Transforming growth factor-β1 (TGF-β1) is secreted as a latent precursor, consisting of a homodimer of the latency-associated peptide and the mature peptide. TGF-β1 can only exert its many functions after going from this latent to an active state, in which the binding site of the mature peptide for its receptor is no longer shielded by the latency-associated peptide. We and others reported that mutations in TGFBI cause Camurati-Engelmann disease, a rare bone disorder. Until now, seven mutations have been published. In this study, we investigate the effect of the LLL12–13ins, Y81H, R218C, H222D, and C25R mutations on the functioning of TGF-β1 in vitro. A luciferase reporter assay specific for TGF-β1-induced transcriptional response with wild type and mutant TGF-β1 constructs showed a positive effect of all mutations on TGF-β1 activity. By way of enzyme-linked immunosorbent assay, we found that in the R218C, H222D, and C25R mutant constructs, this effect is caused by an increase in active TGF-β1 in the medium of transfected cells. The LLL12–13ins and Y81H mutations on the contrary have a profound effect on secretion; a decreased amount of TGF-β1 is secreted, but the increased luciferase activity shows that the intracellular accumulation of (aberrant) TGF-β1 can initiate an enhanced transcriptional response, suggesting the existence of an alternative signaling pathway. Our data indicate that the mutations in the signal peptide and latency-associated peptide facilitate TGF-β1 signaling, thus causing Camurati-Engelmann disease.

Transforming growth factor-β1 (TGF-β1) has a versatile role in our body; it regulates cell proliferation, migration, differentiation, and apoptosis and has an influence on embryogenesis, angiogenesis, wound healing, immune suppression, and many other biological processes (1, 2). TGF-β1 is abundant in skeletal tissue, where it is stored in the bone matrix (3). Resorbing osteoclasts can activate TGF-β1 in the low pH environment of their ruffled border (4). Active TGF-β1 indirectly inhibits further osteoclast differentiation and activation through its action on osteoblasts (5–7) and stimulates osteoblast chemotaxis, proliferation, and differentiation (8, 9), thus coupling bone resorption to new bone formation.

TGF-β1 is part of the TGF-β superfamily, consisting of more than 40 members, including the bone morphogenetic proteins, inhibins, and activins (1, 2). Like almost all other members, TGF-β1 is synthesized as a precursor structure (pre-pro-TGF-β1) and gives rise to its mature peptide by cleaving off the N-terminal part. In the first step, the signal peptide is released during translocation through the endoplasmic reticulum. This is followed by dimerization by way of cysteine bridges and cleavage at the dibasic protease site by furin (10). Other post-translational modifications include glycosylation and phosphorylation (10, 11). This homodimer, in which the latency-associated peptide (LAP) and the mature peptide are non-covalently linked, is secreted and stored in the extracellular matrix as a latent complex, either alone (as small latent complex) or in conjunction with a latent TGF-β-binding protein as a large latent complex (10, 12, 13). In this latent state, TGF-β1 cannot bind to its receptor and hence cannot activate the signaling pathway. Because TGF-β1 and its receptors are ubiquitously expressed, latency is the most important means of controlling the biological effects of this protein. Several activation mechanisms in vivo have been described, including the action of plasmin and thrombospondin (14, 15). Once activated, TGF-β1 can bind to the constitutively active type II receptor (TβRII). This binding recruits the type I receptor (TβRI) and induces the formation of a heteromeric complex (16). TβRI is phosphorylated and activated and in turn phosphorylates the R-Smad2 or 3. Activated R-Smads form heteromeric complexes with Smad4 and accumulate in the nucleus, where they can bind directly to DNA or interact with other transcription factors to regulate gene transcription (17).

Camurati-Engelmann disease (CED) or progressive diaphyseal dysplasia is a rare bone disorder with an autosomal dominant mode of inheritance. Radiologically, it is characterized by hyperostosis and sclerosis of the diaphyses of the long bones.
and sclerosis of the skull base. Patients suffer mainly from bone pain, muscle weakness, a waddling gait, and fatigue (18).

