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(12) (19) (CA) **Brevet-Patent**

(11) (21) (C) **2,034,881**

(22) 1991/01/24
(43) 1991/07/26
(45) 2001/05/29

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(51) Int.Cl.[5] C12N 15/62, C12N 15/57, C12N 9/48, C07K 19/00, A61K 38/46, A61K 38/16
(30) 1990/01/25 (470,289) US

(54) **PROTEINE HYBRIDE FACTEUR X-LACI**
(54) **FACTOR X-LACI HYBRID PROTEIN**

(57) A single-chain hybrid blood coagulation inhibitor is disclosed which has an amino acid sequence composed of two subsequences corresponding to the light chain of Factor X and LACI's first Kunitz domain.

FACTOR X-LACI HYBRID PROTEIN

Abstract of the Disclosure

A single-chain hybrid blood coagulation inhibitor is disclosed which has an amino acid sequence composed of two subsequences corresponding to the light chain of Factor X and LACI's first Kunitz domain.

FACTOR X-LACI HYBRID PROTEIN

Background of the Invention

This invention relates to a novel hybrid protein and, more particularly, to a synthetic blood coagulation inhibitor having an amino acid sequence composed of two subsequences of human plasma Factor X and human Lipoprotein-Associated Coagulation Inhibitor (LACI), respectively. .

The coagulation cascade that occurs in mammalian blood comprises two distinct systems - the so-called intrinsic and extrinsic systems. The latter system is activated by exposure of blood to tissue thromboplastin (Factor III), hereinafter referred to as tissue factor (TF). Tissue factor is a lipoprotein that arises in the plasma membrane of many cell types and in which the brain and lung are particularly rich. Upon coming into contact with TF, plasma Factor VII or its activated form, Factor VII_a, forms a calcium-dependent complex with TF and then proteolytically activates Factor X to Factor X_a, and Factor IX to Factor IX_a, thereby triggering a cascade of events which leads eventually to the formation of thrombin and a fibrin clot.

Plasma contains a Lipoprotein-Associated Coagulation Inhibitor (LACI) which inhibits activated Factor X (Xa) directly and, in a Xa-dependent manner, inhibits VII(a)/TF activity, presumably by forming a quaternary Xa/LACI/VII(a)/TF complex. See, e.g., Broze et al., Blood 71, 335-343 (1988); Sanders et al., Ibid. 66, 204-212 (1985); and Hubbard et al., Thromb. Res. 44, 527-537 (1987).

Sequence analysis of complementary DNA clones has shown that LACI contains three tandemly repeated Kunitz-type serine protease inhibitory domains. See, e.g. Wun et al., J. Biol. Chem. 263, 6001-6004 (1988); Girard et al., Thromb. Res. 55, 37-50 (1988); Girard et al., Nature 338, 518-520 (1989); and European Patent Application No. 318,451, published May 31, 1989. Both the first and second Kunitz domains of LACI are necessary for inhibition of VII(a)/TF. The present inventors have proposed that in the putative Xa/LACI/VII(a)/TF inhibitory complex, LACI's first Kunitz domain is bound to the active site of VII(a)/TF while the second Kunitz domain is bound to Xa's active site as reported in Nature 338, 518-520 (1989).

Coagulation Factor X (Stuart factor) is a two chain molecule covalently linked by a disulfide bridge. Proteolytic release of a peptide from the heavy chain of zymogen Factor X through the action of VII(a)/TF (or Factor IXa with its cofactor VIIIa) produces the active enzyme Xa. The heavy chain of Xa contains the catalytic site whereas Xa's light chain contains an NH_2-terminal, γ-carboxyglutamic acid (gla)-containing domain, which is responsible for Ca^{++} binding, followed by two growth factor-like domains which may in part mediate its interaction with specific coagulation cofactors. Chymotryptic treatment of bovine Xa cleaves the gla-containing domain from the NH_2-terminus of the molecule (residues 1-44), and although LACI binds to and inhibits gla-domainless Xa, Xa(-GD), LACI in the presence of Xa(-GD) does not inhibit VII(a)/TF activity.

Brief Description of the Invention

In accordance with the present invention a novel single-chain hybrid protein is provided which is a potent blood coagulation inhibitor. This hybrid protein has been synthesized whereby it has an amino acid sequence composed of two subsequences of human plasma Factor X and human Lipoprotein-Associated Coagulation Inhibitor (LACI), respectively. In this hybrid, protein, Factor Xa's light chain is fused to LACI's first Kunitz domain.

In a preferred embodiment, a genetically engineered hybrid protein, designated $X_{LC}LACI_{K1}$, consisting of the light chain of Factor X and LACI's first Kunitz domain, which inhibits VII(a)/TF activity directly, was prepared. In contrast to LACI's mode of action, inhibition of VII(a)/TF activity by $X_{LC}LACI_{K1}$ is not Xa dependent. $X_{LC}LACI_{K1}$ expressed by cells grown in the presence of warfarin, which inhibits γ-carboxylation of the hybrid protein, dramatically reduced inhibitory activity, thereby indicating the $LACI_{K1}$ portion of the hybrid protein alone was not sufficient for its inhibitory activity.

In a preferred embodiment to prepare $X_{LC}LACI_{K1}$, conventional recombinant DNA procedures were used to design a hybrid gene encoding for Factor X's proleader sequence (which directs the γ-carboxylation of glutamic acids) and light chain fused to a sequence encoding LACI's first Kunitz domain.

Using a previous numbering system in which amino acid +1 was assigned to the first methionine after a stop codon in the 5'-noncoding region, the first Kunitz domain was originally designated as LACI(47-117). See European Patent Application No. 318,451, published May 31, 1989, and Wun et al., J. Biol. Chem. 263, 6001-6004 (1988). In a preferred

numbering system in which amino acid +1 is assigned to the NH_2-terminus of the 276 amino acid LACI protein, the first Kunitz domain can be designated LACI(19-89). See Girard et al., Nature 338, 518-520 (1989). It will be appreciated, however, that variations in length and composition of the first Kunitz domain or the individual internal amino acids which do not adversely or detrimentally affect the biological activity of the hybrid protein as defined herein are included within the scope of the invention. Thus, in the illustrative embodiment, $X_{LC}LACI_{K1}$, the first Kunitz domain in the hybrid protein is a subsequence of 79 amino acids which includes LACI(12-88) with an additional Met-His at the C-terminus.

In a like manner, the light chain sequence of Factor X in the hybrid protein which includes the proleader sequence (residues -40 to -1) and the light chain sequence (residues 1 to 131) for a total of 171 amino acids in the preferred embodiment, $X_{LC}LACI_{K1}$, can also vary in length and composition or in the individual internal amino acids to the extent that it does not adversely or detrimentally affect the biological activity of the hybrid protein as defined herein.

