Identification, Characterization, and Cloning of TIP-B1, a Novel Protein Inhibitor of Tumor Necrosis Factor-induced Lysis

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ABSTRACT

Some cancer cells evade elimination by virtue of their insensitivity to agents that induce apoptosis. Conversely, the side effects of anticancer agents could be diminished if normal cells were more resistant. To further elucidate the factors that contribute to the susceptibility of a cell to apoptosis, these investigations were designed to identify proteins isolated from cells exposed to low concentrations of tumor necrosis factor (TNF) that, when incubated with normally TNF-sensitive cells, protect these cells from TNF-induced cytotoxicity. TIP-B1, a novel protein, has been identified, purified, and characterized from cytosolic extracts of TNF-treated human fibroblasts. The ~27 kDa ~4.5 TIP-B1 protein is unique based on both the sequence of three internal peptides (comprising 51 amino acids) and the nucleotide sequence of the corresponding 783-bp cDNA partial clone. Western blot analyses using polyclonal antisera raised against both the purified native TIP-B1 and the ~14 kDa product of the cDNA partial TIP-B1 clone, as well as Northern blot analyses using the cDNA insert as a probe, indicate that TIP-B1 may belong to a family of proteins that are expressed in a number of cell lines from diverse tissues. TNF-sensitive cells, when exposed to 4-10 μg/ml concentrations of TIP-B1 prior to the addition of TNF, are completely protected from TNF-induced lysis. Furthermore, TIP-B1 protects cells from apoptotic lysis induced by TNF. Preincubation of TIP-B1 with TNF does not affect the ability of TNF to induce lysis. Moreover, TIP-B1 does not seem to interfere with the interactions between TNF and the TNF receptors, based on a preliminary flow cytometric analysis of the cellular binding of biotinylated TNF. On the basis of these characteristics, TIP-B1 is not a soluble TNF receptor, an anti-TNF antibody, nor a protease that degrades TNF; yet TIP-B1 functions when added exogenously to cells. These characteristics, its novel sequence, and its function when added exogenously to cells indicate that TIP-B1 is unique and is not one of the other proteins reported previously to be involved in resistance to TNF. The ability of TIP-B1 to function after exogenous incubation with target cells makes TIP-B1 a likely candidate for therapeutic manipulation of TNF-induced effects.

INTRODUCTION

TNF is a multifunctional cytokine with important roles in immune responses, inflammation, and response to injury (1, 2). It was identified initially by its ability to cause hemorrhagic necrosis of certain tumors and then was determined to be homologous with the causative agent of cachexia. The overproduction or inappropriate production of TNF has been implicated as having an important, often causative, role in a number of diseases (e.g., autoimmune diseases, parasitic infections, diabetes, multiple sclerosis, rheumatoid arthritis, and AIDS). TNF affects essentially every type of cell in the body. The pleiotropic effects of TNF may be explained in part by the existence of two forms of TNF (secreted [17 kDa] and membrane bound [26 kDa]) and two receptors for TNF [TNFR1 (~55 kDa) and TNFR2 (~75 kDa)]. The relative roles of TNFR1 and R2 signaling in TNF-induced effects, including lysis, differ depending on the cells under investigation and the effect examined. Both TNFRs appear to stimulate lysis (3–9), although it is not clear whether both receptors can induce lysis of the same cell. Additionally, it has been reported that TNF can induce both necrotic and apoptotic lysis (10).

Further investigations of the lytic potential of TNF indicated that some cells possess an innate resistance to TNF-induced lysis. This protection was not attributable to lack of TNFR on the cells but rather appeared to be the consequence of macromolecular synthesis. The production and release from the plasma membrane of TNF has been reported as one mechanism of TNF regulation (1). Moreover, several intracellular proteins have been suggested to be protective against TNF-mediated lysis, based on evidence from either gene transfection and expression of the specific proteins or from surveys that showed increased levels of these specific proteins in subclones with increased resistance. However, in many cases, neither mRNA levels nor activity of these proteins correlate with resistance (11–15). Additionally, in most cases, neither their mechanism(s) of protection nor their physiological significance to TNF resistance have been defined completely. Singly, none of these intracellular proteins appear to completely protect the cells, and it has been suggested that more than one protein is probably involved in protection (16). These data are consistent with the hypothesis that: (a) multiple mechanisms contribute to TNF-induced lysis; (b) only some of these multiple lytic pathways are activated in a given cell; and therefore (c) a specific protein will only protect cells lysed by the pathway with which it interferes.

Proteins that have been linked to protection from TNF-induced lysis include: (a) MnSOD, (15, 16); (b) PAI2 (17); (c) hsp 70 (14, 18) and hsp 27 (19); (d) endogenous TNF (20, 21); (e) bcl-2 and bcl-x (12); (f) several viral proteins [e.g., crmA (22)]; (g) adult T-cell leukemia-derived factor (23) now known to be the hTRX (24); and (h) the ~90 kDa protein encoded by the A20 clone of a TNF subtraction library (25). With the exception of hTRX, the data linking these proteins to TNF resistance involve correlating increases in their intracellular levels, either in selected resistant sublines or after gene transfection and expression, with decreased sensitivity to TNF-induced lysis.

The novel TNF inhibitory protein, TIP-B1, the discovery of which is described here, was isolated from the cytosol of low-dose, TNF-treated fibroblasts based on its ability to render cells resistant when added exogenously. This ability has only been described previously for hTRX. Although it is not yet known whether TIP-B1 is internalized and/or has a specific receptor in the cell membrane, the ability of...
TIP-B1 to function when added exogenously identifies it as a likely candidate for clinical modulation of TNF-induced effects.

