An Inhibitor-Resistant Histone Deacetylase in the Plant Pathogenic Fungus *Cochliobolus carbonum*†

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ABSTRACT: We have partially purified and characterized histone deacetylases of the plant pathogenic fungus *Cochliobolus carbonum.* Depending on growth conditions, this fungus produces HC-toxin, a specific histone deacetylase inhibitor. Purified enzymes were analyzed by immunoblotting, by immunoprecipitation, and for toxin sensitivity. The results demonstrate the existence of at least two distinct histone deacetylase activities. A high molecular weight complex (430 000) is sensitive to HC-toxin and trichostatin A and shows immunoreactivity with an antibody against *Cochliobolus* HDC2, an enzyme homologous to yeast RPD3. The second activity, a 60 000 molecular weight protein, which is resistant even to high concentrations of well-known deacetylase inhibitors, such as HC-toxin and trichostatin A, is not recognized by antibodies against *Cochliobolus* HDC1 (homologous to yeast HOS2) or HDC2 and represents a different and/or modified histone deacetylase which is enzymatically active in its monomeric form. This enzyme activity is not present in the related filamentous fungus *Aspergillus nidulans*. Furthermore, in vivo treatment of *Cochliobolus* mycelia with trichostatin A and analysis of HDACs during the transition from non-toxin-producing to toxin-producing stages support an HC-toxin-dependent enzyme activity profile.

Histone acetylation plays a crucial role in fundamental cellular processes such as transcription, chromatin assembly, and DNA repair. Histone acetyltransferases (HATs)[1] thereby catalyze the transfer of an acetyl group from acetyl-CoA onto the ϵ-amino group of lysine residues in the amino-terminal tail of core histones. The reaction is reversed by the action of histone deacetylases (HDACs). The identification of HATs as transcriptional coactivators and HDACs as transcriptional (co-)repressors has confirmed the importance of these enzymes for gene regulatory processes [reviewed in (*1*−*6*)].

After the identification of a human HDAC with sequence homology to the yeast transcriptional regulator RPD3, termed HDAC1 (*7*), numerous HDACs have been identified in higher eukaryotes. HDACs are classified into four families resulting from sequence homologies to either yeast RPD3 (*7*−*12*), yeast HDA1 (*11, 13, 14*), plant HD2 (*15, 16*), or yeast SIR2 (*17, 18*), the founding members of these families. As a characteristic feature, many of these proteins are components of large multiprotein complexes primarily involved in gene silencing processes. Furthermore, RPD3-, HDA1-, and plant HD2-like HDACs are specifically affected by a number of potent inhibitors, like trichostatin A (TSA) or various cyclic tetrapeptides including trapoxin, HC-toxin, chlamydocin, and apicidin [for review, see (*19*)].

HC-toxin, the host-specific toxin of the maize pathogenic filamentous fungus *Cochliobolus carbonum*, was shown to inhibit HDACs from a variety of species (*20*). As a consequence, during infection, the maize core histones become hyperacetylated in vivo in susceptible, but not in resistant, maize lines (*21*). It was postulated that *C. carbonum* either interferes with the expression of maize defense genes when colonizing the host cells by highly specific inhibition of HDACs or inhibits protein synthesis by interfering with ribosomal gene expression through action on the nucleolar deacetylase HD2. However, like all eukaryotes, *C. carbonum* has histones and HDACs, and it must therefore possess a mechanism to protect itself against HC-toxin. An HC-toxin efflux pump in *C. carbonum* (*22*) and HC-toxin reductase in resistant maize plants for detoxification (*23*) have been demonstrated. However, one could also propose the existence of an inhibitor-resistant HDAC in *C. carbonum*.

Since the majority of hitherto identified HDACs are highly sensitive to various inhibitors, the finding of modified or structurally novel HDACs would be of great importance with respect to the proposed role of HDACs for transcriptional regulation. HDACs have been shown to be part of repressor or corepressor complexes required for the downregulation of specific genes [e.g., ref (*24*)]. In addition to their role as

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[1] Abbreviations: HAT, histone acetyltransferase; HDAC, histone deacetylase; TSA, trichostatin A; HDC1, *Cochliobolus* histone deacetylase 1; HDC2, *Cochliobolus* histone deacetylase 2; PBS, phosphate-buffered saline; MELC, murine erythroleukemia cells; NAD, nicotinamide adenine dinucleotide; ORF, open reading frame.

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transcriptional regulators, HDACs have also been implicated in various diseases (*25*−*28*). Therefore, it is important to study mechanisms leading to aberrant histone acetylation especially with respect to HDACs and the potential of HDAC inhibitors as therapeutic agents; in particular, the structure and function of putative inhibitor-resistant HDACs is an important item. This seems even more attractive in light of the fact that to date only few examples of HDACs are known which show deviating properties against HDAC inhibitors (*18*, *29*, *30*) and which could fulfill different biological functions. Analysis and identification of such undefined HDACs should contribute to a better understanding of the biological role of histone acetylation in general and for the mechanism of pathogenesis with respect to the work presented here.

We report on the identification and characterization of an inhibitor-resistant HDAC of *C. carbonum*. Partial purification, inhibition assays, immunoblotting, and immunoprecipitation experiments revealed the existence of at least two different HDACs, a toxin-resistant and other toxin-sensitive forms. Resistant activity was only present in toxin producing *Cochliobolus* cultures and had a native molecular weight of approximately 60 000. Resistant activity was not due to a putative SIR2-like activity in *Cochliobolus*, since incubation with NAD^+ did not alter the enzyme activity profile. Toxin-sensitive HDAC activity of *C. carbonum* is part of a multiprotein complex sharing properties similar to RPD3-type HDACs. Furthermore, partially purified HDACs of *Aspergillus nidulans*, a closely related filamentous fungus, are toxin-sensitive, indicating that resistance is not a common feature of filamentous fungi. Since resistant HDAC activity did not react with anti-*Cochliobolus* HDC1 and HDC2 antibodies, this and other findings suggest the existence of an additional modified and/or structurally unrelated, yet novel HDAC responsible for resistant activity.

