of Mutagens and Carcinogens, Plenum Press, New York.
Sukumar, S., Notario, V., Martin-Zanca, D., & Barbacid, M. (1983) *Nature (London) 306*, 658–661.
Tabin, C. J., Bradley, S. M., Bargmann, C. I., Weinberg, R. A., Papageorge, A. G., Scolnick, E. M., Dhar, R., Lowy, D. R., & Chang, E. H. (1982) *Nature (London) 300*, 143–149.
Taparowsky, E., Suard, Y., Fasano, O., Shimizu, K., Goldfarb, M., & Wigler, M. (1982) *Nature (London) 300*, 762–765.
Ti, G. S., Gaffney, B. L., & Jones, R. A. (1982) *J. Am. Chem. Soc. 104*, 1316–1319.
Tibanyenda, N., De Bruin, S. H., Haasnoot, C. A. G., van der Marel, G. A., van Boom, J. H., & Hilbers, C. W. (1984) *Eur. J. Biochem. 139*, 19–27.
Toorchen, D., & Topal, M. D. (1983) *Carcinogenesis 4*, 1591–1597.

Isolation of a Human cDNA for α_2-Thiol Proteinase Inhibitor and Its Identity with Low Molecular Weight Kininogen†

Iwao Ohkubo, Kotoku Kurachi,* Toshihide Takasawa, Hiroyuki Shiokawa, and Makoto Sasaki

ABSTRACT: A λgt11 cDNA library containing DNA inserts prepared from human liver mRNA has been screened with an antibody to human α_2-thiol proteinase inhibitor that was isolated from fresh plasma. Eighteen positive clones were isolated from one million phage, and each was plaque purified. The cDNA insert of one of these phage was sequenced and shown to code for α_2-thiol proteinase inhibitor as identified by a partial amino acid sequence of the light chain of α_2-thiol proteinase inhibitor. This cDNA insert contained 1529 base pairs coding for the complete α_2-thiol proteinase inhibitor. It included 45 base pairs of 5′ noncoding sequence, 1281 base pairs that code for pre α_2-thiol proteinase inhibitor, a stop codon, 160 base pairs of 3′ noncoding sequence, and 40 base pairs of poly(A) tail. The noncoding sequence on the 3′ end contained a potential recognition site (AATAAA) for processing and polyadenylation of precursor messenger RNA. The amino acid sequence of α_2-thiol proteinase inhibitor deduced from the cDNA showed a striking similarity (overall homology at 74%) to that of bovine low molecular weight (LMW) kininogen, including two internally repeated sequences and a nonapeptide sequence of bradykinin. These data clearly indicated that α_2-thiol proteinase inhibitor and LMW kininogen are identical. This was further supported by immunological cross-reactivity between α_2-thiol proteinase inhibitor and LMW kininogen. When the amino acid sequence of α_2-thiol proteinase inhibitor was compared with those for several low molecular weight thiol proteinase inhibitors, including human and chicken cystatins, rat liver thiol proteinase inhibitor, human stefin, and rat epidermal thiol proteinase inhibitor, it was obvious that there are clearly homologous structures, including two potential reactive site sequences of Gln-Val-Val-Ala-Gly.

Thiol proteinase inhibitors with high and low molecular weights have been isolated from plasma and various tissues and extensively studied (Sasaki et al., 1977, 1983; Järvinen, 1979; Ryley, 1979; Hirado et al., 1981; Lenney et al., 1982; Wakamatsu et al., 1982; Katunuma et al., 1983). These inhibitors inactivate a number of different thiol proteinases, including cathepsins, ficin, papain, and calpain. Accordingly, their physiological function appears to involve the regulation of the thiol proteinases. We have previously characterized two high molecular weight thiol proteinase inhibitors from human plasma and designated them as α_1-thiol proteinase inhibitor and α_2-thiol proteinase inhibitor (Sasaki et al., 1981). The α_1-thiol proteinase inhibitor is a glycoprotein with a minimum molecular weight (M_r) of about 60 000, as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reduced conditions, while the α_2-thiol proteinase inhibitor is a glycoprotein composed of a heavy chain (app M_r 66 000 or 65 000) and a light chain (M_r 4200). The α_2-thiol proteinase inhibitor with the heavy chain of M_r 66 000 has also been designated α_2-thiol proteinase inhibitor$_1$, while the inhibitor with the heavy chain of M_r 65 000 was called α_2-thiol proteinase inhibitor$_2$. α_1-Thiol proteinase inhibitor and α_2-thiol proteinase inhibitors are closely related proteins since they show immunological cross-reactivity.

Several of the low molecular weight thiol proteinase inhibitors from mammalian tissues and serum have also been shown to be highly homologous by amino acid sequence analysis (Takio et al., 1983, 1984; Turk et al., 1983; Brzin et al., 1983, 1984; Machleidt et al., 1983).

In the present studies, we describe the isolation and characterization of a cDNA coding for human α_2-thiol proteinase inhibitor. Furthermore, the amino acid sequence predicted from the cDNA indicates that human α_2-thiol proteinase inhibitor is identical with LMW kininogen.[1] Cloning and sequencing of cDNAs for bovine LMW kininogen and HMW kininogen have recently been reported by Nawa et al. (1983)

† From the Department of Biochemistry, Nagoya City University Medical School, Nagoya 467, Japan (I.O. and M.S.), the Department of Biochemistry, University of Washington, Seattle, Washington 98195 (K.K.), and the Division of Biochemistry, Institute of Immunological Science, Hokkaido University, Sapporo 060, Japan (T.T. and H.S.).
Received August 22, 1984. This investigation was supported in part by the Ministry of Education, Science and Culture of Japan (Research Grant 59370013 to M.S.), the Naito Foundation (I.O.), and the National Institutes of Health (Research Grant HL 31511 to K.K. and Research Grant HL 16919 to Earl W. Davie).

