The High Molecular Weight Nerve Growth Factor Complex from *Mastomys natalensis* Differs from the Murine Nerve Growth Factor Complex†

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ABSTRACT: Both the male and the female of *Mastomys natalensis*, an African rat, have high levels of nerve growth factor (NGF) in their submaxillary glands. *Mastomys* high molecular weight (HMW) NGF was purified by gel filtration and ion-exchange chromatography and was compared with HMW NGF from the male mouse submaxillary gland. *Mastomys* HMW NGF sediments as a 5S species, does not exhibit esterase activity, and is more difficult to dissociate at acid pH than mouse 7S NGF. The biological activity could be isolated as a purified *Mastomys* βNGF protein identical in size and charge with that purified from male mice. The N-terminal amino acid sequence of the first 20 residues was determined and found to differ from that of mouse only at residue 8. Western blotting of *Mastomys* 5S NGF using antiserum against mouse βNGF indicates that the βNGF subunit of *Mastomys* is very similar to that of the mouse. Southern blots using a mouse kallikrein probe also demonstrate the presence of a large kallikrein family in *Mastomys* similar to that in mouse, and Northern blots verify transcription of kallikreins in *Mastomys* submaxillary gland. SDS–PAGE and isoelectric focusing gels reveal a *Mastomys* subunit that comigrates with mouse α subunit. However, neither oligonucleotide probes directed against mouse α subunit RNA nor antibodies directed against mouse αNGF cross-react strongly with the *Mastomys* material. This indicates that the second subunit of the *Mastomys* complex is not very similar to the mouse α subunit.

Nerve growth factor (NGF)[1] is found in high concentrations in the submaxillary gland of the male mouse. Mouse NGF is isolated as a high molecular weight (7S) complex of three subunits, α, β, and γ (Server & Shooter, 1977). Female mice contain much lower concentrations of NGF than males, because all three subunits of the complex are inducible by testosterone (Ishii & Shooter, 1975). The β subunit of NGF is responsible for the nerve growth promoting activity, while the α and γ subunits belong to the kallikrein group of the serine proteases (Evans & Richards, 1985). The γ subunit is an active protease and has been implicated in the processing of the βNGF precursor (Darling et al., 1983). However, the α subunit has no proteolytic activity, and its role in the mouse 7S complex is not well understood (Isackson et al., 1984).

NGF biosynthesis has recently become an active area of investigation because the cDNA encoding βNGF has been cloned and sequenced (Scott et al., 1983; Ullrich et al., 1983) and concomitantly the NGF protein precursors have been shown to exist as predicted by the cDNA-derived model (Berger & Shooter, 1978; Darling et al., 1983). The availability of cDNA probes for both NGF and its receptor has shown that discrete sites of NGF synthesis can be identified in a wide variety of cell types and developmental stages and that the receptor is likewise more widely distributed than suspected only a few years ago. These findings make it all the more urgent to explain the roles of other NGF-associated proteins in processing and delivery of active nerve growth factor. Our understanding is based almost entirely on the well-documented study of murine 7S NGF.

NGF has also been isolated from snake venom (Perez-Polo et al., 1978), bovine seminal plasma (Harper et al., 1982), and the prostate gland of the guinea pig (Rubin & Bradshaw, 1981). Snake venom NGF is complexed with a γ subunit. While there is some evidence for a high molecular weight NGF from bovine seminal plasma, guinea pig prostate NGF is associated with a kallikrein but does not appear to form a stable complex (Dunbar & Bradshaw, 1985).

It is of interest to identify and characterize other NGF complexes, to determine whether such complexes are specific only to males, and to examine the protein chemistry related to biosynthetic processing. In particular it is important to determine whether α and γ subunits associate with βNGF in other species or tissues and, if so, their biological role.

Mastomys natalensis is a rodent from southern Africa which has been studied due to the presence of an androgen-responsive prostate in the female (Brambell & Davis, 1940). Despite the low concentrations of androgens circulating in the serum of the female (Ghanadian et al., 1977), high levels of NGF are found in the submaxillary glands of both males and females (Aloe et al., 1981). *Mastomys* βNGF exhibits the same biological activity as mouse βNGF in the chick DRG assay and the radioimmunoassay (Darling & Shooter, 1984). We show here that *Mastomys* NGF, like mouse NGF, can be isolated as a high molecular weight (HMW) complex. The similarities and differences between the mouse and *Mastomys* subunits that we report afford an opportunity to study the functions of the subunits in the HMW NGF complex.

Materials and Methods

Materials. Sephadex G-100 and Sephadex G-75 and sulfopropyl-Sephadex SPC-50 were purchased from Pharmacia; DEAE-cellulose (DE-52) was purchased from Whatman; and

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[1] Abbreviations: NGF, nerve growth factor; HMW, high molecular weight; BAPNA, N^{α}-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride; DRG, dorsal root ganglia; RIA, radioimmunoassay; kb, kilobases; bp, base pair; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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Bio-Gel P-100 was purchased from Bio-Rad. N^{α}-Benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA) and carboxypeptidase B were obtained from Sigma. [^{32}P]dCTP was from Amersham. All materials for electrophoresis and isoelectric focusing were purchased from Bio-Rad. Nitrocellulose was BA-85 from Schleicher & Schuell. 7S NGF was isolated from mouse submaxillary gland as described by Varon et al. (1967). All other chemicals and materials were obtained from standard commercial sources.

