BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **249,** 283–286 (1998)
ARTICLE NO. RC989132

Cloning and Characterization of a Transforming Growth Factor β1-Induced Anti-apoptotic Adhesion Protein TIF2[1]

Gregory B. Carey and Nan-Shan Chang[2]
Guthrie Research Institute, Laboratory of Molecular Immunology, Guthrie Medical Center, Sayre, Pennsylvania 18840

Received July 9, 1998

Transforming growth factor-β (TGF-β) antagonizes the cytotoxic function of tumor necrosis factor (TNF). By differential display and library screening, we isolated a murine TIF2 (TGF-β-induced factor 2) cDNA, encoding a putative 15-kDa membrane adhesion protein, which possesses an RGD sequence at the extracellular region. When TNF-sensitive murine L929 fibroblasts were stably transfected with TIF2 cDNA, these cells significantly resisted TNF killing. In contrast, L929 cells, which stably expressed the TIF2 antisense mRNA, acquired enhanced TNF susceptibility. Calculated EC_{50} values, i.e., the amount of TNF needed for killing 50% cells, are 10, 55, and 1.5 ng/ml, respectively, for vector control, sense transfectant, and antisense transfectant. TGF-β1 rapidly induces TIF2 gene expression (~1 hr), which correlates with time-related acquisition of TNF-resistance in TGF-β1-treated L929 cells. Notably, TIF2 gene expression is markedly increased in human breast cancer and lymphoid leukemia cells, compared to normal human cells, suggesting its potential role in cancer development. Together, the anti-apoptotic function of TIF2 is responsible in part for TGF-β-mediated protection of L929 cells against TNF cytotoxicity.

Transforming growth factor beta (TGF-β) family proteins are potent immunoregulators (1-5; reviews). Overexpression of TGF-β and excessive activation of latent TGF-β lead to immune defects and malignancy such as prostate and breast cancers (1-6). The overexpressed TGF-β is utilized by cancer cells to fend off immune attack. Nonetheless, tumor necrosis factor (TNF) counteracts the biologic effects of TGF-β. For example, TGF-β inhibits the development of lymphokine-activated killer cells (7) and cytotoxic T cells (8), whereas TNF suppresses these TGF-β effects.

TGF-β protects several types of cancer cells from TNF-mediated cell death (1, 9-12). How TGF-β counteracts the cytotoxic functions of TNF is largely unknown. However, a likely scenario is that TGF-β1 induces protein tyrosine phosphorylation in murine L929 fibroblasts, and this event appears to interrupt the TNF killing pathway (10). Furthermore, a novel TGF-β1-induced extracellular matrix protein, named TNF-resistance triggering (TRT) protein, protects L929 cells against TNF-mediated cell death (10).

To better understand the protective mechanism of TGF-β1 against TNF cytotoxicity, we have isolated a novel murine cDNA, designated TGF-β-induced factor 2 (TIF2), by differential display (13) and library screening. TIF2 cDNA encodes a putative transmembrane adhesion protein containing an extracellular RGD motif. When stably expressed in TNF-sensitive L929 cells, TIF2 strongly inhibits TNF-mediated cell death.

MATERIALS AND METHODS

Differential display and cDNA library screening. To analyze specific mRNAs induced by TGF-β1, L929 cells were pretreated with or without TGF-β1 (2 ng/ml) for 1 hr, followed by total cellular RNA isolation and processing differential display (13). A 500 base pair (bp) cDNA, amplified by RT/PCR, was found in the TGF-β1-treated cells but not in control cells. This cDNA was labeled with [^{32}P]dCTP and used to screen an NIH/3T3 fibroblast cDNA library (constructed in λgt11; Clontech, Palo Alto, CA) (14). Eighteen positive clones were obtained (from ~2 x 10^6 colonies), which contained only two cDNA inserts of 1.9 and 1.5 kilobases (kb). The 1.9-kb cDNA insert, designated TIF2, was subcloned in a CMV promoter-based mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA). Both the sense and anti-sense cDNA/vector constructs were subjected to fluorescent sequencing analysis (Applied Biosystems, Foster City, CA) and functional characterization.

