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Dual Function of the Propeptide of Prouroguanylin in the Folding of the Mature Peptide

DISULFIDE-COUPLED FOLDING AND DIMERIZATION*

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Guanylyl cyclase activating peptide II (GCAP-II), an endogenous ligand of guanylyl cyclase C, is produced via the processing of the precursor protein (prepro-GCAP-II). We have previously shown that the propeptide in pro-GCAP-II functions as an intramolecular chaperone in the proper folding of the mature peptide, GCAP-II (Hidaka, Y., Ohno, M., Hemmasi, B., Hill, O., Forssmann, W.-G., and Shimonishi, Y. (1998) *Biochemistry* 37, 8498–8507). Here, we report an essential region in pro-GCAP-II for the correct disulfide pairing of the mature peptide, GCAP-II. Five mutant proteins, in which amino acid residues were sequentially deleted from the N terminus, and three mutant proteins of pro-GCAP-II, in which N-terminal 6, 11, or 17 amino acid residues were deleted, were overproduced using *Escherichia coli* or human kidney 293T cells, respectively. Detailed analysis of *in vivo* or *in vitro* folding of these mutant proteins revealed that one or two amino acid residues at the N terminus of pro-GCAP-II are critical, not only for the chaperone function in the folding but also for the net stabilization of pro-GCAP-II. In addition, size exclusion chromatography revealed that pro-GCAP-II exists as a dimer in solution. These data indicate that the propeptide has two roles in proper folding: the disulfide-coupled folding of the mature region and the dimerization of pro-GCAP-II.

Endogenous peptide hormones are often synthesized *in vivo* in the form of precursor proteins with pre- (or signal) and prepro-leader sequences, which are subsequently processed into biologically active mature peptides after their release from the ribosome (1). Little is known, however, concerning the role of the propeptide in the pro-leader sequence in the processing of precursor proteins to the mature peptide hormones or their function in the folding process, which results in the mature hormones. Guanylin and uroguanylin (2–4), endogenous ligands of particulate guanylyl cyclase C (GC-C)[1] (5), are thought to function in regulating the level of cGMP as a second messenger in intestinal and kidney cells, *i.e.* the regulation of chloride and water secretion from the inside of these cells to the outside (6, 7). Guanylin and uroguanylin are generated as precursor proteins (preproguanylin (prepro-GCAP-I) or prepro-urouroguanylin (prepro-GCAP-II), respectively), which contain the prepro-leader sequences which precede the mature portion. After cleavage of the pre-sequence, pro-GCAP-I and/or pro-GCAP-II are further processed to give the mature peptides, guanylin or uroguanylin (Fig. 1). GCAP-II, a plasma form of uroguanylin, is one of the mature forms of pro-GCAP-II *in vivo* (4, 8, 12, 14–16). Recent studies in this laboratory have shown that spontaneous refolding to the native conformation is attained in pro-GCAP-II but not in GCAP-II (17), *i.e.* GCAP-II requires the propeptide, in order to efficiently fold into the bioactive form.

There are a few examples, such as subtilisin, α-lytic protease, etc., in which the peptides of the pro-leader sequences in the precursor proteins aid the mature proteins in the proper assembly of the three dimensional structures *in vitro* and are referred to as "intramolecular chaperones" (18–20). The mature proteins are produced by enzymatic cleavage of the peptides in the pro-leader sequences from the folded precursor proteins. In these examples, the peptides in the pro-leader sequences function not only to diminish the activation energy but also to stabilize the rate-determining transition state(s) in the folding pathway (20, 21). Moreover, the N-terminal peptide in the pro-leader sequence of prosubtilisin, the precursor protein of subtilisin, mediates the folding of the protein intermolecularly. Prosubtilisin exists as homodimer that is assembled during the folding of the protein (21, 22). However, the mechanism, at the molecular level, of the folding of these proteins via the peptides in the pro-leader sequence remains unclear.

