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Alteration of the Kinetics of Type I Procollagen Synthesis in Human Osteosarcoma Cells by 1,25-Dihydroxyvitamin D_3

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Abstract The kinetics of type I procollagen synthesis in a human osteosarcoma cell line, MG 63, were investigated after treatment with 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2$ D_3), a hormonal inducer of phenotypic differentiation. Pulse label and chase experiments demonstrated greatly enhanced production and more rapid reduction of intracellular procollagen molecules in the 1,25-$(OH)_2$ D_3–treated cells as compared to the nontreated case. After a chase for 1 h, labeled procollagen was reduced by nine-tenths in 1,25-$(OH)_2$ D_3–treated cells, while half of the radioactivity still remained in nontreated cells. The expression rate of type I collagen, which was examined by pulse label experiment, was elevated in association with an increase in the mRNA coding for the type I collagen α1 chain by 1,25-$(OH)_2$ D_3 treatment. However, the amount of intracellular procollagen present after 4 h continuous labeling was almost the same, independent of the 1,25-$(OH)_2$ D_3 treatment. Thus, we conclude that strage of the molecule was not affected. The results therefore suggest an increase in both the synthesis and secretion of type I collagen. The 1,25-$(OH)_2$ D_3 treatment was also found to induce the α subunit of prolyl 4-hydroxylase and to be associated with an elevated level of hydroxyproline in the procollagen. Moreover, gelatinase B–resistant procollagen molecules, indicative of intracellular procollagen molecules in the stable triple helical form, were detected only in the 1,25-$(OH)_2$ D_3–treated cells. These data suggest more efficient proline hydroxylation is involved in rapid secretion of procollagen after hormone administration. The present evidence points to posttranslational control of procollagen synthesis. J. Cell. Biochem. 65:542–549.

Key words: procollagen synthesis; human osteosarcoma cells; 1,25-dihydroxyvitamin D_3; type I collagen; proline hydroxylation

A number of studies revealed the effects of 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2$ D_3) on collagen biosynthesis in many kinds of cells. This steroid hormone stimulates collagen synthesis in MC3T3-E1 mouse carvaria–derived cells [Kurihara et al., 1986], primary cultured human bone cells [Beresford et al., 1986], and a human osteosarcoma cell line, MG 63 [Franceschi et al., 1988]. However, this hormone has a catabolic or inhibitory effect on the collagen synthesis in bone organ culture of rat [Raisz et al., 1978; Canalis and Lian, 1985; Owen et al., 1991], chicken osteoblasts [Broess et al., 1995], rat osteosarcoma cells [Kream et al., 1986], and a mouse bone cell line [Walters et al., 1982]. This controversial manner of type I collagen synthesis regulation by 1,25-$(OH)_2$ D_3 is not well resolved, although its effect is considerable depending on the maturational stages of the osteoblast phenotype [Owen et al., 1991].

A human osteosarcoma cell line, MG 63, is considered to possess typical characteristics of

Abbreviations used: BAPN, β-aminopropionitrile; DMEM, Dulbecco's modified Eagle's medium; αPH, α subunit of prolyl 4-hydroxylase; 1,25-$(OH)_2$ D_3, 1,25-dihydroxyvitamin D_3.

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undifferentiated osteoblasts because it synthesizes large amounts of type III collagen but hardly produces alkaline phosphatase or osteocalcin, in line with an osteoprogenitor character [Heremans et al., 1978]. It has been demonstrated that 1,25-$(OH)_2$ D_3 selectively stimulates synthesis of α1 and α2 chains of type I collagen in MG 63 cells with increases in the respective mRNAs, indicating regulation at the transcriptional level [Franceschi et al., 1988]. However, synthesis of the α1 chain of type III collagen was not appreciably affected by the hormone treatment, although its mRNA was increased severalfold [Franceschi et al., 1988], suggesting the possibility of a complex regulation involving changes in translational or posttranslational steps.

