# The proteolytic specificity of the natural enediyne-containing chromoproteins is unique to each chromoprotein

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**Background:** Enediyne chromoproteins are potent antitumor antibiotic agents. They consist of a labile nine-membered enediyne chromophore non-covalently associated with a stabilizing acidic polypeptide. Studies *in vitro* on three members of this superfamily of natural products — kedarcidin, maduropeptin and neocarzinostatin — demonstrated that their chromophores cleave DNA at sites specific to each chromophore. Recently, we showed that these chromoproteins possess proteolytic activity against histones *in vitro*, with histone H1 as a preferred substrate. Based on these results, we speculated that this selective proteolytic activity may be important *in vivo* in the delivery of the enediynes intact to the DNA in chromatin.

**Results:** We show here that each chromoprotein generates a unique set of H1 fragments as revealed by gel analyses of the H1 cleavage reaction products. To probe the observed cleavage specificity, we synthesized a 24-amino-acid peptide representing a basic region of histone H1. This model peptide was incubated individually with similar concentrations of the kedarcidin, neocarzinostatin and maduropeptin chromoproteins as well as the kedarcidin apoprotein. The reaction products were analyzed by electrospray liquid chromatography/mass spectrometry. Our results indicate that all proteins cleave the peptide at selected backbone amides, and that these sites vary according to the chromoprotein used. Moreover, the kedarcidin apoprotein appears to be less specific than the kedarcidin chromoprotein complex.

**Conclusions:** The small size, unique architecture and very acidic nature of the enediyne chromoproteins are highly unusual. These natural products exhibit the dual functionalities of specific DNA cleavage and selective proteolytic activity. This observation adds to the fascinating properties of these molecules and suggests that it is possible not only to design small moieties to cleave DNA but also to conceive of small proteins to deliver these moieties intact to defined areas of chromatin.

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## Introduction

Enediyne chromoproteins are unusually potent antitumor antibiotics with a unique molecular architecture. They consist of a labile nine-membered enediyne-containing chromophore, non-covalently associated with a highly acidic, stabilizing polypeptide. These chromoproteins include kedarcidin (KDC), neocarzinostatin (NCS), macromomycin, actinoxanthin, maduropeptin (MDP) and C1027 [1–16]. A comparison of the amino-acid sequences (Hans Marquardt, personal communication) shows that KDC shares 38.7 % identity with NCS in a 111-residue overlap (four gaps), 36.0 % identity with macromomycin in a 111-residue overlap (two gaps) and 40.2 % identity with actinoxanthin in a 107-residue overlap (five gaps). The amino-acid sequences of MDP and C1027 have not yet been disclosed.

Mechanistic studies have been performed primarily on the chromophores of NCS, KDC, C1027 and MDP [1–20]. These chromophores were shown to cleave DNA *in vitro* at sites specific to each chromophore. This DNA damage was proposed to be responsible for the potent biological activities of the holoantibiotics. Furthermore, it was suggested that the apoprotein component of the complexes served to stabilize and regulate the availability of the labile chromophores. Having observed a 10-fold higher potency for the KDC chromoprotein than the KDC chromophore in cell-based studies, we compared the activity of purified KDC apoprotein, the intact chromoprotein and the purified chromophore against DNA and several proteins *in vitro*. In contrast to the chromophore and the chromoprotein, the apoprotein had no activity against DNA. The chromophore, however, did not exhibit any cleavage activity against any of the proteins tested, whereas the apoprotein and the chromoprotein both cleaved proteins selectively. Among the proteins tested, histones, the most opposite in net charge to the highly acidic apoprotein, were cleaved most readily. Moreover, histone H1, richest in lysines, appeared to be the preferred substrate [20,21]. Similar activity was noted for both MDP and NCS chromoproteins (their corresponding apoproteins were not tested for availability reasons). Histones combine with DNA to form chromatin, the material that makes up chromosomes. Based on our results and on chromatin structure, we speculated that the highly acidic apoproteins may provide the chromophores with targeted delivery *in vivo* to the

