Korean J Biol Sci 1: 463-466, 1997

γ-NGF Produced in CHO Cells Does Not Cleave Mouse *Ren-2* Prorenin

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Key Words:
Submandibular gland
Glandular kallikrein
Prorenin converting enzyme
EGF-BP
Nerve growth factor

We have recently demonstrated, by protein and cDNA sequence analysis, that prorenin converting enzyme (PRECE) in the mouse submandibular gland is identical to the epidermal growth factor-binding protein (EGF-BP) type B. However, type A and C did not show prorenin converting activity. To demonstrate whether γ-NGF is involved in prorenin processing, we have cloned cDNA of γ-NGF and examined prorenin converting activity using the CHO cell expression system. Trypsin converted the 33 kDa γ-NGF precursor produced in CHO cells to a two-chain form, 9.4 and 16.4 kDa polypeptide chains, which has been known as an active form of γ-NGF in mouse SMG (Server and Shooter, 1976). However, the two chain forms of γ-NGF did not reveal prorenin-processing activity. Thus, only PRECE is involved in prorenin processing in mouse SMG. This result shows that their substrate specificities appear to be very strict, although some kallikreins share a high degree of amino acid sequence identity.

Renin, an aspartyl protease, is the key enzyme of the renin-angiotensin, and plays a pivotal role in the regulation of blood pressure (Inagami, 1981). It is produced from a larger, inactive precursor, prorenin, through endoproteolysis at paired basic amino acids, Lys-Arg, during intracellular transport. Although the kidney is the major source of circulating renin, other tissues are also capable of renin synthesis. For example, the submandibular gland (SMG) of male mice produces a large amount of renin. Renins in the kidney and the SMG are encoded by separate genes, *Ren-1* and *Ren-2*, respectively (Holm et al., 1984). In recent years, we have purified and characterized an endopeptidase involved in processing of *Ren-2* prorenin, named prorenin converting enzyme (PRECE), from the mouse SMG (Nakayama et al., 1989, Kim et al., 1990, and Nakayama et al., 1990). It consists of two polypeptide chains of 17 and 10 kDa linked by disulfide bonds (Kim et al., 1990). Protein and cDNA sequence analyses (Kim et al., 1991a) have revealed that PRECE is identical to the epidermal growth factor-binding protein (EGF-BP) type B, the product of the mGK-13 gene identified in Balb/c mouse (Drinkwater et al., 1987). EGF-BPs have been demonstrated to be members of the glandular kallikrein family (Evans et al., 1987), and to be responsible for conversion of the 9 kDa proEGF intermediate to mature EGF (Frey et al., 1979). Thus, PRECE is involved in the maturation of two bioactive polypeptides produced in the mouse SMG, *Ren-2* renin and EGF. However, in the course of cDNA cloning, we noticed the presence of another cDNA type highly homologous but not identical to the PRECE cDNA. The newly identified cDNA was identical to that of the pSGP-2 cDNA cloned from NMRI mice, which encoded a protein similar to EGF-BP type B but different at 9 out of 261 amino acids from the mGK13 product. Also, we had demonstrated that the products of the newly identified cDNA had a prorenin converting enzyme activity (Kim et al., 1991b). Thus, the products of both cDNAs of PRECE and pSGP-2 are involved in maturation of two bioactive polypeptides produced in mouse SMG, *Ren-2* renin and EGF. However, mouse glandular kallikreins, which are involved in the post-translational processing of polypeptide precursors to their biologically active forms, are a highly homologous subfamily of serine proteases, but the substrate specificities of some kallikreins appear to be very strict. For example, it has been reported that γ-NGF cannot cleave proEGF (Frey et al., 1979) and also that EGF-BPs cannot cleave proNGF (Blaber et al., 1989). Also, it was demonstrated that EGF-BP type A and C do not possess the prorenin converting activity (Kim et al., 1996) but EGF-BP type A has the β-NGF endopeptidase activity (Fahnestock et al., 1991). Thus, it is very interesting whether other kallikreins, especially γ-NGF which is involved in the maturation of proNGF, possess prorenin converting activity.

In this study, we have cloned the cDNA of γ-NGF and examined the prorenin converting activity of the encoded protein.

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Materials and Methods

cDNA cloning procedures were described previously (Kim et al., 1991a). The sequences of both strands of the cloned cDNA (γ-NGF) were determined by the dideoxy method (Sanger et al., 1977).