In a recent study, it was demonstrated that guanylin, which is homologous to GCAP-II, requires the assistance of the propeptide of the precursor protein, pro-GCAP-I, not only to achieve correct folding but also for the formation of the native disulfide linkages (23). These findings, and our previous studies, led us to conclude that the propeptide in the pro-leader sequence of pro-GCAP-I and pro-GCAP-II plays a functional role as an intramolecular chaperone in the correct folding of the mature peptide and is also crucial for the disulfide-coupled folding of the reduced precursor (17, 23). Furthermore, these studies have led us to propose that the mature form of pro-GCAP-II, GCAP-II, is not at the thermodynamic ground state

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[1] The abbreviations used are: GC-C, guanylyl cyclase C; GCAP-II, guanylyl cyclase-activating peptide II (the plasma form of uroguanylin); pro-GCAP-I, proguanylin; pro-GCAP-II, prouroguanylin; prepro-GCAP-I, preproguanylin; prepro-GCAP-II, preprouroguanylin; Arg-C, arginylendopeptidase C; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; DTT, dithiothreitol; IGD, insulin-like growth factor.

but, rather, is kinetically trapped in the precursor protein (17). Consequently, these studies raised questions as to which region(s) of the protein, or the manner in which the pro-leader sequence of pro-GCAP-II, contributes to the correct folding of the mature peptide.

In this report, we provide evidence that the amino acid residues at the N terminus of the pro-leader sequence are heavily involved in the correct assembly of the three dimensional structure of pro-GCAP-II and, in turn, of GCAP-II. These residues function to stabilize the bioactive form of the mature portion during the folding of the entire protein. Moreover, we provide evidence that supports the existence of a homodimer, which is stabilized by intermolecular and non-covalent interactions between the region in the pro-leader sequence and, possibly, in the intermediate as well as the final steps of the folding process. The data obtained provide basic information, which is critical for our understanding of the role of the pro-leader sequence of the precursor proteins during the maturation of peptide hormones, such as GCAP-II and guanylin.

EXPERIMENTAL PROCEDURES

Materials —Restriction enzymes were purchased from Toyobo (Osaka, Japan) and New England Biolabs (Beverly, MA). *Taq* polymerase, T4 DNA ligase, and endoproteinase Arg-C were obtained from Takara Shuzo Co. (Kyoto, Japan). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Nissui Pharmaceutical, Co. (Tokyo, Japan) and Dainippon Pharmaceutical, Co. (Osaka, Japan), respectively. Thr-Ile-Ala-uroguanylin and its disulfide isomers were synthesized according to a previously described procedure (17). All other chemicals and solvents were reagent grade. PCR was carried out using a Sanyo DNA amplifier MIR-D30 (Osaka, Japan).

Construction of Expression Vectors of the Deletion Mutants for and Their Expression by E. coli Cells—The cDNAs encoding the deletion mutant proteins were subcloned into a pET17b expression vector (Novagen), following the introduction, by means of PCR, of an *Nde*I site at its 5′ end and a *Xho*I site at its 3′ end using pEX2 as a template. The cDNA sequences of the vectors were confirmed as described above. *E. coli* BL21(DE3) cells, which were transformed with the expression vector, was cultured at 37 °C in Luria broth medium supplemented with ampicillin (50 mg/liter). The production of the mutant proteins was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside at the mid-log phase of cell growth. After incubation at 37 °C for 3 h, the cells were harvested and washed with phosphate-buffered saline without magnesium and calcium ions, containing 1% Triton and 1 mM phenylmethylsulfonyl fluoride. The cells were resuspended in the same buffer, sonicated on ice, and centrifuged (15,000 × *g*, 20 min). The mutant proteins, isolated as an inclusion body, possessed the Met residue at the N terminus derived from the *Nde*I site during subcloning. The proteins thus prepared were characterized by mass spectrometry and amino acid analysis.

Construction of Expression Vectors of Deletion Mutants for Human Embryonic Kidney 293T Cells—The pEX2 vector derived from the pcDNA3 vector (Invitrogen), which contains a strong cytomegalovirus enhancer-promoter sequence for a high level of protein expression in mammalian cells (17), was used in this experiment. The construction of the expression vectors of the N-terminal deletion mutants of pro-GCAP-II was carried out as follows. The pEX2 vector, which carries a cDNA encoding pre-pro-GCAP-II between a *Bam*HI site at its 5′ end and a *Xba*I site at its 3′ end (8), was employed as a template for the construction of the expression vectors in carrying the cDNAs of the N-terminal deletion mutant proteins of pro-GCAP-II. To efficiently use the signal sequence (the pre-region of pre-pro-GCAP-II) for the expression of the mutant proteins, the cDNA fragment from a *Bam*HI site to the end of the signal sequence was amplified by PCR using the pEX2 vector as a template and a sense (ATATAGGATCCAGGGAGCGCGATG) as primer 1 and an antisense (TCTCTCTAGAGAATTCCTCGAGTGACTGTGTGCTCTG) as primer 2. The cDNA fragment encoding the signal sequence was inserted between the *Bam*HI and *Xba*I sites of pEX2, resulting in the construction of pcDNA3H, which contains a unique *Xho*I site after the signal sequence between the *Bam*HI site and the *Xba*I site. The cDNAs encoding the deletion mutant proteins were prepared by PCR and subcloned into the site between the *Xho*I site and the *Xba*I site in pcDNA3H. The resulting expression vectors comprised the cDNA sequences, which encode the signal peptide of pre-pro-GCAP-II and each of the deletion mutant proteins, pro-GCAP-II-(7–86), pro-GCAP-II-(12–86), and pro-GCAP-II-(18–86). The mutant proteins (pro-GCAP-II-(7–86) and pro-GCAP-II-(18–86)) contained two additional amino acid residues, which are derived from the *Xho*I site in the expression vector, at their N termini. The cDNA sequences of the vectors thus constructed were confirmed by analysis using an Applied Biosystems 373A sequencing system.

