FEBS Letters 464 (1999) 164–168 FEBS 23115

Phosphorylation of the Spi-B transcription factor reduces its intrinsic stability

Dominique Ray-Gallet, Françoise Moreau-Gachelin*

INSERM, Unité 248, Institut Curie, 26 rue díUlm, 75248 Paris Cedex 05, France

Received 22 November 1999

Edited by Lev Kisselev

Abstract The Spi-B transcription factor is an Ets protein expressed in B lymphoid cells and closely related to the Spi-1/PU.1 oncoprotein. By mutational analysis, we showed that Spi-B is phosphorylated by casein kinase II in vitro on four serine residues. Mutation of these four serines to alanines prevented the phosphorylation of Spi-B in vivo, increased the ability of Spi-B to transactivate expression of a reporter gene and led to a decrease of Spi-B stability. We propose that the phosphorylation of Spi-B may participate in the modulation of Spi-B functional activity by controlling its intracellular protein level.

Key words: Spi-B transcription factor; Ets protein; Serine phosphorylation; Casein kinase II

1. Introduction

The Spi-B transcription factor [1] is an Ets protein closely related to the Spi-1/PU.1 oncoprotein [2] and to a lesser extent to the recently characterized Spi-C [3]. Spi-B and Spi-1/PU.1 are co-expressed in B lymphoid cells and bind in vitro similar consensus DNA sequences containing a purine-rich central core 5′-A/GGAA-3′ [4]. However, they have not redundant functions in lymphopoiesis in vivo, as suggested by gene targeting experiments. Mice with a null-mutation in the *spi-B* gene develop normally but exhibit a major alteration of the B cell function [5] whereas the PU.1$^{-/-}$ mice present defects in the development of neutrophils and T lymphocytes, an absence of B cells and macrophages and a reduced number of lymphoid-myeloid precursor cells [6,7]. These data raise the question of the regulation process leading to the different biological activities between Spi-B and Spi-1/PU.1.

Phosphorylation is a post-translational modification able to affect the intrinsic activity of a transcription factor by regulating its DNA binding and/or its transcriptional capacity [8]. It can also influence subcellular localization, interaction with some protein partners and proteolysis of the protein. Spi-B is phosphorylated by the casein kinase II (CKII) in vitro [9]. To further investigate the consequences of phosphorylation on Spi-B function, we mapped the phosphorylation sites of Spi-B in vitro and in vivo. By systematic mutational analysis, we showed that Spi-B is phosphorylated by CKII on four serine residues. Mutations of these four serines to alanines prevented Spi-B phosphorylation in vivo and led to an increase of both the stability and the transcriptional activity of the protein. These results indicate that a role of Spi-B phosphorylation might be to control the intracellular level of Spi-B protein and by this way to affect its biological activity.

2. Materials and methods

2.1. Plasmid constructs

pGEX vectors were used to express glutathione-*S*-transferase (GST)-Spi-B fusion proteins in DH5α bacteria (Fig. 1). GST-Spi-B, GST-160, GST-108 and GST-DNA binding domain (DBD) were described [9]. GST-59, GST-60-108 and GST-PEST were constructed and a series of GST fusion proteins with mutation of serine residues to alanine within Spi-B were generated by PCR mutagenesis and confirmed by sequencing.

In eukaryotic cells, pCS3-MT(MycTag) vector, under the control of the CMV promoter, was used to express Myc-tagged proteins recognized by the 9E10 monoclonal antibody (Santa Cruz Biotechnology) [10]. pCS3-MT-Spi-B and pCS3-MT-Spi-BΔPEST encode wild-type Spi-B and Spi-B deleted of the PEST domain, respectively. pCS3-MT-Spi-B(S-37-A), pCS3-MT-Spi-B(S-129,144,146-A) and pCS3-MT-Spi-B(S-37,129,144,146-A) encode Spi-B proteins with the indicated serine to alanine substitutions.

