Histone Deacetylase Inhibitors Regulate Retinoic Acid Receptor β Expression in Neuroblastoma Cells by Both Transcriptional and Posttranscriptional Mechanisms

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The retinoic acid receptor β (RARβ) is a retinoic acid (RA)-inducible tumor suppressor, which plays an important role in the arrest of neuroblastoma cell growth. Using human neuroblastoma SH-SY5Y cells, we have examined the regulation of RARβ expression by histone deacetylase inhibitors (HDACi), considered to be promising agents in anticancer therapy. Our results show that HDACi cooperated with RA to increase RARβ mRNA levels and to activate the RARβ2 promoter in transient transfection assays. Chromatin immunoprecipitation assays showed that the basal RARβ2 promoter that contains the RA response element was refractory to acetylation by both HDACi and RA. In addition, HDACi caused a transient increase in acetylation of a downstream RARβ2 region, even though global histones remain hyperacetylated after a prolonged treatment with the inhibitors. RA potentiated this response and maintained acetylation for a longer period. Despite the cooperation of RA with HDACi to increase transcription of the RARβ gene, these inhibitors caused a paradoxical reduction of the cellular levels of the RARβ protein in cells treated with the retinoid. This reduction is secondary to a change in the protein half-life that is decreased by the HDACi due to increased ubiquitin-independent proteasomal degradation. These results show that HDACi regulate expression of the tumor suppressor gene RARβ by both transcriptional and posttranscriptional mechanisms and might then modulate sensitivity to the retinoid in neuroblastoma cells. (Molecular Endocrinology 21:2416–2426, 2007)

Due to their role in the regulation of cell growth, differentiation, and apoptosis, retinoids are being extensively evaluated in cancer prevention and treatment (1). Retinoic acid (RA) causes differentiation of neuroblastoma cell lines, characterized by the appearance of long neurites and cell growth arrest (2, 3). This observation led to clinical trials in children with advanced neuroblastoma, the most common extracranial solid tumor of early childhood (4), which demonstrated that retinoid therapy significantly improves survival in these patients (5).

The nuclear retinoic acid receptors (RAR) and retinoid X receptors (RXR) are members of the nuclear hormone receptors superfamily (6). These receptors are each encoded by three different genes (α, β, and γ) that give rise to different isoforms due to alternative promoter usage and differential splicing (7, 8). These receptors act as ligand-inducible transcription factors by binding as RAR/RXR heterodimers to RA-responsive elements (RARE), which are normally located in the regulatory region of target genes. RARE typically consist of at least two copies of the consensus sequence PuGG/TTCA, normally configured as direct repeats (DR) spaced by two or five nucleotides (DR2 and DR5, respectively). In the absence of ligand, RA target genes are silenced due to the recruitment of histone deacetylase (HDAC)-containing multicomponent complexes that are tethered through corepressor proteins to the unliganded RAR/RXR heterodimer. Ligand binding causes conformational changes in the receptors that allow the release of corepressors and facilitate the ordered recruitment of coactivator complexes, some of which possess histone acetylase (HAT) activity, which cause chromatin decompaction and transcriptional stimulation (9, 10). Several retinoids are able to inhibit the activator protein-1 (AP-1) transcription pathway, which is activated upon growth factor signaling (6, 10) or even to have nongenomic effects that lead to stimulation of signaling pathways involved in neuronal differentiation (11).
The RARβ gene is itself a retinoid target gene, with a well-characterized DR5 element and an accessory RARE in its 5'-flanking region (12). RARβ has been suggested to play an important role in the biological functions of RA and to be associated with cellular sensitivity to the retinoid in different types of cancer cells. RARβ may act as a tumor suppressor, and there is evidence that RARβ induction by retinoids is important for tumor cell growth inhibition (1). Although most malignant cells have a very low level of RARβ expression, retinoid-sensitive cancer cells are characterized by a marked induction of endogenous RARβ expression after retinoid treatment in vitro. A loss of RARβ expression is associated with retinoid insensitivity, and DNA methylation is at least one contributing factor to RARβ inactivity, which can be alleviated by demethylation using 5-aza-2-deoxycytidine (1).