Mutations in TGFβ1 were found to underlie this bone disorder (19, 20). With one exception, a duplication of three Leu residues in the signal peptide, all of them are missense mutations in the LAP. An earlier study suggests a disruption of the association of the LAP and mature TGF-β in the case of domain-specific mutations in LAP as the cause for CED (21). This study aims at further unraveling the effect of these and other mutations on the action of TGF-β1 in vitro.

EXPERIMENTAL PROCEDURES

**Mutation Analysis**—Primers were designed from intronic sequences flanking exons 1–7 of TGFβ1 to amplify genomic DNA from patients and control individuals. Direct sequencing of the PCR products was performed on both forward and reverse strands on an ABI 377 sequencer using the BigDye Terminator Cycle Sequencing v2.0 ready reaction Kit (PerkinElmer Life Sciences).

**Cell Culture Condition**—HEK293T, transformed primary human embryonal kidney cells, were kindly provided by Dr. D. Huybrechts and were grown in Dulbecco’s modified Eagle’s medium with 4500 mg/l glucose, supplemented with fetal calf serum (15% v/v), penicillin (100 units/ml), streptomycin (100 µg/ml), and l-glutamine (2 mM). Medium and sera were from Invitrogen, Bethesda, MD. The media stably transfected with a CAGA-luciferase reporter construct (Astra Draco, Lund, Sweden) (22) were maintained under the same conditions.

**Construction of Expression Vectors and Transfection**—A 1286-base pair HindIII-EcoRI DNA fragment containing the full-length mutant or WT TGFβ1 was ligated into pcDNA3 (Invitrogen) and transformed in XL1 blue competent cells (Stratagene, La Jolla, CA). For transient expression of the mutant and WT gene products, the constructs were transfected into HEK293T cells, plated at a density of 5 × 10⁵ cells per well in 2 ml Dulbecco’s modified Eagle’s medium, using the transfection reagent Fugene 6 (Roche Molecular Biochemicals). Each transfection was performed in duplicate and repeated several times. HEK293T cells were transfected at 80–90% confluence with 1 µl or 5 µl of Fugene 6, 10 or 50 ng of pRL-TK (Promega, Madison, WI), and 50 ng or 1 µg of the pcDNA3 construct in a total volume of 2 ml for subsequent use of media and/or lysates in reporter assays and ELISAs/Western blots, respectively. For the luciferase assay, 200 ng of a TGF-β responsive reporter construct was cotransfected as well. After 24 h of incubation, the cells were rinsed with phosphate-buffered saline to remove all traces of serum, and the medium was replaced with 2 ml of serum-free Opti-Mem I (Invitrogen) to avoid the influence of TGF-β contained in the fetal calf serum on our measurements. After another 24-h incubation of the transfected cells, the supernatants were collected. The cells were lysed with 500 µl of passive lysis buffer (Promega), and the lysates were centrifuged. The media and lysates were aliquoted and stored at −70 °C and were never thawed more than once.

**Reporter Assay**—A TGF-β responsive reporter construct was provided by Dr. D. Dennler (22). Cotransfection of the pRL-TK vector, containing the herpes simplex virus-thymidine kinase (HSV-TK) promoter region upstream of the Renilla luciferase gene, allowed calibration of the Firefly luciferase activity against the Renilla luciferase activity, providing normalization of transfection efficiency. Cell lysates were assayed for Firefly and Renilla luciferase activity using the dual-luciferase reporter assay system (Promega), following the manufacturer’s instructions. Briefly, 20 µl of lysate was transferred to a luminometer tube, together with 50 µl of luciferase assay reagent II, and Firefly luciferase activity was measured for a period of 10 s. After addition of 10 µl of Stop&Glo reagent, Renilla luciferase activity was measured, again for a period of 10 s. The ratio of both activities was determined.

HT1080 cells were plated in Dulbecco’s modified Eagle’s medium with fetal calf serum at a concentration of 5 × 10⁵ cells per well. After adherence, the cells were washed with phosphate-buffered saline, and the medium was changed for the conditioned medium of the transfected HEK cells. 24 h later, the cells were lysed, and the lysates were collected and assayed for Firefly luciferase activity.

