Converging signals synergistically activate the LAMC2 promoter and lead to accumulation of the laminin γ2 chain in human colon carcinoma cells

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The trimeric extracellular matrix molecule laminin-5 and its constituent chains (α3, β3, γ2) are normally not detectable intracellularly in intestinal epithelial cells but the laminin γ2 chain can be detected in cancer cells at the invasive front of a subset of colon carcinomas. These cells are subjected to cytokines such as transforming growth factor β1 (TGF-β1) and hepatocyte growth factor (HGF), produced by the tumour cells or by the surrounding stromal cells. The purpose of the present work was to investigate whether TGF-β1 and HGF, known to stimulate the LAMC2 gene encoding the laminin γ2 chain, might synergize to activate the LAMC2 promoter, and to identify the promoter elements involved. We find evidence for synergy between TGF-β and HGF with respect to laminin γ2 chain expression and promoter activation and demonstrate that this requires the 5′ activator protein-1 (AP-1) element of the promoter and an additional upstream element which is also responsive to co-expression of the Smad3 protein from the TGF-β signalling pathway. The transcripts encoding the other laminin-5 chains are not synergistically activated by HGF and TGF-β. Thus the synergistic activation of the LAMC2 gene is mediated via different *cis*-elements and results in an overproduction of the laminin γ2 chain relative to the other laminin-5 constituent chains. This difference may explain why laminin γ2 chains accumulate in the cells at the invasive front of colon carcinomas.

Key words: cellular interaction, hepatocyte growth factor, laminin-5, promoter regulation, transforming growth factor β.

INTRODUCTION

Extracellular matrix proteins play a crucial role in the development and maintenance of the gastrointestinal mucosa. During the development of the intestinal mucosa, the extracellular matrix is deposited by the apposed epithelial and stromal cells, resulting in the generation of a basement membrane which separates the two cellular compartments (for reviews see [1,2]). The basement membrane signals to both epithelial and stromal cells via integrin receptors [3,4]. The signalling is an integrated part of mesenchymal and epithelial interactions also involving soluble cytokines such as hepatocyte growth factor (HGF), secreted by the mesenchymal cells [5], and transforming growth factor β (TGF-β), expressed by both the epithelial cells [6] and mesenchymal cells [7]. The degradation of the basement membrane depends also on mesenchymal and epithelial interactions as exemplified by the reciprocal expression of the urokinase-type plasminogen activator (uPA) in stromal cells [8] positioned beneath uPA-receptor-expressing epithelial cells that are about to be shed from the epithelium [9]. During invasion of colorectal cancer cells, changes in the epithelial and mesenchymal interactions contribute to the triggering of matrix degradation [10] and the induction of tumour cell migration [11]. The laminin γ2 chain of the heterotrimeric laminin-5 (α3β3γ2) basement membrane molecule has been identified as a marker of a subset of invasive colorectal carcinoma cells [12,13]. This may have important pathophysiological consequences, as laminin-5 and proteolytically processed forms thereof are known to promote migration of a multitude of epithelial cells [14,15]. Recently it was suggested that the high expression of the laminin γ2 chain in invasive colon carcinoma cells is due to synergistic activation of the LAMC2 gene, which encodes the laminin γ2 chain, by convergence of HGF and WNT (wingless-type murine mammary tumour virus integration-site family member) signalling pathways [16]. HGF activates the LAMC2 promoter via an activator protein-1 (AP-1) site close to the transcription-initiation site [17] and WNT signals activate the promoter via β-catenin and transcription factor 4 (TCF-4) complexes binding to two sites placed upstream and downstream of the 5′ AP-1 site, respectively [16]. TGF-βs (TGF-β1, -β2 and -β3) belong to another class of cytokines which stimulate laminin-5 expression [18,19] and promote migration of intestinal epithelial cells [7]. Moreover, TGF-βs are produced as latent forms which might undergo activation by the plasmin activity which is directed towards the interface between invading carcinoma cells and surrounding stromal cells by the uPA receptor expressed by both stromal and colon carcinoma cells [20]. TGF-βs signal mainly via intracellular so-called Smad proteins. Receptor Smads (Smad2 and 3) are phosphorylated by the serine/threonine kinase activity of the TGF-β type I receptor and subsequently form homo-oligomers and hetero-oligomeric complexes with each other and hetero-oligomeric complexes with the co-mediator Smad4, resulting in nuclear translocation of the complexes (for reviews see [21–23]). The nuclear Smad protein complexes function as sequence-specific

Abbreviations used: AP-1, activator protein-1; APC, adenomatous polyposis coli; CBP, cAMP-response-element-binding protein-binding protein; EMSA, electrophoretic mobility shift assay; HGF, hepatocyte growth factor; LAMA3, gene encoding the laminin α3 chain; LAMB3, gene encoding the laminin β3 chain; LAMC2, gene encoding the laminin γ2 chain; MAP kinase, mitogen-activated protein kinase; TCF-4, transcription factor 4; TGF-β, transforming growth factor β; uPA, urokinase-type plasminogen activator; WNT, wingless-type murine mammary tumour virus integration-site family member.

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DNA-binding transcriptional activators [24,25], as co-activators binding to other DNA-binding transcription factors such as the AP-1 factor [26] and as co-activators binding to the cAMP-response-element-binding protein ('CREB')-binding protein (CBP)/p300 co-activator proteins [27,28]. TGF-β signalling strongly inhibits growth of epithelial cells [29], including intestinal epithelial cells [7]. Colorectal cancer cells are, in contrast, often resistant to the growth-inhibitory effect of TGF-β, which depends on the presence of Smad4 [30]. Moreover, mutations in the Smad4 gene are often found in colorectal cancers [31]. In parallel with the Smad signal transduction pathway, TGF-β can also rapidly activate the Ras and mitogen-activated protein kinase (MAP kinase) pathway, culminating in the generation of mainly JunD/Fra-2 AP-1 heterodimers (for a review see [32]). Thus, whereas TGF-β is known to stimulate the LAMC2 promoter [17], it is currently not known through which elements in the LAMC2 promoter the TGF-β stimulation occurs, whether the signalling depends on the Smad proteins, the AP-1 proteins or both, and how the signalling is integrated on the LAMC2 promoter in invasive carcinoma cells where several signalling pathways converge on the promoter. It is the purpose of the present work to investigate whether HGF and TGF-β synergize in activating the LAMC2 promoter and to identify the *cis*-elements involved.

MATERIALS AND METHODS

Construction of LAMC2 deletions and site-directed mutagenesis

We have described previously the construction by PCR of the -1.2, -0.35, -0.15 and -0.12 kb LAMC2/luciferase constructs [17]. The -0.28 kb (-278 bp) and -0.21 kb (-212 bp) LAMC2/luciferase deletion constructs were constructed by PCR using a similar approach. For construction of point mutations, oligonucleotides were designed to have an *Nhe*I restriction site as the specific mutation, flanked by 12–15 nucleotides of the surrounding wild-type sequence on either site. The mutations were introduced into the 1.2 kb LAMC2/luciferase construct using the GeneEditor system (Promega, Madison, WI, U.S.A.) following the manufacturer's recommendations.

Cell culture, transfections and reporter gene measurements

HT29mtx cells [33] were cultured in minimal essential medium with Earle's salts (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 10 % (v/v) heat-inactivated fetal calf serum and antibiotics (100 i.u./ml penicillin, 100 i.u./ml streptomycin and 40 μg/ml gentamycin).

