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Polypeptide Growth Factors and Phorbol Ester Induce Progressive Ankylosis (Ank) Gene Expression in Murine and Human Fibroblasts

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Abstract Polypeptide growth factors promote cellular proliferation by binding to specific plasma membrane-anchored receptors. This interaction triggers the phosphorylation of signal transducing molecules and the transcriptional activation of numerous genes. We have used a differential display approach to identify fibroblast growth factor (FGF)-1-inducible genes in murine NIH 3T3 fibroblasts. Here we report that one of these genes encodes ank, a type IIIa transmembrane protein reported to function in cells as an inorganic pyrophosphate transporter. FGF-1 induction of ank mRNA expression is first detectable at 2 h after growth factor addition and is dependent on de novo RNA and protein synthesis. Ank gene expression is also upregulated after treating quiescent fibroblasts with several other mitogenic agents (e.g., calf serum or platelet-derived growth factor-BB) or the tumor promoter phorbol 12-myristate 13-acetate. Furthermore, in comparison to parental NIH 3T3 cells, oncogene-transformed NIH 3T3 cells constitutively express elevated levels of ank mRNA. FGF-1 also increases ank gene expression in non-immortalized human embryonic lung fibroblasts. Finally, the murine and human ank genes are expressed in vivo in a tissue-specific manner, with highest levels of mRNA expression found in brain, heart, and skeletal muscle. These results indicate that ank is a growth factor-regulated delayed-early response gene in mammalian cells, and we propose that increased ank expression during cell cycle progression may be necessary to maintain proper intracellular pyrophosphate levels during conditions of high cellular metabolic activity. J. Cell. Biochem. 84: 27–38, 2002.

Key words: fibroblast growth factor; phorbol ester; ankylosis; craniometaphyseal dysplasia; inorganic pyrophosphate

The majority of the cell types within the healthy adult are either terminally-differentiated or in a quiescent, nonproliferative growth state. This latter class of cells can be stimulated to re-enter the cell cycle and proliferate if they are exposed to the appropriate polypeptide growth factors. For example, skin fibroblasts proliferate during wound repair, capillary endothelial cells proliferate during blood vessel formation (angiogenesis), and vascular smooth muscle cell proliferation occurs in response to vessel wall injury. These cellular growth responses in vivo have been primarily studied at the molecular level using growth factor-stimulated mammalian cells cultured in vitro. It is now well established that polypeptide growth factors act on quiescent cells by binding and thereby activating specific transmembrane receptor tyrosine kinases. The activation of cell surface receptors by these polypeptide ligands triggers downstream intracellular events, including the stimulation of protein phosphorylation cascades and the transcriptional activation of specific genes [reviewed in Winkles, 1998; Jones and Kazlauskas, 2000]. Recent DNA microarray hybridization studies have revealed that there are hundreds of growth factor-inducible genes in the mammalian genome, and that these genes encode functionally-diverse polypeptides implicated in a wide variety of cellular processes [Fambrough et al., 1999; Iyer et al., 1999].

Our laboratory has been studying fibroblast growth factor (FGF)-1- and FGF-2-regulated

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gene expression in murine NIH 3T3 fibroblasts. FGF-1 and FGF-2 are the most extensively characterized members of the FGF family of structurally-related, heparin-binding proteins [reviewed in Burgess and Winkles, 1996; McKeehan et al., 1998]. They are potent mitogenic, chemotactic, angiogenic, and neurotrophic factors both in vitro and in vivo. These cellular responses are mediated via high-affinity binding to a family of related membrane-spanning tyrosine kinase receptors [reviewed in Burgess and Winkles, 1996; McKeehan et al., 1998]. We have shown that FGF-1 or FGF-2 stimulation of quiescent NIH 3T3 cells promotes the transcriptional activation of various immediate-early response, delayed-early response, and late response genes [Winkles, 1998]. In addition, we have demonstrated that a differential display approach [Donohue et al., 1997] can be successfully used to discover novel FGF-1- and FGF-2-inducible genes; e.g., we reported previously the identification of the Fnk and Fn14 immediate-early response genes, which encode a serine/threonine protein kinase [Donohue et al., 1995; Chase et al., 1998] and a plasma membrane protein [Meighan-Mantha et al., 1999; Feng et al., 2000], respectively.

In this paper, we report that one of the FGF-1-inducible delayed-early response genes identified using differential display encodes a recently described protein named ank [Ho et al., 2000]. Ank is the product of the mouse progressive ankylosis locus, and a mutation at this locus causes a form of arthritis characterized by excessive mineralization of articular cartilage [Sweet and Green, 1981; Hakim et al., 1984; Mahowald et al., 1988; Mahowald et al., 1989; Sampson and Trzeciakowski, 1990; Sampson et al., 1991]. In addition, heterozygous mutations in the human ank gene are associated with the disease craniometaphyseal dysplasia [Nurnberg et al., 2001]. Ank is a multipass transmembrane protein, and it appears to be involved in the transport of inorganic pyrophosphate from intracellular to extracellular compartments [Ho et al., 2000; Nurnberg et al., 2001]. Since pyrophosphate is a metabolic byproduct of many biosynthetic reactions [Russell, 1976], we propose that increased ank expression during growth factor-stimulated cell cycle progression is required to maintain basal intracellular levels of pyrophosphate during conditions of high metabolic activity.