**ELISA**—The amount of active and total TGF-β1 in sera was activated with 2.5 N acetic acid/10% urea for 10 min and neutralized with 2.7 N NaOH/0.5 M HEPES. Latent TGF-β1 in conditioned media and lysates was acidified with 1 N HCl for 10 min and neutralized with 1.2 N NaOH/0.5 M HEPES. Latent TGF-β1 in sera was activated with 2.5 N acetic acid/10% urea for 10 min and neutralized with 2.7 N NaOH/0.5 M HEPES.

**Preparation of Serum Samples**—Blood samples of patients and control individuals were collected in serum separator tubes. After clotting of the samples for 1 h at room temperature, they were incubated overnight at 4 °C, allowing complete release of TGF-β1. The samples were centrifuged for 10 min at 1000 × g, the serum was removed, and aliquoted and stored at −70 °C. The sera were never thawed more than once.

**Western Blotting**—12 µl of the supernatant or lysate samples was fractionated under reducing or non-reducing conditions by SDS-PAGE on a 7.5 or 12% gel. Following electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad) for 1 h at 0.8 mA. The membranes were blocked by incubation with 3% bovine serum albumin (Sigma) in TBST for 2 h. Staining for immunoreactive mature or precursor forms of the TGF-β1 protein for pSmad2 or total Smad2 was performed overnight at 4 °C using the following primary antibodies: anti-human LAP neutralizing antibody (1/2000 dilution; R&D Systems), anti-TGF-β1 antibody (1/1000 dilution; Promega), anti-pSmad2 antibody (1/1000 dilution; see Ref. 23), and anti-Smad2 monoclonal antibody (1/1000 dilution; New England Biolabs, Beverly, MA) diluted in TBST with 1% bovine serum albumin. After three washes with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies diluted in TBST with 1% bovine serum albumin (Promega) or pSmad2 (1/2000; see Ref. 23). The membranes were washed again three times in TBST. Proteins were visualized using Western blot chemiluminescence reagent (PerkinElmer Life Sciences).

RESULTS

**CED Is Caused by Mutations in the Signal Peptide and LAP of TGFβ1**—With the exception of the H222D mutation, all mutations studied in this paper have been described previously (19). The H222D mutation was found in an isolated patient of German origin and is caused by a C to G transition at position 664. Although CED has been reported to occur both in families and sporadic patients, this is the first report of a mutation in an isolated patient. The observations that the transition could not be found in 208 control chromosomes, that His222 is conserved in all mammals, and that the residue is located in the immediate vicinity of several other residues mutated in CED provide evidence that this is a disease-causing mutation. Fig. 1 shows an overview of all mutations studied in this paper and their localization with regard to functionally important residues in the pre-pro-TGF-β1.

**CED Mutations Lead to Overactivation of the Smad Signaling Pathway**—Each WT or mutant TGF-β1 construct was co-transfected in HEK293T cells with the pRL-TK vector and the TGF-β-responsive reporter construct. Active TGF-β1 would lead to transcription of Firefly luciferase by way of autocrine or paracrine activation of the Smad signaling pathway. Measurement of luciferase activity in the lysates of cells transfected with the reporter construct and the Y81H, R218C, H222D, or C225R mutant construct showed that mutant TGF-β1 was more capable of activating the signaling pathway, leading to a higher increase in luciferase activity of 35.66 ± 6.75 times (mean ± S.D.; p < 0.001) compared with the WT protein. The effect of the L11L12–13ins mutation was smaller but consistent, giving a 2.10 ± 0.14-fold increase in activity (p = 0.002) (Fig. 2A). For none of the mutations, this effect could be attributed to overproduction of the mutant protein (Fig. 2B), indicating the involvement of other mechanisms (see below).

Overproduction of the Smad signaling pathway should be reflected by an increase in phosphorylation of Smad2 by TβRII. Therefore, lysates of the transfected HEK293T cells were probed with an antibody staining for pSmad2. Although this method is not as sensitive as the reporter assay, there is a clear
correlation between the amount of pSmad2 detected and the luciferase activity (Fig. 2C).

