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Identification of a c-*fos*-induced gene that is related to the platelet-derived growth factor/vascular endothelial growth factor family

(Fos/cell transformation)

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ABSTRACT **Using a mRNA differential screening of fibroblasts differing for the expression of c-*fos* we isolated a c-*fos*-induced growth factor (FIGF). The deduced protein sequence predicts that the cDNA codes for a new member of the platelet-derived growth factor/vascular endothelial growth factor (PDGF/VEGF) family. Northern blot analysis shows that FIGF expression is strongly reduced in c-*fos*-deficient cells. Transfection of exogenous c-*fos* driven by a constitutive promoter restores the FIGF expression in these cells. In contrast, both PDGF and VEGF expression is unaffected by c-*fos*. FIGF is a secreted dimeric protein able to stimulate mitogenic activity in fibroblasts. FIGF overexpression induces morphological alterations in fibroblasts. The cells acquire a spindle-shaped morphology, become more refractive, disorganized, and detach from the plate. These results imply that FIGF is a downstream growth and morphogenic effector of c-*fos*. These results also suggest that the expression of FIGF in response to c-*fos* activation induces specific differentiation patterns and its aberrant activation contributes to the malignant phenotype of tumors.**

The c-*fos* protooncogene plays a central role in the nuclear response to stimulatory signals that regulate cellular proliferation and differentiation. It codes for a nuclear protein that belongs to the AP-1 family of transcription factors. AP-1 factors are part of the bZip family of transcription factors which can form homo- and heterodimers and activate transcription by binding the DNA at AP-1 sites (1, 2). AP-1 is composed of dimeric complexes formed between Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra1, and Fra2) proteins which are induced by many cellular stimuli including growth factors, citokines, T-cell activators, and UV irradiation (3). As a member of the immediate-early genes, c-*fos* expression is rapidly and transiently increased in response to extracellular signals. The role of c-*fos* during development has been studied by the generation of c-*fos*-deficient mice (4, 5). c-*fos* knockout mice are viable but show a range of tissue specific developmental defects including osteopetrosis, delayed gametogenesis, and limphopenia.

Continuous expression of c-*fos* causes transformation of fibroblasts and loss of polarity of epithelial cells *in vitro* (6), and induces the formation of condroblastic osteosarcomas when it is expressed under the control of ubiquitous promoters in transgenic mice (ref. 7 and references therein). Tumors obtained from c-*fos*-deficient cells fail to undergo malignant progression even if they are carrying the activated v-H-ras (8). These experiments suggest an essential role of c-*fos* in the malignant tumor development. c-*fos* contribution to differentiation and tumor progression is most probably due to the activation of specific target genes. These may play a role in differentiation, in cell transformation, and/or malignant progression of tumors. A large number of genes have been shown to contain functional AP-1 sites in their regulatory regions. These include c-*jun* (2), the adipocyte P2 gene (9), type I collagenase (10), and stromelysin (11). Different strategies have been adopted to identify new c-*fos* target genes. The generation of a hormonally regulated c-Fos-estrogen receptor chimera allowed the isolation of the c-*fos* responsive gene Fit-1 (12) which codes for a membrane-associated protein. Reversion of the v-*fos*-dependent transformed phenotype in rat cells allowed the isolation of Fte-1 (13), a protein probably involved in protein import into mitochondria.

To isolate new c-*fos*-responsive genes we utilized cells differing only for the expression of c-*fos*. By mRNA differential display we compared the expression pattern of c-*fos*-deficient fibroblasts with cells derived from their wild-type siblings. In this report we describe the isolation of a cDNA that is strongly induced by c-*fos*. The cDNA sequence shows that it codes for a putative growth factor related to the platelet-derived growth factor/vascular endothelial growth factor (PDGF/VEGF) family. The protein, which was named FIGF (for c-*fos*-induced growth factor), is secreted and shows autocrine mitogenic and morphogenic effects on fibroblasts.

MATERIALS AND METHODS

Cells and Cell Culture. c-*fos* (−/−)-deficient fibroblasts, obtained from c-*fos* knockout mice (5, 14) and c-*fos* (+/+) fibroblasts derived from their wild-type siblings were grown in DMEM supplemented with 10% fetal calf serum (FCS). The c-*fos* (−/−)f cells, which express constitutively c-*fos*, were cultured in DMEM supplemented with 10% FCS and G418 (Geneticin; GIBCO/BRL) at 400 μg/ml. Stable clones constitutively expressing FIGF were obtained by cotransfection of an FIGF expression vector together with a plasmid containing the hygromycin resistance gene under the control of the simia virus 40 promoter (SO166). Transfectants were selected in DMEM supplemented with 10% FCS and hygromycin B (Calbiochem) at 300 μg/ml. The FIGF expression vector was constructed by the cloning of the FIGF cDNA under the control of the cytomegalovirus (CMV) promoter in the plasmid pcDNAIII-Δ neo (kindly provided by L. D'Adamio, National Institutes of Health).

Differential Display and Cloning of FIGF cDNA. c-*fos* (−/−) and c-*fos* (+/+) cells were maintained in DMEM containing 0.5% FCS for 48 h and then subjected to serum treatment. After 2 h of 10% serum induction, total cellular

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Abbreviations: PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; FCS, fetal calf serum; CMV, cytomegalovirus; MEF, mouse embryo fibroblast

Data depostion: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U99572).