Stable transfectants and TNF cytotoxicity assays. Stable L929 transfectants, expressing sense or anti-sense TIF2 mRNA, were established as described previously (14, 15). Briefly, L929 cells were electroporated with 10 or 40 μg of empty pcDNA3 vector or TIF2/pcDNA3 (sense or antisense) constructs. These cells were then cultured in the continuous presence of G418 (200 μg/ml). After culturing for 15-30 days, cell colonies (or foci) with acquired neomycin (neo) resistance were obtained. These colonies were harvested, regrown, and examined for the presence of TIF2 mRNA expression by RT-PCR using 1 μg cellular RNA (Access RT-PCR Kit, Promega, Madi-

[1] GenBank accession number for the TIF2 cDNA: AF075717.
[2] To whom correspondence should be addressed. Fax: 717-882-5151. E-mail: nschang@inet.guthrie.org.

0006-291X/98 $25.00

```
MFRGTGRGVC  PPLYCDEHSI  FYVSCNCLLC  RLEEWGREER  AGVEGEPIHA   50
DVTAVPVALS  SLPWEVEQVD  PNKHVAWEQC  RLLGARTPDL  TSVSVLITRSV  100
TM
TLRHELGICG  FPTVKWWGQR  GDPAFLGAVQ  SQQMSTDVL.               139
```

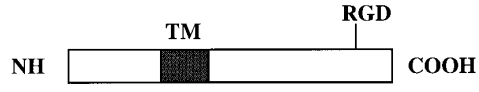

FIG. 1. Deduced TIF2 protein sequence. The deduced murine TIF2 protein sequence from cDNA is shown in the upper panel, and a schematic structure is at the bottom panel. TIF2 amino acid sequence contains a putative transmembrane (TM) region (amino acids #45-65; underlined), an RGD sequence at the extracellular *C*-terminus (underlined and bold), and a conserved threonine (Thr-5) phosphorylation site for protein kinase C (bold) and a conserved glycine (Gly-4) myristoylation site at the cytoplasmic *N*-terminus.

son, WI). Primers used for PCR analyses were: forward primer, 5′-GTCTGAAGGTTGGTGTT; reverse primer, 5′-ATCCACTTGTTC-CACTT. The expected amplified cDNA was 766 bp.

To examine whether TIF2 conferred TNF resistance, these stable transfectants (2.5×10^5/ml) were seeded onto 96-well plates, cultured overnight, and then exposed to recombinant TNF-α (0.1-20 ng/ml; PeproTech Inc., Rocky Hill, NJ) for 16-24 hr. TNF-mediated cell death was measured using crystal violet stain (OD at 590 nm) as previously described (9, 10, 14). The extent of cell death was calculated as follows: % Cell Death = [(OD from control cells − OD from TNF-treated cells)/OD from control cells] × 100.

Northern hybridization. Where indicated, L929 cells were treated with human platelet TGF-β1 (2 ng/ml; Collaborative Research Incorporated, Bedford, MA) for 1-4 hr. To analyze TIF2 gene expression, Northern hybridization was performed (14), which included total cellular RNA isolation (using Trizol; GIBCO/Life Technologies, Gaithersburg MD), 1% formaldehyde-agarose gel electrophoresis (10 μg RNA per lane), capillary blotting onto Zeta-Probe nylon membranes (Bio-Rad, Hercules, CA), and hybridization with [^{32}P]-labeled TIF2 cDNA (Ready to Go random prime labeling kit; Pharmacia, Piscataway, NJ).

