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## Domain-specific Mutations of a Transforming Growth Factor (TGF)- $\beta$ 1 Latency-associated Peptide Cause Camurati-Engelmann Disease Because of the Formation of a Constitutively Active Form of TGF- $\beta$ 1\*

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Transforming growth factor (TGF)- $\beta$ 1 is secreted as a latent form, which consists of its mature form and a latency-associated peptide ( $\beta$ 1-LAP) in either the presence or the absence of additional latent TGF- $\beta$ 1-binding protein. We recently reported that three different missense mutations (R218H, R218C, and C225R) of  $\beta$ 1-LAP cause the Camurati-Engelmann disease (CED), an autosomal dominant disorder characterized by hyperostosis and sclerosis of the diaphysis of the long bones. Pulse-chase experiments using fibroblasts from CED patients and expression experiments of the mutant genes in an insect cell system suggest that these mutations disrupt the association of  $\beta$ 1-LAP and TGF- $\beta$ 1 and the subsequent release of the mature TGF- $\beta$ 1. Furthermore, the cell growth of fibroblasts from a CED patient and mutant gene-transfected fibroblasts was suppressed via TGF- $\beta$ 1. The growth suppression observed was attenuated by neutralizing antibody to TGF- $\beta$ 1 or by treatment of dexamethasone. On the other hand, the proliferation of human osteoblastic MG-63 cells was accelerated by coculture with CED fibroblasts. These data suggest that the domain-specific mutations of  $\beta$ 1-LAP result in a more facile activation of TGF- $\beta$ 1, thus causing CED.

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Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1)<sup>1</sup> is a multifunctional protein acting on cell growth, differentiation, and morphogenesis of many different-type cells. In skeletal tissue, TGF- $\beta$ 1 serves as a systematic regulator that couples bone formation and resorption by regulating the function of the osteoblasts and osteoclasts (1–5).

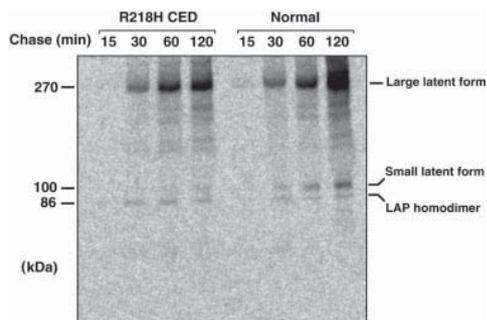
The mature form of TGF- $\beta$ 1 is proteolytically cleaved from the N-terminal remnant of the TGF- $\beta$ 1 precursor, designated as TGF- $\beta$ 1 latency-associated peptide ( $\beta$ 1-LAP) but remains non-covalently associated with the rest of the complex, which plays a role in latency of TGF- $\beta$ 1 (6). TGF- $\beta$ 1 is ubiquitously distributed, and the activation of the latent form is likely an important step, because it exists as either a large latent form, composed of  $\beta$ 1-LAP, TGF- $\beta$ 1, and latent TGF- $\beta$ 1-binding protein (LTBP), or a small latent form, which is devoid of LTBP (7). Under normal conditions, activation of the latent TGF- $\beta$ 1 is strictly controlled as follows. The large latent form is temporarily converted to the small latent form, which is then cleaved by plasmin or the plasmin-like protease to give the mature TGF- $\beta$ 1 (6, 8, 9).

Camurati-Engelmann disease (CED) or progressive diaphyseal dysplasia (DPD1) is an autosomal dominant disorder that is characterized by hyperostosis and sclerosis of the diaphysis of the long bones (10). The onset of CED is often during early childhood with severe pain in the legs, muscle weakness, a waddling gait, and easy fatigability. The patients occasionally suffer from systemic manifestations, such as anemia, leukopenia, or hepatosplenomegaly (11). We, as well as two other groups, previously assigned the locus for CED to chromosome 19q13.1-q13.3 (12–14). By a positional candidate gene approach and haplotype analyses, three different missense mutations (R218H, R218C, and C225R), which are located near the C terminus of  $\beta$ 1-LAP, were found in nine CED families (15, 16). In this paper, we report on the biochemical mechanism by which the domain-specific mutations of  $\beta$ 1-LAP cause CED.