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RNA was extracted by the guanidinium thiocyanate method (15) and subjected to the differential display technique (16–18). The amplified cDNA fragments were compared in nondenaturing gels (19). The differentially expressed cDNAs were reamplified, cloned into pGEM-T vector (Promega), and used as probe in Northern blot assay. A fibroblast cDNA library was generated by oligo-dT reverse transcription of poly(A)$^+$ RNA from a cell clone constitutively expressing c-*fos*, and cloned into Uni-Zap vector (Stratagene). A partial cDNA fragment (273 bp), whose corresponding mRNA was induced by c-*fos*, was labeled with [^{32}P]dCTP by random prime labeling and used to screen the library. The longest cDNA isolated was sequenced on both strands by the dideoxy DNA sequencing method (United States Biochemical).

Northern Blot Analysis. Total RNA (10 μg) was run on denaturing formaldehyde-agarose gel and transferred to nylon membranes. Filters were hybridized with [^{32}P]-labeled probes at 60°C in a buffer containing 0.5 M sodium phosphate (pH 7.2), 7% SDS, and 1 mM EDTA. The filters were washed for two 30-min periods at 60°C in 40 mM sodium phosphate (pH 7.2), 1% SDS, and 1 mM EDTA and exposed to x-ray film or analyzed by using a PhosphorImager (Molecular Dynamics).

Production of Bacterial FIGF and Anti-FIGF Antibodies. The FIGF protein was expressed in *Escherichia coli* under the control of the T5 promoter. The cDNA fragment, from the coding region of FIGF, was generated by PCR from the methionine residue at position +40 and cloned into the pQE-31 vector (Qiagen, Chatsworth, CA) to obtain a fusion protein with a N-terminal histidine tag. The protein was expressed in TG1 bacteria (pREP+) by induction for 4 h at 37°C in the presence of 2 mM isopropyl β-D-thiogalactopyranoside. The recombinant protein was exclusively localized in inclusion bodies and was purified on a column of Ni-NTA-resin under denaturing conditions, according to the manufacturer's protocols (Qiagen). To produce partially refolded FIGF protein, the purified recombinant protein was treated as described (20, 21). Briefly the protein concentration was adjusted to 0.4 mg/ml and completely reduced in the presence of 8 M urea, 2% 2-mercaptoethanol for 1 h at 37°C. The reduced protein was dialyzed against a solution containing 50 mM Tris·HCl (pH 8.0), 1 M urea, 5 mM reduced glutathione, and 0.5 mM oxidized glutathione for 2 days, and against a solution containing 20 mM Tris·HCl (pH 7.5) and 0.2 M NaCl for 1 day. Polyclonal antibodies were raised by injecting New Zealand White rabbits with 200 μg of recombinant FIGF in form of denaturated protein in complete Freund's adjuvant. Antiserum was prepared after four injections in incomplete Freund's adjuvant at 3-week intervals.

Expression of FIGF in COS-7 Cells. COS-7 cells were transfected with an expression vector (pcDNAIII; Invitrogen) containing the FIGF coding sequence by using calcium phosphate precipitation. Cells were metabolically labeled with

A

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1                                                 50
MYGEWGMGNI LMMFHVYLVQ GFRSEHGPVK DFSFERSSRS MLERSEQQIR

51                                               100
AASSLEELLQ IAHSEDWKLW RCRLKLKSLA SMDSRSASHR STRFAATFYD

101                                              150
TETLKVIDEE WQRTQCSPRE TCVEVASELG KTTNTFFKPP CVNVFRCGGC

151                                              200
CNEEGVMCMN TSTSYISKQL FEISVPLTSV PELVPVKIAN HTGCKCLPTG

201                                              250
PRHPYSIIRR SIQTPEEDEC PHSKKLCPID MLWDNTKCKC VLQDETPLPG

251                                              300
TEDHSYLQEP TLCGPHMTFD EDRCECVCKA PCPGDLIQHP ENCSCFECKE

301                                              350
SLESCCQKHK IFHPDTCSCE DRCPFHTRTC ASRKPACGKH WRFPKETRAQ

       358
GLYSQENP
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B

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FIGF    (103) TLKVIDEEWQ RTQCSPRETC VEVASELGKT T..NTFFKPP CVNVFRCGGC
VEGF-C   (49) ILKSIDNEWR KTQCMPREVC IDVGKEFGVA T..NTFFKPP CVSVYRCGGC
VEGF     (39) EVVKFMDVYQ RSYCHPIETL VDIFQEYPDE I..EYIFKPS CVPLMRCGGC
PlGF     (39) EVVPFQEVWG RSYCRALERL VDVVSEYPSE V..EHMFSPS CVSLLRCTGC
VEGF-B   (35) VVPWID.VYA RATCQPREVV VPLSMELMGN V..VKQLVPS CVTVQRCGGC
PDGF-B   (84) GSLTIAEPAM IAECKTRTEV FEISRRLIDR TNANFLVWPP CVEVQRCSGC
PDGF-A   (83) RRKRSIEEAV PAVCKTRTVI YEIPRSQVDP TSANFLIWPP CVEVKRCTGC

