The Transcriptional Repressor Sp3 Is Associated with CK2-phosphorylated Histone Deacetylase 2

Received for publication, June 25, 2002, and in revised form, August 7, 2002
Published, JBC Papers in Press, August 9, 2002, DOI 10.1074/jbc.C200378200

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Sp1 and Sp3 are ubiquitously expressed mammalian transcription factors that function as activators or repressors. Although both transcription factors share a common domain involved in forming multimers, we demonstrate that Sp1 and Sp3 form separate complexes in estrogen-dependent human breast cancer cells. Sp1 and Sp3 complexes associate with histone deacetylases (HDACs) 1 and 2. Although most HDAC2 is not phosphorylated in the breast cancer cells, HDAC2 bound to Sp1 and Sp3 and cross-linked to chromatin in situ is highly enriched in a phosphorylated form that has a reduced mobility in SDS-polyacrylamide gels. We show that protein kinase CK2 is associated with and phosphorylates HDAC2. Alkaline phosphatase treatment of HDAC2 and Sp1 and Sp3 complexes reduced the associated HDAC activity. Protein kinase CK2 is up-regulated in several cancers including breast cancer, and Sp1 and Sp3 have key roles in estrogen-induced proliferation and gene expression in estrogen-dependent breast cancer cells. CK2 phosphorylation of HDAC2 recruited by Sp1 or Sp3 could regulate HDAC activity and alter the balance of histone deacetylase and histone acetyltransferase activities and dynamic chromatin remodeling of estrogen-regulated genes.

Remodeling of chromatin structure mediated by ATP-driven chromatin-remodeling complexes and histone-modifying enzymes has a crucial role in gene expression. Acetylation of the core histones favors decondensation of the chromatin fiber by preventing interfiber interactions, whereas the unacetylated histone state contributes to chromatin condensation (1, 2). Dynamic histone acetylation catalyzed by histone deacetylases (HDACs) and histone acetyltransferases allows the chromatin fiber to rapidly oscillate from the condensed and decondensed states (3, 4). In mammalian cells three classes of HDACs are identified. Class I HDACs, such as HDAC1 and HDAC2, are homologous to yeast RPD3, whereas class II HDACs are similar to yeast HDA1. Class III HDACs are related to yeast SIR2 (5, 6). HDAC1 and -2 are components of large multisubunit complexes called Sin3 or NuRD, which are recruited by transcriptional factors such as Mad, YY1, and Rb (5, 7–9).

Mammalian cells ubiquitously express Sp1 and Sp3. Sp3 has three isoforms, a long (L-Sp3) and two short forms (M1-Sp3, M2-Sp3) that are the products of differential translational initiation (10). Sp3 may act as a repressor or an activator, with the short forms acting only as repressors (10). The protein structure of L-Sp3 is very similar to that of Sp1, except that Sp3 has a repression domain located N-terminal to the zinc finger DNA-binding domain (11). It has been reported that the relative levels of Sp3 forms change with differentiation, with the differentiated Caco-2 cells expressing more long than short forms (12). Further, alterations in the relative levels of Sp1 to Sp3 have been recorded, with Sp3 levels being greater than Sp1 in primary keratinocytes (13).

In this study we investigated the association of histone deacetylase with Sp3 in human breast cancer cells. We found that Sp3 and Sp1 were associated with HDAC1 and a modified form of HDAC2. HDAC2 is shown to be associated with protein kinase CK2 and phosphorylated by this enzyme. The low abundance phosphorylated form of HDAC2 is preferentially associated with chromatin.

**Experimental Procedures**

Cells and Plasmid—Human breast cancer T5 cells, estrogen receptor-positive and hormone-dependent, were grown in Dulbecco’s modified Eagle’s medium and 5% fetal bovine serum as previously described (4). T5 cells were grown under estrogen-depleted conditions, and in some cases estradiol was added for 20 min as reported (4). Plasmid pGST-HDAC2 has been described previously (14).

Cisplatin and Formaldehyde DNA Cross-linking—T5 cells were incubated with 1 μM cisplatin at 37 °C for 2 h or with 1% formaldehyde at room temperature for 10 min as described previously (3, 15). The methods for isolating the proteins cross-linked to DNA in situ are described in detail (16, 17). Briefly, following cross-linking, cells were washed twice with TNM buffer (100 mM NaCl, 300 mM sucrose, 10 mM Tris-HCl, pH 8.0, 2 mM MgCl₂, 1% thiodiglycol) containing 1 mM PMSF, phosphatase inhibitors (25 mM β-glycerophosphate, 10 mM sodium fluoride, 1 mM sodium orthovanadate) and protease inhibitor mixture (Roche Molecular Biochemicals). The cells were resuspended in cross-linking lysis buffer (5 mM urea, 2 mM guanidine hydrochloride, 2 mM NaCl, and 0.2 mM potassium phosphate buffer, pH 7.5) containing 1 mM PMSF, and the lysate was incubated with prehydroylated hydroxyapatite (BioRad). DNA-protein cross-links were reversed, and proteins were isolated.

