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Identification of Ku70/Ku80 as ADD1/SREBP1c Interacting Proteins

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In vertebrates, multisubunit cofactors regulate gene expression through interacting with cell-type- and gene-specific DNA-binding proteins in a chromatin-selective manner. ADD1/SREBP1c regulates fatty acid metabolism and insulin-dependent gene expression through binding to SRE and E-box motif with dual DNA binding specificity. Although its transcriptional and post-translational regulation has been extensively studied, its regulation by interacting proteins is not well understood. To identify cellular proteins that associate with nuclear form of ADD1/SREBP1c, we employed the GST pull-down system with Hela cell nuclei extract. In this study, we demonstrated that Ku proteins interact specifically with ADD1/SREBP1c protein. GST pull-down combined with peptide sequencing analysis revealed that Ku80 binds to ADD1/SREBP1c *in vitro*. Additionally, western blot analysis showed that Ku70, a heterodimerizing partner of Ku80, also associates with ADD1/SREBP1c. Furthermore, co-transfection of Ku70/Ku80 with ADD1/SREBP1c enhanced the transcriptional activity of ADD1/SREBP1c. Taken together, these results suggest that the Ku proteins might be involved in the lipogenic and/or adipogenic gene expression through interacting with ADD1/SREBP1c.

Eukaryotic gene expression is regulated by complex mechanisms in which the recruitment and assembly of the transcriptional machinery are directed by gene- and cell-type-specific transcription factors with DNA-binding ability. When DNA is packaged into chromatin, gene activation requires another class of proteins with chromatin-targeting activity. In vertebrates, several multisubunit cofactors such as TRAP/SMCC and DRIP/ARC mediate selective and efficient gene expression with specific transcriptional factors in a chromatin-selective manner (Ito et al., 2000; Wang et al., 2001; Rachez et al., 1999; Naar et al., 1999a).

Sterol regulatory element binding proteins (SREBPs) play a critical role in lipid homeostasis by regulating genes involved in cholesterol and fatty acid metabolism. Three isoforms of SREBPs have been identified, SREBP1a, SREBP1c (also known as adipocyte determination and differentiation dependent factor 1 [ADD1]) and SREBP2, constituting a family of basic helix-loop-helix (bHLH) transcription factors (Wang et al., 1993; Briggs et al., 1993; Tontonoz et al., 1993). SREBP1a and ADD1/SREBP1c are generated by alternative promoter usage and alternative splicing from a single gene, while SREBP2 is encoded by a distinct gene with 47% identity with SREBP1 (Horton et al., 2002). Unlike other transcription factors, precursor forms of SREBPs are inserted into the endoplasmic reticulum (ER) membrane as a transcriptionally inactive form (Sato et al., 1994; Wang et al., 1994). When cellular cholesterol levels are low, SREBP cleavage-activating protein (SCAP), together with Insig-1 or Insig-2, escorts SREBPs from the ER to the Golgi, where they are sequentially cleaved by Site 1 and Site 2 proteases (Hua et al., 1996; Yang et al., 2002; Yabe et al., 2002; Brown and Goldstein, 1999). Mature forms of SREBPs, with N-terminal fragments, are then released from the Golgi and translocate to the nucleus where they bind to the promoters or enhancers of SREBP target genes (Horton et al., 2002; Inoue et al., 2001; Yang et al., 2002; Yabe et al., 2002, 2003).

