Histone deacetylase inhibitors upregulate p57^Kip2^ level by enhancing its expression through Sp1 transcription factor

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Histone deacetylase inhibitors (HDACs) represent a new class of targeted anticancer agents. Here, we evaluate the effects of butyrate (BuA) and other HDACIs on p57^Kip2^, a cyclin-dependent kinase inhibitor (cki). We observed that inhibitors of class I/II histone deacetylases (HDACs), but not of class III HDACs, induce a remarkable accumulation of p57^Kip2^ in several cells. The cki upregulation is associated with an increased gene expression that was not prevented by cycloheximide, indicating that HDACIs affect directly p57^Kip2^ transcription. The characterization of p57^Kip2^ promoter indicates that the first 165 bp are mostly involved in the BuA effects. Chromatin immunoprecipitation studies demonstrated that the BuA treatment causes the recruitment of Sp1 transcription factor. The Sp1 importance was confirmed by the reduction of BuA effects by mithramycin A (an Sp1 antagonist) and, most stringently, by Sp1 downregulation due to Sp1 siRNA. Moreover, both the treatments reduce the p57^Kip2^ transcription in untreated cells, suggesting that Sp1 is required for the constitutive cki expression. Studies employing plasmids containing parts of the 165 bp of p57^Kip2^ promoter indicate that the promoter region between –87 and –113 bp, which includes two putative Sp1 consensus sequences, plays a critical role in the response to HDACIs. Since this p57^Kip2^ promoter region also embraces the consensus sequence for the transcriptional repressor chicken ovalbumin upstream promoter transcription factor-interacting protein 2 (CTIP2), we evaluated whether this factor is involved into the BuA effect. When CTIP2 was downregulated by a specific siRNA, we observed the enhancement of BuA activity on p57^Kip2^ expression suggesting that CTIP2 might also be involved in HDACIs effects.

Introduction
Acetylation of histones plays a pivotal role in the regulation of gene transcription, modulating the expression of about 4–8% of human genes (1–3).

Modification of the ε-amino group of lysine residues changes the configuration of nucleosomes. The positive charge on unacetylated lysines of histones H3 and H4 is attracted by the negatively charged DNA yielding a compact chromatin state that is repressive for transcription. Acetylation of lysines by histone acetylase removes their positive charge resulting in an open chromatin structure, which facilitates gene expression. Histone deacetylases (HDACs) remove the acetyl groups from lysines, thus reversing this process and silencing gene expression. There are two families of HDACs: the so-called ‘classical’ HDAC family and the recently discovered SIR2 family of NAD+-dependent HDACs (class III HDACs). Members of the classical HDAC family fall into two different phylogenetic classes (4). The class I HDACs (HDAC1, 2, 3 and 8) are most closely related to the yeast transcriptional regulator RPD3. Class II HDACs (HDAC4, 5, 6, 7, 9 and 10) share domains with similarity to HDAC1, another deacetylase found in yeast (4). HDACs of class I are expressed in most cell types, whereas the tissue distribution of class II HDACs is more restricted, suggesting that these enzymes might be involved in cellular differentiation and developmental processes (4).

The function of class III HDACs (corresponding to the sirtuin family in higher eukaryotes) is still unclear, although pieces of evidence correlate the deacetylases to the ageing process (5).

The reprogramming of the histone acetylation/deacetylation process by changes of environment or addition of drugs might result in marked phenotypical changes. This is well illustrated by the antiproliferative and anticancer effects of histone deacetylase inhibitors (HDACIs), which have been demonstrated both in cellular and animal models and in clinical trials (6).

One of the most interesting HDACI is the short-chain fatty acid butyric acid (BuA). The molecule and its derivatives (particularly phenylbutyrate, PBuA) induce important phenotypical changes, which include cell cycle arrest, differentiation and apoptosis. The remarkable in vitro efficacy and the lack of in vivo toxicity of BuA prompted the use of the compound in the treatment of a number of human diseases, including cancers, bowel inflammatory pathologies, β-thalassemia, sickle cell anemia, cystic fibrosis and X-linked adrenoleukodystrophy (7–9). However, the clinical use of BuA is hampered by its in vivo very short half-life.