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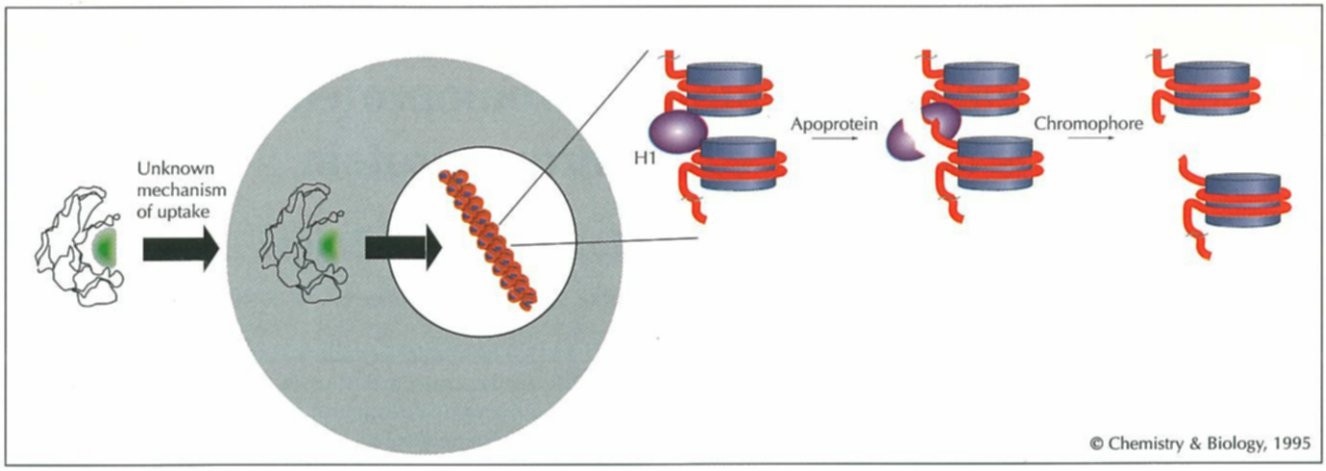


**Fig. 1.** The protein component of the chromoprotein assists in delivery of the chromophore to DNA. Proposed scheme for the way in which the highly acidic apoproteins may provide the chromophores with targeted delivery *in vivo* to the spacer DNA, by cleaving histone H1. The chromophore is shown in green.

spacer DNA, after cleaving H1 (Fig.1). These observations, along with the fact that different chromophores target different DNA sequences, led us to investigate whether these natural proteases exhibit specificity in their H1 cleavage.

Here we show that the chromoproteins described above cleave histone H1 to yield a set of fragments unique to each chromoprotein used. To probe the specificity exhibited by the chromoproteins, we synthesized a 24-amino-acid peptide, representing a particularly basic region of histone H1 [22–28]. The peptide was incubated individually with similar concentrations of the KDC, NCS and MDP chromoproteins as well as the KDC apoprotein. The reaction products were then analyzed by electrospray liquid chromatography/ mass spectrometry (LC/MS) [29]. Our results indicate that each chromoprotein specifically cleaved the peptide bonds at different sites, and that the KDC apoprotein is less specific than the KDC chromoprotein complex.

## Results and discussion

### Cleavage of histone H1 by the KDC, NCS and MDP chromoproteins and by the KDC apoprotein

Incubation of H1 with 10 μg $ml^{-1}$ (≈50:1 molar ratio H1:chromoprotein) of KDC, NCS and MDP chromoproteins results in cleavage profiles that vary according to the chromoprotein used as shown by SDS-PAGE (Fig. 2, NCS and MDP cleavage patterns shown). At this concentration, MDP produces higher molecular weight fragments than do NCS and KDC, suggesting that MDP may be more selective in its H1 cleavage.