Collagen biosynthesis is in fact known to be regulated at the posttranslation as well as transcription and translation levels [Kivirikko and Myllyla, 1987]. It is a general rule that newly synthesized secretory proteins, including procollagen, are not transported from the endoplasmic reticulum (ER) to the Golgi apparatus until they acquire the correct tertiary and quaternary structures [Hurtley and Helenius, 1989]. Collagen is first synthesized as larger preproalphachains with signal sequences and propeptides at both ends. Then sets of three proalphachains are wound around each other to form procollagen molecules with the correct triple helical structure in the lumen of the ER. It has been reported that certain posttranscriptional modifications of the polypeptides are required for the helix formation. The most important modification is the formation of 4-hydroxyprolines in the collagenous domains, because the hydroxyproline residues are indispensable for stable triple helix formation [Kivirikko and Myllyla, 1987]. Thus, procollagen polypeptides without a sufficient number of hydroxyproline components are not normally transported along the secretory pathway. The catalyst for the hydroxylation of proline residues is therefore a key enzyme in collagen biosynthesis. The present study focused on posttranslational processing of type I procollagen during 1,25-$(OH)_2$ D_3–induced phenotypic differentiation of MG 63 cells. Thus, the synthesis and processing of type I procollagen in both differentiated and undifferentiated MG 63 cells were examined using pulse label and chase experiments in relation to the hydroxylation of proline residues.

MATERIALS AND METHODS

Reagents and Antibodies

1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2$ D_3) was obtained from Wako (Tokyo, Japan). β-aminopropionitrile, p-aminophenylmercuric acetate (APMA), and bacterial collagenase (Form I) were purchased from Sigma (St. Louis, MO). Purified bacterial collagenase (Form III) was purchased from Advance Biofactures Co. (Lynbrook, NY). The radioisotope (Tran^{35} S-label™) was obtained from ICN (Irvine, CA). Goat antibody against type I collagen was purchased from Southern Biotechnology Co. Ltd (Birmingham, AL). Mouse monoclonal antibody against the human α subunit of prolyl 4-hydroxylase (αPH) was obtained from Fuji Chemicals (Toyama, Japan). Protein G–conjugated Sepharose Fast Flow was purchased from Pharmacia (Piscataway, NJ).

Cell Culture

MG 63 human osteosarcoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and passed using 0.05% trypsin–0.02% EDTA (Gibco BRL, Gaithersburg, MD).

In all experiments, the treatment of MG 63 cells with 1,25-$(OH)_2$ D_3 was performed as previously described [Franceschi et al., 1988]. Briefly, cells were plated at an initial density of 50,000 cells/cm^2. After 24 h, the medium was changed to DMEM containing 1% FBS and 10^{-7} M 1,25-$(OH)_2$ D_3. Then the cells were incubated in the medium for a further 48 h before use in the experiments.

Metabolic Labeling and Pulse Label and Chase Experiments

At 24 h before the labeling, 50 μg/ml ascorbate and 64 μg/ml β-aminopropionitrile (BAPN) were added to the medium. Then the cells were labeled with 1.85–3.7 Bq/ml Tran^{36}S-label in methionine-free DMEM supplemented with ascorbate, BAPN, and 1,25-$(OH)_2$ D_3 at 37°C.

After labeling, the cells were rinsed four times with phosphate buffered saline (PBS) and then incubated in 0.1% bacterial collagenase (Form III) solution at 37°C for 10 min in order to remove the extracellular collagen. Then 25 mM EDTA was added to block the collagenase activity. Cells were again rinsed with PBS and lysed

in a lysis buffer (2% NP-40, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM *N*-ethylmaleimide). After centrifugation at 12,000*g* for 15 min, the supernatant was used for the immunoprecipitation studies.

For pulse label and chase experiments, the cells were pulse-labeled for 15 min and chased for various times (0, 15, 30, 60 min) in the presence of excess methionine (20 mM).