The cDNA sequence of a hybrid gene encoding for the $X_{LC}LACI_{K1}$ hybrid protein is shown in the following 788 bp sequence which includes portions of the 5'- and 3'-noncoding regions. Nucleotides are numbered above the cDNA sequence. The corresponding 250 amino acids of the hybrid protein are shown below the cDNA sequence.

```
          10          20          30          40          50          60
GGATCCGAATTCGGATCCACCATGGGGCGCCCACTGCACCTCGTCCTGCTCAGTGCCTCC
                          M   G   R   P   L   H   L   V   L   L   S   A   S

          70          80          90         100         110         120
CTGGCTGGCCTCCTGCTGCTCGGGGAAAGTCTGTTCATCCGCAGGGAGCAGGCCAACAAC
 L   A   G   L   L   L   L   G   E   S   L   F   I   R   R   E   Q   A   N   N

         130         140         150         160         170         180
ATCCTGGCGAGGGTCACGAGGGCCAATTCCTTTCTTGAAGAGATGAAGAAAGGACACCTC
 I   L   A   R   V   T   R   A   N   S   F   L   E   E   M   K   K   G   H   L

         190         200         210         220         230         240
GAAAGAGAGTGCATGGAAGAGACCTGCTCATACGAAGAGGCCCGCGAGGTCTTTGAGGAC
 E   R   E   C   M   E   E   T   C   S   Y   E   E   A   R   E   V   F   E   D

         250         260         270         280         290         300
AGCGACAAGACGAATGAATTCTGGAATAAATACAAAGATGGCGACCAGTGTGAGACCAGT
 S   D   K   T   N   E   F   W   N   K   Y   K   D   G   D   Q   C   E   T   S

         310         320         330         340         350         360
CCTTGCCAGAACCAGGGCAAATGTAAAGACGGCCTCGGGGAATACACCTGCACCTGTTTA
 P   C   Q   N   Q   G   K   C   K   D   G   L   G   E   Y   T   C   T   C   L

         370         380         390         400         410         420
GAAGGATTCGAAGGCAAAAACTGTGAATTATTCACACGGAAGCTCTGCAGCCTGGACAAC
 E   G   F   E   G   K   N   C   E   L   F   T   R   K   L   C   S   L   D   N

         430         440         450         460         470         480
GGGGACTGTGACCAGTTCTGCCACGAGGAACAGAACTCTGTGGTGTGCTCCTGCGCCCGC
 G   D   C   D   Q   F   C   H   E   E   Q   N   C   V   V   T   S   C   A   R

         490         500         510         520         530         540
GGGTACACCCTGGCTGACAACGGCAAGTCCTGCATTCCCACAGGGCCCTACCCCCACAGAT
 G   Y   T   L   A   D   N   G   K   A   C   I   P   T   G   P   Y   P   T   D

         550         560         570         580         590         600
ACGGAGTTGCCACCACTGAAACTTATGCATTCATTTTGTGCATTCAAGGCGGATGATGGC
 T   E   L   P   P   L   K   L   M   H   S   F   C   A   F   K   A   D   D   G

         610         620         630         640         650         660
CCATGTAAAGCAATCATGAAAAGATTTTTCTTCAATATTTTCACTCGACAGTGCGAAGAA
 P   C   K   A   I   M   K   R   F   F   F   N   I   F   T   R   Q   C   E   E

         670         680         690         700         710         720
TTTATATATGGGGGATGTGAAGGAAATCAGAATCGATTTGAAAGTCTGGAAGAGTGCAAA
 F   I   Y   G   G   C   E   G   N   Q   N   R   F   E   S   L   E   E   C   K

         730         740         750         760         770         780
AAAATGTGTACAAGAGATAATGCAAACAGGATTATAAAGACAACAATGCATTGATGAAGC
 K   M   C   T   R   D   N   A   N   R   I   I   K   T   T   M   H   *