To ensure that the difference in H1 cleavage pattern is due to a difference in specificity among the three chromoproteins and not to a difference in activity, H1 was incubated with several concentrations of the three chromoproteins, ranging from 10 μg $ml^{-1}$ to 1 mg $ml^{-1}$. This variation in concentration did not alter the above results, that is, the three chromoproteins cleaved H1 to yield a set of fragments unique to each chromoprotein regardless of chromoprotein concentration (Fig. 2).

Incubation of H1 with varying concentrations of the KDC apoprotein (10 μg $ml^{-1}$ to 1 mg $ml^{-1}$) and comparison of the resulting cleavage profiles with those obtained using

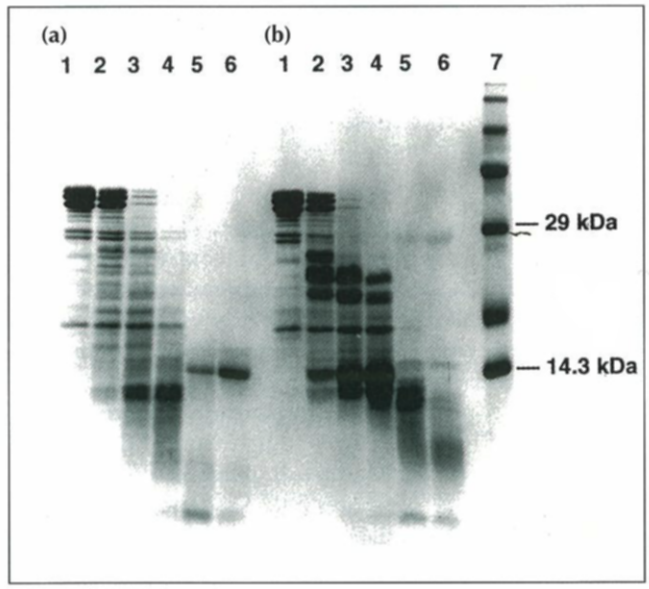


**Fig. 2.** SDS-PAGE of the reaction products of **(a)** NCS and **(b)** MDP with calf thymus histone H1. Reaction conditions: 50 mM Tris-HCl (pH 7.5), 1 mg $ml^{-1}$ H1 and varying concentrations of NCS and MDP in a total volume of 10 ml, 37 °C overnight. The control reactions were carried out under identical conditions except that the chromoproteins were replaced by pure water. Lane 1: control reactions with H1. Lanes 2, 3, 4, 5 and 6: reactions of H1 with 10 μg $ml^{-1}$, 50 μg $ml^{-1}$, 100 μg $ml^{-1}$, 500 μg $ml^{-1}$ and 1 mg $ml^{-1}$, respectively, of NCS (a) and MDP (b). Lane 7: protein size standards from 200 to 14.3 kDa. The band corresponding to NCS protein seen in (a) (lanes 5 and 6) migrates close to the 14.3-kDa marker and that corresponding to MDP protein in (b) (lanes 5 and 6) migrates close to the 29-kDa marker.

**Fig. 3.** Cleavage of peptide substrate by apo/chromoprotein. **(a)** Peptide **1** with numbered residues. The asterisks in (b)–(e) indicate the observed cleavage sites caused by: **(b)** KDC apoprotein, **(c)** KDC chromoprotein, **(d)** NCS and **(e)** MDP.

**(a)** $^{1}$AEKTPVKKKA $^{11}$AKKPAGARRK $^{21}$ASGP-$NH_2$

**(b)** AEKTPVKKK*A AKKPAGAR*R*K*A*SGP-$NH_2$

**(c)** AEKTPVKKK*A AKKPA*GARRK ASGP-$NH_2$

**(d)** AEKTPVKK*K*A AKKPAGARRK* ASGP-$NH_2$

**(e)** AEKTPVKKKA AKKPAGARRK* ASGP-$NH_2$

the KDC chromoprotein suggested a slight difference in their cleavage patterns (data not shown). However, the observation that at equal concentrations there is a difference in activity between the apoKDC and the KDC complex [21] makes it difficult to draw conclusions about their difference in specificity. The specificity difference, however, becomes clearer in the study using the peptide as substrate.

