cDNA Cloning of a Novel, Developmentally Regulated Immediate Early Gene Activated by Fibroblast Growth Factor and Encoding a Nuclear Protein*

(Received for publication, June 5, 1997, and in revised form, July 18, 1997)

Gary D. Paterno, Yu Li‡, H. Artee Luchman, Paula J. Ryan, and Laura L. Gillespie§

From the Terry Fox Cancer Research Laboratories, Division of Basic Medical Sciences, Faculty of Medicine, Memorial University of Newfoundland, St. John’s, Newfoundland A1B 3V6, Canada

We have utilized the polymerase chain reaction (PCR)-based differential display methodology (Liang, P., and Pardee, A. B. (1992) Science 257, 967–969) to identify a novel transcript whose expression levels increased in Xenopus embryo explants during mesoderm induction by fibroblast growth factor. The PCR product was used to clone a 2.3-kilobase pair cDNA representing this transcript, which we have named er1 (early response 1). The er1 cDNA contained a single open reading frame predicted to encode a protein of 493 amino acid residues. A database homology search revealed that the predicted ER1 amino acid sequence contains three regions of similarity to the rat and human proteins encoded by the metastasis-associated gene, mta1, and two regions of similarity to the Caenorhabditis elegans sequence that is similar to mta1. The fibroblast growth factor-induced increase in er1 steady-state levels was not dependent on de novo protein synthesis, demonstrating that er1 is an immediate-early gene. Northern blot analysis revealed a single 2.8-kilobase pair mRNA that was observed predominantly during the initial cleavage and blastula stages of Xenopus development, with little or no detectable mRNA during subsequent development. Quantitative PCR analysis of early developmental stages showed that er1 peaked during late blastula.

Computer-assisted analysis of the predicted ER1 amino acid sequence revealed two putative nuclear localization signals, four highly acidic regions clustered at the N-terminus and a proline-rich region located near the C-terminus. Subcellular localization by immunocytochemistry revealed that the ER1 protein was targeted exclusively to the nucleus. Transactivation assays using various regions of ER1 fused to the DNA binding domain of GAL4 demonstrated that the N-terminal acidic region is a potent transactivator. These data suggest that ER1 may function as a transcription factor.

The family of fibroblast growth factors (FGFs)1 consists of nine members related by sequence and their ability to bind heparin (1). FGFs are involved in a number of cellular activities, including mitogenesis, cell differentiation, and angiogenesis (reviewed in Ref. 2). In addition, overexpression of FGF in various cell lines leads to phenotypic transformation (3–5). To define the mechanisms by which a ligand can have such pleiotropic effects, a better understanding of molecular aspects of the various cellular responses is required.

FGF can induce mesoderm differentiation in Xenopus embryonic tissue (6), and many of the initial events in the cellular response during induction are similar to those previously characterized for the FGF-mediated mitogenic response. During mesoderm induction, FGF binds to high affinity cell surface receptors (7), which in turn become phosphorylated on tyrosine (8). The phosphorylated FGF receptor forms a signaling complex by binding a number of intracellular substrates (9), which results in activation of several well characterized signaling pathways. For instance, protein kinase C becomes activated during FGF-induced mesoderm differentiation (8) as does mitogen-activated protein kinase (10).

The ultimate targets of these signal transduction pathways are the immediate-early genes. To date, very few FGF immediate-early genes have been identified (11, 12). Accordingly, we have utilized the differential display methodology (13) to isolate cDNAs representing such genes. In this paper, we describe the cloning and characterization of a cDNA representing a novel immediate-early gene, er1, whose steady-state levels increased in response to FGF. We show that the ER1 protein is targeted to the nucleus and that the N-terminal acidic region of ER1 can function as a transcriptional activator.

