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Research article

CHARACTERIZATION OF THE 5'-FLANKING REGION OF THE MOUSE ASPARAGINE-LINKED GLYCOSYLATION 12 HOMOLOG GENE

KENTARO OH-HASHI[1,*], TOMOMI TEJIMA[1], YOKO HIRATA[1, 2] and KAZUTOSHI KIUCHI[1, 2]

[1]Department of Biomolecular Science, Faculty of Engineering, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan, [2]United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

Abstract: Recently, we characterized multiple roles of the endoplasmic reticulum stress responsive element (ERSE) in the promotion of a unique head-to-head gene pair: mammalian asparagine-linked glycosylation 12 homolog (ALG12) and cysteine-rich with EGF-like domains 2 (CRELD2). This bidirectional promoter, which consists of fewer than 400 base pairs, separates the two genes. It has been demonstrated that the ALG12 promoter shows less transcriptional activity through ERSE, but its basic regulatory mechanism has not been characterized. In this study, we focused on well-conserved binding elements for the transcription factors for ATF6, NF-Y and YY1 and the Sp1 and Ets families in the 5'-flanking region of the mouse ALG12 gene. We characterized their dominant roles in regulating ALG12 promoter activities using several deletion and mutation luciferase reporter constructs. The ALG12 gene is expressed in three distinct cell lines: Neuro2a, C6 glioma and HeLa cells. The reporter activity in each cell line decreased similarly with serial deletions of the mouse ALG12 promoter. Mutations in the ERSE and adjacent NF-Y-binding element slightly affected reporter activity. Each of the mutations in the GC-rich sequence and YY1-binding element reduced ALG12 promoter activity, and the

* Author for correspondence. e-mail: oohashi@gifu-u.ac.jp, tel: +81-58-293-2659, fax: +81-58-293-2794

Abbreviations used: ALG12 – asparagine-linked glycosylation 12 homolog; ATF6 – activating transcription factor 6; CDG – congenital disorders of glycosylation; CRELD2 – cysteine-rich with EGF-like domains 2; ER – endoplasmic reticulum; ERSE – ER stress response element; Ets – v-ets erythroblastosis virus E26 oncogene homolog; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; HDAC – histone deacetylase; NF-Y – nuclear transcription factor Y; Rb – retinoblastoma; YY1 – Yin Yang 1

combination of these mutations additively decreased reporter activity. Each mutation in the tandem-arranged Ets-family consensus sequences partially attenuated ALG12 promoter activity, and mutations of all three Ets-binding elements decreased promoter activity by approximately 40%. Mutation of the three conserved regulatory elements (GC-rich, YY1 and Ets) in the ALG12 promoter decreased reporter activity by more than 90%. Our results suggest that the promoter activity of the mouse ALG12 gene is regulated in a similar manner in the three cell lines tested in this study. The well-conserved consensus sequences in the promoter of this gene synergistically contribute to maintaining basal gene expression.

Key words: ALG12, CRELD2, ERSE, Ets family, NF-Y, Sp1 family, YY1

INTRODUCTION

The mammalian ALG12 gene is the ortholog of the yeast gene that encodes dolichyl-P-Man:$Man_7GlcNAc_2$-PP-dolichyl α6-mannosyltransferase. An ALG12 T571G point mutation causes a congenital disorder that affects glycosylation in the endoplasmic reticulum (ER) [1, 2]. Clinically, a child with this point mutation shows severe symptoms, such as psychomotor retardation, hypotonia, growth retardation, dysmorphic features and anoxia [2]. The sequential protein modification steps of glycosylation and trimming in the ER are important for maintaining quality control of glycoproteins through protein folding and ER-associated protein degradation [3, 4]. Defects in N-linked glycosylation interfere with the intracellular trafficking and secretion of glycoproteins. Suitable regulation of the ALG12 gene is required to maintain ER homeostasis.

