HDAC6 Is Required for Epidermal Growth Factor-induced β-Catenin Nuclear Localization*

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Nuclear translocation of β-catenin is a hallmark of Wnt signaling and is associated with various cancers. In addition to the canonical Wnt pathway activated by Wnt ligands, growth factors such as epidermal growth factor (EGF) also induce β-catenin dissociation from the adherens junction complex, translocation into the nucleus, and activation of target genes such as c-myc. Here we report that EGF-induced β-catenin nuclear localization and activation of c-myc are dependent on the deacetylase HDAC6. We show that EGF induces HDAC6 translocation to the caveolar membrane and association with β-catenin. HDAC6 deacetylates β-catenin at lysine 49, a site frequently mutated in anaplastic thyroid cancer, and inhibits β-catenin phosphorylation at serine 45. HDAC6 inactivation blocks EGF-induced β-catenin nuclear localization and decreases c-Myc expression, leading to inhibition of tumor cell proliferation. These results suggest that EGF-induced nuclear localization of β-catenin is regulated by HDAC6-dependent deacetylation and provide a new mechanism by which HDAC inhibitors prevent tumor growth.

HDAC inhibitors represent a new class of targeted drugs for cancer therapy (1). However, the role of HDACs in tumorigenesis and the key targets for HDAC action are not yet fully understood, making it difficult to select patients who are most likely to respond to HDAC inhibitors. Historically, histones were perceived to be the most important substrates of HDACs, if not the sole substrate. Recent studies, however, have revealed that HDACs have many non-histone substrates involved in a much broader array of biological processes (2). The best example is the cytoplasmic deacetylase HDAC6, which has been implicated in the regulation of microtubules, growth factor-induced chemotaxis, and misfolded protein stress response (3–8).

Wnt signaling plays a central role in both normal development and tumor formation. Abrupt activation of Wnt signaling has been associated with multiple types of cancer (9, 10). The key player in Wnt cascade is β-catenin, a cytoplasmic protein whose stability is regulated by the destruction complex, consisting of the tumor suppressor adenomatous polyposis coli protein, Axin, casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3). In unstimulated cells, β-catenin is associated with the destruction complex, allowing CK1 to phosphorylate β-catenin at Ser–45, a residue often mutated in cancers (11, 12). Subsequently, GSK3 phosphorylates additional serine and threonine residues near the N terminus of β-catenin, leading to its ubiquitination and degradation. Activation of the Wnt signaling pathway inhibits the kinase activity of the destruction complex by an unknown mechanism. As a consequence, stabilized β-catenin accumulates and translocates to the nucleus, where it forms complexes with the TCF/LEF-1 transcription factors and activates target genes such as c-myc and cyclin D1 (13).

In addition to its function in Wnt signaling, β-catenin also binds to E-cadherin and plays an essential role in cell–cell adherens junctions (14). Recently, it has been shown that EGF induces breakup of cell–cell junctions and increases the level of nuclear β-catenin, either by direct release of the functional β-catenin from the membrane-bound pool or by activating E-cadherin endocytosis (15). EGF receptor overexpression has been reported in many human tumors and correlates with poor clinical prognosis. EGF-induced release of β-catenin from the adherens junction not only contributes to epithelial-mesenchymal transition but also provides an alternative mechanism for activating β-catenin signaling. This cross-talk between EGF and Wnt signaling could represent an important mechanism underlying the effect of EGF during tumor development.

Here we report that EGF-induced β-catenin nuclear localization and c-myc expression are dependent on HDAC6. We found that EGF stimulates HDAC6-dependent deacetylation of β-catenin at Lys–49. This deacetylation leads to a reduction in β-catenin phosphorylation and promotes β-catenin nuclear accumulation. We showed that inactivation of HDAC6 inhibits β-catenin nuclear localization and c-myc induction. Our data suggest that HDAC6 is an important link between EGF and Wnt signaling.