MATERIALS AND METHODS

Cell Culture

Murine NIH 3T3 fibroblasts (American Type Culture Collection) were grown, expanded, and serum-starved as described [Meighan-Mantha et al., 1999]. Serum-starved cells were then either left untreated or treated with either 10 ng/ml human recombinant FGF-1 (kind gift of W. Burgess, Clearant Inc.) in combination with 5 U/ml heparin (Upjohn), 10 ng/ml human recombinant FGF-2 (Bachem), 10 ng/ml human recombinant platelet-derived growth factor-BB (PDGF-BB; Genzyme), 2 ng/ml human recombinant transforming growth factor-β1 (TGF-β1; R&D Systems), 20 ng/ml human recombinant epidermal growth factor (EGF; Genzyme), 20 ng/ml human recombinant insulin-like growth factor-1 (IGF-1; Bachem), 10% calf serum (Hyclone) or 30 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma). In some experiments, NIH 3T3 cells were treated with 2 μg/ml actinomycin D (Calbiochem) or 10 μg/ml cycloheximide (Sigma). NIH 3T3 cell lines transfected with either the pZIP-NeoSV expression vector or expression plasmids encoding activated Ha-Ras (G12R) or c-Raf-1 (ΔRaf-22W) were kindly provided by A. Belkin (Holland Laboratory) and cultured in normal NIH 3T3 cell growth medium as described above. Human M426 embryonic lung fibroblasts (kindly provided by J. Rubin, National Institutes of Health) were grown as described [Feng et al., 2000]. They were incubated in Dulbecco's modified Eagle's medium F-12 medium (Mediatech) containing 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenious acid (ITS; Collaborative Biomedical Products) for 48 h prior to treatment with 10 ng/ml FGF-1, 10% calf serum or 30 ng/ml PMA.

RNA Isolation and Differential Display

Tissue culture cells were harvested by trypsin/EDTA treatment and total RNA isolated using RNA Stat-60 (Tel-Test Inc.) according to the manufacturer's instructions. Tissues from newborn (1–5-day-old) or adult FVB/N mice (Taconic Farms) were obtained and RNA was isolated as above but the samples were initially homogenized using a Tissumizer (Tekmar). RNA concentrations were calculated by measuring UV light absorbance at 260 nm. For the differential display experiment described in this report, RNA (1 μg) isolated from quiescent

or FGF-1-stimulated NIH 3T3 cells was converted into cDNA using M-MLV reverse transcriptase (BRL/Gibco) and random hexamer primers (Boehringer Mannheim) as described previously [Meighan-Mantha et al., 1999]. Polymerase chain reaction (PCR) assays were then performed using a sense protein kinase domain oligonucleotide primer [described in Donohue et al., 1995] and the antisense src homology-2 (SH2) domain oligonucleotide primer 5′-GGTCTCAGACTCCCGCACCAGGAA-3′. This latter primer was designed using codon utilization data [Lathe, 1985] and was based upon a highly conserved region (FLVRESET) found in several SH2 domains [Koch et al., 1991]. These two PCR primers were used in an attempt to identify cDNA fragments encoding proteins containing both a protein kinase catalytic domain and an SH2 protein interaction domain. An equivalent aliquot of each amplification mixture was subjected to electrophoresis in a 2% agarose gel. DNA was visualized by ethidium bromide staining. A ~200-base pair (bp) DNA fragment was excised, recovered using the freeze-squeeze method [Tautz and Renz, 1983], reamplified, and ligated into the vector pCRII using a T/A cloning kit (Invitrogen Corp.).

cDNA Library Screening

A mouse Balb/c 3T3 cell cDNA library (kind gift of T. Lanahan, Johns Hopkins University School of Medicine) was screened with the PCR-derived murine ank DNA fragment to obtain longer cDNA clones. The DNA fragment was labeled with α-^{32}P-dCTP (3,000 Ci/mmol, Amersham) using a random primer DNA labeling kit (Boehringer Mannheim) according to the manufacturer's instructions. Approximately 2×10^5 phage were plated at a density of 2×10^4 plaque-forming units/150-mm dish using *Escherichia coli* C600 Hfl as host. Duplicate plaque lifts (Colony/Plaque screen, DuPont) were prehybridized, hybridized, washed, and exposed to film as described [Meighan-Mantha et al., 1999]. A single positive phage was plaque-purified and amplified on *E. coli* C600 Hfl cells. Plasmid was excised from the phage clone using XL-1 Blue cells and R408 helper phage (Stratagene) as described [Short et al., 1988].

5′-Rapid Amplification of cDNA Ends (RACE) Assays

Several cDNA fragments representing the 5′ region of murine ank mRNA were identified by reverse transcription-PCR (RT-PCR) using mouse brain 5′-RACE-Ready cDNA (Clontech) according to the manufacturer's instructions. The ank antisense oligonucleotide primer used in the PCR amplification step was 5′-CCAGCATGAGTCCACGCCATGGCATCCATA-3′ (nucleotides 450–479 of the murine ank cDNA sequence). Amplification products were subjected to electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining. Two DNA fragments of slightly different sizes were recovered, reamplified, and cloned into pCRII as described above.

