Regulation of Extracellular Matrix Production by Chemically Synthesized Subfragments of Type I Collagen Carboxy Propeptide†

Kou Katayama,* Jerome M. Seyer, Rajendra Raghow, and Andrew H. Kang
Veterans Affairs Medical Center and Departments of Biochemistry, Pharmacology, and Medicine, University of Tennessee, Memphis, Tennessee 38104

Received August 23, 1990; Revised Manuscript Received March 14, 1991

ABSTRACT: The complete COOH-propeptide of human α1(I) procollagen was chemically synthesized as a series of overlapping subfragments which were then tested for their effect on extracellular matrix protein production by subconfluent human lung fibroblasts (HFL-1). One peptide (R11; residues 197–241) stimulated production of both collagen and fibronectin by 6–8-fold while a second peptide with a partial overlap with R11 (R9; residues 182–216) enhanced collagen accumulation. The peptide R12 (residues 197–216), which has a sequence common to both R9 and R11, also stimulated collagen production, suggesting that this 20-residue peptide alone contains the required structure for activity. The other synthetic peptides, R1–R13, were inactive in their ability to alter collagen or fibronectin production. Consistent with previously published data, the COOH-terminal peptide, R14, inhibited extracellular matrix production [Aycock, R. A., Raghow, R., Stricklin, G. P., Seyer, J. M., & Kang, A. H. (1986) *J. Biol. Chem. 261*, 14355–14360]. Both R9 and R11 preferentially stimulated production of collagen types I and III and fibronectin in dose-dependent manner. Elevated collagen and fibronectin production was evident at 4-h posttreatment, and maximal enhancement was seen at 8 h after exposure to peptides. Interestingly, subconfluent cultures of HFL-1 fibroblasts responded vigorously to the stimulatory action of R9 and R11 while confluent cells failed to show any response. Steady-state levels of messenger RNAs encoding type I procollagen and fibronectin were not measurably altered by treatment with R9 or R11, suggesting that the regulation of procollagens and fibronectin by these peptides involves posttranscriptional mechanisms. Finally, we observed that both R9 and R11 inhibited serum-induced stimulation of DNA synthesis in cells grown under serum-deprived conditions. Possible molecular mechanisms by which COOH-terminal peptide fragments regulate cellular proliferation and extracellular matrix biogenesis are considered.

Type I collagen is a major component of the extracellular matrix from skin, bone, and tendon, and is the predominant collagen species produced during the postinflammatory regenerative processes. Human fetal lung fibroblasts (HFL-1) produce the same amount of collagen on a per cell basis during both log and stationary phases of growth (Tolstoshev et al., 1981); under these conditions, the level of type I collagen mRNA is also constant (Voss & Bornstein, 1986). Although type I collagen is constitutively produced by most mesenchymal cells, its rate of production can be altered by a variety of inflammatory mediators (Ignotz & Massague, 1986; Raghow et al., 1984, 1987; Penttinen et al., 1988; Rossi et al., 1988; Goldring & Krane, 1987; Postlethwaite et a., 1988; Solis-Herrozo et al., 1988; Choe et al., 1987; Brenner & Chojkier, 1987), by hormones (Raghow et al., 1986), and by neoplastic transformation (Sandmeyer et al., 1981). Regulation of collagen biosynthesis in response to altered biologic or pharmacologic stimuli occurs by a combination of transcriptional and posttranscriptional mechanisms [see Raghow and Thompson (1989) and Bornstein and Sage (1989) for reviews]. The concept that collagen-producing cells may have autoregulatory feedback control was initially suggested by Wiestner et al. (1979) and extended later by Paglia et al. (1979); they observed that NH_2-propeptides and subfragments preferentially inhibited collagen production by fibroblasts. Since translation of procollagen mRNA in a cell-free translation extract could be inhibited by NH_2-propeptides, it was suggested that the extension peptides were taken up by cells and affected some component of the protein synthesis apparatus. The uptake of colloidal gold labeled peptide via the coated pits into acid phosphatase negative endosomes was demonstrated by Schlumberger et al. (1988). Horlein et al. (1981) showed that the peptides inhibited polypeptide chain elongation. However, interpretation of these observations became complicated since it was found that reductive alkylation converted NH_2-propeptides into nonspecific inhibitors of polypeptide chain initiation. Nonspecific translational chain initiation inhibitory activity was localized to a tetrapeptide, Pro-Thr-Asp-Glu (Horlein et al., 1981).

More recently, analogous experiments aimed at determining the feedback regulation of cellular protein synthesis by COOH-propeptides and subfragments have been reported by Wu et al. (1986) and Aycock et al. (1986). While it was evident from these studies that COOH-propeptides and subfragments were capable of inhibiting collagen synthesis, the underlying mechanisms were different. Thus, Wu et al. (1986) provided evidence suggesting that inhibition of collagen synthesis by intact COOH-propeptides occurred at a pretranslational level; treatment of IMR-90 fibroblasts with COOH-propeptides reduced the level of type I procollagen mRNA but had no effect on β-actin mRNA levels. They likewise found that ^{125}I-labeled COOH-propeptide was taken up by cells during this process (Wu et al., 1991). In related studies, we found that a synthetic homologue of the terminal 22 amino acids of the COOH-propeptide of α2(I) procollagen (T14; residues 225–246) inhibited type I procollagen and fibronectin

† Supported in part by VA Medical Research funds and by a National Institutes of Health grant (AR39166).

