Mouse 7S Nerve Growth Factor: Complete Sequence of a cDNA Coding for the α-Subunit Precursor and Its Relationship to Serine Proteases†

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ABSTRACT: Two synthetic oligonucleotides, one 14-mer and one 15-mer, each containing 32 sequences and corresponding to two regions of the partially determined protein sequence, were utilized to identify three cDNA clones coding for the precursor of the α-subunit of 7S mouse nerve growth factor (NGF). This library, containing 860 clones, had been preselected from a much larger one by low-stringency hybridization using a cDNA probe corresponding to one of the large family of glandular kallikreins expressed in the adult male mouse submandibular gland. Partial sequence analysis had previously established the α-subunit to be a member of this group, albeit with no demonstrable catalytic activity. Nucleotide sequence analysis of the longest of these clones (2A4) predicted the apparent complete amino acid sequence of the 265-residue precursor. One of the other clones (3F2) contained an A → G substitution at position 565 resulting in a Lys → Glu change at position 160 of the mature sequence. These clones probably represent two different alleles. Several amino acid changes, relative to other serine proteases, are evident, which may account for the apparent lack of enzymatic activity. An Arg → Gln substitution at residue −1 would prevent cleavage of the putative activation peptide, and the deletion of residues 2–5 interrupts the highly conserved Ile/Val-Ile/Val-Gly-Gly N-terminal sequence. An Asp → Tyr substitution in the binding pocket and a Gly → His substitution near the active site serine also probably contribute to the inactive structure. The role of this subunit in NGF function remains obscure.

Nerve growth factor (NGF)[1] occurs in the adult male mouse submandibular gland as a high molecular weight complex containing three types of polypeptide subunits (Varon et al., 1968). Designated α, β, and γ, they are associated by noncovalent forces with the principal interactions occurring at the α–β and β–γ interfaces (Silverman & Bradshaw, 1982). Two moles of Zn^{2+} also stabilizes the complex, apparently through binding to the γ-subunit (Pattison & Dunn, 1975). The β-subunit, which is solely responsible for the nerve growth promoting activity, is present as a dimer of two polypeptide chains (M_r 13 250 each). A cDNA clone for mouse βNGF has recently been sequenced, predicting a much larger precursor protein (Scott et al., 1983; Ullrich et al., 1983). Comparison of the pro-βNGF sequence with that of the mature protein (Angeletti & Bradshaw, 1971) indicates that proteolytic processing must occur at both the N- and C-termini.

The γ-subunit has arginine esteropeptidase activity (Greene et al., 1969) and has been proposed as a possible processing enzyme for βNGF precursors (Angeletti & Bradshaw, 1971; Berger and Shooter, 1978). Sequence analysis of γNGF confirmed it to be a serine protease (Thomas et al., 1981) and subsequently placed it in a family of highly homologous proteins, produced in the adult male mouse submandibular gland, that have been designated glandular kallikreins (Mason et al., 1983). Other members of this family for which partial or complete sequence data are available include γ-renin (Poe et al., 1983), two variants of the EGF binding protein (Silverman, 1977; Ronne et al., 1983), and three kallikreins of unknown specificity: pMK1 (Richards et al., 1982) and mGK-1 and mGK-2 (Mason et al., 1983). Mason et al. (1983) have estimated a minimum of 25–30 different genes to be present in this family. We have determined a substantial portion of the amino acid sequence of the α-subunit of 7S NGF and found that it is also closely related to these proteins (Isackson & Bradshaw, 1984).

In view of the postulated processing function of the NGF and EGF binding proteins (Angeletti & Bradshaw, 1971; Savage et al., 1972), this may be a general role for other members of this proteinase family. They appear to have similar catalytic specificities; i.e., they favor basic side chains but differ in respect to their protein substrates. For example, the γ-subunit of NGF does not bind EGF, and EGF-BP does not bind NGF (Server & Shooter, 1977).

Although αNGF is highly homologous to the glandular kallikrein family, it appears unique in having no apparent proteolytic activity. αNGF is unable to cleave N^{α}-benzoyl-DL-arginine-*p*-nitroanilide and tosyl-L-arginine methyl ester and, more importantly, has greatly reduced reactivity with [^{3}H]diisopropyl fluorophosphate ([^{3}H]DFP) relative to γNGF (D. Raben, P. J. Isackson, and R. A. Bradshaw, unpublished results). The partial protein sequence (Isackson & Bradshaw, 1984) showed two amino acid changes compared to γNGF, which could explain the loss of catalytic activity.