EXPERIMENTAL PROCEDURES

Cell Culture—HCT116, SW480, and 293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells were incubated in serum-free medium overnight prior EGFR treatment (1 μM) or preincubated with trichostatin A (TSA) (400 nM) for 8 h.

Reagents—EGF and TSA were from Cell Signaling Technology (CST). Methyl-β-cycloextrin was purchased from Sigma. All antibodies used in this study, except for HDAC6 (Santa Cruz Biotechnology) and Ac-tubulin (Dr. Yao), were from CST. The acetylated β-catenin (Lys–49) was raised by immunizing rabbit with a synthetic peptide containing acetylated lysine 49. β-Catenin plasmids were provided by Dr. C. Neuveut (Institute...
of Pasteur). All siRNAs were from CST. Transfections of siRNA was performed using Mirus reagents. DNA plasmids were transformed with FuGENE 6 from Roche Applied Science.

Cell Fractionation—Nuclear fractionation was performed as described (16). In brief, cells were treated as indicated, washed with phosphate-buffered saline, and swelled in hypotonic buffer (25 mM Tris–HCl, pH 7.5, 5 mM KCl, 1 mM phenylmethanesulfonyl fluoride, 1 mM aprotinin) for 30 min on ice. Following homogenization, the nuclei were pelleted at 800 g for 10 min. The supernatant was centrifuged at 15,000 g for 20 min to remove cell debris and solubilized with SDS lysis buffer, and the resulting lysates were collected as cytosolic lysates.

Immunoblotting, Immunoprecipitation, and Immunostaining—Assays were carried out following the standard protocols of CST.

RESULTS AND DISCUSSION

In the colon cancer cell line HCT116, the protein level of c-Myc was significantly decreased when cells were treated with β-catenin siRNA (Fig. 1a), indicating that c-Myc expression is dependent on β-catenin. As expected, EGF treatment increased c-Myc protein level (Fig. 1b). The basal level of c-Myc under starvation conditions may reflect that HCT116 has a heterozygous serine 45 deletion mutation on β-catenin, which could lead to constitutive transcription of c-Myc (21). Interestingly, this EGF-enhanced c-Myc expression was inhibited by treatment with the HDAC inhibitor TSA, although TSA had no effect on the protein level of β-catenin (Fig. 1b). Immunoprecipitation of β-catenin from these cell lysates revealed the accumulation of acetylated β-catenin after TSA treatment (Fig. 1b), suggesting a role of acetylation in β-catenin-dependent c-Myc expression. Similar results were obtained from another colon cancer cell line, SW480 (Fig. 1b, right panel).

TSA inhibition of c-Myc expression prompted us to test whether TSA blocks EGF-induced β-catenin nuclear translocation. To this end, we examined the subcellular localization of β-catenin after TSA treatment. Immunostaining showed that β-catenin was primarily present at the cell surface in untreated cells (Fig. 1c). Consistent with previous work, β-catenin membrane staining was reduced after EGF treatment, whereas its nuclear staining was increased (Fig. 1c). However, when cells were treated with TSA, EGF-induced redistribution of β-catenin was inhibited. In addition, cellular fractionation showed that EGF treatment increased the level of nuclear β-catenin, and this increase was inhibited by TSA (Fig. 1d). In contrast, the level of cytosolic β-catenin was slightly decreased after EGF treatment, and it is restored by TSA (Fig. 1d).

The effect of TSA suggests that HDACs might be involved in EGF-induced β-catenin nuclear accumulation. To test this, we first asked whether specific HDACs interact with β-catenin. In a time course study, β-catenin was immunoprecipitated from HCT116 cells after EGF treatment and immunoblotted with antibodies against HDACs. Among the HDACs tested (HDAC1–7), only HDAC6 was coimmunoprecipitated with β-catenin (data not shown), and importantly, this interaction was induced by EGF (Fig. 2a).