MATERIALS AND METHODS

Cells and Their Maintenance

Cell lines, with the exceptions noted, were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 25 mM HEPES, and 0.1 mg/ml gentamicin in a humidified atmosphere with 5% CO₂, at 37°C. Cultured cell lines used in the purification, cloning, and activity assays of TIP-B1 included two cultured human foreskin fibroblasts lines derived at Roswell Park Cancer Institute [Hf1 (also known as BG-9) and Hf2], human embryonic lung fibroblasts (HEL cells, provided by Asahi Chemical Industry Co., Ltd.), and the human histiocytic lymphoma U937 (ATCC #CRL-1593). Cell types surveyed by Northern blot analysis for TIP-B1 mRNA expression included: mammary stromal cells and freshly isolated rat mammary epithelial cells, both kindly provided by Dr. M. M. Ip, Roswell Park Cancer Institute; murine mastocytoma P815 (26); murine lymphoma EL4 (27); human breast adenocarcinoma MCF7 (ATCC #HTB-22); human promyelocytic leukemia HL-60 (ATCC #CCL-36); human acute lymphoblastic leukemia (ATCC #CRL-1582); human promyelocytic leukemia HL-60 (ATCC #CCL-240); NIHDF (Clonetics, San Diego, CA); and human malignant melanoma A375 (ATCC #CRL-1619). The latter two cell lines were maintained in DMEM supplemented with 10% Fe₂⁺-enriched calf serum, 4.5 g/l glucose, 25 mM HEPES, and 0.1 mg/ml gentamicin.

TNF-induced Lysis

The TNF (recombinant human, 1 unit = 0.4ng) used in these studies was a gift from Asahi Chemical Industry Co., Ltd. (Shizwoka-ken, Japan). The amount of lysis induced by TNF was examined in the presence of CHX. The addition of CHX accomplishes two things that are most likely not mutually exclusive. CHX decreases the time required for TNF-induced lysis to reach a detectable level (28) and also prevents TNF-induced synthesis of protective proteins that would compromise the interpretation of the data. Controls were: medium (no TNF or CHX), TNF alone, and CHX alone. The specifics of these assays are described below and, in each case, the assay was optimized (e.g., TNF concentration, CHX concentration, time of incubation, time of preincubation, and others) for the target cell type being used. The concentrations of TNF + CHX added were selected to produce a minimal lytic effect of CHX alone but maximal lysis in the presence of TNF. Final volumes were kept constant at 200 μl for assays of total lysis and 1 ml for apoptotic assays.

TIP Protection from TNF-induced Lysis

Protein fractions tested for TIP activity were preincubated at multiple concentrations (serial 2-fold dilutions spanning several log orders (i.e., 1:2, 1:4, 1:8, 1:16, ... 1:65,536)) with target cells for 6–20 h (depending on the cell line/assay) prior to the addition of TNF + CHX to assess TIP sensitivity. In all cases, protection against lysis was determined by comparison with controls that contained all assay components but were incubated with medium in place of putative TIP fractions.

Specific Assays

³¹Cr Release. This assay was used with cells that grew in suspension (e.g., U937). After ³¹Cr labeling (100 μCi Na₂³¹CrO₄/10⁶ cells for 1 h), cells were dispensed into the wells of 96-well plates (2 × 10⁵ cells/well) and preincubated for 18 h with putative TIP-B1 fractions. TNF and/or CHX were added (e.g., for U937: 50 units TNF/ml and 0.25 μg CHX/ml), followed by a 6-h incubation; the cells were pelleted, and lysis was assessed by gamma counting of the supernatant, as has been described previously (26).

Staining of Viable Cells. These assays were used with adherent cells (e.g., HF2). Twenty h after cell plating (1.5–4 × 10⁵ cells/well, 96-well plates), medium was removed, and putative TIP-B1 fractions were added. Ten h later, assay components (e.g., for HF2: 500 units of TNF/ml and 100 μg of CHX/ml) were added. Eighteen h later, the cells were stained with MTT, as described previously (29) or crystal violet as follows. The cells were fixed with 4% formaldehyde for 15 min, the formaldehyde was removed, the cells were stained with crystal violet (0.05%, 100 μl/well) for 15 min, and the plates were washed extensively and air dried. The crystal violet retained by the cells (indicative of survival) was eluted with 100 μl/well of ethanol:H₂O:acetic acid (25:24:1) and monitored by absorbance at 570 nm. Because MTT staining requires active mitochondrial enzymes and TNF has been shown to affect mitochondrial function (30, 31), crystal violet and MTT staining were carried out in parallel in many experiments. The results obtained using these two different dyes did not differ significantly under the conditions used (data not shown).

Staining of Apoptotic Cells. Cells (10⁶ for propidium iodide or 5 × 10⁵ for TD) were preincubated for 18 h with putative TIP-B1 fractions. Then TNF and/or CHX were added (final concentrations of 50 units/ml and 0.25 μg/ml, respectively, for U937 cells); 6 h later, the cells were washed, and apoptosis was assessed by propidium iodide staining or by labeling of DNA strand breaks using exogenous TdT. Propidium iodide staining was carried out as described previously (32). For labeling DNA strand breaks using TdT (33), the manufacturer’s (Boehringer Mannheim, Indianapolis, IN) suggested protocol was followed. The samples were analyzed by using a flow cytometer, and five thousand events per sample were acquired in list mode on FACScan (Becton Dickinson, San Jose, CA) with linear amplification. Data were analyzed with Lysys and Paint-A-Gate (Becton Dickinson, San Jose, CA). The degree of apoptosis was determined from the specific fluorescein-dUTP labeling in the TdT assay and from the subdiploid peak in the propidium iodide assay.

Preparation of a Cytosolic Extract

TNF-treated (1000 units/ml for 18 h) HEL cells were lysed by sonication in ice-cold lysis buffer (20 mM sodium phosphate (pH 7.4), with 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM Na₃-p-tosyl-L-arginine methyl ester, 1 mM phenylmethylsulfonyl fluoride, and 1 mM N-tosyl-L-phenylalanine chloromethyl ketone for 20 ml/10⁶ cells) and clarified by centrifugation (30 min at 12,000 × g), and the resulting supernatant was recentrifuged (100,000 ×g for 60 min). The protein concentration of this cytosolic extract was determined by absorbance (1.55 A₅₃₀ -0.76 A₅₄₆ = mg/ml of protein). An identical procedure was used to prepare cytosolic extracts (100,000 × g supernatant) from other cultured cell lines.