Currently, many powerful and stable HDACIs are available, including trichostatin A (TSA), trapoxin, suberoylanilide hydroxamic acid, MS-275, apicidin and others. Their use in cancer treatment is under evaluation in phase I and II clinical trials (10 and references therein). Additionally, it has been demonstrated that valproic acid, a drug employed in the therapy of epilepsy and bipolar syndrome, inhibits HDACs, thus suggesting its use in chemotherapy (11).

Here, we report a study on the effect of HDACIs on the transcription of p57^Kip2^, a tight-binding cyclin-dependent kinase inhibitor (cki), belonging to the Cip/Kip family.

Materials and methods

Materials
BuA, PBuA, valproic acid, nicotinamide and cycloheximide were supplied by Sigma Chemical Company, St Louis, MO. TSA and mithramycin A were furnished by BioMol Research Laboratories, Plymouth Meeting, PA; MS-275 was from Calbiochem EMD Biosciences, La Jolla, CA. Splitomicin was supplied by Alexis Biochemicals, San Diego, CA. PuGENE 6 transfection reagent was from Roche Applied Science, Monza, Italy, and lipofectamine 2000 by Invitrogen Life Technology, Carlsbad, CA. siRNAs directed against Sp1 and chicken ovalbumin upstream promoter transcription factor-interacting protein 2 (CTIP2) were furnished by Santa Cruz Biotechnologies, Santa Cruz, CA.

Cell line and treatment
The following cell lines were employed in our experiments: K562 (from an erythroleukemia); HL-60 (from a promyelocytic leukemia); HT-29 and CaCO2 (from human colon adenocarcinomas); HeLa (from a cervical cancer) and HEK293 (from human embryonic kidney). The culture conditions of these cell lines were previously reported (12). EPN is a cell line derived from the normal prostate epithelium as described in (13). Cell treatments were described in (14).

Reverse transcription–polymerase chain reaction
Reverse transcription–polymerase chain reaction (PCR) analyses for glyceraldehyde 3-phosphate dehydrogenase, p21^CIP1^, p27^Kip1^, p57^Kip2^, γ-globin and...
**Gpdh** expression were performed as in (14). *Glycerdehyde 3-phosphate dehydrogenase* amplification was used for normalization purposes.

**Antibodies used and immunoblotting**

Monoclonal antibodies to poly (ADP-ribose) polymerase were from BioMol Research Laboratories. Monoclonal antibodies against p27Kip1 were from Transduction Laboratories, Lexington, KY. The polyclonal antibodies to Sp1, Sp3 and p57Kip2 were from Santa Cruz Biotechnologies. Polyclonal antibodies to actin were to be from Sigma Chemical Company. Anticellulase H4 and actin-cellulase H3 were obtained from Upstate (Upstate Biotechnology, Charlottesville, VA). Immunoblotting analyses were performed as described (12,14).

**Firefly luciferase reporter gene plasmid transfection and luciferase activity assay**

Some of the firefly luciferase reporter gene plasmids employed was kindly given by Dr Olivier Delattre (INSERM U509, Laboratoire de Pathologie Moléculaire des Cancers, Institut Curie, Paris, France) and their preparation and features were described in (15). Particularly, one plasmid contains the full-length (2191 bp) p57Kip2 promoter, whereas the other three plasmids contain −1550 bp, −595 bp or −165 bp of the promoter region relative to the transcription start site. Starting from the plasmid containing 165 bp of p57Kip2 promoter, we constructed the following plasmids: S1 (from −1 to −87), S2 (from −1 to −113), S3 (from −59 to −151) and S4 (from −59 to −165). In particular, in order to prepare plasmids S1, S3 and S4, the relative DNA fragments were amplified by PCR employing as template the plasmid containing 165 bp of p57Kip2 promoter. The primers employed for the PCR include a KpnI linker at the 5' end of the sense primer and a HindIII linker at the 3' end of the antisense primer. The PCR products were digested with KpnI and HindIII and then ligated into the pKpn-HindIII site of the plasmid. To prepare the S2, we amplified the DNA fragment from −1 to −113 of p57Kip2 promoter by using primers that have a HindIII linker at the 5' end of the sense and antisense primers. The PCR product was digested with HindIII and then subcloned into the HindIII site of pGL3-basic vector. Details on the primers employed are available on request. The sequence of the prepared plasmids was confirmed by direct sequencing.

