Interleukin-6 Regulation of Transforming Growth Factor (TGF)-β Receptor Compartmentalization and Turnover Enhances TGF-β1 Signaling*

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Transforming growth factor (TGF)-β1 is a key cytokine involved in the pathogenesis of fibrosis in many organs, whereas interleukin (IL)-6 plays an important role in the regulation of inflammation. Recent reports demonstrate interaction between the two cytokines in disease states. We have assessed the effect of IL-6 on TGF-β1 signaling and deﬁned the mechanism by which this occurred. Stimulation of Smad-responsive promoter (SBE)-4-Lux activity by TGF-β1 was signiﬁcantly greater in the presence of IL-6 than that induced by TGF-β1 alone. Augmented TGF-β1 signaling following the addition of IL-6 appeared to be mediated through binding to the cognate IL-6 receptor, the presence of which was conﬁrmed by ﬂuorescence-activated cell sorting and Stat-speciﬁc signaling. TGF-β1 receptors internalize by both caveolin-1 (Cav-1) lipid raft and early endosome antigen 1 (EEA-1) non-lipid raft pathways, with non-lipid raft-associated internalization increasing TGF-β1 signaling. Affinity labeling of TGF-β1 receptors demonstrated that IL-6 stimulation resulted in increased partitioning of TGF-β receptors to the non-lipid raft fraction. There was no change in expression of Cav-1; however, following IL-6 stimulation, co-immunoprecipitation demonstrated decreased association of IL-6 receptor with Cav-1. Increased TGF-β1-dependent Smad signaling by IL-6 was signiﬁcantly attenuated by inhibition of clathrin-mediated endocytosis and augmented by depletion of membrane cholesterol. These results indicate that IL-6 increased trafﬁcking of TGF-β1 receptors to non-lipid raft-associated pools results in augmented TGF-β1 Smad signaling.

MATERIALS AND METHODS

Reagents—All tissue culture plastics were obtained from BD Biosciences. Reporter Lysis Buffer and Bright-Glo Luciferase Assay System were purchased from Promega (Southampton, United Kingdom). FuGENE 6 transfection reagent was bought from Roche Applied Science. [32P]Deoxynucleoside triphosphate and 125I-TGF-β1 were obtained from Amersham Biosciences. Other reagents and sources were as follows: R-phycocerythrin-conjugated mouse anti-human IL-6 receptor monoclonal antibody, R-phycocerythrin-conjugated mouse IgG monoclonal antibody, and mouse anti-human caveolin-1 antibody, BD Biosciences; recombinant IL-6 and recombinant human TGF-β1, R&D Systems Europe Ltd. (Abingdon, United Kingdom); rabbit anti-human

1 The abbreviations used are: TGF, transforming growth factor; IL, interleukin; Cav-1, caveolin-1; Stat, signal transducers and activators of transcription.
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TGF-β receptor type I, Stat1 and Stat3 polyclonal antibodies, normal rabbit IgG, and goat anti-human Smad2, Smad3, and Smad7 polyclonal antibodies, Autogen Bioclear UK Ltd. (Calne United Kingdom).

Cell Culture—HK-2 cells (human renal proximal tubular epithelial cells immortalized by transduction with human papillomavirus 16 E6/E7 genes; Ref. 16) were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 (Invitrogen) supplemented with 10% fetal calf serum (Biological Industries Ltd., Camberra, United Kingdom), 2 μg/l-glutamine (Invitrogen), 20 mM HEPES buffer (Invitrogen), 5 μg/ml insulin, 5 μg/ml transferrin (Sigma), 40 ng/ml hydrocortisone (Sigma), and 5 ng/ml sodium selenite (Sigma). Cells were grown at 37 °C in 5% CO₂ and 95% air. Fresh growth medium was added to the cells every 2–3 days until the cells reached confluence. Cells were grown to confluence and serum-deprived for 48 h prior to experimental manipulation. In all experiments, cells were stimulated with recombinant TGF-β1 and/or IL-6 under serum-free conditions. In all aspects of cell biology that we observed, HK-2 cells behaved in an identical fashion to primary cultures of human proximal tubular cells (17–20). HK-2 cells are therefore a good model from which general conclusions can be drawn in terms of proximal tubular cell biology.

Transient Transfection—The Smad-responsive promoter (SBE)₄-Lux (21) was a gift from Aristidis Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden). For transfection of the reporter construct, 1.3 g confluent monolayers were washed once with cold phosphate-buffered saline, 30% solubilized in detergent buffer (Autogen Bioclear), 25 μg/ml sodium selenite (Sigma) and separated proteins were visualized by autoradiography. In supershift experiments, 20 μg/ml rabbit anti-human Stat1 or Stat3 antibody (Autogen Bioclear) was added and incubated for 20 min before addition of radiolabeled probe.

