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Transforming Growth Factor β Stimulates the Expression of Fibronectin and of Both Subunits of the Human Fibronectin Receptor by Cultured Human Lung Fibroblasts*

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Transforming growth factors of the β-class (TGFs-β) stimulate extracellular matrix synthesis and have been implicated in embryogenesis, wound healing, and fibroproliferative responses to tissue injury. Because cells communicate with several extracellular matrix components via specific cell membrane receptors, we hypothesized that TGFs-β may also regulate the expression of such receptors. We confirmed that TGF-β1 increases the expression of fibronectin, an adhesive glycoprotein expressed during embryogenesis and tissue remodeling. Based upon the 48–72-h period required for a maximal fibroproliferative response to dermal injections of TGF-β1, we exposed human fetal lung fibroblasts (IMR-90) to TGF-β1 for periods up to 48 h *in vitro*. We observed as much as 6-fold increases in fibronectin synthesis by 24 h as previously reported for fibroblastic cells (Ignotz, R. A., and Massagué, J. (1986) *J. Biol. Chem.* 261, 4337–4345; Ignotz, R. A., Endo, T., and Massagué, J. (1987) *J. Biol. Chem.* 262, 6443–6446; Raghow, R., Postlethwaithe, A. E., Keski-Oja, J., Moses, H. L., and Kang, A. H. (1987) *J. Clin. Invest.* 79, 1285–1288), but up to 30-fold increases by 48 h. These increases are accompanied by similar increases in fibronectin mRNA levels which are prevented by actinomycin D treatment. Using a monospecific antibody raised to the human placental fibronectin receptor complex, we found that TGF-β1 stimulated fibronectin receptor synthesis up to 20–40-fold and increases mRNA levels encoding both the α- and β-subunits up to 3-fold, compared to control IMR-90 in serum-free medium. Actinomycin D blocks TGF-β1-mediated increases in receptor mRNA levels. The earliest detectable TGF-β1-mediated increases in fibronectin receptor complex protein synthesis and mRNA levels occur at 8 h, whereas the earliest increases in fibronectin protein synthesis and mRNA levels occur at 12 h. These results demonstrate that TGF-β1 stimulates fibronectin receptor synthesis, extending the diverse stimulatory activities of this polypeptide to matrix receptors. In addition, because fibronectin matrix assembly may involve the fibronectin cell adhesive receptor complex, increased receptor expression may help drive fibronectin deposition into matrix.

Transforming growth factors of the β-class (TGFs-β[1] are dimeric polypeptides of 25 kDa that allow anchorage-independent growth of normal fibroblasts (1). TGFs-β are composed of two closely related subunits, β1 and β2, that are found as homodimers and a heterodimer (2, 3). The β1 homodimer (TGF-β1) was first purified from the α-granules of human platelets where it exists in a latent form and subsequently has been found in many other cell types (4, 5). TGFs-β are members of a larger class of proteins that are involved in tissue differentiation and restructuring, including müllerian inhibiting substance, inhibins, activins, and the decapentaplegic transcript of *Drosophila* (6–8). TGFs-β bind to cell-surface receptors whose mechanism of signal transduction is, as yet, unknown (9). *In vivo*, TGF-β1 causes granulation tissue to form when injected intradermally (10, 11), evoking an altered fibroblast phenotype typical of wound healing.[2] *In vitro*, TGF-β1 stimulates the expression of fibronectin and collagen type I, in part mediated via increased levels of mRNA, and increases the deposition of fibronectin into the pericellular matrix (12–17).

Fibroblasts interact with extracellular fibronectin in part via a specific, dimeric cell-surface receptor complex termed the fibronectin cell adhesive receptor, a member of the "integrin" or very late antigen family of receptors (18–20). This receptor, consisting in mammals of an α- and a β-subunit which are noncovalently associated, recognizes the Arg-Gly-Asp-Ser sequence in fibronectin (18, 21). Recently, we found that this receptor appears to be important in pericellular matrix assembly (22). Based upon this observation and upon the ability of TGF-β1 to stimulate both fibronectin synthesis and its incorporation into the pericellular matrix, we hypothesized that TGF-β1 increases the expression of the fibronectin cell adhesive receptor complex. Accordingly, we studied the

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[1] The abbreviations used are: TGFs-β, transforming growth factors of the β-class; TGF-β1, β1 homodimer of transforming growth factor β; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

[2] W. Garner, C. Kuhn, and J. A. McDonald, unpublished data.

effects of TGF-β1 on the expression of both the fibronectin receptor complex and fibronectin in cultured human lung fibroblasts. We found that TGF-β1 is a potent stimulator of the expression of both the fibronectin receptor complex and fibronectin in these cells. The mechanism for this effect is unknown, but it is mediated in part by increasing the mRNA levels for fibronectin and for both subunits of the fibronectin receptor complex.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise specified, organic chemicals and enzymes were purchased from Sigma. Dulbecco's modified Eagle's medium was purchased from the Tissue Culture Support Center, Washington University School of Medicine, St. Louis, MO; antibiotics/antimycotic 100 × stock solution (10,000 units/ml penicillin, 10,000 μg/ml streptomycin, 25 μg/ml Fungizone) was from Gibco Laboratories; fetal bovine serum was from HyClone Laboratories (Logan, UT); human fetal lung fibroblasts (IMR-90) were from American Type Culture Collection (Rockville, MD); Tran^{35}S-label ([^{35}S]methionine, 1000 Ci/mmol; containing up to 15% [^{35}S]cysteine) was from ICN Biomedicals, Inc. (Costa Mesa, CA); [^{32}P]dCTP (3000 Ci/mmol) was from Du Pont-New England Nuclear; and IgSorb was from the Enzyme Center, Inc. (Maiden, MA). Porcine platelet-derived TGF-β1 was purchased from R & D Systems, Inc. (Minneapolis, MN), and human platelet-derived TGF-β1 was the generous gift of Dr. Michael Sporn (Laboratory of Chemoprevention, National Cancer Institute, National Institutes of Health, Bethesda, MD). We have used both forms in our experiments, and both have given identical results. Prestained high molecular weight protein standards were purchased from Amersham Corp. and from Sigma; RNA molecular weight standards were purchased from Bethesda Research Laboratories. Autofluor autoradiographic image enhancer was from National Diagnostics, Inc. (Somerville, NJ); XAR-5 diagnostic film was from Eastman; and Cronex Lightning Plus intensifying screens were from Du Pont-New England Nuclear. Restriction endonucleases were purchased from New England Biolabs, (Beverly, MA), and low melting temperature agarose was from International Biotechnologies Inc. (New Haven, CT).