Of the three SREBPs, ADD1/SREBP1c is a key transcription factor for fatty acid metabolism and activates several genes responsible for fatty acid synthesis, including fatty acid synthase (FAS), lipoprotein lipase (LPL), acetyl Co A carboxylase (ACC) and steroyl Co A desaturase (SCD) in fat and liver (Horton et al., 2002; Kim and Spiegelman, 1996; Lopez et al., 1996; Shimomura et al., 1998a; Bene et al., 2001). ADD1/SREBP1c is

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predominantly expressed in white adipose tissue, brown adipose tissue and liver, and its mRNA is induced at an early stage of adipocyte differentiation (Tontonoz et al., 1993; Kim et al., 1996). Ectopic expression of ADD1/SREBP1c in preadipocyte cell lines promotes adipocyte differentiation and stimulates the transcriptional activity of peroxisome proliferator activated receptor (PPAR) γ, a master transcription factor for adipogenesis, by producing its activators or endogenous ligands (Kim and Spiegelman, 1996; Kim et al., 1998a). In addition, ADD1/SREBP1c expression is modulated by nutritional status and regulated in an insulin-sensitive manner in which it regulates transcription of insulin-dependent lipogenic genes (Kim et al., 1998b). It is therefore likely that ADD1/SREBP1c is a major transcription factor coordinating fatty acid and glucose metabolism in insulin-sensitive organs. In contrast, several studies have suggested that ADD1/SREBP1c is dispensable for the development of adipose tissue *in vivo* (Shimomura et al., 1998b; Shimano et al., 1997a; Liang et al., 2002). For example, aP2-ADD1/SREBP1c transgenic animals show lipodystrophy rather than increased fat mass (Shimomura et al., 1998b). However, ADD1/SREBP1c-specific knockout mice have clearly decreased fat mass and severely down regulated hepatic and plasma triglycerides, suggesting that ADD1/SREBP1c is critical for lipogenesis (Shimano et al., 1997a; Liang et al., 2002).

Compared to other SREBPs, ADD1/SREBP1c has a relatively weak trans-activity. This can be explained by the fact that it has shorter acidic amino acids in its N-terminal region compared to SREBP1a or SREBP2 (Shimano et al., 1997b). Through long N-terminal acidic domain, SREBP1a and SREBP2 associate with various transcriptional co-activators including p300, CBP and ARC/DRIP (Naar et al., 1998, 1999; Sanchez et al., 1995). However, interaction between ADD1/SREBP1c and co-activators has not been demonstrated. Rather, it has been reported that the transcriptional activities of SREBP1 isoforms are modulated by functional interaction with other trranscription factors such as Sp1 and NF-Y (Sanchez et al., 1995; Dooley et al., 1998; Magana et al., 2000).

Although the transcriptional and post-translational regulation of ADD1/SREBP1c has been extensively investigated, little is known of proteins that interact with it or regulate its transcriptional activity. To address this issue, we used a GST pull-down system followed by peptide sequencing analysis from the purified protein complex. Here, we demonstrate that an about 80 kDa protein(s) from Hela nuclei interacts with ADD1/SREBP1c. Peptide sequencing analysis and the protein size information revealed that the protein is Ku80. Furthermore, western blot analysis revealed that Ku70, a heterodimerizing partner of Ku80, also associates with ADD/SREBP1c *in vitro*. Co-transfection of Ku70/80 with ADD1/SREBP1c enhanced the transcriptional activity of ADD1/SREBP1c. Our results suggest that the Ku70/Ku80 heterodimer would participate in the transcriptional regulation by ADD1/SREBP1c and might regulate lipogenic gene expression via association with ADD1/SREBP1c.

Materials and Methods

Plasmid constructs

The active form of the ADD1/SREBP1c expression vector encoding a.a.1-403 of rat ADD1/SREBP1c was cloned in frame fusion into the pcDNA3.1-Myc/HisA and the pGEX4T-1 vector (Pharmacia). p53 expression vector was cloned into the pGEX4T-1 vector. Expression plasmids for Ku70 and Ku80 were gifts from Dr. H. Kim.

Cell culture and preperation of nuclei extract

Hela cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. HeLa cell nuclear extract was prepared as described previously (Dignam et al., 1983), except that the final dialysis in 0.1 M KCl D buffer (20 mM HEPES) [pH 7.9], 20% [vol/vol] glycerol, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol) was omitted.

