A Nuclear Factor, ASC-2, as a Cancer-amplified Transcriptional Coactivator Essential for Ligand-dependent Transactivation by Nuclear Receptors in Vivo*

(Received for publication, July 6, 1999, and in revised form, August 25, 1999)

Soo-Kyung Lee‡, Sarah L. Anzick§, Ji-Eun Choi‡, Lukas Bubendorf§, Xin-Yuan Guan§, Yong-Keun Jung, Olli P. Kallioniemi§, Juha Kononen§, Jeffrey M. Trent§, David Azorsa§, Byung-Hak Jhun‡, Jae Hun Cheong‡, Young Chul Lee‡, Paul S. Meltzer***, and Jae Woon Lee‡‡‡

From the ¶Center for Ligand and Transcription and §§Hormone Research Center, Chonnam National University, Kwangju 500-757, Korea, and the ¶¶Hormone Research Center, Chonnam National University, Pusan National University, Pusan 609-735, Korea, and the ¶¶¶Department of Life Sciences, Kwangju Institute of Science and Technology, Kwangju 500-303, Korea

Many transcription coactivators interact with nuclear receptors in a ligand- and C-terminal transactivation function (AF2)-dependent manner. We isolated a nuclear factor (designated ASC-2) with such properties by using the ligand-binding domain of retinoid X receptor as a bait in a yeast two-hybrid screening. ASC-2 also interacted with other nuclear receptors, including retinoic acid receptor, thyroid hormone receptor, estrogen receptor α, and glucocorticoid receptor, basal factors TFIIA and TBP, and transcription integrators CBP/p300 and SRC-1. In transient cotransfections, ASC-2, either alone or in conjunction with CBP/p300 and SRC-1, stimulated ligand-dependent transactivation by wild type nuclear receptors but not mutant receptors lacking the AF2 domain. Consistent with an idea that ASC-2 is essential for the nuclear receptor function in vivo, microinjection of anti-ASC-2 antibody abrogated the ligand-dependent transactivation of retinoic acid receptor, and this repression was fully relieved by coinjection of ASC-2-expression vector. Surprisingly, ASC-2 was identical to a gene previously identified during a search for genes amplified and overexpressed in breast and other human cancers. From these results, we concluded that ASC-2 is a bona fide transcription coactivator molecule of nuclear receptors, and its altered expression may contribute to the development of cancers.

The nuclear receptor superfamily is a group of ligand-dep-tent transcriptional regulatory proteins that function by binding to specific DNA sequences named hormone response elements in the promoters of target genes (for a review, see Ref. 1). The superfamily includes receptors for a variety of small hydrophobic ligands such as steroids, T3,1 and retinoids as well as a large number of related proteins that do not have known ligands, referred to as orphan nuclear receptors (reviewed in Ref. 2). Functional analysis of nuclear receptors has shown that there are two major activation domains. The activation function-2 (AF-2) at the extreme C-terminal region of the ligand-binding domain (LBD) exhibits ligand-dependent transactivation, whereas the N-terminal activation function-1 contains a ligand-independent transactivation domain. The AF-2 region is conserved among nuclear receptors, and deletion or point mutations in this region impair transcriptional activation without changing ligand and DNA binding affinities. X-ray crystallographic studies of the LBD of nuclear receptors revealed that the ligand binding induces a major conformational change in the AF-2 region (3–7), suggesting that this region may play a critical role in mediating transactivation by a ligand-dependent interaction with coactivators. As expected, many coactivators fail to interact with AF-2 mutants of nuclear receptors (8–10).

Transcriptional activation of most nuclear receptors involves at least two separate processes as follows: derepression and activation. Repression is mediated in part by interaction of unliganded receptors with corepressors such as N-CoR (11) and SMRT (12). However, ligand binding triggers dissociation of these corepressors and concomitant recruitment of coactivators. Transcription coactivators bridge transcription factors and the components of the basal transcriptional apparatus. In particular, the functionally conserved proteins CREB-binding protein (CBP) and p300 (reviewed in Ref. 13) have been shown to be essential for the activation of transcription by a large number of regulated transcription factors, including nuclear receptors (14–17), CREB (18–20), NFXβ (21), bHLH factors (22), STATs (23, 24), and AP-1 (25, 26). Steroid receptor coactivator-1 (SRC-1) (15–17, 27) and its family member p/CIP (28), originally isolated as nuclear receptor coactivators, were recently shown to coactivate CREB and STATs (28), NFXβ (29), AP-1 (30), SRF (31), and p53 (32). Based on this broad spectrum of action, these proteins (i.e. CBP/p300 and SRC-1) are termed transcription integrators.

The regulation of gene expression by nuclear receptors has

*This work was supported in part by grants from the KOSEF and the MEK (to B. H. J.) and the National Creative Research Initiatives of the Korean Ministry of Science and Technology (to J. W. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**To whom correspondence may be addressed. Tel.: 82-62-530-0910; Fax: +82-62-530-0772; E-mail: jlee@chonnam.chonnam.ac.kr (for J. W. L.) or E-mail: pmeltzer@nhgrl.nih.gov (for P. S. M.).