* Correspondence should be addressed to this author at the Veterans Affairs Medical Center, Research Service (151), 1030 Jefferson Ave., Memphis, TN 38104.

0006-2960/91/0430-7097$02.50/0

production by HFL-1 fibroblasts (Aycock et al., 1986). In contrast to the observations of Wu et al., we proposed that posttranscriptional mechanisms mediated this effect since mRNA levels were unaffected by treatment with T14 peptide. To extend these studies, the complete COOH-propeptide of human α1(I) procollagen was chemically synthesized in a series of overlapping 16–45-residue peptides, and the individual peptide subfragments were tested for biological activity. We observed that although none of the remaining peptides (other than R14) contained inhibitory activity, to our surprise, a 35-residue segment adjacent to R14 stimulated collagen production. This effect was specific for fibronectin and collagen production only in subconfluent HFL-1 fibroblasts, and confluent monolayers were unaffected. There was no apparent change in the steady-state levels of mRNAs encoding type I procollagen or fibronectin in HFL-1 fibroblasts treated with R9 or R11, suggesting that enhanced expression of extracellular matrix proteins in response to COOH-propeptide subfragment occurred by posttranscriptional mechanisms.

Experimental Procedures

Materials and Cell Lines. Reagents, unless specified, were analytical grade and used without further purification. Methylene chloride and dimethylformamide for peptide synthesis was obtained from Baxter Scientific (HPLC grade, Burdick and Jackson). All other peptide synthesis reagents and protected *tert*-butyoxycarboxyl amino acids were from Applied Biosystems Inc., Foster City, CA. EN^{3}HANCE, ^{32}P-labeled nucleoside triphosphates, [^{35}S]methionine, and [^{3}H]proline were purchased from New England Nuclear, Boston, MA. RNAzol was purchased from CINNA/Biotecx, Friendswood, TX. Nitrocellulose paper (BA85) and the slot blot apparatus were bought from Schleicher & Schuell, Keene, NH. Human lung fibroblasts (HFL-1) was purchased from American Type Culture Collection, Rockville, MD. Human rhabdomyosarcoma cell line (A204) was a gift from Dr. Helene Sage, University of Washington, Seattle, WA.

Peptide Synthesis and Analysis. Peptides were synthesized by the solid-phase procedure using an Applied Biosystems automatic peptide synthesizer (Model 430) with anhydride activation. The peptides were recovered from the resin by HF cleavage and extraction with 5% acetic acid. They were purified by gel filtration on Sephadex G-25 columns with 0.1 M acetic acid and 20% acetonitrile as the eluting solvent and by HPLC (Aycock et al., 1986). Their composition was confirmed by amino acid analysis (Beckman Model 121MB) and amino acid sequencing (Applied Biosystems Model 477).

Cell Culture. Human lung fibroblasts (HFL-1, passages 11–20) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum. Cells were incubated in a humidified carbonated atmosphere (7% CO_2, 93% air) at 37 °C. Stock cultures were trypsinized and subcultured in 35-mm-diameter wells or 100-mm-diameter culture plates. Subconfluent cells were grown for 2 days after being seeded at a density of 2×10^5 cells/well or 1×10^6 cells/plate. Cells grown for 5 days after seeding reached superconfluency and were used as previously described (Tolstoshev et al., 1981). Quiescence after serum starvation was determined by counting the cell numbers in individual wells before and after measurements of [^{3}H]thymidine incorporation into cellular DNA.

Treatment with Synthetic Peptides, Radioactive Labeling, and Determination of Protein Synthesis. After removal of the media, the cell layer was washed 2 times with phosphate-buffered saline (PBS). The cell monolayers were then incubated with 1.0 mL of DMEM supplemented with β-aminopropionitrile (80 μg/mL) and the designated concentration of synthetic peptide (determined by amino acid analysis). At various times after treatment, 10 μCi of [^{3}H]proline and fresh ascorbic acid (50 μg/mL) were added to each well, and after a 4-h labeling period, the medium was harvested. Measurements of the intracellular proline pools were done in some cases. At the end of the labeling period, the cell monolayers were washed 2× with PBS, and cells were collected after being scraped. Total ninhydrin-positive proline was quantitated by split-stream amino acid analysis, and the specific activity of the radioactive proline was determined by scintillation spectrometry as described previously (Raghow et al., 1984). There were insignificant changes in the specific activity of the intracellular proline pool after peptide stimulation, and, therefore, such changes in the intracellular pool were not considered in our calculation of the rate of collagen production.

Protease inhibitors (final concentration: 10 mM *N*-ethylmaleimide, 20 mM EDTA, and 0.3 mM phenylmethanesulfonyl fluoride) were immediately added to the media, and proteins were precipitated by adding absolute ethanol to a concentration of 33% (v/v). After precipitation for 15 h at 4 °C, the samples were centrifuged at 10000*g* for 20 min. The pellet was dissolved in Laemmli buffer with or without 5% 2-mercaptoethanol and heated at 100 °C for 5 min; the polypeptide chains were then separated on a 7.5% SDS–polyacrylamide gel (Laemmli, 1970). Following electrophoresis of equal-sized aliquots of the labeled proteins, the gels were sequentially treated with 10% TCA and EN^{3}HANCE, and fluorographed. The fluorograms were scanned with an LKB laser densitometer coupled to a Hewlett-Packard 3390A integrator. For total protein biosynthesis, cells were labeled with [^{35}S]methionine in methione-free media; rates of synthesis of extracellular and cell-associated proteins were quantitated as described in detail previously (Raghow et al., 1986). Intracellular degradation was measured by the method described by Tolstoshev et al. (1981). Medium and cell layers after a 4-h pulse of [^{3}H]proline were combined, and half of the combined material was filtered through a Centricon 30 filter. The other half was dialyzed extensively. Aliquots of both the dialyzate and the filtrate were hydrolyzed with 6 N HCl, and hydroxyl[^{14}C]proline was quantitated by split-stream amino acid analysis (Raghow et al., 1984).

