

# HAMLET Interacts with Histones and Chromatin in Tumor Cell Nuclei\*

Received for publication, June 18, 2003, and in revised form, July 25, 2003  
Published, JBC Papers in Press, July 29, 2003, DOI 10.1074/jbc.M306462200

Caroline Düringer‡, Ali Hamiche§¶, Lotta Gustafsson‡, Hiroshi Kimura||, and Catharina Svanborg‡\*\*

From the ‡Institute of Laboratory Medicine, Section for Microbiology, Immunology and Glycobiology, Lund University, Sölvegatan 23, 223 62 Lund, Sweden, §Equipe Fonction et Dynamique de la Chromatine, CNRS UPR 9079, Institut André Lwoff, Bâtiment O, Premier Etage, 7 rue Guy Moquet, 94800 Villejuif, France, and ||Department of Functional Genomics, Medical Research Institute, Tokyo Medical and Dental University, Yushima 1-5-45, Bunkyo-ku, Tokyo 113-8510, Japan

**HAMLET is a folding variant of human  $\alpha$ -lactalbumin in an active complex with oleic acid. HAMLET selectively enters tumor cells, accumulates in their nuclei and induces apoptosis-like cell death. This study examined the interactions of HAMLET with nuclear constituents and identified histones as targets. HAMLET was found to bind histone H3 strongly and to lesser extent histones H4 and H2B. The specificity of these interactions was confirmed using BLAcore technology and chromatin assembly assays. *In vivo* in tumor cells, HAMLET co-localized with histones and perturbed the chromatin structure; HAMLET was found associated with chromatin in an insoluble nuclear fraction resistant to salt extraction. *In vitro*, HAMLET bound strongly to histones and impaired their deposition on DNA. We conclude that HAMLET interacts with histones and chromatin in tumor cell nuclei and propose that this interaction locks the cells into the death pathway by irreversibly disrupting chromatin organization.**

HAMLET (human  $\alpha$ -lactalbumin made lethal to tumor cells)<sup>1</sup> is a protein-folding variant of  $\alpha$ -lactalbumin with remarkable properties in cellular assays. It forms a molecular complex with oleic acid that induces cell death with selectivity for tumor cells and undifferentiated cells. The apoptotic activity of this complex was discovered by serendipity in a fraction of human milk casein (1), and the structural basis of this novel activity was studied by a combination of spectroscopic techniques and biological assays (2, 3). HAMLET contains partially unfolded  $\alpha$ -lactalbumin with native-like secondary structure but lacking specific tertiary packing of the side chains. Oleic

acid binds to the unfolded protein with a stereo-specific fit, and the hinge region between the  $\alpha$ -helical and the  $\beta$ -sheet domains has been proposed as the fatty acid binding site.<sup>2</sup> The link between apoptosis induction and the folding change was proven by deliberate conversion of native  $\alpha$ -lactalbumin to the apoptosis-inducing form in the presence of oleic acid (3). HAMLET is thus defined as the biologically active conversion product of  $\alpha$ -lactalbumin and oleic acid.

HAMLET triggers cell death in many different tumor cell lines, with morphological features resembling apoptosis. The dying cells show nuclear condensation, cell shrinkage, cytoplasmic blebbing, and DNA fragmentation. Healthy, differentiated cells, in contrast, survive HAMLET challenge and show no apoptotic changes (1). This difference in sensitivity implies that HAMLET reaches unique targets in tumor cells, but not in healthy, differentiated cells, and that cell death programs are activated and executed as a result of these interactions.

One striking feature of HAMLET is the ability to move through the cytoplasm of tumor cells to the nuclei, where HAMLET remains and accumulates. This unusual trafficking behavior was first observed in early studies with the active human milk fraction (2, 4). The nuclear accumulation occurred in the majority of dying tumor cells but not in the healthy cells that remained viable in the presence of HAMLET, showing that the interaction with the nuclear compartment is an important aspect of the tumor cell response. In addition, the nuclear accumulation appeared to be irreversible, suggesting that nuclear target molecules were able to bind and retain the active complex in the nucleus.

The present study identified nuclear target molecules for HAMLET in cancer cells. We present evidence that HAMLET interacts with specific histone proteins and chromatin. The chromatin interaction may indeed mark the irreversible phase of cell death.

## EXPERIMENTAL PROCEDURES

**Purification of  $\alpha$ -Lactalbumin and Conversion to HAMLET**—HAMLET is a folding variant of human  $\alpha$ -lactalbumin stabilized by a C18:1 fatty acid cofactor. In this study, native  $\alpha$ -lactalbumin was purified from human milk and converted to HAMLET on an oleic acid conditioned ion exchange matrix as previously described (3).

**Protein Labeling**—HAMLET was labeled with <sup>125</sup>I (ICN Biomedicals, Irvine, CA) using the lactoperoxidase method as described previously (4). HAMLET was labeled with AlexaFluor 568 according to the manufacturer's instructions (Molecular Probes Inc., Eugene, OR).

