BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **236,** 83–87 (1997)
ARTICLE NO. RC976911

A Splicing Variant of Steroid Receptor Coactivator-1 (SRC-1E): The Major Isoform of SRC-1 to Mediate Thyroid Hormone Action

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Received June 4, 1997

Steroid Receptor Coactivator-1 (SRC-1) interacts with nuclear receptors only when they are bound to the ligands and enhance the transactivation. We identified splicing variants encoding three isoforms, SRC-1, SRC-1(-Q), and SRC-1E, generated by alternative usage of an exon(s) and splicing acceptor sites. RT-PCR analysis showed that SRC-1E was more abundantly expressed than SRC-1 or SRC-1(-Q) at the mRNA level in all the cell lines tested. SRC-1E lacks 56 amino acids of SRC-1 and has unique 14 amino acids at the carboxyl terminus, while SRC-1(-Q) differs from SRC-1 by deletion of only one glutamine residue. Since the C-terminal domain of SRC-1 has been shown to be involved in the interaction with nuclear receptors, the enhancement of transactivation by these three isoforms was tested. SRC-1E enhanced thyroid hormone dependent transactivation of reporter gene expression more profoundly than SRC-1 or SRC-1(-Q). Taken together, it was suggested that SRC-1E is the major isoform of SRC-1 to mediate thyroid hormone action. © 1997 Academic Press

Thyroid hormone plays important roles in differentiation and development of vertebrates. In human, lack of thyroid hormone in early period of life results in irreversible deficits in growth, especially neurological development, known as cretinism. Thyroid hormone action is mediated by its receptor (TR), which belongs to nuclear receptor superfamily. Members of this superfamily regulate the transcriptional rate of target gene through specific *cis*-elements in the promoter region. TR binds to the specific sequence containing AGGTCA half site motifs arranged in palindrome, inverted palindrome or direct repeats with 4 bp spacing as a monomer, a homodimer or a heterodimer with retinoid X receptors (RXR) (1, 2). In the absence of triiodothyronine (T3), an active form of thyroid hormone, TR can suppress the expression of target gene, while binding of T3 converts TR from a silencer to a transactivator.

Recently several cofactors which interact with nuclear receptors in the absence and/or in the presence of the cognate ligands have been identified (3). Coactivators interact with TR bound to T3 and enhances transactivation, while corepressors mediate silencing, by interacting with unliganded TR (4, 5). Steroid Receptor Coactivator-1 (SRC-1) was cloned by two hybrid strategy using ligand binding domain of progesterone receptor as a bait and revealed to interact a variety of nuclear receptors including TR, enhancing ligand-dependent transactivation (6).

We first studied the level of SRC-1 mRNA expression in various cell lines, especially those derived from nervous system. During the course of the study, we identified splicing variants of SRC-1. A splicing variant named SRC-1E is expressed more abundantly than SRC-1. This variant enhances thyroid hormone dependent transactivation more potently than SRC-1.

MATERIAL AND METHODS

Cell culture. The following 8 cell lines were obtained from American Type Culture Collection and used for the present study. They are neuroblastoma derived cell lines, SK-N-SH, NB-1, glioma derived cell lines,T98G, U251MG, U251SP, AJ, hepatoblastoma derived HepG2 and choriocarcinoma derived JEG3. All the cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, Life Technologies Inc.) under the atmosphere of 95% air / 5% CO2 and 100% humidity.

RT-PCR and sequence determination. Total RNA was extracted from cultured cells and cDNA was synthesized using oligo-dT as a primer as described previously (7). Following primers were used for the amplification of various fragments of SRC-1 by PCR. SRC-S: 5′-TTG GCC AAC CGC AAC AGC ATG G-3′, SRC-S2: 5′-CAG CTC TCA TCC ACT GAC CTT CTC-3′, SRC-A: 5′-TTG TTA TTC AGT