FIGF    (151) CNEEGVMCMN TSTSYISKQL FEIS.VPLTS VPELVPVKIA NHTGCKCLPT
VEGF-C   (97) CNSEGLQCMN TSTSYLSKTL FEIT.VPLSQ GPKPVTISFA NHTSCRCMSK
VEGF     (87) CNDEGLECVP TEESNITMQI MRIK..P.HQ GQHIGEMSFL QHNKCECRPK
PlGF     (87) CGDENLHCVP VETANVTMQL LKIR..S.GD RPSYVELTFS QHVRCECRPL
VEGF-B   (82) CPDDGLECVP TGQHQVRMQI LMIQ.YPSSQ ...LGEMSLE EHSQCECRPK
PDGF-B  (134) CNNRNVQCRP TQVQLRPVQV RKIEIVRKKP IFKKATVTLE DHLACKCETV
PDGF-A  (133) CNTSSVKCQP SRVHHRSVKV AKVEYVRKKP KLKEVQVRLE EHLECACATT
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FIG. 1. (*A*) Deduced amino acid sequence of mouse FIGF. The putative secretory signal peptide rich in hydrophobic residues is underlined. (*B*) Alignment of the FIGF protein with the conserved domain of the PDGF/VEGF family of growth factors. Amino acid residues identical to FIGF are boxed. Dots indicate the cysteine residues which are characteristic of these growth factors (22). Numbers on the left indicate amino acid positions relative to the initiator methionine residue of each protein.

[^{35}S]methionine and [^{35}S]cysteine (Amersham) at 100 mCi/ml (1 Ci = 37 GBq) for 1 h and chased with cold methionine and cysteine. After the chase period medium was collected and cells were lysed in 50 mM Tris·HCl (pH 7.5), 1% Nonidet P-40, 150 mM NaCl, and 4 μg/ml phenylmethylsulfonyl fluoride. Conditioned media and cell lysates were immunoprecipitated separately with anti-FIGF polyclonal antibodies. Immune complexes were collected on protein A-Sepharose beads (Pharmacia) and separated by 12% SDS/PAGE in the presence of 2% 2-mercaptoethanol.

Mitogenic Assay for FIGF. Conditioned medium containing FIGF was collected from cells transfected with the appropriate expression vectors or with vector alone. c-*fos* (−/−) cells were plated into 96-well plates at the density of 5 × 10^3 cells/well in DMEM supplemented with 0.5% FCS and incubated for 48 h. Mouse embryo fibroblasts (MEFs) were obtained from 13- to 15-day embryos of B6D2F1 mice. The embryos were sacrificed, rinsed in Hanks' balanced salt solution and trypsinized for 30 min at 37°C. MEFs were grown in DMEM supplemented with 10% FCS. Second-passage MEFs were plated into 96-well plates at the density of 7 × 10^3 cells/well in DMEM containing 0.5% FCS and incubated for 30 h. Conditioned media or purified proteins were added to the wells and cells were stimulated for 14 h. [^{3}H]Thymidine (2.5 μCi/ml) diluted in DMEM without serum was added to the cells for a period of 8 h. Cells were washed with PBS, trypsinized, and the incorporated radioactivity was determined by liquid scintillation counting.

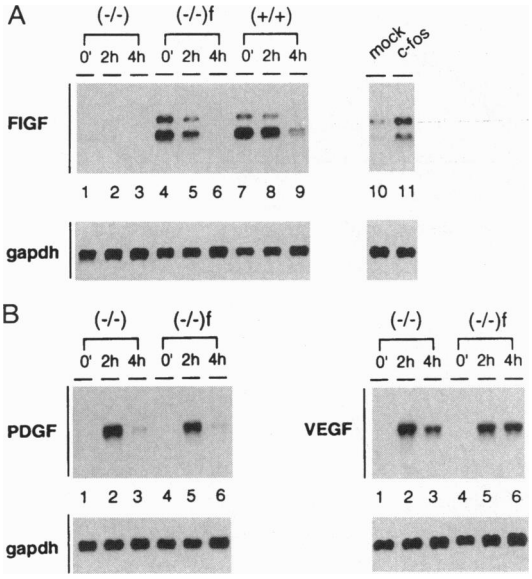

FIG. 2. (*A*) Expression of FIGF in cultured cells. Northern blot analysis of total RNA obtained from c-*fos* (−/−) fibroblasts, a cell line lacking c-*fos* (lanes 1–3); c-*fos* (−/−)f cells, a stable cell line expressing exogenous c-*fos*, obtained transfecting c-*fos* (−/−) cells with c-*fos* under the control of a constitutive promoter (lanes 4–6); wild-type c-*fos* (+/+) fibroblasts (lanes 7–9). Cellular RNA was extracted from cells grown for 48 h in DMEM supplemented with 0.5% FCS (time 0). The serum concentration was increased to 10% and total RNA was collected at 2 h or 4 h as indicated. Lanes 10 and 11 show FIGF expression in c-*fos* (−/−) fibroblasts transiently transfected with the vector alone (mock) or containing c-*fos* under the control of FBJ–LTR constitutive promoter (c-*fos*). The RNAs of the transiently transfected cells were collected 30 h after culturing the cells in DMEM containing 0.5% FCS. Each lane was loaded with 10 μg of total cellular RNA. (*B*) Expression of PDGF or VEGF in cultured cells. Total cellular RNAs from c-*fos* (−/−) cells (lanes 1–3) or from c-*fos* (−/−)f cells (lanes 4–6) were extracted as indicated in *A*. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control for RNA loading.