Isolation and Quantitation of Messenger RNAs. Total cellular RNA was extracted by the RNAsol method (Armendariz-Borunda et al., 1990), and the quality and quantity of RNA were routinely tested by determining the A_{260}/A_{280} ratio and by ethidium bromide fluorescence of RNA electrophoresed in agarose gels. Total mRNA was heat-denatured (60 °C for 5 min) in 50% formamide, 6% formaldehyde, and 20 mM phosphate buffer, pH 7.0. Beginning with 10 μg of mRNA in 150 μL, 2-fold serial dilutions were made by adding 10× NaCl/citrate (3 M NaCl/0.3 M sodium citrate), 3% formaldehyde, and 20 mM phosphate buffer, pH 7.0. Samples were applied to nitrocellulose sheets premoistened with 10× NaCl/citrate using a slot blot apparatus, baked at 80 °C under vacuum, and either stored under vacuum or immediately subjected to prehybridization. The prehybridization and hybridization protocols were essentially as described previously (Raghow et al., 1984). Radiolabeled nick-translated recombinant DNA plasmids [specific activity $(1–5) \times 10^7$ cpm/μg] were hybridized under conditions of excess probe. After removal of the probe (Thomas, 1987), blots were subjected to a second round of prehybridization and hybridization with a second probe. Methodology involved in the analysis of Proα1(I) procollagen, fibronectin, and β-actin mRNAs using

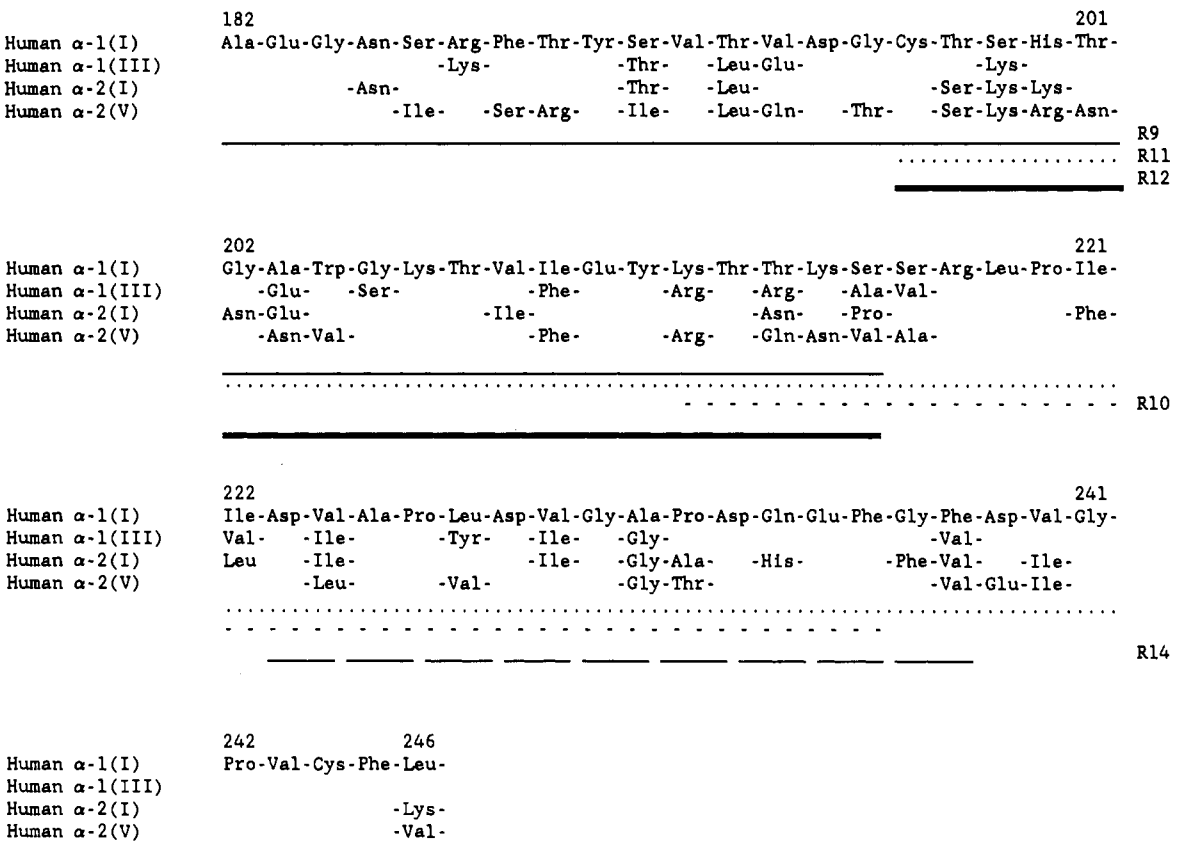

FIGURE 1: Comparative analysis of the sequence of COOH-propeptide subfragments R9 (residues 182–216) and R11 (residues 197–241) with homologous regions from various interstitial collagens. These sequences represent amino acids 182–246 of COOH-propeptides. Amino acid 1 was arbitrarily defined as the NH_2-terminal Asp of the COOH-propeptide cleavage site. Amino acid residues contained within R9 (—), R10 (- - -), R11 (· · ·), R12 (═), and R14 (— —) are highlighted. References for sequence information can be found in the text.

recombinant cDNA probes has been described in previous publications (Raghow et al., 1984, 1986; Aycock et al., 1986).

