Histone deacetylases in replicative senescence: evidence for a senescence-specific form of HDAC-2

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Abstract To analyze mechanisms of senescence-associated gene expression, we have investigated histone deacetylases (HDACs) in human fibroblasts undergoing replicative senescence. We found that the overall acetylation pattern of histones does not vary detectably with replicative senescence. By Northern blot and Western blot, we found a significant decrease in the abundance of HDAC-1 in senescent cells. Biochemical analysis of deacetylase activities in extracts from old and young cells revealed a striking difference. While by anion exchange chromatography we found a single peak of activity in extracts from young cells, which coincided with the elution of both HDAC-1 and HDAC-2, in senescent cells a second peak of activity was found. This second peak of activity is associated with HDAC-2 but does not contain HDAC-1. These results suggest that HDAC-2 is present in at least two distinct forms, one of which is specific for senescent cells. Further biochemical characterization of the enzyme activity revealed that addition of nicotinamide adenine dinucleotide (NAD) did not detectably influence the activity of any fraction, suggesting that NAD is not an essential co-factor for the analyzed HDACs from diploid human fibroblasts. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

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1. Introduction

Replicative senescence of diploid human fibroblasts has been proposed as an experimental model for human ageing (for recent review, see [1]). Cellular ageing is associated with drastic changes in gene expression. On the one hand, a set of genes is downregulated in senescent cells ([2,3]; for review, see [4]). This concerns mainly growth-associated and cell cycle regulatory genes. On the other hand, it was also shown that a group of genes consisting of quite divergent members is upregulated as diploid fibroblasts approach the senescent state. These are referred to as senescence-associated (SA) genes and include the amyloid precursor protein [5], insulin-like growth factor binding protein-3 [6], cyclins D1 and E [7,8], the cdk inhibitors p16INK4a [9,10] and p21WAF-1/Cip1 [11,12] as well as a few other genes ([13], for review, see [14]). While the SA downregulation of growth-regulated proteins can be explained in part by the irreversible cell cycle arrest that pertains in senescent fibroblasts [15], transcriptional upregulation of SA genes is less clear as far as the mechanisms are concerned. In some cases, for example for the genes encoding the cdk inhibitor p16INK4a [16] and the p53-interacting protein p14ARF [17,18], which are colocalized in the INK4 locus (for review, see [19]), a senescence-dependent loss of active repression has been discussed [20]. Upregulation of both the p14 and p16 genes correlates with the loss of bmi1, a transcriptional repressor of the Polycomb group of proteins (for review, see [21]), as evidenced by genetic studies. However, the molecular mechanisms of SA gene regulation remain largely unresolved.

It is known that the transcription of genes in vivo is controlled to some extent by enzymes that modify the chromatin structure, such as histone acetyltransferases and histone deacetylases (HDACs; for review, see [22]). In particular, a prominent role for HDACs has been reported and it was shown that transcriptional repression by HDACs plays a role in growth arrest exerted by the retinoblastoma protein (pRb) [23–25] and other cellular transcriptional repressors [26]. In most instances, HDAC-1 and HDAC-2 are tightly associated with each other in a 'corepressor core complex' that invariably contains also two other closely related proteins, RbAp46 [27] and RbAp48 [28]. The 'corepressor core complex' is a constitutive component of two distinct chromatin-associated transcriptional repressor complexes, referred to as the Sin3 [29] and NURD [30] complexes, respectively. It is believed that these complexes play an essential role in transcriptional repression (for review, see [31]). In senescent fibroblasts, pRb is present in the hypophosphorylated form, which acts as a transcriptional repressor [32]. While it was reported by others that inhibitors of HDAC, such as butyrate or trichostatin A, can induce premature senescence in human fibroblasts [33] and modulate the lifespan of yeast cells [34], the role of HDACs in senescence-specific gene expression requires further investigation.