         790         800         810         820         830         840
TTGGATCC
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In the preferred embodiment illustrated herein, the hybrid gene was inserted into a bovine papilloma virus (BPV) expression vector and the gene was transfected into mouse C127 fibroblasts. Use of BPV in a process for producing recombinant DNA suitable for introduction and replication in eukaryotic cells is well known as can be seen, e.g., from U.S. Patent 4,419,946. It will be appreciated that the novel hybrid gene can be used for transfection of other prokaryotic and eukaryotic cells, e.g. E. coli cells or in Chinese Hamster Ovary (CHO) cells and the like mammalian cells, for expression of the hybrid protein.

An illustrative example of a conventional and suitable BPV expression vector is the plasmid pMON1123. This vector is based on the 100% viral genome ligated to the well-known pBR322 derivative of pML2 and utilizes the mouse metallothionein I promoter and the SV40 Late poly A addition site to regulate the expression of foreign genes. Its use for the expression of LACI is described by Girard et al., Thromb. Res. 55, 37-50 (1989).

Detailed Description of the Invention

While the specification concludes with claims particularly pointing out and distinctly claiming the subject matter regarded as forming the present invention, it is believed that the invention will be better understood from the following detailed description of preferred embodiments of the invention taken in conjunction with the appended drawings, in which, briefly:

FIG. 1 (Fig. 1A & 1B) is a schematic diagram of an illustrative hybrid protein of the invention, designated $X_{LC}LACI_{K1}$. Fig. 1 is split into two parts: Fig. 1A illustrates the carboxy-terminal part of the hybrid molecule, including the growth factor domains and Kunitz domain, linked to the amino-terminal part of the molecule illustrated in Fig. 1B, which includes the Pre-Pro leader sequence, a protease processing site, and a GLA domain. A K residue in Fig. 1A connects with an N residue in Fig. 1B. The layout of Factor X light chain structure is based on Leytus et al, Biochemistry 25, 5098-5102 (1986). The LACI-derived portion of the hybrid protein is shaded and is based on Girard et al., Nature 338, 518-520 (1989). The arrow indicates the probable cleavage site to generate the mature protein.

FIG. 2 shows Western blots which illustrate the expression of the recombinant hybrid protein. Relative molecular masses of standards are shown at the right.

FIG. 3 shows SDS-PAGE and silver staining of the purified $X_{LC}LACI_{K1}$ (250 ng). The reduced sample contained 1.25% 2-mercaptoethanol. Relative molecular masses of standards are shown at the right.

FIG. 4 is a graphical representation which shows inhibition of VII(a)/TF by purified LACI in the presence of (•); or absence (o) of 0.1 μg/ml Factor X.

FIG. 5 is a graphical representation which shows inhibition of VII(a)/TF activity by barium sulfate eluted $X_{LC}LACI_{K1}$ in the presence (•); or absence (o) of 0.1 μg/ml factor X.