Peptide sequencing and peptide sequence analysis

Peptide sequencing analysis was performed by Michael Berne in Tufts University (Applied Biosystem Analysis software). Protein databases were analyzed with the BLAST package of search algorithms (http://ncbi.nlm.nih.gov/cgi.bin/BLAST).

GST pull-down

Hela cell nuclei extract was precleared with excess GST protein and glutathione sepharose beads (Pharmacia) in binding buffer (20 mM HEPES, pH 7.6; 0.1 mM EDTA; 10% glycerol; 0.1% NP-40; 1 mM DTT; 1 mM benzamidine; 0.25 mM PMSF; 2 μg/ml aprotinin). The precleared Hela nuclei extract was mixed with 1 μg of recombinant GST-ADD1/SREBP1c (a.a.1-403) fusion protein and glutathione sepharose beads. Reaction volumes were made up to 150 μl with binding buffer and incubated for 3 h at 4°C. As negative control, GST or GST-p53 protein was also incubated in a reaction mixture as GST-ADD1/SREBP1c. The beads were pelleted and washed with binding buffer. Proteins were eluted by 10 mM glutathione-containing binding buffer and mixed with SDS-gel sample buffer (125 mM Tris/HCl, pH 6.8, 50% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.01% Bromophenol Blue). Boiled samples were subjected to SDS-PAGE and stained with commassie brilliant blue R-250 solution.

Western blot analysis

The protein samples separated by SDS-PAGE were transferred to PVDF membrane (millipore). Monoclonal antibodies for Ku70 and Ku80 were gifts from Dr. I Lee.

Transient transfection assay

HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. HEK293 cells were transfected one day before confluence by the calcium phosphate method described previously (Lee et al., 2003). After incubation for 24 hr, cell extracts were prepared with lysis buffer (25 mM Tris-phosphate pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, and 1% Triton X-100), and activities of β-galactosidase and luciferase determined according to the manufacturer's instructions (Promega). Luciferase activities were normalized to the corresponding β-galactosidase activities. All transfection experiments were performed in duplicate and repeated independently at least three times.

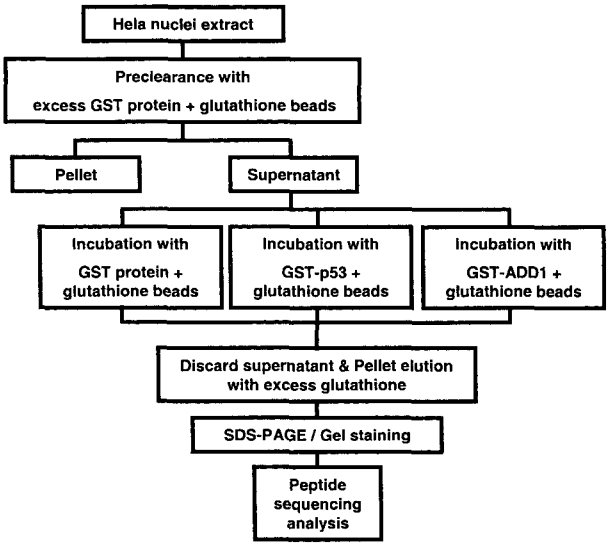

Fig. 1. Experimental schemes for purification of ADD1/SREBP1c interacting proteins.

Results

A putative 80 kDa protein specifically binds to ADD1/SREBP1c in vitro

To purify the nuclear proteins interacting with ADD1/SREBP1c, we adopted a GST pull-down system. In this system, we precleared Hela nuclei extract with excess GST proteins and glutathione sepharose beads, and incubated the precleared Hela nuclei extract with GST-ADD1/SREBP1c protein in the presence of glutathione-conjugated sepharose beads (Fig. 1). As a negative control, we also incubated the precleared Hela nuclei extract with GST alone or GST-p53 protein in place of GST-ADD1/SREBP1c. After washing with excess binding buffer, the proteins associated with the GST proteins were boiled and separated by SDS-PAGE. As shown in Fig. 2, a protein of about 80 kDa was observed to bind specifically to GST-ADD1/SREBP1c, but not to GST alone or GST-p53.

