Fibroblast growth factor (FGF)-9 is a glycosylated neurotrophic polypeptide highly expressed in brain. The mechanism for its secretion from expressing cells is unclear, because its primary structure lacks a cleavable signal sequence. We, therefore, investigated the mechanism and structural requirements for secretion of FGF-9. As with other secreted proteins, in vitro translation of FGF-9 was inhibited by signal recognition particle, which binds to the signal sequence. When translated in vitro, full-length FGF-9 was translocated into microsomes, glycosylated, and protected from trypsin digestion. By using various FGF-9 deletion mutants, we found that two hydrophobic domains, located at the N terminus and at the center of the FGF-9 primary structure, were crucial for translocation. Examination of various point mutants revealed that local hydrophobicity of the central hydrophobic domain, but not the N terminus, was crucial for translocation. Analogous results were obtained with respect to FGF-9 secretion from transfected cells. Upon deletion of the complete sequence preceding it, the previously uncleavable hydrophobic domain appeared to serve as a cleavable signal sequence. Our results suggest that nascent FGF-9 polypeptides translocate into endoplasmic reticulum without peptide cleavage via a co-translational pathway in which both the N terminus and the central hydrophobic domain are important; thereafter, FGF-9 is glycosylated and secreted.

A Hydrophobic Region Locating at the Center of Fibroblast Growth Factor-9 Is Crucial for Its Secretion*

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1 The abbreviations used are: FGF, fibroblast growth factor; SRP, signal recognition particle; PCR, polymerase chain reaction; ER, endoplasmic reticulum; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; PPL, preprolactin; PBS, phosphate-buffered saline.

The presence of heparin/heparan sulfate proteoglycans, FGFs bind with varying affinities to transmembrane tyrosine kinase receptors encoded by four distinct genes (10). The binding induces receptor dimerization, activation of the intracellular kinase domain, and downstream intracellular signaling (11). In this context, we sought to understand better the mechanism by which FGFs are secreted from expressing cells.

FGF-9 was originally purified as a glial cell-activating factor from culture medium conditioned with the human NMCG-1 glioma cell line. Indeed, FGF-9 is a potent mitogen for glia, rat primary cortical astrocytes, BALB/c3T3 fibroblasts, and oligodendrocyte type 2 astrocyte progenitor cells (12). Despite the absence of a typical N-terminal signal sequence, FGF-9 is efficiently secreted following transfection into COS cells (13). Peptide microsequencing revealed that secreted FGF-9 has an alanine as the second amino acid residue at its N terminus (14); it is N-glycosylated following secretion or in vitro translation in the presence of microsomes, which is in good agreement with the presence of an N-glycosylation consensus sequence (13, 15); and deletion of the N-terminal 33 amino acids of FGF-9 abolished the secretion. These characteristics distinguish FGF-9 from FGF-1 and FGF-2, both of which lack a secretion signal and are only inefficiently exported from cells in non-glycosylated forms. In the present study, we investigated the mechanism and structural requirements for the secretion of FGF-9.

EXPERIMENTAL PROCEDURES

Reagents and Cell Culture—Wheat germ extract and rabbit reticulocyte lysate were purchased from Promega Corp. (Madison, WI). COS-1 cells were cultured with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. All restriction enzymes were purchased from Takara Syuzo (Kyoto, Japan).

Construction of Plasmids—Various deletion mutants of FGF-9 were generated by PCR with the primers listed in Table I using FGF-9 cDNA in the pUC118 vector (R-6) as a template. The mutants were cloned into a pBluescriptIIKS(+) expression vector (Stratagene La Jolla, CA) as described previously (16). Briefly, a representative PCR reaction mixture would consist of Vent DNA polymerase (New England Biolabs, Beverly, MA), 20 μM dNTPs, 1 μg of primer (Table I), 1 μg of template DNA (full-length FGF-9) and 1 unit of enzyme in a final volume of 50 μl. The amplification protocol consisted of 30 cycles at 94 °C for 1 min, 72 °C for 2 min, and 50 °C for 2 min (16). The amplified product of each construct was separated by agarose gel electrophoresis, and a fragment with the predicted size was recovered from the gel using GeneClean (BIO 101, Inc., Vista, CA). The fragment was then digested with EcoRI and BamHI and then subcloned into the EcoRI/BamHI site of the pBlueScriptIIKS(+) vector. Nucleotide sequences of all constructs were confirmed by DNA sequencing (Applied Biosystems model 373A).