Preparation of Antibodies—Human fibronectin receptor was purified from placenta essentially as described by Pytela *et al.* (23) with the following minor modifications. The 110-kDa cell-binding domain thermolysin fragment of human plasma fibronectin was isolated according to Zardi *et al.* (24) and coupled to CNBr-Sepharose (Pharmacia LKB Biotechnology Inc.) Human term placenta was homogenized in a blender in Dulbecco's phosphate-buffered saline containing 1.0 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS++), 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Octyl β-D-glucopyranoside (United States Biochemical Corp., Cleveland, OH) was added to a concentration of 75 mM, and the PMSF was increased to 3 mM. The homogenate was stirred for 30 min at 4 °C and then centrifuged at 145,000 × *g* for 30 min. The homogenate was loaded on the 110-kDa fragment affinity column, the column was washed with 1% octyl glucoside in Dulbecco's phosphate-buffered saline, and the receptor was eluted with 10 mM EDTA in the same buffer. Comparisons of elution with 1 mg of the synthetic peptide Gly-Arg-Gly-Asp-Ser/ml yielded identical preparations, whereas the control peptide Gly-Arg-Gly-Glu-Ser was inactive. The receptor preparation was further purified by affinity chromatography on wheat germ agglutinin-agarose (Vector Laboratories, Inc., Burlingame, CA) and eluted with *N*-acetylglucosamine. The higher purified receptor was then purified to electrophoretic homogeneity by fast protein liquid chromatography size exclusion chromatography with a Superose 6 column (Pharmacia LKB Biotechnology Inc.). The final preparation showed only the two receptor subunit bands by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining. Polyclonal IgG was isolated from a rabbit immunized by multiple injections of the purified receptor complex using DEAE-Affi-Gel blue (Bio-Rad) according to the recommendations of the manufacturer. The affinity-purified polyclonal IgG used to immunoprecipitate fibronectin was isolated from rabbits immunized with purified human plasma fibronectin (25).

cDNA Probes—The fibronectin cDNA, pFH6 (26), was a generous gift of Dr. A. Kornblihtt (Instituto de Investigaciones en Ingenieria Genetica y Biologia Molecular, Buenos Aires, Argentina). We used the 700-base pair *Xba*I/*Bam*HI subfragment because of high nonspecific binding with the intact cDNA. The fibronectin receptor cDNAs encoding the α-subunit λP7 (27), and the β-subunit, λP32 (28), were generous gifts of Drs. S. Argraves and E. Ruoslahti, (La Jolla Cancer Research Foundation, La Jolla, CA).

Cell Culture—IMR-90 fibroblasts were grown to confluence in growth medium (Dulbecco's modified Eagle's medium) containing 10% fetal bovine serum, 2 mM glutamine, 1:100 dilution of the antibiotics/antimycotic solution, and 25 μg/ml ascorbic acid. Cells were maintained in an incubator at 37 °C in a 5% CO_2 atmosphere. Confluent monolayers were washed three times with Dulbecco's phosphate-buffered saline. Dulbecco's modified Eagle's medium containing 2 mM glutamine, 1:100 dilution of the antibiotics/antimycotic solution, and 25 μg/ml ascorbic acid was added; and for metabolic labeling, cultures were supplemented with 1) no additions (serum-free medium), 2) 100 pM TGF-β1 (serum-free medium + TGF-β1), 3) 10% fetal bovine serum (serum-containing medium), or 4) 10% fetal bovine serum + 100 pM TGF-β1 (serum-containing medium + TGF-β1). To achieve maximal stimulation, we used TGF-β1 at a concentration of 100 pM based on previously published results (16). The cells were incubated for the indicated length of time, the medium was removed, the monolayer was rinsed twice with Dulbecco's phosphate-buffered saline, and the medium was replaced with medium containing 5% of the normal methionine and cysteine concentrations and 100 μCi/ml Tran^{35}S-label. For pulse-chase experiments, Tran^{35}S-label was increased to 200 μCi/ml. For the Northern blot analyses, cultures were incubated as described above, but the metabolic labeling step was omitted. Actinomycin D and cycloheximide were added concurrently with TGF-β1 to final concentrations of 400 ng/ml and 20 μg/ml, respectively.

