Interactions between Activating Signal Cointegrator-2 and the Tumor Suppressor Retinoblastoma in Androgen Receptor Transactivation*

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Activating signal cointegrator-2 (ASC-2), a cancer-amplified transcription coactivator of nuclear receptors and numerous other transcription factors, was previously shown to contain two LXXLL motifs, each of which interacts with a distinct set of nuclear receptors. In this work, we showed that ASC-2 has an indirect, separate binding site for androgen receptor (AR). Interestingly, this region overlapped with the direct interaction interfaces with the tumor suppressor retinoblastoma (Rb). Although ASC-2 alone stimulated AR transactivation in cotransfections of HeLa cells, ectopic expression of Rb affected ASC-2 to act as a transcription coactivator of AR in Rb-null Saos2 cells. These results, along with the previous report in which AR was shown to directly interact with Rb (Yeh, S., Miyamoto, H., Nishimura, K., Kang, H., Ludlow, J., Hsiao, P., Wang, C., Su, C., and Chang C. (1998) Biochem. Biophys. Res. Commun. 248, 361–367), suggest that the AR-ASC-2 interactions in vivo may involve Rb. Thus, ASC-2 appears to contain at least three distinct nuclear receptor interaction domains.

The nuclear receptor superfamily is a group of proteins that regulate, in a ligand-dependent manner, transcriptional initiation of target genes by binding to specific DNA sequences named hormone response elements (reviewed in Ref. 1). Functional analysis of nuclear receptors has shown that there are two major activation domains. The N-terminal domain (i.e. AF-1) contains a ligand-independent activation function, whereas the ligand-binding domain exhibits ligand-dependent transactivation function (i.e. AF-2). The AF-2 core region, located at the extreme C terminus of the receptor ligand-binding domains, is conserved among nuclear receptors and undergoes a major conformational change upon ligand binding (1). This region has been shown to play a critical role in mediating transactivation by serving as a ligand-dependent interaction interface with many different coactivators (reviewed in Ref. 2). These coactivators, including the SRC/p160 family members, CBP/p300, pCAF, TRAP/DRIP, activating signal cointegrator-2 (ASC-2)1 and many others, bridge nuclear receptors and the basal transcription apparatus and/or remodel the chromatin structures (2).

A distinctive structural feature of the AF-2-dependent coactivators is the presence of LXXLL signature motifs (i.e. NR box) (3, 4). The AF-2 core region (helix 12), upon undergoing a major restructuring upon ligand binding, forms part of a “charged cleft” that accommodates coactivators within a hydrophobic cleft of the receptor ligand-binding domain, through direct contacts with these NR boxes (2). Interestingly, the N-CoR/SMRT nuclear receptor interaction motifs exhibit a consensus sequence of I/LXXLI/II (i.e. CoRN box, in which H indicates hydrophobic residues) (5, 6), which interacts with specific residues in the same receptor pocket required for coactivator binding. Thus, discrimination of the subtle differences between the coactivator and corepressor interaction helices by the nuclear receptor AF-2 core may provide the molecular basis for the exchange of coactivators for corepressors, with ligand-dependent formation of the charged clamp that stabilizes NR box binding and inhibits interaction with CoRN box helix.

ASC-2 (also named AIB3, TRBP, RAP250, NRC, and PRIP), gene-amplified and overexpressed in certain human cancers (7–12), contains two NR boxes (13). The C-terminal NR box specifically interacts with liver X receptors and the N-terminal NR box binds many different nuclear receptors, including retinoic acid receptor (13). Transgenic mice overexpressing ASC-2 fragment DN1 (the ASC-2 residues 489–929 containing the C-terminal NR box) were impaired for transactivation by liver X receptors (15). In addition, single cell microinjection of neutralizing antibodies against ASC-2 abolished transactivation by retinoic acid and other nuclear receptors that interact with ASC-2 (7). More recently, three different groups demonstrated that transactivation by retinoic

1 The abbreviations used are: ASC-2, activating signal cointegrator-2; AR, androgen receptor; GST, glutathione S-transferase; GFP, green fluorescence protein; RFP, red fluorescence protein; Rb, retinoblastoma; MMTV, murine mammary tumor virus; ChIP, chromatin immunoprecipitation.

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X receptor and PPARγ are abolished in mouse embryo fibroblast cells of ASC-2-null mice (16–18). Thus, ASC-2 is likely a bona-fide coactivator molecule of many nuclear receptors in vivo.