RESULTS

By differential display and cDNA library screening, we isolated a novel TIF2 cDNA (1941 bp) from a murine NIH/3T3 fibroblast cDNA library. The full-length cDNA encodes a putative 139-amino-acid transmembrane adhesion protein (15 kDa) (Fig. 1). The open reading frame is at nucleotide #1242-1661, and an upstream stop codon TAG at #1224-1226. Software-based sequence analysis shows that TIF2 possesses a putative transmembrane region (amino acids #45-#65) (16), an RGD sequence at the extracellular *C*-terminus, and a conserved phosphorylation site for protein kinase C (Thr-5) and a conserved myristoylation site (Gly-4) at the cytoplasmic *N*-terminus (Fig. 1). The presence of RGD sequence indicates that the putative TIF2 is a typical adhesion protein (17), whereas its sequence is not homologous to the sequences of known adhesion molecules. No known structural domains are found in the TIF2 protein sequence as compared to the available universal database.

Northern blot analysis confirmed that TGF-β1 induced TIF2 gene expression (Fig. 2). TGF-β1 induced TIF2 gene expression in L929 cells in 1 hr. This early induction correlates with the time-frame of acquisition of TNF-resistance in TGF-β1-treated L929 cells (10). The TGF-β-induced TIF2 mRNA levels remained elevated over controls through 24 hr (data not shown).

L929 cells were electroporated with a sense-oriented TIF2-pcDNA3 construct, an antisense-oriented TIF2-pcDNA3 construct, or an empty pcDNA3 vector, followed by selection of stable neo-resistant transfectants using G418. These established stable transfectants were seeded in 96-well plates, cultured overnight, and challenged with various concentrations of TNF-α for 16-20 hr. Compared to control cells, the TIF2-transfected (sense-producing) cells were resistant to the cytotoxic effect of TNF-α (Figs. 3A and 3B). In contrast, cells, which expressed the antisense TIF2 mRNA, acquired an enhanced susceptibility to TNF cytotoxicity (Fig. 3A). Calculated EC_{50} values for TNF cytotoxicity, i.e. the amount of TNF needed for killing 50% cells, in the cells transfected with 10 μg DNA are 10, 55, and 1.5 ng/ml for vector control, sense transfectant, and antisense transfectant, respectively. Similarly, the calculated EC_{50}s for TNF cytotoxicity in cells transfected with 40 μg vector or TIF2 sense construct are 12 and 200 ng/ml TNF, respectively (2nd order regression analysis).

RT-PCR analysis was used to confirm TIF2 mRNA expression in the L929 transfectants. The results showed that vector control cells constitutively produced a basal level of TIF2 mRNA (Fig. 3C, lane 2). Cells transfected with 10 μg sense-producing TIF2 constructs produced an increased amount of TIF2 mRNA (~100% increase compared to vector control; Fig. 3C, lane 3). Cells transfected with the TIF2-antisense producing constructs produced no detectable TIF2 mRNA (Fig. 3C, lane 4).

Both the transcriptional inhibitor actinomycin D (1

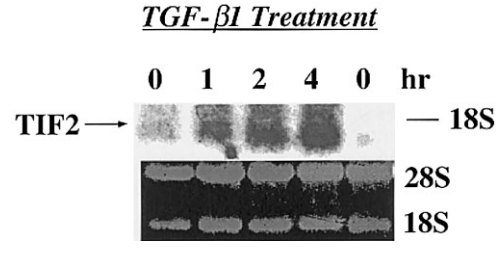

FIG. 2. Induction of TIF2 gene expression by TGF-β1. L929 cells in 100 mm petri dishes were cultured overnight and treated with 2 ng/ml TGF-β1 for 0, 1, 2, and 4 hr. Total RNA was extracted and 10 μg total RNA was loaded per lane on a 1% agarose gel, followed by processing Northern blotting (see Materials and Methods; final washing condition: 2X SSC/1% SDS at 65°C for 1 hr). The RNA gel is shown at the bottom panel, and the Northern blot is shown at the top. Lanes 1 and 5, controls (time 0). Lanes 2, 3 and 4, TGF-β1 treatment for 1, 2, and 4 hr, respectively.

