Purification and Characterization of a High Molecular Weight Histone Deacetylase Complex (HD2) of Maize Embryos[†]

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ABSTRACT: The dynamic state of core histone acetylation is maintained by histone acetyltransferases and deacetylases. In germinating maize embryos, four nuclear histone deacetylases can be distinguished. From a chromatin fraction prepared at 72 h after start of embryo germination, we have purified the nuclear histone deacetylase HD2 to homogeneity. Using a sequence of chromatographic steps, we achieved the purification of an enzymatically active high molecular weight protein complex with an apparent molecular mass of 400 kDa, as determined by gel filtration chromatography. The purified enzyme was characterized in terms of enzymatic and kinetic properties, and sensitivity to several histone deacetylase inhibitors. In SDS−polyacrylamide gels, HD2 split into three polypeptides of 45, 42, and 39 kDa, suggesting that the native enzyme is a multimer−protein complex. Electrophoresis under nondenaturing conditions in combination with second dimension SDS−gel electrophoresis indicated that all three protein components of the HD2 complex were enzymatically active. Polyclonal antibodies against each of the three polypeptides were raised in rabbits. Each antiserum reacted with all three polypeptides on Western blots, suggesting that p45, p42, and p39 are highly homologous. This homology was confirmed by amino acid sequencing of peptides generated from each of the three HD2 components.

Modification of core histones by acetylation of ϵ-amino groups of specific lysine residues in the N-terminal part of histones is a dynamic enzymatic process whose exact biological functions remain unclear (Allfrey et al., 1964; Loidl, 1988, 1994; Ausio, 1992; Bradbury, 1992; Turner, 1993; Wolffe, 1994). Different levels and patterns of acetylation have been correlated with various biological processes, like histone deposition and chromatin assembly during DNA replication, histone replacement during differentiation, or chromatin structural transitions during gene activation or silencing (*e.g.*, Christensen et al., 1984; Ausio & van Holde, 1986; Loidl & Gröbner, 1986; Braunstein et al., 1993; Lee et al., 1993; Juan et al., 1994; Kaufman et al., 1995; Krude, 1995). The dynamic state of core histone acetylation is maintained by two enzyme activities, histone acetyltransferase and histone deacetylase. Both enzyme activities exist as multiple enzyme forms whose activities, location, and substrate specificities are regulated in a complex, cell cycle-related manner (López-Rodas et al., 1991; Georgieva et al., 1991, 1994; Brosch et al., 1992a,b, 1995; Grabher et al., 1994).

A necessary approach for understanding the biological functions of histone acetylation is the investigation of the structure of the involved enzymes and their encoding genes. Recently, major advances have been achieved with respect to our knowledge on histone acetyltransferases. A genetic approach led to the identification of a putative histone H4 acetyltransferase of yeast (HAT1) with partial sequence homology to N-terminal acetyltransferases (Kleff et al., 1995); our laboratory has purified a cytoplasmic histone acetyltransferase B of maize to homogeneity (Eberharter et al., 1996); a nuclear histone acetyltransferase of *Tetrahymena* has been identified as a homolog of the well known yeast transcriptional coactivator Gcn5p (Brownell et al., 1996).

Histone deacetylases have been characterized from a variety of organisms (Hay & Candido, 1983; Alonso & Nelson, 1986; Mold & McCarthy, 1987; Sendra et al., 1988; Sanchez del Pino et al., 1994). In lower eukaryotes and plants, these enzymes exist in multiple forms (Sendra et al., 1988; López-Rodas et al., 1992). In maize, four different histone deacetylases can be distinguished during embryo germination (López-Rodas et al., 1991; Georgieva et al., 1991; Brosch et al., 1992a; Grabher et al., 1994; Lechner et al., 1996); these enzymes differ considerably from each other in terms of substrate specificity, intranuclear location, kinetic properties, and posttranslational modification. In yeast, the nuclear histone deacetylase could be isolated as a high molecular mass complex (>350 kDa) at low ionic strength (Sanchez del Pino et al., 1994), which was converted to a low molecular mass form (∼150 kDa) upon exposure to 0.5 M salt. The two histone deacetylase forms differed significantly in their response to the specific deacetylase inhibitor Trichostatin. A similar high molecular weight histone deacetylase complex was also found in chicken erythrocytes (Li et al., 1996).

We have set out to characterize and purify enzymes involved in histone acetylation in germinating maize embryos, a source particularly rich in these enzymes. We report purification of the chromatin-bound histone deacetylase HD2. Using five successive chromatographies, we achieved the purification of HD2 to homogeneity. The purified enzyme

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had a molecular mass of ~400 kDa, as revealed by gel filtration chromatography, but split into three distinct polypeptides after sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE).[1] It was highly sensitive to deacetylase inhibitors, like Trichostatin or cyclic tetrapeptides. A newly developed PAGE for histone deacetylases under nondenaturing conditions showed that all three HD2 polypeptides were enzymatically active. Immunological studies, as well as peptide sequences, revealed that p45, p42, and p39 are highly homologous and are obviously components of a high molecular weight enzyme complex *in vivo*.

