論文

Identification and Characterization of the SHARP-2-Interacting Proteins

YAMADA Kazuya, MISHIMA Ayumi, HANAOKA Yukina, MISAKI Toshinori, KAMIJO Akane, SAKAMOTO Natsuko, CHINO Yukari, HORIUCHI Suzuka, KONDO Haruna, NAGAI Akeri, MARUYAMA Yukino, YAMANE Yuuna, TANOKUCHI Aika, TSUKADA Akiko, and TAKAGI Katsuhiro

SHARP-2相互作用タンパク質の同定と特性

山田 一哉・三島 歩実・花岡 由紀奈・三崎 紀展・上條 茜・坂本 那津子・茅野 友加里・堀内 涼香・近藤 晴菜・長井 朱里・丸山 ゆきの・山根 優奈・田野口 愛華・塚田 晃子・髙木 勝広

Summary

The rat enhancer of split- and hairy-related protein-2 (SHARP-2) is a basic helix-loop-helix transcriptional repressor. Many transcription factors interact with other proteins to form complex transcriptional regulation networks. To identify a SHARP-2-interacting protein(s), we screened a rat liver cDNA library using a yeast two-hybrid system. Approximately 3.5×10^7 independent clones were screened and 29 positive clones were obtained. Of these, multiple clones contained AT motif-binding factor-1 (ATBF1) and Sex-determining region Y-box 6 (Sox6) which both were transcription factors. We then focused on and analyzed physical protein-protein interaction of SHARP-2 with ATBF1 and Sox6. First, minimal interaction domain of ATBF1 and Sox6 with the SHARP-2 was determined. Amino acid residues between 3,271 and 3,379 of the ATBF1 and those between 201 and 362 of Sox6 were required for an interaction with SHARP-2, respectively. We then examined which domain of SHARP-2 is required for interactions with ATBF1 and Sox6. Amino acid residues between 301 and 355 of the SHARP-2 interacted with the ATBF1 and those between 384 and 397 of the SHARP-2 interacted with the Sox6. Thus, we concluded that both the ATBF1 and Sox6 were identified as the SHARP-2-interacting protein and that the SHARP-2 interacted with them using different domains.

Keywords

transcription factor, protein-protein interaction, SHARP-2, ATBF1, Sox6

Contents

Ⅰ. Introduction

The rat enhancer of split- and hairy-related protein (SHARP) family is a basic helix-loop-helix transcriptional repressor and belongs to the clock gene family regulating the circadian rhythm[1]. Members of the SHARP family are SHARP-1 (also referred to as the human DEC2) and SHARP-2 (also referred to as the human DEC1). These form a homo- or a hetero-dimer each other and bind to the E box sequence (5'-CANNTG-3') locating at the transcription control region of the target gene. The expression of the *SHARP-2* gene is induced in the rat liver after feeding with a high carbohydrate diet[2, 3]. In the rat highly differentiated hepatoma H4IIE cells, insulin induces the *SHARP-2* gene expression via a phosphoinositide 3-kinase (PI3-K)/ atypical protein kinase C (aPKC) λ pathway and a PI3-K/ mammalian target of rapamycin (mTOR) pathway, respectively[3, 4]. SHARP-2 represses transcription of the rat *phosphoenolpyruvate carboxykinase (PEPCK)* gene which encodes a rate-limiting gluconeogenic enzyme[5]. Thus, SHARP-2 is one of the transcription factors involved in lowering the blood glucose level by insulin.

We hypothesized that if we could induce the expression of the *SHARP-2* gene with some ingredients, it might be effective in the prevention and treatment of diabetes. We examined several food ingredients that have been reported to have a hypoglycemic effect, and found that green tea catechin (-)-epigallocatechin-3-gallate (EGCG), soybean isoflavone genistein, and (*S*)-equol (an intestinal bacterial metabolite of soybean isoflavone daidzein) induced the expression of the *SHARP-2* gene[6-8]. We also reported that the EGCG effect occurred via the PI3-K pathway, the genistein effect via the protein kinase C pathway, and the (*S*)-equol effect via the PI3-K/aPKC λ pathway, respectively[6-8]. Therefore, these food components affect the expression of downstream target genes through the action of the SHARP-2 stimulated with various signaling pathways.

Many transcription factors interact with other proteins to form complex transcriptional regulation networks. In this study, to explore which proteins interact with SHARP-2, we screened a rat liver cDNA library using a yeast two-hybrid system. We cloned the ATBF1, Sox6, and so on. We determined the minimum interaction domain between SHARP-2 and these interacting proteins.