Prorenin converting activity of γ-NGF was examined in a manner similar to that employed in the previous study (Kim et al., 1991a). Briefly, the cDNA insert was subcloned into the pcDNA3 expression vector (Kim et al., 1996), and transfected into Chinese hamster ovary (CHO) cells. A cell line stably expressing the highest level of the protein (CHO/γNGF) was then selected. The conditioned medium of CHO/γNGF cells cultured in a serum-free medium was treated with trypsin. The [^{35}S] methionine-labeled medium of CHO/MRB cells (a *Ren-2* prorenin-producing cell line) (Hatsuzawa et al., 1990) was then incubated with the trypsin-treated or untreated conditioned medium of CHO/γNGF cells, and subjected to immunoprecipitation with anti-*Ren-2* renin antiserum and SDS-PAGE analysis.

Results

Although we have shown that PRECE is involved in the processing of prorenin in mouse SMG, we did not exclude the possibility that other members of kallikreins, especially EGF-BPs and γ-NGF, could be involved in prorenin processing. In the previous study, we have demonstrated that EGF-BP type A and C produced in CHO cells do not cleave *Ren-2* prorenin (Kim et al., 1996). Thus, it was assumed that only type B of these EGF-BPs was involved in processing *Ren-2* prorenin in mouse SMG. However, it still remains to be determined that γ-NGF is involved in prorenin processing, since kallikreins and renin are present in the same secretory granules in SMG (Chiang et al, 1968). To address this problem, we screened approximately 1 × 10^5 clones from the cDNA library of male ICR mouse SMG and obtained about 100 positive clones. Twenty

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          -24                                                            -1  1                 6
PRECE :   M  W  F  L  I  L  F  L  A  L  S  L  G  G  I  D  A  A  P  P  L  G  S  R  V  V  G  G  F  N
γ-NGF :   -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  V  Q  -  -  I  -  -  -  -  -
                                                             propeptide  ⇐  ⇒
                10                         20                             30  9.4 kDa chain
PRECE :   C  K  K  N  S  Q  P  W  Q  V  A  V  Y  Y  Q [K] E [H] I  C  G  G  V  L  L  D  R  N  W  V
γ-NGF :   -  E  -  -  -  -  -  H  -  -  -  -  -  -  - [T] Q [-] L  -  -  -  -  -  -  -  P  -  -  -
                40 ★                       50                             60
PRECE :   L  T  A  A  H  C  Y  V  D  Q  Y  E  V  W  L  G  K  N  K  L  F  Q  E  E  P  S  A  Q  H  R
γ-NGF :   -  -  -  -  -  -  -  D  -  N  -  K  -  -  -  -  -  -  N  -  -  K  D  -  -  -  -  -  -  -
                70                         80                             90                   ★
PRECE :   L  V  S  K  S  F  P  H  P  G  F  N  M  S  L  L  M  L  Q  T  I  P  P  Q [A] D  F  S  N  D
γ-NGF :   F  -  -  -  A  I  -  -  -  -  -  -  -  -  -  M  R  K  H  I  R  F  L  E [Y] -  Y  -  -  -
                                                             ⇒
                100                        110                6.8 kDa chain 120
PRECE :   L  M  L  L  R  L  S  K  P  A  D  I  T  D  V  V  K  P  I  A  L  P  T  K  E  P  K  P  G  S
γ-NGF :   -  -  -  -  -  -  -  -  -  -  -  -  -  -  T  -  -  -  -  T  -  -  -  E  -  -  -  L  -  -
                130                        140                            150
PRECE :   K  C  L  A  S  G  W  G [S] I  T  P  T  R  W  Q  K  P  D  D  L  Q  C  V  F  I  T  L  L  P
γ-NGF :   T  -  -  -  -  A  -  - [-] -  -  -  -  K  F  -  F  T  -  -  -  Y  -  -  N  L  -  -  -  -
                                                  ⇒ 11.4 kDa chain
                160                        170                            180
PRECE :   N  E  N  C  A  K  V [Y] L  Q  K  V  Y  D  V  M  L  C  A  G  E  M  G  G  G  K [D  T  C  R]
γ-NGF :   -  -  D  -  -  -  A [H] -  E  -  -  T  -  A  -  -  -  D  -  -  -  D  -  -  - [-  -  -  K]
              ★ 190                        200                            210
PRECE :   D  D  S  G  G  P  L  I  C  D  G  I  L  Q  G  T  T [S  Y  G  P  V  P] C  G  K  P  G  V  P
γ-NGF :   G  -  -  -  -  -  -  -  -  -  -  V  -  -  -  I  - [-  W  -  H  T  -] -  -  E  -  D  M  -
                220                        230                237
PRECE :   A  I  Y  T  N  L  I  K  F  N  S  W  I  K  D  Y  M  M  K  N  A
γ-NGF :   G  V  -  -  K  -  N  -  -  T  -  -  -  -  -  T  -  A  -  -  P
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Fig. 1. Amino acid composition of the γ-NGF and PRECE. The amino acid sequence of the γ-NGF was aligned to the sequences of the PRECE (Kim et al., 1991). Residues of γ-NGF identical to those of PRECE are indicated by hyphens. Residues believed to line the substrate-binding pocket are boxed. The consensus sequences for N-glycosylation are shadowed. The proposed active site amino acid residues, His^{41}, Asp^{95}, and Ser^{189} are indicated by asterisks.