Animals and Tissue. Adult male Swiss–Webster mice and Sprague-Dawley rats were obtained from Simonsen Laboratories. Male and female *M. natalensis* were from a breeding colony established at Stanford University Medical Center. Animals were sacrificed by CO_2 asphyxiation, and submaxillary glands were immediately collected, frozen in liquid nitrogen and stored at −20 °C for not more than 2 months.

Purification of High Molecular Weight NGF from Mastomys. All steps were carried out at 4 °C. The glands were homogenized in cold water in a Waring blender and then centrifuged at 50000*g* for 1 h. The supernatant was filtered through glass wool, lyophilized, and then solubilized in buffer A (25 mM sodium phosphate, pH 6.8, 10 μM $ZnCl_2$). Column chromatography (1.5 × 100 cm for 2.5 g of submaxillary gland starting material) was carried out by using Sephadex G-100 equilibrated with buffer A. Fractions were tested for protein concentration by measuring optical density at 280 nm and for NGF concentration by using both radioimmunoassay and bioassay on chick dorsal root sensory neurons as well as PC-12 cells (Varon et al., 1977). The peak of HMW NGF was collected and stored frozen. After being thawed and cleared by centrifugation (10000*g* for 10 min), the sample was applied to a column of DEAE-cellulose (Whatman) that had been degassed and equilibrated with buffer A. The column was then washed with buffer A, followed by a wash with buffer A containing 10 mM NaCl and elution with buffer A containing 100 mM NaCl. The eluted protein was concentrated in an Amicon concentrator with a PM-10 membrane. The concentrate was applied to a Bio-Gel P-100 column that had been equilibrated with buffer A containing 50 mM NaCl and was eluted with the same buffer.

Sedimentation Analysis. Both mouse high molecular weight NGF and *Mastomys* NGF (DEAE fraction) were subjected to sucrose density gradient sedimentation through a gradient of from 10 to 40% sucrose in 25 mM sodium phosphate, pH 7.2, and 10 μM $ZnCl_2$, with fluoresceinated BSA as an internal standard. The gradients were fractionated, and the fractions were assayed by radioimmunoassay for their NGF content.

Arginine Esteropeptidase Activity. Fractions from each stage of purification were tested for proteolytic cleavage of the synthetic arginine derivative BAPNA according to the method of Nichols and Shooter (1983).

Isoelectric Focusing and Polyacrylamide Gel Electrophoresis. The peak fraction from the Bio-Gel column was dialyzed against water containing 10 μM $ZnCl_2$ and was subjected to isoelectric focusing and polyacrylamide gel electrophoresis (SDS–PAGE) under reducing and nonreducing conditions as described by Moore et al. (1974).

Western Blotting. Proteins were transferred from polyacrylamide gels to nitrocellulose. Filters were probed with rabbit antiserum directed against mouse βNGF and αNGF (Darling et al., 1983; Fahnestock et al., 1986). Bound rabbit antibodies were detected with ^{125}I-Staph A protein.

Isolation of βNGF. βNGF was dissociated from the HMW complex by overnight incubation at room temperature in 0.1 M glycine hydrochloride, pH 3.0, and 3 M urea. The solution was loaded onto a column (0.5 × 4 cm) packed with sulfopropyl-Sephadex SPC-50 which had been equilibrated with 0.1 M glycine hydrochloride, pH 3.0, at a flow rate of 10 mL/h. The column was washed with two column volumes of the equilibrating buffer and then sequentially eluted with 50 mM sodium acetate, pH 4; the same plus 100 mM NaCl; the same plus 200 mM NaCl; 50 mM sodium acetate, pH 5, plus 100 mM NaCl; 50 mM sodium phosphate, pH 6; and 50 mM sodium phosphate, pH 6, plus 6 M guanidine hydrochloride. The last buffer eluted *Mastomys* βNGF, which was dialyzed against 0.1% acetic acid at 4 °C with three changes for 48 h.

Amino Acid Sequencing. The N-terminal amino acid sequence of the *M. natalensis* βNGF was determined with an Applied Biosystems Model 410A gas-phase protein sequencer and Model 120A PTH analyzer.

Plasmids. A cDNA probe for a mouse kallikrein was previously isolated (Fahnestock et al., 1986). The probe for mouse γNGF (Ullrich et al., 1984) was obtained from A. Ullrich at Genentech.

Preparation of Synthetic Oligonucleotides. Oligonucleotides chosen to be specific for mouse α subunit (5′-TTGGTACTTGTCGTT-3′ and 5′-GTCCTCAGGTTG-TGGGGTGTGCTCATTCAG-3′) or γ subunit (5′-ATACTCAAGGAATCGGATGTGCTTCCTCAT-3′) were synthesized by an Applied Biosystems 380A DNA synthesizer. Hydrolysis from the resin and purification were performed according to Applied Biosystems' protocols. The oligonucleotide was then end-labeled with ^{32}P, and the sequence was verified (Maxam & Gilbert, 1980).

