Secretion of FGF-16 Requires an Uncleaved Bipartite Signal Sequence*

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Fibroblast growth factor (FGF)-16 is one of the rare secreted proteins that do not possess a cleavable signal sequence. Here we describe our examination of the mechanism and structural requirements for the secretion of FGF-16 from COS-1 transfectants. Inhibition of its secretion by brefeldin A and identification of an N-glycan on the secreted form confirmed that FGF-16 is secreted by means of the endoplasmic reticulum and Golgi apparatus, as are secreted proteins having a conventional cleavable signal sequence. Deletion of its N terminus abolished secretion of FGF-16. When chimerized with prolactin, however, the N-terminal sequence of FGF-16 was not able to mediate secretion of the chimer. Point mutations that made the N terminus less hydrophobic had little effect on secretion of FGF-16, whereas making the central hydrophobic region less hydrophobic abolished secretion. Within cells, an uncleavable FGF-16 N-terminal deletion mutant was distributed in the perinuclear region and overlapped the distribution of the Golgi apparatus. Mutants with less hydrophobic central regions were distributed evenly throughout the cytosol. Collectively, these results indicate that FGF-16 employs a unique bipartite signal sequence (i.e. both the N-terminal region and central hydrophobic region) that is not cleaved, although it shares the same secretory machinery used by secreted proteins with cleavable signal sequences.

The FGF family is composed of 22 homologous polypeptides (1) involved in a variety of biological processes, including regulation of cell growth, differentiation, migration, angiogenesis, and tissue development (2–6). The biological activity of FGFs is thought to be mediated in part by their interaction with both high and low affinity plasma membrane receptors for FGF family members (9–11). Interaction with FGFs induces autophosphorylation of the receptors and initiates various signal transduction pathways (12). The low affinity receptors consist of heparan sulfate proteoglycans, which function to stabilize the ligands, concentrate them on the cell surface, and present ligand dimers to the tyrosine kinase receptors (13).

To understand fully the biological functions of FGFs and how they are regulated, it is necessary to understand the mechanism and regulation of their secretion. Although many FGFs possess a conventional signal sequence, some do not. FGFs that do possess them are exported through a constitutive secretory pathway that includes the endoplasmic reticulum (ER) and Golgi complex/trans-Golgi network (14). The signal sequence is usually located at the N terminus and is cleaved off by signal peptidase after the protein has crossed the ER membrane (15). The primary structures of signal sequences vary widely, but are typically composed of a hydrophobic region (h-region) of 6–15 amino acids, a short stretch of 2–5 positively charged residues (n-region), and a hydrophilic region (c-region) of about 6 residues terminating at a small uncharged residue that contributes its carboxyl group to the peptide bond cleaved by signal peptidase (16). In contrast, FGF-9 (17), FGF-16 (18), and FGF-20 (19) all seem to be secreted despite their lack of cleavable, N-terminal signal peptides. We reported previously that FGF-9 contains an uncleaved signal sequence that involves both its N-terminal region and central hydrophobic region (20). However, this understanding was challenged by another study, which suggested that the N terminus of FGF-9 is sufficient, by itself, to mediate secretion (21).

The primary structure of FGF-16 shows 73% identity with that of FGF-9 (18). It is expressed mainly in embryonic brown adipose tissue and adult heart and, once secreted, has a mitogenic effect on primary brown adipocytes, which it exerts by binding to and activating FGFR-4 on the plasma membrane (22). In the present study, we investigated the mechanism and structural requirements for the secretion of FGF-16.

EXPERIMENTAL PROCEDURES

Construction of cDNAs—The full-length FGF-16 cDNA was isolated after reverse transcription of total RNA from adult rat heart and then subcloned into eukaryotic expression vector pcDNA3.1 or pcDNA3.1/Myc-His(−) (Invitrogen Japan K.K., Tokyo, Japan). The cDNAs for three N-terminal FGF-16 deletion mutants (i.e. FGF-16A10 (aa 11–207), FGF-16A15 (aa 16–207), and FGF-16A27 (aa 28–207)) were generated from FGF-16ApcDNA3.1 by PCR and subcloned into pcDNA3.1/Myc-His(−). PL and PPL were amplified from bovine PPL (23) by PCR and respectively subcloned into pcDNA3.1/Myc-His(−). The plasmid DNA was amplified from bovine PPL (23) by PCR and respectively subcloned into pcDNA3.1/Myc-His(−).