Affinity Labeling of TGF-β1 Receptors and Assessment of Receptor Turnover—HK-2 cells were incubated with 250 pM ¹²⁵I-TGF-β1 at 37 °C for 1 h, and receptors were cross-linked to ligand with disuccinimidyl suberate (Pierce) as described previously (24). Previous studies suggest that receptor cross-linking itself has no effect on receptor internalization; cells were incubated with either TGF-β1 or the combination of TGF-β1 and IL-6. Following lysis of the cells in Reporter Lysis Buffer, luciferase activity was normalized to β-galactosidase activity. Nuclear extracts were prepared according to the method of Andrews and Faller (23), and protein concentrations were determined using the Bio-Rad protein assay. Nuclear extracts (5 μg) were incubated for 20 min at room temperature with 1.25 ng of the sIE oligonucleotide corresponding to a consensus Stat-binding element, 5'-CGAGTGTCAATTCGTAATCTTGTCTACA-3', in a 20-μl binding reaction buffer and 1 μg of poly(dI/dC) (Sigma-Aldrich). Complexes were resolved on nondenaturing 6% polyacrylamide gels, and separated proteins were visualized by autoradiography. In supershift experiments, 20 μg/ml rabbit anti-human Stat1 or Stat3 antibody (Autogen Bioclear) was added and incubated for 20 min before addition of radiolabeled probe.

Detergent-free Purification of Lipid Raft-rich Membrane Fraction—HK-2 cells were grown to near confluence in 100-mm² dishes and affinity-labeled as described above. Two washes with cold phosphate-buffered saline, two confluent dishes were scraped into 2 ml of 500 mM sodium carbonate, pH 11.0. Homogenization was carried out with the use of 10 strokes of a tight-fitting Dounce homogenizer, followed by three 10-s bursts of a tissue homogenizer (Powergen 125; Fisher Scientific), followed by three 20-s bursts of an ultrasonic disintegrator (Soniprep 150; Fisher Scientific) to disrupt cellular membranes (25). The homogenates were adjusted to 45% sucrose by addition of 2 ml of 90% sucrose prepared in MBS (2-(N-morpholino)ethanesulfonic acid-buffered saline, 25 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.5, and 0.15 M NaCl) and placed at the bottom of an ultracentrifuge tube. A discontinuous sucrose gradient (4 ml of 35% sucrose and 4 ml of 5% sucrose, both prepared in MBS) was formed above and centrifuged at 39,000 rpm for 16–20 h in an SW40 Ti rotor (Beckman Instruments). A light-scattering band was observed at the 3–35% sucrose interface. Twelve 1-ml fractions were collected from the top of the tubes, and a portion of each fraction was analyzed by SDS-PAGE.

Nuclear extracts were pre-cleared with 25 μg/ml rabbit anti-human Stat1 or Stat3 antibody (Autogen Bioclear) was added and incubated for 20 min before addition of radiolabeled probe.

IL-6 Enhances TGF-β1 (Smad-dependent) Signaling—Activation of the Smad signaling pathway was examined using the (SBE)₄-Lux reporter, which contains four repeats of the CA(GA)₄ sequence identified as a Smad-binding element. Stimulation of HK-2 cells transiently transfected with TGF-β1 led to a significant increase in luciferase activity of the reporter construct as described previously (26) (Fig. 1). Addition of IL-6 (0–100 ng/ml) alone did not increase the signal above control values. In contrast, addition of IL-6 in the presence of TGF-β1 led to a dose-dependent increase in luciferase activity that was significantly greater than that induced by TGF-β1 alone (Fig. 1). This effect of IL-6 was statistically significant at IL-6 doses of ≥10 ng/ml.

RESULTS
expression is confined to hepatocytes and leukocyte subpopulations to date documenting that IL-6 cognate receptor is more limited, though expression of gp130 is found in almost all cell types, cellular distribution of IL-6 cognate receptor was quantified as described under “Materials and Methods,” and the results were normalized for transfection efficiency (using β-galactosidase) expressed as the fold increase above the non-stimulated control. Data represent mean ± S.D. (n = 9).

