Evidence against requirement of Ser41 and Ser45 for function of PU.1 – Molecular cloning of rat PU.1 ☆

Chiharu Nishiyama[a,*], Nobutaka Masuoka[b], Makoto Nishiyama[b], Tomonobu Ito[a], Hisakazu Yamane[b], Ko Okumura[a], Hideoki Ogawa[a]

[a] *Atopy (Allergy) Research Center, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan*
[b] *Biotechnology Research Center, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan*

Received 8 June 2004; revised 7 July 2004; accepted 7 July 2004

Available online 23 July 2004

Edited by Takashi Gojobori

Abstract The transcription factor PU.1 plays an important role in the development of the myeloid and lymphoid lineages and regulates the transcription of several genes expressed in these cells. Ser41 is conserved in the acidic region (33–47) of PU.1 from a variety of eukaryocytes and has been reported to be one of the two important Ser residues (S41 and S45) for the function of PU.1. In the present study, however, we found that rat PU.1 has Gly at position 41. To elucidate the role of amino acid residues at 41 and 45 in functions of PU.1, we generated mutants of rat PU.1, G41S, G41A, and S45A, and analyzed their transcription-enhancing activities by using two different systems, transient reporter assay system and retroviral transfection system. The amino acid substitution at 41 of PU.1 causes no effect on both transcription-enhancing activity for M-CSF receptor promoter and the cooperative transcription-enhancing activity with GATA-1 for FcεRI α-chain promoter. Furthermore, the substitution at 41 also had no effect on the activity to induce monocyte-specific gene expression in the bone marrow-derived hematopoietic cells. From these results, we conclude that Ser41 as well as Ser45 are not essential for the promoter-upregulating function of PU.1.

Keywords: PU.1; Transcription factor; Ser; Hematopoiesis

1. Introduction

PU.1, a transcription factor belonging to Ets-family, is critically required for the development of both myeloid and lymphoid lineages and the cell-type specific gene expression. Necessity of PU.1 for generation of these lineages was definitively demonstrated by PU.1 gene disruption that abolishes macrophages and B cells production and delays neutrophils and T cells production [1–5]. According to the analysis using truncation or amino acid replacement of PU.1, the function of each domain has been elucidated; the acidic region and the glutamine-rich region are both involved in transactivation, the PEST region in controlling protein stability [6] and proteolytic degradation [7], and the Ets domain in DNA-binding. In addition, it has been reported that Ser41 is also required for AKT-induced transactivation of κE3′-enhancer activity in B cells [8] and both Ser residues at position 41 and 45 are involved in macrophage proliferation [9]. These results suggest that Ser residues at 41 and 45 are important for PU.1 functions in these cells. We recently found that overexpression of PU.1 in bone marrow-derived cells by retroviral transfection induced the expression of monocyte-specific genes [10,11]. In the analysis, the acidic region (from 33 to 73) was shown to be essential for the induction [11]. Since both Ser residues are contained in the acidic region, we at first thought that the Ser residues were necessary for the function of PU.1. In the present study, however, we found that rat PU.1 possesses Gly but not Ser as the 41st residue. This finding prompted us to examine whether or not Ser41 is indeed necessary for the functions of PU.1. In the present analysis, in addition to general transient expression system using luciferase as the reporter, we used the retroviral transfection system to evaluate the possible role of Ser41 as well as Ser45. In this paper, we describe that Ser residues at 41 and 45 are not essential for the functions of PU.1.

2. Materials and methods

2.1. Culture of cell lines

KU812 and U937 cells were cultured in RPMI 1640 medium (Sigma–Aldrich, St. Louis, MO) supplemented with 10% FCS (Biological Industries, Haemek, Israel), 100 U/ml penicillin, and 100 μg/ml streptomycin. RBL-2H3 and CV-1 cells were grown in α-MEM (Invitrogen, San Diego, CA) and DMEM (Sigma–Aldrich, St. Louis, MO) supplemented with 10% FCS, penicillin, and streptomycin, respectively. Retrovirus packaging cell line, Plat-E [12], was maintained in DMEM supplemented with 10% FCS, penicillin, streptomycin, 1 μg/ml puromycin (Sigma–Aldrich), and 10 μg/ml blasticidin (Funakoshi, Tokyo, Japan).

2.2. Cloning of rat PU.1 cDNA

Total RNA from RBL-2H3 cells was purified using TRIzol reagent (Invitrogen). A DNA fragment containing the coding region of rat PU.1 was prepared by RT-PCR using the total RNA of RBL-2H3 as the template with the following oligonucleotides; PU.1-For, 5′-CCCCACCGAAGCAGGGGATCTGACC-3′, and PU.1-Rev, 5′-CCGGGCGACGGGTTAATGCTATGGCC-3′, which corresponded to just upstream of the translational start codon and downstream of the translational stop codon of mouse PU.1 (Accession Nos. M32370/M38252/NM011355 [13–15]), respectively, as primers. An RT-PCR kit was obtained from TAKARA BIO (Otsu, Japan). PCR products were

☆ This work was supported in part by grant-in-aid for Young Scientists from the Ministry of Education, Culture, Sports, Science and Technology of Japan (C.N.).

* Corresponding author. Fax: +81-3-3813-5512.
E-mail address: chinishi@med.juntendo.ac.jp (C. Nishiyama).

0014-5793/$22.00
doi:10.1016/j.febslet.2004.07.008

inserted to pCR3.1 (Invitrogen) and their nucleotide sequences were determined for several clones to avoid PCR errors. PCR using the same primers was performed with a commercial rat cDNA, a Rat Liver Marathon-Ready cDNA (Clontech, Palo Alto, CA), as a template.

2.3. Plasmid construction

For the expression of PU.1 in mammalian cells, rat PU.1 cDNA inserted into pCR3.1 as described above was ligated into *Eco*RI/*Xho*I-digested pCR-2F ([16] kindly provided by Dr. H. Nakano) after the nucleotide replacements by using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) to introduce *Eco*RI and *Xho*I sites at just upstream of the start codon and downstream of the stop codon of PU.1, respectively. The resulting plasmid, pCR-2F-PU.1, directs the production of PU.1 tagged with 2 × Flag at N-terminus site. The expression plasmid, pCR-GATA-1 [17,18], was also used. For the expression of various truncated PU.1 proteins, a series of expression plasmids, pCR-2F-PU.1-ΔAcid (lacking the acidic region), pCR-2F-PU.1-ΔGln (lacking the Gln-rich region), pCR-2F-PU.1-ΔPEST (lacking the PEST domain), and pCR-2F-PU.1-ΔEts (lacking the Ets domain), were generated from pCR-2F-PU.1 as follows. To construct pCR-2F-PU.1-ΔAcid, pCR-2F-PU.1-ΔGln, or pCR-2F-PU.1-ΔPEST, *Pst*I or *Sma*I site was introduced into pCR-2F-PU.1 by a QuikChange site-directed mutagenesis kit using the following oligonucleotides and their complements;

ΔAcid: 5′-CCAACGTCAAACA**CtgcAg**TACTATTCCTTGATT-3′ (*Pst*I is bold and base substitutions are small letters)

ΔGln: 5′-GCACGTCCTTGAC**ctgCag**ATGGCACCGCCCCATG-3′ (*Pst*I is bold and base substitutions are small letters)

ΔPEST: 5′-CCTTCTGCATGGG**cccggG**GCAGCAAGAAGA-AGA-3′ (*Sma*I is bold and base substitutions are small letters).

