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# Cutting Edge: TRANK, a Novel Cytokine That Activates NF-κB and c-Jun N-Terminal Kinase[1]

Valsala Haridas,* Jian Ni,† Anthony Meager,‡ Jeffery Su,† Guo-Liang Yu,† Yifan Zhai,† Hla Kyaw,† Keith T. Akama,§ Jingru Hu,§ Linda J. Van Eldik,§ and Bharat B. Aggarwal[2]*

**We searched the expressed sequence tag database using sequence homology and identified a novel cytokine, which we have named TRANK (thioredoxin peroxidase-related activator of NF-κB and c-Jun N-terminal kinase). The predicted amino acid sequence of TRANK was highly homologous to that of the thiol-specific antioxidant proteins. Unlike these proteins, however, TRANK had a putative secretory signal polypeptide and was found to be secreted by cells. TRANK was expressed in most tissues and cell lines, and the gene that encodes it was mapped to chromosome Xp21–22.1. TRANK activated NF-κB and induced the degradation of the inhibitory subunit of NF-κB. In addition, TRANK upregulated the expression of NF-κB-dependent gene products, ICAM-1, and inducible nitric oxide synthase. TRANK also activated c-Jun N-terminal kinase and induced the proliferation of normal human foreskin fibroblasts. Its homology with antioxidant proteins, wide distribution in tissues, and ability to activate NF-κB and c-Jun N-terminal kinase suggest that TRANK plays an important role in inflammation.** ***The Journal of Immunology*****, 1998, 161: 1–6.**

Most living organisms have evolved several antioxidant proteins and peptides to counteract the devastating effects of reactive oxygen species. These include superoxide dismutase, glutathione, glutaredoxin, thioredoxin, thioredoxin reductase, and thioredoxin peroxidase. Most of these proteins have been well-conserved during the evolution from yeast to human (see Refs. 1 and 2). NK enhancing factor (NKEF)[3] is another protein that was recently discovered and found to possess an antioxidant property which protects proteins and DNA from oxidative damage (3). NKEF is a 24-kDa protein that was initially found to be abundant in RBCs and to augment NK cell-mediated cytotoxicity (4). This protein is encoded by two distinct genes, *nkef-A* and *nkef-B*, which are 71% identical in their nucleotide sequence and 88% identical in their deduced amino acid (aa) sequence (5). Almost all antioxidant proteins are cytoplasmic proteins; consequently, they can only protect those cells in which they are produced.

To find the antioxidant protein that may be secreted by the cells, we compared the expressed sequence tag database with the signal sequences and the NKEF sequence. This resulted in the identification of a novel gene whose product was named TRANK based on its sequence homology to thioredoxin peroxidase (the product of the *nkef-B* gene) and its ability to activate NF-κB and c-Jun N-terminal kinase (JNK).

## Materials and Methods

### *Identification, cloning, expression, and purification of rTRANK*

TRANK was identified by a systematic comparison of the signal peptide and NKEF sequence homology with the expressed sequence tag database (Human Genome Sciences, Rockville, MD) and by functional screening assays (6–8). The sequence encoding the putative natural TRANK protein (aa 32–271) was amplified employing standard PCR techniques using the following primers: 5′*Nco*I, CGCCCATGGCTGGAGCTGTGCAGGG and 3′*Hin*dIII, CGCAAGCTTCAATTCAGTTTATCGAAATACTTCAGC. The amplified fragment was subsequently purified, digested with *Nco*I and *Hin*dIII, and cloned in a pQE60 *Escherichia coli* expression vector. The cloning, expression, and confirmation of the cloned sequence were performed using standard procedures. The chromosomal localization of the TRANK gene was performed as described previously (9).

The protein was purified using $(NH_4)_2SO_4$ and polyethylene glycol precipitation followed by anion exchange chromatography performed on an HQ50 column (Poros CM20; PerSeptive Biosystems, Hertfordshire, U.K.). The purified protein migrated as a single band by SDS-PAGE, and the N-terminal aa sequence demonstrated that >95% of the purified TRANK contained the expected N-terminal sequence (MAGAVQ). Endotoxin levels assayed by the amebocyte lysate test (BioWhittaker, Walkersville, MD) proved to be <16.5 endotoxin U/mg protein.

