



Modulation of the activation status of Stat5a during LIF-induced differentiation of M1 myeloid leukemia cells

Roland Piekorz, Ralf Rinke, Fabrice Gouilleux, Brigitte Neumann, Bernd Groner, Gertrud Hocke

► To cite this version:

Roland Piekorz, Ralf Rinke, Fabrice Gouilleux, Brigitte Neumann, Bernd Groner, et al.. Modulation of the activation status of Stat5a during LIF-induced differentiation of M1 myeloid leukemia cells. *Biochimica et Biophysica Acta - Molecular Cell Research*, 1998, 1402 (3), pp.313-323. 10.1016/s0167-4889(98)00024-x . hal-02427392

HAL Id: hal-02427392

<https://univ-tours.hal.science/hal-02427392>

Submitted on 4 Nov 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



ELSEVIER

Biochimica et Biophysica Acta 1402 (1998) 313–323



Modulation of the activation status of Stat5a during LIF-induced differentiation of M1 myeloid leukemia cells

Roland P. Piekorz^a, Ralf Rinke^a, Fabrice Gouilleux^b, Brigitte Neumann^a, Bernd Groner^b,
Gertrud M. Hocke^{a,*}

^a Institut für Mikrobiologie, Biochemie und Genetik, University of Erlangen-Nuernberg, Staudtstrasse 5, D-91058 Erlangen, Germany

^b Institute for Experimental Cancer Research, Tumor Biology Center, 79106 Freiburg, Germany

Received 25 November 1997; revised 4 February 1998; accepted 25 February 1998

Abstract

Treatment of M1 myeloid leukemia cells with leukemia inhibitory factor (LIF) causes activation of transcription factors Stat1, Stat3 and Stat5a (signal transducers and activators of transcription). DNA-binding of Stat proteins was detectable for extended periods of time in LIF-treated M1 cells, which simultaneously underwent terminal differentiation. The relative composition of Stat factors in the protein–DNA complexes changed during time. Whereas Stat3 was activated up to 36 h during treatment with LIF, Stat5a was activated only short-termed. Similarly, high expression of the immediate early gene CIS (cytokine-inducible SH2-containing protein), a known target gene of Stat5 in hematopoietic cells, occurred only during the onset of differentiation. This suggests a role of Stat5a in the early phase of LIF-induced differentiation and growth arrest of M1 cells. © 1998 Elsevier Science B.V.

Keywords: M1 myeloid leukemia cell; LIF; Stat factor; Differentiation

1. Introduction

The cytokine leukemia inhibitory factor (LIF) exerts pleiotropic effects on a variety of target cells. One of its characteristic features, from which the name was derived, is its ability to induce differentia-

tion and growth arrest in M1 cells, a myelomonocytic leukemia-derived cell line [1]. M1 cells offer a suitable model system to study the molecular mechanisms of LIF-induced terminal differentiation and growth arrest of myeloid leukemia cells.

LIF belongs to the family of Interleukin-6- (IL-6) type cytokines which utilize a common signal pathway [2]. It involves tyrosine protein kinases of the family of Janus kinases (Jak) and transcription factors of the family of signal transducers and activators of transcription (Stat), the so called Jak/Stat pathway [3,4]. After binding of the cytokine to its specific ligand-binding chain, the ligand–receptor complexes couple to the common signal transducer subunit gp130, thus, activating it in turn. This event leads to the activation of receptor associated Jak kinases which

Abbreviations: α_2 M, α_2 -macroglobulin; CIS, cytokine-inducible SH2-containing protein; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3'-phosphate dehydrogenase; IL-6, Interleukin-6; Jak, Janus kinase; LIF, leukemia inhibitory factor; MGF, mammary gland factor; PCBA, proteolytic clipping bandshift assay; RE, response element; SSI-1, Stat-induced Stat inhibitor-1; SOCS-1, suppressor of cytokine signalling-1; Stat, signal transducers and activators of transcription

* Corresponding author. Fax: +49-9131-85-85-26; E-mail: ghocke@biologie.uni.erlangen.de

phosphorylate cytoplasmic Stat factors at specific tyrosine residues. As a consequence, Stat factors dimerize and translocate to the nucleus to bind to specific DNA response elements in the transcriptional control regions of their respective target genes [3,4].

Incubation of various cell types with LIF leads mainly to the activation of Stat proteins 1 and 3 [5,6]. However, nuclear extracts of M1 cells contained activated Stat5a in addition [7,8]. Stat factors bound to response elements (RE) from various target genes [9] such as the LIF-RE of the rat α_2 -macroglobulin (α_2 M) gene [10,11]. A characteristic protein–DNA complex, referred to as complex II, was assembled between the LIF-RE and nuclear proteins from various cell types after incubation of the cells with LIF [6,7,10].

Differentiation and growth arrest of M1 cells were strictly dependent on activated Stat3 [12,13], but not Stat1 [13]. These results demonstrated a critical and causative role of the Jak/Stat signalling pathway in the induction of differentiation of M1 cells. However, a possible involvement of Stat5a was not addressed. Stat5a/MGF (mammary gland factor) was initially discovered as a prolactin-activated Stat factor in sheep mammary gland epithelial cells [14]. In contrast to other known Stat factors, Stat5 occurs in two forms, Stat5a and Stat5b, and two species of genes coding for Stat5 have been cloned [15–18]. Stat5 proteins are activated in different cell types including hematopoietic and epithelial cells by a variety of different peptide hormones, cytokines and hematopoietic growth factors [19].

Recently, we demonstrated that treatment of the myeloid leukemic cell line M1 with LIF led to activation of Stat5a in addition to Stat1 and Stat3 [7]. Here the special properties of activation of Stat5a compared to Stat3 were analyzed. Our findings suggest a certain role of Stat5a in the early phase of induction of differentiation of LIF-treated M1 cells.

2. Materials and methods

2.1. Cell culture and reagents

M1 murine myelomonocytic cells (American Type Culture Collection) were grown in RPMI medium supplemented with 10% fetal calf serum (Boehringer

Ingelheim, Heidelberg, Germany), 2 mM L-glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin. The supernatant of a stably transfected CHO cell line secreting recombinant human LIF (Genetics Institute, Cambridge, MA) was used as a source of human LIF at a final dilution of 1:100.

