Fibroblast growth factors (FGFs) are secreted regulatory proteins involved in various developmental processes. In vertebrates, the FGF superfamily comprises 22 members. In non-vertebrates, six FGF genes have been identified in Ciona intestinalis, three in Drosophila melanogaster, and two (let-756 and egl-17) in Caenorhabditis elegans. The core of LET-756 shares a 30–50% sequence identity with the various members of the superfamily. The relationships between vertebrate and non-vertebrate FGFs are not clear. We made chimeric FGFs by replacing the core region of LET-756 by the cores of various mammalian, fly, and worm FGFs. LET-756 deleted in its core region was no longer able to rescue the lethal phenotype of a let-756 null mutant, and only chimeras containing the cores of FGFs 9, 16, and 20 showed rescue capacity. This core contains an internal motif of six amino acid residues (EFISIA) whose deletion or mutation abolished both the rescue activity and FGF secretion in the supernatant of transfected COS-1 cells. Chimeras containing the core of C. intestinalis FGF9/16/20, a potential ortholog of FGF9 lacking the complete EFISIA motif, was not able to rescue the lethal phenotype or be secreted. However, the introduction of the EFISIA motif restored both activities. The data show that the EFISIA motif in the core of LET-756 is essential for its biological activity and that FGFs 9, 16, and 20, which contain that motif, are functionally close to LET-756 and may be evolutionary related. This non-classical mode of secretion using an internal motif is conserved throughout evolution.

**EXPERIMENTAL PROCEDURES**

Bioinformatic Methods—All of the FGF sequences used for this study were from GenBank™ with the exception of the D. melanogaster thibe (TH1) and pyramus (PYR) sequences, which have been reported recently (5): human FGF1 (NP_000791); FGF2 (NP_001997); FGF3 (NP_005238); FGF4 (NP_001998); FGF5 (NP_004455); FGF6 (NP_066276); FGF7 (NP_002000); FGF8 (NP_149355); FGF9 (NP_002001); FGF10 (NP_004456); FGF11 (NP_004103); FGF12 (NP_066360); FGF13 (NP_004105); FGF14 (NP_004106); FGF16 (NP_003859); FGF17 (NP_003858); FGF18 (NP_003853); FGF19 (NP_005108); FGF20 (NP_066285); FGF21 (NP_061986); FGF22 (NP_065688); FGF23 (NP_065689); mouse FGF15 (NP_032029); C. intestinalis FGF3/7/10/22 (BAC22066); FGF4/5/6 (BAC22067); FGF8/17/18 (BAC22068); FGF9/16/20 (BAC22069); FGF11/12/13/14 (BAC22070); FGF with large molecular mass (BAC22071); the fruitfly D. melanogaster branchless (BNL) (NP_732453); the nematode C. elegans EGL-17 (NP_508107); and LET-756 (NP_498403).

The boundaries of core regions were determined by SMART (16) (smart.embl-heidelberg.de) and then aligned using ClustalX (17) and by the human eye (see supplementary data for the alignments of the various FGFs). Phylogenetic trees were constructed using both maximal parsimony and bootstrapped neighbor-joining techniques. Branch support for the best-fitting tree was assessed using 1000 bootstrap replicates. Alignments, bootstrap analysis, and neighborhood-joining trees were carried out at default parameters.

Eukaryotic Cells and C. elegans Cells Expression Vectors—The strategy for cloning eukaryotic and C. elegans expression vectors is depicted

The abbreviations used are: FGF, fibroblast growth factor; GFP, green fluorescent protein; HRP, horseradish peroxidase; NLS, nuclear localization signals.

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are designated by using the PvuI/SacI restriction sites. The cDNA fragments containing the N-terminal core vector) and introduced in the cloning region, core and N- and C-terminal regions, and EFISIA/EFVSVA motif.

The chimeric constructs allowing the expression in mammalian cells are referred to as 

In Fig. 1A. Additional details are given as supplementary material, and the sequences of the primers used to amplify the various FGF fragments are available upon request.

