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Selective proteolytic activity of the antitumor agent kedarcidin

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ABSTRACT **Kedarcidin is a potent antitumor antibiotic chromoprotein, composed of an enediyne-containing chromophore embedded in a highly acidic single chain polypeptide. The chromophore was shown to cleave duplex DNA site-specifically in a single-stranded manner. Herein, we report that *in vitro*, the kedarcidin apoprotein, which lacks any detectable chromophore, cleaves proteins selectively. Histones that are the most opposite in net charge to the apoprotein are cleaved most readily. Our findings imply that the potency of kedarcidin results from the combination of a DNA damaging-chromophore and a protease-like apoprotein.**

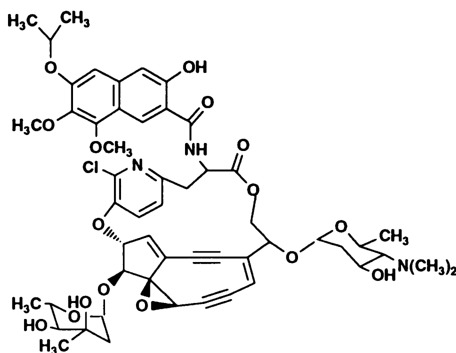
Kedarcidin was recently isolated from fermentations of an actinomycete strain obtained from soils collected in India (1, 2). Kedarcidin exhibits potent *in vivo* antitumor activity against P388 leukemia and B16 melanoma similar to that of the enediyne antitumor agents: neocarzinostatin, esperamicin, and calicheamicin (W. C. Rose, personal communication). Kedarcidin also shows pronounced activity against Gram-positive bacteria. Structural studies demonstrate that kedarcidin is composed of an acidic single chain polypeptide (Structure 1 *Upper*) and a highly labile enediyne-containing chromophore (Structure 1 *Lower*) reminiscent of the neocarzinostatin holomolecule (2–9). DNA experiments indicate

ASAAVSVSPA TGLADGATVT VSASGFATST SATALQCAIL ADGRGACNVA

EFHDFSLSGG EGTTSVVVRR SFTGYVMPDG PEVGAVDCDT APGGCEIVVG

GNTEEYENAA ISFE 114

Apoprotein



Chromophore
Structure 1

that the kedarcidin chromophore cleaves duplex DNA site-specifically in a single-stranded manner (10).

Thus far, it has been suggested that the antitumor activity of calicheamicin γ_1^I, esperamicin A_1, and the neocarzinostatin chromophore involves DNA cleavage, a process about which there is substantial *in vitro* chemical and structural information (4–21). Furthermore, it was proposed that the neocarzinostatin apoprotein stabilizes and regulates the availability of the labile chromophore (4–9). As previously shown for neocarzinostatin (4–9), DNA experiments with the isolated chromophore and the kedarcidin chromoprotein demonstrated that DNA cleavage was mostly due to the chromophore (N.Z. and W.S., unpublished data). However, cytotoxicity assays using human colon cancer cell lines HCT 116 showed the chromophore and the chromoprotein to exhibit similar IC_{50} values of 10^{-9} M (IC_{50} is the drug concentration that causes 50% cell kill), despite the difference in their molecular weights (N.Z. and W.S., unpublished data; R. A. Dalterio, personal communication; refs. 22 and 23). These observations suggested that the apoprotein must be contributing actively to the cytotoxicity of the holomolecule and prompted us to investigate an additional role for the kedarcidin polypeptide beyond that of an inert chromophore stabilizer.

In this paper, we report that the kedarcidin apoprotein possesses selective proteolytic activity. Among the proteins tested, histones that are the most opposite in net charge to the apoprotein are cleaved most readily. Preliminary studies on the neocarzinostatin chromoprotein indicate that this complex also cleaves histones selectively. This selective proteolytic activity may be crucial for the delivery of the nine-membered enediyne chromophores intact to the DNA, *in vivo*.