RESULTS

Isolation and Characterization of the FIGF cDNA. c-*fos*-deficient cells, derived from c-*fos* knockout mice (5), are defective in the induction of AP-1-responsive genes such as stromelysin and type I collagenase (14). To identify new specific c-*fos* target genes, we used the mRNA differential display technique which allowed us to isolate genes differentially expressed in c-*fos* (−/−) versus wild-type c-*fos* (+/+) cells. Few cDNA fragments, corresponding to differentially expressed mRNA, were identified and their expression pattern was confirmed by Northern blot analysis (data not shown). One of these cDNA fragments was FIGF. The full-length FIGF cDNA was isolated by screening a fibroblast cDNA library using as a probe the cDNA fragment corresponding to the 3′ end of FIGF. The nucleotide sequence of the cDNA revealed a single open reading frame coding for a putative protein of 358-amino acid residues (Fig. 1*A*). FIGF presents a hydrophobic sequence of 20 residues at the N terminus which could code for a signal peptide (23). Comparison of the predicted FIGF protein with the SWISS-PROT data bank revealed a significant similarity of FIGF with the PDGF/VEGF family of growth factors (Fig. 1*B*). FIGF contains, at the same relative distance, the eight conserved cysteine residues which are characteristic of this growth factor's family (22, 24–29). These cysteine residues are involved in intra- and interchain disulfide bridges of the active dimeric molecules (30). The long N-terminal region of FIGF does not show significant similarity to known proteins. The C-terminal domain is very long and rich in cysteine residues, some of which occur in repeat units as described in the recently identified VEGF-C molecule (28).

FIGF Is Induced by c-*fos* in Cultured Fibroblasts. The expression of FIGF transcripts was examined in cells differing for the expression of c-*fos*. Northern blot analysis reveals two hybridizing FIGF transcripts of 2.4 and 4 kbp, respectively. Analysis of FIGF gene expression reveals that the FIGF messenger is barely detectable in c-*fos* (−/−) fibroblasts, while its expression is high in wild-type c-*fos* (+/+) fibroblasts (Fig. 2*A*, compare lanes 1 and 7). FIGF expression is completely restored in stable clones derived from c-*fos* (−/−) cells, expressing exogenous c-*fos* under the control of a constitutive

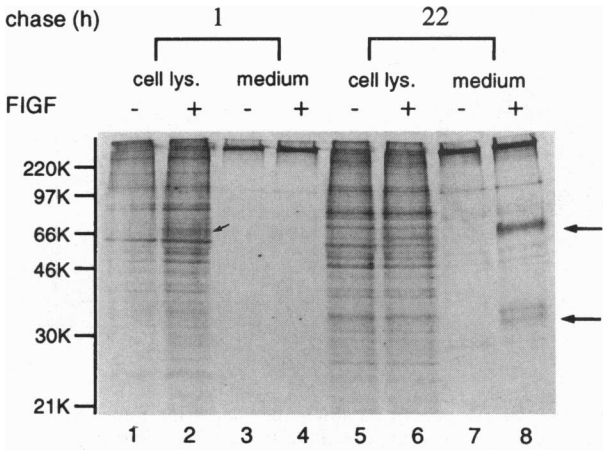

FIG. 3. Immunoprecipitation assay of the FIGF protein. COS-7 cells transiently transfected with the vector alone (−) or with a vector containing the FIGF coding sequence under the control of a CMV promoter (+) were metabolically labeled. After 1-h or 22-h chase, culture supernatants and detergent-solubilized cell lysates were subjected to immunoprecipitation and SDS/PAGE analysis under reducing conditions. Arrows indicate specific bands present only in FIGF transfected cell. The upper arrow indicates a 66-kDa protein which corresponds to the putative dimer and the lower arrow indicates a 33-kDa protein which corresponds to a putative monomer.

promoter (Fig. 2*A*, compare lanes 1 and 4). To exclude that in c-*fos* (−/−) cells the low expression is due to clonal variation, we transiently transfected these cells with c-*fos* under the control of a constitutive promoter. The transient transfection of exogenous c-*fos*, driven by the viral FBJ–LTR promoter (14), results in FIGF induction in c-*fos* (−/−) cells (Fig. 2*A*, lanes 10 and 11). These experiments show that the FIGF expression is induced by c-*fos*.

Since FIGF shows strong sequence similarities with the PDGF and VEGF, we asked whether their expression was affected by c-*fos*. As can be observed in Fig. 2*B*, the regulation of both PDGF and VEGF transcripts is different from that of FIGF. These growth factors are rapidly induced following serum induction and their expression is independent of c-*fos*. These data indicate that c-*fos* is required for the induction of FIGF, while the c-*fos* expression is not required for the PDGF and VEGF induction. FIGF does not differ from PDGF and VEGF in the negative regulation since they all decrease from 4 h after serum induction (Fig. 2*A*, lanes 6 and 9). Moreover, FIGF mRNA accumulates in quiescent cells. This pattern of expression suggests that, besides the expression of c-*fos*, additional regulatory controls are required for FIGF regulation.