In this communication, we have compared the activity and expression levels of human HDACs in diploid fibroblasts undergoing senescence. The expression of HDAC-1 decreases with increasing passage number of senescent cells, whereas expression of HDAC-2 is only slightly reduced. When the enzymatic activities of HDACs in old and young cells were compared, we found that they differed significantly in their chromatographic behavior. In both cell types, a similar peak of activity could be identified which coincides with the distribution of the HDAC-1 protein. For HDAC-2, we found a

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completely different chromatographic behavior in senescent as compared to young cells. While HDAC-2 from young cells elutes as a single peak, HDAC-2 from senescent cells elutes in several different forms, at least one of which is specific for senescent cells. We conclude that the abundance and activity of HDACs is changing significantly with cellular senescence and present direct biochemical evidence for a senescence-specific HDAC activity.

2. Materials and methods

2.1. Cell culture

Normal diploid fibroblasts were isolated from human foreskin [35] and cultured in Dulbecco's modified Eagle's medium (Gibco Life Technologies, Vienna, Austria), supplemented with penicillin/streptomycin solution (Gibco Life Technologies, Vienna, Austria) and 10% fetal calf serum (PAA, Linz, Austria). The cells were subcultured in an atmosphere of 5% CO_2 at 37°C by passaging them at a ratio of 1:5 in regular intervals. At later passages, the splitting ratio was reduced to 1:3 and 1:2, respectively. Cells were passaged such that the monolayers never exceeded 80% confluency. Population doublings (PDL) were estimated using the following equation: $n = (\log_{10}F - \log_{10}I)/0301$ (with n = PDL, F = number of cells at the end of one passage, I = number of cells that were seeded at the beginning of one passage). After roughly 55 PDL, the cells reached growth arrest. The senescent status was verified by in situ staining for SA-β-galactosidase as described [36]. 90–100% of the cells at 55 PDL stained positive for SA-β-galactosidase.

2.2. RNA extraction and Northern blot analysis

Total RNA was isolated using the 'RNeasy kit' (Qiagen, Hilden, Germany), following the instructions of the manufacturer. 10–15 μg of RNA was separated by electrophoresis on gels containing 1.2% agarose, 2.3% formaldehyde, 20 mM 3-*N*-morpholino-propanesulfonic acid, 5 mM Na-acetate, 1 mM EDTA, and transferred onto positively charged nylon membranes (Hybond-N^+, Amersham Life Science, Braunschweig, Germany) by capillary blotting. Membranes were dried and the RNA was fixed by UV-crosslinking, using a Stratagene Crosslinker. Prehybridization was performed for 1 h in 'Quick Hyb solution' (Stratagene, Heidelberg, Germany) at 65°C, then the membranes were hybridized with ^{32}P-labelled cDNA probes, generated by random primed labelling, in fresh solution for 1 h at 65°C. The membranes were washed twice in 2×SSC/0.1% sodium dodecyl sulfate (SDS) at room temperature for 15 min and once in 0.5×SSC/0.1% SDS at 65°C and exposed to Kodak X-ray films with intensifying screens at −70°C. We used the plasmid constructs pcDNA3-HDAC-1 and pcDNA3-HDAC-2 [37] to generate cDNAs for Northern blot hybridization.

2.3. Preparation of cellular extracts

To prepare whole cell extracts, cells were trypsinized from culture flasks, washed with phosphate-buffered saline (PBS) and lysed for 30 min on ice in a high stringency buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% Na-deoxycholate, 0.2 mM phenylmethylsulfonyl-fluoride, 1 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin). After centrifugation at 20 000×*g* for 15 min at 4°C, the supernatants were separated on SDS–polyacrylamide gels, followed by immunoblotting, as described below. Since the total level of protein per cell increases about 2.5-fold with replicative senescence (M. Wagner et al., unpublished), due to an increased size of the senescent cells [38], the loading of the gels was normalized to cell number.