MATERIALS AND METHODS

Kedarcidin Chromophore and Chromoprotein. The kedarcidin chromophore and chromoprotein, prepared as described (1–3), were obtained from the Division of Chemistry, Bristol-Myers Squibb, Wallingford, CT. As was observed with auromomycin chromoprotein, the ratio of apoprotein:chromophore is dependent on fermentation conditions. The apoprotein:chromophore molecular ratio in the kedarcidin complex studied was found to be approximately 16:1 (170:1 mass ratio) as determined by differential UV methodology (R. A. Dalterio, personal communication; refs. 22 and 23).

Kedarcidin Apoprotein. The kedarcidin apoprotein was isolated from the chromoprotein as follows: the purified chromoprotein (1, 2) was incubated in 6 M guanidine hydrochloride for 16 hr at 37°C. The solution was then applied to a Bio-Gel P6 column equilibrated with guanidine hydrochloride. The solution was dialyzed using a Spectra/Por molecular weight cutoff 3500 three different times against 1% ammonium carbonate and then concentrated using an Amicon Centriprep. 3 filter. The Bio-Gel P6 chromatography and the dialysis procedure were repeated twice. The apoprotein was then further purified with a Phenomenex size-exclusion column, Biosep SEC S2000.

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Testing for Residual Chromophore in the Apoprotein. To ensure that the kedarcidin apoprotein activity was not due to the presence of residual chromophore, the kedarcidin chromophore, chromoprotein, and apoprotein were tested for antimicrobial activity vs. *Bacillus subtilis* and *Escherichia coli* in well-agar diffusion assays (S. W. Mamber, personal communication; ref. 24). These agents were also tested for DNA reactivity in the *E. coli* SOS chromotest (S. W. Mamber, personal communication; ref. 25). The apoprotein was inactive in all microbial assays at 1000 μg/ml. In contrast, the chromoprotein exhibited antimicrobial and SOS-inducing activities at concentrations of 4 μg/ml or lower (S. W. Mamber, personal communication). Analysis by electrospray mass spectrometry and tandem mass spectrometry showed that the concentration of chromophore in the apoprotein is at least 100-fold less than in the chromoprotein. Using this method, it was possible to detect chromophore in the chromoprotein even after it had been diluted to a concentration 100-fold less than that of the apoprotein (M. Bolgar, personal communication).

Chemicals. The neocarzinostatin chromoprotein was also obtained from the Division of Chemistry mentioned earlier. The total calf thymus histones, the individual histones H1, H2A, H2B, H3, and H4, the 3′,5′-cyclic AMP-dependent protein kinase, the prostatic phosphatase, the apo- and holotransferrin, the protease inhibitors, the guanidine hydrochloride, and the ammonium carbonate were purchased from Boehringer Mannheim, Sigma, and Baker. The calf brain tubulin and the HCT 116 cell membrane protein extract were obtained from the Division of Experimental Therapeutics, Bristol-Myers Squibb, Princeton, NJ. The Econopac 10DG columns were obtained from Bio-Rad; the Centriprep filters were from Amicon; the dialysis material was from Spectrum Medical Industries.

Histone Cleavage by the Kedarcidin Apoprotein and Chromoprotein and the Neocarzinostatin Chromoprotein. The kedarcidin apoprotein, the kedarcidin chromoprotein, and the neocarzinostatin chromoprotein (1 μl of 0.1–1 mM solution in Millipore-grade water, 10× solution) were incubated with total calf thymus histones and with each of the five calf thymus histones H1, H2A, H2B, H3, and H4 (1 μl of a 10 mg/ml solution in Millipore-grade water) at a 1:1 or 0.1:1 molar ratio in apo/chromoprotein:histone at 37°C in a total volume of 10 μl. The reactions were carried overnight in 50 mM Tris·HCl at pH 7.4 (Tris). The samples were then heated in a denaturing dye for 1 min and analyzed on a 17% SDS/polyacrylamide gel. The protein bands were visualized with Coomassie blue.

Time Study of Histone H1 Cleavage by the Kedarcidin Apoprotein. The kedarcidin apoprotein (6 μl of a 1 mM solution, 10×) was incubated with histone H1 (6 μl of a 10 mg/ml solution) at 37°C in a total volume of 60 μl, in Tris. Aliquots (10 μl) were removed at 1, 3, 5, 7, and 24 hr. The reaction was stopped in each case by adding denaturing loading dye, heated for 1 min at 90°C, and frozen at −20°C. The results were analyzed on a 17% SDS/polyacrylamide gel. The control reaction in this case was carried for 24 hr.