[1] Abbreviations: LMW kininogen, low molecular weight kininogen; HMW kininogen, high molecular weight kininogen; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

0006-2960/84/0423-5691$01.50/0 © 1984 American Chemical Society

and Kitamura et al. (1983), respectively.

Materials and Methods

Purification and Amino Acid Sequence Analysis of α_2-Thiol Proteinase Inhibitor. Human α_2-thiol proteinase inhibitor was purified to homogeneity from human plasma as follows. The protein precipitate obtained by ammonium sulfate fractionation (35–55%) of fresh human plasma (5 L) was dissolved in 500 mL of 20 mM sodium borate, pH 8.0, containing 0.05 M NaCl and 1 mM benzamidine and dialyzed overnight against the same buffer. The solution was then applied to a DEAE-cellulose column (5.5 × 65 cm) previously equilibrated with the same buffer. The column was eluted with a linear gradient of 20 mM sodium borate, pH 8.0, containing 0.05 M NaCl and of 20 mM sodium borate, pH 8.0, containing 0.3 M NaCl. The fractions with α_2-thiol proteinase inhibitor activity were then applied to a second DEAE-cellulose column (3 × 70 cm) and eluted as described above. The fractions containing the α_2-thiol proteinase inhibitor were then applied to a nickel chelate column (3 × 25 cm) previously equilibrated with 20 mM sodium borate, pH 8.0, containing 0.15 M NaCl. The α_2-thiol proteinase inhibitor passed through the column, while a significant amount of contaminating protein was bound to the column. The pass-through solution was then concentrated and applied to a Sephadex G-200 column (3.5 × 140 cm) equilibrated with 20 mM sodium borate–saline buffer, pH 8.0. Fractions with inhibitory activity were dialyzed against 20 mM sodium phosphate, pH 7.2, and applied to a Blue–Sepharose column (3 × 25 cm) previously equilibrated with the same buffer. α_2-Thiol proteinase inhibitor did not bind to the column, while a significant amount of the contaminating proteins was bound to the column. The α_2-thiol proteinase inhibitor was finally applied to a Red–Sepharose column (2 × 20 cm) and eluted with a linear gradient formed with 20 mM sodium phosphate, pH 7.2, and 0.75 M KCl in the same buffer.

Thiol proteinase inhibitor activity of various fractions, as well as purified preparations, was assayed by a modification of the method described by Sasaki et al. (1981). In this assay, ficin (Sigma) was employed as a target thiol proteinase and *N*-benzoyl-L-arginine-*p*-nitroanilide as a substrate for residual ficin activity. The hydrolysis of the synthetic substrate was monitored by following the absorption at 410 nm in a Hitachi spectrophotometer, Model 228. One unit of activity was defined as the amount of inhibitor that inactivates that amount of ficin that hydrolyzes 0.5 μmol of *N*-benzoyl-L-arginine-*p*-nitroanilide min^{-1} mL^{-1} at 30 °C.

For sequence analysis, 20 mg of α_2-thiol proteinase inhibitor was reduced and carboxymethylated according to Crestfield et al. (1963). The heavy chain and the light chain were separated by gel filtration on a Sephacryl S-200 column (1.5 × 140 cm) previously equilibrated with 35% formic acid. The fractions containing the heavy and light chains were then lyophilized. Automated Edman degradations on the heavy chain (1 mg) and the light chain (230 μg) were carried out with a Beckman sequenator, Model 890C, and phenylthiohydantoin amino acids were identified and quantitated by high-performance liquid chromatography (Gasukuro Kogyo Ltd., Tokyo) according to Tarr (1975).

Preparation of Antibodies. Antibodies against human α_2-thiol proteinase inhibitor were prepared from rabbits that had been injected subcutaneously with 150–250 μg of purified α_2-thiol proteinase inhibitor with Freund's complete adjuvant every 2 weeks for 8 weeks. The antisera obtained from the rabbits were partially purified by subsequent ammonium sulfate fractionation and DEAE-cellulose column chromatography. The γ-globulin fraction was then applied to a Sepharose column (1.4 × 9 cm) linked with purified α_2-thiol proteinase inhibitor as ligands. The column was previously equilibrated with 0.1 M Tris-HCl, pH 8.9, containing 0.5 M NaCl. The column was then washed with the same buffer and eluted with 0.1 M glycine hydrochloride, pH 2.75. The fractions containing immunoglobulin were immediately mixed with 1 M Tris-HCl, pH 7.5, followed by dialysis against 0.02 M sodium borate containing 0.15 M NaCl, pH 8.2. The affinity-purified antibody readily inhibited the activity of α_2-thiol proteinase inhibitor as well as α_1-thiol proteinase inhibitor. A double-immunodiffusion test for α_2-thiol proteinase inhibitor and LMW kininogen was carried out by the method of Ouchterlony (1958). Human LMW kininogen and its rabbit antibody were generously provided by Drs. Sakamoto and Nishikaze at Hokkaido University and prepared according to their method (Sakamoto & Nishikaze, 1979).

Screening of λgt11 cDNA Library. A λgt11 cDNA library containing cDNA inserts from human liver mRNA was kindly provided by Dr. Savio L. C. Woo. Approximately one million phage were screened for α_2-thiol proteinase inhibitor by using affinity-purified antibody according to the methods of Young & Davis (1983a,b). The affinity-purified antibody was labeled with ^{125}I to a specific activity of 6×10^7 cpm/μg and used to screen filters containing phage plated at a density of 5×10^4 plaques per 150-mm plate. Positive clones were isolated and plaque purified. Iodine-125 as sodium iodide was purchased from Amersham.