EXPERIMENTAL PROCEDURES

Embryos and Mesoderm Induction—Xenopus laevis were purchased from Nasco. Embryos were obtained and cultured as in Ref. 14. The recombinant Xenopus basic FGF used for induction was prepared as in Ref. 15. Animal pole explants (animal caps) were induced to form mesoderm as described (9), and animal caps were treated for 30 min prior to RNA extraction. For inhibition of protein synthesis during induction, animal caps were pretreated for 30 min with 5 μg/ml cycloheximide (Sigma), cultured with or without FGF for an additional 30 min, and then processed for PCR analysis as described below. Protein synthesis was measured in parallel samples by including 2 μCi/μl of [35S]methionine in the culture medium, and 35S incorporation into trichloroacetic acid precipitable material was determined according to Clemens (16).

Differential Display—RNA was extracted from induced or uninduced animal caps using the NaCl/EDTA/Tris/SDS protocol (17). Reverse transcription (RT) and polymerase chain reaction (PCR) were performed as in Ref. 13 with the following primers: 5′-TACGCT-3′ and 5′-CTGATCCATG-3′. PCR products were separated on a 6% polyacrylamide/6% urea gel; the gel was dried, and the products were visualized by autoradiography. The nt-PCR amplified a cDNA fragment of 2.8 kilobases that was not dependent on de novo protein synthesis, demonstrating that er1 is an immediate-early gene. Northern blot analysis revealed a single 2.8-kilobase pair mRNA that was observed predominantly during the initial cleavage and blastula stages of Xenopus development, with little or no detectable mRNA during subsequent development. Quantitative PCR analysis of early developmental stages showed that er1 peaked during late blastula.

Computer-assisted analysis of the predicted ER1 amino acid sequence revealed two putative nuclear localization signals, four highly acidic regions clustered at the N-terminus and a proline-rich region located near the C-terminus. Subcellular localization by immunocytochemistry revealed that the ER1 protein was targeted exclusively to the nucleus. Transactivation assays using various regions of ER1 fused to the DNA binding domain of GAL4 demonstrated that the N-terminal acidic region is a potent transactivator. These data suggest that ER1 may function as a transcription factor.

The family of fibroblast growth factors (FGFs) consists of nine members related by sequence and their ability to bind heparin. FGFs are involved in a number of cellular activities, including mitogenesis, cell differentiation, and angiogenesis (reviewed in Ref. 2). In addition, overexpression of FGF in various cell lines leads to phenotypic transformation (3–5). To define the mechanisms by which a ligand can have such pleiotropic effects, a better understanding of molecular aspects of the various cellular responses is required.

FGF can induce mesoderm differentiation in Xenopus embryonic tissue (6), and many of the initial events in the cellular response during induction are similar to those previously characterized for the FGF-mediated mitogenic response. During mesoderm induction, FGF binds to high affinity cell surface receptors (7), which in turn become phosphorylated on tyrosine (8). The phosphorylated FGF receptor forms a signaling complex by binding a number of intracellular substrates (9), which results in activation of several well characterized signaling pathways. For instance, protein kinase C becomes activated during FGF-induced mesoderm differentiation (8) as does mitogen-activated protein kinase (10).

The ultimate targets of these signal transduction pathways are the immediate-early genes. To date, very few FGF immediate-early genes have been identified (11, 12). Accordingly, we have utilized the differential display methodology (13) to isolate cDNAs representing such genes. In this paper, we describe the cloning and characterization of a cDNA representing a novel immediate-early gene, er1, whose steady-state levels increased in response to FGF. We show that the ER1 protein is targeted to the nucleus and that the N-terminal acidic region of ER1 can function as a transcriptional activator.

EXPERIMENTAL PROCEDURES

Embryos and Mesoderm Induction—Xenopus laevis were purchased from Nasco. Embryos were obtained and cultured as in Ref. 14. The recombinant Xenopus basic FGF used for induction was prepared as in Ref. 15. Animal pole explants (animal caps) were induced to form mesoderm as described (9), and animal caps were treated for 30 min prior to RNA extraction. For inhibition of protein synthesis during induction, animal caps were pretreated for 30 min with 5 μg/ml cycloheximide (Sigma), cultured with or without FGF for an additional 30 min, and then processed for PCR analysis as described below. Protein synthesis was measured in parallel samples by including 2 μCi/μl of [35S]methionine in the culture medium, and 35S incorporation into trichloroacetic acid precipitable material was determined according to Clemens (16).

