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The sequence of a cDNA clone coding for a novel kallikrein from mouse submaxillary gland

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ABSTRACT

Mouse submaxillary gland contains many proteolytic enzymes, the most widely studied of which are the kallikreins. This gland also contains high levels of nerve growth factor (NGF), which is isolated as a complex of three subunits, α, β, and γ. We report here the cloning and sequence analysis of a novel kallikrein from mouse submaxillary gland. Antibodies directed against the α subunit precipitate the product of this clone, but do not precipitate the homologous γ subunit. This new kallikrein is therefore closely related to αNGF, yet in contrast to the α subunit, its sequence suggests it has proteolytic activity.

INTRODUCTION

The male mouse submaxillary gland contains a large family of serine proteases called the kallikreins. At least 23 closely homologous genes coding for these proteases have been identified (1,2). Certain kallikreins are involved in the processing of the various growth factors found in the submaxillary gland, including epidermal growth factor (EGF) and βNGF (3,4).

The male mouse submaxillary gland contains high levels of NGF. High molecular weight NGF (7S NGF) as isolated from this source consists of three subunits (5). The first, βNGF, is responsible for the nerve growth promoting activity. cDNA clones for the β subunit have been obtained by two separate groups (6,7). The βNGF protein is synthesized as a larger precursor and is processed at both amino- and carboxyl-terminal ends into the mature 13,000 molecular weight protein. However, little is known about the details of biosynthesis and processing of βNGF.

A second subunit of the 7S NGF complex, the γ subunit, has been implicated in the processing of the carboxyl-terminal dipeptide from the βNGF precursor (3). Both the amino acid sequence and the nucleotide sequence of the γ subunit have been determined. These sequences demonstrate that γNGF belongs to the kallikrein family (1,8). The enzymes that process the amino-terminal end of the βNGF precursor have not yet been identified.

The complete nucleotide sequence of the third subunit in the murine NGF complex, the α subunit, has also been determined (1,9), as have partial amino acid sequences of this subunit (10,11). Comparative sequence analysis demonstrates significant homology between the α and γ subunits, although the α subunit completely lacks enzymatic activity, presumably because of amino acid changes near the active site and at the amino-terminus (10). The α subunit consists of two

chains of approximately 16,500 and 10,000 molecular weight, which arise by cleavage of a 32,000 molecular weight precursor (12). The protein is heterogeneous; up to four different forms of the mature protein and two different forms of the precursor are detectable (13, 14, and this report).

The possible role of the α subunit and other mouse kallikreins in the processing and regulation of the 7S NGF complex is of considerable interest. We report here the cloning and sequence analysis of the cDNA coding for a novel murine kallikrein. The protein derived from this cDNA shares immunological determinants and sequence homology with the α subunit of NGF.

MATERIALS AND METHODS

Chemicals

All radioisotopes, with the exception of [^{35}S]methionine, were obtained from Amersham. Labeled methionine was purchased from New England Nuclear as part of an *in vitro* reticulocyte lysate translation kit. *Staphylococcus aureus* cells (Pansorbin) were from Calbiochem. BAPNA (*N*-α-benzoyl-DL-arginine *p*-nitroanilide) was purchased from Sigma. Materials for gel electrophoresis were obtained from Bio-Rad. Enzymes used in cloning and sequencing were supplied by either BRL or New England Biolabs, and nitrocellulose was BA85 from Schleicher and Schuell. All other chemicals were obtained from standard commercial sources.

Preparation of an RNase-Free Anti-α Subunit IgG

α Subunit was purified from 7S NGF by the method of Varon et al. (15) and was examined by isoelectric focusing and by BAPNA assay (16,17) in order to verify the absence of any contamination by the other two subunits of 7S NGF or by other kallikreins. Purified α subunit was emulsified in Freund's adjuvant and injected subcutaneously into New Zealand White rabbits, 1 mg per animal. The rabbits were boosted one month later with 200 μg alum-precipitated α subunit in PBS, and blood was drawn from the ear vein eight days later. An RNase-free IgG fraction was prepared by first diluting the serum with an equal volume of 10 mM sodium phosphate-15 mM NaCl pH 7.2 (Buffer A), precipitating in the cold with 41% ammonium sulfate, and dialyzing the precipitate against the same buffer. The dialyzed sample was loaded onto a 2 × 14 cm column of DEAE-cellulose layered over CM-cellulose (7 cm each). All glassware, resins, and buffers were autoclaved to maintain RNase-free conditions. Chromatography in Buffer A at room temperature resulted in a peak of IgG that was quantitated by absorbance at 280 nm and immunoprecipitation of ^{125}I-labeled α subunit. The IgG fraction was also tested by Ouchterlony double diffusion (18) for precipitation of purified α and γ subunits at various dilutions. No cross-reactivity with γ subunit was detectable.