DNA Sequence Analysis. Phage DNA was prepared from positive clones by the plate–lysate method (Maniatis et al., 1982), followed by banding on a cesium chloride step gradient as described by Degen et al. (1983).

The cDNA insert was isolated by digestion with *Eco*RI and subcloned into plasmid pUC9 (Vieira & Messing, 1982). Appropriate restriction fragments from the insert were subcloned into M13mp18 and M13mp19 for sequencing by the dideoxy method (Sanger et al., 1977). Sequencing reactions were carried out with [^{35}S]dATPαS and run on 0.5–2.5 × 0.089 M Tris–borate, 0.089 M boric acid, and 0.002 M EDTA buffer, pH 8.3, gradient gels (Biggin et al., 1983). Over 90% of each strand of the cDNA insert was sequenced two or more times. M13mp18, M13mp19, and [^{35}S]dATPαS were purchased from Amersham. Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, bacterial alkaline phosphatase, *Escherichia coli* DNA polymerase I (Klenow fragment), and other enzymes were purchased from New England Biolabs, Bethesda Research Laboratories, or Amersham. Deoxynucleotide triphosphates and dideoxynucleotide triphosphates were purchased from P-L Biochemicals. DNA sequences were stored and analyzed by the computer programs of Staden (1977, 1978).

Containment. Experiments were performed in compliance with NIH Guidelines for Recombinant Research.

Results and Discussion

In order to facilitate the cloning of cDNAs and gene(s) coding for plasma thiol proteinase inhibitors, we have isolated human α_2-thiol proteinase inhibitor from fresh human plasma with a newly improved procedure. The overall yield for α_2-thiol proteinase inhibitor was 15–20% and about 17 mg of α_2-thiol proteinase inhibitor was routinely isolated from 1 L of fresh plasma. The purified α_2-thiol proteinase inhibitor had a specific activity of 0.3–0.35 unit/mg and showed a single protein band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis with an approximate molecular weight of 64 000–65 000. We have prepared affinity-purified antibody

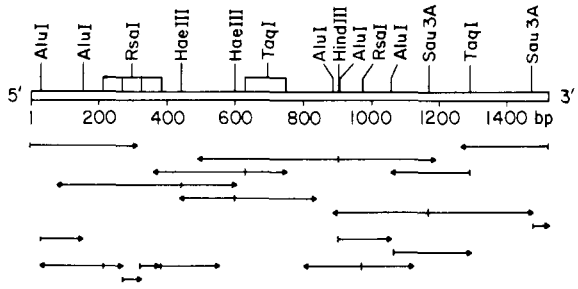

FIGURE 1: Restriction map and sequencing strategy for the cDNA insert in λHTPI·1529. The extent of sequencing is shown by the length of each arrow, and the direction of the arrow indicates the strand that was sequenced from each of the restriction fragments.

against the purified α_2-thiol proteinase inhibitor and also carried out amino acid sequence analyses on the heavy and light chains of α_2-thiol proteinase inhibitor. By automated Edman degradation analyses, the amino terminus of the heavy chain of α_2-thiol proteinase inhibitor was found to be blocked in an unknown manner. Serine, however, was identified as the amino-terminal residue of the light chain. The amino acid sequence of the first 24 residues of the light chain was Ser-Ser-Arg-Ile-Gly-Glu-Ile-Lys-Glu-Glu-Thr-Thr-Ser-His-Leu-Arg-Ser-Cys-Glu-Tyr-Lys-Gly-Arg-Pro. The yield for residues 1–24 was 0.2, 0.2, 0.1, 0.5, 0.4, 0.4, 0.5, 0.2, 0.3, 0.3, 0.1, 0.1, 0.05, 0.05, 0.2, 0.05, 0.02, not determined, 0.1, 0.05, 0.05, 0.05, 0.02, and 0.03 equiv per 4200 g of the light chain, respectively. The affinity-purified antibody and the partial amino acid sequence of α_2-thiol proteinase inhibitor were then used to isolate and identify human cDNA clones as described below.

A human liver cDNA library cloned into a λgt11 phage was screened for α_2-thiol proteinase inhibitor by employing the antibody screening technique of Young & Davis (1983a,b). In these studies, a ^{125}I-labeled affinity-purified rabbit antibody was used to detect phage plaques directing the synthesis of a fusion protein of β-galactosidase and α_2-thiol proteinase inhibitor. Eighteen positive clones were isolated, and each phage was plaque purified. One clone, named λHTPI·1529 (human thiol proteinase inhibitor 1529), gave a strong signal with the antibody probe and contained an insert of approximately 1500 base pairs. It was subcloned into pUC9 and further characterized by restriction mapping and DNA sequencing employing the strategy shown in Figure 1. The DNA sequence analysis indicated that this clone contained DNA sequences corresponding to the amino acid sequence of the amino-terminal region of the light chain of α_2-thiol proteinase inhibitor. These experiments, shown in Figure 2, indicated the presence of a Ser-Ser-Arg-Ile-Gly-Glu-Ile-Lys-Glu sequence starting at amino acid residue 390. This sequence corresponded to the amino-terminal region of the light chain of α_2-thiol proteinase inhibitor.

