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TGF-β-Induced Matrix Proteins Inhibit p42/44 MAPK and JNK Activation and Suppress TNF-Mediated IκBα Degradation and NF-κB Nuclear Translocation in L929 Fibroblasts

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The role of transforming growth factor β1 (TGF-β1)-induced extracellular matrix proteins in the modulation of cellular response to the cytotoxic effect of tumor necrosis factor (TNF) or Fas ligand was investigated. Murine L929 fibroblasts were prestimulated with or without TGF-β1 for 1–24 h and the resulting extracellular protein matrices were prepared. Unstimulated control L929 cells were then cultured on these matrices. Compared to control matrix-stimulated L929 cells, the TGF-β1 matrix-stimulated cells resisted TNF killing in the presence of actinomycin D (ActD), but became more susceptible to killing by anti-Fas antibodies/ActD. The induced TNF resistance is independent of the NF-κB antiapoptotic effect. For example, exposure of TGF-β1 matrix-stimulated L929 cells to TNF failed to result in IκBα degradation and NF-κB nuclear translocation or activation. Also, control matrix stimulated the activation of p42/44 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) in L929 cells, whereas TGF-β1 matrix suppressed the activation. Nonetheless, in response to TNF, JNK activation was restored in the TGF-β1 matrix-stimulated cells. By metabolic labeling, ammonium sulfate precipitation and N-terminal amino acid microsequencing, TGF-β1 was shown to induce a novel matrix protein of 46 kDa (p46) from L929 cells. Adsorption of p46 by peptide antibodies against its N-terminus removed the TGF-β1 matrix protein-mediated protection against TNF/ActD cytotoxicity and its enhancement of anti-Fas/ActD killing, indicating that p46 is responsible for these effects. Immunostaining of L929 cells revealed that the antibodies were bound to a membrane protein of 100 kDa (p100). Thus, the matrix p46 is likely derived from the released membrane p100.

As a potent immunoregulator, transforming growth factor β (TGF-β) counteracts various functions of tumor necrosis factor (TNF or TNF-α) (1–3; reviews). For example, TGF-β inhibits the development of lymphokine-activated killer cells (4) and cytotoxic T cells (5), whereas TNF suppresses these TGF-β effects. Various cancer cells are sensitive to TNF cytotoxicity, and TGF-β effectively protects these cells from TNF killing (6–8).

TGF-β1-mediated protection of murine L929 fibroblasts against TNF cytotoxicity is associated with its induction of protective proteins. These TGF-β1-induced proteins include unidentified extracellular matrix proteins (6), a novel nuclear TIAF1 (TGF-β-induced antiapoptotic factor 1) (8), and a putative transmembrane adhesion protein TIF2 (TGF-β-induced factor 2) (9). In this study, the role of TGF-β1-induced matrix proteins in the regulation of the activation of p42/44 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK) and NF-κB, as well as the expression of apoptosis regulatory and effector proteins, was determined in L929 cells. Also, the regulation of cellular sensitivity to the cytotoxic effect of TNF and anti-Fas antibodies in the presence of actinomycin D (ActD) by these TGF-β1-induced matrix proteins was investigated. Finally, a TGF-β1-induced matrix protein of 46 kDa (p46) from L929 cells was characterized by N-terminal amino acid microsequencing, peptide antibodies, and functional testings. Supporting evidence showed that the matrix p46 is from a released membrane protein p100. p46 contributes to the protection of L929 cells against TNF/ActD cytotoxicity, but sensitizing these cells to anti-Fas/ActD killing.

MATERIALS AND METHODS

Regulation of TNF and Fas cytotoxicity by TGF-β1-induced matrix proteins. Functional analysis of the TGF-β1-induced extracellular matrix proteins versus the control matrix proteins was performed as

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previously described (6). Briefly, L929 cells (3×10^5 cells/ml) were cultured in 96-well plates (Corning Glass Works, Corning, NY) overnight and treated with or without human platelet-derived TGF-β1 (0.25–4 ng/ml; Collaborative Research Incorporated, Bedford, MA) for 1–24 h at 37°C. These cells were then removed from the plates by treating with 50 μl of trypsin/EDTA (Sigma, St. Louis, MO) for 5 min at room temperature. These plates were thoroughly washed 6 times with 150 μl of phosphate-buffered saline (PBS). Freshly harvested control L929 cells (3×10^5 cells/ml) were seeded onto these washed plates, cultured overnight, and exposed to TNF (1000–4000 units/ml; Genzyme, Boston, MA), TNF (2.5–20 units/ml)/ActD (1 μg/ml), or monoclonal anti-Fas antibodies (125–500 ng/ml; Pharmingen, San Diego, CA)/ActD (1 μg/ml) for 16–24 h. The extent of cell death was calculated (6). The percentages of resistance or enhancement to TNF or Fas killing as mediated by TGF-β1-induced matrix proteins were also calculated as: % Resistance (or Enhancement) = [1 − (% killing of TGF-β1 matrix-stimulated cells/% killing of control matrix-stimulated cells)] × 100 (6), where the control or the TGF-β1 matrix is defined as the matrix proteins induced by medium or TGF-β1.