EXPERIMENTAL PROCEDURES

Cultivation of Organisms. Cochliobolus carbonum (strain 377-12; derived from American Type Culture Collection 90305) was maintained on vegetable-juice agar. Small pieces of colonized agar were used to inoculate 125 mL of HMT medium per 1000 mL flask (*31*). *C. carbonum* was grown for 7 days at 25 °C (still culture) or for 2 days at 25 °C (shake culture). *Aspergillus nidulans* strain A4 (Glasgow wild type) provided from the Fungal Stock Center (Kansas City, KS) was grown in minimal medium according to (*32*) for 7 days at 37 °C (still culture) and for 2 days at 37 °C (shake culture), respectively.

Partial Purification of Histone Deacetylase Activity. Protein Extraction. The fungal mats were collected and washed in a sintered glass filter, thoroughly dried with filter paper, and immediately frozen in liquid nitrogen. Thirty grams of frozen mycelia was ground to powder in an IKA grinding machine, and the powder was suspended in 60 mL of buffer A [15 mM Tris-HCl, pH 7.6, 700 mM NaCl, 0.25 mM EDTA, 1 mM β-mercaptoethanol, 10% (v/v) glycerol]. The mixture was stirred for 1 h on ice and then centrifuged for 30 min at 35000*g* at 4 °C. The supernatant was dialyzed against buffer B [15 mM Tris-HCl, pH 7.6, 10 mM NaCl, 0.25 mM EDTA, 1 mM 2-mercaptoethanol, 10% (v/v) glycerol] overnight and used for SourceQ chromatography.

SourceQ Chromatography. The dialyzed extracts were batch-extracted with 10 mL of Source 30Q anion exchange medium (Amersham Pharmacia Biotech, Uppsala, Sweden) for 5 h at 4 °C, equilibrated with buffer B. The matrix resin was pelleted by centrifugation and transferred to an FPLC column (1.6 × 15 cm). Elution of proteins was performed with 60 mL of a linear gradient from 10 mM to 500 mM NaCl in buffer B at a flow rate of 1 mL/min. Fractions of 1.5 mL were collected and assayed for HDAC activity. Fractions with HDAC activity were pooled and concentrated to a final volume of 1 mL by centrifugation (2500*g*, 4 °C) using Amicon Centriprep-10.

Size Exclusion Chromatography (Superdex 200). Concentrates of fractions from SQ-chromatography were applied onto a Superdex 200 FPLC column (2.5 × 100 cm; 120 mL; Amersham Pharmacia Biotech, Uppsala, Sweden), equilibrated with 200 mM NaCl in buffer B. The flow rate was maintained at 1 mL/min, and fractions of 1.5 mL were collected and assayed for HDAC activity. For estimation of the molecular weight of the native enzymes, the Superdex 200 column was calibrated with proteins of known molecular weight.

Histone Deacetylase Assay. HDAC activity was determined as described (*33*) using [^{3}H]acetate-prelabeled chicken reticulocyte histones as substrate. Sample aliquots of 50 μL were mixed with 10 μL of total [^{3}H]acetate-prelabeled chicken reticulocyte histones (1 mg/mL), resulting in a concentration of 11 μM. This mixture was incubated at 37 °C with samples of *C. carbonum* and at 25 °C with samples of *A. nidulans* for 1 h. The reaction was stopped by the addition of 50 μL of 1 M HCl/0.4 M acetyl acetate and 0.8 mL of ethyl acetate. After centrifugation at 10000*g* for 5 min, an aliquot of 600 μL of the upper phase was counted for radioactivity in 3 mL of liquid scintillation cocktail.

Toxins. HC-toxin was purified from 21-day-old still cultures by $CHCl_3$ extraction and reversed-phase chromatography (*34*). Trichostatin A and butyric acid were purchased from Sigma. The hydroxamate (TSA analogue) D85 was synthesized (*35*) and kindly provided by M. Jung (Department of Pharmaceutical Chemistry, University of Münster, Germany). For enzyme inhibition assays, samples with HDAC activity were incubated with different concentrations of TSA, HC-toxin, D85, and butyrate for 15 min on ice prior to the standard HDAC assay.

Protein Analysis. SDS−PAGE was performed in 10% polyacrylamide gels as previously described (*36*). Aliquots (2 μL each) of inhibitor-resistant HDAC activity preparations of different stages of purification were analyzed. After electrophoresis, proteins were stained with silver as described (*37*) with minor modifications; gels were soaked in 50% methanol for 90 min, incubated in 150 mL of staining solution (0.02 N NaOH, 0.37% NH_4OH, and 0.8% $AgNO_3$) for 10 min, washed in distilled water for 10 min, and finally developed in 250 mL of a solution containing 0.01% citrate and 0.037% formaldehyde.

Immunological Analysis. Expression of Recombinant HDC1, HDC2, and A. nidulans SIR2 and Production of Antibodies. Coding sequences of the C-terminal fragment of HDC2 spanning amino acids 449−648 (J. Walton, S. Graessle, and G. Brosch, unpublished results), full-length HDC1 [amino acids 1−505; (*38*)], and full-length *Aspergillus* SIR2 (amino acids 1−383; S. Graessle and G. Brosch, unpublished results)

were amplified from *C. carbonum* and *A. nidulans* cDNAs by PCR. The nucleotide sequences were inserted into a pQE9 expression vector (Qiagen, USA). Expression in *Escherichia coli* (M15) led to recombinant fragments with a 6× His affinity tag on the N-terminus, allowing purification of the fragments on a metal chelate adsorbent Ni−NTA resin. Eluted proteins were detected at a wavelength of 280 nm and were analyzed by SDS−PAGE. Purified recombinant proteins were used for polyclonal antibody production in rabbits. IgG antibodies were purified by protein-G immunoaffinity chromatography.

Immunoprecipitation. For immunoprecipitation experiments, 50 μL of partially purified HDAC activities of *C. carbonum* (SQ-chromatography) was mixed with 2 μg of affinity-purified anti-HDC1 and anti-HDC2 antibodies and 30 μL of protein-A Sepharose, equilibrated with buffer B, and incubated for 2 h by permanent shaking at 4 °C. To avoid unspecific binding, the mixture was adjusted to 150 mM NaCl. After centrifugation for 10 min at 3000*g*, the supernatant was saved for HDAC assay and protein blotting. Pellets were washed 3 times with 500 μL of buffer B and finally resuspended in 20 μL of buffer B. Entire pellets and 50 μL of supernatants were used for standard HDAC assay. For immunoblotting, precipitates were mixed with SDS sample buffer, boiled, and centrifuged for 5 min at 12000*g*, and the resulting supernatant was used for SDS−PAGE.