*Cytokine Research Section, Department of Molecular Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030; †Human Genome Sciences, Inc., Rockville, MD 20850; ‡Division of Immunobiology, The National Institute for Biological Standards and Control, South Mimms, U.K.; and §Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago, IL 60611

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[2] Address correspondence and reprint requests to Dr. Bharat B. Aggarwal, Cytokine Research Section, Department of Molecular Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030. E-mail address: aggarwal@utmdacc.mda.uth.tmc.edu

[3] Abbreviations used in this paper: NKEF, NK enhancing factor; JNK, c-Jun N-terminal kinase; TRANK, thioredoxin peroxidase-related activator of NF-κB and c-Jun N-terminal kinase; iNOS, inducible nitric oxide synthase; IκBα, inhibitory subunit of NF-κB; aa, amino acid; EMSA, electrophoretic mobility shift assay.



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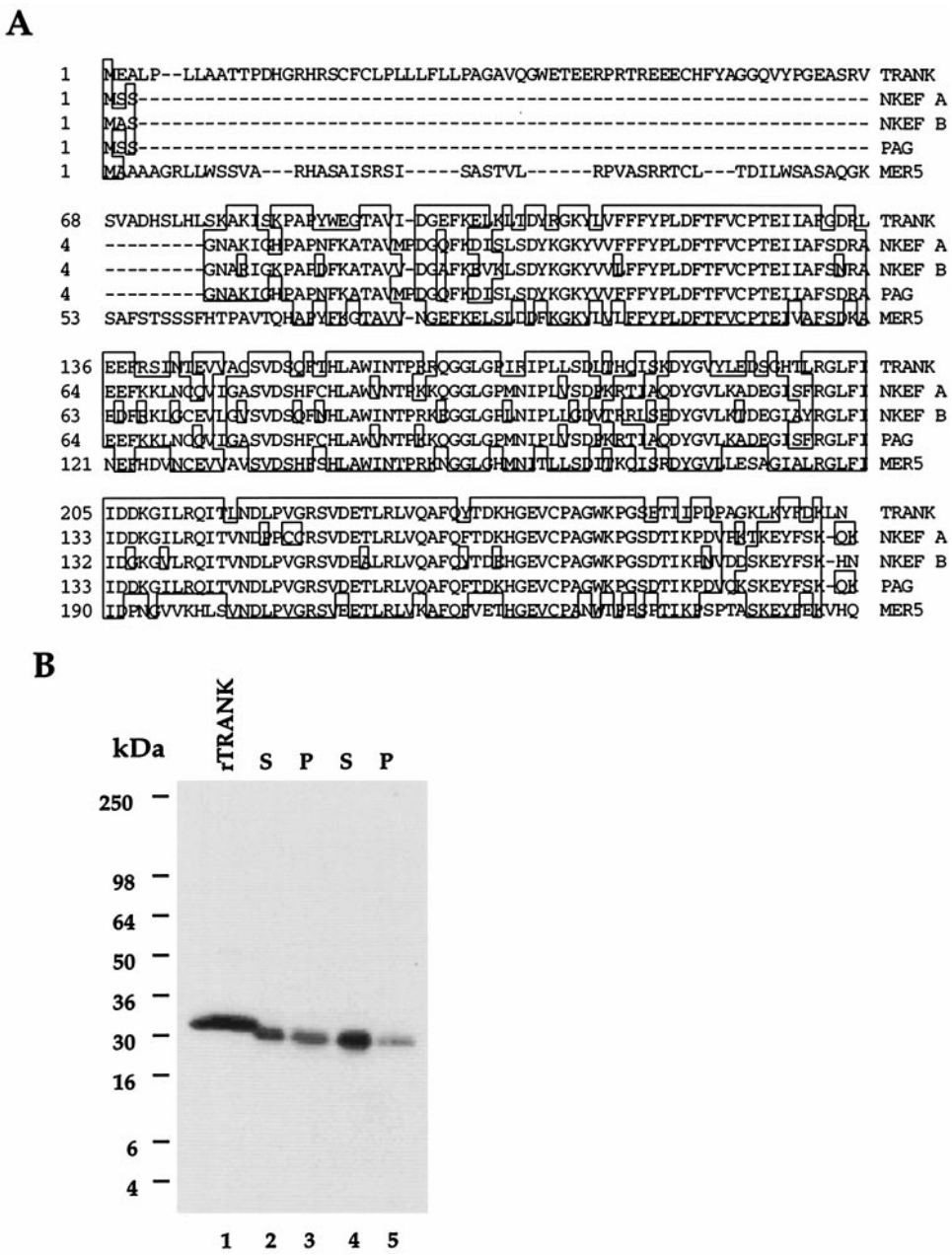