FIG. 6 is a graphical representation which shows inhibition of TF-induced coagulation by purified $X_{LC}LACI_{K1}$ (o); and LACI (•).

FIG. 7 shows a Western blot which illustrates the presence of $X_{LC}LACI_{K1}$ in the media of C10.1 cells cultured with vitamin K or warfarin.

FIG. 8 is a schematic of the proposed inhibitory complexes with LACI and with $X_{LC}LACI_{K1}$. Indentations represent the active sites for VII(a) and Xa; and the protrusions represent LACIs' three Kunitz domains. In the quaternary Xa/LACI/VII(a)/TF complex, Xa is bound at its active site to LACI's second Kunitz domain and VII(a) is bound at its active site to the first Kunitz domain of LACI. Xa's gla domain interacts in a Ca^{++} dependent fashion with an as yet to be identified site of the VIIa/TF complex. The $X_{LC}LACI_{K1}$ hybrid protein likely substitutes for the Xa/LACI complex.

Standard biochemical nomenclature is used herein in which the nucleotide bases are designated as adenine (A); thymine (T); guanine (G); and cytosine (C). Corresponding nucleotides are, for example, deoxyguanosine-5'-triphosphate (dGTP). As is conventional for convenience in the structural representation of a DNA nucleotide sequence, only one strand is shown in which A on one strand connotes T on its complement and G connotes C.

Amino acids are shown herein either by three letter or one letter abbreviations as follows:

Abbreviated Designation		Amino Acid
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

Commonly available restriction endonucleases described herein have the following restriction sequences and (indicated by arrows) cleavage patterns:

<u>Apa</u> I	GGGCCC CCCGGG	
Bam HI	GGATCC CCTAGG	
<u>Eco</u> RI	GAATTC CTTAAG	
<u>Nsi</u> I	ATGCAT TACGTA	

In the sequences shown in FIG. 1, γ refers to γ-carboxyglutamic acid, and β refers to β-hydroxyaspartic acid.

In a preferred embodiment of the invention, a hybrid gene encoding for Factor X's preproleader sequence (which directs the γ-carboxylation of glutamic acids) and light chain fused to a sequence encoding LACI's first Kunitz domain was designed (Fig. 1). Inserted into a bovine papilloma virus expression vector, this gene was transfected into mouse C127 fibroblasts. Expression of the hybrid protein molecules into the media by a transfected clone, designated C10.1, was detected using Western blot analysis. Both a monoclonal antibody to Xa's light chain and a polyclonal IgG fraction to the NH_2-terminal sequence of LACI resulted in similar staining patterns with the dominantly stained material migrating at 30,000 molecular weight (MW) and a less intensely stained band at 28,000 MW (Fig.

2). A diffuse band with MW 52,000 was also observed and although it could represent oligomeric hybrid complexes, the identification of this material remains unclear.

SDS-PAGE and silver staining showed that the purified hybrid protein consists of a dominant 30,000 MW band with minor bands of 31,000 and 28,000 MW. This staining pattern was also observed under reducing conditions, thereby indicating that the multiple bands are not simply due to alternative disulfide bonding (Fig. 3).