Immunoprecipitation

Cell lysates of the same radioactivity were incubated in buffer containing 1% NP-40, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 5 mM EDTA with anti–type I collagen polyclonal antibody (5 µg) or anti-human αPH monoclonal antibody (10 µg) for 1 h at 4°C; then a 20 µl aliquot of protein G–Sepharose Fast Flow was added and incubated in the same condition. After rinsing three times with the same buffer, we eluted immunoprecipitates in Laemmli's sample buffer containing 5% 2-mercaptoethanol by boiling. One-dimensional SDS-PAGE was performed using 4% stacking and 7.5% or 10% resolving slab gels.

The radioactivity of the immunoprecipitated procollagen was quantified using a BAS 2000 RI-imaging system (Fuji Film Co. Ltd, Tokyo, Japan).

Collagenase Treatment

Immune complexes precipitated with protein G–Sepharose Fast Flow were suspended in 40 µl of collagenase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$) containing 20 U/ml of purified bacterial collagenase (Form III) and incubated at 37°C for 90 min. The samples were then boiled for 5 min in the sample buffer and applied to SDS-PAGE.

Gelatinase Treatment

Mouse gelatinase B (95 kDa gelatinase, MMP-9) was immunoaffinity-purified using anti-mouse gelatinase B rat monoclonal antibody conjugated to Sepharose 4B from serum-free conditioned medium of a mouse colon carcinoma cell line [Sakata et al., 1996]. Immune complexes precipitated with protein G–Sepharose were resuspended in buffer of 50 mM Tris-HCl, pH 7.5, and 10 mM $CaCl_2$ containing mouse gelatinase B (95 kDa gelatinase, MMP-9) and then incubated at 22°C for 24 h in the presence of 0.25 mM p-aminophenylmercuric acetate (APMA). The samples were then boiled for 5 min in the sample buffer and applied to SDS-PAGE.

Quantification of Hydroxyproline

Intracellular collagenous molecules, immunoprecipitated using anti–type I collagen antibody, were digested with bacterial collagenase as described above. These solutions were filtered using a 30,000 molecular weight cutting filter. The protein concentrations of collagenase-sensitive fractions were determined by the method of Waddell and Hill [1956] using the formula

$$\text{collagenase-sensitive precipitated protein content} = (\text{absorbance at 225 nm} - \text{absorbance at 215 nm}) \times 144 \text{ (mg/ml)}.$$

The hydroxyproline content in each fraction was quantified according to the method of Firschein et al. Statistical comparisons were made using the unpaired *t*-test.

Assays of mRNAs

Total RNAs were extracted from the cultured MG 63 cells under defined conditions according to the method of Chomczynski and Sacchi [1987]. The human αPH cDNA probe was previously described [Miyaishi et al., 1995]. The C-terminal region of the human α1 chain of type I collagen–specific cDNA probe (1,142 bp) was synthesized from total RNA of the human fibroblasts by reverse transcription using Superscript^{TMII} (Gibco BRL) and amplified by the PCR technique with the specific human α1(I) chain 20 primers: 1C11, 5′-CATAAAGGGTCACCGTGGCT-3′; 1C12, 5′-GTCCATGTGAAATTGTC-TCC-3′, 1C13, 5′-CTGGTGAACAAGGTCCCTCT-3′; and 1C14, 5′-GGTCA-TGTTCGGTTGGTCAA-3′. The amplification consisted of 30 cycles of the following steps: 94°C, 1 min; 54°C, 2 min; 72°C, 3 min. The 1C11 and 1C14 set of primers was used for the first step of amplification and the other set, 1C12 and 1C13, was used for the second amplification. Total RNAs extracted from 1,25-$(OH)_2$ D_3–treated and nontreated MG 63 cell cultures were electrophoresed in formaldehyde-agarose gels (1% w/v) and transferred to nylon membranes (Hybond-N+; Amersham,

Amersham, UK). Blotted membranes were prehybridized at 42°C for 4 h in 5× SSPE (0.15 M NaCl, 10 mM $NaHPO_4$, 1 mM EDTA) containing 50% (v/v) formamide, 5× Denhart's solution, 10% Dextran sulfate, 0.1% SDS (w/v), and 200 μg/ml denatured salmon sperm DNA and then hybridized with ^{32}P-labeled cDNA probes in the same solution for 20 h. After rinsing twice with 5× SSPE containing 0.1% SDS at room temperature, we thoroughly washed the membranes twice again with 1× SSPE containing 0.1% SDS for 30 min at 65°C.