In neuroblastoma cells, RARβ is expressed at a very low level but can be strongly induced by treatment with RA (13). The induction of RARβ is thought to play an important role in the morphological differentiation as well as in the arrest of neuroblastoma cell growth (14–16). The important role of this isoform has been confirmed in studies in which the effects of receptor-selective retinoids on neuroblastoma cells have been analyzed (17–20). It has been reported that low RARβ expression contributes to the malignant phenotype of neuroblastoma and that high-level RARβ expression is a favorable prognostic feature of primary neuroblastoma tumors (21, 22).

HDAC inhibitors (HDACi) are also considered to be among the most promising agents in drug development for cancer therapy (23, 24). Treatments of RA plus HDACi have been successfully used to reduce neuroblastoma tumor growth using xenografts (25, 26), suggesting that this combination may have therapeutic utility for neuroblastoma. We have recently shown that HDACi enhance reduction of human neuroblastoma SH-SY5Y cell growth by RA and that both types of drugs cooperate to activate transcription of cyclin kinase inhibitors (27).

Because transcription of the RARβ gene plays a critical role in mediating ligand-dependent growth inhibition, and HDACi have been found to modulate RARβ2 promoter activation by RA in embryonal carcinoma cells (28, 29), we sought to investigate whether HDACi alone or in combination with RA modulates expression of RARβ in neuroblastoma SH-SY5Y cells. Our results show that HDACi cooperate with RA to induce RARβ2 promoter activity in transient transfection assays and to increase RARβ transcripts. Paradoxically, HDACi reduce induction of RARβ protein by RA. This is a consequence of a decreased half-life of the protein in the presence of HDACi that is in turn due to increased proteasomal degradation that occurs without RARβ ubiquitylation.

RESULTS

Influence of HDACi and RA on RARβ Transcription in SH-SY5Y Cells

To investigate the effects of deacetylase inhibition on regulation of RARβ gene expression in neuroblastoma cells, we used sodium butyrate (But), a natural short-chain fatty acid, and suberoylanilide hydroxamic acid (SAHA), a second-generation HDACi (30) that can suppress growth of different tumor cells. Acetylation of histone H3 and histone H4 tails has been linked to actively transcribed genomic regions. However, inhibition of HDAC alone does not lead to a generalized increase in transcription. As shown in Fig. 1, incubation with But (2 mM) or SAHA (1 μM) alone had at most a weak stimulatory effect on RARβ mRNA levels in SH-SY5Y cells. In contrast, RA caused the expected induction of RARβ transcripts, and the receptor mRNA levels were further induced when the retinoid was combined with either But or SAHA.

Expression of the RARβ gene is controlled by two promoters, and the RARβ2 promoter contains a canonical (proximal) RARE and an auxiliary (distal) RARE that mediate stimulation by RA (31). Cooperation of HDACi and RA to stimulate the activity of a reporter RARβ2 construct containing both RARE in transient transfection assays was also analyzed. As shown in Fig. 2A, suboptimal concentrations of RA (10 nM) or But (0.5 mM), which separately had only a weak stimulatory effect, when combined resulted in a marked synergistic activation of the RARβ2 promoter. A cooperative effect of RA with SAHA and other HDACi, trichostatin A (TSA), was also found (Fig. 2C and D). In contrast, a higher concentration of But (2 mM) that by itself produced a strong induction of promoter activity showed little cooperative effect with 1 μM RA, which also caused a strong stimulation (Fig. 2B).

To test a possible basis for the HDACi-dependent increase in stimulation of RARβ transcription by RA, the effect of these drugs on the level of histone acetylation in the basal RARβ2 promoter region that contains the RARE was examined by chromatin immunoprecipitation (ChIP) assays (Fig. 3A). Analysis with antibodies against acetylated histone H3 demonstrated constitutive histone acetylation in untreated

![Fig. 1. RARβ Transcripts in Neuroblastoma Cells Treated with HDACi and RA](image-url)
cells, and unexpectedly, the amount of acetylated histone H3 bound to the promoter was barely increased after incubation with But, which however increased significantly global levels of histone H3 acetylation (Fig. 3B). Incubation with RA for 60 min, either alone or in combination with the HDACi, did not alter the amount of acetylated histone H3 on the RARβ2 promoter (Fig. 3A). Similar results were obtained after incubation with RA for 30 or 90 min (not illustrated). Therefore, the proximal RARβ2 promoter region appears to be highly refractory to acetylation by these compounds.