Fibronectin Receptor Immunoprecipitation—Except where specified, all procedures were performed at 4 °C, and all centrifugations were performed in a Beckman Microfuge at 10,000 × *g* for 5 min. The cell layer was rinsed five times with PBS++ at 37 °C. Membrane-associated proteins were solubilized by adding, per 35-mm culture dish, 100 μl of a 2% Triton X-100 solution in PBS++ containing 2 mM PMSF and 15 μg/ml leupeptin. After a 10-min incubation, the cell layer was scraped into the Triton solution with a rubber policeman, and the suspension was clarified by centrifugation for 15 min. The supernatant solution was quick-frozen in methanol/dry ice and was stored at −70 °C. The identical procedure was performed with an unlabeled cell monolayer to obtain an unlabeled fibroblast homogenate. After thawing, the supernatant solution was incubated with shaking with one-fifth volume of a 50% Sepharose CL-4B/PBS++ suspension and one-tenth volume of a 50% Protein A-Sepharose CL-4B/PBS++ suspension for 30 min to remove labeled proteins nonspecifically binding to Protein A-Sepharose CL-4B and Sepharose CL-4B. After the suspension was centrifuged, polyclonal rabbit anti-human fibronectin receptor antibody was added to the supernatant solution to a final concentration of 100 μg/ml. The solution was incubated with shaking for 1 h. A 50% Protein A-Sepharose CL-4B/PBS++ suspension was preincubated with shaking with one-third volume of the unlabeled fibroblast homogenate for 30 min (this prevents nonspecific immunoprecipitation of labeled protein by using unlabeled cell homogenate to block possible sites of interaction). After the suspension was centrifuged, the Protein A-Sepharose pellet was washed three times with PBS++ and was resuspended in an equal volume of PBS++. One-fourth volume of this Protein A-Sepharose CL-4B suspension was added to the antibody-containing supernatant mixture. After a 30-min incubation with shaking, the Protein A-Sepharose CL-4B was pelleted by centrifugation. The pellet was washed six times with 1% Triton X-100 solution in PBS++ containing 2 mM PMSF and 15 μg/ml leupeptin. Bound antigen was solubilized by heating the pellet to 100 °C for 4 min in SDS sample buffer which contained either 15 mM iodoacetamide to ensure nonreducing conditions or 50 mM dithiothreitol. Following centrifugation, the supernatant solution was removed and reheated to 100 °C for 4 min before loading on the gel.

Fibronectin Immunoprecipitation—The medium and total cell layer fractions of monolayer cultures were obtained as previously described (22). The immunoprecipitation protocol was a modification of a previously described procedure (29). Briefly, the medium was removed from the monolayer, and PMSF and EDTA were added to a final concentration of 2 and 5 mM, respectively. The cell layer was solubilized with a solution of 1% SDS, 150 mM NaCl, 50 mM Tris-HCl (pH 8.2), 5 mM EDTA, 2 mM PMSF, and 5 mM dithiothreitol for 10 min at room temperature. Iodoacetamide was added to the SDS solution to a final concentration of 15 mM; the solution was diluted with 4 volumes of a solution of 1.25% Triton X-100, 190 mM NaCl, 50 mM Tris-HCl (pH 7.4), and 5 mM EDTA (29). Affinity-purified polyclonal

rabbit anti-human fibronectin IgG was added to the diluted sample to a final concentration of 10 μg/ml, and the sample was incubated for 1 h with shaking at 37 °C. A 50% slurry of IgSorb (one-tenth volume) was added to the sample. The suspension was incubated for 1 h with shaking at 37 °C; the sample was centrifuged in a Beckman Microfuge; and the IgSorb pellet was washed three times with a solution of 0.1% Triton X-100, 0.2% SDS, 150 mM NaCl, 5 mM EDTA, and 50 mM Tris-HCl (pH 7.5). Bound antigen was solubilized by heating the pellet in SDS sample buffer containing 50 mM dithiothreitol at 100 °C for 4 min. Following centrifugation, the supernatant was removed and reheated to 100 °C for 4 min before loading on the gel.

SDS-PAGE and Fluorography—SDS-PAGE was performed according to Laemmli (30) using a 7.5% resolving gel with a 3% stacking gel. Gels were run at a constant current of 40 mA/gel. Gels were fixed for 1 h in a 5:5:90% isopropyl alcohol/glacial acetic acid/H_2O solution and then rinsed for 10 min three times in the same solution and for 15 min in H_2O. The gels were impregnated with Autofluor for 1 h, dried, and exposed to preflashed XAR-5 diagnostic film at −70 °C for appropriate times. Relative intensities of polypeptides were quantified by scanning soft laser densitometry within a predetermined linear dose-response range. When the prestained molecular weight standards were calibrated against a third set of known protein standards, α_2-macroglobulin, reported to be 180 kDa, migrated with an apparent molecular weight of 230,000. The figures are labeled to reflect this recalibration.

Quantification of RNA Levels—IMR-90 fibroblasts were grown to confluence in growth medium and were switched to the defined conditions for the indicated amount of time. Total cellular RNA was isolated by lysing the cell monolayers with a solution of 4 M guanidine thiocyanate, 25 mM sodium acetate (pH 6), 2% Sarkosyl, and 0.835% β-mercaptoethanol, followed by centrifugation through a 5.7 M cesium chloride cushion (31). Ten micrograms of RNA was electrophoresed in a 1% agarose, 1.0 M formaldehyde gel (32) and was electroblotted to a nylon membrane according to the manufacturer's recommendations (GeneScreen *Plus*, Du Pont-New England Nuclear). Equal amounts of RNA were loaded on the basis of absorbance at 260 nm, and equivalency of samples was verified by the intensity of ethidium bromide staining of the 28 S and 18 S rRNA bands. In control experiments, we could discern a 20% difference in sample loading by the intensity of ethidium staining. Efficiency of transfer was judged by viewing the nylon membrane under UV light.