2.4. Preparation of nuclear extracts

Young (20 PDL) and senescent (56 PDL) fibroblasts (equal number of cells) were trypsinized from cell culture dishes, pelleted by centrifugation and washed in PBS. The cell pellets were resuspended in two volumes of hypotonic buffer (10 mM HEPES/KOH pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2 \cdot 6H_2O$, 0.5 mM PMSF, 1.5 mM 2-mercaptoethanol) and incubated for 15 min on ice. The suspension was carefully homogenized, followed by centrifugation (10 min, 1000×*g*). The supernatant was frozen at −20°C, the pellet (= nuclei) was resuspended in four pellet volumes of nuclear extraction buffer (20 mM HEPES/KOH pH 7.9, 10% glycerol, 500 mM NaCl, 0.2 mM EDTA, 0.5 mM PMSF, 1.5 mM 2-mercaptoethanol) and incubated for 30 min at 4°C with agitation. After centrifugation (10 000×*g*, 20 min), the supernatant (= nuclear extract) was used for further analysis.

2.5. SourceQ chromatography

Nuclear extracts dialyzed against column buffer (15 mM Tris–HCl, pH 7.9, 10 mM NaCl, 0.25 mM EDTA, 1 mM 2-mercaptoethanol, 10% (v/v) glycerol) were loaded onto a 2 ml Source 30Q anion exchange column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with the same buffer. Elution of proteins was performed with 60 ml of a linear gradient from 10 mM to 500 mM NaCl in column buffer at a flow rate of 1 ml/min. Fractions of 1.5 ml were collected and assayed for HDAC activity. Aliquots of fractions with HDAC activity were used for immunoblotting after precipitation of protein using trichloro-acetic acid.

2.6. HDAC assay

HDAC activity was determined as described [39], using [^{3}H]acetate-prelabelled chicken erythrocyte histones as substrate. Sample aliquots of 50 μl were mixed with 10 μl of total [^{3}H]acetate-prelabelled chicken reticulocyte histones (1 mg/ml). This mixture was incubated at 30°C for 1 h. The reaction was stopped by addition of 50 μl of 1 M HCl/0.4 M acetate and 800 μl of ethylacetate. After centrifugation at 10 000×*g* for 5 min, an aliquot of 600 μl of the upper phase was counted for radioactivity in 3 ml liquid scintillation cocktail. Nicotinamide adenine dinucleotide (NAD; Sigma, Vienna, Austria) was added at a final concentration of 100 mM.

2.7. Immunoblot analysis

After electrophoresis of cellular or nuclear extracts on SDS–polyacrylamide gels, the proteins were transferred to PVDF membranes by wet electroblotting in a buffer containing 25 mM Tris–HCl, 190 mM glycine, 0.5% SDS, 10% methanol. Transfer was controlled by staining the membrane with Ponceau S. Membranes were blocked by incubation in 5% non-fat dried milk in TBS-T (20 mM Tris–HCl pH 7.6, 137 mM NaCl, 0.1% Tween 20) for 1 h at room temperature. Incubation with the primary antibody (rabbit polyclonal anti-HDAC-1, anti-HDAC-2, and anti-HDAC-3, E. Seto, unpublished) was performed for 60 min at room temperature, then the membranes were washed twice with TBS-T and incubated with the secondary antibody for 30 min. After four washes with TBS-T and one wash with TBS (TBS-T without Tween 20), immunoreactive proteins were detected using an enhanced chemiluminescence system (Amersham Life Science, Braunschweig, Germany). The following antibodies were used: anti-p16 (Pharmingen, Inc.), anti-cullin-1 (a gift from W. Krek, Basel, Switzerland), anti-RbAp46/48 (Signal Transduction Laboratories).