Additional ChIP assays with a DNA fragment located downstream from the basal promoter revealed that, in contrast with the results obtained with proximal promoter sequences, the amount of acetylated H3 was increased significantly after treatment with But (Fig. 3A). Interestingly, the effect of the HDACi on the levels of acetylated histone H3 was transient. But caused a strong increase at 1 and 3 h, but the amount of acetylated H3 decreased thereafter. This occurred despite the sustained increase in bulk H3 acetylation caused by the HDACi (Fig. 3B). Thus, whereas global cellular levels of acetylated H3 histone were strongly elevated after 5 or 7 h of incubation with the HDACi, no increase in the acetylation status of this RARβ2 region was found at these time points. However, the retinoid was able to further induce acetylation caused by But. When RA was present together with the HDACi, acetylation was stronger at 3 h, and this increase remained up to 7 h. As an additional control, we also examined acetylation of a promoter that is not regulated by RA (Fig. 3A). Association of acetylated histone H3 to 5′-flanking sequences of the Na+/P+-cotransporter gene (Na+/P+) was increased by the HDACi with a similar time course as that found with the RARβ2 promoter, but the combination with RA did not further increase acetylation of this nontarget gene.

To analyze whether RA could alter the acetylation pattern in regions different from the immediate vicinity of the promoter, acetylated H3 was examined in fragments located approximately 1 kb upstream and downstream of the transcription initiation site. As shown in Fig. 3C, these regions were also constitutively acetylated, and RA was unable to increase this acetylation. In addition, these regions were also resistant to HDACi treatment. On the other hand, the lower panels in Fig. 3C show that treatment with neither RA nor But altered the amount of total histone H3 bound to the different regions of the RARβ2 promoter.

It has been described that in embryonal carcinoma cells, RA treatment does not significantly modulate acetylation levels of histone H3 (29) but has a strong stimulatory effect on phosphorylation of histone H3 in serine 10 (32). Furthermore, in this cell type, incubation with HDACi causes a large increase in bulk H3 phosphorylation (32). This was not found in neuroblastoma cells where But was unable to increase global H3 phosphorylation and rather caused a decrease of this modification (Fig. 3C). In ChIP assays performed in SH-SYSY cells with antibodies against the modified histone, phosphorylated H3 was detectable in the RARE-containing RARβ2 promoter fragment under basal conditions but was not increased by incubation with RA and/or But. Binding of phosphorylated H3 to the +129/+273 RARβ2 region was essentially undetectable, and the HDACi, either alone or in combination with RA, was unable to increase H3 phosphorylation status (Fig. 3C). In addition, RA did not increase the levels of phosphorylated H3 bound to the fragments −824/−851 and +1140/+1328 that unexpectedly were reduced by the HDACi. Phosphorylated histone H3 was also detectable in the promoters of the Na+/P+ cotransporter and cyclin kinase inhibitor p21^[Cip1] genes and was not increased by treatment with But and/or RA either (not illustrated). There was the possibility that RA could increase H4 acetylation, another mark of active transcriptional activity. However, as shown in Fig. 3D, ChIP assays performed with the different RARβ2 fragments demonstrated that acetylation of histone H4 was similar in RA-treated and untreated cells, a result similar to that found with acetylation of histone H3.
As shown in Fig. 5A, whereas RA caused a dose-dependent induction of RARβ protein in SH-SY5Y cells, incubation with increasing concentrations of But (from 0.5–5 mM) or SAHA (from 0.5–2 μM) was unable to induce RARβ expression. The effectiveness of the HDACi was demonstrated by a strong increase in the cellular levels of acetylated histone H3 and a concomitant reduction in cyclin D1. Furthermore, as shown in Fig. 5B, paradoxically, both HDACi reduced the RARβ2 promoter activity.