**Cell Culture**—A549 (ATCC, CLL 185), Jurkat (European Cell Culture Collection, no. 88042803), and primary human renal tubular epithelial cells were cultured as described (1). HeLa cells were grown in

\* This study was supported by grants from the Lund Family American Cancer Society (SPG-97-157), the Swedish Cancer Society (3807-B01-06XCC), the Swedish Pediatric Cancer Society, the Medical Faculty, Lund University, The Segerfalk Foundation, The Anna-Lisa and Sven-Eric Lundgren Foundation, The Knut and Alice Wallenberg Foundation, and The John and Augusta Persson Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Recipient of grants from the Association de Recherche contre le Cancer, the Ligue Nationale Contre le Cancer, and the French Ministry as an Action Concertée Incitative.

\*\* To whom correspondence should be addressed. Tel.: 46-46-173972; Fax: 46-46-137468; E-mail: Catharina.Svanborg@mig.lu.se.

<sup>1</sup> The abbreviations used are: HAMLET, human  $\alpha$ -lactalbumin made lethal to tumor cells; Alexa-HAMLET, AlexaFluor 568-labeled HAMLET; GFP, green fluorescent protein; <sup>125</sup>I-HAMLET, <sup>125</sup>I-labeled HAMLET; MNase, micrococcal nuclease; MALDI TOF, matrix-assisted laser desorption/ionization time-of-flight; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; NAP-1, nucleosome assembly protein 1.

<sup>2</sup> M. Svensson, A.-K. Mossberg, J. Pettersson, S. Linse, and C. Svanborg, manuscript submitted.

Dulbecco's modified Eagle's medium with glutamax supplemented with penicillin (100 units/ml)-streptomycin (100  $\mu$ g/ml), sodium pyruvate (1 mM) (Invitrogen), 10% fetal calf serum, and for cells expressing green fluorescent protein (GFP)-tagged histones, 2  $\mu$ g/ml blasticidin S (Invitrogen).

**Subcellular Localization of HAMLET**—For subcellular localization by real-time confocal microscopy, A549 or human renal tubular epithelial cells were incubated with AlexaFluor 568-labeled HAMLET (Alexa-HAMLET, 0.1 mg/ml) under cell culture conditions described above and analyzed in a Bio-Rad 1024 laser scanning confocal equipment attached to a Nikon Eclipse 800 microscope (Nikon) with a 60 $\times$  objective (NA 1.40). For *in vivo* co-localization of HAMLET and histones, HeLa cells expressing GFP-tagged histone H3 or H2B (5) were exposed to Alexa-HAMLET for 24 h (H3) or 3 h (H2B) and analyzed by confocal microscopy. For localization of  $^{125}$ I-labeled HAMLET ( $^{125}$ I-HAMLET), HeLa cells were incubated with 0.4 mg/ml unlabeled HAMLET and  $\sim 1.5 \times 10^5$  cpm  $^{125}$ I-HAMLET/ml for 1, 3, or 6 h. The cells were subsequently fractionated into a cytoplasmic and a nuclear fraction. The activity of the fractions was measured using a gamma counter (1282 Compu-gamma, LKB Wallac).

**Cellular and Nuclear Fractionation**—Nuclei from HeLa cells were purified according to *Current Protocols in Molecular Biology* (6). The supernatant remaining after collection of the nuclei was used as the cytoplasmic fraction.

Nuclei were subfractionated by treatment with 1) 0.3 M KCl (6), 2) 0.1 mg/ml RNase I (Sigma) in the presence of 0.25 M KCl for 30 min at 37  $^{\circ}$ C, or 3) 0.5 units of microcococcus nuclease (MNase) to 8 optical units ( $A_{260}$ ) of nuclei in the presence of 0.25 M KCl for 5 min at 37  $^{\circ}$ C. Following treatment, soluble and insoluble fractions were separated by centrifugation at 16,000  $\times$  g for 10 min at 4  $^{\circ}$ C.

To prepare whole nuclear extracts from Jurkat and A549, cells were harvested, washed twice in 1/15 M phosphate-buffered saline, pH 7.2 (PBS), and suspended in homogenization buffer (10 mM Tris-HCl, 5 mM MgCl<sub>2</sub>, and 2 mM CaCl<sub>2</sub> for Jurkat cells and 5 mM EDTA, pH 8, for A549 cells, with 10  $\mu$ g/ml leupeptin, 20  $\mu$ g/ml antipain, and phenylmethyl-sulfonyl fluoride) on ice for 15 min. The cells were homogenized (Dounce homogenizer, pestle size 411), and sucrose was added to a final concentration of 0.25 M. Nuclei were collected by centrifugation at 1000  $\times$  g for 10 min, digested with MNase (7), harvested by centrifugation at 1000  $\times$  g, and lysed in 1 mM EDTA. Protein concentrations were measured with a Bio-Rad DC protein assay kit.