Preparation of an α Subunit-Enriched cDNA Probe from Mouse Submaxillary Gland

Polysomes were prepared by magnesium precipitation (19) of a male mouse submaxillary gland homogenate. Briefly, submaxillary glands from eight mice (2.25 g) were homogenized in 22.5 ml DEP-treated buffer containing 25 mM Tris pH 7.5, 25 mM NaCl, 5 mM $MgCl_2$, 2% Triton X-100, 1 mg/ml heparin, and 1 μg/ml cycloheximide. After pelleting debris, $MgCl_2$ was added (final concentration 0.2 M) and the solution was incubated 1.5 hours on ice. The solution was then centrifuged through 0.2 M sucrose in 25 mM Tris pH 7.5, 25 mM NaCl, 100 mM $MgCl_2$ (10 minutes, 27,000 xg),

and the pellet resuspended in Polysome Buffer, 2.5 ml/g tissue (Polysome Buffer is 50 mM Tris-Cl pH 7.5, 300 mM NaCl, 5 mM $MgCl_2$ 0.1% Nonidet P-40, 100 µg/ml heparin and 1 µg/ml cycloheximide). A brief spin (10 minutes, 12,000 xg) removed any precipitated material. Approximately 13 mg polysome RNA was obtained from eight glands. Immunopurification of polysomes was carried out according to the method of Korman et al. (20). Anti-α subunit IgG (1 mg) was added to the polysomes, and after three hours on ice, the mixture was passed twice through a 0.75 g protein A-Sepharose CL-4B column in Polysome Buffer. The column was washed with 50 column volumes Polysome Buffer, and polysomes were eluted with 20 ml 25 mM Tris-Cl pH 7.5, 20 mM EDTA, 100 µg/ml heparin. Eluted polysomes were heat-denatured (5 minutes at 65°C), NaCl and SDS were added (final concentrations 0.5 M and 0.2%), and polyA$^+$ mRNA was purified by passing the mixture twice over an oligo(dT)-cellulose column. Yields were usually 0.2 µg polyA$^+$ RNA from eight glands. After several phenol-chloroform extractions and precipitation with both ethanol and LiCl, the mRNA was copied by reverse transcriptase (21) into ^{32}P-labeled cDNA. The cDNA was purified over a 1-ml Sephadex G-100 column before use as a probe.

In Vitro Translation of Polysome-Purified mRNA

PolyA$^+$ mRNA purified from α-subunit-enriched polysomes was examined by cell-free translation in a nuclease-treated rabbit reticulocyte lysate translation system (New England Nuclear) in the presence of [^{35}S]methionine. Total RNA isolated by the guanidine-HCl method (22) was also examined in this manner. Translation products were immunoprecipitated where indicated in the figures by incubation for several hours on ice with anti-α subunit IgG and subsequent addition of *Staphylococcus aureus* cells (Pansorbin) with incubation for an additional one-half hour. The cells were spun down, washed 5 times in a buffer containing 0.15M NaCl, 5% Sarkosyl and 1 mg/ml BSA in Tris-EDTA, and then washed another 2 times in 10 mM Tris-Cl, pH 6.8. The cells were resuspended in 15 µl SDS sample buffer, boiled 3 minutes, and the cells spun down. The entire supernatant was loaded on a 15% SDS polyacrylamide gel. Translation products were visualized by autoradiography.

Selection of a cDNA Clone Containing α Subunit Sequences

A pool of over 300 male-specific cDNA clones selected from a mouse submaxillary gland library cloned in ptrpE4 was obtained from James Scott and William J. Rutter (University of California, San Francisco). This pool was transferred to nitrocellulose filters and screened (23) with a cDNA probe made from the polysome-purified mRNA described above. Hybridization was for 60 hours at 42°C with 2×10^5 cpm/ml ^{32}P-labeled cDNA probe in 50% formamide, and washes were in 0.1 × SSPE-0.1% SDS at room temperature.

Preparation of Synthetic Oligonucleotides

Oligonucleotides were synthesized using an Applied Biosystems synthesizer. Hydrolysis from the resin was accomplished by incubation at 55°C in NH_4OH for several hours. NH_4OH was removed by centrifugation overnight in a speed-vac, and the dried residue was then resuspended in 200 µl 10 mM ammonium bicarbonate and passed over a 10-ml Sephadex G-50 column. Peak fractions were further purified on a 20% polyacrylamide gel, and DNA was eluted by the diffusion method of Maxam and Gilbert (24). Further desalting on a Sephadex G-50 column was followed by lyophilization. The

oligonucleotide was then end-labeled with ^{32}P and the sequence was determined (24).