Differential Display—RNA was extracted from induced or uninduced animal caps using the NaCl/EDTA/Tris/SDS protocol (17). Reverse transcription (RT) and polymerase chain reaction (PCR) were performed as in Ref. 13 with the following primers: 5′-TACGCT-3′ and 5′-CTGATCCATG-3′. PCR products were separated on a 6% polyacrylamide/6% urea gel; the gel was dried, and the products were visualized by autoradiography.

RT, reverse transcription; kb, kilobase pair(s); CAT, chloramphenicol acetyltransferase.
FIG. 1. Nucleotide and predicted amino acid sequence of er1.

The nucleotide sequence numbers of the er1 cDNA are shown on the left, and the amino acid sequence numbers of the predicted ER1 protein are shown on the right. The TAA termination codon is indicated by an asterisk. Four stretches of predominantly acidic residues are underlined, the proline-rich region is in bold, and two putative NLS are indicated by double underlines; the second NLS conforms to the consensus for a bipartite NLS.

cDNA Cloning and Sequencing of er1 from Xenopus Embryos—

Purified Xenopus cDNAs were cloned into the pCR II vector using the TA cloning kit (Invitrogen). The sequences for both strands of the initial cDNA were determined as in Ref. 14. A 2.3-kb PCR product and all subsequent cDNA inserts were determined as in a cloning kit (Invitrogen). The sequences for both strands of the initial PCR product were eluted from the gel in 100 mM Tris, pH 8.0, 10 mM EDTA, 1% sarcosyl, and 10% glycerol and were used as templates for sequencing. ELuted PCR products were cloned into the pCR II vector using the TA cloning kit (Invitrogen) and were used to generate overlapping clones for sequencing. The TAA termination codon was added to a 50-μl PCR reaction after the initial cycles, and the annealing temperature was reduced to 56 °C. Histone H4 was used as a control with forward (F) and reverse (R) primers as described (18), and the primer sequences for er1 were as follows:

5′-ACTCACTATAGGG-3′ (R) primers as described (18), and the primer sequences for er1 were as follows:

5′-TGGCTGAAATTCG-3′ (R) primers as described (18), and the primer sequences for er1 were as follows:

5′-GGCTGAAATTCG-3′ (R) primers as described (18), and the primer sequences for er1 were as follows:

5′-GGCTGAAATTCG-3′ (R) primers as described (18), and the primer sequences for er1 were as follows:

5′-GGCTGAAATTCG-3′ (R) primers as described (18), and the primer sequences for er1 were as follows:

5′-GGCTGAAATTCG-3′ (R) primers as described (18), and the primer sequences for er1 were as follows:

5′-GGCTGAAATTCG-3′ (R) primers as described (18), and the primer sequences for er1 were as follows:

5′-GGCTGAAATTCG-3′ (R) primers as described (18), and the primer sequences for er1 were as follows:

FIG. 2. Amino acid comparison of ER1 to the rat and human MTA1 and the C. elegans similar-to-MTA1 protein. A schematic illustrating alignment of the predicted Xenopus ER1 protein sequence with the rat and human MTA1 and the protein from C. elegans that is similar to MTA1. The N termini were aligned, and gaps (black lines) were introduced in the C. elegans and Xenopus proteins to align the regions of similarity (boxed) identified by the BLAST program. White boxes indicate unique regions. B, alignment of the predicted ER1 amino acid sequence with the MTA1 amino acid sequences in the regions of similarity illustrated in A. Identities are indicated by the one-letter amino acid code, conservative changes are indicated by a plus sign (+), and dashes (−) indicate nonconservative changes. The amino acid sequence numbers of the ER1 protein are shown on the right.

