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Cloning of a Growth Arrest-Specific and Transforming Growth Factor β-Regulated Gene, TI 1, from an Epithelial Cell Line

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By cDNA cloning and differential screening, five genes that are regulated by transforming growth factor β (TGFβ) in mink lung epithelial cells were identified. A novel membrane protein gene, TI 1, was identified which was downregulated by TGFβ and serum in quiescent cells. In actively growing cells, the TI 1 gene is rapidly and transiently induced by TGFβ, and it is overexpressed in the presence of protein synthesis inhibitors. It appears to be related to a family of transmembrane glycoproteins that are expressed on lymphocytes and tumor cells. The four other genes were all induced by TGFβ and correspond to the genes of collagen α type I, fibronectin, plasminogen activator inhibitor 1, and the monocyte chemotactic cell-activating factor (JE gene) previously shown to be TGFβ regulated.

Cell growth is believed to be controlled by a complex balance of stimulatory and inhibitory factors. Among factors with dual effects, transforming growth factor β (TGFβ) constitutes a family of pleiotropic cytokines with physiological importance. Three different forms of TGFβ have been identified in mammals, and homologs have been found in evolutionarily distant species (for a review, see reference 27).

TGFβ is a strong inhibitor of proliferation of most epithelial, endothelial, and lymphoid cells, whereas it stimulates growth of mesenchymally derived cells, probably through induction of platelet-derived growth factor (4, 23). In addition to its role in cell proliferation, TGFβ has been implicated in early embryo development, immunomodulation, stimulation of angiogenesis, and wound healing (41, 51; for a review, see reference 31). TGFβ obviously regulates expression of many genes, including those coding for extracellular matrix proteins, proteases, protease inhibitors, and acute phase-proteins (22, 25, 29). More directly, TGFβ can also induce expression of some serum-induced early genes; however, the expression pattern is not always related to growth control (36, 46, 49).

Reduction of *myc* gene expression and posttranslational modification of the retinoblastoma gene product have both been proposed as possible ways for TGFβ to exert its growth-inhibiting effect (21, 37, 38; for a review, see reference 30), although these events are not observed in all cell lines, indicating that TGFβ may act through several different pathways in inhibiting cell growth (40).

Although little is known about the mechanism of growth arrest by TGFβ, it is well documented that microinjection of mRNA from arrested cells into growing cells can induce growth arrest, suggesting that growth arrest may be mediated by specific mRNA species expressed during arrest (24, 35). One such cDNA has recently been cloned (32). Entry into quiescence or the G_0 phase is furthermore accompanied by the expression of a complex set of genes. From growth-arrested mouse fibroblasts, six genes, termed growth-arrest-specific (*gas*) genes 1 to 6, were isolated whose mRNAs accumulated when cells exit from the cell cycle (44). Another, apparently nonoverlapping, set of five genes termed *gadd* genes induced after exposure of cells to UV irradiation also proved to be specifically expressed in the G_0 phase (12).

To better understand the mechanism of the TGFβ response, we isolated and characterized TGFβ-regulated genes in growth-arrested mink lung epithelial cells.

MATERIALS AND METHODS

Tissue culture. The TGFβ-sensitive mink epithelial cell line CCL64 (21) was maintained in Dulbecco modified Eagle's medium with 10% fetal calf serum (FCS), penicillin, and streptomycin. The cells were passaged twice weekly by trypsinization and reseeded at a 10-fold dilution. For induction of quiescence and preparation of RNA, cultures were grown in 24- by 24-cm plates (Nunc, Roskilde, Denmark). After 3 days, the confluent cultures were shifted to fresh medium with 0.5% FCS and incubated for 2 additional days. Cells harvested at this point are termed arrested. TGFβ-treated cells were obtained by further incubation for 24 h in the presence of human TGFβ1 (1 ng/ml; R & D Systems, Minneapolis, Minn.). Serum-stimulated cells were obtained by exposure of arrested cells to 10% FCS for 4 h. DNA synthesis was scored by incorporation of bromodeoxyuridine for 6 h and detecting the incorporated nucleoside by a monoclonal antibody (Partec, Arlesheim, Switzerland) as previously described (26). Cycloheximide treatment of arrested cells was performed by treatment with cycloheximide (10 μg/ml), TGFβ1 (1 ng/ml), or a combination of the two.