Purification of TIP-B1 from a Cytosolic Extract (100,000 × g Supernatant)

Proteins (50 mg) in the 100,000 × g supernatant from HEL cells were separated into 20 fractions by isoelectric focusing on a Rotofor apparatus (Bio-Rad, Hercules, CA) using their pl 3–10 amphyoles according to the manufacturer’s instructions. Fractions from multiple Rotofor separations were pooled based on similar TIP activity, pH range and protein profiles on silver-stained SDS-PAGE (PAGE, 12.5% acrylamide gels with a running buffer of Tris/SDS/glycine). The amphyole-bound proteins within the lowest pl (pH < 4.5) pool of fractions that contained TIP activity were then refocused without additional amphyoles, again yielding 20 fractions. The bound amphyoles were removed from each fraction by incubation in 1 M NaCl and repeated buffer exchange (10 mM NaPO₄, pH 7.4). Protein concentrations were then determined using either the Bio-Rad Protein Assay reagent and/or by densitometric scans of silver-stained SDS-PAGE gels of the various fractions and comparison with the density of known amounts of BSA resolved on the same gel.

Generation of an Antisera to TIP-B1 Purified from Eukaryotic Cells

After SDS-PAGE and Coomassie blue staining, the ~27 kDa TIP-B1 band was excised from the gel. Two female NZW rabbits were injected intradermally at multiple sites with a sonicated suspension of the pulverized gel slice (containing TIP-B1) and Freund’s adjuvant. The initial injection contained 2.5 μg of TIP-B1 in complete adjuvant; the subsequent boost injection contained 1.0 μg of TIP-B1 in incomplete adjuvant. The rabbits were boosted at 3-week intervals for a total of three injections. A serum sample was tested 7 days after the last injection using Western blot analysis; reactivity to specific protein bands was apparent, and the rabbits were exsanguinated 7 days later.
Primary Amino Acid Sequence Analysis of TIP-B1

Two independently purified protein preparations were subjected to amino terminal sequencing. For analysis of the internal amino acid sequence of TIP-B1, ~5 μg (~200 pmol) of one of the two purified TIP-B1 preparations were digested with endoproteinase Lys C (Boehringer-Mannheim; 24 h at 37°C per Stone et al. (34)). The peptides generated were separated by reverse phase HPLC (Vydac C18), and peptide peaks were collected (35). Fractions containing single peaks were repurified by a similar HPLC procedure. Four of the resulting peptides were analyzed by mass spectroscopy, and amino acid sequences of the three were determined. All amino acid sequence analyses were performed by the W. M. Keck Foundation Biotechnology Resource Laboratory (New Haven, CT).

Construction of a TNF-induced HF1 cDNA Library and Isolation of a Partial TIP-B1 cDNA Clone

A cDNA phagemid library was constructed from 5 μg of poly(A)⁺ RNA (Poly(A) Tract System; Promega, Madison, WI) from TNF-treated (1000 units/ml for 18 h) HF1 cells using the AZAP cDNA synthesis kit (Stratagene, La Jolla, CA).

On the basis of the codons of the amino acid sequences of the three internal peptides, mixtures of degenerate oligonucleotides were designed to serve as primers. The oligonucleotide synthesis was performed by the Biopolymer Facility (Roswell Park Cancer Institute). These primers were used in various combinations in RT-PCR amplifications of poly(A)⁺-enriched RNA from total RNA (36) of TNF-treated (1000 units/ml for 18 h) HF1 cells. Primer extension of the product cDNA was followed by design of new primers. Utilization of these primers in the PCR amplification (95°C for 5 min, then 35 cycles of 95°C for 30 s, 45°C for 1 min, and 72°C for 3 min, with a final extension at 72°C for 7 min) of plasmid DNA (isolated by max excision of cDNA library) led to the isolation of a 274-bp cDNA product, which was cloned onto the T/A vector (Invitrogen, La Jolla, CA). Nucleotide sequencing of this cDNA (DNA Sequencing Facility, Roswell Park Cancer Institute) demonstrated that it encoded the three peptides arranged in tandem (ABC). This 274-bp cDNA was used to rescreen the cDNA library. Several positive clones were identified; the clone with the longest cDNA insert (~0.8 kb) was sequenced and found to consist of 783 bp and to contain the sequence of the 274-bp cDNA. This clone was designated tip-SN. The cDNA insert from this clone was recloned in a prokaryotic expression vector (pET Z4a-; Novagen, Madison, WI) containing an IPTG-inducible promoter. DNA sequencing confirmed that this clone contained the tip-SN cDNA insert in the reading frame corresponding to that of the peptides and, it was predicted that upon translation, its product would be an ~14 kDa protein.

Production and Purification of Recombinant Partial TIP-B1 Protein (rTIP-B1p)

After 0.4 mM IPTG induction (for 2 h) of bacteria transfected with the tip-SN expression plasmid, the bacteria were lysed by sonication in 100 mM Tris-HCl (pH 8.3; 5°C), 1 mM EDTA, 100 mM phenylmethylsulfonyl fluoride, and a bacterial protein extract (100,000 × g supernatant) was generated, essentially as described for the purification of TIP-B1 protein from eukaryotic cells. The proteins present in extracts from uninduced and induced tip-SN-transfected bacteria were resolved by SDS-PAGE and visualized by Coomassie blue staining; the levels of an ~14 kDa protein were increased in the extract from IPTG-induced tip-SN-transfected bacteria. The proteins that precipitated from the supernatant upon 40% ammonium sulfate saturation of the 100,000 × g supernatant from IPTG-induced tip-SN-transfected bacteria were resuspended, dialyzed into column running buffer (PBS; pH 8.3) and separated by gel filtration (G75 Sephadex; Pharmacia, Piscataway, NJ) using column chromatography (~100 mg protein in 3 ml column; column size, 2.5 cm × 60 cm; flow rate, 1.75 ml/min). The fractions containing the 14 kDa protein (rTIP-B1p), as determined by Coomassie blue staining after SDS-PAGE analysis of an aliquot of each of the column fractions, were pooled and concentrated.