Metabolic labeling, ammonium sulfate precipitation, N-terminal amino acid microsequencing, antibody production, and functional testings. To examine the specific matrix protein(s) induced by TGF-β1, L929 cells (1×10^6 cells/ml) were cultured on 60 mm petri dishes using RPMI-1640 medium (containing cold methionine) supplemented with 10% FBS, treated with or without TGF-β1 (2 ng/ml) for 16 h, and simultaneously labeled with 500 μCi [S^{35}]methionine (ICN, Costa Mesa, CA) on a competitive basis. The cells were then removed from the petri dishes by cell scrapers, and the petri dishes were washed thoroughly with PBS. The plastic adhered matrix proteins were treated with 500 μl of an SDS-PAGE sample buffer (60 mM Tris buffer, pH 6.8, 0.1% glycerol, 2.0% SDS and 5% β-mercaptoethanol), and removed by scraping the petri dishes with cell scrapers. SDS-PAGE and autoradiography were performed to analyze the TGF-β1-induced matrix proteins, as compared to control matrix proteins.

Previously we showed that the inhibitory activity of TGF-β1-induced matrix proteins against TNF/ActD killing could be found in the culture medium only when L929 cells were grown under non-adherent, *roller* culture conditions (6). Briefly, L929 cells (2×10^6 cells/ml) were labeled with 500 μCi [S^{35}]methionine (on a competitive basis) and cultured in roller bottles in the presence or absence of TGF-β1 (2 ng/ml) for 16 h. The culture supernatants were then harvested, and treated with 2 mM phenylmethylsulfonyl fluoride (PMSF; Sigma). Proteins in the supernatants were precipitated by ammonium sulfate in a stepwise manner at 10, 15, 20, 25, 30 and 40% of saturation. Each precipitated protein fraction was resuspended in 5 ml PBS and dialyzed exhaustively against PBS. Thirty μl of the protein fractions were coated (non-dry coating) onto the 96-well plates for 1–3 h in a 37°C humidified incubator. Similarly, the post 40% soluble protein fraction was also dialyzed and aliquots of this fraction were coated onto 96-well plates. These plates were then washed 5 times with 150 μl of PBS, and seeded with 100 μl aliquots of L929 cells (3×10^5/ml). After overnight culture, the cells were exposed to TNF/ActD or anti-Fas/ActD for 16–24 h. The protein fraction(s) which contained the inhibitory activity for TNF/ActD killing was then analyzed by SDS-PAGE and autoradiography. These separated proteins in polyacrylamide gels were then electrotransferred onto polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA), and subjected to N-terminal gas-phase amino acid microsequencing (10). A peptide was synthesized corresponding to the determined N-terminal amino acid sequence (Genemed Biotechnologies Inc., South San Francisco, CA). The peptide was covalently conjugated with KLH (Keyhole Limpet Hemocyanin) for polyclonal antibody production in rabbits according to the instruction of Pierce antibody production kit (Pierce, Rockford, IL).

Western blotting and immunofluorescence. Whether TGF-β1-induced matrix proteins modulated the expression of apoptosis regulatory and effector proteins was examined. L929 cells were grown overnight on the control or TGF-β1 matrix, as prepared on 60 mm petri dishes as described above. Western blotting was utilized to examine the expression of the following proteins using specific antibodies, respectively, against the tumor suppressor p53, CAS (cellular apoptosis susceptibility protein) (11), TIAR (an RNA-binding protein) (12), ICH-1L (caspase 2) (13), CPP32 (caspase 3) (14), FasL (Fas ligand) (15), RIP (receptor interacting protein) (16) (the above antibodies from Transduction Laboratories, Lexington, KY), PARP (poly(ADP-ribose) polymerase) (17) (Biomol, Plymouth Meeting, PA), FLICE (caspase 8) (18), NF-κB and IκBα (19) (the last three antibodies from Santa Cruz Biotechnology, Santa Cruz, CA). Additionally, antibodies against phosphopeptides of p42/44 MAPK and p46 JNK (New England Biolab, Beverly, MA) were used. For protein loading controls, antibodies against the house-keeping protein α-tubulin (Accurate Chemical & Scientific Corp., Westbury, NY) were used.