The ability of the expressed $X_{LC}LACI_{Kl}$ molecules to inhibit VII(a)/TF activity was investigated. VII(a)/TF activity was determined by the release of the activation peptide from its substrate factor IX [Girard et al., Thromb. Res. 55, 37-50 (1988)]. Assaying extracted gel slices following SDS-PAGE of the C10.1 cells' conditioned media showed that functional inhibitory activity co-migrates with immuno-stained material having a MW of 30,000. No inhibitory activity was observed for the 28,000 or 52,000 MW materials. As previously demonstrated [Broze et al., Blood 71, 335-343 (1988)], Fig. 4 illustrates that the inhibition of VII(a)/TF activity by LACI is dependent upon the presence of Factor Xa. In contrast, inhibition of VII(a)/TF activity by the $X_{LC}LACI_{Kl}$ hybrid was not dependent on Xa (Fig. 5).

$X_{LC}LACI_{Kl}$ was compared to LACI for the ability to inhibit TF-induced coagulation of normal plasma as measured in a modified prothrombin time assay. Fig. 6 shows that 50% apparent TF inhibition occurred with 35 ng/ml $X_{LC}LACI_{Kl}$ while 2.5 µg/ml LACI was required for the equivalent effect. The greater relative inhibitory activity of $X_{LC}LACI_{Kl}$ in this system is believed to reflect its ability to directly

inhibit VIIa/TF while LACI requires the generation of Xa before its inhibition of VIIa/TF becomes manifest. Further, the inhibitory effect of LACI in the prothrombin time assay is at least partly due to its direct inhibition of Xa since at the same concentrations, LACI also prolongs surface contact activated coagulation of normal plasma, as measured by the activated partial thromboplastin time (aPTT).

The γ-carboxylation of glutamic acid residues on Factor X's light chain requires a vitamin K dependent process which is inhibited by warfarin. Replacement of vitamin K with warfarin in the C10.1 culture media results in an ~80 fold reduction in VII(a)/TF inhibitory activity (Table 1), while reducing the quantity of $X_{LC}LACI_{K1}$ in the media only ~ two fold (Fig. 7). Barium sulfate, which selectively adsorbs gla-containing molecules [Kisiel & Davies, Biochemistry 14, 4928-4934 (1975)], binds the VII(a)/TF inhibitory activity expressed by C10.1 cells grown in the presence of vitamin K, indicating that the functional $X_{LC}LACI_{K1}$ is γ-carboxylated (Table 1). Thus the γ-carboxylation of $X_{LC}LACI_{K1}$ appears essential for its VII(a)/TF inhibitory activity, and it is concluded by the inventors that the $LACI_{K1}$ portion of the hybrid protein alone is not sufficient for the observed inhibitory activity. In addition, neither Factor X, Xa nor inactivated Xa (Xa treated with dansyl-L-glutamyl-L-glycyl-L-arginine chloromethyl ketone) possessed VII(a)/TF inhibitory activity though each contains the X light chain found in the $X_{LC}LACI_{K1}$ hybrid. These results indicate both the X light chain and $LACI_{K1}$ portions of the hybrid protein are necessary for VII(a)/TF inhibitory activity.

