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Amino-terminal extension of 146 residues of L-type GATA-6 is required for transcriptional activation but not for self-association

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ABSTRACT

Transcription factor GATA-6 plays essential roles in developmental processes and tissue specific functions through regulation of gene expression. GATA-6 mRNA utilizes two Met-codons *in frame* as translational initiation codons. Deletion of the nucleotide sequence encoding the PEST sequence (Glu^{31}–Cys^{46}) between the two initiation codons unusually reduced the protein molecular size on SDS–polyacrylamide gel-electrophoresis, and re-introduction of this sequence reversed this change. The long-type (L-type) GATA-6 containing this PEST sequence self-associated similarly to the short-type (S-type) GATA-6, as determined on co-immunoprecipitation of Myc-tagged GATA-6 with HA-tagged GATA-6. The L-type and S-type GATA-6 also interacted mutually. The L-type GATA-6 without the PEST sequence also self-associated and interacted with the S-type GATA-6. The transcriptional activation potential of L-type GATA-6 is higher than that of S-type GATA-6. When the PEST sequence (Glu^{31}–Cys^{46}) was inserted into the L-type GATA-6 without Arg^{13}–Gly^{101}, the resultant recombinant protein showed significantly higher transcriptional activity, while the construct with an unrelated sequence exhibited lower activity. These results suggest that the Glu^{31}–Cys^{46} segment plays an important role in the transcriptional activation, although it does not participate in the self-association.

1. Introduction

Transcription factor GATA-6 containing tandem zinc fingers (CVNC-X17-CNAC)-X29-(CXNC-X17-CNAC) recognizes a canonical DNA motif, (T/A)GATA(A/G) [1,2], and regulates the expression of various genes required for developmental processes and tissue-specific functions [3,4]. Among mammalian GATA factors, GATA-6 is distinct in that it has a 146 extra-amino terminal extension compared with the other five members [3,5]. Interestingly, both L-type and S-type GATA-6 are translated from a single mRNA [5,6] due to the leaky scanning of Met codons by ribosomes; the introduction of a Kozak sequence, 5′GCCACCaugG3′ [7], around the upstream initiation codon 5′CCGUGGaugG3′ by means of site-directed mutagenesis produced only L-type GATA-6 [5].

GATA-6 has been identified as an essential gene since a knock-out mouse showed embryonic lethality [8,9]. However, the roles of translational isoforms of GATA-6 have not been examined although both L-type and S-type GATA-6 are produced under *in vivo* conditions [10,11], and the molecular activity of L-type GATA-6 is suggested to be higher than that of the S-type [5]. Similar to other GATA factors, GATA-6 might have a function in regulation of cell type-specification or determination through unique interactions with other semi-restricted transcription factors [2]. Actually, GATA-6 was shown to physically interact with a coactivator, p300/CBP, which results in transcriptional synergy for the smooth muscle-myosin heavy chain gene and maintenance of the differentiated phenotype in vascular smooth muscle cells [12]. Furthermore, CHF1/Hey2 interacts directly with GATA-6 and prevents triggering of the smooth muscle-heavy chain transcription upon de-differentiation of smooth muscle cells [13]. In the lungs,

Abbreviations: HA, human influenza hemagglutinin; PBS, 10 mM sodium phosphate buffer (pH 7.2), 137 mM NaCl, 3 mM KCl; PBS(+), PBS containing 0.1% (v/v) Tween 20; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TNE buffer, 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 10 μg/μl leupeptin, 10 μg/μl pepstatin A; TNE(+ NP40), TNE buffer containing 1% (w/v) NP-40; Tris, tris(hydroxymethyl)aminomethane.

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GATA-6 physically interacts with homeobox factor TTF1 and activates the surfactant protein-C gene [14]. It has also been demonstrated that cooperative interaction between GATA-4 and GATA-6 affects transcription of atrial natriuretic factor and B-type natriuretic peptide genes in cardiomyocytes positively [15].

Although such heterotypical interaction with other regulatory proteins has been described, a homotypic interaction has not been demonstrated for GATA-6. As for GATA-1, self-association synergistically stimulated transcription [16]. In this study, we examined whether GATA-6 interacts mutually and whether such an interaction may be affected by the presence of the amino-terminal 146 residues in the L-type or not. Here we show that GATA-6 self-associates, although the L-type specific sequence does not affect the self-association of GATA-6. Furthermore, we demonstrated that a PEST sequence [17] in the amino-terminal extension causes the higher transcriptional activity of L-type GATA-6.

2. Materials and methods

2.1. Expression plasmids for GATA-6 with a Myc-tag or HA-tag

The construction of expression plasmids for GATA-6 with a Myc-tag is shown in Fig. S1. The resulting expression plasmids for L-type GATA-6 with a carboxyl-terminal Myc-tag with or without the sequence between Arg^{13} and Gly^{101} (Fig. 1) were named pME-hGT1L5′KMyc and pME-hGT1L5′ΔEKMyc, respectively. An expression plasmid for S-type GATA-6 (pME-hGT1SMyc) was similarly constructed. All the expression plasmids were derivatives of pME18S [18]. The expression plasmids for L-type GATA-6 with an amino-terminal HA-tag were constructed by introduction of a CTC codon instead of an ATG codon corresponding to the S-type initiation codon (Fig. S2). The DNA sequence was confirmed by the dideoxy chain-termination method [19]. The molecular biological techniques were performed by published methods [20].