Effect of the Mutations on the Activation and Secretion of TGF-β1—To unravel the underlying mechanism leading to increased activation of the TGF-β1 signaling pathway, ELISA on the collected supernatants of cells transfected with a WT or mutant construct was performed. All values were corrected for the transfection efficiency and recalculated with regard to the WT construct to eliminate differences between successive experiments.

The missense mutations in exon 4, R218C, H222D, and C225R, resulted in higher levels of active, mature TGF-β1 in the medium, being on average 2.10 ± 0.76-fold compared with controls (p < 0.001). Representative results are shown in Fig. 3A. Measurement of total TGF-β1 concentration after full activation showed that constructs mutant in exon 4 secreted 1.08 ± 0.31-fold the amount of total TGF-β1 secreted by the WT construct (p = 0.777) (Fig. 3B), indicating that the difference in active TGF-β1 concentrations observed was not because of an effect on production or secretion of the mutant protein but rather reflected an abnormality in the activation mechanism of the latent TGF-β1. In Fig. 3C, the ratios of mature to total TGF-β1 are given. Concentrations measured after transfection of an empty pcDNA3 vector as negative control were very low, for both not and fully activated samples, showing that the amount of endogenous TGF-β1 produced by the HEK293T cells is negligible (data not shown).

The mutation in the signal peptide exerted its effect on the secretion of TGF-β1; as measured by fully activating the samples, decreased amounts of TGF-β1 were being secreted, ranging from 22 to 56% of the normal values (average 38%) (Fig. 3B). The diminished secretion was reflected by an accumula-
tation of total TGF-β1 intracellularly as measured by ELISA on the lysate; the concentration was 2- to 7-fold the normal value (average four) (Fig. 3D).

As with the LLL12–13ins mutation, the Y81H mutation resulted in an extremely low secretion of total TGF-β1, varying from 3 to 31% of the normal value, with an average of 14% (Fig. 3B). Expecting an intracellular accumulation of TGF-β1, ELISA on the lysate of the Y81H transfectant cells was performed, but no such accumulation could be found. The intracellular concentration of the mutant TGF-β1 seemed even decreased with regard to the other constructs (Fig. 3D). Keeping in mind the possibility of a conformational change of the LAP, making the epitope on the mature peptide unreachable for the Dusset’s antibody, we performed both ELISAs with the Quantikine kit in which TβRII is used as the capture antibody but obtained the same results. The expression of the Y81H TGF-β1 mRNA, however, appeared to be normal (data not shown).

Confirmation of Aberrant Secretion of LLL12–13ins and Y81H Mutants—Analysis of not and fully activated samples by SDS-PAGE revealed that SDS breaks the non-covalent bonds between LAP and mature TGF-β1, making it impossible to see a difference in activation efficiency between control and mutant constructs. The results for the LLL12–13ins mutation confirmed the data obtained by ELISA; almost no mature TGF-β1 was detected in the medium because of the diminished secretion (Fig. 4A), but a dense band could be seen in the lysate (Fig. 4B). As expected based on the ELISA, we could hardly detect any mature TGF-β1 in the conditioned medium or lysate of cells transfected with the Y81H construct (Fig. 4, A and B). However, a clear band of the LAP dimer was detected in the lysate (Fig. 4C).

To confirm the results obtained with the overexpression constructs in the patients themselves, an ELISA was performed on the acidified sera of patients with the LLL12–13ins or Y81H mutation and age- and sex-matched controls. In this way, we could prove that the effect of the duplication and the Y81H mutation results in mean decreases of total TGF-β1 in the serum of 41 and 26%, respectively (Fig. 5). The concentration of mature TGF-β1 in non-acidified serum is too low to make a comparison of TGF-β1 activity between healthy individuals and patients with an exon 4 mutation possible (data not shown).