EXPERIMENTAL PROCEDURES

Materials. Trichostatin was purchased from Wako Pure Chemicals (Osaka, Japan). Maize seeds (*Zea mays*, strain Cuzco) were germinated in darkness for 72 h on cotton layers soaked with water at 28 °C. The endosperm-free seedlings were harvested into liquid nitrogen. Cellular fractionation was performed as described (Grabher et al., 1994; Steinmüller & Apel, 1986). Frozen tissue (1 kg) was ground to powder in an Ika grinding machine and then quickly suspended in 4 L of precooled solubilization buffer [20 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 5 mM KCl, 0.25 M sucrose, 0.25% (v/v) Triton X-100, 0.1% (v/v) 2-mercaptoethanol, and 40% (v/v) glycerol]. The mixture was stirred until the temperature was raised to −10 °C and filtered through 200 and 100 μm (pore size) nylon membranes. After centrifugation at 10000*g* for 15 min at 0 °C, the supernatant was decanted. The resulting pellet was resuspended in 1 L of solubilization buffer and centrifuged at 6000*g* for 15 min at 4 °C (twice). After the second wash, the pellet was resuspended in 1 L of buffer B [15 mM Tris-HCl, pH 7.9, 10 mM NaCl, 0.25 mM EDTA, 10 mM 2-mercaptoethanol, and 10% (v/v) glycerol], stirred on ice for 2 h, and centrifuged at 12000*g* for 15 min at 4 °C. The resulting supernatant is referred to as the "chromatin" fraction and contains chromatin proteins. For purification of histone deacetylase HD2, the chromatin fraction of a total of 12 kg (wet weight) of embryo tissue was used.

Chromatographic Protein Purification. (A) Q-Sepharose. Q-Sepharose chromatography was used as a first step to separate HD1-B and HD2, both chromatin-bound histone deacetylases (Grabher et al., 1994). For processing of 12 kg of starting material, six Q-Sepharose chromatographies were performed: the chromatin portion (2 L) was loaded onto a 200 mL Q-Sepharose Fast Flow column (Pharmacia Biosystems, Uppsala, Sweden), equilibrated with buffer B, and elution of bound proteins was performed with 1.4 L of a linear gradient from 10 mM to 500 mM NaCl in buffer B at a flow rate of 3 mL/min. Fractions (20 mL) were assayed for histone deacetylase activity. After this first step of purification, two peaks with enzymatic activity could be detected, corresponding to HD1-B and HD2, eluting at 230 (HD1-B) and 380 mM (HD2) NaCl, respectively. Fractions with high HD2 activity (fractions 67−78) of six chromatographies were pooled, concentrated in an Amicon ultrafiltration cell (cutoff 30 kDa) to a final volume of 30 mL, and then dialyzed against buffer B.

(B) Poly(lysine)−Agarose. The dialyzed pool of six Q-Sepharose chromatographies was applied onto four poly(lysine)−agarose (high molecular weight; Sigma Chemicals, St. Louis, MO) columns (2.5 × 10 cm; 50 mL), equilibrated with buffer B. The flow rate was 48 mL/h. Proteins were eluted with 400 mL of a linear gradient from 10 mM to 1 M NaCl in buffer B. Fractions (5 mL) with high HD2 activity (fractions 82−96) were pooled and concentrated to a final volume of 20 mL by centrifugation (3000*g*; 4 °C) using Amicon Centriprep-30. The concentrate was dialyzed against buffer B.

(C) Histone−Agarose. Poly(lysine)−agarose pools (20 mL) were applied to three histone−agarose (Sigma) columns (1.5 × 20 cm; 20 mL) equilibrated with buffer B at a flow rate of 48 mL/h. Elution was performed with 120 mL of a linear gradient from 10 mM to 0.8 M NaCl in buffer B; fractions of 3 mL were assayed for HD2 activity. Fractions 36−44 were pooled and concentrated to a final volume of 1 mL by centrifugation (3000*g*) in Amicon Centriprep-30.

(D) Size Exclusion (Superdex S-200). Each of the histone−agarose concentrates was applied onto a Superdex S-200 FPLC column (2.5 × 100 cm; 120 mL; Pharmacia), equilibrated with 0.2 M NaCl in buffer B. The flow rate was maintained at 1 mL/min; fractions of 1 mL were assayed for HD2 activity. Fractions 50−54 of three chromatographic runs were pooled.

(E) Heparin−Sepharose. Pools of the three S-200 chromatographies were directly loaded onto a heparin−Sepharose HiTrap FPLC column (Pharmacia; 5 mL), equilibrated with 0.2 M NaCl in buffer B. Proteins were eluted with 40 mL of a linear gradient from 200 mM to 600 mM NaCl in buffer B. Using a flow rate of 1 mL/min, fractions of 1.5 mL were assayed for HD2 activity.

Histone Deacetylase Assay. Histone deacetylase activity was determined as described (Sendra et al., 1988) using [^{3}H]-acetate-prelabeled chicken reticulocyte histones as substrate. Sample aliquots of 25 μL were mixed with 10 μL of total [^{3}H]acetate prelabeled chicken reticulocyte histones (1.5 mg/mL). After incubation at 30 °C for 20 min, the reaction was stopped by addition of 36 μL of 1 M HCl/0.4 M acetate and 800 μL 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.

For determination of HD2 activity in gel slices after PAGE under nondenaturing conditions, the assay conditions were slightly modified. Each gel piece (0.5 × 0.5 cm) was suspended in 150 μL of buffer B and incubated with 10 μL of [^{3}H]acetate-prelabeled chicken reticulocyte histones as substrate for 1 h at 30 °C under permanent shaking. The reaction was stopped by addition of 72 μL of 1 M HCl/0.4 M acetate, and released [^{3}H]acetate was extracted with 0.8 mL of ethyl acetate by head-over-head shaking for 1 h at room temperature. After centrifugation at 10000*g* for 5 min, an aliquot (600 μL) of the upper phase was counted for radioactivity in 3 mL of liquid scintillation cocktail.

Determination of Enzymatic Properties of HD2. The pH optimum and temperature optimum were tested by incubating the purified enzyme under standard assay conditions (see above) at different pH values (from pH 6.0 to 10.5; 0.5 pH unit intervals) and different temperatures (from 10 to 45 °C; 5°C intervals). To determine the k_M value of HD2 for core histones, purified HD2 was incubated with increasing

[1] Abbreviations: FPLC, fast performance liquid chromatography; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

concentrations of core histones at the optimum pH. Results were plotted on Lineweaver−Burk diagrams, and the k_M value was determined from the plot. Analysis of substrate specificity (individual core histone species) was performed as described by Brosch et al. (1992a).