Expression of Deletion Mutants in 293T Cells—Human embryonic kidney 293T cells (24) were maintained in 10% FBS/DMEM and transferred in a 10-cm diameter plate at 60–80% confluence with 20 μg of each of the expression vectors and the SuperFect reagent (Qiagen, Hilden, Germany) according to the manufacturer's specifications. After incubation for 16 h, the medium was replaced by DMEM (10 ml/plate) without FBS and the cells were incubated for an additional 2 days at 37 °C in a CO_2 incubator.

Purification of the Recombinant Proteins Expressed by E. coli Cells or Human Kidney 293T Cells—The recombinant proteins, which were expressed as inclusion bodies in *E. coli* cells, were treated with 20 eq of DTT in 50 mM Tris/HCl (pH 8.0) (200 μl) containing 6 M guanidine HCl under an N_2 atmosphere at 50 °C for 1 h. The supernatant of the reaction mixture or the culture medium (20 ml) of the 293T cells were applied to a column of Cosmosil 140C_{18}-OPN (1 ml) (Nacarai Tesque Inc., Kyoto, Japan) pre-equilibrated with and washed with 20 ml of solvent A (20% CH_3CN in 0.05% trifluoroacetic acid). The adsorbed proteins, which were eluted with solvent B (80% CH_3CN in 0.05% trifluoroacetic acid), were collected and lyophilized. The protein was purified by HPLC and analyzed by mass spectrometry, as described previously (17). The yield of the purified protein was 0.5–1 nmol/10 ml of the culture medium of 293T cells, as estimated by amino acid analyses.

Endoproteinase Arg-C Digestion of the Recombinant Proteins—The recombinant protein (1 nmol) was incubated with endoproteinase Arg-C (50 pmol) in 0.1 M Tris/HCl (pH 8.0) (200 μl) at 37 °C for 18 h. The digest was treated with anhydrotrypsin agarose as described previously (17), and the supernatant was subjected to HPLC. The eluates were analyzed by mass spectrometry and amino acid analysis.

Gel Filtration Chromatography —The HPLC apparatus consisted of a Waters 600 multisolvent delivery system (Bedford, MA) equipped with a Hitachi L-3000 photodiode array detector and a D-2000 chromato-integrator (Tokyo, Japan). The protein (1 nmol) was dissolved in 50 mM Tris/HCl (pH 7.4) (50 μl) containing 0.2 M NaCl and chromatographed on a TSK-Gel G3000SW_{XL} column (7.8 × 300 mm; Tosoh, Tokyo, Japan). The protein was eluted with 50 mM Tris/HCl (pH 7.4) containing 0.2 M NaCl at a flow rate of 0.8 ml/min, and the eluate was monitored at 220 nm. The molecular mass of the protein was calibrated using a gel filtration calibration kit (Amersham Pharmacia Biotech) containing bovine serum albumin (67 kDa), ovalbumin (43 kDa), RNase A (13.7 kDa), and thioredoxin (20 kDa). Thioredoxin was prepared from *E. coli* cells transformed with pET32b (Novagen), which possesses the cDNA encoding thioredoxin, purified on a nickel-nitrilotriacetic acid resin (Qiagen), and identified by mass spectrometric analysis.