As a result of the similarities with other glandular kallikreins, apparent unique sequences were selected from the partial structure and used to predict the mRNA nucleotide sequence for the preparation of oligonucleotide probes. Two of these identified three clones, including a full-length cDNA whose nucleotide sequence reveals the complete amino acid sequence of the α-subunit precursor.

Materials and Methods

Colonies were grown on nitrocellulose filters in ordered arrays and processed for colony hybridization (Grunstein & Hogness, 1975). Filters were prehybridized 15 h at room temperature in 5 mM EDTA/100 mM Tris-HCl (pH 7.5)/100 μg/mL poly(A)/0.5% NP-40/1 × Denhardt's solution/1 M NaCl/1 mM sodium pyrophosphate/0.1 mM ATP. Oli-

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[1] Abbreviations: NGF, nerve growth factor; mGK-1, murine glandular kallikrein 1; EGF, epidermal growth factor; EGF-BP, epidermal growth factor binding protein; DFP, diisopropyl fluorophosphate; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate.

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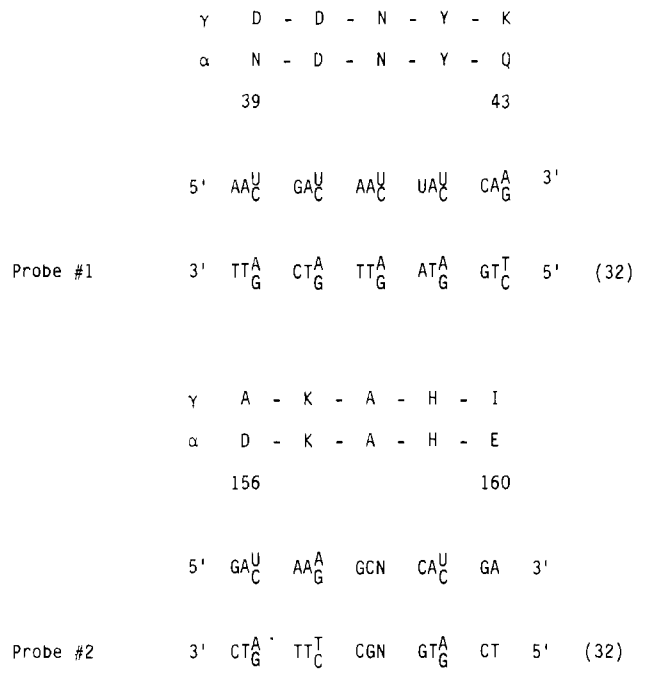

FIGURE 1: αNGF-specific oligonucleotide probe sequences. The amino acid sequence of the γ- and α-subunits of 7S NGF are shown along with the predicted mRNA sequence for αNGF (Thomas et al., 1981; Isackson & Bradshaw, 1984). Both probes were synthesized as equimolar mixtures of 32 sequences. Amino acid residue 41 was shown to be lysine rather than asparagine after the synthesis of probe 1 (see text). The amino acid numbering is from the α-subunit (see Figure 3).

gonucleotide probes were labeled at their 5'-end with [γ-^{32}P]ATP by using T4 polynucleotide kinase and added directly to the prehybridization mixture without separation from unincorporated [^{32}P]ATP (Ullrich et al., 1984). Sets of two filters each containing 96 colonies were hybridized with approximately 50 ng of probe in 10 mL for 24 h at room temperature. The filters were washed 2 times for 15 min at 48 °C in 3 M tetramethylammonium chloride/50 mM Tris-HCl (pH 8)/0.1% SDS/2 mM EDTA (W. I. Wood, unpublished results; Ullrich et al., 1984) followed by air-drying and autoradiography.

Oligonucleotide probes were manually synthesized by the phosphotriester method on a solid support (Ike et al., 1983). Blocked dimer precursors were obtained from Bachemgentec, Inc.

cDNA was prepared from poly(A$^+$)–RNA of adult male Swiss Webster mouse submandibular glands and inserted into the *Pst*I site of pBR322 (Ullrich et al., 1984). A library of 10 000 clones was obtained and screened for kallikrein-like sequences with a ^{32}P-labeled restriction fragment of a glandular kallikrein cDNA clone as described elsewhere (Ullrich et al., 1984). A total of 860 kallikrein-related cDNA clones selected under low-stringency conditions was further screened for αNGF sequences.