**FIGURE 1.** *A*, Deduced aa sequence of TRANK and its homology with NKEF-A, NKEF-B, proliferation-associated gene product (PAG), and murine erythroleukemia-related (MER)5. *B*, Results of Western blot analysis showing purified TRANK (*lane 1*) and its secretion by Jurkat cells (*lanes 2* and *3*) and HL-60 cells (*lanes 4* and *5*). S and P are conditioned media and cell extracts, respectively.

### *Northern blot analysis*

Two filters containing ~2 $\mu$g of poly(A)$^+$ RNA per lane from various human tissues (Clontech, Palo Alto, CA) were probed with [$^{32}$P]-labeled TRANK cDNA. The RNA from a selected panel of human cell lines was also probed according to a similar method.

### *Immunoprecipitation and Western blot analysis of TRANK*

HL-60 and Jurkat cells ($2 \times 10^7$) were grown in culture for 24 h. Conditioned media (10 ml) and cell lysate (2.25 ml) were subsequently immunoprecipitated and analyzed by Western blot using anti-TRANK polyclonal antisera with rTRANK (100 ng) as a control. Blotting and detection were performed as described in the protocol for the enhanced chemiluminescence Western blotting kit (Boehringer Mannheim, Arlington Heights, IL).

### *Electrophoretic mobility shift assays (EMSAs)*

NF-$\kappa$B activation was analyzed by electrophoretic mobility shift assay as described previously (10).

### *Western blot analysis of inhibitory subunit of NF-$\kappa$B (I$\kappa$B$\alpha$)*

Cytoplasmic extracts of U-937 cells that had been treated for different lengths of time with TRANK were used to examine I$\kappa$B$\alpha$ degradation by Western blot analysis as described previously (11).

### *NF-$\kappa$B luciferase assay*

In the NF-$\kappa$B luciferase assay, a consensus NF-$\kappa$B element was cloned into the pSEAP-Basic vector (Clontech). A stable Jurkat cell line (Jurkat/NF-$\kappa$B-SEAP) that showed good activation in response to TNF was used in the assay. Test samples were added to Jurkat/NF-$\kappa$B-SEAP cells, and the alkaline phosphatase activity was measured at 405 nm after 72 h.

### *Determination of ICAM-1 expression*

ICAM-1 expression was induced in the EAhy926 hybrid cell line (human vascular endothelial cell line $\times$ human A549 lung carcinoma cell line) and in the U-138 MG human umbilical vein endothelial cell line as described previously (12). The levels of induced ICAM-1 were measured by ELISA