A schematic illustrating the formation of the Xa/LACI/VII(a)/TF and $X_{LC}LACI_{Kl}$/VII(a)/TF inhibitory complexes is shown in Fig. 8. It is believed by the inventors that the $X_{LC}LACI_{Kl}$ mimics the Xa/LACI complex in binding to and inhibiting VIIa/TF. LACI's inhibition of VII(a)/TF activity occurs by a novel feedback inhibition mechanism requiring the generation of Xa, a product of VII(a)/TF catalytic activity. In contrast, the $X_{LC}LACI_{Kl}$ hybrid protein inhibits VII(a)/TF activity directly.

In order to illustrate the invention in greater detail the following preparative laboratory work was carried out to generate the results set forth herein, including the appended drawings. The methods described are subdivided for relation to the results shown in FIGS. 1 to 7 and Table 1. It will be appreciated, however, that the invention is not limited to these specific examples.

EXAMPLES

FIG. 1

Site-directed mutagenesis was performed on a modified LACI cDNA insert [Girard et al., Nature 338, 518-520 (1989)] to create a Nsi I site between LACI's first and second Kunitz domains' coding sequences. A naturally occurring Nsi I site exists upstream from the first Kunitz domain's coding sequence. Following Nsi I digestion, the region encoding LACI's first Kunitz domain was isolated and ligated into the Nsi I site of $pGEMX_{LC}$ to create $pGEMX_{LC}LACI_{Kl}$. $pGEMX_{LC}$ was derived from a commercially available plasmid, pGEM (Promega, Madison, WI) containing a cDNA insert encoding for

human Factor X with the naturally occurring Eco R1 site eliminated by a modification which does not alter the encoded amino acid sequence (from A. Strauss, Washington University, St. Louis, MO). $pGEMX_{LC}$ contains a modified partial cDNA encoding for the light chain of Factor X lined via an Apa I site to complementary synthetic oligomers. The synthetic oligomers encode for additional Factor X light chain sequence, followed by LACI NH_2-terminal sequence which precedes the first Kunitz domain and includes the naturally occurring Nsi I site, followed by two stop codons and convenient restriction site palindromes. The $pGEMX_{LC}LACI_{K1}$ insert was excised with Bam HI and ligated into the bovine papilloma virus expression vector pMON1123 and the resulting plasmid, $pMONX_{LC}LACI_{1}$, was used with pSV2neo to co-transfect C127 mouse fibroblasts [Girard et al., Thromb. Res. 55, 37-50 (1988)].

FIG. 2

For the Western blots, five fold concentrated serum-free conditioned media (25 µl) from a transfected cloned cell line, designated C10.1, and 40 ng purified LACI or 60 ng Factor X were run on a 15% polyacrylamide gel, transferred to nitrocellulose, probed with anti-$LACI_{(peptide)}$ IgG or anti-X monoclonal antibody and colorimetrically developed by conventional procedure as described by Girard et al., Thromb. Res. 55, 37-50 (1988).

FIG. 3

$X_{LC}LACI_{K1}$ was purified from C10.1 serum free conditioned media by barium sulfate adsorption and elution [Kisiel & Davies, Biochemistry 14, 4928-4934 (1975)], followed by anhydrotrypsin affigel affinity chromatography [Ishii et al., Meth. Enzymol. 91, 378-383 (1983)], and Mono Q (quaternary amine anion exchange resin; a trademark of Amersham Pharmacia Biotech Ltd., Buckinghamshire, England, HP7 9NA) anion exchange chromatography [EP 0 300 988, filed July 22, 1988 and Broze et al., Blood 71, 335-343 (1988)].

