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β-NGF-endopeptidase: Structure and Activity of a Kallikrein Encoded by the Gene *mGK-22*†

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ABSTRACT: Mouse nerve growth factor (NGF) is cleaved at a histidine–methionine bond to release an NH_2-terminal octapeptide (NGF^{1-8}). The enzyme responsible, β-NGF-endopeptidase, is structurally and functionally similar to γ-NGF and epidermal growth factor-binding protein (EGF-BP) and cleaves mouse low molecular weight kininogen to produce bradykinin-like activity. These data have suggested that, like γ-NGF and EGF-BP, β-NGF-endopeptidase is a mouse glandular kallikrein. Evidence for a physiological role for NGF^{1-8} encouraged studies to further characterize the structure and function of this enzyme. Purified β-NGF-endopeptidase migrated as a single band on isoelectric focusing and reducing SDS–polyacrylamide gels. As was expected, it removed NGF^{1-8} from NGF. Interestingly, enzymatic activity on an artificial substrate, and on NGF, was inhibited by NGF^{1-8} and by bradykinin. These studies further supported the view that β-NGF-endopeptidase acts on both NGF and kininogen. The first 30 NH_2-terminal amino acids of β-NGF-endopeptidase were sequenced. This analysis demonstrated that the enzyme is encoded by the gene designated *mGK-22* (Evans et al., 1987). The sequence of this gene corresponds to that of EGF-BP type A (Anundi et al., 1982; Drinkwater et al., 1987), and so studies were performed to determine whether or not β-NGF-endopeptidase participates in EGF complex formation. Chromatographic and kinetic data gave no evidence that β-NGF-endopeptidase is an EGF-binding protein. Our studies suggest that contamination of high molecular weight (HMW) EGF preparations with β-NGF-endopeptidase erroneously led to earlier designation of the product of *mGK-22* as an EGF-BP. We conclude that *mGK-22* codes for β-NGF-endopeptidase and that this enzyme is a kallikrein active on both NGF and kininogen. These data suggest that the enzyme may have a biological role through production of NGF^{1-8} and kinins.

Mouse glandular kallikreins comprise a multigene family located on chromosome 7. The glandular kallikreins are serine proteases, structurally similar to trypsin; however, unlike trypsin, they have been reported to exhibit highly defined substrate specificities (Mason et al., 1983). Kallikreins may play an important role in enzymatically processing the precursors of polypeptide hormones. Twenty-four separate mouse kallikrein genes have been sequenced, and as many as 14 different active gene products may be produced (Evans et al., 1987). Members of the kallikrein family produced in the mouse submandibular gland include the α and γ subunits of 7S NGF[1] (Thomas et al., 1981; Isackson & Bradshaw, 1984), glandular kallikrein (Drinkwater & Richards, 1987), renal kallikrein (van Leeuwen et al., 1986), γ-renin (Poe et al., 1983; Drinkwater et al., 1988), and a number of other kallikreins whose functions have not been defined (Fahnestock et al., 1986; Drinkwater & Richards, 1988). In addition, three separate members of the kallikrein family have been identified as mouse EGF-binding proteins (EGF-BP). These proteins are EGF-BP type A, a single-chain protein (Anundi et al., 1982; Ronne et al., 1983), EGF-BP type B, a two-chain protein (Ronne et al., 1983), and EGF-BP type C, a three-chain protein and the only

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[1] Abbrevations: NGF, nerve growth factor; NGF^{1-8}, NH_2-terminal octapeptide of NGF; des^{1-8}NGF, NGF chain lacking the first eight NH_2-terminal amino acids; EGF, epidermal growth factor; HMW-EGF, high molecular weight complex containing EGF; EGF-BP, epidermal growth factor binding protein; SDS-PAGE, SDS–polyacrylamide gel electrophoresis; BAPNA, N^{α}-benzoyl-DL-arginine-*p*-nitroanilide; BK, bradykinin; Lys-BK, lysylbradykinin; CM-Cys, (carboxymethyl)cysteine. As used herein, NGF refers to NGF molecules irrespective of the protocol used for purification.

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one yet shown to form a high molecular weight complex with EGF (Isackson et al., 1987).

β-NGF-endopeptidase is a mouse submandibular gland enzyme that removes an NH_2-terminal octapeptide (NGF^{1-8}) from NGF (Wilson & Shooter, 1979). β-NGF-endopeptidase cleaves NGF at an unusual site, a His–Met bond, suggesting a high degree of substrate specificity. This enzyme is secreted into saliva together with NGF, and there is evidence that release of NGF^{1-8} occurs during secretion (Burton et al., 1978). The physiological relevance of NGF^{1-8} production is uncertain; however, this peptide has recently been shown to induce hyperalgesia in the rat paw (Levine et al., 1988). Interest in β-NGF-endopeptidase actions encouraged studies to define further enzyme structure and function. Prior studies have shown β-NGF-endopeptidase is a single-chain protein that cross-reacts immunologically with γ-NGF and EGF-BP type C and has a molecular weight and amino acid composition similar to these two kallikreins (Wilson & Shooter, 1979; Bothwell et al., 1979). It was also reported to cleave kininogen, releasing bradykinin-like activity (Bothwell et al., 1979). These data suggested that β-NGF-endopeptidase was a member of the kallikrein family. Herein, we identify the gene encoding this enzyme and we further characterize the activity of β-NGF-endopeptidase. These studies suggest a biological role for the enzyme and a mechanism for regulating its activity.