Generation of anti-rTIP-B1p Antiserum

Two NZW rabbits were injected at multiple sites intradermally with purified rTIP-B1p protein (500 μg in Complete Freund’s adjuvant), and the rabbits were boosted at weeks 3 and 5 with one-half of the initial dose in incomplete adjuvant. Rabbits were bled 7 days after each boost, and the titers of these antisera were assessed using serial dilutions of each primary antiserum in a series of Western blots. Injections of antigen and bleeding were a service of Roswell Park Cancer Institute’s Large Animal Facility (Springville Labs, Springville, NY).

RNA Extraction and Northern Blot Analysis

For HF1, HEL, and P815 cells, total RNA was isolated by lysis in guanidinium isothiocyanate and pelleting through CsCl (36). For all other cell lines, total RNA was purified using the Trizol reagent according to the manufacturer’s (Life Technologies, Inc., Grand Island, NY) instructions. Northern blotting was performed according to standard methods (37) using Genescreen membrane (NEN Research Products/DuPont, Boston, MA) and detection by hybridization to a [³²P]cDNA-labeled TIP-B1 783-bp clone tip-SN cDNA insert. The blots were stripped andrehybridized with a [³²P]glyceraldehyde-3-phosphate dehydrogenase probe (Clontech, Palo Alto, CA).

Western Blot Analysis

Protein (5–20 μg) was separated by SDS-PAGE (15% acrylamide gels). The proteins were transferred from the gels to Immobilon P membranes (Millipore, New Bedford, MA) by electroblotting in 25 mM Tris base, 250 mM glycine, and 15% v/v methanol (5°C, 90 V, 2 h). Immunostaining of the membrane was then used to detect TIP-B1. Briefly, the membrane was blocked with 3% dry milk in TST [50 mM Tris-HCl (pH 7.6) at 37°C, 150 mM NaCl, and 0.1% v/v Tween 20] for 30 min and then incubated for 1 h with the primary polyclonal antiserum (1:10,000) in TST. The blot was washed with TST three times, incubated for 1 h with goat anti-rabbit IgG linked to alkaline phosphatase (Jackson Immunological, West Grove, PA; 1:50,000) in TST, and washed three times with TST prior to chemiluminescent detection according to the manufacturer’s (Tropix, New Bedford, MA) directions.

GST γ protein samples used were a HeLa cell extract (Transduction Laboratories, Lexington, KY) and GST γ purified from human placenta (Sigma Chemical Co., St. Louis, MO). The latter was also tested for TIP activity using the procedure described above.
replicate microwells; with crystal violet and monitored by absorbance. Each point represents the mean of six results for TNF preincubation from 2.5–40 min on an expanded time scale.

normally sensitive human fibroblasts from lysis induced at high concentrations of TNF. To identify and investigate the factor(s) responsible, normally sensitive cells from TNF-induced lysis. The profile of the resulting 20 fractions was analyzed as above for its ability to protect TNF-sensitive cells from lysis when added exogenously to the cells.

Isoelectric Focusing of the Cytosolic Extract Generated Three Protein Fractions with the Ability to Protect Cells from TNF-induced Lysis. The TIP-containing cytosolic extract from TNF-treated HEL cells was fractionated by isoelectric focusing. Each of the resulting 20 fractions was analyzed as above for its ability to protect normally sensitive cells from TNF-induced lysis. The profile of the activity (Fig. 2B) suggested that three peaks of apparent TIP activity were resolved (pIs of 4.5, 5.2, and 8.0) and, to a first approximation, the levels of activity in the three pools were similar. The fractions encompassed by each peak were pooled and were evaluated by silver-stained SDS-PAGE analysis (not shown). The pool (fractions 2 + 3) having the lowest pI (pI ~4.5) contained the fewest proteins (i.e., two predominant protein bands) and, therefore, was selected as the first to be examined further.

Association of Protective Activity in the Lowest pI Fraction with a Single Protein Band. To further purify the pI ~4.5 TIP protein, an aliquot of fractions 2 + 3 pooled from multiple Rotofor separations, with the ampholytes from the original focusing still bound, was refocused. This resulted in a very shallow pH gradient (Fig. 3A) and further resolution of the fractions 2 + 3 proteins across the 20 resulting fractions. However, protein was only detectable in fractions 9–11. Equal volumes of each of these nine fractions were assayed for TIP activity using HF2 (Fig. 3A) and U937 (not shown) cells or analyzed by silver-stained SDS-PAGE (Fig. 3B). The TIP activity (Fig. 3A) correlated with the amount of the ~27 kDa protein (Fig. 3B) and not with that of the other detectable protein (~37 kDa; Fig. 3B). However, these data do not exclude the possibility that the ~37 kDa protein also has some TIP activity. The ~27 kDa and ~37 kDa proteins were electroeluted after their separation by SDS-PAGE to generate a preparation of essentially pure ~27 kDa protein and one of pure ~37 kDa protein (data not shown). After removal of SDS, these two proteins were assayed in parallel for TIP activity. The ~27 kDa protein protected NHDF cells from TNF + CHX cytotoxicity (32 ± 7% protection at 1 µg/ml; however, comparable amounts of the ~37 kDa protein provided no protection (0 ± 6%). A standard protein purification table showing the fold increase in specific activity at each step is not valid in this instance because the activity in the original cytosolic extract (100,000 × g supernatant) is apparently attributable to multiple proteins, one of which is TIP-B1.