Six FGF-16-PL chimeras (i.e. FGF-16 (1–27)-PL (FGF-16 (aa 1–27) and PL (aa 1–229)), FGF-16 (1–57)-PL (FGF-16 (aa 1–57) and PL (aa 1–229)), FGF-16 (1–83)-PL (FGF-16 (aa 1–83) and PL (aa 1–229)), FGF-16 (1–27)-PPL (FGF-16 (aa 1–27) and PPL (aa 31–229)), FGF-16 (1–57)-PPL (FGF-16 (aa 1–57) and PPL (aa 31–229)), and FGF-16 (1–83)-PPL (FGF-16 (aa 1–27) and PPL (aa 31–229))) were constructed.

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FGF-16 is secreted from COS-1 transfectants with N-glycosylation. A, secretion and heparin affinity of wild-type and tagged FGF-16. Medium conditioned by COS-1 transfectants expressing wild-type or Myc-His<sub>6</sub>-tagged FGF-16 was absorbed onto a Hi-Trap heparin HP column, and the bound proteins were eluted with a linear NaCl gradient in 10 mM phosphate buffer (pH 7.4) containing 0.01% CHAPS. Proteins in fractions 11, 12, and 13 were resolved by SDS-PAGE and stained with Coomassie Blue: lanes 1, 4, and 7, medium conditioned by transfectants expressing wild-type FGF-16; lanes 2, 5, and 8, medium conditioned by transfectants expressing tagged FGF-16; lanes 3, 6, and 9, medium conditioned by mock transfectants. Wild-type FGF-16 and the tagged form were recovered at similar heparin affinities (black and white arrows, respectively). Positions of the molecular mass markers are indicated by the bars on the left. B, N-glycosylation of secreted FGF-16. COS-1 transfectants expressing Myc-His<sub>6</sub>-tagged FGF-16 were lysed, after which the proteins were resolved by SDS-PAGE, transferred to a membrane, and visualized with anti-His<sub>6</sub> antibody (lane 1). Conditioned medium obtained from the same number of transfectants was concentrated and analyzed without (lane 2) or after (lane 3) digestion with N-glycosidase. Glycosylated and non-glycosylated forms of FGF-16 are indicated by the white and black arrows, respectively. C, amino acid sequence of wild-type rat FGF-16. The SignalP-HMM software predicted that there was no cleavable signal sequence. A possible N-glycosylation site is underlined.
transfectants on glass coverslips were fixed with 4% paraformaldehyde for 10 min at room temperature and permeabilized with PBS containing 0.2% Triton X-100 for 6 min at room temperature. First, the cells were incubated for 1 h with a mixture of polyclonal rabbit anti-c-Myc antibody (1:50 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and monoclonal mouse anti-Golgi-58K antibody (1:50 dilution; Sigma-Aldrich Japan K.K.). Then, after washing, the cells were incubated for 30 min with Texas Red-conjugated donkey anti-rabbit Ig (1:50 dilution; Amersham Biosciences K.K.) and fluorescein-conjugated goat anti-mouse IgG (1:800 dilution; Molecular Probes Inc., Eugene, OR). The cells were observed under a confocal microscope (Zeiss LSM 510, invert mode).