The ALG12 gene is adjacent to the CRELD2 gene in a head-to-head configuration in some species. Analyses of the human and mouse genomes revealed that more than 10% of genes that are arranged as bidirectional gene pairs are separated by less than 1 kb of genomic DNA [5-7]. Some of these gene pairs could have evolved from a common ancestral gene during duplication. However, other gene pairs do not have any genetic relationship and are thought to have different biological functions within the same cell. We recently demonstrated that the ERSE motif located within the 360-bp intergenic region asymmetrically participates in the activation of the CRELD2 promoter in response to ER stress or ATF6-overexpression [8]. We also reported on the molecular features of CRELD2 as a novel ER stress-inducible secretory factor [8-10]. However, the precise functions of CRELD2 in the intra- and extra-cellular spaces remain to be determined [9-11]. It is unclear whether there is any functional relationship between CRELD2 and ALG12 as ER stress-inducible genes.

To further characterize the transcriptional regulation of ALG12, we focused on well-conserved transcription factor-binding elements for ATF6 [12, 13], NF-Y [14], the Sp1 family [15, 16], YY1 [17, 18] and the Ets family [19, 20] in the 360-bp intergenic region of the mouse ALG12 gene, including several deletions and mutations of the

5'-flanking region upstream of the initiation site of this gene. We evaluated their roles in regulating promoter activity using luciferase reporter constructs.

MATERIALS AND METHODS

Construction of plasmids

To generate reporter constructs of the mouse ALG12 promoter, genomic DNA from Neuro2a cells was extracted, and the mouse ALG12 promoter (-335/+45) was amplified using PCR and cloned into a pGL3-Basic vector as described previously [8]. Other constructs containing deleted and mutated mouse ALG12 promoters were also prepared via PCR. The promoter region was defined using the latest database for the mouse ALG12 gene (NM_001142357), which has a transcriptional start site 34 bp upstream of the previous data of this gene (BC021379) [8], based on the RIKEN functional annotation of a full-length mouse cDNA collection (FANTOM).

Cell culture and treatment

Neuro2a and HeLa cells were maintained in Dulbecco's modified Eagle's minimum essential medium containing 8% fetal bovine serum (FBS) or 10% bovine serum. C6 glioma cells were cultured in HAM F10 medium containing 3% horse serum and 7% FBS. The transfection of each vector used in this study was performed using Lipofectamine-Plus Reagent (Invitrogen) according to the manufacturer's instructions.

Reverse transcription polymerase chain reaction

To estimate the expression level of each gene, total RNA was extracted from cells lysed with TRIzol reagent and converted to cDNA by reverse transcription using random ninemers to prime SuperScript III Reverse Transcriptase (Invitrogen) as previously described [8]. Each specific cDNA was mixed and amplified with a PCR mixture (Taq PCR Kit, Takara). The RT-PCR primers used in this study were:
ALG12 sense primer – 5'-GTGATTTCTGGACTCTGGAC-3';
ALG12 antisense primer – 5'-GGGGTATGAAGAGAAGGCTGCA-3';
GAPDH sense primer – 5'-ACCACAGTCCATGCCATCAC-3'; and
GAPDH antisense primer – 5'-TCCACCACCCTGTTGCTGTA-3'
The typical reaction cycling conditions were 30 sec at 96°C, 30 sec at 60°C and 30 sec at 72°C. The results represent 20 or 30 cycles of amplification, after which the products were separated via electrophoresis on 2.0% agarose gels and visualized using ethidium bromide. The relative expression level of ALG12 mRNA in each cell line was calculated by comparison with that of GAPDH using NIH imaging, and each value was normalized to that in the Neuro2a cells.

Reporter assay

The reporter constructs and the pGL4.70 vector (Renilla luciferase), an internal control, were transfected into the cell lines in 48-well plates. Thirty-six hours

after transfection, the cells were lysed, and the luciferase activity in each lysate sample was measured using a Dual-Luciferase assay system (Promega). The reporter activity in each lysate was normalized to the co-transfected Renilla luciferase activity, and results are shown as the relative luciferase activity.