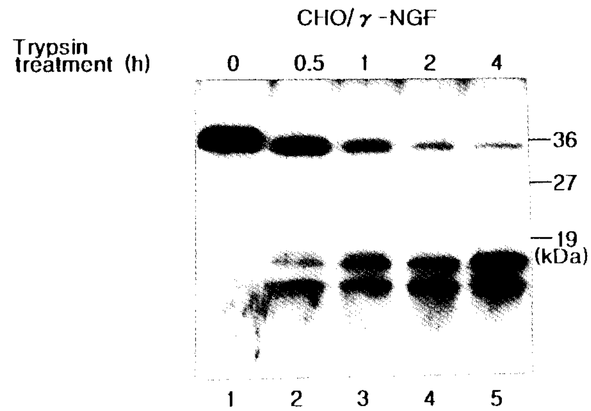

Fig. 2. Trypsin treatment of the conditioned medium of CHO/γNGF. The conditioned medium of CHO/γNGF cells were treated with trypsin for 4 h, electrophoresed in the SDS-polyacrylamide gel under reducing condition, and analyzed by Western blotting with anti-PRECE antiserum.

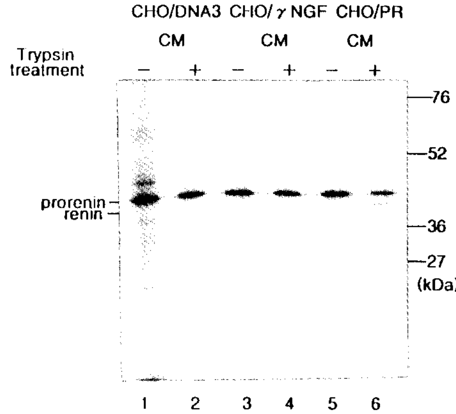

Fig. 3. Prorenin converting activity of recombinant γ-NGF. The radiolabeled conditioned medium(CM) of CHO/MRB cells (100 μl) was incubated with the trypsin-treated (lanes 2, 4, and 6) or untreated (lanes 1, 3, and 5)- conditioned medium of CHO/DNA3 (lanes 1 and 2), CHO/γNGF (lanes 3 and 4) or CHO/PR (lanes 5 and 6) cells (100 μl) in a final volume 500 μl of o.1 M Tris/HCl (pH 8.0) at 37℃ for 4 h, immunoprecipitated with anti-Ren-2-renin antiserum, and analyzed by SDS-PAGE under reducing conditions followed by fluorography.

out of the longest positive clones were sequenced and the cDNAs of the γ-NGF were cloned. As shown in Fig. 1, γ-NGF and PRECE share a high degree of amino acid sequence identity (74%). To examine whether γ-NGF has a prorenin converting activity, CHO cells were transfected with pcDNAγNGF expression plasmids, and the stable cell line CHO/γNGF expressing a high level of the γ-NGF was identified by Northern and Western blot analysis (data not shown). γ-NGF was synthesized as an inactive precursor of approximately 33 kDa single chain in these transfected cells. The precursor form of γ-NGF appeared to have higher molecular weight than that of the expected one which is 28 kDa polypeptide chain. This molecular weight difference could be explained by N-glycosylation, since γ-NGF has a potential N-glycosylation site (see Fig. 1). The conditioned medium CHO/γNGF was then treated with trypsin, since trypsin activated proPRECE effectively (Kim et al., 1991). However, it has been known that active γ-NGF is present in both two or three chain forms, presumably arising from limited, perhaps autocatalytic, proteolysis in mouse SMG (Server and Shooter, 1976, Stach et al., 1976), i.e., there are three autocatalytic sites, Arg^{-1}, Arg^{83}, and Lys^{140} (Fig. 1). Cleavage sites were between the propeptide (residues 18-24) and 9.4 kDa chain (residues 25-83), 9.4 and 6.8 kDa chains (residues 84-140), and 6.8 and 11.4 kDa chains (residues 141-237) at Arg^{-1}, and Arg^{83}, and Lys^{140} residues, respectively (Server and Shooter, 1976, Tomas et al., 1981). As shown in Fig. 2, trypsin converted the 34 kDa precursor form of γ-NGF to 17 and 15 kDa polypeptide chains. The 17 kDa band can be obtained by the cleavages between 9.4 and 6.8 kDa chains occuring after the Arg^{83} residue, but not the Lys^{140} residue. Also, the 15 kDa bands could be explained by N-glycosylation, since the 9.8 kDa chain has the potential N-glycosylation site, and these molecules treated with endoglycosidase F migrated as a 10 kDa protein on SDS-polyacrylamide gel (data not shown). From this observation, we speculated that trypsin preferred to cleave after Arg^{-1} and Arg^{83} residues of the 33 kDa γ-NGF precursor form, and then converted to two chain forms, 10 kDa and 17 kDa chains. This result is consistant with the presence of 9.4 and 16.4 kDa chains in mouse SMG as an active form γ-NGF (Server and Shooter, 1976). Subsequently, the trypsin-treated or untreated- conditioned medium of CHO/DNA3 (cells transfected with the control plasmid lacking the cDNA insert), CHO/γNGF and CHO/PR (cells stably express PRECE, Kim et al., 1991) were incubated with ^{35}S-labeled conditioned medium from CHO/MBR cells, which stably express *Ren-2* prorenin (Hatsuzawa et al., 1990). As shown in Fig. 3, trypsin-treated conditioned medium (lane 6) of CHO/PR cells caused conversion of prorenin to renin, but trypsin-untreated one (lane 5) and trypsin-treated (lane 4) or untreated-(lane 3) conditioned medium of CHO/γNGF cells did not. The conditioned medium of CHO/DNA3 cells had no prorenin converting activity. These observations indicate that the two chain form γ-NGF produced in CHO cells do not possess prorenin converting activity.