For transfections, exponentially growing cells were seeded (3×10^4 cells/well) in 96-well culture plates (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) and cultured for 24 h before transfection. For each well the LAMC2/luciferase construct (32 fmol), transfection control construct (SEAP-control; Clontech, Palo Alto, CA, U.S.A.), filler DNA (pBluescript KS+; Stratagene, La Jolla, CA, U.S.A.) and in some cases Smad3/4 expression vectors were mixed to give a total of 0.5 μg of DNA. The transfection was carried out using the Exgen500 (Fermentas, Vilnius, Lithuania) polyethylenimine transfection reagent (1.25 μl/well) according to the manufacturer's recommendations. When used, TGF-β1 and HGF were added 24 h after DNA/Exgen500 addition to final concentrations of 10 and 50 ng/ml respectively. Following transfection (48 h) the medium was collected, and the cells washed once in PBS and lysed with 20 μl of luciferase lysis buffer (Promega). Luciferase activity was determined by injecting 100 μl of substrate solution (Promega) directly into the wells followed by measurements of luminiscense using the Ascent FL luminometer (Labsystems, Finland). The SEAP-control plasmid directs the synthesis of a secreted form of the human placental alkaline phosphatase [34] and was used to correct for variations in transfection efficiency. Measurement of secretory alkaline phosphatase activity was performed using the chemiluminescent Great Escape SEAP system (Clontech) according to the manufacturer's recommendations.

Design of PCR primers and verification of reverse transcriptase-PCR product identity

Primer pairs for the LAMA3 (the gene encoding the laminin α3 chain), LAMB3 (the gene encoding the laminin β3 chain) and LAMC2 transcripts were planned using the nucleotide sequences taken from the Refseq database: LAMA3/NM_000227, LAMB3/NM_000228 and LAMC2/NM_005562. The sequences of the primers were as follows. LAMA3 (305 bp product), 5′-GGGACTACATCGGCATGGCA-3′ and 5′-AAAATCAGGTGGGTAACCTCCA-3′; LAMB3 (263 bp product), 5′-ACCTGACAGGACTGGAGAAGCG-3′ and 5′-ATTGGCTCAGGCTCAGCTGC-3′; LAMC2 (340 bp product), 5′-GTATGTGAACCCACAACCCACAA-3′ and 5′-TGTCCACTGGCTTCTCAGGGT-3′.

The primer pairs were used in reverse transcriptase-PCR reactions using the Access RT-PCR system (Promega) with 0.5 μg of HT29mtx RNA as a template, following the manufacturer's recommendations. PCR products were gel-purified and cloned into the pCR2.1-TOPO vector using the topoisomerase cloning system (Invitrogen). The cloned PCR products were sequenced to verify the identity of the inserts.

Expression analysis by real-time PCR

HT29mtx cells were seeded into a total of 16 wells on a 24-well culture plate at 5×10^4 cells/well. After 24 h of culture four wells were left untreated. TGF-β1 was added to each of four wells (10 ng/ml total concentration), HGF was added to each of four wells (50 ng/ml total concentration) and to the remaining four wells TGF-β1 (10 ng/ml) and HGF (50 ng/ml) were added simultaneously. After 20 h in the presence of cytokines, total RNA was extracted from the cells in each well using a total RNA isolation kit (Sigma, St. Louis, MO, U.S.A.). The RNA concentration was determined with a fluorometric procedure using the fluorescent dye RiboGreen according to the manufacturer's instructions (Molecular Probes, Eugene, OR, U.S.A.). The fluorescence was determined using an Ascent FL fluorometer (Labsystems) with a 485 nm excitation filter and a 525 nm emission filter.

Total RNA (1 μg) was used for first-strand cDNA synthesis in a 20 μl reaction containing 50 mM Tris/HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 500 μM each of dATP, dCTP, dGTP and dTTP, 0.5 μg of oligo$(dT)_{17}$ and 10 units of Superscript reverse transcriptase (Invitrogen). The reaction was incubated at 42 °C for 1 h.

For real-time PCR, the LightCycler DNA master SYBR green I system (Roche, Mannheim, Germany) was used. Reactions were assembled in LightCycler capillary tubes (Roche) and 1 μl of first-strand cDNA was used as the template. For standardization, 1 μl serial dilutions of the cloned PCR products were used as templates in separate reactions. The optimal $MgCl_2$ concentrations were determined to be 3 mM for the LAMA3 PCR product and 4 mM for the LAMB3 and LAMC2 PCR products. The standard amplification programme (Roche) of denaturation at 95 °C, annealing at 55 °C and extension at 72 °C was used. The quantification was done using the second derivative method option in the LightCycler software (Roche).

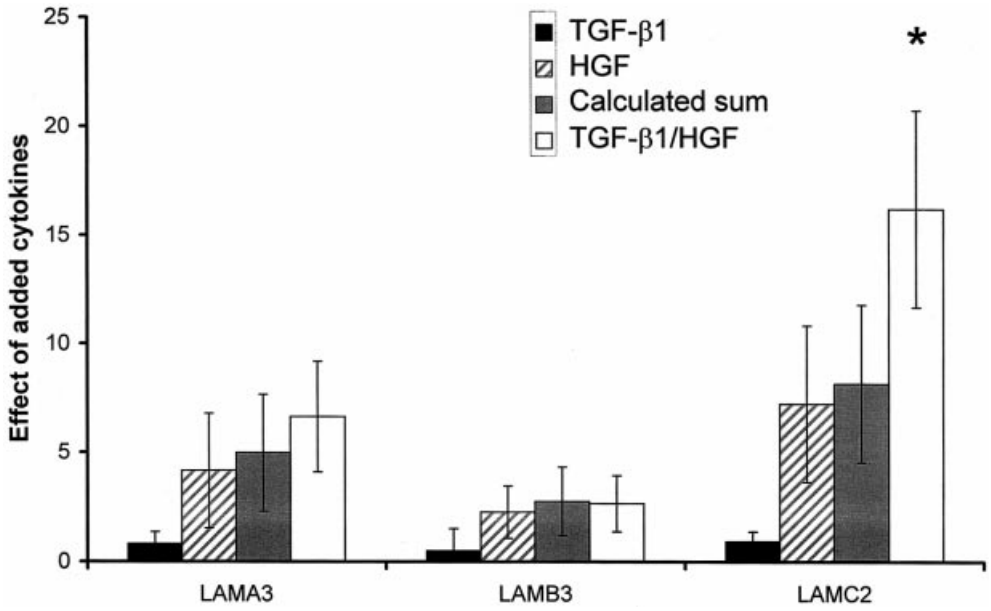

Figure 1 HGF and TGF-β1 synergistically stimulate the expression of endogenous laminin γ2 mRNA

HT29mtx cells were left untreated or cultured in the presence of TGF-β1, HGF or both cytokines simultaneously (TGF-β1/HGF). RNA was extracted, and first-strand cDNA produced and used as a template in individual PCR reactions with primer pairs directed against portions of the transcripts from the LAMA3, LAMB3 and LAMC2 genes. The PCR reaction was carried out using the LightCycler real-time PCR system. The copy number of each transcript in the sample was measured using DNA standards and the fold stimulation relative to the untreated condition was calculated. From the fold stimulation, the effect was calculated as the difference in fold stimulation with and without cytokine additions. The sum of the effects of TGF-β1 and HGF could then be calculated and plotted along with the effects of the cytokines added individually or together. Only for the LAMC2 transcript was the effect of the cytokines added simultaneously significantly higher ($^*P < 0.05$) than the calculated sum of the individual treatments. Thus TGF-β1 and HGF synergize in stimulating the expression of the LAMC2 transcript. Four independent experiments were carried out for each gene and each treatment.