Other Methods

SDS polyacrylamide gels were performed as described by Laemmli (25). Northern and Southern analyses were carried out according to protocols obtained from Schleicher and Schuell. Hybrid selection was carried out as described by Parnes et al. (26). Nucleotide sequencing was by the method of Maxam and Gilbert (27).

RESULTS

Production of a cDNA Probe Enriched for α Subunit Sequences

α Subunit was purified from male mouse submaxillary gland, high molecular weight NGF and was shown to be completely free of βNGF or γ subunit contamination. IgG fractions were obtained from antisera prepared in rabbits against this α subunit. One IgG fraction was used in further experiments after it was shown to precipitate the α subunit and to have no cross-reactivity with the γ subunit in Ouchterlony double diffusion tests (Figure 1). This IgG also bound to purified α subunit in a Western blot (data not shown).

This IgG was used to isolate polysomes enriched for α subunit mRNA, and the enriched mRNA was reverse transcribed to obtain a ^{32}P-labeled α-subunit-enriched cDNA probe.

Selection of α Subunit-Related cDNA Clones

A pool of over 300 male-specific clones selected from a male mouse submaxillary gland cDNA library was screened using the ^{32}P-labeled α-subunit-enriched cDNA probe described above. One clone (pMF-1) was selected on the basis of its exceptionally strong hybridization signal. DNA from this clone hybridized to a single band from male mouse mRNA much more strongly than from female

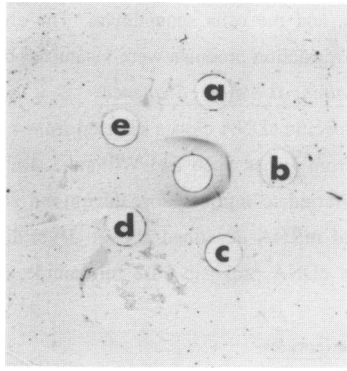

Figure 1. Ouchterlony immunodiffusion of anti-α subunit antiserum against α and γ subunits and 7S NGF. 30 μg of anti-α subunit IgG was placed in the center well of an Ouchterlony plate, and the following samples were placed in the surrounding wells: a) 0.7 ng dissociated 7S NGF; b) 3 ng purified α subunit; c) 0.6 ng purified α subunit; d) 4 ng purified γ subunit; e) 0.8 ng purified γ subunit. The plate was incubated for 24 hours at room temperature, washed exhaustively with 2% NaCl, stained with amido black, and destained with acetic acid.

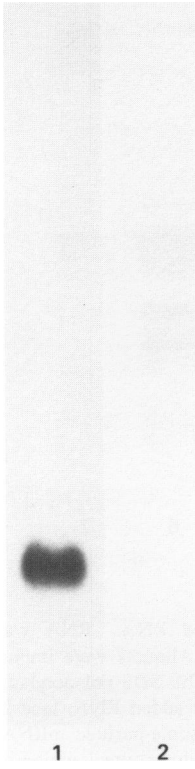

Figure 2. Northern analysis of mouse RNA using pMF-1 as a probe. Total RNA was purified from both male and female mouse submaxillary glands by guanidine-HCl extraction. This RNA was electrophoresed on a 1% formaldehyde-agarose gel and transferred to nitrocellulose paper. The filter was hybridized to purified pMF-1 plasmid DNA which had been nick-translated to high specific activity. The filter was then washed, dried, and autoradiographed. Lane 1, 10 μg total RNA from male mouse submaxillary gland; lane 2, 10 μg total RNA from female mouse submaxillary gland.

(Figure 2). The size of the RNA (approximately 1000 bp) was sufficient to code for an α subunit precursor, which has a molecular weight of approximately 32,000 (12,14).

Characterization of pMF-1

The complete sequence of the pMF-1 was determined by the method of Maxam and Gilbert (27). The cDNA was inserted in the PstI site of ptrpE4 with subsequent loss of one of the PstI sites flanking the insert by exonucleolytic removal of one nucleotide during tailing of the vector. A restriction map

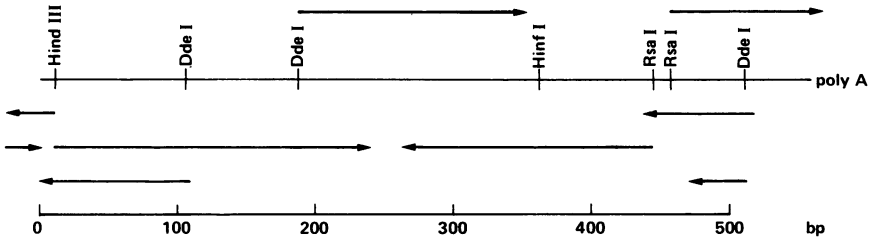

Figure 3. Restriction map of pMF-1 and diagram of sequencing strategy.