Statistical analysis

The results are expressed as means ± SD of the indicated number. Statistical analyses were carried out by one way-ANOVA followed by Fischer's PLSD test. $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

To characterize the transcriptional regulation of the bidirectional promoter between ALG12 and CRELD2, we analyzed the 400-bp promoter and the 5'-flanking region sequences of ALG12 of mouse, rat and human using

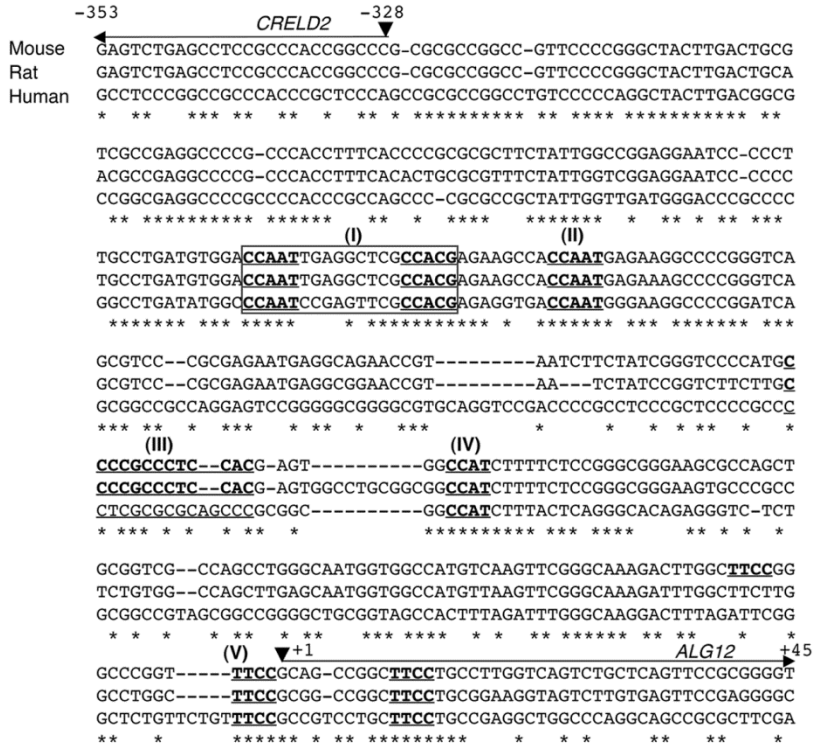

Fig. 1. Alignment analysis of the mouse, rat and human CRELD2-ALG12 gene pairs. Nucleotide sequences conserved among the mouse, rat and human CRELD2-ALG12 gene pairs are shown with asterisks. The conserved ERSE motif (I, boxed) and adjacent NF-Y-binding site (II) are indicated in bold letters with an underline. The GC-rich (III) and putative YY1- and Ets family-binding sequences (IV and V) are also indicated in bold types with an underline. Arrows and arrowheads respectively mark the transcriptional direction and putative transcriptional start sites of the mouse CRELD2 and ALG12 genes.

SwissRegulon software, which has an algorithm for searching for regulatory motifs. We identified the consensus sequences for ATF6, NF-Y and YY1 and the Sp1 and Ets families. These are well conserved in sets of orthologous sequences (Fig. 1).

We then examined the expression level of ALG12 mRNA in the three cell lines using RT-PCR. Because the sequential transfer of each sugar moiety to the dolichol-PP-oligosaccharide precursor is ubiquitous and crucial to maintaining the quality of glycoproteins through protein folding and ER-associated protein degradation [3, 4], ALG12 mRNA is expressed in the Neuro2a, C6 glioma and HeLa cells (Fig. 2A). To evaluate the roles of these putative elements in

