Inhibitory Activity of a Heterochromatin-associated Serpin (MENT) against Papain-like Cysteine Proteinases Affects Chromatin Structure and Blocks Cell Proliferation*

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MENT (Myeloid and Erythroid Nuclear Termination stage-specific protein) is a developmentally regulated chromosomal serpin that condenses chromatin in terminally differentiated avian blood cells. We show that MENT is an effective inhibitor of the papain-like cysteine proteinases cathepsins L and V. In addition, ectopic expression of MENT in mammalian cells is apparently sufficient to inhibit a nuclear papain-like cysteine proteinase and prevent degradation of the retinoblastoma protein, a major regulator of cell proliferation. MENT also accumulates in the nucleus, causes a strong block in proliferation, and promotes condensation of chromatin. Variants of MENT with mutations or deletions within the M-loop, which contains a nuclear localization signal and an AT-hook motif, reveal that this region mediates nuclear transport and morphological changes associated with chromatin condensation. Non-inhibitory mutants of MENT were constructed to determine whether its inhibitory activity has a role in blocking proliferation. These mutations changed the mode of association with chromatin and relieved the block in proliferation, without preventing transport to the nucleus. We conclude that the repressive effect of MENT on chromatin is mediated by its direct interaction with a nuclear protein that has a papain-like cysteine proteinase active site.

MENT (Myeloid and Erythroid Nuclear Termination stage-specific protein), a developmentally regulated nuclear protein, is present in three main avian blood cell types (erythrocytes, lymphocytes, and granulocytes) where it is the predominant non-histone protein associated with compact heterochromatin (1). In vitro, MENT brings about a dramatic remodeling and condensation of chromatin higher order structure by forming protein “bridges” connecting separate nucleosomes in nucleosome arrays (for review see Ref. 2). MENT has no homology with other known chromatin proteins but belongs to the intracellular branch of the serpin superfamily (3). Serpins were originally characterized as serine proteinase inhibitors; however, more recently certain members have been shown to be capable of inhibiting other proteinase classes such as caspases (the viral serpin crmA (4)) and papain-like cysteine proteinases (SCCA-1 (5)). The inhibitory members of the serpin family are notable for their ability to undergo a large scale conformational transition (for review see Ref. 6) that is critical for inhibition of target proteinases (7, 8) and for self-association or polymerization (9–11). Multiple sequence alignments and comparison with known serpin structures reveal that MENT contains a large insertion, the “M-loop,” between the C- and D-helices. This loop contains two critical functional motifs as follows: a classical nuclear localization signal (NLS) that is required for nuclear import, and an AT-hook motif that is involved in chromatin and DNA binding (3). Like other serpins, MENT possesses a reactive center loop (RCL) through which interaction with a cognate proteinase occurs. The presence of an inhibitory hinge motif (12) in its RCL suggests that MENT may retain the propensity for serpin-like conformational transitions and hence proteinase inhibition.

An important question is whether MENT is indeed a proteinase inhibitor, and if so, whether this activity plays any role in the interaction between MENT and chromatin or fulfills an unrelated second function of the protein. We show that MENT is an effective inhibitor of the papain-like cysteine proteinase cathepsins K, L, and V, as well as a cysteine proteinase from CV-1 cells with characteristics similar to the recently described nuclear proteinase, SPase (13). Thus MENT is the first known chromatin-associated cysteine proteinase inhibitor. We demon-

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1 The abbreviations used are: NLS, nuclear localization signal; RCL, reactive center loop; SI, stoichiometry of inhibition; ORF, open reading frame; PBS, phosphate-buffered saline; MES, 2-(N-morpholino)ethanesulfonic acid; BrdUrd, 5-bromo-2′-deoxyuridine-5′-monophosphate; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight analyzer; BSA, bovine serum albumin; Rb, retinoblastoma protein; Cbz, benzoxycarbonyl.

2 Numbering of the reactive center loop is according to the standard nomenclature defined by Schecter and Berger (48), in which the amino acids N-terminal to the site of cleavage are denoted P1, P2, . . . , PM, and those C-terminal to it are denoted P1′, P2′, . . . , PP′.
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Experimental Procedures

Materials—Unless otherwise noted, all fine chemicals were from Sigma. Sepharose resins and Hitrap columns were from Amersham Biosciences AB. Human cathepsins K, L, and V were expressed in Ficha pastoris and purified as described previously (14, 15). Papain was purchased from ICN (New South Wales, Australia). Sheep cathepsin L was purified from sheep liver as described previously (16). Active enzyme concentration was determined by titration with the inhibitor E-64.

MEN'T Expression Constructs—A wild-type MEN'T (MEN'TWT)-expressing plasmid based on the pRc/cytomegalovirus vector (Invitrogen), pSG109, has been described previously (3). Nucleotide substitutions and new endonuclease restriction sites were introduced into the MEN'T ORF by site-directed mutagenesis PCR (17). The PCR amplifications employed the following primers encoding the 5′ and the 3′ ends of the MEN'T ORF: M49 (5′, forward), AGCAAGCTT-GATCTCGGATGAAAACCACTGTGCATACG, and MENT ATHOOK-reverse, TCAGCTTTGTTCGAAATGAAGAACCTCTGCCA; and MENTMLOOP-reverse, AGCTGTCGCCCCACCTCTTCTATTCAATCGTC; MEN'TMLOOP-forward, TTCAATTGCGACAACTGAAAACTCTACCTCCT, and MEN'THOOK-forward, TCAGCTTTGCTAAGAAATTGACCTTCCGA; and MEN'THOOK-reverse, TCAGCTTTGCTAAGAAATTGACCTTCCGA, and MEN'THOOK-forward, AAAGGCAGCTGGCCACGAAAGAAAAGAACATTGACCC, and MEN'THOOK-reverse, TTCGCC- TTGGCCGCTTTGGCCACAGAAGAGCTTTCAG.