Examination of TIP-B1 Purity and Partial Amino Acid Sequencing of TIP-B1. The resolution of the ~27 kDa protein, as shown in Fig. 3B and on other SDS-PAGE gels, suggested that it might contain more than one ~27 kDa protein. To address this possibility, an aliquot of pooled fractions 9–11 (Fig. 3), in which only proteins of ~27 kDa were detected, was analyzed by two-dimensional gel electrophoresis (Fig. 4A, inset). The presence of a single spot indicated that this preparation contained only a single protein (~27 kDa, pl ~4.5), which was designated TIP-B1 and used for protein sequencing. Two independent TIP-B1 preparations were subjected to NH2-terminal sequencing. An identical sequence was obtained with

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Fig. 1. Treatment with low concentration of TNF induces resistance of HF1 cells to TNF. HF1 cells were incubated with TNF at the indicated concentrations for various times ranging from 2.5 to 1440 min prior to the addition of TNF + CHX (at final concentrations of 500 units/ml and 200 µg/ml, respectively). Eighteen h later, viable cells were stained with crystal violet and monitored by absorbance. Each point represents the mean of six replicate microwells; bars, SD. Similar results were obtained in two experiments. Inset, results for TNF preincubation from 2.5–40 min on an expanded time scale.

Fig. 2. The 100,000 × g supernatant from fibroblasts contains TIP activity that can be separated into three protein pools, A, HF1 cells were preincubated with multiple dilutions of HF1 100,000 × g supernatant for 16 h prior to the addition of TNF and CHX and assessment of lysis (as described in Fig. 1). Each point represents the mean of three microwells; bars, SD. At the 1:4 dilution, the final concentration of 100,000 × g protein in the assay was 1.9 µg/ml. B, 50 µg of cellular protein extract (100,000 × g supernatant) from TNF-treated HEL cells were separated by isoelectric focusing on the Bio-Rad Rotofor apparatus using ampholytes ranging in pl from 3 to 10. The bound ampholytes were removed. Each fraction was adjusted to the same final volume, and a series of serial dilutions of each fraction was assayed (in duplicate) for TIP activity in U937 cells. The maximum percentage of protection provided by each fraction is shown. The highest protection observed in this experiment was 25%; however, concentrations of protein greater than those in a 1:4 dilution were not tested. Representative data from one of three experiments are shown; a similar profile of activity was evident when proteins from TNF-treated HF1 cells were analyzed.
Investigation of the Mechanism of Action of Purified TIP-B1

Cells Must Be Preincubated with TIP-B1 to Be Protected. A number of characteristics of the protection of TIP-B1 against TNF-induced lysis were investigated further to provide information pertaining to its mechanism of action. As shown in Fig. 5, preincubation of TIP-B1 with the target cells was found to be required for protective activity; when TIP-B1 was preincubated with TNF prior to the addition of target HF2 cells and CHX, no inhibition of TNF-induced lysis was detected. This indicates that TIP-B1 is neither one of the previously characterized soluble receptors for TNF (consistent with its novel sequence), nor does it neutralize TNF by binding or degrading it. The results shown in Fig. 5 also demonstrate that, when assayed in the standard TIP assay, there was a clear concentration dependency to the protective activity. Furthermore, the specific activity (\(~8.75\%\) protection/\(\mu\)g \(~27\) kDa TIP-B1 protein) of fractions 9–11 and fraction 13 are the same. This provides further evidence that the TIP activity in these fractions is attributable to the \(~27\) kDa (and not the \(~37\) kDa) protein.

TIP-B1 Protects against Total Lysis and Apoptosis. The assays of TIP activity (Figs. 2, 3A, and 5) measured total lysis and could not distinguish TNF-induced necrosis from apoptosis. Flow cytometric analyses were used to determine whether TIP-B1 specifically blocked TNF-induced apoptosis. TIP-B1 was capable of protecting U937 cells against TNF-induced apoptosis in a concentration-dependent manner when measured with a flow cytometer by assessing either: (a) the subdiploid peak of propidium iodide stained DNA (data not shown); or (b) the amount of DNA-associated fluorescent labeling observed using a standard TdT assay [per Gorczyca et al. (33); Table 1]. Similarly, protective activity was found in parallel assays evaluating total lysis (i.e., \(^{51}\)Cr release) in the presence of TNF + CHX.

Molecular Biological Characterization of TIP-B1

Development of a Probe and Isolation of a Partial TIP-B1 cDNA Clone (tip-SN). As described in “Materials and Methods,” a combination of RT-PCR and primer extension reactions led to the isolation of a partial TIP-B1 cDNA clone (tip-SN). As described in “Materials and Methods,” a combination of RT-PCR and primer extension reactions led to the isolation of a partial TIP-B1 cDNA clone (tip-SN).
generation of a 274-bp cDNA probe found to encode all three internal peptides. This 274-bp cDNA probe was used to screen a cDNA library produced from RNA of TNF-treated HF1 cells. A clone was identified (clone tip-SN, 783 bp), and sequencing confirmed that it contained the 274-bp sequence (Fig. 6). DNA sequence database searches (the most recent in December 1998) have not identified homology of this sequence with any sequence that encodes a protein of known function. However, the clone tip-SN cDNA sequence is 98–99% homologous to ~200 nucleotide sequences of unknown function arising from the sequencing of cDNA libraries (ESTs). All of these EST sequences are included within the tip-SN sequence. The nucleotide sequence of the clone tip-SN cDNA sequence is 98–99% homologous to ~200 nucleotide sequences of unknown function arising from the sequencing of cDNA libraries (ESTs). All of these EST sequences are included within the tip-SN sequence. The nucleotide sequence of the clone tip-SN cDNA sequence is 98–99% homologous to ~200 nucleotide sequences of unknown function arising from the sequencing of cDNA libraries (ESTs). All of these EST sequences are included within the tip-SN sequence. The nucleotide sequence of the clone tip-SN cDNA sequence is 98–99% homologous to ~200 nucleotide sequences of unknown function arising from the sequencing of cDNA libraries (ESTs). All of these EST sequences are included within the tip-SN sequence.

Table 1  

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<th>Assay</th>
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<td>TIP-B1 (3.12 μg/ml) + TNF + CHX</td>
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<sup>a</sup> ND, not determined.