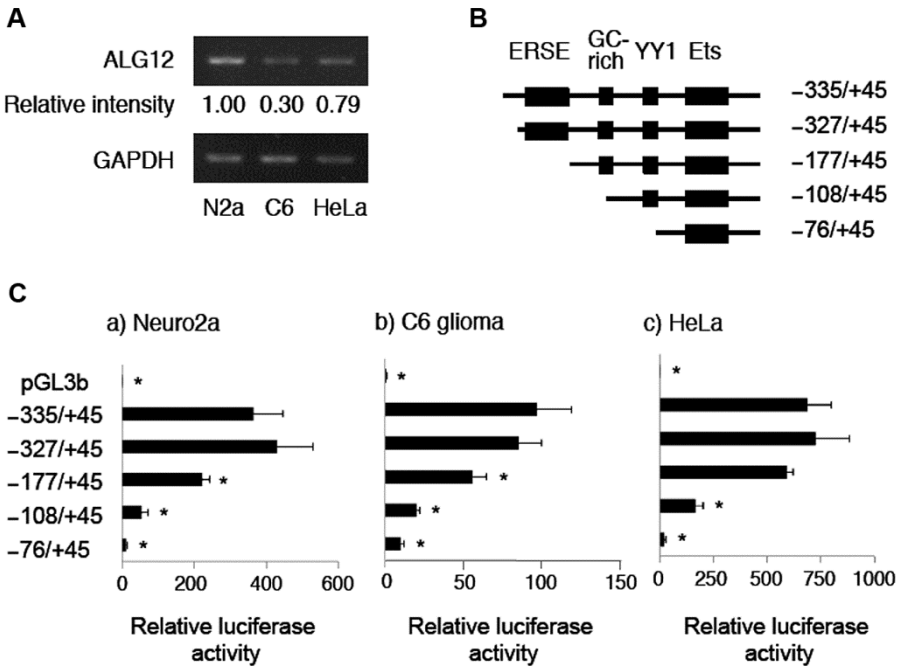

Fig. 2. Expression levels of ALG12 mRNA and promoter activity of the mouse ALG12 gene in Neuro2a, C6 glioma and HeLa cells. A – The expression level of each mRNA of interest was evaluated using RT-PCR as described in the Materials and Methods section. The expression level of ALG12 mRNA in each cell line was calculated by comparison with that of GAPDH, and the relative values by normalization with that in Neuro2a cells (shown below each lane). B, C – The indicated luciferase reporter constructs containing serial deletions of the mouse ALG12 promoter region were transfected into Neuro2a (a), C6 glioma (b) and HeLa (c) cells. After 36 h, the cells were lysed, and luciferase activities were measured as described in the Materials and Methods section. Values represent the means ± SD from more than three independent cultures and are expressed relative to the activity of the pGL3-Basic vector. Data were analyzed by one way-ANOVA followed by Fischer's PLSD test to evaluate the effect of deletion or mutation of ALG12 promoter (-327/+45) on the ALG12 promoter activity. Values marked with an asterisk are significantly different from the ALG12 (-327/+45) promoter activity ($p < 0.05$).

regulating ALG12 promoter activity, several luciferase reporter constructs containing serial deletions of the promoter of the mouse ALG12 gene were transfected into the three cell lines. As shown in Fig. 2C, the luciferase activity in each cell line was similarly decreased with each deleted region. These results suggest that the consensus sequences for ATF6, NF-Y, YY1 and the Sp1 family are responsible for the stable and ubiquitous expression of the ALG12 gene.

Deletion of the ERSE and the adjacent NF-Y-binding element in the distal region of the ALG12 promoter (-177/+45) reduced reporter activity to approximately half in Neuro2a cells, whereas mutations in these sequences had little effect on the promoter activity in this cell line (Fig. 3). We previously demonstrated that the ERSE motif of the same mouse ALG12 reporter barely responded to thapsigargin treatment and ATF6-overexpression despite that of the CRELD2 promoter in the opposite direction being functional [8]. Considering the asymmetry of the ERSE in the bidirectional CRELD2–ALG12 gene pair, the sequence adjacent to ERSE in the proximal region of the CRELD2 promoter seems to influence both the basal and ER stress-inducible transcription level of this gene to a greater degree than the ALG12 gene. However, the precise mechanisms for the different transcriptional regulation between the CRELD2 and ALG12 gene promoters still need to be clarified.