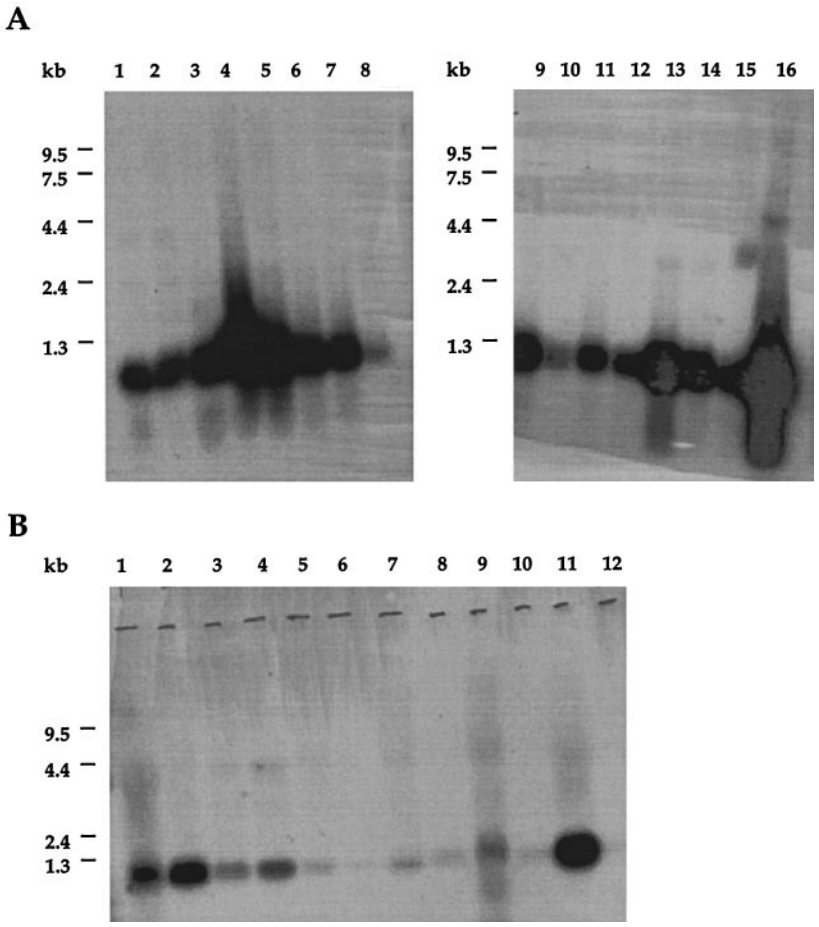


**FIGURE 2.** *A*, Tissue distribution of TRANK mRNA as shown by Northern blot analysis that was performed as described in *Materials and Methods*. *Lane 1*, spleen; *lane 2*, thymus; *lane 3*, prostate; *lane 4*, testis; *lane 5*, ovary; *lane 6*, small intestine; *lane 7*, colon; *lane 8*, peripheral blood leukocytes; *lane 9*, heart; *lane 10*, brain; *lane 11*, placenta; *lane 12*, lung; *lane 13*, liver; *lane 14*, skeletal muscle; *lane 15*, kidney; *lane 16*, pancreas. *B*, Expression of TRANK mRNA in various cell lines. *Lane 1*, Jurkat (T cell leukemia); *lane 2*, A293 (embryonic kidney); *lane 3*, HL60 (promyelocytic leukemia); *lane 4*, VE 11 (venous endothelium); *lane 5*, A431 (epidermoid carcinoma); *lane 6*, VE 9 (venous endothelium); *lane 7*, Raji (Burkitt lymphoma); *lane 8*, AE (arterial endothelium); *lane 9*, THP-1 (monocytic leukemia); *lane 10*, BUD 8 (skin fibroblast); *lane 11*, Chang liver (liver); *lane 12*, CCD-29 (lung fibroblast).

using a mAb to human ICAM-1 (CD54) (Camfolio; Becton Dickinson, Oxford, U.K.).

### *Measurement of inducible nitric oxide synthase (iNOS) and nitrite*

Rat cortical astrocyte cultures were treated with various concentrations of TRANK for 12 h, RNA was isolated, and iNOS mRNA levels were measured by slot blot analysis as described previously (13). The levels of the stable nitric oxide metabolite, nitrite, were measured in the conditioned medium of TRANK-treated cells (48 h) after any nitrate had been converted into nitrite with nitrate reductase and NADPH at 37°C for 1 h (13).

### *JNK assay*

The JNK assay was performed according to the method that was developed in our laboratory and recently described (14).

### *Proliferation assays*

The effect of TRANK on the proliferation of human diploid foreskin fibroblasts was examined as described previously (15). Fibroblasts (5000/0.1 ml) were cultured with different concentrations of TRANK for 72 h at 37°C in 96-well plates, and proliferation was examined by the tritiated thymidine uptake method.