2.8. Preparation of histones

Young (20 PDL) and senescent (56 PDL) fibroblasts (equal number of cells) were trypsinized from cell culture dishes and washed in a buffer containing 10 mM Tris–HCl, 10 mM NaCl, and 5 mM $MgCl_2$. The cell pellet was resuspended in five pellet volumes of lysis buffer (10 mM Tris–HCl, 10 mM NaCl, 5 mM $MgCl_2$, 0.5% (v/v) Triton X-100) and incubated for 1 h at 4°C. Trichostatin A (100 ng/ml) was added to prevent deacetylation of histones. After careful homogenization in a potter, lysis of the cells was controlled by microscopy. After centrifugation (1000×*g*, 10 min) the supernatant was stored at −20°C; the pellet was incubated with four pellet volumes of 0.5 M HCl overnight at 4°C with agitation. The suspension was centrifuged (8000×*g*, 15 min) and the supernatant (= S1) was mixed with eight volumes of acetone and incubated overnight at −20°C for precipitation. After centrifugation (15 000×*g*, 4°C, 15 min) the pellet was vacuum-dried, then resuspended in 30 μl H_2O, followed by centrifugation (15 000×*g*, 10 min). The supernatant was removed and loaded on a SDS–polyacrylamide gel (14%) and on an acid–urea–Triton (AUT) gel (12%) [40] for analysis of the histones.

3. Results and discussion

Human diploid fibroblasts were explanted from neonatal foreskin and grown in culture until they reached the senescent status. As was described by others before, the cells grew exponentially in early passages and reached a plateau after

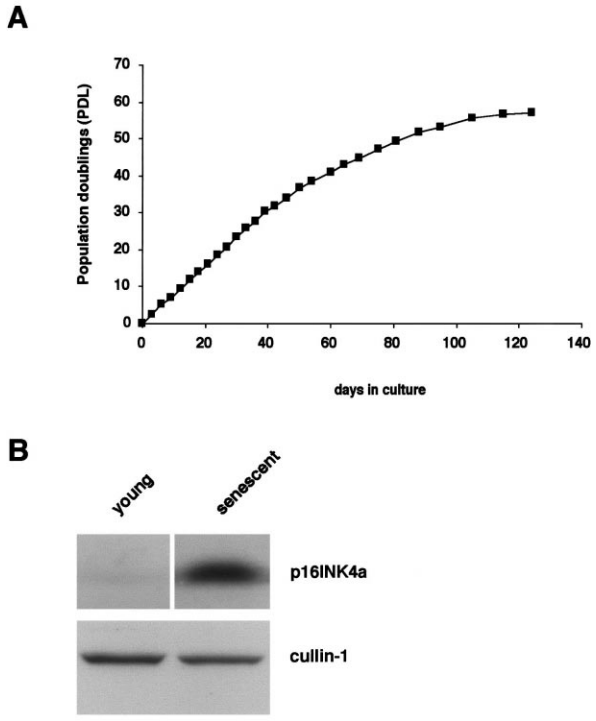

Fig. 1. Growth characteristics of human primary fibroblasts. A: Human diploid fibroblasts were isolated from neonatal foreskin and grown in serial passage. A representative cumulative growth curve is shown. B: The level of p16INK4a protein was determined in extracts from young (13 PDL) and senescent (55 PDL) fibroblasts by Western immunoblotting. Cullin-1 served as loading control.

about 55 PDL (Fig. 1A). At this point, >95% of the cells were arrested in the G1 phase of the cell cycle and stained positive for SA-β-galactosidase (data not shown), confirming that all the cells were in the senescent state. Entering senescence correlated with a downregulation of cyclin A gene expression (data not shown) and the upregulation of the p16(INK4a) cdk inhibitor (Fig. 1B). Expression of cullin-1 [41], another nuclear protein assayed as control, was unaltered during senescence (Fig. 1B).