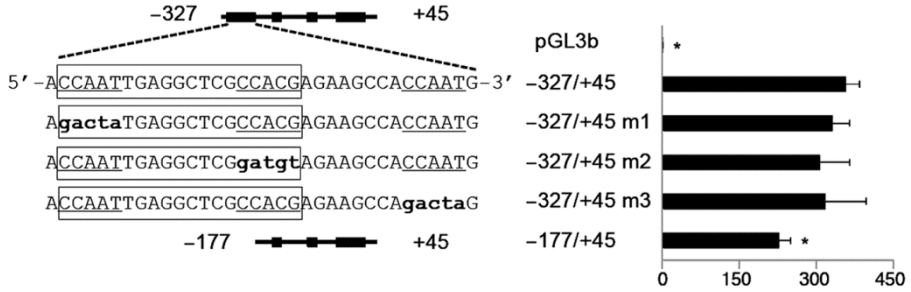

Fig. 3. Effect of ERSE and its flanking region on promoter activity of the mouse ALG12 gene in Neuro2a cells. Thirty-six hours after transfection of the indicated reporter construct, luciferase activity was measured as described in the Materials and Methods section. Values represent the means ± SD from three independent cultures and are expressed relative to the activity of the pGL3-Basic vector. ATF6- and NF-Y-binding sequences (underlines) around the ERSE motif (boxes) and their mutated sequences (small letters) are shown. Data were analyzed by one way-ANOVA followed by Fischer's PLSD test to evaluate the effect of deletion or mutation of ALG12 promoter (-327/+45) on the ALG12 promoter activity. Values marked with an asterisk are significantly different from the ALG12 (-327/+45) promoter activity ($p < 0.05$).

Next, we studied the roles of the putative Sp1- and YY1-binding elements in regulating the expression of the mouse ALG12 gene. As shown in Fig. 4A, deletion of both elements (-327/-118 plus -77/+45) markedly reduced reporter activity in Neuro2a cells. Mutations of each individual motif also decreased promoter activity, but the YY1 motif mutation produced a more drastic effect

than that of the Sp1 motif mutation (Fig. 4B). In addition, mutation of both motifs (-327/+45 m6) additively decreased promoter activity, as in the case of the two-motif deletion (-327/-118 plus -77/+45).