**HK-2 Cells Express Functional IL-6 Cognate Receptor**—Although expression of gp130 is found in almost all cell types, cellular distribution of IL-6 cognate receptor is more limited, with studies to date documenting that IL-6 cognate receptor expression is confined to hepatocytes and leukocyte subpopulations. Enhanced TGF-β1-dependent Smad signaling seen following addition of IL-6 suggests that this is the result of binding of IL-6 directly to its cognate receptor and the resulting dimerization of gp130.

Flow cytometry was used to confirm the expression of IL-6 cognate receptor on the cell surface of HK-2 cells (Fig. 2A). Functional integrity of the IL-6 signaling pathway was examined by gel shift assays performed with nuclear proteins and a Stat site-specific consensus probe (Fig. 2B). Stat activation was seen following addition of IL-6 (Fig. 2B). We verified activation of both Stat1 and Stat3 following addition of IL-6 alone to HK-2 cells by supershift assay. Incorporation of an antibody to either Stat1 or Stat3 interfered with the formation of the probe-protein complexes because band intensities were reduced with inclusion of antibody, and an additional retarded band was seen in the presence of the Stat3 antibody (Fig. 2C).

**Smad Protein Expression and Association**—Previous studies have demonstrated cooperative binding of the Stat and Smad proteins between lipid and non-lipid membrane pools (34). To examine alterations in receptor partitioning, endogenous TGF-β receptors on HK-2 cells were affinity-labeled using 125I-TGF-β1. Subsequently, rafts were fractionated by sucrose density centrifugation (30). Following the addition of 125I-TGF-β1 (250 pM), receptors were found in both the raft and non-raft fractions, as we demonstrated previously (Fig. 4A). Scanning densitometry of the results of three separate experiments confirmed that following the addition of 125I-TGF-β1, 38% of the total TGF-β receptor partitioned into the non-raft fractions (Fig. 4B). Addition of 25I-TGF-β1 in the presence of IL-6 led to a significant increase in TGF-β receptor detected in the non-raft fraction. Under these conditions, 62% of total TGF-β receptor partitioned into the raft fractions (mean of n = 3, p = 0.02).

The sterol-binding antibiotic nystatin specifically interacts with cholesterol to sequester it in the membrane, thereby effectively reducing the ability of cholesterol to interact with and exert its effects on other membrane components (31, 32). Disruption of cholesterol by pre-treatment of cells with 50 µg/ml nystatin (29) at 37 °C for 1 h led to an increase of TGF-β receptor partitioning into the non-raft fraction (Fig. 4A). The increase in trafficking of TGF-β receptor into the non-lipid raft membrane fraction was associated with a further increase in TGF-β1-dependent Smad signaling following the addition of TGF-β1 together with IL-6 (Fig. 5A). The effect of nystatin was dependent on cholesterol sequestration because it was prevented by addition of cholesterol (25 µg/ml) together with nystatin to cells at 37 °C for 1 h prior to addition of TGF-β1 together with IL-6 (Fig. 5A). Next we examined the effect of inhibition of clathrin-mediated endocytosis by K+ depletion, which prevents clathrin lattice assembly and has been shown to inhibit endosome-dependent TGF-β1 signaling (8). Activation of the TGF-β1 signaling pathway following addition of TGF-β or TGF-β1 in combination with IL-6 was assessed by using the (SBE)4-Lux reporter (Fig. 5B). The augmentation of TGF-β1-dependent Smad signaling by IL-6 was significantly attenuated when carried out in minimal, K+-depleted medium (Fig. 5B). Cellular cholesterol disruption has been previously demonstrated to trigger shedding of membrane proteins (33). In our experimental system, addition of 50 µg/ml nystatin at 37 °C for 1 h to confluent monolayers of HK-2 cells did not induce shedding of either TGF-β type I receptor as assessed by Western analysis of cell culture supernatant or IL-6 as assessed by enzyme-linked immunosorbent assay (data not shown).

**Growth factor signaling may be modulated by alteration of caveolin-1 expression** which is likely to influence trafficking of receptors between lipid and non-lipid membrane pools (34). Total caveolin-1 expression as assessed by Western analysis was unaltered following stimulation with either TGF-β1 or TGF-β1 + IL-6 (Fig. 6). In contrast, following stimulation with TGF-β1 in the presence of IL-6, there was a decrease in the association of Cav-1 with the TGF-β receptor compared with addition of TGF-β1 alone, as assessed by immunoprecipitation of TGF-β receptor type I and immunoblot analysis for Cav-1 (Fig. 6).