The complete sequence for the insert in λHTPI·1529 showed considerable amino acid sequence homology to bovine LMW kininogen (Figure 3), a plasma protein recently cloned by Nawa et al. (1983). A comparison of the human and bovine cDNAs then made it possible to identify the initiator methionine in λHTPI·1529 (Figure 2). The cDNA insert in λHTPI·1529 was composed of 45 base pairs of 5′ noncoding sequence, 1281 base pairs coding for pre α_2-thiol proteinase inhibitor, a stop codon, 160 base pairs of 3′ noncoding sequence, and 40 base pairs of poly(A) tail. The polyadenylation or processing sequence of AATAAA was present 18 base pairs upstream from the poly(A) tail. A detailed comparison of the human and bovine proteins indicated 110 changes in amino acid sequence, in addition to one insertion (Gln, residue 22) in the human α_2-thiol proteinase inhibitor (Figure 3). The overall homology between human α_2-thiol proteinase inhibitor and bovine LMW kininogen, including the signal peptide, is approximately 74%. Human α_2-thiol proteinase inhibitor, however, was 10 amino acid residues shorter than bovine LMW kininogen at its carboxyl-terminal end. Other than this difference, their homologous sequences were spread uniformly throughout both molecules, including the nonapeptide of bradykinin.

In a double-immunodiffusion experiment, human α_2-thiol proteinase inhibitor and human LMW kininogen formed fused precipitin lines with each other. Furthermore, human LMW kininogen, isolated by using an assay for released bradykinin, also showed a strong inhibitory activity toward ficin. Thus, α_2-thiol proteinase inhibitor was shown to be identical with LMW kininogen in amino acid sequence and biological activity.

In the presence of kallikrein, HMW kininogen is converted to a two-chain form with a heavy chain and a light chain held together by a disulfide bond. This results in the release of bradykinin and fragment 1.2, a histidine-rich fragment (Kato et al., 1981; Muller-Estrel et al., 1983). LMW kininogen, which lacks a histidine-rich sequence, is also converted by kallikrein to a two-chain form with a heavy chain and a light chain held together by a disulfide bond. In this case, however, only bradykinin is released. Bradykinin moieties and the heavy chains of bovine HMW kininogen and LMW kininogen are identical. The light chains, however, are different in size and only the first 12 amino acid residues at the amino-terminal end are identical (Nawa et al., 1983; Kitamura et al., 1983). Kitamura et al. (1983) also suggested that bovine HMW kininogen and LMW kininogen are encoded by a single gene with alternative expression mechanisms. Although only a limited amount of information on human HMW kininogen is available at the present time (Kato et al., 1981; Muller-Estrel et al., 1983), it appears that a similar structural relationship exists for human LMW kininogen and HMW kininogen. The light chain of human HMW kininogen, however, seems to retain its histidine-rich region during its reaction with kallikrein. The blocked amino-terminal amino acid residue of human HMW kininogen was reported to be pyroglutamic acid (Mori et al., 1981). Accordingly, Nawa et al. (1983) suggested that a possible site for cleavage of the signal peptide in bovine LMW kininogen is between Ser-22 and Gln-23, since the amino-terminal sequence is known to be pyroglutamic acid–glutamic acid (pyroGlu-Glu) (Kato, 1980). It seems likely, however, that the cleavage site in human α_2-thiol proteinase inhibitor by signal peptidase is between Thr-18 and Gln-19, giving rise to an amino-terminal sequence of pyroGlu-Glu. A stop codon of TAG is present in the 5′ end of the cDNA starting with nucleotide 40. This indicates that the Met codon at nucleotide residue 46 is the first amino acid to be translated, yielding a signal peptide that is probably 18 amino acid residues long. On the basis of these data, a tentative amino acid composition for the α_2-thiol proteinase inhibitor was calculated as follows: Asp_{20}, Asn_{23}, Thr_{34}, Ser_{32}, Glu_{32}, Gln_{22}, Pro_{22}, Gly_{20}, Ala_{25}, Val_{25}, Met_3, Ile_{22}, Leu_{22}, Tyr_{15}, Phe_{18}, Lys_{32}, His_5, Arg_{16}, Trp_3, and $^1/_2$-Cys_{18}. This corresponds to a molecular weight of 45 806 without carbohydrate and 51 761 with the addition of 13% carbohydrate. The total number of amino acid residues is 409.

Human α_2-thiol proteinase inhibitor contains four potential sites for attachment of carbohydrate chains to asparagine. These four sequences are located at residues 48, 169, 205, and 294 (Figure 2). The same sequences are also present in the