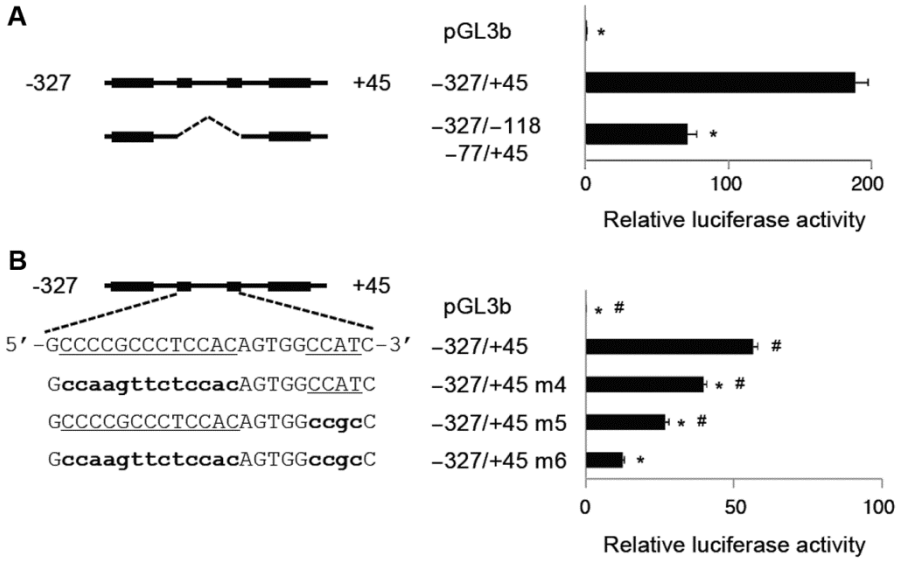

Fig. 4. Effect of the GC-rich element and YY1 consensus sequence on promoter activity of the mouse ALG12 gene in Neuro2a cells. Thirty-six hours after transfection of the indicated reporter construct, luciferase activity was measured as described in the Materials and Methods section. Values represent the means ± SD from three independent cultures and are expressed relative to the activity of the pGL3-Basic vector (A, B). The GC-rich element and YY1 consensus sequence and their mutated sequences are shown with underlines and their mutated sequences are shown with small letters. Data were analyzed by one way-ANOVA followed by Fischer's PLSD test to evaluate the effect of deletion or mutation of ALG12 promoter (-327/+45) on the ALG12 promoter activity. Values marked with an asterisk or # are respectively significantly different from the ALG12 (-327/+45) or (-327/+45 m6) promoter activity ($p < 0.05$).

A large number of genes are known to have GC-rich promoters, and Sp1 recognizes the GC-rich sequence through its C-terminal zinc finger domain [15, 16, 21]. It has been suggested that Sp1 binds a variety of gene promoters, including those for genes regulating cell proliferation, differentiation and metabolism, because a heterozygous deletion of Sp1 in fertilized mouse eggs causes embryonic malformation and is embryonic lethal [22]. By contrast, Sp1-like transcription factors containing a highly homologous C-terminal domain have been identified, and some are thought to bind an overlapping subset of GC-rich promoters with Sp1 [15, 16]. YY1 is also reported to regulate the transcription of several genes [17, 18] and play an essential role in embryonic development, similar to that of the Sp1 family [23]. YY1 recognizes two sequences, CCAT and ACAT, in the consensus element of various promoters

and has been demonstrated to either activate or repress gene expression, depending on the co-factors involved. These co-factors include HDACs, Rb, p53 and caspases, which are recruited to YY1 [18, 24, 25].