The coding region of let-756, FGF-E, and FGF9 was amplified with F1-R1 primers (containing BamHI and BglII restriction sites) and inserted in the BglII/BamHI sites of pEGFP-N2-eukaryotic cell expression vector (underlined restriction sites shown in Fig. 1 are introduced by mutagenesis). The cores of various FGFs are amplified with P2-R2 primers (using as template the FGF coding region inserted in a cloning vector) and introduced in the plet containing the BssHII and SpeI restriction sites. The cDNA fragments containing the N-terminal core or core-C-terminal coding regions are amplified with F3-R2 and F2-R3 primers and inserted by using NheI/SpeI and BssHII/XmaI restriction sites. The core and N- and C-terminal regions are made in the eukaryotic cell expression vector transferred to the C. elegans expression vector by using the PvuII/SacI restriction sites.

The chimeric constructs allowing the expression in mammalian cells are designated PCMV::let-756/FGF9/let-756: GFP in which in the bracketed region is the core-coding region and n stands for different FGFs as follows: mammalian FGF23/5/9/12/16/20/26, and Fgfl/11/12/13/14 (referred to as FGF-D, FGF-E, and FGF-F, respectively); C. elegans let-756 and egl-17, and D. melanogaster bal. The C. elegans expression vectors are designated accordingly with the exception that Pbal::let-756 for the let-756 promoter replaces Pcmv::let-756. Other designations as follows. The constructs containing the N-terminal and core-coding regions of FGF9 fused to the C-terminal coding region of let-756 is PCMV::FGF9/let-756: GFP, and the constructs containing the N-terminal coding region of let-756 fused to the core and C-terminal coding regions of FGF9 is PCMVC::FGF9/let-756: GFP. Mutations or deletions in the FGF molecule are indicated as exons. As an example, the constructs deleted for the EFISIA-coding region are referred as PCMVC::let-755EFVSVA::gfp or PCMVC::let-756/FGF9/EFVSVA let-756: GFP.

Nematode Culture and Transformation Rescue Experiments—C. elegans Bristol (N2) strain nematodes were cultured using standard techniques (18). let-756-rescuing activity was assayed by injecting tester DNA at 50 ng/µl into gravid worms (FGF1/2); B (FGF4/6); C (FGF7/10/22); D (FGF8/17/18); E (FGF11/12/13/14) as well as the strategy for the cloning of the various constructs (A) as well as C. elegans LET-756 and human FGF9 proteins (B) including NLS, R318 mutation, core and N- and C-terminal regions, and EFISIA/EFVSVA motif.

Microscopy—F1 GFP-expressing nematodes were selected under a stereomicroscope (Leica MZ6) equipped with a GFP fluorescence module. When necessary, individual nematodes were picked from plates onto 2% agar pads containing 10 µl of 1x levanasome as an anesthetic and observed with a Leica TCS NT confocal microscope. Transfected COS-1 were grown on coverslips, fixed with 3.7% paraformaldehyde, and mounted in Dako. In some experiments, fixed cells were incubated with anti-giantin antibodies (a gift of H. F. Hauri, Biozentrum) followed by Texas Red-coupled secondary antibodies.

Western Blot—To assay secreted proteins, a 48-h conditioned medium from COS-1-transfected cells were collected, cleared by centrifugation, and immunoprecipitated with polyclonal rabbit anti-GFP antibodies (Abcam, Cambridge, United Kingdom) and protein A-Sepharose beads (Amersham Biosciences). To study cell-associated proteins, lysates from transfected cells were, depending on the experiment, either immunoprecipitated or boiled for 5 min in 2x SDS/PAGE and, after blotting, revealed by anti-mouse anti-GFP antibody (Roche Applied Science). The signal was detected using HRP-conjugated anti-mouse IgG and ECL kit (Pierce, Rockford, IL). Brefeldin A was used at a 2.5 µg/ml concentration, and tunicamycin was used at 5 µg/ml.