## Results

### *Identification, sequencing, and purification of TRANK*

We identified, cloned, and expressed a new protein called TRANK. This protein exhibits significant sequence homology to several antioxidant proteins, including NKEF-A, NKEF-B (thioredoxin peroxidase), proliferation-associated gene product, and murine erythroleukemia-related 5 (see Refs. 2 and 5) (Fig. 1*A*). Specifically, the nucleotide sequence of TRANK cDNA was 58% and 56% identical to that of NKEF-A and NKEF-B, respectively. The deduced aa sequence showed a 66% and 68% homology to that of NKEF-A and NKEF-B, respectively (see Fig. 1*A*). Unlike NKEF-A and NKEF-B, however, TRANK had a putative N-terminal secretory signal sequence. The results of the Western blot analysis of the conditioned media suggested that TRANK is indeed secreted from Jurkat and HL-60 cells (Fig. 1*B*).

The gene encoding the putative TRANK protein (aa 32–271) (Fig. 1*A*) was amplified using standard PCR techniques and then cloned and expressed in *E. coli*; the recombinant protein was subsequently purified. SDS-PAGE analysis of TRANK showed a single band at around 30 kDa. The size of rTRANK appeared to be slightly greater than the natural protein, which could be due to the difference in cleavage site at the amino terminal.

### *Tissue distribution of TRANK and its production in different human cell lines*

The gene that encodes for human TRANK was mapped to human chromosome Xp21–22.1. Northern blot analysis showed that TRANK was highly expressed in the testis, ovary, heart, liver, skeletal muscle, and pancreas. Moderate expression was seen in the spleen, thymus, prostrate, small intestine, colon, placenta, and lung, while low expression was seen in peripheral blood leukocytes and the brain (Fig. 2*A*). Almost all types of cells among human tumor cell lines transcribed TRANK mRNA. However, T cells (Jurkat), kidney cells (A-293), endothelial cells (VE 11), and Chang liver cells showed relatively high expression (Fig. 2*B*).

### *TRANK activates NF-κB*

Treating human myeloid U-937 cells with TRANK for 30 min revealed a dose-dependent activation of NF-κB by EMSA (Fig.