Plasmid DNA of selected clones was purified by a scaled-up version of the procedure of Birnboim & Doly (1979) followed by CsCl gradients. After restriction endonuclease mapping, fragments were labeled at their 3'-ends with [α-^{32}P]deoxynucleotides and the Klenow fragment of *Escherichia coli* DNA polymerase I followed by sequencing by the method of Maxam & Gilbert (1978). Additional sequence information was obtained by the dideoxy method (Sanger et al., 1977; Messing et al., 1981).

Results

Partial protein sequence analysis of the α-subunit (Isackson & Bradshaw, 1984) showed a high degree of similarity with the γ-subunit of 7S NGF as well as a number of other glandular kallikreins of the mouse submandibular gland. It was therefore not surprising that initial attempts to identify α-clones in a cDNA library by filter hybridization-translation selection were unsuccessful due to cross-hybridization of similar sequences (data not shown). Three cDNA clones identified in this manner, which hybridized to RNAs producing polypeptides that comigrated with α-precursors but not γ-precursors, were sequenced and found to be similar but clearly different from either α- or γNGF (P. J. Isackson, D. Raben, S. J. Nisco, D. K. Hopson, and R. A. Bradshaw, unpublished results).

As an alternative approach, two oligonucleotides were prepared complementary to codons for regions of amino acid sequence that appeared to be unique to the α-subunit as compared to the other sequences available (Figure 1). These probes were used to screen a cDNA library of 860 kallikrein-related clones that were previously selected from a much larger library of 10 000 clones, prepared from adult male mouse submandibular gland mRNA, by colony hybridization under low-stringency conditions to a kallikrein cDNA clone (Ullrich et al., 1984).

Under high-stringency hybridization conditions (see Materials and Methods), oligonucleotide probe 1 selected four cDNA clones. Hybridization with oligonucleotide probe 2, however, resulted in greater than 200 positives, including three of the four clones selected by probe 1. Restriction mapping

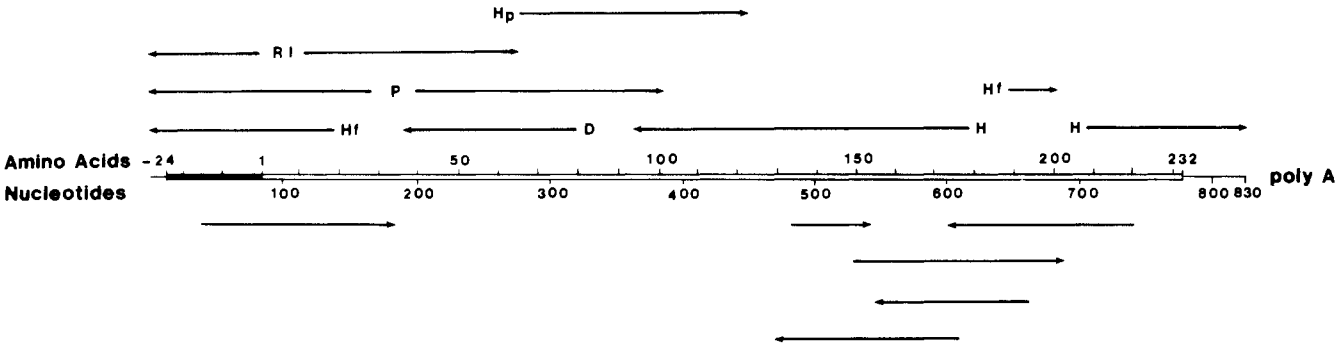

FIGURE 2: Sequencing strategy for prepro-αNGF cDNA clone 2A4. The shaded area of the amino acid coding region aligns with residues of other kallikreins that encode the signal and activation peptides. Horizontal arrows above the cDNA indicate sequence data obtained by the method of Maxam & Gilbert (1980). Fragments for sequencing were prepared by cleavage of the purified *Pst*I insert by the following enzymes: *Eco*RI, RI; *Pvu*II, P; *Hae*III, H; *Hpa*II, Hp; *Hinf*I, Hf; *Dde*I, D. All fragments were labeled with ^{32}P at the 3'-end; the arrows indicate the direction and length of the sequencing runs. The horizontal arrows below the cDNA represent sequence information obtained by the method of Sanger et al. (1977). *Pst*I inserts were cleaved with *Sau*3A1 and subcloned into the *Bam*HI site of M13 mp 11 (Messing et al., 1981) or cut with *Alu*I and subcloned into *Sma*I-cleaved M13 mp 11 to obtain templates for dideoxy sequencing.