Materials and Methods

β-NGF-endopeptidase was purified by the method of Wilson and Shooter (1979). On occasion, the last step of the purification protocol (carboxymethyl cellulose chromatography) was repeated in order to increase sample purity. NGF was purified directly from the homogenate of the adult male mouse submandibular gland via ion-exchange chromatography (Mobley et al., 1986) or, using the same procedures, from the 7S NGF-containing peak of the Sephadex G-100 column used to prepare β-NGF-endopeptidase. High molecular weight EGF (HMW-EGF), EGF, and EGF-BP type C were isolated from epinephrine-stimulated saliva (Burton et al., 1978) or from the submandibular gland homogenate via the methods of Taylor et al. (1970). N^{α}-Benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA) was purchased from Sigma (St. Louis, MO). The extinction coefficient of the cleaved product was taken as 8800 cm^{-1} M^{-1} (Nichols & Shooter, 1983); this was not adjusted for the percentage of the L isomer present. Molecular weight marker proteins for gel filtration were obtained from Sigma. Bradykinin (BK) and lysylbradykinin (Lys-BK) were obtained from Peninsula Laboratories Inc. (Burlingame, CA). NGF^{1-8} (S-S-T-H-P-V-F-H; i.e., ending as a free carboxy acid) and its analogues were synthesized by solid-phase methods (Merrifield, 1986) using commercially available reagents and instrumentation (Applied Biosystems 430A; Applied Biosystems, Foster City, CA). Peptides were applied to a reversed-phase HPLC column [radial pak C18 (Waters, Milford, MA) or Vydac C18 (Separations Group, Hesperia, CA)] in 0.1% TFA (Pierce, Rockford, IL) and eluted in a gradient of acetonitrile (Fisher Scientific, Pittsburgh, PA) in 0.1% TFA. The concentration of all samples was determined by amino acid analysis.

Isoelectric focusing was performed on β-NGF-endopeptidase as described (Greene et al., 1971; Server & Shooter, 1976) with a gradient of pH 3.5–10 ampholines (LKB, Gaithersburg, MD). p*I* marker proteins were obtained from Calbiochem (La Jolla, CA). Gels were fixed in 20% trichloroacetic acid (TCA), stained in 0.1% Naphthol Blue Black (Bio-Rad, Richmond, CA) in 10% acetic acid, and destained in the same solvent. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli (1970) using 15% or 10–20% gradient gels. Molecular weight markers were from Sigma or LKB. Gels were fixed in 20% TCA, stained in 1% Fast Green FCF (Bio-Rad) in acetic acid–methanol–H_2O (10:45:45), and destained in the same solvent (Mobley et al., 1986). To examine β-NGF-endopeptidase and EGF-BP type C for their ability to form a complex with EGF, we used a nondenaturing gel electrophoresis system in 12% polyacrylamide, pH 7.05 (Server et al., 1976). Gels were stained with 1% Fast Green FCF in 10% acetic acid and destained in the same solvent.

Processing of NGF by β-NGF-endopeptidase. NGF was lyophilized and incubated with β-NGF-endopeptidase in 50 mM sodium phosphate buffer, pH 6.8, at room temperature. At intervals, the reaction was stopped by freezing. Samples were analyzed by SDS–PAGE (Laemmli, 1970) after first adding 2X SDS–PAGE treatment buffer (0.125 M Tris-HCl buffer, pH 6.8, 4% SDS, 10% β-mercaptoethanol) and boiling for 90 s. Stained gels were densitometrically scanned (LKB-Ultroscan). $Des^{1-8}NGF$ was purified by ion-exchange chromatography with the second carboxymethyl cellulose column of Mobley et al. (1976), after first dialyzing vs 50 mM sodium acetate, pH 4.0, containing 0.4 M NaCl. For studies demonstrating inhibition of NGF cleavage by NGF^{1-8} or BK, the peptide was first added to enzyme in 50 mM sodium phosphate, pH 6.8, and preincubated at room temperature for 15–30 min. After NGF was added, the reaction proceeded at room temperature for 30 min and was stopped by freezing the mixture or by adding an equal volume of 2X SDS–PAGE treatment buffer. Samples were then analyzed by SDS–PAGE.

Processing of BAPNA by β-NGF-endopeptidase. Enzyme (20 μg) was incubated with BAPNA (0.02–2.8 mM) in 50 mM sodium phosphate buffer, pH 6.8, at room temperature while the change in absorbance at 410 nm was recorded. The rate of BAPNA cleavage was expressed in relation to the amount of enzyme in micrograms. Kinetic data were analyzed with Enzfitter software (Robin Leatherbarrow, Cambridge, U.K.). In inhibition experiments β-NGF-endopeptidase was preincubated with NGF^{1-8}, NGF^{1-8} analogues, BK, or Lys-BK in 50 mM sodium phosphate, pH 6.8, for 15 min at room temperature prior to BAPNA addition. The percent inhibition of BAPNA cleavage was determined from the average rate of BAPNA cleavage per minute, over the first 10–30 minutes.

Esteropeptidase Activity Measurements in the Presence of EGF. Twenty micrograms of EGF-BP type C was incubated in the absence or the presence of 4.1 μg of EGF in a final volume of 100 μL of 50 mM sodium phosphate, pH 6.8, overnight at 4 °C. The final concentration of both EGF and EGF-BP was 6.8 μM. Following incubation, the solution was brought to room temperature, 10 μL was added to 20 μM BAPNA in 50 mM sodium phosphate, pH 6.8 (total volume 1074 μL), and the reaction was allowed to proceed with monitoring at 410 nm. In one experiment an additional 4.1 μg of EGF was added to the cuvette at the time of assay. Incubations were also conducted with β-NGF-endopeptidase (17.7 μg) plus or minus EGF (4.1 μg), as described above. For the assay, a 40-μL aliquot was added to the cuvette. In one experiment an additional 16 μg of EGF was added to the cuvette at the time of assay.

Gel Filtration of Mouse Salivary Proteins. Mouse saliva (4.4 mL) obtained after epinephrine stimulation (Burton et al., 1978) was dialyzed against 25 mM sodium phosphate buffer, pH 6.8, overnight at 4 °C, and centrifuged at 10000*g* for 30 min, and the supernatant was made 10% in sucrose and