The cDNA probes were radiolabeled with [^{32}P]dCTP in low melting agarose using the random primer technique as described by the manufacturer (Amersham Corp.). Unincorporated nucleotides were removed by centrifuging the reaction mixture in a Centricon 30 microconcentrator (Amicon Corp.). The blots were prehybridized in a solution of 5 × SSC (1 × SSC = 0.15 M sodium chloride, 0.15 M sodium citrate), 5 × Denhardt's (1 × Denhardt's = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 50% formamide, 7.5% dextran sulfate, 0.1 mg/ml denatured salmon sperm DNA, 1% SDS, 1 μg/ml poly(A), and 20 mM $NaPP_i$ (pH 6.5). After 1 h at 42 °C, the labeled probe was added (500,000 cpm/ml of hybridization buffer), and the hybridization was continued overnight. The blots were washed twice in 2 × SSC at room temperature for 5 min, then twice in a solution of 2 × SSC and 1% SDS at 60 °C for 30 min, and then twice in 0.1 × SSC at room temperature for 30 min. Blots were exposed to Kodak XAR-5 film with an intensifying screen at −70 °C. The relative intensities of RNA bands were quantified by scanning densitometry. To strip off the hybridized probe, the blots were boiled in a solution of 0.1% SSC and 1% SDS for 15 min.

RESULTS

IMR-90 Fibroblasts Synthesize a Dimeric Fibronectin Receptor Complex—The polyclonal rabbit anti-human placenta fibronectin receptor IgG immunoprecipitates four polypeptides of 110, 125, 155, and 185 kDa (reduced) from IMR-90 metabolically labeled for 16 h (Fig. 1). Following the nomenclature used by other groups studying the family of receptors recognizing ligands containing the Arg-Gly-Asp sequence (18), we shall refer to the 125-kDa polypeptide as the β-subunit and to the 155-kDa polypeptide as the α-subunit. The 110-kDa polypeptide will be termed the pre-β-subunit (see below). The relationship of the less abundant 185-kDa polypeptide to the other polypeptides is not known. This polypeptide was not precipitated by nonimmune rabbit IgG and might be a distinct α-subunit (18, 20).

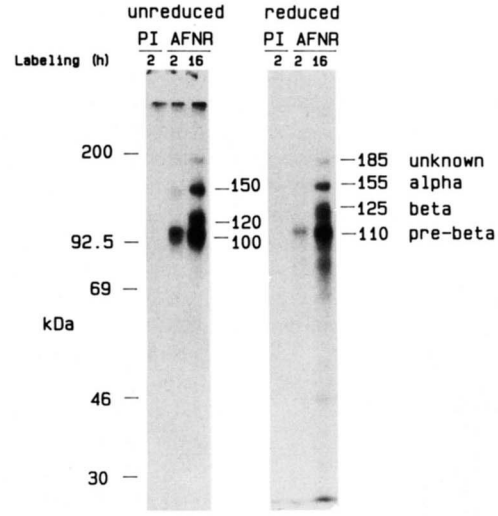


FIG. 1. **Immunoprecipitation of human fibronectin receptor complex.** IMR-90 fibroblasts were labeled with 100 μCi/ml [^{35}S] methionine for 2 or 16 h. Triton extracts of the cell layer were immunoprecipitated with preimmune normal rabbit IgG (*PI*) or polyclonal rabbit anti-human fibronectin receptor (*AFNR*) as described under "Experimental Procedures." Immunoprecipitates were separated by SDS-PAGE in gels containing 7.5% acrylamide. Prestained molecular mass standards include myosin (200 kDa), phosphorylase *b* (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), and carbonic anhydrase (30 kDa).

The optimal antibody concentration for immunoprecipitation was between 50 and 100 μg/ml, and the freezing of the initial supernatant and the preabsorptions of the labeled homogenate with the Protein A-Sepharose CL-4B/Sepharose CL-4B mixture and of the Protein A-Sepharose CL-4B with the unlabeled homogenate were essential to prevent immunoprecipitation of extraneous polypeptides (not shown). The antibody preparation recognizes primarily β- and pre-β-subunits, but has limited reactivity to the α-subunit. When IMR-90 cell homogenates are immunoblotted, 110- and 125-kDa polypeptides (pre-β- and β-subunits), but no α-sized component, react with the antibody. However, when high concentrations (approximately 1–5 μg/lane) of the isolated, purified human placental fibronectin receptor complex are immunoblotted, both α- and β-subunits are recognized (data not shown).

Because the predominant 125-kDa polypeptide present in the immunoprecipitates from cells labeled for 16 h was not present in samples labeled for 2 h, we studied possible processing of the fibronectin receptor complex by pulse-chase labeling (Fig. 2). This analysis revealed that the β-subunit is synthesized as an abundant 110-kDa pre-β-subunit which is processed slowly ($t_{½}$ ~ 4–6 h) to a 125-kDa form. Only about one-third of the pre-β-form is processed; the remainder apparently is degraded. The α-subunit appears to be synthesized as a 150-kDa precursor which is processed completely after 2 h to the 155-kDa α-subunit. Both the α- and β-subunits migrate more slowly when reduced than when unreduced (125 *versus* 120 kDa and 155 *versus* 150 kDa), compatible with intramolecular disulfides. The ratio of the completely processed β-subunit to the α-subunit is approximately 3:1 in methionine-labeled samples. After correcting for the methionine ratio of 2.6:1 in the mature β-subunit:α-subunit of the human fibronectin receptor (28), the ratio becomes 1.2:1, suggesting a 1:1 stoichiometry in the fully processed receptor complex.