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Table 1  TIP-B1 protects U937 cells against TNF-mediated lysis, including apoptosis

The degree of lysis was determined by the release of radioactivity from 31Cr-labeled U937 cells. Briefly, U937 cells were labeled with 31Cr and plated in 96-well plates. TIP-B1 or medium alone was added (final volume, 100 μl) as indicated, and the plates were incubated for 18 h. Then, the lysis assay was initiated by the addition of TNF (at a final concentration of 50 units/ml) and CHX (at a final concentration of 0.25 μg/ml) where indicated (in a final volume of 200 μl). Six or 10 h later, the cells were pelleted, and the amount of radioactivity in the supernatant was assessed by gamma counting. The percentage of specific 31Cr release was calculated and is expressed as the average ± SD (n = 5). Apoptosis was measured by flow cytometric analysis of the specific fluorochrome label incorporated after fixation in formaldehyde and incubation with TdT enzyme and fluorescein labeled-dUTP. Five thousand events (cells) were measured.

Expression of TIP-B1 mRNA. To examine the expression of TIP-B1 message, RNA blots prepared from a variety of human cell lines were probed with the tip-SN cDNA insert. The Northern blot analysis of HF1 RNA revealed a strong 1.1 ± 0.1-kb message. Two weaker bands of about 1.7 and 3.0 kb were revealed upon more prolonged exposure of the autoradiograms (Fig. 7A). The 1.1-kb message, when normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA, was induced 1.7-
fold by TNF treatment of HF1 cells (Fig. 7A). The same TNF treatment of cells in flasks (1000 units/ml) that was used in the protein purification was used in these experiments analyzing mRNAs that hybridize to tip-SN (Fig. 7A). A 1.1-kb RNA that hybridized to the tip-SN probe was also detected in a number of other human cells, including those of the HEL, MCF7, U937, HL-60, Reh, Molt 4, NHDF, and A375 cell lines (Fig. 7B).

The clone tip-SN insert hybridized to a 1.1-kb RNA present in rat cells (stromal and epithelial) isolated from mammary glands and murine (P815 and EL4) cultured cell lines (data not shown). The weaker signals (i.e., 1.7- and 3.0-kb messages) were detected in some, but not all, of the cell lines examined.

Production of the Recombinant Protein Product from Clone tip-SN. The tip-SN insert cloned in-frame into an IPTG-inducible prokaryotic expression vector was used to transfect bacteria. SDS-PAGE analysis of cytosolic extracts from IPTG-induced bacteria contained a protein of the predicted size (~14 kDa), designated rTIP-B1p. This protein was purified by gel filtration (Fig. 8A). Purified rTIP-B1p possessed protective activity against TNF-induced lysis of both HF1 (Fig. 8B) and U937 cells (not shown), as has been demonstrated for TIP-B1. The maximal activity obtained with rTIP-B1p was ~22% ± 7% protection against TNF-induced lysis at ~50 μg/ml. This represents ~40% of the specific activity of TIP-B1 (compare Figs. 5 and 8B). In addition, unlike TIP-B1, increasing amounts of rTIP-B1p did not result in greater amounts of protection (compare Figs. 5 and 8B). The differences in activity between the two proteins are likely related to the fact that rTIP-B1p represents only ~50% of TIP-B1 and/or, because rTIP-B1p is bacterially produced, certain posttranslational modifications, which may be required for full TIP-B1 activity, would not have occurred.

Polyclonal Antiserum Raised against TIP-B1 and Antiserum Raised against the Recombinant Partial TIP-B1 Protein React with the Same Proteins. NZW rabbits were immunized with purified rTIP-B1p, and the reactivity of the resulting polyclonal serum was examined by Western blotting. The antiserum reacted with a number of proteins in the semipurified rTIP-B1p fraction (Fig. 9A). Experiments demonstrated that the majority of the immunoreactive bacterial proteins, but not the ~14 kDa protein, were adsorbed by incubation of the serum with proteins extracted from an IPTG-induced bacterial culture that had been transfected with the empty vector (Fig. 9B). A second adsorption of this “vector adsorbed serum” with purified rTIP-B1p specifically eliminated reactivity with the ~14 kDa band.
Fig. 10. Antibodies to TIP-B1 react with an ~27 kDa protein in 100,000 x g supernatants from HFI and HEL cells. Duplicate samples of 5 μg of cytosolic protein (100,000 x g supernatant) from several sources were separated by SDS-PAGE (12.5% gel) and electotransferred to polyvinylidene difluoride membranes. The blot was blocked, hybridized with serum obtained prior to inoculation with TIP-B1 (Pre-immune, left panel) or anti-TIP-B1 antiserum (1:1000; Immune, right panel), hybridized with the secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit IgG), and reacted with chemiluminescent substrate prior to visualization on film. Cytosolic protein samples were obtained from: untreated HF1 cells (Lane 1), TNF-treated (1000 units/ml; 18 h) HEL cells (Lane 2), and a fraction enriched for TIP-B1 from HEL cells (Lane 3).

Fig. 11. Anti-TIP-B1 antiserum and anti-rTIP-B1p antiserum have similar reactivities. Western blot analysis was performed with protein extracted from HEL cells. Two primary antisera were used: antiserum against TIP-B1 (Lane 1) and antiserum against rTIP-B1p (Lane 2).

Western blot analysis using a different polyclonal antiserum generated in rabbits injected with purified TIP-B1 resulted in detection of a protein of the anticipated size (~27 kDa) in both HFI and HEL cells; another protein of ~34 kDa was also detected with this antiserum in both cell lines (Fig. 10). Furthermore, based on these analyses, it can be suggested that the immunoreactive ~27 kDa protein is induced in HFI cells by TNF treatment. However, none of these stocks of this human fibroblast line have been exhausted, and further studies of this cell line are not possible. The reactivity of the anti-TIP-B1 antiserum and the antiserum raised against the recombinant ~14 kDa protein product of the tip-SN clone were compared. Each serum was used independently to develop a Western blot containing protein extracted from HEL fibroblasts (which contain RNAs that hybridize to the clone tip-SN cDNA insert, Fig. 7B). Both antisera detected proteins of identical size (P27, P32 and P34; Fig. 11). Anti-TIP-B1 antiserum did not react with two different GSTp preparations, despite their use at amounts comparable with those at which TIP-B1 is easily detectable (Fig. 12); both GSTp preparations were readily detected by immunoblot analysis using a monoclonal anti-GSTp antiserum (data not shown). Additionally, GSTp did not protect NHDF cells from TNF-induced lysis (0 ± 3% protection at the concentration of 100 μg/ml and lower concentrations were also inactive), whereas purified TIP-B1 did (94 ± 0.3% protection at 3 μg/ml).