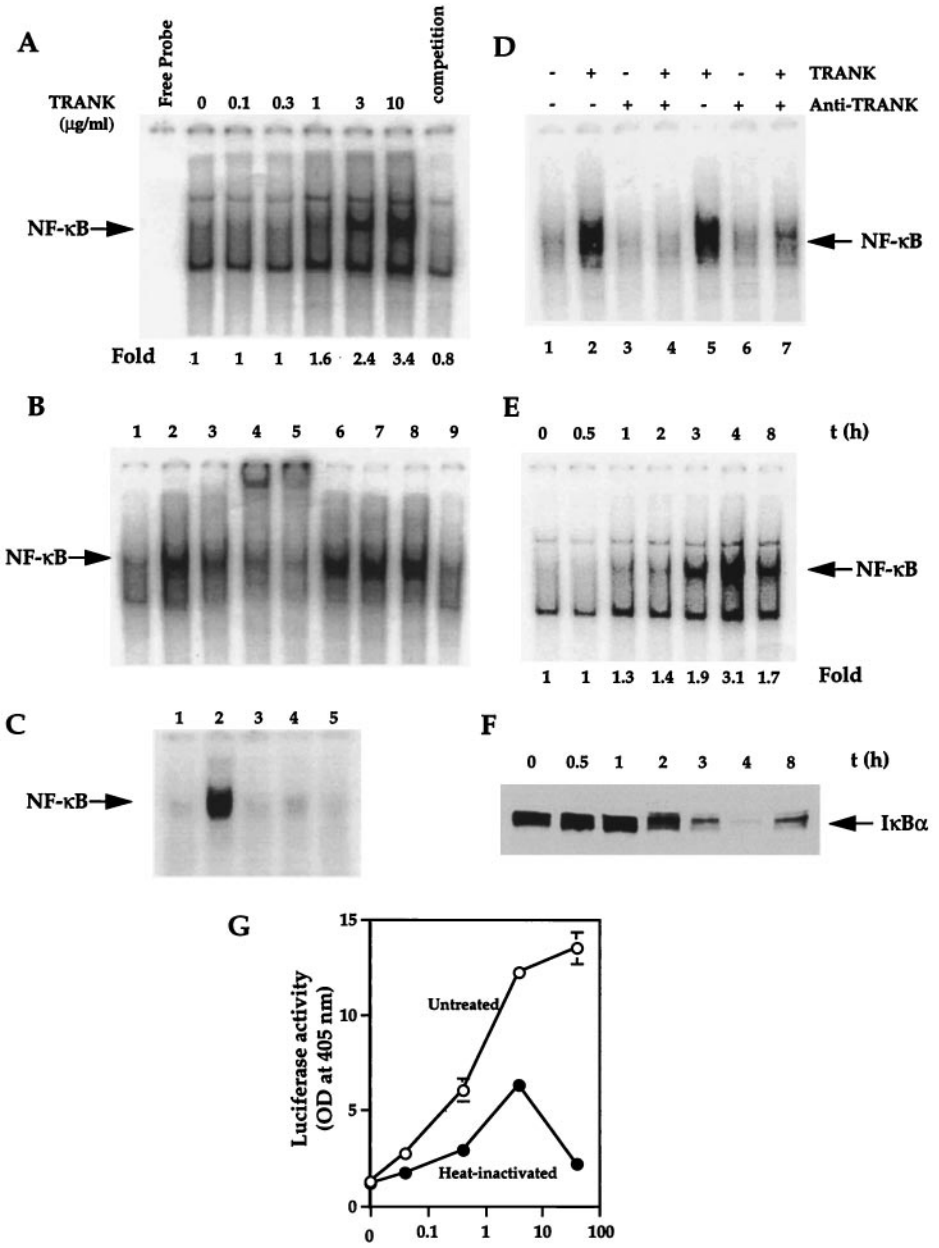


**FIGURE 3.** *A*, Dose response of TRANK-induced NF-κB activation. U-937 cells ($2 \times 10^6$/ml) were treated with different concentrations of TRANK for 30 min at 37°C and then assayed for NF-κB by EMSA as described in *Materials and Methods*. *B*, Supershift and specificity of NF-κB. Nuclear extracts from TRANK-treated (*lane 2*) cells were incubated for 15 min with anti-p50 (*lane 3*), anti-p65 (*lane 4*), anti-p50 plus anti-p65 (*lane 5*), anti-cyclin D1 (*lane 6*), anti-c-Rel (*lane 7*), preimmune serum (*lane 8*), and cold NF-κB oligo probe (*lane 9*) and then assayed for NF-κB as described. *Lane 1* shows the TRANK-untreated control. *C*, Effect of trypsinization and boiling on the ability of TRANK to activate NF-κB. TRANK was treated with 1% trypsin for 1 h at room temperature and then checked for its ability to activate NF-κB in U-937 cells (*lane 4*). The effect of trypsin alone is shown in *lane 3*. TRANK was also boiled at 100°C for 10 min and then checked for activity (*lane 5*). *Lanes 1* and *2* show the NF-κB status in untreated and TRANK-treated cells, respectively. *D*, Effect of anti-TRANK polyclonal Abs on TRANK-induced NF-κB in U-937 cells. TRANK was preincubated with anti-TRANK Abs at a dilution of 1:100 (*lanes 3* and *4*) or 1:1000 (*lanes 6* and *7*) before exposure to cells. *E*, U-937 cells were treated with 1 μg/ml TRANK for different lengths of time at 37°C and then assayed for NF-κB activity. *F*, Time course of TRANK (1 μg/ml) -induced IκBα degradation in U-937 cells. *G*, TRANK is shown to activate NF-κB-dependent gene transcription in Jurkat cells. The effect of heat-denatured TRANK on this activity is also shown. NF-κB-driven luciferase activity was measured as described in *Materials and Methods*.