**Effect of HDACi on RARβ Stability**

The discrepancies between the effect of the HDACi on RARβ protein levels and those observed on RARβ mRNA induction and promoter activity could be explained by changes in the stability of the protein. To analyze this point, RARβ levels were determined in cells induced with RA in the presence and absence of HDACi for 24 h and then incubated for varying times with cycloheximide. As illustrated in Fig. 6B, the half-

Transcriptional stimulation by RA involves the release of HDAC-containing corepressor complexes (10). To analyze whether corepressor release from the RARβ2 promoter could also participate in stimulation by HDACi, ChIP assays were performed with antibodies against the corepressor silencing mediator of retinoid and thyroid receptors (SMRT). As shown in Fig. 3C, the corepressor bound to the basal RARβ2 promoter in SH-SY5Y cells, and incubation with RA caused corepressor release, whereas But did not reduce SMRT binding. In addition, other RARβ2 regions bound more weakly the coregulator, and in contrast with the results obtained with the basal promoter, But was as efficient as RA to release SMRT. This appears to represent a rather generalized effect of HDACi, because incubation with another inhibitor, SAHA, also caused the release of SMRT from different promoters, where a strong binding of the corepressor was found (Fig. 4).

**Effect of HDACi on RARβ Protein Levels**

As shown in Fig. 5A, whereas RA caused a dose-dependent induction of RARβ protein in SH-SY5Y cells, incubation with increasing concentrations of But (from 0.5–5 mM) or SAHA (from 0.5–2 μM) was unable to induce RARβ expression. The effectiveness of the HDACi was demonstrated by a strong increase in the cellular levels of acetylated histone H3 and a concomitant reduction in cyclin D1. Furthermore, as shown in Fig. 5B, paradoxically, both HDACi reduced the RARβ response to RA. This finding was not a generalized effect of HDACi, because as shown in Fig. 5C, the increase of p21Waf1/Cip1 caused by RA was not reduced but rather enhanced in the presence of these compounds.

**HDACi Decrease RARβ Stability**

The discrepancies between the effect of the HDACi on RARβ protein levels and those observed on RARβ mRNA induction and promoter activity could be explained by changes in the stability of the protein. To analyze this point, RARβ levels were determined in cells induced with RA in the presence and absence of HDACi for 24 h and then incubated for varying times with cycloheximide. As illustrated in Fig. 6B, the half-
clearly contributes to the reduced RARα protein destabilization with an apparent half-life of 5–8 h in cells treated with SAHA, or TSA. Thus, protein destabilization of the RARα protein was shorter in the HDACi-treated cells. Whereas in cells incubated with RA alone, RARβ was very stable and only a slight reduction on the levels of this protein was found even after 8 h treatment with the inhibitor, RARβ disappeared with an apparent half-life of 5–8 h in cells treated with But, SAHA, or TSA. Thus, protein destabilization clearly contributes to the reduced RARβ protein response to RA in cells incubated with HDACi. In contrast, RARβ mRNA half-life, determined after incubation of cells in the presence of actinomycin D, was not altered by the HDACi. Figure 6A shows that RARβ mRNA disappeared with a similar half-life (approximately 3 h) in cells treated with RA alone or in combination with But, SAHA, or TSA, showing that mRNA stabilization is not involved in the induction of RARβ transcripts by HDACi.

HDACi have been recently demonstrated to cause the proteasomal degradation of several proteins (33–35). To investigate the underlying mechanisms of HDACi-mediated reduction of RARβ protein induction by RA, we tested whether this effect required an active proteasome. Figure 7A shows that the proteasome inhibitor MG132 significantly reversed the inhibitory effect of But and SAHA on RARβ expression. The finding that the inhibitor restored RARβ levels indicates that HDACi affect RARβ degradation through the proteasome system.

