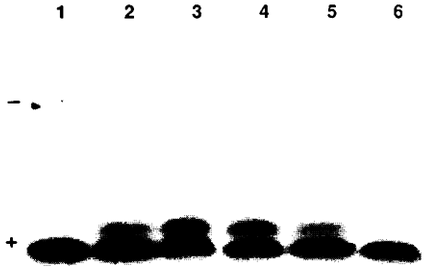

Fig. 5. Expression and phosphorylation of Gα2 from an extrachromosomal plasmid in a Gα2$^-$ cell line. Lanes 1 to 6 correspond to sampling times of 0, 30 s, 1 min, 2 min, 5 min, and 15 min after the addition of 1 μ*M* cAMP to basal state cells. The Gα2$^-$ cell line was generated by homologous recombination with a truncated Gα2 sequence. These cells were complemented by means of a full-length, wild-type Gα2 sequence inserted into an extrachromosomal vector (*16*). The cells were differentiated as described in Fig. 1, except that they were pulsed with 50 n*M* cAMP at 6-min intervals as previously reported (*19*). Immunoblots prepared as described in Fig. 2. The minus sign indicates the Gα2$^-$ cell line; the plus sign indicates the addition of the Gα2 plasmid.

tion occurs through conformational changes induced by GTP binding and hydrolysis. Our results suggest that, in vivo, there may exist an additional level of regulation of this pathway, α-subunit phosphorylation.

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Tick Anticoagulant Peptide (TAP) Is a Novel Inhibitor of Blood Coagulation Factor Xa

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A low molecular weight serine protease inhibitor (TAP) was purified from extracts of the soft tick, *Ornithodoros moubata*. The peptide is a slow, tight-binding inhibitor, specific for factor Xa (K_i = 0.588 ± 0.054 n*M*). The inhibitor also acts as an anticoagulant in several human plasma clotting assays in vitro. Its amino acid sequence (60 residues) has limited homology to the Kunitz-type inhibitors. However, unlike other inhibitors of this class, TAP inhibits only factor Xa. It had no effect at a 300-fold molar excess on factor VIIa, kallikrein, trypsin, chymotrypsin, thrombin, urokinase, plasmin, tissue plasminogen activator, elastase, or *Staphylococcus aureus* V8 protease. TAP's specificity and size suggest that it may have therapeutic value as an anticoagulant.

TICKS ARE HEMATOPHAGOUS ARthropods that must overcome host hemostasis in order to locate blood and maintain its flow during ingestion. Antihemostatic factors in the saliva of these and other blood-feeding organisms are probably directed toward platelet aggregation, coagulation, and vascular contraction. Although the mechanics and physiology of the blood-sucking process in ticks has been well studied (*1*), little is known about the sites of action or the molecular properties of the anticoagulant substances produced by these animals.

Inhibitors of factor Xa (*2, 3*) and thrombin (*4*) have been identified and partially purified from tick saliva. Tick saliva also blocks clotting via the intrinsic pathway (*5*), perhaps by inhibiting factor IXa (*4*). Antiplatelet activity that blocks platelet aggregation induced by adenosine diphosphate, collagen, or platelet-activating factor is also present (*5*).

To better define the anticoagulant activities present in ticks, a crude soluble extract of whole ticks was prepared and fractionated by gel filtration on Sephadex G-50. When selected column fractions were assayed, a peak with the ability to inhibit factor Xa (*6*) eluted with an apparent molecular size of 8,000 to 10,000. Peaks of both thrombin and platelet aggregation inhibitory activity were identified that were somewhat larger in size, 15,000 to 20,000. Trypsin inhibitory activity was also monitored, and was present throughout the column. Plasminogen activator activity was not detected in crude extracts or in column fractions.

The factor Xa inhibitory activity was purified by a combination of gel filtration, anion-exchange chromatography, and reversed-phase high-performance liquid chromatography (RP-HPLC). Most of the inhibitory activity eluted in one peak on RP-HPLC (Fig. 1A). This material was rechromatographed to obtain a homogeneous preparation of the protein (Fig 1B). We estimate that 200 to 250 μg of the purified tick anticoagulant peptide (TAP) could be obtained from 500 ticks. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) showed a single polypeptide with a molecular size of 6000 (Fig. 1C).