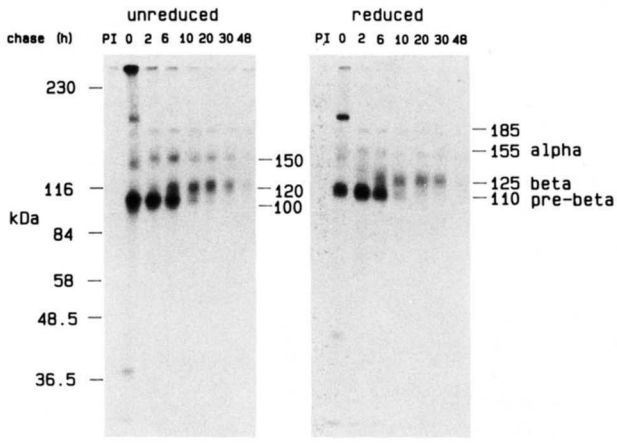
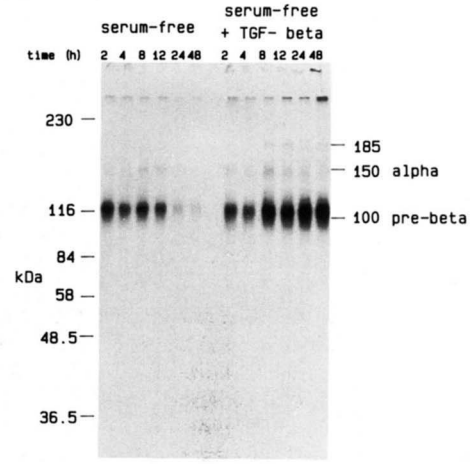


FIG. 2. **Human fibronectin receptor complex processing.** IMR-90 fibroblasts were pulse-labeled with 200 μCi/ml [^{35}S]methionine for 1 h and then chased with growth medium for the indicated times. Triton extracts of the cell layer were immunoprecipitated with preimmune normal rabbit IgG (*PI*) or polyclonal rabbit anti-human fibronectin receptor as described under "Experimental Procedures." Immunoprecipitates were separated by SDS-PAGE in gels containing 7.5% acrylamide. Prestained molecular mass standards include α_2-macroglobulin (migrating at 230 kDa as described under "Experimental Procedures"), β-galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa), pyruvate kinase (59 kDa), fumarase (48.5 kDa), and lactate dehydrogenase (36.4 kDa). The 48-h samples were partly lost during handling, so their intensity does not reflect actual quantity.

Regulation of the Fibronectin Receptor Complex by TGF-β1—IMR-90 fibroblasts incubated in serum-free medium alone or supplemented with 100 pM TGF-β1 for varying periods were labeled with [^{35}S]methionine for 2 h, and the fibronectin receptor complex was immunoprecipitated. As shown in Fig. 3 (*upper*), in serum-free medium, TGF-β1 stimulates pre-β-subunit synthesis 2-, 20-, and 40-fold compared to serum-free controls at 8 and 12, 24, and 48 h, respectively. Much of the late increase is attributable to the fact that in control cultures maintained in serum-free medium, fibronectin receptor complex synthesis has decreased significantly by 24 h (compare *2* and *24 h lanes* in serum-free group of Fig. 3, *upper*). In serum-containing medium, TGF-β1 stimulates synthesis of the fibronectin receptor complex 2-fold at 24 and 48 h compared to serum-containing controls (Fig. 3, lower; see below).

Regulation of Fibronectin and Total Protein Synthesis by TGF-β1—In parallel experiments, we found that TGF-β1 also increases fibronectin synthesis. Fig. 4*A* (*upper*) displays fibronectin synthesis in cells cultured with 100 pM TGF-β1 in serum-free medium. The earliest increase in fibronectin synthesis is detectable at 12 h and is 2-fold greater than control. By 24 and 48 h, fibronectin synthesis is increased 20- and 30-fold, respectively, compared with controls. Fibronectin synthesis in cells cultured in serum-containing medium supplemented with TGF-β1 is 3-fold above control at 24 and 48 h (Fig. 4, *center* and *lower*).

To determine if the stimulatory effect of TGF-β1 on the synthesis of fibronectin and the fibronectin receptor complex is selective or is the result of a general increase in protein synthesis, we scanned fluorograms of total cell layer polypeptides from control and TGF-β1-treated fibroblasts. In serum-free medium, 100 pM TGF-β1 stimulated [^{35}S]methionine incorporation into most discrete polypeptides (see Fig. 4, *upper*, *cell layer lanes*). The magnitude of TGF-β1-mediated increases in fibronectin and in the fibronectin receptor complex labeling was similar to that seen for most polypeptides. This is compatible with a general increase in protein synthesis



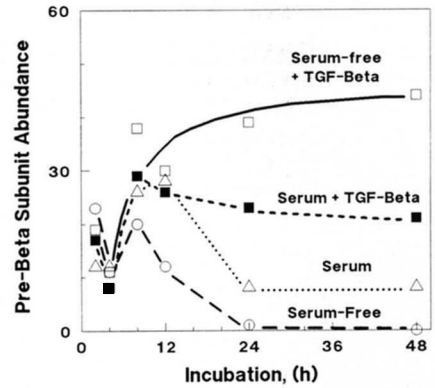


FIG. 3. **Effect of TGF-β1 on fibronectin receptor complex expression.** *Upper,* fluorogram of immunoprecipitations of cell extracts. IMR-90 fibroblasts incubated with or without 100 pM TGF-β1 were labeled with 100 μCi/ml [^{35}S]methionine for the last 2 h of each period of time. Triton extracts of the cell layer were immunoprecipitated with polyclonal rabbit anti-human fibronectin receptor as described under "Experimental Procedures." Immunoprecipitates were separated by SDS-PAGE under nonreducing conditions in gels containing 7.5% acrylamide. Molecular mass standards are those described in the legend of Fig. 2. *Lower,* quantitation of immunoprecipitated samples by scanning soft laser densitometry. Fluorograms were scanned for the pre-β-subunit quantitation. ○, serum-free; □, serum-free + TGF-β1; △, serum alone; ■, serum + TGF-β1.

in fibroblasts treated with TGF-β1 in the presence of low serum or serum-free medium as previously described (12, 14). In contrast, IMR-90 fibroblasts treated with TGF-β1 in serum-containing medium increase their synthesis of fibronectin 3-fold and of the fibronectin receptor complex about 2-fold without increasing overall protein synthesis (Fig. 4, *lower*), demonstrating a selective stimulatory effect.