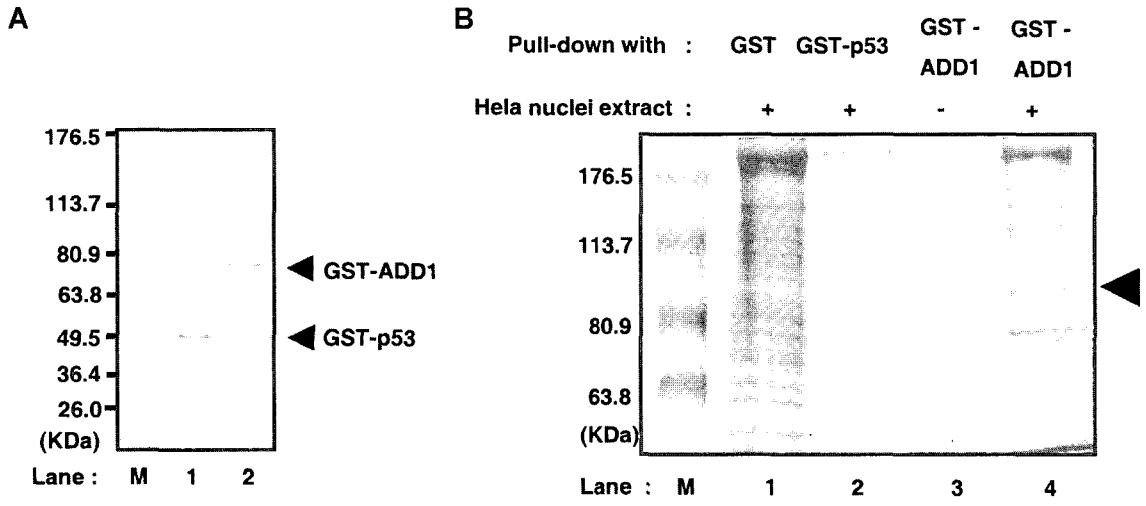

Fig. 2. Isolation of ADD1/SREBP1c interacting proteins via GST-pulldown system. A, Purified GST proteins were eluted from glutathione-coated beads with elution buffer containing 10 mM glutathione, and subjected to SDS-PAGE. B, Several GST proteins were used to purify the proteins specifically interacting with ADD1/SREBP1c as illustrated in Fig. 1, and subjected to SDS-PAGE. The gels were stained with commassie brilliant blue. The specific band is indicated by an arrowhaed.

A

Aacid #	Aacid ID	R. Time (min)	C.Time (min)	Pmol (raw)	Pmol (-bkgd)	Pmol (+1ag)	Pmol Ratio	Aacid ID
1	G	9.02	8.98	364.52	347.32	388.43	1300.00	GLY
2	G	9.02	8.98	341.80	326.55	320.46	1072.51	GLY
3								
4	G	9.02	8.98	33.64	21.58	13.30	44.51	GLY
5	N	6.82	6.80	3.07	1.01	1.19	3.67	ASN
6	K	26.53	26.48	3.47	1.01	1.19	3.43	LYS
7	A	12.58	12.55	5.93	1.72	2.05	16.18	ALA
8	A	12.58	12.55	6.30	1.87	1.79	14.17	ALA
9	V	20.77	20.73	5.24	0.98	1.17	7.55	VAL
10	V	20.77	20.73	5.96	1.47	1.52	9.80	VAL
11	L	27.07	27.03	6.35	0.63	0.76	2.93	LEU
12	F	25.27	25.23	3.19	0.17	0.21	1.58	PHE
13	G	9.02	8.98	8.32	0.79	0.71	2.39	GLY
14	Q	8.32	8.27	4.38	0.16	0.14	0.86	GLN
15	P	19.22	19.18	4.00	0.28		1.28	PRO