The plasmids (0.5 μg per 1.0 × 106 cells) were transiently transfected in K562 cells as described previously (14). Briefly, 5 h following the transfection, the medium was changed, and 2–3 h later, the transfected cells were treated as reported in the text. The luciferase activities of the cell lysates were measured and normalized as reported (14).

The treatments with siRNA were performed as follows: 24 h before siRNA addition, HeLa or EPN cells were seeded in six-wells tissue culture plates and cultured in Dulbecco modification of Eagle’s medium/F12-HAM (Sigma Chemical Company) supplemented with 5% fetal bovine serum without antibiotics. Transfection was performed by lipofectamine 2000 following manufacturer’s instructions and using a 40 nM siRNA final concentration. After 72 h, the cells were treated as reported in the text.

**Chromatin immunoprecipitation assays**

Chromatin immunoprecipitation (ChIP) assays were performed exactly as reported (14). Briefly, treated and control K562 cells (1.2 × 106 cells) were collected by low-speed centrifugation, re-suspended in phosphate-buffered saline and cross-linked with 1% formaldehyde at room temperature. After 10 min, cells were washed twice with ice-cold phosphate-buffered saline, then placed on ice, lysed and sonicated. Insoluble materials were removed and the supernatants pre-cleared by 30 μl of packed protein A agarose beads (pre-treated with sheared DNA salmon sperm). The supernatants were immunoprecipitated with the specific antisense primer plus protein A agarose beads. After extensive washing, the beads were mixed with 100 μl of 1% sodium dodecyl sulfate/10mM Tris-HCl, 1.0 mM EDTA and incubated at 65°C for 10 min, centrifuged and the eluates were transferred to fresh tube. The beads were then washed with 150 μl of 0.67% sodium dodecyl sulfate/10mM Tris-HCl, 1.0 mM EDTA, centrifuged again and the washes were added to eluates. To reverse the formaldehyde cross-links, the samples were then incubated overnight at 65°C in 0.05 M NaCl (final concentration). After this step, the samples were treated with 50 μg Proteinase K solution and incubated for 2 h at 37°C. Finally, DNA was extracted, precipitated and recovered in 20 μl of sterile H2O. Five micro-liters of DNA samples were then subjected to amplification by employing the following primers, which amplify the promoter regions of p57Kip2 (from −160 to +87): 5'-CGCCAATGCGCGTTGATTGC-3' (sense) and 5'-GACTTCCTCGTGTCGCTGTC-3' (antisense), hot start of 5 min at 95°C, 30–35 cycles composed of steps at 95°C for 1 min, 60°C for 1 min, 72°C for 1 min and a final elongation step at 72°C for 7 min. For the amplification of the negative control region (exon XI of the urokinase-type plasminogen activator gene), the following primers were used: 5'-TGGATATCTTGGTGCAGG-3' (sense) (position 5262 of the gene) and 5'-CATCTCTCCTCTGTTGGT-3' (antisense) (position 5444). For the negative control region, PCR was performed as follows: hot start 95°C, 3 min; 95°C, 1 min, 58°C, 1 min; 72°C, 1 min (repeated for 33 cycles) and 72°C, 7 min (final extension) (16).

**Results**

**HDACIs inhibit K562 cells growth, induce G1 phase arrest and upregulate p21cip1 and p57Kip2 levels**

As reported in Figure 1A, 1 mM BuA or 2 mM PBuA inhibited the growth of K562 cells. Higher concentration of the molecules (5 mM) resulted in a progressive decrease of cell number associated to clear morphological changes (cell shrinkage and lysis), which represent putative signs of apoptotic or necrotic events. Thus, we choose to employ in our study the HDACIs amounts that exert only an antiproliferative activity and do not induce complex phenotypes.