In Vitro Complementary Refolding of Pro-GCAP-II-(7–86) and Pro-GCAP-II-(12–86)—The fully reduced pro-GCAP-II-(12–86) was prepared as follows. The protein (2 nmol) was incubated with 20 eq of DTT in 50 mM Tris/HCl (pH 8.0) (200 μl) containing 6 M guanidine HCl under an N_2 atmosphere at 50 °C for 1 h. The reduced pro-GCAP-II-(12–86) was purified by HPLC, as described above, and lyophilized. The reduced pro-GCAP-II-(12–86) (1 nmol) was dissolved in 0.05% trifluoroacetic acid (20 μl) and mixed with 9 volumes of 50 mM Tris/HCl (pH 8.0) in the presence of 2 mM GSH and 1 mM GSSG as described previously, and incubated at room temperature for 2 days. The oxidative refolding experiment was also carried out in the redox buffer in the presence of the synthetic complementary N-terminal peptides (VYIQYQ or VYIQYQGFRVQ). The reaction mixture was analyzed by HPLC. All solutions used for the refolding experiment were flushed with N_2, and the reaction was carried out in a sealed vial under an atmosphere of N_2.

RESULTS AND DISCUSSION

Mutational Analysis of the N-terminal Amino Acids for a Role in the in Vitro Folding of Pro-GCAP-II—In a previous report, we demonstrated that the mature form of GCAP-II does not possess sufficient information to permit for its correct folding and that the propeptide in pro-GCAP-II aids in the folding process, yielding only the bioactive form of GCAP-II (17). This result provided confirmation that the function of the propeptide in the pro-leader sequence of pro-GCAP-II was to serve as an intramolecular chaperone in the folding of GCAP-II, and con-

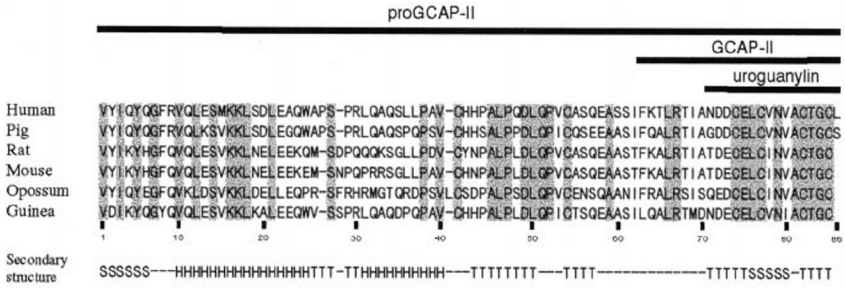

FIG. 1. **Primary and predicted secondary structures of prouroguanylin for human (8), pig (EMBL/GenBank/DDBJ accession number O13009), rat (EMBL/GenBank/DDBJ accession number P70668), mouse (11), opossum (12), and guinea pig (EMBL/GenBank/DDBJ accession number P70107).** *Numbers* on figure refer to the amino acid sequence of human prouroguanylin. Completely matched amino acid residues are *shaded*. Disulfide linkages are between positions Cys[41] and Cys[54], Cys[74] and Cys[82], and Cys[77] and Cys[85] (17). *Single-letter codes* for amino acid residues are used. The *H*, *S*, and *T* in the secondary structure represent α-helix, β-sheet, and β-turn, respectively.

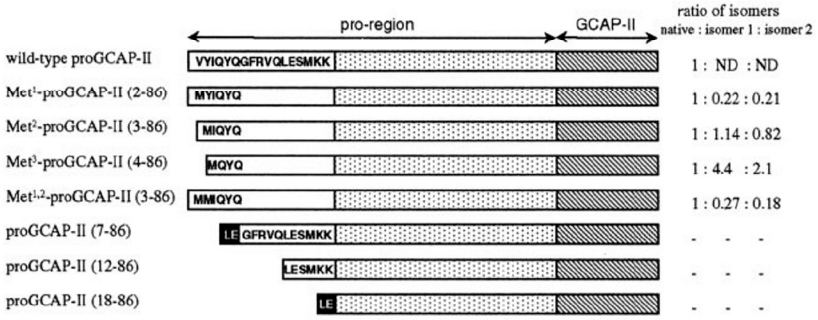

FIG. 2. **Schematic representation of the recombinant wild-type prouroguanylin and N-terminal deletion mutant proteins prepared in this study.**

sequently raised a number of questions, such as (i) which region(s) in the pro-leader sequence of pro-GCAP-II contribute to the correct folding of the mature peptide, and (ii) how does the propeptide play a role in the folding of GCAP-II *in vivo* and *in vitro*?