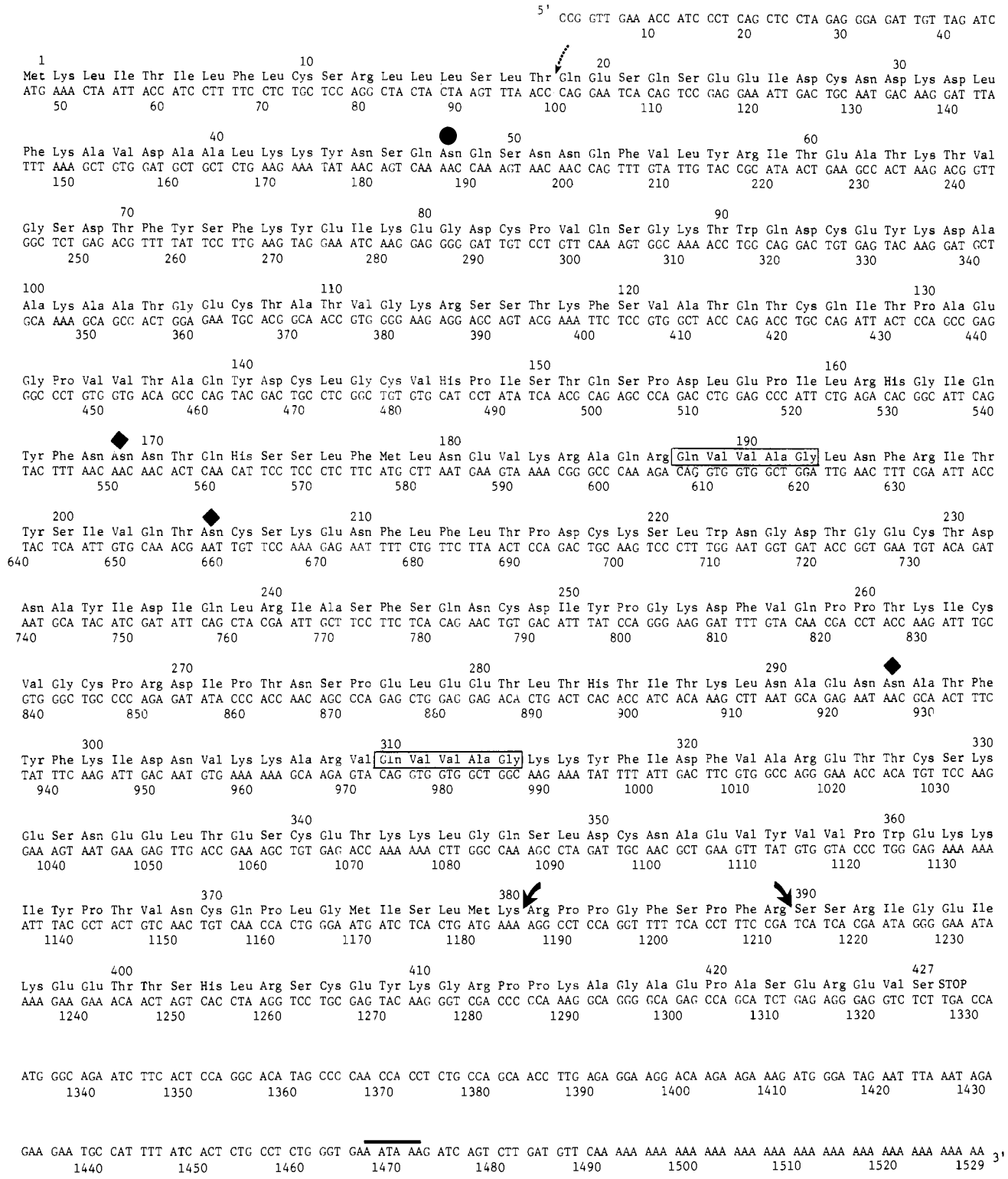

FIGURE 2: Complete nucleotide sequence of the cDNA insert in λHTPI-1529. The predicted amino acid sequence is shown starting with a methionine residue that is encoded by the first ATG codon following a stop codon (TAG) in the same reading frame. The proposed cleavage site by signal peptidase is shown by a dotted arrow. Potential carbohydrate binding sites to Asn residues are shown by solid diamonds. The solid heavy arrows show the sites cleaved by kallikrein to release bradykinin, a vasoactive nonapeptide. The boxed sequences indicate the proposed reactive sites of the inhibitor. The polyadenylation or processing sequence of AATAAA is shown by a bar.

corresponding positions of bovine LMW kininogen except at residue 294. The human molecule lacks the potential carbohydrate binding sites that are present in bovine LMW kininogen at amino acid residues 88, 170, and 198 (Figure 3). The locations of other carbohydrate chains, if any, that are attached to threonine or serine residues are not clear at the present time. A number of such carbohydrate chains are found in bovine HMW kininogen (Kato et al., 1981).

The amino acid sequence of α_2-thiol proteinase inhibitor contained two repeat sequences that occur in tandem (Figure 4). Both sequences (amino acid residues 104–225 and 226–347) consist of 122 amino acid residues with 42 identical amino acids. This corresponds to a homology of 34%. All six cysteine residues in the two repeat sequences are conserved. This observation suggests that gene duplication has occurred in this protein during evolution. The same gene duplication