Measurements of the Rate of [^{3}H]Thymidine Incorporation in Cells Treated with COOH-Propeptide Subfragments. For determining [^{3}H]thymidine incorporation, we used a procedure described previously (Postlethwaite et al., 1988) with minor modifications. Briefly, HFL-1 fibroblasts were plated in 96-well plates (10000 cells/well) in media containing 10% FCS. Two days later, cells were washed with PBS and incubated for 48 h in media either with 0.2% serum or without serum. Quiescent cell cultures were washed twice with PBS and incubated in media supplemented with different concentrations of serum, with or without the propeptide subfragment. Cells were incubated for an additional 24 h, the last 8 h of which were in the presence of [^{3}H]thymidine (0.5 μCi/well). Monolayers were washed 2× in phosphate-buffered saline, trypsinized, and harvested mechanically. Cell-associated radioactivity was determined by scintillation spectrometry.

Results

Synthesis and Characterization of the Subfragments of COOH-Propeptide. A series of 16–45-residue subfragments (R1–R14) encompassing the complete 246 amino acid sequence of the COOH-propeptide from human procollagen α1(I) chain were chemically synthesized and tested for their biological activities (Table I). The peptides were designed to contain at the most only one Cys residue to limit mixed-disulfide formation. Some peptides (R10–R13) contained overlapping sequences and were designed to identify the specific structure required for stimulatory activity. Consistent with previously published data, the COOH-terminal subfragment of procollagen α2(I) (T14) (Aycock et al., 1986) and the COOH-terminal subfragment of procollagen α1(I) (R14) inhibited collagen production. A control peptide representing residues 1–16 had no effect as has been previsouly demonstrated (Aycock et al., 1986). Of all the remaining synthetic peptides tested, two, designated R9 and R11, showed significant biological activity and were studied more extensively. R12 which contained the common sequences in R9 and R11 was also stimulatory but was not examined further. These two peptides are located adjacent to and overlapping with peptide R14. The regions of the COOH-propeptide represented in peptides R14, R9, and R11 are highly conserved. The analogous sequences of Proα1(I), Proα2(I), Proα1(III), and Proα2(V) from humans are compared in Figure 1. Proα1(I) sequences represented in peptides R9 and R11 share strong sequence identities with human Proα1(III) (69 and 56%, respectively); the analogous regions in Proα2(V) are more diverged (i.e., 46 and 54%, respectively). The COOH-peptide regions of types I and III collagen chains also share strong interspecific sequence homologies; thus, there are 91 and 86% amino acid identities between human Proα1(I) and chicken Proα1(I) regions encompassed in R9 and R11, respectively (data not shown).

Production of Types I and III Procollagen and Fibronectin Is Enhanced by R9 and R11 in Subconfluent Fibroblasts. Synthetic peptides were added to subconfluent cultures of HFL-1 fibroblasts, and accumulation of extracellularly released polypeptides was evaluated at various intervals after [^{3}H]proline radiolabeling. A comparative analysis of the effect of collagen and fibronectin synthesis in HFL-1 fibroblasts treated with R9, R11, or an inactive 32-residue peptide, R1 (residues 16–47), is shown in Figure 2. On the basis of our previous experience with T14, we chose to treat cells with these

Table I: Biological Activity of Chemically Synthesized Subfragments of the COOH-Propeptide from Human α1(I)[a]

peptide synthesized	residues	peptide length (residues)	activity
R1	16–47	32	none
R2	43–63	21	none
R3	57–73	17	none
R4	66–81	16	none
R5	76–113	38	none
R6	108–137	30	none
R7	132–160	29	none
R8	155–188	34	none
R9	182–216	35	stimulate
R10	212–236	25	none
R11	197–241	45	stimulate
R12	197–216	20	stimulate
R13	178–198	21	none
R14	223–238	16	inhibit
T14[b]	225–246	22	inhibit

[a] The peptides were synthesized by the Merrifield solid-phase procedure, cleaved with liquid HF, and purified. Synthesis of extracellular matrix production by HFL-1 fibroblasts in response to a given peptide was determined as outlined under Experimental Procedures. Residue 1 was arbitrarily assigned to Asp at the COOH-propeptide cleavage site of α1(I) procollagen. [b] T14 peptide was a synthetic copy from α2(I) procollagen previously shown to inhibit extracellular matrix production. The sequence of T14 is homologous to R14 in Proα1(I); earlier studies also showed that a peptide containing residues 1–16 contained no regulatory activity (Aycock et al., 1986).