Flow cytometry analyses showed minor profile changes after 24 h treatment, whereas a G1 phase accumulation of cells was gradually observed after 48 and 72 h (Figure 1B). Moreover, a low percentage of apoptotic cells occurred at all times intervals (Figure 1B). The absence of poly (ADP-ribose) polymerase cleavage, a marker of programmed cell death, after 72 h of BuA or PBuA treatment, confirmed the lack of apoptosis (Figure 1C).

Since K562 cells differentiate towards two different phenotypes, we analyzed the expression of genes specific of erythroid and mega-karyocytic phenotypes, namely γ-globin and Gpdh, respectively (14). BuA and PBuA upregulated the expression of γ-globin gene, whereas Gpdh expression was not modified by the addition of cycloheximide (Figure 2B), thus indicating that the effect was not mediated by the synthesis of new proteins. Analysis by immunoblotting confirmed, at protein level, the increase of p21Cip1 (data not shown) and p57Kip2 involved in the control of G1 → S transition. When their expression was evaluated by semi-quantitative reverse transcription–PCR after 8 h treatment (Figure 2A), we observed a significant increase of both p21Cip1 and p57Kip2 transcription, whereas a small decrease of p27Kip1 expression was evidenced. The upregulation of p21Cip1 and p57Kip2 expression was not modified by the addition of cycloheximide (Figure 2B), thus indicating that the effect was not mediated by the synthesis of new proteins. Primarily, data suggest that such an effect is due to a decreased amount and activity of p27Kip1 ubiquinylation complex (data not shown). Since the effect of BuA on p21Cip1 has been investigated previously by others, we focused our interest on p57Kip2 gene modulation.

**The induction of p57Kip2 expression by HDACs is associated with histone hyperacetylation**

We tested the effect of various HDACIs acting either on class I/II HDACs or on class III HDACs. TSA, MS-275 and valproic acid, which inhibit the activity of HDACs of class I and II, significantly upregulated p57Kip2 level in K562 cells (Figure 2D). Conversely, the addition of splotomicin or nicotinamide, two powerful inhibitors of SIR2 family of NAD+-dependent HDACs, did not modify p57Kip2 cellular content (Figure 2D).

In order to assess the general value of the findings obtained in K562 cells, we extend our study to other cell lines. As shown in Figure 2E and F, BuA caused the cki accumulation in HT-29, CaCo2, EPN, Lan-5 and SK-N-SH cells. Similar results were also obtained in HeLa and HEK293 cells (data not shown). Conversely, in one cell line, i.e. SK-N- BE, butyric acid was unable to induce p57Kip2 expression (Figure 2F).

When, we compared the kinetics of BuA-dependent p57Kip2 induction in K562 and CaCo2 cells, we observed a significant difference that may reflect distinct transcriptional (or translational) machineries (Figure 2G versus Figure 2C).

It has been reported that the methylation of Cpg islands localized at the p57Kip2 promoter region causes a strong downregulation of its transcription (17). Particularly, HL-60 cells, which has a fully
methylated p57Kip2 promoter, do not express the cki (17). Accordingly, we observed in HL-60 cells, both at mRNA (data not shown) and protein level (Figure 3A), the complete absence of p57 Kip2. The addition of BuA and other HDACIs caused an early re-expression of p57Kip2 in HL-60 cells (Figure 3A and B). The demethylation of the promoter, obtained by 5-azacytidine treatment, also allowed the re-expression of the cki but at time intervals later than that observed with HDACIs (Figure 3C). Then, we investigated in K562 cells the effect of BuA on the luciferase expression driven by constructs containing different elements of p57Kip2 gene promoter. As shown in Figure 3D, the minimal region of promoter that is strongly activated by BuA included 165 bp before the starting point of transcription. Interestingly, we did not observe significant differences between the expression of the various constructs in untreated cells suggesting that the first 165 bp probably include consensus sequences pivotal for p57Kip2 expression.