loaded under the eluant onto a Sephadex G-100 column equilibrated in the same buffer (2.5 × 100 cm; flow rate = 20 mL/h). Two-milliliter fractions were assayed for absorbance at 280 nm, for β-NGF-endopeptidase protein by SDS–PAGE, for β-NGF-endopeptidase activity, and for EGF-like immunoreactivity. β-NGF-endopeptidase activity was assayed by adding 10 μL of individual fractions, plus 15 μL of the same buffer, to 20-μg samples of NGF dried by lyophilization. The reaction proceeded for 30 min at room temperature and was stopped by the addition of an equal volume of 2X treatment buffer followed by SDS–PAGE. For the EGF immunoassay, individual column fractions or known amounts of HMW-EGF were acidified with an acetic acid containing buffer (final pH 4.5–5.1) and spotted onto nitrocellulose paper (BA85; Schleicher & Schuell, Keene, NH). For HMW-EGF, 5 μL containing 0.36–11.67 μg was used to generate a standard curve. Sephadex G-100 column fractions were diluted with an equal volume of the buffer before applying 5 μL. Blots were dried for $^1/_2$ h at 37 °C, blocked in 5% Carnation nonfat dry milk in 10 mM PBS for 2 h at room temperature, rinsed in 10 mM phosphate-buffered saline containing 0.05% Tween (Bio-Rad) (PBS–Tween), and incubated overnight at 4 °C in a 1:50 dilution of rabbit anti-EGF antiserum (Collaborative Research, Bedford, MA) or normal rabbit serum (1:50) in 0.5% bovine serum albumin (BSA) (Sigma) in PBS–Tween containing 1% normal sheep serum. Following a series of three rinses in PBS–Tween, blots were incubated with biotinylated sheep anti-rabbit IgG (1:500) (Sigma) in 0.5% BSA–PBS–Tween for 3 h at room temperature. After another rinse series, the blot was incubated in streptavidin–horseradish peroxidase (1:2000) (Zymed, San Francisco, CA) in 0.5% BSA–PBS–Tween for 1 h at room temperature. After a final rinse series the blot was incubated in a solution containing 4-chloro-1-naphthol (0.09 g) in 30 mL of methanol, 90 mL of PBS, and 50 μL of 30% H_2O_2. Blots were scanned, and the immunoreactivity present in the dots from individual fractions was determined from the HMW-EGF standard curve.

Amino Acid Sequence Analysis. βNGF-endopeptidase was prepared for sequence analysis by dissolving in 0.5 M Tris, 10 mM EDTA, 6 M guanidinium, pH 8.5, reduced with 10 mM dithiothreitol under nitrogen for 2 h at 50 °C, and carboxymethylated with 20 mM iodoacetic acid containing 0.1 mL (50 μCi) of [^{3}H]iodoacetic acid. The reaction was allowed to proceed at room temperature for 15 min followed by dialysis against 0.1 M ammonium bicarbonate and lyophilization. The resulting product was subsequently redissolved in 200 μL of 70% formic acid and gel filtered on a Superose 12 column (Pharmacia) equilibrated in the same buffer (Shishikura et al., 1987). Four hundred picomoles of carboxymethylated protein was applied to an Applied Biosystems 470A gas-phase sequencer equipped with a 120A phenylthiohydantion (PTH) analyzer and 900A data module. PTH derivatives were separated by using the Applied Biosystems program 1 as previously described (Shishikura et al., 1987). Quantitation was by direct comparison with 250 pmol of PTH standard as calculated by the systems data module. Cysteine residues at positions 7 and 26 were confirmed by liquid scintillation counting of ([^{3}H]carboxymethyl)cysteine (CM-Cys). Des$^{1-8}$NGF prepared by enzymatic digestion was purified as indicated above and stored in 0.2% acetic acid prior to sequence analysis by using the same sequencer and analysis system. A single preparation of NGF$^{1-8}$ was also sequenced in this manner.

Results

Characterization of Purified β-NGF-endopeptidase. β-NGF-endopeptidase was purified via the method of Wilson and Shooter (1979). A single major band on isoelectric focusing gels comprised 95% of the protein. The isoelectric point of this band, determined in relation to protein standards, was 5.9 (data not shown). A single band was present on reducing SDS–PAGE and migrated at an apparent molecular mass of 26 kDa (data not shown). This result indicates that β-NGF-endopeptidase is a single polypeptide chain, in agreement with the observations of Wilson and Shooter (1979). As expected, β-NGF-endopeptidase was active in cleaving the NH_2-terminal octapeptide of NGF. This was apparent in conversion of NGF chains to a slightly more rapidly migrating species in SDS–PAGE (data not shown) and by NH_2-terminal sequence analysis of purified des$^{1-8}$NGF chains. The sequence of the first 10 residues conformed to expectations for NGF residues 9–18 with an initial yield of 54%.

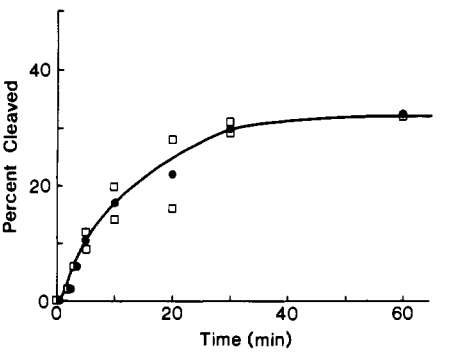

FIGURE 1: Time course of cleavage of NGF by β-NGF-endopeptidase. To 25-μg aliquots of lyophilized NGF was added 2.5 μg of β-NGF-endopeptidase (in 9.3 μL of 50 mM sodium phosphate, pH 6.8) at room temperature. The reactions were stopped at various times by addition of 50 μL of 2X SDS–PAGE treatment buffer. Samples were analyzed by SDS–PAGE as described under Materials and Methods. The mean (●) of one or more individual measurements (□) is given.

Enzymatic Activity of β-NGF-endopeptidase. The time course of cleavage of NGF by β-NGF-endopeptidase (Figure 1) demonstrated relatively rapid cleavage for the first 10 min; approximately 20% of the NGF molecules were cleaved during this period. Specific activity was 13.7 pmol of NGF cleaved min^{-1} μg^{-1} of enzyme. Thereafter, the rate slowed considerably. Between 10 and 30 min, the rate was one-third that recorded earlier even though only about 30% of the substrate was consumed during this period. Between 60 and 180 min the rate of cleavage averaged 0.85 pmol min^{-1} μg^{-1}, only 6% of the rate for the first 10 min. Approximately 46% of the NGF chains were cleaved by 180 min. These data suggested that NGF$^{1-8}$, des$^{1-8}$NGF, or both inhibited further cleavage.