Northern Blot Analysis of Fibronectin and the Fibronectin Receptor Complex mRNAs—mRNAs encoding fibronectin and the α- and β-subunits of the fibronectin receptor complex were quantified by Northern blot analysis of total RNA. Exposure to 100 pM TGF-β1 increases steady-state mRNA levels encoding all three proteins compared to mRNA levels in control cells cultured in serum-free medium (Fig. 5). The stimulatory effect of TGF-β1 on the α- and β-subunits of the fibronectin receptor becomes apparent by 8 h and results in 3-fold increases in their steady-state mRNA levels by 24 h, with no further increase at 32 h. TGF-β1 increases fibronectin mRNA levels 5-, 10-, and 20-fold compared to levels in cells cultured in serum-free medium for 12, 24, and 32 h, respectively. The increase in mRNAs encoding the fibronectin receptor complex subunits occurs prior to any detectable

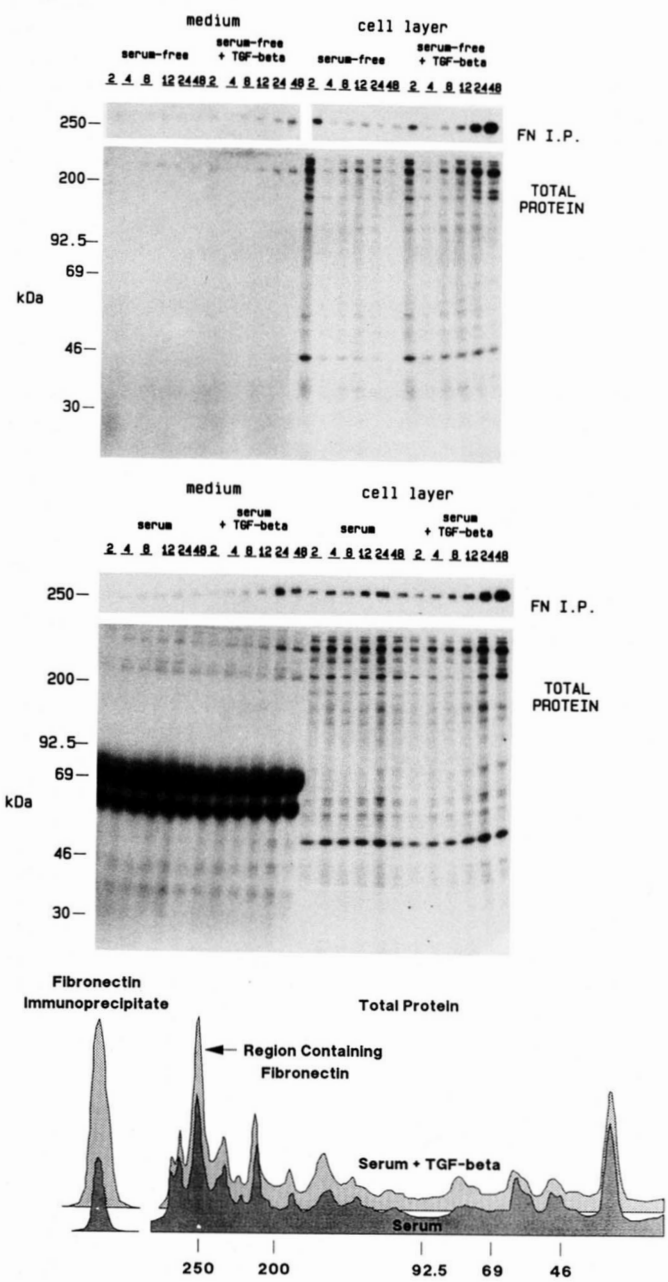


FIG. 4. **Effect of TGF-β1 on fibronectin and total protein expression.** IMR-90 fibroblasts incubated with or without 100 pM TGF-β1 in either serum-free (*upper*) or serum-containing (*center*) medium were labeled with 100 μCi/ml [^{35}S]methionine for the last 2 h of each period, as indicated by the label over each lane. The medium and the cell layer extracts were immunoprecipitated with polyclonal rabbit anti-human fibronectin IgG (*FN I.P.*) as described under "Experimental Procedures." Immunoprecipitates as well total protein samples were separated by SDS-PAGE under reducing conditions in gels containing 7.5% acrylamide. *Lower,* scanning densitometry of the 24-h bands of fibronectin immunoprecipitates and total cell layer proteins from *center*. The immunoprecipitate profiles are plotted to a different scale than the total protein profiles. The fibronectin-containing region around 250 kDa contains non-fibronectin polypeptides, as under conditions in which fibronectin is quantitatively immunoprecipitated, a larger difference is seen in the amount of fibronectin synthesized in cells treated with or without TGF-β1 in serum-containing medium than is reflected in the total protein fluorogram profiles in this region. Thus, although overall polypeptide intensities are similar between the two profiles aside from a 1.5-2 fold increase in the 250-kDa region, there is a 3-fold increase in fibronectin labeling in cells treated with TGF-β1 compared to control. Molecular mass standards are those described in the legend of Fig. 1.