RESULTS

Identification of FGF Gene Families—FGF sequences share identity and similarity in a central core region of the molecule (10). We constructed a phylogenetic tree of the FGF core sequences (Fig. 2). The tree included core sequences from human, C. intestinalis, D. melanogaster, and C. elegans FGFs. We added the FGF15 mouse sequence, because it is not present in the human genome, whereas reciprocally, FGF19 is not found in the mouse. The phylogenetic tree was constructed using the distance matrix (Blossum 30 matrix) and neighbor-joining algorithms implemented in ClustalW. A total of 1000 bootstrapped replicates were run. The tree showed that the FGF superfamily could be divided into seven evolutionary divergent families: A (FGF1/2); B (FGF4/6); C (FGF7/10/22); D (FGF8/17/18); E
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(FGF9/16/20); F (FGF11–14); and G (FGF15/19/21/23). Based only on high bootstrap values (above 700), it was not possible to firmly include in any of these families the following: human FGF3 and FGF5; C. intestinalis FGF3/7/10/22, FGF4/5/6, FGF8/17/18, FGF9/16/20, and FGF with a large molecular mass; D. melanogaster FGFs; and C. elegans LET-756 and EGL-17. The grouping of the non-vertebrate sequences remained non-conclusive, and we could not establish relationships of direct orthology between human, fly, and worm FGFs.

During evolution, the formation of the FGF families may have occurred before and/or after the separation of protostomians from deuterostomians. If they formed before this separation, a non-vertebrate FGF should belong to a given family and be the respective ortholog of its mammalian members. If they formed after this separation, non-vertebrate FGFs should be orthologous to all of the deuterostomian FGFs. Documenting this relationship may help to understand the FGF features and functions and the reason for FGF gene expansion.

To address this question, we studied the LET-756 molecule. We previously characterized the let-756 locus (7). In a severely affected strain (s2887), a loss-of-function let-756 allele causes developmental arrest early in the larval stages. In a partial loss-of-function allele (s2613), a mutation introduces a stop codon at the Arg-318 position leading to a truncated protein for Rescuing Activity—The let-756 gene has rescuing activity when expressed under the control of its own promoter in the strain carrying the s2887 null allele (7).

This activity is conserved when LET-756 is tagged with GFP in the C-terminal region. In contrast, a construct deleted of the core region of LET-756 could not rescue the let-756(s2887) mutants (Table I). To determine whether LET-756 is functionally closer to any of the FGF families, we replaced the LET-756 core sequence with that of other FGFs including one from each human family (FGF2, FGF3, FGF6, FGF7, FGF8, FGF9, FGF12, and FGF21) and with that of Drosophila BNL and C. elegans EGL-17. The sequence alignments are shown in Fig. 4A. Only the core of FGF9 replaced efficiently the LET-756 core for rescuing ability (Table I).

Family E Groups FGF9, FGF16, and FGF20—Similar to the FGF9 core, the cores of FGF16 and FGF20, introduced in the LET-756 background, rescued the let-756 phenotype (s2887). Because FGF-E from C. intestinalis is supposed to be orthologous to FGF9 (8), we also tested the Plet-756::let-756 [Fgf-E]::let-756 construct. This chimera was unable to rescue the lethal let-756 phenotype as did two other chimeras containing cores of Ciona FGF-D and FGF-F. In control experiments, wild-type worms were transformed with FGF9, FGF12, and FGF16 chimeras (Fig. 3, b–d). GFP expression was detected in neurons and muscle cells, indicating that the lack of rescuing activity was not linked to a defect in protein expression.

Residues in the Core Region Are Necessary but Not Sufficient for Rescuing Activity—These results suggest that LET-756 could resemble FGF family E members FGF9, FGF16, and FGF20. We looked for what LET-756 and this family have in common and do not share with the other families. An examination of the alignment of the various FGFs pointed to the presence of a highly conserved motif (shaded in Fig. 4A) located in the core region. It was only present in LET-756 (EFVSVA: amino acids 116–120), FGF9, (EFISIA), FGF16 (EFISLA), and FGF20 (EFISVA). This sequence is slightly different in Ciona FGF-E, because only four of six conserved amino acids are present (EFISTG).