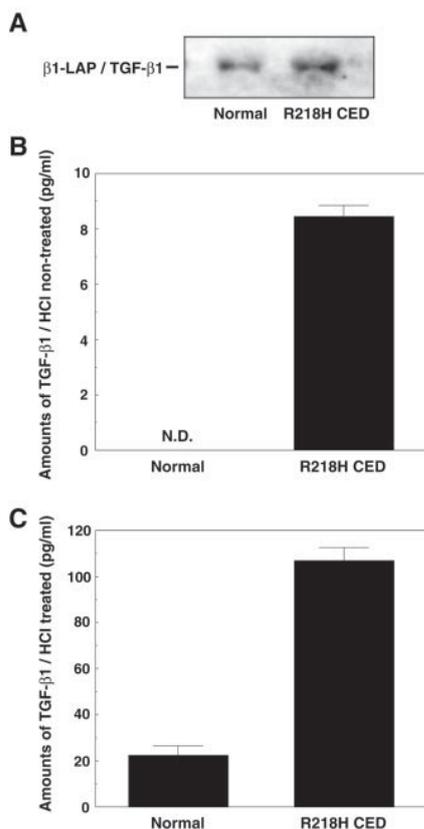
### EXPERIMENTAL PROCEDURES

**Pulse-Chase Analysis**—R218H CED skin fibroblasts and normal skin fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), inoculated in 60-mm dishes, and then grown to near confluency. Metabolic labeling and immunoprecipitation procedures were performed as previously described (7), with slight modifications. Cells were placed in a cysteine- and methionine-free medium containing 10% fetal calf serum and incubated overnight. For pulsing, the cell culture medium was replaced with a medium containing [<sup>35</sup>S]cysteine (100  $\mu$ Ci/ml) and [<sup>35</sup>S]methionine (100  $\mu$ Ci/ml), incubated for 15 min, washed with fresh DMEM, and then incubated for various periods. Media were collected, and 10  $\mu$ l/ml of normal rabbit serum was added, followed by a further incubation for 2 h at 4 °C, and 15  $\mu$ l/ml of rProtein A-Sepharose (Amersham Pharmacia Biotech) was then added. The resulting mixture was incubated for an additional 45 min at 4 °C with gentle mixing. The beads were spun down by centrifugation, and the supernatants were collected. Immunoprecipitation of these samples was carried out by adding 15  $\mu$ l of LT-1 ( $\beta$ 1-LAP antibody)-conjugated rProtein A-Sepharose, followed by an overnight incubation at 4 °C with gentle mixing. The beads were collected by centrifugation, washed three times with 1% Triton X-100 in PBS, twice with 0.2% Triton X-100 in PBS, and once with PBS. The

<sup>1</sup> The abbreviations used are: TGF, transforming growth factor; LAP, latency-associated peptide; LTBP, latent TGF- $\beta$ 1-binding protein; CED, Camurati-Engelmann disease; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay.



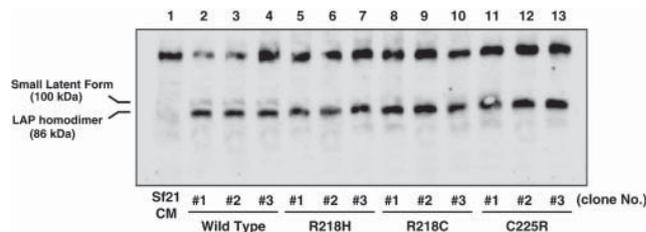
**FIG. 1. A pulse-chase analysis of latent TGF- $\beta$ 1 forms of R218H CED and normal fibroblasts.** Both R218H CED and normal fibroblasts were pulse-labeled for 15 min with [ $^{35}$ S]cysteine and [ $^{35}$ S]methionine and then chased for the indicated times. Conditioned media were reacted with an antibody against  $\beta$ 1-LAP (7), and the immunoprecipitates were subjected to SDS-PAGE in a 5–20% gradient gel under non-reducing conditions.