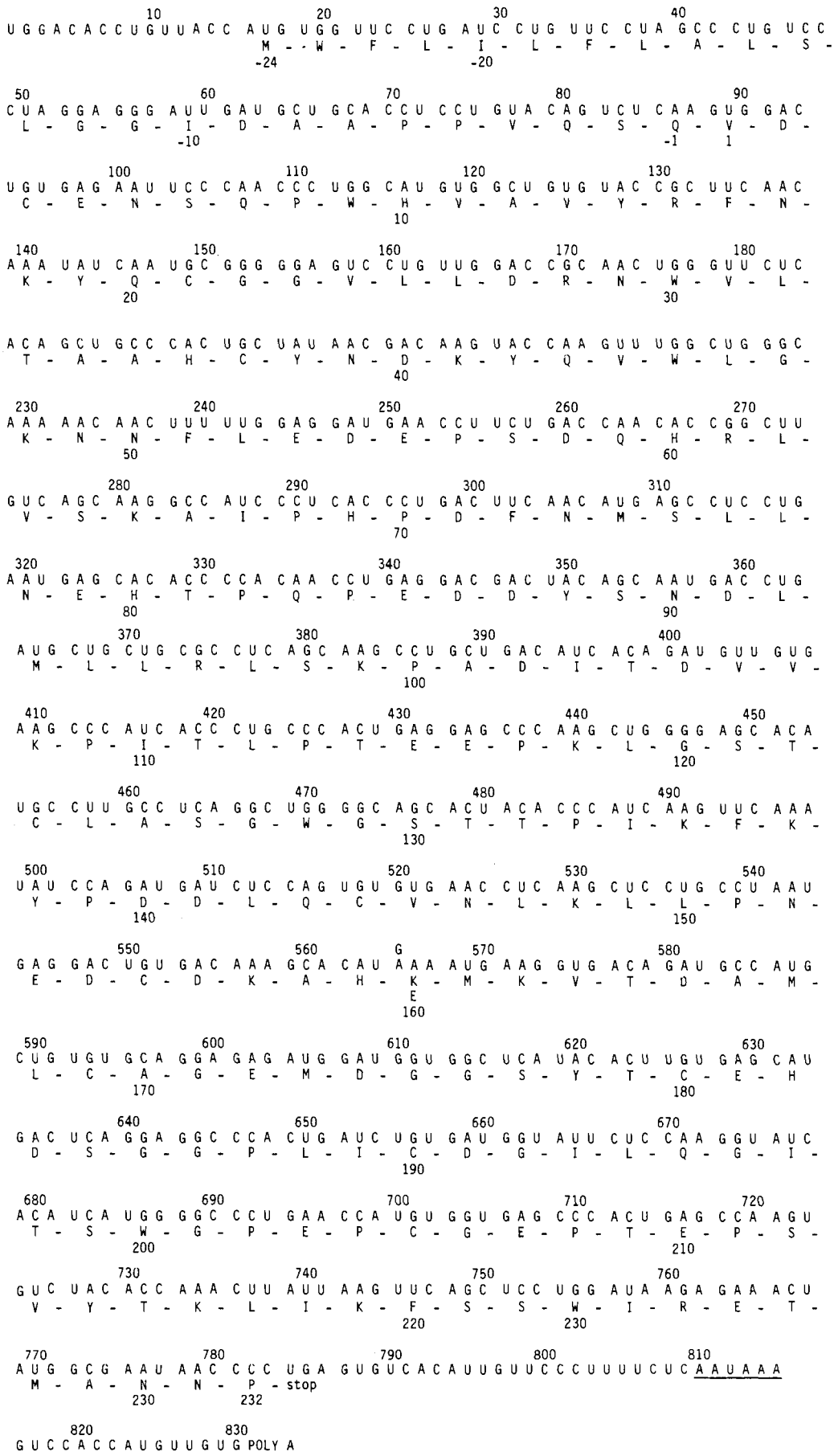

FIGURE 3: Nucleotide sequence of prepro-αNGF mRNA and the predicted amino acid sequence of the protein. The numbering of amino acid residues begins at the valine that corresponds to the N-terminal of the γ-subunit of 7S NGF. The base substitution at position 565 that is present in clone 3F2 is shown above the sequence of clone 2A4. The conserved sequence AAUAAA in the 3′-noncoding region is underlined.