Preparation of RNA and Northern (RNA) blots. For harvest of cells, the medium was aspirated and the cells were washed twice in phosphate-buffered saline, lysed in guanidine thiocyanate, and loaded onto a cushion of 4 ml of 5.7 M CsCl–1 mM EDTA in SW40 tubes (6). Total cellular RNA was pelleted by centrifugation at 33,000 rpm for 18 h; the pellet was resuspended in 10 mM Tris HCl (pH 7.5)–1 mM EDTA–0.5% sodium dodecyl sulfate, extracted with phenol once, and precipitated with ethanol. Poly(A) mRNA was prepared by passage over oligo(dT)-cellulose twice, and RNA was resuspended at a concentration of around 1 mg/ml in sterile distilled water. For Northern blot analyses, 10-μg aliquots of

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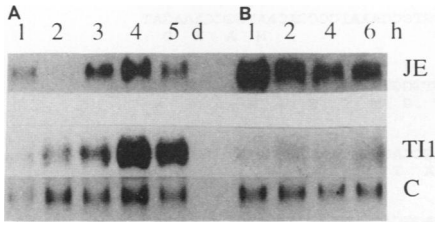

FIG. 1. Northern blot hybridization analysis of RNA from CCL64 cells harvested daily for 1 to 5 days (d) after passage as indicated in panel A. On day 3, the cultures were shifted to medium with 0.5% FCS, leading to arrest of DNA synthesis. After 2 days in low serum, the cells were fed with fresh medium containing 10% FCS, and cells were harvested 1, 2, 4, or 6 h later, as indicated in panel B. The blots were probed with the cDNA clone for the TI 1 gene and with the JE gene as a control. C corresponds to a mink cDNA clone hybridizing with an 800-bp mRNA, the expression of which was only moderately affected by cell growth or TGFβ exposure.

total cellular RNA were denatured with formamide and formaldehyde and then loaded onto 1.4% agarose gels. The RNA was transferred onto GeneScreen Plus hybridization membranes (Du Pont, Wilmington, Del.), and hybridization was performed as recommended by the supplier.

Construction and screening of cDNA libraries. cDNA was synthesized from 2.5 μg of poly(A)$^+$ mRNA from TGFβ-treated cells, using an oligo(dT) primer as previously described (14, 15). The cDNA was adapted with *Eco*RI linkers and subcloned into lambda gt10 arms (Stratagene, La Jolla, Calif.). The library contained about 4.5×10^5 recombinants and had an average insert size of about 1 kb (library I). Before screening, the library was amplified in *Escherichia coli* C600hfl. Screening was performed in *E. coli* C600. A second library was prepared with a directional cloning strategy (15) and cloned in plasmid pUEX (47). This second library had about 2.5×10^5 recombinants and an average insert size of 1.4 kb. A third library was prepared in lambda gt10 from CCL64 cells that after 36 h of culture were treated with TGFβ for 2 h in the presence of 10% FCS. This library contained about 10^5 recombinants, and about 2×10^4 phages were used for screening.

For screening, an aliquot of the amplified library I was plated at a density of 800 PFU/150-mm petri dish. A total of 10,000 plaques were screened. Two lifts were made from each petri dish, using nylon membranes (Duralon; Stratagene). The first lift from each plate was probed with a single-stranded cDNA probe from serum-stimulated CCL64 cells; the second lift was probed with cDNA from TGFβ-

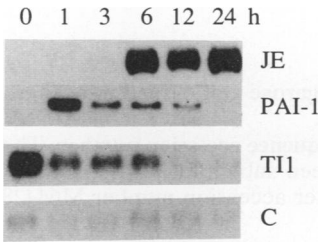

FIG. 2. Northern blot analysis of the effect of TGFβ on expression of the TI 1 gene in arrested CCL64 cells. Cells were harvested at the indicated hour following addition of TGFβ (1 ng/ml). The JE, PAI 1, and C clones were included as controls.