By self-ligation of each mutant plasmid after *Pst*I-digestion (ΔAcid and ΔGln), or *Sma*I-digestion (ΔPEST), each of pCR-2F-PU.1-ΔAcid, pCR-2F-PU.1-ΔGln, or pCR-2F-PU.1-ΔPEST was obtained.

To construct pCR-2F-PU.1-ΔEts, a DNA fragment amplified by PCR using wild-type PU.1 as the template and 5′-GCCC**TGAATTC** ATGTTACAGGCGTGC-3′ (*Eco*RI is bold) and 5′-CTT**Ctc-gag***tCa*GCCTGTCTCCCCATG-3′ (*Xho*I is bold, stop codon is italic, and base substitutions are small letters) as primers was introduced into *Eco*RI/*Xho*I-digested pCR-2F after *Eco*RI/*Xho*I-digestion.

Expression plasmids of PU.1 mutants, pCR-2F-PU.1-G41S, pCR-2F-PU.1-G41A, and pCR-2F-PU.1-S45A, were constructed as follows. To replace Gly41 by Ser, for example, two DNA fragments were amplified by general PCR method using pCR-2F-PU.1 as template with primers (shown below) SDM-N plus G41S-2 and with SDM-C plus G41S-1, respectively. These two PCR products were denatured at 94 °C and annealed by cooling at room temperature. The resulting partially double-stranded DNA was subjected to PCR using primers SDM-N and SDM-C. The PCR product was digested with *Hin*dIII/*Xba*I to replace *Hin*dIII/*Xba*I fragment of pCR-2F-PU.1. The following primers were used to construct pCR-2F-PU.1-G41A and pCR-2F-PU.1-S45A as well as pCR-2F-PU.1-G41S.

SDM-N: 5′-CTGGCTTATCGAAATTAATACGACTCACTATA-GGG-3′,

SDM-C: 5′-AACAGATGGCTGGCAACTAGAAGGCACAGTC-GAGG-3′,

G41S-1: 5′-CTACTATTCCTTGATTGGTAGCGATGGAGACA-GCCATAGCG-3′,

G41S-2: 5′-CGCTATGGCTGTCTCCATCGCTACCAATCAAG-GAATAGTAG-3′,

G41A-1: 5′-CTACTATTCCTTGATTGGTGCGGATGGAGACA-GCCATAGCG-3′,

G41A-2: 5′-CGCTATGGCTGTCTCCATCCGCACCAATCAAG-GAATAGTAG-3′,

S45A-1: 5′-GATTGGTGGTGATGGAGACGCGCATAGCGAT-CACTATTGGG-3′,

S45A-2: 5′-CCCAATAGTGATCGCTATGCGCGTCTCCATCA-CCACCAATC-3′.

As a reporter plasmid, pGV-B2-αNN0.6 [17,18] carrying luciferase gene under the control of human FcεRI α promoter (−605/+29) was used. Another reporter plasmid, pGV-B2-M-CSFR, was constructed as follows. The human M-CSF receptor promoter region (−87/+72), including repeated PU.1 motifs [19], was amplified by PCR using human genomic DNA purified from THP-1 cells (human monocyte cell line) as the template and the following oligonucleotides as the primer sets; 5′-**AGAtCt**AAGATTTCCAAACTCTGTGGTTG-3′ and 5′-**aaGCtt**AGAGCTCTCAGCTACTAGCTCCGCAG-3′. The replaced nucleotides to introduce *Bgl*II and *Hin*dIII sites (shown as bold), respectively, are shown as small letters. The resulting DNA fragment was introduced into *Bgl*II/*Hin*dIII-digested pGL3-Basic (Promega, Madison, WI) after *Bgl*II/*Hin*dIII-digestion.

From pCR-2F-PU.1 derivatives, a fragment encoding 2 × Flag-tagged PU.1 or its mutant was isolated and subcloned into pMX-puro ([20] moloney murine leukemia virus-based retroviral vector) by using restriction endonucleases to generate the plasmids for expression of PU.1 mutants, pMX-puro-PU.1-ΔAcid, pMX-puro-PU.1-G41S, pMX-puro-PU.1-G41A, and pMX-puro-PU.1-S45A.

The plasmids for the expression of PU.1 mutants, pMX-puro-PU.1-S131A, pMX-puro-PU.1-S132A, pMX-puro-PU.1-S141A, and pMX-puro-PU.1-S147A, were constructed by site-directed mutagenesis as described above using the following synthesized oligonucleotides.

S131A-1: 5′-GTCCCCAGCCCACCAGCAGGCGTCAGATGAG-GAGGAGGGTG-3′,

S131A-2: 5′-CACCCTCCTCCTCATCTGACGCCTGCTGGTGG-GCTGGGGAC-3′,

S132A-1: 5′-CCAGCCCACCAGCAGAGCGCGGATGAGGAG-GAGGGTGAG-3′,

S132A-2: 5′-CTCACCCTCCTCCTCATCCGCGCTCTGCTGGT-GGGCTGG-3′,

S141A-1: 5′-GGAGGAGGGTGAGAGGCAGGCGCCTCCACT-GGAGGTGTCTG-3′,

S141A-2: 5′-CAGACACCTCCAGTGGAGGCGCCTGCCTCTC-ACCCTCCTCC-3′.

S147A-1: 5′-CCCTCCACTGGAGGTGGCGGATGGAGAAGC-TGATG-3′,

S147A-2: 5′-CATCAGCTTCTCCATCCGCCACCTCCAGTGGA-GGG-3′.

2.4. Transfection and luciferase assay

U937 cells and CV-1 cells were cotransfected with a mixture of reporter plasmid pGV-B2-M-CSFR (5 μg), PU.1-expression plasmid (5 μg), and pRL-CMV (25 ng; Promega) as internal control. KU812 was cotransfected with a mixture of pGV-B2-αNN0.6 (5 μg), PU.1-expression plasmid (5 μg), and pRL-CMV (25 ng) in the presence or absence of pCR-GATA-1 (GATA-1 expression plasmid). Transfection and luciferase assay were performed as described previously [17,18].