In this report, we found that ASC-2 has an indirect, separate binding site for androgen receptor (AR). This region, distinct from two previously characterized LXXLL motifs (13), overlapped with the newly defined, direct interaction interfaces with the tumor suppressor retinoblastoma (Rb). In addition, Rb appeared to be required for ASC-2-mediated transactivation of AR in cotransfections. Along with the previous report in which AR was shown to directly interact with Rb (19), these results suggest that the AR-ASC-2 interactions in two contexts are mediated by Rb. Overall, these results suggest that 1) ASC-2 is likely an essential coactivator of AR, and 2) ASC-2 contains at least three distinct nuclear receptor-interacting domains.

EXPERIMENTAL PROCEDURES

Plasmids—For glutathione S-transferase (GST) fusion vectors, PCR-fragments encoding Rb, Rbm1, Rbm2, and Rbm3 were cloned into EcoRI and XhoI restriction sites of pGEX4T (Amersham Biosciences). For VP16 fusion vectors, PCR-fragments for AR and Rb were cloned into EcoRI and XhoI restriction sites of pCMX-VP16. Similarly, a PCR-fragment encoding ASC2–2b was cloned into EcoRI and XhoI restriction sites of pCDNA3-HA-NLS. The mammalian expression vectors for ASC-2, Gal4/ASC2–2, Gal4/ASC2–1, Gal4/ASC2–2, Gal4/ASC2–3,5, Gal4/ASC2–4, Gal4/ASC2–2a, Gal4/ASC2–2b, Gal4/ASC2–2c, AR, and Rb, along with the transactivation construct pcRSV-β-gal and reporter constructs MMTV-luciferase and Gal4-luciferase, were as previously described (7, 13).

GST-Pull Down Assays—The GST-fusions or GST alone were expressed in Escherichia coli, bound to glutathione-Sepharose 4B beads (Amersham Biosciences), and incubated with labeled proteins expressed by in vitro translation by 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 previously (20).

Cell Culture, Transfections, Co-immunoprecipitation, and Immunohistochemistry—HeLa and CV-1 cells were grown in 24-well plates with medium supplemented with 10% fetal calf serum for 24 h and transfected with 100 ng of LacZ expression vector pRSV-β-gal and 100 ng of an indicated reporter gene, along with indicated amounts of various mammalian expression vectors. Total amounts of expression vectors were kept constant by adding pcDNA3. Transfections and luciferase assays were done as described (20), and the results were normalized to the LacZ expression. Similar results were obtained in more than two similar experiments. For co-immunoprecipitation experiments, 293T cells were cotransfected with mammalian expression vectors for Rb, ASC-2, and AR, as indicated. HL60 cells were also employed. Antibody coupling to protein G agarose and immunoprecipitation were executed as previously described (21). Immunohistochemistry was performed as previously described (22). Briefly, adult mouse testes were fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.6) overnight. Paraflin sections (5 μm in thickness) were prepared. Deparaffinized and hydrolyzed sections were incubated overnight with ASC-2 antibody as previously described (22) or AR antibody (mouse anti-AR prediluted antibody, Zymed Laboratories, Inc., San Francisco, CA), followed by secondary biotinylated antibody (22). Negative control staining was performed with pre-absorbed anti-ASC-2 serum (22) or non-immune mouse IgG at 1:200 dilutions (Vector Laboratories, Inc., Burlingame, CA).

Single-Cell Microinjection Assay—CV-1 fibroblast cells, made quiescent by incubating in serum-free medium for 24 h, were microinjected with either preimmune IgG or the affinity-purified αASC-2 IgG (13) along with reporter and indicator constructs (25 μg/ml each). The reporter construct consisted of the MMTV promoter driving the expression of green fluorescence protein (MMTV-GFP), whereas the cytomegalo virus promoter drives the expression of red fluorescence protein in microinjection indicator construct CMV-RFP. About 1 h after injection, cells were stimulated, where indicated, with 0.1 μM of testosterone. After 18 h incubation, cells were examined for GFP and RFP expressions as previously described (23).

Chromatin Immunoprecipitation (ChIP)—HEK293 cells overexpressing AR were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% charcoal-dextran-stripped fetal bovine serum, and were treated with 0.1 μM of testosterone. ChIP assays were essentially done as described (24). The primers used were 5′-TCGCCCCTTGTCCCCCTAGAT-3′ and 5′-AACCTTATAAGACCCCGACTCTC′.