Where indicated, L929 cells were cultured on the control or TGF-β1 matrix overnight, and exposed to TNF (4000 units/ml; Genzyme) for various durations (0–4 h), followed by determining IκBα degradation by Western blotting. To determine TNF-mediated NF-κB nuclear translocation, L929 cells were grown on coverslips overnight, which were precoated with the control or TGF-β1 matrix proteins. These cells were stimulated with TNF (4000 units/ml) for 15–120 min, fixed with 3.7% formaldehyde (Sigma) for 15 min, and then permeabilized with 0.1% Triton X-100 (Sigma) for 2 min at room temperature. The cells were stained with anti-p65 NF-κB antibodies (Santa Cruz Biotechnology) and FITC-conjugated anti-goat antibodies (Protos Immunoresearch, San Francisco, CA). Nuclear localization of NF-κB was examined by fluorescence microscopy.

RESULTS

TGF-β1-induced matrix proteins (TGF-β1 matrix) stimulated L929 cells resistant to TNF/ActD cytotoxicity but increased cellular sensitivity to anti-Fas/ActD killing. In agreement with the previous observations (6), seeding and culturing of L929 cells on the TGF-β1 matrix, but not on the control matrix, enabled these cells to resist killing by TNF/ActD (Fig. 1A). In contrast, the TGF-β1 matrix failed to protect L929 cells against death by TNF alone (Fig. 1A). The TGF-β1 matrix was normally prepared by exposure of L929 cells to TGF-β1 for at least 1 h, following by removing cells using trypsin/EDTA and washing the 96-well plates thoroughly with PBS. Prolonging the treatment of L929 cells with TGF-β1 for 24–48 h could not reduce the protective function of the resulting extracellular matrix.

In contrast, TGF-β1 matrix-stimulated L929 cells became more susceptible to anti-Fas/ActD killing than the control matrix-stimulated cells (Fig. 1B). In this experiment, the TGF-β1 matrix was prepared from L929 cells, which were pretreated with TGF-β1 for 2 and 4 h. This enhancing activity was not reduced by examining a TGF-β1 matrix derived from L929 cells pretreated with TGF-β1 for 24 h.

TGF-β1 matrix suppressed p42/44 MAPK and p46 JNK activation, and inhibited TNF-mediated IκBα degradation and NF-κB nuclear translocation in L929 cells. To examine the mechanisms whereby TGF-β1 matrix mediated TNF/ActD resistance and enhanced anti-Fas/ActD cytotoxicity, L929 cells were cultured on the control or TGF-β1 matrix overnight. Activation of

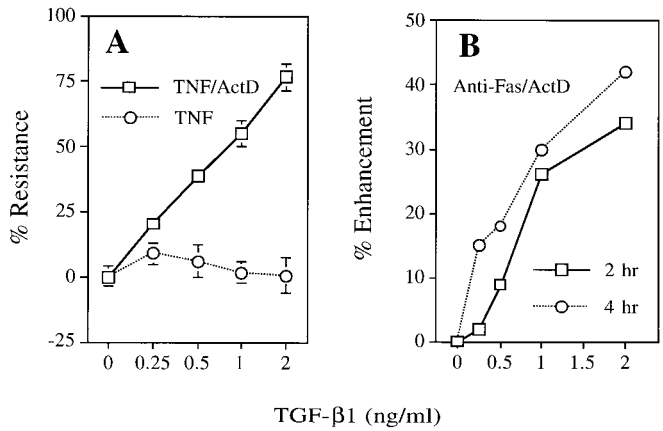

FIG. 1. Modulation of TNF-, TNF/ActD-, and anti-Fas/ActD-mediated L929 cell death by TGF-β1-induced or control matrix proteins (defined as TGF-β1 or control matrix). (A) L929 cells were cultured in 96-well plates overnight and treated with or without TGF-β1 for 4 h, followed by removal with trypsin/EDTA. The plates were washed thoroughly with PBS and seeded with freshly harvested control L929 cells. Following overnight culture, these cells were exposed to either TNF (5 units/ml)/ActD (1 μg/ml) or TNF (1000 units/ml) alone for 16–24 h. Approximately 50% death of control L929 cells occurred using the indicated concentrations of TNF in the presence or absence of ActD. Compared to control matrix-stimulated cells, TGF-β1 matrix-stimulated L929 cells resisted TNF/ActD cytotoxicity (mean ± standard deviation; n = 8). In contrast, these TGF-β1 matrix-stimulated cells failed to resist cytolysis by TNF alone. (B) Similar experiments were performed by treating L929 cells with TGF-β1 for 2 and 4 h, followed by preparing the extracellular matrices and examining the TGF-β1 matrix-mediated enhancement of anti-Fas antibodies (125 ng/ml)/ActD (1 μg/ml) cytotoxicity in L929 cells. Compared to controls, L929 cells acquired an enhanced sensitivity to anti-Fas/ActD killing when grown on the TGF-β1 matrix (mean ± standard deviation; n = 8).