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0006-291X/97 $25.00

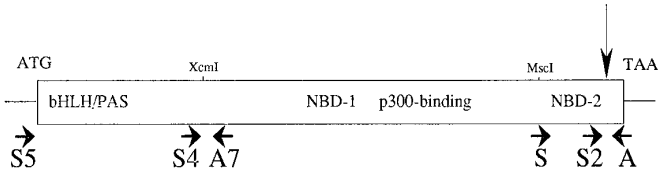

FIG. 1. Functional domains of SRC-1 and location of the primers. Location of the primers are indicated as horizontal arrows. The location of insertion/deletion generating splicing variants characterized in the present study is indicated as a vertical arrow. Restriction sites used for the construction of the expression plasmid for SRC-1 isoforms are also indicated (see materials and methods). Domains of SRC-1 summarized by Yao et al (12)are shown. bHLH/PAS: basic helix-loop-helix/PAS, NBD: nuclear receptor binding domain.

CAG TAG CTG CTG-3′, SRC-S4 (5′-TCG ACA GGG AGC ACA GTG GGC-3′) and SRC-A, SRC-S5 (5′-GCT ACC CTC TGG AAC TCA AGA TTT),and SRC-A7 (5′-TGA CTG CTG CTA TGA AGG TCT GAG-3′) Location of these primers in SRC-1 cDNA are depicted in Fig. 1. Typically, PCR was carried out for 30 cycles consisting of 96° denature for 10 sec. and 68° annealing/extension, the time of which varied from 30 sec to 4 minutes depending on the length of predicted size of DNA fragments. PCR products were ligated to pGEM-T vectors (Promega, Madison, WI) according the instruction. The sequences of the inserts were determined by using PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Inc., Foster City, CA) and DNA sequencer model 373A-35 (Applied Biosystems Inc.).

Plasmids. The plasmids expressing SRC-1 variants were constructed as follows. A cDNA insert, which encoded C-terminal two third region of SRC-1 variant molecule named SRC-1E and was obtained from RT-PCR with primers S4 and A (see Fig. 1), was inserted into pRc/CMV (Invitrogen, San Diego, CA) to generate pRc/CMV-ΔN-SRC-1E. For this procedure, restriction sites for Not I and Apa I, present in both multiple cloning sites (MCS) of pGEM-T and pRc/CMV were used. Another cDNA insert encoding an N-terminal fragment, which was obtained with primers S5 and A7, was excised at Not I site in MCS and Xcm I site between primers S4 and A7 in SRC-1 cDNA. This fragment was ligated into the same sites in pRc/CMV-ΔN-SRC-1E to generate pRc/CMV-SRC-1E. The cDNA inserts encoding fragments of SRC-1 or 1(-Q), obtained with primers S and A, were excised at MscI site in primer S and ApaI in MCS. The corresponding part of pRc/CMV-SRC-1E was replaced by these fragments to generate pRc/CMV-SRC-1 and 1(-Q). It was confirmed that these cDNA inserts encoded 160 kD proteins by using in vitro transcription coupled translation using TNT-Lysate system (Promega, Madison, WI). Two reporter plasmids palx3-Luc and F2x3-Luc have been described previously (8). Palx3-Luc contains 3 copies of palindromic T3 response element (TRE) in the promoter region, and F2x3-Luc contains 3 copies of inverted palindromic TRE derived from chicken lysozyme silencer. An expression plasmid for TR, pcDNAI/Amp-TR has been described (8).

Transfection and reporter assays. For the transactivation study, HepG2 cells cultured in 12-well plates were transfected using calcium phosphate precipitation method (9), with 500 ng of reporter plasmid, 250 ng of pSV-βGal (Promega), 50 or 250 ng of pcDNAI/Amp-TR, and 200 or 1000 ng of pRc/CMV-SRC-1 splicing variants per well. The total amount of plasmids was adjusted by pcDNAI/Amp or pRc/CMV carrying no cDNA inserts. After overnight exposure to DNA-$CaPO_4$ precipitate, the cells were washed and incubated in the medium supplemented with 5% charcoal stripped FBS with or without 100 nM of T3. Forty-eight hours after the hormonal treatment, the cells were lysed by the addition of 100μl glycylglycine buffer (25 mM glycylglycine pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT) containing 0.2% TritonX 100. Twenty μl aliquots of cell lysate were mixed with 300μl of glycylglycine buffer containing 2 mM ATP /15 mM potassium phosphate. One hundred μl of glycylglycine buffer containing 0.2 mM d-luciferine was injected to the mixture using automatic device in LUMAT LB9501 (Berthold Japan KK, Tokyo, Japan) and luciferase activity was measured for 10 sec. The activity of β-galactosidase was measured using Luminescent β-gal detection kit (CLONTECH, Palo Alto, CA) and LUMAT LB9501 according to the instruction. Luciferase activity was corrected by β-galactosidase activity and the data from triplicate determinations were expressed as mean ± SD.