The purified TAP was used to characterize its inhibitory properties. The inhibition of factor Xa activity by TAP was concentration-dependent (Fig. 2). Preliminary evidence suggested that the inhibition of factor Xa was reversible and stoichiometric. An intrinsic inhibition constant K_i of 0.588 ± 0.054 n*M* was calculated by the Morrison method for tight-binding inhibi-

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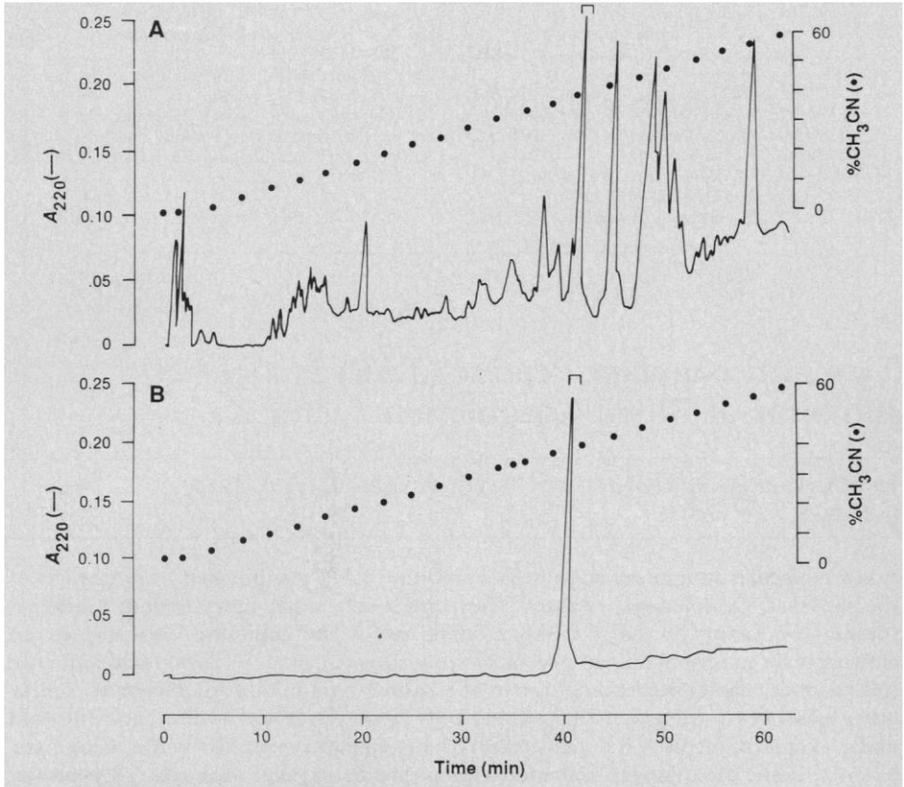

Fig. 1. Purification of the tick factor Xa inhibitor. Five hundred ticks (~8 g) were homogenized in a ground glass homogenizer in 20 m*M* Bis-tris (*p*H 7.0) containing 0.15*M* NaCl and 50 μ*M* E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane], 50 μ*M* pepstatin, and 50 μ*M* chymostatin. The homogenate was centrifuged, and the resultant pellets were reextracted in 3 ml of buffer and recentrifuged. The combined supernatants were diluted four times with water and applied to a 20-ml column of Fast Q (Pharmacia) equilibrated with 20 m*M* Bis-tris-HCl (*p*H 6.0). Bound proteins were eluted with 0.6*M* NaCl, sucrose was added to a final concentration of 10 mg/ml, and the eluate was lyophilized. This material was dissolved in water and applied to a column of Sephadex G-50 (Pharmacia) equilibrated in 20 m*M* tris-HCl (*p*H 7.4) containing 25 m*M* NaCl. Fractions were collected and samples from selected fractions were assayed for their effect on factor Xa. The fractions containing factor Xa inhibitory activity were pooled and applied to a column of Mono Q equilibrated in 20 m*M* Bis-tris-HCl (*p*H 6.0). The column was eluted with a gradient of NaCl (0 to 0.6*M*). The material that inhibited factor Xa eluted at 0.25*M* NaCl, was dialyzed, lyophilized, and dissolved in water and applied to a Vydac C_{18} column equilibrated in 0.1% trifluoroacetic acid. (**A**) The proteins were eluted with a gradient of acetonitrile (0 to 60%) in 0.1% trifluoroacetic acid. (**B**) The active region (in brackets) was rechromatographed under the same conditions. (**C**) SDS-PAGE (*20*) of purified tick factor Xa inhibitor (TAP). The purified inhibitor (3 μg) was detected by staining with Coomassie blue. The gel was calibrated with prestained proteins whose apparent molecular sizes (kilodaltons) were supplied by the manufacturer, Bethesda Research Labs.