For the first PCR amplification step was conducted between either M49 and one of the reverse primers or between M36 and one of the forward primers. Following this, the PCR products from the two reactions were mixed and the internal primers removed, and the PCR was continued using the flanking primers M49 and M36. PCR amplifications were carried out using Taq DNA polymerase (Invitrogen). Reaction cycles were carried out in a 30-μl volume using highmelting wax for “hot start” conditions with 25 cycles each containing three 1-min steps at 94, 55, and 72 °C and finally 10 min at 72 °C. The PCR products were inserted between the HindIII and BstBI restriction sites in pSG109, replacing the MEN'TWT ORF. MEN'TWT was amplified using the reverse primer M36 and a pair of “nested” oligonucleotide primers: Mov2, GCTGAATGCTGCTGCTCGCTGCGCTGCG; and Mov1, AACGACGGATGACCTTACGTTACAGAAGCTTGTTGC. The nested PCR product was used to replace MEN'TMLOOP by cutting and ligating at the NruI and BstBI restriction sites. To verify the DNA sequences, all plasmids were digested with EcoRI and BamHI and ligated into EcoRI/BamHI-cut pET-15b vector (Novagen) using EcolR/BamHI linker DNA (GeneWorks, Australia). The location of the mutations is shown in Fig. 1.

Expression and Purification of MEN'T and Mutants—The pET-15b constructs were used to express MEN'T and its variants in the BL21 Escherichia coli strain according to the vendor’s protocol (18), with the exception that expression was induced at an A600 of 1–1.1. Harvested cells were resuspended in lysis buffer (50 mM NaPO4, pH 6.3, 2 mM NaCl, 1% (v/v) Triton X-100) and disrupted using a final concentration of 1 mg/ml lysozyme and sonication. Following centrifugation, the soluble fraction was applied to nickel-chelated chelating Sepharose resin and washed with 10 column volumes of lysis buffer. A buffer containing 50 mM NaPO4, pH 8.0, 0.5 mM NaCl, 25 mM imidazole was applied to the resin until the A600 returned to base line, and protein was eluted with 50 mM HEPS, pH 7.0, 50 mM NaCl, 250 mM imidazole, pH 7.4. The eluate was diluted 2-fold into buffer A (50 mM HEPS, pH 7.4, 50 mM NaCl) and loaded onto a 5-ml Hitrap SP column. After washing in the same buffer, a salt gradient was applied over 12 column volumes using 50 mM HEPS, pH 7.4, 1 mM NaCl; intact MEN'T eluted in the middle of the gradient. These fractions were diluted 1:10 into 25 mM HEPS, pH 7.8, 1.7 mM (NH4)2SO4, loaded onto a 5-ml Hitrap SP column, and eluted using a gradient over 10 column volumes into 25 mM HEPS, pH 7.8. The (single band) purity of the proteins was verified using SDS-PAGE and their monomeric nature by using acid-PAGE (method appears below). The concentration of the protein was determined using an A280 measurement with reference to the predicted extinction coefficient (19). After dialysis into buffer A and concentration to ∼1 mg/ml, protein was stored at −80 °C.

Formation of Peptide-complexed MEN'T—A 20 μM concentration of MEN'TWT was incubated with a 2 mM solution of a peptide corresponding to the P1–P3 residues of the anthithrombin RCL (sequence SEAAAS, Auspex, Parkville, Australia) in buffer A at 37 °C for 1 h. A sample of MEN'TWT incubated in the absence of peptide did not lose activity under these conditions. None of the peptides employed the following primers encoding the complex with the peptide and, in conjunction with gel filtration, that peptide-complexed MEN'T did not polymerize. The gel system consisted of a 10% running gel (no stacking gel) with 260 mM Tris, pH 7.8, running gel, and 1× TBE tank buffer, pH 8.0, with migration of the protein toward the cathode.

Determination of Kinetic Parameters—Enzyme assays were undertaken in pH 5.5 buffer (0.1 mM acetate, pH 5.5, 1 mM EDTA, 0.1% (w/v) Brij-35, 10 mM cysteine) or an AMT buffer at pH 7.0 (0.05 mM acetate, 0.05 mM MES, 0.1 mM Tris, pH 7.0, 1 mM EDTA, 0.1% (w/v) Brij-35, 10 mM cysteine). The stability of cathepsin proteinases at neutral pH is often regarded as being low, although more recent studies (20) have indicated that the proteinases are more stable than previously thought in some buffer systems. Under the assay conditions used here, the cathepsins were found to be stable over the entire period of the experiment.