p42/44 MAPK and p46 JNK was observed when L929 cells were grown on the control matrix, but not on the TGF-β1 matrix (Fig. 2A). Also, TGF-β1 matrix-mediated regulation of cellular sensitivity to TNF/ActD or anti-Fas/ActD killing was not related with the expression of the following apoptosis regulatory and effector proteins: FasL (Fas ligand), NF-κB (p65), RIP, PARP, TIAR (an RNA binding protein), p53, CAS (cellular apoptosis susceptibility), CPP32 (caspase 3), FLICE (caspase 8), and ICH-1L (caspase 2) (Fig. 2A). As a control for equal protein loading, the housekeeping protein α-tubulin was examined (Fig. 2A).

TNF restored p46 JNK activation in the TGF-β1 matrix-stimulated L929 cells. Exposure of TGF-β1 matrix-stimulated L929 cells to TNF resulted in a rapid activation of p46 JNK (Fig. 2B). However, TNF failed to further increase the level of p46 JNK activation in the control matrix-stimulated cells (Fig. 2B).

Compared to control matrix-stimulated L929 cells, TGF-β1 matrix-stimulated cells had a reduced expression of IκBα (by ~35%) (Fig. 2C). Exposure of the control matrix-stimulated L929 cells to TNF resulted in degradation of cytosolic IκBα (Fig. 2C), and nuclear translocation or activation of p65 NF-κB (Fig. 2D). However, these events were suppressed in the TGF-β1 matrix-stimulated L929 cells in response to TNF (Fig. 2C and 2D). These results are in parallel with our previous observations, which demonstrated that TGF-β1 downregulates IκBα expression in L929 cells, and IκBα degradation is suppressed when the TGF-β1-treated L929 cells are exposed to TNF (8). TGF-β1 alone fails to mediate NF-κB nuclear translocation (8).

TGF-β1 induction of an extracellular matrix 46-kDa protein. To identify the TGF-β1-induced matrix protein(s) that conferred resistance to TNF/ActD (and enhanced anti-Fas/ActD killing), L929 cells (2×10^6 cells/ml) were metabolically labeled with [^{35}S]methionine (on a competitive basis) under roller culture conditions in the presence or absence of TGF-β1 for 16 h. The culture supernatants were harvested and subjected to stepwise ammonium sulfate precipitation. The precipitated protein fractions were resuspended in PBS, dialyzed and coated onto 96-well plates. L929 cells were cultured on these plates overnight and exposed to TNF/ActD. Cellular resistance to TNF/ActD killing was observed when L929 cells were grown on the matrices of the 25% precipitation protein, as induced by TGF-β1 (Fig. 3A). Also, this 25% precipitation fraction enhanced cell death by anti-Fas/ActD (Fig. 3A). No activities were found at 10, 15, 20, 30, 40% and the post 40% fractions (Fig. 3A; data not shown for the 15, 20, 30% and post 40%). Without coating, the 25% precipitation fraction failed to induce TNF-resistance or enhance anti-Fas/ActD killing in L929 cells (data not shown). Protein bound to each well of the 96-well plates was less than 10 ng per well, which was approximately ~0.1% of the total protein added to each well.

As analyzed by SDS-PAGE and autoradiography, TGF-β1 was shown to induce the release of a 46-kDa protein (p46) from L929 cells (Fig. 3B). p46 was found in the 25% precipitation fraction. To confirm the presence of p46 in the extracellular matrix, both control and TGF-β1-treated L929 cells were cultured on petri dishes overnight and metabolically labeled, and the extracellular proteins were harvested from the matrices of these cells. Again, p46 was found in the matrix of TGF-β1-treated L929 cells (Fig. 3C).