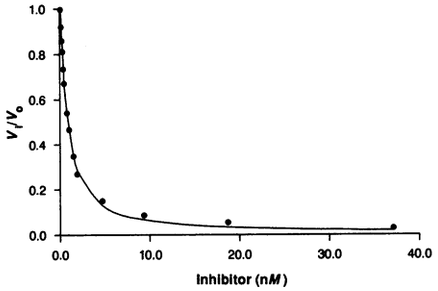

Fig. 2. Concentration-dependent inhibition of human factor Xa by TAP. Factor Xa (500 p*M*) was incubated for 30 min with increasing amounts of purified inhibitor (90 p*M* to 37 n*M*), and the residual activity was determined by measuring an initial velocity for 5 min following the addition of substrate (Spectrozyme Xa) at a concentration equal to five times its K_m. Determination of the intrinsic K_i was done by fitting the observed data (●) with nonlinear regression analysis to the ratio of initial velocity (V_i) to observed velocity (V_o) described by the equation: $V_i/V_o = [E_t - I_t - K_i^*] + [(I_t + K_i^* - E_t)^2 + 4K_i^*E_t]^{1/2}$ derived by Morrison for tight-binding inhibition (*7*) (solid line), where E_t and I_t are the total amount of enzyme and inhibitor, respectively, included in the assay. Because an equilibrium between the inhibitor and enzyme was established before the addition of the substrate and the dissociation of the enzyme-inhibitor complex is slow, the value of K_i^* in the above equation represents the intrinsic K_i, regardless of whether the inhibitor acts competitively or noncompetitively. In addition, K_i^* represents a thermodynamically appropriate binding constant for the enzyme-inhibitor complex.

tion (*7*). Inhibition of factor Xa was also characterized by a slow onset that required at least a 15-min preincubation for maximal inhibition. Thus, TAP can be classified as a slow, tight-binding inhibitor (*8*).

The effect of TAP on various proteases also was tested. Even at a 300-fold molar excess of TAP over each protease, no inhibition of factor VIIa, thrombin, chymotrypsin, elastase, trypsin, kallikrein, urokinase, plasmin, tissue plasminogen activator, or *Staphylococcus aureus* V8 protease was detected when we used the hydrolysis of chromogenic peptides to monitor enzyme activity (*6*). The inhibitor was also active in several human plasma–based clotting assays (Table 1). Prothrombin clotting time, activated partial thromboplastin time, and modified Stypven time were prolonged in a concentration-dependent manner.

Because of its small size, it was possible to determine most of the primary structure of the tick peptide by direct sequence analysis (Fig. 3). The peptide contains 60 amino acid residues and has a calculated molecular weight of 6850, a size consistent with that determined by SDS-PAGE (Fig. 1) and on gel filtration under nondenaturing conditions.