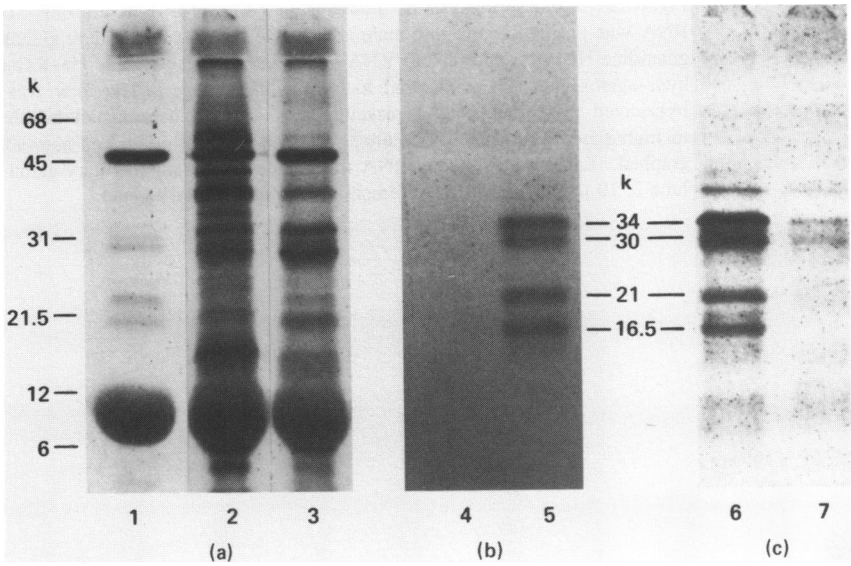

Figure 4. *In vitro* translation and immunoprecipitation of polysome-purified RNA. RNA was translated in a rabbit reticulocyte system in the presence of [^{35}S] methionine. Aliquots were immunoprecipitated where indicated with anti-α subunit IgG. Samples were run on 10% SDS polyacrylamide gels (25) and detected by autoradiography. Lane 1, translation mix without added RNA; lane 2, translation of total mouse submaxillary gland RNA; lane 3, translation of polysome-purified mRNA; lane 4, immunoprecipitation of reticulocyte lysate with no added RNA; lane 5, immunoprecipitation of *in vitro*-translated, polysome-purified mRNA; lane 6, immunoprecipitation of *in vitro*-translated, polysome-purified mRNA; lane 7, same as lane 6 but immunoprecipitation of translated proteins was in the presence of 5 μg purified α subunit.

of the insert and a diagram of the sequencing strategy is shown in Figure 3. The clone consists of a 558 base pair cDNA sequence plus approximately 200 base pairs in GC tails. The cDNA sequence is not full-length; i.e., it does not extend to the 5'end of the mRNA (see Figure 8).

Comparison of the pMF-1 Protein Product to the α Subunit of NGF

Total RNA from male mouse submaxillary gland was translated in an *in vitro* cell-free translation assay in the presence of [^{35}S]methionine using nuclease-treated reticulocyte lysate. In addition, polyA$^+$ mRNA that had been enriched for α subunit sequences by passage of polysomes mixed with anti-α subunit IgG over a protein A-Sepharose column (see Methods section) was translated in the same system. Analysis by SDS polyacrylamide gel electrophoresis and subsequent autoradiography revealed approximately five non-lysate-coded protein bands in the enriched mRNA fraction, as compared with approximately a dozen ^{35}S-labeled proteins coded for by total gland RNA (Figure 4a).

Immunoprecipitation of the translated proteins by anti-α subunit IgG in the presence of *Staphylococcus aureus* cells and analysis by SDS polyacrylamide gel electrophoresis revealed four polypeptides of molecular weights ranging from 16,500 to 34,000 (Figure 4b). An excess of purified α subunit