We next examined RARβ stability in cells incubated in the presence of MG132. For this purpose, cells were incubated with RA, SAHA, and/or the inhibitor for 24 h in the presence and absence of cycloheximide during the last 8 h (Fig. 7B). In agreement with the results shown in Fig. 6B, cycloheximide produced a stronger decrease of RARβ levels in SAHA-treated cells. However, MG132 reversed again this inhibitory effect, reinforcing the idea that proteasomal-mediated degradation underlies HDACi-mediated reduction of RARβ receptor levels. In most cases, ubiquitylation is required to undergo proteasomal degradation, although non-ubiquitin-mediated proteasomal degradation has been described for several proteins (36). Therefore, we also measured the effect of MG132 and cycloheximide on total levels of ubiquitylated proteins, as well as in the expression of β-catenin, which is known to be degraded by the ubiquitin-proteasome pathway (37). As expected, treatment with the proteasomal inhibitor greatly enhanced in the levels of ubiquitylated proteins both in the presence and absence of cycloheximide. In addition, β-catenin was very stable because cycloheximide did not significantly reduce the levels of this protein in cells treated with RA and/or the HDACi. In contrast with RARβ, both MG132 and SAHA caused the appearance of additional β-catenin bands that may represent modified forms of the protein. These species were more prominent in cells treated with the HDACi, where with the combination of cycloheximide and MG132, new bands with slower mobility were
detected. These results suggest that whereas β-catenin degradation is indeed dependent on ubiquitylation, RARβ could be degraded in a ubiquitin-independent manner. However, it was also possible that the levels of ubiquitylated protein were too low to be detected by this method. To further examine whether HDACi-induced RARβ degradation depends on the ubiquitin system, cells were transfected with expression vectors for ubiquitin and the receptor and treated with But in the presence and absence of the inhibitor MG132. RARβ was immunoprecipitated and immunoblotted with anti-ubiquitin antibodies. As an additional control, the influence of the HDACi on ubiquitylation of RXRα, which is known to be degraded by the proteasome system (38), was also analyzed. When immunoprecipitated RXRα was immunoblotted with anti-ubiquitin antibodies, higher molecular weight species could be detected in transfected cells (Fig. 8A), and these species increased in intensity in cells treated with the MG132 inhibitor, showing that this receptor is ubiquitylated in SH-SY5Y cells. In addition, RXRα ubiquitylation was not increased by HDACi treatment and the inhibitor did not enhance ubiquitylation in the presence of MG132. In contrast with RXRα, higher molecular weight species of immunoprecipitated RARβ were barely detectable, and these species did not increase in cells treated with MG132 and/or But (Fig. 8A). Thus, HDACi-induced degradation of RARβ cannot be correlated to an increase in the ubiquitylation of this receptor.

There was also the possibility that RARβ itself could be acetylated in response to HDACi treatment. To analyze this point, cells were treated with RA in the presence and absence of SAHA for 36 h, and proteins were immunoprecipitated with anti-RARβ antibodies. When immunoprecipitates were immunoblotted with an antibody recognizing acetyl-lysines (Ac-lys), a band corresponding to the receptor could be observed, suggesting that RARβ is acetylated in SH-SY5Y cells (Fig. 8B). A similar result was obtained when RARβ was detected in Ac-lys immunoprecipitates. The amount of acetylated receptor increased in RA-treated cells in parallel with the total amount of RARβ, suggesting that RA does not increase acetylation. In addition, SAHA caused a detectable increase, and despite the reduction in total receptor levels observed in cells incubated with RA plus SAHA, the levels of modified receptor were maximal under these conditions. These results indicate that RA, although ineffective by itself, could cooperate with the HDACi to induce RARβ acetylation.

**DISCUSSION**

Our results indicate that variations in histone acetylation have a regulatory effect on the transcription of the RARβ gene in response to RA in neuroblastoma cells. As expected, the retinoid caused a significant increase in RARβ mRNA levels, and this response was stronger in cells cotreated with HDACi. This potentiation was also observed in transient transfection assays with the
By ChIP analysis, we have also analyzed the relationship between histone posttranslational modifications in the RARβ2 promoter and transcription of this gene in neuroblastoma cells. Treatment with RA did not cause a detectable increase of histone H3 or histone H4 acetylation of the RARE-containing proximal RARβ2 promoter. The result that acetylation of the promoter is insensitive to RA differs from that described for other promoters regulated by nuclear receptors but is in agreement with that found with the RARβ2 promoter in embryonal carcinoma cells, which are also highly sensitive to RA, where the retinoid does not affect acetylation (29, 32). Remarkably, treatment with HDACi results in only a weak increase in the amount of acetylated histone H3 associated with the basal RARβ2 promoter. This shows that this region of the gene that is already constitutively acetylated is refractory to further modification even under conditions in which global cellular levels of acetylation are significantly increased. The same occurred with regions located approximately 1 kb upstream and downstream of the transcription initiation site. Enhanced acetylated histone H3 binding to region +129/+273 of the RARβ2 gene was, however, detectable after incubation of SH-SYS5Y cells with HDACi. This modification alone is not sufficient to cause a strong RARβ2 gene transcription, as demonstrated by the finding that HDACi by themselves caused little if any stimulation of RARβ mRNA levels. Interestingly, acetylation of this region increases initially but then decreases with time even though global histones remain hyperacetylated after a prolonged treatment with HDACi, demonstrating that this region can also become unresponsive to acetylation. This also appears to occur with other promoters examined in this study, showing that this is not a specific phenomenon for the RARβ2 gene. In addition, although RA alone does not increase acetylation, it is able to potentiate the response to HDACi and to maintain acetylation of this RARβ2 region for a longer period. This change correlates with the cooperative activation of RARβ2 transcription found with the combination of HDACi and the retinoid. These results indicate that the effect of these compounds does not involve a local acetylation of the basal RARβ2 promoter but could be associated with hyperacetylation of regions located downstream of the promoter.