A comparison of the amino acid sequence of this new inhibitor with other polypeptide inhibitors of serine proteases showed that it may be related to the Kunitz family, but there are important differences (Fig. 4). Although similar in size to the Kunitz-type protease inhibitors, TAP is an acidic protein (*p*I 4.5), whereas the Kunitz inhibitors are generally highly basic (*9*). TAP appears to be specific for factor Xa, because it did not inhibit trypsin or any other serine protease tested. In contrast, Kunitz inhibitors, such as bovine pancreatic trypsin inhibitor (BPTI) and isoinhibitor K from snails, have a broad specificity (*9*). Trypstatin, a Kunitz inhibitor from mast cells, is a potent inhibitor of factor Xa ($K_i = 1.2 \times 10^{-10} M$) and also inhibits trypsin ($K_i = 1.4 \times 10^{-8} M$) (*10*). The leech protein antistasin, which has no homology to the inhibitor from ticks or Kunitz-type inhibitors, inhibits both factor Xa (*11*) and trypsin (*12*).

When compared with the sequence of snail isoinhibitor K, there are 14 identical residues as well as four conservative substitutions (Fig. 4). However, the invariant

Table 1. Effect of tick anticoagulant peptide on various plasma-based clotting assays (*21*). Values given are those obtained in a typical experiment.

TAP added (pmol)	Time to clot (s)		
	Prothrombin time	Activated partial thromboplastin time	Modified Stypven time
None	14.6	31.3	10.3
28.9	28.9	45.2	17.7
57.8	31.2	57.7	31.2
115	48.7	92.2	65.1

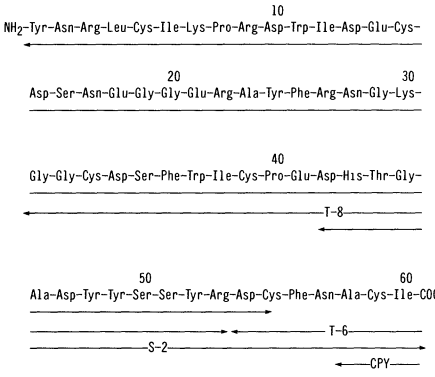

Fig. 3. Amino acid sequence of the factor Xa inhibitor from ticks. The sequence of 60 residues (*18*) results from analysis of the reduced and carboxamidomethylated peptide that provided good yields for 53 residues on an Applied Biosystems gas-phase sequenator. Two tryptic fragments and an overlapping peptide from a *S. aureus* V8 protease digest were isolated by fractionating digests by RP-HPLC on a Vydac C_{18} column and used to obtain the complete sequence. To confirm the order of the peptides shown, the COOH-terminal sequence of the intact peptide was determined by treatment of the performic acid–oxidized inhibitor with carboxypeptidase Y.

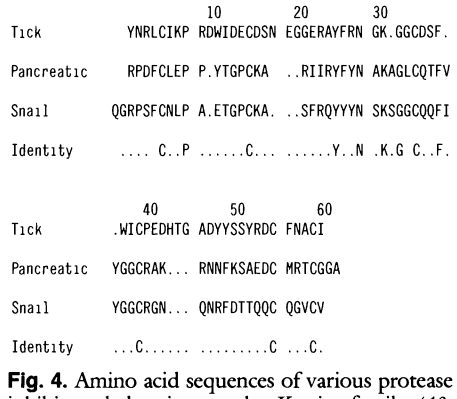

Fig. 4. Amino acid sequences of various protease inhibitors belonging to the Kunitz family (*13, 18*).

spacing of the Cys residues (*13*) is not maintained; only Cys^{55} and Cys^{59} have the same spacing as seen in the other Kunitz inhibitors. A Kunitz inhibitor from *Drosophila* that lacks the typical spacing between its first two Cys residues inhibits both trypsin and acrosin (*14*). A comparison with other Kunitz inhibitors provides an explanation for the inability of TAP protein to inhibit trypsin or kallikrein. BPTI, as well as other members of this family, forms a complex with trypsin or kallikrein in which a Lys or Arg residue that is always adjacent to the second Cys in the sequence is inserted into the active site of the protease where it interacts with the Asp residue in the enzyme's catalytic triad. Two potent Kunitz-type inhibitors of factor Xa, the peptide trypstatin (*10*) and the second domain of the lipoprotein-associated coagulation inhibitor (LACI) (*15*) present in plasma, both have an Arg residue after the second Cys. In TAP the corresponding amino acid is an Asp, having a negatively charged side chain that would not be readily accommodated by proteases that hydrolyze proteins on the COOH-terminal side of basic residues. TAP also did not inhibit *S. aureus* V8 protease, a serine protease that cleaves on the COOH-terminal side of acidic amino acids. Because TAP contains no Cys residues that are followed by an Arg, it is not possible to assign an active site in analogy to other members of this inhibitor family.