To determine the effect of various ions on HD2 activity, the purified enzyme was incubated under standard assay conditions in the presence of buffered solutions of 5 mM Fe^{3+}, Zn^{2+}, Cu^{2+}, Mg^{2+}, and Ca^{2+} in the form of various salts. To test the inhibitory effect of several well-known histone deacetylase inhibitors on purified HD2, we performed the standard assay in the presence of different concentrations of sodium acetate, sodium *n*-butyrate, Trichostatin A, HC-toxin, or chlamydocin. Inhibitor concentrations for 90% inhibition were determined.

Protein Analysis. SDS−PAGE was performed in 10% polyacrylamide gels as previously described (Laemmli, 1970). Aliquots of HD2 preparations of different stages of purification were analyzed. After electrophoresis, proteins were stained with silver as described (Oshawa & Ebata, 1983) 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.

Molecular Weight Analysis. Molecular weight determination of the native enzyme was done by gel filtration chromatography. A Superdex S-200 column (2.5 × 100 cm; 120 mL; Pharmacia), equilibrated with 0.2 M NaCl in buffer B, was calibrated with proteins of known molecular weight. The flow rate was 1 mL/min; fractions of 1.5 mL were collected. The molecular weight of the purified enzyme was determined under denaturing conditions by SDS−PAGE.

PAGE under Nondenaturing Conditions. Six percent polyacrylamide gels (80 × 80 × 1 mm) were prepared as described except that SDS was omitted. An equal volume of sample buffer (0.1 M Tris-HCl, pH 8.5, 10% glycerol) was added to HD2 preparations from different stages of purification. Electrophoresis was carried out at constant current (2 mA) for 17 h at 4 °C [running buffer (25 mM Tris-HCl, 190 mM glycine, pH 8.5)]. After electrophoresis, the gel lane was vertically cut in two halves. One half was sliced into 0.5 cm pieces from top to end, and each gel piece was assayed for HD2 activity as described above. If PAGE was carried out for protein separation and immunodetection only (no enzyme activity assay), running conditions were 125 V constant voltage for 2.75 h at 4 °C.

For second dimension SDS−PAGE, the remaining half of the lane of the first dimension gel was incubated in SDS−sample buffer [62 mM Tris-HCl, pH 6.8, 0.1 M SDS, 10% (v/v) glycerol, and 5% (v/v) 2-mercaptoethanol] for 5 min at room temperature. The gel lane was then immediately placed onto an SDS−10% polyacrylamide gel (80 × 80 × 1 mm) with an agarose bridge [1% (w/v) agarose, 125 mM Tris/phosphate, pH 6.8, and 0.1% (w/v) SDS]. Running conditions of SDS−PAGE were 5 min at 35 V, 10 min at 55 V, and finally 100 V constant.

Antibodies. Polyclonal antibodies were raised against p45, p42, and p39. For this purpose, purified HD2 after heparin−Sepharose chromatography was subjected to SDS−10% PAGE and subsequent blotting on nitrocellulose membrane. Coomassie-stained protein bands were excised, destained, minced into micro-fibers, and used for intracutaneous immunization of rabbits following standard protocols.

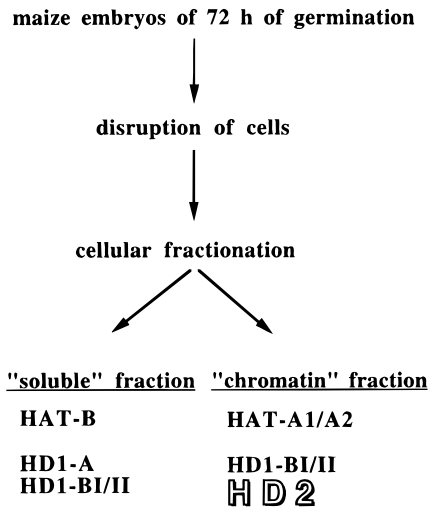

FIGURE 1: Distribution of histone deacetylases (HD) and histone acetyltransferases (HAT) in the "soluble" and "chromatin" fraction after cellular fractionation of maize embryos.

Immunoglobulin fractions of antisera against the different polypeptides were purified by HiTrap Protein-G Sepharose (Pharmacia; 1 mL) chromatography and analyzed by SDS−PAGE.

Immunoblotting. Pure HD2 was analyzed by SDS−PAGE (10%) or two-dimensional PAGE (see above), followed by immunoblotting (Towbin et al., 1979). After incubation with anti-HD2 antibodies, immunocomplexes were visualized with alkaline phosphatase-conjugated second antibody using 5-bromo-4-chloro-3-indolyl disodium phosphate and nitro-blue-tetrazolium chloride as substrate solution.

Peptide Sequencing. p45, p42, and p39 were digested with Lys-C-endoprotease on Immobilon membrane. Resulting peptides of each protein were separated by reverse-phase HPLC. Peptides were subjected to modified Edman degradation (Hunkapiller et al., 1983).