2.2. Preparation of protein extracts

Nuclear and cytoplasmic protein extracts were prepared according to published procedures [20,21]. Briefly, after homogenization of the cells and pelleting of the nuclei, cytosolic extracts were obtained. The supernatant was cleared by centrifugation ($14\,000 \times g$ for 10 min at 4°C) and cytoplasmic proteins were precipitated with ammonium sulfate. Proteins from the nuclei were extracted under high salt conditions (0.42 M NaCl) and concentrated by precipitation with ammonium sulfate. Extraction buffers were supplemented with 0.5 mM phenylmethyl-sulfonylfluoride (PMSF) and 100 kallikrein-inhibitory (KI) units/ml of aprotinin (Roth, Karlsruhe, Germany).

Whole cell extracts were prepared essentially as described [22]. The extraction buffer was supplemented with 3 μ g/ml aprotinin, 1 mM PMSF, 7 μ g/ml leupeptin, 1 μ g/ml pepstatin and 1 mM sodium orthovanadate. Protein concentrations were determined using the colorimetric assay of Bradford [23].

2.3. Electrophoretic mobility shift assay (EMSA)

EMSA was performed following published procedures [24]. Binding reactions were carried out for 20 min at 25°C in a 20 μ l reaction volume containing 10 mM HEPES, 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 6 mM $MgCl_2$, 1.2 mM $CaCl_2$, 2 KI units of aprotinin, 2.5 μ g of poly(dI:dC), 2.5 μ g of single-stranded salmon sperm DNA, 10% glycerol, variable amounts of nuclear protein and approximately 7 fmol of the double-stranded, 32 P-labelled oligonucleotide TB2. This probe was end-labelled with polynucleotide kinase and used at a specific activity of $\approx 3 \times 10^6$ cpm/pmol, corresponding to 2×10^4 cpm per reaction. TB2 included two copies of the core-element of the IL-6-response element (IL-6-RE) from the rat α_2 -macroglobulin gene which

also acts as a LIF-RE (TB2: 5'-GATCATCCTTCTGGGAATTCTGATATCC TTCTGGGAATTCTG-3'; Stat binding sequence underlined) [10,20,25]. Its sequence represents a binding site for Stat3 and resembles the prolactin response element of the β -casein promoter, a binding site for Stat5, and the so called γ -activating-site, a binding site for Stat1.

EMSA-competition analysis was performed with a 100-fold molar excess of double-stranded oligonucleotides representing wild type or mutant sequences of the LIF-RE [20,25].

EMSA-supershift assays were performed by preincubation of extracts with antibodies for 1 h at 4°C prior to DNA-binding. The following antibodies were used: mouse antibodies directed against the C-terminal part of human Stat1 α (from Dr. J.E. Darnell, New York, NY); rabbit antibodies against the C-terminal part of murine Stat3 (C-20; Santa Cruz Biotechnology, Heidelberg, Germany); chicken antibodies against the N-terminus of ovine Stat5a/MGF [14,17]; and appropriate control antibodies.

2.4. Proteolytic clipping bandshift assay (PCBA)

Constant amounts of nuclear protein extract (5 μ g) were incubated with the 32 P-labelled probe TB2 for 10 min as described above. Thereafter increasing amounts of proteinase type XVII from *Staphylococcus aureus* V8 (Sigma, Deisenhofen, Germany) were added and the reaction mixture was incubated for another 10 min at 37°C [26]. Proteolytic fragments were analyzed by electrophoresis in a 5% polyacrylamide gel and visualized by autoradiography.

2.5. Immunoprecipitation and Western blotting

Stat3 was immunoprecipitated from whole cell extracts using antibody C-20. Stat5a was precipitated using a polyclonal rabbit anti-peptide serum raised against the C-terminal part of murine Stat5a (AA 774–793). Immune complexes were collected by incubation with protein A-agarose, washed and analyzed by SDS-PAGE and Western blotting. Signals were visualized using an enhanced chemiluminescent system (Amersham, Braunschweig, Germany). Antibodies against phosphotyrosine (pY20) were obtained from Santa Cruz Biotechnology.

2.6. RNA extraction and Northern blot analysis

RNA was isolated by the guanidine isothiocyanate–cesium chloride method [27]. Poly(A)⁺-enriched RNA was purified on oligo-dT-cellulose. Three μ g of poly(A)⁺-enriched RNA per sample was separated by 1% agarose–formaldehyde gels, transferred to Hybond N-membranes (Amersham) and fixed by UV-crosslinking. Prehybridization was performed in 50% formamide/5 $\times$ SSC (1 $\times$ SSC is 150 mM sodium chloride/15 mM sodium citrate)/1% SDS/2 $\times$ Denhardt's (1 $\times$ Denhardt's is 20 mg/ml each of polyvinylpyrrolidone/Ficoll 400/bovine serum albumin) at 42°C. Filters were hybridized with 50 ng of 32 P-labelled cDNA fragments overnight. Probes were radiolabelled with [α - 32 P]dCTP (Amersham) to specific activities of 2–6 $\times 10^8$ cpm/ μ g DNA using a commercial kit (Pharmacia, Freiburg, Germany). cDNA fragments for murine Stat1 (nucleotides 1835 to 2228; Ref. [28]), Stat3 (nucleotides 479 to 997; Ref. [5]) and Stat5a (nucleotides 1192 to 1717; Ref. [14,16]) were generated by reverse transcriptase–polymerase chain reaction (RT–PCR) using RNA from M1 cells and the following primers: Stat1, sense (S): 5'-GCCATCACATTACATGGG-3', antisense