To analyze histone acetylation, core histones were isolated from young and senescent diploid human fibroblasts. When the histone acetylation status was analyzed by electrophoresis on AUT gels [40], we found that histone H4 extracted from diploid fibroblasts shows a low degree of acetylation in that basically the mono- and non-acetylated forms of H4 are present. In these experiments, we detected no significant change in the overall acetylation pattern when young and senescent cells were compared (data not shown). To determine if cellular senescence is accompanied by changes in the expression level of the known HDACs, we analyzed the expression of HDACs 1, 2, and 3 during passaging of diploid human fibroblasts. While we were unable to detect any specific signal with the HDAC-3 antibodies available to us (data not shown), HDAC-1 and HDAC-2 were easily detected. Specificity of the antibodies was confirmed in control experiments where HDAC-1, -2, and -3 were transiently overexpressed in U2-OS cells (data not shown). The level of HDAC-1 protein decreased about 3-fold when cells reached the senescent state (Fig. 2A), whereas the level of cullin-1 assayed as control was not significantly altered. The decreased abundance of HDAC-1 in senescent cells was correlated with a similar decrease in the level of the corresponding mRNA (Fig. 2B), indicating that with increasing passage number the expression of the HDAC-1 gene is significantly reduced. We also noted a slight reduction of HDAC-2 protein with increasing passage number (Fig. 2A), which was however less pronounced than in the case of HDAC-1; the HDAC-2 mRNA levels were not reduced in senescent cells (Fig. 2B).

We then analyzed the enzymatic activity of HDACs in extracts of young versus old cells. In these experiments we detected slightly more (~1.5-fold) HDAC activity in nuclear extracts of senescent cells than in extracts from young cells. Since the protein levels for HDAC-1 and HDAC-2 are somewhat lower in senescent cells (Fig. 2A), this result suggests that besides HDAC-1 and HDAC-2 other HDACs may be present in senescent cells, which are not detected by the antibodies available to us. To obtain further information on changes in HDACs during replicative senescence, nuclear extracts from old and young cells were separated on an anion exchange chromatography column where elution was per-

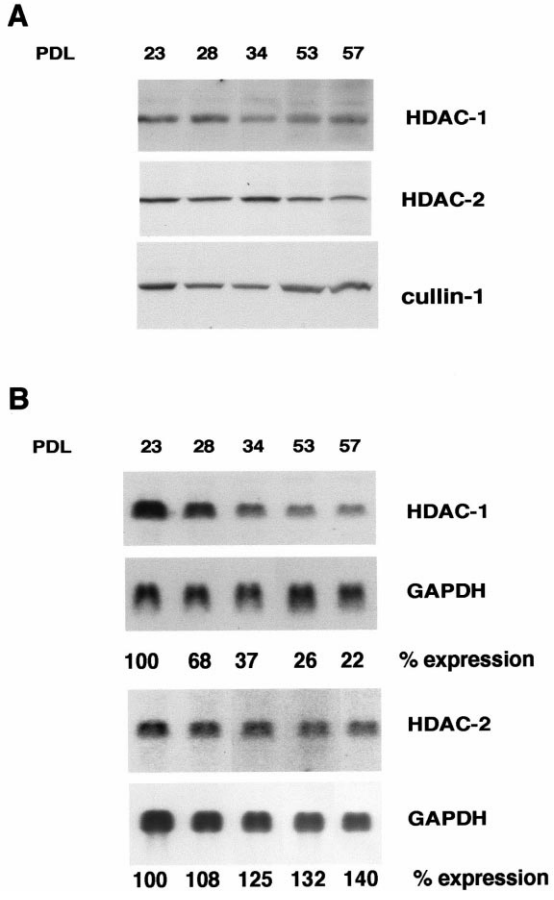

Fig. 2. Analysis of the expression of HDAC-1 and HDAC-2. Primary human fibroblasts were grown in cell culture until they reached replicative senescence. A: Cellular extracts were prepared after various numbers of PDL (as indicated) and samples corresponding to equal cell numbers were separated by SDS–PAGE and analyzed by Western blot, using antibodies to HDAC-1, HDAC-2, and cullin-1, respectively. B: RNA was prepared after various numbers of PDL and analyzed by Northern blot with probes specific for HDAC-1 and HDAC-2, as indicated. For loading control, the membranes were reprobed for glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). Expression levels were determined relative to GAPDH by densitometric scanning; values are provided in %.