RESULTS

Solubilization of HD2 from Chromatin. Since maize embryos contain four histone deacetylases (HD1-A, HD1-BI, HD1-BII, HD2), an important initial step was the quantitative solubilization of HD2 under conditions that minimize total protein content as well as contamination with other histone deacetylases of the sample for chromatographic purification. When total homogenates were extracted with high ionic strength buffers (0.5 M NaCl), the resulting solution contained all four histone deacetylases. We therefore employed a cellular fractionation procedure for preparation of plant chromatin to separate chromatin-bound histone deacetylases from soluble nuclear enzyme forms (Figure 1). Plant material was homogenized in liquid nitrogen and suspended in low salt buffer containing 40% glycerol at a temperature less than −10 °C during the solubilization period. This was a crucial step for further purification. After centrifugation, the supernatant contained HD1-A, a small proportion of HD1-B (Grabher et al., 1994), and the majority of total cellular protein. The chromatin fraction contained HD2 and part of HD1-B. HD2 could then be solubilized from the chromatin fraction.

Purification of HD2 by a Series of Chromatographic Steps. We started purification of HD2 from 12 kg of maize

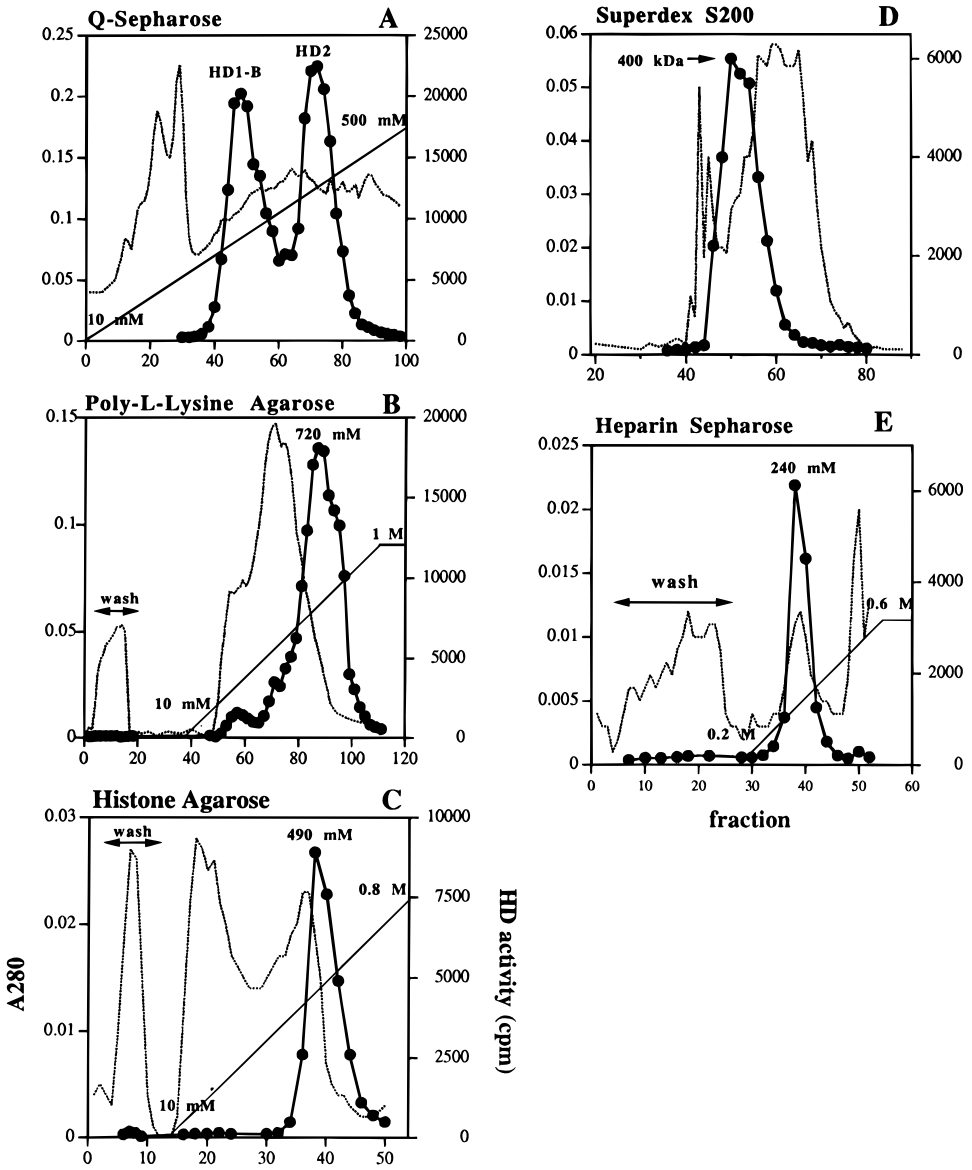

FIGURE 2: Chromatographic steps for purification of maize HD2. The chromatin fraction of maize embryos at 72 h of germination was subjected to five consecutive chromatographies. (A) Q-Sepharose. Proteins solubilized from the chromatin fraction were loaded at a flow rate of 3 mL/min and eluted with a linear gradient (1.4 L) from 10 mM to 0.5 M NaCl in buffer B. Fractions of 20 mL were collected. (B) Poly(lysine)−agarose. Dialyzed fractions (67−78) of the Q-Sepharose chromatography were loaded at a flow rate of 48 mL/h. Proteins were eluted with a linear gradient (400 mL) from 10 mM to 1 M NaCl in buffer B. Fractions of 5 mL were collected. (C) Histone−agarose. The dialyzed poly(lysine)−agarose concentrate (fractions 82−96) was loaded at a flow rate of 48 mL/h. Proteins were eluted with a linear NaCl gradient (120 mL) from 10 mM to 0.8 M NaCl. Fractions of 3 mL were collected. (D) Superdex S-200. Fractions 36−44 of the histone−agarose chromatography were concentrated and applied at a flow rate of 1 mL/min. Fraction size was 1 mL. HD2 eluted in a volume corresponding to a molecular mass of ∼400 kDa. (E) Heparin−Sepharose. Fractions 50−54 of the Superdex S-200 chromatography were loaded at a flow rate of 1 mL/min. Proteins were eluted with a linear gradient (40 mL) from 0.2 to 0.6 M NaCl in buffer B (fractions of 1.5 mL). Enzyme activity (●—●) was measured in the standard assay and is expressed as cpm. Protein content (···) was recorded by the absorption at 280 nm. Gradients with the starting and final concentrations as well as salt concentration of the maximum enzyme activity peak are indicated.