of the four clones positive for probe 1 demonstrated that three of the clones were identical except for length. These clones corresponded to the same three recognized by probe 2. The lengths of these clones, designated 2A4, 5H8, and 3F2, were 850, 800, and 700 bp, respectively. The complete nucleotide sequence of 2A4 was determined by a combination of the methods of Maxam & Gilbert (1980) and Sanger (1979). The strategy for sequencing is indicated in Figure 2, and the nucleotide sequence and predicted protein sequence of the prepro-α-subunit are shown in Figure 3.

Discussion

The sequence of the precursor to the α-subunit of 7S NGF has been determined from an apparent full-length cDNA clone. The cDNA clone was identified from a library of 860 cDNA clones containing kallikrein-like sequences by screening with two oligonucleotide probes under highly selective conditions, demonstrating the power and great selectivity of this approach for isolating specific cDNA clones of similar sequences from cDNA libraries. At the amino acid level, the α-subunit sequence is 78% identical with the mature protein sequence of γNGF, 75% identical with the precursor sequence of mGK-1, and 30% identical with rat trypsinogen (Figure 4).

Both oligonucleotide probes contained a single base-pair mismatch. In the case of probe 1, this resulted from an initial incorrect protein sequence assignment of asparagine rather than lysine at position 41 arising from the ϵ-succinyllysine derivatives used (Figure 1). This error was uncovered by additional protein sequence analyses after synthesis of the oligonucleotide had been completed. Nucleotide sequence analysis through the region corresponding to probe 2 predicted a lysine rather than a glutamic acid at position 160. Reexamination of the protein sequence assignment of glutamic acid at this position, however, showed it to be unequivocal. This ambiguity was resolved by nucleotide sequence analysis of clone 3F2 through this region, which revealed an A → G substitution at position 565 that predicts the expected glutamic acid. A difference in the intensity of the hybridization signal was not detectable between these two clones. Furthermore, under the conditions used, strong positive hybridization signals were obtained for both probes even though probe 1 contained a mismatch in the middle and probe 2 contained a mismatch at the end. At higher temperatures, the hybridizations would have undoubtedly reflected these differences. These data suggest there are at least two alleles for the prepro-α-subunit differing at least at position 565. Our sequencing (Isackson & Bradshaw, 1984) studies only revealed one of these forms although both were probably present in the preparations of α-subunit used. The determined cDNA and amino acid sequences were otherwise in complete agreement.

The αNGF sequence, being very similar to γNGF and the other glandular kallikreins that have been sequenced, retains a number of features characteristic of serine proteases. In particular, the charge-relay complex of His-36, Asp-91, and Ser-184 is present. A signal peptide is predicted that is identical with that of mGK-1 (Figure 5) and γNGF (Ullrich et al., 1984) followed by a sequence probably representing the activation peptide. We have numbered the valine, which aligns with the first residue of the other kallikreins, as residue 1.

The α-subunit is peculiar as a serine protease homologue in that it has no detectable proteolytic activity and a greatly reduced ability to bind DFP. Four notable sequence changes are evident that may explain this lack of activity. First, there is an Arg → Gln substitution at residue −1 as compared to mGK-1 (Mason et al., 1983) and γNGF (Ullrich et al., 1984) (Figure 5). This presumably prevents cleavage of the activation peptide in a fashion identical with other serine proteases. In all other members of this family, removal of this peptide is followed by the formation of a salt linkage between the new free N-terminal and Asp-183 (Stroud et al., 1971). The concomitant conformational change is necessary for the stabilization of the catalytic site.