RESULTS

To estimate the level of SRC-1 mRNA expression in various cell lines, we at first amplified 3′-region of SRC-1 cDNA using primers, SRC-S and SRC-A (see Fig. 1). From all the cell lines tested, DNA fragment with size similar to the predicted size could be amplified (data not shown), indicating the expression of SRC-1 in a broad range of cell lines. However, sequence analysis of a cloned cDNA fragment revealed that it was 54 bps longer than the sequence originally reported by Onate et al (6). The difference was due to insertion of 57 bps unique sequence and deletion of 3 bps encoding glutamine at the same position. Since an in-frame termination codon was present in the inserted 57 bps fragment, the protein encoded by this splicing variant was predicted to lack 56 C-terminal amino acids and to have unique 14 amino acids (see Fig. 3A). During the course of the present study, Kamei et al cloned full length mouse SRC-1 cDNA and several splicing variants. The present variant was named human SRC-1E, according to the nomenclature by Kamei et al (10).

We then estimated the relative abundance of SRC-1 and SRC-1E mRNAs. For this purpose, another primer named SRC-S2 was designed so that the presence or absence of inserted sequence could be readily detected by agarose electrophoresis. As shown in Fig. 2, all the tested cell lines expressed SRC-1E mRNA more abundantly than that of SRC-1.

We further sequenced the cDNA inserts amplified from RNA in SK-N-SH cell line using primer S2 and A. As depicted in Fig. 3A, 4 different cDNA species, SRC-1, SRC-1(-Q), SRC-1E and SRC-1E(+cag) were ob-

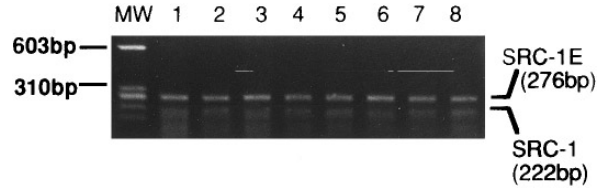

FIG. 2. RT-PCR analysis of SRC-1 splicing variants. RNA from various cell lines was analyzed by RT-PCR using primer SRC-S2 and SRC-A. MW:φX174 molecular weight marker. lane 1:SK-N-SH, 2: NB-1, 3:T98G, 4:U251MG, 5:U251SP, 6:AJ, 7: HepG2, 8:JEG3, 9:blank. Arrows indicate SRC-1 and the splicing variant, SRC-1E.