RT-PCR Amplification of a Human Ank cDNA

Human fetal brain poly(A) + RNA (1 μg; Clontech) was converted to cDNA and PCR was performed for 35 cycles as described [Brogi et al., 1993]. The ank sense oligonucleotide primer was 5′-TGGGATGTGCCTCAATCTCAGATG-3′ (nucleotides 512–535 of the murine ank cDNA sequence) and the antisense oligonucleotide primer was 5′-TGTGACCTCCTCTGTTGGAG-3′ (nucleotides 1457–1476 of the murine ank cDNA sequence). Amplification products were subjected to electrophoresis in a 1.2% agarose gel and visualized by ethidium bromide staining. The predicted 965-bp fragment was recovered, reamplified, and cloned into pCRII as described above.

cDNA Sequence Analysis

Plasmid DNA was purified using a Magic Miniprep kit (Promega Corp.) and both strands of the entire ~2.2-kilobase (kb) murine ank cDNA clone were sequenced by the dideoxynucleotide chain termination method. Both strands of the two murine ank cDNA fragments isolated by the 5′-RACE technique were also completely sequenced. The identity of the RT-PCR-generated ~1.0-kb human ank cDNA was confirmed by sequencing the 5′ and 3′ ends. Sequencing was either done automatically using an Applied Biosystems model 373A DNA sequencer and a Dye Terminator Cycle Sequencing kit (Perkin Elmer) or manually using a Sequenase 2.0 kit (U.S. Biochemical Corp.) and α-^{35}S-dATP (1,000 Ci/mmol, Amersham Corp.). The nucleotide and deduced protein sequence for murine ank has been deposited in the GenBank database under GenBank Accession Numbers AF001532 and AF001533. It should be noted that this gene was named Fn54 at the

time when these sequences were submitted to the database.

Northern Blot Hybridization

Each RNA sample (10 μg) was denatured and subjected to electrophoresis in 1.2% agarose gels containing 2.2 M formaldehyde. The gels were stained with ethidium bromide to verify that each lane contained similar amounts of undegraded rRNA. RNA was transferred onto Zetabind nylon membranes (Cuno Inc.) by electroblotting and then cross-linked to the membrane by UV light irradiation using a Stratalinker (Stratagene). Northern blots containing 2 μg of poly(A) + RNA isolated from either mouse embryos at different developmental stages or various human tissues were purchased from Clontech. Membrane prehybridization, hybridization, and washing conditions were as described [Meighan-Mantha et al., 1999]. The four cDNA hybridization probes were: (a) mouse ank, ~2.2-kb EcoRI/XhoI fragment of pBluescript/mAnk, (b) human ank, ~1.0-kb EcoRI fragment of pCRII/hAnk, (c) mouse Fn14, ~1.0-kb EcoRI/XhoI fragment of pBluescript/mFn14 [Meighan-Mantha et al., 1999], and (d) human glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ~0.8-kb Pst1/Xba1 fragment of pHcGAP (American Type Culture Collection). The probes were radiolabeled with α-^{32}P-dCTP as described above. The blots were air-dried and autoradiography performed using Kodak X-Omat AR film.

Western Blot Analysis

Cells were washed with phosphate-buffered saline (PBS), collected by scraping with a rubber policeman, and pelleted by centrifugation at 500*g*. Cell pellets were resuspended in ~200 μl of 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1 × protease inhibitor mixture (PharMingen) and lysed on ice for 20 min by the addition of Triton X-100 to a final concentration of 1.2%. Lysates were clarified by centrifugation at 2,000*g* for 5 min at 4°C. The protein concentration of each supernatant was determined using the BCA assay kit (Pierce). An equal amount of each sample was mixed with 2 × gel-loading buffer and subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) using a 10% polyacrylamide gel without heating of the sample. Proteins were transferred to a nitrocellulose membrane and the membrane was blocked as previously described [Meighan-Mantha et al., 1999]. The membrane was then incubated for 1 h at room temperature in Tris–buffered saline with Tween-20 (TBST) containing 5% bovine serum albumin and a 1:500 dilution of anti-ank polyclonal serum (kind gift of D. Kingsley, Stanford University School of Medicine), washed three times in TBST, and incubated for 1 h at room temperature in TBST containing 5% nonfat dry milk and a 1:10,000 dilution of goat anti-rabbit Ig-horseradish peroxidase (BioRad). The blot was again washed three times with TBST and bound secondary antibody was detected using the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

RESULTS

Identification of an FGF-1-Inducible Gene by mRNA Differential Display

We have described previously a differential display approach which employs random hexamer-primed cDNA templates, PCR oligonucleotide primers designed to anneal with DNA sequences encoding particular protein domains (i.e., motif primers), and agarose gel electrophoresis [reviewed in Donohue et al., 1997]. In the present experiments, RNA was isolated from quiescent or FGF-1-stimulated NIH 3T3 cells and converted into cDNA. PCR assays were then performed using sense protein kinase domain and antisense SH2 domain oligonucleotide primers. Amplification products were displayed using agarose gel electrophoresis and ethidium bromide staining. The pattern of amplified cDNAs obtained from quiescent or FGF-1-stimulated cellular RNA were, for the most part, similar (Fig. 1). However, an ~200-bp DNA fragment was amplified to a greater degree when cDNA representing the RNA isolated from cells treated with FGF-1 for 8 or 12 h was used as a template. This cDNA fragment was isolated, cloned, and used as a probe in preliminary Northern blot hybridization experiments to confirm that it did indeed represent an FGF-1-inducible mRNA.