Incubation with Various Protease Inhibitors. Leupeptin, antipain, trypsin inhibitor, pepstatin, and benzamidine were added separately at a final concentration of 20 μg/ml to the apoprotein/total histone reaction mixture described above.

Effect of the Kedarcidin Apoprotein and Chromoprotein on Different Proteins Other than Histones. The kedarcidin apoprotein and the kedarcidin chromoprotein (1 μl of a 1 mM solution, 10×) were incubated separately with each of the following individual proteins: apo- and holotransferrin, calf brain tubulin, 3′:5′-cyclic AMP-dependent protein kinase, prostatic phosphatase, HCT 116 cell membrane protein extract (1 μl of a 10 mg/ml solution) in a total volume of 10 μl as described above. The results were analyzed with 10%, 12%, and 17% SDS/polyacrylamide gels depending on the protein studied.

Reaction of the Kedarcidin Chromophore with Various Proteins. The kedarcidin chromophore (1 μl of a 1–10 mM solution, 10×) was allowed to react with 1 μl of a 10 mg/ml solution of each of the above-mentioned proteins in a total volume of 10 μl, at 37°C in 10:90 (vol/vol) dimethyl sulfoxide/Tris overnight, in the absence and in the presence of 2-mercaptoethanol. The results were analyzed as described above.

Reaction of the Kedarcidin Chromophore with the Kedarcidin Apoprotein. The kedarcidin chromophore (1 μl of a 10× solution, 10 mM) was allowed to react with 1 μl of a 10 mg/ml solution of the kedarcidin apoprotein in the absence and in the presence of 2-mercaptoethanol, in a total volume of 10 μl, at 37°C in dimethyl sulfoxide/Tris overnight. The results were analyzed as described above.

RESULTS AND DISCUSSION

Histone Cleavage by the Kedarcidin Apoprotein and Effect of Protease Inhibitors. The reaction of the kedarcidin apoprotein with total calf thymus histones demonstrated the formation of low molecular mass peptides. Incubation of each individual calf thymus histone (H1, H2A, H2B, H3, and H4) with the apoprotein showed that all histones were cleaved. The relative susceptibility to cleavage was H1 > H2B, H3, H2A > H4 (Fig. 1). It is interesting to note that histone H1 is the richest histone in lysines followed by H2A, H2B, H3, and H4. A time course experiment with histone H1 revealed the formation of low molecular mass bands within 3 hr of incubation. Addition of the protease inhibitors leupeptin, antipain, and trypsin inhibitor inhibited the cleavage reactions, whereas pepstatin and benzamidine had no effect.

Purity of the Kedarcidin Apoprotein. Amino acid sequencing of the apoprotein, purified as described above, showed it

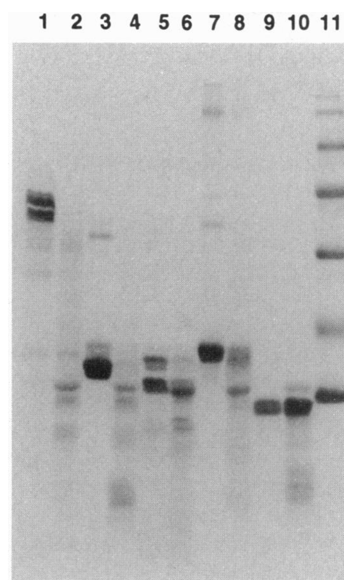

FIG. 1. A 17% SDS/polyacrylamide gel of the reaction of kedarcidin apoprotein with calf thymus histones. Reaction conditions: 50 mM Tris·HCl (pH 7.5), 1 mg of individual histones per ml, and 1 mg of apoprotein per ml in a total volume of 10 μl, 37°C overnight. The control reactions were carried out under identical conditions except that the apoprotein was replaced by pure water. Lanes 1, 3, 5, 7, and 9, control reactions with H1, H2A, H2B, H3, and H4, respectively; lanes 2, 4, 6, 8, and 10, reactions of the apoprotein with H1, H2A, H2B, H3, and H4, respectively; lane 11, protein size standards from 200 to 14.3 kDa. The band corresponding to the apoprotein migrates close to the 14.3-kDa marker.