Plasmid Construction and Transient Transactivation Assays—

Plasmid construction and transient transactivation assays were performed as described in Ref. 19 using the 2.3-kb er1 or histone H4 cDNA as a probe.

Immunocytochemistry and Protein Analysis—

Immunocytochemistry and Protein Analysis—

Immunocytochemistry and Protein Analysis—

Immunocytochemistry and Protein Analysis—

Immunocytochemistry and Protein Analysis—

Immunocytochemistry and Protein Analysis—

Immunocytochemistry and Protein Analysis—

Immunocytochemistry and Protein Analysis—

Immunocytochemistry and Protein Analysis—

Immunocytochemistry and Protein Analysis—

Immunocytochemistry and Protein Analysis—

Immunocytochemistry and Protein Analysis—

Immunocytochemistry and Protein Analysis—

Immunocytochemistry and Protein Analysis—
immunosorbent assay kit (Boehringer Mannheim) according to the manufacturer's directions.

RESULTS AND DISCUSSION

In our efforts to elucidate the molecular mechanisms of FGF-induced mesoderm differentiation in Xenopus, we employed the PCR-based differential display method (13) to identify and characterize genes that are expressed early during the cellular response to FGF. RNA was isolated and reverse-transcribed from five individual sets of 30-min FGF-treated or control animal pole explants (animal caps) from Xenopus blastulae. PCR products from the five sets were separated on a 6% polyacrylamide/urea gel. Only those bands that were differentially expressed in all five sets were chosen for further analysis. A total of eleven differentially expressed bands were identified, and one of these was eluted from the gel, cloned, and sequenced. A search of the database for similarity to known sequences revealed that this cDNA represented a novel Xenopus gene, which we have named er1 (early response 1).

The sequence of the er1 PCR product was used to obtain a 2.3-kb cDNA from a Xenopus blastula library (14). This cDNA consisted of a single 1497-base pair open reading frame, bracketed by a 214-base pair 5'-untranslated region that contained several stop codons in all three frames and a 626-base pair 3'-untranslated region (Fig. 1). The ATG initiation codon is predicted to be at nucleotides 233–235, because this site is positioned within a Kozak consensus sequence for the start of translation (21), with a purine in the −3 position and a guanine in the +4 position. The open reading frame is predicted to encode a protein of 493 amino acids, beginning at nucleotide 233 and ending with an in-frame TAA stop codon at position 1712 (Fig. 1).

Computer-assisted analysis of the deduced amino acid sequence revealed that this cDNA represented a novel Xenopus gene, which we have named er1 (early response 1).

![Fig. 3. er1 is an FGF immediate-early response gene. A, FGF-stimulated increase in steady-state levels of er1. Experiments were set up in the presence (lane 2) or absence (lane 1) of 100 ng/ml Xenopus basic FGF. Total RNA was extracted, and RT-PCR analysis was performed as described under “Experimental Procedures.” B, FGF-stimulated increase of er1 in the absence of protein synthesis. Experiments were performed as described in lane 3, except that 5 µg/ml cycloheximide was added to the samples in lanes 2 and 4, and all samples were incubated for an additional 30 min. Extraction and analysis were performed as described for A. CHX, cycloheximide.](image)

![Fig. 4. Expression of er1 is restricted to early developmental stages in Xenopus. A, Northern blot analysis of er1 expression. Total RNA was isolated from the following developmental stages: stage 2 (2-cell; lane 1), stage 6 (64-cell; lane 2), stage 7 (early blastula; lane 3), stage 8 (mid-blastula; lane 4), stage 12 (mid-gastrula; lane 5), stage 17 (neurula; lane 6), stage 22 (tailbud; lane 7), stage 30 (stage 8), and stage 41 (tadpole; lane 9). Northern analysis was performed as in Sambrook et al. (20) using 32P-labeled 2.3-kb er1 cDNA as a probe. The blot was stripped and reprobed with 32P-labeled histone H4 cDNA. B, quantitative PCR analysis of er1 levels during blastula and gastrula stages of development. Total RNA was isolated at 1-h intervals during blastula and gastrula stages, beginning at stage 7 (lane 1) and ending with stage 9 (lane 4). For gastrula stages in lanes 5–7, RNA was isolated at stages 10, 10.5, and 12, respectively, according to morphological criteria (29). RT-PCR and analysis were performed as described in the legend to Fig. 3.](image)