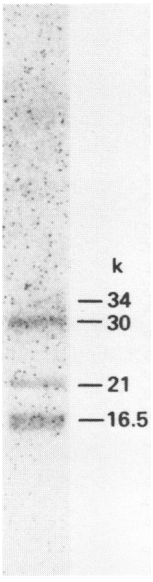

Figure 5. Hybrid selection and translation of mouse RNA by pMF-1 DNA. 50 μg pMF-1 DNA was denatured and immobilized on a 24 mm nitrocellulose filter. This filter was hybridized with 22 μg polyA$^+$ mRNA from male mouse submaxillary gland. After extensive washing of the filter and elution of bound RNA, this specifically bound material was translated in an *in vitro* reticulocyte lysate assay and immunoprecipitated as in Figure 4. SDS polyacrylamide gel analysis and autoradiography was also as in Figure 4.

added along with anti-α subunit IgG blocked precipitation of all four of these polypeptides (Figure 4c), demonstrating that immunoprecipitation was specific for all of them. The two higher molecular weight bands consistently appeared as a doublet with molecular weights between 30,000 and 34,000, consistent with the size previously reported for the α subunit precursor (12,14). The lowest molecular weight band is the same size as the mature α subunit large chain (16,500). The identity of the 21,000 molecular weight band is unknown; however, if other kallikrein proteins are synthesized during *in vitro* translation, the α subunit precursor or other precursors in the reaction mix may be processed to an intermediate size.

pMF-1 DNA was used to hybrid select mRNA from mouse submaxillary gland; when this RNA was translated *in vitro*, immunoprecipitation using anti-α subunit IgG resulted in the same four polypeptides observed previously. The two lower molecular weight bands were enriched relative to the upper two, however (Figure 5).

Figure 6 compares the pMF-1-derived amino acid sequence to the amino acid sequences of the α and γ subunits of NGF (8,9), mouse glandular kallikrein (2), and a kallikrein described first by Ronne et al. (28) and later by Lundgren et al. (29). pMF-1 shares a 70% homology at the amino acid sequence level with members of the kallikrein arginyl esteropeptidase group of serine proteases, demonstrating that this clone codes for a member of the kallikrein family. However, the sequence of pMF-1 is unique and differs from all other known members of the family, including the α subunit of NGF.

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pMF-1          L V S K S F P H P G F N M S L[L T L K E I P]P G A D
αNGF           L V S K A I P H P D F N M S L[L N E H T P Q]P E D D
γNGF           F V S K A I P H P G F N M S L[M R - - - - F]L E Y D
Kallikrein     L V S K S F L H P C Y N M S L[H R N R I E N]P Q D D
Kall (Ronne)   L V S K S F P H P G F N M S L[L M L Q T T P]P G A D

pMF-1          F S N D L M L L R L S K P A D I T D A V K P I T L P
αNGF           Y S N D L M L L R L S K P A D I T D V V K P I T L P
γNGF           Y S N D L M L L R L S K P A D I T D T V K P I T L P
Kallikrein     Y S Y D L M L L R L S K P A D I T D V V K P I A L P
Kall (Ronne)   F S N D L M L L R L S K P A D I T D V V K P I A L P

pMF-1          T K E S K L G S T C L A S G W G S I T P T K W Q K P
αNGF           T E E P K L G S T C L A S G W G S T T P I K F K Y P
γNGF           T E E P K L G S T C L A S G W G S I T P T K F Q F T
Kallikrein     T E E P K L G S T C L A S G W G S I I P V K F Q Y A
Kall (Ronne)   T K E P K P G S T C L A S G W G S I T P T R W Q K S

pMF-1          D D L Q C V F L K L L P I K N C I E N H N V K V T D
αNGF           D D L Q C V N L K L L P N E D C D K A H E M K V T D
γNGF           D D L Y C V N L K L L P N E D C A K A H I E K V T D
Kallikrein     K D L Q C V N L K L L P N E D C D K A Y V Q K V T D
Kall (Ronne)   D D L Q C V F I T L L P N E N C A K V Y L Q K V T D

pMF-1          V M L C A G E M S G G K N I C K G D S G G P L I C D
αNGF           A M L C A G E M D G G S Y T C E H D S G G P L I C D
γNGF           A M L C A G E M D G G K D T C K G D S G G P L I C D
Kallikrein     V M L C A G V K G G G K D T C K G D S G G P L I C D
Kall (Ronne)   V M L C A G E M G G G K D T C A G D S G G P L I C D

pMF-1          S V L Q G I T S T G P I P C G K P G V P A M Y T N L
αNGF           G I L Q G I T S W G P E P C G E P T E P S V Y T K L
γNGF           G V L Q G I T S W G H T P C G E P D M P G V Y T K L
Kallikrein     G V L Q G L T S W G Y N P C G E P K K P G V Y T K L
Kall (Ronne)   G I L Q G T T S N G P E P C G K P G V P A I Y T N L

pMF-1          I K F N S W I K D T M T K N S
αNGF           I K F S S W I R E T M A N N P
γNGF           N K F T S W I K D T M A K N P
Kallikrein     I K F T S W I K D T L A Q N P
Kall (Ronne)   I K F N S W I K D T M M K N A
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Figure 6. Comparison of the derived amino acid sequence of pMF-1 with amino acid sequences for the α and γ subunits of NGF (8,9) mouse glandular kallikrein (2), and a kallikrein previously thought to be the EGF-binding protein (28,29). A seven amino acid stretch of pMF-1 which differs from the other sequences and which was used to isolate pMF-2 is marked in brackets.