The desired point mutants were constructed using the overlap extension method (17) with the primers listed in Table II; they include Leu(1)→Asn, Gly(1)→Tyr, Gly(2)→Tyr, Ile(9)→Asn, Ile(10)→Asn, and Ala(11)→Asn. Briefly, PCR was first carried out with primers I and II and then with primers III and IV. Primer I corresponds to the 5′-end of FGF-9; primers II and III contain the sense and antisense strands of the

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The cDNA cloned into the pGEM-T vector was digested with EcoRI and recovered and then subcloned into a pGEM-T vector (Promega Corp.). The PCR amplification protocol consisted of 30 cycles at 94 °C for 1 min, 72 °C for 2 min, and 50 °C for 2 min (16). The amplified product was separated by agarose gel electrophoresis and Geneclean (Bio 101). Both products were then mixed and subjected to PCR using primers I and IV. Each PCR reaction mixture consisted of AmpliTaq DNA polymerase (Perkin-Elmer Applied Biosystems, Forster City, CA), and the corresponding buffer was provided by the manufacturer (20 μM dNTPs, 1 μg of each primer (Table II), and 1 unit of enzyme) in a final volume of 50 μL. The amplification protocol consisted of 30 cycles at 94 °C for 1 min, 72 °C for 2 min, and 50 °C for 2 min (16). The amplified product was separated and recovered and then subcloned into a pGEM-T vector (Promega Corp.). The cDNA cloned into the pGEM-T vector was digested with EcoRI and BamHI and subcloned into the EcoRI/BamHI site of the pBluescriptIIKS(+) vector. All nucleotide sequences were confirmed by DNA sequencing (Applied Biosystems model 373A).

For expression of FGF-9 in COS-1 cells, the various FGF-9 cDNAs in pBluescriptIIKS(+) were digested with EcoRI and XbaI and subcloned between the EcoRI and XbaI sites of a pSVK3 vector (Amerham Pharmacia Biotech). The expression vector for FGF-9 N33(34–208) was previously constructed (14).

In vitro translation and microsomal translocation of FGF-9 using the reticulocyte lysate system—The plasmids constructed for the in vitro translation assay were linearized by digestion with BamHI, and each cDNA was transcribed using the mMESSAGE mMACHINE T3 in vitro transcription kit (Ambion Inc.). The transcript was then translated using nuclease-treated rabbit reticulocyte lysate (Promega Corp.) at final concentration of 33%, with or without canine pancreatic rough microsomes prepared as described previously (18). Translation was performed at 30 °C for 30 min, and [3H]leucine was included in the reaction mixture. In some cases, aliquots of the translation products were further treated with 0.25 mg/ml trypsin (Sigma) for 1 h, on ice, in the presence or absence of 0.6% Triton X-100 (Nacalai Tesque, Tokyo, Japan). The translation products of the deletion mutants were precipitated by adding (NH₄)₂SO₄ to 65% saturation, washed with 5% trichloroacetic acid, and dissolved in SDS-PAGE sample buffer by sonication. They were then resolved on SDS-polyacrylamide gels and fixed. The gels were soaked in ENLIGHTENING (NEN Life Science Products) for fluorography, dried, and the translation products were visualized by Biomax MS film (Kodak Co.).

Analysis of FGF-9 expressed by COS-1 transfectants—COS-1 cells were transfected using the DEAE-dextran method. Cells were plated on 10-cm dishes and cultured until 40% confluent. They were then washed with phosphate-buffered saline (PBS) and incubated in 3.5 ml of DNA mixture (20 μg of DNA in 0.4 mg DEAE-dextran/ml of Tris-supplemented, serum-free medium (Tris-SFM, 50 mM Tris-HCl, pH 7.5) in DMEM) for 4 h at 37 °C; after that the cells were washed again in PBS and placed in 7 ml of 100 μM chloroquine in Tris-SFM. After incubating for 4 h at 37 °C, the cells were once again washed in PBS and further cultured for 3 days in 4 ml of DMEM supplemented with 10% fetal bovine serum. The conditioned medium was collected, centrifuged (ENLIGHTENING, NEN Life Science Products), and exposed to Biomax MS film (Eastman Kodak Co.).