The tick peptide is also missing the Arg residues at positions 17 and 39 that in BPTI are responsible for maintaining electrostatic interactions between trypsin and this inhibitor (*16*). Since conversion of the active site Lys to a neutral residue preserves the inhibitory activity of BPTI (*17*), these other basic amino acids are also essential to inhibitor function. It is therefore possible that TAP does not interact with a P_1-reactive amino acid within the specificity pocket of factor Xa. Alternatively, it could contain a sequence that resembles the physiological substrate for factor Xa and thus compete for the enzyme's active site. However, an inspection of the primary structure shows no sequences that resemble one of the cleavage sites in prothrombin (YIDGRIVG or AIEGRTATS) (*18*). Thus, like the thrombin inhibitor hirudin, the interaction of TAP with factor Xa may be more complex, perhaps involving high and low affinity sites (*19*), in order to account for its high degree of specificity.

Ticks are vectors for numerous bacterial and viral infections, including Lyme disease, from which there is often no protection. It is not yet known whether antibodies to the purified peptide will block successful feeding of the tick or transmission of the infectious agent. The tick peptide (termed tick anticoagulant peptide, TAP) represents a novel anticoagulant whose effectiveness in vitro also makes it attractive to test in various in vivo models of occlusive vascular disease.

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18. Single-letter abbreviations for the amino acid resi-

dues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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22. We thank M. Sardana, J. Rodkey, and L. Wassel for amino acid and sequence analyses, C. Bennett for helpful discussions, and C. Hannan for helping to prepare this manuscript. R. Endris provided the identification of the species of tick used in this study.

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L-Cysteine, a Bicarbonate-Sensitive Endogenous Excitotoxin

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After systemic administration to immature rodents, L-cysteine destroys neurons in the cerebral cortex, hippocampus, thalamus, and striatum, but the underlying mechanism has never been clarified. This neurotoxicity of L-cysteine, in vitro or in vivo, has now been shown to be mediated primarily through the *N*-methyl-D-aspartate subtype of glutamate receptor (with quisqualate receptor participation at higher concentrations). In addition, the excitotoxic potency of L-cysteine was substantially increased in the presence of physiological concentrations of bicarbonate ion. L-Cysteine is naturally present in the human brain and in the environment, and is much more powerful than β-*N*-methylamino-L-alanine, a bicarbonate-dependent excitotoxin, which has been implicated in an adult neurodegenerative disorder endemic to Guam. Thus, the potential involvement of this common sulfur-containing amino acid in neurodegenerative processes affecting the central nervous system warrants consideration.