Histone H3 phosphorylation has been associated with RA-dependent activation of the RARβ2 promoter in P19 embryonal carcinoma cells, and incubation with HDACi was shown to cause an important increase of bulk H3 phosphorylation in this cell type (32). However, HDACi did not significantly increase global histone H3 phosphorylation in neuroblastoma SH-SYS5Y cells, showing that this is a cell-type-specific effect. Furthermore, neither RA nor HDACi caused recruitment of phosphorylated histone H3 to the RARβ2 promoter in SH-SYS5Y cells.

We also tested the possibility that HDACi might alter the recruitment of HDAC-containing corepressor complexes to the promoter. It has been recently shown...
that SMRT occupancy of the basal RARβ2 promoter was not found in P19 cells, whereas the corepressor was constitutively loaded at this promoter in poorly retinoid-responsive HeLa cells. Furthermore, the phosphoinositide 3-kinase/Akt signaling pathway leads to an increased tethering of the corepressor SMRT to the RARβ2 promoter in P19 cells, decreasing histone acetylation and gene expression (41). Our results show that SMRT binds to the RARβ2 promoter in RA-responsive neuroblastoma cells and that this ligand but not HDACi treatment, causes the release of the corepressors from the promoter. This differential effect could be involved in the different potency of both compounds to stimulate transcription from this promoter. In contrast, the corepressor bound very weakly to the downstream RARβ2 fragment, and this binding was also released by HDACi. This also occurred with other cellular promoters that bound SMRT strongly, suggesting that HDACi not only inhibit activity of deacetylases but also can dislodge corepressors and their associated HDACs. This release might contribute to transcriptional stimulation of HDACi target genes, independently of ligand-dependent receptor actions in the promoter.

From these results, as well as from previous reports (27, 32, 42), it can be deduced that chromatin structure of the proximal RARβ2 promoter differs in many aspects from other regulatory regions of other genes or even from other regions of this gene and that this occurs in a cell-dependent manner, making this promoter a good model for additional studies of chromatin covalent modifications and remodeling.

A most unexpected finding in our work was that, despite the cooperation of RA with HDACi to increase transcription of the RARγ gene, the cellular levels of this protein were paradoxically reduced in cells treated with the retinoid in the presence of the inhibitors. This reduction is due to a change in the protein half-life that is decreased by the HDACis. Many transcription factors are degraded by the proteasome system and our results suggest that HDACi trigger a selective degradation of RARγ via the proteasomal pathway, because the amount of protein can be restored by treatment with the peptidyl aldehyde MG132, a proteasome inhibitor. It has been previously demonstrated that RARs are degraded by the proteasome in response to RA or cellular stress. This has been shown for RARα and RARγ (37, 42–44) but has not been described for RARβ. Proteasomal degradation by RA appears to involve the ubiquitylation of RARs and the recruitment of the proteasome through SUG-1 (45), one of the components of the 19S regulatory complex of the 26S proteasome. It has been proposed that this degradation process would provide a mechanism to control the magnitude and the duration of retinoid-mediated transcription (9). It was possible that the effect of HDACi on RARβ half-life might be also caused by increased ubiquitylation of the receptor that would be then targeted for destruction by the 26S proteasome. Alternatively, HDACi could induce a ubiquitin-independent proteasomal degradation of RARβ. Our results suggest that this is indeed the case, because ubiquitylation of the receptor was not detected, and HDACi did not increase this modification for RXRα. This process of ubiquitin-independent proteasomal degradation is mediated by the free 20S proteasome that normally degrades naturally unfolded or damaged proteins. Reversible protein acetylation has been characterized as linked to histone and chromatin-dependent processes. Recent studies, however, have revealed a much broader array of biological processes that involve protein acetylation (46). Inhibition of HDAC activity can then lead to changes in protein degradation through acetylation of different proteins. Interestingly, HDACi cause proteasomal degradation of the transcription factor hypoxia-inducible factor-1α in a ubiquitin-independent manner, and this effect appears to be mediated by the activity of HDAC-6 (33). Non-ubiquitin-mediated proteasomal degradation has been described for several other proteins, including ornithine decarboxylase, p21Waf1/Cip1, c-Jun, and p53 (36). Targeting of a substrate to the 20S proteasome can be brought about by accessory molecules or by sequences within the substrate itself (36). Thus, Tax increases the binding of inhibitor-κB to the B7 subunit of the 20S proteasome, whereas p21Waf1/Cip1 degradation is mediated by the binding of the terminus of the protein to the C8-α subunit (47). Interestingly, we have observed that RARβ can be acetylated in SH-SY5Y cells and that RA appears to cooperate with HDACi to increase acetylation. Whether this modification could affect activity and/or degradation of the receptor as well as the exact mechanism by which HDACi cause RARβ degradation remains to be established.