Ⅱ. Materials and methods

Materials

The pGBKT7 (pDBD) and pACT2 (pAD) plasmids, AH109 and SFY526 yeast cells, and X-α-gal were purchase from Clontech (Palo Alto, U.S.A.). 3-aminotriazole and *o*-nitrophenyl-β-D-galactoside were purchased from Sigma-Aldrich Co. (Saint Louis, U.S.A.).

Construction of plasmids

The pCI-SHARP-2 plasmid was previously described[5]. A 1.2 kb *Eco*RI/*Sal*I fragment of the pCI-SHARP-2 plasmid was inserted into the *Eco*RI/SalI sites of the pDBD to produce pDBD-SHARP2 (1-411). This plasmid expresses the entire coding sequence of SHARP-2 fused to the DNA-binding domain (DBD) of transcription factor GAL4. The pAD plasmid expresses the activation domain (AD) of the GAL4. Also, several truncated forms of ATBF1 or Sox6 fused to the GAL4AD were prepared using polymerase chain reaction (PCR) or annealing, kination, and ligation of double stranded oligonucleotides. These plasmids were named as the pAD-ATBF1 (3030-3150), pAD-ATBF1 (3151-3270), pAD-ATBF1 (3271-3379), pAD-ATBF1 (3271-3306), pAD-ATBF1 (3307-3343), pAD-ATBF1 (3271-3343), pAD-ATBF1 (3344-3379), pAD-ATBF1 (3307-3379), pAD-Sox6 (144-362), pAD-Sox6 (363-580), pAD-Sox6 (581-787), pAD-Sox6 (144-200), pAD-Sox6 (201-362), pAD-Sox6 (201-262), and pAD-Sox6 (263-362), respectively.

Several truncated forms of SHARP-2 fused to

the GAL4DBD were prepared using the PCR or annealing, kination, and ligation of double stranded oligonucleotides. These plasmids were named as the pDBD-SHARP2 (1-120), pDBD-SHARP2 (121-300), pDBD-SHARP2 (301-411), pDBD-SHARP2 (301-355), pDBD-SHARP2 (356-411), pDBD-SHARP2 (301-328), pDBD-SHARP2 (329-355), pDBD-SHARP2 (316-341), pDBD-SHARP2 (356-383), pDBD-SHARP2 (384-411), pDBD-SHARP2 (384-397), pDBD-SHARP2 (392-403), and pDBD-SHARP2 (398-411), respectively.

Library screening with the yeast two-hybrid system and liquid β-galactosidase assays

Construction of rat liver cDNA library were described previously[9]. AH109 yeast cells were transformed with the pDBD-SHARP2 (1-411) plasmid. The yeast strain was employed as a bait to screen a rat liver cDNA library using a high-efficiency transformation method[10]. Approximately 3.5×10^7 independent clones were screened on histidine-, tryptophan-, and adenine-free synthetic dextrose plates supplemented with 1 mM 3-aminotriazole and X-α-gal, respectively. Positive clones were obtained from the primary transformants. The yeast strain SFY526, which contains a quantifiable *lacZ* reporter gene, and either pDBD or pDBD-SHARP2 (1-411) plasmid, was transformed with plasmids isolated from positive clones in primary screening or the parent vector, the pAD. In the second screening, 29 clones specifically showed reproducible high β-galactosidase activity.

In the case of minimal domain mapping, the pDBD-SHARP2 derivative plasmids which express various truncated forms of the SHARP-2 fused to the GAL4DBD and the pAD-ATBF1 or pAD-Sox6 derivative plasmids which express various truncated forms of the ATBF1 or Sox6 fused to the GAL4AD were transformed into the SFY526 strain.

Quantitative β-galactosidase assays, using *o*-nitrophenyl-β-D-galactoside, were carried out on permeabilized cells, as described previously[11].

Statistical analysis

All experiments were performed at least three times, and the obtained data was subjected to statistical analysis by one-way analysis of variance to calculate the average value and standard error.