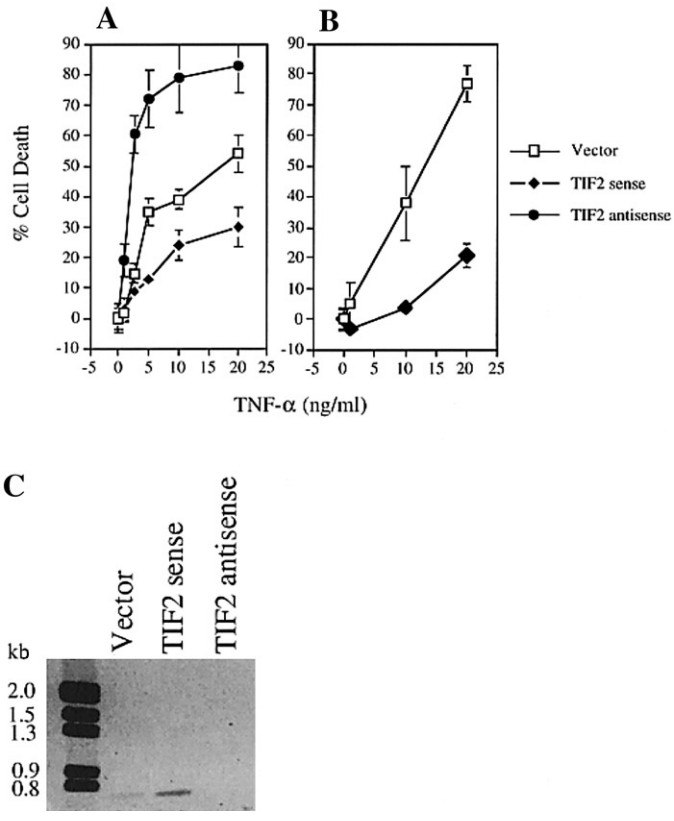

FIG. 3. TIF2 inhibition of TNF cytotoxicity in L929 cells. L929 cells were transfected with either 10 μg (A) or 40 μg (B) TIF2 constructs (sense or antisense) or empty vector DNA alone, and stable transfectants were established by G418 (see Materials and Methods). These cells were seeded onto 96-well plates and challenged with recombinant human TNF-α for 16-20 hr, and the extent of lysis determined by crystal violet staining (9,10,14). Note that TIF2 protected L929 cells against TNF cytotoxicity, whereas ablation of TIF2 expression by antisense mRNA resulted in enhanced TNF susceptibility in L929 cells. Confirmation of TIF2 expression in these L929 transfectants were determined by RT-PCR (see Materials and Methods; C). Lane 1, DNA size markers. Lanes 2, 3, 4, L929 cells transfected with 10 μg pcDNA3 vector, TIF2 sense construct, or TIF2 antisense construct, respectively.

μg/ml) and the protein synthesis inhibitor cycloheximide (20 μg/ml) abolished the protective effect of TIF2 against TNF killing (data not shown). These data further support the above observations that TIF2 cDNA is integrated in the chromosome of L929 transfectants and is translated into mRNA for protein synthesis.

Finally, we examined TIF2 gene expression in various cell lines. Compared to normal human bronchial epithelium cells (lane 8, Fig. 4), TIF2 mRNA is expressed at a relatively high level (~200-300% increases) in human leukemia (Molt 4, CEM and Daudi) and human MCF-7 breast cancer cells (transfected with or without protein kinase C alpha cDNA; 18). Notably, the protein kinase C-overexpressing metastatic human breast cancer cells express more TIF2 message than their non-metastatic counterparts (Fig. 4, lanes 2 and 1, respectively). Also, a smaller TIF2 mRNA transcript was found in the non-metastatic MCF-7 cells (lane 1). Interestingly, cervical HeLa cells failed to express the TIF2 gene (lane 7, Fig. 4).