Northern Analysis. Total RNA was isolated from mouse, *Mastomys*, and rat submaxillary glands by the method of Chirgwin et al. (1979). Ten to twenty micrograms of each RNA was electrophoresed through a formaldehyde–agarose gel and transferred to nitrocellulose (Thomas, 1980). Filters were prehybridized overnight and hybridized with the nick-translated probe (Fahnestock et al., 1986) for 48 h at 42 °C in the presence of 50% formamide. Washes were at room temperature first in 2 × SSPE–0.1% SDS and then in 0.1 × SSPE–0.1% SDS. (1 × SSPE is 10 mM sodium phosphate, pH 7.7, 0.18 M NaCl, and 1 mM EDTA.) Autoradiography was from 4 to 17 days at −80 °C. When an oligonucleotide probe was used, 10 μg of poly(A+) mRNA purified by chromatography over oligo(dT)–cellulose (Aviv & Leder, 1972) was electrophoresed and blotted as above, and the oligonucleotide was 5′-end labeled with polynucleotide kinase. Filters were hybridized as above but at room temperature and washed in 5× SSPE–0.1% SDS.

Southern Analysis. HMW DNA was isolated from the livers of both mouse and *Mastomys* (Maniatis et al., 1982), and 10-μg aliquots were digested with various restriction enzymes. DNA was electrophoresed through a 0.8% agarose gel and transferred to nitrocellulose (Thomas, 1980). Hybridization was carried out as for Northern analysis above, with a cDNA probe for a mouse kallikrein, pMF-1 (Fahnestock et al., 1986).

RESULTS

We confirmed the finding of Aloe et al. (1981) that homogenates of gland obtained from both male and female *Mastomys* contain NGF biological activity, which can be blocked by anti-NGF antibodies. The competitive radioimmunoassay indicated that the amounts of NGF (0.17 mg/g wet weight in male submaxillary gland, 0.13 mg/g in female glands) were equivalent in the two sexes and similar to the levels found in male mouse submaxillary glands (Darling & Shooter, 1984). HMW NGF from both male and female

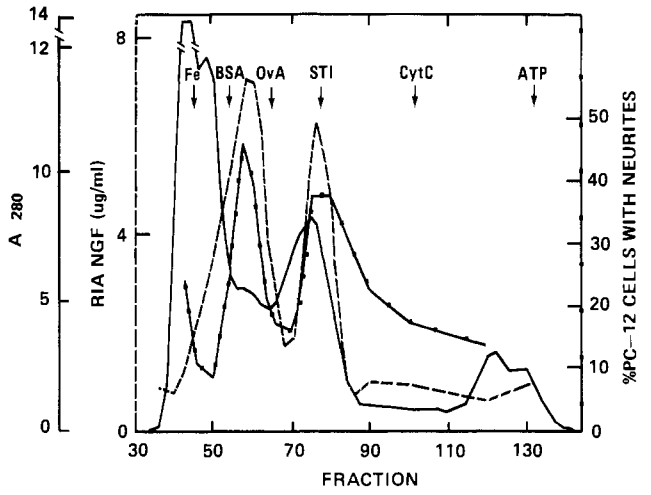

FIGURE 1: Chromatography of *Mastomys* proteins on Sephadex G-100. Chromatography was as described under Materials and Methods. (—) Total protein as measured by absorbance at 280 nm (--) NGF activity as measured by radioimmunoassay. (-●-) NGF activity as measured by percent of PC-12 cells with neurites in the bioassay.

Mastomys exhibited similar properties during purification.

The lyophilized gland extracts were solubilized in a buffer containing 10 μM $ZnCl_2$, which is known to stabilize murine 7S NGF, specifically the interaction of the γ subunit. Chromatography of *Mastomys* submaxillary gland supernatant proteins on Sephadex G-100 yields two peaks defined by both RIA and bioassay with DRG neurons as well as PC-12 cells (Figure 1). Submaxillary glands obtained from females as well as from males yielded identical chromatographic profiles. The high molecular weight (HMW) early-eluting material was always detected and always contained NGF biological activity that could be suppressed by antibody. The HMW peak did not coincide with a peak of esterase activity. In sharp contrast, the second peak of apparent M_r 25 000 was quite variable. This peak often had no biological activity but was detected as a positive response in the radioimmunoassay. The M_r 25 000 peak coincided exactly with a peak of esterase activity and probably contains considerable kallikrein. Expressed as a percentage of total RIA activity, this material varied between 15 and 50% in extracts prepared from either male or female tissue. The low molecular weight RIA-positive material will be considered in a separate paper.

The elution of murine 7S NGF from Sephadex gives an incorrect estimate of apparent molecular weight: 90 000 instead of the well-documented 135 000 (Burton et al., 1978), a result attributable to the interaction of 7S NGF with chromatographic resins. As an alternative, sedimentation through sucrose density gradients was used to size the *Mastomys* HMW NGF.

The protein peak representing the HMW *Mastomys* complex was pooled and chromatographed on DEAE-cellulose as shown in Figure 2. Only the second peak, eluting with 0.1 M NaCl, exhibited NGF activity by both RIA and bioassay. This peak was pooled. Sucrose density gradient sedimentation of the pooled, DEAE-fractionated material (Materials and Methods) yielded a sedimentation coefficient for mouse 7S NGF of 6.9 S, and for *Mastomys* HMW NGF of 5.1 S (Figure 3). The HMW NGF pool from Sephadex G-100 and the Bio-Gel P-100 pool (below) showed similar sedimentation profiles, in each case migrating as 5.1S NGF compared to 7S NGF and the fluoresceinated BSA internal standard.