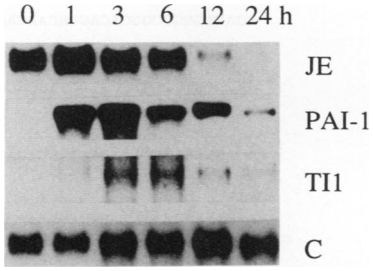

FIG. 3. Northern blot analysis of the effect of TGFβ on expression of the TI 1 gene in growing cells in the presence of 10% FCS (see Materials and Methods). Cells were harvested at the indicated hour after addition of TGFβ. The JE, PAI 1, and C clones were included as controls.

treated cells. Plaques that specifically hybridized with the cDNA probe from TGFβ-treated cells were isolated and rescreened twice. Inserts from purified lambda phages were excised with *Eco*RI and subcloned into the Bluescript pKS M13+ vector. Purified subcloned inserts were labeled (11) and used for cross-hybridization of all phages isolated and for probing of Northern blots with RNA from TGFβ-treated, arrested, and serum-stimulated CCL64 cells. Clones that specifically hybridized with RNA from TGFβ-treated cells were sequenced from both termini.

Subcloning, DNA sequencing, and sequence comparison. Dideoxy DNA sequencing was performed with use of a Sequenase kit (U.S. Biochemical, Cleveland, Ohio) or an automated sequencing protocol (20). The TI 1 clone was subcloned at seven unique sites in the 5′ half of the insert. The 3′ half of the clone was sequenced by using walking primers. Sequence analysis and comparison were performed with the University of Wisconsin Genetics Computer Group sequence analysis software package (9, 34).

Primer extension. A total of 10^5 cpm of ^{32}P-labeled primer complementary to positions 230 to 276 of TI 1 was annealed to 5 μg of poly(A) RNA from CCL64 cells in 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4)–0.4 M NaCl–1 mM EDTA–80% formamide for 16 h at 37°C, precipitated with ethanol, and resuspended in 20 μl of reverse transcription buffer. The annealed primer was extended at 42°C for 1 h with RNase H$^-$ mouse mammary tumor virus reverse transcriptase (Bethesda Research Laboratories, Bethesda, Md.). After RNase treatment, phenol-chloroform extraction, and ethanol precipitation, one-third of the reaction was analyzed on a 6% acrylamide–urea gel

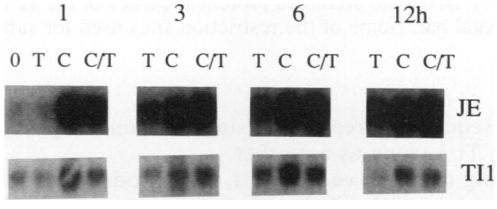

FIG. 4. Northern blot analysis of the effect of cycloheximide on the expression of two TGFβ-regulated genes in arrested CCL64 cells. Cells were grown to saturation density and shifted to low serum as described in the legend to Fig. 1. Cycloheximide at 10 μg/ml (C), TGFβ (T), or a combination of the two (C/T) was added, and cells were harvested at the indicated hour after addition of the reagents. The JE and TI 1 clones were used as probes.