The stoichiometry of inhibition (SI) was determined in pH 5.5 buffer. Briefly, 10 μl of 20 nm enzyme was mixed with varied concentrations of inhibitor, incubated for 45–100 min at 30 °C, diluted to 200 μl in buffer containing a final concentration of 10 μM N-Cbz-Ph-Arg-methylcoumarin substrate, and the residual enzyme activity measured using a Fluostar Galaxy plate reader (BMG Labtechnologies, Germany) with excita-

tion/emission wavelengths of 370/460 nm, at 30 °C. Data analysis was carried out as described previously (21).

The second-order association rate constant (kobs) was determined under pseudo-first-order conditions using continuous kinetics. Within a given assay, the enzyme concentration was held constant, at a value between 0.025 and 0.5 μM, and the inhibitor concentration was always at least 20-fold higher, varying between 0.5 and 100 μM. All assays were conducted at 30 °C, using a 20 μM concentration of N-Cbz-Ph-Arg-methylcoumarin substrate in a total volume of 200 μl. Upon addition of enzyme to the inhibitor/substrate mixture, cleaved substrate fluorescence was monitored continuously using excitation/emission wave-
lengths of 370/460 nm. These fluorescence data were satisfactorily fitted, using least squares regression in PRISM (GraphPad Software), to Equation 1 describing slow, tight inhibitor binding (22).

\[ F = V_0 + t \cdot \left( V_0 - V_i \right) \cdot \frac{1}{k_{\text{obs}}} \cdot e^{-k_{\text{obs}} t} \]  

where F is the absolute fluorescence value at time t; V0 is the velocity at time 0; V is the residual velocity once the reaction has run to completion; and kobs is the apparent second-order rate constant. The uncorrected second-order association rate constant (kunc) was obtained from the slope of the linear regression through a plot of kobs against inhibitor concentration. The substrate-decay corrected rate constant was finally obtained as shown in Equation 2.

\[ k_{\text{obs}} = k_{\text{unc}} \cdot \left( 1 + \frac{[S]}{K_m} \right) \]  

Km values for human cathepsin L and human cathepsin V were determined in this study for the hydrolysis of the fluorogenic substrate and were found to be essentially identical in both buffer systems. The Km values of 7.5 μM for human cathepsin K and 9.8 μM for sheep cathepsin L were obtained from previous studies (23, 24). Preliminary experiments showed that proteinase activity did not drop below 95% over the time course of the assays.

Assessment of Complex Stability—Complex stability was assessed by incubating human cathepsin V with a 2-fold molar excess of MEN'TWT with pH 5.5 buffer at room temperature. Samples were removed at 0, 12, 24, and 48 h, and activity was measured using the same conditions as when determining the SI (see above). An inhibitor-free control was also used and remained >90% active throughout the course of the assay.

Mass Spectrometry—Molecular weight determinations of purified MEN'TWT and its products following digestion with cathepsin L were conducted using a linear MALDI-TOF mass spectrometer (Perseptive
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RESULTS

MENT Is an Efficient Inhibitor of the Cysteine Proteinases Cathepsin L and Cathepsin V—Previous studies (1, 3) demonstrated that MENT was unable to inhibit a variety of serine proteinases. We predicted that the bulky hydrophobic phenylalanyl residue in the putative P2 position would allow MENT to inhibit members of the papain-like cysteine proteinase family. This is not unprecedented, because previous work by Schick et al. (29) showed that the serpin SCCA-1 was capable of inhibiting cathepsins K, L, and S. It was found that recombinant MENT is an effective inhibitor of the human cysteine proteinases cathepsin L and V and sheep cathepsin L at pH 5.5, with $k_a$ values of 1.4 $\times 10^4$ M$^{-1}$ s$^{-1}$, 4.0 $\times 10^4$ M$^{-1}$ s$^{-1}$, and 2.2 $\times 10^4$ M$^{-1}$ s$^{-1}$, respectively (Tables I and II; Fig. 2a, right panel). The stoichiometry of inhibition was 1.0 with all three proteinases (Table I; Fig. 2a, left panel). MENT was also capable of inhibiting human cathepsins L and V at neutral pH (Table I). Intact MENT purified from chicken granulocyte nuclei (MENT$nuk$) demonstrated similar inhibitory behavior (Table I) and was as efficient in immunoprecipitating sheep cathepsin L protein using anti-MENT antibodies as recombinant material, visualized by Western blot with antibodies against sheep cathepsin L (Fig. 2b, top left panel). A mutant lacking the M-loop (MENT$nuk$-M) (Fig. 1) gave almost identical SI and $k_a$ values to wild-type material, confirming that this region is not important for inhibitory activity (Table I).