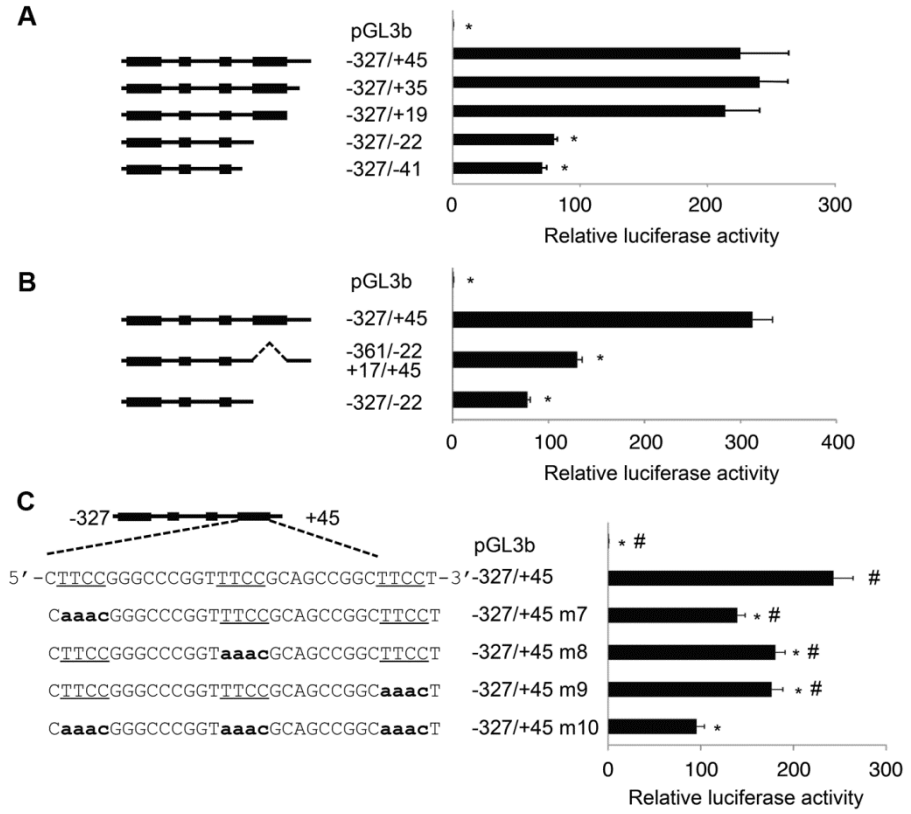

Fig. 5. Effect of the proximal Ets-family consensus sequence on the promoter activity of the mouse ALG12 gene in Neuro2a cells. Thirty-six hours after transfection with the indicated reporter construct, luciferase activity was measured as described in the Materials and Methods section. Values represent the means ± SD from more than three independent cultures and are expressed relative to the activity of the pGL3-Basic vector (A, B and C). The Ets family consensus sequence and their mutated sequences are indicated with underlines and small letters. Data were analyzed by one way-ANOVA followed by Fischer's PLSD test to evaluate the effect of deletion or mutation of ALG12 promoter (-327/+45) on the ALG12 promoter activity. Values marked with an asterisk or # are respectively significantly different from the ALG12 (-327/+45) or (-327/+45 m10) promoter activity ($p < 0.05$).

In addition to the above-mentioned motifs, putative binding elements for the Ets family transcription factors in the ALG12 promoter are well conserved in the human, rat and mouse genomes. Three putative Ets-family consensus sequences (TTCC) are aligned in tandem, but the most distal one is not conserved in human and rat promoters. Therefore, we made additional reporter constructs to include

serial deletions of the three Ets-family motifs and individual mutations of each consensus. The deletion of the proximal ALG12 promoter region including all three tandem motifs (-327/-22 plus +17/+45) reduced reporter activity by more than half in Neuro2a cells (Fig. 5A and B). These results suggest that the well-conserved Ets family consensus sequences within this proximal region might partly contribute to sustaining the basal ALG12 promoter activity.

We characterized each of the Ets family consensus sequences by preparing mutated ALG12 promoter reporter constructs. As shown in Fig. 5C, individual mutations of each Ets-family consensus sequence in the ALG12 promoter partially decreased reporter activity (Fig. 5C). All three consensus sequences might synergistically regulate ALG12 promoter activity because the mutation of all of the elements (-327/+45 m10) decreased reporter activity to a similar degree to the triple deletion (-327/-22 plus +17/+45). The Ets family consists of approximately 30 members that are highly conserved in various species including human, mouse, *Xenopus*, *Drosophila* and nematode [19, 20]. Ets factors also regulate a variety of biological processes, such as development, differentiation and inflammatory responses [20, 26, 27]. Like YY1 and the Sp1 family, the Ets family has been reported to interact with several other transcription factors such as Smad2/3, Smad4 and Sp1 [28].