**FIG. 2. The amounts of mature TGF- $\beta$ 1 protein in the conditioned media from R218H CED and normal fibroblasts.** Conditioned media were collected at 48-h. The amounts of TGF- $\beta$ 1 protein in these samples were assayed using a TGF- $\beta$ 1-ELISA kit (R & D Systems Inc.). *A*, Northern blot analysis to confirm the expression level of TGF- $\beta$ 1 in R218H CED and normal fibroblasts. *B*, the amount of mature TGF- $\beta$ 1 protein in HCl non-treated conditioned media. *N.D.*, not detected. *C*, the amount of mature TGF- $\beta$ 1 protein in HCl pre-treated conditioned media (9).

proteins bound to the beads were eluted by the addition of 15  $\mu$ l of SDS sample buffer, and the eluates were heated to boiling for 5 min at 95  $^{\circ}$ C. Samples were then subjected to SDS-PAGE using 5–20% polyacrylamide gradient gels (Bio-Rad) under non-reducing conditions. The gels were dried and exposed to an imaging plate (Fuji Film).  $^{35}$ S radioactivity was detected using a BAS2500 system (Fujix).

**Enzyme-linked Immunosorbent Assay (ELISA)**—To activate the latent TGF- $\beta$ 1 to the immunoreactive form, conditioned media were treated with 1N HCl for acidic activation and then neutralized with NaOH, according to the manufacturer's instructions (R & D Systems Inc.).



**FIG. 3. Effect of the mutations on the expression of recombinant latent TGF- $\beta$ 1.** Samples were subjected to Western blot analysis using an anti-LAP monoclonal antibody (R & D Systems Inc.). *Lane 1*, conditioned medium from uninfected Sf21 cells; *lanes 2–4*, wild-type TGF- $\beta$ 1 baculovirus clones; *lanes 5–7*, R218H-TGF- $\beta$ 1 baculovirus clones; *lanes 8–10*, R218C-TGF- $\beta$ 1 baculovirus clones; *lanes 11–13*, C225R-TGF- $\beta$ 1 baculovirus clones.

**Preparation of Recombinant Latent TGF- $\beta$ 1**—A 1195-base pair *EcoRI-XbaI* cDNA fragment containing a full-length of R218H-, R218C-, C225R-, and wild-type-TGF- $\beta$ 1 were ligated into pFast Bac vector (Life Technologies, Inc.) and transfected to DH10Bac competent cells for recombination. Recombined Bacmid DNAs were transfected into Sf21 insect cells, and after 72 h, the resultant virus was amplified for 4 days on Sf21 monolayers in Grace medium (Life Technologies, Inc.) that contained 10% FBS. Virus stocks were then reamplified in Sf21 monolayers for 4 days. Fresh Sf21 cells were infected with reamplified virus and grown in serum-free Sf-900 (Life Technologies, Inc.). After 4 days, the recombinant protein was harvested from the Sf21-conditioned medium.

Samples of each conditioned medium were concentrated to 3 mg/ml of protein using a Centricon-10 (Amicon; Millipore), and 10  $\mu$ l of the concentrated conditioned media were subjected to SDS-PAGE in an 8% gel under non-reducing conditions.