RESULTS AND DISCUSSION

ASC-2 as an Essential Coactivator of AR—The nuclear receptor coactivator ASC-2 functions with two steroid receptors (i.e. estrogen and glucocorticoid receptors) via its N-terminal NR box (7, 13). To test whether or not the target steroid receptors of ASC-2 extend to AR, we microinjected IgG or monoclonal antibody against ASC-2 into the nuclei of CV-1 cells, along with AR-expression vector as well as CMV-RFP indicator and MMTV-GFP reporter constructs. Notably, this proviral MMTV promoter contains the well characterized cis-elements that are responsive to glucocorticoid, mineralocorticoid, progesterone, and testosterone (25). With IgG microinjected, the percentage of green cells (i.e. GFP-positive cells indicating of activated AR) over the total number of red cells (i.e. RFP-positive cells indicative of microinjected cells) increased from 0 to almost 75% upon addition of 0.1 μM of testosterone (Fig. 1A). In the presence of microinjected aASC-2 antibody and testosterone, however, it dropped to less than 20%, indicating that the endogenous ASC-2 in CV-1 cells is important for AR transactivation. To confirm these results, we executed transient cotransfection assays by employing a luciferase reporter construct driven by MMTV proviral promoter (i.e. MMTV-luciferase). As shown in Fig. 1B, increasing amounts of ASC-2-expression vector cotransfected into HeLa cells stimulated testosterone-dependent transactivation by AR in a dose-dependent manner. Notably, ASC-2 had no significant effect on the basal level of transactivation. Finally, we have employed ChIP assays to directly examine whether ASC-2 is recruited to endogenous AR target.
genes. Indeed, ASC-2 was recruited to the androgen response element in the prostate-specific antigen (PSA) gene (24) in a testosterone-dependent manner, along with AR itself (Fig. 1C). Taken together, these results strongly suggest that ASC-2 is an essential transcriptional coactivator protein of AR.

**Indirect Binding of ASC-2 and AR**—To understand the mechanistic basis of this ASC-2 recruitment to AR (Fig. 1C), we tried to localize the putative interaction interfaces between ASC-2 and AR. Surprisingly, the mapped AR-interacting region of ASC-2 (ARID in Fig. 2A) did not coincide with either of the two NR boxes. As demonstrated with the mammalian two hybrid tests (Fig. 2B), AR interacted with ASC2–2b that consists of the ASC-2 residues 622–849 (13) in a testosterone-independent manner, whereas it did not interact with other regions of ASC-2 including the N-terminal and C-terminal NR boxes (data not shown). Furthermore, from whole cell lysates of HEK293 cells cotransfected with expression vector for AR, αAR and αASC-2 antibodies co-immunoprecipitated ASC-2 and AR, respectively (Fig. 2C and data not shown). Consistent with the mammalian two hybrid tests, these interactions were also ligand-independent. To further probe the physiological significance of these interactions, we examined whether these two proteins are co-localized within the known target cells of AR in vivo. Indeed, we have found that ASC-2 is co-expressed with AR in the nuclei of Sertoli and Leydig cells, the major target cells of AR in testis (22) (Fig. 2D). However, it is important to note that we failed to detect any direct interaction between radio-labeled ASC-2 or ASC2–2b and GST fusion to AR as well as radio-labeled AR and GST-ASC2–2b (data not shown). These results imply that AR likely binds to ASC-2 via other adaptor protein(s) in vivo; i.e. the AR-ASC-2 interactions are indirect.

**Rb as a Putative Adaptor between ASC-2 and AR**—Rb was previously shown to function as a coactivator of AR via direct protein-protein interactions (19). Notably, these interactions were testosterone-independent similar to the indirect interactions of AR and ASC-2 (Fig. 2). Thus, we explored the possibility of Rb being the putative adaptor protein that mediates the AR-ASC-2 interactions in vivo. To test this idea, we transfected 293T cells with expression vectors for ASC-2, AR, and Rb. From the whole cell lysates of these cells, Rb was co-immunoprecipitated by αASC-2 antibody, and αAR antibody readily co-immunoprecipitated Rb in a ligand-independent manner (data not shown), consistent with the reported results (19). Similarly, the endogenous Rb and ASC-2 were co-immunoprecipitated from HL60 cells (Fig. 3A). Indicative of direct protein-protein interactions, radio-labeled ASC-2 readily interacted with GST fusion to Rb in the GST-pull down assays, and these interactions were localized to two independent subregions of Rb (Fig. 3B). We further localized the Rb-interacting region of ASC-2 to ASC2–2b in the mammalian two hybrid tests (Fig. 3C). It is important to note that this region of ASC-2 is identical to the indirect, AR-interacting domain we have localized (Fig. 2B). Taken together, these results suggest that Rb may act as an adaptor protein between AR and ASC-2.