**Gel Electrophoresis**—The histones and nuclear extracts were separated on Tris-glycine polyacrylamide gels (15 or 16%) on a Novex Nu-Page Mini Cell II (Novex, San Diego, CA). Protein bands were visualized with Coomassie Blue or silver staining (8). Chromatin samples were electrophoresed at room temperature in 4% polyacrylamide (acrylamide:bisacrylamide, 29:1 (w/w)) in TE buffer (10 mM Tris-HCl (pH 7.5) and 1 mM EDTA) (9). The chromatin was stained with SYBR green (Molecular Probes) and detected with a FluorImager (Amersham Biosciences).

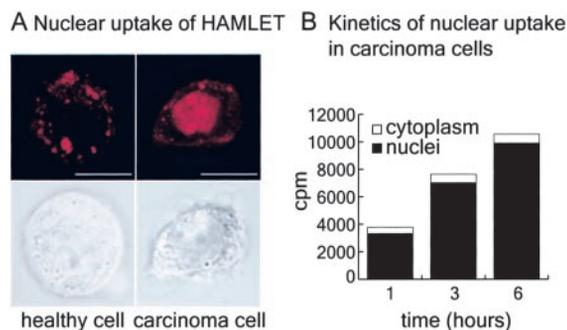
**Overlay**—Nuclear extracts or commercial histones were separated by PAGE and blotted to a polyvinylidene difluoride (PVDF) membrane. After blocking with solutions Sat 1 (ethanolamine 6.1 g/liter, glycine 9 g/liter, polyvinylpyrrolidone 10 g/liter, methanol 25%) and Sat 2 (ethanolamine 6.1 g/liter, glycine 9 g/liter, Tween 20 1.25 g/liter, gelatina hydrolysate 5 g/liter, methanol 25%) for 15 min each, the membrane was washed three times for 15 min with PBS-T (PBS and 0.05% Tween 20), incubated with  $^{125}$ I-HAMLET in PBS overnight, washed in PBS-T six times for 15 min, and dried. Bound HAMLET was detected using a STORM 840 PhosphorImager (Amersham Biosciences).

**Protein Sequencing and Identification**—Nuclear extracts were blotted to PVDF membranes and stained with Coomassie Blue, and bands to be sequenced were excised and subjected to N-terminal amino acid sequencing by Edman degradation in an Applied Biosystems model 477 A peptide sequencer. Sequences were compared with the Swiss Protein Database with PatScan software ([www.unix.mcs.anl.gov/compbio/PatScan/HTML/patscan.html](http://www.unix.mcs.anl.gov/compbio/PatScan/HTML/patscan.html)).

**Mass Spectrometry**—The 12-, 14-, 16-, and 17-kDa bands from the nuclear extract were excised from the gel and prepared for mass spectrometry with a Bruker Scout 384 Reflex III matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (10).

**Histones**—Individually purified bovine histones H1, H2A, H2B, H3, and H4 were purchased from Roche Diagnostics (Bromma, Sweden). Native, folded histones were obtained from duck erythrocyte nuclei (11). *Drosophila melanogaster* histones were expressed in *Escherichia coli*, purified, and assembled into octamers (12).

**Affinity Chromatography**—HAMLET was immobilized on CNBr-acti-



**FIG. 1. HAMLET accumulates in tumor cell nuclei.** *A*, the intracellular trafficking of HAMLET was examined in A549 lung carcinoma cells, which are sensitive to HAMLET, and a primary culture of human kidney cells (*healthy cell*), which are resistant to HAMLET treatment (1). Real-time confocal microscopy images (*upper panels*) showed differences in nuclear localization between kidney cells and carcinoma cells after 24 h of exposure to Alexa-HAMLET (red). The *lower panel* shows light transmission images of the same cells. Bars represent 10  $\mu$ m. *B*, kinetics of nuclear accumulation in HeLa cells treated with  $^{125}$ I-HAMLET. Nuclear and cytosolic fractions were prepared, and the radioactivity associated with each fraction was measured.

tivated Sepharose 4B<sup>TM</sup> (Amersham Biosciences) according to manufacturer's instructions. The gel was mixed with core histones in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.2 M NaCl for 2 h at room temperature and washed, and bound material was eluted by boiling in SDS loading buffer. The eluted material was analyzed by PAGE.

**Surface Plasmon Resonance**—Biotinylated HAMLET was immobilized on a SA sensor chip in a BIAcore X facility (BIAcore AB, Uppsala, Sweden). Histone octamers were applied in serial dilutions. A flow rate of 5  $\mu$ l/min was used during the immobilization and 20  $\mu$ l/min during the analysis. PBS with 0.05% Tween 20 and 1 M NaCl was used as the flow buffer. The surface was regenerated with 10 or 20 mM HCl. The data were analyzed with the BIAevaluation 2.2 software.

**Precipitation of Histones with HAMLET**—Total core histones (native from cells, 2.5  $\mu$ g) were mixed with HAMLET (2.5  $\mu$ g) in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.2 M NaCl, and 2 mM CaCl<sub>2</sub> at room temperature for 5 min, and the precipitate was collected by centrifugation at 7000  $\times$  g for 2 min and analyzed by 15% Laemmli SDS-PAGE and silver staining.