![Fig. 5. Nuclear localization of ER1. A, immunoprecipitation of in vitro translation products with anti-ER1. Rabbit reticulocyte lysates programmed with er1 cDNA in pcDNA3 were immunoprecipitated with either preimmune (lane 2) or anti-ER1 (lane 3) serum prepared in our laboratory. Total translation products representing one-half of the input into each immunoprecipitation are shown in lane 1. B, ER1 is localized within the nucleus in transfected NIH 3T3 cells. NIH 3T3 cells were transfected with either the pcDNA3 vector alone (top) or er1-pcDNA3 (bottom). After 48 h, cells were fixed and stained with anti-ER1 as described under “Experimental Procedures.”](image)
FIG. 6. The N terminus of ER1 functions as a transcriptional activator. NIH 3T3 cells were transiently transfected with various GAL4-ER1 fusion constructs along with a CAT reporter plasmid. After 48 h, CAT enzyme levels were measured as described under “Experimental Procedures.” Vector denotes the control pM plasmid, containing only the GAL4 DNA binding domain, whereas the numbers indicate the amino acids of ER1 encoded by each construct. The value for each construct represents the fold activation relative to the pM plasmid, averaged from 3 to 12 independent transfections.

A data base homology search using the National Center for Biotechnology Information BLAST Network Service revealed that ER1 does not contain an N-terminal signal sequence for transfer into the endoplasmic reticulum or a hydrophobic domain characteristic of transmembrane proteins. However, ER1 does contain two potential nuclear localization signals (NLS): RPRR and KKKRYDFFAQQTRFGKKK (Fig. 1); the latter conforms to the consensus sequence for a bipartite NLS (22). ER1 also contains a proline-rich sequence near the C terminus that corresponds to the PXXP motif found in all high affinity SH3-domain binding ligands (23). The N terminus of ER1 includes several highly acidic stretches (Fig. 1), characteristic of the acidic activation domains of many transcription factors (24).

Our sequence analysis revealed two putative nuclear localization signals, suggesting that ER1 is targeted to the nucleus. We investigated the subcellular localization of the ER1 protein using a polyclonal anti-ER1 antibody to stain transfected NIH 3T3 cells expressing ER1. This antibody, directed against a synthetic C-terminal peptide, recognizes full-length ER1 protein synthesized in vitro (Fig. 5A, lane 3) and specifically stains the nuclei of cells expressing ER1 (Fig. 5B). Cells transfected with the pcDNA3 vector alone (Fig. 5B) as well as pcDNA3-er1 transfected cells stained with preimmune serum (not shown) gave similar patterns and showed no specific nuclear staining.

The facts that ER1 is targeted to the nucleus and that its N terminus contains stretches of acidic residues characteristic of acidic activation domains (25) suggest that ER1 may function as a transcription factor. We investigated this possibility by testing the transactivation potential of various regions of the ER1 protein. Constructs containing different portions of er1
fused to the GAL4 DNA binding domain were used along with a CAT reporter plasmid in transient transfections. Assays of CAT enzyme levels revealed that although full-length ER1 did not activate transcription, the N-terminal region (ER 1–175) containing all four acidic stretches (Fig. 1) stimulated transcription 10-fold (Fig. 6). The complementary C-terminal portion, ER 176–493, on the other hand, had no transactivational activity. It is unclear why full-length ER1 was unable to stimulate transcription, but one possible explanation is that fusion of ER1 to GAL4 may alter the tertiary structure of the ER1 protein, affecting its activity. A similar observation was made with the ETS transcription factor ER81, which when fused to the GAL4 DNA binding domain lost its ability to activate transcription (28).