**Rb Effects ASC-2 to Mediate AR Transactivation**—In cotransfections of HeLa cells, overexpression of ASC2–2b suppressed the AR transactivation (Fig. 4A), consistent with the importance of the ASC-2–AR/ASC2–2b interactions. Similarly, coexpression of Gal4 fusion to ASC2–2b also inhibited the AR transactivation (data not shown). Moreover, ectopic expression of Rb appeared to effect the ability of ASC-2 to mediate the AR transactivation in Saos2 cells that lack Rb (Fig. 4B). Taken together, these results further suggest that Rb is important for ASC-2 to mediate the AR transactivation. It is also possible that the previously described, AR-coactivator function of Rb (19) could have resulted from this indirect recruitment of ASC-2 to AR via Rb. Overall, our results strongly suggest that ASC-2 acts as a specific coactivator of AR in conjunction with Rb.

We have recently found that ASC-2 exists as a steady-state complex of ~2 MDa (26). This complex named ASC2–complex contains ALR-1 and its splicing isofrom ALR-2, ALR-like protein HALR, ASH2, the Rb-binding protein RBQ-3, and αβ-tubulins (26). In particular, ALR-1/ALR-2 and HALR exhibit specific histone methyltransferase (HMT) activity toward histone H3-lysine 4, and thus ASCOM represents the first nuclear receptor coactivator complex associated with this important histone modification function (26). Therefore, our working model (Fig. 4C) predicts that a ternary interaction of AR, Rb, and ASC-2 effects the recruitment of this ASC-2 complex/AS-
Fig. 3. Interactions of ASC-2 and Rb. A, co-immunoprecipitation of Rb and ASC-2. The whole cell lysates from HL60 cells were immunoprecipitated by αRb and IgG antibodies (IP) and probed with αASC-2 antibody (WB). − and + indicate the absence and presence of 0.1 μM of testosterone, respectively. B, The full-length Rb and a series of three Rb fragments, along with the amino acid numbers for each construct, are as depicted. Three pocket domains of Rb (A, B, and C) that mediate numerous protein-protein interactions are as indicated. The full-length ASC-2 was labeled with [35S]methionine by in vitro translation and incubated with glutathione beads containing GST alone, GST/Rb1, GST/Rb2, and GST/Rb3, as indicated. Beads were washed, and specifically bound material was eluted with reduced glutathione and resolved by SDS-polyacrylamide gel electrophoresis. Approximately 15% of the total reaction mixture was loaded as input.

Fig. 4. Rb effects ASC-2 to mediate AR transactivation. A and B, testosterone-responsive MMTV-luciferase reporter construct was cotransfected into HeLa (A) or Saos2 (B) cells, along with LacZ expression vector (100 ng) and expression vectors for ASC-2, ASC2-2h, AR, and Rb, as indicated. Closed and open boxes indicate the presence and absence of 0.1 μM of testosterone, respectively. Normalized luciferase expressions from triplicate samples were calculated relative to the LacZ expression. C, the working model for the Rb-ASC-2 interactions in AR transactivation. ASC-2, a component of a steady-state complex ASCOM (26), is recruited to AR via Rb, which results in the recruitment of ASCOM to AR.

COM to AR. Notably, other proteins such as the Rb-binding protein RBQ-3 (27) may also provide additional interaction interfaces to stabilize this AR/Rb/ASC2/ASCOM supramolecular complex. In turn, ASCOM is expected to exert its yet unknown regulatory functions on AR transactivation, which may involve chromatin remodeling of target genes via its histone modification activities. We have recently established mouse genetic models that specifically inactivate the methylation function of ASCOM2 and these mice may provide important insights into the exact regulatory functions of ASC-2/ASCOM with AR and other nuclear receptors. Finally, it should be noted that the ASC-2-Rb interactions we defined in this report may also play an important role with other target transcription factors of ASC-2.