Ⅲ. Results and discussion

Screening of the SHARP-2-interacting proteins

To search the SHARP-2-interacting proteins, we used the yeast two-hybrid system. An entire coding region of the rat SHARP-2 was fused to the GAL4DBD and this chimeric protein was employed as the bait to screen rat liver cDNA library. Approximately 3.5×10^7 independent clones were screened, 327 clones showed reproducible His^+, Trp^+, and Ade^+, and X-α-gal-positive properties. After determination of their nucleotide sequences, they were compared with the GenBank database using the BLAST search program. The SHARP-2-interacting proteins which multiple clones were obtained were listed in the Table 1. AT motif-binding factor-1 (ATBF1) was originally cloned as a factor that represses transcription by binding to an AT-rich sequence in the transcriptional regulatory region of the *α-fetoprotein (AFP)* gene which encodes a hepatocellular cancer marker molecule[12, 13]. ATBF1 is a huge protein with a molecular weight of about 406 kDa, which has twenty-three zinc-finger motifs and four homeodomains. As abnormalities in the *ATBF1*

Table 1. The SHARP-2-interacting proteins

AT motif-binding factor (ATBF1)
Amino-terminal enhancer of split (AES)
ATP citrate lyase (ACL)
Sex-determining region Y-box 6 (Sox6)
Tumorous imaginal discs protein

gene cause cancer, it functions as an anti-oncogene[14-17] . Amino-terminal enhancer of split (AES) belongs to the Groucho/TLE family of transcriptional co-repressor[18] . ATP citrate lyase (ACL) is a lipogenic enzyme. Sex-determining region Y-box 6 (Sox6) is a member of the Sox transcription factor family, which shares a DNA-binding region called the high mobility group (HMG) box[19] . Eight members of the Sox family are divided into groups based on structural characteristics, and Sox6 belongs to group D, which contains functional domains related to protein-protein interaction. Furthermore, it has been reported that Sox6 binds to the transcription factor pancreatic-duodenal homeobox factor-1 (PDX1) through protein-protein interaction in pancreatic cells and represses transcription of the *insulin* gene[19] . Tumorous imaginal discs protein encodes a tumor suppressor[20]. While clones of the ATBF1, ACL, Sox6, and tumorous imaginal discs protein encoded the GAL4AD-fusion proteins, clones from the AES did not encode the GAL4AD-fusion proteins. In addition, as both the ATBF1 and Sox6 were transcription factors as well as SHARP-2, these proteins were further analyzed in this study. These clones contained the amino acid sequences between 3,030 and 3,379 of the ATBF1 and between 144 and 787 of the Sox6, respectively. Plasmids extracted from the positive clones were named as the pAD-ATBF1 (3030-3379) and pAD-Sox6 (144-787) plasmid, respectively. First, we confirmed a specificity of protein-protein interaction between SHARP-2 with these proteins. As shown in Fig. 1, when a yeast strain SFY526 was transformed with a combination of the pDBD-SHARP2 (1-411) and pAD plasmid, β-galactosidase activities were quite low levels. When the yeast was transformed with a combination of the pDBD-SHARP2 (1-411) and pAD-ATBF1 (3030-3379) or pAD-Sox6 (144-787) plasmid, β-galactosidase activities significantly increased. In contrast, when SFY526 were transformed with a combination of

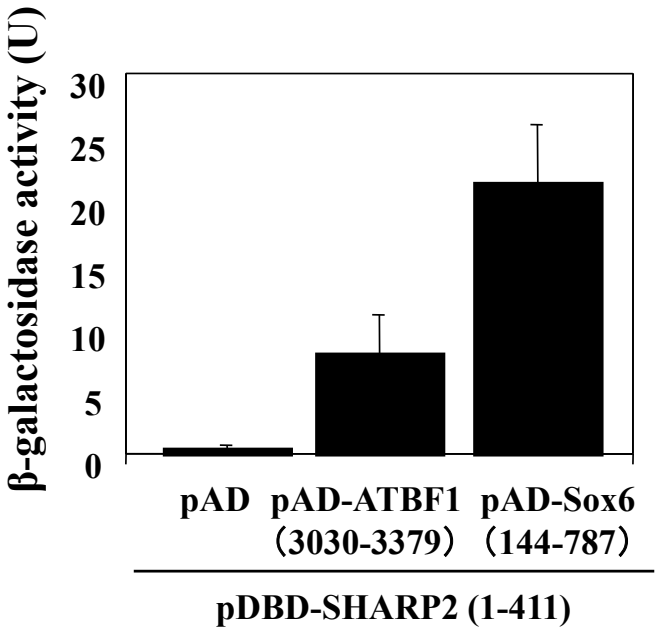

Fig. 1. SHARP-2 specifically interacts with ATBF1 and Sox6.