FIGS. 4 and 5

The purification of LACI was carried out by conventional procedure as described by Broze et al., Thromb. Res. 48, 253-259 (1988). $X_{LC}LACI_{K1}$ was partially purified from 200 ml of C10.1 serum-free conditioned media by barium sulfate adsorption [Kiesiel & Davies, supra] and eluted with 0.2 M sodium citrate. The preparation was then concentrated and dialyzed into TS buffer (100 mM NaCl, 50 mM tris-HCl, pH 7.4); final volume = 1 ml. VII(a)/TF inhibition was assayed using a previously described ^{3}H-IX activation peptide release assay [Girard et al., Thromb. Res. 55, 37-50 (1988)] except that the Factor VII in the assay was replaced with VIIa since in some assays no X (or Xa) is present to activate the VII to VIIa. Heparin (2 units/ml) was also present in the assay system. In this assay the activation of IX by VII(a)/TF is measured as release of TCA soluble tritiated activation peptide from radiolabeled IX and VII(a)/TF inhibitory activity results in decreased TCA soluble cpm. [TCA = trichloroacetic acid]. Nanograms LACI equivalents were determined from a standard LACI concentration curve.

FIG. 6

TF-induced coagulation was measured by a modified prothrombin time assay in a fibrometer (BBL, Cockeysville, MD) by incubating 60 µl rabbit brain cephalin, 60 µl crude EDTA-washed TF [Broze & Majerus, J. Biol. Chem. 255, 1242-1247 (1980)], 10 µl sample and 60 µl normal human plasma (George King, Overland Park, KS) at 37°C. After 30 seconds, 60 µl $CaCl_2$ (25 mM) was added and the time to clot formation determined. In the absence of the inhibitors the clotting time of the assay was 29 seconds. A standard curve plotting TF concentration versus clotting time (log-log plot) was used to determine apparent TF activity at various inhibitor concentrations in the assay.

Table 1

This table shows the effects of vitamin K versus warfarin on the expression of VII(a)/TF inhibitory activity and the ability of barium sulfate to adsorb this activity.

Comparison of VII(a)/TF inhibitory activity expressed by C10.1 cells grown in the presence of vitamin K versus warfarin

sample	expressed inhibitory activity (ng LACI equivalence/ml media)
vitamin K	
media	590
$BaSO_4$ nonadsorbed	3
$BaSO_4$ adsorbed	420
warfarin	
media	7
$BaSO_4$ nonadsorbed	1
$BaSO_4$ adsorbed	0

Upon reaching confluence, C10.1 cells expressing $X_{LC}LACI_{Kl}$ were transferred to serum-free conditioned media containing vitamin K (1 µg/ml) or warfarin (20 µg/ml). The culture medias were replaced on days 0, 1 and 2 and harvested on day 5. Ten ml of each media were barium sulfate adsorbed as described under Fig. 3, above, and adsorbed proteins were eluted with 0.2 M sodium citrate. The barium sulfate adsorbed and eluted materials, barium sulfate nonadsorbed materials and 10 ml of additional conditioned medias were each dialyzed into TS buffer and concentrated; final volumes = 1 ml each. Samples were diluted appropriately in TBSA buffer and assayed for inhibition of VII(a)/TF activity. Nanograms LACI equivalents were determined from a standard LACI concentration curve.