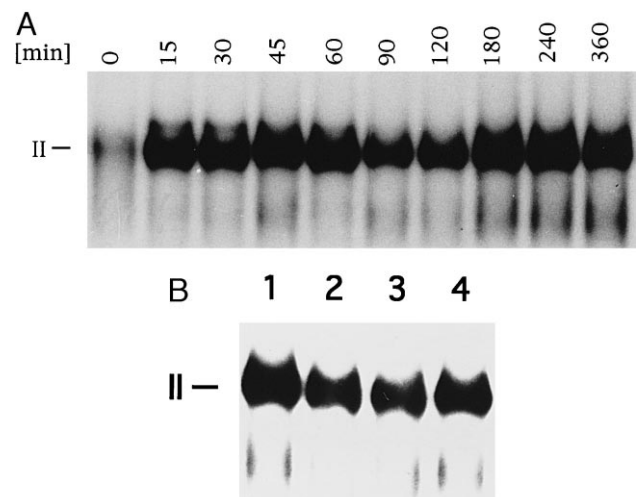
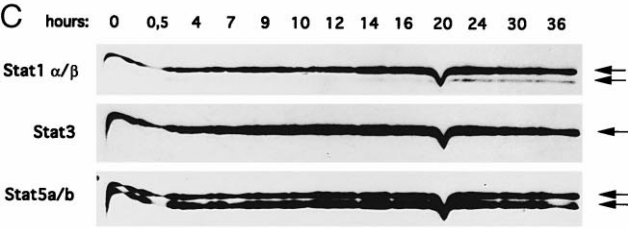
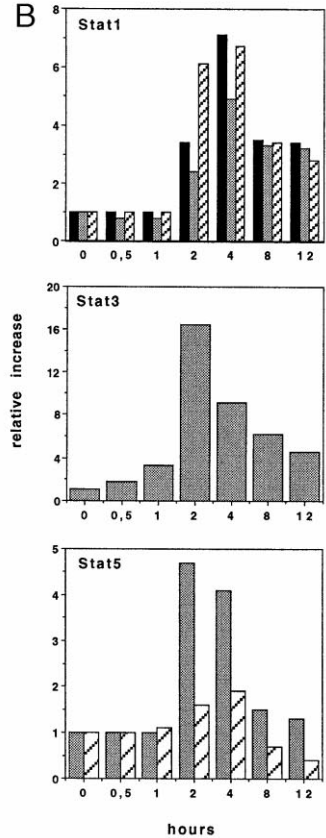
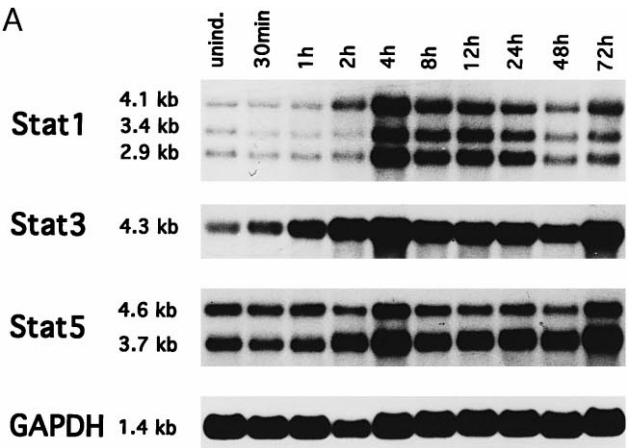


Fig. 1. The protein–DNA complex II (II) stays detectable in LIF-treated M1 cells for several hours without a major decrease in intensity. (A) Nuclear proteins from M1 cells treated with LIF for the indicated lengths of time were prepared and EMSA experiments were performed. (B) Nuclear proteins of M1 cells treated with LIF for 3 h (track 1), 8 h (track 2), 10 h (track 3) and 36 h (track 4) were analyzed in EMSA experiments.



(AS): 5'-CTACAGAGCCCACTATCCG-3'; Stat3 (S): 5'-GACAGAGAAGCAGCAGATG-3', (AS): 5'-ACGATCCTCTCCTCCAGC-3'; and Stat5 (S): 5'-AACAACTGCTGCGTG/CATGGA-3', (AS): 5'-ACTGCCAGAAGGTA/GTAA/GTTC-3'. Hybridization with a radiolabeled 0.8 kb *Pst*I cDNA fragment of rat GAPDH (glyceraldehyde-3'-phosphate dehydrogenase) [29] was used as loading control. An *Eco*RI–*Not*I murine cDNA fragment was used as a CIS (cytokine-inducible SH2-containing protein)-specific hybridization probe [30]. Filters were washed with high stringency and autoradiographs were evaluated using a laser densitometer (Pharmacia).

3. Results

3.1. DNA-binding of Stat proteins stays detectable for a prolonged time after treatment of M1 cells with LIF

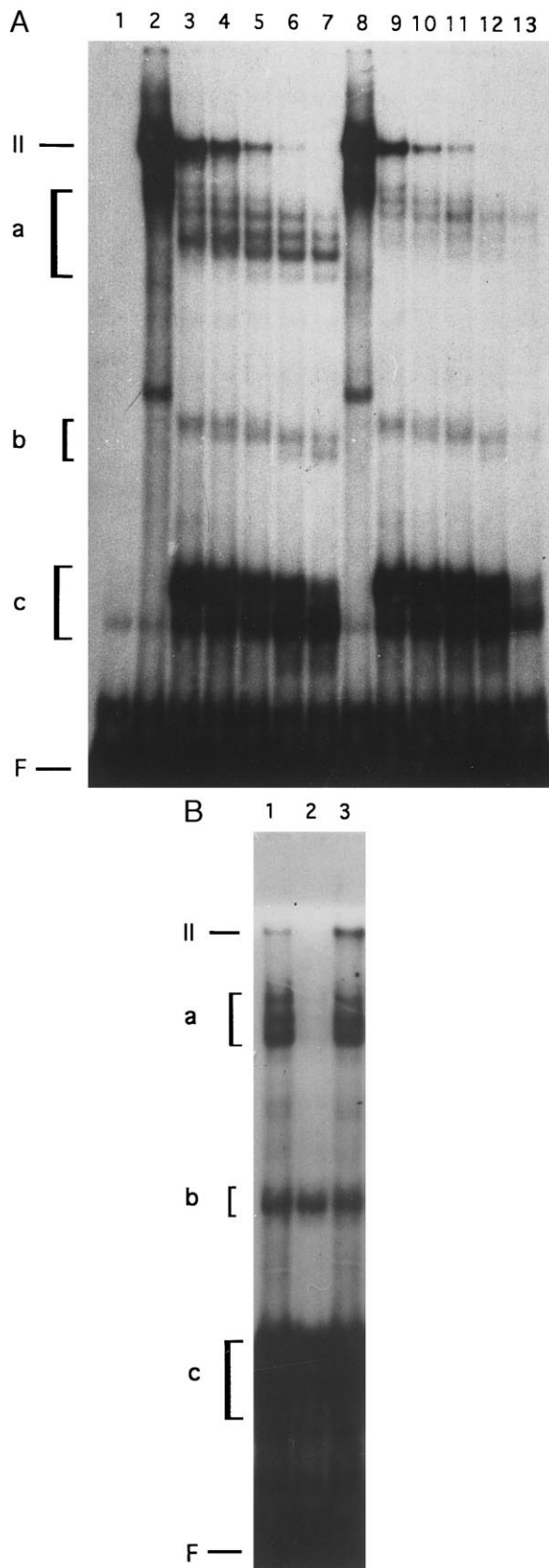
The LIF-RE of the α_2 M-gene represents a binding site for Stat3 and resembles bindings sites for Stat5 and Stat1. Using a dimer of its binding site as a probe (TB2), a characteristic gel mobility shift complex, complex II, was formed with nuclear proteins from M1 cells after incubation of the cells with LIF. This protein–DNA complex contained Stat1, Stat3 and Stat5a, but not Stat5b [7]. As Stat5b was not contained in complex II, further studies focused on Stat5a. DNA-binding of Stat factors occurred rapidly and intensities of complex formation stayed unchanged for up to 6 h (Fig. 1A and Ref. [31]). Activation of Stat factors was detectable for an extended period of time (Fig. 1B). After 2 days of treatment with LIF,