Immunoblotting. Enzyme fractions of immunoprecipitation experiments and fractions from SQ- or S200-chromatography were electrophoresed in precast 10% polyacrylamide gels (Novex, USA) at 35 mA for 1.5 h at room temperature. Gels were blotted onto a nitrocellulose membrane (Schleicher & Schuell, Germany) at 350 mA for 2 h, and membranes were blocked with 2% (w/v) skim milk in phosphate-buffered saline (PBS, 20 mM, at pH 7.4) for 2 h. Membrane strips were incubated with a 1:1000 dilution of affinity-purified antibodies against HDC1 and HDC2 in 1% skim milk in PBS at 4 °C overnight. After being washed, strips were incubated for 2 h with horseradish peroxidase-conjugated secondary anti-rabbit Ig (Amersham Pharmacia Biotech), and immunodetection was performed using the enhanced chemiluminescence detection system (ECL) following the manufacturers' instructions (Amersham Pharmacia Biotech).

Nuclear Isolation and Analysis of Histone H4 Acetylation. For analysis of histone H4 acetylation, *C. carbonum* was grown in still culture for 2 or 7 days. Five hours prior to harvesting, TSA was added to the culture medium to reach a final concentration of 1 μM. Isolation of nuclei was performed according to a modified protocol of (*39*). All steps were performed at 4 °C. Eight grams of frozen mycelia was ground to powder in an IKA grinding machine, and the powder was suspended in 400 mL of homogenization buffer [10 mM PIPES, pH 6.9, 5 mM $CaCl_2$, 5 mM $MgSO_4$, 0.5 M sucrose, 1 mM PMSF, 10 mM β-mercaptoethanol, 1 μM TSA (no TSA was added to the control)]. The suspension was homogenized by 10 strokes at 500 rpm in a Potter−Elvehjem homogenizer and centrifuged for 5 min at 100*g*. The supernatant was filtered and centrifuged for 20 min at 6000*g*. The supernatant was decanted, and the pellet was resuspended in 15 mL of 2.1 M sucrose in homogenization buffer. After centrifugation for 10 min at 3000*g*, the resulting supernatant was again centrifuged at 161000*g* for 50 min to pellet the nuclei. For disruption of nuclei, the pellet was resuspended in 3 volumes of 8 M urea/0.9 M acetic acid and subjected to sonification. Aliquots of nuclear extracts were analyzed by SDS−PAGE (precast 14% gels) with subsequent Coomassie blue staining.

For immunological analysis, equal amounts of nuclear extracts (estimated by laser densitometry) were electrophoresed in precast 14% polyacrylamide gels, and blotted onto a nitrocellulose membrane. Membrane strips were incubated with an antibody against acetylated peptide corresponding to amino acids 2−19 of *Tetrahymena* histone H4 (Upstate Biotechnology, USA). This antibody recognizes highly acetylated histone H4 isoforms (di-, tri, and tetraacetylated). Immunodetection was performed using the ECL system. For quantification of results, blots were stripped, and immunodetection was performed with alkaline phosphatase conjugated secondary antibody.

RESULTS

Partial Purification of HDACs in C. carbonum under HC-toxin-Producing and -Nonproducing Conditions. For the analysis of HDACs in *C. carbonum* in the presence and absence of HC-toxin, two different growth conditions were chosen. Whereas growth in still culture leads to production of HC-toxin, toxin is not synthesized in shake culture due to inhibition of apressorium development (J.D. Walton, personal communication). To compare results, we used the closely related filamentous fungus *Aspergillus nidulans* as a reference system, which does not produce an HDAC-inhibiting toxin.

Dialyzed protein extracts were subjected to SourceQ (SQ)-anion exchange chromatography. In still cultures, HDAC activity of *Cochliobolus* eluted as a single peak at 150 mM NaCl (Figure 1A), whereas the corresponding activity of *Aspergillus* eluted at a high salt concentration of 290 mM NaCl (Figure 1B). No further activities could be observed at lower or higher salt concentrations, respectively.

When analyzing shake culture mycelia of *C. carbonum* in the same experimental setting, the elution profile differed from the still culture profile (Figure 1A). Although only one single sharp peak could be detected, this activity eluted at a higher salt concentration of 280 mM NaCl as compared to 150 mM for still culture. At 150 mM NaCl, no significant HDAC activity could be observed in shake culture. The elution profile of *Aspergillus* shake culture, however, was consistent with the profile of still culture (Figure 1B) with a minor peak of approximately 5% of the total HDAC activity eluting at 120 mM NaCl.

C. carbonum Expresses Inhibitor-Resistant and Inhibitor-Sensitive HDACs. HDAC activities were then analyzed for their sensitivity to various HDAC inhibitors. SQ-chromatography fractions (fraction 25 of *C. carbonum* and fraction 35 of *A. nidulans* still cultures) were incubated with different concentrations of HC-toxin, TSA, D85, and butyrate. As a reference, inhibition assays were also performed with partially purified nucleolar HD2 from *Zea mays*, which was shown to be highly sensitive to HC-toxin and TSA (*20*). Figure 2 shows that *Cochliobolus* HDAC was resistant to high concentrations of TSA, HC-toxin, and D85. A concentration of 300 nM TSA, leading to an inhibition of more than 98% of the maize enzyme, had only a negligible effect on *Cochliobolus* HDAC. Similar results were obtained when