RESULTS

FGF-16 Protein Is Secreted as an N-Glycosylated Form without Predictable Signal Cleavage—In an earlier study, Miyake et al. (18) showed that tagged rat FGF-16 is secreted from insect Sf9 cells transfected with its cDNA. Our first aim was to confirm secretion from FGF-16 from the mammalian COS-1 cell line (Fig. 1, A and B). cDNAs encoding FGF-16 (Fig. 1C) with or without a C-terminal Myc-His6 tag were expressed in COS-1 cells, after which post-heparin-Sepharose fractions from the conditioned medium were examined for the presence of secreted FGF-16. We found that both intact wild-type FGF-16 (Fig. 1A, lanes 1, 4, and 7) and its tagged form (Fig. 1A, lanes 2, 5, and 9) were secreted into the culture medium and that the addition of a tag did not affect secretion or heparin affinity (Fig. 1A, compare lanes 4 and 5). Analysis of equal aliquots of cell lysate and conditioned medium from COS-1 transfectants expressing FGF-16-Myc-His6 confirmed the secretion of FGF-16 (Fig. 1B, lanes 1 and 2). Notably, FGF-16 was detected in the cell lysate mainly in a 30-kDa form, which is consistent with the calculated molecular mass of the tagged, full-length FGF-16 (lane 1), and in a 34-kDa form in the conditioned medium (lane 2).

Prediction of a signal sequence in FGF-16 yielded negative results. By using SignalP-HMM software, which is available on the Internet (25) and has a substantially greater ability to discriminate between signal peptides and uncleaved signal peptides.
chors than its predecessor (SignalP-NN), FGF-16 was predicted to be a non-secretory protein (i.e. there was no predictable, cleavable signal sequence). We attempted to confirm that finding by directly sequencing the N terminus of the secreted FGF-16 protein present in medium conditioned by FGF-16-expressing COS-1 cells, but we found that the N terminus of the secreted FGF-16 was blocked. Still, treatment of the 34-kDa-secreted FGF-16 with N-glycosidase F yielded a 30-kDa form (Fig. 1B, lane 3, black arrow), suggesting that the 34-kDa form was composed of a 30-kDa simple polypeptide modified with a 4-kDa N-linked glycan. The only possible N-glycosylation site within the FGF-16 primary structure is the sequence Asn78-Gly79-Thr80, which, consistent with the idea that FGF-16 is not cleaved upon its secretion from cells, precedes the central hydrophobic region (Fig. 1C).

Secretion of FGF-16 Is Potently Inhibited by Brefeldin A—In mammalian cells, most proteins with conventional signal sequences are secreted by means of the ER and Golgi apparatus. To determine whether FGF-16 makes use of the same secretion pathway, we investigated whether the secretion of FGF-16 was affected by brefeldin A, which accentuates the retrograde transport of Golgi components (proteins and lipids) to the ER, thereby causing disassembly of the Golgi apparatus and inhibition of protein secretion (26). When COS-1 transfectants expressing FGF-16 were labeled with [35S]Met/[35S]Cys and then incubated for various times in the presence of 25 μg/ml brefeldin A, FGF-16 secretion was potently inhibited (Fig. 2).

Both the N Terminus and Central Hydrophobic Region of FGF-16 Are Required for the Secretion, but the Hydrophobicity of the N Terminus Is Not—Although N-terminal signal sequences typically include a strongly hydrophobic region, the N-terminal region of FGF-16 does not (Fig. 3A). To determine whether the N-terminal region of FGF-16 is necessary nevertheless to the secretion of FGF-16, we expressed in COS-1 cells three serial FGF-16 N-terminal deletion mutants tagged with Myc-His6 at their C termini (to enable immunological detection) (Fig. 3A). Deletion of the first 15 or 27 amino acids caused a profound reduction in the secretion of FGF-16 (Fig. 3B, lanes 9 and 10, respectively), as compared with the full-length protein (lane 6). Apparently, the N-terminal region of FGF-16 is indispensable for its secretion, despite its lack of a strongly hydrophobic region.