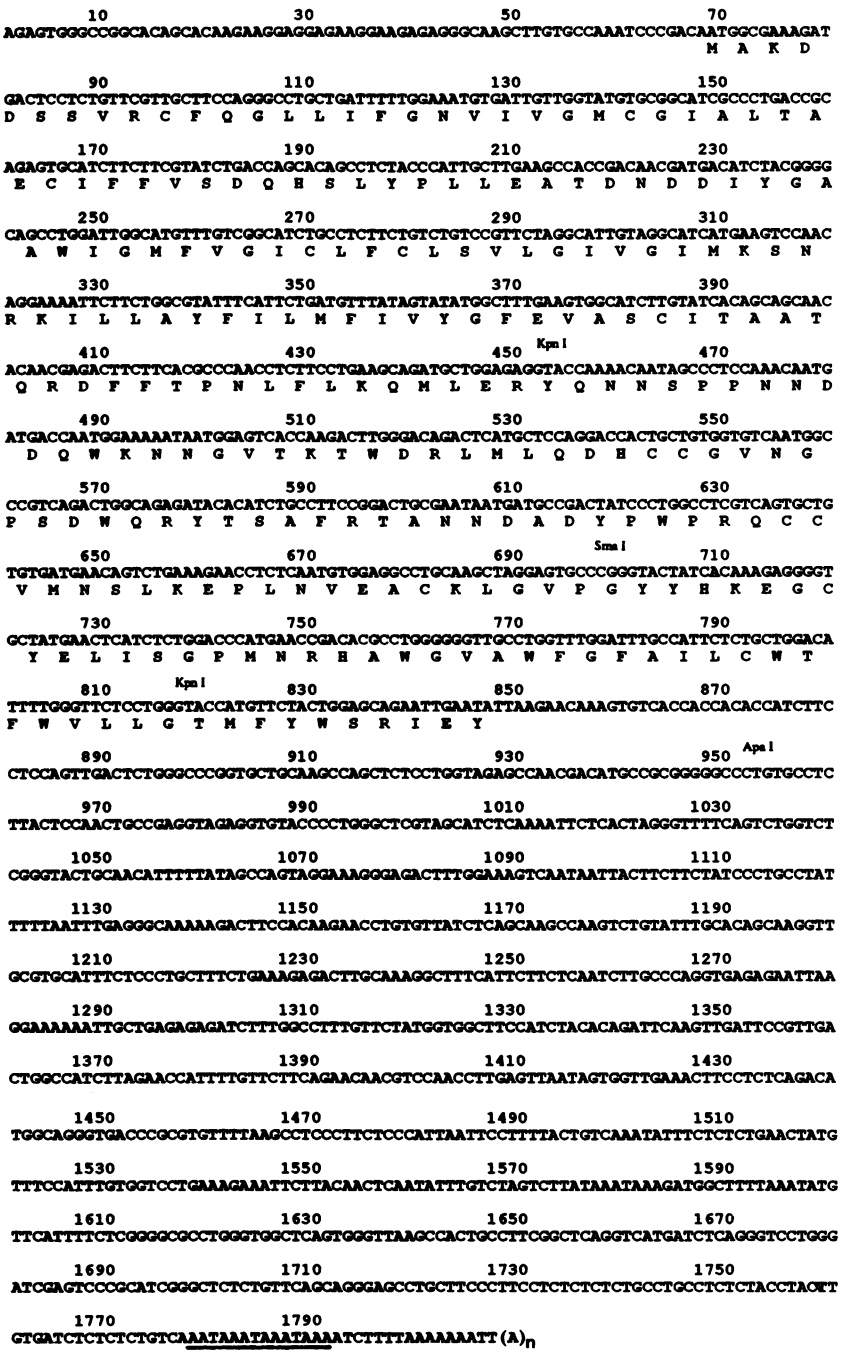

FIG. 5. DNA and predicted protein sequence of the TI 1 cDNA clone. The positions of the triple polyadenylation signals are indicated by a horizontal bar. Some of the restriction sites used for subcloning are indicated.

with a sequencing reaction, using the same oligonucleotide and the TI 1 clone as a marker.

Cloning of the 5′ end of TI 1. The product of the primer extension was dC tailed with terminal transferase (Boehringer, Mannheim, Germany) and amplified by 35 cycles of a polymerase chain reaction (94°C for 1 min; 50°C for 1.5 min; 72°C for 1.5 min), using an oligonucleotide complementary to positions 69 to 86 of the TI 1 sequence and oligo(dG) as primers. After filling in and kinasing, DNA of approximately 100 bp was separated from the primers on a 1.5% low-melting-point agarose gel, cloned into *Sma*I-cut pKSM13+, and sequenced.

Nucleotide sequence accession number. The sequence of TI 1 cDNA has been submitted to the EMBL/GenBank/DDBJ data banks under accession number M64428.