RESULTS

The kinetics of newly synthesized procollagen in 1,25-$(OH)_2$ D_3–treated and nontreated MG 63 osteosarcoma cells were analyzed by pulse and chase experiments, in which the amounts of procollagen were examined by an immunoprecipitation method. After cells were radiolabeled for 15 min, chased with excess methionine, and lysed, analysis of type I collagen was performed using an immunoprecipitation technique. The supernatant after immunoprecipitation contained no more detectable antigen as revealed by reprecipitation with the same antibody (data not shown), ensuring the accuracy of the quantitative analysis. Since extracellular collagen on the cell surface had been removed by collagenase treatment before lysis of the cells, the bands detected in this analysis represent only intracellular procollagen. The radioactivity incorporated into intracellular procollagen bands within 15 min labeling in the 1,25-$(OH)_2$ D_3–treated cells was much higher than in nontreated cells (Fig. 1A, lanes 1,5). However, after a chase period of 60 min, the residual radioactivity in intracellular procollagens had become almost equal (Fig. 1A, lanes 4,8), indicating more rapid decrease of intracellular procollagens in the 1,25-$(OH)_2$ D_3–treated case. Radioactivities of procollagen bands were measured with an image analyzing system, and the results were summarized in Figure 1B. After a 60 min chase, the labeled procollagens decreased to one-tenth of the initial level in the 1,25-$(OH)_2$ D_3–treated cells, whereas about a half was still retained in the nontreated cells.

In order to estimate the amounts of intracellular procollagen in the steady state, we continuously radiolabeled 1,25-$(OH)_2$ D_3–treated and nontreated MG 63 cells for 4 h. Procollagen bands, detected as two bands for the α1 and α2 chains with molecular weights of 160 and 140 kDa, respectively, displayed similar intensities independent of the hormone treatment (Fig. 2, lanes 5,6), in clear contrast to the marked difference with 15 min pulse labeling (Fig. 2, lanes 2,3). These bands were completely digested by bacterial collagenase (Fig. 2, lanes 4,7), and these results suggest that the procollagen content of within cells in steady state is constant regardless of the 1,25-$(OH)_2$ D_3 treatment. The molecular weights of the procollagen polypeptides after 4 h labeling (Fig. 2, lanes 5,6) were larger than those observed with the 15 min pulse labeling (Fig. 2, lanes 2,3), presumably reflecting differences intracellular processing such as glycosylation.

Procollagen biosynthesis is regulated at many intracellular processing steps. The most crucial and rate limiting is hydroxylation of proline residues in the collagenous domain of the polypeptides that are cotranslationally catalyzed by the enzyme, prolyl 4-hydroxylase. We therefore assessed the hydroxyproline contents of the procollagen polypeptides in 1,25-$(OH)_2$ D_3–treated and nontreated cells. The concentration of hydroxyproline in immunoprecipitated type I procollagen was spectrometrically quantified according to the method of Firschein and Shill (1966). The 1,25-$(OH)_2$ D_3–treated cells demonstrated a value of 5.19 ± 0.46% (w/w) of collagenase-sensitive fractions, while in nontreated cells it was 4.50 ± 0.16%. This difference was statistically significant ($P < 0.01$).