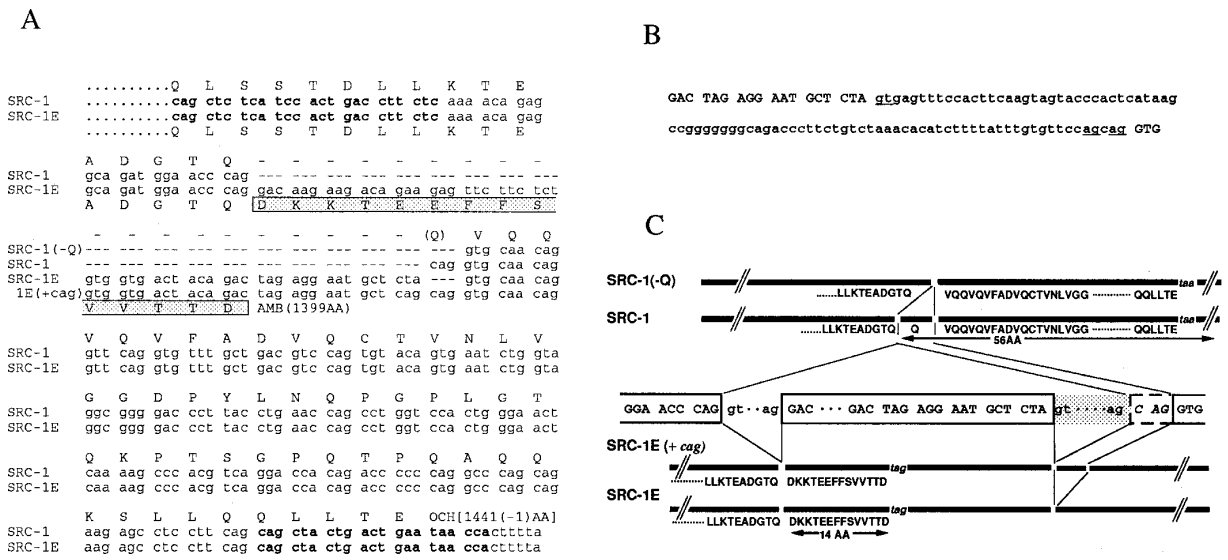

FIG. 3. (A) Nucleic acid and deduced amino acid sequence of the SRC-1 splicing variants. (B) Sequence of the intron downstream of alternatively used exon. (C) Structure of intron-exon junction of SRC-1 generating the splicing variants. (A)Nucleic acid sequences in bold indicate the primers, SRC-S2 and SRC-A. Amino acid sequence is shown in one letter code. Glutamine in bracket is present in SRC-1 but not in SRC-1(-Q), thus total amino acid residues are 1441 for SRC-1 and 1440 for SRC-1(-Q). SRC-1E and SRC-1E(+cag) encode an identical polypeptide with 1399 amino acid residues. The 14 amino acid sequence unique to SRC-1E is shaded. (B) Nucleic acid sequence of exons are shown in uppercase and that of intron lowercase. Splicing sites are underlined. Note that two splicing acceptors are present at 5′-end of down stream exon. See also Fig. C. (C) The shaded region in the nucleic acid sequence corresponds to the intron sequence shown in Fig. B. Partial amio acid sequences are also shown in one letter code along with spliced transcripts (bold lines).

tained. Their difference were due to presence or absence of 57 bp-long exon(s) insertion in addition to alternative usage of a CAG triplet. SRC-1E and SRC-1E(+cag) encodes an identical polypeptide, since the alternatively used CAG triplet is in the 3′-untranslated region in SRC-1E and SRC-1E+cag. Thus, these four cDNAs encodes three isoforms of SRC-1 at the protein level (Fig. 3A). The presence of BsmI site in SRC-1E unique sequence allowed us to estimate the abundance of each isoform by restriction analysis. The numbers of clones encoding SRC-1E were twice as abundant as those encoding SRC-1 or SRC-1(-Q) in SK-N-SH cell. Sequencing of 9 clones which lack 57 bps insertion revealed that 7 clones were SRC-1 while 2 were SRC-1 (-Q). Collectively, the relative frequency of SRC-1, SRC-1(-Q), and SRC-1E/SRC-1E(+cag) was estimated to be 23%, 10% and 67%, respectively. Along with these clones, we obtained a clone containing intron sequence downstream of the alternative exon(s) probably arising from an incompletely spliced transcript. The intron sequence is presented in Fig. 3B and shows that the production of mRNAs with or without the CAG triplet can be explained by the alternative usage of splice acceptor site. Figure 3C depicts the structure of exon intron junction producing the present isoforms.