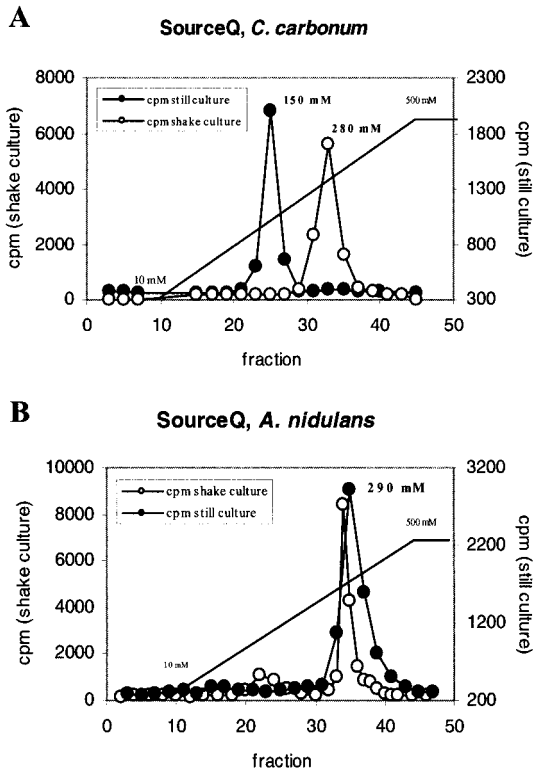

FIGURE 1: HDAC activities of *C. carbonum* and *A. nidulans* grown in still culture or shake culture. Dialyzed salt extracts were mixed with 10 mL of Source 30Q anion exchange medium for 5 h at 4 °C equilibrated with buffer B. The matrix was pelleted by centrifugation and transferred to an FPLC column (1.6 × 15 cm). Elution of proteins was performed with 60 mL of a linear gradient from 10 to 500 mM NaCl in buffer B at a flow rate of 1 mL/min. Fractions of 1.5 mL were collected and assayed for HDAC activity. Salt concentrations of eluting peak fractions are indicated in panels A and B.

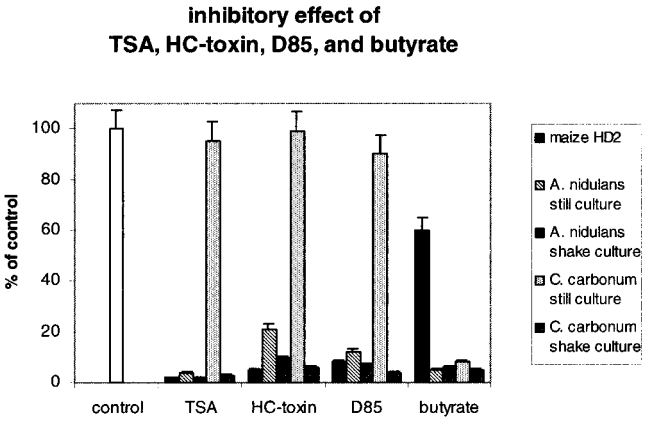

FIGURE 2: Inhibitory effect of HC-toxin, TSA, D85, and butyrate on HDAC activities. SourceQ chromatography peak fractions (see Figure 1) of *C. carbonum* still and shake cultures (fractions 25 and 33, respectively), *A. nidulans* still and shake cultures (fractions 35 and 33, respectively), and maize nucleolar HD2 were incubated with inhibitors (300 nM TSA, 200 μM HC-toxin, 2 μM D85, 5 mM butyrate) for 15 min at 4 °C before the standard HDAC assay. Inhibitory effect is shown as percent of control. SD values are indicated for 5 independent detections.

the synthetic TSA analogue D85 was used for inhibition assays. Resistance was even more pronounced for HC-toxin, where a concentration of 200 μM HC-toxin had no inhibitory effect on *Cochliobolus* HDAC activity at all. Interestingly, the *Cochliobolus* HDAC activity is sensitive to 5 mM butyrate, indicating a different mode of action of this agent (Figure 2). In contrast to *Cochliobolus*, HDAC activity of *Aspergillus* still culture was sensitive to HDAC inhibitors, although in a slightly less pronounced manner compared to maize HD2. For HC-toxin, 80% inhibition was obtained at 200 μM compared to 94% for the maize enzyme.

When we analyzed shake culture SQ-chromatography peak fractions (fraction 33 of *C. carbonum* and *A. nidulans* shake cultures) for sensitivity to HDAC inhibitors, both the *Aspergillus* and also the *Cochliobolus* HDACs were toxin-sensitive. TSA (30 nM) resulted in approximately 60% inhibition of HDAC activities, and 300 nM TSA caused complete inhibition (Figure 2).

Resistant HDAC Activity Is Present as a Protein Monomer. To further characterize HDAC activities of *Cochliobolus* and *Aspergillus* still culture mycelia, peak fractions of SQ-chromatographies were pooled, concentrated, and subjected to S200 gel filtration chromatography. Whereas the inhibitor-resistant *Cochliobolus* HDAC activity eluted at an apparent molecular mass of approximately 60 kDa (Figure 3A), the *Aspergillus* activity eluted as a high molecular mass complex of approximately 390 kDa (Figure 3B).

Peak fractions of SQ-chromatography of shake culture extracts with HDAC activity were concentrated and loaded onto an S200 column (Figure 3). HDAC activity of *Cochliobolus* shake culture eluted at a molecular weight of 430 000, in contrast to *Cochliobolus* still culture where HDAC activity was shown to be active in a low molecular weight form (Figure 3A). The HDAC activity of *Aspergillus* shake culture eluted at a molecular weight of approximately 390 000, which is consistent with results obtained from still culture (Figure 3B). These data indicate that the resistant activity of *C. carbonum* obviously is active as a protein monomer.

When peak fractions of S200-chromatographies (Figure 3) were treated with different inhibitors, HDAC activity of *Cochliobolus* still culture was still resistant, and a similar inhibition pattern as shown in Figure 2 was obtained (data not shown). Therefore, progressive purification did not change its sensitivity to toxins. Considerable purification was achieved after two chromatographic steps. Figure 3C shows the reduction of proteins in the S200 peak fraction in comparison to the protein extract. However, further purification steps always led to a drastic loss of enzymatic activity. Until now we were not able to purify the resistant activity to homogeneity although we have a promising 60 kDa protein whose abundance corresponds to the enzyme activity profile after chromatographic elution.