Isolation of Longer cDNA Clones and cDNA Sequence Analysis

A mouse fibroblast cDNA library was then screened with the ~200-bp DNA fragment in order to isolate longer cDNA clones. One positive phage was isolated that contained an ~2.2-kb cDNA insert. The nucleotide sequence

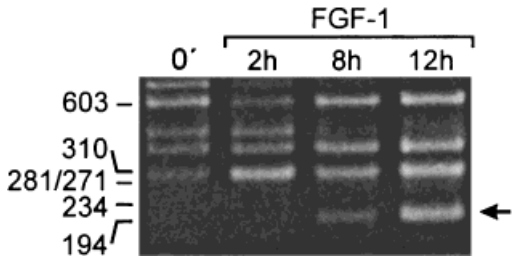

Fig. 1. Identification of an FGF-1-inducible mRNA in NIH 3T3 cells by differential display. Serum-starved cells were either left untreated or treated with FGF-1 for 2, 8, or 12 h. RNA was isolated, cDNA was synthesized, and PCR was performed using sense protein kinase domain and antisense src homology-2 domain primers. Amplification products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. DNA size markers (in bp) are shown on the left. The arrow denotes the DNA fragment that was recovered and cloned.

of this cDNA contained a long open-reading frame and a 1239-nucleotide (nt) 3′-untranslated region with a typical polyadenylation signal followed by a poly(A) tract. However, an initiating ATG methionine codon was not present. Therefore, we used the 5′-RACE method to isolate cDNA fragments encoding additional amino-terminal protein sequence. DNA sequence analysis of the longest 5′-RACE-derived cDNA clone (479-bp) indicated that the 3′ region was identical to the 5′ region of the ~2.2-kb cDNA and that the additional 5′ sequence contained an in-frame ATG codon flanked by a favorable sequence for translation initiation [reviewed in Kozak, 1991]. Also, an in-frame TAG termination codon was present 30-nt upstream of the presumed initiating ATG. The composite cDNA nucleotide sequence is predicted to encode a 492-amino acid (aa) protein with a predicted molecular mass of 54,221 Da and an estimated isoelectric point of 7.56. Analysis of the predicted protein sequence using several proteomics tools accessible through the ExPasy Molecular Biology Server (e.g., SignalP, ScanProsite, PSORT II, HMMTOP, SOSUI, TMAP, TMpred) revealed that this protein could be classified as a type IIIa transmembrane protein with 8–12 hydrophobic membrane-spanning regions. The predicted protein also contains three potential N-glycosylation sites and numerous putative serine/threonine phosphorylation sites. A recent search of the GenBank database indicated that this cDNA had 100% nucleotide sequence identity to the murine progressive ankylosis (ank) gene recently described by Ho et al. [2000].

Regulation of Ank mRNA Expression in NIH 3T3 Cells

We initially examined the kinetics of ank mRNA accumulation following FGF-1 stimulation of cell growth. Quiescent NIH 3T3 cells were either left untreated or treated with FGF-1 for various lengths of time. RNA was then isolated and Northern blot hybridization analysis was performed. Two ank transcripts of ~3.7- and ~3.3-kb in size were detected in FGF-1-treated cells (Fig. 2A). Increased ank mRNA levels were first evident at 2 h after FGF-1 addition and maximal levels were present at 12 h.

The effect of the RNA synthesis inhibitor actinomycin D on FGF-1 induction of ank mRNA levels was then examined. Quiescent cells were either left untreated or treated with FGF-1 alone, both FGF-1 and actinomycin D, or actinomycin D alone for 8 h. Cells were collected, RNA was isolated, and ank mRNA levels analyzed by Northern blot hybridization. Actinomycin D treatment prevented FGF-1 induction of ank mRNA (Fig. 2B); thus, the increase in ank mRNA expression after FGF-1 addition is likely to be due, at least in part, to transcriptional activation of the ank gene.

We next used the protein synthesis inhibitor cycloheximide to determine whether FGF-1 induction of ank mRNA levels was dependent on de novo protein synthesis. Quiescent cells were either left untreated or treated with FGF-1 alone, both FGF-1 and cycloheximide, or cycloheximide alone for 8 h. Cells were collected, RNA was isolated, and Northern blot hybridization analysis was performed. FGF-1 induction of ank mRNA levels did not occur in the presence of cycloheximide (Fig. 2C). This indicates that FGF-1-induced ank mRNA expression requires the synthesis of intermediary proteins; therefore, ank can be classified as a delayed-early response gene.

We then determined whether FGF-2, PDGF-BB, TGF-β1, EGF, IGF-1, calf serum, or the phorbol ester PMA could also increase ank mRNA levels in NIH 3T3 fibroblasts. Quiescent cells were either left untreated or treated with FGF-1 (as a positive control) or each of the above agents for 8 h. Cells were collected, RNA was isolated, and ank mRNA levels analyzed by Northern blot hybridization. All of these agents were able to increase ank mRNA expression