Serpins inhibit serine proteinases using a unique “inhibition by distortion” mechanism (8), in which the RCL is cleaved in a central bond in serpins. As analyzed by acid denaturation and by electrophoresis on 12% SDS-PAGE (28), the stoichiometry of inhibition was 1.0 with all three proteinases (Table I; Fig. 2a, left panel). MENT was also capable of inhibiting human cathepsins L and V at neutral pH (Table I). Intact MENT purified from chicken granulocyte nuclei (MENT$nuk$) demonstrated similar inhibitory behavior (Table I) and was as efficient in immunoprecipitating sheep cathepsin L protein using anti-MENT antibodies as recombinant material, visualized by Western blot with antibodies against sheep cathepsin L (Fig. 2b, top left panel). A mutant lacking the M-loop (MENT$nuk$-M) (Fig. 1) gave almost identical SI and $k_a$ values to wild-type material, confirming that this region is not important for inhibitory activity (Table I).
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The inhibitory properties of MENT<sub>wt</sub> and its mutants at pH 5.5 and pH 7.0

The<sub>K<sub>m</sub></sub> values of the enzymes for N-Cbz-Phe-Arg-methylcoumarin, determined in this study and essentially identical at both pH values, are shown. All errors were below 5%.

| Inhibitor form | Cathepsin L (SI) | K<sub>m</sub> = 15 μM | Cathepsin V (SI) | K<sub>m</sub> = 15 μM |
|----------------|-----------------|----------------------|-----------------|----------------------|
| pH 5.5         |                 |                      |                 |                      |
| MENT<sub>WT</sub> | 1.0             | 1.4 × 10<sup>6</sup> | 1.0             | 4.0 × 10<sup>5</sup> |
| MENT<sub>MLOOP</sub> | 1.2             | 1.2 × 10<sup>6</sup> | 1.1             | 3.2 × 10<sup>5</sup> |
| MENT<sub>TA</sub> | 1.0             | 3.9 × 10<sup>6</sup> | 1.0             | 2.3 × 10<sup>5</sup> |
| MENT<sub>P14R</sub> | ND<sup>a</sup> |                      | 50              | 3.4 × 10<sup>5</sup> |
| MENT<sub>R</sub> | ND              |                      | 112             |                      |
| MENT<sub>6-mer</sub> | ND              |                      | ND              |                      |
| pH 7.0         |                 |                      |                 |                      |
| MENT<sub>WT</sub> |                 | 2.6 × 10<sup>6</sup> |                 | 2.0 × 10<sup>5</sup> |
| MENT<sub>MLOOP</sub> |                 | 1.9 × 10<sup>6</sup> |                 | 1.5 × 10<sup>5</sup> |

<sup>a</sup> ND, not detectable.

FIG. 1. Molecular model of MENT protein and its mutations. A homology model of MENT is shown, constructed using the MODELLER component (49) of the QUANTA package (MSI Inc.) and based on the structure of ovalbumin (Protein Data Bank code 1OVA (50)). No attempt was made to model the M-loop, which is represented by a dashed blue line (not to scale). Color designations are as follows: A β-sheet, red; B β-sheet, green; C β-sheet, yellow; reactive center loop, purple; C- and D-helices are in orange, all other helices are in gray; and the P<sub>1</sub> residue appears as an aqua-blue sphere. The site of the modifications in each MENT variant is indicated.

Molecular model of MENT protein and its mutations. MENT<sub>OV</sub>, replacement with P<sub>15-P<sub>4</sub></sub> sequence of ovalbumin (GREVQGSAEAGVDAAVSVE).

MENT<sub>P14R</sub> T→R substitution
MENT<sub>MLOOP</sub> deletion of M-loop
MENT<sub>ATIEOK</sub> RPSRGP KGAKAG

Table I: The inhibitory properties of MENT<sub>wt</sub> and its mutants at pH 5.5 and pH 7.0

| Inhibitor form | Cathepsin L (SI) | K<sub>m</sub> = 15 μM | Cathepsin V (SI) | K<sub>m</sub> = 15 μM |
|----------------|-----------------|----------------------|-----------------|----------------------|
| pH 5.5         |                 |                      |                 |                      |
| MENT<sub>WT</sub> | 1.0             | 1.4 × 10<sup>6</sup> | 1.0             | 4.0 × 10<sup>5</sup> |
| MENT<sub>MLOOP</sub> | 1.2             | 1.2 × 10<sup>6</sup> | 1.1             | 3.2 × 10<sup>5</sup> |
| MENT<sub>TA</sub> | 1.0             | 3.9 × 10<sup>6</sup> | 1.0             | 2.3 × 10<sup>5</sup> |
| MENT<sub>P14R</sub> | ND              |                      | 50              | 3.4 × 10<sup>5</sup> |
| MENT<sub>R</sub> | ND              |                      | 112             |                      |
| MENT<sub>6-mer</sub> | ND              |                      | ND              |                      |
| pH 7.0         |                 | 2.6 × 10<sup>6</sup> |                 | 2.0 × 10<sup>5</sup> |
| MENT<sub>MLOOP</sub> | 1.9 × 10<sup>6</sup> | 1.5 × 10<sup>5</sup> |

<sup>a</sup> ND, not detectable.

that the physiological target of MENT is a papain-like cysteine proteinase. MENT<sub>WT</sub> was also able to inhibit other members of the cysteine proteinase family. It was found to be an effective inhibitor of human cathepsin K and a relatively less effective inhibitor of papain but not an inhibitor of human cathepsin B (Table II).