Immunocytochemistry

HT29mtx cells were seeded (1×10^5 cells/chamber) in chamber slides (Invitrogen) and cultured for 24 h. The cells were then either left untreated or treated with TGF-β1 (10 ng/ml), HGF (50 ng/ml) or both for 20 h. The medium was removed, and the cells washed twice in PBS (room temperature) and fixed in PBS containing paraformaldehyde (3.7% v/v) at 4 °C for 10 min. The fixative was removed and the cells washed three times for 2 min in water and air dried. The cells were permeabilized by saponin (0.05%) at 4 °C for 10 min. The slides were incubated with an anti-γ2-chain mouse monoclonal antibody (MAB19562; Chemicon, Temecula, CA, U.S.A.) at a 1:50 dilution in PBS plus 0.05% saponin at 4 °C overnight. Following three washes in PBS plus 0.05% saponin the slides were incubated with a FITC-conjugated rabbit anti-mouse polyclonal antibody (Dako, Copenhagen, Denmark) for 1 h at room temperature, washed three times in PBS and mounted with an anti-fading solution.

Metabolic labelling of HT29mtx cells: laminin-5 immunoprecipitation

HT29mtx cells seeded at high density (1×10^6 cells/12 cm^2) were cultured for 1 day in Dulbecco's modified Eagle's medium containing 10% inactivated fetal calf serum. Cells were then radiolabelled for 24 h with 100 μCi/ml trans-^{35}S-label™ metabolic labelling reagent containing [^{35}S]L-methionine and [^{35}S]L-cysteine (ICN Biomedicals, Orsay, France) in methionine/cysteine-free Dulbecco's modified Eagle's medium medium containing 2% fetal calf serum. The cytokines were added alone or in combination at final concentrations of 10 ng/ml for TGF-β and 50 ng/ml for HGF. Then, 24 h later, the cells were scraped and ground into a small volume of RIPA buffer (radioimmunoprecipitation assay buffer; 10 mM Tris/HCl, pH 7.4, 2 mM EDTA, 2 mM cysteine, 2 mM methionine, 250 μM PMSF, 1 mM *N*-ethylmaleimide, 0.5% Nonidet P-40, 0.1% SDS, 0.05% Triton X100, 0.3% sodium desoxycholate, 0.1% BSA and 150 mM NaCl). The cell homogenates were centrifuged at 330 ***g*** for 10 min.

For immunoprecipitation, samples were first preincubated with Protein G–Sepharose beads (Sigma) for 1 h at 4 °C. In parallel, the monoclonal anti-laminin-5 antibodies (MAB1947; Chemicon) were incubated with Protein G–Sepharose beads for 1 h at 37 °C. The pellets containing the antibodies bound to the beads were washed twice with RIPA buffer and incubated with the precleared samples for 18 h at 4 °C. Immune complexes were washed five times with RIPA buffer and resuspended in Laemmli buffer. Pellets were boiled and subjected to SDS/PAGE (5%) gels under non-reducing conditions. The gels were then dried and exposed for 1–2 days at −80 °C to X-ray film (Kodak, U.S.A.) using an intensifying screen (Biomax, Transcreen LE; Kodak, Rochester, NY, U.S.A.). Gels were calibrated with ^{14}C-labelled protein standards (Amersham Biosciences, Little Chalfont, Bucks., U.K.).

Electrophoretic mobility shift assays (EMSAs)

Nuclear extracts were prepared from untreated and TGF-β1- and HGF-treated cells as described previously [17]. EMSA was performed as described in [35]. We used 8 fmol of 5′ AP-1/Sp1 probe [17] end-labelled with [γ-^{32}P]ATP (4000 Ci/mmol), 500 ng

of poly(dI-dC), 100 ng of sonicated salmon sperm DNA and 4 μg of protein extracted from HT29mtx nuclei. For the reactions where antibodies against JunD (sc-74; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or Fra-2 (sc-604; Santa Cruz Biotechnology) were added, 1 μg of IgG was used.

Statistical calculations

Student's t tests were performed according to the principles outlined in [36]. For evaluation of the synergistic effect, the reporter gene measurements for each construct were first normalized to the average activity measured for the construct in question without addition of cytokines. The effect was calculated by subtracting the normalized reporter gene activity without cytokine addition from the normalized reporter gene activity with cytokine addition. The variances for the effects were calculated as the sums of the individual variances. The sum of the effects of the individual TGF-β1 and HGF treatments could then be calculated and their variances likewise. The difference between the sum of the individual treatments and the treatment with TGF-β1 and HGF simultaneously was calculated together with its confidence interval. In case of synergy this difference should be statistically significantly greater than zero. When more than three different constructs were tested a significance level of 1 % was chosen to take into account the effect of multiple testing; otherwise a 5 % significance level was used.

RESULTS

Simultaneous treatment of HT29mtx cells with HGF and TGF-β1 results in synergistic activation of the LAMC2 promoter and intracellular accumulation of the laminin γ2 chain

We chose to use the HT29mtx cell line [33] for the present work because it expresses laminin-5 and the LAMC2 promoter displays responsiveness to HGF and TGF-β in these cells [17]. Moreover, the parental HT29 cell line from which the HT29mtx cell line originates contains a mutation in both alleles of the adenomatous polyposis coli (APC) gene, resulting in C-terminally truncated proteins [37], whereas β-catenin is in its wild-type form [38]. These cells therefore have constitutively high activity in the β-catenin/TCF-4 pathway [39]. The HT29mtx cells were cultured in the absence or presence of TGF-β1, HGF or both cytokines simultaneously. The expression of mRNA transcripts from the three genes LAMA3, LAMB3 and LAMC2, encoding the α3, β3 and γ2 chains of laminin-5 respectively, were measured by real-time PCR and the effect of cytokine addition was calculated. The definition of the effect of a treatment is the difference between the activities before and after the treatment. We therefore calculated the effects of the treatments as the difference between the fold stimulation with addition of cytokines and the fold stimulation without cytokine addition. As seen in Figure 1, both TGF-β1 and HGF increased the amounts of all three individual mRNAs and HGF was the more potent of the two cytokines. We next wanted to evaluate whether the observed difference reflected a synergistic effect of HGF and TGF-β1 when added simultaneously, and therefore we calculated the sum of the effects (Figure 1). For the LAMC2 transcript the sum of the effects from the addition of the cytokines individually was significantly lower ($P < 0.05$) than the effect when both cytokines were added simultaneously. Thus TGF-β1 and HGF stimulate synergistically the production of the endogenous LAMC2 transcript in HT29mtx cells. This is not the case, however, for the LAMA3 and the LAMB3 transcripts (Figure 1).