to be 92.6% pure. Amino acid compositions and partial amino acid sequencing of the contaminating proteins revealed no homology with any of the proteases listed in the Protein Identification Resource of the National Biomedical Research Foundation. The specific proteolytic activity of the kedarcidin apoprotein on a per weight basis appeared to be the same before and after the size-exclusion chromatography. The minor contaminants, isolated by size-exclusion chromatography, were found not to possess any proteolytic activity when allowed to react with histones at 1 mg/ml. Several preparations of the protein were individually tested in the histone H1 cleavage assay and the activity was consistent from batch to batch. Visualization of the protein by staining with Coomassie blue showed a single band on the polyacrylamide gel, even when overloaded. For reasons unknown to us, the more sensitive silver staining failed to stain our protein but also failed to detect any other contaminating proteins. Although we cannot rule out the possibility of a minor contaminating protease, the above data suggest, within reasonable limits, that the proteolytic activity observed is due to kedarcidin and not to a minor protease contaminant.

The Kedarcidin and Neocarzinostatin Chromoproteins Cleave Histones Similarly to the Kedarcidin Apoprotein. The kedarcidin chromoprotein complex reacted with the histones in a manner similar to that of the apoprotein. Interestingly, the neocarzinostatin chromoprotein also showed a similar behavior to that of the kedarcidin apo/chromoprotein (Fig. 2).

The amount of kedarcidin chromoprotein used to damage H1 was 10-fold less than that needed for the kedarcidin apoprotein. It is possible that the denaturation procedure, essential for the removal of the chromophore, may have resulted in a decrease in the apoprotein activity. A more appealing thought would be that the presence of chromophore in the complex causes a more compact structure, resulting in a more efficient binding between the kedarcidin and the histone.

In contrast to a previous report that showed that macromomycin, an enediyne-containing protein, acts as an aminopeptidase (26), our results suggest that the kedarcidin apoprotein and the neocarzinostatin chromoprotein¶ possess proteolytic activity similar to that of an endoprotease since they are able to cleave proteins generating small peptides. In addition, whereas macromomycin was unable to degrade acetylated proteins, kedarcidin and neocarzinostatin efficiently cleaved histones H1 and H2A, both of which have acetylated N termini.

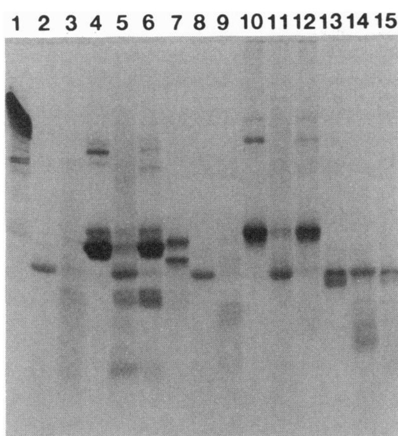

FIG. 2. A 17% SDS/polyacrylamide gel of the reaction of neocarzinostatin chromoprotein with calf thymus histones. Reaction conditions were identical to those in Fig. 1 with 1 mg/ml and 0.1 mg/ml concentrations in drug. Lanes 1, 4, 7, 10, and 13, control reactions with H1, H2A, H2B, H3, and H4, respectively; lanes 2, 5, 8, 11, and 14, reactions of the chromoprotein at 1 mg/ml with H1, H2A, H2B, H3, and H4, respectively; lanes 3, 6, 9, 12, and 15, reactions of the chromoprotein at 0.1 mg/ml with H1, H2A, H2B, H3, and H4, respectively.