embryos. Proteins of the chromatin fraction were subjected to Q-Sepharose chromatography (Figure 2A). HD2 was separated from HD1-B and eluted at ∼0.4 M NaCl. Fractions 67−78 were applied to a poly(L-lysine)−agarose affinity column (Figure 2B). HD2 quantitatively bound to the matrix and was eluted at ∼0.7 M salt. Chromatography resulted in good separation of HD2 from contaminating protein. Fractions 82−96 were applied to a histone−agarose column (Figure 2C). Again, good separation of HD2 from the bulk of proteins was achieved; HD2 eluted at ∼0.5 M salt. Fractions with maximum activity were concentrated and subjected to Superdex S-200 chromatography (Figure 2D). The enzyme eluted in a volume equivalent to a molecular mass of ∼400 kDa. Chromatography with higher ionic strength buffer (up to 0.8 M) did not change the apparent molecular mass of the enzyme (result not shown). The peak fractions (50−54) were finally loaded onto heparin−Sepharose (Figure 2E) where HD2 was completely separated from contaminating protein and eluted as a defined peak at ∼0.25 M salt. This peak fraction was analyzed by SDS−PAGE (Figure 3). The silver-stained electrophoretic pattern revealed three proteins at molecular masses of 45, 42, and 39 kDa (lane 5). Since native HD2 eluted at a molecular mass of ∼400 kDa in gel filtration chromatography, we assume that it is a high molecular weight complex, built up by three polypeptides. In separate small-scale

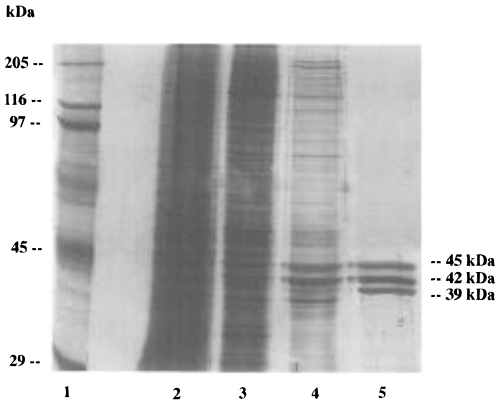

FIGURE 3: Electrophoretic analysis of proteins at different steps of HD2 purification on SDS−polyacrylamide gels. Samples of Q-Sepharose (fraction 70; lane 2), poly(L-lysine)−agarose (fraction 88, lane 3), histone−agarose (fraction 40, lane 4), and heparin−Sepharose (fraction 38, lane 5) were subjected to SDS−10% PAGE. The gel was stained with silver. Molecular mass of marker proteins (lane 1) is indicated (kDa).

experiments, we loaded the heparin−Sepharose peak onto a second gel filtration column (Superdex S-200) to check whether the high molecular weight complex was still intact after heparin−Sepharose chromatography. Indeed, HD2 eluted at ~400 kDa, irrespective of the order and number of chromatographic steps (result not shown).

Enzymatic Properties of Maize HD2. Purified HD2 was used to determine the usual enzymatic parameters. The rather pronounced pH optimum was 7.5, the optimum temperature 30 °C, and the k_M value for core histones 55 μmol/L. The enzyme was negatively affected (50% inhibition) by Fe^{3+}, Zn^{2+}, Cu^{2+}, Mg^{2+}, and Ca^{2+} (5 mM). HD2 accepted all core histones as substrate *in vitro*, with a significant preference for H2B, as determined with purified individual core histone species in the enzymatic assay. Highly purified HD2 was strongly inhibited (90%) by less than 0.1 μM of the specific deacetylase inhibitor Trichostatin A (Yoshida et al., 1990), cyclic tetrapeptides (<10 μM), like HC-toxin or chlamydocin (Brosch et al., 1995), butyrate (110 mM), and acetate (80 mM). Enzymatic parameters of purified HD2 were almost identical with those of partially purified HD2, as previously determined (López-Rodas et al., 1991; Lechner et al., 1996).

Electrophoretic Identification of Proteins with Histone Deacetylase Activity under Nondenaturing Conditions. Samples with histone deacetylase activity were electrophoresed in 6% polyacrylamide slab gels (see Experimental Procedures). After electrophoresis, the lane was cut in two halves; one half was sliced for determination of enzyme activity within the gel (Figure 4A), and the other half was put on top of an SDS gel for resolution of proteins in the second dimension (Figure 4B). Figure 4 shows results using a purified HD2 preparation (pooled peak fractions after heparin−Sepharose chromatography). Enzyme activity was measured in the gel slices at 1−3 cm of migration (Figure 4A). The second dimension SDS gel allowed identification of proteins with defined molecular mass in regions of the first dimension gel, where enzyme activity had been detected. It can clearly be seen that the three protein spots of 45, 42, and 39 kDa were present in that region of the first dimension gel that contained histone deacetylase activity (Figure 4B). Note that the two-dimensional electrophoresis confirmed the