DISCUSSION

In this study we have isolated the TIF2 cDNA, which encodes a putative transmembrane adhesion protein. Functional analysis showed that TIF2 inhibits TNF-mediated cell death. In contrast, antisense expression of TIF2 enhances cellular sensitivity to TNF. These observations indicate that TIF2 plays a critical role in modulating the TNF killing pathway. TIF2 is rapidly induced by TGF-β1 in L929 cells. Similarly, upon stimulation of L929 cells with TGF-β1, L929 cells rapidly develop TNF resistance (10). Thus, one of mechanisms by which TGF-β1 protects L929 cells against TNF cytotoxicity is via its induction of TIF2.

Structurally, TIF2 possesses a cytoplasmic myristoylation site at the *N*-terminus. Post-translational myristoylation is important for protein membrane anchor (19). Additionally, a conserved Thr-5 phosphorylation site for protein kinase C is found at the cytoplasmic *N*-terminus, suggesting that TIF2 may be able to transduce signals. The presence of an RGD motif at the extracellular *C*-terminus supports that TIF2 is an adhesion protein. Thus, binding of a specific receptor or integrin to TIF2 could result in transducing a signal and regulation of cellular responses. A likely candidate integrin for binding to TIF2 is β1 integrin, which recognizes the RGD motif (20).

Adhesion proteins play an important role in cellular differentiation, death and metastasis for cancer cells (20-22). For example, perturbation of β1-integrin func-

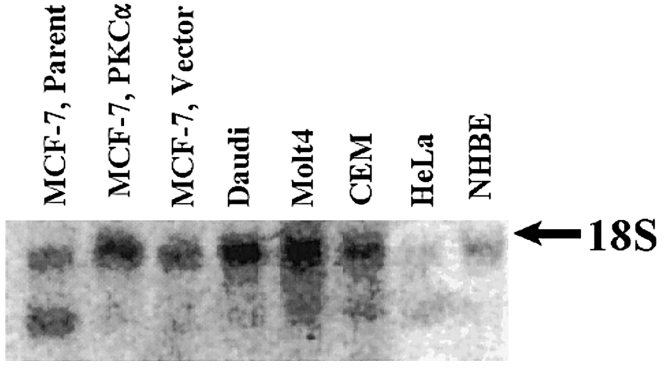

FIG. 4. TIF2 expression in various human cancer cell lines. Total RNA was extracted from various human cancer cell lines. Ten micrograms was run on a 1% RNA gel, followed by processing for Northern Blot analysis. Cells: lane 1, MCF-7 (non-metastatic human breast cancer); lane 2, PKCα-overexpressing metastatic MCF-7; lane 3, non-metastatic MCF-7 vector controls; lanes 4, 5, 6: Molt 4, CEM and Daudi leukemias, respectively; lane 7, HeLa, cervical carcinoma; lane 8, NHBE (normal human bronchial epithelium).

tion alters the development of murine mammary gland (23). TNF and interferon-γ reduce $\alpha_V\beta_3$ integrin activation in endothelial cells that lead to decreased cell adhesion and survival (24). The pattern of expression and function of integrin extracellular matrix receptors may affect macrophage apoptosis (25). In this study we first demonstrate an inhibitory function of the adhesion protein TIF2 against TNF cytotoxicity. Notably, a raised level of TIF2 gene expression is found in highly metastatic protein kinase C-overexpressing MCF7 breast cancer cells, compared to non-metastatic MCF7 cells. These observations suggest that TIF2 may play a role in breast cancer metastasis or aggressiveness.

How TIF2 inhibits TNF killing is unknown. We believe that in addition to TIF2, TGF-β1 probably induces a ligand for binding to TIF2, so that an anti-apoptotic or survival signal is introduced into cells. Alternatively, TIF2 may be able to abolish TNF signaling pathway, such as disruption of the binding of TNF receptors to intracellular death domain adapter proteins TRADD (TNF receptor associated death domain protein) and FADD (Fas associated death domain protein) (26). These likely scenarios are being investigated in our laboratory.

Taken together, a novel transmembrane adhesion protein TIF2 is shown to inhibit cell death by TNF. Compared to normal cells, TIF2 is overexpressed in several cancer cells. These observations indicate that TIF2 is a survival factor necessary for cancer development and metastasis.