Immunoprecipitation—T5 cells were lysed in immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA) containing 1 mM PMSF, phosphatase inhibitors, and protease inhibitors. The cells were sonicated twice for 15 s. The cell lysate was collected by centrifugation at 10,000 × g for 10 min at 4 °C and incubated with anti-Sp1 or anti-Sp3 antibodies for 16 h at 4 °C. The
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Fig. 1. Sp1 is not associated with Sp3. Two A549 of T5 cell lysate were incubated with 4 μg of anti-Sp1 antibodies, and the immunoprecipitation (IP, lane 2) and immunodepletion (ID, lane 3) fractions were collected. The immunodepleted fraction was next incubated with anti-Sp3 antibodies, yielding IP (lane 4) and ID (lane 5) fractions. Ten μl of cell lysate (lane 1), IP, and ID fractions were loaded onto a SDS-10% polyacrylamide gel, transferred to nitrocellulose membranes, and immunologically stained with anti-Sp1 and anti-Sp3 antibodies. The long (L) and short (M1 and M2) forms of Sp3 are identified.

Sequential Immunoprecipitations—Sequential immunoprecipitations were done as described above. Briefly, cell lysates were incubated with anti-Sp1 antibodies. The immunoprecipitated and immunodepleted (supernatant) fractions were collected. Secondary immunoprecipitations were done with anti-Sp1 antibodies, and the immunoprecipitated and immunodepleted fractions were collected. Sequential immunoprecipitations and immunoblotting experiments tested whether Sp1 associated with Sp3 but not Sp3 associated with Sp1. The immunodepleted supernatant was next incubated with anti-Sp3 antibodies, and the immunoprecipitate was harvested. Fig. 1 shows the analyses of the fractions immunologically stained with anti-Sp1 or anti-Sp3 antibodies. Anti-Sp1 antibodies efficiently immunoprecipitated Sp3 but not Sp3 (lane 2), whereas antibodies against Sp3 immunoprecipitated Sp3 but not Sp1 (lane 4). The results demonstrated that Sp1 does not associate with Sp3 in T5 cells. Further, the immunoblot results show that T5 breast cancer cells express the L-, M1-, and M2-Sp3 forms, with the latter lower molecular mass forms of Sp3 predominating. Quantification of Sp1 and Sp3 levels on immunoblots indicated that Sp1 was 3-fold more abundant than Sp3.

RESULTS AND DISCUSSION

Sp1 and Sp3 contain a similar D domain that is required for the proteins to form multimers (22–24). In vitro evidence suggests that Sp1 and Sp3 may form heteromultimers (24). Sequential immunoprecipitations and immunoblotting experiments tested whether Sp1 associated with Sp3 in situ in T5 human breast cancer cells. A T5 cell lysate was incubated with anti-Sp1 antibodies, and the immunoprecipitate was collected. The immunodepleted supernatant was next incubated with anti-Sp3 antibodies, and the immunoprecipitate was harvested. Fig. 1 shows the analyses of the fractions immunologically stained with anti-Sp1 and anti-Sp3 antibodies. Anti-Sp1 antibodies efficiently immunoprecipitated Sp1 but not Sp3 (lane 2), whereas antibodies against Sp3 immunoprecipitated Sp3 but not Sp1 (lane 4). The results demonstrated that Sp1 does not associate with Sp3 in T5 cells. Further, the immunoblot results show that T5 breast cancer cells express the L-, M1-, and M2-Sp3 forms, with the latter lower molecular mass forms of Sp3 predominating. Quantification of Sp1 and Sp3 levels on immunoblots indicated that Sp1 was 3-fold more abundant than Sp3.

It has been reported that Sp1 recruits HDAC1 to repress transcription (25). It is conceivable that Sp3 also recruits HDAC to repress transcription. Fig. 2A shows that Sp1 and Sp3 were associated with HDAC activity. Culturing breast cancer cells in the absence or presence of estradiol did not affect the HDAC activity associated with these transcription factors. The sequential immunoprecipitation strategy with anti-Sp1 followed by anti-Sp3 antibodies was applied to decide which of the HDACs was bound to Sp1 or Sp3. Fig. 2B shows that Sp1 and Sp3 were associated with HDAC1 and HDAC2. Both immunoprecipitates were highly enriched in a slower migrating form of HDAC2. Neither Sp1 nor Sp3 was associated with HDAC3 (data not shown).