The 0.1 M NaCl eluate pooled after the DEAE step was subjected to chromatography on Bio-Gel P-100 as shown in

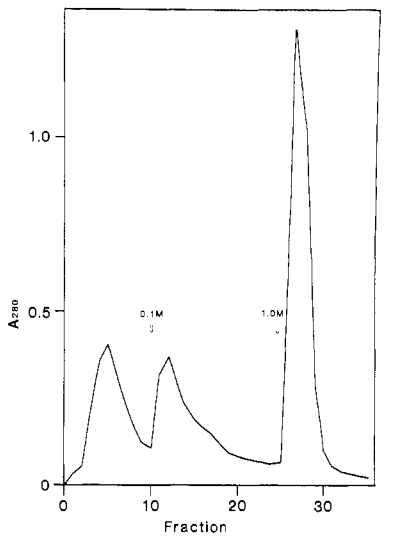

FIGURE 2: Chromatography of *Mastomys* high molecular weight NGF on DEAE-cellulose. The early-eluting peak from Figure 1 was subjected to chromatography on DEAE-cellulose as described under Materials and Methods. The profile represents absorbance at 280 nm. Only the second peak, eluting with 0.1 M NaCl, exhibited NGF activity by both RIA and bioassay.

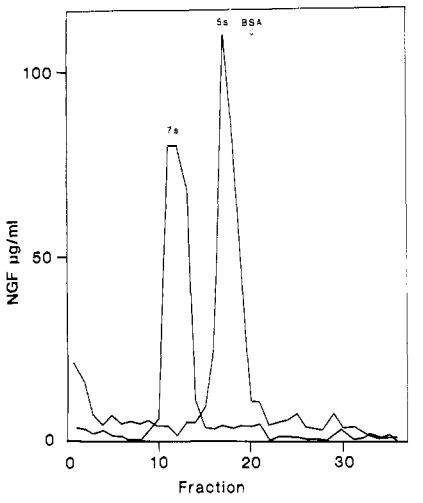

FIGURE 3: Sedimentation of mouse and *Mastomys* HMW NGF. Sucrose gradients were run as described under Materials and Methods. Fractions were assayed by radioimmunoassay. Purified mouse NGF sedimented as the peak labeled 7S and *Mastomys* NGF from the DEAE-cellulose column (0.1 M NaCl peak) sedimented as the peak marked 5S.

Figure 4. Only the major peak, estimated to have a molecular weight of 70 000–80 000 and comprising fractions 21–24, exhibited NGF activity by bioassay. These fractions were pooled. When the fractions collected from the Bio-Gel P-100 column were assayed for arginylesteropeptidase activity with the substrate BAPNA, some activity was found in the small peak which eluted after the HMW NGF; however, no activity was detected in the HMW NGF peak itself.

The Bio-Gel HMW NGF was analyzed by SDS–PAGE (Figure 5). Under nonreducing conditions three bands were observed: M_r 70 000, 27 000, and 13 000. The M_r 13 000 and 27 000 bands comigrate with mouse β and α subunits, respectively. When reducing conditions were employed, the M_r 13 000 band migration was unchanged, which is typical of βNGF; the M_r 70 000 band exhibited some heterogeneity; and the M_r 27 000 band was lost, with new bands at less than M_r 20 000 and at approximately 10 000, consistent with an α subunit. When the HMW NGF Bio-Gel peak was subjected to SDS–PAGE under nonreducing conditions and transferred

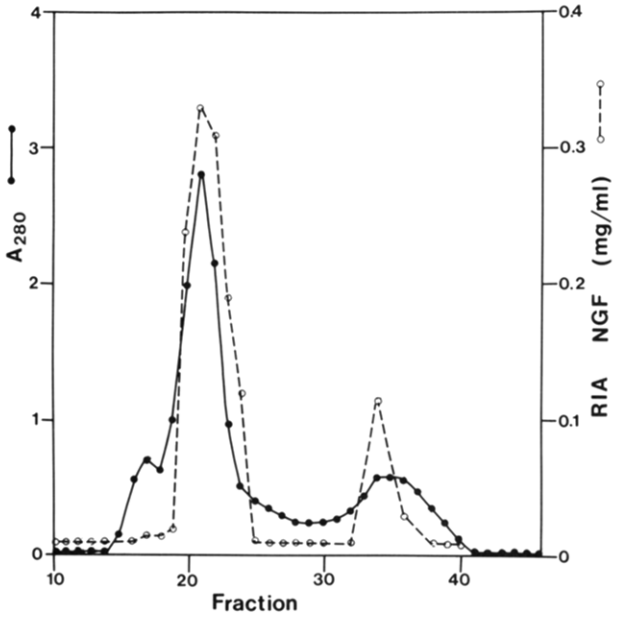

FIGURE 4: Chromatography of *Mastomys* HMW NGF on Bio-Gel P-100. The peak fraction (0.1 M NaCl) from Figure 2 was subjected to chromatography on Bio-Gel P-100 as described under Materials and Methods. (−●−) Total protein as measured by absorbance at 280 nm. (−○−) NGF activity, as determined by radioimmunoassay. Only the major peak (fractions 21–24) exhibited NGF activity by bioassay.