Interestingly, deletion of the N-terminal region to produce a construct containing only the first three acidic stretches (ER 1–98), resulted in a much more potent transactivator that stimulated transcription 80-fold (Fig. 6). This suggests that a negatively acting domain is located between amino acids 99–176. Further truncation of the N terminus to generate ER 1–57 and ER 1–25 completely abolished transactivation. These results demonstrate that the ER1 protein contains regions with transcription transactivating activity and that ER1 has the potential to function as a transcription factor.

REFERENCES
1. Fernig, D. G., and Gallagher, J. T. (1994) Prog. Growth Factor Res. 5, 353–377
2. Baird, A., and Klagsburn, M. (1991) The Fibroblast Growth Factor Family, The New York Academy of Sciences, New York
3. Delli-Bovi, P., and Basilico, C. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 5660–5664
4. Sasada, R., Kurokawa, T., Iwane, M., and Igarashi, K. (1988) Mol. Cell Biol. 8, 588–594
5. Neufeld, G., Mitchell, R., Poste, P., and Gospodarowicz, D. (1988) J. Cell Biol. 106, 1385–1394
6. Slack, J. M. W., Darlington, B. G., Heath, J. K., and Godsave, S. F. (1987) Nature 326, 197–200
7. Gillespie, L. L., Paterno, G. D., and Slack, J. M. W. (1989) Development 106, 203–208
8. Gillespie, L. L., Paterno, G. D., Mahadevan, L. C., and Slack, J. M. W. (1992) Mech. Dev. 38, 99–108
9. Ryan, P. J., and Gillespie, L. L. (1994) Develop. Biol. 166, 101–111
10. Hartley, R. S., Llewellyn, A. L., and Maller, J. L. (1994) Dev. Biol. 163, 521–524
11. Smith, J. C., Price, B. M. J., Green, J. B. A., Weigel, D., and Herrmann, B. G. (1991) Cell 67, 79–87
12. von Dassow, G., Semidt, J. R., and Kimelman, D. (1993) Genes Dev. 7, 355–366
13. Liang, P., and Pardee, A. B. (1992) Science 257, 967–969
14. Gillespie, L. L., Chen, G., and Paterno, G. D. (1995) J. Biol. Chem. 270, 22758–22763
15. Kimelman, D., Abraham, J. A., Haaparanta, T., Palisi, T. M., and Kirschner, M. W. (1988) Science 242, 1053–1056
16. Clemens, M. J. (1984) Transcription and Translation: A Practical Approach, pp. 231–270, IRL Press, Oxford
17. Evans, J. P., and Kay, B. K. (1991) Methods Cell Biol. 36, 133–148
18. Niehrs, C., Steinbeisser, H., and De Robertis, E. M. (1994) Science 263, 817–820
19. Reynolds, J. N., Prasad, A., Gillespie, L. L., and Paterno, G. D. (1996) Mol. Brain Res. 35, 11–18
20. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., pp. 7.43–7.52, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. Kozak, M. (1986) Cell 44, 283–292
22. Robbins, J., Dilworth, S. M., Laskey, R. A., and Dingwall, C. (1991) Cell 64, 615–623
23. Cohen, G. B., Ren, R., and Baltimore, D. (1995) Cell 80, 237–248
24. Ptashne, M. (1988) Nature 335, 683–689
25. Toh, Y., Pencil, S. D., and Nicolson, G. L. (1994) J. Biol. Chem. 269, 22958–22963
26. Pathak, S., Siciliano, M. J., Caileau, R., Wiseman, C. L., and Hsu, T. L. (1979) J. Natl. Cancer Inst. 62, 263–271
27. Newport, J., and Kirschner, M. (1982) Cell 30, 687–696
28. Janneke, R. (1996) Mol. Cell. Biol. 16, 1550–1556
29. Nieuwkoop, P. D., and Faber, J. (1967) Normal Table of Xenopus laevis, North-Holland Publishing Co., Amsterdam, Holland