Resistant HDAC Activity of Cochliobolus Is Not Due to SIR2. The yeast HDAC SIR2 was shown to be resistant to high concentrations of TSA. To decide whether the resistant HDAC activity in *Cochliobolus* is due to an NAD^+-dependent SIR2 enzyme activity, we incubated fractions of SQ-chromatography of still culture extracts with different amounts of NAD^+ (0.01, 0.1, and 1 mM) and performed our standard HDAC assay. However, neither significant differences in the elution profile of HDAC activity nor an increase in the activity of fractions could be obtained (results not shown). Furthermore, chromatographic fractions containing resistant HDAC activity were also analyzed by an antibody against *A. nidulans* SIR2 protein. Since *A. nidulans* is a closely related filamentous fungus, an anti-*Aspergillus*

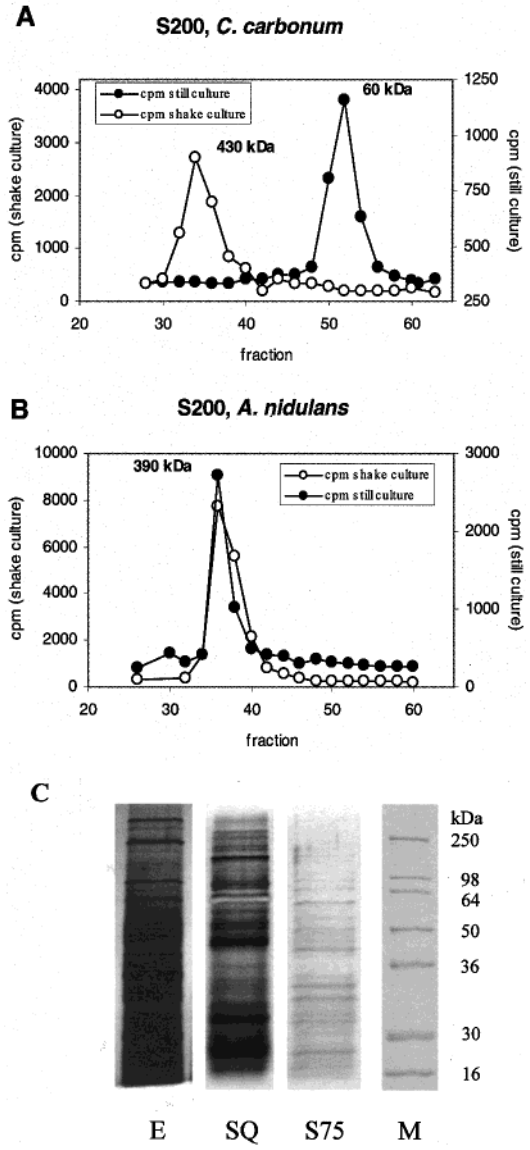

FIGURE 3: Size exclusion chromatography (Superdex 200) of HDACs and electrophoretic analysis of proteins at different steps of purification of inhibitor-resistant HDAC activity on SDS−polyacrylamide gels. SourceQ chromatography peak fractions of *C. carbonum* and *A. nidulans* still and shake cultures (Figure 1A,B) were concentrated and applied to a Superdex 200 FPLC column equilibrated with 0.2 M NaCl in buffer B. The flow rate was 1 mL/min, and fractions of 1.5 mL were collected and assayed for HDAC activity. Estimated native molecular masses are indicated in panels A and B. For SDS−PAGE, 2 μL of crude extract (E), SourceQ (SQ, fraction 25), and Superdex 75 (S75, fraction 52) were adjusted to 10 μL with sample buffer and subsequently subjected to SDS−10% PAGE. The gel was stained with silver. The molecular mass of marker proteins (M) is indicated (kDa) (panel C).

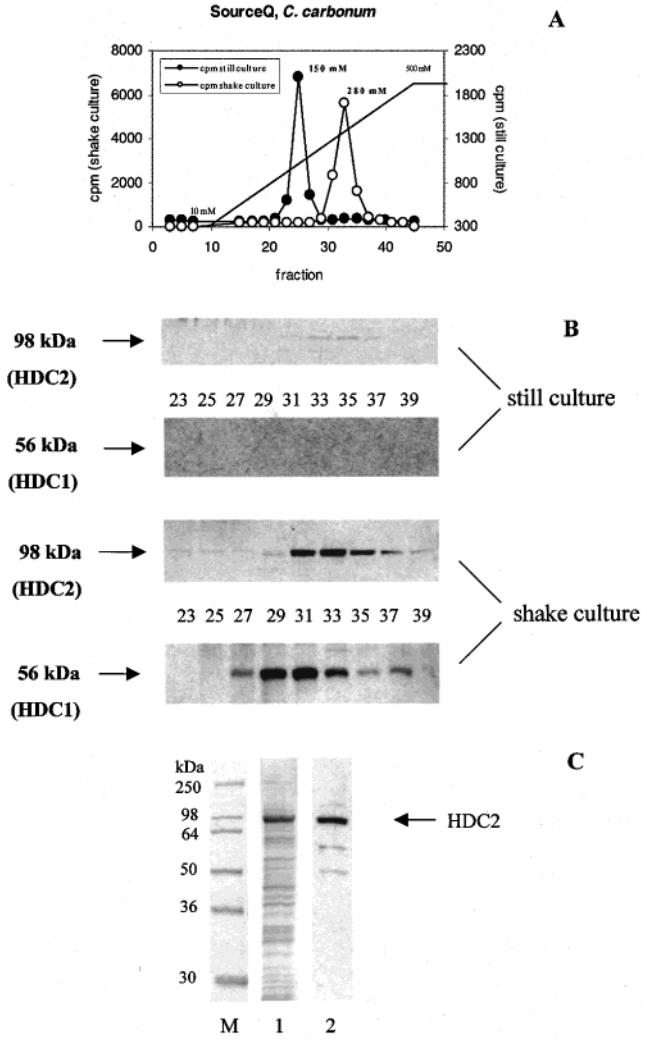

FIGURE 4: Immunological identification of *Cochliobolus* proteins related to yeast RPD3 (HDC2) and yeast HOS2 (HDC1) and electrophoretic mobility of recombinant HDC2. SQ-chromatography of extracts of *C. carbonum* mycelia grown in still culture and shake culture was performed as described in Figure 1 (A). Aliquots of each fraction from SQ-chromatography of (A) were subjected to SDS−PAGE with subsequent blotting onto nitrocellulose membranes. Antibodies against recombinant HDC1 and HDC2 were used for immunodetection. Detection was performed by the ECL detection system (Amersham International plc). Molecular masses of recombinant HDC1 and HDC2, estimated by SDS−PAGE, are indicated (B). 15 μL of *E. coli* extract containing recombinant HDC2 was subjected to SDS−10% PAGE. The gel was stained with Coomassie blue (1), or in case of immunoblotting, the gel was transferred to a nitrocellulose membrane with subsequent immunodetection using anti-HDC2 antibodies (2). Detection was performed by the ECL detection system. Molecular mass of marker proteins (M) and position of recombinant HDC2 are indicated (C).