FIGF Is a Secreted Protein. To verify that FIGF is a secreted protein, we transfected COS-7 cells with an expression vector containing the FIGF cDNA under the control of the CMV immediate-early gene promoter. Polyclonal antibodies, raised against recombinant FIGF produced in *E. coli*, immunoprecipitated a specific band that is observed in both the cell lysates and the conditioned media of the FIGF transfected COS-7 cells. After 1-h labeling followed by 1-h chase a specific band was mainly present in the cell lysate (Fig. 3, lane 2) while, after a chase longer than 4 h, the protein accumulated in the cell supernatant (lane 8). Under nondenaturing conditions FIGF aggregated into a multimeric form (not shown). Addition of 2-mercaptoethanol resulted in partial denaturation of the secreted protein which migrated mostly as a 66-kDa band. This corresponds to the migration of the putative dimeric form. Only a minor fraction of the secreted protein can be found at 33 kDa of molecular mass. This should correspond to the expected migration of FIGF in the monomeric form (Fig. 3, lane 8). Dimerization of FIGF could be predicted since the FIGF central domain is highly conserved and contains the eight cysteine residues involved in the dimerization of all the other known members of the PDGF and VEGF family (28–30).

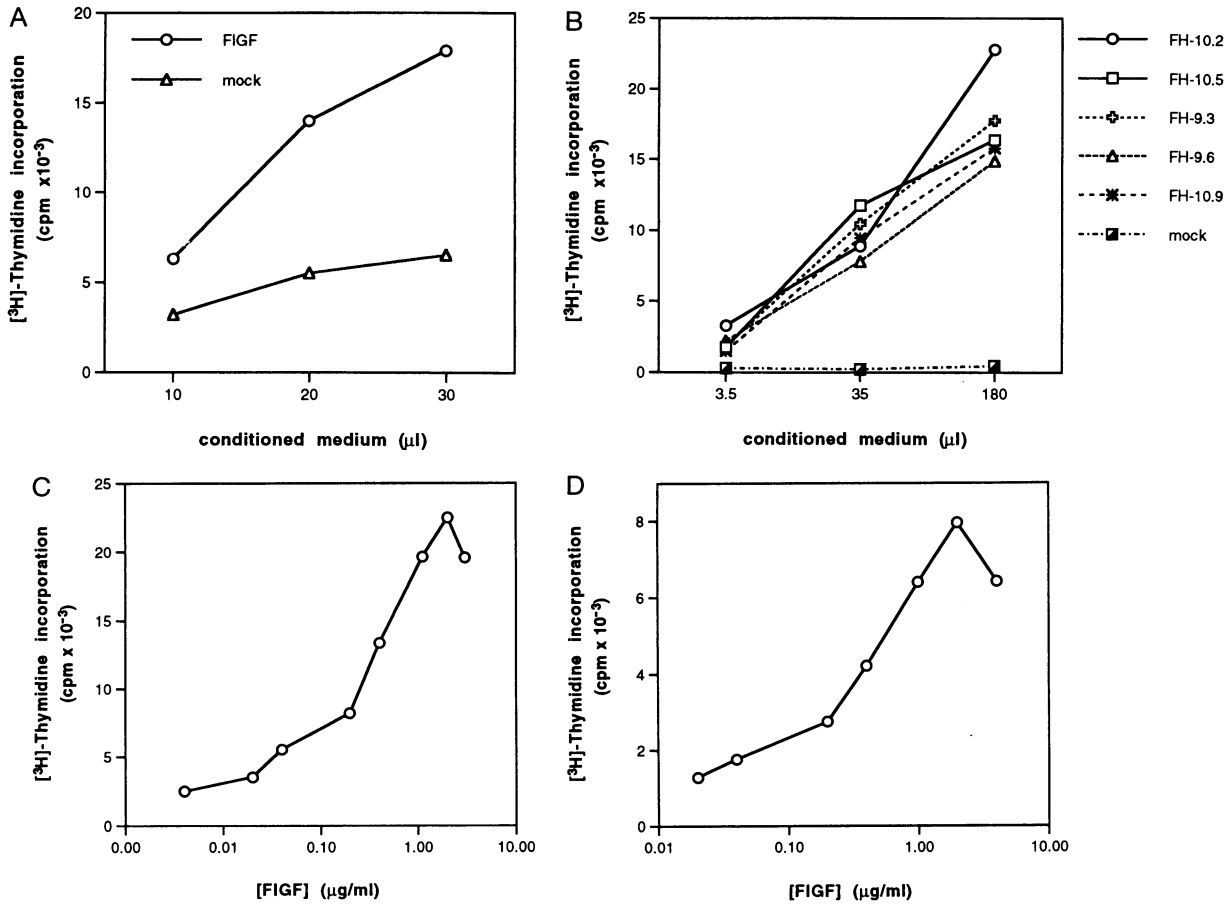

FIG. 4. FIGF induces [³H]thymidine incorporation into fibroblasts. (*A*) Mitogenic activity on c-*fos* (−/−) fibroblasts. Starved cells were stimulated with conditioned medium from COS-7 cells transiently transfected with the FIGF expression vector or with the vector alone (mock). One day after transfection COS-7 cells were split and mantained in 2% serum and conditioned media were collected after 120 h. (*B*) Mitogenic activity on c-*fos* (−/−) fibroblasts. Starved fibroblasts were stimulated with conditioned media obtained from c-*fos* (−/−) stable clones, named FH-10.2, FH-10.5, FH9.3, FH-9.6, FH-10.9, and c-*fos* (−/−) cells (mock), constitutively expressing exogenous FIGF under the control of the CMV promoter. Culture supernatants were collected from cells maintained for 48 h in DMEM supplemented with 0.5% FCS. (*C*) Mitogenic activity induced by the recombinant FIGF protein on c-*fos* (−/−) fibroblasts. Starved cells were incubated with partially renatured recombinant FIGF. Under the same conditions, incubation with PDGF-BB (10 ng/ml; Sigma), used as a positive control, induces about 30% higher [³H]thymidine incorporation, while VEGF (10 ng/ml; Sigma) does not induce incorporation above the background (not shown). The data shown are the mean of six experiments performed with three different FIGF preparations. (*D*) Mitogenic activity on MEFs. Starved MEFs were stimulated with partially renatured recombinant FIGF. Under the same conditions, incubation with PDGF-BB (10 ng/ml; Sigma), used as a positive control, induces about 30% higher [³H]thymidine incorporation, while VEGF (10 ng/ml; Sigma) does not induce incorporation above the background (not shown). The data shown are the mean of six experiments performed with three different FIGF preparations. The background values were subtracted in each experiment.