ACKNOWLEDGMENTS

Research was supported by the Wendy Will Case Cancer Fund, Pharmakon Inc., National Cancer Institutes Grants R01-CA61879 and R55-CA64423, and the Guthrie Foundation for Education and Research. We thank Drs. John Noti and Indira Carey of the Guthrie Research Institute for kindly providing the RNA blot using various cancer cells.

REFERENCES

1. Shepard, H. M., and Lewis, G. D. (1988) *J. Clin. Immunol.* **8,** 333–341.
2. Letterio J. J., and Roberts, A. B. (1998) *Annu. Rev. Immunol.* **16,** 137–162.
3. Wojtowicz-Praga, S. (1997) *J. Immunother.* **20,** 165–177.
4. Weller, M., and Fontana, A. (1995) *Brain Res. Brain Res. Rev.* **21,** 128–151.
5. Steiner, M. S. (1995) *World J. Urol.* **13,** 329–336.
6. Steiner, M. S., Zhou, Z. Z., Tonb, D. C., and Barrack, E. R. (1994) *Endocrinology* **135,** 2240–2247.
7. Espevik, T., Figari, I. S., Ranges, G. E., and Palladino, M. A., Jr. (1988) *J. Immunol.* **140,** 2312–2316.
8. Ranges, G. E., Figari, I. S., Espevik, T., and Palladino, M. A., Jr. (1987) *J. Exp. Med.* **166,** 991–998.
9. Chang, N.-S. (1997) *Am. J. Physiol.* **273,** C1987–C1994.
10. Chang, N.-S. (1995) *J. Biol. Chem.* **270,** 7765–7772.
11. Kamijo, R., Takeda, K., Nagumo, M., and Konno, K. (1989) *Biochem. Biophys. Res. Commun.* **158,** 155–162.
12. Sugarman, B. J., Lewis, G. D., Eessalu, T. E., Aggarwal, B. B., and Shepard, H. M. (1987) *Cancer Res.* **47,** 780–786.
13. Liang, P., and Pardee, A. B. (1997) *Methods Mol. Biol.* **85,** 3–11.
14. Cao, H., Mattison, J., Zhao, Y., Joki, N., Grasso, M., and Chang, N. S. (1996) *Biochem. Biophys. Res. Commun.* **227,** 266–272.
15. Chang, N.-S., and Mattison, J. (1993) *BioTechniques* **14,** 342–346.
16. von Heijne, G. (1992) *J. Mol. Biol.* **225,** 487–494.
17. Ruoslahti, E. (1997) *Annu. Rev. Cell Dev. Biol.* **12,** 697–715.
18. Ways, D. K., Kukoly, C. A., deVente, J., Hooker, J. L., Bryant, W. O., Posekany, K. J., Fletcher, D. J., Cook, P. P., and Parker, P. J. (1995) *J. Clin. Invest.* **95,** 1906–1915.
19. Boutin, J. A. (1997) *Cell Signal* **9,** 15–35.
20. Akiyama, S. K. (1996) *Hum. Cell* **9,** 181–186.
21. Akiyama, S. K., Olden, K., and Yamada, K. M. (1995) *Cancer Metastasis Rev.* **14,** 173–189.
22. Lafrenie, R. M., Buchanan, M. R., and Orr, F. W. (1993) *Cell Biophys.* **23,** 3–89.
23. Faraldo, M. M., Deugnier, M. A., Lukashev, M., Thiery, J. P., and Glukhova, M. A. (1998) *EMBO J.* **17,** 2139–2147.
24. Ruegg, C., Yilmaz, A., Bieler, G., Bamat, J., Chaubert, P., and Lejeune, F. J. (1998) *Nat. Med.* **4,** 408–414.
25. Judware, R., McCormick, T. S., Mohr, S., Yun, J. K., and Lapetina, E. G. (1998) *Biochem. Biophys. Res. Commun.* **246,** 507–512.
26. Darnay, B. G., and Aggarwal, B. B. (1997) *J. Leukocyte Biol.* **61,** 559–566.