**DNA**—A 256-bp fragment containing a sea urchin 5 S RNA gene (13) was gel-purified from an *Eco*RI or *Nci*I digest of plasmid pLV405-10 (14).

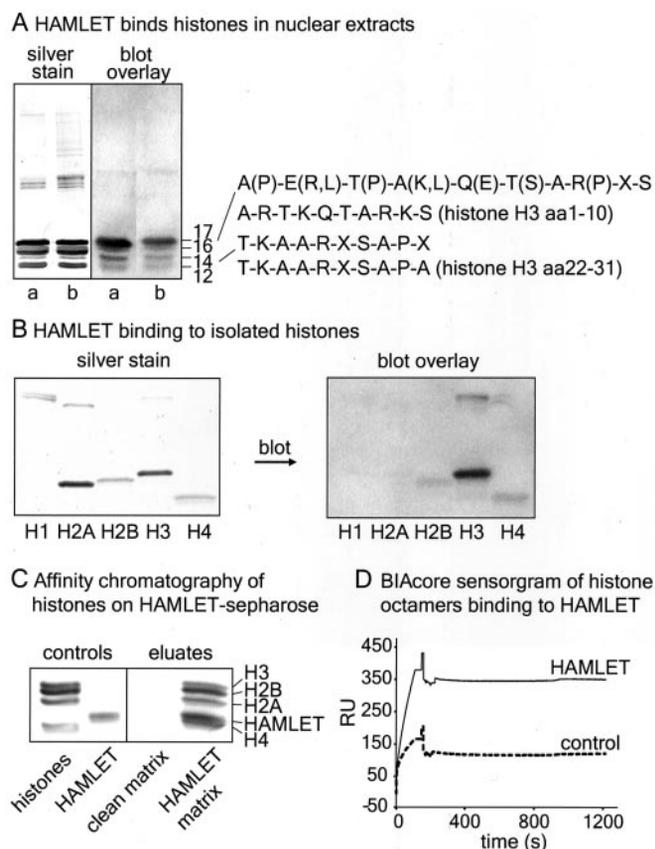
## RESULTS

**HAMLET Targets Tumor Cell Nuclei**—The accumulation of HAMLET in tumor cell nuclei was demonstrated by real-time confocal microscopy (Fig. 1A). Alexa-HAMLET was shown to translocate into the cytoplasm of the tumor cells and to accumulate in their nuclei. In healthy cells, HAMLET entered the cytoplasm but did not travel further, and no nuclear accumulation was detected (Fig. 1A).

The nuclear accumulation of HAMLET was quantified using radiolabeled protein. Nuclear fractions were obtained from  $^{125}$ I-HAMLET-treated carcinoma cells after 1, 3, or 6 h of incubation. About 90% of the cell-associated radioactivity was in the nuclear fraction after 1 h, with a little further increase at the later time points, and 0.2–0.4% of the total added radioactivity was incorporated into the cells (Fig. 1B). After 6 h, cell viability had decreased to 67%.

**HAMLET Binds to Specific Histones in Nuclear Extracts**—Molecular targets for HAMLET were identified in nuclear extracts separated by SDS-PAGE and overlaid with  $^{125}$ I-HAMLET. Experiments were performed in parallel in one human lymphoma (Jurkat) and one carcinoma (A549) cell line, and similar results were obtained using nuclear extracts from both cell lines. HAMLET was shown to recognize four distinct bands of  $\sim$ 12, 14, 16, and 17 kDa molecular mass (Fig. 2A).

The 17-, 16-, 14-, and 12-kDa bands were identified as histones H3, H2B, H3, and H4, respectively, by MALDI-TOF. The

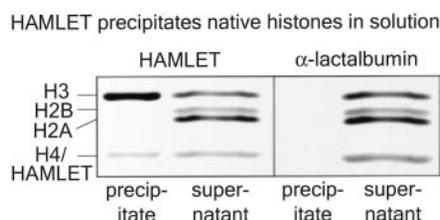


**FIG. 2. HAMLET binds histones in nuclear extracts and interacts with purified histones.** *A*, nuclear extracts from Jurkat (lanes *a*) or A549 cells (lanes *b*) were run on polyacrylamide-SDS gels, blotted to PVDF membranes, and exposed to  $^{125}\text{I}$ -HAMLET. The four bands interacting with HAMLET were identified as histones by N-terminal amino acid sequencing and MALDI-TOF. N-terminal sequences of the 17- and 14-kDa bands are shown (alternative amino acids in parentheses) with the known histone sequences as controls. *B*, binding of  $^{125}\text{I}$ -HAMLET to purified bovine histones H1, H2A, H2B, H3, and H4 after SDS-PAGE and blotting to a PVDF membrane. A parallel gel was silver-stained. *C*, affinity chromatography of histones on HAMLET-Sepharose in comparison with a clean matrix control. Bound proteins were eluted by boiling in SDS. *D*, binding of histone octamers to biotinylated HAMLET coupled to a BIAcore streptavidin sensor chip. Native core histones octamers (100  $\mu\text{g}/\text{ml}$ ) were flowed over the chip, and the binding was measured in resonance units (RU) (solid line) and compared with an uncoated surface (dashed line).