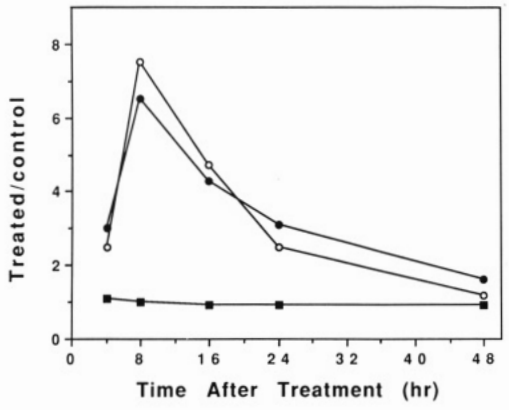

FIGURE 2: Regulation of procollagen production in HFL-1 fibroblasts incubated with COOH-propeptide subfragments. Subconfluent HFL-1 fibroblasts were cultured in medium without or with peptides R9 and R11 and a control peptide (R1) for the indicated times. After 4-h labeling with [^{3}H]proline (10 μCi), the radiolabeled polypeptides released in the media were processed for SDS–polyacrylamide gel electrophoresis, fluorographed, and quantitated by densitometry. Results were expressed as treated/control. Effect of COOH-propeptide 182–216 (R9) on Proα1(I) production (○); effect of COOH-propeptide 197–241 (R11) on Proα1(I) production (●); effect of control peptide 16–43 (R1) on Proα1(I) production (■). Under these conditions of electrophoresis, Proα1(I) and Proα1(III) comigrate, and thus the effect of the peptide on either type I or type III procollagen alone cannot be distinguished. Standard error of triplicate <10%.

peptides at a concentration of 45 μM (Aycock et al., 1986). Stimulated production of both procollagens and fibronectin was apparent within 4 h, and the maximum effect occurred by 8 h (6–8-fold, Figure 2); by 24 h, the overall rate had declined to about 2–3-fold compared to untreated cells. The temporal pattern of maximum stimulation observed at 8 h followed by a decline to near-base-line synthesis was highly reproducible. Administration of additional peptide and/or vitamin C into pretreated cells failed to restimulate collagen and fibronectin production (data not shown). There was no significant change in the level of collagen and fibronectin production between cells treated or untreated with the control peptide R1. Even more significantly, there were differences

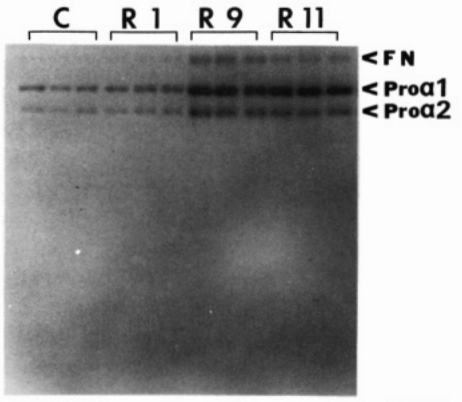

FIGURE 3: COOH-Propeptide subfragments R9 and R11 stimulate procollagen and fibronectin production. Triplicate cultures of subconfluent cells were incubated with 45 μM COOH-propeptide fragment R9, R11, or a control peptide R1. Untreated parallel cultures were incubated in growth medium only (C) for 8 h. In the final 4 h of incubation, cells were labeled with 10 μCi of [^{3}H]proline. Extracellularly released polypeptides in the medium were analyzed by SDS–polyacrylamide gel electrophoresis and fluorographed.

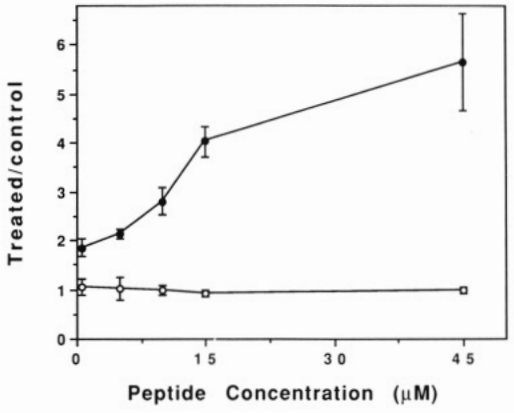

FIGURE 4: Carboxy propeptide subfragment R11 stimulates procollagen production in a dose-dependent manner. Subconfluent HFL-1 fibroblasts were incubated with 0, 0.5, 5, 10, 15, and 45 μM either R1 or R11 for 8 h, the last 4 h of which were in the presence of 10 μCi of [^{3}H]proline. Polypeptides released in the media were analyzed by SDS–polyacrylamide gel electrophoresis, fluorographed, and quantitated by densitometry. Results are expressed as the ratio of collagen production in treated/untreated cultures. Effect of R11 on Proα1(I) production (●); effect of R1 on Proα1(I) production (○). Under the conditions of electrophoresis, Proα1(I) and Proα1(III) comigrate, and thus the effect of the peptides on either type I or type III procollagen alone cannot be distinguished. Data are shown in mean ± standard error (n = 3).

neither in the specific activity of [^{3}H]proline nor in the rates of intracellular degradation after treatment with R9 or R11 (data not shown).

A comparison of the effect of three different peptides (i.e., R1, R9, and R11) on triplicate cultures is presented in Figure 3 in order to demonstrate the consistency and reproducibility of this effect. Densitometric quantitation of these autoradiographs indicated that while R1 did not affect collagen biosynthesis significantly, R9 and R11 enhanced collagen production by more than 6-fold; furthermore, a quantitative variation of less than 10% was observed in the triplicate cultures shown in Figure 3 (data not shown).