At the protein level, culture of HT29mtx cells in the presence of TGF-β1 alone or in combination with HGF led to a slight increase in the cellular pool of laminin-5 as compared with the control situation without cytokine or with HGF treatment (Figure 2). As far as the secreted laminin-5 is concerned, no major differences were observed between the different cytokine treatments (results not shown). Thus there is no evidence for a synergistic effect of TGF-β1 and HGF on the production of the trimeric laminin-5 molecule in HT29mtx cells. We then analysed the expression of intracellular laminin γ2 using a monoclonal antibody. As seen in Figure 2, only trace amounts of the laminin γ2 chain could be detected in HT29mtx cells cultured with or without TGF-β1 or HGF. Clear intracellular staining

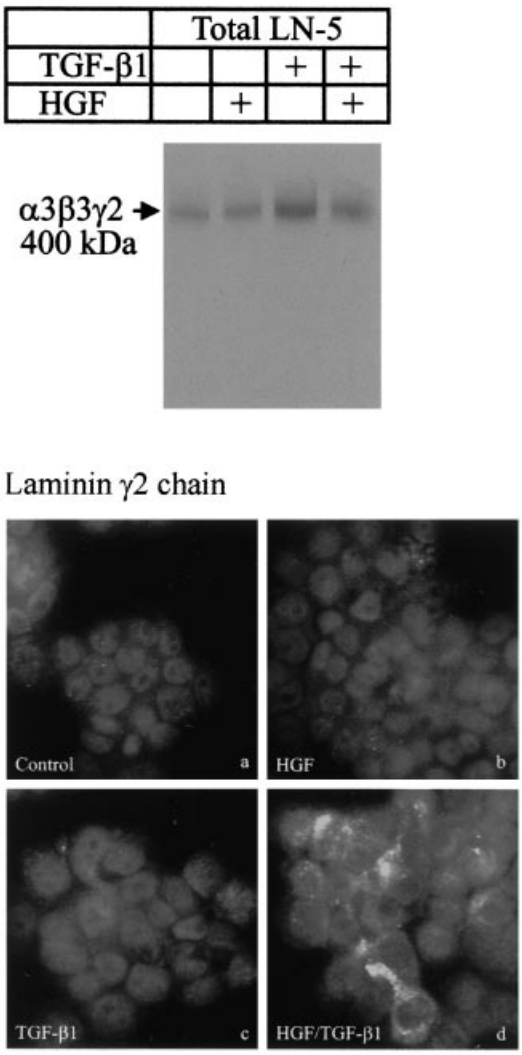

Figure 2 The laminin γ2 chain, but not total laminin-5, accumulates in HGF/TGF-β1-treated HT29mtx cells

Upper panel: after a 24 h metabolic labelling period, cell lysates from HT29mtx cells cultured in standard medium or in medium containing HGF (50 ng/ml), TGFβ1 (10 ng/ml) or both were immunoprecipitated with the anti-laminin-5 monoclonal antibody. The immunoprecipitated materials were analysed by SDS/PAGE on a 5% acrylamide gel under non-reducing conditions. The radioactive bands were revealed by fluorography and corresponded to the native $\approx$ 400 kDa $\alpha3\beta3\gamma2$ trimeric laminin-5 (LN-5) protein. Lower panel: in a separate experiment HT29mtx cells were cultured on coverslips and left untreated (**a**) or treated with HGF (**b**), TGF-β1 (**c**) or TGF-β1/HGF (**d**). The cells were fixed and incubated with a monoclonal antibody raised against the human laminin γ2 chain. Bound antibody was detected using an FITC-conjugated secondary antibody. Only following simultaneous addition of TGF-β1 and HGF (**d**) could the laminin γ2 chain be detected intracellularly in HT29mtx cells.

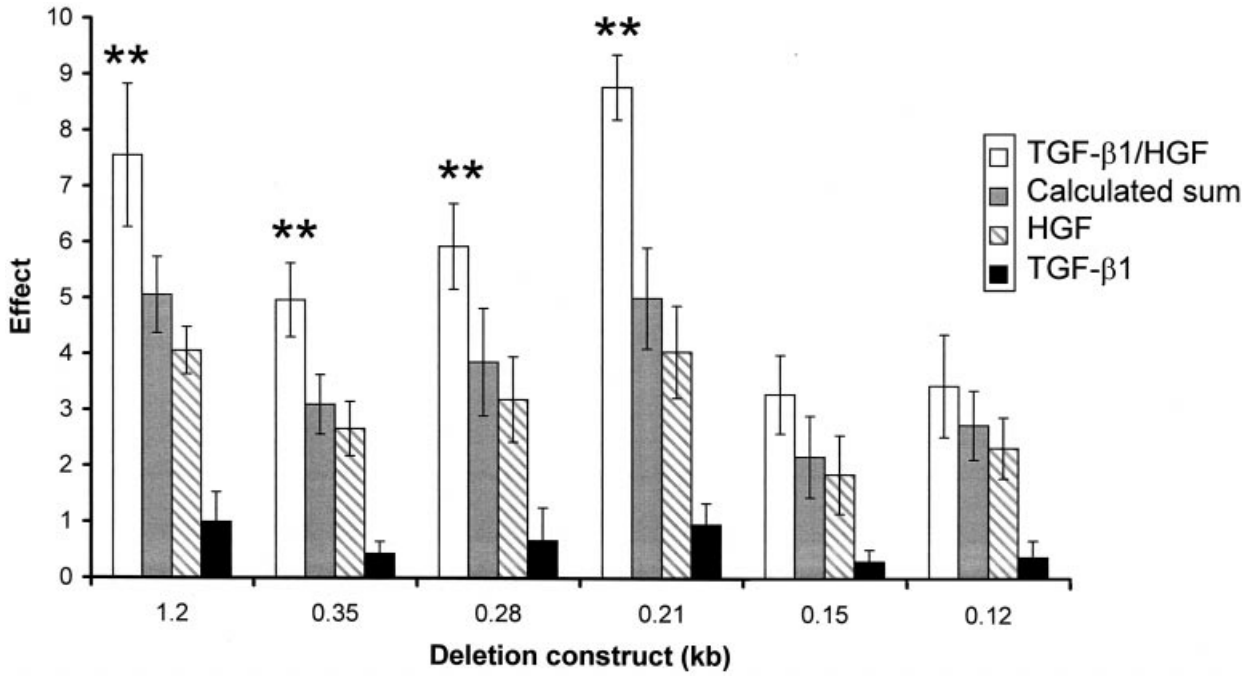

Figure 3 Synergistic activation of the LAMC2 promoter by TGF-β1 and HGF

HT29mtx cells were transfected with the indicated deletion construct of the LAMC2 5′-flanking region placed in front of the luciferase gene. Cytokines TGF-β1 and HGF were added separately or together followed by reporter gene measurements. The effect of the cytokines on reporter gene expression was calculated as the difference in fold stimulation with and without cytokine addition for each construct. The sum of the effects following the addition of the cytokines individually was also calculated. S.D.s are indicated by error bars. Only the longer constructs displayed a significantly higher (**$P < 0.01$) effect of both cytokines added simultaneously compared with the sum of the individual treatments. These constructs thus display a synergistic response to the simultaneous addition of TGF-β1 and HGF.

for the laminin γ2 chain was, however, detected in cells cultured in the presence of both cytokines. Thus simultaneous stimulation with TGF-β1 and HGF leads to a situation very similar to that reported for invading colon carcinoma cells, where clear laminin γ2 staining is found intracellularly [12,13].