Numerous studies of serpins that inhibit serine proteinases have shown that mutation of the P<sub>1</sub> position to a large polar residue results in substrate-like rather than inhibitory behavior (33), presumably because the mutation interferes with RCL insertion. To determine whether such a structural transition was required in the inhibition of cathepsin proteinases by MENT, a P<sub>1</sub>Asp→Arg mutant (MENT<sub>P14R</sub>) was constructed. Although some residual inhibitory activity was observed against cathepsin V (k<sub>in</sub> of 3.4 × 10<sup>3</sup> M<sup>–1</sup>s<sup>–1</sup>), MENT<sub>P14R</sub> acted predominantly as a substrate, with an SI of 50. The limited residual inhibitory activity observed is consistent with previous studies (33) on the interaction between P<sub>1</sub>Asp and serine proteinases. Correction of the k<sub>in</sub> value for the high SI (by multiplying the two) yielded a value close to that seen with MENT<sub>WT</sub> (1.7 × 10<sup>5</sup> M<sup>–1</sup>s<sup>–1</sup>). This indicates that the P<sub>1</sub>Asp mutation does not interfere significantly with the initial docking between MENT and cathepsin V but does perturb the transition to an irreversible complex. Consequently, the MENT<sub>P14R</sub> mutant interacted with but was unable to sustain an interaction with cathepsin proteinases; it did not co-precipitate sheep cathepsin L protein in the immunoprecipitation assay (Fig. 2b, top left panel, lanes 5 and 6).

Incubation of wild-type MENT with a 100-fold molar excess of the peptide SEAAAS (corresponding to the P<sub>15-P<sub>4</sub></sub> residues of the RCL of antithrombin) resulted in a MENT-peptide complex (Fig. 2b, top right panel) that demonstrated no inhibitory activity (Table I). It is suggested that the 6-mer peptide inserts into the A-sheet in a similar fashion to that seen in the structure of PAI-1 in complex with two pentapeptides, preventing RCL insertion (34).

Finally, it was found that a construct of MENT in which the P<sub>15-P<sub>4</sub></sub> residues of the RCL were replaced with those of the non-inhibitory serpin ovalbumin (MENT<sub>OV</sub>) was also unable to inhibit cathepsin L and was an extremely poor cathepsin V inhibitor (Table I).

ECTOPIC EXPRESSION OF MENT IN CV-1 CELLS IS SUFFICIENT TO PROTECT Rb1 PROTEIN FROM DEGRADATION BY SPase, A NUCLEAR PAPAIN-TYPE CYSTEINE PROTEINASE—Although cathepsins L and V are lysosomal proteins (35), cysteine proteinases with properties very similar to cathepsin L have been reported to be localized to the nucleus and involved in the degradation of a number of important nuclear regulators (35–38). In particular, work by Nishimakia et al. (13) demonstrated the presence in CV-1 cells...
and several other cell lines of a cathepsin L-like proteinase, SPase, whose activity correlated with degradation of underphosphorylated Rb protein (39), a factor that plays a pivotal role in regulating the cell cycle (40, 41).

To examine the influence of MENT on SPase activity in vitro, we prepared nuclear extracts from wild-type (non-transfected) CV-1 and NIH/3T3 cells. Fig. 3a shows that the nuclear extract from wild-type CV-1 cells contained a strong cathepsin-like protease (SPase) activity. A nuclear extract prepared from NIH/3T3 cells, which do not express SPase (13), did not demonstrate such enzymatic activity. Addition of either E-64d or bacterially expressed MENT<sub>WT</sub> to the CV-1 extract completely abolished the SPase activity, whereas addition of bacterially expressed MENT<sub>P14R</sub> had no effect (Fig. 3a).

To monitor the stability of Rb protein, we then analyzed the CV-1 nuclear proteins using SDS-PAGE followed by Western blotting with an anti-Rb antibody. In the nuclear extract of CV-1 cells, the bands at around 105 kDa correspond to intact and multiphosphorylated forms of Rb and the 50-kDa band to the main product of Rb proteolysis by SPase (13). This latter degradation product was observed in the nuclei of control CV-1 cells but was dramatically reduced in cells isolated in the

FIG. 2. MENT inhibits and forms stable complexes with cathepsins K, L, and V. a, determination of the MENT<sub>WT</sub>-to-proteinase ratio (SI) required to fully inhibit human cathepsins L and V (open square and filled triangle, left panel). The plot of k<sub>obs</sub> against inhibitor concentration used to derive the second-order rate constant (k<sub>i</sub>) is shown at the right, including that for cathepsin K (open diamond). b, top left panel, in vitro co-immunoprecipitation, using antiment antibodies, of purified chicken MENT (MENT<sub>Nat</sub>), recombinant wild-type MENT (MENT<sub>WT</sub>), mutant MENT (P14R), or control without MENT (Cont.) with (+) or without (−) sheep cathepsin L (Cat L). Immunoprecipitated protein was electrophoresed on a 13% SDS-PAGE gel and subjected to detection by Western blot with antibodies against cathepsin L. Top right panel, native gel electrophoresis of MENT<sub>WT</sub> (M) and MENT<sub>WT</sub> complexed with antithrombin RCL peptide (M+P), which migrates more slowly due to the negative charge of the acetylated peptide. Bottom left panel, complex of cathepsin V with MENT<sub>WT</sub>, visualized using 10% native acid PAGE electrophoresis. A constant amount of MENT<sub>WT</sub> was pre-incubated with increasing ratios of cathepsin V, indicated below the gel. Control lanes containing MENT alone (M) and cathepsin V alone (V) are shown. Bottom right panel, MENT<sub>WT</sub> alone (M), complex of MENT<sub>WT</sub> incubated with an equal amount of human cathepsin L (M+L), and cathepsin L alone (L). Cathepsin L alone runs slower under the acid gel system due to its low pI of −5.7, compared with MENT (9.2), cathepsin V (−8.6), or MENT-cathepsin L complex (−8.9). Position of complex is indicated. c, determination of molecular masses of peptides using MALDI-TOF. A mass spectral range of 0–20 kDa is shown for sheep cathepsin L alone, recombinant wild-type MENT alone, and the product of a reaction between cathepsin L and MENT (1:1 ratio). d, SDS-PAGE gel of the interaction between MENT<sub>WT</sub> and cathepsins L and V. Lanes shown are MENT<sub>WT</sub> alone (M), sheep cathepsin L alone (L), MENT incubated with a 0.5 molar ratio of cathepsin L for 10 min at room temperature (M+L), cathepsin V alone (V), MENT incubated with 0.5 molar ratio of cathepsin V (M+V). Positions of intact and cleaved inhibitor, and protease, are indicated.
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The inhibitory properties of MENT<sub>WT</sub> against several members of the papain-like cysteine proteinase superfamily, at pH 5.5, including (where applicable) k<sub>a</sub> values corrected for inhibitor turnover (SI)