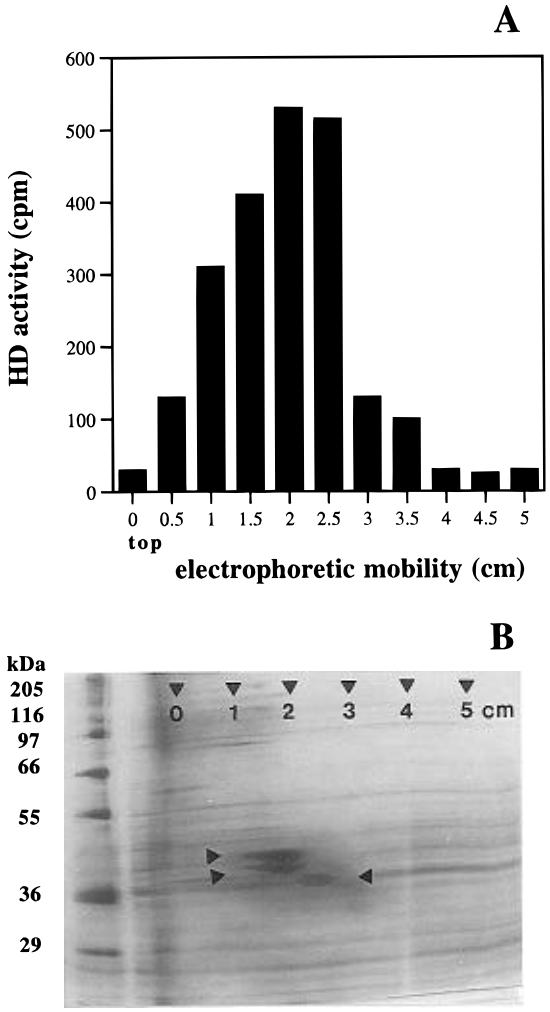

FIGURE 4: Electrophoretic analysis of HD2 and determination of enzymatic activity in a gel activity assay. HD2 (aliquot with high enzyme activity from pooled peak fractions after heparin−Sepharose chromatography) was subjected to 6% PAGE under nondenaturing conditions. After electrophoresis, the lane was cut in two halves. One half was sliced (0.5 cm slices), and enzyme activity was determined in the gel pieces (A). Note that column 0 represents the slice from 0 to 0.5 cm, column 0.5 represents the slice from 0.5 to 1.0 cm, etc. The second half of the lane was put on top of a second dimension SDS−10% polyacrylamide gel (B). The gel was stained with silver. Molecular mass marker proteins (kDa) in polyacrylamide gel pieces were applied into a separate lane and coelectrophoresed in the second dimension gel (B). Arrows mark p45, p42, and p39. Distances of the first dimension gel are given in centimeters from the top.

SDS gel of Figure 3, since almost no other proteins than p45, p42, and p39 could be detected. Analysis of less purified HD2 fractions (after Q-Sepharose chromatography) revealed enzyme activity and faint spots of 45, 42, and 39 kDa at the same positions in the gels (results not shown).

Immunological Studies. The purified high molecular mass enzyme complex was subjected to SDS−PAGE with subsequent blotting onto nitrocellulose membrane. The three protein bands at 45, 42, and 39 kDa were excised from the blot and minced to fibers in liquid nitrogen. The fibers were suspended in PBS and used for immunization of rabbits. Immunoglobulin fractions were purified from each of the raised polyclonal antisera and tested for reactivity against purified HD2 on Western blots. Figure 5 shows that each of the antibodies reacted with all three proteins (p45, p42, p39), suggesting the three proteins to be highly homologous.

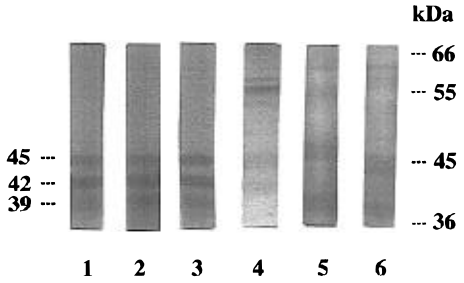

FIGURE 5: Immunological cross-reactivity of antibodies against p45, p42, and p39. Purified HD2 complex (corresponding to fractions 38 and 39 after heparin−Sepharose chromatography, lanes 1−3) and crude chromatin extract (lanes 4−6) were subjected to SDS−PAGE with subsequent blotting onto nitrocellulose membrane. Immunodetection of HD2 was performed with anti-p45 (lanes 1, 4), anti-p42 (lanes 2, 5), and anti-p39 (lanes 3, 6). Molecular mass markers (kDa) are indicated.

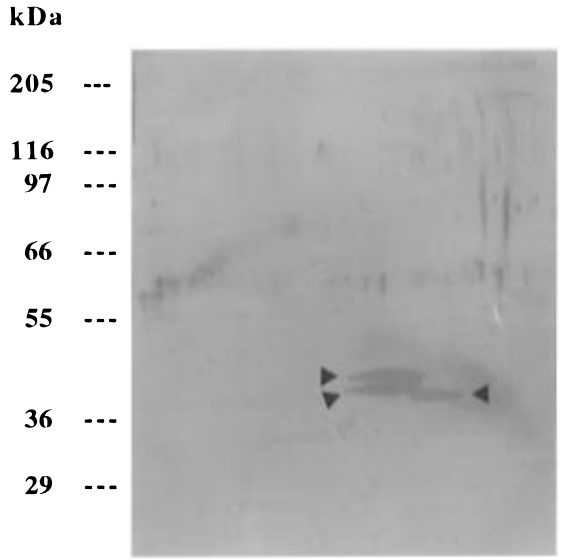

FIGURE 6: Immunodetection of HD2 in a partially purified enzyme preparation by a combination of antibodies against p45, p42, and p39. An aliquot of the pooled peak fractions after Superdex S-200 chromatography was subjected to two-dimensional gel electrophoresis. After blotting onto a nitrocellulose membrane, immunoreactive proteins were detected by a combination of equal amounts of purified immunoglobulins (0.1 mg/mL) against p45, p42, and p39. The proteins are marked with arrows. Molecular mass markers (kDa) are indicated.