3*A*). The gel shift band was specific, as it could be competed out with an unlabeled oligonucleotide and was supershifted by anti-p50 or anti-p65 Ab only (Fig. 3*B*) indicating that it is composed of p50 and p65 subunits (16). To rule out the possibility that the activity observed was not the result of a contaminant, TRANK was inactivated either by treatment with 1% trypsin or by heat denaturation (100°C for 10 min) (Fig. 3*C*). Both treatments abolished TRANK-induced NF-κB activity, indicating that a protein is responsible for its activation. We also pretreated TRANK with polyclonal Abs to the protein. This blocked NF-κB activation (Fig. 3*D*), thereby indicating that the effect is specific. TRANK activated NF-κB in a time-dependent manner, reaching a peak by 4 h and declining thereafter (Fig. 3*E*). The degradation of IκBα in cells treated with TRANK for different lengths of time was also examined using Western blot analysis. The analysis showed that IκBα started to degrade at 3 h, had almost completely degraded by 4 h, and began to be resynthesized at 8 h (Fig. 3*F*). We also examined the effect of TRANK on NF-κB-dependent luciferase gene transcription. The results shown in Figure 3*G* reveal that TRANK induced an approximately sevenfold increase in luciferase activity. Similar to NF-κB activation, heat-denaturing TRANK significantly abolished its ability to induce luciferase activity (Fig. 3*G*).

### *TRANK stimulates iNOS and ICAM-1 expression*

Because the expression of iNOS and ICAM-1 can be regulated by NF-κB (17, 18), we examined the effect of TRANK on the expression of these molecules. Treating rat astrocyte cultures with TRANK resulted in a stimulation of iNOS mRNA levels (Fig. 4*A*) and a dose-dependent increase in nitrite accumulation (Fig. 4*B*). TRANK also stimulated ICAM-1 expression in EAhy926 cells in a dose-dependent manner (Fig. 4*C*), and polyclonal antiserum to TRANK partially neutralized the TRANK-mediated enhancement of ICAM-1 expression (data not shown).