In summary, our results show the importance of posttranscriptional regulation in modulation of RARβ expression by HDACi. Proteasomal degradation of the receptor counteracts stimulation of transcription and leads to a net decrease of RARβ protein levels in response to RA. Given the importance of this receptor in neuroblastoma growth and differentiation, regulation of RARβ levels by HDACi might alter cellular sensitivity to retinoids and the subsequent biological responses.

MATERIALS AND METHODS

Western Blot and Immunoprecipitations

Cell lysates were obtained as previously described (27, 48). Proteins were separated in SDS-PAGE and transferred to PVDF membranes (Immobilon; Millipore, Bedford, MA) that were blocked for 1 h at room temperature with 4% BSA. Incubation with primary antibodies was performed overnight at 4 C and with the secondary antibody for 1 h at room temperature. Blots were visualized with ECL (Amersham, Piscataway, NJ). Antibodies against RARβ, cyclin D1, and p21 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and used at a 1:2000 dilution. The antibody for
acetylated histone H3 in lysines 9 and 14 and H3 phosphor-
ylated in serine 10 (Upstate Biotechnology, Lake Placid, NY) were used at a 1:5000 and 1:1000 dilution, respectively. Anti-ubiquitin antibody (z-0458’ Dako, Carpenteria, CA) was used at a 1:1000 dilution. Membranes were stripped, blocked, and reprobed with antibodies against human actin (Santa Cruz) or b-catenin (BD Biosciences Pharmingen, San Diego, CA). For determination of RAR? half-life, cells pre-
treated for 24 h with RA in the presence and absence of HDACi were incubated for different time periods in the presen-
tence of 10 ?/ml cycloheximide. Western blots for RAR? were scanned, quantitated by densitometry with the NIH Image 1.59 program, and corrected by the amount of actin. The results are shown as percentage of the values obtained in cells at time zero of treatment with the inhibitor. For im-
munoprecipitations, the cells grown in p150 dishes were transfected with expression vectors for RAR?, RXR?, and HA-ubiquitin (30 ?g). Whole-cell extracts were prepared in RIPA buffer and were precleared with protein A-Sepharose beads for 1 h at 4 °C. Subsequently, 500 ?g cell extracts were incubated overnight at 4 °C with 3 ?g of the appropriate antibodies (RAR?, sc-552; RXR?, sc-553; Santa Cruz). Im-
mune complexes were washed four times with single-deter-
gen lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 ?g/ml aprotinin, and 1 ?g/ml pepstatin]. The immunopre-
cipitated proteins were eluted in Laemmli buffer and detected by immunoblotting with a 1:1000 dilution of anti-ubiquitin antibody and chemiluminescence. Alternatively, cell extracts (500 ?g) were immunoprecipitated with 2 ?g of the RAR? antibody and immunoblotted with a 1:2000 dilution of an antibody recognizing Ac-ly s (no. 9441; Cell Signaling Technology, Beverly, MA), and vice versa. Twenty-five micro-
grams of protein extracts were immunoblotted with the RAR? antibody and used as inputs.