In order to determine whether the NGF$^{1-8}$ inhibited cleavage by β-NGF-endopeptidase, we examined the activity of β-NGF-endopeptidase on its artificial substrate BAPNA (Figure 2). The K_m for cleavage of BAPNA by β-NGF-endopeptidase was determined to be 5.1 × 10^{-4} M (±0.36 × 10^{-4} M), and the V_{max} was 0.170 μM/min (±0.01 μM/min). Next, NGF$^{1-8}$ at a final concentration of 20 μM was incubated with the enzyme prior to BAPNA addition. Figure 2 shows that NGF$^{1-8}$ competitively inhibited β-NGF-endopeptidase activity. The apparent K_m at this concentration of NGF$^{1-8}$ was 15.1 × 10^{-4} M (±2.0 × 10^{-4} M), and the K_i was 10.25 × 10^{-6} M.

NGF$^{1-8}$ also inhibited enzyme activity on NGF. In these experiments, β-NGF-endopeptidase (136 μg/mL in 25 mM sodium phosphate, pH 6.8) was preincubated for 30 min with NGF$^{1-8}$ prior to addition of NGF (1.36 mg/mL final concentration), and the reaction was allowed to proceed for 30 min at room temperature. Results were assessed by SDS–PAGE and densitometry (data not shown). NGF$^{1-8}$ inhibited

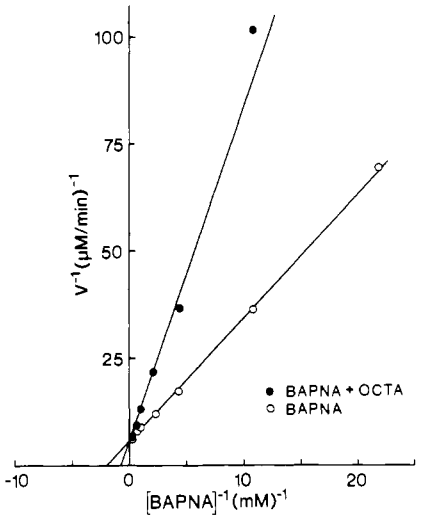

FIGURE 2: Inhibition of β-NGF-endopeptidase by NGF^{1-8}. The activity of β-NGF-endopeptidase on BAPNA was examined (○). The enzyme (20 μg) was incubated with BAPNA (concentration range 0.02–2.8 mM) in a final volume of 1074 μL in 50mM sodium phosphate buffer, pH 6.8, at room temperature while the change in absorbance was recorded at 410 nm. The effect of NGF^{1-8} on enzyme activity was also examined (●). NGF^{1-8} (20 μM) was added to enzyme in the reaction cocktail described above, except that BAPNA was withheld until after preincubating NGF^{1-8} and the enzyme for 15 min at room temperature. The rate of BAPNA cleavage was expressed per microgram of enzyme.

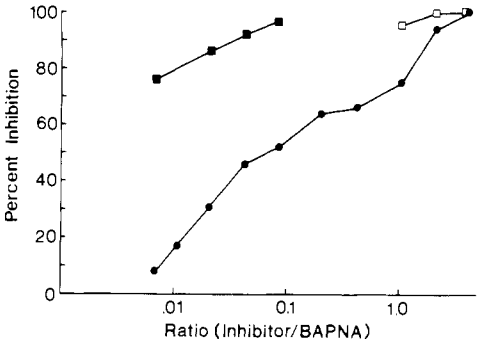

FIGURE 3: Peptide-mediated inhibition of β-NGF-endopeptidase. NGF^{1-8} (●), BK (■), and Lys-BK (□) were tested for inhibition of BAPNA cleavage. The percent inhibition of BAPNA cleavage is plotted vs the ratio of the inhibitor to BAPNA concentration. β-NGF-endopeptidase (20 μg) was incubated with or without peptide (20 μM) in 50 mM sodium phosphate buffer, pH 6.8 (volume = 1044–1072 μL), for 15 min at room temperature prior to addition of BAPNA (2–30 μL to achieve a final volume of 1074 μL) at a concentration sufficient to achieve the molar ratio indicated. In experiments where the molar ratio of NGF^{1-8} or Lys-BK to BAPNA was 1 or greater, BAPNA was held constant at 0.0186 mM and peptide concentration was increased to achieve the desired ratio. The change in OD at 410 nm was recorded over the first 10–30 min.

NGF cleavage in a dose-dependent manner. At a 1:1 molar ratio of added NGF^{1-8} to NGF, the cleavage was inhibited by 46%, whereas at a 4:1 molar ratio the cleavage was inhibited by 72%. The inhibition was comparable to that seen for BAPNA cleavage with respect to the molar ratio of NGF^{1-8} to substrate (Figure 3, see below). These data suggest that a majority of the inhibition of NGF cleavage seen in Figure 1 is due to generation of NGF^{1-8} with resulting product inhibition.

To demonstrate that interaction of NGF^{1-8} with β-NGF-endopeptidase was sequence-specific, we examined an analogue of NGF^{1-8} that was composed of the same eight amino acids but in random sequence (S-F-T-V-S-H-H-P). β-NGF-endopeptidase (20 μg) was preincubated with peptide (20 μM) for 15 min at room temperature prior to the addition of BAPNA (0.46 mM). Whereas NGF^{1-8} inhibited β-NGF-endopeptidase activity on BAPNA by 56.5 ± 5%, the randomized peptide inhibited BAPNA-cleaving activity by only 2.2% For synthetic peptides that omitted either the C-terminal histidine or the entire C-terminus (V-F-H), inhibition of BAPNA cleavage was dramatically less (8 ± 5.5% and 10 ± 2.8%, respectively) than for NGF^{1-8}. This indicated that the C-terminal histidine of NGF^{1-8} was a critical determinant for enzyme inhibition.

All BAPNA-cleaving activity in the β-NGF-endopeptidase preparation could be inhibited by preincubating the enzyme with NGF^{1-8}. BAPNA cleavage was inhibited 75% at an approximately equimolar ratio of NGF^{1-8} to BAPNA; 100% inhibition was found at higher ratios (Figure 3). Together with results for gel electrophoresis and amino acid sequence analysis (see below) on the β-NGF-endopeptidase preparation, the kinetic data suggest that all the BAPNA-cleaving activity present is due to β-NGF-endopeptidase.