The second change is a deletion of four residues at the N-terminal of the α-subunit corresponding to residues 2–5 of

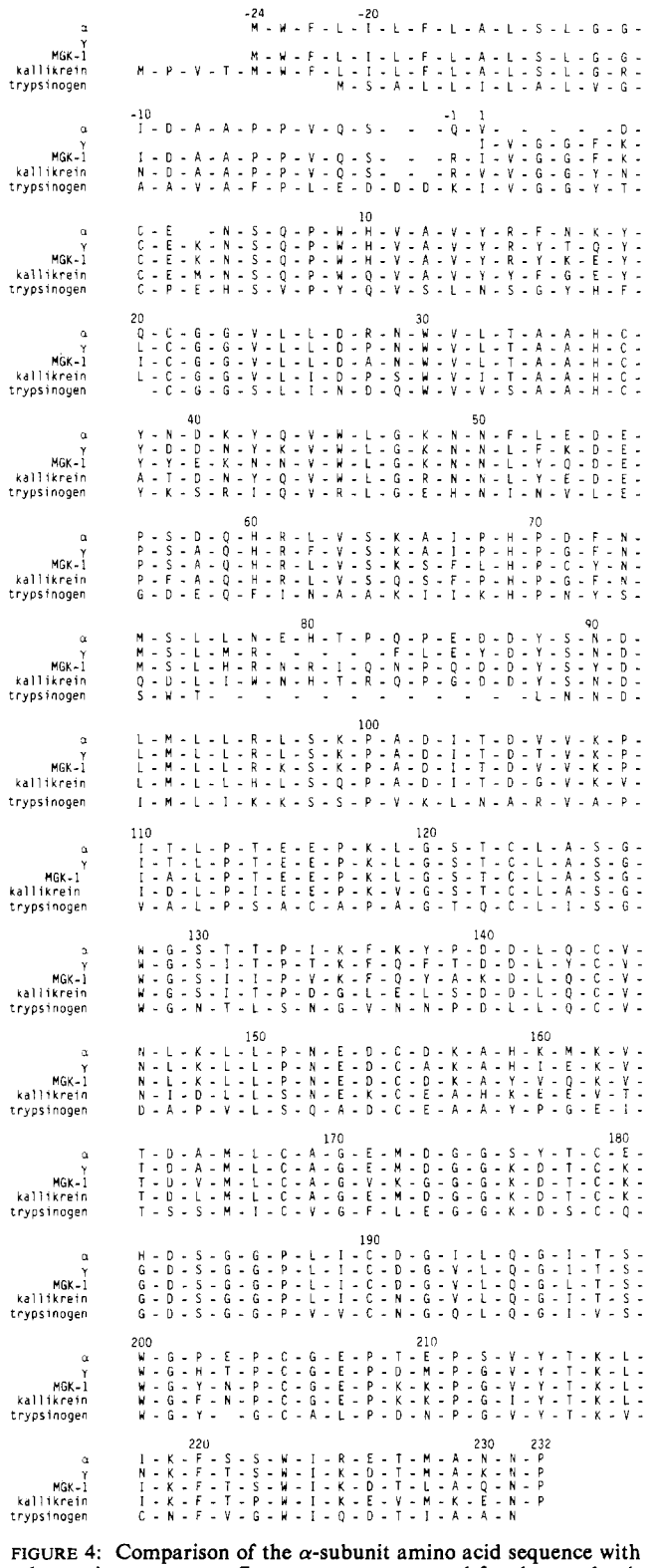

FIGURE 4: Comparison of the α-subunit amino acid sequence with other serine proteases. Sequences are presented for the γ-subunit of 7S NGF (Thomas et al., 1981), mouse glandular kallikrein (mGK-1) (Mason et al., 1983), rat pancreatic kallikrein (Swift et al., 1982), and rat trypsinogen I (MacDonald et al., 1982a).