2.5. Transfection of bone marrow-derived cells by retrovirus vectors

Infection of bone marrow-derived cells prepared from Balb/c (Japan SLC, Hamamatsu, Japan) was performed as follows according to the method previously reported [10,21]. To prepare retrovirus, a series of pMX-puro plasmids to express wild-type PU.1 and its mutants were introduced into Plat-E [12] with Fugene6 (Roche Diagnostics, Indianapolis, IN). Culture media including the virus after 24 h- and 48 h-incubation were harvested and concentrated to 1/10 volume by centrifugation. Bone marrow cells cultured under the previously described conditions [10] were incubated with infectious supernatants for 2 days in the presence of 10 μg/ml of polybrene (Sigma–Aldrich). Infected cells were selected by cultivating in the presence of 1.2 μg/ml puromycin for 10–20 days.

2.6. Flow cytometric analysis

Fc receptors on the cell surface were blocked with 2.4G2 (BD PharMingen, San Diego, CA) before staining. Antibodies used were FITC-conjugated anti-I-A^d, anti-CD11b, anti-CD11c, anti-F4/80, and PE-conjugated anti-c-kit, all of which were purchased from BD PharMingen. For staining of FcεRI, FITC-conjugated mouse IgE (BD PharMingen) antibodies were used. Staining and analysis by flow cytometry on FACSCalibur (BD) of cells were performed as described previously [22].

2.7. Cytochemical analysis

Cells were cytocentrifuged onto glass slides and were stained by May–Grünwald–Giemsa's staining solution (Muto Pure Chemicals Co., Ltd., Tokyo).

2.8. Western blotting analysis

Whole cells (2.5×10^6) from each transfectant were subjected to Western blotting analysis. Rabbit polyclonal antibody against PU.1 (Stanta Cruz Biotechnology, Santa Cruz, CA) or mouse monoclonal antibody against Flag-tag (Sigma–Aldrich) was used as the primary

antibody. Alexa Fluor 680 goat anti-rabbit IgG or Alexa Fluor 680 goat anti-mouse IgG (Molecular Probes, Inc., Eugene, OR) was used as the secondary antibody, respectively. Infrared fluorescence on membranes was detected by Odyssey infrared imaging system (Model ODY-9201-SC, LI-COR, Inc., Lincoln, NE).

3. Results

3.1. Cloning of rat PU.1 cDNA and its nucleotide sequence

To clone rat PU.1 cDNA, we performed PCR by using two oligonucleotides corresponding to the sequences of mouse PU.1 cDNA as primers and cDNA prepared from RBL-2H3 cells or rat intestine cDNA as template. An amplified DNA fragment with expected size (about 900 bp) was cloned into pCR3.1 and several clones were sequenced. Nucleotide sequence of rat PU.1 cDNA and its deduced amino acid sequence are shown in Fig. 1. An insertion of 3 bp, which caused a single Gln insertion between Pro15 and Pro16, was observed in one of the five clones from RBL-2H3 and in one of the five clones from rat intestine (Fig. 1). When cDNA sequences were compared between PU.1s from RBL-2H3 and intestine, a source-specific silent nucleotide substitution was found at the third position of Thr32 codon (aca/acg) in cDNA of rat PU.1; aca in RBL-2H3 and acg in rat intestine (see Fig. 1). Nucleotide sequences of all the other portions were completely identical among the cloned fragments. The nucleotide sequence of

```
   1                                                  aacctggagctcagctgg   18
                                                        cag
                                                         Q
  19 atgttacaggcgtgcaaaatggaagggtttccctcgtcgccctccatcggatgagctg   78
   1  M  L  Q  A  C  K  M  E  G  F  P  L  V  A  P  P  S  D  E  L   20
                                       g
  79 gttacttatgaatcagaactataccaacgtcaaacacatgactactattccttgattggt  138
  21  V  T  Y  E  S  E  L  Y  Q  R  Q  T  H  D  Y  Y  S  L  I  G   40

 139 ggtgatggagacagccatagcgatcactattgggatttctccacacaccatgtacacagc  198
  41  G  D  G  D  S  H  S  D  H  Y  W  D  F  S  T  H  H  V  H  S   60

 199 gagtttgagagcttccctgagaaccacttcacggagctgcagagcgtgcagccccgcag  258
  61  E  F  E  S  F  P  E  N  H  F  T  E  L  Q  S  V  Q  P  P  Q   80

 259 ctacagcagctctaccgccacatggagctggagcagatgcacgtccttgacaccccatg  318
  81  L  Q  Q  L  Y  R  H  M  E  L  E  Q  M  H  V  L  D  T  P  M  100

 319 gcaccgcccatgccagcctcagtcaccaggttcctacatgcccgggtgtgcttccct  378
 101  A  P  P  H  A  S  L  S  H  Q  V  S  Y  M  P  R  V  C  F  P  120

 379 tatccacccttgtccccagcccaccagcagagctcagatgaggaggagggtgagaggcag  438
 121  Y  P  P  L  S  P  A  H  Q  Q  S  S  D  E  E  E  G  E  R  Q  140

 439 agccctccactggaggtgtctgatggagaagctgatggcttggagcctgggccaggcctt  498
 141  S  P  P  L  E  V  S  D  G  E  A  D  G  L  E  P  G  P  G  L  160

 499 ctgcatgggagacaggcagcaagaagaagattcgtttgtaccagttcctgctggacctg  558
 161  L  H  G  E  T  G  S  K  K  K  I  R  L  Y  Q  F  L  L  D  L  180

 559 ctgcgtagcggagacatgaaggacagcatctggtgggtggacaaggacaaaggtaccttc  618
 181  L  R  S  G  D  M  K  D  S  I  W  W  V  D  K  D  K  G  T  F  200

 619 cagttctcgtccaagcacaaggaggcgctggcgcaccgctggggcatccagaagggaaac  678
 201  Q  F  S  S  K  H  K  E  A  L  A  H  R  W  G  I  Q  K  G  N  220

 679 cgcaagaagatgacctaccagaagatggcgcgcgcgctgcgcaactacggcaagacgggc  738
 221  R  K  K  M  T  Y  Q  K  M  A  R  A  L  R  N  Y  G  K  T  G  240

 739 gaggtgaagaaagtcaagaagaagctcacttaccagttcagcggcgaggtgctgggccgt  798
 241  E  V  K  K  V  K  K  K  L  T  Y  Q  F  S  G  E  V  L  G  R  260

 799 ggggggcctggctgagcggcgcctcccgccccactgatcgcctgcagagcccgccaggctc  858
 261  G  G  L  A  E  R  R  L  P  P  H  *                          271

 859 ccggcccccgcc                                                  870
```

Fig. 1. Nucleotide sequence of rat PU.1 cDNA and its deduced amino acid sequence. The nucleotide sequence of rat PU.1 was registered in Genbank/EMBL/DDBJ under accession number AB154364. An insertion of three nucleotides resulting in addition of Gln was observed in two from ten clones and are shown as attached boxed sequence. A nucleotide substitution observed between cDNA prepared from RBL-2H3 and that from rat intestine cDNA library are also boxed. All clones have Gly as 41st residue (boxed).

rat PU.1-coding region was highly homologous to those of mouse PU.1 (93.4%) and human PU.1 (88.0%).