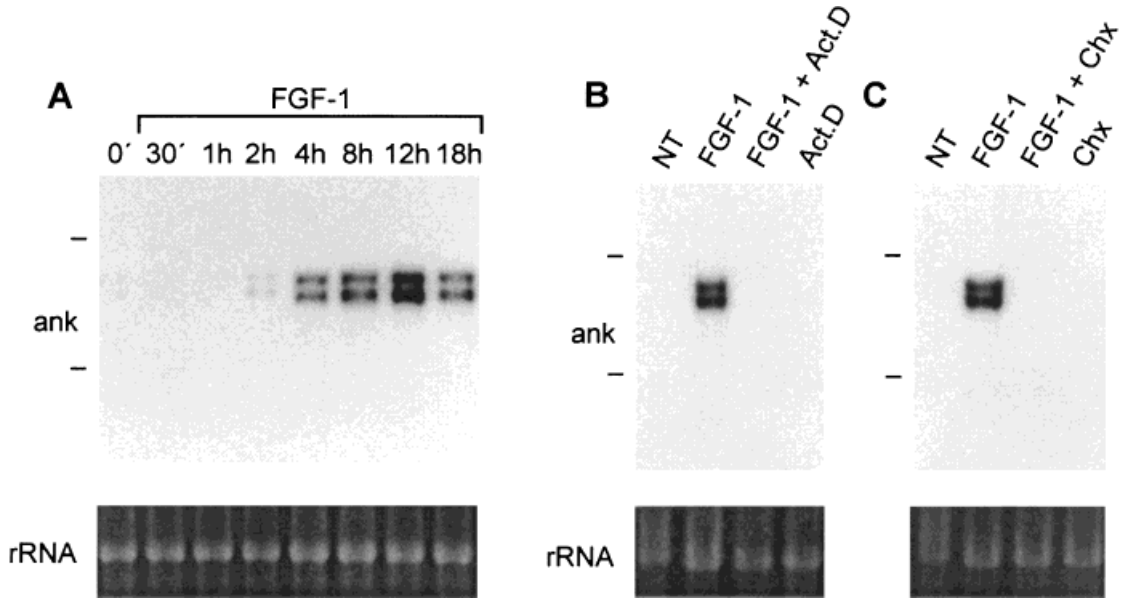

Fig. 2. Effect of FGF-1 on ank mRNA levels in NIH 3T3 cells. **A:** Serum-starved cells were either left untreated or treated with FGF-1 for the indicated time periods. RNA was isolated and equivalent amounts of each sample were analyzed by Northern blot hybridization. The upper and lower bars on the left side of the top panel represent the positions of 28S and 18S rRNA, respectively. In the bottom panel, a photograph of the 18S rRNA band is shown to demonstrate that equivalent amounts of RNA were present in each gel lane. **B:** Serum-starved cells were either left untreated (NT, no treatment) or treated with FGF-1, FGF-1 and actinomycin D (Act.D), or actinomycin D alone for 8 h. RNA was isolated and equivalent amounts of each sample analyzed as described above. **C:** Serum-starved cells were either left untreated (NT, no treatment) or treated with FGF-1, FGF-1 and cycloheximide (Chx), or cycloheximide alone for 8 h. RNA was isolated and equivalent amounts of each sample analyzed as described above.

(Fig. 3). PMA, which mimics diacylglycerol and thereby activates members of the protein kinase C family, was the strongest inducer, while FGF-1, FGF-2, PDGF-BB, and calf serum were slightly less potent. FGF-1 or FGF-2 treatment induced ank mRNA expression to a similar extent, consistent with the ability of these structurally-related proteins to bind the same tyrosine kinase receptors and promote similar biological effects on any given cell type [reviewed in Burgess and Winkles, 1996; McKeehan et al., 1998]. EGF treatment also increased ank mRNA expression, while TGF-β1 and IGF-1 had only a small stimulatory effect on ank mRNA levels.

Regulation of Ank Protein Expression in NIH 3T3 Cells

We next investigated whether growth factor stimulation of quiescent NIH 3T3 cells also resulted in elevated levels of the ank protein. For these experiments, we used FGF-2 as the mitogenic agent. Serum-starved cells were either left untreated or treated with FGF-2 for different lengths of time. Cell lysates were prepared and Western blot analysis was conducted using anti-ank polyclonal antiserum. Several NIH 3T3 cell proteins were recognized by the ank antiserum but FGF-2 stimulation increased the expression level of only one protein of ~42-kDa in size (Fig. 4). The temporal expression kinetics of this protein after FGF-2 addition were consistent with the ank mRNA expression pattern observed after FGF-1 addition (Fig. 2A). This immunoreactive protein was not detected when Western blot analysis was performed using these same protein samples and pre-immune serum as the primary antibody reagent (data not shown).

Ank mRNA Expression Levels in Transformed NIH 3T3 Cell Lines

Some growth factor-inducible genes are constitutively expressed at relatively high levels in rapidly proliferating transformed cell lines. We investigated whether ank gene expression was upregulated in NIH 3T3 cell lines stably expressing constitutively activated forms of either the c-Ha-Ras or c-Raf-1 proteins. Previous studies have demonstrated that each of these cell lines display a highly transformed

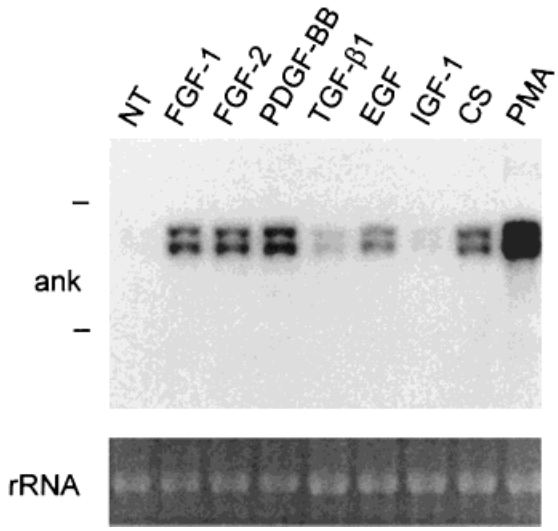

Fig. 3. Effect of various polypeptide growth factors, calf serum, or PMA on ank mRNA levels in NIH 3T3 cells. Serum-starved cells were either left untreated (NT, no treatment) or treated with FGF-1, FGF-2, PDGF-BB, TGF-β1, EGF, IGF-1, calf serum (CS), or PMA for 8 h. RNA was isolated and equivalent amounts of each sample analyzed by Northern blot hybridization. The upper and lower bars on the left side of the top panel represent the positions of 28S and 18S rRNA, respectively. In the bottom panel, a photograph of the 18S rRNA band is shown to demonstrate that equivalent amounts of RNA were present in each gel lane.