| Proteinase     | SI | K<sub>a</sub> (mM) | k<sub>a</sub> (s<sup>-1</sup>) | SI-corrected |
|----------------|----|-------------------|-----------------------------|--------------|
| Ovine cathepsin L | 1.0| 9.8              | 2.2×10<sup>5</sup>         | 2.9×10<sup>4</sup> |
| Human cathepsin K | 1.3| 7.5              | 2.2×10<sup>5</sup>         | 2.9×10<sup>4</sup> |
| Papain         | 3.2| 160              | 2.3×10<sup>5</sup>         | 7.2×10<sup>4</sup> |
| Human cathepsin B | ND |                 |                            |              |

* Determined elsewhere (see "Materials and Methods").
* ND, not detectable.

All errors were below 5%.

To characterize the potential influence of MENT on the stability of Rb protein, CV-1 cells were transfected with expression vectors containing MENT and mutant MENT ORFs, transfected with control vector (lanes 6–20), or transfected with control vector (lanes 16–20). It was thus concluded that ectopic expression of MENT is sufficient to inhibit the nuclear cysteine proteinase activity that potentially plays a role in regulating the metabolism of the key proliferation control factor, Rb protein, in vivo.

The Inhibitory Reactive Center but Not the M-loop Domain of MENT Is Essential for a Proliferation Block—It was noted that during ectopic expression experiments all MENT<sub>WT</sub>-expressing cells ceased to proliferate and did not give rise to stable colonies producing even a low level of MENT. Normally, MENT is expressed in terminally differentiated blood cells, which may reflect a role in the repression of cell division during terminal differentiation. We therefore sought to determine which structural elements of MENT could be responsible for the cellular repression. Two unique, putatively important structural regions in this regard are MENT’s chromatin-binding domain (the M-loop (3)) and the RCL, shown above to be essential for cysteine protease inhibition and capable of influencing the level of cell cycle regulators such as Rb. We used the murine fibroblast NIH/3T3 cells, which lack SPase activity (Fig. 3a), for comparison with CV-1 cells. Cells transiently expressing wild-type and MENT variants (Fig. 1) were identified by cell staining with anti-MENT antibodies and subsequent immunofluorescent microscopy (Fig. 4). The proliferation rates were monitored by co-immunofluorescence with antibodies against MENT and BrdUrd, a marker of DNA replication (43), and quantitation of the double positive cells with Image-Pro Plus software (Fig. 5).

In transfected CV-1 cells, MENT<sub>WT</sub> showed a predominantly nuclear localization as revealed by immunofluorescence with anti-MENT antibodies and counterstaining with a DNA-specific dye, Hoechst 33258 (Fig. 4, panels 1 and 2). This is consistent with the presence of a putative NLS in the MENT M-loop (3). Nuclei of MENT<sub>WT</sub>-expressing CV-1 cells (arrows on panels 1 and 2) showed an altered chromatin organization with more prominent Hoechst-positive foci and diminished nuclear diameters indicating partial chromatin condensation. Similar results were obtained with NIH/3T3 cells (Fig. 4, arrows on panels 3 and 4). In contrast to cells transfected with vector alone, there was a very low rate of proliferation in either CV-1 or NIH/3T3 cells expressing MENT<sub>WT</sub> on the second day after transfection (Fig. 5A). Less than 3% of the MENT<sub>WT</sub>-positive CV-1 or NIH/3T3 cells survived a 30-day incubation in the culture media, and none gave rise to stable MENT<sub>WT</sub>-expressing colonies under neomycin selection.

To determine the role of the AT-hook (42), which lies within the M-loop, a variant of MENT lacking this motif was constructed by replacing the surrounding residues RPSRGRP with a similarly charged sequence, KGAKAKG (MENT<sub>ATHOOK</sub>).
This mutation significantly affected the association of MENT with nuclear chromatin, causing the localization of a substantial portion of the protein to the cytoplasm (Fig. 4, panel 5) but failed to rescue the block in proliferation (Fig. 5A). The mutant in which the M-loop was completely deleted, MENTMLOOP-, was more abundant in the cytoplasm than the nucleus in both CV-1 (Fig. 4, panel 6) and NIH/3T3 cells (Fig. 4, panel 8). This is consistent with the deletion of the NLS motif found in the MENT M-loop (3). Although the nuclear morphology of CV-1 cells expressing MENTMLOOP- (panel 7 on Fig. 4) did not show the signs of chromatin condensation observed with MENTWT (panel 2), the deletion of the M-loop did not rescue the proliferation block in either CV-1 or in NIH/3T3 cells (Fig. 5A).