Alteration of prolyl 4-hydroxylase expression associated with 1,25-$(OH)_2$ D_3 treatment was therefore investigated. The enzyme is a tetramer (α2β2) composed of α subunits with the catalytic sites and β subunits that are identical to the multifunctional enzyme, protein disulfide isomerase (PDI), synthesized in excess over the α subunit [Kivirikko et al., 1989, 1990]. Since the activity of the enzyme is therefore considered to be defined by the expression level of the α subunit of prolyl 4-hydroxylase (αPH), this latter was examined by immunoprecipitation using an anti-αPH monoclonal antibody. It was demonstrated that the expression of αPH protein was induced by 1,25-$(OH)_2$ D_3 treatment (Fig. 3), although nonspecifically bound polypeptides were present. In addition, elevation of the αPH mRNA level in 1,25-$(OH)_2$ D_3–treated cells could be clearly demonstrated by Northern blot hybridization (Fig. 4). We also examined type I collagen α1 chain expression and confirmed the induction by 1,25-$(OH)_2$ D_3

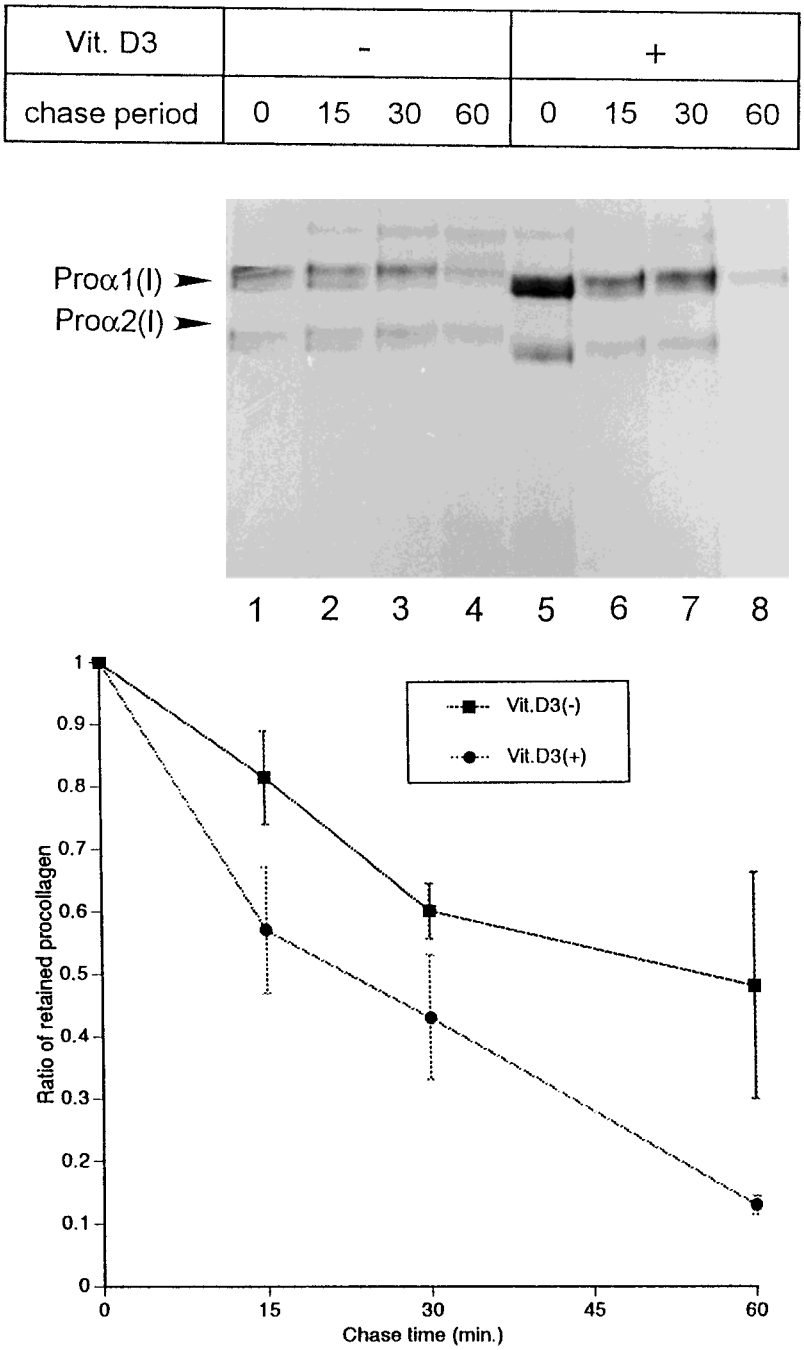

Fig. 1. Pulse and chase analysis of type I procollagen in 1,25-$(OH)_2$ D_3–treated and nontreated MG 63 human osteosarcoma cells. 1,25-$(OH)_2$ D_3–treated and nontreated MG 63 cells were labeled with Tran^{35}S-label for 15 min and chased in the presence of excess methionine for 15, 30, and 60 min. Cells were lysed, and the lysates were analyzed by SDS-PAGE after immunoprecipitation using anti–type I collagen goat polyclonal