Alignment of amino acid sequences of rat, mouse, and human PU.1 is shown (Fig. 2A). The amino acid sequences of the Ets domain and the Gln-rich region are extremely conserved among rat, mouse, and human PU.1 proteins, while the amino acid sequence of the acidic region is not so highly homologous, compared to those of other regions. Among six Ser residues that were suggested to be involved in the function in previous reports (boxed with yellow in Fig. 2A), the 41st residue is replaced by Gly in rat PU.1, although Ser at this position is also conserved in non-mammals, Fowl and Caiman (Fig. 2B) [23].

3.2. Effect of deletion of functional domains of PU.1 on the transcription activity

To evaluate the role of the previously defined regions in the transcription-enhancing activity of PU.1, we at first performed a transient reporter assay for wild-type or various truncated mutants of PU.1 using the luciferase gene under the control of M-CSF receptor promoter that is positively regulated by PU.1 [19]. In U937 cells, M-CSF receptor promoter is known to be activated by PMA stimulation via activation of PU.1 [24]. Consistent with the report, significant upregulation of the promoter activity was observed when wild-type PU.1 was co-introduced with the reporter plasmid (Fig. 3B). Both mutants PU.1-ΔGln and PU.1-ΔPEST lacking the Gln-rich region and the PEST domain, respectively, possessed transactivation-enhancing activity no less than that of wild-type (Fig. 3B). However, PU.1-ΔAcid and PU.1-ΔEts, lacking the acidic region and the Ets domain, respectively, possessed decreased activities (Fig. 3B).

3.3. Effect of replacement of Ser residues in acidic region on transactivation of M-CSF receptor promoter

The above result indicates that PU.1 binds the target sequence by its Ets domain and exerts the ability to transactivate the promoter by the acidic region. The acidic region contains two Ser residues, Ser41 and Ser45, which are reported to be involved in the function of PU.1 [8,9]. To analyze whether or not these Ser residues are involved in the transcription activity of PU.1, we constructed three mutants of rat PU.1, G41S, G41A, and S45A and analyzed their promoter-transactivating activity by transient reporter assay. As shown in Fig. 3C, all of the three mutants possessed transactivation activity almost the same as that of wild-type. Even when similar reporter assay was performed in CV-1 cells, where PU.1 co-upregulates M-CSF receptor promoter with endogenously expressed c-Jun [24], no apparent difference was observed between the wild-type and the mutants (Fig. 3D).

3.4. Effect of replacement of Ser41 and Ser45 on transcription activity in mast cells

We have previously found that PU.1 transactivates FcεRI α-chain promoter cooperatively with GATA-1 [18]. Then, we analyzed the transcription-enhancing activity of wild-type, G41S, G41A, and S45A by using the α-chain/mast cell system, to investigate whether or not Ser residues at the 41st and 45th positions are indeed required for the cooperation with GATA-1. The α-chain promoter was upregulated about 1.5-fold by exogenously expressed wild-type PU.1, and no significant difference was observed in the luciferase activity, when G41S,

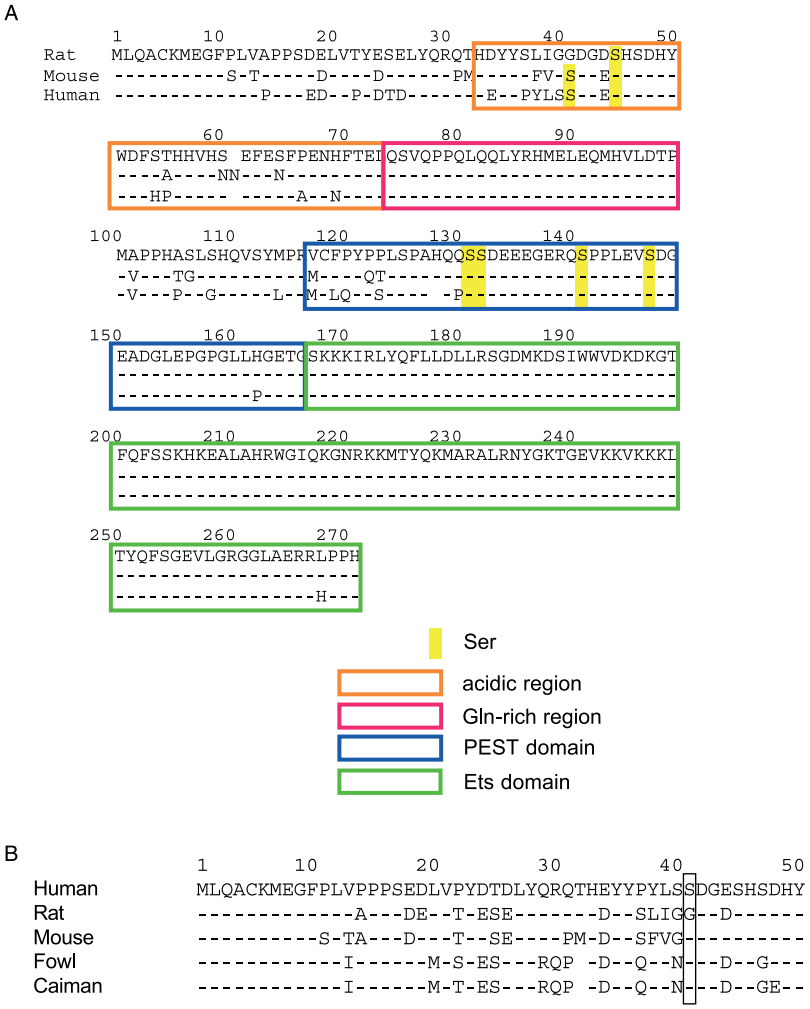

Fig. 2. Amino acid alignment of PU.1. (A) Amino acid sequences of rat, mouse, and human PU.1. Each of the acidic region, the Gln rich region, the PEST domain, and the Ets domain are boxed. Ser residues previously supposed to be important are also shown. (B) Amino acid alignment of human, rat, mouse, fowl, and caiman PU.1 (1–50th residues) [23]. As 41st residue, Ser is conserved between human, mouse, fowl, and caiman, while Gly is used in rat.

G41A, or S45A was overproduced in place of wild-type PU.1 (Fig. 4). The α-chain promoter was markedly upregulated, over 40-fold, by simultaneous overproduction of GATA-1 and wild-type PU.1. However, similar to the case with the wild-type PU.1, all the other mutants also activated the α-chain promoter cooperatively with GATA-1 (Fig. 4). Therefore, we conclude that residues at 41st and 45th positions are not essential for the cooperative activation of the α-chain promoter with GATA-1.