Yeast SFY526 cells were transformed with the pDBD-SHARP2 (1-411) which expresses fusion protein of GAL4DBD and full-length SHARP-2. The reporter yeast was further transformed with the pAD which expresses the GAL4AD, pAD-ATBF1 (3030-3379) which expresses fusion protein of GAL4AD and the amino acid sequences between 3,030 and 3,379 of the ATBF1, or pAD-Sox6 (144-787) which expresses fusion protein of GAL4AD and the amino acid sequences between 144 and 787 of the Sox6. The β-galactosidase activity of each yeast was measured. Each column and bar represent the mean and standard error of the β-galactosidase activity of at least three independent experiments.

the pDBD and pAD-ATBF1 (3030-3379) or pAD-Sox6 (144-787) plasmid, β-galactosidase activities were quite low levels (data not shown). These results indicated that SHARP-2 specifically interacted with ATBF1 and Sox6.

Mapping of the minimal domain of ATBF1 and Sox6 interacting with SHARP-2

We then examined which domains of ATBF1 and Sox6 is required for an interaction with SHARP-2. Hereafter, in a yeast harboring a combination of a pDBD-SHARP-2 (1-411) plasmid and various truncated pAD-ATBF1 or pAD-Sox6 plasmids, significant increased and unchanged levels of β-galactosidase activity, respectively, compared with a yeast harboring a combination of the pDBD-SHARP-2 (1-411) plasmid and the pAD plasmid depicted the interaction (+) and no interaction (-).

The ATBF1 protein was divided into three domains with amino acid sequences 3,030 to 3,150, 3,151 to 3,270, and 3,271 to 3,379, respectively. Only the 3,271 to 3,379 amino acid sequences showed an interaction with SHARP-2 (Fig. 2). Next, this domain was divided into two amino acid sequences 3,271 to 3,306 and 3,307 to 3,379. Neither domain showed an interaction with the SHARP-2. Furthermore, to identify the minimal interaction domain of ATBF1, some constructs contained various amino acid sequences were transformed into the reporter yeast. None of yeasts harboring these plasmids showed an interaction with the SHARP-2 (Fig. 2). These results indicated that the amino acid residues between 3,271 and 3,379 of the ATBF1 was a minimal interaction domain with the SHARP-2. The amino acid sequence of this