No other band was decorated with the antibodies in gels of partially or highly purified HD2. When crude chromatin preparations were subjected to SDS−PAGE with subsequent blotting, no prominent bands could be detected by the antibodies at the same dilution as used for immunodetection in the highly purified HD2 preparations (Figure 5; lanes 4−6). One cross-reactive protein band was detected by the anti-p45 antibody at a molecular mass of ~55 kDa which disappeared during the subsequent chromatographic purification steps. We also used a combination of identical amounts of all three antisera to immunodetect proteins on a two-dimensional gel of a less purified HD2 preparation (peak fraction after Superdex S-200 chromatography). The antibody mixture decorated the p45, p42, and p39 spots (Figure 6). This pattern clearly resembled the staining pattern of Figure 4B and confirmed the immunodetection pattern of Figure 5 (lanes 1−3).

Peptide Sequences. Attempts to microsequence the N-terminus of p45, p42, and p39 failed due to blocking.

HISTONE DEACETYLASE HD2

45 kD protein

peptide 9: K T T S V F F **S** G Y K

42 kD protein

peptide 19: K T T S V F F **T** G Y K

peptide 25: K N P H I Q F D L I F D

39 kD protein

peptide 14: K T T S V F F **T** G Y K

peptide 22: K N P H I Q F D L I F D

FIGURE 7: Amino acid sequences of peptides derived from p45, p42, and p39. p45, p42, and p39 were digested with Lys-C endoprotease. Resulting peptides were separated by reverse-phase HPLC, and selected peptides were subjected to Edman degradation. The listed sequences are identical (peptides 19 and 14; 25 and 22) or highly homologous (peptides 9 and 19).

Therefore, protein bands were excised from Immobilon blots and subjected to Lys-C endoprotease digestion. After reverse-phase HPLC of the resulting peptides, selected peptides were sequenced. Among the amino acid sequences we obtained, there was one sequence present in p45, p42, as well as p39, and a second sequence in p42 as well as in p39 (Figure 7). These overlapping, identical peptide sequences and the cross-reactivity of anti-HD2 antibodies suggest a high structural homology among p45, p42, and p39.

DISCUSSION

For more than 30 years, it has been recognized that ϵ-amino groups of N-terminal lysine residues of core histones are subject to posttranslational acetylation (Allfrey et al., 1964). However, a detailed understanding of the biological functions of this modification has proven rather difficult to establish. Especially, the comparison of the physicochemical properties of acetylated and hyperacetylated chromatin has not revealed major alterations of DNA−histone interactions due to an elevation of the acetate content. Previous experimental results from our laboratory (Loidl, 1988; Loidl & Gröbner, 1986, 1987) have indicated that the absolute level of acetylation *per se* is less important for the regulation of nuclear processes than the acetylation/deacetylation of distinct lysine residues. This was substantiated by the use of site-specific antibodies against ϵ-acetyllysines of H4 in different experimental systems (*e.g.*, Turner et al., 1992). A genetic approach in yeast recently demonstrated that the acetylation of each lysine residue had a distinct function in epigenetic signaling in chromatin (Megee et al., 1995). These results implicate site-specific histone acetyltransferases and deacetylases in chromatin; indeed, multiple histone acetyltransferase and deacetylase forms exist (*e.g.*, Georgieva et al., 1991, 1994; Brosch et al., 1992a,b; Grabher et al., 1994; Lechner et al., 1996).

If one assumes site-specific functions of acetylation, the involved enzymes must differ from each other in certain properties, like substrate specificity or position specificity, and act in a nonrandom, coordinated way. For the understanding of the regulatory role of specific acetyllysine sites, we have to gain information on histone acetyltransferases and deacetylases, in particular on the DNA sequence of the encoding genes. Purification and molecular characterization of histone acetylation enzymes and their encoding genes will

enable us to study site-specificity and subcellular and tissue distribution during the cell cycle and differentiation, as well as the functional consequences of acetylation on development in genetically manipulated organisms as a final goal. Recently, major "breakthroughs" have been achieved in the acetylation field. In yeast, a genetic study led to the identification of a B-type histone acetyltransferase gene (Kleff et al., 1995). In the ciliate protozoan *Tetrahymena*, an A-type histone acetyltransferase with a molecular mass of 220 kDa has been identified (Brownell & Allis, 1995). The p55 subunit of this complex has been cloned and sequenced, and identified as a homologue of yeast Gcn5, a well-known transcriptional coactivator (Brownell et al., 1996). In germinating maize embryos, a cytoplasmic histone acetyltransferase B has been purified and characterized (Eberharter et al., 1996); the enzyme had a molecular mass of 90 kDa and was composed of two distinct subunits with molecular masses of 45 and 50 kDa.