†The abbreviations used are: T3, triiodothyronine; AF-2, activating function-2; LBD, ligand-binding domain; CBP, CREB binding protein; SRC-1, steroid receptor coactivator-1; RXR, retinoid X receptor; ASC-2, activating signal cointegrator-2; DBD, DNA-binding domain; TR, thyroid hormone receptor; ER, estrogen receptor; ERE, estrogen response element; RAR, retinoic acid receptor; RARE, retinoic acid response element; GR, glucocorticoid receptor; 9-cis-RA, 9-cis-retinoic acid; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; FISH, fluorescence in situ hybridization; CREB, cAMP-response element-binding protein; FITC, fluorescein isothiocyanate; PCR, polymerase chain reaction; VDR, vitamin D receptor; TRAPs, thyroid hormone receptor-associated proteins; GST, glutathione S-transferase; TBP, TATA-binding protein.
Transcription Coactivator ASC-2 Amplified in Cancers

been postulated to involve targeted changes in chromatin structure (33–35). In particular, recent biochemical and genetic studies support the notion that hyperacetylation of core histones is characteristic to gene activation, whereas histone deacetylation is involved with transcriptional repression (36, 37). SRC-1 (38) and its homologue ACTR (39), along with CBP/p300 (40, 41), were recently shown to contain potent histone acetyltransferase activities themselves and associate with the histone acetyltransferase protein P/CAF (42). CBP/p300 also forms a complex with SRC-1 (15–17, 27). These results suggest that nuclear receptors target at least three different histone acetyltransferase activities (SRC-1 or related proteins CBP/p300 and P/CAF) to promoters (39). In contrast, it was shown that SMRT and N-CoR, nuclear receptor corepressors, form complexes with Sin3 and histone deacetylases proteins, suggesting that chromatin remodeling by cofactors contributes to receptor-mediated transcriptional regulation (43–46).

Genomic instability in human cancers commonly results in gene amplification and the consequent overexpression of specific genes. Breast cancers frequently exhibit increased copy number of chromosomal segments that encode genes related to tumor growth such as ERBB-2, MYC, and cyclin D1 (47). The application of molecular cytogenetic technology to breast cancers has identified several regions of increased DNA copy number whose target genes remain unknown (48, 49). In order to isolate candidate genes from one of these regions (20q), chromosome microdissection and hybrid selections were used to isolate amplified cDNAs. In particular, three novel amplified genes of potential biological relevance to cancer progression (termed AIB1, AIB3, and AIB4) have been isolated from a cDNA library constructed from the 20q amplified breast cancer cell line BT-474 (50). These genes were mapped to 20q11–12 (AIB3 and AIB4) and 20q12 (AIB1) by fluorescence in situ hybridization. AIB1 was subsequently shown to be a member of the SRC-1 family and a coactivator of ERα (51).

In this study, we report the isolation of a novel nuclear protein-activating signal cointegrator 2 (ASC-2) based on its interaction with retinoid X receptor (RXR) which on sequence analysis is identical to AIB3. Consistent with specific interactions with basal factors TFIIA and TBP, ASC-2 exhibits an autonomous transactivation function in yeast. ASC-2 also interacts with transcription integrators SRC-1 and CBP and stimulates transactivation by nuclear receptors in conjunction with these proteins. Remarkably, microinjection of anti-ASC-2 antibody blocks the ligand-dependent transactivation of nuclear receptors, and this repression is fully relieved by coinjection of ASC-2 expression vector. Thus, ASC-2 is a novel transcriptional coactivator molecule required for the ligand-dependent transactivation of nuclear receptors in vitro, and its altered expression may contribute to the development of cancers.

EXPERIMENTAL PROCEDURES

Hormones, Yeast Cells, and Plasmids—Estrogen, T3, and 9-cis-RA were obtained from Sigma. Y190 cells and a parental vector pAS2–1 are described (CLONTECH). A chimeric protein consisting of Gal4 DNA-binding domain (DBD) fused to the LBD of human RXRα was constructed by PCR (Gal4-DBD/RXR-LBD). EGY48 cells, the LexA-β-galactosidase reporter construct, the LexA and B42 parental vectors were as reported (52). LexA fusions to the LBDs of RXRα and its AF-2 mutant and thyroid hormone receptor β (TRβ) and its AF-2 mutant as well as full-length TRα, estrogen receptor α (ERα), RXRα, and retinoic acid receptor α (RARα) were as described previously (52, 30, 39, 50). Activation domain fusions to Asc2-A, Asc2-B, Asc2-C, CBP-A, CBP-B, CBP-C, CBP-D, and CBP-E as well as LexA fusions to Asc2-1, Asc2-2, Asc2-3, Asc2-4, Asc2-5, SRC-N, SRC-M, and SRC-C were constructed by either using PCR or appropriate restriction sites. GST vectors encoding glucocorticoid receptor (GR/LBD, ERα/LBD, RARα, TRβ, RXRα, RXRα/ABC, RXRα/LBD, RXRβ/LBDΔAF, CBP1, CBP2, CBP3, CBP4, CBP5, ASC-1, TBP, and TFIIA as well as a T7 vector encoding c-Jun, along with B42 fusions to SRC-N, SRC-B, SRC-C, SRC-D, and SRC-E, were as described (28–30, 53, 54). Vectors expressing GST fusion to the LBDs of RXRα, RXRβ, and RXRα/β were similarly constructed into EcoRI and XhoI restriction sites of pCDNA for T7-directed in vitro transcription/translation and mammalian expression. Mammalian expression vectors for SRC-1, p300, TRβ1, ERαAF2, ERα, RXRα, and RXRαAF2, the reporter constructs ERE-Luc, MMTV-LUC, βARE-TK-LUC, and TRPα1-TK-LUC, and the transfection indicator construct pHSV-β-gal were as described (29–31, 55).