when differentiation already was detectable, complex II was still formed (R. Piekorz and G. Hocke, unpublished results).

3.2. The levels of Stat mRNAs, but not of Stat proteins, increase in M1 cells after treatment with LIF

mRNAs for Stat1, Stat3 and Stat5 were already present in untreated M1 cells (Fig. 2A). Three different variants of Stat1 mRNA (4.1, 3.4 and 2.9 kb) and two variants of Stat5 mRNA (4.6 and 3.7 kb) most likely representing Stat5b and Stat5a [17], respectively, were detected as well as one species of Stat3 mRNA (4.3 kb). After addition of LIF, the concentration of mRNAs of all three Stat factors increased. Maximum levels were reached after 4 h of LIF-treatment of M1 cells for Stat1 and after 2 h for Stat3 and Stat5 (Fig. 2B). At later times, the concentrations decreased, but remained higher during the entire period of observation compared to the initial values (Fig. 2A,B). The relative increases were approximately 16-fold for Stat3 and 5- to 6-fold for Stat1. The 4.6 kb mRNA for Stat5b increased only 1.5-fold, but the 3.7 kb species coding for Stat5a increased 4-fold. Despite of the different amounts of Stat mRNA the concentrations of Stat proteins did not change significantly during treatment of the cells with LIF (Fig. 2C). The increased intensities seen in the Western blot experiment were due to unequal loading, demonstrated by incubation of the membrane with antibodies against β -actin (Hocke, unpublished result). As the intensities of complex II did not vary significantly during time of cytokine treatment, the features of the proteins contained in complex II of

Fig. 2. The concentrations of mRNAs but not proteins for Stat1, Stat3 and Stat5 increase after treatment of M1 cells with LIF. (A) Concentration of mRNAs for Stat1, Stat3 and Stat5 increase after treatment of M1 cells with LIF. Northern blots were prepared using poly(A)⁺-RNA from M1 cells incubated with LIF for increasing lengths of time (as indicated). Specific cDNA fragments for murine Stat1, Stat3 and Stat5 were used as hybridization probes as described in Section 2. Hybridization with a cDNA probe for (GAPDH) was performed as loading control. (B) Intensities of mRNAs were evaluated by laser densitometry of autoradiographs and corrected for constant loading by normalization to the GAPDH signals. Relative intensities are the ratios of the signals from LIF-treated M1 cells divided by the corresponding signals from untreated cells. The result for the mRNA species for Stat1 are presented in the top panel: black bar: 4.1 kb; dotted bar: 3.4 kb; striped bar: 2.9 kb; center panel: 4.3 kb mRNA for Stat3; bottom panel presents the mRNA species for Stat5: striped bar: 4.6 kb; dotted bar: 3.7 kb. (C) Amounts of Stat1, Stat3 and Stat5 stay constant after treatment of M1 cells with LIF. Cell extracts were prepared from M1 cells treated with LIF for the indicated times. A total of 30 μ g protein per lane was separated by SDS-polyacrylamide gel electrophoresis and blotted to a nitrocellulose membrane. Western blotting was performed using antibodies specific for the indicated Stat proteins.



differentiating M1 cells were investigated in greater detail.

3.3. The quantitative composition of Stat factors of complex II changes in M1 cells with increasing time of incubation with LIF

Nuclear extracts from M1 cells treated with LIF for 15 min and 4 h, referred to as 'early' and 'late' extracts, respectively, were compared in gel mobility shift experiments after preincubated with a protease (Fig. 3A). This proteolytic clipping bandshift assay (PCBA) produced three blocks of proteolytic fragments (marked a, b and c in Fig. 3A). The fragments of group 'a' retained sequence-specificity for the LIF-RE as determined by competition gel-mobility shift analysis (Fig. 3B). The intensity of the faster migrating subset of proteolytic fragments of group 'a' was clearly reduced in the 'late' nuclear extracts (Fig. 3B, tracks 8–13). The 'late' complex II contained proteins reacting in a different manner to proteolytic clipping than the 'early' complex II probably due to different relative amounts or biochemical differences of the proteins. Simultaneously, the dependence of the formation of complex II on protein synthesis changed. Whereas the 'early' activation of Stat proteins was independent of protein synthesis, the appearance of the 'late' complex II was prevented by continuous treatment of the cells with cycloheximide (R. Piekorz and G. Hocke, unpublished observations). Therefore, although the intensity of complex II did

Fig. 3. Proteins of complex II display a different proteolytic sensitivity depending on the length of treatment of the cells with LIF. (A) Nuclear extracts of M1 cells incubated with LIF for 15 min (tracks 2–7) or 4 h (tracks 8–13) were used. Constant amounts (5 μ g) of nuclear proteins were incubated with increasing amounts of V8 proteinase for 10 min. Thereafter EMSA experiments were performed. Tracks: (1) without protein; (2, 8) no proteinase; (3–7) and (9–13) 2.5, 5, 10, 20 and 30 μ g of V8 proteinase, respectively. a, b, c: regions of proteolytic fragments; F: free probe. (B) Proteolytic fragments of region 'a' bind sequence-specific to the Stat binding site. PCBA was performed with nuclear extracts from LIF-treated M1 cells as outlined in track 5 of (A). The binding reactions contained: (1) no competitor; (2) 100-fold excess of unlabelled double-stranded wild type oligonucleotide [20,25]; (3) 100-fold excess of the mutant oligonucleotide [20,25] II: complex II; a, b, c: regions of proteolytic fragments; F: free probe.

not change to a great extent during treatment with LIF (Fig. 1A,B) the biochemical features of factors binding to the LIF-RE varied during time. Consequently, we investigated the composition of complex II.