| Table I | PCR primers for full-length and deletion mutants of FGF-9 |
| Construction | Primer sequence (5’ → 3’) |
| FGF-9 | Sense \( \text{cggagttcaccatgctcccttaggtgaagttgggagc} \) |
| dFGF-9(1–104) | Antisense \( \text{cgccggatccaccatggctcccttaggtgaagttgggagc} \) |
| dFGF-9(105–208) | Sense \( \text{cggagttcaccatgctcccttaggtgaagttgggagc} \) |
| dFGF-9(93–156) | Antisense \( \text{cgccggatccaccatggctcccttaggtgaagttgggagc} \) |
| dFGF-9(1–156) | Sense \( \text{cggagttcaccatgctcccttaggtgaagttgggagc} \) |
| dFGF-9(209–91) | Antisense \( \text{cgccggatccaccatggctcccttaggtgaagttgggagc} \) |
| TABLE II | PCR primers for FGF-9 point mutants |
| Construction | Primer sequence (5’ → 3’) |
| FGF-9(I25-N) I | \( \text{cggagttcaccatgctcccttaggtgaagttgggagc} \) |
| II | \( \text{ctgctaccacggtacccgtcgc} \) |
| III | \( \text{ctgctacactgccctgtg} \) |
| IV | \( \text{cgtcgcgatctccatcttctg} \) |
| FGF-9(G71-Y) I | \( \text{cggagttcaccatgctcccttaggtgaagttgggagc} \) |
| II | \( \text{ctgctaccacggtacccgtcgc} \) |
| III | \( \text{ctgctacactgccctgtg} \) |
| IV | \( \text{cgtcgcgatctccatcttctg} \) |
| FGF-9(G80-Y) I | \( \text{cggagttcaccatgctcccttaggtgaagttgggagc} \) |
| II | \( \text{ctgctaccacggtacccgtcgc} \) |
| III | \( \text{ctgctacactgccctgtg} \) |
| IV | \( \text{cgtcgcgatctccatcttctg} \) |
| FGF-9(I100-N,A101-N) I | \( \text{cggagttcaccatgctcccttaggtgaagttgggagc} \) |
| II | \( \text{ctgctaccacggtacccgtcgc} \) |
| III | \( \text{ctgctacactgccctgtg} \) |
| IV | \( \text{cgtcgcgatctccatcttctg} \) |
RESULTS

FGF-9 Lacks a Predictable Signal Sequence but Is Secreted as an N-Glycosylated Form—We analyzed the primary structure of FGF-9 for the presence of a signal sequence using the latest prediction software available on the web server (SignalP (19)). Consistent with previous experimental results, we found that FGF-9 lacked a predicted signal sequence.

To confirm the secretion of FGF-9, COS cells were transfected with an expression vector encoding the full-length FGF-9 cDNA. Subsequently, cell lysate and conditioned medium were then analyzed for FGF-9 content (Fig. 1). The conditioned medium (lane 2) contained high levels of FGF-9, as compared with cell lysates (lane 1); secretion was apparently quite efficient. Two forms of FGF-9 with molecular masses of 30 and 27 kDa were present in the conditioned medium (lane 2, closed and open arrowheads, respectively). Levels of 30-kDa FGF-9 were diminished by treating the conditioned medium with N-glycanase (lane 3, open arrowhead), which suggests that the 30-kDa moiety is the N-glycosylated form of the 27-kDa FGF-9 simple polypeptide and is consistent with the presence of an N-glycosylation consensus sequence within its primary structure (Asn-Gly-Thr).