17-kDa band showed N-terminal homology to histone H3, and the identity was verified by immunoblot using monoclonal anti-H3 antibodies (not shown). The 14-kDa band showed sequence homology with H3 but lacked the first 21 amino acids of the N-terminal tail (Fig. 2A). This form of H3 corresponds to a fragment obtained after proteolytic degradation, and as a consequence, the band was not recognized by the anti-H3 antibody, which is directed to the tail region (not shown). HAMLET, in contrast, bound to this form of H3, suggesting that the interaction is independent of the histone tail.

**HAMLET Interacts with Purified Histones**—These interactions were further examined using purified histone proteins. As a first step, purified bovine histones H1, H2A, H2B, H3, and H4 were separated by SDS-PAGE and blotted onto PVDF membranes, and the blots were overlaid with radiolabeled HAMLET (Fig. 2B). High affinity binding to H3 and weak binding to H4 and H2B were observed. HAMLET did not bind to bovine H1 or H2A in the overlay assay.

The histone specificity of HAMLET was examined further using natively folded histones in affinity chromatography. A mixture of core histones (H2A, H2B, H3, and H4) purified from



**FIG. 3. HAMLET precipitates core histones from solution.** Native core histones were mixed with HAMLET or  $\alpha$ -lactalbumin. The precipitates and supernatants were subjected to SDS-PAGE and silver staining.

duck erythrocyte nuclei was allowed to interact with HAMLET immobilized on CNBr-activated Sepharose. Proteins eluted with SDS loading buffer were identified by SDS-PAGE. The four histones were retained on the column in approximately equal amounts, but there was no binding to the clean Sepharose matrix (Fig. 2C).

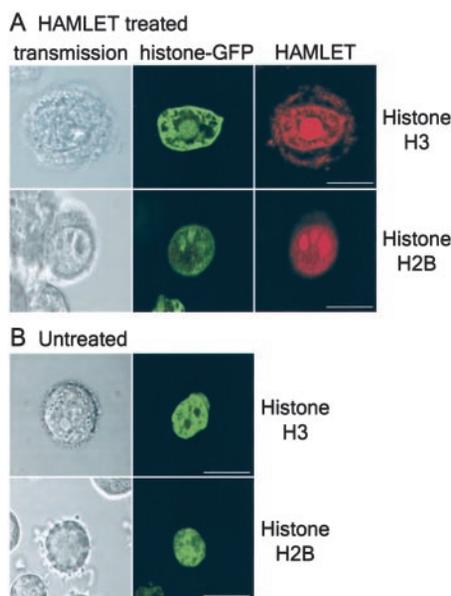
The affinity of HAMLET for isolated histones was studied by surface plasmon resonance using HAMLET-coated BIAcore sensor chips and bovine or natively folded duck histone preparations. Bovine histone H3 showed very rapid binding kinetics and remained bound with no evidence of dissociation during the experimental period, suggesting virtually irreversible binding to HAMLET (data not shown). H3 could not be forcibly eluted from the chip when detergents, salt, or acid were used. The native core histones bound rapidly to the HAMLET coated surface (Fig. 2D), and the dissociation was very slow.

**HAMLET Precipitates Histones from Solution**—In preparation for studies of nucleosomes and chromatin, native core histones (H2A, H2B, H3, and H4) were mixed with HAMLET in solution. To our surprise, the solution immediately turned opalescent, and with time a white precipitate accumulated at the bottom of the test tube. The precipitate was analyzed by SDS-PAGE and was shown to contain mainly histones H3 and H4 and minor amounts of H2A and H2B (Fig. 3). HAMLET was also present in the precipitates (not shown). Native  $\alpha$ -lactalbumin was used as a control and did not form precipitates with the histones (Fig. 3); the histones did not precipitate in the absence of HAMLET (not shown).

**HAMLET Co-localizes with Histones H2B and H3 in Vivo**—The interaction of HAMLET with histones was further examined in the nuclei of intact, living cells. Stably transfected HeLa cell lines expressing GFP-H3 or GFP-H2B were exposed to Alexa-HAMLET. By real-time confocal microscopy (Fig. 4A), HAMLET was shown to co-localize with both histones in the HeLa cell nuclei.

In addition, the global chromatin structure was perturbed by HAMLET treatment. In HAMLET-treated cells, the chromatin was condensed to the nuclear periphery, and new, spherical structures appeared. Both HAMLET and the histones were present in those structures. Control cells showed normal chromatin distribution (Fig. 4B).