The EFISIA/EFVSVA sequence is a hydrophobic stretch within the LET-756 and FGF9 molecules (Fig. 4B). To test for the importance of this motif on the function of LET-756, we made a construct where let-756 under the control of its own promoter was deleted in the sequence encoding these six residues (Plet-756::let-756 [EFVSVA]::gfp construct) and tested the rescuing activity in let-756(s2887) animals. The construct deleted...
of the sequence was devoid of rescuing activity (Table I). No other mutation in the core impaired the rescue of the lethal phenotype. As an example, LET-756 deleted from the NLS4–6 region was still able to rescue. When the region coding for the EFISIA motif was deleted from the core region of the LET-756/FGF9 chimera (P\textsubscript{let-756}::let-756[FGF9\textsubscript{LEFISIA}]/let-756:gfpm::construct), the rescue was abolished.

To confirm the importance of this region in the function of LET-756, we exchanged the corresponding regions in LET-756 and Ciona FGF-E. When the EFISIA stretch replaced EFISTG in the FGF-E chimera (P\textsubscript{let-756}::let-756[FGF-E\textsubscript{EFISIA}]/let-756:gfpm::construct), the rescuing activity was restored. Conversely, the replacement of EFVSVA by EFISTG in LET-756 (P\textsubscript{let-756}::let-756[FGF-E\textsubscript{EFISIA}]/let-756:gfpm::construct) abolished the rescuing activity. However, the role of the EFISIA motif worked only in the context of the FGF-E family. Appending an EFISIA motif to the LET-756/FGF2 chimera (P\textsubscript{let-756}::let-756[FGF2\textsubscript{EFISIA}]/let-756:gfpm::construct) did not confer rescuing activity (Table I). Thus, an EFISIA motif was necessary for the FGFs of the E family but was not sufficient for the other FGFs.

The EFISIA Motif Is Essential for Secretion—To understand how this motif influences the rescue activity, we tested its role in secretion. COS-1 cells were transfected with constructs including or not including the EFISIA motif. The P\textsubscript{cam}::let-756:gfpm::construct expressed GFP in the nucleus as speckles. A faint staining of the Golgi apparatus was also observed (Fig. 5a), which colocalized with the Golgi marker giantin (Fig. 5d). The fusion protein corresponding to the partial loss-of-function allele (R318Stop) was found in the nucleoli as well as in the Golgi (Fig. 5, c and f). The protein deleted of the EFISIA motif exhibited the same speckle staining as the wild-type molecule but no staining of the Golgi apparatus (Fig. 5, b and e). GFP9 was mostly found in the Golgi apparatus (Fig. 5g), in agreement with previous findings (22). By contrast, the various mammalian FGF chimeras, including the FGF9 chimera, were localized at speckles in the nucleus (Fig. 5, h, k, and l). Replacing the C-terminal region of LET-756 by that of FGF9 abolished this localization (Fig. 5i), whereas replacing the N-terminal region of LET-756 by that of FGF9 was without effect on the chimera localization (Fig. 5j), pointing out the crucial role of the C-terminal region of LET-756 for nuclear sublocalization.

Immunoblots were done with supernatants and cell lysates from transfected cells. LET-756 was efficiently secreted (Fig. 6a). This secretion was dependent on the Golgi-associated system, because it was inhibited by the addition of brefeldin A (Fig. 6a).


tables

| Rescue ability and secretion of LET-756 mutants and chimeras |
|-----------------|-----------------|-----------------|
| LET-756          | +               | +               |
| LET-756\textsuperscript{core} | -               | -               |
| LET-756\textsubscript{EFISIA} | -               | -               |
| LET-756\textsubscript{EFIST} | -               | -               |
| LET-756\textsubscript{AAAARRRKK} | -               | -               |
| LET-756\textsubscript{EFISIA} | +/−             | +/−             |
| LET-756\textsubscript{FGF9}\textsubscript{16/20}/LET-756 | +               | +               |
| LET-756\textsubscript{FGF9}\textsubscript{LEFISIA}/LET-756 | NT              | -               |
| LET-756\textsubscript{FGF9} | +               | +               |
| FGF9[FGF9]/LET-756 | +               | +               |
| LET-756\textsubscript{FGF3}\textsubscript{6–8}/LET-756 | −               | −               |
| LET-756\textsubscript{FGF-E\textsubscript{EFISIA}}/LET-756 | +               | +               |
| LET-756\textsubscript{FGF2}/LET-756 | −               | −               |
| LET-756\textsubscript{FGF2\textsubscript{LEFISIA}}/LET-756 | −               | −               |
| FGF9 | −               | −               |