It was thus concluded that the M-loop was not essential for the repressive effect imposed by MENT. Because the MENTMLOOP-mutant retains full cathepsin inhibitory activity (Table I), we set out to determine whether this inhibitory activity could be linked to the effect on chromatin structure and cell proliferation.

The RCL mutations in MENTP14R and MENTOV were demonstrated above to virtually abolish the ability of MENT to inhibit cathepsin proteinases in vitro (Table I). Upon expression of MENTP14R or MENTOV in CV-1 cells, the mutants demonstrated a prominent nuclear localization (Fig. 4, panels 11–13). However, in contrast with cells expressing either MENTWT or MENTMLOOP-, there was a dramatic increase in the rate of BrdUrd incorporation among CV-1 and NIH/3T3 cells expressing MENTP14R or MENTOV (Fig. 5A). A slightly (but significantly) lower BrdUrd incorporation in the presence of MENTP14R than of MENTOV was most probably due to residual inhibitory activity of the former. More than 10% of NIH/3T3 cells expressing MENTP14R or MENTOV survived a 30-day incubation in cell culture, and using neomycin selection, stable colonies expressing the mutant MENT could be obtained. These experiments demonstrated that mutations in the RCL were able to rescue the MENT-imposed proliferation block without preventing intranuclear transport or inhibiting the binding of MENT to chromatin or DNA. Because the very same mutations have been shown to compromise the ability of MENT to inhibit cysteine proteinases in vitro and SPase in the CV-1 nuclear extracts (see above), these results for the first time link a proliferation block imposed by a nuclear serpin to its cysteine proteinase inhibitory activity.

To test if papain-like cysteine proteinase inhibition could induce the proliferative block independently of MENT, MENT-transfected cells were incubated with 50 μg/ml cell-penetrating inhibitor, E-64d, by adding the inhibitor to the media. At this concentration, E-64d has been shown to block the intranuclear degradation of Rb by SPase (13). E-64d did not significantly inhibit BrdUrd incorporation in control CV-1 and NIH/3T3 cells (Fig. 5A), thus showing that inhibitory activity per se is not sufficient to block proliferation.

RCL Mutations and E-64d Affect Intranuclear Localization of MENT, Evidence for Its Interaction with a Papain Protease-like Reactive Center in Vivo—NIH/3T3 have a well defined focal distribution of nuclear heterochromatin that can be easily visualized using Hoechst 33258, a fluorescent dye that preferentially stains AT-rich DNA in murine pericentromeric heterochromatin (44). By staining with anti-MENT antibodies, we observed that in NIH/3T3 cells expressing a low level of MENT,
the protein had a focal distribution in the nucleus co-localizing with heterochromatin (arrowheads on Fig. 4, panels 3 and 4). In moderate-to-high MENT-expressing cells the protein was distributed throughout total nuclear chromatin (arrows on Fig. 4, panels 3 and 4; see also Fig. 5B).

A dramatically different pattern of immunostaining was observed in cells expressing MENTP14R and MENTOV. In these cells, the protein accumulated in a number of foci confined exclusively to the Hoechst-positive pericentromeric heterochromatin (Fig. 4, panels 9, 10, and 14), even at high levels of expression (Fig. 5B). This result shows that a single amino acid mutation abolishing cysteine proteinase inhibition leads to a large scale rearrangement of the distribution of MENT inside nuclear chromatin. We thus concluded that the organization of nuclear chromatin, the distribution of MENT, and repression of proliferation can all be affected by amino acid substitutions in the RCL of MENT that affect cysteine proteinase inhibition.

To investigate whether an exogenous cysteine proteinase inhibitor could substitute for the loss of the inhibitory activity, cells expressing MENTP14R were incubated in the presence of E-64d. This treatment, however, restored neither the anti-proliferative effect nor the chromatin localization of MENTP14R (Fig. 4, panel 15).

Cells expressing MENTWT were incubated in the presence of E-64d to test whether a cysteine proteinase is involved in the MENT-chromatin interaction. Remarkably, after a 24-h treatment with E-64d, the majority of MENTWT was relocalized to the heterochromatic regions, especially noticeable in cells expressing high levels of MENT (Fig. 4, panel 16; Fig. 5B), making the protein distribution similar to that of MENTP14R and MENTOV (Fig. 4, panels 9, 10 and 14; Fig. 5B). The E-64d-treated nuclei also had a normal morphology and shape clearly distinct from untreated, MENTWT-expressing cells (Fig. 4, panel 3). The E64d-treated cells, however, showed a stronger background staining of euchromatic regions than with MENTP14R and MENTOV (panels 9 and 14). This residual staining probably reflects a less efficient disruption of the interaction between MENTWT and its euchromatic target by E-64d, which relies on competition between the molecules for a binding site.

**DISCUSSION**

The only heterochromatin protein identified to date that can bring about large scale chromatin condensation both in vitro and in vivo is an abundant, developmentally regulated nuclear protein, MENT, which is also a member of the serpin superfamily.