Isolation of a Full-Length Copy of pMF-1

In order to further characterize this clone, a full-length version extending to the 5′end was needed. For this purpose, a seven amino acid sequence was chosen near the amino-terminal end of the derived pMF-1 sequence. The sequence in this region differs significantly from the known amino acid sequences of other members of the kallikrein family (Figure 6). A synthetic oligonucleotide was synthesized (5′–GACTGTGAGTTTCTCTATGGA–3′) that was identical to the pMF-1 nucleotide sequence over this region. This oligonucleotide was end-labeled using polynucleotide kinase and was used as a hybridization probe to screen the male-specific mouse submaxillary gland library from which pMF-1 had been isolated (see above). A full-length clone that hybridized to the synthetic oligonucleotide was isolated and was designated pMF-2. This clone was sequenced and was shown to be identical to pMF-1 except that it extends for an additional 289 bases at the 5′end. The sequencing strategy for the 5′end of pMF-2 is shown in Figure 7, and the complete nucleotide sequence and derived amino acid sequence of the clone is shown in Figure 8.

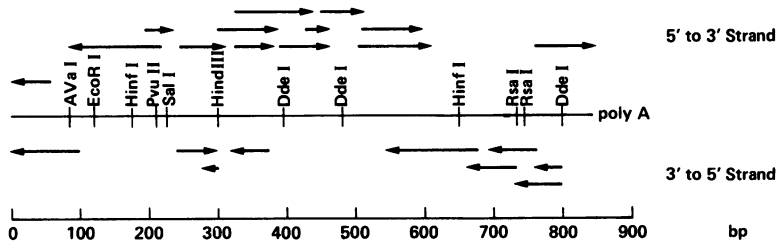

Figure 7. Restriction map and sequencing strategy of pMF-2.

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                                    MetArgPheLeuIleLeuPheLeuAlaLeuSerLeu
GGGGGGGGGCTCCTGCACCCTGTTACCATGAGGTTCCTGATCCTGTTCCTAGCCCTGTCCCTA

GlyGlyIleAspAlaAlaProProLeuGlnSerArgValValGlyGlyPheAsnCysGluLys
GGAGGGATTGATGCTGCACCTCCTCTCCAGTCTCGGGTGGTTGGAGGATTTAACTGTGAGAAG

AsnSerGlnProTrpGlnValAlaValTyrAspAsnLysGluHisIleCysGlyGlyValLeu
AATTCCCAACCCTGGCAGGTGGCTGTGTATGACAACAAGGAACACATTTGTGGGGGAGTCCTG
EcoRI

LeuGluArgAsnTrpValLeuThrAlaAlaHisCysTyrValAspGlnTyrGluValTrpLeu
TTGGAACGCAACTGGGTTCTCACAGCTGCCCACTGCTATGTCGACCAGTATGAAGTTTGGCTG
                        PvuII               SalI
                                            ↓
GlyLysAsnLysLeuPheGlnGluGluProSerAlaGlnHisArgLeuValSerLysSerPhe
GGCAAAAACAAGTTATTCCAAGAGGAACCCTCTGCTCAACACCGATTGGTCAGCAAAAGCTTC
                                                    HindIII

ProHisProGlyPheAsnMetSerLeuLeuThrLeuLysGluIleProProGlyAlaAspPhe
CCTCACCCTGGCTTCAACATGAGCCTCCTGACACTCAAAGAGATACCTCCTGGGGCTGACTTC

SerAsnAspLeuMetLeuLeuArgLeuSerLysProAlaAspIleThrAspAlaValLysPro
AGCAATGACCTGATGCTGCTCCGCCTCAGCAAGCCTGCTGACATCACAGATGCTGTGAAGCCC
                    DdeI

IleThrLeuProThrLysGluSerLysLeuGlySerThrCysLeuAlaSerGlyTrpGlySer
ATCACCCTGCCCACGAAGGAGTCAAAACTGGGGAGCACATGCCTAGCCTCAGGCTGGGGCAGC
                                                DdeI