The optimal stimulatory response of HFL-1 fibroblasts to treatment with R9 and R11 peptides at 8-h posttreatment was dose-dependent in the concentration range of 0–50 μM as shown in Figure 4. Although data for only R11 are presented in Figure 4, both R9 and R11 stimulated collagen production at all concentrations tested; a control peptide R1 failed to exhibit this effect, demonstrating the specificity of the R9 and

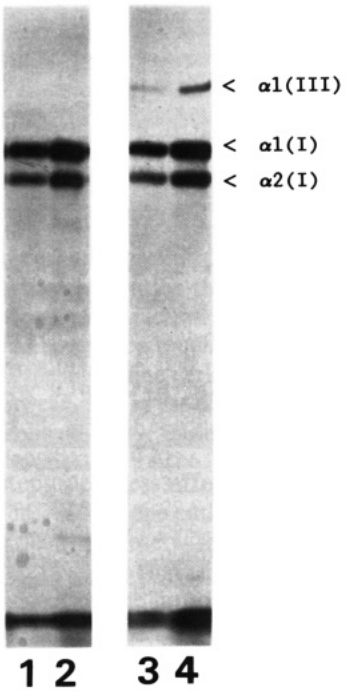

FIGURE 5: Treatment of HFL-1 fibroblasts with R11 enhances biosynthesis of both type I and type III procollagens. Subconfluent monolayers were incubated with 45 μM concentration of R11 for 8 h, the last 4 h of which were in the presence of [^{3}H]proline (10 μCi/well). Extracellular polypeptides were precipitated, treated with pepsin (1 mg/mL), and analyzed by interrupted gel electrophoresis to resolve types I and III α-chains. Polypeptide chains were visualized by fluorography. Pepsin-treated collagenous proteins from control (lanes 1 and 3) and R11-treated (lanes 2 and 4) analyzed by either conventional (lanes 1 and 2) or interrupted gel electrophoresis (lanes 3 and 4) are shown.

R11 subfragments. Additional information not presented in Figure 4 is the effect of R9 and R11 on fibronectin production, which was similarly enhanced by both peptides; a 2–3-fold enhancement at 15 μM and a 5–6-fold stimulation at 45 μM of both peptides were observed (data not shown).

We also investigated whether types I and III collagens were similarly affected by R9 and R11 subfragments. In order to evaluate the effect of COOH-terminal propeptide fragments on collagen types I versus III, extracellularly released polypeptides were processed and separated by interrupted gel electrophoresis (Sykes et al., 1976), and autoradiograms were quantitated by densitometry. We found that R11 treatment of HFL-1 cells induced a greater than 4-fold increase in the production of both interstitial collagen types (Figure 5). On the basis of densitometric quantitation, we determined that the relative proportion of types I versus III remained the same (95:5%) in R11-treated cells (data not shown); therefore, we conclude that both R9 and R11 had an equal effect on the production of the two collagen types. Although not presented here, a similar effect of R9 on types I and III collagen production was also observed.

Production of Procollagen and Fibronectin in Confluent Fibroblasts Is Unaltered by R9 and R11 Subfragments. In a preliminary experiment, we observed that if confluent fibroblast cultures of HFL-1 cells were treated with either 45 μM R9 or 45 μM R11, contrary to the results with subconfluent cultures (Figures 2–5), no elevation in collagen or fibronectin production was apparent even at very high concentrations (135 μM) or R9 or R11 (data not shown). This was somewhat unexpected and of considerable interest to us since in previous studies we had not considered the effect of cell density on cellular response to COOH-terminal propeptide

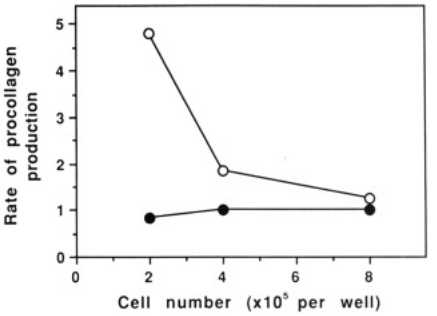

FIGURE 6: COOH-Propeptide subfragment R11 affects type I procollagen production in a density-dependent manner. HFL-1 fibroblasts were cultivated at a density of 2×10^5, 4×10^5, or 8×10^5 cells/well. After 2 days in culture with DMEM alone, cells were further incubated in media with or without COOH-propeptide 197–241 (R11) for 8 h, the last 4 h of which included [^{3}H]proline. Extracellularly released polypeptides were electrophoretically separated, fluorographed, and quantitated by laser densitometry. Rates of procollagen production in cells treated with (○) or without (●) R11 are shown. The respective cell numbers in wells plated at different densities at the end of the experiment are the following: control, $(2.02 \pm 0.46) \times 10^5$; treated, $(1.89 \pm 0.30) \times 10^5$; control. $(4.72 \pm 0.56) \times 10^5$; treated, $(4.00 \pm 0.42) \times 10^5$; control, $(8.24 \pm 0.68) \times 10^5$; treated, $(7.52 \pm 0.09) \times 10^5$. Cell viability as determined by trypan blue was >95% in all cultures. The rate of procollagen production in control cells is expressed as 1, which represents a ratio of densitometry unit/final cell number. Standard error ($n = 3$) <10%.

fragments (Aycock et al., 1986). Therefore, we reexamined the influence of the inhibitory peptide R14 [the Proα1(I) homologue corresponding to T14; Aycock et al., 1986] in subconfluent cultures and observed that the inhibitory effect occurred regardless of the state of confluency of the HFL-1 monolayers.

To further substantiate and examine this phenomenon in greater detail, the stimulating effects of R9 and R11 on procollagen and fibronectin production were systematically assessed in cells seeded at predetermined densities. These analyses revealed that the ability of COOH-terminal propeptide fragments to regulate procollagen production in HFL-1 fibroblasts was remarkably density-dependent. Thus, cells seeded at a density of 2×10^5 cells per well were optimally stimulated by peptide R11 while cells seeded at higher densities showed little or no response (Figure 6). The effect of these peptides on fibronectin production was similarly dependent on cell density (data not shown). On the basis of these analyses, we conclude that in contrast to the inhibitory effect of R14 (and T14; Aycock et al., 1986), the stimulatory effects of R9 and R11 on extracellular matrix production are quite specific for subconfluent fibroblasts.