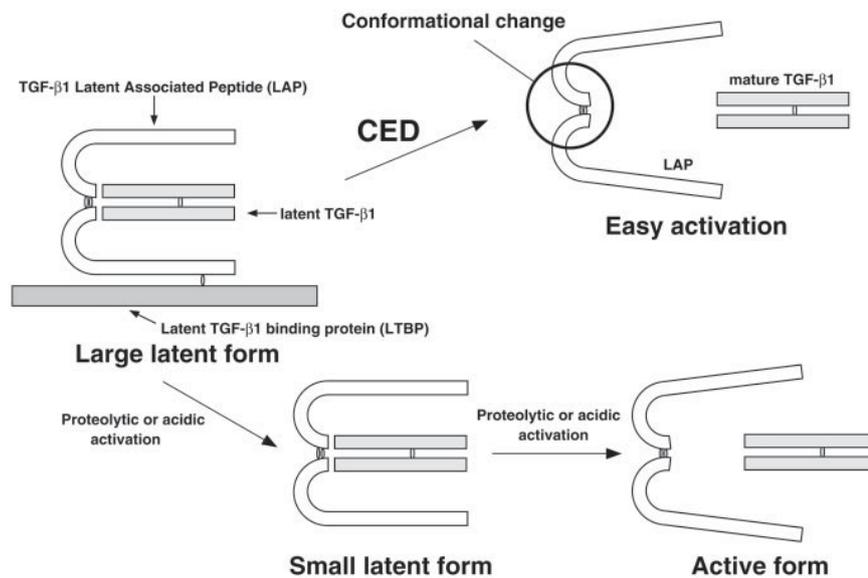
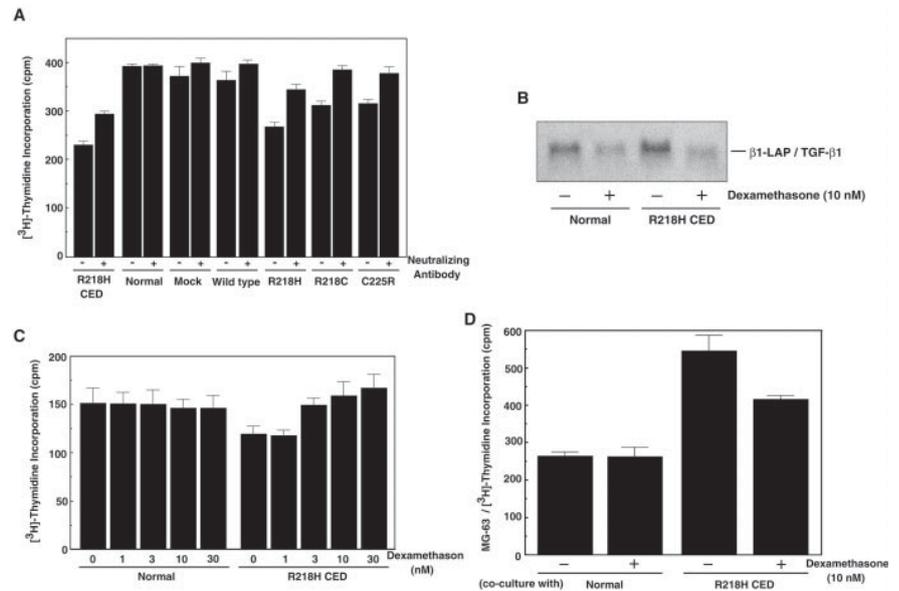
**Effect of the Mutant-gene Transfection on Cell Proliferation**—Each construct was recombined in a pRc/CMV vector. These vectors were transiently expressed in normal skin fibroblasts. The resulting cells were inoculated on a 96-well plate ( $2 \times 10^3$  cells/well) with or without a neutralizing antibody against TGF- $\beta$ 1 (R & D Systems Inc.). R218H CED skin fibroblasts were also cultured in the presence or absence of dexamethasone. After 48-h, 5  $\mu$ Ci/ml of [ $^3$ H]thymidine was added, and 6 h later the cells were collected. [ $^3$ H]Thymidine incorporation was determined with a  $\beta$ -plate counter.

**Human Osteoblastic MG-63-cell Proliferation Assay**—MG-63 cells, a transformed human osteoblastic cell line, were seeded at  $1 \times 10^4$  cells/well (24-well plate) as the bottom wells, and R218H CED and normal fibroblasts were cocultured in DMEM supplemented with 2% FBS at  $5 \times 10^3$  cells/well using the cell culture inserts (pore size 3  $\mu$ m; Falcon) as the upper wells. After 36 h, 5  $\mu$ Ci/ml of [ $^3$ H]thymidine was added and incubated for 6 h. The MG-63 cells were then collected, and [ $^3$ H]thymidine incorporation was determined with a  $\beta$ -plate counter.