**HAMLET Is Not a Chromatin Assembly Protein**—The results suggested that HAMLET may interact directly with soluble histones and chromatin in tumor cells. HAMLET was compared with the chromatin assembly protein nucleosome assembly protein 1 (NAP-1), which binds histones and delivers them to DNA, thereby enhancing nucleosome formation. NAP-1 was mixed with histones, DNA fragments were added to the mixture, and the assembled nucleosomes were detected by native PAGE (Fig. 5A). The pure histone-DNA mixture formed unspecific aggregates, but after the addition of NAP-1, a concentration-dependent nucleosome assembly was observed. Depending on the position of the histone octamer on the DNA fragment, two mononucleosome species were formed (Fig. 5A, bands  $N_1$  and  $N_2$ ). The same assay



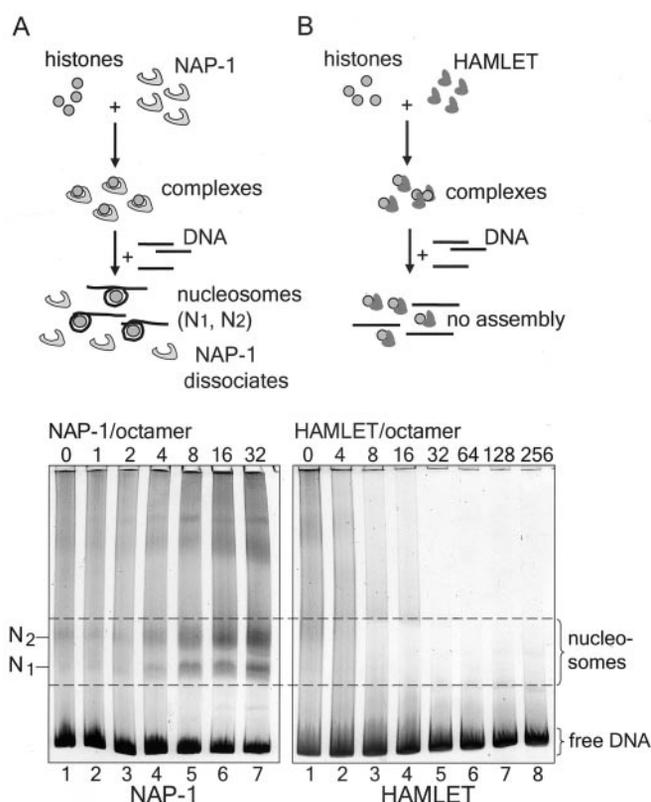
**FIG. 4. HAMLET co-localizes with histones in tumor cell nuclei.** HeLa cells expressing GFP-tagged histones H3 or H2B were treated with Alexa-HAMLET and fluorochromes were localized by confocal microscopy. GFP-histones are shown in *green*, HAMLET in *red*, and light transmission images in *gray*. Bars indicate 10  $\mu\text{m}$ . *A*, HAMLET co-localizes with histones in HAMLET-treated cells and perturbs chromatin structure. *B*, histone staining and chromatin structure of untreated cells.

system was used to test how HAMLET affected the assembly of nucleosomes (Fig. 5B). No nucleosome assembly was detected in the presence of HAMLET. In contrast, HAMLET prevented the histones from binding to the DNA.

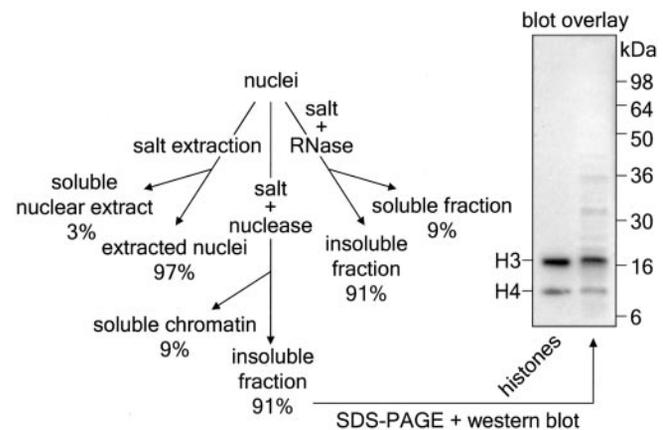
**HAMLET Forms an Insoluble, Histone-containing Complex in Tumor Cell Nuclei**—Nuclei were purified from  $^{125}\text{I}$ -HAMLET-treated HeLa cells, and HAMLET-containing nuclear fractions were purified (Fig. 6). After measurements of total nucleus-associated radioactivity, nuclei were extracted with 0.3 M KCl to release soluble nuclear proteins. 97% of the radioactivity remained in the insoluble nuclear fraction. The nuclei were further solubilized by treatment with RNase I and 0.25 M NaCl. Labeled HAMLET (91%) remained in the insoluble fraction. Finally, the chromatin was solubilized by MNase cleavage in the presence of 0.25 M KCl. This treatment released chromatin from the nuclei (not shown), but the majority of HAMLET (91%) and some chromatin (not shown) remained in the insoluble fraction. This fraction was forced into solution at 95  $^{\circ}\text{C}$  in SDS loading buffer and analyzed by SDS-PAGE. Molecular species interacting with HAMLET were identified by blotting with the radiolabeled protein. HAMLET recognized histones H3 and H4 on the blot (Fig. 6).