FIGF Shows Mitogenic Activity on Fibroblasts. The above experiments show that FIGF is a secreted protein. We further investigated whether the conditioned medium of cells producing FIGF could promote cell growth *in vitro*, assayed as [^{3}H]thymidine incorporation. Conditioned medium was obtained either from transiently transfected COS-7 cells or from stable clones, obtained from c-*fos* (−/−) fibroblasts, expressing FIGF under the CMV promoter. The mitogenic activity of the conditioned medium containing FIGF was first tested on c-*fos* (−/−) fibroblasts. Both conditioned medium obtained from transfected COS-7 or constitutive FIGF expressing clones induced DNA synthesis on c-*fos* (−/−) fibroblasts (Fig. 4 *A* and *B*). We also tested the mitogenic activity of the recombinant FIGF protein expressed in *E. coli*. To obtain a biologically active recombinant protein, FIGF was purified from inclusion bodies and partially renatured in the presence of a mixture of reduced and oxidized glutathione. The partially refolded recombinant FIGF-induced DNA synthesis on c-*fos* (−/−) fibroblasts in a dose-dependent manner (Fig. 4*C*). As expected, c-*fos* (−/−) cells were also responsive to PDGF-BB, while the treatment with VEGF did not induce [^{3}H]thymidine incorporation (not shown). We also tested the mitogenic activity of the recombinant FIGF on mouse embryo fibroblasts. As shown in Fig. 4*D*, FIGF-induced DNA synthesis on these cells to levels comparable to those induced by PDGF.

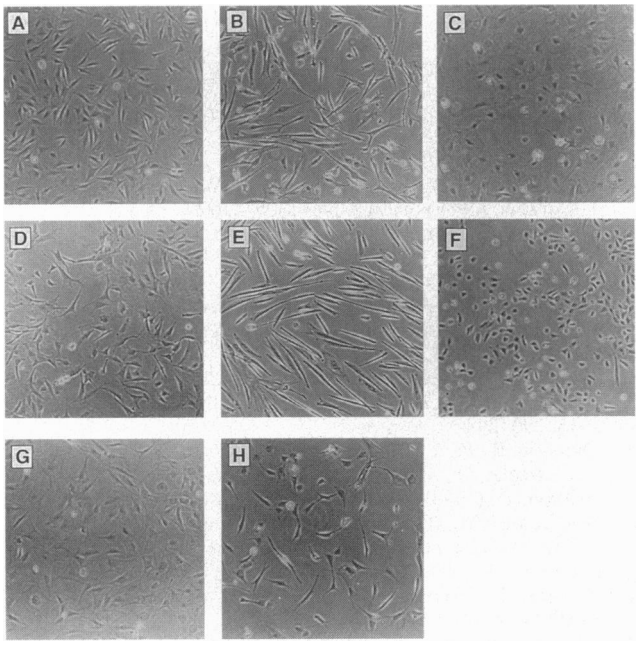

FIG. 5. (*A*) Morphology of c-*fos* (−/−) cells stably transfected with the vector alone. (*B*) Morphology of a cell clone derived from c-*fos* (−/−) cells stably transfected with the expression vector containing FIGF under the control of the CMV promoter. (*C*) Morphology of cells stably transfected with an expression vector containing the FIGF cDNA in the antisense orientation under the control of the CMV promoter. (*D*) Morphology of cells stably transfected with the expression vector containing c-*fos* under the control of the FBJ–LTR promoter. (*E*) A cell clone derived from the same cells as in *D* (expressing c-*fos* contitutively) transfected with an expression vector containing FIGF under the control of the CMV promoter. (*F*) A cell clone derived from the same cells as in *D* (expressing c-*fos* contitutively) transfected with an expression vector containing the FIGF cDNA in the antisense orientation under the control of the CMV promoter. (*G*) c-*fos* (−/−) fibroblasts cultured for 120 h in DME medium containing 0.5% serum. (*H*) Cells as in *G* but treated for 120 h with partially renatured recombinant FIGF at 2 μg/ml. Ten independent clones obtained from three independent transfections were analyzed. All showed morphological changes similar to those observed in the figure.