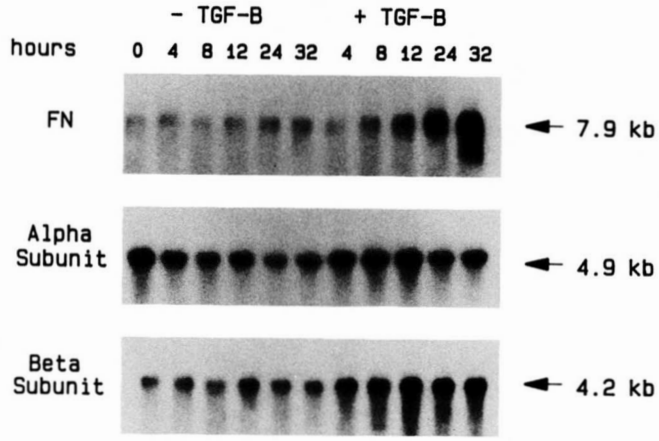


FIG. 5. **Effect of TGF-β1 on fibronectin and fibronectin receptor complex mRNA levels.** IMR-90 fibroblasts were grown in serum-free medium in the presence (+*TGF-β*) or absence (−*TGF-β*) of 100 pM TGF-β1 for the indicated periods prior to isolation of RNA. The same Northern blot of total RNA (10 μg/lane) was hybridized to a labeled cDNA, stripped of that probe, and rehybridized to another labeled cDNA, as described under "Experimental Procedures." *Upper,* fibronectin (*FN*) mRNA (*XbaI/BamHI* subfragment of pFH6) (26); *center,* the α-subunit of the fibronectin receptor complex mRNA (λP7) (27); *lower,* the β-subunit of the fibronectin receptor complex mRNA (λP32) (28). The sizes in kilobases (*kb*) of the mRNAs are indicated as deduced from comparison with molecular mass standards.

increase in fibronectin mRNA (Fig. 5).

The effect of TGF-β1 and serum starvation on mRNAs encoding fibronectin and the fibronectin receptor subunits is complex. The fibronectin mRNA level remains constant during serum starvation (Fig. 5, *upper*), and TGF-β1 increases this steady-state level dramatically. The steady-state level of mRNA encoding the α-subunit decreases steadily in the absence of serum. TGF-β1 prevents this decrease, but does not increase the α-subunit steady-state mRNA level (Fig. 5, *center*) above that found in cells maintained in serum-containing medium (*center, 0 h lane*). In contrast, the β-subunit mRNA level remains relatively constant in the absence of serum, and TGF-β1 increases this steady-state level above that present in serum-starved or serum-containing control cells (Fig. 5, *lower*). Thus, the mRNA for the β-subunit responds similarly to that for fibronectin.

Actinomycin D blocks the TGF-β1-mediated increase in all three mRNAs (Fig. 6). Cycloheximide at a concentration completely inhibiting protein synthesis (not shown) blocks the TGF-β1-mediated increase in the fibronectin mRNA level (Fig. 6) and prevents the decline in α-subunit mRNA levels in cells cultured in serum-free medium. The addition of TGF-β1 to cycloheximide-treated cells does not further increase α-subunit levels. The β-subunit mRNA responds to cycloheximide in a similar fashion as the α-subunit. The observation that actinomycin D inhibits TGF-β1-mediated increases in fibronectin and fibronectin receptor complex expression could argue for a transcriptional effect. However, because cycloheximide blocks the TGF-β1-mediated increase in fibronectin mRNA, the induction of fibronectin by TGF-β1 may be indirect. Thus, TGF-β1 may stimulate the synthesis of another protein or proteins that mediate the accumulation of fibronectin mRNA.

DISCUSSION

TGFs-β, dimeric polypeptide growth hormones found in α-granules of platelets and produced by immune cells, appear to play a key role in wound healing, cellular differentiation,

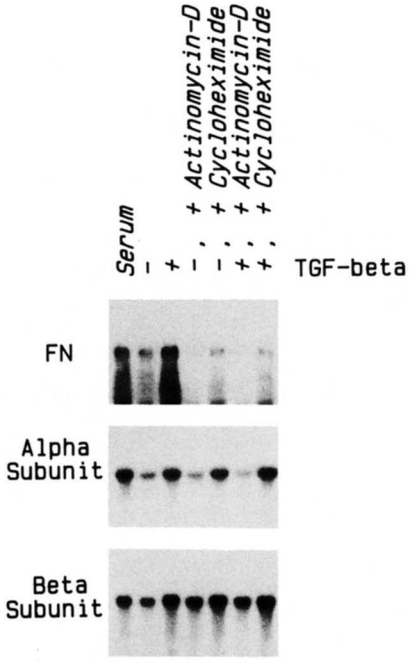


FIG. 6. **Effect of actinomycin D and cycloheximide on fibronectin and fibronectin receptor complex mRNA levels with or without addition of TGF-β1.** IMR-90 fibroblasts were grown in serum-containing medium (*Serum, first lane*, 0 h) or serum-free medium (*remaining lanes*) in the presence (+) or absence (−) of 100 pM TGF-β1 and either 400 ng/ml actinomycin D or 20 μg/ml cycloheximide as indicated for 24 h prior to RNA isolation. Northern blot analysis was carried out as described in the legend of Fig. 5. *Upper*, fibronectin (*FN*) mRNA; *center*, the α-subunit of the fibronectin receptor complex mRNA; *lower*, the β-subunit of the fibronectin receptor complex mRNA.

and regulation of connective tissue synthesis by mesenchymal cells (2, 3). Ignotz and Massagué (13) demonstrated that TGF-β1 stimulates fibronectin synthesis by a variety of fibroblasts and increases the deposition of fibronectin into the pericellular matrix.