Signalizing by the LLL12–13ins and Y81H Mutant Constructs Does Not Occur through the Extracellular Pathway—Puzzled by the contrast between their ability to induce the TGF-β1 signaling pathway and the low levels of active TGF-β1 in the media of cells transfected with the LLL12–13ins or Y81H mutant construct, HT1080 cells, stably transfected with a TGF-β1 responsive construct, were grown in conditioned medium from the transfected HEK293T cells. In this experiment, the Firefly luciferase activity in the lysates of the HT1080 cells is a measure for extracellular but not intracellular active TGF-β1 produced by the transfected HEK293T cells. As can be seen in Fig. 6, luciferase activity drops most for the WT, LLL12–13ins, and Y81H constructs and to a lesser degree for the constructs with a missense mutation in exon 4. This indicates that in the case of the latter constructs induction of the signaling pathway is accomplished by way of the active TGF-β1 present in the conditioned medium, whereas this is not the case for the former constructs. This observation suggests the existence of an alternative pathway for signaling in the case of the LLL12–13ins and Y81H mutant constructs.

DISCUSSION

The results presented in this paper illustrate that the pathologic mechanism underlying the sclerosing bone phenotype in CED patients is increased TGF-β1 signaling as a result of disturbed activation or secretion of the mutant protein. The
experimental results suggest the existence of two distinct mechanisms leading to increased TGF-β1 activity in CED patients depending on the underlying mutation. In the first, illustrated by the R218C, H222D, and C225R mutations in exon 4 of TGFβ1, secretion is normal, but the percentage active TGF-β1 is elevated. These mutations are located close to the two cysteine residues (Cys223 and Cys225) that form the intrachain disulfide bonds. We hypothesized that the resultant destabilization of the dimerization process would facilitate the activation of latent TGF-β1. Our assumption was partly based on a study by Brunner et al. (24), who showed that site-directed mutagenesis of Cys223 or Cys225 led to a 2.4- to 4.3-fold increase in active TGF-β1 prior to acidification without altering secretion and was recently suggested by Saito et al. (21). Our hypothesis was confirmed by the luciferase reporter assay, the immunostaining for pSmad2 in cell lysates, and the ELISA on supernatants of transfected cells; when corrected for the transfection efficiency and the total amount of TGF-β1 secreted, a significant greater amount of TGF-β1 already active prior to acidification is present in the supernatants of cells transfected with a construct mutated in exon 4. The minor variations in total TGF-β1 production show that this is not the result of overproduction of the mutant protein but reflects the instability of the precursor protein, facilitating activation. The observation that the presence of approximately two times more active TGF-β1 leads to such a strong Smad-dependent transcriptional response can be explained by the fact that in the ELISA, the active TGF-β1 present in the medium of the transfected cells at the moment of collection is measured, whereas the Firefly luciferase activity measured in the reporter assay reflects the amount of active TGF-β1 produced by the cells and bound to its receptor over a time period of 24 h.

In the second mechanism, illustrated by the LLL12-13ins and Y81H mutations, secretion is disturbed, leading to intracellular accumulation of TGF-β1. This is not unexpected for the mutation in the signal peptide. The first step in the processing of the pre-pro-TGF-β1 is the signal peptide cleavage at the Gly29-Leu30 peptide bond during transit through the rough endoplasmic reticulum. Although insertion of three Leu residues does not change the predicted site of signal peptide cleavage (25), this calculation does not take into account the critical length of the central hydrophobic stretch (h region). Nilsson et al. (26) constructed signal sequences with poly-Leu h regions
the requirement for receptor internalization in TGF-β1. The membrane bound receptor. Our theory is partly based on the signaling pathway without the need for ligand binding to count for the increased transcriptional response. Induced glycosylation signal and is supported by the finding by based on the presumed influence of the mutation on the first covalent bond is broken by furin; this assumption is again longer able to bind the mature peptide, releasing it as soon as the Y81H mutation, we assume that the mutated LAP is no longer. In the case of the Y81H mutation, we were unable to experimentally prove the intracellular accumulation of the protein, but several arguments are in favor of a disturbed secretion. The Y81H mutation is located next to the first of three glycosylation signals in the LAP. Mutagenesis of this mannose 6-phosphate-containing site results in an 85% decrease in the amount of TGF-β1 secreted (27). Additionally, by using different inhibitors of glycosylation or maturation of oligosaccharide chain formation, Sha et al. (28) showed that these events play an important role in secretion. Furthermore, substitution of Tyr81 for a stretch of five amino acids led to a decrease in secretion to 13–33% of the normal values (29).