SIR2 antibody is likely to react with a homologous protein in *C. carbonum.* However, immunoblotting revealed no signal in inhibitor-resistant enzyme fractions (data not shown).

HDC1 and HDC2 Are Part of an Inhibitor-Sensitive, High Molecular Weight Complex. Biochemical characterization of HDAC activities indicated the presence of two distinct HDAC activities in *C. carbonum*, dependent on growth conditions. To further substantiate this finding and to characterize these activities, each fraction from SQ-chromatography (still and shake cultures) was subjected to immunoblotting with anti-HDC1 and anti-HDC2 antibodies (see Experimental Procedures). Figure 4 shows that anti-HDC2 antibodies detected a single protein band at a molecular weight of 98 000 in the sensitive HDAC activity of *Cochliobolus* shake culture. The intensity of the immunosignal corresponded to the pattern of HDAC activity in the chromatography elution. Although the predicted molecular weight of HDC2 is 71 000 (J. Walton, S. Graessle, and G. Brosch, unpublished results), the enzyme migrates at a much higher apparent molecular weight in SDS−PAGE; this

finding was substantiated by testing recombinant HDC2 which also exhibits this unusual electrophoretic mobility (Figure 4C).

Incubation of the same blots with anti-HDC1 antibodies resulted in detection of a single protein band at a molecular weight of 56 000 which coincides with the predicted size of HDC1 (*38*). However, the maximum intensity of the immunosignal (fractions 29−31) did not exactly correspond to HDAC activity with the maximum in fraction 33 (Figure 4).

When HDACs of still culture mycelia were partially purified by SQ-chromatography and then analyzed by immunoblotting using anti-HDC2 antibodies, a very faint signal at a molecular weight of 98 000 could be detected in fractions 31−37, that lacked HDAC activity. In fractions containing resistant HDAC activity (fractions 23−27), no immunosignal was detectable (Figure 4B). Moreover, no immunosignal could be obtained using the anti-HDC1 antibody, neither in fractions 23−27 nor in fractions 31−37 (Figure 4B).

Immunoprecipitation of HDC2. To further demonstrate a direct link between HDC2 protein and HDAC activity in extracts and SQ-chromatography fractions of shake culture of *C. carbonum*, we performed immunoprecipitation experiments. For this purpose, samples were incubated with anti-HDC1 and anti-HDC2 antibodies, respectively; proteins were precipitated using protein-A Sepharose. Resulting supernatants and pellets were tested for HDAC activity and were subjected to immunoblotting. Figure 5A shows that in the peak fraction of SQ-chromatography (fraction 33) of *Cochliobolus* shake culture significant HDAC activity was precipitated by HDC2 antibodies (P1) with concomitant depletion from the supernatant (S1), whereas HDC1 antibodies could not precipitate any HDAC activity (data not shown). In a control reaction (without antibody), neither depletion of HDAC activity from the supernatant nor precipitation of HDAC activity was observed (Figure 5A, S2, P2).

Subsequent immunoblotting of the samples of Figure 5A using anti-HDC2 antibodies yielded a specific immunosignal at 98 kDa when immunoprecipitation was performed in the presence of the antibody (Figure 5B, P1), whereas the control precipitate (no antibody) did not react with the antibodies (P2). Apparently, HDC2 protein was removed from the supernatant, since no residual protein could be detected (S1). However, immunoprecipitation experiments revealed the depletion of only a minor part of HDAC activity. Residual activity may be due to an additional HDAC form which is present in an HDC2 multiprotein complex but was not separated by our purification procedure. It is unlikely that the putative HDAC HDC1 (*11*) is responsible for this activity for several reasons. Although anti-HDC1 antibodies clearly detect a single 56 kDa protein in the chromatography fractions, this immunosignal does not exactly match with the detected levels of HDAC activity in the HDAC activity peak. Furthermore, no additional enzyme activity could be precipitated either by the HDC1 antibody or by HDC2 co-immunoprecipitation.

HDAC activities of crude extracts and of partially purified chromatography fractions of *Cochliobolus* still culture (grown for 7 days) could not be precipitated by anti-HDC2 antibodies. Thus, extracts of *Cochliobolus* mycelia grown in still culture contain a different, toxin-resistant HDAC activity.