Nuclear extracts from M1 cells from 'early' and 'late' time points after LIF-treatment were compared in EMSA experiments using Stat-specific antibodies. Incubation with anti-Stat1 α , anti-Stat3 and anti-Stat5a antisera revealed the presence of these Stat factors in both extracts (Fig. 4A). However, the reactivity of 'late' nuclear extracts with anti-Stat1 α and anti-Stat5a antisera was less pronounced compared to the 'early' extracts (Fig. 4A, left and right panel, tracks 3 and 6). At the same time, the reactivity with anti-Stat3 antiserum was increased. Complex II assembled with nuclear extracts of M1 cells treated with LIF for 36 h contained no Stat1 and Stat5a, but only Stat3 (Fig. 4B). In summary, although the intensity of complex II did not change to a great extent during treatment with LIF (Fig. 1) the composition of Stat proteins binding to the LIF-RE varied significantly during time.

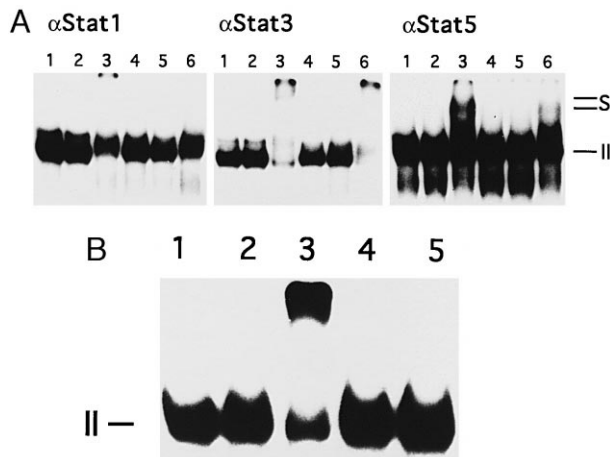


Fig. 4. The amounts of Stat factors contained in complex II (II) vary during LIF-treatment of M1 cells. (A) Nuclear extracts from M1 cells treated with LIF for 15 min (1–3) or 4 h (4–6) were preincubated with control antibodies (2, 5) or specific antibodies against Stat1 α , Stat3 or Stat5a (3, 6) and analyzed by EMSA. (1, 4) without antibody treatment. (B) Nuclear extracts of M1 cells treated with LIF for 36 h were analyzed as in (A): (1) without antibody treatment; (2–5) after preincubation with antibodies against Stat1 α (2), Stat3 (3), Stat5a (4) and control antibodies (5). S: supershifted complexes.

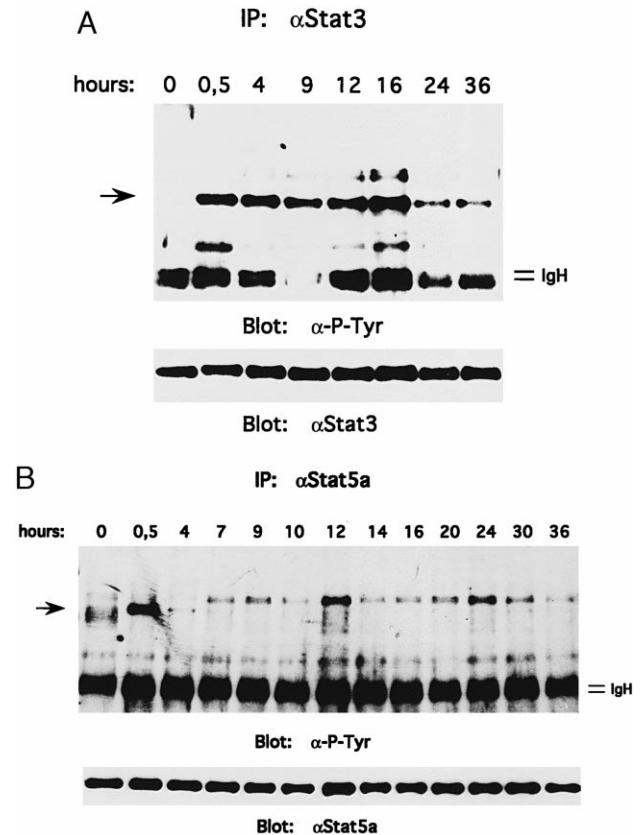


Fig. 5. Stat3 (A) and Stat5a (B) show different kinetics of tyrosine phosphorylation during treatment of M1 cells with LIF. Stat factors were immunoprecipitated (IP) from M1 cells and analyzed by Western blotting using anti-phosphotyrosine (α -P-Tyr) and Stat-specific antibodies as indicated. IgH: Immunoglobulin heavy chains.

3.4. The kinetics of the activation status of Stat5a differs compared to Stat3

A parallel development of the tyrosine phosphorylation status of Stat3 and Stat5a in time was observed compared to the activation measured by specific binding to DNA (Fig. 4). Stat3 remained tyrosine phosphorylated at least up to 36 h (Fig. 5A), whereas Stat5a was phosphorylated only short-termed after treatment with LIF. After 30 min, a strong signal was visible which decreased completely during the next 6 h, although the amount of Stat5a stayed constant (Fig. 5B). The different course of phosphorylation of Stat3 and Stat5a explains the change in composition of the characteristic protein–DNA complexes demonstrated in Fig. 4.