We could purify maize HD2 to homogeneity. Histone deacetylases have been partially purified and characterized in a variety of organisms (Hay & Candido, 1983a,b; Alonso & Nelson, 1986; Mold & McCarthy, 1987; Sendra et al., 1988; Sanchez del Pino et al., 1994; López-Rodas et al., 1991, 1992; Brosch et al., 1992a, 1995; Grabher et al., 1994; Lechner et al., 1996). A number of investigations presented evidence that histone deacetylase exists as a high molecular weight complex (Hay & Candido, 1983a,b; Sanchez del Pino et al., 1994). The histone deacetylase complex of yeast with a molecular mass of >350 kDa disintegrated into smaller components upon exposure to 0.5 M salt or upon chromatography on ion exchange matrices. Recently, a high molecular mass histone deacetylase (~220 kDa) and a low molecular mass form (~55 kDa) were characterized in terms of properties and substrate specificity in chicken erythrocytes (Li et al., 1996). In maize, we also found a complex of a corresponding molecular weight, but this complex was stable and resisted chromatographic purification steps as well as exposure up to 0.8 M salt. However, HD2 lost its activity after exposure to salt concentrations higher than 1 M or after contact with even very low concentrations of detergents (Triton, SDS, Nonidet), probably due to the disintegration of the high molecular weight complex. Our immunological data, as well as peptide sequences of p45, p42, and p39, suggest that the enzymatically active high molecular weight complex is built up by the three protein components. Although we have purified HD2 to homogeneity, we cannot exactly calculate the extent of purification. We had a substantial increase of the specific activity during the first steps of purification, but as purification proceeded the specific activity stayed almost constant despite a considerable loss of contaminating proteins during these chromatographies. This is most likely due to the fact that the organization of this enzymatically active complex is sensitive, and though one purifies histone deacetylase proteins, one more and more disturbs subtle arrangements among the different components of the complex or the exact combination of the enzymatically active complex. This was also indicated by the relatively low enzyme activity that we measured in gel slices after nondenaturing electrophoresis. The yield of activity was ~10%, which is probably due to disintegration of the high molecular weight complex. At present, it is difficult to assess whether all three polypeptides are enzymatically active, since our antibodies, which have been raised against SDS−denatured subunit proteins, did not immunoprecipitate the native enzyme complex satisfactorily. However, the distribution of enzyme activity in the native gel suggests that all three polypeptides have catalytic activity, because we can detect activity in the region of the p45 and p42 spots, and also over p39. It is possible that one of the polypeptides is further responsible for the association of the complex to the proper nucleosome area or for binding of additional factors. We rule out the possibility that an enzyme form with high molecular weight is degraded by an endoprotease, thus giving rise to the three polypeptides; then we would neither expect an enzymatically active 400 kDa complex until the end of our extensive purification procedure, nor would we expect immunological cross-reactivity between the three proteins, roughly equal amounts of the three proteins, and identical amino acid sequences of peptides generated from p45, p42, and p39. Furthermore, rechromatography of purified native HD2 (after heparin−Sepharose) in a further gel filtration column again resulted in elution of the enzyme at 400 kDa. The proportion of p45, p42, and p39 on SDS gels was identical in different stages of HD2 purification. Moreover, the electrophoretic pattern of the three polypeptides did not indicate proteolytic degradation. According to our concept HD2 is a multimer, assembled by homologous subunits which may differ from each other with respect to substrate specificity or lysine position specificity. One has to postulate such a specificity if deacetylases contribute to establish a specific acetylation pattern on nucleosomes for regulation of certain nuclear processes (López-Rodas et al., 1993; Brosch et al., 1995). It is interesting in this context that maize HD1-A and HD1-B are absolutely different proteins (Brosch et al., 1996; Lechner, unpublished results); both are monomeric proteins with a native molecular mass of 45 kDa (HD1-A) or 50 kDa (HD1-B). They differ in enzymatic properties (Lechner et al., 1996) and sensitivity toward Trichostatin A, being less inhibited by this compound than HD2. Moreover, the chromatographic properties of HD1-A and HD1-B are completely different from those of HD2, a biochemical fact that argues against a close structural relationship between these different deacetylase forms. Interestingly, a high molecular weight histone deacetylase of yeast that could be converted to a low molecular weight form differed considerably in sensitivity toward Trichostatin A (Sanchez del Pino et al., 1994). Recently, Carmen et al. (1996) reported the purification of two high molecular weight deacetylase complexes in yeast that also differed in sensitivity toward Trichostatin. Although we strongly suggest one high molecular weight HD2 complex, composed of three highly homologous polypeptide subunits, we cannot rule out the possibility of two high molecular mass complexes (each ~400 kDa), one assembled as a multimer of p42 and p45 and another one composed of p39; since p39 is migrating slightly faster than p42 and p45 in native gel electrophoresis, this possibility may be considered. However, due to the homogeneous chromatographic HD2 peaks during purification and the fact that highly purified HD2 has one clear-cut k_M value for core histones, we favor the concept of one HD2 complex; moreover, we always got maximum histone deacetylase activity in our native gels in exactly that region of the gel where the p42 and p45 bands overlapped with the p39 band (Figure 4). In independent, repetitive electrophoretic analyses of HD2 of different stages of purification, we noticed that the enzyme activity in relation to the amount

of protein on the gel is higher for p39 than p42 and p45. This can also be seen for highly purified HD2 in Figure 4.

During preparation of this paper, Taunton et al. (1996) reported the purification and cloning of a human deacetylase (HD1) which is related to the yeast transcriptional regulator Rpd3p. Mammalian HD1 is a 55 kDa protein. The protein could also be a subunit of the high molecular weight deacetylase complexes of yeast (Sanchez del Pino et al., 1994) and chicken (Li et al., 1996), since these complexes disintegrate upon exposure to high ionic strength or ion exchange chromatography. Maize HD2 behaves differently, since neither high salt nor repeated ion exchange chromatography disintegrated the 400 kDa complex. None of our HD2 peptide sequences showed homology to mammalian HD1 (Taunton et al., 1996). At present we assume that maize HD1-A and/or HD1-B are homologues of human HD1, but maize HD2 represents a distinct enzyme; we could show that HD1-A is a monomer of 45 kDa (Brosch et al., 1996). In yeast, Carmen et al. (1996) have purified 2 deacetylases (HDA, HDB) with molecular masses of ~350 and 600 kDa; the 350 kDa complex split into polypeptides of around 70 kDa under denaturing conditions. Immunological data indicated that the polypeptides are homologous or closely related. In this respect, maize HD2 may be comparable to the yeast HDA complex. Forthcoming characterization of the multiple deacetylases at a genetic level will hopefully unfold their interrelation and individual roles in modulation of chromatin structure and function.

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