3.5. *Effect of replacement of residues at 41st and 45th positions on monocyte specific gene expression*

Recently, it was reported that human CD34+ hematopoietic progenitor cells were developed to Langerhans cells by enforced expression of PU.1 [25]. In addition, we recently found that when PU.1 was overproduced by retrovirus vector in mouse bone marrow-derived mast cell progenitors, monocyte-specific gene expression was induced in the cells [10]. We also found that the acidic region was critically required for the monocyte-specific gene expression [11]. To examine the possible involvement of 41st and 45th residues in the monocyte-specific gene expression, we overproduced wild-type PU.1, G41S, G41A, or S45A by using retrovirus system and characterized development of the bone marrow-derived cells. The progenitor overproducing wild-type PU.1, G41S, G41A, or S45A equally expressed MHC class II, CD11b, CD11c, and F4/80, all of which were not detected at all on surfaces of the cells infected with the virus carrying ΔAcid or mock vector (Fig. 5A). Suppression of c-kit expression was caused by overexpression of G41S, G41A, and S45A as well as wild-type PU.1 (Fig. 5A). Expression level of PU.1 protein in each transfectant was analyzed by Western blotting analysis (Fig. 5B). The level of exogenously produced PU.1 was almost the same in every transfectant, suggesting that the profile of the cells transfected with the retrovirus carrying cDNA of PU.1-ΔAcid was not due to lack or low-level production of the PU.1 mutant. Only a faint band was observed in mock transfectant when detection was performed under overexposure condition (bottom of Fig. 5B). These data coincide well with our previous observation [11] and indicate that the level of exogenously produced protein is much higher than that of endogenous PU.1 protein.

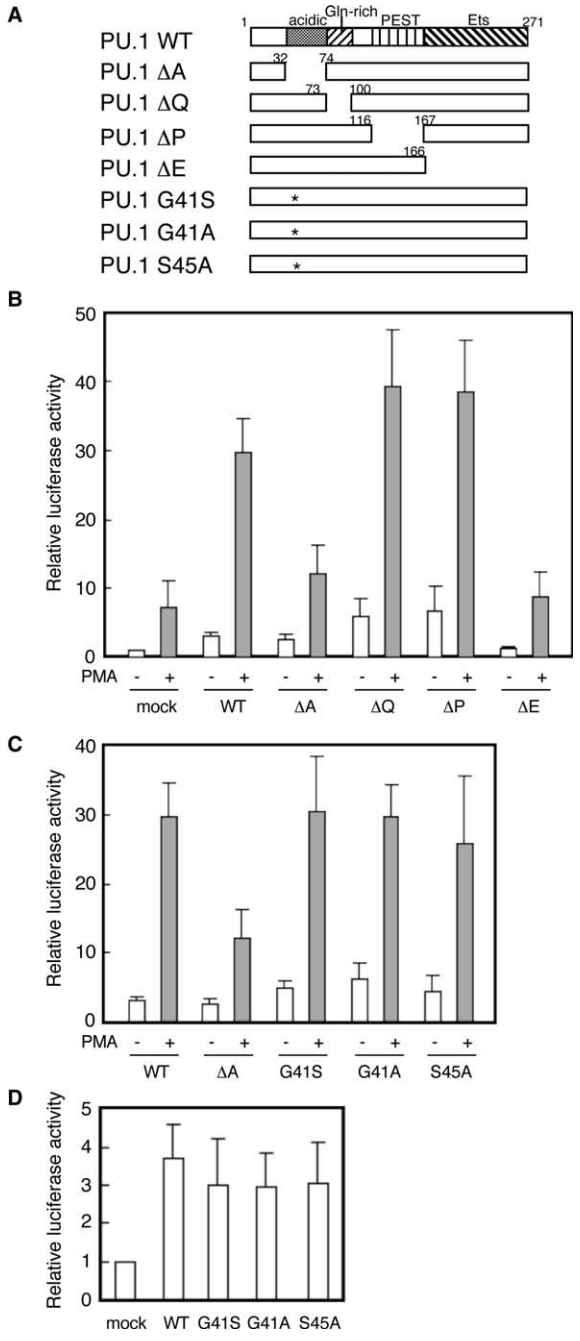

Fig. 3. Transactivation activity of PU.1 and its derivatives. (A) Structure of wild-type or various mutants of PU.1. (B) Transactivation activity of wild-type or truncated mutants of PU.1. U937 cells were transfected with pGV-MCSFR and expression plasmid. Relative luciferase activity is represented as the ratio to that of pGV-MCSFR with mock plasmid and the results are expressed as means ± S.E. for more than three independent experiments in (B)–(D). (C) Effect of amino acid substitution of residues at 41st and 45th positions for the transactivation activity. (D) PU.1 mutants, G41S, G41A, and S45A showed almost equal degree of transactivation activity in CV-1 cells.

The morphology of each infected cell was analyzed by May–Grünwald–Giemsa stain (Fig. 6). Control cells (infected by the mock virus) were developed to the cells containing many granules, which are a typical feature of mast cells. However, overexpression of wild-type PU.1 caused a decrease in amount of the granules and converted the cells with eccentric nuclei

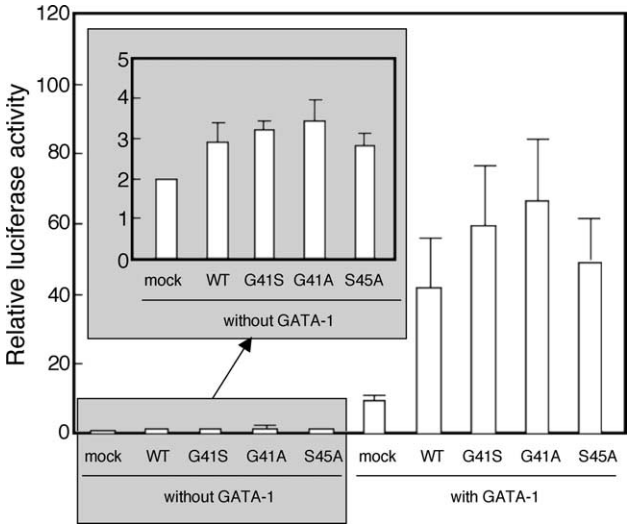

Fig. 4. Transactivation activity of wild-type, G41S, G41A, and S45A PU.1 in mast cells. Reporter plasmid carrying human FcεRI α-chain promoter and PU.1-expression plasmid are transiently introduced into KU812 cells with or without GATA-1-expression plasmid. Relative luciferase activity is represented as the ratio to that of pGV-B2αNN0.6 with mock plasmid and the results are expressed as means ± S.E. for more than three independent experiments.

and polarized lamellipodia, which are monocyte-specific features. The cells overexpressing G41S, G41A, and S45A exhibited the morphology similar to that of the cells overexpressing wild-type PU.1, while the cells overexpressing PU.1-ΔAcid were developed to the cells like mast cells with many granules.

3.6. *Effect of replacement of other serine residues in the PEST domain on monocyte specific gene expression*

Four additional Ser residues at 132, 133, 142, and 148 (residue numbers according to mouse and human sequences and corresponding to 131, 132, 141, and 147 of rat sequence) in the PEST domain are proposed to be involved in the function of PU.1 [26,27]. To analyze the possible role of these Ser residues in myeloid specific gene expression, PU.1 mutants, in which Ser residue at 131, 132, 141, or 147 of rat PU.1 was replaced with Ala, were overexpressed in bone marrow-derived cells by using retrovirus system. The expression levels of overproduced PU.1 were almost the same among transfectants by Western blotting analysis (data not shown). All the cells overproducing S131A, S132A, S141A, and S147A expressed MHC class II, CD11c, CD11b, and F4/80 and gave suppressed expression of c-kit, which was similar to the case of wild-type PU.1 transfectant (Fig. 7), suggesting the unobvious role of these Ser residues in monocyte-specific gene regulation.