RESULTS

Genes specifically expressed in growth-arrested and TGFβ-treated cells. For the isolation of TGFβ-regulated genes,

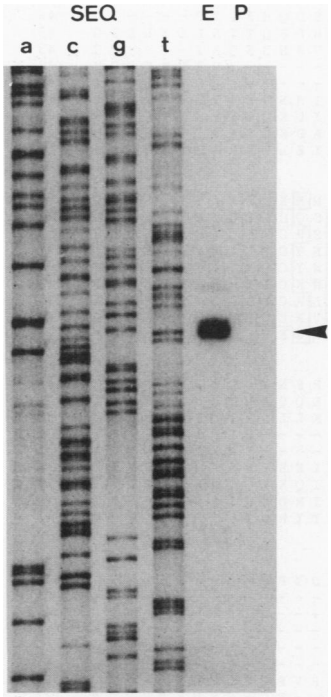

FIG. 6. Primer extension analysis of TI 1 RNA. E, extended primer (see arrow); P, unextended primer which has migrated out of the gel; SEQ, sequence of TI 1 with the same primer (ACGT).

cDNA library I was prepared in lambda gt10. The RNA was from mink lung epithelial cells that had first been arrested by exposure of confluent cultures to low serum for 48 h and then exposed to TGFβ at 1 ng/ml for 24 h in the same medium. In the TGFβ-treated cells, 0.1% of the cells incorporated bromodeoxyuridine into DNA, whereas the corresponding figure for arrested cells was around 1% (not shown). This library was differentially screened with a total cDNA probe from TGFβ-treated cells and a probe from serum-stimulated cells. By differential screening of 10,000 plaques, 32 clones that preferentially hybridized with cDNA from TGFβ-treated cells were recovered. Following subcloning into the Bluescript pKS M13+ plasmid and cross-hybridization, purified cDNA inserts were used to probe Northern blots of RNA from arrested, serum-stimulated, and TGFβ-treated cells. cDNA that specifically hybridized with RNA from arrested or TGFβ-treated cells was further examined by DNA sequence analysis and additional Northern blot analyses.

Seven cross-hybridizing clones detected a 5-kb mRNA expressed at high levels in TGFβ-treated cells. Upon DNA sequencing, one of them was found to be similar to human fibronectin mRNA. Another clone with a similar expression pattern was similar to collagen α type I mRNA. The fibronectin and collagen clones were not studied further since it has been amply demonstrated that TGFβ induces both collagen and fibronectin mRNA (for references, see reference 27). Likewise, two clones were isolated which upon sequencing were found to be identical to the plasminogen activator inhibitor 1 (PAI 1) gene (25) and the JE gene (42), respectively. The former clone was isolated by screening the third library prepared from RNA of rapidly growing CCL64 cells that had been treated for 2 h with TGFβ. Both RNAs were induced by TGFβ and used as internal controls in Northern blot analysis.

Finally, two cross-hybridizing clones hybridized with a 1.8-kb mRNA in arrested cells. The larger of these clones (TI 1), 1.4 kb in length, was used as a probe in subsequent Northern blot experiments.

Expression pattern of the TGFβ-regulated TI 1 gene. To determine how the TI 1 gene was affected by growth condition and TGFβ treatment, three different experiments were performed.

In the first type of experiment, the effect of serum starvation was analyzed. RNA was harvested daily for 5 days after reseeding of the cells. After day 3, the cultures were confluent and the cells were shifted to fresh medium containing 0.5% FCS. After day 5, the cultures were shifted back to fresh medium with 10% FCS, and cells were harvested after 1, 2, 4, and 6 h. The relatively abundant 1.8-kb mRNA detected by the TI 1 probe accumulated as the cells became confluent and remained at high levels after the shift to low serum. Addition of serum after 2 days in low serum resulted in a rapid decrease of RNA to a basal level which remained constant for the next 6 h (Fig. 1). A parallel blot (Fig. 1) probed with the JE clone as a control identified a 0.8-kb mRNA which was detectable at low levels during establishment of confluence. The shift to 10% FCS led to a sharp increase of mRNA which peaked after only 1 h and then slowly declined.