As noted, β-NGF-endopeptidase preparations have been reported to act on kininogen, releasing bradykinin-like activity (Bothwell et al., 1979). To ask whether inhibition of the enzyme might also be found with BK, we studied the effect of BK and Lys-BK on BAPNA-cleaving activity. Both peptides inhibited activity (Figure 3). Activity was completely blocked at a molar ratio of Lys-BK:BAPNA of 2:1. BK was especially potent; 80% inhibition was achieved at a molar ratio of BK to BAPNA of 1:100, a potency approximately 100-fold greater than for NGF^{1-8}. BK also inhibited β-NGF-endopeptidase activity on NGF. Following a 10-min preincubation of the enzyme and BK, and with the same experimental conditions as for NGF^{1-8}, cleavage of NGF was inhibited by 87% at a molar ratio of BK to NGF of 1:1. Inhibition of β-NGF-endopeptidase activity by both NGF^{1-8} and BK peptides gives further support to the assertion that both NGF and kininogen are substrates for this enzyme.

Amino Acid Sequence of β-NGF-endopeptidase. The first 30 NH_2-terminal amino acids of β-NGF-endopeptidase were determined. A single identifiable sequence was observed. The sequence demonstrated that β-NGF-endopeptidase is highly homologous to members of the mouse kallikrein family such as γ-NGF and EGF-BP type C (Figure 4). The nucleotide sequences and the derived amino acid sequences of 24 mouse kallikrein genes have been published (Evans et al., 1987). The sequences of these kallikreins differ at their NH_2-terminal ends. By comparison with the published sequences, it was determined that β-NGF-endopeptidase is encoded by the gene designated *mGK-22* (Figure 4). The NH_2-terminal sequence of β-NGF-endopeptidase is also identical with the amino acid sequence of a kallikrein that has been called EGF-BP type A (Anundi et al., 1982; Ronne et al., 1983) (Figure 4). This sequence is found in no other member of the mouse kallikrein gene family.

Comparison of β-NGF-endopeptidase and EGF-Binding Protein. To determine whether β-NGF-endopeptidase is an EGF-BP, three series of experiments were conducted. As a control for these studies we used EGF-BP type C. This kallikrein has been shown to cofractionate with EGF during purification and to bind to EGF when the purified molecules are recombined (Isackson et al., 1987). In the first series of experiments, we attempted to detect complex formation between β-NGF-endopeptidase and EGF on nondenaturing polyacrylamide gels. EGF forms a high molecular weight complex with EGF-BP type C, thus slowing its migration in the gel (Figure 5, lanes 1–4). In contrast, preincubation with EGF did not alter the banding position of β-NGF-endopeptidase (Figure 5, lanes 5–7). No evidence for complex formation was

Amino acid sequence comparison of βNGF-endopeptidase and EGF-BP

	5				10				15				20				25				30
βNGF-endopeptidase	I	L	G	G	F	K	C	E	K	N	S	Q	P	W	Q	V	A	V	Y	Y	L	D	E	Y	L	C	G	G	V	L
EGF-BP TYPE A[1]	I	L	G	G	F	K	C	E	K	N	S	Q	P	W	Q	V	A	V	Y	Y	L	D	E	Y	L	C	G	G	V	L
mGK-22[2]	I	L	G	G	F	K	C	E	K	N	S	Q	P	W	Q	V	A	V	Y	Y	L	D	E	Y	L	C	G	G	V	L
EGF-BP Type B[3]	V	V	G	G	F	N	C	E	K	N	S	Q	P	W	Q	V	A	V	Y	Y	Q	K	E	H	I	C	G	G	V	L
EGF-BP TYPE C[4]	I	V	G	G	F	K	C	E	K	N	S	Q	P	W	H	V	A	V	Y	R	Y	N	E	Y	I	C	G	G	V	L
γ-NGF[5]	I	V	G	G	F	K	C	E	K	N	S	Q	P	W	H	V	A	V	Y	R	Y	T	Q	Y	L	C	G	G	V	L

Boxed regions represent amino acid positions which differ from those of βNGF-endopeptidase

1. Anundi et al., Eur. J. Biochem. 129,365 (1982)
2. Drinkwater et al., Biochemistry 26,6750 (1987)
3. Ronne et al., EMBO J. 2,1561 (1983)
4. Isackson et al., Biochemistry 26,2082 (1987)
5. Thomas et al., J. Biol. Chem. 256,9156 (1981)

FIGURE 4: Amino acid sequence determination of β-NGF-endopeptidase and comparison with mouse kallikreins.

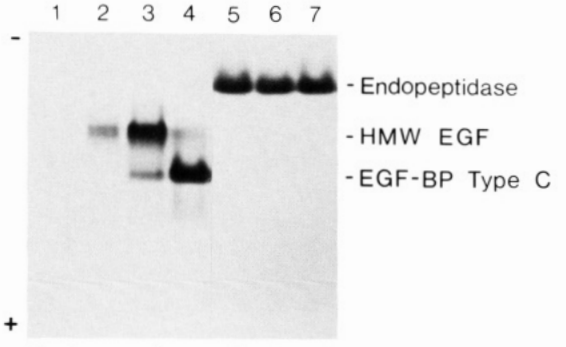

FIGURE 5: Attempted recombination of β-NGF-endopeptidase and EGF. Various mixtures of EGF, EGF-BP type C, and β-NGF-endopeptidase were incubated overnight at 4 °C in 50 mM sodium phosphate buffer, pH 6.8, in a total volume of 180 μL. Following incubation, the entire mixtures were loaded in individual lanes on discontinuous 12% polyacrylamide gels (pH 7.05), and electrophoresis was carried out as indicated under Materials and Methods. Lanes: 1, 20 μg of EGF; 2, 20 μg of EGF plus 20 μg of EGF-BP type C; 3, 20 μg of EGF plus 100 μg of EGF-BP type C; 4, 100 μg of EGF-BP type C; 5, 100 μg of β-NGF-endopeptidase; 6, 20 μg of EGF plus 100 μg of βNGF-endopeptidase; 7, 100 μg of β-NGF-endopeptidase. Note that EGF would be expected to enter the gel but does not stain under these conditions.

found even when β-NGF-endopeptidase was incubated with a 4-fold molar excess of EGF (data not shown).