The preferential association of the slower migrating form of HDAC2 with the transcription factors Sp1 and Sp3 suggested that this form of HDAC2 may be selectively in contact with chromatin. T5 cells were incubated with the cross-linker cisplatin, and the proteins cross-linked to nuclear DNA in situ were isolated. Unlike formaldehyde, cisplatin does not form protein-protein cross-links. Fig. 3A shows that the slower migrating form of HDAC2 was enriched in the proteins cross-linked to DNA. Similar results were obtained with the cross-linker formaldehyde. HDAC3 was not cross-linked to nuclear DNA with cisplatin.

As HDAC1 is phosphorylated (26, 27), we determined whether the slower migrating form of HDAC2 was phosphorylated by incubating the proteins cross-linked to DNA with alkaline phosphatase. Fig. 3B demonstrates that incubation of the protein sample with alkaline phosphatase resulted in the disappearance of the slower migrating form of HDAC2 and the appearance of a band co-migrating with the major HDAC2 band present in the cell lysate. Identical results were obtained when the Sp1 or Sp3 immunoprecipitates were incubated with alkaline phosphatase (data not shown). The phosphorylated form of HDAC1 has a reduced mobility on SDS gels (27). However, we did observe an enrichment of the slower migrating HDAC1-phosphorylated form in the Sp1 or Sp3 immunoprecipitates or in the protein fraction cross-linked to DNA with cisplatin. Treatment of these fractions with alkaline phosphatase did not alter the mobility of HDAC1, ruling out the possibility that the entire HDAC1 population was phosphorylated.

Recently, HDAC1 was shown to be phosphorylated by CK2 (26, 27). The CK2 phosphorylation sites located in the C-terminal region of HDAC1 are conserved in HDAC2 (27). Fig. 4A illustrates that incubation of HDAC2 in the cell lysate with CK2 and ATP generates the slower migrating HDAC2 form. Inhibition of CK2 with apigenin prevented the appearance of this band. Further, GST-HDAC2 and casein but not GST were radiolabeled with CK2 and [32P]ATP (Fig. 4B). To decide if CK2
was associated with HDAC2, HDAC2 and HDAC1 immunoprecipitates were analyzed by immunoblotting with anti-CK2α or anti-CK2α' antibodies (Fig. 4C). HDAC2 and to a lesser extent HDAC1 were bound to CK2. Immunoblotting experiments of Sp1 and Sp3 immunoprecipitates revealed the presence of CK2 (data not shown).

In immunoprecipitation and immunoblotting experiments we determined that most HDAC2 was in complex with HDAC1 in T5 breast cancer cells. HDAC1, which was more abundant than HDAC2, was in complex with HDAC2 and with other complexes not containing HDAC2. Incubation of HDAC1 and HDAC2 immunoprecipitates with alkaline phosphatase reduced the HDAC activity of the complexes (Fig. 5). The associated HDAC activities with Sp1 and Sp3 immunoprecipitates were also reduced when incubated with alkaline phosphatase.

In summary, we demonstrate that Sp3 and Sp1 are associated with HDAC1 and CK2-phosphorylated HDAC2. Although most HDAC2 is in an unmodified state, phosphorylated HDAC2 is preferentially associated with Sp1, Sp3, and chromatin in human breast cancer cells. CK2 is up-regulated in several cancers including breast cancer, and there is evidence that CK2 may promote breast cancer by deregulating key transcription processes (28–30). Many estrogen-induced genes (e.g., cathepsin D, c-fos, adenine deaminase, and c-myc) in human breast cancer cells have a half-life estrogen response element positioned next to a Sp1 binding site (31–33). Sp3 would compete with Sp1 to bind the regulatory regions of these genes. Both Sp1 and Sp3 may recruit HDAC1 and phosphorylated HDAC2 to these sites, whereas the estrogen receptor recruits histone acetyltransferases CBP (cAMP-response element-binding protein (CREB)-binding protein) and p300, resulting in dynamic acetylation of histones and transcription factors located at the promoters of these genes (4, 34, 35). CK2 phosphorylation of HDAC2 recruited by Sp1 or Sp3 would regulate HDAC activity and alter the balance of histone deacetylation and histone acetyltransferase activities and dynamic chromatin modeling of these estrogen-regulated promoters.

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FIG. 3. Phosphorylated HDAC2 is preferentially associated with chromatin. T5 cells were incubated with 1 mM cisplatin, and proteins cross-linked to DNA were isolated. Ten μg of protein was separated on a SDS-12% polyacrylamide gel, transferred onto nitrocellulose membranes, and immunochemically stained with anti-HDAC2 and anti-HDAC3 antibodies (A). Protein cross-linked to DNA was incubated with or without alkaline phosphatase, separated on a SDS-12% polyacrylamide gel, and immunochemically stained with anti-HDAC2 antibodies (B).
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