TGF-β stimulation of the LAMC2 promoter occurs through the 5′ AP-1 element, but synergy with HGF requires additional upstream sites

A series of deletion contructs with fragments of the LAMC2 upstream region placed in front of the luciferase gene were transfected into HT29mtx cells followed by cytokine addition. The effect of the cytokines on reporter gene expression was calculated as the difference in fold stimulation with and without cytokine addition. Thus when a cytokine does not affect reporter-gene activity, the effect will be zero. The sum of the effects observed with the cytokines added individually was calculated and plotted together with the measured effects of the different treatments (Figure 3). TGF-β1 addition resulted in a significant effect on all constructs (Figure 3; $P < 0.05$). The effect obtained with the -1.2 kb construct was 1-fold, which is comparable with the effect on the endogenous LAMC2 mRNA expression following TGF-β1 addition (Figure 1). When both TGF-β1 and HGF were added simultaneously the effect was significantly greater than the sum of the individual treatments ($P < 0.01$) on the promoter activity from the four longest deletion constructs only. Thus these constructs are stimulated synergistically by HGF and TGF-β1. There was, however, no synergy between TGF-β1 and HGF on the -0.15 kb and -0.12 kb deletion constructs (Figure 3).

We have shown previously that the LAMC2 promoter contains two AP-1 sites within the first 120 bp upstream of the transcription-initiation site and that the most 5′ AP-1 site overlaps with a binding site for Sp1 [17]. This 5′ AP-1 site is crucial for both the basal activity of the promoter and the HGF responsiveness of the promoter [17]. Mutations of the three sites were introduced into the -1.2 kb construct. As seen in Figure 4, mutation of the 5′ AP-1 site resulted in a dramatic 10-fold drop in promoter activity. Moreover, mutation of the 5′ AP-1 site abolished TGF-β1 responsiveness. Mutation of the 3′ AP-1 site alone also reduced promoter activity, but to a lesser extent, and this construct still displayed significant ($P < 0.01$) TGF-β1 responsiveness. The double mutant, containing both the 5′ and 3′ AP-1 sites mutated, was also unresponsive to TGF-β1 stimulation. Mutation of the Sp1 site, which overlaps with the 3′ part of the 5′ AP-1 site, increased the promoter activity, which confirms our previous prediction that Sp1 and AP-1 proteins compete for binding to this region. It is also clear, however, that the Sp1-binding site plays no part in the TGF-β1 response as mutation of this site does not affect the TGF-β1 responsiveness. Thus mutation of the 5′ AP-1 site in the context of the long -1.2 kb deletion construct renders this construct unresponsive to TGF-β1. The -0.15 kb deletion, on the other hand, contains the intact 5′ AP-1 site and is responsive to TGF-β1 but is not synergistically stimulated by TGF-β1 and HGF (Figure 3). From this it can be concluded that the 5′ AP-1 site is essential for the TGF-β1 responsiveness of the promoter, but it is not sufficient for synergy with HGF stimulation.

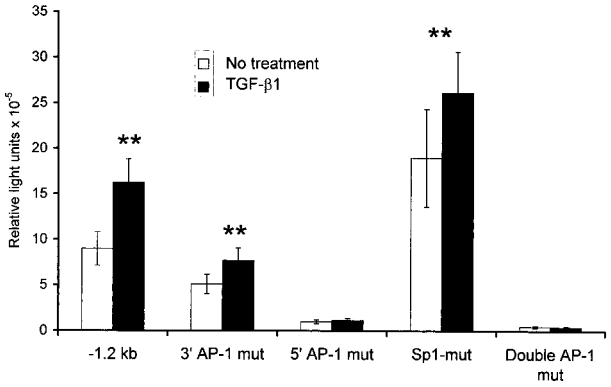

Figure 4 Mutational analysis of the LAMC2 promoter TGF-β1 response

HT29mtx cells were transfected with the 1.2 kb LAMC2 5′-flanking region placed in front of the luciferase gene or with versions thereof carrying mutations (mut) in the 5′ AP-1 site, the 3′ AP-1 site, the Sp1 site in the 5′ AP-1/Sp1 region or a double mutation of the AP-1 sites. The cells were cultured in the presence or absence of TGF-β1 followed by luciferase measurements. The measured relative light units are indicated together with S.D. Student's *t* tests were carried out to evaluate the significance of the effect of TGF-β1 treatment for each construct. Constructs demonstrating significant stimulation (**$P < 0.01$) are indicated.

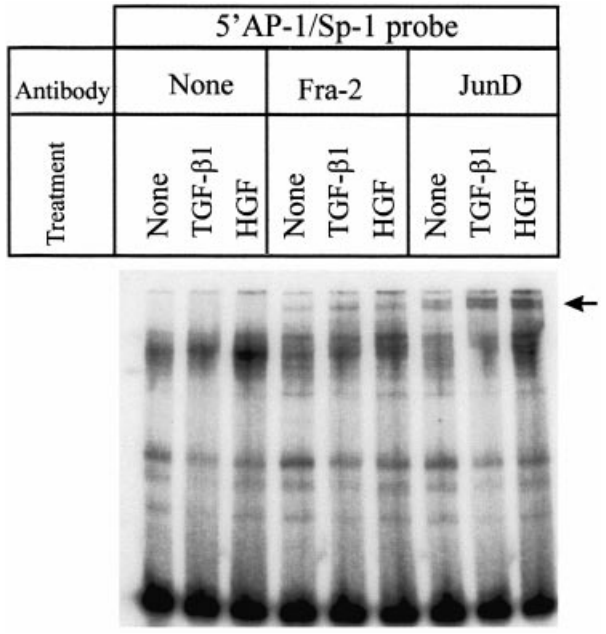

Figure 5 JunD and Fra-2 proteins bind to the 5′ AP-1 site following TGF-β1 treatment

Nuclear extracts (4 μg) were prepared from HT29mtx cells which had been cultured for 4 h with or without the addition of TGF-β1 or HGF and used together with a double-stranded oligonucleotide spanning the LAMC2 5′ AP-1/Sp1 region (see Figure 8A) for EMSA analysis. The arrow indicates supershifted bands following incubation with antibodies against Fra-2 and JunD.

During HGF stimulation, JunD-containing AP-1 dimers are the dominating species binding to the 5′ AP-1 site [17]. A screening (results not shown) using a panel of Jun, Fos and Fra antibodies in combination with EMSA suggested that JunD- and Fra-2-containing dimers are also the dominating AP-1 dimers that bind to the 5′ AP-1/Sp1 region during TGF-β1 stimulation. As seen in Figure 5, Fra-2 can be detected as a protein binding to the 5′ AP-1/Sp1 probe irrespective of the addition of either of the two cytokines, but it was consistently observed that the supershifted band (Figure 5, arrow) was strongest with nuclear extracts from TGF-β1- and HGF-stimulated cells. With JunD antibodies the supershifted band was strongest with nuclear extracts from HGF-stimulated cells, but the supershifted band with extracts from TGF-β1-stimulated cells was also stronger than the band observed with nuclear extracts from untreated cells. These results suggest that TGF-β1 stimulation induces the binding of JunD/Fra-2 heterodimers and probably JunD homodimers to the 5′ AP-1 site.