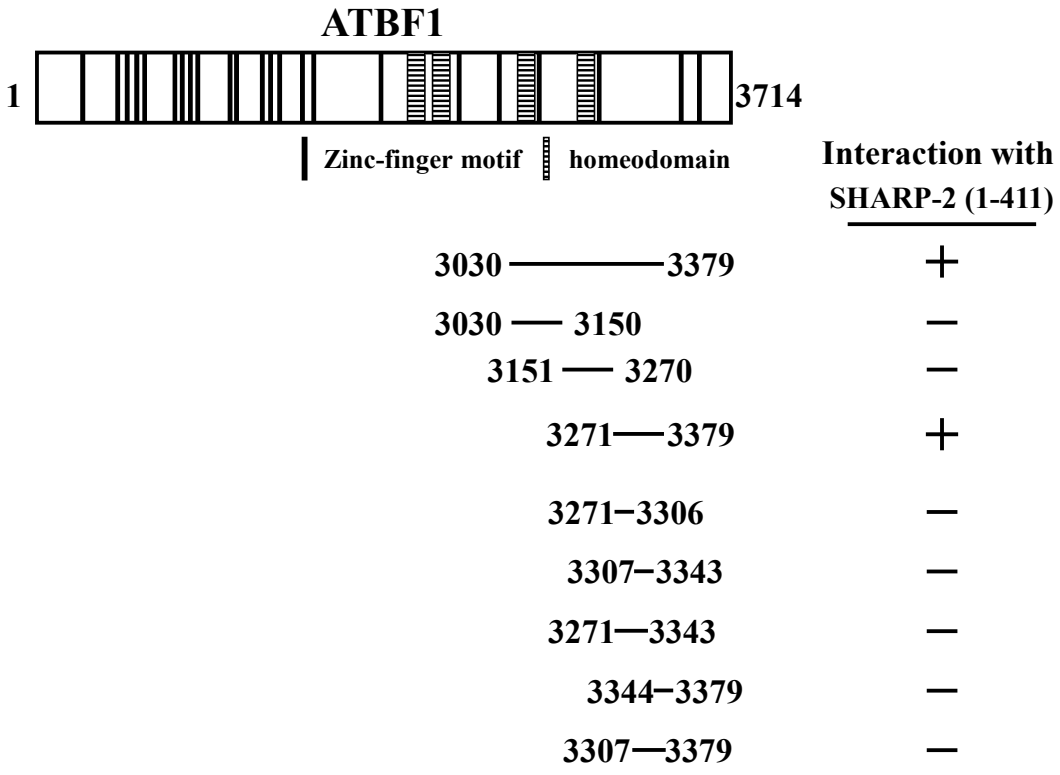

Fig. 2. Identification of the minimal interaction domain of ATBF1 with SHARP-2 using the yeast two-hybrid system.

A schematic representation of ATBF1 and the GAL4AD-ATBF1 fusion constructs are depicted on the left. The + and - symbols indicate significant increased and unchanged levels of β-galactosidase activity, respectively, compared with a yeast harboring a combination of pDBD-SHARP-2 (1-411) and the pAD.

region was scanned using the Motif Scan database, but no characteristic amino acid sequence was included.

Next, we analyzed the minimal domain of Sox6, which is required for an interaction with SHARP-2. Sox6 was divided into three regions such as amino acid sequences 144 to 362, 363 to 580, and 581 to 787, respectively. Of these, amino acid sequences 144 to 362 showed an interaction with SHARP-2 (Fig. 3). We further divided this domain into two regions, amino acid sequences 144 to 200 and 201 to 362. Amino acid sequences containing 201 to 362 revealed an interaction with SHARP-2 (Fig. 3). When this region was divided into small regions, no interaction with SHARP-2 was observed (Fig. 3). These results indicate that the amino acid sequences between 201 and 362 of Sox6 was a minimal interaction domain with the SHARP-2. In this region, there was a glutamine (Q)-rich domain which is required for transcriptional activity in most transcription factors. It raises a possibility that the SHARP-2 regulates transcriptional activity of Sox6 via a protein-protein interaction with this domain and *vice versa*. It has been reported that the HMG box of Sox6 is required for an interaction with PDX1[19]. Therefore, Sox6 has multiple interaction domains. It remains to be determined whether the use of each domain of Sox6 has any physiological roles.

Mapping of the minimal domain of SHARP-2 interacting with ATBF1 or Sox6

We then examined the issue of which domain of SHARP-2 is required for an interaction with these proteins. Hereafter, in a yeast harboring a combination of various truncated pDBD-SHARP-2 plasmids and the pAD-ATBF1 (3030-3379) or pAD-Sox6 (144-787) plasmid, significant increased