2.2. Expression plasmids for GATA-6 to measure luciferase activity

Expression plasmid, pME-hGT1S, pME-hGT1L5′K and pME-hGT1L5′ΔEK were described previously [5]. pME-hGT1L5′ΔAK and pME-hGT1L5′ΔA(−2)K were constructed in this study (Fig. S3). In the latter plasmids, nucleotide sequences encoding the PEST sequence and an unrelated sequence, respectively (Fig. 1), were introduced into pME-hGT1L5′ΔEX together with a Kozak sequence, GCCACCatgG [7], around the upstream initiation codon for L-type GATA-6. The introduction of a Kozak sequence results in inhibition of leaky scanning by ribosomes and the production of only L-type GATA-6 [5].

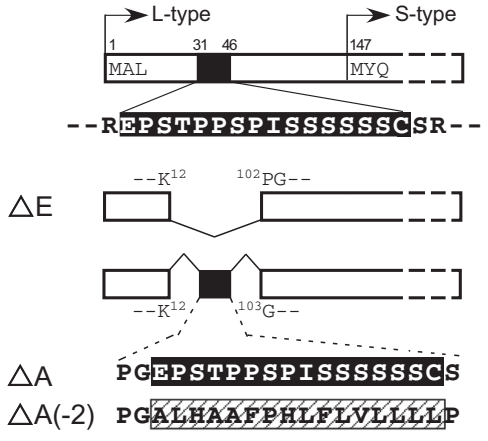

Fig. 1. Schematic representation of human GATA-6 expressed in this study. L-type GATA-6 has an extension of 146 amino acid residues in front of S-type GATA-6. Numericals indicate the amino acid residue numbers from the amino terminus of L-type GATA-6. The 16 residue segment between Glu^{31} and Cys^{46} was determined to affect the mobility of GATA-6 on SDS–polyacrylamide gel-electrophoresis [5]. The ΔE protein was derived from L-type GATA-6 by deletion of the region between Arg^{13} and Gly^{101}. The ΔA protein was constructed by addition of the DNA segment encoding Glu^{31}–Cys^{46}. The ΔA(−2) protein was constructed by adding essentially the same DNA segment to produce a frame shift of two bases.

2.3. Expression of GATA-6 tagged with a Myc-tag or HA-tag, and its immunoprecipitation

Cos-1 cells (ATCC) were grown in Dulbecco's modified Eagle medium (GIBCO). An expression plasmid was introduced into the cells by means of the diethylaminoethyl-dextran method, as described previously [5], and then cells were grown for 48 h before harvest. Protease inhibitors [20 μM benzyloxycarbonyl-Leu-Leu-norvalinal (Peptide Institute), 1 mM PMSF (Sigma), and 50 μM L-trans-epoxysuccinyl-Leu-3-methylbutylamide-ethyl ester (Peptide Institute)] were added at 24 h before harvesting as a dimethyl sulfoxide solution (10, 25 and 25 μl/5 ml medium, respectively). All the media were supplemented with 7% (v/v) fetal bovine serum (JRH Biosciences).

A cell extract was prepared by the published method [10,21]. Briefly cells from two dishes (Φ 10 cm) were collected in 1 ml of ice-cold TNE (+ NP-40) and kept on ice for 30 min. After sheering the suspension 10 times through a 25G needle, a supernatant (12,000*g*, 30 min) was obtained. Protein G Sepharose beads (GE Healthcare) were prewashed with TNE (+ NP-40). The cell extract (1 ml) was treated with the prewashed Protein G Sepharose beads (30 μl bed volume) for 1 h, and then centrifuged (2000*g*, 5 min). The supernatant was treated with 5 μl of anti-HA (F-7) antibodies (Santa Cruz) for 1 h, and further incubated with Protein G Sepharose beads (50 μl bed volume) for 1 h. The beads were precipitated (2000*g*, 5 min) and then washed five times with 200 μl of TNE buffer without NP-40. The recovered immuno-complex was heated at 95 °C for 5 min after the addition of 10 μl of 2× sample buffer [22]. The solubilized protein was subjected to SDS–polyacrylamide gel-electrophoresis, and then to Western blotting.

2.4. SDS–polyacrylamide gel-electrophoresis and Western blotting

A cell extract (20 μg protein) or immuno-precipitate (25 μl) was subjected to SDS–polyacrylamide gel-electrophoresis [7.5% (w/v), 1 mm thickness], and then electro-blotted (200 mA, 90 min.; ATTO Model-AE6675) onto an Immobilon™-P membrane [Millipore PVDF membrane (0.45 μm), IPVH00010] [5]. The membrane was washed with PBS, and then blocked for 1 h at 4 °C with PBS(+) containing 5% (w/v) skim milk (DIFCO). The Myc-tag was reacted with the peroxidase-linked mouse anti-c-Myc antibodies (MC045, Nacarai Tesque) (8000× diluted). Chemiluminescence was detected with an ECL Western blotting kit (GE Healthcare) using Scientific Imaging Film (KODAK).

Reprobing was carried out as follows. The membrane was treated with the buffer [2% (w/v) SDS, 100 mM β-mercaptoethanol, 62.5 mM Tris–HCl (pH 6.7)] for 30 min at 50 °C, and then blocked overnight at 4 °C. The HA-tag was detected with HA-7 (10,000× diluted) (Sigma), followed by horseradish peroxidase-linked anti mouse IgG (GE Healthcare) (4000× diluted) as the second antibody. Protein concentrations were determined with a BioRad Protein Assay Kit using bovine serum albumin (Fraction V, Sigma) as a standard [23].

2.5. Reporter gene assay

The reporter gene assay was carried out as described previously [5]. Into each well of a 6-well culture plate, 1×10^5 CHO-K1 cells were seeded. Lipofectamine™ (Invitrogen) was used for