Yeast Two-hybrid Screening and Yeast β-Galactosidase Assay—Gal4-DDB/RXR-LBD was used as bait to screen a Xenopus oocyte cDNA library in pGAD10 vector (CLONTECH) for RXR-interacting proteins, according to the manufacturer's protocols. However, the screening was executed either in the presence or absence of 1 μM 9-cis-RA as described (52). The library plasmids from positive clones that expressed both HIS3 and lacZ reporters were rescued and retransformed into yeast cells, together with the original or other bait constructs, to test specificity of the interactions. The cotransformation and β-galactosidase assays in yeast were performed as described (52). For each experiment, at least three independently derived colonies expressing chimeric receptors were tested.

GST Pull Down Assays—The GST fusions or GST alone was expressed in Escherichia coli, bound to glutathione-Sepharose-4B beads (Amersham Pharmacia Biotech), and incubated with labeled proteins expressed by in vitro translation using the TNT-coupled transcription-translation system, with conditions as described by the manufacturer (Promega, Madison, WI). Specifically bound proteins were eluted from beads with 40 mM reduced glutathione in 50 mM Tris (pH 8.0) and analyzed by SDS-PAGE and autoradiography as described (56).

Cell Culture and Transfection—CV-1 or HeLa cells were grown in 24-well plates with medium supplemented with 10% charcoal-stripped serum. After 24 h incubation, cells were transfected with 100 ng of β-galactosidase expression vector pRSV-β-gal and 100 ng of an indicated reporter gene, along with hRXRα-, ERα-, ERαAF2, RXRαAF2, ASC-2-, SRC-1-, or p300-expression vectors. Total amounts of expression vectors were kept constant by adding decreasing amounts of the CMV expression vector (i.e. pCDNA3) to transfections. Twelve hours later, cells were washed and refed with Dulbecco's modified Eagle's medium containing 10% charcoal-stripped fetal bovine serum. After 12 h, cells were left unstimulated or stimulated with 0.1 μM 9-cis-RA as described (57). ASC-2 expression. Consistent results were obtained in more than two similar experiments.

Fluorescence in Situ Hybridization (FISH)—AIB3-specific oligonucleotide primers, G3F1 (TTTATTCACTGGTCCATTTCTACA) and G3R2 (AGTTTTCACATTTCCTAAGC), were used to screen a human genomic BAC library (Research Genetics). A positive BAC clone was labeled with Spectrum Orange-DUTP (Vysis) using the BioPrime DNA labeling system (Life Technologies, Inc.), purified through a Bio-Spin 6 chromatography column (Bio-Rad), and ethanol-precipitated. Hybridizations of breast cancer cell lines were performed as described (58). The 12 breast cancer cell microarray specimens included in the multicancer microarray were colon (32), stomach (12), renal cell (24), testis (19), non-small cell lung (78), prostate (19), bladder (34), endometrial (8), ovarian (22), head/neck (15), carcinoma (3), phyllodes (4), pheochromocytoma (2), thyroid (2), and melanoma (4).

Northern Blot Analysis—Breast cancer cell lines were obtained from the American Type Culture Collection (BT-474, T-47D, MCF-7, BT-20, 31, 55).
few different human cDNA libraries. A number of full-length cDNA clones encoding 2,063 amino acids were obtained in which the initiator methionine is preceded by a few in-frame stop codons. The amino acid sequence (Fig. 2) shows that ASC-2 contains an acidic domain at the N terminus, two glutamine-rich domains, and a serine/threonine-rich domain at the C terminus. It also contains two separate basic domains, potential nuclear localization signals, and two copies of a sequence motif LXXLL (where L and X denote leucine and any amino acid, respectively), recently shown to function in ligand-dependent interaction with the AF-2 domain of nuclear receptors (28, 63).