The association between HDAC6 and β-catenin raises the possibility that β-catenin might be a substrate of HDAC6. To test this, we used siRNA to inhibit HDAC6 expression. As shown in Fig. 2b, β-catenin acetylation levels were significantly increased in HCT116 cells transfected with HDAC6 siRNA, indicating that
ACCELERATED PUBLICATION: HDAC6 Deacetylates β-Catenin

FIGURE 2. HDAC6 deacetylates β-catenin at Lys-49 and inhibits phosphorylation of β-catenin at Ser-45.

a, immunoblot analysis of HCT116 cells. Cells were serum-starved overnight and treated with EGF for the indicated times, and β-catenin was immunoprecipitated (IP) followed by Western blot with antibodies to β-catenin and HDAC6. b, immunoblot analysis of HCT116 cells transfected with HDAC6 siRNA. 48 h after transfection, lysates were immunoprecipitated with β-catenin antibody and immunoblotted with acetylated lysine antibody (AcK). c, alignment of N-terminal amino acid sequences of β-catenin. d, immunoblot analysis of Myc immunoprecipitates from 293T cells expressing Myc-β-catenin or Myc-β-catenin (K49R), using antibodies to Ac-β-catenin (Lys-49) and p-β-catenin (Ser-45). e, immunoblot analysis of HCT116 cells expressing HA-CBP, wild-type (wt) FLAG-HDAC6, or catalytically dead (mut) FLAG-HDAC6. f, immunoblot analysis of HCT116 and SW480 cells transfected with HDAC6 siRNA or treated with TSA. Cells were serum-starved overnight and treated with EGF for 4 h. g, immunoblot analysis of 293T cells expressing Myc-β-catenin, together with FLAG-HDAC6. h, immunoblot analysis of SW480 cells expressing FLAG-HDAC6. 16 h after transfection, cells were serum-starved overnight before cell lysates were collected.

HDAC6 can function as a β-catenin deacetylase in vivo. We next attempted to identify which lysine residues of β-catenin were deacetylated by HDAC6. Four lysines on β-catenin have been identified as acetylation sites (17–19). In this study, we focused on lysine 49 for the following reasons. 1) Lys-49 of β-catenin is conserved from Drosophila to human (Fig. 2c). 2) Lys-49 is adjacent to the highly conserved regulatory serine and threonine residues in the N terminus. 3) Lysine to arginine transition at Lys-49 (K49R) is the most frequent mutation in thyroid carcinomas patients (20). 4) K49R mutation enhances the ability of β-catenin to activate c-myc (17).

A polyclonal antibody specifically recognizing Lys-49 acetylated β-catenin was generated and tested for specificity. 293T cells were transfected with Myc-tagged wild-type and K49R β-catenin. Myc immunoprecipitates were subjected to Western blot with the acetylated-lysine-specific antibody. Fig. 2d shows that wild-type β-catenin, but not K49R mutant, was detected by the Ac-β-catenin (Lys-49) antibody, demonstrating the specificity of this antibody. We then used this antibody to determine whether acetylation of Lys-49 is regulated by HDAC6. Consistent with previous work (17), acetylation of β-catenin at Lys-49 was increased by transfecting cells with HA-CBP (Fig. 2e). The amount of Ac-β-catenin (Lys-49), however, was significantly reduced when cells were cotransfected with wild-type HDAC6 but not with a catalytically inactive HDAC6 mutant (Fig. 2e). We also examined the ability of endogenous HDAC6 to deacetylate β-catenin at Lys-49. As shown in Fig. 2f (left panel), Lys-49 was deacetylated after EGF treatment in HCT116 cells, and this EGF-induced Lys-49 deacetylation was blocked by HDAC6 siRNA. Consistently, EGF-mediated Lys-49 deacetylation was also blocked by TSA treatment in SW480 cells (Fig. 2f, right panel). These results demonstrate that HDAC6 is an endogenous β-catenin deacetylase.