**Peptide cleavage by the KDC, NCS and MDP chromoproteins**

LC/MS analyses show that incubation of peptide **1** (Fig. 3a) with KDC, NCS and MDP chromoproteins at a concentration of 10 μg $ml^{-1}$ results in peptide bond cleavage at specific sites.

The LC/MS results are summarized in Figure 3 and Table 1. As expected from the H1 study described above, the cleavage sites vary according to the chromoprotein used. The KDC chromoprotein cleaves the peptide after two major sites, Lys9 and Ala15. The points of attack by NCS are after Lys8, Lys9 and Lys20. MDP cleaves the peptide at one major site, which is after Lys20. These cleavage sites resemble those found for proteases in that their substrates have common P1 sites (nomenclature of Schechter and Berger [30]) for cleavage, specifically lysine and arginine (trypsin-like). Thus, the proteins appear to exhibit a sequence-dependent substrate specificity.

To ensure that the difference in peptide cleavage is due to a difference in specificity among the chromoproteins and not to a difference in activity, peptide **1** was incubated with either 100 μg $ml^{-1}$ of KDC or 100 μg $ml^{-1}$ of NCS. This 10-fold increase in protein concentration did not alter the cleavage data. MDP was not assayed at the higher concentration due to lack of material.

The results obtained in the peptide-cleavage reactions mirror those observed in the H1 cleavage experiments. Each of the proteolytic proteins gives a unique set of cleavage products. Although the active-site residues

**Table 1.** Cleavage sites of the chromoproteins.

| Chromoprotein | Retention time | MW | Sequence | | Cleavage site |
|---|---|---|---|---|---|
| KDC | 14:45 | 897.9 | 16–24 | GARRKASGP[b] | A*G[c] |
| | 14:51 | 1028 | 1–9 | AEKTPVKKK | K*A |
| | 16:05 | 1594.6 | 1–15[a] | AEKTPVKKKAAKKPA | A*G |
| | 16:20 | 1464.4 | 10–24[a] | AAKKPAGARRKASGP | K*A |
| | 17:06 | 2474.7 | 1–24[a] | | – |
| NCS | 4:51 | 329.2 | 21–24[a] | ASGP | K*A |
| | 15:35 | 1028.1 | 1–9 | AEKTPVKKK | K*A |
| | 16:08 | 899.7 | 1–8 | AEKTPVKK | K*K |
| | 16:48 | 1592.7 | 9–24[a] | KAAKKPAGARRKASGP | K*K |
| | 16:52 | 1464.4 | 10–24[a] | AAKKPAGARRKASGP | K*A |
| | 16:57 | 2163.5 | 1–20 | AEKTPVKKKAAKKPAGARRK | K*A |
| | | | | or[d] | or |
| | | | 2–21 | EKTPVKKKAAKKPAGARRKA | A*E; A*S |
| | 17:31 | 2475.0 | 1–24[a] | | – |
| MDP | 4:45 | 329.3 | 21–24[a] | ASGP | K*A |
| | 16:45 | 2163.5 | 1–20 | AEKTPVKKKAAKKPAGARRK | K*A |
| | | | | or[e] | or |
| | | | 2–21 | EKTPVKKKAAKKPAGARRKA | A*E; A*S |
| | 17:17 | 2474.7 | 1–24[a] | | |

[a]Unique sequence based on MW.
[b]Underlined portions of sequence were deduced by tandem mass spectrometry.
[c]Asterisk indicates site of cleavage.
[d]For retention time (R.T.) 16:57, there are two possible sequences based on molecular weight; distinction by MS/MS was unsuccessful. However, since fragment 21–24 at R.T. 4:51 was observed, it is most likely that the fragment at R.T. 16:57 corresponds to 1–20.
[e]For R.T. 16:45, there exist two possible sequences based on molecular weight; distinction by MS/MS was unsuccessful. However, since fragment 21–24 at R.T. 4:45 was observed, it is most likely that the fragment at R.T. 16:45 corresponds to 1–20.