**DISCUSSION**

The studies described herein were designed to identify and then further characterize cellular proteins that protect normally sensitive cells from TNF-induced lysis. These investigations have led to the discovery of a novel cytosolic protein, designated TIP-B1, which possesses protective activity against TNF-induced cell death, including protection from TNF-induced apoptosis when given exogenously.

**TIP-B1 Is a Novel Protein.** The sequence of TIP-B1 is unique at both the amino acid (Fig. 4) and cDNA (Fig. 6) levels based on lack of homology to sequences in several protein databases and to those in nucleotide databases identified as encoding a protein of known function. The cDNA clone (clone tip-SN) putatively encodes ~70% of the TIP-B1 mRNA and ~50% of the TIP-B1 protein (the carboxyl portion), including the 51 amino acids determined by micro amino acid sequencing of the three internal peptides isolated after enzymatic digestion of purified TIP-B1. Specific sequence comparisons between the TIP-B1 protein and proteins that are believed to be involved in TNF signaling revealed that there was no apparent homology between TIP-B1 and proteins reported to associate with the cytoplasmic domains of TNFR superfamily members (i.e., TRAFs, TRADD, MACH1, and others) including inhibitors of apoptosis proteins (40, 41). As far as it can be determined, TIP-B1 and hsp 27, a protein that has also been implicated in resistance to TNF-induced lysis (19), have no amino acid sequence homology. However, they have similar molecular weights and isoelectric points. As an additional method to confirm their independent identities, Western blotting analysis was performed using antibodies to hsp 27 (StressGen Biotechnologies Corp., Victoria, British Columbia, Canada), and no reactivity with purified TIP-B1 was detected (data not shown). It has been reported previously that three proteins are induced in A375 melanoma cells by TNFR1 cross-linking: MnSOD, PAI2, and an ~28 kDa protein (pl ~5.6) protein, which is also not immunologically related to hsp 27 (42). On the basis of the paucity of published information on the amino acid structure of the 28/5,6 protein, it is not possible to say what relation, if any, exists between that 28 kDa protein and either TIP-B1 or the other TIP fractions described herein (Fig. 2B). Taken together, these data indicate that TIP-B1 is a previously unreported protein inhibitor of TNF-induced lysis.

**TIP-B1 Shares Homology with Cellular Proteins Involved in Reduction/Oxidation.** Although TIP-B1 has a unique sequence, it does contain regions that share homology with proteins involved in modulating the reduction/oxidation (redox) state of the cell via GSH...
TIP-B1 protein (determined from sequencing of two independently purified protein preparations) are homologous to the NH2-terminal sequences of GST\(\pi\) and FAEES III (26 kDa, pI 4.9). FAEES III catalyzes the addition of ethanol to fatty acids, shares 98% homology with GST\(\pi\), and also has GST activity. Both FAEES III and GST\(\pi\) have a tyrosine at position 7, also present in the TIP-B1 sequence, which has been demonstrated to bind the sulfhydryl group of GSH (43). TIP-B1, however, is NOT GST\(\pi\) based on: (a) the lack of TIP activity of a purified GST\(\pi\) preparation; and (b) the inability of anti-TIP-B1 antiserum to react with GST\(\pi\) (Fig. 12). An additional connection between TIP-B1 and reduction/oxygenation is that the TIP-B1 partial cDNA clone encodes a protein sequence that shares homology (19 amino acids of a 51-residue sequence are identical) with bacterial glutaredoxin, an enzyme that catalyzes thiol transfer (44). Although the human homologues of glutaredoxin are known, they do not share homology with the TIP-B1 sequence (45). Despite the implication that TIP-B1 could conceivably be involved in oxidation/reduction of GSH, purified TIP-B1 lacked activity when assayed for the ability to transfer GSH to a standard substrate. Additional evidence suggesting that protection against TNF-induced lysis may be conferred by proteins that modulate the redox state of the cell via GSH metabolism is the report that hTrX protects against TNF-induced lysis when added exogenously (23). A recent hypothesis pertaining to the activities of TNF is that TNF induces proteins, via activation of NF-\(\kappa\)B, which inhibit TNF-induced lysis (46). In light of this theory, it is interesting that hTrX has been reported to activate NF-\(\kappa\)B (47). Finally, computer protein motif searches of the predicted NH2 acid sequence of the 14 kDa rTIP-B1p indicated that it contained a casein kinase II phosphorylation, an amidation, and three \(N\)-myristoylation consensus sequence sites (Fig. 6). This suggests that TIP-B1 may undergo considerable posttranslational modification.