Gas-phase microsequencing analysis determined that p46 possesses a unique N-terminal sequence of N-P-L-D-P-V-A-H-R-D-E-P-P-R-C-D-H, as compared to the universal protein databases. This sequence has 44.4% homology (in 18 amino acid overlap) to a conserved region in the family proteins of the p60^{SRC} protein tyrosine kinase (20) (Fig. 4A). Polyclonal antibodies against a synthetic peptide of this determined sequence were generated in rabbits. Removal of p46 from the 25% precipitation fraction by adsorption with the anti-p46 antibodies and anti-rabbit IgG agarose beads resulted in depletion of the functional activity in

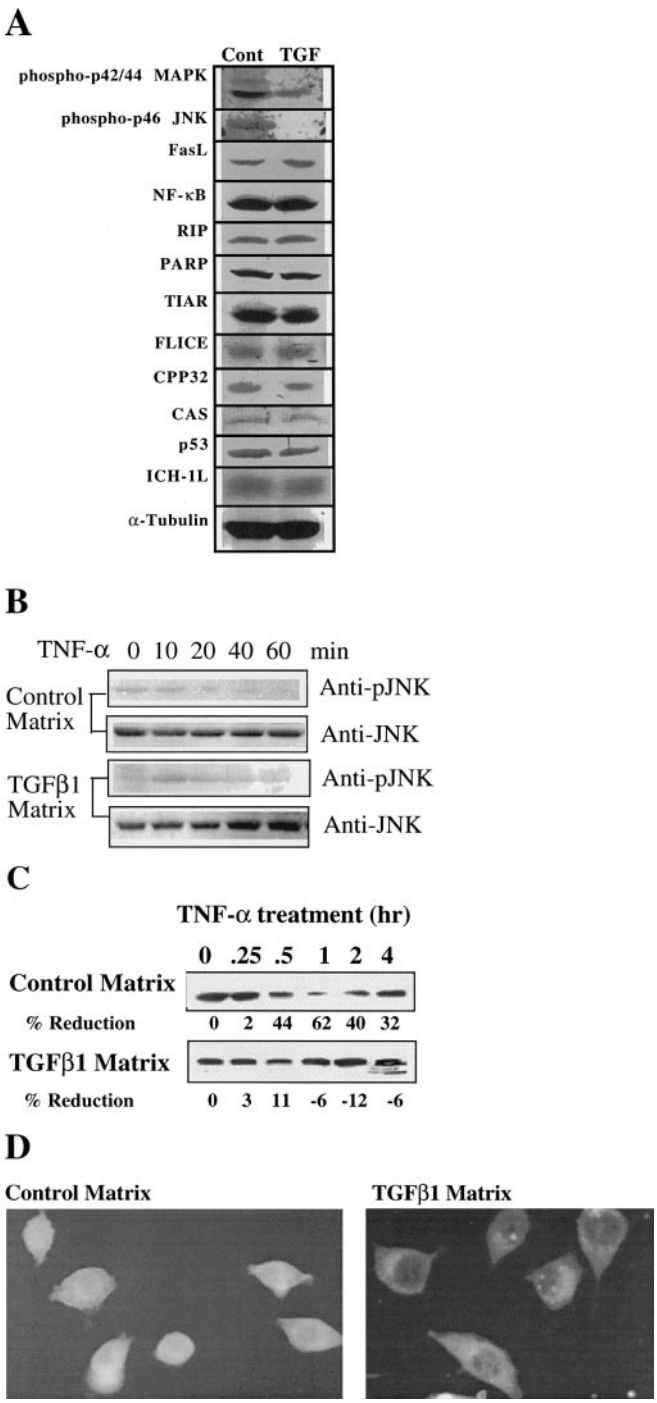

FIG. 2. TGF-β1 matrix suppressed p42/44 MAPK and p46 JNK activation, and inhibited TNF-mediated IκBα degradation and NF-κB activation in L929 cells. (A) L929 cells were cultured overnight on the control or TGF-β1 matrix, as prepared in 60 mm petri dishes (see Materials and Methods). The control matrix stimulated phosphorylation or activation of p42/44 MAPK and p46 JNK in L929 cells, as determined using anti-phosphopeptide antibodies. The phosphorylation was suppressed in the TGF-β1 matrix-stimulated cells. Also, TGF-β1 matrix failed to alter the expression of the following proteins which are involved in apoptosis: FasL, NF-κB, RIP, PARP, TIAR, FLICE (caspase 8), CPP32 (caspase 3), CAS, p53, and ICH-1L (caspase 2). As protein loading control, the level of α-tubulin was examined. (B) Exposure of the TGF-β1 matrix-stimulated cells to TNF (4000 units/ml) resulted in restoration of p46 JNK activation. However, in response to TNF, there was no increase in JNK activation in the control matrix-stimulated cells. (C) Both the control and TGF-β1 matrix-stimulated L929 cells were exposed to TNF (4000 units/ml) for various durations. Compared to control cells, TNF-mediated degradation of IκBα was blocked in TGF-β1 matrix-stimulated cells. Also, the level of IκBα was reduced by ~35% in TGF-β1 matrix-stimulated cells, as compared to control cells. (D) Upon stimulation with TNF (4000 units/ml) for 30 min, cytosolic p65 NF-κB of the control matrix-stimulated L929 cells translocated to the nuclei, whereas no nuclear translocation of NF-κB was observed in the TGF-β1 matrix-stimulated cells. These cells were stained with anti-p65 NF-κB and anti-goat IgG conjugated with FITC.