Northern Blot
Total RNA from cells grown in 90-mm dishes was isolated with TriReagent (Sigma Chemical Co., St. Louis, MO), following the manufacturer’s protocol. Total RNA (15 ?g) was run in 1% formaldehyde-MOPS agarose gels and transferred to nylon Nytren-N (Schleicher & Schuell, Keene, NH) as de-
scribed (15). Then RNA was linked to membrane using UV-
Stratalinker and stained with 0.02% methylene blue to assess equal loading. The RNA was hybridized with a probe for RAR?, labeled by random primer, as described (15). For determination of RAR? mRNA half-life, the cells were incu-
bated with RA alone or in combination with HDACi for 24 h and with 5 ?g/ml actinomycin D thereafter. At different time points, RAR? levels were quantified by densitometric scan of the autoradiograms. The values obtained were corrected by the amount of 18S RNA loaded, which was determined by densitometry of the stained membranes.

Transient Transfections
SH-SY5Y cells (1 3 105) were plated in 60-mm wells, changed to serum-free medium 24 h later, and transfected with 5 ?g of a luciferase reporter plasmid containing se-
quences –124 to +14 of the RAR?2 promoter (15, 49). Trans-
fec tion was obtained by incubation with a mixture of cationic liposomes (1.5 ?g/3?g DNA) for 6 h. Cells were then treated for 48 h with RA and/or HDACi and activity determined. Each experiment was performed in triplicate and was repeated at least three times. Data are mean ± so and are expressed as fold induction over the values obtained in the untreated cells.

ChIP Assays
SH-SY5Y cells growing in p150 dishes were maintained in depleted medium for 48 h. Cells were washed twice in serum-
free medium and treated for 2.5 h with 2.5 ?g/ml ?-amanitin (Sigma). Cells were then washed and treated with RA and/or HDACi. At the indicated time points, cells were fixed with 1% formaldeh yde for 15 min at 37 °C. The Chromatin Immuno-
precipitation Assay kit from Upstate (catalog item 17-295) was used. Sonication was performed using a Bioruptor UCD-
200TM (Diagenode, Liège, Belgium) following manufacturer’s s-
tions. For each immunoprecipitation, 2.5–3.0 3 106 cells were used and 2 ?g of the following antibodies: anti-acety-
lated histone H3 (catalog item 06-599; Upstate), anti-acety-
lated histone H4 (catalog item 06-598; Upstate); anti-phos-
phorylated histone H3 (06-570; Upstate), anti-SMRT (catalog item PA1-842; Affinity BioReagents, Golden, CO), and normal rabbit serum Ig (sc-2027; Santa Cruz). DNA were subjected to 35 cycles of PCR with primers 5'-GGGAGTTTTT-
AGCTCTGTGAG-3' and 5'-TGAACAGCTACCTCTCCTACT-3' that amplify the fragment –123 to +33 of the human RAR?2 promoter that contains the RARE. The upstream –824 to –581 was amplified with primers 5'-CACAGGTGGTAATGG-GAGA-3' and 5'-TTGACTGACGTGTTGCTC-3'.
The downstream –129 to –273 region of this promoter was amplified with primers 5'-GGCGAGAAGCGGAGGATCC-3' and 5'-GGGCAAATACGCGGGG-3' and fragment –1140 to +1328 with primers 5'-GCTGCTCCCGGAAGTCTCATAA-3' and 5'-AATGGATGCGTCACTGACAG-3'. Additional primers pairs were used: 5'-GGCTTAGTGTGGAGATTGTA-3’ and 5'-GTTTCCTCCCTCCCTCACCTC-3'; to amplify a 245-bp frag-
ment of the Na+/K cotransporter gene promoter (from –2031 to –1888); 5'-GGCTAGTGGGAGATCTAG-3' and 5'-
CACGGAAAACAGAAAAGATGGA-3' to amplify a 155-bp fragment of the p21/Waf1 (G1p1 gene promoter (from –877 to –722); 5'-GGCATCTCTCCTATAGAATC-3' and 5'-
GGATTGGTGTAGACACAG-3' to amplify a 193-bp fragment of the p21 gene promoter (from –352 to –159). These primers amplify fragments containing regulatory sequences that are bound by other nuclear receptors but do not contain a RARE.

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