Effect of R9 and R11 Subfragments Is Specific for Procollagen and Fibronectin. To determine if the COOH-propeptide subfragments R9 and R11 affected extracellular matrix proteins preferentially or influenced the overall rate of protein synthesis of HFL-1 fibroblasts, subconfluent cultures were treated as before and labeled with [^{35}S]methionine or [^{3}H]proline. Radiolabeled cell-associated or extracellularly released polypeptides were electrophoretically size-fractionated, fluorographed, and densitometrically quantitated. As shown in Table II, the rate of production of procollagen and fibronectin in cultures treated with R9 or R11 was enhanced, regardless of the radioactive precursor used to analyze the extracellularly released polypeptides. We noticed that accumulation of two unknown, cell-associated polypeptides (56 and 46 kDa) and one extracellularly released polypeptide (46 kDa) remained unchanged after treatment with R11. Thus, there were little differences between control and treated samples with regard to [^{35}S]methionine-labeled cell-associated polypeptides.

Table II: Production of Cell-Associated and Extracellularly Released Matrix Polypeptides Is Enhanced by COOH-Propeptide Subfragment R11 (Residues 197–241) in Human Lung Fibroblasts[a]

protein	relative absorbance units: control	relative absorbance units: treated	x-fold change
intracellular ([^{35}S]methionine)			
fibronectin	1.474	1.494	1.0
56 kDa	3.496	3.269	0.9
46 kDa	0.964	1.031	1.2
extracellular ([^{35}S]methionine)			
fibronectin	4.519	7.039	1.6
proα1(I)	2.144	5.067	2.4
46 kDa	2.534	2.081	0.8
extracellular ([^{3}H]proline)			
fibronectin	0.130	0.799	6.1
Proα1(I)[b]	1.156	3.836	3.3

[a] HFL-1 monolayers containing equal numbers of cells were treated with R11 (45 μM) for 4 h; cultures were supplemented with either [^{3}H]proline or [^{35}S]methionine during the last 4 h of incubation. Labeled cellular or extracellular proteins were size-fractionated on Laemmli gel and scanned with an LKB laser densitometer and signals processed on a Hewlett-Packard 3390A integrator. Multiple exposures of the fluorographs were scanned to assure that the polypeptide bands selected for quantitation were within the linear range of detection. Relative absorbance units represent the areas determined by the integrator. Results are expressed as the ratio of treated versus control. The 56- and 46-kDa bands were selected as representatives of prominent noncollagenous components synthesized by HFL-1 cells. [b] Under the electrophoretic conditions used here, Proα1(I) and Proα(III) comigrate, and, therefore, these values represent combined accumulation of these two polypeptides.

A substantial enhancement of collagen and fibronectin production in response to R9 (2–8-fold) was similarly observed (data not shown). Enhancement in the production of extracellular matrix polypeptides in response to R9 or R11 was also observed when much shorter pulses (e.g., 15 min to 1 h) of radioactivity were used. On the basis of these data, we conclude that (i) the effect of COOH-propeptide fragments is restricted to extracellular matrix polypeptides and (ii) the predominant step of their action is synthesis rather than export or degradation.

COOH-Propeptide Subfragments Do Not Alter Steady-State Levels of Procollagen and Fibronectin mRNAs. The levels of procollagen α1(I) and fibronectin mRNAs were quantitated to determine the mechanism of the altered rates of protein production in subconfluent HFL-1 fibroblasts. We quantitated the relative levels of Proα(I), fibronectin, and β-actin mRNAs in R9- and R11-treated and in untreated fibroblasts. Serially 2-fold-diluted samples of total cellular RNA were immobilized on nitrocellulose and hybridized to nick-translated recombinant plasmids containing appropriate cDNA sequences. In some experiments, total mRNA from control or treated HFL-1 fibroblasts was transferred to nitrocellulose for Northern blotting and similarly hybridized (data not shown). The relative steady-state levels of procollagen and fibronectin mRNA did not change after treatment with R9 (Table III) or R11 (data not shown). Results from three independent measurements of steady-state levels of mRNAs show that the action of COOH-propeptide subfragments does not involve an increase in the level of cognate mRNAs of the target extracellular matrix proteins.

COOH-Propeptide Subfragments Inhibit Serum-Induced DNA Synthesis in HFL-1 Cells Made Quiescent by Growth in Serum-Depleted Culture Medium. Since the extracellular matrix regulatory property of the COOH-propeptide subfragments was found to be dependent on the density of the cells in culture (Figure 6), we were curious to know if DNA synthesis and cellular proliferation were also affected by these peptides. Thus, we imposed quiescence on HFL-1 fibroblasts by incubating cells in serum-free medium for 48 h. Following serum deprivation, the cells were reincubated in media containing different concentrations of serum with or without 45 μM R11, and the incorporation of [^{3}H]thymidine was determined. As depicted in Figure 7, although R11 substantially inhibited serum-induced DNA synthesis at all serum concentrations, the inhibitory activity of R11 was partially reversed by higher concentrations of serum in the media. Thus, when cells were grown in 2.5% serum, R11 reduced [^{3}H]-thymidine incorporation by more than 80% whereas when the cells were incubated in media containing 20% FCS, the inhibitory activity of R11 was significantly less (35%). A similar pattern of inhbition of DNA synthesis was also observed when we used R9; the specificity of R9- or R11-mediated inhibition of DNA synthesis was substantiated by the observations that the control peptide R1 failed to exhibit a similar effect (data not shown). How the inhibition of serum-induced DNA synthesis and cellular proliferation are related to the post-transcriptional enhancement of collagen production by COOH-peptide fragments is currently unclear.