We also evaluated whether the short-chain fatty acid increased histone acetylation at the proximal region of p57Kip2 promoter by means of ChIP approach. Particularly, in the experiment reported in Figure 3E, we evaluated, by means of a specific PCR, the occurrence of the 165 bp of p57Kip2 promoter in the DNA precipitated with antibodies against acetyl-histone H4. As shown in Figure 3E, the addition of BuA increased the amount of immunoprecipitated DNA. This pattern was also observable, although with a minor intensity, by employing in the ChIP experiment antiacetyl-histone H3 antibodies.

Fig. 1. Effect of BuA and PBuA on K562 cells proliferation and poly (ADP-ribose) polymerase proteolysis. (A) K562 cells were incubated without control, (Con) and with BuA or PBuA. Cells were then counted daily. (B) Effect of 1 mM BuA and 2 mM PBuA on cell cycle progression of K562 cells. Flow cytometry analyses were performed, as described in ref. 14, on cells treated for 24, 48 and 72 h with the reported HDACIs. The control sample corresponds to untreated cells at 24 h. (C) K562 cells were incubated without (Con) and with BuA or PBuA for 72 h. Cell extracts were then prepared and analyzed by a specific antiserum to poly (ADP-ribose) polymerase. The cleavage of poly (ADP-ribose) polymerase was demonstrated by the accumulation of the 85 kDa fragment with respect of the intact protein (signal at 115 kDa). (D) The expression of γ-globin and GpIIb genes was evaluated by reverse transcription–PCR after 3 days incubation, as reported in Materials and Methods. In this experiment, K562 cells were treated with 1 mM BuA.

Sp1 but not Sp3 or Egr1 modulates p57Kip2 promoter activity
A computer-aided analysis (by the AliBaba 2 software) of the proximal region of p57Kip2 promoter showed the presence of several putative consensus regions for transcriptional factors localized in the initial 165 bp (Figure 4A). We investigated the role of some of them that are particularly frequent in this region and are also important in the control of cell growth, i.e. Egr1 and Sp1/Sp3. Recently, we demonstrated that resveratrol, a polyphenol endowed with antiproliferative activity, strongly upregulates Egr1 level (14). Thus, we evaluated in K562 cells the effect of resveratrol on the expression of p57Kip2 gene. Although the polyphenol remarkably increased Egr1 content, the molecule did not affect p57Kip2 cellular level (Figure 4B) and did not increase the luciferase expression driven by the p57Kip2 gene promoter (Figure 4C). These findings suggest that Egr1 is not involved in p57Kip2 gene expression.

Figure 4A shows that the 165 bp region of p57Kip2 promoter embraces nine putative Sp1/Sp3 consensus sequences. Therefore, we evaluated the importance of these transcription factors in the control of the cki expression. ChIP experiments demonstrated that the DNA fragments immunoprecipitated by an anti-Sp1 antiserum contained the proximal region of p57Kip2 promoter (Figure 4D and E). Importantly, the amount of the immunoprecipitated promoter increased after BuA addition (Figure 4D and E). In addition, an anti-Sp3 antiserum did not precipitate significant amount of p57Kip2 promoter (Figure 4E). Anti-Sp1 antibodies did not precipitate the exon XI of urokinase-type...
plasminogen activator gene, a genomic region that does not contain Sp1 consensus sequences (Figure 4F) (16). This finding demonstrates the absence of non-specific DNA in the genomic fragments precipitated by anti-Sp1 antibodies.

In order to confirm the role of Sp1 in the HDACI activity on p57Kip2 promoter, they were treated with mithramycin A, a molecule that prevents the activation of Sp1 consensus sequences. As shown in Figure 5A, mithramycin A hampered the BuA-dependent activation of the luciferase expression driven by 165 bp of the p57Kip2 gene promoter. Moreover, the addition of mithramycin A prevented p57Kip2 protein accumulation in K562 cells treated with BuA (Figure 5B). Intriguingly, the molecule downregulated the expression of the gene in the untreated cells (Figure 5A and B).