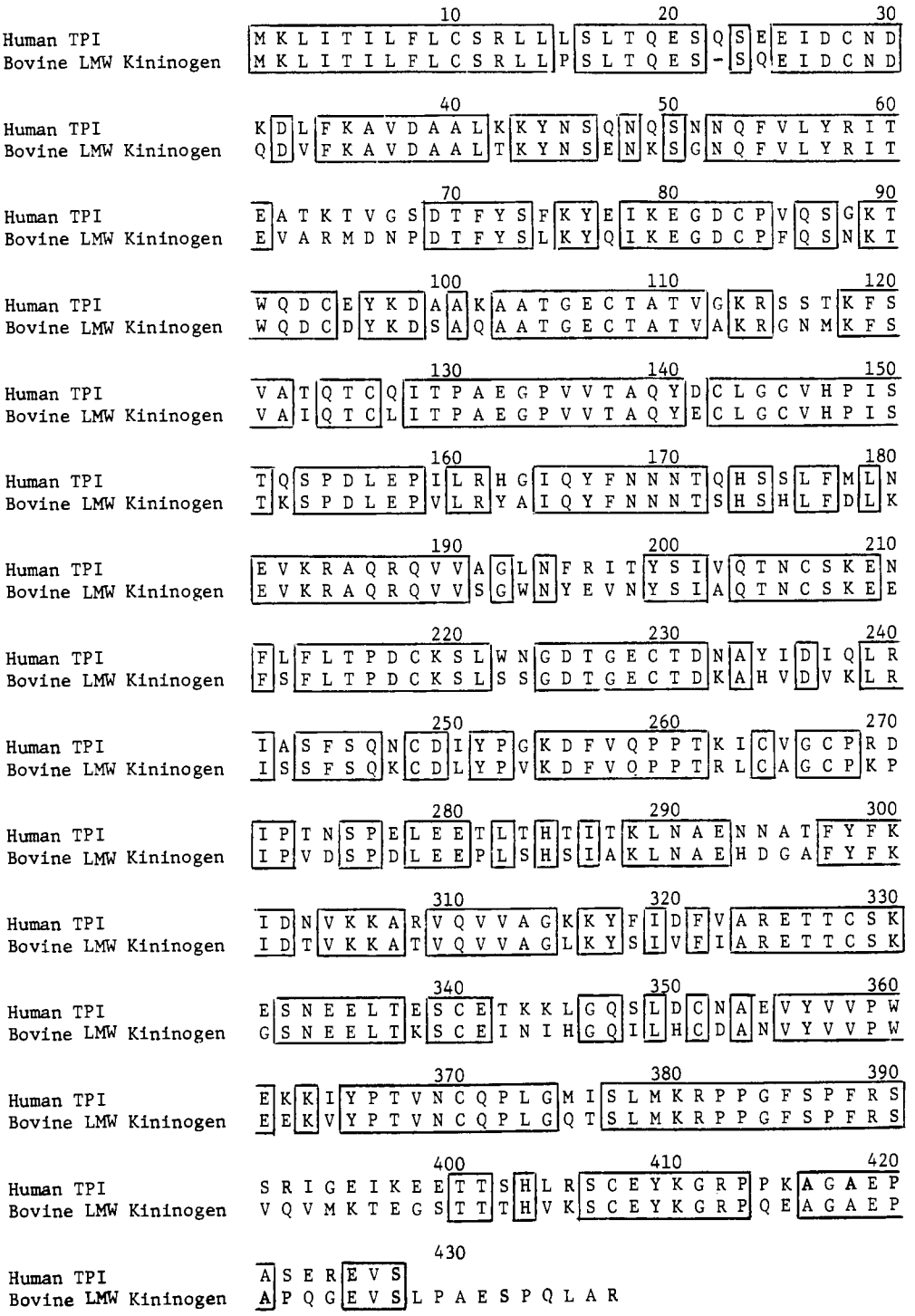

FIGURE 3: Amino acid sequences of human α_2-thiol proteinase inhibitor and bovine low molecular weight kininogen. The sequence of bovine LMW kininogen is from Nawa et al. (1983). The sequences are positioned with the appropriate gaps to show maximal homology. Common residues are enclosed in boxes. Amino acid sequences are shown with the standard single letter for amino acid residues as follows: Ala, A; Arg, R; Asn, N; Asp, D; Cys, C; Gln, Q; Glu, E; Gly, G; His, H; Ile, I; Leu, L; Lys, K; Met, M; Phe, F; Pro, P; Ser, S; Thr, T; Trp, W; Tyr, Y; Val, V.

was also observed in the gene for bovine LMW kininogen (Nawa et al., 1983). Nawa et al. (1983) also reported that bovine LMW kininogen is encoded by at least two very similar but distinct mRNAs. Whether or not this is also true for human kininogen is not clear at the present time.

Recently, several different thiol proteinase inhibitors with low molecular weights (10000–14000) have been isolated from human serum (human cystatin), human polymorphonuclear granulocyte (stefin), chicken egg white (chicken cystatin), rat liver, and rat epidermis. The complete amino acid sequences of these inhibitors have also been established (Brzin et al., 1983, 1984; Machleidt et al., 1983; Turk et al., 1983; Gauthier et al., 1983; Takio et al., 1983, 1984).

When the two tandemly repeating sequences of α_2-thiol proteinase inhibitor were compared with these low molecular weight proteinase inhibitors, clear homologies were found (Figure 5). Among these sequences, the highly conserved amino acid sequences for these inhibitors were Gln-Val-Val-Ala-Gly. A clear conservation of the sequence strongly suggests that this site may be the reactive site for these thiol proteinase inhibitors. This further suggests that α_2-thiol proteinase inhibitor may have two reactive sites per molecule

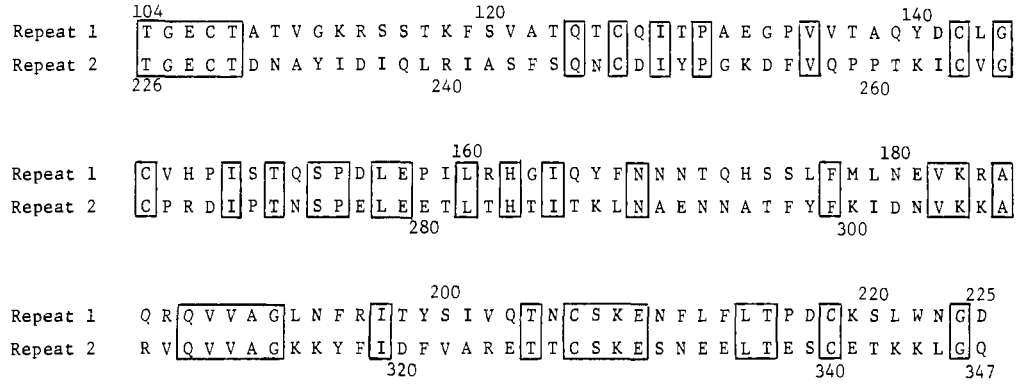

FIGURE 4: Comparison of amino acid sequences of the two internal repeats in α_2-thiol proteinase inhibitor. The internally homologous sequences are designated as repeats 1 and 2. The numbering system for amino acid residues is as in Figure 2. Amino acid residues that are identical in the two repeated regions are enclosed in boxes.