4. Discussion

Transcription factor PU.1 belonging to Ets-family is involved in the development of myeloid and lymphoid cells and regulates the transcription of many genes expressed in these cells. PU.1 is composed of the following four functional domains (regions), the acidic region, the Gln-rich region, the PEST domain, and the Ets domain. The first two regions, the acidic region and the Gln-rich region, are involved in trans-

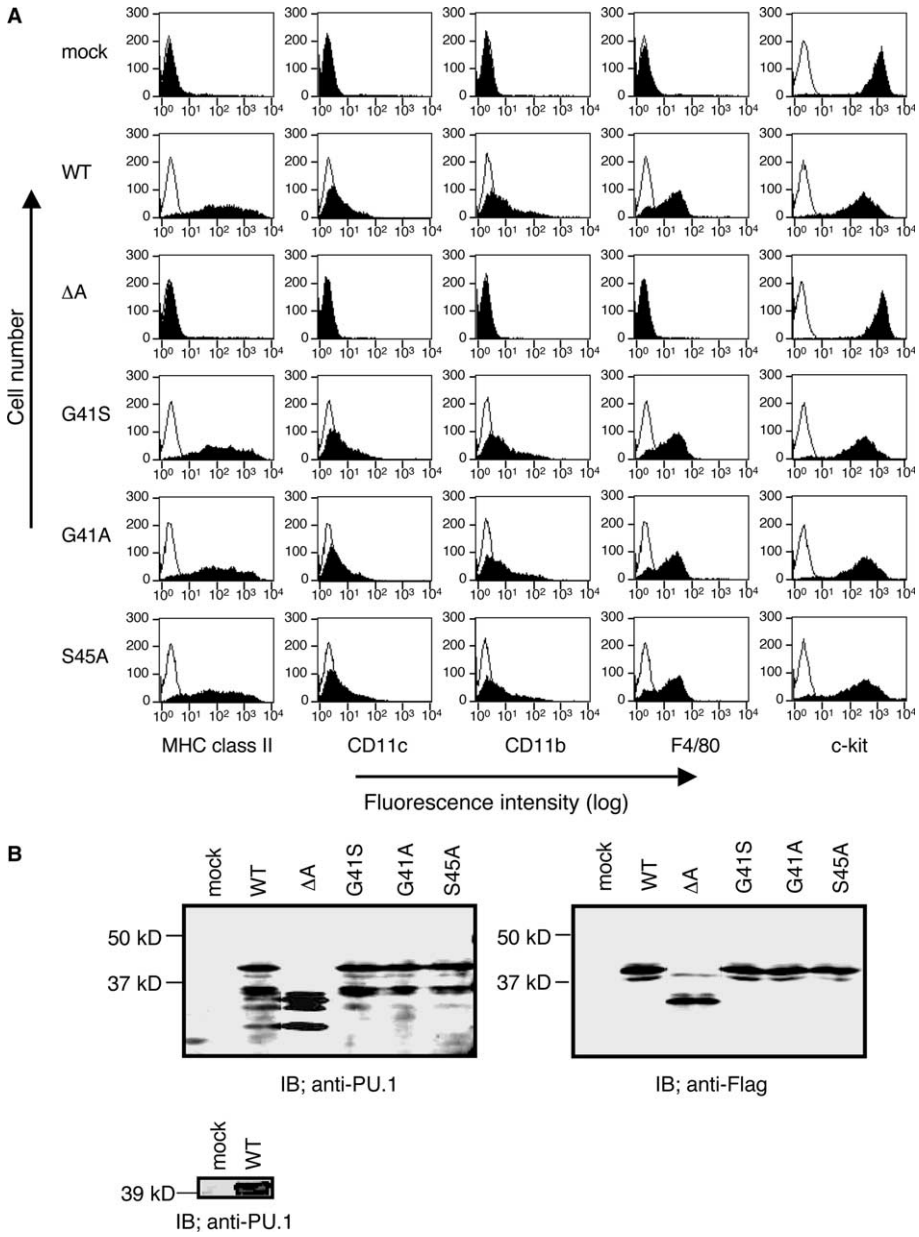

Fig. 5. Phenotype of bone marrow-derived cells transfected with retrovirus carrying wild-type or various mutant PU.1 cDNA (PU.1-ΔAcid, G41S, G41A, or S45A). (A) Flow cytometric analysis of monocyte- and mast cell-specific gene expression. (B) Western blotting analysis to detect the expression level of PU.1 proteins. Lysates (2.5×10^6 cells per lane) were analyzed by using anti-PU.1 (top left) or anti-Flag (top right) antibody. Detection was also performed under overexposure condition (bottom).

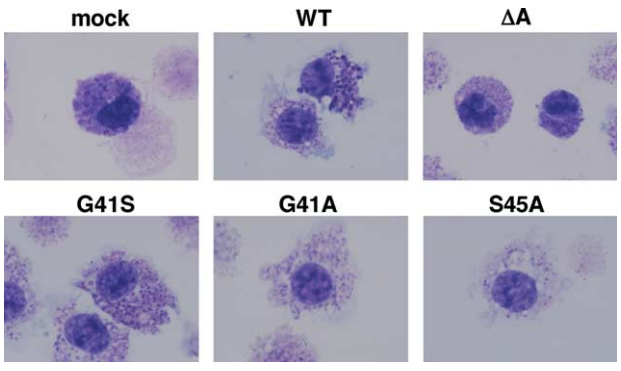

Fig. 6. May–Grünwald–Giemsa stain of the cells cytospun, 400×.

activation function, and the PEST domain functions for both regulation of protein degradation and interaction with other transcription factors. The Ets domain is responsible for the DNA-binding ability of PU.1. In the previous studies, six Ser residues at 41, 45, 132, 133, 142, and 148 are proposed to be involved in the function of PU.1 [26,27]. Among them, until now, Ser at position 148 is the only residue whose function has been definitively demonstrated [28]. In that study, Ser148 is shown to be critical for association with IRF-4 in B cells, and the function of PU.1 is suggested to be regulated by phosphorylation of Ser148. However, the involvement of other candidate Ser residues in PU.1 function is still under controversy. Celada et al. [9] proposed that Ser41 and Ser45 were required for macrophage proliferation in response to M-CSF.