antibody as described in Materials and Methods. **A:** A representative fluorography of the results of SDS-PAGE. Type I procollagen was immunoprecipitated from nontreated cells (*lanes 1–4*) and 1,25-$(OH)_2$ D_3–treated cells (*lanes 5–8*). Immunoprecipitates from pulse-labeled cells (lanes 1,5) and the cells chased for 15 min (lanes 2,6), for 30 min (lanes 3,7), and for 60 min (lanes 4,8) are shown. **B:** Kinetics of procollagen synthesis in 1,25-$(OH)_2$ D_3–treated and nontreated cells. Pulse and chase experiments were performed as described above, and the procollagen radioactivities in three independent experiments were quantified using a BAS 2000 image analyzing system. Mean values and the standard deviations for the ratio of the radioactivity to that before chasing are shown.

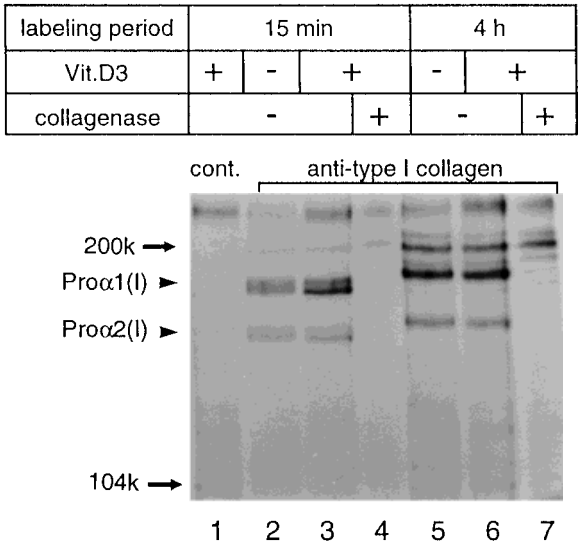

Fig. 2. Expression of type I procollagen in 1,25-$(OH)_2$ D_3–treated and nontreated MG 63 cells. Cells were radiolabeled with Tran^{35}S-label for 15 min or 4 h, and the expression of procollagen was investigated using immunoprecipitation with anti–type I collagen antibody. Immunoprecipitates from 1,25-$(OH)_2$ D_3–treated cells labeled for 15 min (*lanes 1,3,4*) and for 4 h (*lanes 6,7*) and from nontreated cells labeled for 15 min (*lane 2*) and for 4 h (*lane 5*) were analyzed by SDS-PAGE. Lane 1: control with nonimmune goat IgG; lanes 2–7: fractions binding to anti–type I collagen antibody; lanes 4,7: treated with bacterial collagenase.