#### DISCUSSION

HAMLET causes apoptosis-like death of tumor cells and accumulates in their nuclei. In this study, histones and chromatin were found to be the nuclear target molecules involved in this process. HAMLET was shown to bind a discrete set of proteins in nuclear extracts, which were identified as histones H3, H4, and H2B. The histone specificity of HAMLET was confirmed using isolated histone proteins, and both denatured and natively folded histones were found to bind HAMLET. High affinity interactions were detected by BIAcore methodology, and surprisingly, HAMLET was found to form macroscopically visible precipitates with histones in solution. The *in vivo* correlate of the HAMLET-histone interactions was studied by real-time confocal microscopy. Using H3 and H2A GFP-reporter constructs, fluorochrome-labeled HAMLET was shown



**FIG. 5. HAMLET is not a chromatin assembly protein.** Core histones were incubated with NAP-1 or HAMLET followed by the addition of DNA fragments, and the products were analyzed by PAGE. The histone-DNA mixture formed unspecific aggregates (*lanes 1* in *A* and *B*). The addition of NAP-1 (*A*, *lanes 2–7*) caused a concentration-dependent nucleosome assembly (bands  $N_1$  and  $N_2$ ). Nucleosomes were not formed after the addition of HAMLET (*B*, *lanes 2–8*).



**FIG. 6. HAMLET-histone interactions *in vivo*.** Nuclei from  $^{125}\text{I}$ -HAMLET-treated HeLa cells (6 h) were either subfractionated by salt extraction or solubilized with RNase I or MNase in the presence of salt to release soluble proteins. Soluble and insoluble fractions were separated by centrifugation, and the radioactivity associated with each fraction was measured in percent of total nucleus-associated radioactivity. The insoluble material remaining after MNase cleavage contained most of the cell-associated HAMLET and was solubilized and subjected to a blot overlay assay with  $^{125}\text{I}$ -HAMLET. Core histones were used as a control.

to co-localize with histones in tumor cell nuclei and to perturb the global chromatin structure. HAMLET-treated cells showed condensation of the chromatin, a feature that normally is associated with apoptosis (15). Spherical structures were formed within the nuclei, and both HAMLET and histones were present in those structures. The effect on chromatin was confirmed

*in vitro*, and HAMLET was found to differ from other histone-binding proteins like NAP-1, in that it blocked rather than promoted assembly of chromatin. Finally, HAMLET and histones were identified in an insoluble nuclear fraction isolated from dying cancer cells. The results demonstrate that core histones, and especially H3, are targets for HAMLET in tumor cell nuclei, suggesting that the affinity for histones perturbs the chromatin structure in tumor cells. In addition, HAMLET binding to free histones may influence their function and transport to the nuclear compartment. By these mechanisms, HAMLET may force the tumor cells into the irreversible phase of cell death.

HAMLET interacted with histones in a dose-dependent manner but differed from other known histone-binding proteins. The nucleosome assembly protein, NAP-1 (16), and other histone-binding proteins act as chaperones during chromatin assembly and remodeling (17). Their binding to histones is reversible, allowing them to deliver the histones from the site of synthesis in the cytoplasm to the nucleus. This effect of NAP-1 was reproduced in the present study, but HAMLET failed to induce nucleosome assembly under the same conditions. When mixed with histones prior to the addition of DNA, HAMLET instead prevented nucleosome formation. Rather than delivering the histones to the DNA, HAMLET thus appeared to sequester them and prevent their deposition on DNA. This is consistent with the high affinity interactions and lack of reversibility of binding that was observed *in vitro*, in the BIAcore and precipitation assays, and with the presence of HAMLET and histones in an insoluble nuclear fraction from dying cancer cells. The results show that HAMLET differs not only in structure but also in function from other histone-binding proteins.

HAMLET was found to bind with high affinity both to native and denatured histone proteins, suggesting that HAMLET recognizes molecular motifs that are maintained regardless of the histone fold. Interestingly, the interaction with H3 was independent of the functionally important histone tail, further supporting the notion that conserved epitopes are involved in binding. This ability of HAMLET to interact with different folding variants of histones is likely to have implications for the effects of HAMLET on histone metabolism and function *in vivo*. HAMLET may bind histones at the time of synthesis in the cytoplasm and disturb the folding process as well as the nuclear transport. HAMLET could also interfere with the association of correctly folded histones with each other and, ultimately, their deposition on DNA, as evidenced by the present study.