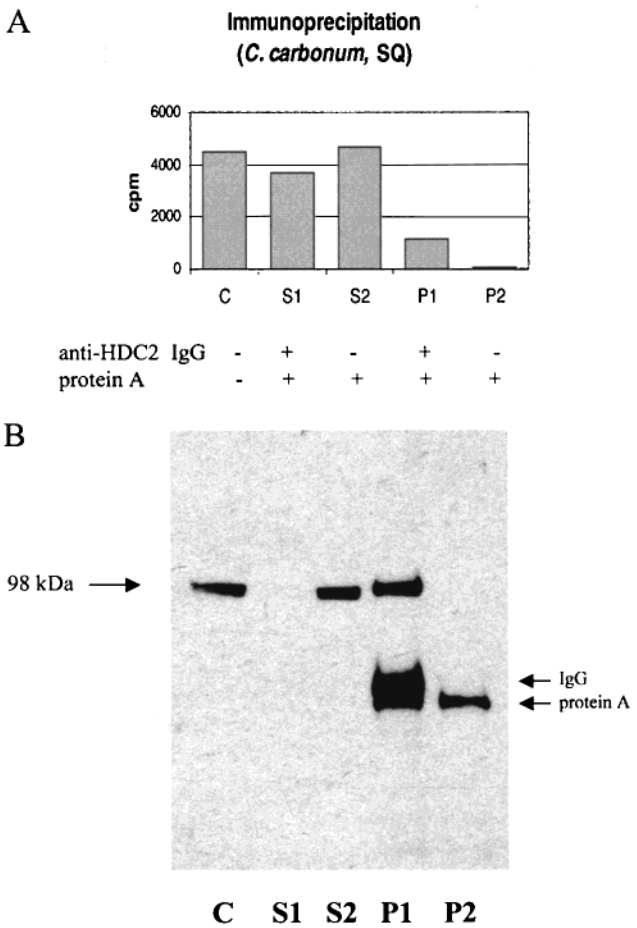

FIGURE 5: Immunoprecipitation of HDC2 protein. 50 μL of fraction 33 from SQ-chromatography of *C. carbonum* shake culture was mixed with or without (control) 2 μg of immunopurified anti-HDC2 antibodies and 30 μL of protein-A Sepharose, equilibrated with buffer B, and was incubated for 2 h by continuous shaking at 4 °C. After centrifugation, the supernatant (S) was saved for HDAC assay and immunoblotting. Pellets (P) were washed 3 times and then resuspended in 20 μL of buffer B. The entire pellets and 50 μL of each supernatant were used for HDAC assay. C, supernatant of control (−HDC2, −protein A); S1, supernatant (+HDC2, +protein A); S2, supernatant (−HDC2, +protein A); P1, pellet (+HDC2, +protein A); P2, pellet (−HDC2, +protein A) (A). For immunoblotting, 20 μL of supernatants and entire pellets were applied to SDS−PAGE with subsequent immunoblotting using anti-HDC2 antibodies. Molecular mass of recombinant HDC2 is indicated. Positions of cross-reacting heavy chains of antibodies and protein A are shown. Data represent results of three independent experiments (B).

HDAC Activity in C. carbonum Is Associated with HC-toxin Synthesis. As pointed out in Figure 1, resistant and sensitive HDAC activity profiles depend on distinct growth conditions by which synthesis of HC-toxin is affected. Therefore, expression profiles of these enzymes may depend on toxin production during growth of the fungus. To address this question, *C. carbonum* was harvested after 2, 4, or 7 days of still culture growth, proteins were extracted, and HDAC activities were analyzed by SQ-chromatography and immunoblotting as described above. Figure 6A shows that during this time course, resistant activity (eluting at 150 mM NaCl) increased to reach a maximum when the fungus was grown for 7 days. Sensitive activity (eluting at 280 mM NaCl), however, continuously decreased until 7 days.

These findings were supported by immunoblotting results using anti-HDC2 antibodies. Immunoblots in Figure 6B

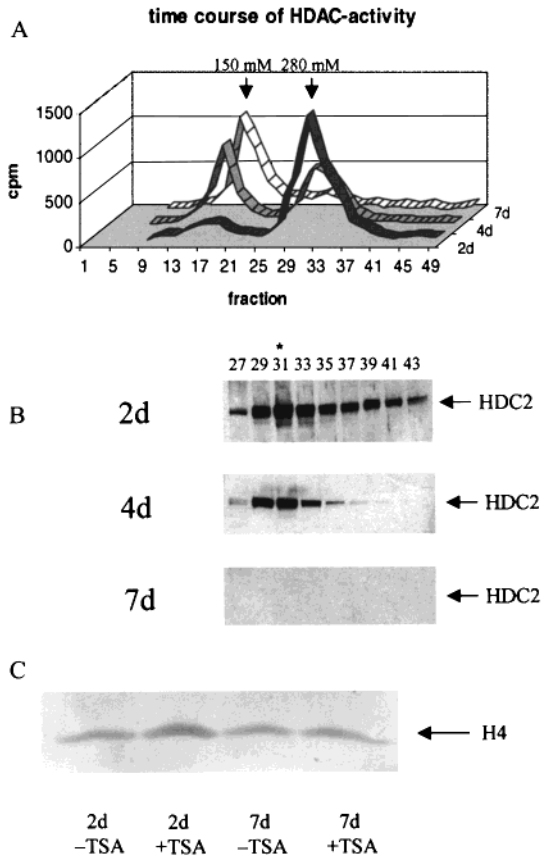

FIGURE 6: Time course of HDAC activity and acetylation of histone H4 in vivo. *Cochliobolus* was grown in still culture for 2, 4, or 7 days. Equal amounts of mycelia were extracted, and HDAC activities were fractionated by SQ-chromatography (see Experimental Procedures and Figure 1 legend). Salt concentrations of eluting peak fractions are indicated (A). Aliquots of fractions 27−43 of different SQ-chromatographies (2d, 4d, 7d) were subjected to SDS−PAGE with subsequent immunoblotting using anti-HDC2 antibodies. The position of HDC2 is shown, and the peak fraction of SQ-chromatography (corresponding to 280 mM NaCl) is indicated by an asterisk. Immunodetection was performed with the ECL detection system (Amersham) (B). For analysis of H4 acetylation, *C. carbonum* was grown in still culture for 2 and 7 days, and TSA was added 5 h prior to harvesting. After isolation of nuclei, nuclear extracts were loaded on SDS−14% PAGE, and histones were estimated by densitometry. Equal amounts of nuclear extracts were then subjected to SDS−14% PAGE and blotted onto nitrocellulose membranes. Membrane strips were incubated with antibodies against acetylated histone H4. The position of acetylated H4 is indicated. Immunodetection was performed with alkaline phosphatase conjugated secondary antibody (C).

demonstrate that HDC2 protein is most prominent at 2 days but it had disappeared in mycelia of cultures grown for 7 days; this is in agreement with the detected HDAC activities (Figure 6A).

TSA Treatment of C. carbonum Mycelia Causes Histone H4 Hyperacetylation in Young Still Cultures but Not after 7 Days of Growth. We studied the effect of treatment of *C. carbonum* still culture mycelia with the HDAC inhibitor TSA on acetylation patterns of histone H4 in vivo. For this purpose, *C. carbonum* was grown for 2 or 7 days in still culture, and TSA (1 μM) was added 5 h prior to harvesting. After isolation of nuclei, nuclear extracts were subjected to SDS−PAGE and immunoblotting. The degree of H4 acetylation was analyzed by antibodies against acetylated histone H4 peptide which recognizes di-, tri-, and tetraacetylated H4. Estimation by laser densitometry revealed that TSA treatment of mycelia grown for 2 days (Figure 6C, lane 2) leads to a 2−3-fold increase of H4 acetylation compared to control mycelia (no TSA added; lane 1). No significant difference between TSA- and non-TSA-treated cells could be obtained when the fungus was grown for 7 days in still culture (lanes 3 and 4). These results are in line with the disappearance of the inhibitor-sensitive HDC2 during the transition from 2 day still culture to 7 day still culture. Since at 7 days the inhibitor-sensitive enzyme is replaced by the inhibitor-resistant form, no hyperacetylation of H4 resulted from treatment of cultures with TSA.