Smad3 and Smad4 activate the LAMC2 promoter via two different upstream regions

Co-transfection of expression plasmids for Smad3 and Smad4, which are important mediators of TGF-β signalling, resulted in stimulation of the basal activity from the −1.2 kb LAMC2 promoter construct (Figure 6). The stimulation was strongest when the expression vectors were added separately; the TGF-β1 response diminished when the Smad3 and Smad4 expression vectors were co-transfected simultaneously ($P < 0.01$). This argues against a role for Smad3/4 heterodimers in the TGF-β response of the promoter and suggests a role for Smad3 or Smad4 acting individually as co-activators. This can be accomplished if Smad homo-oligomers can be recruited to the LAMC2 promoter by other DNA-binding transcription factors. To investigate this, a deletion analysis was carried out. Because Smad3 and Smad4 acted in a similar fashion in the co-transfection experiment (Figure 6) and since Smad4 is often inactivated in colorectal carcinoma cells, whereas intact Smad3 expression is most frequently maintained [31], we decided to focus on the Smad3 protein in the following experiments. As seen in Figure 7, the response to Smad3 co-transfection declined in two discrete steps. There was a 1.4-fold drop ($P < 0.05$) in response between the −1.2 kb and the −0.35 kb constructs and another almost 1.4-fold drop ($P < 0.05$) in the response between the −0.21 kb and the −0.15 kb constructs. The −0.12 kb construct, which harbours the two AP-1 sites, was not significantly stimulated by Smad3 co-transfection. Thus the −1.2 kb promoter construct contains at least two Smad3-responsive elements. One of these is located within the minimal region between positions −0.21 kb and −0.15 kb, which maintains synergistic responsiveness to HGF and TGF-β1.

Synergy between TGF-β1 and HGF stimulation requires a Smad3-responsive element located between positions −216 and −169

In the preceding experiments we have demonstrated that the region between positions −0.21 kb and −0.15 kb is required for synergy between HGF and TGF-β1 stimulation of the LAMC2 promoter and that this region contains at least one Smad3-responsive element. We therefore wanted to investigate if this synergy and the Smad3 response mapped to the same or to different elements within the −0.21 kb to −0.15 kb region. In our previous work on the LAMC2 promoter we have published a DNase I footprint of a LAMC2 promoter fragment including this region [17] and indeed there is a region between positions −216 and −169 which is protected on the lower strand only with nuclear extracts from HT29mtx cells. We therefore focused on this region and introduced seven mutations into it by site-

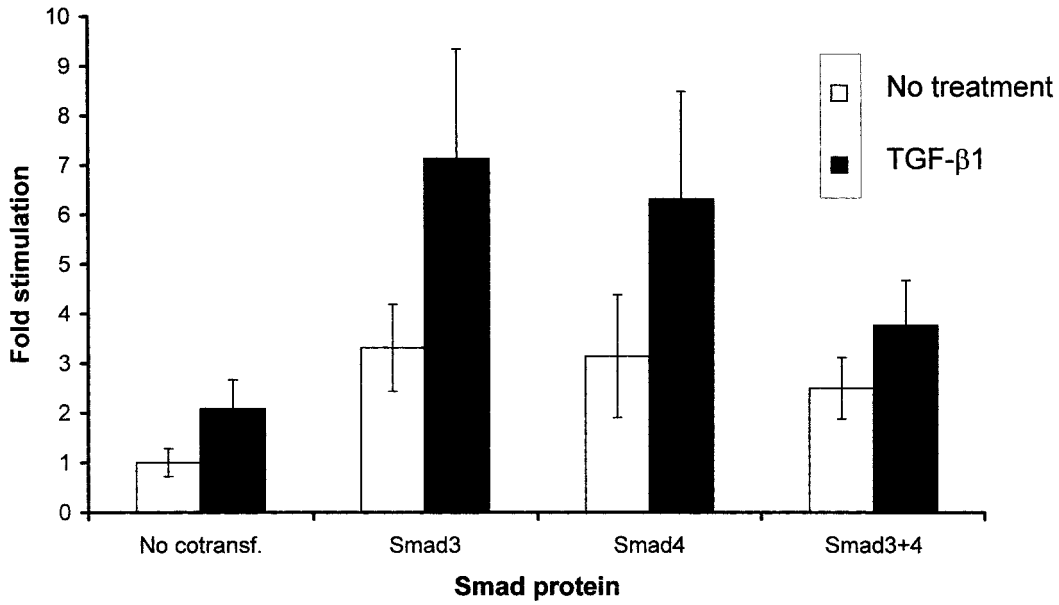

Figure 6 Smad3 and Smad4 stimulate the LAMC2 promoter

HT29mtx cells were transfected with 1.2 kb of the LAMC2 5′-flanking region placed in front of the luciferase gene without or with co-transfection of expression vectors for Smad3 or Smad4. For each construct two experimental conditions were carried out, one with and one without TGF-β1 addition. The results are normalized to the average activity obtained with the 1.2 kb construct without co-transfection or TGF-β1 stimulation. For each condition eight independent experiments were performed.

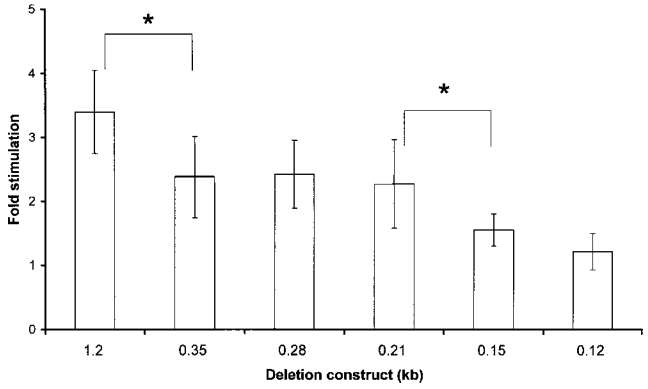

Figure 7 Deletion analysis of the Smad3 response of the LAMC2 promoter

The indicated deletion constructs of the LAMC2 5′ flanking region were co-transfected together with an expression vector for Smad3. The reporter gene luciferase activity is normalized to the average activity obtained for each construct without co-transfection and expressed as fold stimulation. The S.D.s are shown as error bars. Eight independent experiments were performed for each condition. The decreases in activity (indicated by brackets) occurring between the −1.2 and −0.35 kb and the − 0.21 and −0.15 kb constructs were statistically significant ($^*P < 0.05$).

directed mutagenesis (Figure 8A) in the background of the −1.2 kb construct. As seen in Figure 8(B) the response to Smad3 co-transfection was significantly diminished for the MUT.3, MUT.5 and MUT.7 constructs. The strongest effect was observed for the MUT.5 construct, which displayed a 50% reduction in co-transfection with the Smad3 expression vector. Moreover, the same three constructs (MUT.3, MUT.5 and MUT.7) all failed to mediate the synergy of the simultaneous addition of TGF-β1 and HGF (Figure 8C). Thus the Smad3 responsiveness in the −0.21 to −0.15 kb region correlates with the synergistic response to TGF-β1 and HGF.

DISCUSSION

The laminin γ2 chain has been proven to be important due to its described potential implication in cell migration (for a review see [40]). Yet, regulatory elements that control basal and cytokine-activated transcriptional expression of the LAMC2 gene, which encodes the laminin γ2 chain, have so far only been studied a little [16,17]. Our previous study points to a positive effect of HGF or TGF-β1 on the LAMC2 promoter [17]. We show in the present paper that these two cytokines exert their effects at different sites in the LAMC2 promoter and act in a synergistic manner via a Smad3-responsive element (see Figure 9).