In the second series of experiments we examined the effect of EGF on the enzymatic activities of EGF-BP type C and β-NGF-endopeptidase. An interesting and characteristic feature of EGF-BP type C is inhibition of its esterase activity when complexed with EGF (Server et al., 1976). If β-NGF-endopeptidase interacts with EGF in a similar fashion, then one would expect to see inhibition of enzyme activity by EGF. The time course of cleavage of BAPNA was examined in the presence and absence of EGF and was compared to that for EGF-BP type C (data not shown). EGF-BP type C activity was inhibited 20% by an equimolar amount of EGF. Even greater inhibition (45%) was produced when an additional 4.1 μg of EGF (10-fold molar excess) was added to the cuvette at the time of assay. In contrast, there was no inhibition of β-NGF-endopeptidase activity by an equimolar amount of EGF; even when an additional 16 μg of EGF (10-fold molar excess) was added at the time of the assay, there was little, if any, evidence of inhibition.

In the third assay, we asked whether a β-NGF-endopeptidase–EGF complex exists under physiological conditions. Proteins produced in the mouse submandibular gland are secreted into saliva in response to treatment with adrenergic agonists (Wallace & Partlow, 1976; Burton et al., 1978). Among these proteins are 7S NGF, HMW-EGF, and β-NGF-endopeptidase. If β-NGF-endopeptidase and EGF are present as a complex in saliva, we might expect to find comigration of these proteins in gel filtration chromatography. Mouse saliva induced by epinephrine treatment was fractionated on Sephadex G-100 (Figure 6). Protein eluted in three main peaks. The last of these contained β-NGF-endopeptidase activity as determined by using NGF as substrate and β-NGF-endopeptidase protein as determined from SDS–PAGE analysis of individual column fractions. EGF immunoreactivity was present in the second peak, which is known also to contain EGF-BP type C (Burton et al., 1978). There was very little overlap in the migration of β-NGF-endopeptidase and EGF. These data provided no evidence for the existence of an EGF–β-NGF-endopeptidase complex in saliva.

Discussion

We report studies on the structure and activity of purified β-NGF-endopeptidase that demonstrate that (a) the enzyme is active in cleaving the NH_2-terminal octapeptide from NGF; (b) the product of the cleavage, NGF^{1-8}, specifically inhibits the activity of β-NGF-endopeptidase on both NGF and its artificial substrate BAPNA; (c) BK and Lys-BK, the products of kininogen cleavage, also inhibit enzyme activity on NGF and BAPNA; (d) the NH_2-terminal amino acid sequence of this protein identifies *mGK-22* as the gene encoding β-NGF-endopeptidase; and (e) in spite of previous designation of the product of *mGK-22* as EGF-BP type A, β-NGF-endopeptidase exhibits no EGF-binding activity. These studies support the view that β-NGF-endopeptidase is active on two natural substrates, NGF and kininogen. They raise the possibility that this kallikrein has a physiological role through the production of bioactive peptides from each of its substrates.

The many kallikreins expressed in the mouse submandibular gland demonstrate a high degree of sequence homology; correspondingly, they exhibit similar physical properties such as molecular weight, amino acid composition, and isoelectric point. Also, they often cross-react immunologically and may copurify with one another. Ideally, knowledge of the sequence of an individual kallikrein could be used to predict substrate specificity. Unfortunately, as yet this has not proven possible. Therefore, structural and functional studies on purified enzymes are necessary to conclusively link a gene product with

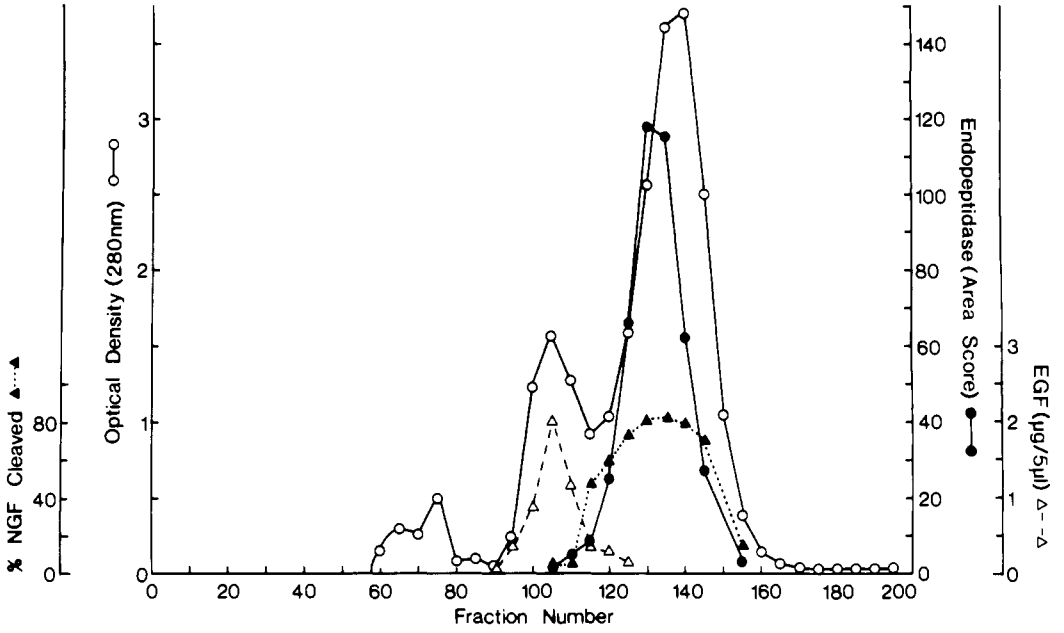

FIGURE 6: Gel filtration of mouse salivary proteins on Sephadex G-100. Protein was measured by optical density at 280 nm (○); β-NGF-endopeptidase was measured by SDS–PAGE analysis of fractions (●); β-NGF-endopeptidase activity was measured by cleavage activity on NGF (▲); EGF was measured by immunoassay (△). All procedures were as indicated under Materials and Methods.

a specific enzymatic activity. Given the structural relatedness of kallikreins, efforts directed at achieving and demonstrating enzyme purity are important.