## RESULTS AND DISCUSSION

A hydropathy plot analysis of the domain involved in the mutations (R218H, R218C, and C225R) of  $\beta$ 1-LAP suggested a working model in which CED mutations make conformational abnormalities of the C terminus of  $\beta$ 1-LAP (data not shown). This domain is closely associated with the formation of intermolecular disulfide bonds between two  $\beta$ 1-LAPs at Cys-223 and Cys-225 that are needed to form a latent TGF- $\beta$ 1. To investigate the possibility that conformational abnormalities play a role in the processing of latent TGF- $\beta$ 1 in CED, the conditioned medium of skin fibroblasts bearing the R218H mutation, which had been metabolically labeled, was immunoprecipitated with an antibody for  $\beta$ 1-LAP (7). Analysis of the samples by SDS-PAGE under non-reducing conditions revealed that a 100-kDa TGF- $\beta$ 1/ $\beta$ 1-LAP complex (*i.e.* the small latent form) disappeared within 30 min, and only an 86-kDa  $\beta$ 1-LAP homodimer band was detected in the R218H medium. Both bands were present up to 120 min in the normal medium (Fig. 1). However, neither the intracellular assembly nor its ability to secrete TGF- $\beta$ 1 appeared to be affected in the mutant fibroblasts, as judged from the manner in which the large latent

**FIG. 4. Effect of the domain-specific mutations in  $\beta$ 1-LAP on the suppression of cell proliferation via TGF- $\beta$ 1.** A, R218H CED and normal fibroblasts and plasmid-transfected cells were cultured for 48 h, and their proliferation rates were determined by [ $^3$ H]thymidine incorporation (18). A neutralizing antibody against TGF- $\beta$ 1 was added to the samples in a final concentration of 5  $\mu$ g/ml. B, Northern blot analysis of the expression level of TGF- $\beta$ 1 mRNA. R218H CED and normal fibroblasts were treated with dexamethasone (10 nM). C, R218H CED and normal fibroblasts were treated with dexamethasone at the indicated dose for 48-h, and their proliferation rates were determined by [ $^3$ H]thymidine incorporation. These data (A and C) are depicted as the mean  $\pm$  S.E. ( $n = 8$ ). D, the proliferation of MG-63 cells was determined by [ $^3$ H]thymidine incorporation under the coculture system with CED fibroblast in the presence or absence of dexamethasone (10 nM). These data show the mean  $\pm$  S.E. ( $n = 4$ ).



**FIG. 5. Schematic illustration of the activation of latent TGF- $\beta$ 1.** The activation of latent TGF- $\beta$ 1 normally occurs via a three-step mechanism (large latent form  $\rightarrow$  small latent form  $\rightarrow$  mature form). In the mutants of CED, TGF- $\beta$ 1 does not form the small latent form. Therefore, the mutant TGF- $\beta$ 1 may undergo rapid activation and/or rapid turnover.

form was processed. Moreover, no differences in plasmin or plasmin like-proteolytic activity, in terms of the conversion of the small latent TGF- $\beta$ 1 into the mature TGF- $\beta$ 1 (6, 9), were noted in either types of cells (data not shown). These observations suggest that the R218H mutation leads to the vulnerability of the small latent TGF- $\beta$ 1 and the subsequent release of the mature TGF- $\beta$ 1.

To assess this hypothesis, the amount of mature TGF- $\beta$ 1 protein in the conditioned media of R218H and normal fibroblasts was determined by an enzyme-linked immunosorbent assay, which detects only the mature form (R & D Systems Inc.). Although there were no differences in secretion of the large latent form (Fig. 1) or the expression level of TGF- $\beta$ 1 mRNA (Fig. 2A) between R218H and normal fibroblasts, the medium from the R218H fibroblasts contained higher levels of mature TGF- $\beta$ 1 protein after a 48-h incubation than the normal fibroblasts (Fig. 2B). Because the mature TGF- $\beta$ 1 has a half-life of only about 2 min (17), its concentration was very small. When the latent TGF- $\beta$ 1 was partially activated to give the immunoreactive form by acid treatment (9), the detectable TGF- $\beta$ 1 in the R218H medium was more than five times that in

the normal one (Fig. 2C). These results strongly suggest that the R218H mutation facilitates the dissociation of the mature TGF- $\beta$ 1 from  $\beta$ 1-LAP, thus leading to the accumulation of mature TGF- $\beta$ 1.