FIGF Induces Morphological Alterations on Fibroblasts. The induction of transformed foci by v-H-ras, v-src, Polyoma middle T antigen, and simian virus 40 large T is not impaired in c-*fos*-deficient cells (14). Rather, c-*fos* has been implicated in tumor progression (8) and its overexpression induces a transformed cell morphology in fibroblasts and epithelial cells (6). As FIGF codes for a c-*fos*-induced growth factor, we analyzed whether its overexpression could induce a fibroblast's morphological transformation. Several independent clones overexpressing FIGF were isolated and all showed morphological alterations. Fig. 5 shows typical morphological changes observed. Cells overexpressing FIGF acquire a spindle-shaped morphology, become more refractive, and detach from the plate (Fig. 5 *B* versus *A*). The constitutive expression of c-*fos* induces morphological changes in c-*fos*-deficient cells which are similar, although less evident, to the alterations observed with the FIGF overexpression (Fig. 5*D*). The overexpression of both c-*fos* and FIGF leads to the same phenotype determined by FIGF overexpression and cells become extremely long (Fig. 5*E*). The depletion of FIGF, obtained by the expression of FIGF in the antisense orientation in c-*fos* constitutive cells, causes the complete loss of the elongated phenotype (Fig. 5*F*). These data show that FIGF is able to induce morphological changes on fibroblasts and suggest that FIGF is the morphological effector of c-*fos*. Cells expressing the FIGF in the antisense orientation show a slow growth rate (not shown).

To verify whether FIGF induces morphological changes on fibroblasts *in vitro* we also treated c-*fos* (−/−) fibroblasts with recombinant FIGF. As can be observed in Fig. 5*H*, that shows cells treated with recombinant FIGF for 120 h, the cell treatment with recombinant FIGF induces morphological alterations similar to the ones observed with the FIGF overexpression.

DISCUSSION

Nuclear oncogenes contribute to the cancerous state by directly altering gene regulation. The missing link between oncogenes and tumors has been the identification of oncologically relevant genes regulated by oncogenes. Some Fos target genes have already been cloned, but we are still far from understanding their role in tumor progression. To identify c-*fos*-responsive genes we isolated differentially expressed genes in cells differing for the expression of c-*fos*. Here we describe the cloning and characterization of the cDNA coding for the gene referred as FIGF. FIGF codes for a 358 amino acid residues long secreted protein. Its deduced amino acid sequence indicates that FIGF is strongly related to the PDGF/VEGF growth factors. FIGF contains in its central region the signature sequence which is characteristic for this family of growth factors. This region contains eight cysteine residues which are important for dimerization. We provide evidence that FIGF acts as a growth and morphogenic factor on fibroblasts *in vitro*. The mechanism of c-*fos* induction in response to PDGF has been well characterized (31, 32). The finding that a growth factor can be induced by c-*fos* allows to put in a consequential order of activation different growth factors of the PDGF family. Thus, the c-*fos* induction in response to PDGF or other growth stimuli may lead to the induction of other growth factors, FIGF being one of them, which most probably allows the cells to differentiate through a specific pathway.

The FIGF expression pattern analyzed by Northern blot showed a reduced expression of FIGF in c-*fos*-deficient cells. That FIGF low expression is due to the lack of c-*fos* rather than to clonal variations is demonstrated by the restoration of FIGF mRNA upon induction of exogenously expressed c-*fos* in these cells. FIGF is expressed at elevated levels, within 2 h after serum induction. This corresponds to the expression of a c-*fos*-induced gene. However, FIGF transcripts accumulates

during the quiescent phase as well. Thus, the FIGF induction is likely to require other regulatory mechanisms, probably connected with the cell cycle, in addition to the requirement for c-*fos*.

The FIGF pattern of expression differs considerably from the expression of its related genes PDGF and VEGF. These growth factors are induced rapidly after serum stimulation and their expression is not affected by c-*fos*. FIGF pattern of expression is most similar to *gas 6*. This gene, which acts as a growth factor, is abundantly expressed in serum starved cells (33, 34).

The FIGF induction by c-*fos* appears quite specific since c-*fos* cannot be substituted by other AP-1 transcription factors. In fact, in c-*fos*-deficient cells all AP-1 transcription factors except c-*fos* are normally expressed (14). In addition, the transfection of the aspecific AP-1 transcriptional activator GCN4 into these cells fails to induce FIGF (data not shown). In mammalian cells GCN4 is able to activate most AP-1 target genes, but it is nononcogenic (35). Taken together, these observations suggest that FIGF is involved in c-*fos*-dependent cell transformation. c-*fos* does not seem to be necessary for early proliferative steps of tumor formation, but it is required for malignant tumor conversion (8). Therefore FIGF could play a role in tumor progression. In this respect FIGF would not differ from VEGF. VEGF plays a role in tumor angiogenesis (36). It has recently been observed that its mRNA is elevated in papillomas originating from c-*fos* wild-type cells with respect to papillomas originating from c-*fos*-deficient cells (8). This contrasts with our results which demonstrate that, *in vitro*, the VEGF mRNA level is not affected by c-*fos*. It is likely that other events must happen before VEGF is induced during tumor progression since this effect can only be observed *in vivo*.

It has been shown that continuos c-*fos* expression induces morphological transformations *in vitro*. These morphological modifications require at least 24 h of continuous c-*fos* expression (6). We observed that FIGF is induced with a slow kinetics in response to c-*fos* and its overexpression induces morphological transformations in fibroblasts. These morphological alterations are similar to those induced by the overexpression of c-*fos* in fibroblasts. Taken together, these data suggest that FIGF is a mitogenic and morphogenic effector of c-*fos*. Thus, the role of c-*fos* in the activation of the malignant phenotype is due, at least in part, to the induction of FIGF. The involvement of FIGF in tumor progression could represent a promising step toward the therapeutic prevention of neoplastic diseases.