**Table 2.** Cleavage sites of the KDC apoprotein.

| Retention Time | MW | | Sequence | Cleavage Site |
|---|---|---|---|---|
| 4:56 | 258.1 | 22–24[a] | SGP | A*S[c] |
| 5:08 | 329.1 | 21–24 | ASGP | K*A |
| 5:14 | 457.4 | 20–24[a] | KASGP | R*K |
| 15:30 | 1028.1 | 1–9 | AEKTPVKKK[b] | K*A |
| 16:47 | 2163.7 | 1–20 | AEKTPVKKKAAKKPAGARRK | K*A |
| 16:55 | 1879.1 | 1–18[a] | AEKTPVKKKAAKKPAGAR | R*R |
| 17:03 | 2035.1 | 1–19[a] | AEKTPVKKKAAKKPAGARR | R*K |
| 17:24 | 2474.7 | 1–24[a] | | – |

[a]Unique sequence based on MW.
[b]Underlined portions of sequence were deduced by tandem mass spectrometry.
[c]Asterisk indicates site of cleavage.

remain to be identified, our results are consistent with the low degree of homology shared between the three chromoproteins. The amino-acid sequences of KDC and NCS share only 38.7 % sequence identity. In addition, preliminary data on the amino-acid sequence of MDP show that it shares no homology with KDC or NCS (H. Marquardt, personal communication). These observations suggest that at least some of the amino acids involved in the protease active sites are different and raise interesting questions regarding the evolutionary implications of the different specificities observed.

#### Peptide cleavage by the KDC apoprotein

Incubation of the KDC apoprotein (10 $\mu g\ ml^{-1}$; 9 x $10^{-7}$ M) with peptide **1** (9 x $10^{-4}$ M) results in several major cleavage sites. As can be seen from Figure 3 and Table 2, these sites are after the following amino acids: Lys9, Arg18, Arg19, Lys20 and Ala21. These results indicate that the apoprotein cleaves the peptide at more sites than its corresponding chromoprotein. This difference in proteolytic specificity between the apo and the chromokedarcidin is intriguing. In the absence of NMR data and a crystal structure of the KDC chromoprotein, it is difficult to offer a definitive interpretation of this result. An appealing explanation, however, is that the chromophore induces a small conformational change on the protein such that the structure of the active site of the chromoprotein is slightly different from that of the apoprotein.

## Significance

**Many antitumor antibiotics mediate their effects by damaging DNA. Often, the efficacy of these agents suffers from their poor solubility, low stability and inefficient cellular uptake. The enediyne chromoproteins seem to have naturally overcome these problems, thanks to their apoprotein component. The apoprotein serves multiple purposes: it solubilizes the chromophore, shields it from deactivation and apparently facilitates its delivery to the DNA in the nucleus. A more thorough understanding of the substrate specificity of these proteins and their mechanisms of proteolytic activity together with a knowledge of the chemistry of their corresponding enediyne chromophores could be invaluable for the design of targeted antitumor agents.**

## Materials and methods

### *Reagents*

KDC chromoprotein and chromophore, MDP and NCS chromoproteins, prepared as described [2,12,21], were kindly provided by J.E. Leet, D.R. Schroeder and J. Golik, respectively (Division of Chemistry, Bristol-Myers Squibb, Wallingford, CT). The 24-amino-acid peptide was synthesized by the solid-phase method using Boc/Benzyl chemistry [28]. The chemicals and solvents used in peptide synthesis were purchased from Applied Biosystems. Calf thymus histone H1 was obtained from Boehringer Mannheim.