blocking TNF/ActD killing and enhancing anti-Fas/ActD killing (Fig. 4B).

To further confirm the presence of p46 in the matrix by the produced antibodies, adherent L929 cells were stimulated with or without TGF-β1 for 1 h, followed by isolation of matrix proteins. Western blotting analysis showed that TGF-β1 induced the secretion of p46 to the matrix (Fig. 4C). Furthermore, TGF-β1 induced a cellular protein of approximately 100-kDa (p100), as determined by Western blotting using whole cell lysate and the anti-p46 antibodies (Fig. 4C). Immunostaining of TGF-β1-stimulated L929 cells with the anti-p46 antibodies and FITC-conjugated anti-rabbit IgG showed the presence of cell surface fluorescence in non-permeabilized cells, as well as the whole cell fluorescence in the Triton X-100-permeabilized cells (data not shown). Western blotting using solubilized membrane proteins also revealed the presence of p100 from TGF-β1-treated cells (data not shown). These observations suggest that the matrix p46 is derived from the released membrane p100.

DISCUSSION

In this study, TGF-β1-induced extracellular matrix protein(s) was shown to inhibit p42/44 MAPK and JNK activation, and suppress TNF-mediated IκBα degradation and NF-κB nuclear translocation in L929 cells. These observations suggest that the TNF signaling pathway which leads to IκBα degradation and NF-κB activation is blocked in the TGF-β1 matrix-stimulated cells. Adsorption studies support the notion that p46 plays a significant role in conferring cellular resistance to TNF/ActD cytotoxicity, as well as increasing cellular susceptibility to Anti-Fas/ActD killing. Whether p46 inhibits the activation of p42/44 MAPK and JNK and suppresses TNF-mediated IκBα degradation and NF-κB activation remains to be established. Supporting evidence shows that the matrix p46 is derived from the released cell membrane p100.

As determined previously, TGF-β1-induced matrix proteins are relatively stable. Their function in block-