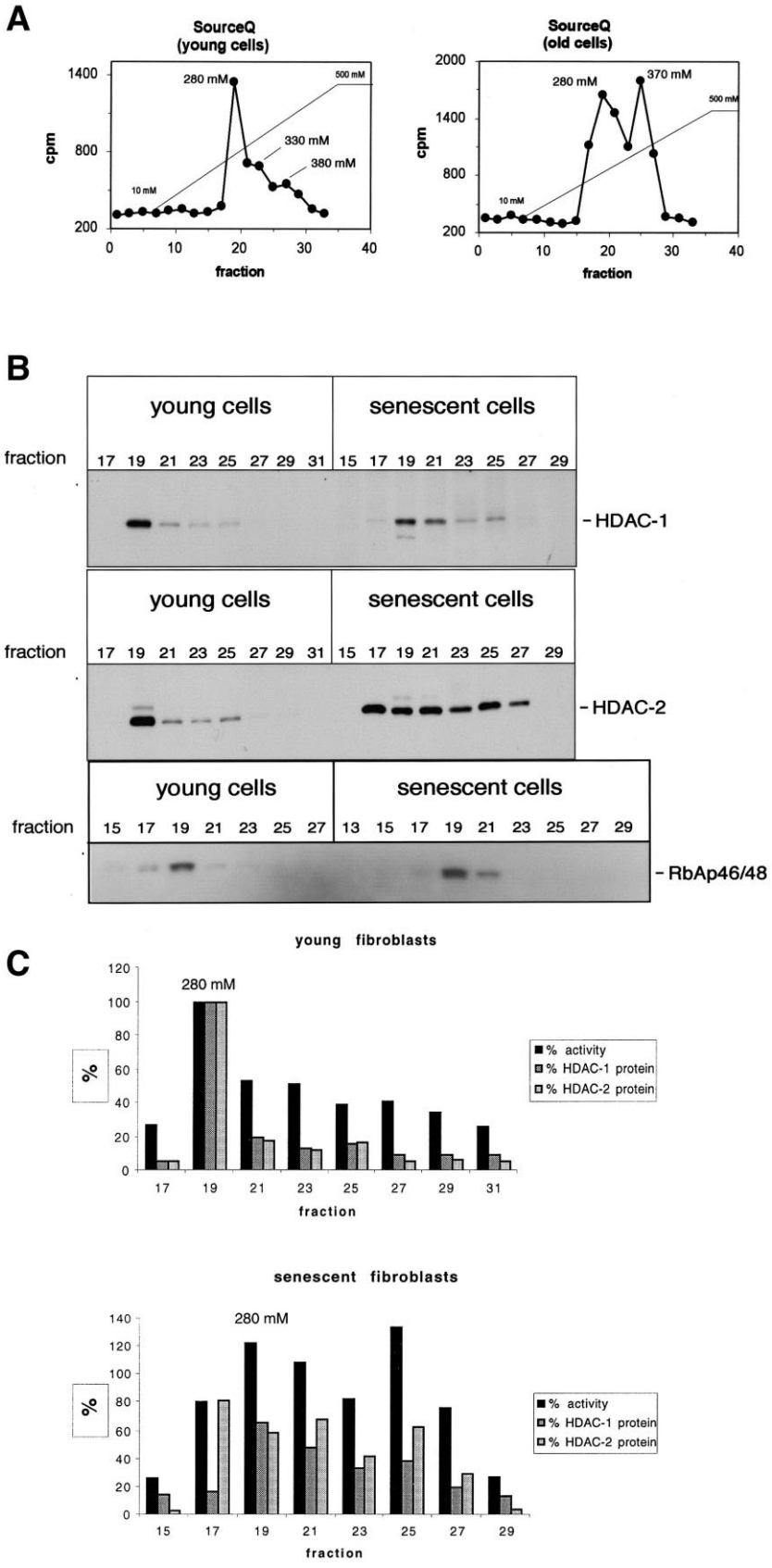

Fig. 3. Analysis of HDACs by anion exchange chromatography. Nuclear extracts from young (~20 PDL) and old (~56 PDL) fibroblasts were prepared and fractionated by SourceQ anion exchange chromatography. A: Every second fraction was analyzed for HDAC activity. B: The same fractions as in A were analyzed for HDAC-1, HDAC-2, and pRbAp46/48 protein levels by immunoblot analysis. C: The levels of HDAC activity and of densitometrically scanned HDAC-1 and HDAC-2 protein are plotted; each signal was normalized to the respective level found in fraction 19 from young cells.