To address these problems, we first searched the sequence motif(s) in the pro-leader sequence of pro-GCAP-II in the primary structures of pro-GCAP-IIs, which have been determined thus far, and then deduced the secondary structure of pro-GCAP-II using the Chou-Fasman method (25), as shown in Fig. 1. The amino acid sequences of the N-terminal region (amino acid residues 1–23) and the C-terminal region (amino acid residues 38–65) in the pro-leader sequence of pro-GCAP-II are highly homologous in all species, whereas that in the central region (amino acids 24–37) is diverse. This raises the possibility that the N-terminal region (amino acid residues 1–23), along with the C-terminal region (amino acid residues 38–65), acts as an intramolecular chaperone for the correct folding of pro-GCAP-II to yield the bioactive conformation of the mature peptide, GCAP-II. Further, the secondary structure prediction implied that the N-terminal region (amino acids 1–6) and the C-terminal region (LCVNV, amino acid residues 76–80) in the mature region exist as β-strands, not only in pro-GCAP-II, but also in pro-GCAP-I. Schulz *et al.* (23) recently demonstrated that the N-terminal region (amino acids 1–5) is in close proximity to the C-terminal region (a portion of guanylin) in the solution structure of pro-GCAP-I, as evidenced by NMR measurement. This may be extended to the speculation that the N-terminal region shares a characteristic secondary structure of pro-GCAP-I with the C-terminal mature region. A similar molecular conformation may be imagined in the structure of pro-GCAP-II, since it is likely that pro-GCAP-II has a conformation similar to that of pro-GCAP-I, *i.e.* it is possible that the N-terminal region (amino acids 1–23) in the pro-leader sequence of pro-GCAP-II interacts with the C-terminal mature region. This interaction may lead to the proper folding of pro-GCAP-II and contribute to the stabilization of the three dimensional structure of the mature portion of pro-GCAP-II, GCAP-II.

To examine the nature of the participation of the N-terminal region in the pro-leader sequence of pro-GCAP-II in terms of its correct folding, we prepared a series of mutant proteins of pro-GCAP-II, in which the N-terminal amino acid residues were sequentially deleted from the N terminus of pro-GCAP-II (Fig. 2). The recombinant proteins were generated using *E. coli* BL21(DE3) cells. All mutant proteins were expressed with an additional Met residue at their N termini, which originated from the starting codon. For example, the deletion of a Val residue at the N terminus of pro-GCAP-II resulted in the production of the mutant protein, Met[1]-pro-GCAP-II-(2–86) (Fig. 2). Since the mutant proteins were produced as inclusion bodies in the bacterial cells, as is the case for the expression of many eukaryotic proteins by *E. coli* cells, we were not able to define the conformational states of the mutant proteins immediately after expression in the cells. As a result, the recombinant proteins were purified by HPLC in the reduced form and then oxidatively refolded to the oxidized forms in the presence of 2 mM GSH and 1 mM GSSG following previously reported procedures (17). The refolded proteins were subjected to HPLC, which indicated the presence of a few isomers, which comprised different disulfide linkages (data not shown). It was not possible to completely separate these isomers from each other under the conditions used in this experiment. The refolded proteins were then directly digested by endoproteinase Arg-C and the resulting digests were separated by HPLC, as previously reported (17). The ratios of the disulfide isomers in the digests, which have different disulfide linkages and were clearly separated on HPLC as is the case in our paper (17), were estimated by measurement of their peak areas on HPLC and shown in Fig. 2. The mutant protein, Met[1]-pro-GCAP-II-(2–86), consisted predominantly of the native-type Thr-Ile-Ala-uroguanylin (the native-type Thr-Ile-Ala-uroguanylin contains two disulfide linkages between Cys[74] and Cys[82] and Cys[77] and Cys[85]