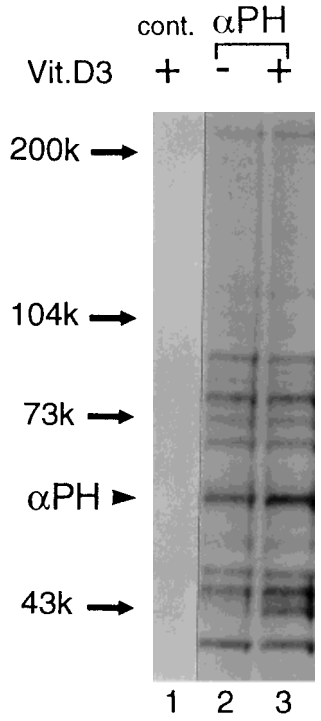

Fig. 3. Induction of the α subunit of prolyl 4-hydroxylase. 1,25-$(OH)_2$ D_3–treated and nontreated MG 63 cells were radiolabeled with Tran^{35} S-label for 4 h. SDS-PAGE was performed after immunoprecipitation using mouse monoclonal antibody against the human α subunit of prolyl 4-hydroxylase. *Lane 1:* control with nonimmune mouse IgG; *lanes 2,3:* fractions from nontreated cells and 1,25-$(OH)_2$ D_3–treated cells, respectively, binding to anti-αPH mouse monoclonal antibody.

observed in a previous study [Franceschi et al., 1988].

In order to investigate whether the molecular structure of intracellular procollagen was changed by 1,25-$(OH)_2$ D_3 treatment, we examined the sensitivity to digestion with gelatinase B (95 kDa gelatinase, MMP-9), which can cleave nonhelical collagenous polypeptides such as gelatin but not triple helical molecules [Amies and Quigley, 1995]. As shown in Figure 5 (lanes 1,4), heat-denatured intracellular procollagen was completely digested by gelatinase B irrespective of 1,25-$(OH)_2$ D_3 treatment. Without heat denaturation a faint band of procollagens resistant to gelatinase B digestion was detected in the 1,25-$(OH)_2$ D_3–treated cells (Fig. 5, lane 5) but not in the nontreated cells (Fig. 5, lane 2).

DISCUSSION

The present study clearly demonstrated increased production and reduction of type I procollagen of MG 63 cells when treated with 1,25-$(OH)_2$ D_3. Pulse label and chase experiments provided an evidence of enhanced incorporation and disappearance of the radioactivity (Fig. 1A,B), suggesting that newly synthesized procollagens are more effectively processed in the 1,25-$(OH)_2$ D_3–treated cells. Procollagen molecules, like other secretory proteins, are not secreted unless they are folded to correct structure [Kielty et al., 1993]. It is reasonable to suppose that the amount of pulse-labeled procollagen reflects the translation rate of the molecule and radiolabeled procollagen by continuous labeling reflects the stable-state level of intracellular procollagen. The results therefore indicate that 1,25-$(OH)_2$ D_3 treatment upregulates the flow rate of procollagen metabolism. One pathway by which procollagen disappears from cells is secretion via the Golgi apparatus and secretory vesicles, and the other is intracellular degradation [Bienkowski, 1983]. This latter could be increased in 1,25-$(OH)_2$ D_3–treated cells, but it is unlikely since no change in the degradation rate was observed in an earlier study of an osteoblastic cell line [Kurihara et al., 1986].

The induction of αPH and the increased content of hydroxyproline in the collagenous frac-

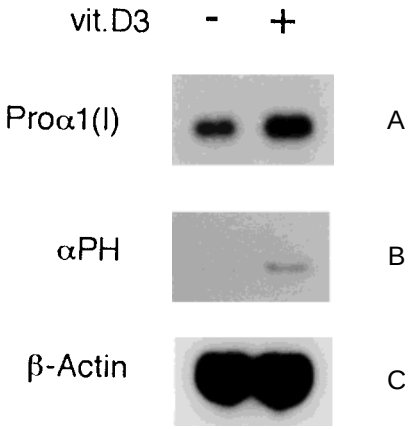

Fig. 4. Northern blot analysis of αPH and the α1 chain of type I collagen in 1,25-$(OH)_2$ D_3–treated and nontreated MG 63 cells. Total RNAs were extracted from 1,25-$(OH)_2$ D_3–treated and nontreated MG 63 cells, and expression of the α1 chain of type I collagen and αPH was analyzed. **A,B,C:** Northern blots of human α1 chain of type I collagen, αPH, and β-actin, respectively. For panels A and C, a 20 μg aliquot of total RNA was applied, and for panel B the amount was 50 μg.