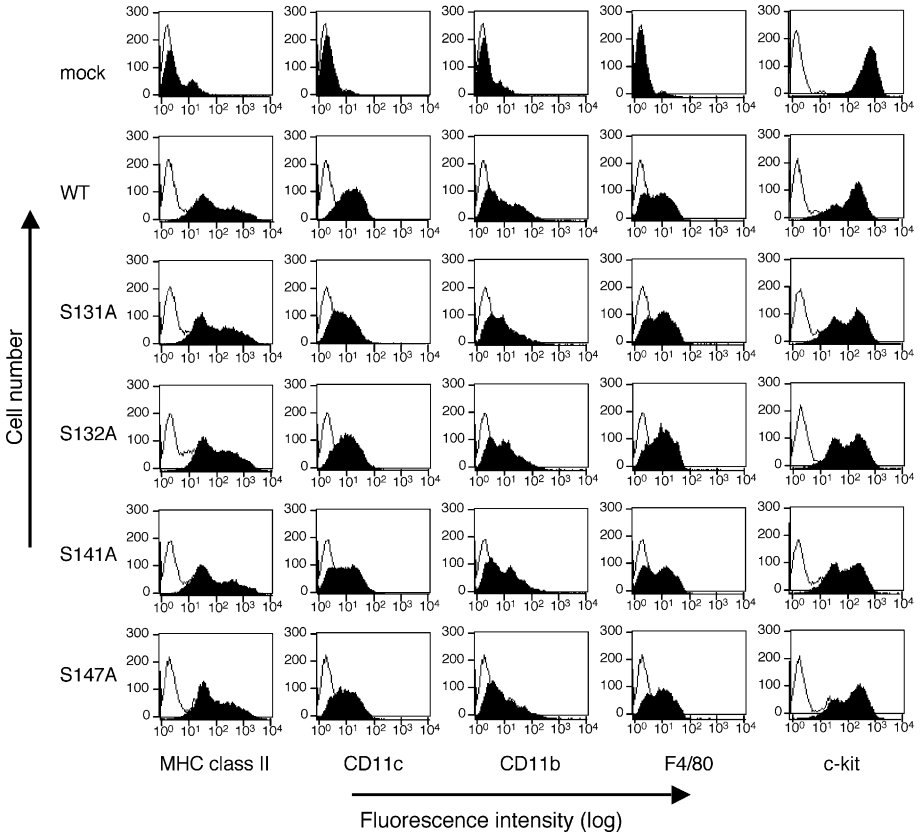

Fig. 7. Effect of Ser residue replacement (S131A, S132A, S141A, and S147A) on monocyte- and mast cell-specific gene expression.

Rieske and Pongubala [8] showed that Ser41 was necessary for the activation of PU.1 in response to AKT. Fisher et al. [29] suggested that only Ser133, but not Ser41, Ser45, nor Ser148, was required for macrophage development. Recently, Wang et al. [27] reported that phosphorylation of PU.1 at Ser142 by a p38 MAPK-dependent pathway was involved in the activation of PU.1. Among them, two Ser residues, Ser41 and Ser45, in the acidic region have been presumed to be the most probable residues that are involved in function and its regulation of PU.1 protein [8,9]. In the present study, we have found that rat PU.1 possesses Gly but not Ser residue at position 41. This indicates that Ser41 is not essential for function at least in rat PU.1. In this study, we analyzed transcription activity of wild-type or mutants of PU.1 in various systems; transactivation of M-CSFR promoter, cooperative transactivation with GATA-1, and induction of monocyte-specific gene expression and morphological change in bone marrow-derived mast cell progenitors. All of the mutant rat PU.1 proteins, G41S, G41A, and S45A, possessed the activities comparable to those of wild-type in all assay systems. Thus, all our results were negative in requirement of Ser41 and Ser45 for the function of PU.1. Considering that the acidic region was critically required for the function of PU.1 (Figs. 3, 5 and 6), we may assume that other residues are responsible for the function, though they have not yet been analyzed. Interestingly, the homology of the amino acid sequence in the acidic region is considerably lower than other regions (Fig. 2). Further analysis using the retrovirus transfection and reporter assay systems used in this study will provide useful information on the residues critical for the transactivation function of PU.1.

Rat PU.1 mutant S147A (corresponding to S148A of mouse and human PU.1) lacks a critical Ser residue whose phosphorylation is essential for interaction with IRF-4 [28]. In this study, Ala-replacement of four Ser residues in the PEST domain, Ser131, Ser132, Ser141, and Ser147 (residue numbers are according to rat PU.1 sequence and corresponding to Ser132, Ser133, Ser142, and Ser147 of mouse and human sequences), did not affect the function of PU.1 as a transactivator. We previously reported that overproduction of PU.1-mutant lacking the PEST domain also induced the expression of monocyte-specific gene, such as MHC class II, CD11c, CD11b, and F4/80 [11], indicating that the PEST domain has no apparent function in our assay systems. Therefore, no obvious effect of Ala-replacement at Ser131, Ser132, Ser141, or Ser147 in the PEST domain found in the present study is quite reasonable. PU.1 is capable of regulating the target genes by binding to its cognate DNA site by itself, while PU.1 is required to form a heterodimer with IRF-4 to activate Igκ 3′ gene expression [30]. Therefore, the present result suggests that PU.1 functions to upregulate the expression of the monocyte-specific genes without forming a heterodimer with IRF-4 at least in our assay system.

Various stimulation signals such as colony-stimulating factors, PMA, and LPS, all of which are capable of inducing myeloid differentiation, are suggested to be transduced to their targets via PU.1 activation [31–34]. It has been reported that stimulation by LPS caused phosphorylation of Ser residues of PU.1 to induce conformational change of the transcription factor suitable for controlling gene expression [32]. Furthermore, by the PMA-stimulation novel slow-migrating PU.1-DNA complex(s) was observed in EMSA [31,33], and PU.1 sometimes exhibited different mobility in SDS–PAGE depending

on the cell lines used as the source of PU.1 [35]. These observations indicate that PU.1 is regulated by post-transcriptional modification. Further analysis using various PU.1 mutants, which possess amino acid substitution other than the 41st and 45th residues in the acidic region, will be required to determine the residues essential for regulating the functions of PU.1. Especially, function of PU.1 mutants, in which all the Ser residues in the acidic region are replaced by Ala, should be analyzed in the near future. Until now, three-dimensional structural information of PU.1 was available only for its DNA-binding Ets domain. Therefore, determination of three-dimensional structure of whole PU.1 molecule could provide useful information to elucidate the regulatory mechanism of PU.1.

Acknowledgements: We thank Dr. T. Kitamura (The Institute of Medical Science, The University of Tokyo) and Dr. H. Nakano (Department of Immunology, Juntendo University School of Medicine) for providing PLAT/E and pCR-2F, respectively, and Dr. K. Yokomizo for May–Grünwald–Giemsa staining. We are grateful to the members of Biotechnology Research Center (The University of Tokyo), Atopy (Allergy) Research Center, and Department of Immunology (Juntendo University School of Medicine) for helpful discussion. We thank Drs. N. Nakano, H. Kawada, A. Takagi, and Ms. T. Tokura for technical assistance, and Ms. M. Matsumoto and Ms. E. Kawasaki for secretarial assistance.