B

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Query      5  - - - - - NKAAVVL S G D/Q G/V  15
                       | | | | | | |   |   |
Sbjct(Ku)  6  MVRSGNKAAVVL C M   D     V   16
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Fig. 3. Peptide sequencing analysis of the isolated protein(s). After separation of the same protein samples used in Fig. 2 with SDS-PAGE, the proteins were transferred to PDVF membrane and stained with commassie brilliant blue R-250 solution. The specific band around 80 kDa was cut out and subjected to peptide sequencing analysis. A, Peptide sequence analysis revealed N-terminal 9 amino acid sequence of the 80 kDa protein. B, Sequence analysis with BLAST algorithm indicated that the 80 kDa protein has nine amino acids matching with amino-terminal region of ku proteins.

Peptide sequencing analysis of the selected protein with 80 kDa

To elucidate the identity of the 80 kDa protein, we performed N-terminal peptide sequencing analysis of the protein (Fig. 3a). The protein samples prepared as described above were separated by SDS-PAGE and transferred to PVDF membrane. After stained with commassie brilliant blue R-250 solution, the band of 80 kDa was cut and subjected to peptide sequencing analysis. This revealed eleven amino-terminal amino acid sequence; NKAAVVLSG(D/Q)(G/V), and the data were analyzed with the BLAST package of search algorithms (Fig. 3A). This revealed that the protein has nine matching amino acid sequence with the conserved region of human ku proteins (NKAAVVLCMDV) (Fig. 3B). In human, Ku is composed of two subunits, Ku70 and Ku80 (deVris et al., 1989; Knuth et al., 1990; Falzon et al., 1993). Because molecular mass of the purified protein was about 80 kDa, this result indicated that Ku80 associated with ADD1/SREBP1c *in vitro*.

Interaction between Ku70 and ADD1/SREBP1c in vitro

Ku80 forms a heterodimer with Ku70 to make a functional unit (deVris et al., 1989; Knuth et al., 1990; Falzon et al., 1993). To test whether Ku70 also associates with ADD1/SREBP1c *in vitro*, we performed the GST pull-down assay in the same way illustrated in Fig. 1 and the protein samples were subjected to the Western blot analysis with anti-Ku70 antibodies. As shown in Fig. 4, Ku70 bound to the immobilized GST-ADD1/SREBP1c, indicating that Ku70 also participated in the ADD1/SREBP1c complex *in vitro*. Ku70 was barely detected with GST alone or GST-p53 (Fig. 4, lane 1 and lane 2).

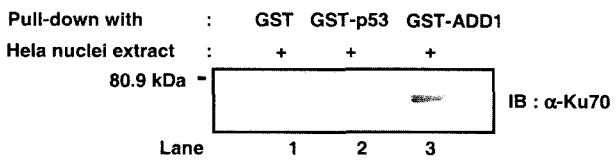

Fig. 4. Western blot analysis shows that Ku70 interacts with ADD1/SREBP1c *in vitro*. The same protein samples used in Fig. 2 were analyzed by Western blot analysis with anti-Ku70 antibody.

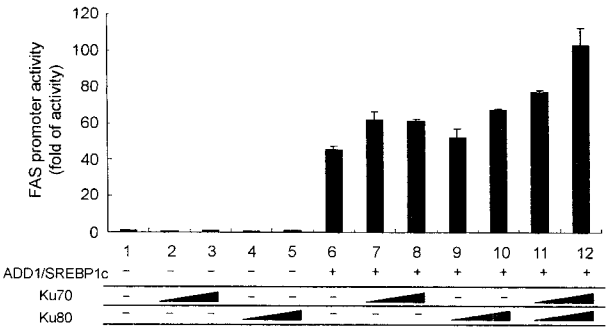

Fig. 5. Transcriptional activity changes of ADD1/SREBP1c with Ku proteins. HEK293 cells were co-transfected with FAS-luciferase reporter and ADD1/SREBP1c expression plasmid in the presence or absence of Ku proteins. Amount of the DNAs used for the reporter assay is as follows; FAS-luciferase (200 ng), ADD1/SREBP1c (200 ng), Ku70/80 (100 ng, 1000 ng for dose-dependent expression).