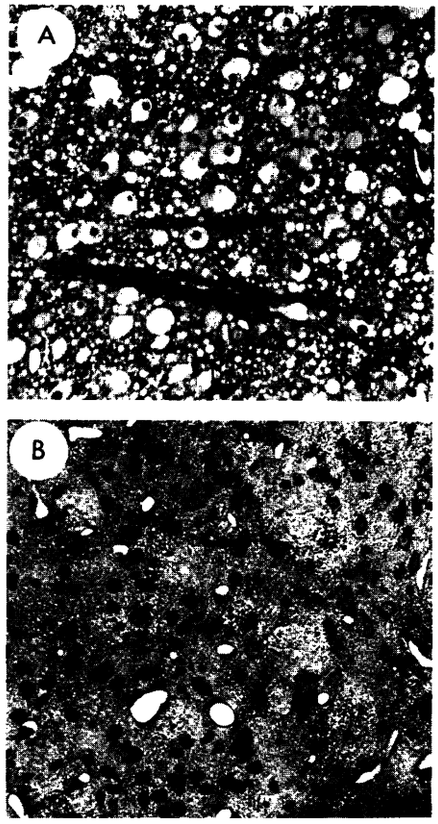

Fig. 1. (**A**) The caudate nucleus of a 4-day-old rat containing many acutely necrotic neurons ("bull's eye" profiles) 6 hours after L-Cys (1 g/kg) was administered subcutaneously. (**B**) In contrast, the caudate nucleus appears entirely normal in a 4-day-old rat 6 hours after a treatment consisting of the same dose of L-Cys accompanied by MK-801 (1 mg/kg). Magnification, ×180.

WHEN ADMINISTERED ORALLY IN high doses to infant mice, L-cysteine (L-Cys) reproduces the type of brain damage that is caused by glutamate (Glu) (*1*). This type of damage is typically restricted to circumventricular organ brain regions that lack blood-brain barriers and evolves very rapidly to reach end-stage neuronal necrosis within 2 to 3 hours. Paradoxically, lower doses of L-Cys resulted in a more devastating neurotoxic syndrome, which developed more slowly (4 to 6 hours) but damaged many more regions of the brain, including the cerebral cortex, hippocampus, caudate, and thalamus (*2*). L-Cys also causes a similarly widespread pattern of damage in the fetal rodent brain when administered orally or subcutaneously to the pregnant dam in late gestation (*2*). Curiously, neurons undergoing L-Cys–induced degeneration have an identical appearance by light or electron microscopy to those undergoing degeneration after exposure to Glu or various excitatory neurotoxic (excitotoxic) analogs of Glu. However, L-Cys has not been considered an excitotoxin because it lacks the Ω acidic group shared by all other excitotoxic analogs of Glu and it was not found to mimic the neuroexcitatory properties of Glu when administered microelectrophoretically onto spinal neurons (*3, 4*). The present experiments were undertaken to reexamine the neurotoxic properties of L-Cys in light of new information pertaining to excitotoxic mechanisms.

To determine whether a glutamate antagonist might protect against L-Cys neurotoxicity in vivo, we assigned 4-day-old Sprague-Dawley rat pups to one of two groups (ten per group) and treated one group subcutaneously (sc) with MK-801 (1 mg/kg), an antagonist of the *N*-methyl-D-aspartate subtype of glutamate receptor, and the other group with saline, then 30 min later we administered L-Cys (1 g/kg, sc) to both groups. The pups were returned to their mothers and observed for 6 hours, then anesthetized with halothane, and perfused with an aldehyde fixative solution; their brains were processed for histopathological evaluation (*4, 5*). All ten of the control pups had acute lesions in several brain regions; typically the most severely affected were the frontoparietal neocortex, hippocampus, thalamus, and caudate nucleus (Fig. 1A). None of the ten experimental pups sustained damage in any brain region (Fig. 1B). These findings implicate the NMDA receptor–ionophore complex in the in vivo neurotoxicity of L-Cys.

To evaluate mechanisms underlying L-Cys neurotoxicity in greater detail, we used the in vitro chick embryo retina, a preparation that is valuable for studying excitotoxic phenomena (*6*). Because the excitotoxic Glu agonists, NMDA, quisqualate (Quis), and kainic acid (KA), interact with different Glu-receptor subtypes that are distributed differently among retinal neurons, each agonist induces an acute cytopathological reaction in the chick retina that has its own distinctive cellular pattern. The typical pattern of neuronal degeneration induced by NMDA (80 μ*M*) in the chick retina is illustrated in Fig. 2A. Although L-Cys was nontoxic at low concentrations, it consistently induced a lesion pattern indistinguishable from the NMDA pattern at a concentration of 2 m*M* (Fig. 2B). The competitive NMDA antagonist, D-2-amino-5-phosphonopentanoate (D-AP5), at a concentration (50 μ*M*) known to block the neurotoxic action of NMDA (80 μ*M*) (*6*), completely prevented

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