(Ref. 17)), along with small amounts of biologically inactive isomers 1 and 2 (the positions of the disulfide bonds of isomer 1 are between Cys^{74} and Cys^{85} and Cys^{77} and Cys^{82}, and isomer 2 between Cys^{74} and Cys^{77} and Cys^{82} and Cys^{85} (Ref. 17)), as found in the folding of the wild-type pro-GCAP-II (17). This indicates that the mutation of the Val residue to Met at the N terminus had no significant effect on the folding of pro-GCAP-II. The mutant proteins, Met^{2}-pro-GCAP-II-(3–86) and Met^{3}-pro-GCAP-II-(4–86), were composed of the native-type Thr-Ile-Ala-uroguanylin, isomer 1 and isomer 2 in ratios of 1: 1.14: 0.82 and 1: 4.4: 2.10, respectively. These data suggest that the deletion of the amino acid residue at the N terminus greatly affects the construction of the native tertiary structure in the mature region of pro-GCAP-II, because the native-type disulfide pairing comprises only one-third of the mutant protein (Met^{2}-pro-GCAP-II-(3–86)). Further, the mutant protein, Met^{3}-pro-GCAP-II-(4–86), in which two amino acid residues were deleted from the N terminus, nearly completely lacked the ability to form the correct disulfide pairing in the mature region and, thus, was devoid of the chaperone function in the pro-leader sequence of pro-GCAP-II, because the ratio of the native type to isomers was comparable with that in the folding of the mature hormone, GCAP-II (17).

To further investigate whether the Tyr residue at position 2 from the N terminus is involved in the folding of pro-GCAP-II, the mutant protein, $Met^{1,2}$-pro-GCAP-II-(3–86), was prepared in which the Tyr residue was replaced by Met. The result indicates that the distribution of the native-type isomers 1 and 2 in $Met^{1,2}$-pro-GCAP-II-(3–86) were nearly the same as in the case of Met^{1}-pro-GCAP-II-(2–86) and, therefore, that the replacement of the Tyr residue with Met had no effect on the function of the propeptide in the pro-leader sequence. Collectively, these results indicate that the two N-terminal amino acid residues in length, in particular the N-terminal residue, play an important role in the formation of the correct disulfide linkages of the mature portion of pro-GCAP-II *in vitro* and, thus, in the function of the intramolecular chaperone of the propeptide in the pro-leader sequence of pro-GCAP-II.

Expression of the N-terminal Deletion Mutants of Pro-GCAP-II in 293T Cells—Since the mutant proteins were expressed as inclusion bodies in the *E. coli* cells, we were not able to estimate the effect of the deletion of the N-terminal residue on the *in vivo* folding of pro-GCAP-II. Therefore, we prepared the wild-type pro-GCAP-II in human embryonic kidney 293T cells, as well as the N-terminal deletion mutants, in which the N-terminal amino acid residues were sequentially deleted from the N terminus, and three types of mutant proteins of pro-GCAP-II, which are devoid of the N-terminal region, as shown in Fig. 2: 1) pro-GCAP-II-(7–86), which is deprived of the 6 N-terminal amino acid residues; 2) pro-GCAP-II-(12–86), which lacks the 11 N-terminal residues; and 3) pro-GCAP-II-(18–86), which lacks the 17 N-terminal residues that comprise an invariant region (amino acid residue sequence 12–17) in both pro-GCAP-II and pro-GCAP-I. The N-terminal deletion mutant proteins, which lack the N-terminal Val or Val-Tyr residues, could not be isolated from 293T cells, although the reason for this is not clear at present. The mutant proteins, which were deleted in a portion of the peptide in the pro-leader sequence of pro-GCAP-II, might be due to its failure to fold in the endoplasmic reticulum, resulting in a protein that is susceptible to degradation by proteases and is not secreted from the endoplasmic reticulum (26). The other deletion mutants and the wild-type pro-GCAP-II were expressed in human kidney 293T cells via the expression vector, secreted into the culture media, and then purified by HPLC (Fig. 3) and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Fig. 4). The wild-type pro-GCAP-II showed a signal at m/z = 9487.0, which is consistent with the mass value (9487.9) calculated from the amino acid sequence. In contrast, pro-GCAP-II-(7–86), pro-GCAP-II-(12–86), and pro-GCAP-II-(18–86) exhibited mass spectral signals at m/z = 17859.5, 16203.0, and 15248.0, respectively, which are twice the theoretical values, calculated from their amino acid sequences. No monomeric forms of the N-terminal deletion mutant proteins were detected on Fig. 3. These results indicate that the wild-type pro-GCAP-II was prepared as a monomer and, conversely, that the mutant proteins, which lack the N-terminal amino acid residues, were expressed as dimers. This dimer appears to be composed of two monomer units, which are connected via a covalent linkage(s), perhaps intermolecular disulfide linkage(s), because the dimer could be converted into a monomer by treatment with DTT, as described below.