For luciferase assays, ptk-luc-*c-fes* plasmid was used as a reporter [11] in which the expression of the firefly luciferase reporter gene is under the control of the thymidine kinase minimal promoter and three copies of an oligonucleotide exhibiting the Spi-1/PU.1 responsive element from the *c-fes* promoter [4]. Expression of the renilla luciferase from the pRL-CMV vector (Promega) was used to assess transfection efficiencies.

2.2. Protein analysis

Nuclear extracts were prepared from HeLa cells [12] and Western blotting was performed as described elsewhere [4].

For immunoprecipitation, nuclear extracts were diluted in phosphate buffer (10 mM sodium phosphate (pH 7.4), 100 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM NaF, 5 mM Na_3VO_4, 2 mM PMSF and antiproteases), incubated with the anti-myc (9E10) antibody for 2 h at 4°C and immunoprecipitates were collected on protein A–Sepharose beads.

2.3. In vitro protein kinase assay

10 μg of GST-fusion proteins bound to glutathione–Sepharose beads were resuspended in 200 μl of kinase buffer (20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 0.2 mM EGTA, 1 mM DTT) containing 50 μM ATP, 5 μCi of γ-[^{32}P]ATP and 0.1 mU of CKII (Boehringer Mannheim). After incubation for 30 min at 30°C, the beads were washed with washing buffer (20 mM HEPES, pH 7.5, 100 mM NaCl, 0.4% Triton X-100). The phosphorylated proteins were eluted from the beads, resolved on 0.1% SDS–10% PAGE and visualized by autoradiography. The amounts of GST fusion proteins were monitored by Coomassie Blue staining of the gel (not shown).

2.4. In vivo phosphorylation of Spi-B protein in HeLa cells

HeLa cells were plated at 4×10^5 cells per 60-mm-diameter dish 1 day before transfection. They were transfected by the calcium phosphate co-precipitation procedure with 5 μg of pCS3-MT-Spi-B or pCS3-MT-Spi-B(S-37,129,144,146-A) plasmids. 40 h after transfection, the cells were incubated for 1 h in 1 ml of PO_4^- Dulbecco's modified Eagle medium containing 10% fetal calf serum. Then,

*Corresponding author. Fax: (33)-1-42 34 66 50.
E-mail: francoise.moreau-gachelin@curie.fr

PII: S0014-5793(99)01687-7

0.5 mCi of [^{32}P]orthophosphate were added in the medium and the incubation was continued during 3 h. Nuclear extracts were prepared and the wild-type and mutant myc-tagged Spi-B proteins were immunoprecipitated with the anti-myc (9E10) antibody. After SDS–PAGE, the immunocomplexes were analyzed by autoradiography and Western blotting with anti-Spi-B antibodies [4].

2.5. In vitro dephosphorylation and phosphorylation assay

After immunoprecipitation of nuclear extracts from myc-tagged Spi-B transfected HeLa cells with the anti-Myc (9E10) antibody, the immunoprecipitates were collected on protein A–Sepharose beads and resuspended in 50 μl of dephosphorylation buffer (50 mM Tris, pH 7.8, 0.1 mM EDTA, 1 mM DTT). The dephosphorylation was carried out for 1 h at 37°C with 50 units of calf intestinal alkaline phosphatase (CIP) (Boehringer Mannheim). The immunoprecipitates were then used as substrates in an in vitro kinase assay and analyzed as described above.

2.6. Luciferase assay

HeLa cells were co-transfected with 2.5 μg of a pCS3-MT (Myc-Tag) Spi-B expression vector, 0.5 μg of the ptk-luc-*c-fes* plasmid and 0.5 μg of the renilla luciferase control vector (pRL-CMV). The cells were harvested 48 h after transfection and the luciferase activities were determined by using a Dual-Luciferase Reporter Assay system (Promega). The transfection experiments were repeated three times and two plasmid DNA preparations were used for each Spi-B expression vector.