Amplification and Overexpression of ASC-2 in Human Cancers—Remarkably, ASC-2 is identical to AIB3, a gene previously identified during a search for amplified and overexpressed genes mapping to chromosome 20q in breast cancers (50). ASC-2 maps to 20q11 and defines a distinct region of amplification (64). To determine the frequency of ASC-2 amplification in breast cancer, we examined 335 specimens by FISH using the recently developed tissue microarray technology (59) (Fig. 3A). In this series, ASC-2 copy number was increased to a moderate level (4–6 copies) in 14/335 (4.2%) cases and to a high level (>6 copies) in 15/335 (4.5%) cases (Table I). We further extended our search for ASC-2 amplification to 284 specimens of various cancer types using tissue microarrays. A pattern of recurrent ASC-2 amplification was also observed in colon and non-small cell lung cancer (Table I) with the frequency of increased copy number in colon cancer being the highest observed in any cancer studied. The presence of amplification in colon, breast, and lung cancers suggests that increased quantities of ASC-2 may contribute to tumor growth in these diseases. In addition, ASC-2 mRNA expression was detected in each of 11 breast cancer cell lines with the highest level of expression in BT-474 (Fig. 3B). Western analyses also showed that ASC-2 protein was differentially expressed in various breast cancer cell lines (Fig. 3C). A relatively high expression of ASC-2 in MDA-MB-231 cells and a low level expression in HT29 or HeLa cells were also observed (data not shown). Interestingly, two protein bands appear to exist, which could have resulted from post-translational modification such as phosphorylation or represent distinct isoforms. It is also notable that high levels of expression are not strictly correlated with ERα positivity. Although ASC-2 is expressed in three of four ERα-positive cell lines, significant expression is apparent in two ERα-negative cell lines BT20 and MDA-MB-453 and with very low expression in ERα-positive MCF7 cell lines.

Interactions of ASC-2 with Nuclear Receptors—The original Xenopus ASC-2 isolates P1, P4, and P5 were isolated from the yeast two-hybrid screening in the presence of the RXR ligand 9-cis-RA, whereas M8, identical to P1, was independently isolated in the absence of 9-cis-RA (Fig. 2A). All of these proteins showed a strong ligand dependence in interactions, i.e. their interactions with RXR were significantly stimulated in the presence of 9-cis-RA (data not shown). Consistent with these results, activation domain fusions to the ASC-2 amino acids 438–1041 (ASC-2c) showed strong ligand-dependent interactions with LexA-RXRA/LBD in yeast (Fig. 4A). These proteins also showed ligand-dependent interactions with LexA fusions to ERα (LexA-ERα), RARα (LexA-RARα), and TRα (LexA-TRα) as well as the LBDs of TRβ (LexA-TRβ/LBD) and GR (LexA-GR/LBD). These interactions, however, were completely abolished by a mutation in the AF-2 domain (see the results with LexA-RXRA/LBDΔAF and LexA-TRβ/LBDΔAF). Similar results were also obtained with the ASC-2 amino acids 586–1041 (ASC-2a) except that interactions with RXRA/LBD and RARα were not as evident. Interestingly, the ASC-2 amino acids

FIG. 1. Northern blot analysis of ASC-2. Northern analysis was performed with Xenopus oocyte cDNA library using the LBD of RXRα as a bait in the yeast two-hybrid system (62). Among 11 isolates that showed 9-cis-RA- and AF-2-dependent interactions, two were found to encode sSRC-3, a new member of the SRC-1 family (54). Four independent isolates encoded various lengths of an identical protein that showed ability to interact functionally with nuclear receptors (this paper) and a series of distinct transcription factors. Accordingly, we named this protein ASC-2 (for activating signal cointegrator). ASC-2 is encoded by a gene that showed 94% identity and 65% similarity in amino acid level (GenBankTM accession number D80003). The 5’ 325-base fragment of this human clone was used as a probe to screen a
586–860 (ASC-2b) readily interacted with TRα and TRβ/LBD but not with RXRα/LBD and RARα. These yeast results were further confirmed by direct in vitro binding (56). ASC-2 and ASC-2DC that includes only ASC-2 amino acids 1–928 interacted relatively weakly with a full-length RXRα-glutathione S-transferase fusion protein (GST-RXRα) (Fig. 4B) but did not bind luciferase and other control proteins (data not shown). These weak interactions were significantly stimulated by 9-cis-RA. A similar ligand-dependent interaction was also observed with a GST fusion to the LBD of RXR (GST-RXRα/LBD), which was completely abolished by a mutation in the AF-2 domain (55). In addition, ASC-2 and ASC-2ΔC independently interacted with the N-terminal ABC domains of receptors, TBP and TFIIA, CBP, and SRC-1 as are indicated. B, two potential nuclear localization signals are italicized, whereas glutamine-rich- and glutamine/proline-rich domains are in bold. Two copies of the LXXLL motif (28, 63) are underlined and in bold. The N-terminal acidic residues are underlined and the C-terminal serine/threonine-rich sequences are indicated with arrows.