IleThrProThrLysTrpGlnLysProAspAspLeuGlnCysValPheLeuLysLeuLeuPro
ATTACACCCACGAAATGGCAAAAGCCAGATGATCTCCAGTGTGTGTTCCTCAAGCTCCTGCCT

IleLysAsnCysIleGluAsnHisAsnValLysValThrAspValMetLeuCysAlaGlyGlu
ATTAAGAACTGTATAGAAAACCACAATGTGAAGGTGACAGATGTCATGCTGTGTGCAGGAGAG

MetSerGlyGlyLysAsnIleCysLysGlyAspSerGlyGlyProLeuIleCysAspSerVal
ATGAGTGGAGGCAAAAACATTTGCAAGGGTGACTCCGGAGGCCCATTGATCTGTGATAGTGTT
                                HinfI

LeuGlnGlyIleThrSerThrGlyProIleProCysGlyLysProGlyValProAlaMetTyr
CTCCAAGGTATCACATCAACAGGCCCTATACCATGCGGTAAACCTGGTGTACCAGCCATGTAC
                                                RsaI        RsaI

ThrAsnLeuIleLysPheAsnSerTrpIleLysAspThrMetThrLysAsnSerTer
ACCAACCTTATTAAGTTCAACTCCTGGATAAAAGATACTATGACGAAAAATTCCTGAGTGTCA
                                                        DdeI

CATTATCCCATGTTCTCAATAAAACCCACCATGAAGCpolyACCCCCCCCCCCCCCCCCCCC
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Figure 8. Nucleotide sequence and derived amino acid sequence of pMF-2. Restriction sites are marked, as well as the 5'end of pMF-1 (arrow). The polyadenylation signal is underlined, and a potential glycosylation site is boxed.

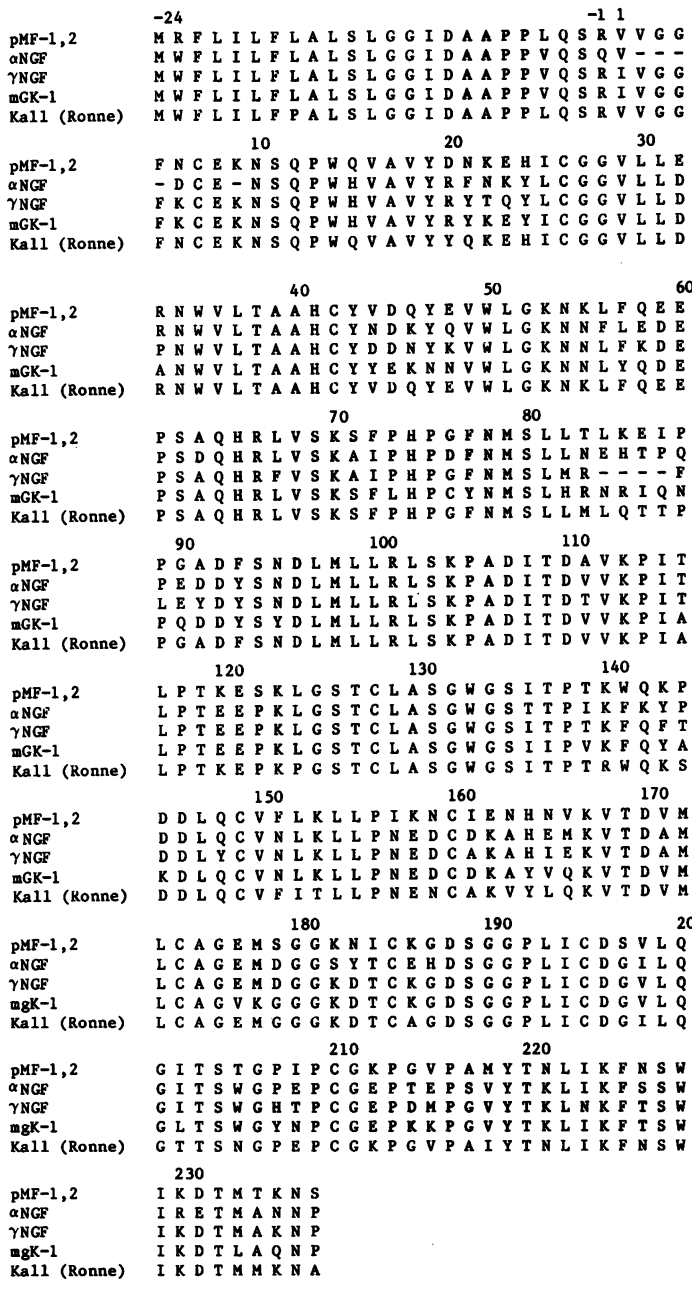

Figure 9. Comparison of derived amino acid sequences from pMF-1 and pMF-2 with sequences of α and γ subunits (8,9), the derived sequence from the mouse glandular kallikrein clone mGK-1 (2) and the sequence of a kallikrein previously thought to be EGF-binding protein (28,29).