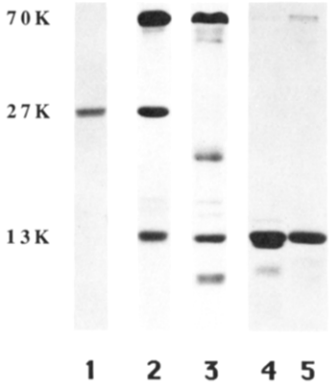

FIGURE 5: SDS–PAGE of mouse and *Mastomys* NGFs. Mouse and *Mastomys* samples were subjected to SDS–PAGE under nonreducing (lanes 1 and 2) and reducing (lanes 3–5) conditions. Lane 1: Purified mouse α subunit. Lanes 2 and 3: *Mastomys* HMW NGF from the peak fractions of the Bio-Gel P-100 column. Lane 4: Mouse βNGF. Lane 5: Purified *Mastomys* βNGF.

to nitrocellulose for a Western blot (Figure 6), the presumptive βNGF protein bound anti-NGF antibodies, as visualized by ^{125}I-Staph A protein binding autoradiography. The M_r 27 000 protein transferred poorly and demonstrated weak binding of anti-α antibody, which is consistent with the lack of a precipitin line in immunodiffusion carried out with this HMW NGF and anti-α sera. The M_r 70 000 band also transferred poorly but demonstrated no binding with either antibody. Several methods were employed to dissociate the M_r 70 000 band, which might have been interpreted as undissociated complex; this material was resistant to 0.1 N HCl, in the presence and absence of urea. Tentatively, the M_r 70 000 species has been interpreted as a contaminant.

Isoelectric focusing and heavy staining of the pooled, Bio-Gel-purified material showed two prominent bands which focused identically with mouse α and β subunits, as well as some fainter bands in the region in which the γ subunits routinely focus (Figure 7).

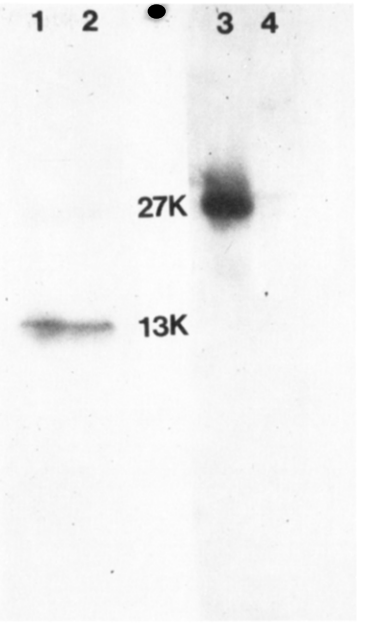

FIGURE 6: Western blotting of mouse and *Mastomys* HMW NGF. Western blotting was as described under Materials and Methods. Lanes 1 and 3: Mouse NGF. Lanes 2 and 4: *Mastomys* NGF. Lanes 1 and 2 were probed with anti-βNGF serum. Lanes 3 and 4 were probed with anti-αNGF serum.

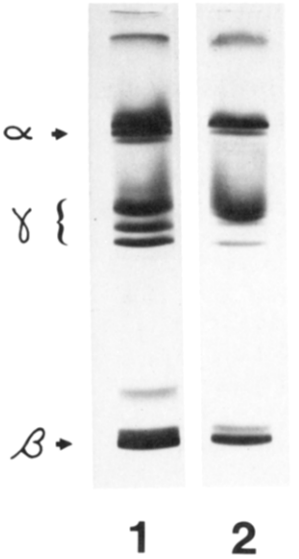

FIGURE 7: Isoelectric focusing of mouse and *Mastomys* HMW NGF. Purified mouse 7S NGF and *Mastomys* NGF from the peak fraction of the Bio-Gel P-100 column were subjected to isoelectric focusing from pH 3 to pH 10. Lane 1: Mouse 7S NGF proteins (α, γ, and β subunits are indicated). Lane 2: *Mastomys* NGF proteins. pH 3 is at the top of the figure and pH 10 is at the bottom.

The βNGF protein of *Mastomys* could not be isolated from the 5S NGF complex by the procedure utilized with mouse 7S NGF, which calls for dissociation at pH 4 followed by adsorption and elution from (carboxymethyl)cellulose equilibrated at pH 4 (Varon et al., 1967). Dissociation of 5S NGF was accomplished by overnight incubation at pH 3.0 in the presence of urea, after which the βNGF subunit was isolated by chromatography on sulfopropyl-Sephadex (Materials and Methods).