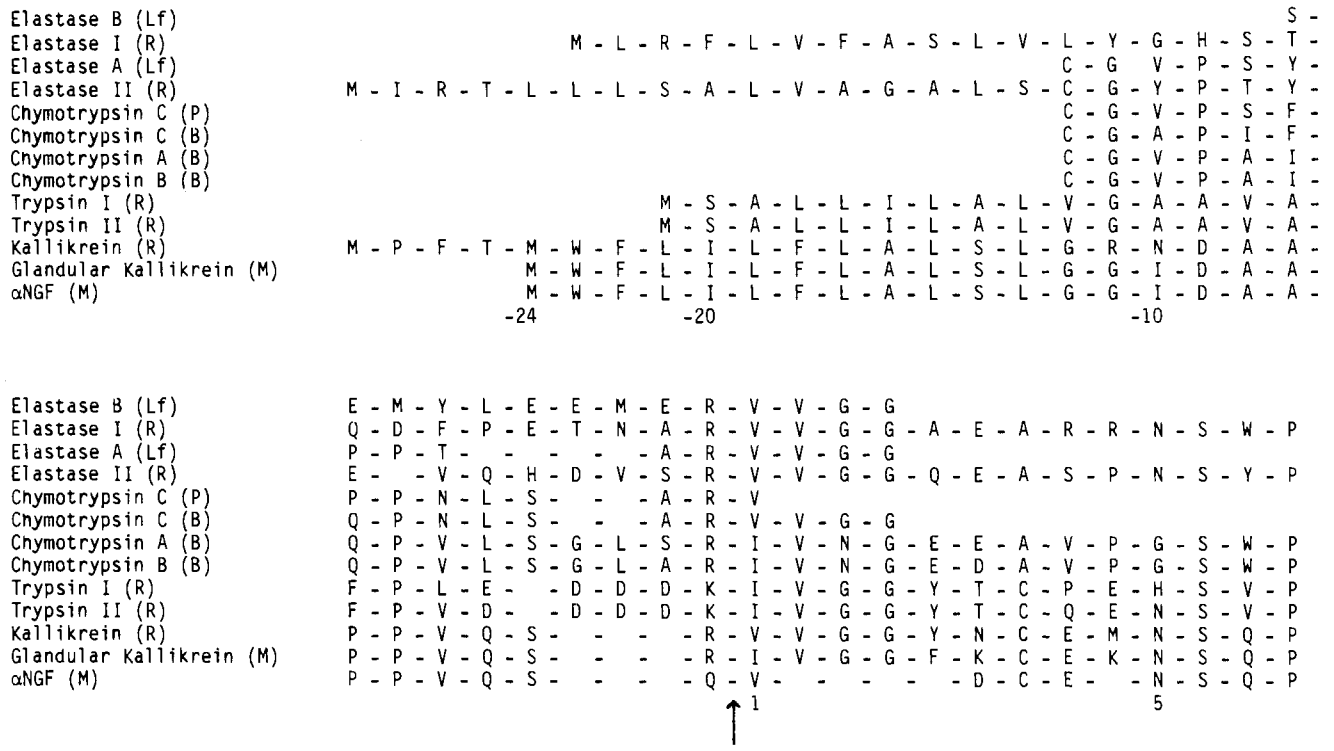

FIGURE 5: Comparisons of N-terminal prepro sequences of several serine proteases with the corresponding region of α-subunit of 7S NGF. The numbering shown is for the α-subunit. Sequences shown are for rat (R) proelastases I and II (MacDonald et al., 1982b), African lungfish (Lf) proelastases A and B (deHaen & Gertler, 1974), porcine (P) chymotrypsinogen C, bovine (B) chymotrypsinogen C, bovine chymotrypsinogen A, and bovine chymotrypsinogen B (Dayhoff, 1972), rat trypsinogens I and II (MacDonald et al., 1982a), rat pancreatic kallikrein (Swift et al., 1982), mouse (M) glandular kallikrein (mGK-1) (Mason et al. 1983), and the α-subunit of 7S NGF. The vertical arrow indicates the bond cleaved on activation (except αNGF).

the γ-subunit. The first four residues of γNGF, Ile-Val-Gly-Gly, are characteristic of serine proteases, and Gly-4 is absolutely conserved in all other serine proteases that have been sequenced (Dayhoff, 1982). Thus, in conjunction with the Arg → Gln substitution, this deletion suggests that the N-terminal region is grossly perturbed relative to active serine proteases.

The other two amino acid changes, Gly → His at position 182 and Asp → Tyr at 178, were previously noted from the amino acid sequence analysis (Isackson & Bradshaw, 1984). The substitution of Gly → His at 182 is at a critical position close to the reactive Ser-184 residue. Gly-182 and Gly-185 form important hydrogen bonds with Asp-183, which stabilize the active site structure (Stroud et al., 1971). This substitution is also interesting in view of the apparent lack of activation and subsequent probable loss of the salt linkage involving Asp-183.

Asp-178, present in the other glandular kallikreins and trypsin, is thought to be responsible for the specificity of these enzymes for substrates with basic side chains, acting through electrostatic interactions. The bulky phenol group of tyrosine would result in a much different specificity, perhaps one that would favor small side chains such as in elastase. However, elastase has a serine residue at the equivalent position, which has a much smaller side chain than tyrosine. Thus the aromatic side chain may be too large to allow any binding within the active site.