SRP Inhibits In Vitro Translation of FGF-9—The transport of proteins containing signal sequences across the endoplasmic reticulum (ER) membrane in mammalian cells generally occurs in a co-translational manner. The process begins in the cytosol with a targeting phase; the signal sequence of a nascent chain, it halts or slows elongation of the polypeptide until contact is made with a docking protein (SRP receptor) in the membrane (22, 23). Consequently, in the absence of membrane, SRP strongly suppresses translation of proteins with signal sequences. To examine the involvement of SRP in FGF-9 synthesis, we used a wheat germ, in vitro translation system in which neither SRP nor microsomes were present. As shown in Fig. 2, addition of SRP strongly inhibited translation of preprolactin (PPL), a well-characterized, secreted protein containing a signal sequence. In contrast, translation of ΔSP-PL, which lacks a signal sequence, was unaffected by SRP. Interestingly, SRP clearly inhibited translation of FGF-9 in a manner similar to PPL (Fig. 2). Thus, SRP appears to bind to a putative signal sequence in FGF-9.

Both the N Terminus and the Central Hydrophobic Region of the FGF-9 Primary Structure Are Indispensable for Translocation across Microsomal Membranes—To determine the sequence in FGF-9 responsible for its secretion, we conducted a microsomal translocation assay using a rabbit reticulocyte lysate, in vitro translation system containing endogenous SRP and other components. As shown in Fig. 3A, FGF-9 was translated as a 27-kDa polypeptide (open arrowhead), which is in good agreement with the calculated molecular mass of the protein (lanes 1 and 2). When microsomes were added to the translation reaction, an additional 30-kDa translation product resulting from N-glycan modification at the Asn-Gly-Thr sequence was observed (Fig. 3A, lanes 3 and 4, closed arrowhead, and Fig. 3G). With the exception of the 30-kDa product (lane 7), all of the translation products were digested upon addition of trypsin to the reaction mixture (lanes 5–8). Furthermore, pre-treatment with Triton X-100 prior to trypsinization resulted in complete degradation of the translate, including the 30-kDa product (lane 8). This confirmed that the 30-kDa protein in lane 7 was a glycosylated form of FGF-9 that was protected from trypsin digestion by translocation into the microsomes.

By using the same protocol with five deletion mutants, we then identified the structural requirements for FGF-9 translocation (Fig. 3G). The dFGF-9-1(1–104) mutant, which lacked the C-terminal half of the molecule, was translated as a 11-kDa polypeptide (Fig. 3B, lane 1, open arrowhead). This translation product was not protected from trypsinization (Fig. 3B, lane 7), indicating that it was not translocated into microsomes. This conclusion was confirmed by the absence of a glycosylated form of the polypeptide (Fig. 3B, lanes 3 and 4), even though the N-glycosylation sequence was present. Both dFGF-9-105(105–208) and dFGF-9-153(153–156) were also not translocated into microsomes (Fig. 3, C and D). On the other hand, dFGF-9-1(1–156) was both glycosylated (Fig. 3E, lanes 3, 4, and 7) and protected from trypsinization (lane 7) in a manner similar to full-length FGF-9. Thus, both the N terminus and the central hydrophobic...
region of the FGF-9 primary structure appear to be indispensable to translocation, whereas the C-terminal one-fourth of the full-length FGF-9 is apparently not required.

Interestingly, deletion of amino acid residues 1–90 preceding the central hydrophobic domain yielded a polypeptide that was not glycosylated yet was capable of being translocated (Fig. 3F). Although the dFGF-9-(91–208) translation product has a molecular mass of 13 kDa (lanes 1 and 2, open arrowhead), in the presence of microsomes, another molecular moiety of 11-kDa was produced (lanes 1, 3, 5, and 7) or presence of Triton X-100 (lanes 2, 4, 6, and 8). These results, along with the observation that FGF-9N33-(34–208) was neither secreted nor glycosylated (Fig. 5, lanes 11 and 14), are summarized in Fig. 3G.