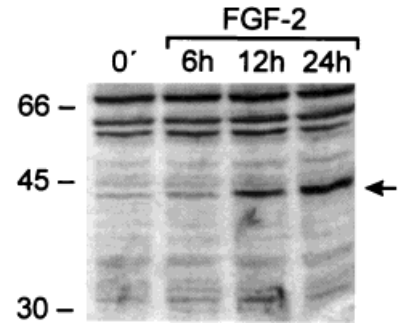

Fig. 4. Regulation of ank protein expression in NIH 3T3 cells. Serum-starved cells were either left untreated (0′) or treated with FGF-2 for 6, 12, or 24 h. Cell lysates were prepared, and equivalent amounts of protein were subjected to SDS–PAGE and Western blot analysis using anti-ank polyclonal antiserum. Molecular masses of protein size standards (in kDa) are shown on the left. The arrow denotes the position of ank protein.

phenotype (morphological alterations, rapid growth kinetics in serum-depleted medium, colony formation in soft agar, etc.) [Clark et al., 1995; Oldham et al., 1996; Belkin and Retta, 1998]. Control, vector-transfected NIH 3T3 cells as well as the Ras- and Raf-expressing transformed cell lines were grown to a similar cell density in the same growth medium and then harvested. RNA was isolated and Northern blot hybridization analysis was performed. Ank mRNA expression levels were significantly higher in the two transformed NIH 3T3 cell lines relative to vector-transfected NIH 3T3 cells (Fig. 5). In order to determine whether the overexpression of all growth factor-regulated genes was a general property of the oncogene-transformed lines, we also examined the expression level of the Fn14 gene. Fn14 is a mitogen-inducible, immediate-early response gene in NIH 3T3 cells and it too encodes a plasma membrane-anchored protein [Meighan-Mantha et al., 1999]. We found that Fn14 mRNA was expressed at a similar level in both the non-transformed and transformed NIH 3T3 cell lines.

Ank mRNA Expression in Mouse Embryos and Tissues

We next analyzed ank gene expression during mouse development and in various mouse tissues. A blot containing RNA isolated from four different developmental stages was obtained and ank mRNA levels were examined by Northern hybridization analysis. Ank mRNA expression was detected throughout development, and the level of expression was highest at

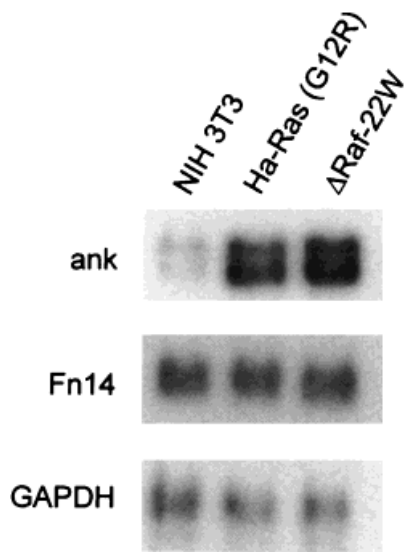

Fig. 5. Ank mRNA expression levels in Ha-Ras- and Raf-1-transformed NIH 3T3 cells. A control, vector-transfected NIH 3T3 cell line or transformed NIH 3T3 cell lines expressing either the activated Ha-Ras or Raf-1 proteins were grown to the same cell density (~70% confluence) in identical culture medium and then harvested. RNA was isolated and equivalent amounts of each sample analyzed by Northern blot hybridization using the cDNA probes indicated on the left.

17.5 days post-coitum (Fig. 6A). The tissue distribution of ank mRNA in both newborn and adult mice was also assayed by Northern blot hybridization. In newborn animals, ank transcripts were expressed at relatively high levels in heart, intestine, and skin (Fig. 6B). In adult mice, ank mRNA was expressed at the highest level in brain, heart, and skeletal muscle and at an intermediate level in intestine and skin (Fig. 6C). Ank mRNA was expressed at a relatively low level in the other seven adult tissues examined.

Ank mRNA Expression in Human Fibroblasts and Tissues

We then determined whether FGF-1 treatment also increased ank gene expression in human embryonic lung fibroblasts. First, we isolated an ~1.0-kb human ank cDNA clone using an RT-PCR approach. DNA sequence analysis of the 5′ and 3′ ends of this cDNA indicated that these regions had 100% nucleotide sequence identity to the human ank cDNA clone described in Ho et al. [2000]. Second, quiescent human M426 fibroblasts were either left untreated or treated with FGF-1 for various lengths of time. RNA was isolated and equivalent amounts of each sample were used for Northern blot hybridization analysis using the human ank cDNA as the radiolabeled probe. A major ank transcript of ~3.5-kb and a minor transcript of ~2.2-kb were detected in human fibroblasts (Fig. 7A). Increased ank mRNA levels were first evident at 4 h after FGF-1 addition and maximal levels were present at 8 h. Calf serum or phorbol ester treatment of M426 cells also induced ank mRNA expression (data not shown). These results demonstrate that ank is a mitogen-inducible gene not only in the immortalized NIH 3T3 cell line but also in diploid, non-immortalized human cells.

Ank mRNA levels were then examined in various human tissues in order to determine whether ank mRNA had the same tissue distribution in the human and mouse. A blot containing RNA isolated from eight different tissues was obtained and ank mRNA levels assayed by Northern hybridization. Ank mRNA was expressed at the highest level in heart, brain, and skeletal muscle and at significantly lower levels in placenta, lung, liver, kidney, and pancreas (Fig. 7B). This overall expression pattern is similar to that described above for mouse-derived tissues. In human tissues, as in murine tissues, two major ank transcripts were detected.