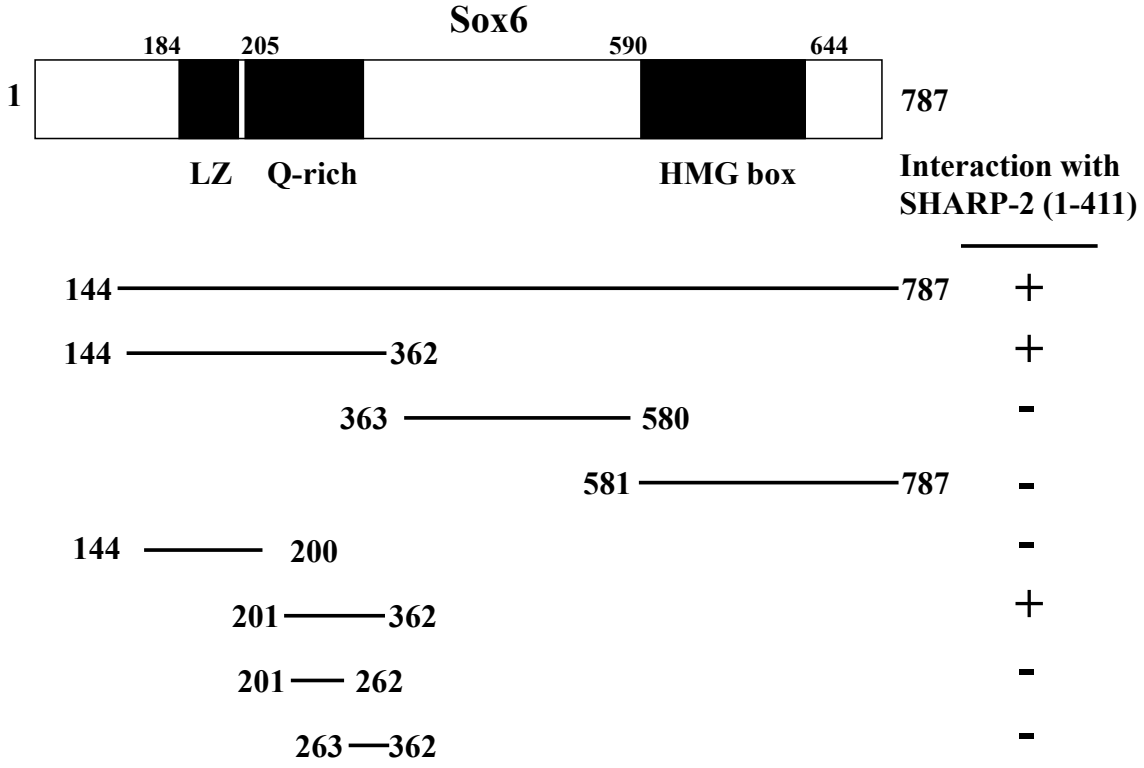

Fig. 3. Identification of the minimal interaction domain of Sox6 with SHARP-2 using the yeast two-hybrid system.

A schematic representation of Sox6 and the GAL4AD-Sox6 fusion constructs are depicted on the left. Details for symbols is described in the legend of Fig. 2. LZ, leucine zipper motif; Q-rich, glutamine-rich domain; HMG box, high mobility group box.

and unchanged levels of β-galactosidase activity, respectively, compared with a yeast harboring a combination of the various truncated pDBD-SHARP-2 plasmids and the pAD plasmid depicted the interaction (+) and no interaction (-).

SHARP-2 consists of 411 amino acids. First, the SHARP-2 protein was divided into three domains: amino acid sequences 1 to 120 including the basic helix-loop-helix region, amino acid sequences 121 to 300 including the orange domain, and amino acid sequences 301 to 411 including the proline-rich region (Figs. 4 and 5). These plasmids were transformed with the pAD or the pAD-ATBF1 (3030-3379) plasmid into SFY526 cells. Of these, a yeast harboring only a plasmid including amino acid sequences 301 to 411 showed an interaction (Fig. 4). Next, this domain was divided into two amino acid sequences 301 to 355 and 356 to 411. A region containing amino acid sequences 301 to 355 showed an interaction with the ATBF1. Further, this region was divided into three amino acid sequences 301 to 328, 329 to 355, and 316 to 341, respectively. None of yeasts harboring these plasmids showed an interaction with the ATBF1. These results indicated that the amino acid sequences between 301 and 355 of the SHARP-2 interacts with the ATBF1.

We then determined the minimal domain of SHARP-2 required for an interaction with Sox6. First, we examined amino acid sequences 1 to 120, 121 to 300, and 301 to 411 of the SHARP-2, respectively. Of these, a yeast harboring only a plasmid including amino acid sequences 301 to 411 showed an interaction (Fig. 5). Next, this domain was divided into two amino acid sequences 301 to 355 and 356 to 411. A region containing amino acid sequences 356 to 411 showed an interaction with the Sox6. This region was then divided into two