2.7. Spi-B degradation analysis

2.5×10^6 HeLa cells were transfected with 30 μg of each pCS3-MT expression vector encoding the various Myc-tagged Spi-B proteins. 16 h after transfection, the cells were harvested by trypsinization and equal number of cells were replated into five 60-mm-diameter dishes. The cultures were treated 24 h after replating either with cycloheximide (Sigma) at 50 μg/ml or with cycloheximide and ALLN (*N*-acetyl-Leu-Leu-norleucinal) (Sigma) at 0.1 mM or with cycloheximide and clasto-lactacystin β-lactone (Calbiochem) at 10 μM. At the indicated times, nuclear extracts were prepared and analyzed by Western blotting.

3. Results

3.1. Serine residues 37 in the transactivation domain and 129, 144 and 146 in the PEST domain of Spi-B are phosphorylated by CKII in vitro

To identify the regions and the amino acids targeted by CKII within Spi-B, various truncated GST fusion proteins

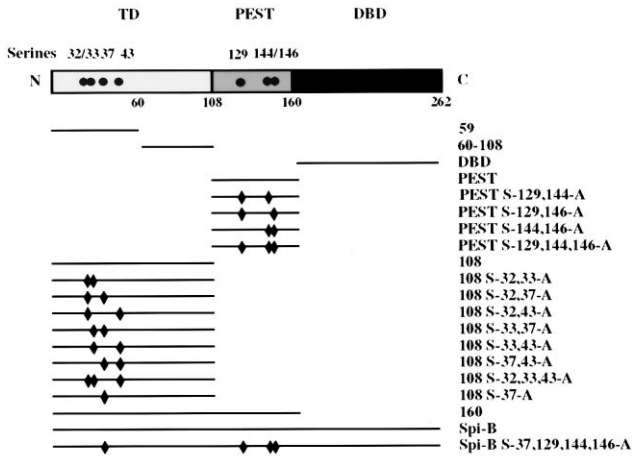

Fig. 1. Schematic representation of Spi-B and GST fusion proteins. The open box contains the transactivation domain (TD), the gray box represents the PEST sequence and the black box contains the DNA binding domain (DBD) or Ets domain. The serine to alanine substitutions are shown by a lozenge.

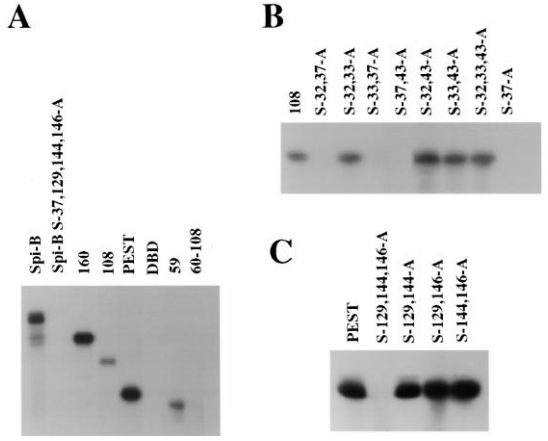

Fig. 2. CKII phosphorylates Spi-B within both the transactivation domain at serine 37 and the PEST motif at serines 129, 144 and 146 in vitro. GST fusion proteins were used as substrates for in vitro kinase assays with purified CKII in the presence of γ-[^{32}P]ATP. The labeled proteins were visualized by autoradiography after SDS–PAGE.

(Fig. 1) were used as substrates for in vitro kinase assays with purified CKII in the presence of γ-[^{32}P]ATP. The results in Fig. 2A showed that GST-Spi-B, GST-160, GST-108, GST-59 and GST-PEST were phosphorylated. No phosphorylation was detected in GST-60-108 and GST-DBD. Thus, phosphorylation sites for CKII appeared located in the NH_2-terminal part of the transactivation domain (amino acids positions from 1 to 59) and in the PEST domain (amino acids positions from 108 to 160) of the Spi-B protein.