**FIG. 2. Structure and amino acid sequences of ASC-2.** A, schematic diagram of ASC-2 is shown, in which the N-terminal acidic, glutamine-rich, glutamine/proline-rich, and serine/threonine-rich domains as well as two basic domains and two LXXLL motifs (28, 63) are as indicated. Three original isolates of Xenopus ASC-2 from the yeast two-hybrid screening, activation domain, and LexA fusions of ASC-2 and in vitro translated ASC-2 constructs are schematically drawn with amino acid numbers at junctions indicated. The deduced interaction domains with nuclear receptors, TBP and TFIIA, CBP, and SRC-1 are as indicated. B, two potential nuclear localization signals are italicized, whereas glutamine-rich- and glutamine/proline-rich domains are in bold. Two copies of the LXXLL motif (28, 63) are underlined and in bold. The N-terminal acidic residues are underlined and the C-terminal serine/threonine-rich sequences are indicated with arrows.
activities associated with ASC-2, we expressed a series of ASC-2 fragments fused to a heterologous DNA-binding protein LexA in yeast, along with a lacZ reporter construct controlled by upstream LexA-binding sites (57). As shown in Fig. 5A, ASC2-1 consisting of the ASC-2 residues 1–557 (as depicted in Fig. 2A) exhibited a very strong transactivation function. In addition, ASC2–2 (the ASC-2 residues 391–1057) showed a relatively strong transactivation activity, whereas more C-terminal fragments of ASC-2 were transcriptionally inert. These results suggest that ASC-2 may directly associate with RNA polymerase II pre-initiation complex by recruiting basal transcription factors such as TBP and TFIIA. Consistent with this idea, both ASC-2 and ASC-2ΔC readily interacted with TBP and TFIIA in vitro, whereas SRC-1 interacted only with TBP, and IκBβ did not interact with either protein (Fig. 5B). Thus, we concluded that the N-terminal subregions of ASC-2 have autonomous transactivation function, at least in yeast, probably through recruitment of basal transcription factors TBP and TFIIA.

**TABLE I**

| Cancer (n) | Moderate (4–6 copies) | High (>6 copies) |
|------------|-----------------------|------------------|
| Breast (335) | 14 (4.2%) | 15 (4.5%) |
| Colon (32) | 6 (18.8%) | 4 (12.5%) |
| Lung (78) | 7 (9%) | 3 (3.8%) |

Fig. 3. Amplification and overexpression of ASC-2 in human cancers. A, interphase FISH demonstrates increased copy number of ASC-2 in nuclei of breast cancer cell line MDA-MB-453 (a) and a breast cancer biopsy (c and d). Biopsy specimens were analyzed by tumor microarray FISH (59). A portion of a hematoxylin and eosin-stained tumor microarray section (b) and individual tumors with high (c) or low (d) ASC-2 copy numbers are illustrated. FISH was performed using a Spectrum Orange-labeled ASC-2 BAC. B, expression levels relative to MCF-10 cells and modal ASC-2 gene copy numbers are indicated. Total RNA (15 μg) from established breast cancer cell lines was size-fractionated by gel electrophoresis, transferred to a nylon membrane, and hybridized with a 4.2-kilobase pair ASC-2 cDNA. A β-actin probe was used to normalize loading. C, ASC-2 protein expression levels were examined by Western analysis using a monoclonal antibody raised against the C-terminal peptide epitope of ASC-2. Nuclear extracts from different breast cancer cell lines have been examined as indicated. Tubulin expression was examined as a loading control.
a LexA fusion to the ASC-2 amino acids 586–1310 (i.e., LexA-ASC2–3) interacted only with SRC-D. In contrast, more C-terminal ASC-2 fragments (i.e., ASC2–4 and ASC2–5) didn’t show interactions with none of the SRC-1 fragments tested. Consistent with these results, ASC-2 and ASC2–ΔC specifically bound to GST fusions to SRC-M (i.e., the SRC-1 residues 782–1139) and SRC-C (i.e., the SRC-1 residues 1107–1441) but not to SRC-N (i.e., the SRC-1 residues 1–415), as shown by the in vitro GST-pull down assays (Fig. 6B). In contrast, c-Jun interacted only with GST-SRC-C, as we recently reported (30). These results indicate that multiple interactions occur between the ASC-2 amino acids 1–1310 and various SRC-1 subregions.

ASC-2, as a Transcription Coactivator of Nuclear Receptors—To assess the functional consequences of these interactions, ASC-2 was cotransfected into CV-1 cells along with a reporter construct ERE-TK-LUC. Increasing amounts of co-transfected ASC-2 enhanced the E2-dependent transactivation of this reporter in a dose-dependent manner, with cotransfection of 200 ng of ASC-2 increasing the fold activation approximately 3-fold (Fig. 7A). Interestingly, the ASC-2 enhancement of reporter gene expression was further stimulated with co-transfected SRC-1 or p300. Consistent with the protein interaction data, however, ASC-2 was not able to coactivate ERαΔAF-driven transactivations. Similar results were also obtained with a series of different reporter constructs responsive to GR, progesterone receptor, RXRs, TRs, or RARs in various cell lines. The results suggest that multiple interactions occur between the ASC-2 amino acids 1–1057 and various CBP subregions.
cell types (Fig. 7, B–D, and data not shown). In contrast, ASC-2 had minimal effects on the basal expression of the reporter in the absence of ligand, expression of the control plasmids pRSV-b-gal or TK-LUC, or GAL4-VP16-mediated transactivation of the GAL4-TK-LUC reporter construct (Fig. 7 and data not shown). These results strongly support the notion that ASC-2 is a bona fide transcription coactivator of nuclear receptors.