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1. Ransone, L. & Verma, I. M. (1990) *Annu. Rev. Cell Biol.* **6,** 539–557.
2. Angel, P. & Karin, M. (1991) *Biochim. Biophys. Acta* **1072,** 129–157.
3. Karin, M. (1995) *J. Biol. Chem.* **270,** 16483–16486.
4. Wang, Z.-Q., Ovitt, C., Gregoriadis, A. E., Möhle-Steinlein, U., Rüther, U. & Wagner, E. F. (1992) *Nature (London)* **360,** 742–745.
5. Johnson, R. S., Spiegelman, B. M. & Papaioannou, V. E. (1992) *Cell* **71,** 577–586.
6. Miao, G. G. & Curran, T. (1994) *Mol. Cell. Biol.* **14,** 4295–4310.
7. Grigoriadis, A. E., Wang, Z.-Q. & Wagner, E. F. (1995) *Trends Genet.* **11,** 436–441.
8. Saez, E., Rutemberg, S. E., Mueller, E., Oppenheim, H., Smoluk, J., Yuspa, S. H. & Spiegelman, B. M. (1995) *Cell* **82,** 721–732.
9. Distel, R., Ro, H. S., Rosen, B. S., Groves, D. & Spiegelman, B. M. (1987) *Cell* **49,** 835–844.
10. Schontal, A., Herrlich, P., Rahmsdorf, H. J. & Ponta, H. (1988) *Cell* **54,** 325–334.
11. Kerr, L. D., Holt, J. T. & Matrisian, L. M. (1988) *Science* **242,** 1424–1427.
12. Superti-Furga, G., Berges, G., Picard, D. & Busslinger, M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5114–5118.
13. Kho, C.-J. & Zarbl, H. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 2200–2204.
14. Hu, E., Mueller, E., Oliviero, S., Papaioannou, V., Johnson, R. & Spiegelman, B. (1994) *EMBO J.* **13,** 3094–3103.
15. Chomzyski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
16. Liang, P. & Pardee, A. B. (1992) *Science* **257,** 967–971.
17. Liang, P., Averboukl, L. & Pardee, A. B. (1993) *Nucleic Acids Res.* **21,** 3269–3275.
18. Liang, P. & Pardee, A. B. (1995) *Curr. Opin. Immunol.* **7,** 274–280.
19. Bauer, D., Müller, H., Reich, J., Riedel, H., Ahrenickiel, V., Warthoe, P. & Strauss, M. (1993) *Nucleic Acids Res.* **21,** 4272–4280.
20. Hoppe, J., Weilch, H. A., Eichner, W. & Tatje, D. (1990) *Eur. J. Biochem.* **187,** 207–214.
21. Hoppe, J., Weich, H. A. & Eichner, W. (1989) *Biochemistry* **28,** 2956–2960.
22. Betsholds, C., Johnsson, A., Heldin, C.-H., Westermark, B., Lind, P., Urdea, M. S., Eddy, R., Shows, T. B., Philphott, K., Mellor, A. L., Knott, T. J. & Scott, J. (1986) *Nature (London)* **320,** 695–699.
23. von Heijne, G. (1986) *Nucleic Acids Res.* **14,** 4683–4690.
24. Leung, D. W., Cachianes, G., Kuang, W., Goeddel, D. V. & Ferrara, N. (1989) *Science* **246,** 1306–1309.
25. Keck, P. J., Hauser, S. D., Krivi, G., Sanzo, K., Warren, T., Feder, J. & Connolly, D. T. (1989) *Science* **246,** 1309–1312.
26. Maglione, D., Guerriero, V., Viglietto, G., Delli-Bovi, P. & Persico, M. G. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 9267–9271.
27. Claffey, K. P., Wilkinson, W. O. & Spiegelman, B. M. (1992) *J. Biol. Chem.* **267,** 16317–16322.
28. Joukov, V., Pajusola, K., Kaipainen, A., Chilov, D., Lathinen, I., Kukk, E., Saksela, O., Kalkkinen, N. & Alitalo, K. (1996) *EMBO J.* **15,** 290–298.
29. Olofsson, B., Pajusola, K., Kaipainen, A., von Euler, G., Joukov, V., Saksela, O., Orpana, A., Petterson, R. F., Alitalo, K. & Erikson, U. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 2576–2581.
30. Andersson, M., Östman, A., Bächström, G., Hellman, U., George-Nascimento, G., Wastermark, B. & Heldin, C.-H. (1992) *J. Biol. Chem.* **267,** 11260–11266.
31. Wagner, B. J., Hayes, T. E., Hoban, C. J. & Cochran, B. (1990) *EMBO J.* **9,** 4477–4484.
32. Silvennoinen, O., Schindler, C., Schessinger, J. & Levy, D. E. (1993) *Science* **261,** 1736–1738.
33. Manfioletti, G., Brancolini, C., Avanzi, G. & Schneider, G. (1993) *Mol. Cell. Biol.* **13,** 4976–4985.
34. Schneider, C., King, R. M. & Philipson, L. (1988) *Cell* **54,** 787–793.
35. Oliviero, S., Robinson, G., Struhl, K. & Spiegelman, B. M. (1992) *Genes Dev.* **6,** 1799–1809.
36. Kim, K., Li, B., Winer, J., Armanini, M., Gilleet, N., Philips, H. S. & Ferrara, N. (1993) *Nature (London)* **362,** 841–844.