To determine whether the N terminus of FGF-16 can, itself, function as a signal sequence, and whether it can affect the
activity of a pre-existing signal peptide, we expressed in COS-1 cells a group of reporter proteins in which FGF-16 N termini of various sizes were chimerized with prolactin (PL) or preprolactin (PPL) (Fig. 4A). The selected N-terminal sequences (FGF-16-(1–27), FGF-16-(1–57), and FGF-16-(1–83)) all preceded the hydrophobic domain of FGF-16 (aa 90–110; hatched region in Fig. 4A). PPL/PL protein was selected for this experiment because both its secretion and the underlying mechanism have been well characterized. PPL is the full-length translation product of the prolactin gene; it contains an N-terminal signal sequence that is cleaved during the secretion of the molecule, yielding mature PL. There is no consensus sequence for N-linked glycosylation in the primary structure PPL/PL. Consequently, the sole N-glycosylation site is mapped to Asn79-Gly80-Thr80 of FGF-16 (marked by Y).

We found that, irrespective of its length (27, 57, and 83 amino acids), the N terminus of FGF-16 was unable to serve as a secretion signal peptide for the PL chimeric proteins (Fig. 4B, lower panel, lanes 3–5); moreover, it did not obstruct the secretion and normal cleavage of the endogenous signal sequence of PPL (Fig. 4B, upper and lower panels, lanes 7–9).

Interestingly, lysates from cells expressing the PPL chimera harboring the 83-amino acid FGF-16 N terminus (FGF-16-(1–83)-PPL) yielded 38- and 42-kDa bands in addition to the 26-kDa band representing the processed form of PL (Fig. 4B, lane 9, black, white, and hatched arrowheads, respectively). The 42-kDa protein disappeared after digestion with N-glycosidase F and endoglycosidase H (Fig. 4C, lanes 2 and 4, white arrowhead), indicating that it was composed of the full-length 38-kDa simple polypeptide modified with a high mannose type N-glycan, almost certainly attached at the Asn79-Gly79-Thr80 glycosylation site in the FGF-16 fragment (Fig. 4A, marked with Y). Apparently, the N-terminal domain (1–86) of FGF-16 was translocated across the ER membrane along with the signal sequence of PPL, as such N-linked high mannose glycosylation occurs at the inside of the ER membrane. Moreover, the fact that FGF-16-(1–83)-PPL was readily secreted into the conditioned medium as plain PL (Fig. 4B, lower panels) indicates that the N-terminal sequence (1–83) of FGF-16 did not interfere with the signal sequence function of PPL.

A strongly hydrophobic motif is distinctive of conventional N-terminal signal sequences of secreted proteins, and it is known to be important for translocation of secreted proteins across the ER membrane. Therefore, we examined the extent to which the hydrophobic regions of FGF-16 contribute to its secretion. Hydropathy plots of FGF-16 showed that its N terminus is weakly hydrophobic and that its central region is strongly hydrophobic (Fig. 5A). With that in mind, we constructed four FGF-16 point mutants in which local hydrophobicity was reduced by amino acid substitution (Fig. 5A). By contrast, FGF-16(I97N) and FGF-16(L99N) were neither glycosylated nor secreted (Fig. 5B, C).

Analysis of the medium conditioned by the respective COS-1 transfectants revealed that both FGF-16(V4N) and FGF-16(V7N) were glycosylated and secreted from the cells (Fig. 5B, lower panels). Apparently, the N-terminal domain (1–86) of FGF-16 was translocated across the ER membrane along with the signal sequence of PPL, as such N-linked high mannose glycosylation occurs at the inside of the ER membrane. Moreover, the fact that FGF-16-(1–83)-PPL was readily secreted into the conditioned medium as plain PL (Fig. 4B, lower panels) indicates that the N-terminal sequence (1–83) of FGF-16 did not interfere with the signal sequence function of PPL.