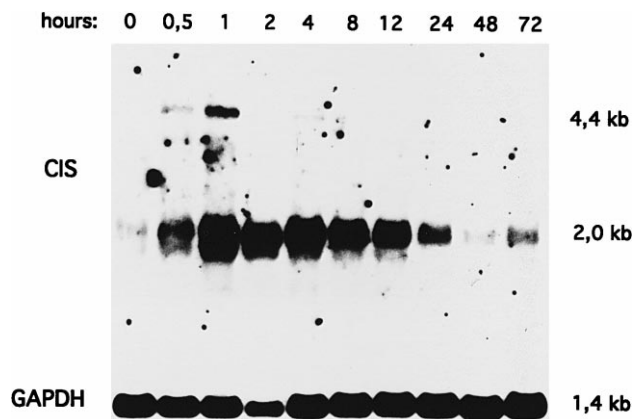


Fig. 6. Immediate early expression of mRNAs for CIS in M1 cells treated with LIF for increasing lengths of time. Hybridization with a GAPDH probe was used as loading control.

3.5. Expression of the immediate early gene *CIS* parallels the activation status of *Stat5a*

Northern blot analysis revealed that the mRNA for CIS was hardly detectable in proliferating M1 cells, but the expression increased enormously already 30 min after incubation of the cells with LIF. Maximal mRNA levels were reached after 1 h, demonstrating that CIS behaves as an immediate early gene in LIF-treated M1 cells (Fig. 6). In accordance with Yoshimura et al. [30] two mRNA species were found, a major transcript of 2.0 kb and a minor transcript of 4.4 kb. The amount of CIS mRNAs declined during time and after 24 h of treatment of the cells with LIF the concentration of CIS transcripts was clearly reduced and declined even thereafter. The kinetics of the levels of mRNAs for CIS apparently followed the activation status of Stat5a in M1 cells (Fig. 5B).

4. Discussion

The main conclusions drawn from the presented data were the following.

(1) The formation of a characteristic protein–DNA complex, complex II, remained detectable for extended periods of time after treatment of M1 myeloid leukemia cells with LIF and correlated with the differentiation process of the cells.

(2) During treatment of M1 cells with LIF the mRNA levels of Stat factors increased, whereas the

corresponding protein concentration did not change significantly.

(3) The composition of Stat factors present in complex II changed during treatment of M1 cells with LIF. The amounts of Stat1 and Stat5a decreased during time and after 36 h of LIF-treatment only Stat3 was detectable in the protein–DNA complex.

(4) The course of tyrosine phosphorylation of Stat3 and Stat5a induced by treatment of M1 cells with LIF differed greatly. Whereas Stat5a was activated only during the first 6 h, phosphorylation of Stat3 stayed detectable at least up to 36 h.

(5) CIS, a known target gene for Stat5, was expressed as an immediate early gene in LIF-treated M1 cells and its expression level apparently followed the activation status of Stat5a.

The critical role of the Jak/Stat signalling pathway in terminal differentiation and growth arrest of M1 cells was recently established. After activation and function of Stat3 were prevented, cytokine-treated M1 cells failed to undergo differentiation [12,13]. In addition, differentiation was blocked in M1 cells overexpressing a Stat-induced Stat inhibitor (SSI-1), also called SOCS-1 (suppressor of cytokine signalling-1) [32,33]. This inhibitor was capable of binding to activated Jak kinases, thereby reducing the amounts of tyrosine-phosphorylated gp130 and Stat3. These findings demonstrated that the level of activation of Stat factors was not only controlled by Jak kinases but also by inhibitors of the Jak/Stat signal pathway.

The formation of the protein–DNA complex II in cytokine-treated M1 cells was not transient but remained elevated for extended periods of time (Fig. 1 and Ref. [31]). This result was rather unexpected, since the formation of complex II was clearly decreased in other cell types during treatment with LIF, such as embryonic stem cells and HepG2 hepatoma cells (Ref. [6] and G. Hocke, unpublished data). In particular, this downregulation was influenced by the activity of one or more tyrosine phosphatases. Interestingly, dephosphorylation was also involved in the regulation of the amounts of activated Stat factors in LIF-treated M1 cells, because inhibition of tyrosine phosphatases by treatment of the cells with orthovanadate caused an increase in the levels of phosphorylated Stat3 and Stat5a (R. Piekorz and G. Hocke, unpublished observation). However, the amounts of

activated phosphatases and probably also of the Jak-inhibitor SSI-1/SOCS-1 [32,33] might be too low to lead to a complete decrease of the intensity of complex II in cytokine-treated M1 cells (Fig. 1).

Despite of the sustained formation of complex II in M1 cells, the composition of Stat factors binding to the LIF-RE changed with time. A difference between the composition of proteins binding to the LIF-RE 'early' and 'late' after treatment with LIF was observed in PCBA experiments (Fig. 3). These differences could either be due to different proteolytic sensitivities of the DNA-binding proteins caused by biochemical differences or a variation of the composition of Stat factors contained in complex II. The amounts of Stat1 and Stat5a present in complex II were diminished, whereas the contribution of Stat3 increased and at later time points only activated Stat3 was detectable (Figs. 4 and 5A). This activation course of Stat3 explained the kinetics of expression of *junB*, a target gene of Stat3 [9,34] and a characteristic marker of myeloid leukemic differentiation [35]. Expression of *junB* started immediately early after exposure of M1 cells to LIF and stayed high during the whole differentiation process. In contrast to these findings, the kinetics of expression of CIS, a target gene of Stat5 [36,37], was different. Transcription of CIS was induced immediately early after treatment of M1 cells with LIF and maximal amounts of CIS mRNAs were observed after 1 h (Fig. 6 and Ref. [33]). In contrast to *junB* [35] the levels of CIS mRNAs declined after prolonged cytokine treatment (Fig. 6). The amount of the 4.4 kb species decreases rapidly already after 2 h, whereas the amount of the 2.0 kb species was high up to 12 h but declined strongly thereafter. Therefore, the kinetics or expression of CIS matches better the activation status of Stat5a than of Stat3. We currently cannot exclude that other factors might influence the expression of CIS during differentiation of M1 cells. Nevertheless, different reports have clearly shown that activation of Stat5 is crucial for expression of CIS in hematopoietic cells [37,38] and, thus, the situation might be similar in the myelomonocytic cell line M1. To demonstrate a major involvement of Stat5a its function needs to be inhibited by overexpression of a dominant negative mutant of Stat5a in M1 cells and, thus, should lead to impaired expression of CIS. Here, we demonstrated in LIF stimulated M1 cells

that a target gene for Stat5 was activated and, thus, Stat5 seems to be involved in the modulation of gene expression during the onset of differentiation.