C-terminal 200 amino acids of SRC-1 have been reported to contain a domain interacting with multiple nuclear receptors (6). Since SRC-1E lacks 56 C-terminal amino acids and has unique 14 amino acids, it is likely that SRC-1E has a differential interaction with nuclear receptors compared to SRC-1. Thus, we constructed expression plasmids for SRC-1, SRC-1(-Q) or 1E and tested their activity to enhance thyroid hormone dependent transactivation mediated through two kinds of artificial TREs, palx3 (Fig. 4A) or F2x3 (Fig. 4B). When no exogenous TR was cotransfected, both addition of 100 nM of T3 and cotransfection of SRC-1 isoforms had little effect on the luciferase (Luc) gene expression. (data not shown) TR strongly transactivated luciferase expression in T3 dependent manner on both reporters. In the absence of T3, additional cotransfection of expression plasmids carrying SRC-1 isoforms had little effect on luciferase expression. In the presence of T3, however, cotransfection of SRC-1 isoforms enhanced the TR-mediated transactivation of luciferase expression in a different manner. On palx3 Luc (Fig. 4A), SRC-1E significantly enhanced the transactivation in a dose dependent manner, while both SRC-1 and SRC-1 (-Q) exerted little effect. On F2x3 Luc (Fig. 4B), both SRC-1 and SRC-1(-Q) showed a significant enhancement of transactivation only when they were cotransfected at a dose of 200 ng per well. Yet, SRC-1E showed stronger enhancement of transactivation than SRC-1 or SRC-1(-Q). Thus we concluded that SRC-1E enhances transactivation more efficiently than SRC-1 or SRC-1(-Q).

DISCUSSION

In the present report, we showed that 4 different mRNA species are present for human SRC-1 and they

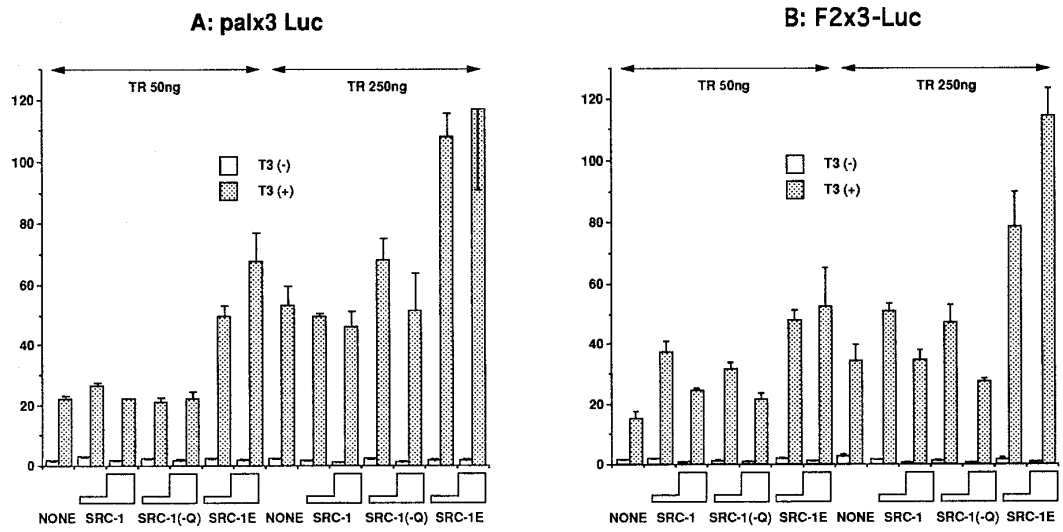

FIG. 4. Enhancement of thyroid hormone dependent transactivation by SRC-1 variants. HepG2 cells were transfected with reporter plasmid, (A) palx3 Luc or (B) F2x3 Luc, and β-galactosidase plasmid to correct the transfection efficiency. The amount of pcDNAI/Amp-TR was shown on the top of the fiture. pRc/CMV-SRC-1 isoforms were cotransfected at doses of 200 ng or 1000 ng (shown at the bottom). After the transfection, the cells were incubated in the absence, (−) or presence (+) of 100 nM T3 for 48 hours. The luciferase activity was corrected by β-galactosidase activity. The data from triplicate determinations were expressed as mean ± SD.

are generated by alternative exon(s) and splicing acceptor sites usage. The four different mRNAs encode three different polypeptides namely SRC-1, SRC-1(-Q) and SRC-1E. SRC-1E revealed to be more abundantly expressed than other isoforms in all the cell lines tested. We also showed that SRC-1 has a stronger potential to enhance T3/TR dependent transactivation than SRC-1 or SRC-1(-Q).

