using stably transfected NALM6 cells (Figure 6a) and in transduced TAT-DN-STAT5A NALM6 and Reh cell extracts (Supplementary Figure S4a; Figure 4). This protein has been described as tumor suppressor, anti-proliferative protein or regulatory molecule of cell-cycle progression and apoptosis.[36] Accordingly, we showed that NALM6Δ5A cells grew more slowly than NALM6neo cells with an estimated doubling time of 30.2 versus 25.1 h, respectively ($P<0.01$; Figure 6b).

DISCUSSION

In keeping with our previous findings with the 697 human pre-B cell line,[22] we showed in this study that expression of a dominant negative form of STAT5A in a stable manner or via TAT-fusion proteins in two other leukemic pre-B cell lines, NALM6 and Reh, led to an increased spontaneous cell death and Fas-induced apoptosis. Similar findings were observed following knockdown of STAT5A by RNA interference, confirming that these changes were due to a loss of STAT5 functions. However, the mechanisms by which pre-B cell growth and survival were altered following inhibition of STAT5A functions remain enigmatic. Interestingly, no tyrosine phosphorylation of the different forms of STAT5A was

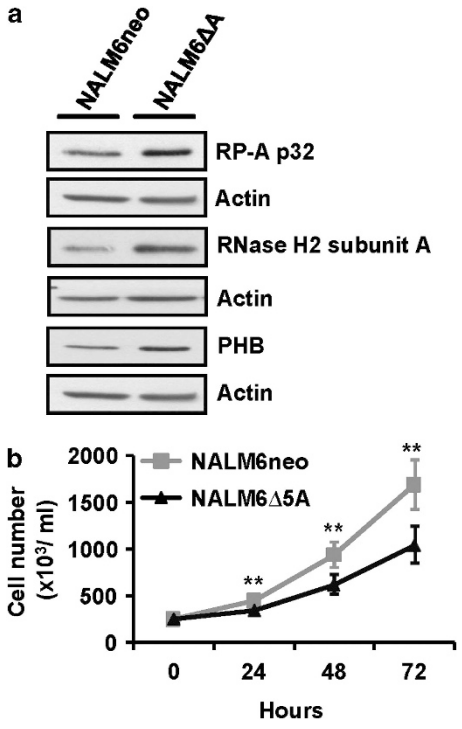

Figure 6. DN-STAT5A increases the expression of RP-A p32, RNase H2 subunit A and PHB and decreases the proliferation of NALM6 cells. (**a**) Western blot analysis with anti-RP-A p32, -RNase H2 and -PHB Abs were performed on NP40 protein extracts from NALM6neo and NALM6Δ5A cells. (**b**) NALM6neo and NALM6Δ5A cells were incubated as triplicates in flat bottom plates and *in vitro* proliferation was evaluated by counting viable cells using the trypan blue dye exclusion assay at 24, 48 and 72 h. Data are the mean of three independent experiments (**$P < 0.01$ Student's *t*-test).

detected in NALM6Δ5A and 697Δ5A cells,[22] whether or not they were stimulated via Fas. In addition, there were no changes in the expression of Fas, FADD, c-Flip, caspase 8, IAP or Bcl-2 family members (namely Bcl-2, Bcl-x, Bax, Bim, A1 and Mcl-1) in unstimulated NALM6Δ5A cells. Therefore, we undertook a more global approach consisting in the differential proteome analysis of NALM6 cells expressing or not the DN-STAT5A mutant. The statistical analysis of all 2D gels showed significant changes in the expression of 14 proteins which identification was obtained by mass spectrometry. Upregulation or downregulation of nine proteins for which specific Abs were available was confirmed by western blot in NALM6Δ5A cells. Changes were also confirmed after transduction of TAT-DN and TAT-WT-STAT5A fusion proteins and following STAT5A knockdown by means of shRNA, strengthening the idea that these changes could be attributed to the loss of STAT5 functions. By western blot, eight out of the nine proteins were found to be also modulated in the 697 and/or Reh pre-B cells following STAT5A inhibition. Strikingly, changes in the expression of these proteins were not systematically accompanied by changes at the transcriptional level (Supplementary Figures S6 and S7), suggesting that STAT5 may also regulate post-transcriptional modifications of proteins via direct and/or indirect mechanisms. One possibility is that STAT5 affects the stabilization or degradation of these proteins through protein–protein interactions or through the regulation of genes involved in the ubiquitin/proteasome degradation pathway. In keeping with this hypothesis, STAT5 was shown to regulate the expression of the E3 ubiquitin ligase Skp2 in mouse pro-B cells.[37]