Our previous data showed that GST-108 was mainly phosphorylated on serine residues [9]. The phosphorylated NH_2-terminal part, spanning amino acids from 1 to 59, contains two serines 33 and 43 that lie within a consensus CKII phosphorylation site (S*/T*-X-X-E/D). However, GST-108(S-33,43-A), in which both serines 33 and 43 were mutated to alanines, appeared phosphorylated as efficiently as the wild-type GST-108 suggesting that serines 33 and 43 were not substrate for CKII (Fig. 2B). In contrast, the mutated proteins GST-108(S-32,37-A), GST-108(S-33,37-A) and GST-108(S-37,43-A) appeared not phosphorylated whereas GST-108(S-32,33-A) and GST-108(S-32,43-A) were phosphorylated (Fig. 2B). These experiments showed that the absence of phosphorylation is correlated to the substitution of the serine 37 to alanine, suggesting that the serine 37 might be the CKII phosphorylation site in the in vitro kinase assay. As expected, CKII failed to phosphorylate GST-108(S-37-A) in contrast with GST-108(S-32,33,43-A) (Fig. 2B). These results demonstrated that the non-CKII consensus serine 37 is the CKII phosphorylation site within the NH_2-terminal part of the transactivation domain of Spi-B protein.

The PEST domain contains three serine residues 129, 144 and 146 that belong to a consensus CKII phosphorylation site. No phosphorylation was observed in the mutant GST-PEST(S-129,144,146-A) whereas GST-PEST(S-129,144-A), GST-PEST(S-129,146-A) and GST-PEST(S-144,146-A) were phosphorylated by CKII (Fig. 2C). These data allowed to conclude that these three serines are CKII phosphorylation sites in vitro.

Finally, the full length GST Spi-B with mutations (S-37,129,144,146-A) appeared not phosphorylated (Fig. 2A).

This result confirmed that the serine residues 37, 129, 144 and 146 are the in vitro CKII phosphorylation sites in Spi-B protein.

3.2. The CKII phosphorylation sites mapped in vitro are phosphorylated in vivo

To determine whether the CKII target serines identified in vitro were also phosphorylated in vivo, we performed metabolic ^{32}P-labeling of HeLa cells transfected with plasmids expressing either wild-type or mutated (S-37,129,144,146-A) Spi-B. As compared to the immunoblotting with anti-Spi-B antibodies showing protein expression, only wild-type Spi-B appeared phosphorylated (Fig. 3A). Moreover, we compared the ability of CKII to phosphorylate untreated and phosphatase-treated Spi-B from transfected HeLa cells. In Fig. 3B, the phosphorylation of Spi-B by CKII was observed only after phosphatase treatment. Although we cannot determine whether each of the four serines is a phosphoacceptor site in vivo, these data strongly suggested that at least one of the identified CKII sites was phosphorylated in vivo.

3.3. Mutations of the CKII phosphorylation sites increase the ability of Spi-B to transactivate

To investigate whether CKII phosphorylation may be involved in Spi-B function, we studied the capacity of Spi-B mutants to transactivate expression of a luciferase reporter gene in HeLa transfected cells. As compared to wild-type Spi-B, we observed an increase in reporter transactivation with both the mutants Spi-B(S-37,129,144,146-A) and Spi-B(S-129,144,146-A) whereas no change in transactivation efficiency was detected for Spi-B(S-37-A) and Spi-BΔPEST (Fig. 4). These results suggested that prevention of CKII phosphorylation in the PEST domain led to the increased ability of Spi-B to transactivate a reporter gene.

3.4. Spi-B phosphorylation by CKII reduces its stability

Three of the four serines phosphorylated by CKII are located within the PEST sequence of Spi-B. PEST sequences are proposed to serve as signals for proteolytic degradation and

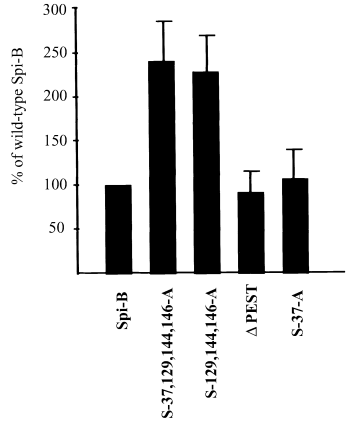

Fig. 4. Increased transactivating abilities of both the mutants Spi-B(S-37,129,144,146-A) and Spi-B(S-129,144,146-A). HeLa cells were co-transfected with a vector expressing the indicated Spi-B protein and the firefly luciferase reporter vector (ptk-luc-*c-fes*). Cells were harvested 48 h post-transfection and luciferase activities were determined. The data are presented as percentages of the transactivation ability of wild-type Spi-B, which was set to 100%. The results shown are means ± S.D. of three separate experiments.