We have already shown the crucial importance of the 5′ AP-1 site in the activation of the LAMC2 promoter by HGF, a scattering factor implicated in cell migration [17]. Here we provide evidence that this site is also implicated in the TGF-β1 effect on the transcriptional activity of the LAMC2 promoter. Interestingly, both treatments result in virtually the same increase in JunD-containing AP-1 dimers binding to the 5′ AP-1 site. Smad3/4 are normally involved in the TGF-β signalling pathway and have been shown to interact directly with Jun and Fos proteins [24,26]. However, we did not find any strong evidence that such interactions are important for the TGF-β1 response of the LAMC2 promoter. The −0.12 kb construct displays only a weak and not statistically significant response to co-transfection with a Smad3 expression vector and, moreover, mutations of both AP-1 sites in the promoter do not lead to a decreased

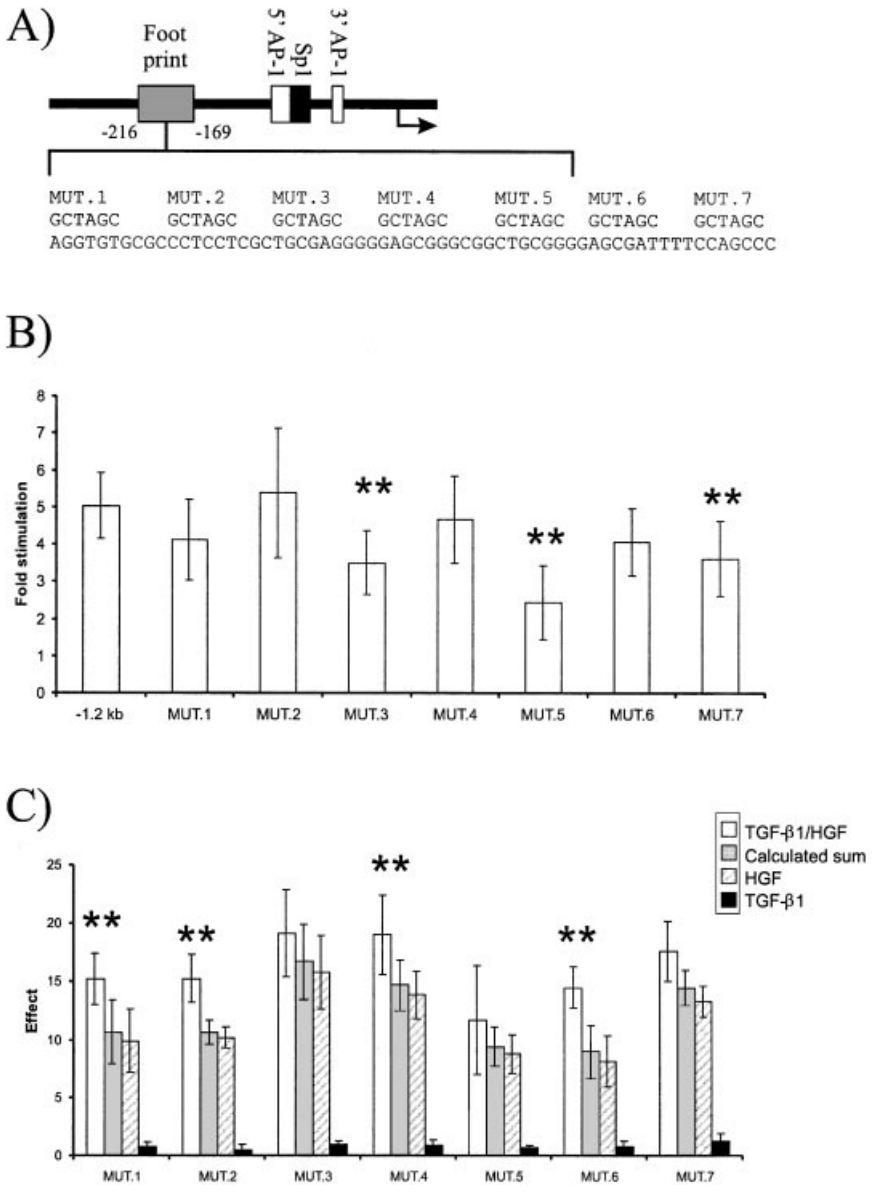

Figure 8 Mutational analysis of the Smad-responsive region

(**A**) The first 350 bp of the LAMC2 region upstream of the major transcription-initiation site (arrow) is illustrated. The overlapping 5′ and 3′ AP-1 sites are shown (white boxes) as is the Sp1 site (black box), which overlaps with the 5′ AP-1 site. The upstream grey box represents a footprinted region detected previously [17] and which is found in the Smad-responsive region located between positions −0.21 kb and −0.15 kb. Within this region, seven individual mutations (MUT.1–7) were introduced into the 1.2 kb LAMC2 promoter construct. (**B**) The 1.2 kb LAMC2 promoter construct or the versions carrying the mutations indicated were transfected into HT29mtx cells with or without co-transfection with the Smad3 expression construct in one experimental series. (**C**) In a separate experimental series the effect of cytokine treatments on the mutation constructs was calculated as the difference in fold stimulation with and without cytokine addition. The sum of the effects following the addition of the cytokines individually was also calculated. S.D.s are indicated. Only mutations 1, 2, 4 and 6 maintained a significantly higher ($^{**}P < 0.01$) effect of both cytokines added simultaneously compared with the sum of the individual treatments. These constructs maintained a synergistic response to the addition of TGF-β1 and HGF despite the introduced mutations. The constructs that failed to mediate a synergistic response (mutations 3, 5 and 7) were the same as those that displayed a reduced Smad3 response (**B**).

response to co-transfection with Smad3 or Smad4 expression vectors (results not shown). Instead, the induction of JunD dimers by TGF-β1 stimulation much more resembles the TGF-β1 response described as a result of activation of the Ras and MAP kinase pathway by this cytokine (reviewed in [32]). Furthermore, the HGF activation of the promoter is much stronger than the TGF-β1 activation, with equivalent amounts of JunD binding to the 5′ AP-1 site, however. The most likely explanation for this finding is that HGF induces the activation of the Jun N-terminal kinase (JNK), which subsequently phosphorylates the JunD protein as described for phorbol ester (PMA)-induced JunD binding to an enhancer regulating the uPA gene [41].

Although the 5′ AP-1 element is required for the TGF-β1 response of the LAMC2 promoter it is not sufficient to achieve a synergistic regulation under the influence of both TGF-β1 and HGF. One possible explanation for this synergy is that TGF-β1 and HGF signalling pathways induce different transcription factors or co-activators that target different parts of the promoter and the 5′ upstream region. The Smad factors are likely candidates for participating in such co-ordinated effects as numerous

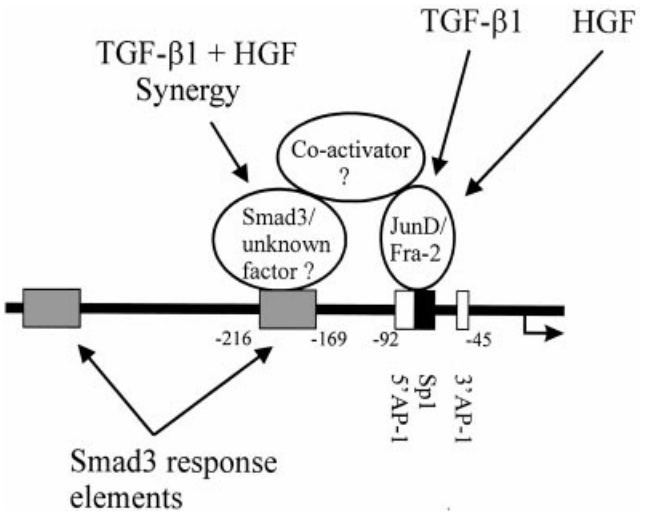

Figure 9 Model for the mechanism behind the synergistic action of HGF and TGF-β1 on the LAMC2 promoter