ASC-2 Is Required for the Nuclear Receptor Function in Vivo—We raised and affinity purified a rabbit polyclonal antibody against the nuclear receptor binding domain of ASC-2, which specifically detected, in Western analyses, either bacterially expressed and purified ASC-2 or endogenous/cotransfected ASC-2 in mammalian cells (Fig. 8A and data not shown). In contrast, this ASC-2 antibody was not able to detect GST alone, GST/ASC-1, a novel transcription coactivator we have...
Rat-1 fibroblast cells with preimmune serum as well as phase contrast 
cates nuclear localization of endogenous ASC-2. Immunostainings of 
1009), as indicated.

not GST alone, GST/ASC-1, or GST/CBP2 (i.e. the CBP residues 451–

i.e. ASC-2-receptor interaction domain specifically recognizes ASC-2 but

various proteins shows that the polyclonal antibody raised against the

CBP residues 451–

Western analysis with

preimmune serum as well as phase contrast images are as indicated.

recently reported (65), or CBP2 (i.e. the CBP residues 451–1009) (Fig. 8A). Immunostaining of Rat-1 fibroblast cells with this antibody revealed that ASC-2 is a nuclear protein, as expected (Fig. 8B). Microinjection techniques were further utilized to investigate the function of ASC-2 \textit{in vivo} (61). Reporter genes were placed under the control of an SV40 minimal promoter containing 9-cis-RA-responsive βRARE sites (16). Remarkably, microinjection of anti-ASC-2 IgG almost completely prevented 9-cis-RA from activating an RAR-dependent transcription unit (Fig. 9) but had no effect on a promoter under the control of the cytomegalovirus promoter (data not shown). The percentage of cells that expressed the lacZ reporter (i.e. blue cells) was not observed among cells microinjected with control IgG in the absence of ligand but increased to approximately 70% in the presence of 9-cis-RA. However, only approximately 10% of cells turned blue even in the presence of 9-cis-RA, when microinjected with anti-ASC-2 IgG (Fig. 9). When cojected with ASC-2-expression vector, however, anti-ASC-2 IgG was not able to prevent 9-cis-RA-dependent transactivation. In contrast, cojection of pcDNA3 or SRC-1-expression vector was without any significant effects. However, cojection of p300 expression vector resulted in approximately 40% of blue cells. These results suggest that p300 but not SRC-1 is functional with receptor-mediated ligand-dependent transactivation in the absence of ASC-2 and also implicate multiple or redundant activation pathways in transactivating nuclear receptors, in which distinct sets of coactivator molecules are employed. Similar results were also obtained with RXR response elements

data not shown). Taken together with the transient transcription data, we concluded that ASC-2 is a molecule essential for the function of nuclear receptors \textit{in vivo}.

**DISCUSSION**

In this report, we have described the initial characterization of a novel coactivator protein ASC-2, which shows a strong ligand and AF-2 dependence in interactions with nuclear receptors (Fig. 4) and an autonomous transactivation function (Fig. 5A). Transcriptional activation of nuclear receptors involves at least two classes of cofactors, corepressors and coactivators (reviewed in Ref. 66). Corepressors that associate with unliganded nuclear receptors mediate repression, whereas coactivators are recruited upon ligand binding and concomitant dissociation of the corepressors. Currently, at least two mechanisms have been proposed to describe the function of these coactivators. First, they are postulated to function to transmit the signal of ligand-induced conformational change to the basal transcription machinery. Second, they have been associated with targeted alterations of chromatin structure (33–35).

Several groups of different macromolecular complexes containing transcription coactivators have been described. First, the TAF components of TFIID (reviewed in Ref. 67) and the SRB/MED components associated with polymerase II (reviewed in Ref. 68) comprise those that are ultimately associated with the general transcription machinery. The CBP-p300-SRC-1 coactivator complex defines the second coactivator complex that directly binds and coactivates a wide spectrum of different transcription factors. In particular, CBP/p300 was recently found to be complexed with a series of cellular proteins with relative molecular masses ranging from 44 to 270 kDa (69). Purification and analysis of various proteins in this group revealed that they are components of the human SWI-SNF complex and that p270 is an integral member of this complex. Interestingly, heterogeneity appears to exist among the CBP-p300-containing complexes. Different classes of mammalian transcription factors (nuclear receptors, CREB, and STATs) were recently shown to require functionally distinct components of the CBP-p300 coactivator complex, based on their platform or assembly properties (70). RAR, CREB, and STATs were further demonstrated to require different histone acetyltransferase activities within the CBP/p300 complex to activate transcription. In addition, p300 and CBP, despite their similarities, have been recently shown to have distinct functions during retinoid-induced differentiation of embryonic carcinoma F9 cells (71). Finally, B cell-specific OCA-B (72), a group of distinct nuclear proteins termed thyroid hormone receptor-associated proteins (TRAPs) (73), and a transcriptionally active nuclear complex that interacts only with liganded vitamin D receptor (VDR) (DRIPs) (74) define a group of coactivator complexes with rather specific functions. TRAPs purified from HeLa cells grown in the presence of thyroid hormone (T3) were found to markedly activate transcription by liganded TR \textit{in vitro}, whereas DRIPs consisting of a complex of at least 10 different proteins ranging from 65 to 250 kDa were found to coactivate the VDR-dependent transactivation. DRIPs and TRAPs are similar to each other, sharing common components, but distinct from the CBP-p300 complex, although like these coactivators, their interaction also required the AF-2 transactivation motif of VDR and TR (73, 74).