The clone extends from 19 nucleotides upstream of the first methionine to 42 nucleotides downstream from the 3'termination codon. The 5'start site of pMF-1 is indicated, as well the termination codon, polyadenylation signal, and all relevant restriction sites. In addition, several members of the kallikrein family, such as the α subunit of NGF, are reported to be glycosylated, and one potential N-glycosylation site is indicated on the pMF-2 sequence.

Homology with the Kallikreins

A comparison of the derived amino acid sequence of pMF-2 with the known sequences of several other members of the kallikrein family is shown in Figure 9. The sequence of pMF-2 is highly homologous to the other sequences shown, proving that the protein coded for by this clone is a member of the kallikrein family. However, the pMF-2 sequence is not identical to these or any other known sequences of members of the family (30). It differs significantly from other members of the kallikrein family in regions known to contribute to the substrate-binding pocket (2). It is most closely related to a kallikrein previously thought to be EGF-binding protein B (28,29), with which it shares 91% homology at the nucleotide sequence level, and is 88 to 90% homologous to two mouse glandular kallikrein clones, pMK-2 and pMK-1 (2,31). It codes for a protein that contains the typical serine protease charge-relay complex (32) but does not exhibit the active site substitutions characteristic of the αNGF subunit (1,10).

DISCUSSION

We have used purified anti-α subunit IgG to select a population of polysomes that were synthesizing proteins immunologically related to the α subunit of NGF. The anti-α subunit IgG is highly specific, since it does not cross-react with the closely-related γNGF subunit. When used to make a cDNA hybridization probe to screen a male mouse submaxillary gland library, the polysome-derived messages selected a kallikrein cDNA closely related to the α subunit of NGF.

The cDNA clones that were selected for study, pMF-1 and pMF-2, code for a protein that is a member of the kallikrein group of serine proteases, based on the high degree of sequence homology that these clones share with other members of the family. pMF-1 and pMF-2 sequences are most closely related to mouse glandular kallikrein and to another kallikrein (28,29) previously thought to be EGF-binding protein but now known to differ from the EGF-binding protein amino acid sequence (33).

By several criteria, the pMF-1 and pMF-2 protein product is also closely related to the α subunit of nerve growth factor: (a) pMF-1 DNA hybrid-selects mRNA coding for four α subunit IgG-precipitable proteins seen in *in vitro* translation assays; (b) anti-α subunit IgG selects polysomes containing pMF-1 mRNA; and (c) a comparison of the derived amino acid sequences of pMF-1 and pMF-2 and the α subunit sequence demonstrates a similarity in protein structure that is responsible for the immunological cross-reactivity between these proteins. Although the kallikrein coded for by pMF-1 and pMF-2 shares at least one immunological determinant with the α subunit, a determinant that is not shared by the γ subunit, it is not identical to the α subunit in amino acid sequence. In particular, the sequence of the protein coded for by pMF-1 and pMF-2 suggests that, unlike the α subunit, it is an active serine protease. pMF-1 and pMF-2 sequences do not contain deletions in the 5'end that are

characteristic of the α subunit and that, presumably, interfere with its enzymatic activity (10).

One possible function of the protein coded for by pMF-1 and pMF-2 is that it is involved in the processing of the βNGF precursor. The processing of βNGF requires a number of cleavages to reduce a 33,800 dalton precursor to its mature size of 13,000 daltons (7). Considerable evidence supports the idea that one of the submaxillary gland kallikreins, the γ subunit, is responsible for the cleavage of a carboxyl-terminal dipeptide from the βNGF precursor; however, other enzymes that process the βNGF precursor have not been identified. The α subunit binds βNGF, but, because it is inactive, it cannot process the βNGF precursor. It is possible that the antigenic determinant that the pMF-2 protein and the α subunit share is also a βNGF binding site; if this is so, then the pMF-2 protein may have the ability to bind to the βNGF precursor and perhaps be involved in its processing.

Processing pathways for other growth factors from the submaxillary gland, EGF for example, have not been extensively studied. The inability of γNGF and EGF-binding protein to substitute for each other (5) demonstrates, however, that these processing steps are highly specific. The product of this new kallikrein cDNA may process EGF or another factor from the submaxillary gland. Expression of the pMF-2 protein should allow a determination of its natural substrate.

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