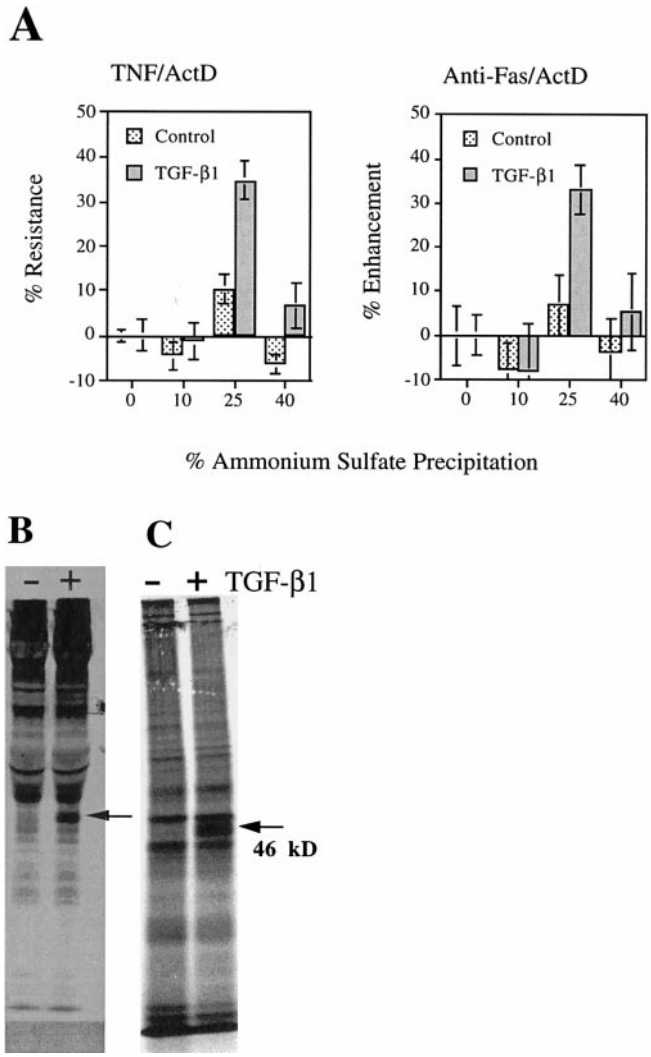

FIG. 3. TGF-β1 induction of a matrix protein p46. (A) L929 cells were metabolically labeled with [^{35}S]methionine and cultured under roller conditions in the presence or absence of TGF-β1 (2 ng/ml) for 16–24 h. Sequential ammonium sulfate precipitation of the culture supernatants and coating of the resuspended protein precipitation fractions onto 96-well plates were performed (see Materials and Methods). PBS was coated onto the plates as in the "0%" ammonium sulfate precipitation. Functional analysis revealed that TGF-β1 induced matrix proteins, which inhibited L929 cell death by TNF (5 units/ml)/ActD (1 μg/ml), or enhanced the cell death by anti-Fas (250 ng/ml)/ActD (1 μg/ml), was present in the 25% precipitation fraction. (B) In this fraction, a TGF-β1-induced 46-kDa protein (p46; see arrow) was found, as analyzed by reducing SDS–PAGE and autoradiography. (C) To examine whether p46 is located in the extracellular matrix, L929 cells in petri dishes were treated with or without TGF-β1 and simultaneously labeled with [S^{35}]methionine for 16 h. The cells were then removed with cell scrapers, and the petri dishes were washed thoroughly with PBS. Matix proteins were harvested by scraping the dishes using an SDS–PAGE sample buffer (containing β-mercaptoethanol) and separated in reducing SDS–PAGE. Compared to control cells, TGF-β1 induced L929 cells to secrete the p46 protein.

ing TNF/ActD cytotoxicity could not be abolished by treatment with heat, collagenase, limited trypsinization, heparin, or α2-macroglobulin (6). Also, neutralizing antibodies against TGF-β1 failed to abrogate the protective function of TGF-β1 matrix, suggesting that there is little or no residual TGF-β1 remained in the matrix (6).

TGF-β1 is known to suppress IκBα expression in L929 cells, and in response to TNF, the TGF-β1-treated L929 cells have a retarded IκBα degradation