REFERENCES

1. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) Cell 83, 835–839
2. McKenna, N. J., Lanz, R. B., and O’Malley, B. W. (1999) Endocr. Rev. 20, 231–244
3. Heery, D. M., Kalkhoven, E., Hoare, S., and Parker, M. G. (1997) Nature 387, 733–736
4. Torchia, J., Rose, D. W., Inostroza, J., Kamei, Y., Westin, S., Glass, C. K., and Rosenfeld, M. G. (1997) Nature 387, 677–684
5. Hu, X., and Lazar, M. A. (1999) Nature 402, 93–96
6. Periacci, V., Staszewski, L. M., McInerney, E. M., Kurokawa, K., Krones, A., Rose, D. W., Lambert, M. H., Milburn, M. V., Glass, C. K., and Rosenfeld, M. G. (1999) Genes Dev. 13, 3198–3208
7. Lee, S.-K., Anzick, S. L., Choi, J. E., Bubendorf, L., Guan, X. Y., Jung, Y. K., Kallioniemi, O. P., Kanonen, J., Trent, J. M., Arozarena, D., Jhun, B. H., Cheong, J. H., Lee, Y. C., Melzner, F. S., and Lee, J. W. (1999) J. Biol. Chem. 274, 34283–34293
8. Tannner, M. M., Tirkkonen, M., Kallioniemi, A. I., Javaherifar, T., Collins, C., Rowbel, D., Guan, Y. Y., Trenth, J., Gray, J. W., Melzner, F. S., and Kallioniemi, O. P. (1996) Cancer Res. 56, 3441–3445
9. Zhu, Y., Kan, L., Qi, C., Kanwar, Y. S., Yeldandi, A. V., Rao, M. S., and Reddy, J. K. (2000) J. Biol. Chem. 275, 13510–13516
10. Mahajan, M. A., and Samuels, H. H. (2000) Mol. Cell. Biol. 20, 5408–5403
11. Ko, L., Cardona, G. R., and Chin, W. W. (1999) Proc. Natl. Acad. Sci. U. S. A. 97, 6212–6217
12. Caira, F., Antonsen, P., Pelto-Huikko, M., Treuter, E., and Gustafsson, J. A. (2000) J. Biol. Chem. 275, 5308–5317
13. Lee, S.-K., Jung, S. Y., Kim, Y. S., Na, S. Y., Lee, Y. C., and Lee, J. W. (2001) Mol. Endocrinol. 15, 241–254
14. Kim, S. W., Cheung, C., Solan, Y. C., Goo, Y.-H., Oh, W. J., Park, J. H., Joe, S. Y., Kang, H.-S., Kim, D.-K., Lee, J. W., and Lee, H.-W. (2002) Mol. Cell. Biol. 22, 8409–8414
15. Kim, S. W., Park, K., Kwak, E., Choi, E., Lee, S., Ham, J., Kang, H., Kim, J. M., Hwang, S. Y., Kang, Y. Y., Lee, K., and Lee, J. W. (2003) Mol. Cell. Biol. 23, 3583–3592
16. Kuang, S. Q., Liao, L., Zhang, H., Pereira, F. A., Yuan, Y., DeMayo, F. J., Ko, L., and Xu, J. (2002) J. Biol. Chem. 277, 45596–45600
17. Zhu, Y. J., Crawford, S. E., Stelmach, V., Dwivedi, R. S., Rao, M. S., Gonzalez, F. J., Qi, C., and Reddy, J. K. (2003) J. Biol. Chem. 278, 1986–1990
18. Antonsen, P., Schuster, G. U., Wang, L., Rosell, B., Holter, E., Flodby, P., Treuter, E., Holmgren, L., and Gustafsson, J. A. (2003) Mol. Cell. Biol. 23, 1260–1268
19. Yeh, S., Miyamoto, H., Nishimura, K., Kang, H., Ludlow, J., Hsiao, P., Wang, D. K. Lee and J. W. Lee, unpublished results.
20. Na, S. Y., Kim, H. J., Lee, S. K., Choi, H. S., Na, D. S., Lee, M. O., Chung, M., Moore, D. D., and Lee J. W. (1998) *J. Biol. Chem.* 273, 3212–3215
21. Lee, Y. C., and Kim, Y. J. (1998) *Mol. Cell. Biol.* 18, 5364–5370
22. Zhang, H., Liao, L., Kuang, S. Q., and Xu, J. (2003) *Endocrinology* 144, 1435–1443
23. Jhun, B. H., Rose, D. W., Seely, B. L., Rameh, L., Cantley, L., Saltiel, A. R., and Olefsky, J. M. (1994) *Mol. Cell. Biol.* 14, 7466–7475
24. Shang, Y., Myers, M., and Brown, M. (2002) *Mol. Cell.* 9, 601–610
25. Truss, M., Chalepakis, G., and Beato, M. (1992) *J. Steroid Biochem. Mol. Biol.* 43, 365–378
26. Goo, Y. H., Sohn, Y. C., Kim, D. H., Kim, S. W., Kang, M. J., Jung, D. J., Kwak, E., Barlev, N. A., Berger, S. L., Chow, V. Y., Roeder, R. G., Azarsa, D. O., Meltzer, P. S., Suh, P. G., Song, E. J., Lee, K. J., Lee, Y. C., and Lee, J. W. (2003) *Mol. Cell. Biol.* 23, 140–149
27. Saijo, M., Sakai, Y., Kishino, T., Niikawa, N., Matsuura, Y., Morino, K., Tamai, K., and Taya, Y. (1995) *Genomics* 27, 511–519
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