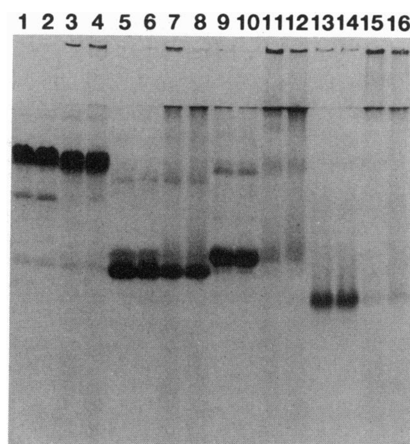

FIG. 3. A 17% SDS/polyacrylamide gel of the reaction of kedarcidin chromophore with calf thymus histones. Reaction conditions were identical to those in Fig. 1 with 1 mg/ml concentration in drug. Lanes 1, 5, 9, and 13, control reactions with H1, H2A, H3, and H4, respectively; lanes 2, 6, 10, and 14, control reactions with H1, H2A, H3, and H4, respectively, in the presence of 2-mercaptoethanol; lanes 3, 7, 11, and 15, reactions of the chromophore with H1, H2A, H3, and H4, respectively, in the absence of 2-mercaptoethanol; lanes 4, 8, 12, and 16, reactions of the chromophore with H1, H2A, H3, and H4, respectively, in the presence of 2-mercaptoethanol.

The Kedarcidin Apoprotein Proteolytic Activity Is Selective. Reaction of the less basic proteins (3′:5′-cyclic AMP-dependent protein kinase and prostatic phosphatase) with the kedarcidin apoprotein/chromoprotein did not lead to any noticeable damage to the proteins. The results were also negative when the apoprotein was incubated with calf brain tubulin and apo- and holotransferrin. Though we cannot rule out specific cleavage of a small number of proteins, kedarcidin apoprotein seemed to have no effect on an HCT 116 cell membrane protein extract. Of all proteins tested, H1, the most basic and the most opposite in net charge to the apoprotein (Structure **1** *Upper*) (27–30), was cleaved the most readily.

The Kedarcidin Chromophore Induces Protein Agglomeration at High Levels. Reaction of the isolated kedarcidin chromophore with histones did not result in protein cleavage, as was the case with the apo/chromoprotein. Rather, it caused the formation of high molecular mass aggregates, after 15 hr of incubation and at high levels of chromophore. Histone H4, richest in arginines, seemed to be the most sensitive to the enediyne (Fig. 3). Protein aggregation was also observed when high levels of kedarcidin chromophore were allowed to react with all of the other proteins tested with the apoprotein. However, the kedarcidin chromophore had no effect on its apoprotein even at the highest concentrations used. The observed protein agglomeration is compatible with the formation of an enediyne-radical intermediate, which leads to a cascade of reactions resulting in protein intermolecular crosslinks. The pronounced acidity of the apoprotein may explain its resistance to such damage.

It should be noted that the kedarcidin chromophore damages DNA extensively at a three-order of magnitude dilution,

¶The neocarzinostatin apoprotein was not tested for availability reasons.

suggesting the DNA to be a preferred substrate for this natural enediyne (11). A similar exclusive "attraction" to the DNA has been previously observed for calicheamicin γ_1^I (16), another natural enediyne-containing chromophore.

CONCLUSION

Our results show that the kedarcidin apo/chromoprotein and the kedarcidin chromophore have different damaging effects on proteins. The kedarcidin chromophore causes protein agglomeration at high concentrations. In contrast, the apo/chromoprotein cleaves certain proteins into low molecular mass peptides. Although we have not yet determined the requirements for the activity or specificity, the apo/chromoproteins clearly are selective in the proteins they cleave. Histones that are most opposite in net charge to the highly acidic apoprotein are damaged most readily. Histone H1, richest in lysines, seems to be the most susceptible to cleavage. Though the biological significance of our data is to be investigated, the results imply that the apoprotein has at least two principal roles in the complex. The first role, as suggested previously for the neocarzinostatin apoprotein (4–9), is that of a host for otherwise highly unstable nine-membered enediyne rings such as neocarzinostatin and kedarcidin chromophores. The second and possibly more significant role is the proteolytic activity. It is attractive to speculate that these highly acidic apoproteins, resistant to proteases, protect their chromophores from deactivation and deliver them to the spacer DNA after cleaving histone H1. Histones combined with DNA form chromatin, the material that makes up the chromosomes. A "targeted delivery" to the chromatin (30) may explain the unusual potency of these natural enediyne-containing chromoproteins.

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