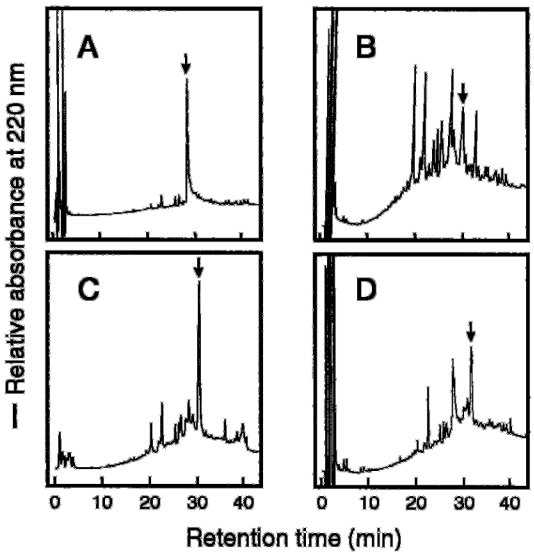

FIG. 3. **HPLC profiles of the culture supernatants of 293T cells, which express the wild-type pro-GCAP-II (*A*), pro-GCAP-II-(7–86) (*B*), pro-GCAP-II-(12–86) (*C*), and pro-GCAP-II-(18–86) (*D*).** The target proteins are indicated by *arrows*. HPLC was performed as described under "Experimental Procedures."

To determine the location of the intermolecular disulfide linkage(s) found in the recombinant proteins, which are devoid of the N-terminal region, the wild-type pro-GCAP-II (as a control) and pro-GCAP-II-(12–86) were each digested with endoproteinase Arg-C and the hydrolysates examined by HPLC (Fig. 5). A comparison of the HPLC profiles of the digests of the wild-type pro-GCAP-II and pro-GCAP-II-(12–86) revealed that the wild-type pro-GCAP-II comprises the native-type Thr-Ile-Ala-uroguanylin covering the mature GCAP-II (observed mass value, 1953.0; theoretical value, 1953.2), whereas pro-GCAP-II-(12–86) contains a dimer (observed mass value, 3906.9; theoretical value, 3906.5) of Thr-Ile-Ala-uroguanylin. Two Cys residues at positions 41 and 54 in the pro-leader sequence of pro-GCAP-II were correctly bridged in both the wild-type pro-GCAP-II and pro-GCAP-II-(12–86). This clearly shows that the intermolecular disulfide linkage(s) in the recombinant pro-GCAP-II-(12–86) were connected in its mature region. The disulfide pairing in the dimer of Thr-Ile-Ala-uroguanylin in pro-GCAP-II-(12–86) could not be further defined, because the peptide was not soluble after purification by HPLC. The other N-terminal deletion proteins also gave the same results as found in pro-GCAP-II-(12–86) (data not shown). Consequently, these data indicate that the deletion of the N-terminal region in the pro-leader sequence of pro-GCAP-II greatly influenced the

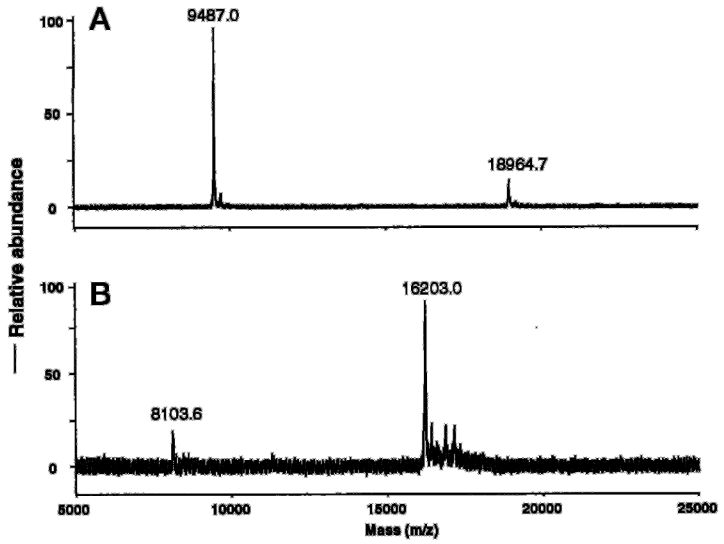

FIG. 4. **Matrix-assisted laser desorption/ionization time-of-flight mass spectra of the wild-type pro-GCAP-II (*A*) and the mutant protein, pro-GCAP-II-(12–86) (*B*).** Mass spectra were obtained using a Voyager Elite XL TOF mass spectrometer equipped with a delayed-extraction system (PerSeptive Biosystems, Framingham, MA).