In order to definitely establish the role of Sp1 in the control of p57Kip2 expression, we treated cells with Sp1 siRNA. However, as also reported by others (18), K562 cells appear difficult to transfect with a good efficiency. Thus, we decided to use two different model systems, i.e. HeLa and EPN cells, in the experiment employing interfering RNAs. We observed, in both the cell lines, that Sp1 siRNA induces a remarkable transcription factor decrease (Figure 5C) paralleled by a downregulation of p57Kip2 level and a diminished activation by BuA (Figure 5C). Accordingly, the decrease of Sp1 protein (by siRNA treatment) downregulated about 10- to 15-fold the luciferase expression driven by the 165 bp of p57Kip2 promoter in untreated cells (Figure 5D). The reduction of Sp1 content also strongly hindered the BuA stimulation of the luciferase expression (Figure 5D).

Sp1 sequences putatively involved in the HDACI activity on p57Kip2 promoter

In order to define the region of the promoter mostly involved in the HDACI response, we constructed four deleted mutants starting from the plasmid containing the 165 bp of promoter (Figure 6A). Two mutants contained 89 bp (from +1 to +87, S1 plasmid) or 115 bp (from +1 to +113, S2 plasmid). The two other deleted mutants included p57Kip2 promoter region from –59 to –151 (excluding the region containing the last Sp1 site, S3 plasmid) or from –59 to –165 (S4 plasmid). The starting point of these two mutants (+59) was chosen in order to include the TATA box.

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This result suggests that the region localized between the $-87$ and $-113$ bp plays an important role in the HDACI regulation of p$57^{kip2}$ gene. Recently, it has been demonstrated that the sequence $-102/-107$ of the p$57^{kip2}$ promoter might represent the consensus for the CTIP2 factor (19). This protein functions as a repressor of transcription and its activity is hindered either by inhibitor of HDAC of class I/II or of class III, depending on the promoter type. In the case of p$57^{kip2}$ gene, the activity of CTIP2 is reverted by TSA, an inhibitor of HDAC of class I/II, whereas the effect of sirtuin inhibitors was not investigated (19). Our finding that the region of p$57^{kip2}$ promoter required for HDACIs response includes not only two putative Sp1 sequences but also the CTIP2 binding sequence appears of interest.

Therefore, we evaluated the effect of CTIP2 downregulation on the BuA activity. As shown in Figure 6C, the treatment of HeLa and EPN cells with CTIP2 siRNA results in a remarkable increase of BuA response. Moreover, a decrease of p$57^{kip2}$ content was observed in HeLa control cells after the treatment with the CTIP2 siRNA.

Discussion

In this paper, we investigated the effects of HDACIs on the expression of p$57^{kip2}$, a powerful inhibitor of cyclin-dependent kinases. HDACIs represent a new class of promising targeted anticancer agents. Several of these compounds are in clinical trials with significant activity.
against a spectrum of both hematological and solid tumors at doses that are well tolerated by the patients (10 and references therein).

The characterization of HDACi mechanism of action represents a main goal of the studies on these molecules, particularly in view of identifying novel targets for therapy. The most widely demonstrated molecular effect is the regulation of gene transcription. Therefore, the analysis of expression profiles is thought as the key methodology to identify the HDACi cellular effectors. The modulation of gene expression appears due to the relaxation of nucleosome structure and/or the acetylation of non-histone proteins. Indeed, the function of a number of transcription factors (including p53, GATA-1, FOXO proteins, estrogen and androgen receptors and others) is controlled by their acetylation status (20–22).

p57Kip2 is a cki strongly involved in differentiation. Differently from p21Cip1 and p27Kip1 gene ablated mice, which show scarce phenotypic defects, p57Kip2 null mice die after birth and display severe developmental abnormalities with varying degree of penetrance (23). p57Kip2 is also required for myogenesis, cardiomyogenesis, osteoblast differentiation, lens morphogenesis, retina maturation and podocyte growth maturation (24–26).