Discussion

Evans and his colleagues (Evans et al., 1987) reported that the mouse glandular kallikrein gene family consists of at least 25 members, designated mGK-1 to mGK-25, including some pseudogenes, and that ten of them are expressed in the SMG. These kallikreins show a high degree of sequence homology and they exhibit similar molecular properties such as molecular weight, amino acid composition, and isoelectric point. Also, they often cross-react immunologically and may be copurified with one another. It has been reported that γ-NGF could not cleave proEGF and it has been proposed that EGF-BP could not cleave proNGF, although these two kallikreins shared a high degree of amino acid sequence identity. Only type B/PRECE of the EGF- BPs is involved in prorenin processing (Kim et al., 1996.). In addition, kidney kallikrein could not cleave *Ren-2* prorenin (Kim et al., 1991). Thus, the substrate specificities of some kallikreins appear to be very strict. In recent years, Fahnestock et al. (1991) demonstrated that EGF-BP type A has a β -NGF-endopeptidase, i.e. contamination of high molecular weight (HMW) EGF precipitations with β -NGF-endopeptidase erroneously led to earlier designation of the product of mGK-22 as an EGF-BP type A and that the β-NGF- endopeptidase do not form a high molecular weight complex with EGF. Thus, it is perplexing whether a particular kallikrein is involved in processing of only one given precursor. In this context, it is of particular interest that other kallikreins could be involved in prorenin processing. In this study, to examine whether γ-NGF is involved in the processing of prorenin, we have cloned the cDNA of the γ-NGF from a library of male ICR mouse submandibular gland and investigated its prorenin converting activity using the mouse *Ren-2* prorenin as a substrate. However, as shown in Fig. 3, the prorenin converting activitiy of γNGF was not detected (lane 2). In serine protease, serine residue of active site forms a triad with histidine and aspartic acid residues which in the correct spatial configuration contitutes the catalytic triad necessary for serine protease activity. Although γ-NGF and PRECE share a high degree of amino acid sequence identity, 7 residues (Lys^{22}, Ala^{91}, Tyr^{164}, Arg^{186}, Tyr^{205}, Pro^{208} and Val^{209}) among the 15 amino acid residues (Lys^{22}, His^{24}, Ala^{91}, Ser^{135}, Tyr^{164}, Asp^{183}, Thr^{184}, Cys^{185}, Arg^{186}, Ser^{204}, Tyr^{205}, Gly^{207}, Pro^{208}, Val^{209} and Pro^{210} in PRECE) that are believed to line the substrate-binding pocket, were replaced by Thr^{22}, Tyr^{91}, His^{164}, Lys^{186}, Trp^{205}, His^{208} and Thr^{209} in γ -NGF, respectively (Mason et al., 1983, Fig. 1). Also, it was reported that γ-NGF cannot cleave proEGF (Frey et al., 1979) and proposed that EGF-BP cannot cleave proNGF (Blaber et al., 1989). Thus, it was assumed that the substrate specificity of γ-NGF appears to be as strict as other glandular kallikreins. However, it is not clear whether the two chain form γ-NGF which is produced in CHO cells posseses the enzyme activity as a serine protease. Thus, there still remains a possibility that the three chain form γ-NGF could be involved in prorenin processing.

Acknowledgements

We would like to thank Dr. K. Nakayama and Dr. K. Murakami for kindly providing CHO cells and anti-renin antiserum and their encouragement. This work was supported by the Genetic Engineering Research Grant (1995), Ministry of Education, Korea.

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[Received May 8, 1997; accepted July 10, 1997]