### *Cleavage of histone H1 by the KDC, NCS and MDP chromoproteins and by the KDC apoprotein*

Histone H1 (4 x $10^{-5}$ M) was incubated separately with the KDC apoprotein, KDC, NCS and MDP chromoproteins (10 $\mu g\ ml^{-1} \approx 10^{-7}$ M; 50 $\mu g\ ml^{-1}$; 100 $\mu g\ ml^{-1}$; 500 $\mu g\ ml^{-1}$; 1 $mg\ ml^{-1}$), in 50 mM Tris-HCl buffer at pH 7.5. The incubation was carried out at 37 °C overnight at ≈ 50:1 to 0.5:1 molar ratio in histone: apo/chromoprotein in a total volume of 10 µl. The samples were then heated in a denaturing dye for one minute and analyzed on a 17 % SDS polyacrylamide gel. The protein bands were visualized with Coomassie blue.

### *Reaction of the chromoproteins/apoprotein with the synthetic peptide*

Peptide **1** (9 x $10^{-4}$ M) was reacted individually with the KDC chromoprotein (10 $\mu g\ ml^{-1} \approx 8 \times 10^{-7}$ M and 100 $\mu g\ ml^{-1} \approx 8 \times 10^{-6}$ M), the NCS chromoprotein (10 $\mu g\ ml^{-1} \approx 8 \times 10^{-7}$ M and 100 $\mu g\ ml^{-1} \approx 8 \times 10^{-6}$ M), the MDP chromoprotein (10 $mg\ ml^{-1} \approx 3 \times 10^{-7}$ M) and the apokedarcidin (10 $\mu g\ ml^{-1}$; 9 x $10^{-7}$ M) in 50 mM Tris-HCl buffer at pH 7.5 at 37 °C overnight.

### *Purity of the chromoproteins studied*

HPLC analyses and/or visualization by gel-staining with Coomassie blue showed single bands corresponding to the chromoproteins. Different preparations of the chromoproteins were tested in the histone H1 cleavage assay [21] and the activity was consistent from batch to batch. As an additional check that the proteolytic activity is associated with

the chromoprotein, KDC was chromatographed using a size exclusion column and fractions were collected throughout the entire run. Each fraction was then assayed for H1 cleavage activity [21]. The fraction in which the chromoprotein eluted contained essentially all of the activity placed on the column (D.W. Phillipson, unpublished results).

### *Electrospray liquid chromatography/mass spectrometry (LC/MS) analyses of the reaction mixtures*

The reaction mixtures were analyzed by LC/MS [29]. All LC/MS data was obtained on a Finnigan TSQ700 (San Jose, CA) mass spectrometer equipped with a Finnigan electrospray source interfaced to a Waters 600MS gradient HPLC (Millipore Corp., Milford, MA). Chromatographic separations were achieved using a 2.0 x 150 mm Partisil C18 column (Keystone Scientific, Bellefonte, PA) with gradient elution at 0.3 ml $min^{-1}$. A binary linear gradient was formed from 100 % to 70 % mobile phase A in 15 min and then from 70 % to 30 % A in 10 min, where mobile phase A was water with 0.05 % trifluoroacetic acid and mobile phase B was acetonitrile with 0.05 % trifluoroacetic acid. Full-scan mass spectra were obtained by scanning the mass spectrometer from 250–1250 u in 2 sec. Electrospray ionization produces multiply charged ions of the type $(M+nH)^{n+}$, where n is related to the number of basic sites on the peptide. In many cases $(M+H)^{+}$ was not observed. Instead, most peptides yield two or more higher charge states, such as $(M+2H)^{2+}$ and $(M+3H)^{3+}$ [29]. Product ion spectra were obtained using argon as the collision gas at a pressure of 2.5–3 mtorr. Collision energies of 15–25 eV were used.

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