formed with a NaCl gradient, followed by measurement of deacetylase activity in the eluted fractions. In these experiments, we reproducibly observed the following chromatographic patterns: in young cells, there was a major peak of activity which eluted at 280 mM NaCl (fraction 19) from the anion exchange column. There were also two minor peaks at 330 mM and 380 mM NaCl. When extracts from senescent cells were analyzed the same way, two major peaks of activity were present: the highest peak occurred in fraction 25, which was eluted at 370 mM NaCl. We also observed a second peak in fraction 19, which eluted at the same salt concentration (280 mM) as the peak obtained in the young cells. The chromatogram shown in Fig. 3A has been independently observed in two experiments which gave identical patterns. This finding indicates that both young and senescent cells contain similar amounts of a common HDAC activity which can be eluted from a SourceQ column at 280 mM NaCl. In addition to this common type of enzyme, senescent cells contain an additional, biochemically distinct HDAC activity, which is much less abundant in young cells.

To investigate if HDAC-1 or HDAC-2 are associated with any of the enzymatic activities shown in Fig. 3A, we probed fractions obtained by the anion exchange chromatography with antibodies to either HDAC-1 or HDAC-2 (Fig. 3B). This experiment revealed that in young cells the major peak of activity associated with a strong peak of HDAC-1 in fraction 19. In senescent cells, the bulk of HDAC-1 also eluted at 280 mM NaCl and was also present in fraction 19. As in the young cells, traces of HDAC-1 from senescent cells were also present in the three consecutive fractions (21–25). When these blots were probed with an antibody to HDAC-2, we found that in extracts from young cells, the bulk of the HDAC-2 protein eluted as a sharp peak at 280 mM NaCl coeluting with HDAC-1 in fraction 19. In chromatographic fractions derived from senescent cells, on the other hand, HDAC-2 shows a much broader distribution. First, HDAC-2 clearly eluted before HDAC-1, at a salt concentration of 240 mM (fraction 17). Second, significant amounts of HDAC-2 were present in six consecutive fractions from 17 to 27 (Fig. 3B). A clear-cut peak for HDAC-2 is seen at 370 mM NaCl (fraction 25). This result suggests that in senescent cells HDAC-2 is present in two major complexes of different chromatographic behavior.

The relative expression level of HDAC-1 and HDAC-2 in individual fractions was quantified by densitometric scanning and plotted against the catalytic activity present in these fractions. Comparing the relative activity and the relative protein levels (Fig. 3C), we concluded that the activity peak eluting at 280 mM NaCl is most likely due to both HDAC-1 and HDAC-2, which are both present in the respective fractions derived from both old and young cells. While the chromatographic behavior of HDAC-1 from young and senescent fibroblasts is quite similar, a significant difference was observed for HDAC-2. In young cells, HDAC-2 is present in a single peak, eluting at 280 mM NaCl. In senescent cells, on the other hand, at least two additional peaks of HDAC-2 exist, eluting at 240 mM and 370 mM, respectively.