To evaluate the importance of the IL-6-dependent trafficking of TGF-β receptors between the lipid raft and non-raft-associated...
Our interest in the regulation of TGF-β1 function in the kidney stems from the overwhelming evidence that implicates this pro-fibrotic factor in the pathogenesis of renal fibrosis (1–5). TGF-β1 also plays a role in fetal development, wound healing, and regulation of inflammatory processes (35, 36). Furthermore, it has an anti-proliferative effect on normal epithelial cells and acts as tumor suppressor, yet it also functions as a promoter of cancer progression and metastasis at later stages of disease (37). It is also clear, therefore, that understanding the processes that modulate TGF-β1 activity has a much wider implication than renal disease.

TGF-βs elicit their signaling effects by binding mainly to three cell surface receptors: type I, type II, and type III. Type I and II receptors are serine/threonine kinases that form heteromeric complexes and are necessary for TGF-β signaling. These are initiated when the ligand induces assembly of a heteromeric complex of type II and type I receptors. The receptor type II kinase then phosphorylates receptor type I on a conserved glycine/serine-rich domain. This activates the receptor type I kinase, which subsequently recognizes and phosphorylates members of the intracellular receptor-regulated Smad signal transduction pathway. For TGF-β1, these include Smad2 and Smad3. This causes dissociation of the intracellular receptor-regulated Smads from the receptor, stimulates the assembly of a heteromeric complex between the phosphorylated intracellular receptor-regulated Smad and the co-Smad Smad 4, and induces nuclear accumulation of this heteromeric Smad complex (reviewed in Ref. 38).

Endocytosis of cell surface receptors is an important regulatory event in signal transduction. TGF-β1 receptors internalize into both caveolin- and EEA-1-positive vesicles and reside in both lipid raft and non-raft membrane domains (8). Clathrin-dependent internalization into the EEA1-positive endosome promotes TGF-β1 signaling. In contrast, the lipid raft-caveolar internalization pathway contains Smad7-bound receptor and is required for receptor turnover. TGF-β receptor type II internalization is unaffected by ligand stimulation, suggesting that its role is not to regulate trafficking but to act to recruit the type I receptor and stabilize heterotrameric receptor complexes during constitutive trafficking events. Subsequently, the activated type I receptor signals in the EEA1-positive endosome by phosphorylating Smad2 or directing degradation through lipid raft pathways by binding Smad7-Smurf2 complexes. We have recently identified a novel mechanism by which this process may be regulated that is dependent on the interaction between distinct receptor populations on the cell surface. These studies demonstrate that co-localization of the hyaluronan receptor CD44 and TGF-β receptors facilitates modulation of both Smad and non-Smad-dependent TGF-β1-mediated events by hyaluronan (26). Engagement of CD44 by hyaluronan in proximal tubular epithelial cells (PTC) attenuates TGF-β1 signaling by increasing trafficking of TGF-β receptors to non-signaling lipid raft associated pools (9).

In the current study we have demonstrated that, in addition to the negative regulation of TGF-β1 receptor function described previously (9, 26), an augmented response to TGF-β1 may also occur as the result of alteration of membrane trafficking of the receptor complex between the lipid and non-lipid-associated membrane fraction. As with our previous observations, these effects were unrelated to the binding of the TGF-β receptor and its ligand but rather related to the engagement of another membrane receptor, in this case, the cognate IL-6 receptor, by its ligand, IL-6. The results demonstrate that an
increase in the association of the TGF-β receptor complex with non-lipid raft membrane fractions is associated with reduced receptor turnover and decreased association of the receptor with Cav-1. This is consistent with the previously published hypothesis that targeting the receptor away from the Cav-1, lipid-associated membrane pool facilitates TGF-β1 signaling and reduces TGF-β1 receptor degradation (8). It has been suggested that regulation of caveolin-1 may be a critical step in modulating cellular responses to growth factors. In support of this, recent studies have demonstrated that

**FIG. 4.** Distribution of TGF-β receptors. A, TGF-β receptor distribution in lipid raft and non-raft fractions was analyzed in HK-2 cells affinity-labeled with [125I]-TGF-β1 and subjected to sucrose gradient subcellular fractionation to separate lipid rafts from other cellular components. The effect of IL-6 on trafficking of TGF-β receptors was assessed by comparison of distribution in HK-2 cells exposed to either 250 pM radiolabeled TGF-β1 alone (Control) or radiolabeled TGF-β1 in the presence of 10 ng/ml IL-6. In all experiments, subcellular fractionation was performed 24 h after addition of the stimuli. Characterization of the contribution of lipid rafts was examined by pre-treatment of cells with 50 μg/ml nystatin for 1 h at 37 °C prior to affinity labeling of TGF-β receptors (Nystatin). An equal volume from each fraction was analyzed by SDS-PAGE electrophoresis followed by autoradiography. B, following scanning densitometry of autoradiographs, the distribution of TGF-β receptor into the raft (fractions 5 and 6) and non-raft fractions (fractions 7–10) was quantified, and the data from three separate experiments are expressed graphically.