In a second experiment, TGFβ was added to the cultures after 2 days in low serum (day 5), and the cells were harvested 1, 3, 6, 12, and 24 h later. Addition of TGFβ led to a gradual reduction of the TI 1 mRNA, reaching a steady-state level after 12 h (Fig. 2). In contrast, the JE mRNA, present at low levels in control cells, remained unchanged for the first hours in TGFβ. This mRNA increased in abundance between 3 and 6 h and remained unchanged thereafter (Fig. 2). The PAI 1 clone, which was isolated from a cDNA library prepared from mRNA of actively growing TGFβ-treated cells, identified a 3.5-kb mRNA that reached its highest abundance after only 1 h of TGFβ exposure and then declined within 24 h to the level before addition of TGFβ (Fig. 2).

To establish whether the effect of TGFβ observed in Fig. 2 was associated with the quiescence of the cells, a third experiment was performed with actively growing cells. TGFβ was added 36 h after passage, and the cells were harvested at various times thereafter. Figure 3 shows that the TI 1 mRNA which was inhibited by TGFβ in arrested cells (Fig. 2) was transiently induced by TGFβ in growing cells, and maximal expression was observed 3 to 6 h after addition. After 24 h with TGFβ, the level of TI 1 expression had returned to the low levels observed before addition (Fig. 3). Expression of the JE mRNA was higher than in arrested cells (Fig. 1), with maximal induction after 1 h followed by a gradual decline (Fig. 3). The effect of TGFβ on the PAI 1 mRNA in growing cells followed the same time course as in arrested cells.

To determine whether the effect of TGFβ on expression of the TI 1 gene is dependent on ongoing protein synthesis, arrested cells were treated in the absence and presence of cycloheximide at 10 μg/ml (Fig. 4). The downregulation of TI 1 expression in arrested cells was partially blocked by the addition of cycloheximide (Fig. 4). The JE mRNA was induced by cycloheximide alone, without addition of TGFβ as previously demonstrated (42).

Sequence comparison of the TGFβ-regulated genes. The original TI 1 (1.4 kb) clone subcloned into Bluescript was sequenced from both termini, as were the subfragments generated by cleavage with *Kpn*I, *Sma*I, and *Apa*I (Fig. 5).

FIG. 7. Sequence alignment of the p28 protein encoded by the TI 1 cDNA clone and the family of related transmembrane glycoproteins. Co-029, colon tumor antigen (48); Me491, melanoma-associated antigen (16); Sm23, antigen from *S. mansoni* (52); CD37 (8), CD53 (2), R2 (13), and Tapa-1 (33), leukocyte antigens; Rds, protein encoded by a gene responsible for the retinal degeneration slow phenotype in mice (50). Amino acid identities and conservative substitutions are boxed.

An open reading frame extending 380 bp into the 5′ end was identified, and the 3′ end carried a 47-bp poly(A) tail. Two larger clones were isolated from a cDNA library in plasmid pUEX from TGFβ-treated cells. The larger of these clones was completely sequenced, and about 400 bases were sequenced for the shorter clone. The sequence of the full-length clone of TI 1 (1,807 bp) is shown in Fig. 5. An open reading frame starts at base 69 and ends at base 848, followed by 951 bp in the 3′ untranslated region and then a poly(A) tail. Since the initiator methionine at base 69 is not preceded by a stop codon, we analyzed whether we had obtained a full-length TI 1 clone. Primer extension using an oligonucleotide complementary to bases 230 to 276 of TI 1 was carried out. As shown in Fig. 6, the extension gives one major band corresponding to the first nucleotide in TI 1. In addition, the sequence of the 5′ end was confirmed by cloning the product of the primer extension by PCR and sequencing (data not shown).

The open reading frame codes for a hypothetical polypeptide of 260 amino acids with a molecular weight of ca. 28,500 (referred to as p28). The predicted p28 protein has an unusually balanced amino acid composition but has a relatively high (5%) cysteine content, including two cysteine doublets. Near the C terminus and at residues 60 to 100, clusters of hydrophobic amino acids are found. Charged residues are dispersed over the N-terminal 60 amino acids and residues 101 to 229. A potential site for N-linked glycosylation is found at residues 130 to 132.