Previous reports of β-NGF-endopeptidase activity (Bothwell et al., 1979; Wilson & Shooter, 1979) pointed to an unusual cleavage specificity, the His^8–Met^9 bond of NGF. Neither carboxymethylated NGF nor RNase A, which contains the same peptide bond, is a substrate for β-NGF-endopeptidase (Wilson & Shooter, 1979). These findings suggested a high degree of substrate specificity, consistent with studies of the activity of other mouse glandular kallikreins such as γ-NGF and EGF-BP. Remarkably, β-NGF-endopeptidase was also reported to cleave kininogen and to do so with a specific activity manyfold greater than for γ-NGF or EGF-BP (Bothwell et al., 1979). We considered the possibility that contamination by another kallikrein may have obscured the pattern of substrate specificity for β-NGF-endopeptidase. Given this concern, it was important to further characterize the purified enzyme.

Highly purified β-NGF-endopeptidase was shown to have the enzymatic properties expected. In addition, product inhibition was demonstrated for NGF^{1-8}. This result extends findings for the inhibition of kallikreins by their natural products. Following processing of proNGF to NGF, γ-NGF forms a complex with NGF that is thought to involve binding of the C-terminus of NGF within the active site of γ-NGF, thus inactivating this enzyme (Bothwell & Shooter, 1978). A very similar situation may characterize the interaction of EGF-BP type C with EGF (Server et al., 1976). Inhibition of β-NGF-endopeptidase by NGF^{1-8} was sequence-specific and dependent on the C-terminal histidine. These results and the competitive inhibition demonstrated for NGF^{1-8} in kinetic studies made it likely that NGF^{1-8} was acting through inhibition of the enzyme at its active site. Complete inhibition of activity against BAPNA with Lys-BK, a product of kininogen cleavage, suggested interaction of this peptide at the same active site as NGF^{1-8}. This view was strengthened in studies demonstrating that both NGF^{1-8} and BK inhibited β-NGF-endopeptidase activity against NGF. Moreover, as was true for BAPNA, BK was more potent than NGF^{1-8} in inhibiting NGF cleavage. In light of an earlier demonstration that NGF cleavage is also inhibited by kininogen (Wilson & Shooter, 1979), the evidence presented here supports the view that β-NGF-endopeptidase is active on both NGF and kininogen.

The possible physiological significance of β-NGF-endopeptidase action is of interest. The enzyme is present in mouse saliva and cleaves approximately one-third of NGF peptide chains during adrenergically mediated salivation (Burton et al., 1978). The discovery that NGF^{1-8} induces hyperalgesia in a dose-dependent and sequence-specific manner in injured skin (Levine et al., 1988) suggests that the enzyme has a role in the response to tissue injury. Furthermore, the ability of β-NGF-endopeptidase to cleave kininogen (Bothwell et al., 1979) may allow for induction of hyperalgesia via a mechanism distinct from that of NGF^{1-8} (Taiwo, Levine, and Mobley, unpublished observations). Thus, β-NGF-endopeptidase may mediate kinin-like effects via different substrates with different mechanisms. Inhibition of the enzyme by each of its products would allow for regulation of its activity, including potential cross-regulation of production of NGF^{1-8} by BK and vice versa. Given the greater inhibition of enzyme activity by BK, BK-mediated inhibition may dominate.

We demonstrate, via NH_2-terminal amino acid sequencing, that β-NGF-endopeptidase is encoded by the mouse kallikrein gene *mGK-22*. The derived NH_2-terminal amino acid sequences of all 24 mouse kallikrein genes are known (Evans et al., 1987). Although the sequences are highly conserved, there are several nonconserved regions within the first 30 amino acids, most notably residues 1–2, 6, 15, and 20–25. The NH_2-terminal amino acid sequence of β-NGF-endopeptidase is distinctive at these residues; in particular, the leucine at residue 2, the glutamine at residue 15, and the sequence Tyr-Leu-Asp-Glu-Tyr-Leu at residues 20–25 unambiguously indicate that this protein is coded for by *mGK-22*.

The derived amino acid sequence of *mGK-22* corresponds to a product previously identified as EGF-BP type A (Drinkwater et al., 1987). Three different proteins have been sequenced and reported as EGF-BP: type A, partially sequenced by Anundi et al. (1982) and coded for by the gene *mGK-22*; type B (Anundi et al., 1982; Ronne et al., 1983; Lundgren et al., 1984) coded for by the gene *mGK-13*; and

type C, partially sequenced by Isackson et al. (1987) and coded for by the gene *mGK-9*. The latter is the only one shown to bind EGF in vitro. Furthermore, a kallikrein called protease A (Schenkein et al., 1981) has been partially sequenced and is nearly identical with EGF-BP type A. In view of confusion in the literature over which genes code for actual EGF-BPs, we felt it necessary to examine more closely whether β-NGF-endopeptidase exhibited EGF-binding activity.

By three independent measures β-NGF-endopeptidase is not an EGF-binding protein. First, no complex was detectable by nondenaturing PAGE when β-NGF-endopeptidase was incubated with EGF. Second, the enzymatic activity of EGF-BP, but not β-NGF-endopeptidase, was inhibited by EGF. Third, when mouse saliva was subjected to gel exclusion chromatography, the β-NGF-endopeptidase activity did not comigrate with EGF activity, as would be expected of an EGF-BP.

Failure to detect a complex involving β-NGF-endopeptidase and EGF suggested that earlier designation of this enzyme as an EGF-BP was incorrect. One possible explanation for this error is that β-NGF-endopeptidase contaminated the HMW-EGF preparation used in earlier studies. Neither Anundi et al. (1982) nor Schenkein et al. (1981) tested their protein for EGF-binding activity. Furthermore, Anundi et al. (1982) did not purify their protein to homogeneity; two or more kallikreins were present in the material analyzed. They found that their EGF-BP comigrated with EGF during gel exclusion chromatography; however, a slight loss of resolution could easily have led to overlap of EGF and β-NGF-endopeptidase (EGF-BP type A), since the Sephadex G-100 peaks for HMW-EGF and β-NGF-endopeptidase are adjacent. Also, it was not clear that subsequent steps used to purify HMW-EGF would exclude β-NGF-endopeptidase. To test this possibility, we used Sephadex G-100 gel chromatography to fractionate the submandibular gland homogenate via the method of Taylor et al. (1970). Fractions were pooled so as to deliberately contaminate HMW-EGF with β-NGF-endopeptidase. Importantly, subsequent purification steps normally used to prepare HMW-EGF (Taylor et al., 1970) did not separate β-NGF-endopeptidase from HMW-EGF even though β-NGF-endopeptidase was not present in an EGF-containing complex (Woo, Fahnestock, and Mobley, unpublished observations). We conclude that EGF-BP type A (and protease A) is actually β-NGF-endopeptidase and that this enzyme is not an EGF-BP.