have been frequently reported to contain consensus site(s) for phosphorylation by CKII, suggesting that CKII phosphorylation might be a regulation mechanism of PEST-mediated protein degradation [13]. In order to determine whether the PEST motif plays a role in the intrinsic stability of Spi-B, we examined the half-life of wild-type and ΔPEST Spi-B in HeLa co-transfected cells after cycloheximide treatment. In Fig. 5A Spi-B and Spi-BΔPEST appeared to be degraded similarly revealing that deletion of the PEST sequence did not change Spi-B stability. The half-lives of both the wild-type Spi-B and Spi-BΔPEST proteins were estimated to be 60 min. We further investigated by the same experimental approach whether the phosphoserines 37, 129, 144 and 146 might be involved in Spi-B stability. No alteration of protein stability was observed for Spi-B(S-37-A) as compared to SpiBΔPEST (Fig. 5C). In contrast, Spi-B(S-37,129,144,146-A) was found more stable than

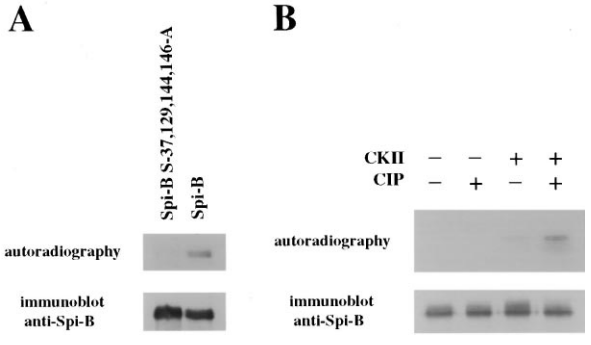

Fig. 3. In vivo phosphorylation of Spi-B. A: HeLa cells, transiently transfected with vectors expressing either wild-type or (S-37,129,144,146-A) Spi-B, were metabolically labeled with [^{32}P]orthophosphate. The transfected proteins were immunoprecipitated from nuclear extracts and visualized by autoradiography and immunoblotting with anti-Spi-B antibodies. B: Spi-B was expressed in HeLa cells and immunoprecipitated from nuclear extracts. The immunocomplexes were incubated with or without CIP, then used as substrates in an in vitro kinase assay with or without CKII in the presence of γ-[^{32}P]ATP. The resolved proteins were visualized by autoradiography and Western blotting with anti-Spi-B antibodies.

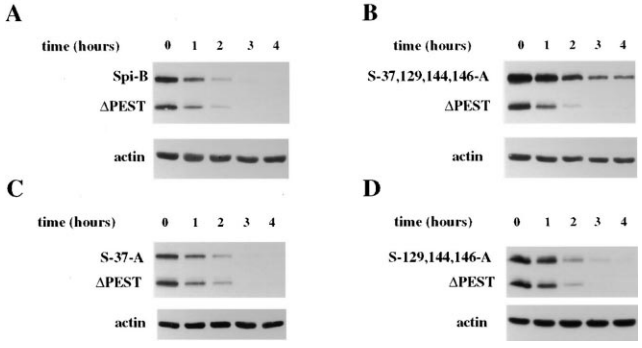

Fig. 5. Increased stabilities of both the mutants Spi-B(S-37,129,144,146-A) and Spi-B (S-129,144,146-A). HeLa cells, transiently co-transfected with vectors expressing ΔPEST and either (A) wild-type or (B) (S-37,129,144,146-A) or (C) (S-37-A) or (D) (S-129,144,146-A) Spi-B proteins, were treated with cycloheximide for the indicated times. The transfected proteins were detected in nuclear extracts by Western blotting analysis with the anti-Myc antibody. Identical blots were subjected to Western analysis with an anti-actin (I-19) antibody (Santa Cruz Biotechnology) to control sample loading.