In this paper we demonstrate that MENT is an efficient inhibitor of cathepsins K, L, and V. Along with SCCA-1 (29), it therefore represents only the second serpin shown to be capable of effective inhibitory activity against cathepsin proteinases. It is worthwhile noting that both proteins arise from the same branch of the superfamily and that both proteins are predominantly intracellular, suggesting that there may be a growing subclass of serpins that, along with cystatins, perform a significant role as physiological intracellular modulators of cathepsin activity. The results presented here also have implications for the nature of the serpin-cysteine protease interaction. The MENT66rd data (as well as that from MENTP14R and a corresponding SCCA-1 mutant (5)) support the hypothesis that the conformational changes required to inhibit both serine and cysteine proteinases are similar (45).

Given the specific nature of the interaction of MENT with certain papain-like cysteine proteinases, and given its predominantly nuclear localization, it was reasoned that MENT may be involved in regulating the activity of an intracellular, nuclear cysteine proteinase in vivo, in particular one similar to the recently identified enzyme SPase. SPase has been shown previously (39) to regulate levels of the cell cycle control protein Rb, which would therefore be expected to be modulated by the presence of a cysteine proteinase inhibitor. It was found that MENT, expressed in CV-1 cells or added exogenously to a nuclear extract, was capable of inhibiting SPase and preventing Rb degradation. Thus, in addition to inhibitory activity seen in vitro, MENT is capable of acting as a proteinase inhibitor in vivo and is able to inhibit an intracellular proteinase with the characteristics of the cysteine proteinase SPase.

Wild-type MENT was capable of exerting a strong proliferation block in CV-1 and NIH/3T3 cells. We do not believe that this effect is due to the inhibition of SPase; however, MENT was found to block the proliferation of CV-1 cells, which contain a relatively high level of SPase, as well as NIH/3T3 cells, which lack nuclear SPase (13). Furthermore, it was found that treating cells with E-64d, an inhibitor of papain-like cysteine proteinases, in the absence of MENT, was incapable of inducing a proliferation block. E-64d has been shown previously (13) to prevent the degradation of signaling molecules and transcription factors, including the degradation of Rb by SPase, and this
The presence of E-64d, the cysteine proteinase is inhibited; excess MENTWT of MENT in euchromatin apparently causes its condensation. This cooperativity may be induced by the MENT-proteinase complex, although its mechanism is presently unknown. Accumulation chromatin. This cooperativity may be induced by the MENT-proteinase and also accumulates in heterochromatin, rather than in euchromatin.

We were intrigued by the possibility that a nuclear cysteine proteinase may be involved in targeting MENT to specific regions of the nucleus. As described previously, in avian erythrocytes expressing low levels of MENT, the protein has a focal distribution in the nucleus (27), whereas at a high expression level, MENT is distributed throughout total nuclear chromatin (1). In NIH/3T3 cells we have also observed an expression level-dependent variation in nuclear localization of ectopically expressed MENTWT (Fig. 4, panel 3; Fig. 5B) but a dramatically different pattern of immunostaining when we expressed the non-inhibitory variant MENTP14R (Fig. 4, panels 9 and 10). In particular, this variant localized strongly to pericentromeric heterochromatin foci and failed to localize to euchromatin, even in cells expressing a high level of MENT (Fig. 5B). Similar results were obtained for the MENTα variant. Furthermore, it was found that treatment with E-64d caused the distribution of MENTWT to resemble more closely that of MENTP14R (Fig. 4, panel 16; Fig. 5B). E-64d is known to inhibit specifically papain-like cysteine proteinases by forming a covalent complex with the reactive center thiol (46) and blocking the S-S subsites (47). It is therefore likely that the treatment with E-64d counteracts the repressive effect of MENT on nuclear chromatin by blocking the active site of a MENT-interacting factor. The specificity of E-64d as well as the requirement for an inhibitory reactive center loop in MENT strongly suggest that one or more cysteine proteinases ubiquitously expressed in different cell types are directly involved in the association of MENT with chromatin in the nucleus, and may thus mediate or regulate the repression of cell proliferation caused by MENT.

These data suggest a scenario in which an active turnover of MENT in complex with a proteinase partner is competed for by the E-64d inhibitor. We therefore propose that the ability of MENT to associate with and repress euchromatin is mediated by its ability to interact and form a complex with a nuclear cysteine proteinase. We suggest that the association of MENT with AT-rich DNA in heterochromatin, in contrast, is mediated by the M-loop and AT-hook and thus does not require an active RCL. This would explain why mutations that impair MENT-proteinase interactions simultaneously push the protein to heterochromatin and prevent the repression of proliferation. A scheme for the proposed targeting of MENT by cysteine proteinase that accounts for the effects of E-64d and the RCL mutations is shown in Fig. 6. We suggest that the MENT-proteinase complex may in some way act as a “seed” for the cooperative binding of additional MENT molecules. The recruited MENT molecules would, in turn, form internucleosome bridges connecting chromatin fibers (2) in a conformation-dependent or concentration-dependent fashion. Such a mechanism would lead to the progressive condensation of neighboring euchromatin (Fig. 6). The nature of MENT conformational transitions triggered by the putative protease and the mechanism of its propagation remain to be characterized.

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