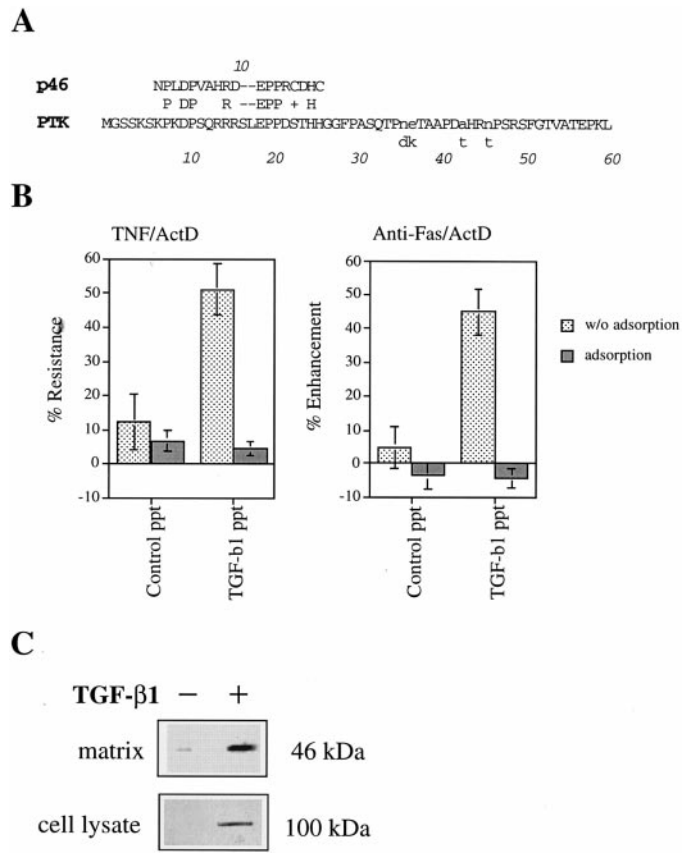

FIG. 4. Characterization of the matrix p46. (A) A unique 18-amino-acid N-terminal sequence of p46 was determined by gas-phase microsequencing. Database searching shows that this sequence has 44.4% homology (in 18 amino acid overlap) to a conserved region in the family proteins of the p60^{SRC} protein tyrosine kinase (PTK). Shown below the PTK sequence are the alternative amino acids of this conserved region. (B) The 25% precipitation protein fraction (from TGF-β1-treated L929 cells as described in the legend to Fig. 3) was adsorbed with or without anti-p46 antibodies and anti-rabbit IgG agarose beads to remove p46, followed by preparing protein matrices in 96-well plates (see Materials and Methods). Removal of p46 from the 25% precipitation fraction resulted in depletion of the functional activity in blocking TNF/ActD killing and enhancing anti-Fas/ActD killing (see the "TGF-β1 ppt" on the *X* axis). In controls, the 25% precipitation fraction from untreated control cells was adsorbed by anti-p46 antibodies and coated onto 96-well plates for functional testing (see the "Control ppt" on the *X* axis). (C) L929 cells in petri dishes were treated with or without TGF-β1 (2 ng/ml) for 1 h, followed by isolation of matrix proteins. The TGF-β1-induced p46 was recognized by the peptide antibodies. Furthermore, the whole cell lysates of control and TGF-β1-treated L929 cells were separated by SDS–PAGE and subjected to immunostaining with the anti-p46 antibodies. Compared to control cells, TGF-β1-treated cells expressed a 100 kDa protein.

and NF-κB activation (8). We have determined that the nuclear TIAF1 participates in the TGF-β1 inhibition of IκBα expression and suppression of TNF-mediated IκBα degradation (8). In parallel with these observations, the TGF-β1 matrix partially inhibits IκBα expression and blocks IκBα degradation in L929 cells upon exposure to TNF. Whether the TGF-β1 matrix upregulates TIAF1 expression or induces TIAF1 nuclear translocation remains to be established.

Recently we have shown that IκBα is essential for maintaining the basal level of JNK activation and regulating JNK-mediated resistance to TNF cytotoxicity in L929 cells (21). Thus, the suppression of JNK activation in the TGF-β1 matrix-stimulated L929 cells is probably due to partial inhibition of IκBα expression in these cells. Additionally, TNF rapidly stimulated JNK activation in the TGF-β1 matrix-stimulated L929 cells. Presumably, this event contributes to the observed cellular resistance to TNF/ActD cytotoxicity, since early activation of JNK increases cell survival to the TNF challenge (22).

The role of TGF-β1 matrix-mediated suppression of p42/44 MAP kinase activation in L929 cells is unknown. TGF-β1 has been shown to rescue serum deprivation-induced apoptosis via the MAPK pathway in macrophages (23). Also, TGF-β1 rapidly induces the activation of the Raf-MEK-MAPK signaling pathway in rat lung fibroblasts via a PKC-dependent mechanism (24). Conceivably, the TGF-β1-induced matrix proteins provide a feedback inhibition mechanism to turn off the activation of MAPK pathway.

TGF-β1 protects L929 cells from TNF killing in the presence or absence of ActD (6, 7). The TGF-β1-induced p46 contributes to the blocking of cell death by TNF/ActD, but not by TNF alone. ActD inhibits gene transcription. Thus, p46 protection of L929 cells against TNF/ActD cytotoxicity is independent of NF-κB-mediated transcription of protective proteins. However, the failure of p46 in inhibition of L929 cell death by TNF alone suggests that TNF induces other proapoptotic proteins which override the p46 protection.

How p46 increases Fas/ActD susceptibility in L929 cells is unknown. Although both TNF receptor 1 and Fas signaling pathways utilize a nearly identical signaling cascade in mediating apoptosis, various distinct adaptor proteins are associated with both receptors and the signaling cascades (25). Differences in the biologic effects of TNF receptor and Fas signaling have been demonstrated. Cytosolic phospholipase A2 which is involved in TNF-killing pathway does not contribute to Fas-mediated cell death (26). TNF cytotoxicity is mediated by reactive oxygen intermediates generated during mitochondrial respiration, in which these events do not occur in the Fas-mediated cell death (27). p46 probably modulates the expression of certain known or unknown proteins which are associated with the TNF or Fas signaling pathway, thereby changing the eventual death-inducing event.

In addition to its expression in L929 cells, p46 is constitutively expressed in cervical ME-180 and HeLa carcinoma cells, Cos7 fibrosarcoma cells and A431 epidermoid carcinoma cells (Chang *et al.*, unpublished). TGF-β1 upregulates p46 expression in these cells. Functionally, p46 from HeLa and LNCaP cells restricts TNF/ActD killing of L929 cells, whereas it has no effect on TNF killing (without ActD). In contrast, p46 and related proteins from ME-180 cells inhibit the cytotoxic effects of TNF/ActD and TNF alone. Molecular cloning and functional characterization of p46 is being underway in this laboratory.

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