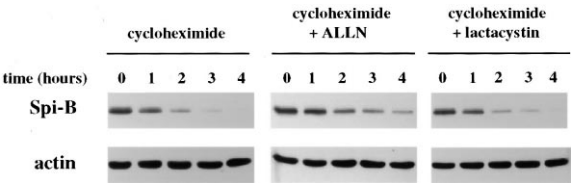

Fig. 6. Spi-B was stabilized by the cysteine protease inhibitor ALLN. HeLa cells, transiently transfected with a vector expressing Spi-B, were treated for the indicated times with either cycloheximide or cycloheximide in the presence of ALLN or cycloheximide in the presence of clasto-lactacystin β-lactone. Spi-B was detected in nuclear extracts by Western blotting as in Fig. 5.

SpiBΔPEST, its half-life being estimated to be increased to 120 min (Fig. 5B). The stability of Spi-B(S-129,144,146-A) appeared also increased as compared to SpiBΔPEST but intermediate between those of wild-type and mutated (S-37,129,144,146-A) Spi-B proteins (Fig. 5D). These results suggested that Spi-B phosphorylation could be involved in the control of the Spi-B intracellular proteolysis.

3.5. *Spi-B degradation is reduced in the presence of a cysteine protease inhibitor*

PEST sequences are most frequently proposed to target proteins for degradation by the 26S proteasome [13]. We tested whether the proteasome may be involved in the degradation of Spi-B in vivo by using two proteasome inhibitors: (1) the clasto-lactacystin β-lactone which is a specific inhibitor of the 26S proteasome (2) the ALLN (*N*-acetyl-Leu-Leu-norleucinal) which inhibits both the 26S proteasome and cysteine proteases including calpains. HeLa cells were transfected with the Spi-B expression vector and then incubated with cycloheximide in the presence of either ALLN or clasto-lactacystin β-lactone. ALLN treatment induced an increase in the stability of Spi-B whereas treatment with the clasto-lactacystin β-lactone inhibitor did not (Fig. 6). These data suggested that the degradation of Spi-B does not proceed by the proteasome pathway but more probably involves a cysteine protease.

4. Discussion

To investigate the potential role of CKII phosphorylation on Spi-B activity, we first identified serine residues that are phosphorylated by CKII and then examined their role in Spi-B transcriptional activity. We showed that the serine residue 37 in the NH_2-terminal transactivation domain and the serine residues 129, 144 and 146 in the PEST domain of Spi-B are phosphorylated by CKII in vitro. These phosphoserines are located at positions very similar to those characterized in the highly related Spi-1/PU.1 oncoprotein [14]. We also showed that the mutation of the four serines to alanines dramatically reduced Spi-B phosphorylation in vivo and increase the transactivation capacity of Spi-B. CKII phosphorylation has been already reported to modulate the transcriptional activity of Spi-1/PU.1. In particular, the physical association between Spi-1/PU.1 and Pip in B lymphoid cells depends on the phosphorylation of the serine 148 within the Spi-1/PU.1 PEST sequence and increases the transcriptional activity of Spi-1/PU.1 [15]. Similar transcriptional synergy, depending of the phosphorylation of S144 was also recently reported for Spi-B [16].

Spi-B containing three serines phosphorylated by CKII in its PEST domain, it could be presumed that such phosphorylation might be needed for degradation of Spi-B. Although deletion of the PEST region does not affect protein turnover (Fig. 5A), mutation of the three CKII sites in the PEST domain was found to slightly increase its stability. However, a greater increase in Spi-B stability was observed when all of the four serines were mutated to alanines. This suggests that the CKII phosphorylation of Spi-B within both the NH_2-terminal and PEST domains is implicated in the constitutive turnover of the protein. The reduced degradation of Spi-B in the presence of the ALLN proteasome inhibitor suggests that Spi-B might be degraded by a cysteine protease. Such a role of CKII phosphorylation in Spi-1/PU.1 stability has not been reported

The biological activities of numerous cellular proteins have been reported to be regulated by the modulation of their protein level through a degradation mechanism [17–19]. The increased stabilities of the above Spi-B mutants are likely to be responsible for their increased transactivation capacities (Fig. 4). However, although Spi-B(S-37,129,144,146-A) exhibited a longer half-life than Spi-B(S-129,144,146-A), they both showed similar transactivating abilities. Thus, the increased transcriptional activity of Spi-B(S-129,144,146-A) is probably not the only consequence of protein stabilization. The lack of CKII phosphorylation in the PEST domain might also induce a conformational change and/or a modification of protein-protein interaction that modulate Spi-B transcriptional activity.