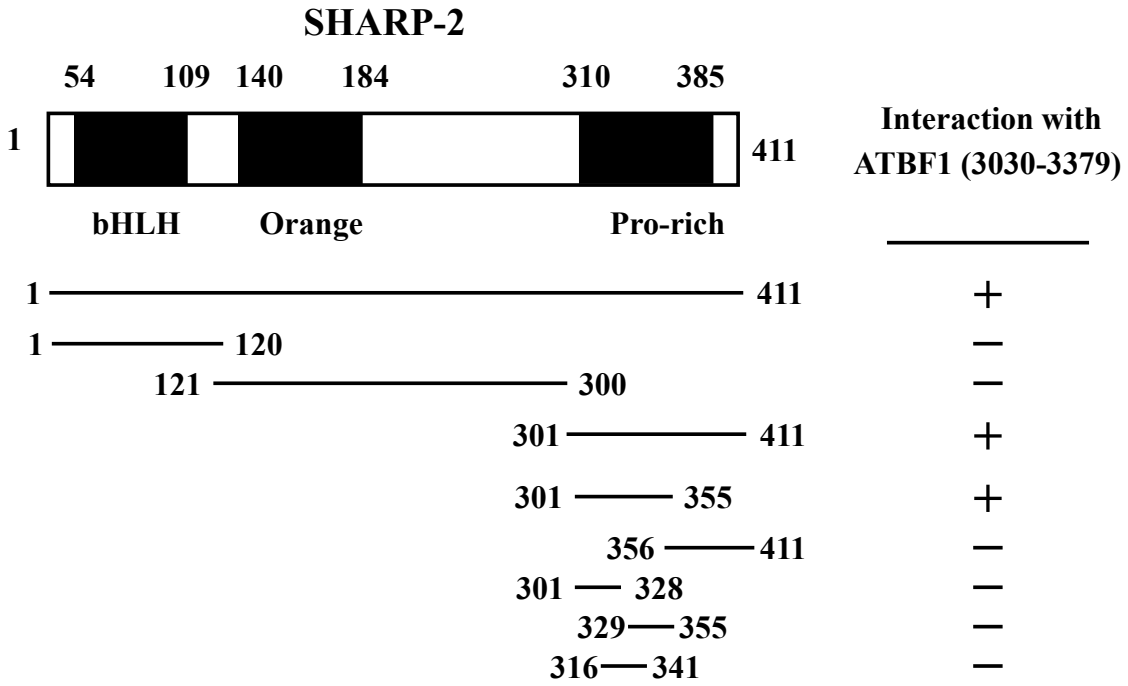

Fig. 4. Identification of the minimal interaction domain of SHARP-2 with ATBF1 using the yeast two-hybrid system.

A schematic representation of SHARP-2 and the GAL4DBD-SHARP-2 fusion constructs are depicted on the left. The + and - symbols indicate significant increased and unchanged levels of β-galactosidase activity, respectively, compared with a yeast harboring a combination of pDBD and pAD-ATBF1 (3030-3379). bHLH, basic helix-loop-helix; Orange, orange domain; Pro-rich, proline-rich domain.

amino acid sequences 356 to 383 and 384 to 411. A region containing amino acid sequences 384 to 411 showed an interaction with the Sox6. Further, this region was divided into three amino acid sequences 384 to 397, 392 to 403, and 398 to 411, respectively. Only a yeast harboring a plasmid including amino acid sequences 384 to 397 showed an interaction with the Sox6. These results indicated that the amino acid sequences between 384 and 397 of the SHARP-2 interacts with the Sox6.

SHARP-2 interacted with ATBF1 and Sox6 using different domains, respectively (Fig. 6). We searched the amino acid sequence of this region in the Motif Scan database. We found that amino acid sequences between 301 and 355 contained a proline-rich region which is required for transcriptional activity and that those between 384 and 397 contain a SSLD sequence that can be phosphorylated by casein kinase II (Fig. 7).

Thus, we concluded that both the ATBF1 and Sox6 were identified as the SHARP-2-interacting protein and that the SHARP-2 interacted with them using different domains.

Ⅳ. Acknowledgements

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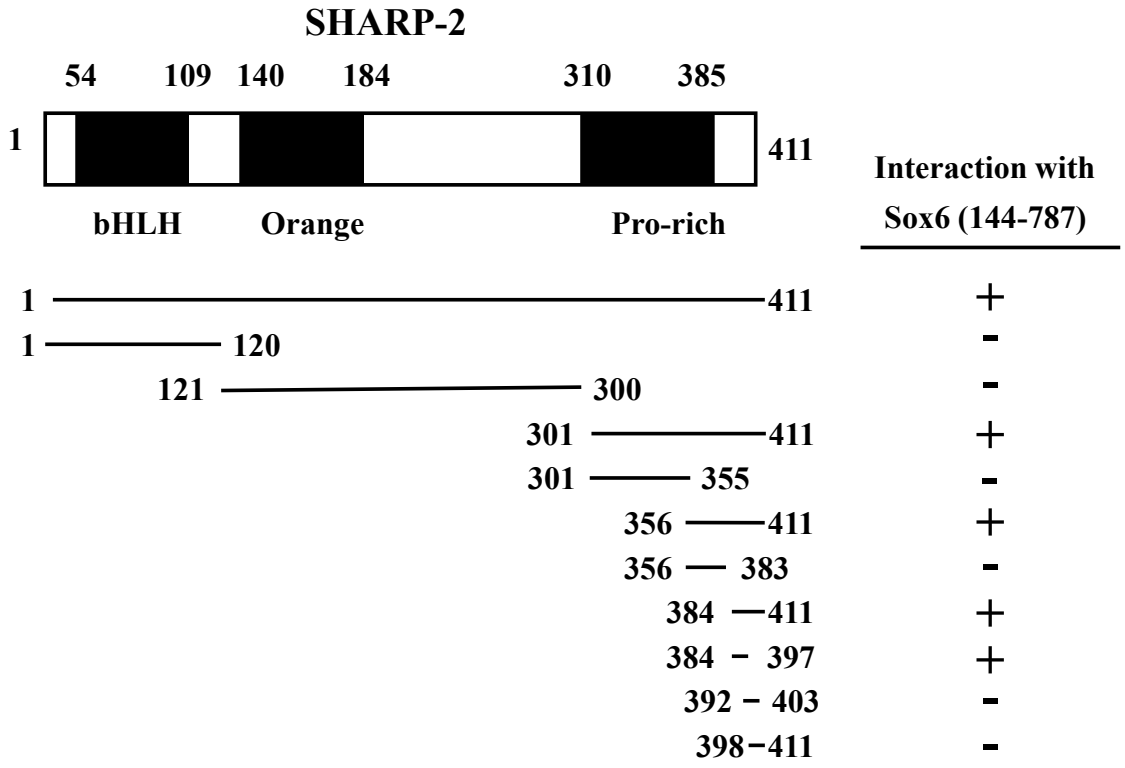