Properties of the Clone tip-SN and the rTIP-B1p It Encodes. The cDNA clone tip-SN, which putatively encodes \(~50\text{--}70\%\) of TIP-B1, has been used in the investigations to probe RNA expression (Fig. 7) and for production of TIP-B1p protein for generation of an antiserum (Fig. 9) because a full-length TIP-B1 cDNA clone has not yet been obtained. Further attempts to obtain a full-length TIP-B1 clone by traditional cloning methods have been unsuccessful to date, presumably because of a secondary structure interfering with either the initial reverse transcription and/or the subsequent PCR amplification. Methods that have been unsuccessful included screening a number of cDNA libraries, primer extension, RT-PCR (using the Tth enzyme or standard reverse transcriptases followed by Taq polymerase) with primers based on the NH2-terminal sequence of TIP-B1, and 5' rapid amplification of cDNA ends with the addition of DMSO and GC melt (Clontech) to disrupt secondary structure. Investigations are now under way to determine the sequence of the TIP-B1 gene. In light of the difficulty encountered during these full-length TIP-B1 cloning efforts, it should be noted that none of the \(~200\text{ EST cDNAs that are homologous to clone tip-SN contain sequence information that is not found in clone tip-SN. Thus, none of these ~200 ESTs extend any further toward the 5' end of the mRNA than does the cDNA sequence of clone tip-SN.}

Despite the partial nature of its sequence, the identification of the tip-SN has allowed for the production of an antisera that reacts with TIP-B1 (Fig. 11) and has provided important information regarding the expression of TIP-B1 mRNA (Fig. 7). There are three mRNAs that hybridize to the insert from clone tip-SN (1.1, 1.7, and 3.0 kb); these three mRNAs are expressed in different proportions in the different cells examined (Fig. 7). The intensity of the signal suggests that the 1.1-kb message shares more homology with tip-SN and/or is more predominant; this mRNA is induced \(~1.5\text{--}2\) fold by TNF in HF1 cells (Fig. 7A). Although this is only a modest TNF induction of mRNA, it is possible that TNF induction of TIP expression occurs at the protein and/or activity level without a major increase in the steady-state levels of the corresponding mRNA(s); the data suggest that this is the case in HF1 cells (compare Figs. 7A and 10). An antiserum raised against rTIP-B1p reacts with three cytosolic proteins (P27, P32, and P34); proteins of identical sizes are detected with an antiserum raised against purified cellular TIP-B1 (Fig. 11). This observation, together with the findings that three pools of cellular proteins with different isoelectric points that possessed TIP activity could be separated during the purification of TIP-B1 (Fig. 2B) and that tip-SN cDNA hybridized to mRNAs of three different sizes (Fig. 7), suggest that TIP-B1 may be a member of a protein family.

Biological Properties of TIP-B1. These investigations have revealed several interesting features of the inhibition of TNF-induced lysis by TIP-B1. Purified TIP-B1 protects against TNF-induced lysis of HF2 (Figs. 3A and 5), U937 (Table 1), and NHDF and HF1 (not shown) cells, suggesting that the TNF inhibitory activity of TIP-B1 is not strictly cell type specific. Although protection by TIP-B1 was qualitatively similar between the various target cell lines used, quantitative differences between the fibroblast cell lines and the U937 hematopoietic cell line in the extent of protection afforded by a given fraction were noted. For example, maximal TIP-B1 protection against total lysis was 100% for HF2 cells (Fig. 3A), whereas it was only \(~50\%\) for U937 cells (Table 1). This might indicate that the TNF-mediated lytic pathway inhibited by TIP-B1 is responsible for a different proportion of the TNF-mediated lysis in the two cell lines. In addition, the concentration of TIP-B1 that resulted in 100% protection against apoptosis of U937 cells by TNF alone protected only 50% against total lysis of these cells. This is consistent with the hypothesis that TNF induces both apoptotic and necrotic lysis of U937 cells [as has been reported in the literature (10)] and that TIP-B1 is capable of inhibiting apoptosis but not necrosis (or at least not to the same extent). Future experiments will examine whether TIP-B1 is capable of protecting cells from cytotoxicity induced by agents (e.g., Fas/Fas ligand, radiation, and chemotherapeutic drugs) other than TNF.

In addition to its unique sequence, the characteristics of the protective activity of TIP-B1 are also unique among those of other proteins that have been reported to be associated with resistance to TNF-induced lysis. TIP-B1, unlike most of the other putative inhibitor proteins, was shown to be active when added exogenously. Proteins such as MnSOD, PAI2, and A20, the increased endogenous expression of which has been correlated with TNF resistance, have not been reported to be active when added exogenously. It is assumed that modulation of TNF resistance by these proteins requires their intracellular expression. It should be noted that a preliminary flow cytometric study showed that preincubation of target U937 cells with TIP-B1 at concentrations inhibiting TNF cytotoxicity by 20% did not affect the subsequent binding of biotinylated TNF to the cells (data not shown). This finding indicates that TIP-B1 most likely does not act by directly interfering with ligand/receptor binding. Although this finding needs extensive verification in planned future experiments it, together with the fact that incubation of TIP-B1 with TNF for up to 24 h does not alter the ability of TNF to induce cytolysis (Fig. 5), would suggest that TIP-B1 may have individual cellular receptor(s).

The ability of TIP-B1 to induce protection after exogenous incubation provides a unique opportunity for modulation of TNF sensitivity by TIP-B1. Thus, delivery of TIP-B1 may provide a more therapeutically useful method for influencing TNF-induced lysis than the genetic manipulations necessitated by the properties of the other proteins associated with TNF resistance. Moreover, unlike the other protective proteins described in the literature, in certain cells TIP-B1 can provide 100% protection against TNF-induced apoptosis and lysis (Table 1 and Figs. 3...
EFFECT OF TIP-B1 ON TNF-INDUCED APOPTOSIS

and 5). Therefore, rather than manipulating the levels of several proteins to change the sensitivity of a cell to TNF-induced lysis, possibly only changes in TIP-B1 expression may be required.

In summary, this report describes the discovery, initial characterization, and investigation of the action of TIP-B1, a novel protein inhibitor of TNF-induced lysis. The data obtained suggest that TIP-B1 has a unique and possibly therapeutically relevant (to a number of diseases associated with TNF) ability to prevent TNF-induced lysis when preincubated with normally sensitive cells. The properties of the activity of TIP-B1 are consistent with the hypothesis that it inhibits a specific pathway of TNF-induced apoptosis. On the basis of the data described, studies are planned to further examine the specificity of TIP-B1 activity and the possible signal transduction pathway(s) that are involved in the protective effect of TIP-B1 against TNF-induced lysis.

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Identification, Characterization, and Cloning of TIP-B1, a Novel Protein Inhibitor of Tumor Necrosis Factor-induced Lysis

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