Based on its direct interaction with CBP and SRC-1 (Fig. 6), it is possible that ASC-2 could be included in the putative CBP-p300-SRC-1 complex. Consistent with this proposal, ASC-2 cooperated with SRC-1 and p300 to coactivate nuclear receptor transactivation (Fig. 7). Furthermore, ASC-2 also

**FIG. 8. ASC-2, as a nuclear protein.** A, Western analysis with various proteins shows that the polyclonal antibody raised against the ASC-2-receptor interaction domain specifically recognizes ASC-2 but not GST alone, GST/ASC-1, or GST/CBP2 (i.e. the CBP residues 451–1009), as indicated. B, immunostaining with anti-ASC-2 antibody indicates nuclear localization of endogenous ASC-2. Immunostainings of Rat-1 fibroblast cells with preimmune serum as well as phase contrast images are as indicated.
forms a complex with ASC-1 both in vitro and in vivo, a novel transcription coactivator that in turn forms a complex with CBP and SRC-1 in vivo (65). The microinjection results in which anti-ASC-2 antibody completely abolished the 9-cis-RA-dependent transactivation (Fig. 9) suggest that ASC-2 should be integral to sustain the function of this putative transcriptional coactivator complex. The associations with multi-functional CBP/p300/SRC-1 (Fig. 6) also suggest that ASC-2 may function with transcription factors other than nuclear receptors. Indeed, we recently found that ASC-2 is required for transactivation by a group of other transcription factors. Alternatively, ASC-2 may form a distinct coactivator complex in vivo and functionally associates/communicates with other co-activator complexes such as CBP-p300, SRC-1, and ASC-1. Consistent with this notion, our recent preliminary data indicated that ASC-1, ASC-2, CBP/p300, or SRC-1 appears to elute as a distinct complex of proteins from Superose 6 gel filtration column. It is also interesting to note that ASC-2, CBP/p300, and SRC-1 target identical receptor sites (i.e., the AF-2 domains), posing an interesting problem whether all of these factors co-occupy the same sites or assemble in an orderly fashion. The former possibility seems unlikely considering the relatively confined structure of the AF-2 core domain (3–7). Thus, it will be interesting to unravel their putative assembly order or the spatial relations between all of these distinct coactivator molecules in mediating transactivation of nuclear receptors.

Overexpression of amplified genes may provide a selective advantage for tumor growth. Recently, AIB1, an SRC-1 family member, was identified as a gene amplified and overexpressed in breast cancer (51), along with genes designated AIB3 and AIB4 (50). In this study, we found that ASC-2 is identical to AIB3, demonstrating that two distinct coactivator molecules of nuclear receptors can be co-amplified in cancer cells. ASC-2 maps to 20q11, substantially centromeric to AIB1 (which maps to 20q12) (51). Therefore, it is important to note that ASC-2 defines an independent region of amplification in tumors that have been surveyed for their pattern of amplification with probes mapping along 20q (64). Nonetheless, co-amplification of AIB1 and ASC-2 as observed in BT-474 is remarkable considering the related function of these proteins. This is consistent with a model in which the intrachromosomal amplicons (contained within homogeneously staining regions characteristically found in breast cancers) evolve from large chromosomal regions by a process favoring retention of target genes and deletion of biologically irrelevant intervening segments. Such a co-selection process may favor genes, which impinge on the same cellular processes. An analogous situation has been observed in amplicons from 12q which contain two genes, CDK4 and MDM2, which affect the G1 to S phase transition of the cell cycle without amplification of the region between these genes (75). Co-amplification of AIB1 and ASC-2 may result in significant effects on transcriptional regulation within tumor cells. Overexpression of these multifunctional coactivators could potentially perturb signal integration by these proteins and affect multiple signal transduction pathways. It will be of considerable interest to identify the putative transcription factors.
Transcription Coactivator ASC-2 Amplified in Cancers

which may be specifically targeted by increased levels of these coactivator proteins in vivo to sustain tumor growth.