We recently found that fibronectin assembly into pericellular matrix appears to involve the fibronectin receptor complex (22). Thus, we hypothesized that TGF-β1 might drive fibronectin matrix assembly in part by increasing the expression of the fibronectin receptor complex as well as of fibronectin. After exposing IMR-90 to TGF-β1 in serum-free medium *in vitro*, we observed 6-fold increases in fibronectin synthesis by 24 h as previously reported for fibroblastic cells (13, 15, 16) and up to 30-fold increases by 48 h. These increases are accompanied by similar increases in fibronectin mRNA levels which are prevented by actinomycin D treatment. The late increase in the synthesis of fibronectin in serum-free medium is accompanied by a general stimulation of fibroblast protein synthesis, as found previously (12, 14). However, in serum-containing medium, TGF-β1 selectively increases fibronectin synthesis. TGF-β1 also caused similar increases in the synthesis of both components of the fibronectin receptor complex. This increase was accompanied by increased steady-state concentrations of mRNA. The actinomycin D inhibition of this increase in fibronectin and the fibronectin receptor complex mRNA levels suggests that the TGF-β1 effect is in part transcriptionally mediated, but we cannot exclude the possibility that other mechanism(s), *e.g.* stabilization of mRNA, may be involved. Interestingly, the time course of increased fibronectin expression correlates closely with the appearance of granulation tissue following intradermal injection of TGF-β1 (10, 11).

The mammalian fibronectin receptor complex consists of an α- and β-subunit which are noncovalently linked (18). We studied the processing of the human receptor in fetal lung fibroblasts using pulse-chase labeling, and found that the α-subunit appears to be synthesized as a precursor which is completely processed within 2 h. The β-subunit is synthesized as a precursor which undergoes processing over an 8-h period to a higher molecular weight form, consistent with other β-subunits in the family of receptors recognizing the Arg-Gly-Asp sequence in their ligands (33, 34). For other receptors in this family, this increase in β-subunit molecular weight has been shown to be due to glycosylation. The pre-β-subunit contains high mannose asparagine-linked oligosaccharides. Processing to the mature β-subunit appears to follow removal of mannose residues and subsequent addition of more complex oligosaccharides (33–36). Interestingly, this process is quite lengthy compared with other proteins. Moreover, only about one-third of the pre-β-subunit is processed to the mature form. Although we do not have an explanation for the apparent excess synthesis of the precursor form, there is precedent for one or more subunits of multisubunit receptors to be synthesized in excess of their final assembled stoichiometry (37, 38). Thus, in the case of the fibronectin receptor, availability of the α-subunit may limit assembly of the receptor complex.

Both the α- and β-subunits migrate more slowly when reduced than when unreduced, compatible with intramolecular disulfides. The α-subunit is often found to consist of a disulfide-cross-linked heavy (140 kDa) and light (20 kDa) chain that are separated by the cleavage of an internal peptide bond (18). Thus, upon reduction, the α-subunit typically migrates faster on SDS-PAGE. We did not identify faster migration of the α-subunit in immunoprecipitated samples from IMR-90, suggesting that the α-subunit is not cleaved. Little is known about the mechanism(s) leading to or the purpose of the cleavage of the peptide bond in the α-subunit; it is possible that in IMR-90 this cleavage occurs variably.

While this manuscript was in review, Ignotz and Massagué (39) reported that TGF-β1 increased the expression of the fibronectin receptor complex under serum-free conditions in 3T3-L1 cells and increased the level of mRNA encoding the β-subunit of the fibronectin receptor complex in 3T3-L1, NRK-49F, and chick embryo fibroblasts. In metabolic labeling experiments, they found little effect of TGF-β1 upon fibronectin receptor complex expression by WI38 human lung fibroblasts. In contrast, under our experimental conditions TGF-β markedly increased the expression of fibronectin and of the fibronectin receptor complex by IMR-90, another human lung fibroblast strain. In addition, we found that the effect of TGF-β1 on fibronectin and fibronectin receptor expression varies according to the culture and metabolic labeling conditions. Under serum-free conditions, TGF-β stimulates the expression of fibronectin and of the fibronectin receptor complex greatly in comparison to control cells. However, based upon SDS-PAGE and fluorography, metabolic labeling of most secreted and cell-associated proteins including fibronectin and the fibronectin receptor complex declines in the serum-free control cells. We found that TGF-β1 prevents this decline and increases the labeling of most polypeptides including fibronectin and the fibronectin receptor complex. Thus, under serum-free conditions, TGF-β1 stimulates labeling of fibronectin and of the fibronectin receptor complex, but the increase is similar to that of many other polypeptides. Other investigators have found a similar pattern of increased metabolic labeling by diploid fibroblasts exposed to TGF-β1 in serum-free conditions (12, 14). However, in the

presence of serum, we found TGF-β1 to stimulate selectively expression of fibronectin and of the fibronectin receptor complex. Ignotz and Massagué did not report the effects of TGF-β1 upon mRNA encoding the α-subunit of the fibronectin receptor complex, which we demonstrate here is subject to a distinct form of regulation by TGF-β1, namely prevention of the decline in steady-state levels associated with serum starvation.

In conclusion, we postulate that one of the actions of TGF-β1 during tissue remodeling is to increase the synthesis of fibronectin and of the fibronectin receptor complex by fibroblasts, possibly as part of a more generalized response characterized by increased protein synthesis. As evidence mounts that cells respond to their surrounding pericellular matrix via specific receptors, this result strengthens the emerging evidence that TGF-β1 plays a pivotal role in matrix synthesis characterizing embryonic development, wound healing, and fibroproliferative responses to tissue injury such as liver and lung fibrosis.

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