To study further the effect of mutations on the formation of the small latent form of TGF- $\beta$ 1, recombinant latent TGF- $\beta$ 1 proteins were prepared using a baculovirus expression system. In the conditioned media of Sf21 cells that had been infected with viruses containing the R218H, R218C, and the C225R mutant TGF- $\beta$ 1, the 100-kDa small latent complex was undetectable, and only the 86-kDa  $\beta$ 1-LAP homodimer was observed (Fig. 3), whereas a recombinant wild-type TGF- $\beta$ 1 produced the small latent form. These results suggest that conformational changes of  $\beta$ 1-LAP as a result of these domain-specific mutations lead to instability of the small latent form, which is consistent with the results observed in the CED cells (Fig. 1). An alteration in the activation of latent TGF- $\beta$ 1 in the CED cells is schematically presented in Fig. 5.

Finally, to study the effects of the  $\beta$ 1-LAP mutations on TGF- $\beta$ 1 activity, the cell growth of the CED and normal fibroblasts and the transfectants that produce mutant  $\beta$ 1-LAPs was

determined by [ $^3$ H]thymidine incorporation into DNA (18). The growth of R218H CED fibroblasts was slower than that of the normal cells. Although the mRNA expression levels of the introduced TGF- $\beta$ 1 gene were similar (data not shown), the proliferation rates of R218H-, R218C-, and the C225R-TGF- $\beta$ 1 gene-transfected cells were slower than the empty vector or wild-type-TGF- $\beta$ 1 gene-transfected cells (Fig. 4A). Furthermore, the suppression of growth in the CED cells and the cells to which mutant genes had been introduced were attenuated by a neutralizing antibody against TGF- $\beta$ 1 (Fig. 4A). There are some reports that experiential administration of dexamethasone and other steroids is found to be effective to CED patients (19). In addition, *in vivo* and *in vitro* studies have indicated that treatment of dexamethasone brings about decrease of mRNA level of TGF- $\beta$ 1 (20, 21) and that glucocorticoids alter bone cell activity and decrease bone mass by changing the local TGF- $\beta$ 1 actions in skeletal tissues (1, 2). Therefore, we studied the effect of dexamethasone on the mRNA level of TGF- $\beta$ 1 and the proliferation of R218H CED fibroblasts. The mRNA levels of TGF- $\beta$ 1 in R218H CED and normal fibroblasts were reduced (Fig. 4B), and the suppression of growth in the CED fibroblasts was attenuated by the treatment of dexamethasone in a dose-dependent manner (Fig. 4C). On the other hand, the proliferation of human osteoblastic MG-63 cells (22), which were cocultured with R218H CED fibroblasts, was accelerated, and this acceleration was attenuated by treatment of dexamethasone (Fig. 4D). Dexamethasone did not affect the growth of MG-63 cells when they were cultured alone (data not shown). These results suggest that TGF- $\beta$ 1 released from the CED fibroblast stimulated the cell growth of osteoblasts. Overall, these data indicate that the  $\beta$ 1-LAP/TGF- $\beta$ 1 mutations observed in CED patients modulate TGF- $\beta$ 1 activity (Fig. 5). It is conceivable that a similar situation exists in bone tissues.

Previous studies have indicated a strict regulation of osteoblasts and osteoclasts in bone tissues and that many factors are involved in this process (23–25). TGF- $\beta$ 1 plays an important role in promoting bone matrix synthesis and in reducing matrix degradation and resorption in the diaphysis of long bones by inhibiting both the formation and activity of osteoclasts (1–5). The observations herein point to the importance of factors that regulate the activation of TGF- $\beta$ 1 in the bone matrix.

In conclusion, the domain-specific mutations in  $\beta$ 1-LAP/TGF- $\beta$ 1 play a role in Camurati-Engelmann disease by modifying the activation of TGF- $\beta$ 1. The present study may provide a new stimulus for investigating the pathophysiology and

treatment of CED, as well as related disorders such as osteoporosis.

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