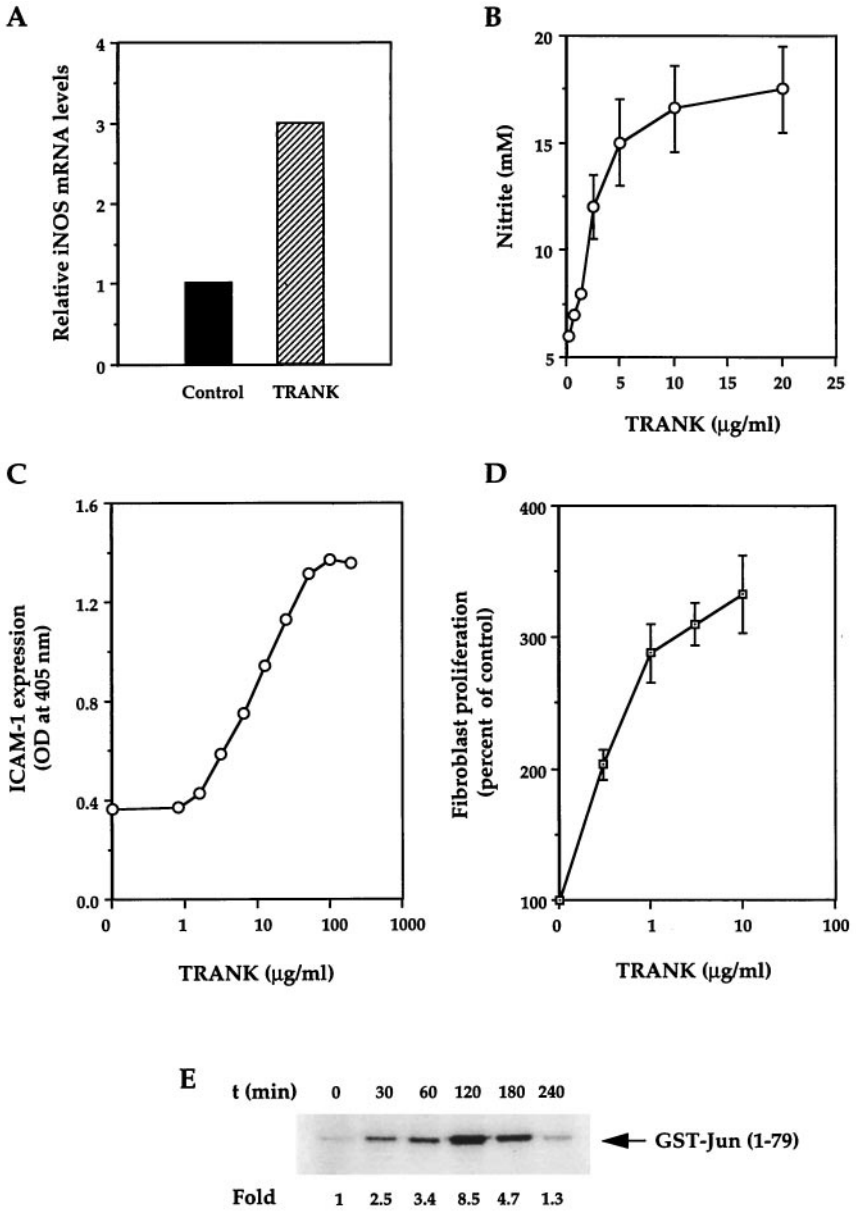


**FIGURE 4.** *A*, Effect of TRANK on iNOS mRNA levels. The induction and measurement of iNOS mRNA is described in *Materials and Methods*. *B*, TRANK-induced release of nitric oxide from rat astrocytes. *C*, Effect of increasing doses of TRANK on the expression of ICAM-1 in EAhy926 cells. *D*, Effect of TRANK on the proliferation of human diploid foreskin fibroblasts. *E*, TRANK-induced JNK activation. U-937 cells ($3 \times 10^6$/ml) were treated with 10 $\mu$g/ml TRANK for different lengths of time, and JNK was subsequently assayed as described in *Materials and Methods*.

*TRANK regulates cell growth*

Although TRANK was found to have no cytotoxic effect on most cells, it did induce the proliferation of normal human diploid fibroblasts in a dose-dependent manner (Fig. 4*D*). A more than threefold increase in fibroblast proliferation over the untreated control was observed. This finding indicated that TRANK is a growth factor for normal human fibroblast cells.

*TRANK activates JNK*

The activation of JNK is another early event that is initiated by many other stress stimuli, including cytokines, via reactive oxygen species. Treating U-937 cells with TRANK led to an increase in JNK activity in a time-dependent fashion; this activity peaked at 120 min (8.5-fold increase) and gradually declined thereafter (Fig. 4*E*).

## Discussion

Our results show that TRANK is structurally highly homologous to various antioxidant proteins, including NKEF-A and NKEF-B. Unlike these proteins, however, TRANK is a secreted protein. The function of TRANK also differs from that of various antioxidant proteins, in that it behaves much like inflammatory cytokines, such as TNF and IL-1. For example, similar to TNF, TRANK was found to activate NF-$\kappa$B and JNK, induce ICAM-1 and iNOS expression, and stimulate the proliferation of normal human fibroblasts (see Ref. 14). Recently, it has been shown that transfecting cells with the thioredoxin peroxidase gene (*nkef-B*) can block apoptosis (19). Similarly, the overexpression of another thioredoxin peroxidase gene, *A0E372*, blocked the activation of NF-$\kappa$B and the degradation of I$\kappa$B$\alpha$ that were induced by TNF and phorbol ester (2), consistent with the antioxidant properties of this gene product. (1). However, these results differ from ours in that TRANK induces

NF-κB and JNK and consequently may play a role in the induction rather than the inhibition of inflammation.

Our results also suggest that there are specific receptors through which TRANK interacts with cells. Thioredoxin (also called adult T cell leukemia-derived factor), an antioxidant protein, blocks TNF-mediated cytotoxicity when exposed to U-937 cells from outside (20) and inhibits PMA-induced NF-κB activation in HeLa cells (21). Although the inhibitory effect of thioredoxin occurred at concentrations similar to that used with TRANK in our studies (21), thioredoxin did not activate NF-κB. Even though thioredoxin was identified more than 10 years ago, no thioredoxin receptor has yet been identified. How thioredoxin is taken up by cells is therefore completely unknown. Like thioredoxin, TRANK is also a secreted protein. The wide tissue distribution of TRANK, its secretion by cells, and its ability to activate NF-κB and JNK and induce fibroblast proliferation together suggest that it plays an important role in inflammation.

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