The purified *Mastomys* βNGF was greater than 95% pure when analyzed by electrophoresis through polyacrylamide gels in the presence of SDS (Figure 5, lane 5) and is identical in apparent size with murine NGF. This *Mastomys* βNGF subunit has a biological activity identical with that of murine βNGF (Darling & Shooter, 1984). Residues 1–20 of the

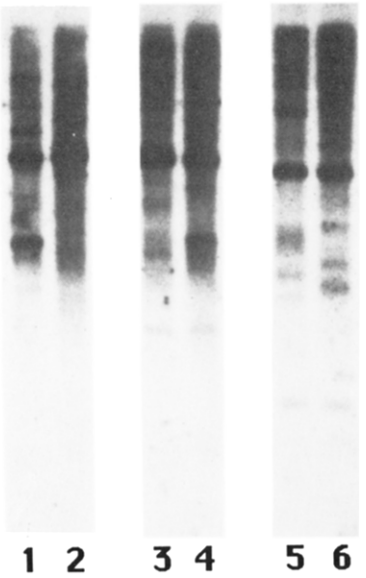

FIGURE 8: Southern analysis of mouse and *Mastomys* DNA. A total of 10 μg each of mouse and *Mastomys* DNA was digested with *Bam*H1 (lanes 1 and 2), *Hin*dIII (lanes 3 and 4), or *Eco*RI (lanes 5 and 6) and electrophoresed on an 0.8% agarose gel. After transfer to nitrocellulose filters, the DNA was probed as described under Materials and Methods with ^{32}P-labeled pMF-1, a plasmid coding for a mouse kallikrein. Lanes 1, 3, and 5: Mouse DNA. Lanes 2, 4, and 6: *Mastomys* DNA.

amino-terminal amino acid sequence of the *Mastomys* β subunit were determined (Materials and Methods): Ser-Ser-Thr-His-Pro-Val-Phe-Gln-Met-Gly-Glu-Phe-Ser-Val-Cys-Asp-Ser-Val-Ser-Val.

The protein chemistry of *Mastomys* NGF indicates that βNGF has several features which are strongly conserved across species lines. The 5S complex has some similarities to the murine 7S NGF complex, but the chemistry of the second protein, despite its charge similarity to the murine α subunit, is obscure because of a lack of strong cross-reactivity with an antiserum that reacts with the mouse αNGF. For these reasons we utilized mouse cDNA and oligonucleotide probes to compare sequences coding for the mouse NGF subunits with the mRNAs expressed in the *Mastomys* submaxillary gland and the genetic information contained in the *Mastomys* genomic DNA. These experiments allow some comparisons to be made immediately and lead directly to the cloning and sequencing of the cDNA coding for the cognate proteins.

The probe used in the Southern blot shown in Figure 8 is pMF-1 (Fahnestock et al., 1986), a cDNA clone coding for a mouse kallikrein. The mouse kallikrein family contains approximately 25 members that share 80–90% sequence homology, whereas the rat contains fewer kallikrein genes (Mason et al., 1983). pMF-1 cross-hybridizes with a number of members of the kallikrein family in both mouse and *Mastomys*. Although the sizes of the bands are not identical, indicating some sequence diversity, the strength and numbers of bands are similar, indicating once again a very high degree of sequence homology between mouse and *Mastomys* and the presence of a large kallikrein gene family in *Mastomys*.

Northern blots of mouse, *Mastomys*, and rat RNA (Figure 9) with the murine kallikrein cDNA pMF-1 as a probe revealed hybridization with a band of the same size (0.95 kb) in both *Mastomys* and mouse but not in rat. Half the amount of RNA was loaded in the *Mastomys* lane as compared to mouse, and the hybridization signal is correspondingly half as strong. The pMF-1 probe hybridizes to a number of kallikreins in both mouse and *Mastomys*, as shown in the

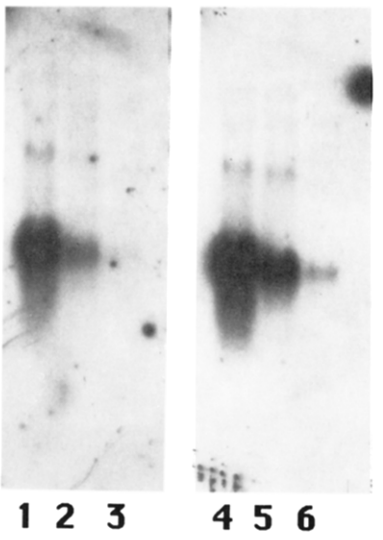

FIGURE 9: Northern analysis of mouse, *Mastomys*, and rat RNA. A total of 20 μg of mouse RNA, 10 μg of *Mastomys* RNA, and 10 μg of rat submaxillary gland RNA were prepared for Northern blotting as described under Materials and Methods. The filter was probed with ^{32}P-labeled pMF-1 cDNA, then washed, and reprobed with ^{32}P-labeled γNGF cDNA. Lanes 1 and 4: Mouse RNA. Lanes 2 and 5: *Mastomys* RNA. Lanes 3 and 6: Rat RNA. Lanes 1–3 probed with pMF-1 cDNA. Lanes 4–6 probed with γNGF cDNA.

Southern blot (Figure 8). Therefore, the band in the Northern blot is a mixture of kallikrein species. We can conclude that both the transcript size and amount of kallikreins transcribed in mouse and *Mastomys* submaxillary gland are similar.

When a cDNA coding for mouse γNGF is used to probe the blot, the same results are obtained as with the kallikrein probe for mouse and *Mastomys*. However, the γ subunit cDNA also hybridizes with kallikrein transcripts in the rat RNA. The weak signal is probably primarily due to the reduced amount of kallikrein transcription in the rat submaxillary gland rather than a lack of homology with the mouse probe.