HGF and TGF-β1 both induce JunD/Fra-2 AP-1 dimers, which bind to the 5′ AP-1 element and stimulate transcription. Deletion analysis of the LAMC2 promoter has demonstrated that this results in an effect which is additive. The presence of the region between −216 and −169 allows synergy to occur between the HGF and TGF-β1 signalling pathways. The elements in this region that are required for synergy are also responsive to Smad3 co-expression. A possible explanation could be that Smad3, when phosphorylated by the TGF-β type I receptor, is recruited to a co-activator complex containing, e.g., CBP/p300. This co-activator complex can concomitantly interact with the the AP-1 proteins bound at the 5′ AP-1 site in the LAMC2 promoter. The interaction between Smad3 and the −216 to −169 LAMC2 promoter region may be either direct or via as-yet-uncharacterized DNA-binding proteins. The other upstream Smad3-responsive region that was also detected in the present work is not required for the synergy.

reports have demonstrated interactions of Smads with the CBP/p300 co-activator [27,28,42], which also interacts with AP-1 proteins [43,44]. The −1.2 kb LAMC2 construct indeed displayed a significant response to co-transfection with expression vectors for Smad3 and Smad4, but the response did not seem to depend on the formation of Smad3/4 heterodimers. The −1.2 kb LAMC2 construct contains at least two different SMAD3-responsive regions and the most 3′ of these was detected in a region (positions −216 to −169) which displayed synergy to TGF-β1 and HGF treatments. This region binds nuclear proteins, as shown by DNase I footprinting assays, but this binding is not essential for the basal LAMC2 promoter activity [17]. Although this region does not contain a Smad-binding consensus sequence, the mutation analysis of this region showed that all the mutations that reduced the Smad3 response also abolished the synergistic response. Altogether this suggests a model where (i) TGF-β1 and HGF both target the 5′ AP-1 site and (ii) TGF-β1 acts specifically via induced Smad3 proteins on the −216 to −169 *cis*-region, resulting in the synergistic effect of HGF and TGF-β1 signalling (Figure 9). Deletion analysis also showed the possible involvement of a further 5′ Smad-responsive element. Therefore, it is likely that one or more DNA-binding proteins link to Smad3 via protein–protein interactions. One possibility is that Smad3 establishes a link between the 5′ AP-1 site and the −216 to −169 *cis*-region via protein–protein interactions as shown in Figure 9. According to this model a co-activator, like the CBP/p300 protein mentioned above, is recruited to the JunD-containing AP-1 dimers binding at the 5′ AP-1 site. Smad3 also interacts with the co-activator proteins and makes the contact with the −216 to −169 *cis*-region. In the absence of a Smad-binding consensus site within this region, this contact might be mediated via an interaction between Smad3 and another as-yet-unidentified protein that will bind directly to the DNA. In the scenario described in Figure 9 the strength of the interaction between such a DNA-binding protein and the −216 to −169 *cis*-region may be weak, as it is stabilized by the protein–protein interactions linking to the AP-1 proteins bound at the 5′ AP-1 site. If this is the case it may explain the observation that proteins binding to the −216 to −169 region can be detected by footprinting using the whole promoter as a probe [17], whereas EMSA experiments failed to produce shifted bands with the −216 to −169 region as a probe (results not shown).

Several studies have focused on the up-regulation of LAMC2 gene expression at the invasive front of colon carcinomas. These invading cells do not typically display a basement-membrane-like deposition of the laminin γ2 chain, but rather showed an intracellular staining for this laminin-5 constituent chain [13]. We show here that HT29mtx colon carcinoma cells accumulate intracellular laminin γ2 chain protein during simultaneous HGF and TGF-β1 treatment, whereas they secrete laminin-5 at similar levels under these conditions. Therefore, one can conclude from our experiments that the level of the secreted laminin-5 molecule is not the sole phenomenon that could lead to cell migration, although it was demonstrated that this substratum is able to trigger cell motility (for a review see [40]). What could be the physiological consequences of such an intracellular accumulation of γ2 chains in cells? First, the overproduction of laminin γ2 chain may affect the association of α, β and γ chains in laminin isoforms other that laminin-5, although the interaction of the γ2 chain with laminin α and β chains other than the α3 and β3 chains has not been reported so far in epithelial cells. Secondly, it cannot be excluded that the laminin γ2 chain, when overexpressed in particular conditions, can be secreted as a monomer, in addition to the hetrotrimer with the α3 and β3 chains, as shown in gastric carcinoma cells [45]. In this case, the monomer could modify the organization of the secreted extracellular matrix (as the laminin γ2 chain is also able to bind fibulin or type VII collagen) or, indirectly, the integrin repertoire, receptors known to play a central role in cell migration and invasion [46]. Furthermore, the possible biological activity of the laminin γ2 chain as a motility factor may be linked to expression of metalloproteases. Of particular interest was the finding of Koshikawa et al. [47] showing that the surface membrane type 1 matrix metalloprotease ('MT1-MMP') was able to cleave the laminin γ2 chain, its activation requiring contact with environmental factors such as the presence of stromal or inflammatory cells. In tumours, the lack of basement-membrane-like staining seen does not seem to be due to a defect in the assembly of the laminin-5 chain but is most likely due to proteolysis, which also takes place in the vicinity of the invading cells [48]. The proteolytic activity at the invasive front might activate matrix-bound TGF-β1, which could limit the TGF-β1 signal to invading cells. HGF is known to be secreted by stromal fibroblasts at contacts between epithelium and mesenchyme [49]. In the HT29mtx cells, the WNT/β-catenin/TCF-4 pathway is constitutively active due to mutations in the APC gene, as in most colon carcinomas [50]. As an effector of WNT signalling, β-catenin is translocated to the nucleus and is able to bind to the TCF-4 transcription factor in intestinal epithelial cells [39]. Interestingly, like the intracellular laminin γ2 chain accumulation, nuclear localization of β-catenin was mainly found at the invasive front of colon carcinomas [16,51]. Recently it was suggested that the high expression of the laminin γ2 chain in invading colon carcinoma cells is due to

convergence of HGF and WNT signalling on the LAMC2 promoter [16].

Altogether, our present data and those published recently [16] allow us to conclude that three pathways converge on the LAMC2 promoter in cancer cells: HGF and TGF-β1 target the 5′ AP-1 element via JunD/Fra2 AP-1 dimers, TGF-β1 targets a Smad3-responsive region between positions -212 and -169, and the β-catenin/TCF-4 pathway targets TCF binding sites in the LAMC2 promoter [16]. Thus the model of the regulation of laminin γ2 gene expression in invading carcinoma cells has to be broadened, allowing room for at least three different mechanisms operating at the invasive front. These include stromal-derived growth factors and activators of the MAP kinase pathway such as HGF, proteolytically activated TGF-β1 and nuclear β-catenin/TCF-4 complexes.

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