**FIG. 5.** Attenuation of TGF-β1 receptor signaling. A, sequestration of TGF-β receptor from lipid rafts. HK-2 cells were transiently transfected with the Smad-responsive promoter (SBE)-luc prior to addition of 0.1 ng/ml TGF-β1 + 10 ng/ml IL-6 as indicated for 24 h. The role of lipid rafts in Smad signaling was examined by pre-treatment of transfected cells with 50 μg/ml nystatin at 37 °C for 1 h prior to addition of TGF-β1 together with IL-6. The effect of nystatin was dependent on chelation of cholesterol, as demonstrated by the addition of nystatin to cells at 37 °C for 1 h prior to addition of TGF-β1 together with IL-6 and cholesterol (25 μg/ml). B, inhibition of endosomal internalization. HK-2 cells transfected with the reporter construct were incubated in medium (Dulbecco’s modified Eagle’s medium/Ham’s F-12)/water (1:1) for 5 min at 37 °C, followed by incubation in minimal medium (K+-depleted serum-free medium containing 20 mM HEPES, pH 7.5, 140 mM sodium chloride, 1 mM calcium chloride, 1 mM magnesium sulfate, and 5.5 mM glucose) or full medium containing 10 mM potassium chloride for 1 h at 37 °C prior to stimulation with TGF-β1 (0.1 ng/ml) + IL-6 (10 ng/ml) for 24 h. For both experimental protocols, luciferase content was quantified as described under “Materials and Methods,” and the results were normalized for transfection efficiency (using β-galactosidase) expressed as the fold increase above the non-stimulated control. The data represent the mean ± S.D. of six individual experiments.
caveolin-1 suppresses mitogen-activated protein kinase activation and cell proliferation induced by basic fibroblast growth factor and platelet-derived growth factor in mesangial cells (34). Previous work has also demonstrated that ectopic expression of Cav-1 enhances TGF-β receptor turnover (8). Although caveolin-1 may facilitate receptor turnover, it is important to note that TGF-β receptor may still partition to lipid raft membrane fractions and that TGF-β1 receptor turnover continues in the absence of Cav-1 (8). The data presented in our study support the notion that TGF-β receptor turnover may be regulated independently from that of Cav-1 because we have shown a decrease in receptor turnover in the absence of changes in caveolin-1 expression, and we propose a model whereby the interaction between distinct receptors at the cell surface orchestrates the trafficking of the receptors, which dictates receptor fate.

The receptor complex mediating the biological activities of TGF-β1 receptor was examined by immunoprecipitation of TGF-β receptors, and the association of Cav-1 to total cell lysates was examined independently from that of Cav-1 because we have shown a decrease in receptor turnover in the absence of changes in caveolin-1 expression, and we propose a model whereby the interaction between distinct receptors at the cell surface orchestrates the trafficking of the receptors, which dictates receptor fate.

**FIG. 6.** Expression of Cav-1 and association with TGF-β receptor. Growth-arrested HK-2 cells were stimulated with combinations of 0.1 ng/ml TGF-β1 and 10 ng/ml IL-6 as indicated. 24 h after stimulation, total cell lysates were prepared. Total Cav-1 expression was examined by Western analysis of cell lysates, and the association of Cav-1 to TGF-β receptor was examined by immunoprecipitation of TGF-β receptor from the cell lysates and determination of Cav-1 expression in the precipitate by Western analysis. Results of three separate experiments are shown.

**FIG. 7.** TGF-β1-dependent TGF-β receptor turnover is decreased by IL-6. Growth-arrested HK-2 cells were incubated with 250 pm TGF-β1 and 4 °C followed by cross-linking. Following incubation in either serum-free medium alone, 10 ng/ml IL-6, or 50 μg/ml nystatin as indicated at 37 °C for the indicated times, cells were lysed and processed for SDS-PAGE, and endogenous receptors were visualized by autoradiography (A). Three separate experiments were performed, quantified by scanning densitometry of autoradiographs, and graphed as receptor quantity (percentage of time 0) versus time (B). Each point represents mean ± S.D. ○, control; ●, IL-6; ■, nystatin; *, p < 0.05, nystatin versus control; #, p < 0.05, IL-6 versus control.
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