Local Hydrophobicity of the Central Hydrophobic Domain, but Not of the N Terminus, Is Important for FGF-9 Translocation—A hydropathy plot of FGF-9 showed that two major hydrophobic regions are contained within its primary structure as follows: a weak hydrophobic domain at the N terminus and a strong hydrophobic domain at the center of the polypeptide (Fig. 4C). Because the aforementioned results suggested the involvement of SRP in the secretion of FGF-9 (Fig. 2), we constructed various FGF-9 point mutants in which local hydrophobicity was significantly altered by amino acid substitution (Fig. 4, A and C). Substituting Asn for Leu 25 makes the N terminus relatively less hydrophobic (Fig. 4C). Unexpectedly, FGF-9(L25N) was protected from trypsinization, indicating translocation of the mutant into microsomes (Fig. 4B); in contrast, FGF-9(G71Y) and FGF-9(G80Y) were not protected (Fig. 4B). Again, the protected bands were observed as the glycosylated forms of the molecules (lane 7 of wt FGF-9; FGF-9(L25N)), which means that the translated proteins were fully accessible to the glycosylation machinery. To ascertain whether a structural interaction between the N terminus and the central hydrophobic domain affected translocation, point mutations (FGF-9(G71Y) and FGF-9(G80Y)) were introduced that caused decreased flexibility of the polypeptide. Despite the reduced flexibility, these mutants were also protected from trypsinization (Fig. 4B).

**FGF-9 Mutants Capable of Microsomal Translocation Are Processed in and Secreted from COS Transfectants—** After con-

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**Fig. 3.** Translocation of FGF-9 and its deletion mutants into microsomes. A, translocation of full-length, wild-type FGF-9 into microsomes. FGF-9 was translated in vitro in the presence of [3H]leucine using reticulocyte lysate in the absence (lanes 1, 2, 5, and 6) or presence of microsomes (lanes 3, 4, 7, and 8) and in the absence (lanes 1, 3, 5 and 7) or presence of Triton X-100 (lanes 2, 4, 6, and 8). The translation products were then digested with trypsin (lanes 1–4) or not treated (lanes 5–8). The reaction products were resolved by SDS-PAGE, and fluorography was performed as described in the legend to Fig. 2. The glycosylated and non-glycosylated forms of FGF-9 are indicated by closed and open arrowheads, respectively. Positions of the molecular mass standards are indicated by bars on the left. B–F, microsomal translocation of various deletion mutants. B, dFGF-9-(1–104); C, dFGF-9-(105–208); D, dFGF-9-(53–156); E, dFGF-9-(1–104); and F, dFGF-9-(91–208). Experiments were performed as in A, except that the dFGF-9-(1–104), dFGF-9-(105–208), and dFGF-9-(53–156) were precipitated by trichloroacetic acid prior to SDS-PAGE. The glycosylated and non-glycosylated forms of the mutants are indicated as closed and open arrowheads, respectively. The arrow in F indicates a form of dFGF-9-(91–208) after signal cleavage. G, diagram showing the various deletion mutants aligned with the FGF-9 hydrophobic plot, as well as the summarized results of their glycosylation and translocation. Hydrophathy plots were generated using the Kyte-Doolittle hydrophobic scale and an interval of 10 amino acids. NGT indicates the position of the N-glycosylation consensus sequence. The asterisk denotes incapability of secretion. wt, wild type.
firming that FGF-9 and FGF-9(L25N) were translocated into microsomes and that FGF-9(I100N, A101N) was not, secretion of these mutants from COS transfectants was examined. We found that secretion from COS cells corresponded with in vitro microsomal translocation. When culture supernatants of the COS transfectants were adsorbed onto heparin-Sepharose beads and resolved by SDS-PAGE, glycosylated forms of FGF-9 and FGF-9(L25N) were clearly detected (Fig. 5, lanes 5 and 6, closed arrowhead). FGF-9(I100N, A101N), by contrast, was not detected in the supernatant (lane 7), although its simple protein was translated in the cells (lane 3, open arrowhead). We also confirmed the previous observation that FGF-9N33-(34–208) (Fig. 5, lane 11, open arrowhead) was neither secreted nor glycosylated (Fig. 5, lanes 11 and 14). In addition, the dFGF-9-(91–208) expressed in COS transfectants was indeed detected at 13 and 11 kDa (Fig. 5, lane 15, arrowhead and arrow, respectively), the same sizes as those observed in in vitro translation/translocation experiment (Fig. 3F, lane 3). By in vitro translation without inclusion of microsomes, only the unprocessed 13-kDa form was detected (Fig. 5, lane 16, arrowhead).