Interestingly, Stat5a was associated with terminal differentiation of human U-937 myeloid leukemia cells and primary chicken myeloid progenitor cells [39,40]. On the other hand, murine myeloid leukemic cell lines unresponsive to IL-6-type cytokines displayed various defects in the Jak/Stat signalling pathway. Especially expression, activation and binding of Stat5a to the LIF-RE were altered in various lines compared to M1 cells [41]. Although Stat5a-deficient mice displayed a normal process of hematopoiesis [42] recent data demonstrate that Stat5 was required for erythroleukemic differentiation [38]. In summary, our data and the findings from others indicate a function of Stat5 in the differentiation of leukemic cells.

Acknowledgements

Gifts of anti-Stat1 α antibodies (from Dr. J.E. Darnell, Jr., New York), the CHO-LIF cell line (from Genetics Institute, Cambridge, MA) and the CIS probe (from A. Miyajima, Tokyo) are gratefully acknowledged. Especial thanks to Dr. G. Fey for critical discussion. This work was supported by research grant Deutsche Forschungsgemeinschaft (DFG) Hi 291/5-5 TP6 and by the Wilhelm Sander-Stiftung awarded to G.M.H.

References

- [1] D.J. Hilton, LIF: lots of interesting functions, *TIBS* 17 (1992) 72–76.
- [2] T. Kishimoto, S. Akira, M. Narazaki, T. Taga, Interleukin-6 family of cytokines and gp130, *Blood* 86 (1995) 1243–1254.
- [3] J.E. Darnell, I.M. Kerr, G.R. Stark, Jak-Stat pathways and transcriptional activation in response to IFNs and other extracellular signalling proteins, *Science* 264 (1994) 1415–1421.
- [4] J.N. Ihle, I.M. Kerr, Jaks and Stats in signalling by the cytokine receptor superfamily, *TIG* 11 (1995) 69–74.
- [5] S. Akira, Y. Nishio, M. Inoue, X.-J. Wang, S. Wei, T. Matsusaka, K. Yoshida, T. Sudo, M. Naruto, T. Kishimoto, Molecular cloning of APRF, a novel IFN-stimulated gene factor 3 p91-related transcription factor involved in the gp130-mediated signalling pathway, *Cell* 77 (1994) 63–71.

- [6] G.M. Hocke, M.-Z. Cui, G.H. Fey, The LIF response element of the α_2 -macroglobulin gene confers LIF-induced transcriptional activation in embryonal stem cells, *Cytokine* 7 (1995) 491–502.
- [7] R.P. Piekorz, C. Nemetz, G.M. Hocke, Members of the family of IL-6-type cytokines activate Stat5a in various cell types, *Biochem. Biophys. Res. Commun.* 236 (1997) 438–443.
- [8] R. Starr, U. Novak, T.A. Willson, M. Inglese, V. Murphy, W.S. Alexander, D. Metcalf, N.A. Nicola, D.J. Hilton, M. Ernst, Distinct roles for leukemia inhibitory factor receptor α -chain and gp130 in cell type-specific signal transduction, *J. Biol. Chem.* 272 (1997) 19982–19986.
- [9] J. Yuan, U.M. Wegenda, C. Lütticken, J. Buschmann, T. Decker, C. Schindler, P.C. Heinrich, F. Horn, The signalling pathways of interleukin-6 and γ -interferon converge by the activation of different transcription factors which bind to common responsive DNA elements, *Mol. Cell. Biol.* 14 (1994) 1657–1668.
- [10] G.M. Hocke, M.-Z. Cui, J.A. Ripperger, G.H. Fey, Regulation of the rat α_2 -macroglobulin gene by interleukin-6 and leukemia inhibitory factor, in: A. Mackiewicz, I. Kushner, H. Baumann (Eds.), *Acute Phase Proteins: Molecular Biology, Biochemistry, Clinical Applications*, CRC Press, Boca Raton, FL, 1993, pp. 467–494.
- [11] C.-F. Lai, J. Ripperger, K. Morella, Y. Wang, D. Gearing, N.D. Horseman, S.P. Campos, G.H. Fey, H. Baumann, Stat3 and Stat5b are targets of two different signal pathways activated by hematopoietin receptors and control transcription via separate cytokine response elements, *J. Biol. Chem.* 270 (1995) 23254–23257.
- [12] M. Minami, M. Inoue, S. Wei, K. Takeda, M. Matsumoto, T. Kishimoto, S. Akira, Stat3 activation is a critical step in gp130-mediated terminal differentiation and growth arrest of a myeloid cell line, *Proc. Natl. Acad. Sci. U.S.A.* 93 (1996) 3963–3966.
- [13] K. Nakajima, K. Yamanaka, H. Kojima, M. Ichiba, N. Kiuchi, T. Kitaoka, T. Fukada, M. Hibi, T. Hirano, A central role for Stat3 in IL-6-induced regulation of growth and differentiation in M1 leukemia cells, *EMBO J.* 15 (1996) 3651–3658.
- [14] H. Wakao, F. Gouilleux, B. Groner, Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response, *EMBO J.* 13 (1994) 2182–2191.
- [15] M. Azam, H. Erdjument-Bromage, B.L. Kreider, M. Xia, F. Quelle, R. Basu, C. Saris, P. Tempst, J.N. Ihle, C. Schindler, Interleukin-3 signals through multiple isoforms of Stat5, *EMBO J.* 14 (1995) 1402–1411.
- [16] A.L.-F. Mui, H. Wakao, A.-M. O'Farrell, N. Harada, A. Miyajima, Interleukin-3, granulocyte-macrophage colony stimulating factor and interleukin-5 transduce signals through two Stat5 homologues, *EMBO J.* 14 (1995) 1166–1175.
- [17] X. Liu, G.W. Robinson, F. Gouilleux, B. Groner, L. Hennighausen, Cloning and expression of Stat5 and an additional homologue (Stat5b) involved in prolactin signal transduction in mouse mammary tissue, *Proc. Natl. Acad. Sci. U.S.A.* 92 (1995) 8831–8835.
- [18] J.A. Ripperger, S. Fritz, K. Richter, G.M. Hocke, F. Lottspeich, G.H. Fey, Transcription factors Stat3 and Stat5b are present in rat liver nuclei late in an acute phase response and bind interleukin-6 response elements, *J. Biol. Chem.* 270 (1995) 29998–30006.
- [19] J.N. Ihle, Signalling by the cytokine receptor superfamily in normal and transformed hematopoietic cells, *Adv. Cancer Res.* 68 (1996) 23–65.
- [20] G.M. Hocke, D. Barry, G.H. Fey, Synergistic action of interleukin-6 and glucocorticoids is mediated by the interleukin-6 response element of the rat α_2 -macroglobulin gene, *Mol. Cell. Biol.* 12 (1992) 2282–2294.
- [21] D.J. Shapiro, P.A. Sharp, W.W. Wahli, M.J. Keller, A high-efficiency HeLa nuclear transcription extract, *DNA* 7 (1988) 47–55.
- [22] A. Eilers, D. Georgellis, B. Klose, C. Schindler, A. Ziemiecki, A.G. Harpur, A.F. Wilks, T. Decker, Differentiation-regulated serine phosphorylation of Stat1 promotes GAF activation in macrophages, *Mol. Cell. Biol.* 15 (1995) 3579–3586.
- [23] M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.* 72 (1976) 248–254.
- [24] M. Fried, D.M. Crothers, Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis, *Nucleic Acids Res.* 9 (1981) 6505–6525.
- [25] T. Brechner, G. Hocke, A. Goel, G.H. Fey, Interleukin-6 response factor binds cooperatively at two adjacent sites in the promoter upstream region of the rat α_2 -macroglobulin gene, *Mol. Biol. Med.* 8 (1991) 267–285.
- [26] E. Schreiber, P. Matthias, M.M. Müller, W. Schaffner, Identification of a novel lymphoid specific octamer binding protein (OTF-2B) by proteolytic clipping bandshift assay (PCBA), *EMBO J.* 7 (1988) 4221–4229.
- [27] J. Sambrook, E.F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbour Laboratory, Cold Spring Harbour, NY, 1989.
- [28] X.-Y. Fu, A transcription factor with SH2 and SH3 domains is directly activated by an interferon- α -induced cytoplasmatic protein tyrosine kinase(s), *Cell* 70 (1992) 323–335.
- [29] P. Fort, L. Marty, M. Piechaczyk, S. ElSabrouty, C. Dani, P. Jeanteru, J.M. Blanchard, Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate dehydrogenase, *Nucleic Acids Res.* 13 (1985) 1431–1441.
- [30] A. Yoshimura, T. Ohkubo, T. Kiguchi, N.A. Jenkins, D.J. Gilbert, N.G. Copeland, T. Hara, A. Miyajima, A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin-3 and erythropoietin receptors, *EMBO J.* 14 (1995) 2816–2826.
- [31] R.P. Piekorz, R. Bläsius, G.H. Fey, G.M. Hocke, Kinetics of the activation of the LIF-response factor in M1 myeloid