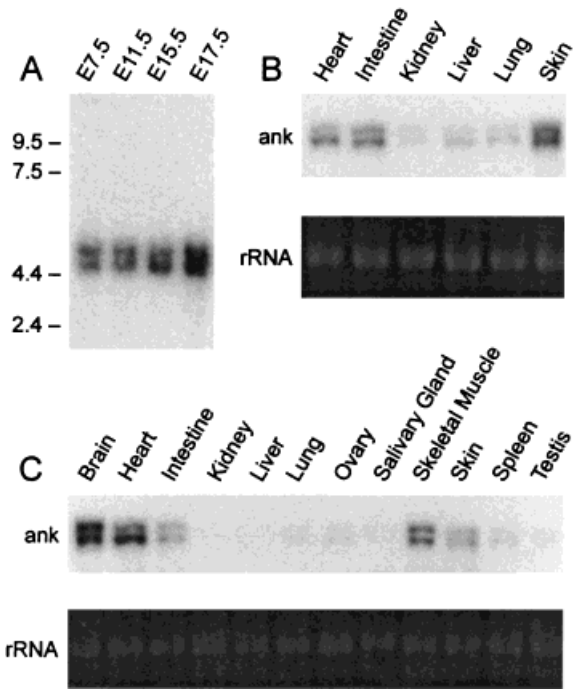

Fig. 6. Ank mRNA expression levels during mouse embryonic development and in various mouse tissues. **A:** A Northern blot containing RNA isolated from 7.5-, 11.5-, 15.5- or 17.5-day post-coitum mouse embryos was obtained and hybridization analysis was performed. RNA size markers (in kb) are shown on the left. **B:** RNA was isolated from the indicated newborn mouse tissues and equivalent amounts of each sample were analyzed by Northern blot hybridization. In the bottom panel, a photograph of the 18S rRNA band is shown to demonstrate that equivalent amounts of RNA were present in each gel lane. **C:** RNA was isolated from the indicated adult mouse tissues and equivalent amounts of each sample analyzed as described above.

DISCUSSION

The addition of serum or purified, individual polypeptide growth factors to quiescent fibroblasts promotes the transcriptional activation of numerous genes encoding proteins with diverse functions. Some of these proteins actually control cell cycle progression while others are required for specific cellular functions (e.g., energy production, biogenesis of organelles, nucleotide and DNA synthesis) or, in the case of serum-stimulated skin-derived fibroblasts, the wound healing response [Winkles, 1998; Iyer et al., 1999]. We have used a differential display approach to isolate

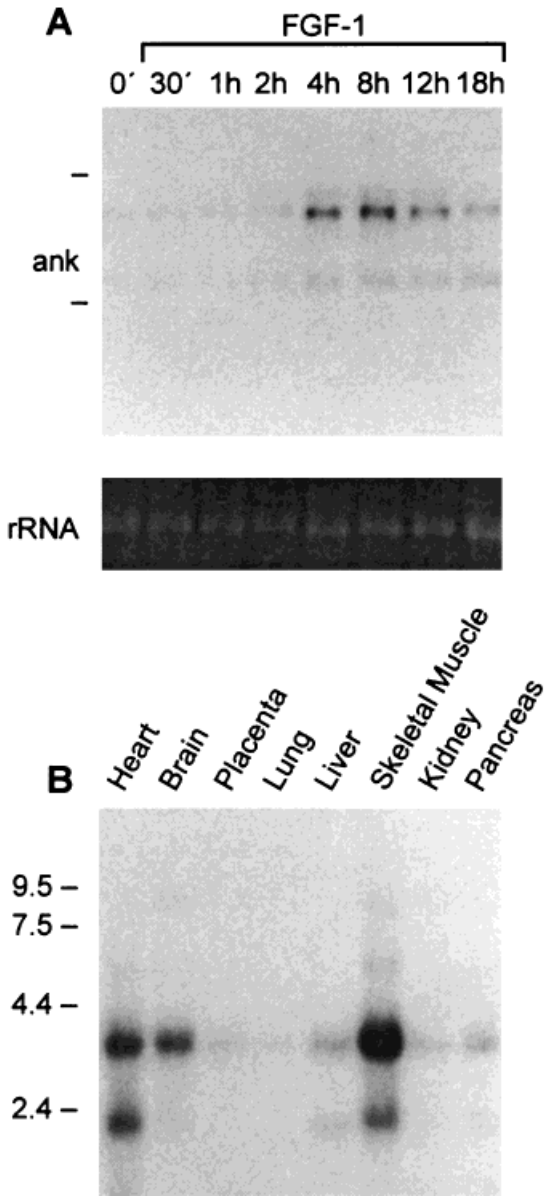

Fig. 7. Ank mRNA expression levels in FGF-1-stimulated M426 cells and in various human tissues. **A:** Serum-starved cells were either left untreated or treated with FGF-1 for the indicated time periods. RNA was isolated and equivalent amounts of each sample were analyzed by Northern blot hybridization using a human ank cDNA probe. The upper and lower bars on the left side of the top panel represent the positions of 28S and 18S rRNA, respectively. In the bottom panel, a photograph of the 18S rRNA band is shown to demonstrate that equivalent amounts of RNA were present in each gel lane. **B:** A Northern blot containing RNA isolated from the indicated human tissues was obtained and hybridization analysis was performed using a human ank cDNA probe. RNA size markers (in kb) are shown on the left.