Finally, it is notable that the LXXLL motif (28, 63) were not included in the minimum receptor-interaction domain mapped (Fig. 4). This was surprising since ASC-2 appeared to bind specifically the AF-2 domains of receptors in a ligand-dependent manner, also substantiated from the results in which these interactions were entirely abolished with mutations in the AF-2 domain (Fig. 4). In particular, the RXR mutant deleted for the AF-2 domain (i.e. RXRΔAF) was previously shown to bind 9-cis-RA with wild type affinity (55), excluding the possibility of other nonspecific effects such as inhibiting ligand binding. However, ASC-2 may contain other subregions that could independently associate with receptors. In particular, RXR appeared to weakly bind ASC2–4 (ASC-2 residues 1172–1729), which contains a singly copy of the LXXLL motif, in a 9-cis-RA-dependent manner, as demonstrated in the GST-pull down assays. Thus, this motif could still be involved with the receptor interactions under certain conditions. More detailed analyses to map these interactions are currently under way.

In conclusion, we identified a novel coactivator ASC-2, which is essential for the receptor function in vivo. ASC-2 is amplified and overexpressed in various human cancers and displays various properties of transcriptional coactivator, including an autonomous transactivation potential, the capacity for ligand-dependent interactions with the receptors, as well as interactions with the basal transcription factors and transcription integrators SRC-1 and CBP/p300. Further characterization of ASC-2 should provide important insights into the molecular mechanisms by which nuclear receptors modulate transcriptions as well as the tumorigenesis processes.

Acknowledgments—We thank Dr. Ming Tsai for SRC-1 clones; Dr. K. Y. Lee, S.-Y. Na, and J. W. Lee, manuscript in preparation.

Goodman, R. H. (1993) Nature 365, 855–859

Kowk, R. P. S., Lundland, J. R., Chrivia, J. C., Richards, J. P., Bachinger, H. P., Brennan, R. G., Roberts, S. G. E., Green, M. R., and Goodman, R. H. (1994) Nature 370, 223–226

Perkins, N. D., Felzien, L. K., Bettis, J. C., Leung, K., Beach, D. H., and Nabel, G. J. (1997) Science 275, 523–527

Eckner, R., Yao, T.-P., Oldread, E., and Livingston, D. M. (1996) Genes Dev. 10, 2478–2490

Bhattacharyya, S., Eckner, R., Grossman, S., Oldread, E., Arany, Z., Andreada, A., and Livingston, D. M. (1998) Nat. Struct. Biol. 5, 341–347

Zhang, J., Vinkemeier, U., Gu, W., Chakravarti, D., Horvath, C. M., and Darnell, J. E. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 15029–15034

Arias, J., Alberts, A. S., Brindle, P. C., Clarck, F. X., Smeal, T., Karin, M., Feramisco, J., and Montminy, M. (1994) Nature 370, 226–229

Bannister, A. J., and Kouzarides, T. (1995) EMBO J. 14, 4758–4762

Onate, S. A., Teai, S. Y., Tsai, M. J., and O'Malley, B. W. (1995) Science 270, 1354–1357

Torchia, J., Rose, D. W., Inostroza, J., Kamei, W., Westin, S., Glass, C. K., and Rosenfeld, M. G. (1997) Nature 387, 677–684

Na, S.-Y., Lee, S.-Y., Han, S.-J., Choi, H.-S., Im, S. Y., and Lee, J. W. (1999) Biol. Chem. 275, 10803–10808

5 S.-K. Lee, S.-Y. Na, and J. W. Lee, manuscript in preparation.
67. Burley, S. K., and Roeder, R. G. (1996) *Annu. Rev. Biochem.* **65**, 769–799
68. Myers, L. C., Gustafsson, C. M., Bushnell, D. A., Lui, M., Erdjument-Bromage, H., Tempst, P., and Kornberg, R. D. (1998) *Genes Dev.* **12**, 45–54
69. Dallas, P. R., Cheney, I. W., Liao, D. W., Bowrin, V., Ryan, W., Pacchione, S., Kobayashi, R., Yaciuk, P., and Moran, E. (1998) *Mol. Cell. Biol.* **18**, 3596–3603
70. Korzus, E., Torchia, J., Rose, D. W., Xu, L., Kurokawa, R., McInerney, E. M., Mullen, T. M., Glass, C. K., and Rosenfeld, M. G. (1998) *Science* **279**, 703–707
71. Kawasaki, H., Eckner, R., Yao, T.-P., Taira, K., Chiu, R., Livingston, D. M., and Yokoyama, K. K. (1998) *Nature* **393**, 284–289
72. Luo, Y., Fujii, H., Gerster, T., and Roeder, R. G. (1992) *Cell* **71**, 231–241
73. Fondell, J. D., Ge, H., and Roeder, R. G. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 8329–8333
74. Rachez, C., Sudan, Z., Ward, J., Chang, C. P. B., Burakov, D., Erdjument-Bromage, H., Tempst, P., and Freedman, L. P. (1998) *Genes Dev.* **12**, 1787–1800
75. Elkahloun, A. G., Bittner, M., Hoskins, K., Gemmill, R., and Meltzer, P. S. (1996) *Genes Chromosomes Cancer* **17**, 201–214
76. Altschul, S. F., Boguski, M. S., Gish, W., and Wootton, J. C. (1994) *Nat. Genet.* **6**, 119–129