Structural relationship between the hypothetical protein encoded by the TI 1 cDNA and a family of transmembrane glycoproteins. The sequence of the putative protein (designated p28) encoded by the TI 1 mRNA was compared with entries in the EMBL (release 26) and PIR (release 27) sequence libraries with the program FASTA (34). The search revealed a family of related transmembrane proteins, among them the membrane-bound glycoprotein Me491, which is

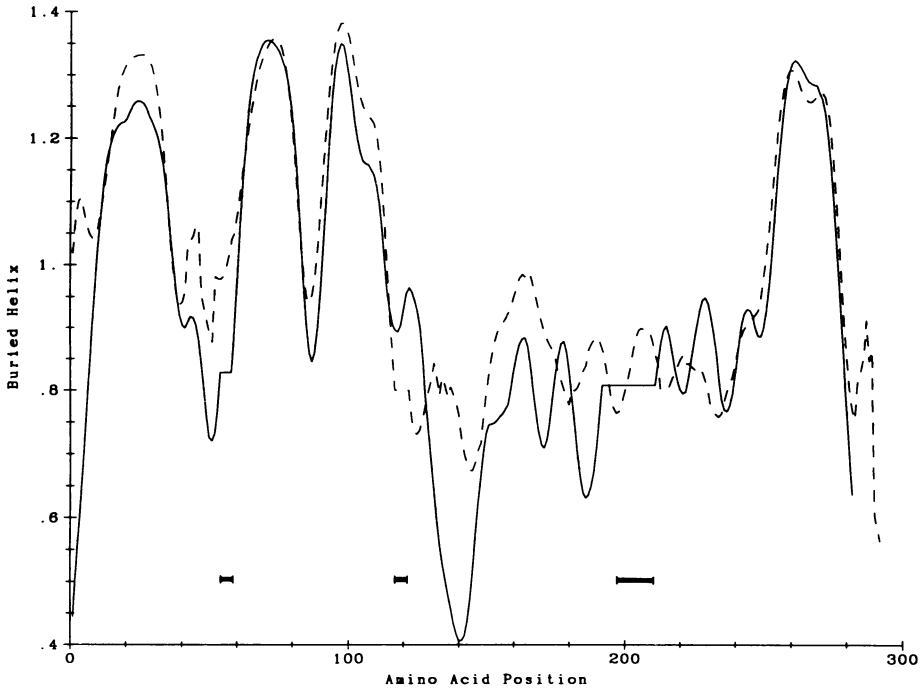

FIG. 8. Plot of amino acid sequence number versus membrane buried-helix parameter for each residue (see reference 28 for method) of TI 1 (solid line) and the average values of the other members (excluding Rds) of the glycoprotein family (dashed line). Bars indicate positions of gaps introduced into the sequences in the alignment.

expressed in carcinomas and particularly in the early stages of melanomas (16), the colon-associated tumor antigen Co-029 (48), the *Schistosoma mansoni* antigen Sm23 (52), and the leukocyte antigens CD53 (2), CD37 (8), R2 (13), and Tapa-1 (33). A more sensitive sequence comparison method (Profilesearch of the University of Wisconsin Genetics Computer Group program package) using only conserved regions in the alignment of the protein family detected a more distant homology to the bovine, murine, and rat Rds (retinal degradation slow) proteins (50). This homology has not been demonstrated previously. As shown in Fig. 7, identical and similar residues are clustered in four regions. Further analysis by the sensitive sequence comparison method (3) confirmed the significance of the relation of the individual family members (data not shown).

Similarities were striking when we compared plots of buried-helix parameter (28) between TI 1 and other members of the glycoprotein family, showing strict conservation of all four putative transmembrane domains (Fig. 8). The potential N-linked glycosylation site in TI 1 is conserved in the same region of Co-029, Me491, CD53, and R2.