DISCUSSION

Our data strongly suggest that an HDAC activity of the plant pathogenic fungus *Cochliobolus carbonum* represents a new type of enzyme since it is highly resistant to well-known HDAC inhibitors such as TSA or HC-toxin and differs significantly in biochemical and immunological properties compared to RPD3/HDA-type HDACs. It is noteworthy that the resistant HDAC of *C. carbonum* is obviously not associated with other proteins in high molecular weight complexes but in contrast to most other HDACs is enzymatically active in its monomeric form.

The finding of a toxin-resistant HDAC activity in *C. carbonum* is important since it indicates the presence of an hitherto unknown class of HDACs; furthermore, it represents a so far undefined mechanism of enzyme protection against HC-toxin in this fungus. Although the most promising approach for the final identification of the enzyme, the isolation of the polypeptide(s) responsible for resistant activity by advanced purification, failed due to instability and low level of enzyme activity in toxin-producing mycelia, two different modes with respect to the underlying mechanism of resistance are conceivable. One possibility is that resistance is extrinsic, which means it is due to another factor that could mask or modify the HDAC and hence prevent binding or action of the toxin. Such a factor, which could be a secondary metabolite, may chemically modify and thus detoxify HC-toxin, or its binding to HDACs could cause inaccessibility of the enzyme for HC-toxin. However, purification of the resistant *Cochliobolus* HDAC activity by a series of chromatographic steps including affinity chromatography did not change resistance to inhibitors; this argues against an extrinsic factor, which would rather be lost during purification. An alternative possibility is that resistance is an inherent feature of the HDAC itself. One could think of a variant form of an already known HDAC of the RPD3-, HDA-, or plant HD2-type. It is well-known that mutations of enzymes can lead to inhibitor resistance. A mammalian cell line that was selected for resistance to trichostatin was at least 20 times more resistant than the parental line to both trichostatin and trapoxin; the same is true for a partially purified HDAC of this cell line (*40*, *41*). However, an HDC1 antibody against the deacetylase core domain conserved in HDACs of the RPD3/HDA1-type (*42*) did not yield any signal in immunoblots of chromatography fractions containing resistant enzyme activity. These results are in line with the observation that resistant activity could be detected in *C. carbonum* strains specifically mutated in HDC1 and HDC3 (homologous to yeast HDA1), respectively (J. Walton, S. Graessle, and G. Brosch, unpublished results).

Therefore, resistant activity of *Cochliobolus* may represent a completely different type of HDAC. In yeast, the formerly identified ORF of HOS3 was shown to be resistant to even high concentrations of TSA (*29*). In the case of HOS3, it was speculated that a difference in structure between the catalytic subunit and that of RPD3 and HDA1 is responsible for its unique behavior toward TSA. More recently, a novel inhibitor-resistant deacetylase was identified in *Drosophila* (*30*). dHDAC6, a homologue to human HDAC6, was highly resistant to TSA and HC-toxin and was exclusively located in the cytosol, indicating a function different from that of nuclear HDACs. Similar to HOS3/dHDAC6, the *Cochliobolus* enzyme could have an altered structure causing reduced inhibitor sensitivity. This assumption is supported by its molecular weight (60 000) in gel filtration chromatography. This indicates that the resistant enzyme is active as a monomer whereas Rpd3/HDA1-type HDACs exert their function as components of large multiprotein complexes. In comparison to HDC2 which is part of a high molecular weight complex, the toxin-resistant enzyme activity is likely to have distinct functional roles.

Due to its insensitivity to TSA (*18*), SIR2 was a putative candidate for the resistant enzyme in *Cochliobolus*. We can rule out this possibility because addition of various concentrations of NAD^+ to chromatography peak fractions did not change HDAC activity and no specific immunosignal could be obtained in resistant enzyme fractions using an anti *Aspergillus* SIR2 antibody.

Expression of the different HDACs in *C. carbonum* obviously depends on synthesis of HC-toxin. Resistant activity is only present in still cultures when toxin is synthesized. Moreover, this enzyme activity increased with fungal growth whereas the sensitive HDC2 decreased and finally disappeared. The loss of sensitive activity was not due to inhibition of HDC2 by residual HC-toxin after extraction since decreasing enzyme activity was accompanied by a decrease of HDC2 protein, as demonstrated by immunoblotting. Moreover, inhibition of HDACs by HC-toxin is reversible (*20*), and protein extracts were dialyzed prior to chromatography.

Our assumption of an "HC-toxin-induced HDAC" is further substantiated by the finding that TSA-induced H4 hyperacetylation varies with aging of the mycelia; at early stages of growth, hyperacetylation is due to inhibition of HDC2. However, no such effect could be observed after aging of mycelia. The decline of sensitive HDC2 in *Cochliobolus* is obviously balanced by resistant HDAC activity, as a consequence of HC-toxin production. HC-toxin thereby may act as a signal to turn off HDC2 and to induce resistant activity.

Our data contribute to a better understanding of the basis of interaction between the plant pathogen *C. carbonum* and its host maize. Although it is not clear how inhibition of maize HDAC allows disease to develop, it is suggested that inhibition of histone deacetylation blocks transcriptional activation of defense genes in maize (*20*, *43*). The fact that *C. carbonum* possesses an HC-toxin-resistant HDAC further supports the idea that maize HDACs are the main target of HC-toxin during pathogenesis.

Several HDAC inhibitors have potential as therapeutic agents. The fungal metabolite apicidin, a cyclic peptide related to HC-toxin, exhibits strong, but selective antiprotozoal (Apicomplexa) activity by inhibition of HDACs in parasites (*44*). Interestingly, apicidin does not affect growth of all protozoan organisms, which could be due to a detoxifying system in these organisms or a specific difference in their HDACs. HC-toxin was shown to have comparable in vivo and in vitro inhibitory efficiency as apicidin (*44*). Since inhibition of histone deacetylation might interfere with transcriptional control and thus cell proliferation, the antiproliferative activity of HC-toxin or apicidin implies that HDACs represent targets for the development of therapeutic agents. Therefore, the study and structural analysis of inhibitor-resistant HDACs could lead to the design of other potent HDAC inhibitors with potential application in chemoprevention and chemotherapy of malignant diseases.

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