Mutations at the N Terminus and Central Hydrophobic Region of FGF-16 Result in Very Different Subcellular Distributions—Because the biochemical data indicated that both the
The central hydrophobic region of FGF-16 is crucial for its secretion. Our results suggest that the central hydrophobic region of FGF-16 is recognized by the SRP during the secretion process. Among FGF family members, the primary structure of FGF-16 shows the highest identity with FGF-9 (73%). Hydropathy plots indicate the central hydrophobic region of FGF-16 is very similar to that of FGF-9, which Miyamoto et al. (17) showed contains an uncleavable signal sequence. Moreover, we observed that both the N-terminal region and central hydrophobic region of FGF-9 are crucial for its translocation into microsomes and secretion from cells and that the translocation was possibly mediated by SRP (20). On the other hand, based on their examination of chimeric proteins composed of FGF-9 N termini and green fluorescent protein, Revet et al. (21) concluded that the N-terminal 28 amino acids of FGF-9 were sufficient to function as an uncleavable signal sequence. Arguing against this interpretation, however, is the fact that the green fluorescent protein construct used in that study was secretable by itself, joining the FGF-9 N terminus to the N terminus of green fluorescent protein only enhanced that secretion. It was never demonstrated that the FGF-9 N terminus could, by itself, convert an otherwise uncleavable protein into a secretable one. We have shown in the present study, however, that the N-terminal region of FGF-16 is not, by itself, sufficient to function as a signal sequence when fused to mature PL lacking its endogenous signal peptide. Still, our observation of a unique FGF-16 (1–83)-PPL form modified with a high mannose N-glycan, which are immediately attached to proteins as they migrate into the ER (28), indicates that the N-terminal FGF-16 region of the chimera had migrated into the ER membrane.

The subcellular distribution of uncleavable FGF-16 mutants provides additional insight into the respective functions of the N-terminal region and central hydrophobic region in the secretion of FGF-16. Like that of wild-type FGF-16, the distribution of FGF-16Δ27 in the perinuclear region significantly overlapped the distribution of the Golgi apparatus. By contrast, FGF-16 mutants with less hydrophobic central regions (FGF-16(L99N,A100N)) were evenly distributed throughout the cytosol. These results strongly suggest that the N-terminal region and central hydrophobic region of FGF-16 cooperate to enable its translocation into the ER during the secretory process. We suggest that the central hydrophobic region is important for concentration of the polypeptide in the approximate ER-Golgi region or for presentation of the polypeptide to the secretion machinery on the ER membrane and that the N-terminal region is important for the polypeptide to cross through the ER membrane.

Other secreted proteins with signal peptides known not to be cleaved include plasminogen activation inhibitor (PAI)-2, ovalbumin (serpin family), and retinoic acid-binding protein. Both PAI-2 and ovalbumin contain internal hydrophobic regions to direct their translocation into the ER (29–31) where their hydrophobic N termini are not cleaved. PAI-2 relies on a cleavable signal sequence. Moreover, we observed that both the N-terminal region and central hydrophobic region of FGF-9 are crucial for its translocation into microsomes and secretion from cells and that the translocation was possibly mediated by SRP (20). On the other hand, based on their examination of chimeric proteins composed of FGF-9 N termini and green fluorescent protein, Revet et al. (21) concluded that the N-terminal 28 amino acids of FGF-9 were sufficient to function as an uncleavable signal sequence. Arguing against this interpretation, however, is the fact that the green fluorescent protein construct used in that study was secretable by itself, joining the FGF-9 N terminus to the N terminus of green fluorescent protein only enhanced that secretion. It was never demonstrated that the FGF-9 N terminus could, by itself, convert an otherwise uncleavable protein into a secretable one. We have shown in the present study, however, that the N-terminal region of FGF-16 is not, by itself, sufficient to function as a signal sequence when fused to mature PL lacking its endogenous signal peptide. Still, our observation of a unique FGF-16 (1–83)-PPL form modified with a high mannose N-glycan, which are immediately attached to proteins as they migrate into the ER (28), indicates that the N-terminal FGF-16 region of the chimera had migrated into the ER membrane.

DISCUSSION

We have shown that FGF-16 secretion can proceed in mammalian cells, even without peptide cleavage. Like proteins with a cleavable signal sequence, FGF-16 was modified with an N-glycan and transported by means of the ER-Golgi apparatus. Although widely separated within the primary structure of the protein, both the N-terminal region and central hydrophobic region of FGF-16 were crucial for its secretion.