Given the known biological properties of the identified proteins, differential expression of 11 of them might explain the effects of DN-STAT5A and HSP might represent one major link between DN-STAT5A and apoptosis. Various studies have highlighted the protective effects of HSP against apoptosis in response to various stimuli such as hyperthermia, oxidative stress or staurosporine[38] From this point of view, the decreased expression of Hsp27 and Hsp70 might provide some cues to explain the susceptibility of NALM6Δ5A cells to apoptosis. We believe that the Hsp27 effect is mediated by Daxx, a protein involved in Fas-mediated cell death[31] given the increased proportion of Daxx in the cell cytoplasm together with the downregulation of Hsp27 expression and phosphorylation. Downregulation of Hsp70 expression could promote the formation of apoptosome and results in hypersensitivity to apoptosis. Changes in Hsp70 expression have been previously shown to confer resistance to apoptosis in leukemic cells expressing the Bcr-Abl oncogene.[39] Unexpectedly, we bring evidence for the first time of a possible link between STAT5 and the oxidative metabolism in pre-B cells. This is suggested by the downregulation of transaldolase, GSH synthetase DJ-1 and Proteins 1–4 expression and the upregulation of QPRTase and DDAH2 expression. Some of these proteins have been shown to control the levels of ROS and might regulate apoptosis as well. Normal reducing atmosphere, that is required for cellular integrity, is provided by reduced GSH which protects cells from ROS[40] NADPH maintains GSH in the reduced state which is then effective in the protection against apoptosis. The GSH synthetase and transaldolase are enzymes involved in the generation of GSH[32] and NADPH, respectively.[41] The down-modulation of these two proteins in cells expressing the DN-STAT5A mutant could be responsible for their sensitivity to apoptosis via the decrease of their protective effect against oxygen-free radicals. The promoter of the GSH synthetase gene contains multiple binding sites for STAT family members including STAT5, suggesting that STAT5 might regulate the transcriptional activation of this gene.[42] Moreover, the synthesis of GSH or the activity of antioxidant enzymes such as GSH reductase and GSH peroxidase are modulated by the Hsp27 and Hsp70. Thus, it is likely that STAT5 modulates expression or activity of these enzymes via an indirect effect on Hsp27 and Hsp70 expression.[43,44] In addition to these proteins, DJ-1, originally identified as an oncogene product, is a protein with anti-apoptotic activity and its main action is to protect cells from oxidative stress.[33] DJ-1 plays a major role in the removal of hydrogen peroxide, a powerful oxidant[33] and in the protection against oxidative stress by increasing intracellular levels of GSH and Hsp70.[45] The downregulation of DJ-1 protein level in pre-B cells may therefore increase cell death susceptibility through oxidative metabolism. Finally, among the downregulated proteins, we identified the proteins 1–4 whose exact functions remain to be determined. However, the latter has a thioredoxin domain, in the C-terminal part of the protein that could confer to the proteins 1–4, antioxidant properties.[46] Among the overexpressed proteins, we identified the DDAH2 an enzyme that has the capacity to hydrolyze ADMA (asymmetric dimethylarginine), an inhibitor of nitric oxide synthase, and thereby plays an important role in the generation of nitric oxide.[47] Although the factors determining the cell sensitivity to nitric oxide are not clearly understood, it is commonly assumed that high dose of nitric oxide induces DNA damage and accumulation of p53.[48] Overexpression of DDAH2 in NALM6Δ5A cells might also contribute to the enhancement of cell death. Thus, the majority of proteins that we identified by proteomic analysis are involved in defense against oxidative stress. It is highly possible that the down-modulation of these proteins in NALM6Δ5A cells is involved in the accumulation of intracellular ROS, as evidenced by flow cytometry. Indeed, we detected a threefold increase in ROS amounts in NALM6Δ5A as compared with NALM6neo cells. Increase in ROS concentrations was also observed in 697 and Reh cells, though at lower levels as compared with Nalm6Δ5A. This may reflect differences in the way DN-STAT5A was expressed in these cell lines: use of TAT-fusion

proteins in 697 and Reh cells versus stable expression in Nalm6Δ5A cells. Although the exact nature of these oxygen radicals was not determined, their accumulation was likely implicated in the increased Fas-induced cell death, as suggested its prevention with the antioxidant GSH tripeptide. ROS are involved in the activation of stress pathways inducing cell death as illustrated by the activation of p53 and the increased expression of proapoptotic members of the Bcl-2 family, in response to oxidative stress.[49,50] Our results suggest that this state of oxidative stress is probably linked to reduced expression of antioxidant proteins such as GSH synthetase, DJ-1 or chaperones Hsp27 and Hsp70 in our pre-B cells.

It is well established that besides promoting cell death, oxidative stress induces DNA damage.[51] It is therefore tempting to hypothesize a link between this biological process and two other proteins identified by proteomic analysis: RNase H2 subunit A and RP-A p32. The role of RNase H2 subunit A is particularly tied to the process of DNA replication and repair.[34] The RP-A p32 plays a coordinating role between replication and DNA repair.[35] In response to oxidative stress, cells activate DNA repair mechanisms to maintain genome integrity.[52] Thus, it is conceivable that overexpression of one or both of these two proteins is also a consequence of oxidative stress observed in cells expressing DN-STAT5A. Finally, prohibitin was one of the proteins that were found to be upregulated using our approach. The role of this protein in cell survival is not well characterized, though it has been shown to exert an anti-apoptotic effect in some embryonic cells.[36] Interestingly, prohibitin has been demonstrated to inhibit cell proliferation[36] and it is therefore tempting to speculate that it might contribute to the decrease in NALM6Δ5A cell proliferation.

Our study describes a new set of functional partners and mechanisms of action of STAT5 in human leukemic pre-B cells that interfere with both cell proliferation and survival. It also points out to a so far undescribed link between STAT5 and oxidative metabolism and shed light onto new mechanisms, including oxidative stress, by which STAT5 might contribute to leukemogenesis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)