Ku enhances the transcriptional activity of ADD1/SREBP1c

In order to investigate the consequences of the interaction between ADD1/SREBP1c and Ku proteins, we examined the transcriptional activity of ADD1/SREBP1c in the presence and absence of Ku proteins. Previously, we and others have demonstrated that the promoter of the FAS gene has specific cis-element for ADD1/SREBP1c and that they are transactivated by ectopic expression of ADD1/SREBP1c (Horton et al., 2002; Kim et al., 1998b). Expression of ADD1/SREBP1c activated the promoter of FAS and co-expression of Ku proteins enhanced the transcriptional activity of ADD1/SREBP1c, while Ku proteins on their own had no effect (Fig. 5). This observation is consistent with the recent report that Ku is involved in the transcriptional activation of p50, a subunit of NF-kB, through interaction with recombination signal binding protein-Jk (RBP-Jk) in human gastric cancer cells (Lim et al., 2004).

Discussion

Ku is an evolutionarily well-conserved protein with abundant expression in the nucleus and is present in vertebrates, insects, yeast, and worms (Tuteja et al., 2000). Binding of Ku was shown to occur at the ends of double stranded DNA, independently of DNA sequence, but it binds also to nicks, gaps, hairpins and the ends of telomeres (Tuteja et al., 2000). Once bound to DNA ends, Ku is able to translocate along the DNA fragments. These properties are important for functional roles of Ku proteins, including V(D)J recombination and DNA double-strand break repair. It has also been claimed that Ku plays a role in DNA replication (Tuteja et al., 2000). Furthermore, it has been suggested that Ku could bind to specific DNA sequence to regulate gene expression (Tuteja et al., 2000; Giffin et al., 1996; Falzon and Kuff, 1989; Quinn and Farira, 1991; Kim et al., 1995). For example, it is involved in enhancement or repression of gene expression mediated by Pol I and Pol II. In addition, Ku binding to the NRE1 sequence (negative regulatory element 1) in the long terminal repeat of mouse mammary tumor virus represses glucocorticoid-induced MMTV transcription (Griffin et al., 1996). Ku also binds specifically to the HSE motif involved in the regulation of heat shock protein HSP70 expression and down-regulates HSP70 gene transcription (Kim et al., 1995).

Ku is composed of two subunits, p70 and p80, with apparent molecular masses of 70 and 80 kDa, respectively (deVries et al., 1989; Knuth et al., 1990; Falzon et al., 1993). It forms a complex which has DNA-dependent kinase activity when associated with a third catalytic subunit (p350) (Dvir et al., 1992; Gottlieb et al., 1993). Ku70/80 heterodimer is a regulatory subunit of the DNA-dependent protein kinase that phosphorylates many proteins including SV40 large T antigen, p53, RNA-polymerase II, RP-A, topoisomerases, hsp90, and many transcription factors such as c-Jun, oct-1, sp-1, c-Myc, TFIID (Dvir et al., 1992; Lees-Miller et al., 1990).

Here, we revealed that Ku70/Ku80 heterodimer interacts with ADD1/SREBP1c *in vitro*. GST pull-down assay combined with peptide sequencing analysis demonstrated that Ku80 associates with ADD1/SREBP1c *in vitro*, and western blot analysis revealed that Ku70 is also interacting with ADD1/SREBP1c complex *in vitro*. Furthermore, overexpression of Ku70/80 in HEK293 cell enhanced the transcriptional activity of ADD1/SREBP1c in FAS promoter. Although the regulatory mechanism for the transcriptional activation of ADD1/SREBP1c by ku proteins remains to be elucidated, our results suggest that Ku protein might participate in the regulation of transcriptional activity of ADD1/SREBP1c and finally regulate lipogenic gene expression.

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