cDNA fragments representing FGF-1-inducible genes in NIH 3T3 cells. In this paper, we report that the progressive ankylosis (ank) gene is a growth factor-inducible gene in both murine and human fibroblasts. Progressive ankylosis is an autosomal recessive mutation on chromosome 15 that arose spontaneously in the mouse colony at the Jackson Laboratory in the early 1970s [Sweet and Green, 1981]. Homozygous ank/ank mice develop a generalized, progressive form of arthritis that normally results in death by the age of 6 months [Sweet and Green, 1981; Hakim et al., 1984; Mahowald et al., 1988; Mahowald et al., 1989; Sampson and Trzeciakowski, 1990; Sampson et al., 1991]. Ho et al. [2000] used a positional cloning strategy to identify the mouse ank gene and show that the ank mutation creates a nonsense codon; consequently, ank/ank mice express a C-terminal truncated ank protein of 440 amino acid residues (instead of 492). This group also reported that the wild-type ank protein is present on the plasma membrane and is likely to function as an inorganic pyrophosphate transporter. The C-terminal truncated ank protein is less active than wild-type ank when these proteins are expressed at similar levels in transfected cells. Recently, it has been reported that patients with craniometaphyseal dysplasia, a rare condition characterized by overgrowth and sclerosis of the craniofacial bones and abnormal remodeling of the tubular bones, have mutations within the ank locus [Nurnberg et al., 2001]. These mutations predict changes in the ank protein sequence (single amino acid substitutions, insertions or deletions) and Nurnberg et al. [2001] speculate that they may be gain-of-function mutations.

Ank was identified as an FGF-1-inducible gene using a differential display approach that used protein motif-based PCR oligonucleotide primers [Donohue et al., 1997]. The original ~200-bp murine ank cDNA fragment was amplified using primers designed to recognize DNA sequences encoding proteins with both a protein kinase and a SH2 domain; however, these domains are not present in the predicted 492-aa protein sequence. This was not unexpected, since comparison of the primer sequences with the appropriate regions in the cDNA sequence revealed that, under the PCR conditions used, these primers were able to anneal to regions of relatively low nucleotide sequence identity (~56%). Indeed, the majority of the genes that we have identified using this differential display strategy do not encode proteins with the targeted structural motifs [Donohue

et al., 1994, 1996; Hsu et al., 1996a,b; Meighan-Mantha et al., 1999]. It is possible that the targeting aspect of this method could be improved by designing primers that are less degenerate in sequence and/or by increasing the PCR annealing temperature.

We demonstrated by Northern blot hybridization analysis that ank gene expression increased after FGF-1 stimulation of quiescent NIH 3T3 cells, with maximal ank mRNA levels detected at 12 h post-stimulation. This response did not occur in the presence of either an RNA or a protein synthesis inhibitor; thus, ank can be classified as a transcriptionally-regulated, delayed-early response gene. FGF-2 treatment induced ank mRNA expression to a similar extent as FGF-1, consistent with previous studies demonstrating that, in general, these structurally-related proteins promote similar biological effects on any given cell type [reviewed in Burgess and Winkles, 1996; McKeehan et al., 1998]. Ank mRNA expression levels also increased when murine fibroblasts were treated with other polypeptide growth factors or the tumor promoter PMA. Western blot analysis revealed that ank protein levels also increased after mitogenic stimulation of quiescent NIH 3T3 cells. In these experiments, we found that the apparent molecular mass of murine ank was ~42-kDa, ~20% less than the mass deduced from the cDNA sequence (~54-kDa). Western blot experiments analyzing ank expression in ank cDNA-transfected cells also revealed that the apparent molecular mass of this protein is not identical to its predicted mass [Ho et al., 2000]. Ank may migrate anomalously on SDS–polyacrylamide gels due to its high percentage of hydrophobic amino acid residues.

The ank gene is expressed in a tissue-specific manner in vivo, with highest levels of ank mRNA present in brain, heart, and skeletal muscle. Our Northern blot hybridization results examining ank gene expression levels in adult mouse tissues are not in total agreement with the findings of Ho et al. [2000], who reported a more ubiquitous pattern of ank mRNA distribution. This inconsistency may reflect differences in the mice used for tissue isolation (e.g., strain, sex, age). In both mouse and human tissues, two major ank mRNA species were detected and it has been proposed that the smaller transcript is generated via usage of an alternative polyadenylation site in the ank mRNA 3′-untranslated sequence [Ho et al., 2000]. Interestingly, the relative expression level of these two mRNAs in any one individual human tissue varied to a greater extent than observed for the murine tissues. For example, in the murine brain, the ~3.7- and ~3.3-kb ank transcripts were present at similar levels, but in the human brain, the ~3.5-kb transcript was clearly more abundant than the ~2.2-kb transcript. These results indicate that in humans there may be more tissue-specific utilization of the two ank mRNA alternative polyadenylation sites.

In summary, we have found that the progressive ankylosis gene recently described by Ho et al. [2000] and Nurnberg et al. [2001] is a polypeptide growth factor-, serum-, and tumor promoter-inducible gene in both immortalized and non-immortalized fibroblasts. In addition, we observed an increase in the basal level of ank gene expression in oncogene-transformed fibroblasts. Ank is a type IIIa transmembrane protein thought to primarily function in the transport of pyrophosphate from intracellular compartments to the extracellular environment. It is well established that mitogen treatment of quiescent cells stimulates an overall increase in metabolic activity. Transformed cells, with enhanced proliferation rates, are also very metabolically active. The cellular biosynthesis of proteins, lipids, nucleotides, and nucleic acids requires reactions that consume ATP and lead to pyrophosphate production. The observed increase in ank transporter expression during mitogen-stimulated cell cycle progression and following oncogenic transformation may be necessary for the efficient removal of the excess intracellular inorganic pyrophosphate generated in these cells.

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