Based on these findings, two potentially important cellular effects of HAMLET may be discussed. First, the studies offer a molecular explanation for the accumulation of HAMLET in the nuclei of tumor cells, as the high affinity binding to histones and nucleosomes may cause HAMLET to remain in the nuclear compartment. Second, this effect of HAMLET may represent a novel mechanism of nuclear attack during programmed cell death. The results suggest a new mechanism of chromatin disruption and propose HAMLET as an agonist with this effect. By binding to histones, HAMLET disrupts chromatin assembly and interferes with intact chromatin, thus preventing the cell from transcription, replication, and recombination. Kinetic studies have confirmed that DNA and RNA synthesis come to a halt within minutes in cells treated with HAMLET.<sup>3</sup> As a consequence, HAMLET causes irreversible damage and cell

death. This mechanism has not been described previously, but chromatin assembly proteins in yeast have been proposed to be involved in cell death (18). Deletion of the histone chaperone ASF1/CIA1 stimulated an active, apoptosis-like cell death mechanism. It is possible that HAMLET may act through a similar mechanism in tumor cells. We propose that HAMLET offers a novel solution to ensure tumor cell death through disruption of the chromatin and speculate that the chromatin interaction marks the irreversible phase of tumor cell death induced by HAMLET. The specificity for histones *per se* does not explain the selectivity of HAMLET for tumor cells, however. The decisive step is the active transport of HAMLET from the cytoplasm into the nuclear compartment.<sup>4</sup> We have observed that this nuclear accumulation occurs in tumor cells and not in healthy differentiated cells, but further studies are required to understand the transport of HAMLET to the nuclear compartment and the molecular basis of selectivity.

Selective induction of apoptosis in tumor cells would be a highly desirable outcome of cancer therapy. Normally, however, tumor cells are refractory to the apoptosis signals that limit the longevity of healthy, differentiated cells. For example, *p53* mutations impair the sensing of DNA damage, and altered expression of the anti-apoptotic *bcl-2* family members inhibits the mitochondrial response to various apoptosis agonists (19). Still, the molecular executors of apoptosis remain intact in many tumor cells, and the task is to find ways of inducing apoptosis by circumventing the roadblocks that prevent them from being activated. HAMLET may offer new solutions to this problem. By attacking the chromatin assembly machinery, HAMLET appears to upset fundamental mechanisms available in all tumor cells, potentially explaining why HAMLET has such a broad anti-tumor spectrum.

*Acknowledgments*—We thank Mikael Danfelter and Prof. Dick Heinegård's group for help with the mass spectrometry and Henrik Svensson and Prof. Lars Björck for assistance with the BIAcore.

#### REFERENCES

- Håkansson, A., Zhivotovsky, B., Orrenius, S., Sabharwal, H., and Svanborg, C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 8064–8068
- Svensson, M., Sabharwal, H., Håkansson, A., Mossberg, A. K., Lipniunas, P., Lefler, H., Svanborg, C., and Linse, S. (1999) *J. Biol. Chem.* **274**, 6388–6396
- Svensson, M., Håkansson, A., Mossberg, A. K., Linse, S., and Svanborg, C. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 4221–4226
- Håkansson, A., Andreasson, J., Zhivotovsky, B., Karpman, D., Orrenius, S., and Svanborg, C. (1999) *Exp. Cell Res.* **246**, 451–460
- Kimura, H., and Cook, P. R. (2001) *J. Cell Biol.* **153**, 1341–1353
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1993) *Current Protocols in Molecular Biology*, Vol. 2, pp. 12.0.3–12.1.9, Wiley Interscience, Hoboken, NJ
- Bates, D. L., Butler, P. J., Pearson, E. C., and Thomas, J. O. (1981) *Eur. J. Biochem.* **119**, 469–476
- Wray, W., Boulikas, T., Wray, V. P., and Hancock, R. (1981) *Anal. Biochem.* **118**, 197–203
- Hamiche, A., and Richard-Foy, H. (1998) *J. Biol. Chem.* **273**, 9261–9269
- Lorenzo, P., Aspberg, A., Onnerfjord, P., Bayliss, M. T., Neame, P. J., and Heinegård, D. (2001) *J. Biol. Chem.* **276**, 12201–12211
- Simon, R. H., and Felsenfeld, G. (1979) *Nucleic Acids Res.* **6**, 689–696
- Hamiche, A., Kang, J. G., Dennis, C., Xiao, H., and Wu, C. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 14316–14321
- Simpson, R. T., and Stafford, D. W. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80**, 51–55
- Simpson, R. T., Thoma, F., and Brubaker, J. M. (1985) *Cell* **42**, 799–808
- Kerr, J. F. R., Wyllie, A. H., and Currie, A. R. (1972) *Br. J. Cancer* **26**, 239–257
- Ishimi, Y., Kojima, M., Yamada, M., and Hanaoka, F. (1987) *Eur. J. Biochem.* **162**, 19–24
- Ito, T., Tyler, J. K., and Kadonaga, J. T. (1997) *Genes Cells* **2**, 593–600
- Yamaki, M., Umehara, T., Chimura, T., and Horikoshi, M. (2001) *Genes Cells* **6**, 1043–1054
- Johnstone, R. W., Ruefli, A. A., and Lowe, S. W. (2002) *Cell* **108**, 153–164

<sup>3</sup> O. Hallgren and C. Svanborg, unpublished data.

<sup>4</sup> L. Gustafsson, C. Düringer, and C. Svanborg, unpublished data.