Poly(A+) mRNA from both mouse and *Mastomys* was used when the Northern blot was probed with oligonucleotides. The first oligonucleotide probe tested, having the sequence 5′-TTGGTACTTGTCGTT-3′, is specific for the α subunit of mouse 7S NGF. The probe covers the region at the N-terminal end of the α subunit precursor from amino acid −1 to amino acid 4, a region which contains a deletion and amino acid substitution relative to other mouse kallikreins (Isackson et al., 1984). The oligonucleotide gave a strong hybridization signal with the mouse mRNA at approximately 950 bp (Figure 10a), but gave an undetectable signal at that molecular weight with *Mastomys* mRNA. Both species showed some nonspecific hybridization between the oligonucleotide probe and ribosomal RNA bands with this probe.

A second probe, complementary to the region coding for amino acids 15–20 of mouse α subunit (5′-GTCCTCAGGTTGTGGGGTGTGCTCATTCAG-3′), hybridized to both mouse and to *Mastomys* mRNA (Figure 10b). This probe also covers a region that is variable among the kallikreins, although the probe is homologous to sequences for mGK-5 and mGK-6 in addition to the α subunit (Evans et al., 1987). Hybridization to *Mastomys* mRNA was weaker than to mouse mRNA, indicating either some sequence divergence or a less prevalent mRNA species in *Mastomys*.

A third oligonucleotide complementary to the γ subunit over the same region (5′-ATACTCAAGGAATCGGATGTG-CTTCCTCAT-3′) hybridized strongly to mouse mRNA but not at all to *Mastomys* mRNA (Figure 10c).

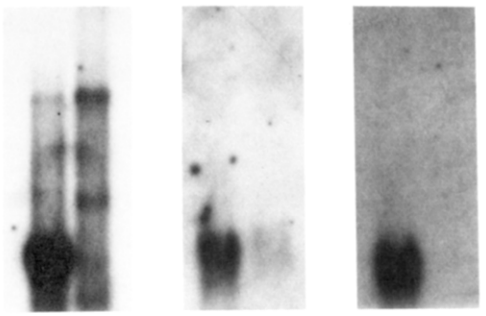

FIGURE 10: Northern analysis of mouse and *Mastomys* RNA. A total of 10 μg each of poly(A+) mRNA from mouse and *Mastomys* were prepared for Northern blotting as described. The filter was probed with (a) an 18 base long synthetic oligonucleotide specific for mouse αNGF, (b) a 30-mer specific for mouse αNGF, and (c) a 30-mer specific for γNGF. Lane 1: Mouse mRNA. Lane 2: *Mastomys* mRNA.

Discussion

High molecular weight NGF can be purified from the submaxillary glands of both male and female *M. natalensis* by successive chromatography over Sephadex G-100, DEAE-cellulose, and Bio-Gel P-100. The material obtained by these procedures sediments at 5.1 S as distinguished from the mouse NGF complex which sediments at 7 S. The *Mastomys* complex contains proteins which migrate identically with mouse α and β subunits on SDS–PAGE and isoelectric focusing gels. This complex is more difficult to dissociate than the mouse 7S complex, however, suggesting the existence of altered subunit contacts. Furthermore, the complex may lack γ subunits, as shown by its lack of esteropeptidase activity.

M. natalensis HMW NGF may be composed of a β dimer and possibly two α subunits. Such a composition would be reasonable in light of a sedimentation coefficient of 5.1 S, which would be appropriate for a complex having M_r 75 000. However, an alternative explanation is that *Mastomys* HMW NGF may exist in the gland as a 7S complex (Burcham & Shooter, 1987) but that its dissociation characteristics are different from those of mouse 7S NGF. The *Mastomys* three-subunit complex may dissociate easily to form a tightly bound two-subunit complex, in contrast to the three subunits strongly bound in the mouse 7S complex.

Our purification utilizes a phosphate buffer, pH 6.8, whereas Burcham and Shooter in a recent abstract (1987) describe the isolation of a *Mastomys* 7S NGF with a Tris buffer, pH 7.4, at 25 °C. A buffer of higher pH could stabilize the γ subunits in a 7S complex, which we cannot positively identify in our preparation. The precise purification scheme may influence the recovery of subunits, as has been documented for mouse 7S NGF. However, the sedimentation coefficient of 5.1 S, which the *Mastomys* NGF complex demonstrates at all stages of purification, suggests a βNGF dimer with two M_r 27 000 proteins which may or may not be identical. The sedimentation profile was reproducible even in the presence of EDTA and pancreatic trypsin inhibitor which are known to disrupt the binding of murine γ subunit in the 7S NGF complex. Furthermore, no peak of esterase activity could be detected copurifying with the *Mastomys* NGF complex during purification. The Bio-Gel-purified NGF complex had no esterase activity even when assayed in the presence of EDTA, despite the presence of stained bands in the γ region of the isoelectric focusing gels.