- leukemic cells, *Ann. New York Acad. Sci.* 762 (1995) 452–454.
- [32] T. Naka, M. Narazaki, M. Hirata, T. Matsumoto, S. Minamoto, A. Aono, N. Nishimoto, T. Kajita, T. Taga, K. Yoshizaki, S. Akira, T. Kishimoto, Structure and function of a new Stat-induced Stat inhibitor, *Nature* 387 (1997) 924–929.
- [33] R. Starr, T.A. Willson, E.M. Viney, L.J.L. Murray, J.R. Rayner, B.J. Jenkins, T.J. Gonda, W.S. Alexander, D. Metcalf, N.A. Nicola, D.J. Hilton, A family of cytokine-inducible inhibitors of signalling, *Nature (London)* 387 (1997) 917–921.
- [34] T. Decker, P. Kovarik, A. Meinke, GAS elements: A few nucleotides with a major impact on cytokine-induced gene expression, *J. Interferon Cytokine Res.* 17 (1997) 121–134.
- [35] K.A. Lord, A. Abdollahi, B. Hoffman-Liebermann, D.A. Liebermann, Proto-oncogenes of the *fos/jun* family of transcription factors are positive regulators of myeloid differentiation, *Mol. Cell. Biol.* 13 (1993) 841–851.
- [36] D. Wang, D. Stravopodis, S. Teglund, J. Kitazawa, J.N. Ihle, Naturally occurring dominant negative variants of Stat5, *Mol. Cell. Biol.* 16 (1996) 6141–6148.
- [37] A. Matsumoto, M. Masuhara, K. Mitsui, M. Yokouchi, M. Ohtsubo, H. Misawa, A. Miyajima, A. Yoshimura, CIS, a cytokine-inducible-SH2 protein, is a target of the Jak/Stat5 pathway and modulates Stat5 activation, *Blood* 89 (1997) 3148–3154.
- [38] K. Iwatsuki, T. Endo, H. Misawa, M. Yokouchi, A. Matsumoto, M. Ohtsubo, K.J. Mori, A. Yoshimura, Stat5 activation correlates with erythropoietin receptor-mediated erythroid differentiation of an erythroleukemia cell line, *J. Biol. Chem.* 272 (1997) 8149–8152.
- [39] A. Meinke, F. Barahmand-pour, S. Wöhr, D. Stoiber, T. Decker, Activation of different Stat5 isoforms contributes to cell-type-restricted signalling in response to interferons, *Mol. Cell. Biol.* 16 (1996) 6937–6944.
- [40] I. Woldman, G. Mellitzer, M. Kieslinger, D. Buchhart, A. Meinke, H. Beug, T. Decker, Stat5 involvement in the differentiation response of primary chicken myeloid progenitor cells to chicken myelomonocytic growth factor, *J. Immunol.* 159 (1997) 877–886.
- [41] R.P. Piekorz, G.M. Hocke, Induction of differentiation by IL-6-type cytokines is impaired in myeloid leukemia cells unable to activate Stat5a, *Cytokine* 9 (1997) 639–649.
- [42] X. Liu, G.W. Robinson, K.-U. Wagner, L. Garrett, A. Wynshaw-Boris, L. Henninghausen, Stat5a is mandatory for adult mammary gland development and lactogenesis, *Genes Dev.* 11 (1997) 179–186.