CK1-mediated phosphorylation of β-catenin at serine 45 is required for β-catenin degradation, and mutations at this site frequently occur in colon cancer (11, 12, 21). Given the proximity of Lys-49 to Ser-45, we examined whether HDAC6 affects the phosphorylation of Ser-45. 293T cells were transfected with Myc-tagged wild-type β-catenin and FLAG-tagged HDAC6. Myc immunoprecipitates were subjected to Western blotting with pS45 phospho-specific antibodies. Fig. 2g showed that overexpression of HDAC6 suppressed Ser-45 phosphorylation in vivo. Consistently, phosphorylation of Ser-45 is also blocked by the K49R mutation (Fig. 2d), suggesting that Lys-49 deacetylation...
by HDAC6 had an inhibitory effect on Ser-45 phosphorylation. Furthermore, we observed increased levels of c-Myc in serum-starved SW480 cells overexpressing HDAC6 (Fig. 2h). Together, these results show that deacetylation of Lys-49 by HDAC6 inhibits β-catenin phosphorylation at Ser-45, leading to β-catenin-dependent c-Myc induction.

How does EGF regulate HDAC6-dependent β-catenin deacetylation? It has been shown that β-catenin is concentrated in caveolae membranes (22). Therefore, we investigated the possibility of a caveolae-dependent mechanism for EGF-induced HDAC6/β-catenin association. To this end, we examined the effect of EGF on HDAC6 interaction with caveolin-1, a major component of caveolae membranes. After EGF treatment, we immunoprecipitated caveolin-1 and immunoblotting with HDAC6 and β-catenin antibodies. Although association of β-catenin and caveolin-1 was not affected by EGF treatment, the level of coimmunoprecipitated HDAC6-caveolin-1 complex was significantly enhanced by 30 min of EGF treatment, and this complex dissociated 60 min after EGF stimulation (Fig. 3a). Interaction between HDAC6 and caveolin-1 was blocked by treatment with the cholesterol-sequestering reagent β-cyclodextrin, which is known to disrupt cholesterol-rich caveolae (Fig. 3b). Thus, we conclude that EGF regulates HDAC6 function by recruiting HDAC6 to caveolae membranes.

To confirm that HDAC6 is required for EGF-induced β-catenin nuclear localization, HDAC6 siRNA was introduced into HCT116 cells, and the nuclear fraction was analyzed. As shown in Fig. 3c, HDAC6 siRNA blocked EGF-induced deacetylation of Lys-49 and reduced the amount of nuclear β-catenin in EGF-treated cells. Subsequently, HDAC6 reduction resulted in decreased c-Myc expression (Fig. 3c) and an ~50% decrease in cell number (Fig. 3d), demonstrating that HDAC6 is required for cell proliferation. The significant reduction of cell number does not appear to be due to apoptosis since we did not detect any caspase activation in HDAC6 siRNA-transfected cells (data not shown). In addition, both HDAC6 and β-catenin siRNAs exhibited similar effects on growth inhibition, and cotransfection of both siRNAs gave no additive effect (Fig. 3d), suggesting that both genes function in the same pathway.

CONCLUDING REMARKS

Recent studies suggest that protein deacetylation has diverse biological functions beyond histone modification (2). Here we found that HDAC6 is involved in EGF-induced β-catenin nuclear localization; EGF stimulates HDAC6 translocation to the plasma membrane, where it deacetylates β-catenin at Lys-49, inhibits its phosphorylation at Ser-45, and promotes its nuclear accumulation. Our data identified HDAC6 as a link between EGF and Wnt signaling that has been implicated in tumor progression. Given that HDAC6 is not an essential gene in mouse,3 we suspect

3 Y. Gao and T. P. Yao, personal communication.
that HDAC6 might not play a dominant role in the canonical Wnt-β-catenin signaling during normal development. If true, our study would suggest that pharmacological inactivation of HDAC6 could offer therapeutic effects to tumors with deregulated EGF-β-catenin signaling.

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