Discussion

From a functional point of view, it is hard to draw parallels among FGF activities in different species. For example, LET-756 shares nuclear localization with FGF1, FGF2, FGF3, and FGFs (for review see Ref. 24) and muscle expression with FGF5, FGF6, and FGF9 (24–26). From an evolutionary point of view, two striking features are associated with the FGF superfamily. First, there has been an important expansion of the number of FGF genes in vertebrates. Second, there is no clear orthology relationship between mammalian and non-vertebrate FGFs. This expansion could be explained by the series of duplications that have accompanied vertebrate evolution, but the absence of direct orthology makes difficult to reconstitute the different steps. This prompted us to study the interchangeability of FGFs from various species with LET-756 as a "functional phylogenetic" approach. We have shown that LET-756 is related to FGF family E. This has two broad implications: 1) biology and 2) evolution.
Among the chimeric constructs tested, only those containing the core of FGF9, FGF16, or FGF20, which belong to the same FGF family (FGF-E family), were capable of a functional substitution. Most FGFs, such as FGF3, FGF6, FGF8, FGF10, FGF17, FGF18, and FGF22, contain a signal sequence for cell export. However, FGF3 is not secreted and is retained in the Golgi complex. FGF1 and FGF2, which do not contain such sequence, are released from cells by a mechanism independent of the ER/Golgi secretory pathway. FGF9, FGF16, and FGF20 do not have a classical signal sequence. They are efficiently secreted in an ER/Golgi-dependent pathway because of the presence of a high hydrophobic N-terminal region and a six amino acid-long region located within the core. We have shown here that LET-756, which like FGF9, FGF16, and FGF20 does not have a typical N-terminal signal sequence, is efficiently secreted by a Golgi-associated mechanism dependent of the ER/Golgi secretory pathway. FGF9, FGF16, and FGF20 do not have a classical signal sequence. They are efficiently secreted in an ER/Golgi-dependent pathway because of the presence of a high hydrophobic N-terminal region and a six amino acid-long region located within the core. We have shown here that LET-756, which like FGF9, FGF16, and FGF20 does not have a typical N-terminal signal sequence, is efficiently secreted by a Golgi-associated mechanism dependent of the ER/Golgi secretory pathway. FGF9, FGF16, and FGF20 do not have a classical signal sequence. They are efficiently secreted in an ER/Golgi-dependent pathway because of the presence of a high hydrophobic N-terminal region and a six amino acid-long region located within the core. We have shown here that LET-756, which like FGF9, FGF16, and FGF20 does not have a typical N-terminal signal sequence, is efficiently secreted by a Golgi-associated mechanism dependent of the ER/Golgi secretory pathway.
and EFISIA motif (b and munostaining (c). GFP is found in the Golgi apparatus of wild-type (a red families is not sufficient to confer rescuing ability. ever, inserting this motif in the cores of FGFs from other core sequences from members of the FGF-E family that have fent on the presence of the same six core residues as for the FGF-E family that are also essential for rescue activity. Only core sequences from members of the FGF-E family that have this sequence are able to rescue the worm null mutant. However, inserting this motif in the cores of FGFs from other families is not sufficient to confer rescuing ability. C. intestrinalis FGF9/16/20 (designated FGF-E in this paper) is supposed to be orthologous to mammalian FGF9, FGF16, and FGF20 (8). It contains an incomplete EFISIA motif that does not allow secretion and rescue in the LET-756 background. Replacement of EFISTG by EFISIA is sufficient to confer both rescuing activity and secretion to the protein. Here we have shown that the atypical mechanism of secretion of LET-756 and FGF-E members that utilizes that internal hydrophobic motif is conserved in different species. Such atypical signal peptides have been found in some other proteins in vertebrates but remain rare (for review see Refs. 22–24). It is the first time that such a mechanism is demonstrated in the nematode. In present-day Ciona FGF-E EFISTG, it is not efficient for export, which uses a classical peptide signal. For FGF9, in addition to the EFISIA motif, the N-terminal portion of the molecule is also important for secretion, whereas it is not the case for LET-756. It is possible that the routing of the proteins associated with this mode of export is different from those using a classical peptide sequence. In this case, interacting proteins could be specific of this pathway but they remain unidentified. Work is in progress to determine whether secretion of LET-756 and subsequent interaction with its membrane FGF receptor, EGL-15 5B (28), is the only activating process or whether the nuclear pool of LET-756 exerts any other biological activity.