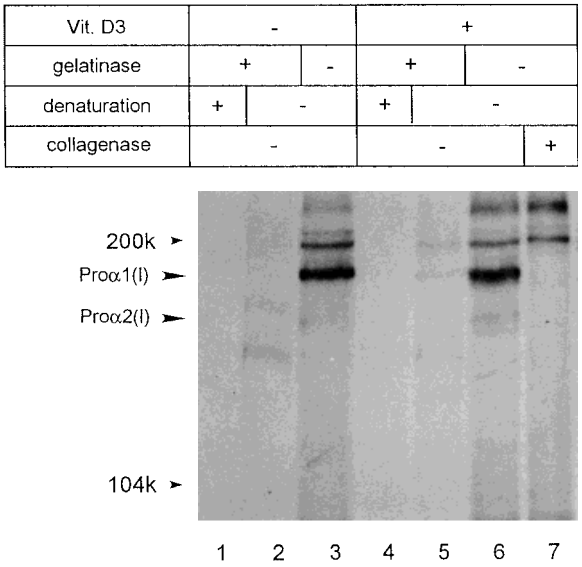

Fig. 5. Gelatinase digestion of intracellular procollagen. 1,25-$(OH)_2$ D_3–treated and nontreated MG 63 cells were labeled with $Tran^{35}$ S-label for 1 h, and cell lysates were immunoprecipitated with anti–type I collagen antibody. The immune complexes were treated with gelatinase B in 50 mM Tris-HCl buffer, pH 7.5, supplemented with 10 mM $CaCl_2$ and 0.25 mM of APMA at 22°C for 24 h and then analyzed by SDS-PAGE. *Lanes 1–3:* immunoprecipitates from nontreated cells; *lanes 4–7:* 1,25-$(OH)_2$ D_3–treated cells. Gelatinase digestion was performed after denaturation at 65°C for 15 min (lanes 1,4) and without denaturation (lanes 2,5). Lanes 3 and 6 show immunoprecipitates without gelatinase digestion, and lane 7 shows immunoprecipitate after collagenase digestion. Note the faint α1 chain band in lane 5.

tion of 1,25-$(OH)_2$ D_3–treated cells provide strong evidence of enhanced triple helix formation and subsequent rapid transport and secretion of correctly folded procollagen molecules. The finding of gelatinase B–resistant procollagen, although in small quantities, supports this conclusion. Correctly folded procollagen is in fact thought to be transported immediately from the ER to the Golgi apparatus and secreted without delay [Kielty et al., 1993], which might explain the faintness of the observed band and the fact it was observed only in the 1,25-$(OH)_2$ D_3–treated case. We conclude that all stages of procollagen processing are enhanced by the hormone.

It was previously reported that differentiation of mouse F9 teratocarcinoma cells involves an increase in mRNAs for the α and β subunits of prolyl 4-hydroxylase as well as marked increments in the mRNA and the rate of synthesis of type IV collagen [Helaakoski et al., 1990]. A differentiation-deficient mutant of the F9 teratocarcinoma cell line was found to produce prolyl hydroxylase with less activity, interfering with normal type IV collagen secretion and phenotypic expression after retinoic acid treatment, indicating that posttranslational modification of collagen is important for complete differentiation [Wang et al., 1989]. On the other hand, induction of differentiation features such as alkaline phosphatase and osteocalcin mRNA in MC3T3-E1 cells requires the presence of appropriately processed type I collagen as extracellular matrix [Franceschi and Iyer, 1992; Franceschi et al., 1994]. In another culture system using chicken embryo osteoblasts, extracellular matrix maturation was found to influence the processing kinetics of the collagen molecules [Gerstenfeld et al., 1988]. Thus, a complex relationship between the differentiation of osteoblastic cells and the posttranslational processing of collagen molecules may exist. For further clarification of the effects of 1,25-$(OH)_2$ D_3 on collagen biosynthesis, the role of the extracellular matrix, particularly with regard to prolyl 4-hydroxylase expression, therefore requires further investigation.

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