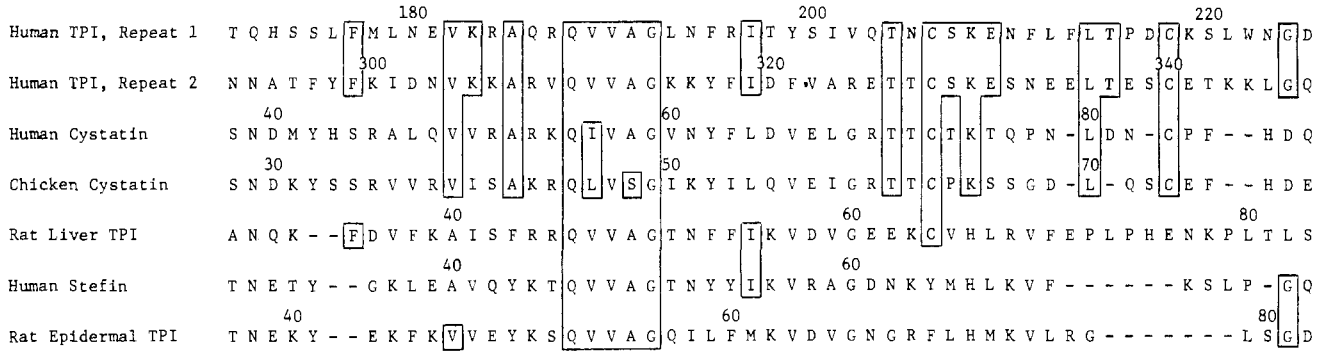

FIGURE 5: Comparison of amino acid sequences of repeat 1 and repeat 2 in human α_2-thiol proteinase inhibitor with the corresponding sequences of human and chicken cystatins, rat liver thiol proteinase inhibitor, human stefin, and rat epidermal thiol proteinase inhibitor. Only the regions that contain significant homologies are shown for each inhibitor. The amino acid residues that are identical with those in repeats 1 and 2 of α_2-thiol proteinase inhibitor are enclosed in boxes. The numbering systems for the amino acid residues are as in the original report for each protein (see below). The sequences are positioned with the appropriate gaps to show maximal homology. The sequences of human cystatin, chicken cystatin, rat liver thiol proteinase inhibitor, human stefin, and rat epidermal thiol proteinase inhibitor are quoted from Brzin et al. (1984), Turk et al. (1983), Takio et al. (1983), Machleidt et al. (1983), and Takio et al. (1984), respectively.

starting with Gln-188 and Gln-310.

A kinetic analysis of the inhibition of thiol proteinase inhibitors with α_2-thiol proteinase inhibitor has shown that 1 molecule of inhibitor will inactivate 1.6 molecules of ficin (data not shown). A further detailed kinetic analysis is in progress in our laboratory.

As shown in Figure 5, the middle part of the repeat sequences of α_2-thiol proteinase inhibitor shows the highest homology to human cystatin. It is also interesting to note that repeat 1 has a higher level of homology (33%) than repeat 2 (24%) within their limited region surrounding the proposed reactive site. The well-conserved amino acid sequences also included two Cys residues, as shown in Figure 5. These data suggest that the two inhibitory domains of α_2-thiol proteinase inhibitor belong to the cystatin type.

It is also very interesting to note that HMW kininogen appears to lack inhibitory activity toward the thiol proteinase, ficin (unpublished data). Further studies are required to determine whether or not the much longer light chain of HMW kininogen, compared to that of LMW kininogen, is covering up the inhibitory domains in the heavy chain, thus inhibiting a free access of reactive sites to the active site of thiol proteinases.

At the present time, it is not clear whether or not α_1-thiol proteinase inhibitor is a derivative of LMW kininogen or HMW kininogen. α_2-Thiol proteinase inhibitor$_1$ appears to be a molecular species with one cleavage of a peptide bond (Arg_{389}-Ser_{390}) between its bradykinin and light chain, and α_2-thiol proteinase inhibitor$_2$ is a molecular species that has a heavy chain and a light chain held together by a disulfide bond but no bradykinin moiety.

As discussed above, LMW kininogen is a thiol proteinase inhibitor in addition to its function as a source for vasoactive bradykinin. It is extremely intriguing to speculate as to its physiological function, particularly in inflammation in which many thiol proteinases, including lysosomal cathepsins, are released and must be regulated.

Acknowledgments

We thank Drs. Savio L. C. Woo and Vincent Kidd for kindly providing the λgt11 cDNA library. We also thank Drs. Earl W. Davie and Akira Makita for their continuous encouragement and support and Dr. Torben Petersen for suggesting the similarities between α_2-thiol protease inhibitor and LMW kininogen. Thanks are also due to Drs. Dominic Chung, Shinji Yoshitake, Akitada Ichinose, and Steve Leytus for their help and discussions and to Drs. Wataru Sakamoto and Osamu Nishikaze for kindly providing human low molecular weight kininogen and its antibody.

References

Biggin, M. D., Gibson, T. J., & Hong, G. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3963.

Brzin, J., Kopitar, M., & Turk, V. (1983) *Hoppe-Seyler's Z. Physiol. Chem. 364*, 1475.

Brzin, J., Popovic, T., Turk, V., Borchart, U., & Machleidt, W. (1984) *Biochem. Biophys. Res. Commun. 118*, 103.

Crestfield, A. M., Moore, S., & Stein, W. H. (1963) *J. Biol. Chem. 238*, 622.

Degen, S. J. F., MacGillivray, R. T. A., & Davie, E. W. (1983) *Biochemistry 22*, 2087.