Fig. 5. Identification of the minimal interaction domain of SHARP-2 with Sox6 using the yeast two-hybrid system.

A schematic representation of SHARP-2 and the GAL4DBD-SHARP-2 fusion constructs are depicted on the left. The + and - symbols indicate significant increased and unchanged levels of β-galactosidase activity, respectively, compared with a yeast harboring a combination of pDBD and pAD-Sox6 (144-787). bHLH, basic helix-loop-helix; Orange, orange domain; Pro-rich, proline-rich domain.

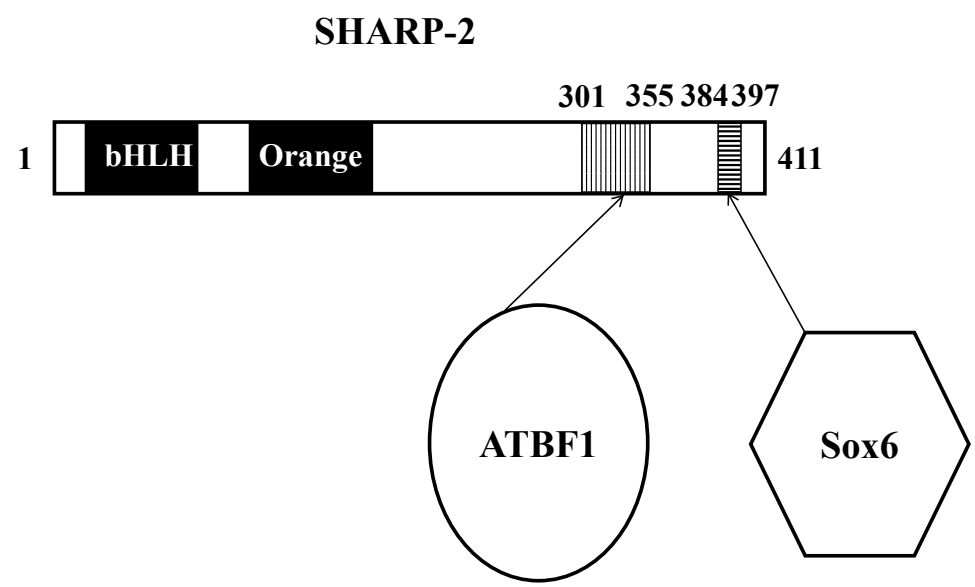

Fig. 6. Schematic representation of the interaction domains of SHARP-2.
SHARP-2 interacts with ATBF1 and Sox6 using different domains.

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       310        320        330        340        350        360
FVGSDLMGSP FLGPHPHQPP FCLPFYLIPP SATAYLPMLE KCWWPTSVPL LYPGLNTSAA
       370        380        390        400        410        420
ALSSFMNPDK IPTPLLLPQR LPSPLAHSSL DSSALLQAAK QIPPLMLETK D
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Fig. 7. Amino acid sequences between 301 and 411 of SHARP-2.
A SSLD sequence, a putative phosphorylation site by casein kinase II, is underlined.

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