In this study, we evaluated the synergistic promoter activity-regulating effects of well-conserved consensus sequences in the intergenic region of the mouse ALG12 gene (Fig. 6). Mutation of the Ets-family consensus sequences (-327/+45 m10) decreased ALG12 promoter activity most strikingly, whereas mutation of the GC-rich sequence (-327/+45 m4) was rather modest. Mutations in two motifs of Sp1, YY1 and Ets further decreased ALG12 promoter activity, and mutations in all three motifs (-327/+45 m13) reduced the promoter activity by more than 90%. These results suggest that these three well-conserved consensus sequences in the ALG12 promoter might co-operatively participate in regulating the expression of the ALG12 gene. On the other hand, the distal region of the ALG12 promoter, with the exception of the ERSE and NF-Y motifs, only partially contributes to ALG12 gene regulation. However, it is unclear if and which transcription factors might form a heterocomplex to bind the ALG12 promoter because the Sp1 and Ets families consist of various members [16, 19, 20, 27]. Moreover, YY1 and some members of the Sp1 and Ets families have been reported to interact with the HDAC family of co-factors to regulate gene expression [18, 24, 29, 30]. YY1 has also been reported to behave like a scaffold for several transcription-related factors that are recruited to and assembled at the promoter region without directly binding to the consensus sequence for YY1 [18, 25]. However, treatment with tricostatin A, an inhibitor of HDACs, does not affect the expression of ALG12 mRNA in Neuro2a cells (data not shown). Therefore, it is likely that protein modification by HDACs might be excluded from the heterocomplex that recognizes the consensus sequences of YY1 and the Sp1 and Ets families in the mouse ALG12 promoter.

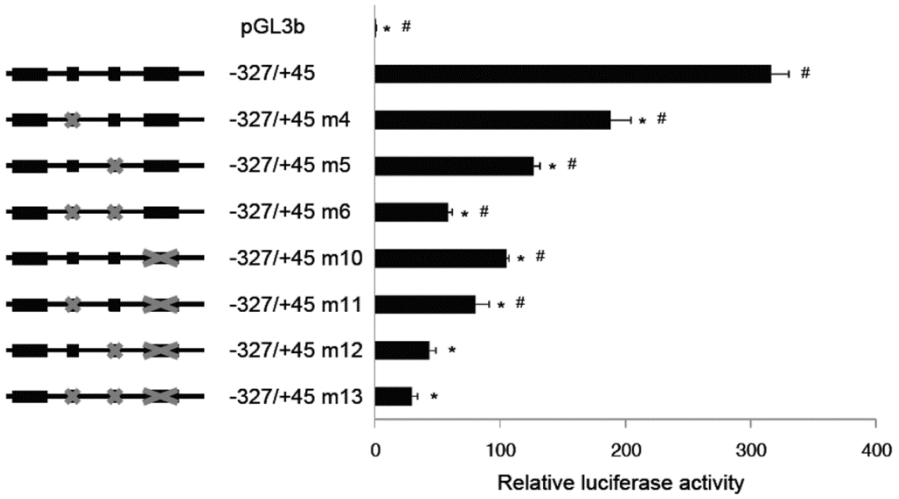

Fig. 6. Synergistic effects of the GC-rich element and YY1 and Ets family consensus sequences on promoter activity of the mouse ALG12 gene in Neuro2a cells. Thirty-six hours after transfection with the indicated reporter construct, luciferase activity was measured as described in the Materials and Methods section. Values represent the means ± SD from three independent cultures and are expressed relative to the activity of the pGL3-Basic vector. Data were analyzed by one way-ANOVA followed by Fischer's PLSD test to evaluate the effect of each mutation of ALG12 promoter (-327/+45) on the ALG12 promoter activity. Values marked with an asterisk or # are respectively significantly different from the ALG12 (-327/+45) or (-327/+45 m13) promoter activity ($p < 0.05$).

As with other ALG members [3, 31], an understanding of the transcriptional regulation of the ALG12 gene is important because of the congenital disorders of glycosylation (CDG) observed in patients with mutations in this gene [1, 2]. In this study, we have evaluated the crucial roles of three conserved consensus sequences in the 5'-flanking region upstream of the initiation site in the mouse ALG12 gene, which might be synergistically recognized by YY1 and the Sp1 and Ets families. It has been reported that the transcriptional activity of the Sp1 and Ets families is modified by several pathophysiological stimuli, which results in phosphorylation of these transcription factors [32, 33]. Therefore, further characterization of ALG12 gene expression during aging and under pathological conditions might provide novel insight into understanding the role of ALG12 gene in the pathology of CDG.

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