Despite the decreased secretion in this second group of mutations, our experimental data show that there is enhanced signaling by the mutant TGF-β1 proteins (Fig. 2A). This observation led us to formulate a hypothesis of intracrine signaling; the intracellular accumulation of (aberrant) TGF-β1 can account for the increased transcriptional response by inducing the signaling pathway without the need for ligand binding to the membrane bound receptor. Our theory is partly based on the requirement for receptor internalization in TGF-β signaling. Although receptor down-regulation requires binding of ligand to a heteromeric receptor complex (30, 31), several researchers report the constitutive endocytosis of the TGF-β receptor complex in the absence of ligand (32, 33). In the case of the Y81H mutation, we assume that the mutated LAP is no longer able to bind the mature peptide, releasing it as soon as the covalent bond is broken by furin; this assumption is again based on the presumed influence of the mutation on the first glycosylation signal and is supported by the finding by Miyazono et al. (34) that treatment of latent TGF-β1 with endoglycosidase F, which removes complex and oligomannose N-linked carbohydrate complexes, generates active TGF-β1, demonstrating the importance of these side chains in latency. Once released, the active, mature dimer could then form an intracellular complex with the internalized receptor complex, preventing it from recycling back to the plasma membrane. The absence of mature TGF-β1 in the lysate and the presence of LAP (Fig. 4C) could thus be explained by the instability and rapid degradation of this complex through lysosomes or the proteasomal pathway. Future experiments will have to prove the presence of such an intracellular complex with the receptor or show whether mature TGF-β1 forms a complex with another yet unidentified protein. Although intracrine signaling is a rare phenomenon, it has been described previously for the v-sis oncogene, whose gene product, p28v-sis, is homologous to the platelet-derived growth factor B chain. Several investigators (35–37) have reported that its transforming capacities are at least partly attributable to internal activation of the platelet-derived growth factor receptor. Another report (38) makes mention of the formation of an intracellular TGF-β1-TβRII complex in plasma cell tumors. Despite the absence of mutations in TGF-β1 or TβRII and normal expression of TβRII, the receptor does not localize to the plasma membrane but is abundant in the cytosol, were it is captured by active, intracellular TGF-β1. The absence of phosphorylated Smad2 led them to conclude that this intracellular complex is incapable of signaling (38). Our experimental results show, however, that signaling does occur through the Smad pathway (Fig. 2C).

In the case of the LLL12–13ins mutation, a comparable intracrine signaling mechanism might exist, but the presence of a major part of the intracellular TGF-β1 in the latent state could account for the limited enhanced transcriptional response. Our hypothesis of intracrine signaling is experimentally supported by the data presented in Fig. 6; the absence of signaling in the case of the Y81H and LLL12–13ins mutations shows the need for the intracellular TGF-β1 generated by these mutant constructs. However, extensive experimental evidence is still needed to further prove this theory, and alternative mechanisms cannot be excluded at present.

Because TGF-β1 acts as a coupling factor between bone resorption and formation, the presence of activating mutations in TGF-β1 in CED patients will unbalance this process, resulting in decreased bone resorption and increased bone formation. This is in agreement with the radiological observations in CED patients; the narrowing of the medullary canal at the endosteal side and the modeling defect at the periosteal side of the diaphyses of the long bones suggest that both the resorption capacity of the osteoclasts and the bone formation process by the osteoblasts are disturbed. Although we mainly looked at the effect of the mutations after overexpression, the results obtained with the sera of patients with the LLL12–13ins or Y81H mutation illustrate that the mutations do have a physiological effect (Fig. 5). Further experiments will have to be performed to establish the exact role of the mutant protein in vivo. Because of the diverse nature of the mutations, we looked at the genotype-phenotype relationship in our families but found no evidence for a correlation. The occurrence of high intrafamilial variability indicates that other factors might influence the outcome of this disorder.

In conclusion, the mutations in the signal peptide and LAP of TGF-β1 give rise to the pathologic phenotype in Camurati-
Engelmann disease by altering the activation or secretion of the protein, leading to enhanced signaling. The present study shows that this not only occurs through the well known extracellular pathway but suggests the existence of an alternative, possibly intracrine pathway for TGF-β1 signaling.

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