The amino-terminal amino acid sequence determined for the *Mastomys* β subunit is identical with that of mouse βNGF except for residue 8. At this position the *Mastomys* protein has a glutamine which replaces the histidine found in murine βNGF. The 20-residue sequence also agrees with the sequence derived from a cDNA clone coding for *Mastomys* βNGF (Fahnestock & Bell, 1988). Murine βNGF can be cleaved by a submaxillary gland kallikrein (Mobley et al., 1976) at the His–Met bond (residues 8–9) to yield an octapeptide and a fully active des(octa)NGF. As others have noted (Harper et al., 1982), it is interesting that the octapeptide sequence is conserved so well in several species, even though murine NGF, at least, is fully active without the octapeptide. *Mastomys* βNGF, however, may be resistant to cleavage at residues 8–9 because of the glutamine substitution at residue 8. This hypothesis is supported by two experimental results. First, the *Mastomys* βNGF exhibits no heterogeneity when subjected to N-terminal amino acid sequencing. Second, SDS–PAGE (Figure 5) demonstrates that βNGF purified from mouse submaxillary gland consists of a mixture of full-length and des(octa)NGF while the purified *Mastomys* βNGF appears to consist of only a single specie.

The *Mastomys* β subunit is highly homologous to the mouse βNGF. This is demonstrated at the protein level by biological activity, cross-reactivity in the radioimmunoassay, identical charge characteristics in isoelectric focusing gels, and both identical size characteristics and immunological cross-reactivity on Western gels. However, the mouse and *Mastomys* βNGFs differ in their susceptibility to carboxypeptidase B, the *Mastomys* βNGF being resistant to cleavage of the C-terminal arginine (data not shown). This difference raises an important question about the structure of the carboxy terminus whose sequence in the mouse derives from specific cleavage of a precursor protein by the γ subunit.

It is clear that a cleavage of the sort carried out by the γ subunit requires a basic residue such as arginine or lysine, both of which should be susceptible to removal by carboxypeptidase B. Indeed, recent nucleotide sequence data of *Mastomys* βNGF (Fahnestock & Bell, 1988) demonstrate the presence of the sequence Arg-Arg-Gly at the carboxy terminus of this molecule. There is in addition, however, a substitution of a proline residue for the threonine-117 found in the mouse sequence. Both the sequences Pro-Arg-Arg-Gly-COOH and Pro-Arg-COOH would be resistant to carboxypeptidase B. Since both mouse and *Mastomys* βNGFs show identical charge characteristics in isoelectric focusing gels, we feel that Pro-Arg-COOH is the most likely sequence for mature *Mastomys* βNGF. In addition, if such a sequence resulted from the cleavage of a precursor by an enzyme of the kallikrein family the proline residue might not allow the subsequent binding of the γ-like enzyme to the site of cleavage or might reduce the affinity of binding greatly, even in the presence of zinc. Since the exact role of zinc and its specific contacts in the interaction are not identified, the presence of the proline residue might specifically inhibit the interaction which in the mouse 7S complex is so greatly stabilized by zinc.

The mouse and *Mastomys* βNGF subunits are also highly homologous at the nucleic acid sequence level. Bowcock et al. (1988) reported the similarity of mouse and *Mastomys* βNGF nucleic acid sequence by Northern and Southern blotting. Fahnestock and Bell (1988) verified the homology by sequencing a cDNA clone for *Mastomys* βNGF.

The identity of the second *Mastomys* subunit is somewhat less clear. In isoelectric focusing gels, a subunit of the *Mastomys* complex migrates identically with mouse α subunit. The lack of any esteropeptidase activity in the complex further suggests that the *Mastomys* subunit is α-like rather than

γ-like. However, anti-α subunit IgG does not cross-react significantly with *Mastomys* HMW NGF in Western blots, and oligonucleotide probes specific for the mouse α subunit hybridize weakly, at best, to *Mastomys* poly(A+) mRNA.

The lack of esteropeptidase activity exhibited by the HMW complex and the lack of hybridization of *Mastomys* mRNA with a γ-specific oligonucleotide argue against the existence of a γ-like subunit in the *Mastomys* complex. However, the lack of γ-like protein in the *Mastomys* HMW complex does not mean that *Mastomys* lack γ protein altogether. A Southern blot using a mouse kallikrein probe (pMF-1) demonstrates that *Mastomys* contains a large number of homologous genes coding for kallikreins, and a Northern blot using the same probe demonstrates that high concentrations of these (levels similar to the mouse and greater than rat) are transcribed in the *Mastomys* submaxillary gland. The nucleotide sequence of a *Mastomys* γ subunit could differ from the mouse sequence in the region covered by the synthetic oligonucleotide used as a probe. Thus, γ subunit or γ-like proteins could be present in the submaxillary gland but not as part of the HMW complex, or alternatively, the *Mastomys* γ subunit could be more loosely bound in the complex than the mouse γ in the 7S complex and, thus, easily lost during the purification procedure. The latter explanation is supported by evidence suggesting altered dissociation characteristics in the *Mastomys* HMW complex and isoelectric focusing gels showing some evidence of γ-like subunits.

In summary, the *Mastomys* NGF complex differs in its properties, especially its behavior during purification and sedimentation, from murine NGF. A protein similar to the murine α subunit can be detected, but the presence of a γ subunit has not been demonstrated. This is surprising, particularly since β–γ complexes have been described before, such as the NGF of *Crotalus* snake venom (Perez-Polo et al., 1978). However, Rubin and Bradshaw (1981) could not find evidence for esterase activity in a putative guinea pig NGF complex. In *Mastomys*, an α-like and a βNGF species can be identified, but the composition of the major complex of *Mastomys* NGF is equivocal thus far. The precise nature of the *Mastomys* NGF subunit contacts and the identity of other subunit(s) in the 5S complex will require further study.

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