References

[1] Scott, E.W., Simon, M.C., Anastasi, J. and Singh, H. (1994) Science 265, 1573–1577.
[2] McKercher, S.R., Torbett, B.E., Anderson, K.L., Henkel, G.W., Vestal, D.J., Baribault, H., Klemsz, M., Feeney, A.J., Wu, G.E., Paige, C.J. and Maki, R.A. (1996) EMBO J. 15, 5647–5648.
[3] Scott, E.W., Fisher, R.C., Olson, M.C., Kehrli, E.W., Simon, M.C. and Singh, H. (1997) Immunity 6, 437–447.
[4] Anderson, K.L., Perkin, H., Surh, C.D., Venturini, S., Maki, R.A. and Torbett, B.E. (2000) J. Immunol. 164, 1855–1861.
[5] Guerriero, A., Langmuir, P.B., Spain, L.M. and Scott, E.W. (2000) Blood 95, 879–885.
[6] Rogers, S., Wells, R. and Rechsteiner, M. (1986) Science 234, 364–368.
[7] Rechsteiner, M. and Rogers, S.W. (1996) Trends Biochem. Sci. 21, 267–271.
[8] Rieske, P. and Pongubala, J.M.R. (2001) J. Biol. Chem. 276, 8460–8468.
[9] Celada, A., Borràs, F.E., Soler, C., Lloberas, J., Klemsz, M., Beveren, C.V., McKercher, S. and Maki, R.A. (1996) J. Exp. Med. 184, 61–69.
[10] Nishiyama, C., Nishiyama, M., Ito, T., Masaki, S., Maeda, K., Masuoka, N., Yamane, H., Kitamura, T., Ogawa, H. and Okumura, K. (2004) Biochem. Biophys. Res. Commun. 313, 516–521.
[11] Nishiyama, C., Nishiyama, M., Ito, T., Masaki, S., Masuoka, N., Yamane, H., Kitamura, T., Ogawa, H. and Okumura, K. (2004) FEBS Lett. 561, 63–68.
[12] Morita, S., Kojima, T. and Kitamura, T. (2000) Gene Therapy 7, 1063–1066.
[13] Klemsz, M.J., McKercher, S.R., Celada, A., van Beveren, C.P. and Maki, R.A. (1990) Cell 61, 113–124.
[14] Paul, R., Schuetze, S., Kozak, S.L., Kozak, C.A. and Kabat, D. (1991) J. Virol. 65, 464–467.
[15] Moreau-Gachelin, F., Ray, D., Mattei, M.G., Tambourin, P. and Tavitian, A. (1989) Oncogene 4, 1449–1456.
[16] Akiba, H., Nakano, H., Nishinaka, S., Shindo, M., Kobata, T., Atsuta, M., Morimoto, C., Ware, C.F., Malinin, N.L., Wallach, D., Yagita, H. and Okumura, K. (1998) J. Biol. Chem. 273, 13353–13358.
[17] Nishiyama, C., Yokota, T., Okumura, K. and Ra, C. (1999) J. Immunol. 163, 623–630.
[18] Nishiyama, C., Hasegawa, M., Nishiyama, M., Takahashi, K., Akizawa, Y., Yokota, T., Okumura, K., Ogawa, H. and Ra, C. (2002) J. Immunol. 168, 4546–4552.
[19] Zhang, D.E., Hetherington, C.J., Chen, H.M. and Tenen, D.G. (1994) Mol. Cell. Biol. 14, 373–381.
[20] Onishi, M., Kinoshita, S., Morikawa, Y., Shibuya, A., Phillips, J., Lanier, L.L., Gorman, D.M., Nolan, G.P., Miyajima, A. and Kitamura, T. (1996) Exp. Hematol. 24, 324–329.
[21] Hata, D., Kawakami, Y., Inagaki, N., Lantz, C.S., Kitamura, T., Khan, W.N., Maeda-Yamamoto, M., Miura, T., Han, W., Hartman, S.E., Yao, L., Nagai, H., Goldfeld, A.E., Alt, F.W., Galli, S.J., Witte, O.N. and Kawakami, T. (1998) J. Exp. Med. 187, 1235–1247.
[22] Hasegawa, M., Nishiyama, C., Nishiyama, M., Akizawa, Y., Takahashi, K., Ito, T., Furukawa, S., Ra, C., Okumura, K. and Ogawa, H. (2003) J. Immunol. 170, 3732–3738.
[23] Shintani, S., Terzic, J., Sato, A., Saraga-Babic, M., O'hUigin, C., Tichy, H. and Klein, J. (2000) Proc. Natl. Acad. Sci. USA 97, 7417–7422.
[24] Behre, G., Whitmarsh, A.J., Coghlan, M.P., Hoang, T., Carpenter, C.L., Zhang, D.-E., Davis, R.J. and Tenen, D.G. (1999) J. Biol. Chem. 274, 4939–4946.
[25] Iwama, A., Osawa, M., Hirasawa, R., Uchiyama, N., Kaneko, S., Onodera, M., Shibuya, K., Shibuya, A., Vinson, C., Tenen, D.G. and Nakauchi, H. (2002) J. Exp. Med. 195, 547–558.
[26] Lloberas, J., Soler, C. and Celada, A. (1999) Immunol. Today 20, 184–189.
[27] Wang, J.-M., Lai, M.-Z. and Yang-Yen, H.-F. (2003) Mol. Cell. Biol. 23, 1896–1909.
[28] Pongubala, J.M.R., vanBeveren, C., Nagulapalli, S., Klemsz, M.J., McKercher, S.R., Maki, R.A. and Atchison, M.L. (1993) Science 259, 1622–1625.
[29] Fisher, R.C., Olson, M.C., Pongubala, J.M.R., Perkel, J.M., Atchison, M.L., Scott, E.W. and Simon, C. (1998) Mol. Cell. Biol. 18, 4347–4357.
[30] Pongubala, J.M.R., Nagulapalli, S., Klemsz, M.J., McKercher, S.R., Maki, R.A. and Atchison, M.L. (1992) Mol. Cell. Biol. 12, 368–378.
[31] Kim, J. and Feldman, R.A. (2002) Mol. Cell. Biol. 22, 1903–1918.
[32] Lodie, T.A., RicardoSavedra, J., Golenbock, D.T., Beveren, C.P.V., Maki, R.A. and Fenton, M.J. (1997) J. Immunol. 158, 1848–1856.
[33] Carey, J.O., Posekany, K.J., deVente, J.E., Pettit, G.R. and Ways, D.K. (1996) Blood 87, 4316–4323.
[34] Shibata, Y., Berclaz, P.-Y., Chroneos, Z.C., Yoshida, M., Whitsett, J.A. and Trapnell, B.C. (2001) Immunity 15, 557–567.
[35] Ford, A.M., Bennett, C.A., Healy, L.E., Towatari, M., Greaves, M.F. and Enver, T. (1996) Proc. Natl. Acad. Sci. USA 93, 10838–10843.