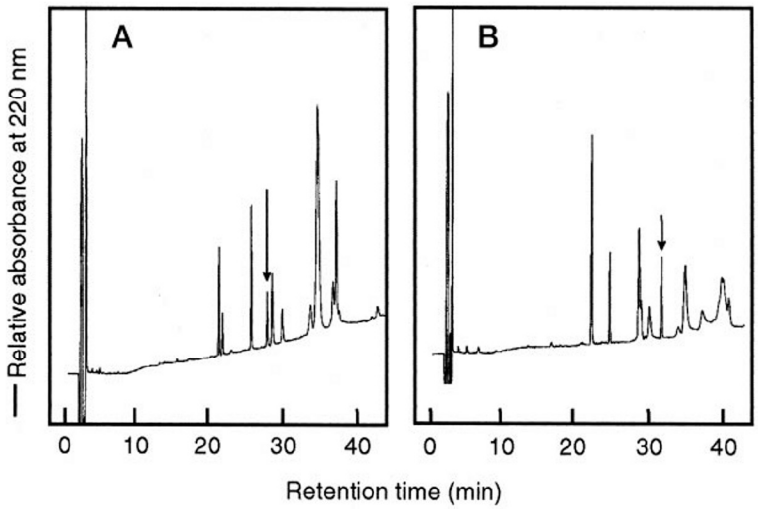

FIG. 5. **Arg-C digestion of wild-type pro-GCAP-II (*A*) and pro-GCAP-II-(12–86) (*B*).** The position of Thr-Ile-Ala-uroguanylin is indicated by *arrows*.

linking of the disulfide bonds in the mature peptide of pro-GCAP-II, GCAP-II. In other words, the N-terminal region (amino acid residues 1 to 6) in the pro-leader sequence of pro-GCAP-II plays a critical role in the formation of the three dimensional structure of pro-GCAP-II *in vivo* and in turn, the folding of the mature peptide, GCAP-II.

In Vitro Disulfide-coupled Folding of the N-terminal Deletion Mutants Expressed in 293T Cells—The S-protein (amino acid sequence 22–124) of RNase A folds complementarily with the S-peptide (amino acid sequence 1–21) to a stable conformation similar to that of intact RNase A (27–29). This led us to determine if pro-GCAP-II-(7–86) or pro-GCAP-II-(12–86) were able to adopt a tertiary structure similar to that of the intact protein by the aid of the complementary N-terminal peptides, which consist of 6 or 11 amino acid residues, respectively. The relative abundance of three disulfide isomers of the refolded proteins, pro-GCAP-II-(7–86) or pro-GCAP-II-(12–86), was nearly identical with those found in the refolding of the mature GCAP-II (17) and Met^{3}-pro-GCAP-II-(4–86) (data not shown). Next, these N-terminal deletion proteins were reduced with DTT and, after removal of the reducing reagent, incubated with the corresponding peptides under the same conditions that were used for the refolding of the full-length protein. The N-terminal deletion proteins did not efficiently adopt a native conformation in the presence of the complementary peptides, as is the case for RNase A (data not shown). It should be noted, however, that the possibility that the complementary refolding of the N-terminal deletion proteins with the rest of the peptides could occur under conditions different from those used in this experiment cannot be excluded. Insulin and chymotrypsin, in contrast to their precursor proteins, have been observed to become thermodynamically unstable after the release of their propeptides. In this case, the mature proteins are required to fold to the proper structure in the original single-chained precursor proteins with the propeptides, which aid in guiding the proteins to the native conformation (30, 31). It is likely in the case of those proteins that the N-terminal portion in the pro-leader sequence of pro-GCAP-II functions to stabilize the three-dimensional structure of pro-GCAP-II.

In eukaryotic translation systems, a protein initiates co-translational folding from the N-terminal region prior to the release of the nascent chain from the ribosome, and achieves a native conformation after the synthesis of the entire protein (32). As described above, the disulfide bond between two Cys residues at positions 41 and 54 in the pro-leader sequence of pro-GCAP-II was correctly formed in the N-terminal deletion proteins both *in vitro* and *in vivo*. This implies that the propeptide region in these proteins assemble the three-dimensional structure in a manner similar to that in the wild-type pro-GCAP-II. However, the N-terminal deletion proteins of pro-

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