In several mammalian cell types, HDAC-1 and HDAC-2 are tightly associated with each other in a 'corepressor core complex' that invariably contains also two other closely related proteins, RbAp46 [27] and RbAp48 [28] (for review, see [31]). Since in senescent cells HDAC-2 is found in fractions devoid of HDAC-1, it appears that the association of both proteins is partly abolished in senescent cells (Fig. 3B). To better characterize the HDAC-containing fractions described here, we also analyzed the distribution of RbAp46/48 in the chromatographic fractions, using a polyclonal antiserum that recognizes both RbAp46 and RbAp48. RbAp46/48 proteins are expressed in young fibroblasts and their abundance is not significantly altered in senescent cells (data not shown). When we probed the HDAC-2-containing fractions with antibodies to RbAp46/48, we found that the RbAp proteins coelute with both HDAC-1 and HDAC-2 in young cells. In senescent cells, however, the senescent-specific forms of HDAC-2 (namely in fractions 17, 25, and 27) are devoid of RbAp46/48 (Fig. 3B). This finding indicates that an age-dependent remodelling of HDAC-2-containing complexes occurs in human fibroblasts.

As was also mentioned above, it is quite likely that not all of the catalytic activity shown in Fig. 3A can be ascribed to either HDAC-1 or HDAC-2. This raises the possibility that some of the fractions contain unidentified HDAC(s), which correspond neither to HDAC-1 nor HDAC-2. However, the identity of such proteins remains to be determined. To address this point, the fractions were also probed with antibodies to HDAC-3, -4, -5, -6, -7, and -8; however, none of the antibodies available to us revealed a specific band in any of the fractions (data not shown). Furthermore, the detection of HDAC-1 or HDAC-2 protein in a fraction does not necessarily mean that the protein contributes to the enzymatic activity that can be measured. Besides a potential modulation of HDAC activity through associated proteins (see above), the detectable enzymatic activity may also depend on the presence of inhibitors and/or essential co-factors. While endogenous inhibitors of HDACs are not known in mammalian cells, it was reported recently that a HDAC from yeast, which plays a role in age-dependent gene expression, depends on NAD as a

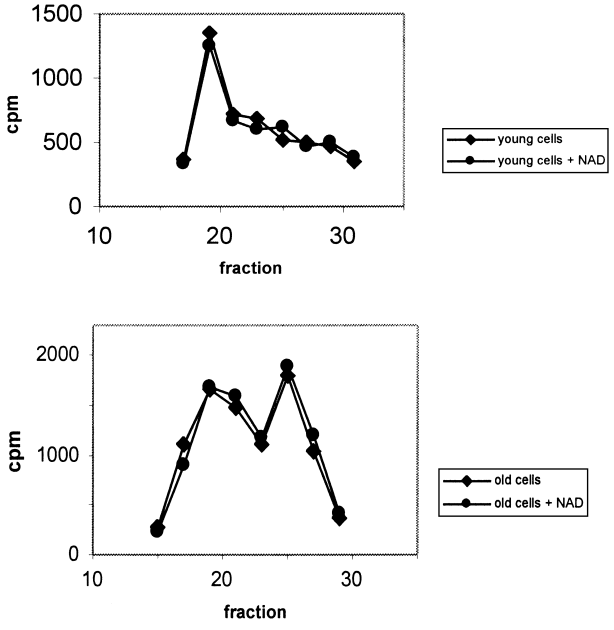

Fig. 4. Influence of NAD on HDAC activity in chromatographic fractions. Nuclear extracts from young (~20 PDL) and old (~56 PDL) fibroblasts were prepared and fractionated by anion exchange chromatography. Every second fraction was analyzed for histone acetylase activity in the absence of exogenous NAD (♦) or after addition of 100 mM NAD (●).

co-factor for activity. NAD-dependent deacetylases were also described in other systems [42–44] (for review, see [45]). To determine whether any of the activities detected in our cell extracts may depend on NAD as a co-factor, chromatographic fractions derived from young and old cells were reassayed for their enzymatic activity, after addition of 100 mM NAD. This experiment revealed that the catalytic activity of the enzyme(s) present in all fractions was not changed by the addition of NAD (Fig. 4). From these data we conclude that NAD is not a limiting factor for the HDAC activities that are detectable in vitro when acetylated histones are used as substrate and raises the question whether, by analogy to yeast ageing, NAD-dependent HDACs play an important role for age-dependent gene expression in human fibroblasts.

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