There may have been a limited conservation of function within the FGF superfamily during evolution. The three mammalian FGFs of the E family and Ciona FGF-E share a common function in the nervous system (9, 27, 29, 30). FGF9 directs function in the nervous system (9, 27, 29, 30). FGF9 directs

![Panel A](image1.png) ![Panel B](image2.png) ![Panel C](image3.png) ![Panel D](image4.png)

**Fig. 5.** Expression of various deleted LET-756 GFP-tagged proteins and chimera in COS-1 cells. The central hydrophobic region of LET-756 contributes to the subcellular distribution of LET-756 protein in COS-1 transfectants. a–f, GFP expression (green). d–f, giantin immunostaining (red). GFP is found in the Golgi apparatus of wild-type (a and d) and B318Stop (c and f) but not if the protein is deleted in the EFISIA motif (b and e). As opposed to LET-756 (a), FGF9 (g) is found exclusively associated to the Golgi apparatus. Replacing the core of LET-756 by the core of any FGF (h, k, and l) restitutes a wild-type phenotype to the chimera. The chimera containing the C-terminal region of FGF9 is uniformly expressed in the nucleus (i), whereas the chimera containing the N-terminal region of FGF9 is expressed as speckles (j).

**Fig. 6.** Expression and secretion of the various mutants and chimeras. Equal aliquots of cell lysates and anti-GFP immunoprecipitated conditioned medium from COS-1 transfectants expressing the various GFP-tagged proteins were resolved by SDS-PAGE, and the blot was revealed with anti-GFP antibodies. Panel A, immunoblot analysis of the wild-type LET-756 protein recovered from supernatant (S) and cell extracts (C) of untreated (0) or treated cells for 24 h with tunicamycin (T) or brefeldin A (B). Panel B, expression in supernatant and cell extracts of LET-756 WT (1), LET-756 EFISIA (2), or LET-756 R318 (3). Panel C, effect of the EFISIA motif on secretion: LET-756 WT (lane 1); LET-756 EFISIA (lane 2); LET-756 R318 (lane 3); LET-756 deleted in the NLS4–6 region (LET-756 ΔNLS4–6) (lane 4); LET-756 swapped with the FIST sequence of FGF-E (LET-756 ΔFIST) (lane 5); LET-756 AAA mutation in the EFISIA motif (LET-756 AAA) (lane 6); FGF9 core chimera (LET-756[FGF9]LET-756) (lane 7); FGF9 core chimera deleted for EFISIA (LET-756[FGF9][EFISIA]LET-756) (lane 8); FGF-E core chimera (LET-756[FGF-E]LET-756) (lane 9); FGF-E core chimera swapped with the EFISIA motif of LET-756 (LET-756[FGF-E][EFISIA][LET-756]) (lane 10); FGF2 core chimera (LET-756[FGF2]LET-756) (lane 11); and FGF2 core chimera swapped with the EFISIA motif of LET-756 (LET-756[FGF2][EFISIA][LET-756]) (lane 12). D, in the FGF9 chimera, the sole core of FGF9 is necessary for secretion: LET-756 WT (lane 1); LET-756[FGF9]LET-756 (lane 2); chimera containing the N-terminal region of LET-756 and core + C-terminal region of FGF9 (LET-756[FGF9]LET-756) (lane 3); chimera containing the N-terminal region + core of FGF9 and C-terminal region of LET-756 (FGF9 [LET-756]) (lane 4); the full FGF9 protein (lane 5).
ancestry of each of the two *C. elegans* FGF with a given family would signify that a first expansion of the FGF genes has taken place early before the protostomian/deuterostomian split, but gene losses have been important in the protostomian branch, and that only two FGF families, E and D, are represented in the present-day nematode. However, LET-756, in addition to a

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