In conclusion, our data argue for a role of CKII phosphorylation in the transcriptional activity of Spi-B by controlling the intracellular level of Spi-B protein through a regulation of its degradation.

Acknowledgements: The research was supported by the Institut National de la Santé et de la Recherche Médicale and the Association pour la Recherche sur le Cancer. We thank Jean de Gunzburg for helpful discussion, and Stéphane Barnache, Laurent Delva and Armand Tavitian for their critical review of the manuscript.

References

[1] Ray, D., Bosselut, R., Ghysdael, J., Mattei, M.G., Tavitian, A. and Moreau-Gachelin, F. (1992) Mol. Cell. Biol. 12, 4297–4304.
[2] Moreau-Gachelin, F., Ray, D., Mattei, M.G., Tambourin, P. and Tavitian, A. (1989) Oncogene 4, 1449–1456.
[3] Bemark, M., Martensson, A., Liberg, D. and Leanderson, T. (1999) J. Biol. Chem. 274, 10259–10267.
[4] Ray-Gallet, D., Mao, C., Tavitian, A. and Moreau-Gachelin, F. (1995) Oncogene 11, 303–313.
[5] Su, G.H., Chen, H.M., Muthusamy, N., Garrett-Sinha, L.A., Baunoch, D., Tenen, D.G. and Simon, M.C. (1997) EMBO J. 16, 7118–7129.
[6] McKercher, S.R. et al. (1996) EMBO J. 15, 5647–5658.
[7] Scott, E.W., Fisher, R.C., Olson, M.C., Kehrli, E.W., Simon, M.C. and Singh, H. (1997) Immunity 6, 437–447.
[8] Hunter, T. and Karin, M. (1992) Cell 70, 375–387.
[9] Mao, C., Ray-Gallet, D., Tavitian, A. and Moreau-Gachelin, F. (1996) Oncogene 12, 863–873.
[10] Hallier, M., Lerga, A., Barnache, S., Tavitian, A. and Moreau-Gachelin, F. (1998) J. Biol. Chem. 273, 4838–4842.
[11] Kherrouche, Z., Beuscart, A., Huguet, C., Flourens, A., Moreau-Gachelin, F., Stehelin, D. and Coll, J. (1998) Oncogene 16, 1357–1367.
[12] Lassar, A.B., Davis, R.L., Wright, W.E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D. and Weintraub, H. (1991) Cell 66, 305–315.

[13] Rechsteiner, M. and Rogers, S.W. (1996) Trends Biochem. Sci. 21, 267–271.
[14] Pongubala, J.M., Van Beveren, C., Nagulapalli, S., Klemsz, M.J., McKercher, S.R., Maki, R.A. and Atchison, M.L. (1993) Science 259, 1622–1625.
[15] Perkel, J.M. and Atchison, M.L. (1998) J. Immunol. 160, 241–252.
[16] Rao, S., Matsumura, A., Yoon, J. and Simon, M.C. (1999) J. Biol. Chem. 274, 11115–11124.
[17] Song, A., Wang, Q., Goebl, M.G. and Harrington, M.A. (1998) Mol. Cell. Biol. 18, 4994–4999.
[18] Tsurumi, C., Ishida, N., Tamura, T., Kakizuka, A., Nishida, E., Okumura, E., Kishimoto, T., Inagaki, M., Okazaki, K. and Sagata, N. (1995) Mol. Cell. Biol. 15, 5682–5687.
[19] Bies, J. and Wolff, L. (1997) Oncogene 14, 203–212.