In addition, a clear correlation exists between the decrease of p57Kip2 content and human tumor progression. Particularly, low levels of the cki have been demonstrated in gastric cancers, esophageal squamous cell carcinomas, human bladder cancers, head and neck carcinomas, hepatocellular carcinomas, pancreatic adenocarcinomas, duct carcinomas and intrahepatic cholangiocellular carcinomas, leukemias and thyroid tumors (27,28).

Although these observations point to p57Kip2 as a protein vital in the process of differentiation and cancerogenesis, few information is available on the mechanisms controlling the cki gene expression. p57Kip2 promoter silencing by hypermethylation has been demonstrated in cancer cells (17) but the importance of DNA methylation in the physiological control of p57Kip2 gene expression is still unclear. Moreover, the fusion protein EWS-FLI-1 has been reported to down-regulate p57Kip2 expression in Ewing tumor (15). Finally, CTIP2, a transcriptional repressor, has been recently reported to hinder p57Kip2 gene transcription (19).

So far, only scarce and descriptive data on the effects of HDACi on p57Kip2 expression are available in literature (29), whereas the precise mechanism of their action has not been investigated. Here, we reported that all the tested inhibitors of class I and II HDACs strongly and rapidly induce the upregulation of p57Kip2. Conversely, we demonstrated that the addition of inhibitors of SIR2 family of NAD+-dependent HDACs, i.e. sirtuin and nicotinamide, does not affect the cki expression. These findings have been observed in numerous cell lines of different origin, thus representing a general response to HDACi addition.

A detailed analysis of p57Kip2 promoter demonstrates, for the first time, that the minimal region responsive to HDACi includes the initial 165 bp of p57Kip2 promoter. The nine Sp1 consensus sequences are also shown as well as the TATA box. (B) The four deleted plasmids as well as the wild-type plasmid described in (A) were transiently transfected into K562 cells and luciferase activities were analyzed after 24 h treatment with 1 mM BuA. Luciferase activity is reported as shown. On the left, the dimension of the cloned promoter sequence is reported. Data are the mean (bars, standard deviation) of at least four different experiments. White box, control; black box, BuA-treated cells.
presumed binding sites for Egr1 and Sp1/Sp3. The role of Egr1 has been ruled out since a substantial upregulation of this transcription factor does not modify p57Kip2 level. On the other hand, ChIP experiments indicate that HDACIs addition cause a remarkable recruitment of Sp1 (but not Sp3) on the 165 bp region of the promoter, thus suggesting a role of this transcription factor in the response to the inhibitors of HDACs.

The finding was strongly confirmed by the observation that the forced decrease of Sp1 levels, obtained by Sp1 siRNA, results in a significant loss of HDACIs activity (Figure 5C). Interestingly, in the untreated cells, the addition of the mithramycin A and Sp1 siRNA also downregulates the level of p57Kip2 expression arguing that Sp1 is also vital for the constitutive cki gene transcription.

The role of Sp1 has also been demonstrated in the control of the expression of other two cks, p21Cip1 (30) and CDKN2D (31), thus indicating that the transcription factor might act as a critical regulator of cell cycle and phenotype by influencing contemporaneously several cki genes.

Experiments with different deleted constructs of the 165 bp promoter demonstrated that the region between –87 and –113 is necessary for the activating effect of HDACIs. Intriguingly, this region includes not only two putative Sp1 consensus sequences but also the putative binding site of CTIP2 on p57Kip2 promoter (19). CTIP2 is a novel C2H2 zinc finger protein that was first isolated and identified of HDACIs on (i) early promoters, (ii) downregulation of Sp1 hinders BuA stimulation, (iii) downregulation of Sp1 (but not Sp3) factor plays an important role in the cki expression and in the response to HDACIs. Moreover, we demonstrate that the region between –87 and –113 that includes 2 Sp1 binding sites and the CTIP2 interacting region probably represents a pivotal site of HDACIs regulation of p57Kip2 gene.

Due to the importance of HDACIs in new therapeutic approaches, our data contribute to unravel their mechanism of action identifying p57Kip2 as a major molecular target.

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References