During this investigation, three groups reported the cloning of SRC-1 independently in addition to the first report by Onate et al (6). Kamei et al characterized CREB-binding protein (CBP)/p300 as a coactivator of nuclear receptors, identified a protein with molecular mass of 160 kD as another coactivator, which was cloned and revealed to be mouse SRC-1 cDNA (10). Their cDNA corresponds to the SRC-1(-Q) in the present report. They also reported the presence of several splicing variants and their nomenclature was incorporated for the human SRC-1E identified in the present study. Takeshita et al cloned full-length human SRC-1(-Q) by far western approach using the ligand binding domain of thyroid hormone receptor as a probe (11). Yao et al cloned mouse SRC-1E by two hybrid strategy using p300 as bait (12). Yet, neither relative abundance nor functional properties of SRC-1 splicing variants were studied in these reports.

Since SRC-1E is active in enhancing T3 dependent transactivation, it was suggested that the very C-terminal part of SRC-1 is not required to enhance transactivation by TR. Recently several candidates for coactivators that interact with nuclear receptors in the presence of the cognate ligands were identified. These include TIF1(13, 14), RIP-140(15), TRIP-1/Sug1 (16), TIF2/GRIP1(17, 18), and ARA70 (19). Low similarity among these molecules suggests that a considerable divergence is allowed for the domain structure to interact with liganded nuclear receptors. Only exception is TIF2/GRIP1, which shows nearly 60% similarity with SRC-1(17). Interestingly, C-terminal end of TIF2/GRIP1 corresponds to the position where SRC-1 and SRC-1E diverge, and yet TIF2/GRIP1 interact with nuclear receptors. This finding is in a good accordance with our assumption that SRC-1 variants having different C-terminus interact with TR. Recently another nuclear receptor interacting domain was identified in the middle part of SRC-1 protein by Yao et al (12), in addition to the one in the C-terminal by Onate et al (6). Yao et al also characterized the domain that interact with p300 in the middle part of the molecule (12), which is retained in all the presently reported SRC-1 isoforms (see Fig. 1).

RT-PCR analysis showed that SRC-1E is more abundantly expressed than SRC-1 or SRC-1(-Q) in all the cell line tested herein. More detailed analysis in SK-N-SH cell line led us to estimate SRC-1E transcripts comprises 67% of the mRNA coding SRC-1 isoforms. Although the relative abundance of SRC-1 isoforms at the protein level is a matter of conjecture, the present three isoforms of SRC-1 could be translated in similar efficiency in *in vitro* translation system (data not shown), supporting that the abundance of mRNA reflects the abundance at the protein level. Since SRC-1 and SRC-1E differs each other by less than 50 amino acid residues in length, discrimination of these molecules by SDS-PAGE was very difficult. We believe that the proteins with molecular mass of 160 kD identified

by western blotting (12) or the interaction with CBP/p300 (10) contains all the three isoforms identified in the present study. Development of isoform-specific anti SRC-1 antibodies is required to determine the expression of SRC-1 isoforms at the protein level. On the other hand, other splicing variants (SRC-1b, c, and d) identified by Kamei et al (10) are shorter in size than SRC-1 by more than 200 amino acids, suggesting these splicing variants are expressed much less compared to three isoforms identified in this study.

Our study suggested that SRC-1E is expressed more abundantly in a broad range of the cells and that SRC-1E can enhance T3/TR mediated transactivation more efficiently than other isoforms. Although a major determinant of tissue sensitivity to thyroid hormones is the abundance of thyroid hormone receptor (20), some part of the tissue sensitivity to thyroid hormone can be modified by the abundance of cofactors mediating transcriptional regulation (21). We speculate that the control of expression and alternative splicing of SRC-1 play some physiological roles through the modification of tissue sensitivity to thyroid hormone.

ACKNOWLEDGMENTS

The present work was supported by a research fellowship from Japan Society for the promotion of the Science (for Y.H.) and by grant-in-aids from Ministry of Health and Welfare (for H.S.).

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