FIGURE 7

For the Western blot, 20 µl of each concentrated media was electrophoretically fractionated by SDS-PAGE, transferred to nitrocellulose, probed with an anti-X-monoclonal antibody followed by an anti-mouse IgG-alkaline phosphatase conjugate and developed colorimetrically.

Various other examples will be apparent to the person skilled in the art after reading the present disclosure without departing from the spirit and scope of the invention. It is intended that all such other examples be included within the scope of the appended claims.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A single-chain hybrid blood coagulation inhibitor having an amino acid sequence composed of two subsequences corresponding to the light chain of Factor X and the first Kunitz domain of LACI (lipoprotein-associated coagulation inhibitor).

2. A single-chain hybrid blood coagulation inhibitor having an amino acid sequence as shown in Figs. 1A and 1B of the drawings.

3. Use of an inhibitory effective amount of the hybrid blood coagulation inhibitor of claim 1 to inhibit factor VII(a)/TF activity.

4. Use of an inhibitory effective amount of the hybrid blood coagulation inhibitor of claim 2 to inhibit factor VII(a)/TF activity.

5. A DNA sequence comprising a sequence encoding the hybrid protein of claim 1.

6. A DNA sequence comprising a sequence encoding the hybrid protein of claim 2.

7. The single-chain hybrid protein of claim 1 or claim 2 for use as a medicament.

8. The single-chain hybrid protein of claim 1 or claim 2 for use as a blood coagulation inhibitor.

9. The single-chain hybrid protein of claim 1 or claim 2 for use as a blood coagulation inhibitor in a mammal.

10. Use of a single-chain hybrid protein according to claim 1 or claim 2 for the manufacture of a medicament for use as a blood coagulation inhibitor in a mammal.

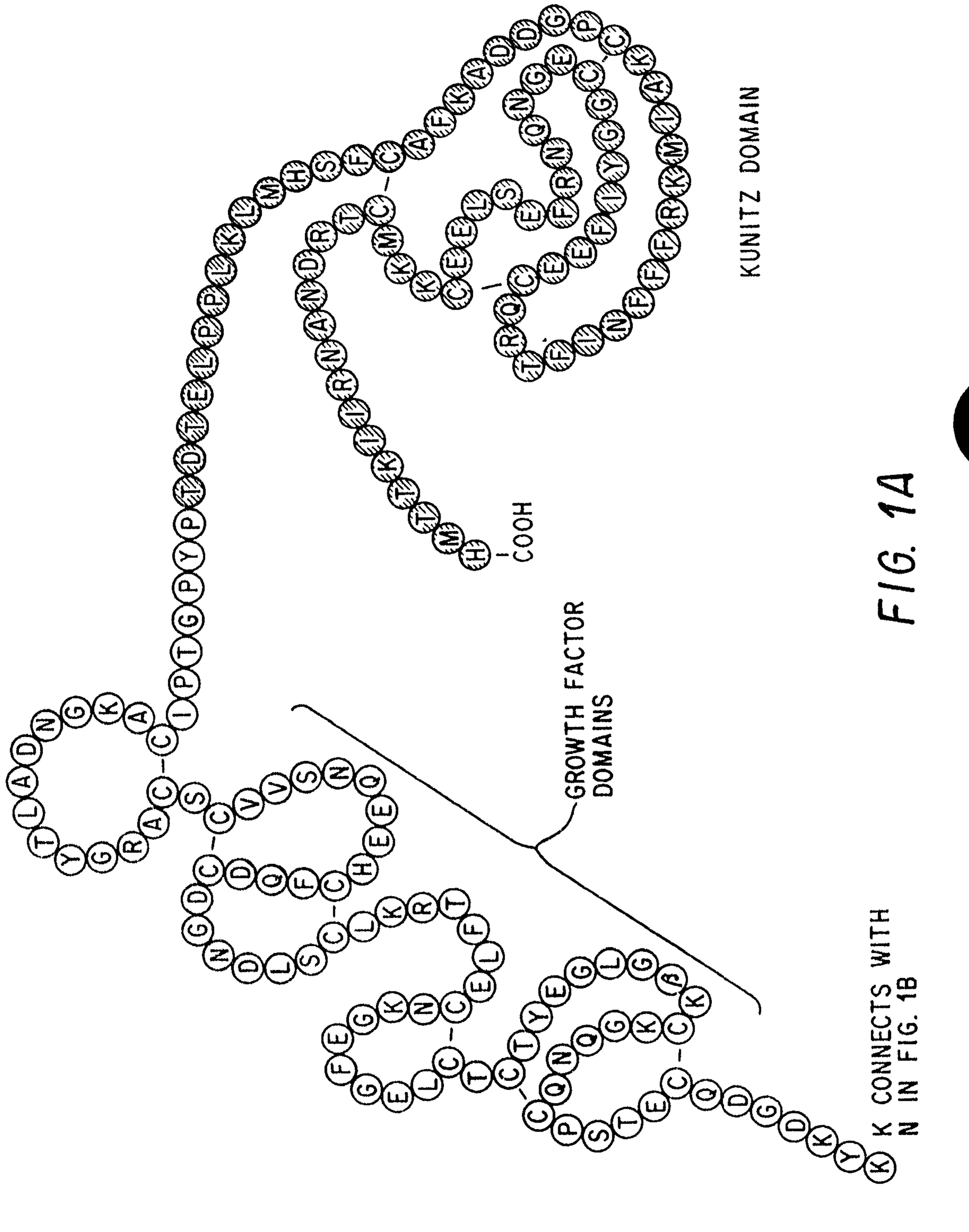

KUNITZ DOMAIN
COOH
GROWTH FACTOR
DOMAINS
K CONNECTS WITH
N IN FIG. 1B

FIG. 1A

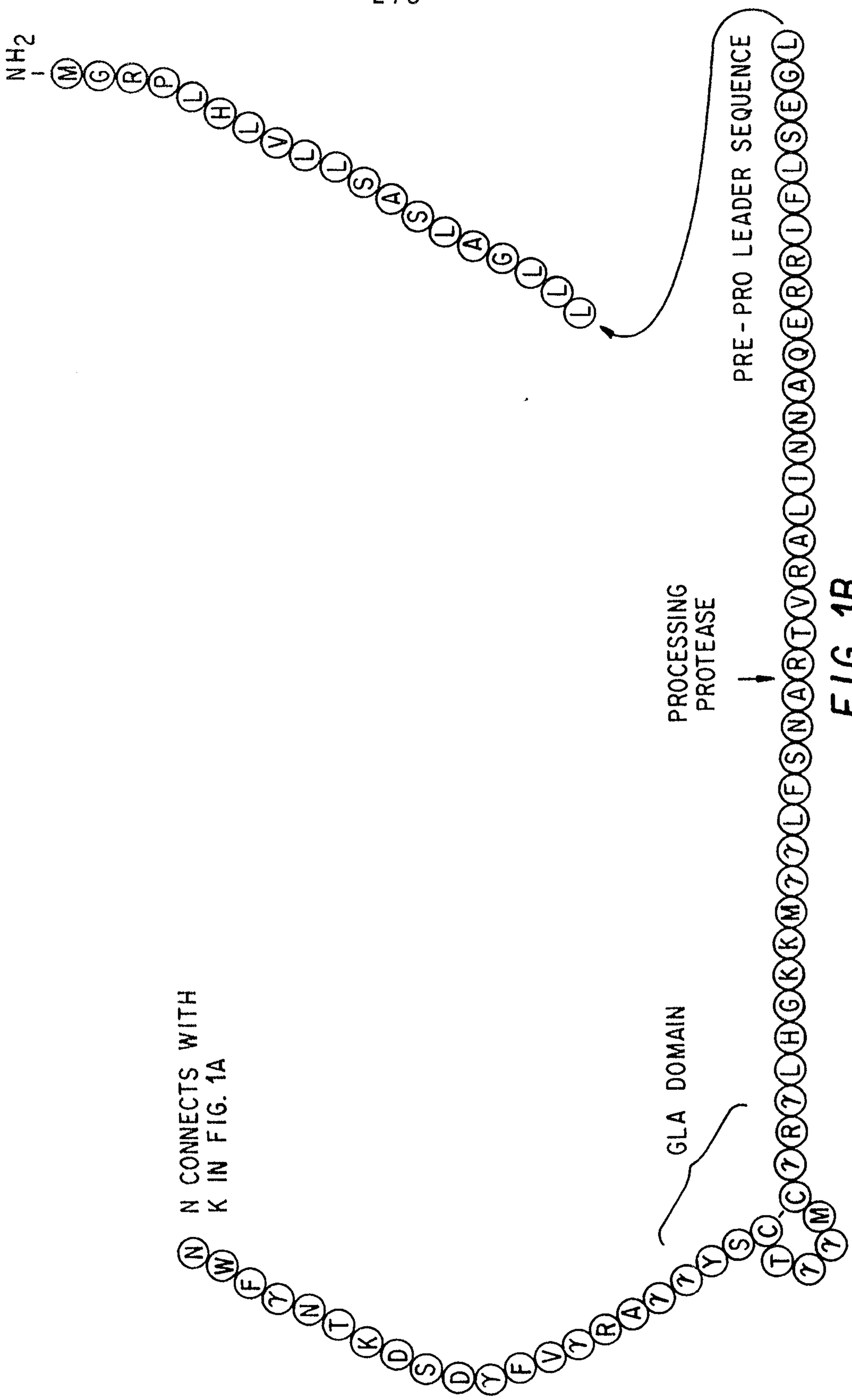


FIG. 1B

McFadden, Fincham, Marcus & Allen

FIG. 2

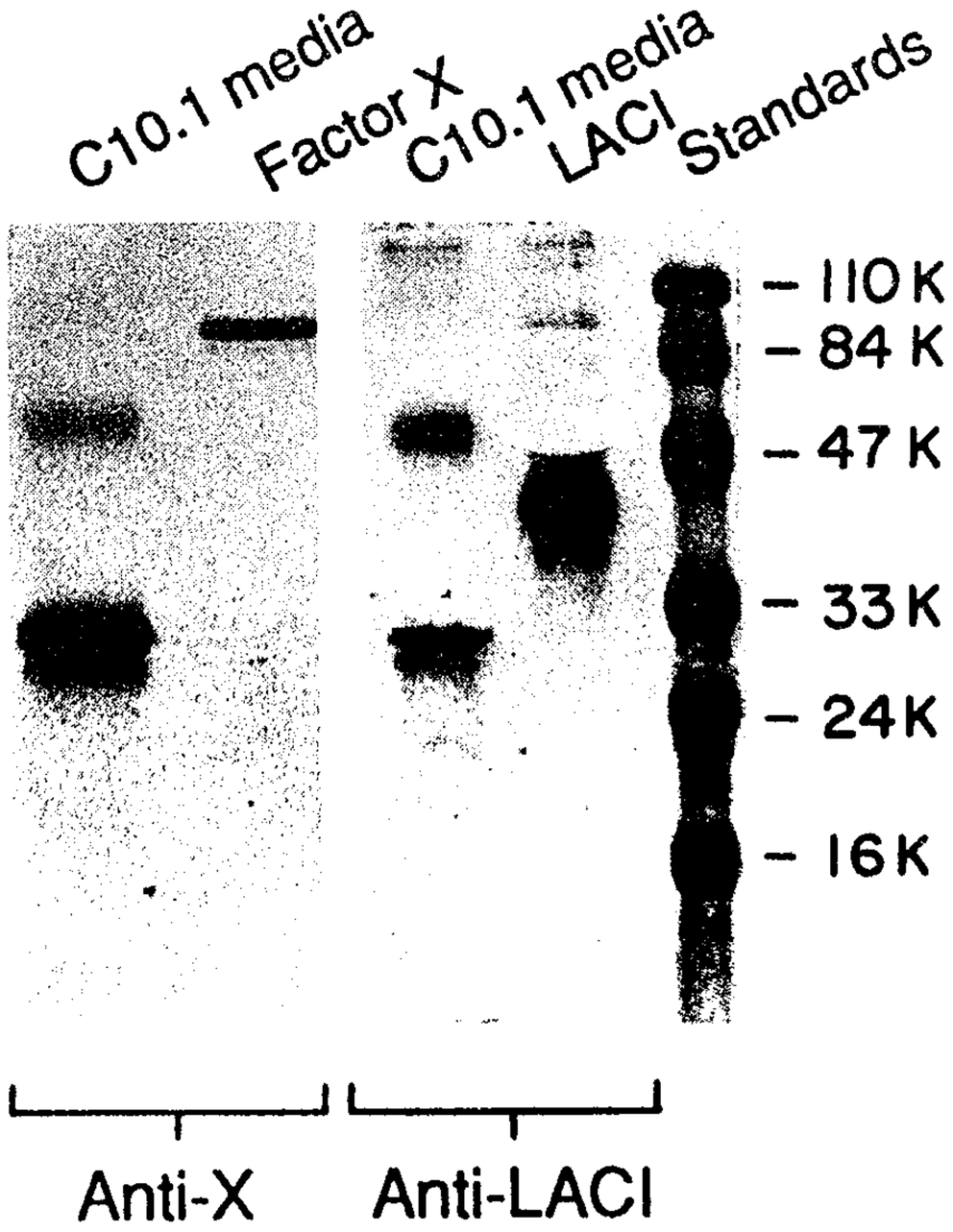
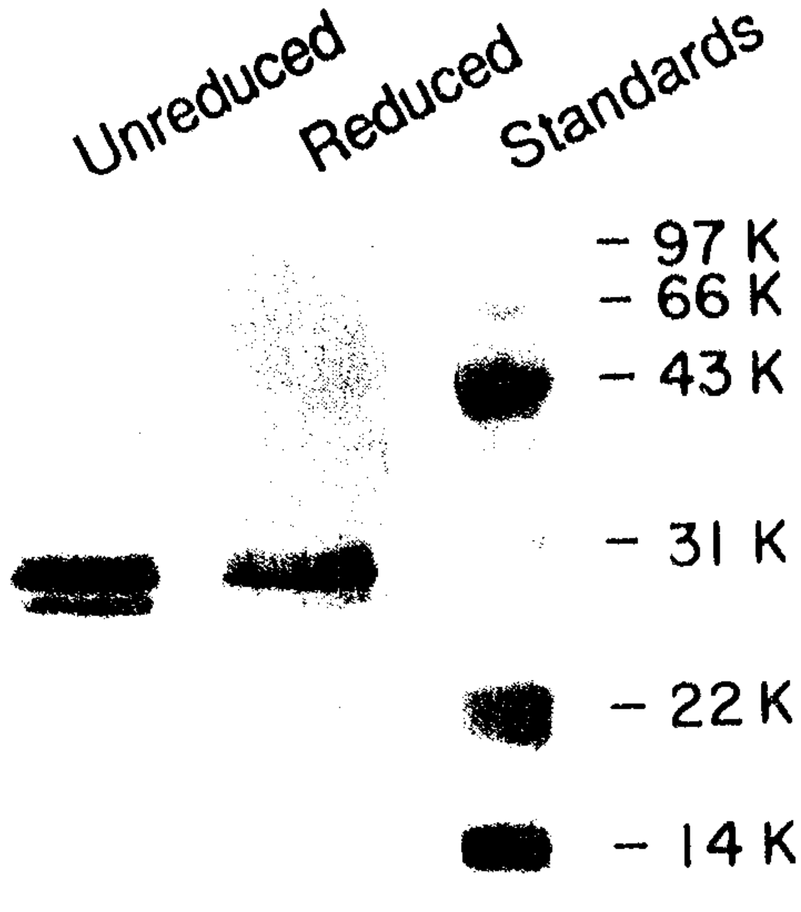

C10.1 media
Factor X
C10.1 media
LACI
Standards
– 110K
– 84K
– 47K
– 33K
– 24K
– 16K
Anti-X
Anti-LACI

FIG. 3


Unreduced
Reduced
Standards
– 97K
– 66K
– 43K
– 31K
– 22K
– 14K

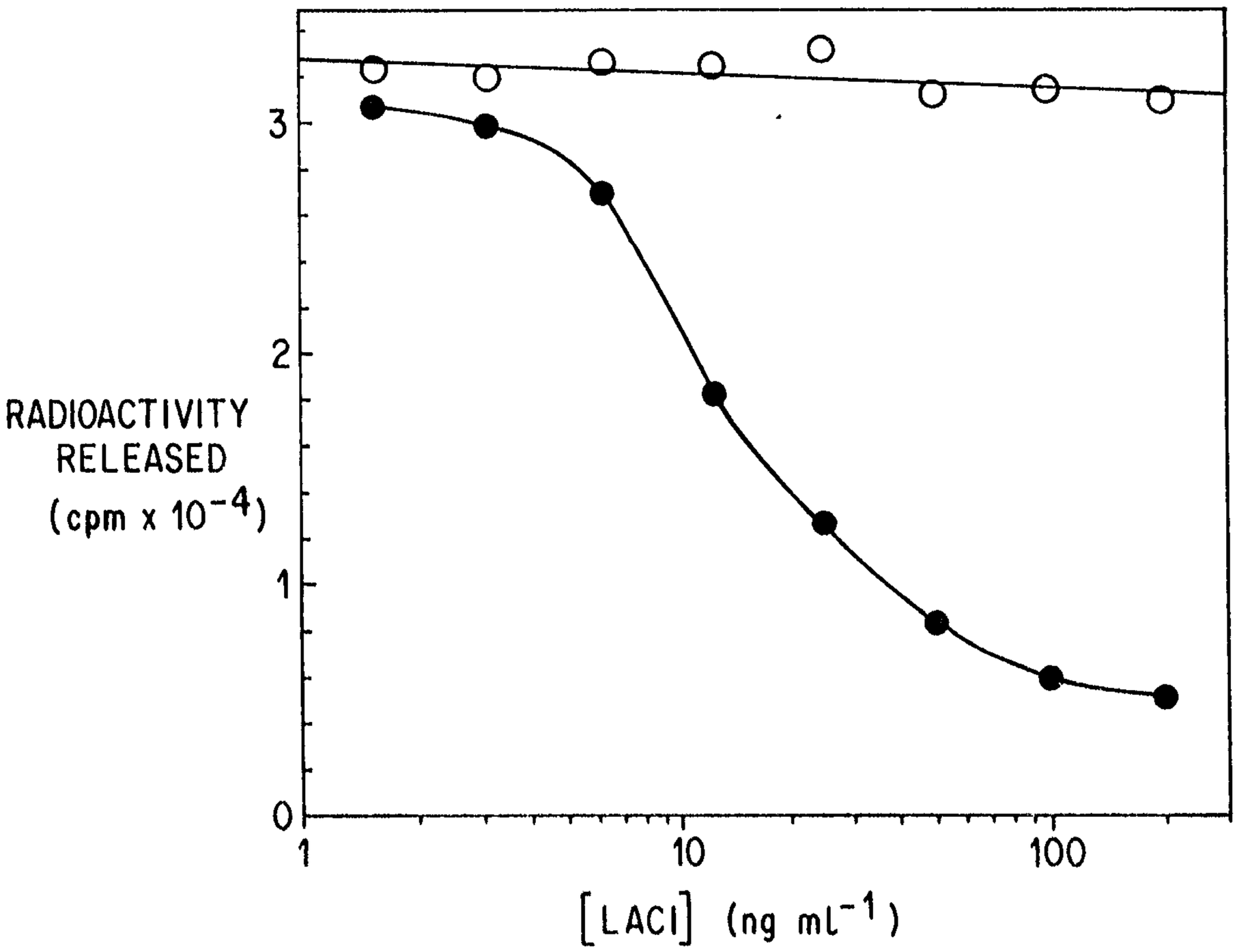


FIG. 4

McFadden, Zinchan, Marcus & Allen

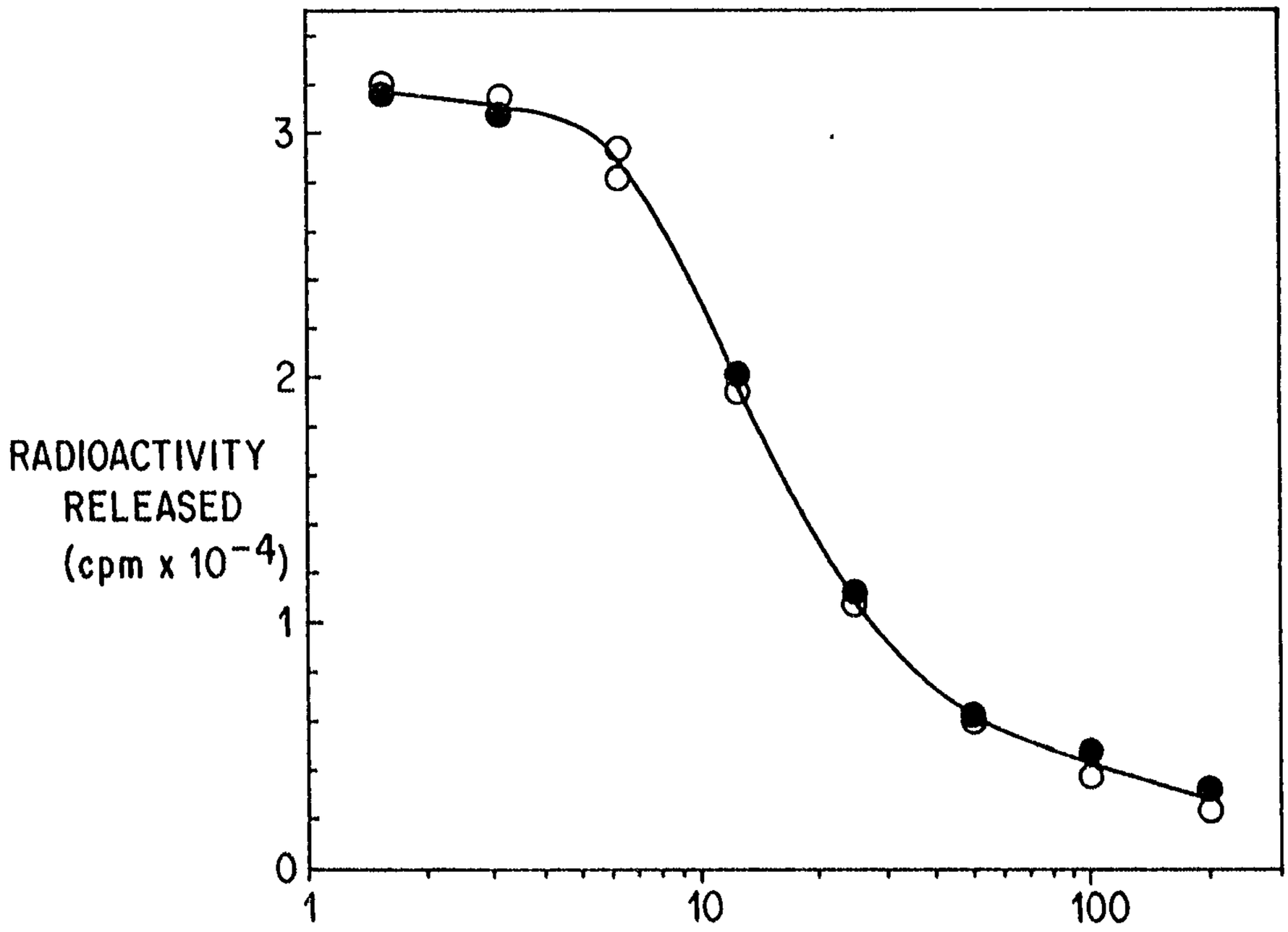


FIG. 5

McFadden, Fincham, Marcus & Allen

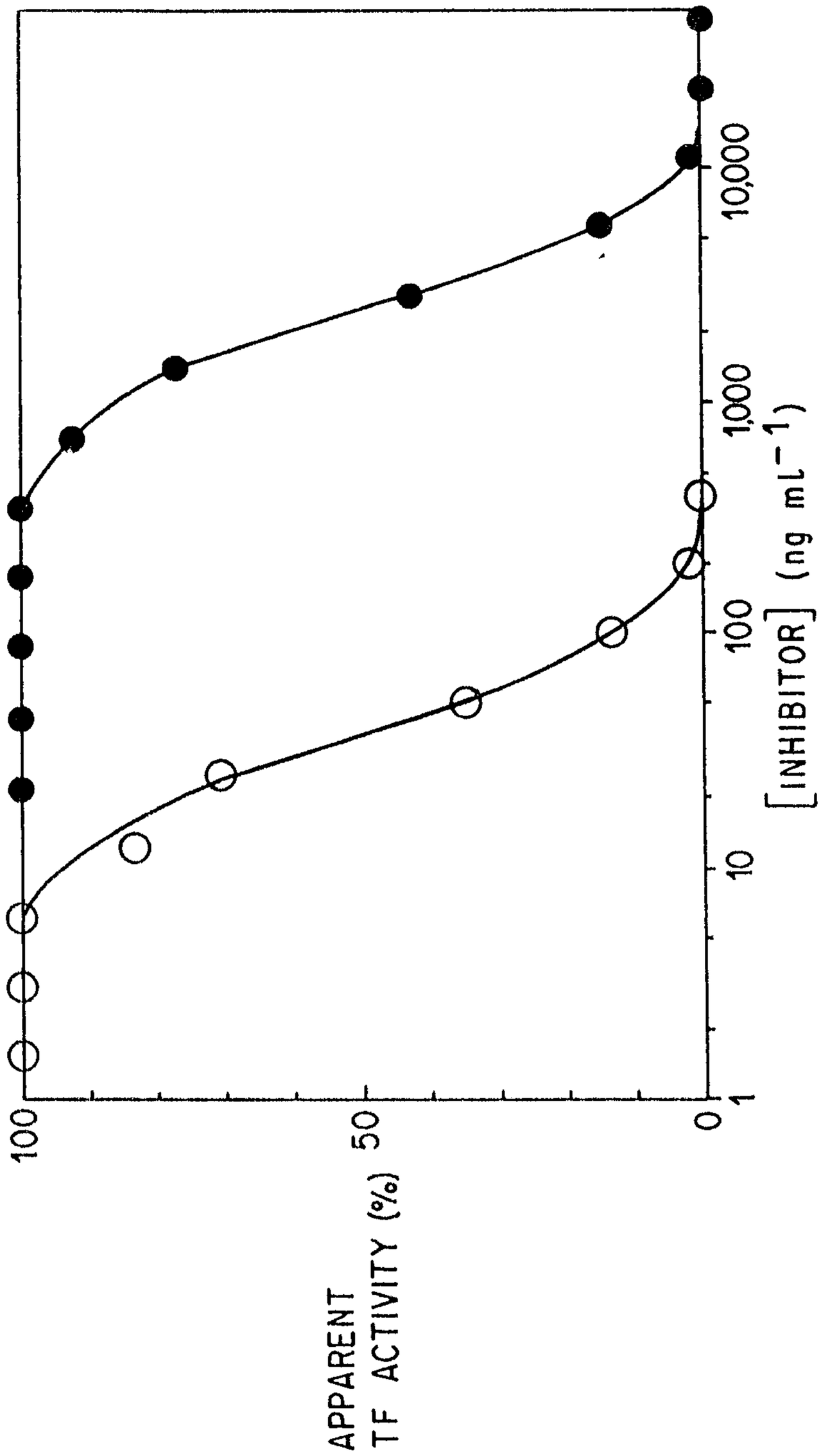

APPARENT TF ACTIVITY (%)
100
50
0
[INHIBITOR] (ng mL⁻¹)
1
10
100
1,000
10,000

FIG. 6

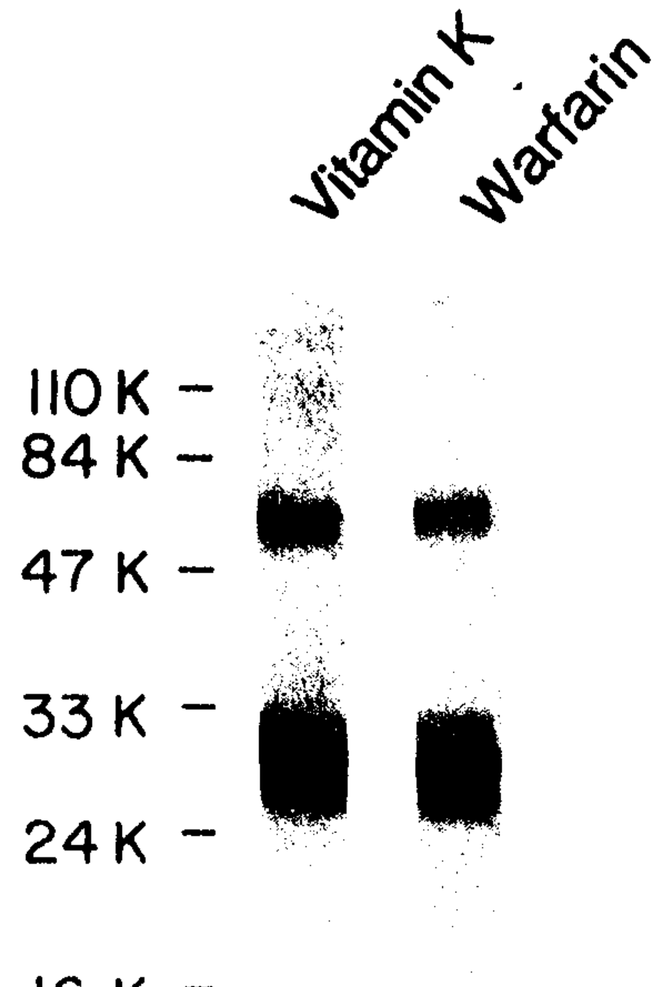


FIG. 7

McFadden, Fincham, Marcus & Allen

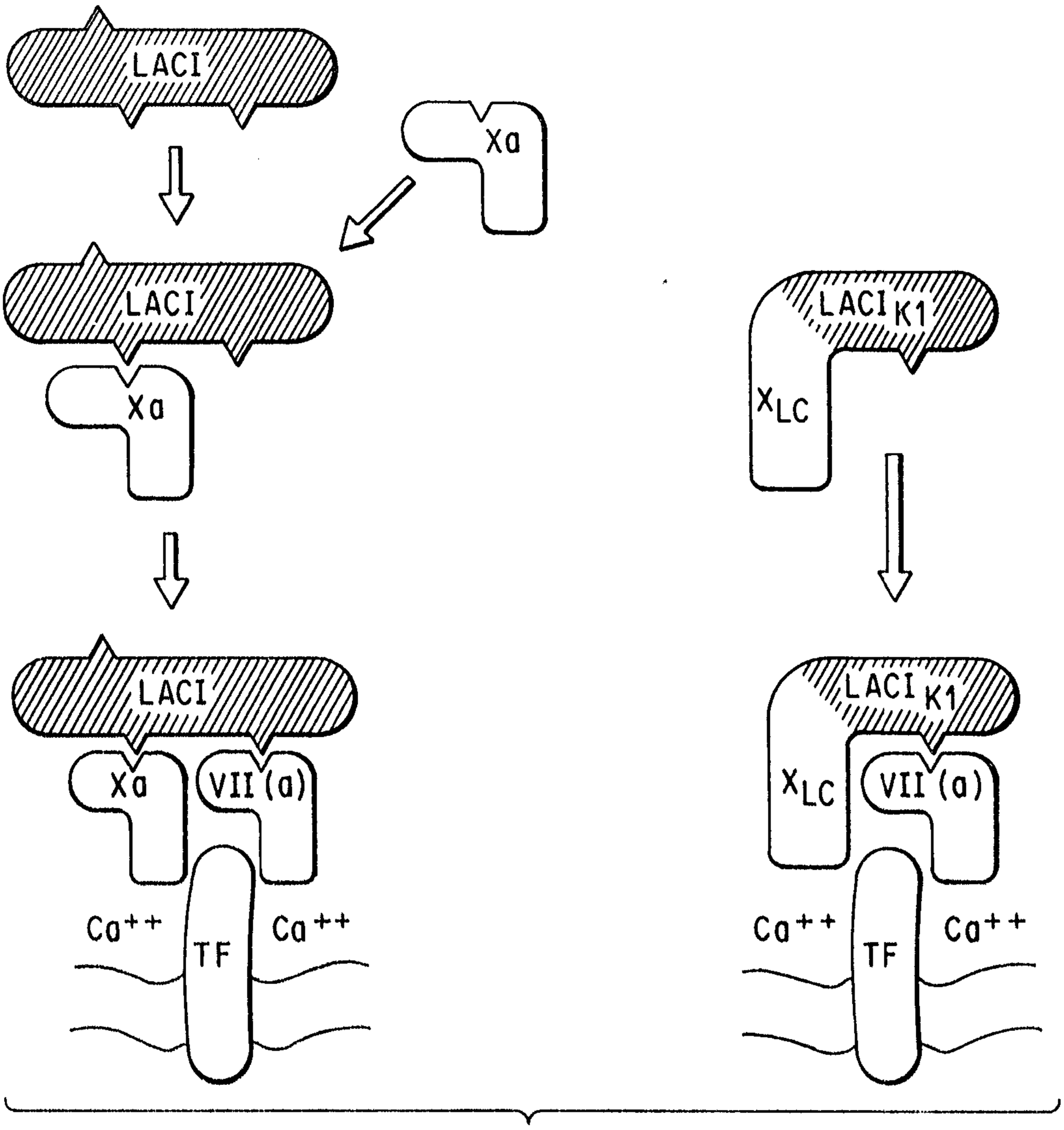

LACI
Xa
LACI
Xa
LACI K1
X_{LC}
LACI
Xa
VII (a)
Ca^{++}
TF
Ca^{++}
LACI K1
X_{LC}
VII (a)
Ca^{++}
TF
Ca^{++}

FIG. 8