At present, defining the substrate specificity of individual kallikreins must rely on colocalization of the enzyme and its potential substrates as well as evidence of their interaction. Substrate availability may allow individual kallikreins to exhibit different biological functions in different tissues. The demonstration that β-NGF-endopeptidase exhibits activity against both NGF and kininogen, as well as binding to protease nexin I (Rosenblatt, Woo, and Mobley, manuscript in preparation) and protease nexin II (Sinha et al., 1990), suggests a broad biological role for this enzyme. Perhaps the substrate specificities and the tissue distributions of the mouse kallikreins should be reexamined to more fully describe their biological roles.

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Rotational Dynamics of the Fc_ϵ Receptor on Mast Cells Monitored by Specific Monoclonal Antibodies and IgE†

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ABSTRACT: The rotational motions of the type I receptor for the Fc_ϵ domains ($Fc_\epsilon RI$) present on mast cells were investigated by measuring the phosphorescence emission and anisotropy decay kinetics of erythrosin (Er) covalently bound to several $Fc_\epsilon RI$-specific macromolecular ligands. The latter consisted of three murine monoclonal antibodies (IgG class) raised against the $Fc_\epsilon RI$ of rat mast cells (RBL-2H3 line), their Fab fragments, and a murine monoclonal IgE. Different anisotropy decay patterns were observed for the three monovalent Er–Fab fragments bound to the $Fc_\epsilon RI$, reflecting the rotational motion of the $Fe_\epsilon RI$ reported by each specific macromolecular probe bound to its particular epitope. Internal motions of the tethered Er-labeled ligands may also contribute to the observed anisotropy decay, particularly in the case of cell-bound IgE. The results corroborate an earlier study with rat Er–IgE in which the $Fc_\epsilon RI$–IgE complex was shown to be mobile throughout the temperature range examined (5–37 °C). The anisotropy decays of the three Er-labeled, $Fc_\epsilon RI$-specific intact mAbs bound to cells also differed markedly. Whereas the decay curves of one mAb (H10) were characterized by temperature-dependent positive amplitudes and rather short rotational correlation times, the decay of a second mAb (J17) showed complex qualitative variations with temperature, and in the case of the third antibody (F4), there was no apparent decay of anisotropy over the time and temperature ranges examined. The spectrum of rotational mobility data obtained for the complexes of the $Fc_\epsilon RI$ with the mAbs and their Fab fragments is consistent with the suggestion that the orientations of the antibody probes with respect to the Fc_ϵ receptor, and thus to the cell membrane, are nonrandom and different. This feature may be decisive in the $Fc_\epsilon RI$-cross-linking process induced by each of the mAbs and in the resulting cellular response culminating in exocytosis. A possible inverse correlation between the rate of rotational depolarization of a given Er–mAb cross-linking $Fc_\epsilon RI$ and its secretion-inducing capacity can be proposed.

The type I receptor for Fc_ϵ domains of IgE ($Fc_\epsilon RI$)[1] is an integral plasma membrane complex of four polypeptide chains present only in mast cells and basophils (Froese, 1984; Metzger et al., 1986; Kinet, 1989). The minimal $Fc_\epsilon RI$ unit is thought to be a complex ($\alpha\beta\gamma_2$) of one α chain (50 kDa), one β chain (35 kDa), and a disulfide-linked γ chain dimer (14 kDa) (Blank et al., 1989; Metzger et al., 1986). $Fc_\epsilon RI$ binds a single IgE molecule primarily via its $C_\epsilon 3$ domain, although the $C_\epsilon 2$ and $C_\epsilon 4$ domains are also involved (Helm et al., 1988; Schwarzbaum et al., 1989). The β and γ polypeptide chains are associated noncovalently with the IgE-binding α subunit and are therefore coisolated from mast cell (RBL-2H3 line) membranes in an affinity-based purification process (Rivnay et al., 1982). No biochemical or biological effects can be detected following the monovalent binding of IgE to $Fc_\epsilon RI$ on the cell surface. Hence this step may be regarded as the priming process of the system, endowing it with antigen-binding specificity corresponding to the variable regions of the IgE. It is the clustering of the $Fc_\epsilon RI$, induced either directly by specific antibodies or via the bound IgE by specific polyvalent antigens or anti-IgE antibodies, that provides the signal initiating the cascade of processes culminating in mediator secretion (Ishizaka & Ishizaka, 1984). A number of biochemical processes have been observed following $Fc_\epsilon RI$ aggregation that are assumed to constitute the cascade coupling this stimulus to secretion. However, the actual involvement and temporal sequence of these steps in the overall mechanism are not clearly resolved and constitute topics of major research activity (Ishizaka & Ishizaka, 1984; Metzger et al., 1986; Siraganian, 1988).

Plasma membrane receptor clustering as the initial step of a cellular stimulus is a general phenomenon observed for a diversity of signaling mechanisms in mammalian cells (De Lisi, 1979). In particular, immunological stimuli are characterized by a requirement for receptor aggregation, as illustrated by different types of antigen–receptors cross-linked by multiple

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[1] Abbreviations: $Fc_\epsilon RI$, type I receptor for Fc_ϵ domains; RBL-2H3, rat basophilic leukemia cells, subline 2H3; Er, erythrosin (tetraiodofluorescein); mAb, monoclonal antibody; DNP, 2,4-dinitrophenyl; FCS, fetal calf serum; PBS, phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.4 mM sodium/potassium phosphate, pH 7.2).

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