The comparison with elastase is, however, interesting with regard to the unprocessed activation peptide. Proelastase, without cleavage of its activation peptide, binds specifically to its substrate elastin (Shotton & Watson, 1970). Furthermore, chemical modification experiments suggest that the N-terminal Val-1–Asp-194 (elastase numbering) salt linkage of elastase is not necessary for catalytic activity (Kaplan & Dugas, 1969). The same salt linkage of α-chymotrypsin (Ile-1–Asp-194), on the other hand, is critical for activity (Oppenheimer et al., 1966).

The N-terminus of purified αNGF is blocked to Edman degradation (Isackson & Bradshaw, 1984). Inspection of the N-terminal sequence predicted from the cDNA does not provide an obvious explanation for this problem. The site of cleavage of signal peptide sequences generally follows a small amino acid side chain such as glycine, serine, alanine, or cysteine (Carne & Scheele, 1982). There are four such sites in the αNGF sequence preceding the putative activation peptide, Gly-(−12), Gly-(−11), Ala-(−8), and Ala-(−7) (Figure 5). Only a cleavage after Ser-(−2) would result in a pyroglutamic acid at the N-terminal, which would explain the intranscientness to Edman reaction. Ronne et al. (1983) have alluded to sequence data indicating that the N-terminal of mature αNGF begins with alanine at −7. Thus, it is unclear whether the α-subunit occurs in a blocked state in the 7S complex or whether it has become blocked in our preparations during isolation or other manipulations.

Because of the four amino acid deletion at the N-terminal of αNGF, it is possible that the unprocessed activation peptide could fold into the interior of the molecule similarly to the N-terminal of a normal processed serine protease. Cleavage of the signal peptide after Gly-(−11) would produce an N-terminal sequence Ile-Asp-Ala-Ala that could potentially act in this manner. Studies performed by Bode (1979) demonstrated that Ile-Val, Val-Val, or Ile-Ile interacts very specifically with Asp-194 of trypsinogen to stabilize the active domain. However, the Val → Asp replacement at residue 2 in this case seems plausible because of the Gly → His substitution at 182 of αNGF.

We have previously identified two forms of αNGF that differ in size by approximately 1000 daltons (Isackson et al., 1983). The expression of one of these forms is under the

control of testosterone, while the other is not. The difference appears to be due to an additional 7–10 amino acids present in one form and does not appear to be due to posttranslational proteolytic processing since the size differences are observable in cell-free translations. Our failure to detect two distinct cDNAs (aside from the allelic substitution) for αNGF in this study may show that there is indeed only one form of αNGF mRNA. Alternatively, there may be additional base substitutions in the region that includes the oligonucleotide probe.

While the amino acid changes in αNGF can easily be expected to have drastic effects on the catalytic efficiency of the enzyme, their importance relative to the interaction of α-subunit with βNGF is less clear. Both α- and γ-proteins bind specifically but to different sites on βNGF (Silverman & Bradshaw, 1982); γNGF is known to bind the C-terminal arginine residue of the β-subunit (Moore et al., 1974) in a manner consistent with an active site interaction. The binding site for the α-subunit is not known. The discovery that α-subunit is a serine protease, albeit inactive, raises the intriguing question of whether it is bound to the β-subunit through what would correspond to the active site or whether this interaction occurs in an entirely different region. In vitro mutagenesis of the cDNAs for α- and γNGF may lead to a better understanding of their interaction with βNGF.

The availability of cDNAs for each of the NGF subunits will also allow the preparation of specific probes for quantitating mRNA levels. This will provide the means to determine their sites of synthesis and to study the regulation of their expression including the coordination of their synthesis.

After this paper was submitted, Ronne et al. (1984) reported a partial amino acid sequence of the α-subunit corresponding to 76 residues in three different segments, and Evans & Richards (B. A. Evans and R. I. Richards, unpublished results) communicated to us the genomic structure of murine glandular kallikrein 4 (mGK-4), identified as α-NGF from the partial protein sequence provided to them (Ronne et al., 1984; Isackson & Bradshaw, 1984). Their results are identical with those reported herein (except for five silent mutations that clearly reflect subspecies differences). Both nucleic acid sequences are in complete agreement with the partial protein sequence of Isackson & Bradshaw (1984) but differ from that reported by Ronne et al. (1984) at positions 20 (Leu → Gln) and 116 (Cys → Glu) (Figure 3).

Acknowledgments

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