Gauthier, F., Pagano, M., Esnard, F., Moury, H., & Engler, R. (1983) *Biochem. Biophys. Res. Commun. 110*, 449.
Hirado, M., Iwata, D., Niinobe, M., & Fujii, S. (1981) *Biochim. Biophys. Acta 669*, 21.
Järvinen, M. (1979) *FEBS Lett. 108*, 461.
Kato, H. (1980) *Seikagaku 52*, 1173.
Kato, H., Nagasawa, S., & Iwanaga, S. (1981) *Methods Enzymol. 80*, 172.
Katunuma, N., Wakamatsu, K., Takio, K., Titani, K., & Kominani, E. (1983) in *Proteinase Inhibitors* (Katunuma, N., Umezawa, H., & Holzer, H., Eds.) pp 135–145, Japan Scientific Society Press, Tokyo.
Kitamura, N., Takagaki, Y., Furuto, S., Tanaka, T., Nawa, H., & Nakanishi, S. (1983) *Nature (London) 305*, 545.
Lenney, J. F., Liao, J. R., Sagg, S. L., Gopalakrishnan, V., Wong, H. C. H., Ouge, K. H., & Chan, P. W. H. (1982) *Biochem. Biophys. Res. Commun. 108*, 1581.
Machleidt, W., Borchat, U., Fritz, H., Brzin, J., Ritonja, A., & Turk, V. (1983) *Hoppe-Seyler's Z. Physiol. Chem. 364*, 1481.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) in *Molecular Cloning, A Laboratory Manual*, pp 371–372, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Mori, K., Sakamoto, W., & Nagasawa, S. (1981) *J. Biochem. (Tokyo) 90*, 503.
Muller-Estrel, M., Dittmann, B., Fritz, H., Lottspeich, F., & Henschen, A. (1983) *Adv. Exp. Med. Biol. 156A*, 157.
Nawa, H., Kitamura, N., Hirose, T., Asai, M., Inayama, S., & Nakanishi, S. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 90.
Ouchterlony, O. (1958) *Prog. Allergy 5*, 1–78.
Ryley, M. C. (1979) *Biochem. Biophys. Res. Commun. 89*, 871.
Sakamoto, W., & Nishikaze, O. (1979) *J. Biochem. (Tokyo) 86*, 1549.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463.
Sasaki, M., Minakata, K., Yamamoto, H., Niwa, M., Kato, T., & Ito, N. (1977) *Biochem. Biophys. Res. Commun. 76*, 917.
Sasaki, M., Taniguchi, K., & Minakata, K. (1981) *J. Biochem. (Tokyo) 89*, 169.
Sasaki, M., Taniguchi, K., Suzuki, K., & Imahori, K. (1983) *Biochem. Biophys. Res. Commun. 110*, 256.
Staden, R. (1977) *Nucleic Acids Res. 4*, 4037.
Staden, R. (1978) *Nucleic Acids Res. 5*, 1013.
Takio, K., Kominani, E., Wakamatsu, N., Katunuma, N., & Titani, K. (1983) *Biochem. Biophys. Res. Commun. 115*, 902.
Takio, K., Kominami, E., Bando, Y., Katunuma, N., & Titani, K. (1984) *Biochem. Biophys. Res. Commun. 121*, 149.
Tarr, G. E. (1975) *Anal. Biochem. 63*, 361.
Turk, V., Brzin, J., Longer, M., Ritona, A., Eropkin, M., Borchart, U., & Machleidt, W. (1983) *Hoppe-Seyler's Z. Physiol. Chem. 364*, 1487.
Vieira, J., & Messing, J. (1982) *Gene 19*, 259.
Wakamatsu, N., Kominami, E., & Katunuma, N. (1982) *J. Biol. Chem. 257*, 14653.
Young, R. A., & Davis, R. W. (1983a) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1194.
Young, R. A., & Davis, R. W. (1983b) *Science (Washington, D.C.) 222*, 778.

Articles

Effect of Polyamines and Basic Proteins on Cleavage of DNA by Restriction Endonucleases†

Alfred Pingoud,* Claus Urbanke, Jürgen Alves, Hans-Jürgen Ehbrecht, Marc Zabeau,‡ and Claudio Gualerzi§

ABSTRACT: We have investigated the effect of the polyamines spermine, spermidine, and putrescine and the prokaryotic histone-like proteins NS1 and NS2 on the restriction endonuclease *Eco*RI catalyzed cleavage of plasmid and bacteriophage DNAs. At low concentrations of spermine and spermidine, the rate of DNA cleavage by *Eco*RI is increased, while high concentrations of spermine as well as of spermidine are inhibitory. These phenomena are also observed with other restriction endonucleases. They are, therefore, probably due to the interaction of the polyamines with the DNA. Putrescine does not have such an effect within the concentration range investigated. Remarkably, low concentrations of spermine and spermidine very efficiently suppress *Eco*RI* activity. An inhibition of the *Eco*RI-catalyzed cleavage of DNA is also observed with NS1 and NS2, an effect that can be mimicked with other basic proteins that interact with DNA. The results are discussed in terms of the mechanism of restriction in vivo.

Class II restriction endonucleases cleave DNA with very high specificity at their respective recognition sites. Few enzyme activities have been described so far that discriminate as effectively between similar substrates as restriction endonucleases: it has been estimated for the *Eco*RI endonuclease that its reactivity toward its recognition site is at least 10^7 times higher than that at alternative sequences (Halford, 1980). The enormous specificity of restriction endonuclease is presumably due to several hydrogen bonds formed between the enzyme and the nucleotide bases of its substrate during the process of enzymatic catalysis (Seeman et al., 1976; Smith, 1979). A

† From the Abteilung Biophysikalische Chemie, Zentrum Biochemie, Medizinische Hochschule Hannover, D-3000 Hannover, FRG. *Received January 18, 1984.* This work was supported by grants from the Deutsche Forschungsgemeinschaft (Pi 122/2-1) and the Fonds der Chemischen Industrie. This paper is dedicated to Prof. Dr. Günter Maass on the occasion of his 50th birthday.
‡ EMBL Heidelberg, D-6900 Heidelberg, FRG.
§ Max-Planck Institut für Molekulare Genetik, D-1000 Berlin, FRG.

0006-2960/84/0423-5697$01.50/0 © 1984 American Chemical Society