DISCUSSION

Several cytokines, such as interferons, TGFβ, and tumor necrosis factor α, are antiproliferative for some cell types. Each cytokine regulates several induced and suppressed genes, of which only a small fraction have been isolated. It remains unclear whether any of the known cytokine-regulated genes relate to growth suppression. However, a subset of these genes, or other similar, not yet identified genes, may play an important role in negative growth regulation. The antiproliferative action of alpha interferon in B-cell lines is blocked by the expression of the Epstein-Barr virus immortalizing gene EBNA 2 (1). This function of EBNA 2 seems to be mediated through its ability to block the induction of interferon-induced genes at the transcriptional level (17a). Because of the inherent difficulty in specifically cloning genes that act in an antiproliferative fashion, the mechanism of growth arrest is largely unknown. Decreased expression of c-*myc* is, however, related to the antiproliferative effect of interferon (19), and recently it has been demonstrated that TGFβ-induced growth arrest may be associated with a moderate reduction of c-*myc* expression (37). A posttranslational effect on the retinoblastoma protein has also been proposed (21, 38). During the preparation of this report, Howe et al. (17) showed that TGFβ induces G_1 arrest in CCL64 cells and that TGFβ blocks the phosphorylation of the mink homolog of yeast p34*cdc2*. Again, a posttranslational control may be involved.

The intention of this work was to isolate genes that are associated with the induction of growth arrest by TGFβ in an epithelial cell line. Three of the genes identified, those for fibronectin, collagen, and PAI 1, are known to be upregulated by TGFβ in human lung fibroblasts (27). However, in these cells, maximal induction of PAI 1 is observed after 10 h and expression remains high after 2 days (25). In the mink cell homolog, a transient induction is observed (Fig. 2 and 3). It is, however, still unresolved whether matrix proteins and protease inhibitors play a role in growth regulation.

The JE cDNA clone isolated contained several small open reading frames but showed an overall homology of 75% with the human monocyte chemotactic and activating factor (JE) gene (43), which is transiently expressed following cytokine stimulation (42). Compared with the human sequence, the mink JE clone had two inserted sequences and thus appears to represent an incompletely processed JE mRNA. Our JE clone had a structure surprisingly similar to that of a human gamma interferon-induced gene called gamma 1 (10), which

is 98% homologous to the JE cDNA clone. The hypothetical mink protein was 83% similar and 72% identical over 80 residues to the human protein. Thus, the gamma 1 cDNA is probably an unspliced precursor of the JE transcript (45). The JE gene is obviously regulated by many factors, not all able to stimulate cell growth. Its expression may, however, relate to the chemotactic properties of TGFβ (39, 51).

The regulation of expression of the TI 1 clone is interesting. It is induced 3 to 6 h after addition of TGFβ in growing cells, corresponding to the intermediate time of induction of growth arrest in CCL64 cells by TGFβ (Fig. 3). Its expression is lower in actively growing cells than in quiescent cells, in which its expression is downregulated by serum stimulation (Fig. 1). Expression of the TI 1 gene is also downregulated by TGFβ in quiescent cells (Fig. 2). Previously isolated genes that are negatively regulated by TGFβ include those encoding extracellular proteases such as transin, urokinase, elastase, and collagenase but also genes such as the proliferin gene and c-*myc* (18, 30). These genes have a common regulatory element, and inhibition of the transin gene seems to be mediated by a *fos*-binding sequence (18). The TI 1 gene therefore seems to be similar to the recently described mouse *gas* genes (44), which accumulate in quiescent cells and are rapidly downregulated by serum. All but one of the *gas* genes are regulated by a posttranscriptional mechanism (7). None of the *gas* genes are expressed in epithelial cells, and thus it appears that TI 1 is a candidate for an epithelial *gas*-like gene. The TI 1 open reading frame shows homology with eight members of a family of transmembrane glycoproteins expressed on leukocytes and several types of tumor cells. Although very little is known about their function, the Me491 antigen might possibly serve as a rapid-growth-inhibitory gene (16). Moreover, Tapa-1, expressed on hematopoietic, neuroectodermal, and mesenchymal cells, is the target of an antiproliferative monoclonal antibody (33). The expression of CD37 is high in resting B cells but is rapidly downregulated following induction of mitosis and differentiation with phorbol esters (5). Clearly, at least some members of the TI 1-related gene family are expressed at growth arrest. Given the similarity between these proteins, they may have similar functions. Further experiments are required to evaluate a possible causative role of the TI 1 protein in growth regulation.

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