DISCUSSION

In the present study we showed that 1) SRP arrests translation of FGF-9; 2) FGF-9 is translocated into microsomes where it is glycosylated; 3) both the N terminus and central hydrophobic regions are important for translocation; 4) the hydrophobicity of the central hydrophobic region, but not that of the N terminus, is crucial for translocation; 5) the central hydrophobic region appears to serve as a cleavable, N-terminal signal sequence in an artificial construct; and 6) point mutations that abolished microsomal translocation of FGF-9 also abolished its secretion from cells. These results indicate that despite the absence of a predictable, cleavable signal sequence, secretion of FGF-9 from expressing cells is mediated via co-translational translocation into ER in a manner very similar to that of other secreted proteins.

Proteins secreted by co-translational transport generally contain N-terminal signal sequences. When translated on rough ER, nascent proteins to be secreted are targeted to translocation channels located in the ER membrane, and the signal sequence is cleaved by a signal peptidase (24). The specific...
structures of signal sequences are diverse; nonetheless, they have certain features in common as follows: 1) a net positive charge at the N terminus, 2) a hydrophobic region, and 3) a C-terminal region with small, nonpolar amino acids at positions –1 and –3 (signal peptides are numbered negatively from the cleavage site toward the N terminus of the precursor; Ref. 25). The weak hydrophobic region at its N terminus notwithstanding, FGF-9 lacks these characteristics, as confirmed by the signal sequence prediction software. Indeed, the N terminus was intact in the secreted form of FGF-9. Instead, based on SignalP analysis, the central hydrophobic domain was predicted to serve as a cleavable signal sequence, a prediction that was confirmed by our experimental results.

Our findings that the C-terminal hydrophilic region (i.e. amino acids 157–209) was not required for FGF-9 translocation (Fig. 3E) and that translation of FGF-9 was inhibited by SRP, which generally binds hydrophobic motifs in signal sequences, prompted us to characterize the contribution made by the FGF-9 hydrophobic regions to its translocation into microsomes. FGF-9 deletion mutants that lacked any part of the major hydrophobic regions were not translocated (Fig. 3G). Moreover, as substituting Asn for Ile\textsuperscript{36} or Asn-Asn for Ile\textsuperscript{100}, Ala\textsuperscript{101} blocked translocation, local hydropathy of the central side the SERPIN family, of a secreted protein that translocates is important for the translocation of the protein into microsomes. FGF-9 deletion mutants that lacked any part of the major hydrophobic regions were not translocated (Fig. 3G). Moreover, as substituting Asn for Ile\textsuperscript{36} or Asn-Asn for Ile\textsuperscript{100}, Ala\textsuperscript{101} blocked translocation, local hydropathy of the central hydrophobic domain is apparently crucial for translocation. Analogous observations were made with plasminogen activator inhibitor-2 and ovalbumin, SERPIN family proteins which are the only other known examples of secreted proteins lacking cleavable signal sequences (26–29). With respect to plasmino- gen activator inhibitor-2, it was shown that SRP recognizes the second hydrophobic region in its primary structure and that this region was important for translocation into the ER (30). Similarly, the second hydrophobic region was also necessary for efficient secretion of ovalbumin (28). In contrast, despite the finding of a previous study (14) that the N-terminal 33 amino acids were indispensable for FGF-9 secretion, making this region less hydrophobic by substituting Asn for Leu\textsuperscript{36}, did not affect translocation. Thus, whereas both the N terminus and central hydrophobic region (amino acids 95–120) were required for translocation, the specific requirement for hydrophobicity was only demonstrated for the central region.

Point mutations (G71Y and G80Y) that should have restricted local flexibility of the polypeptide did not affect translocation. It is unlikely, therefore, that an interaction between the N terminus and the central hydrophobic domain is important for translocation. Consequently, it is still unclear how the N terminus of FGF-9 is involved in the secretion mechanism. Our findings that the central hydrophobic domain is able to function as a signal sequence, but that the presence of excess amino acids preceding this region (amino acids 34–90) abolished the secretion, suggest that the N terminus is involved in the effective presentation of the central hydrophobic region to the translocation machinery. Similar observations have been made with plasminogen activator inhibitor-2, where a weakly hydrophobic region preceding a more strongly hydrophobic one is important for the translocation of the protein into microsomes (31).

Finally, we have identified FGF-9 as the first example, outside the SERPIN family, of a secreted protein that translocates into microsomes with a clearly uncleavable signal sequence.