The cleavable signal sequences characterized to date are located at the N termini of secreted and transmembrane proteins. Although signal sequences vary widely, they generally include a hydrophobic region of 6–15 amino acids that directs the nascent polypeptide chain to the ER membrane with the aid of a signal recognition particle (SRP) (27). Our results suggest that the central hydrophobic region of FGF-16 is recognized by the SRP during the secretion process. Among FGF family members, the primary structure of FGF-16 shows the highest identity with FGF-9 (73%). Hydropathy plots indicate the central hydrophobic region of FGF-16 is very similar to that of FGF-9, which Miyamoto et al. (17) showed contains an uncleavable signal sequence. Moreover, we observed that both the N-terminal region and central hydrophobic region of FGF-9 are crucial for its translocation into microsomes and secretion from cells and that the translocation was possibly mediated by SRP (20). On the other hand, based on their examination of chimeric proteins composed of FGF-9 N termini and green fluorescent protein, Revet et al. (21) concluded that the N-terminal 28 amino acids of FGF-9 were sufficient to function as an uncleavable signal sequence. Arguing against this interpretation, however, is the fact that the green fluorescent protein construct used in that study was secretable by itself, joining the FGF-9 N terminus to the N terminus of green fluorescent protein only enhanced that secretion. It was never demonstrated that the FGF-9 N terminus could, by itself, convert an otherwise uncleavable protein into a secretable one. We have shown in the present study, however, that the N-terminal region of FGF-16 is not, by itself, sufficient to function as a signal sequence when fused to mature PL lacking its endogenous signal peptide. Still, our observation of a unique FGF-16 (1–83)-PPL form modified with a high mannose N-glycan, which are immediately attached to proteins as they migrate into the ER (28), indicates that the N-terminal FGF-16 region of the chimera had migrated into the ER membrane.

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Other secreted proteins with signal peptides known not to be cleaved include plasminogen activation inhibitor (PAI)-2, ovalbumin (serpin family), and retinoic acid-binding protein. Both PAI-2 and ovalbumin contain internal hydrophobic regions to direct their translocation into the ER (29–31) where their hydrophobic N termini are not cleaved. PAI-2 relies on a cleavable signal sequence. Moreover, we observed that both the N-terminal region and central hydrophobic region of FGF-9 are crucial for its translocation into microsomes and secretion from cells and that the translocation was possibly mediated by SRP (20). On the other hand, based on their examination of chimeric proteins composed of FGF-9 N termini and green fluorescent protein, Revet et al. (21) concluded that the N-terminal 28 amino acids of FGF-9 were sufficient to function as an uncleavable signal sequence. Arguing against this interpretation, however, is the fact that the green fluorescent protein construct used in that study was secretable by itself, joining the FGF-9 N terminus to the N terminus of green fluorescent protein only enhanced that secretion. It was never demonstrated that the FGF-9 N terminus could, by itself, convert an otherwise uncleavable protein into a secretable one. We have shown in the present study, however, that the N-terminal region of FGF-16 is not, by itself, sufficient to function as a signal sequence when fused to mature PL lacking its endogenous signal peptide. Still, our observation of a unique FGF-16 (1–83)-PPL form modified with a high mannose N-glycan, which are immediately attached to proteins as they migrate into the ER (28), indicates that the N-terminal FGF-16 region of the chimera had migrated into the ER membrane.

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amino acid sequences spanning the central core region are highly conserved. On the other hand, their N and C termini are quite divergent. Although many members of the FGF family contain a classical N-terminal signal sequence, several do not. The prototype members of the FGF family, FGF-1 and FGF-2, which primarily localize in the cytoplasm and cell nucleus, do not possess a signal sequence but are nevertheless released and have been visualized in the extracellular matrix of various tissues (34, 35). The mechanism of their release is not yet completely understood, although FGF-1 is known to be released in response to heat shock (36), and FGF-2 may be released through exocytosis (37), as evidenced by the fact that depleting tissues (34, 35). The mechanism of their release is not yet well understood, although FGF-1 is known to be released in response to heat shock (36), and FGF-2 may be released through exocytosis (37), as evidenced by the fact that depleting tissues (34, 35).

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