Table III: Steady-State Levels of Proα1(I), Fibronectin, and β-Actin mRNA Are Not Altered by Treatment with COOH-Propeptide Subfragment R9 (Residues 182–216)[a]

probe	% of control
Proα1(I)	113 ± 23
fibronectin	104 ± 16
β-actin	101 ± 12

[a] Total mRNA was isolated from fibroblasts incubated for 8 h with or without 45 μM R9, and serially diluted samples of mRNA were blotted onto nitrocellulose. Specific mRNAs were measured by hybridizing the blots to nick-translated cDNA probes representing Proα1(I), fibronectin, and β-actin. After autoradiography, the bands were quantitated by laser densitometry. Data averaged (± standard deviation) from three independent experiments are shown.

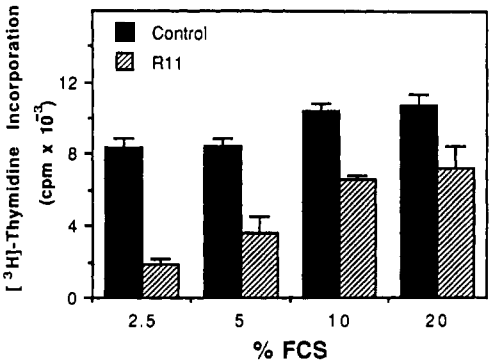

FIGURE 7: Effect of the COOH-propeptide subfragment 198–241 (R11) on DNA synthesis of HFL-1 fibroblasts. HFL-1 fibroblasts were seeded in 96-well plates (1×10^4 cells/well) using media containing 10% fetal calf serum. Two days later, cells were washed with PBS and incubated for 48 h in media without serum. Quiescent cells were washed with PBS, incubated in media containing 2.5, 5, 10, and 20% FCS without (open bars) or with 45 μM R11 (hatched bars) for 24 h, the last 8 h of which were in the presence of [^{3}H]thymidine (0.5 μCi/well). Monolayers were washed with PBS, trypsinized, and harvested onto paper filters with a multiple harvester. Final [^{3}H]-thymidine incorporation is expressed as the mean counts per minute with the standard deviation derived from triplicate samples. cpm of medium only = 466 ± 12. cpm of R11 only = 400 ± 10.

Discussion

A number of studies have suggested that the NH_2- or COOH-terminal propeptides of collagens, once removed from their parent procollagen molecules, may exhibit specific regulatory activities; included among these is the influence of propeptides on the synthesis of collagen itself. Intact NH_2- or COOH-propeptides of either Proα1(I) or Proα(III) chains

and enzymatically derived subfragments were shown to regulate collagen biosynthesis in cultured cells (Krieg et al., 1978; Wiestner et al., 1979; Paglia et al., 1979; Horlein et al., 1981; Goldenberg & Fine, 1985; Wu et al., 1986; Schlumberger et al., 1988). In a previous report, we presented data that a synthetic copy of residues 225–246 (T14) of the COOH-propeptide of human Proα2(I) inhibited collagen and fibronectin biosynthesis (Aycock et al., 1986). In the process of extending these studies, we noticed that a homologous peptide, R14 from the COOH-propeptide of human Proα1(I), also exhibited similar inhibitory properties while the NH_2-terminal fragment of the COOH-propeptide containing the first 16 residues had no activity. To further explore the sequence specificity needed for inhibition and to explain why the synthetic peptides were active posttranscriptionally as opposed to the transcriptional inhibition by intact COOH-propeptide (Wu et al., 1986), we synthesized a series of overlapping peptides spanning the complete COOH-propeptide of Proα1(I) and tested their biological effects. To our surprise, we found that two subfragments, referred to as R9 (residues 182–216) and R11 (residues 197–241), stimulated production of type I procollagen and fibronectin in human lung fibroblasts. A 20-residue peptide, R12, containing the overlapping sequences of R9 and R11 likewise stimulated collagen production. This observation is in contrast to all previous studies that showed inhibition of collagen production by the intact COOH-propeptides or its subfragments. To our knowledge, this is the first demonstration of a propeptide subfragment which is capable of stimulating extracellular matrix biosynthesis. The synthetic propeptide subfragments R9 and R11, in addition to stimulating procollagen (types I and III) biosynthesis, enhance the synthesis of fibronectin to an even greater extent. Fibronectin and collagen accumulation has been shown to be coordinately regulated in response to TGFβ (Ignotz & Massague, 1986; Raghow et al., 1987; Penttinen et al., 1988), to transformation by Rous sarcoma virus (Sandmeyer et al., 1981; Tyagi et al., 1982) or in cells treated with a synthetic subfragment of the COOH-terminal propeptide of the α2(I) chain (Aycock et al., 1986).

The propeptide subfragments studied here stimulate extracellular matrix proteins within a narrow window of time, i.e., maximally by 8 h with a rapid reversal of these rates to base-line levels by 48 h. Thus, kinetics of this response are in variance with TGFβ-treated fibroblasts in which enhanced rates of synthesis of extracellular matrix proteins occurred maximally at 24 h and effects persisted after 48–72 h (Fine & Goldstein, 1987). Interestingly, incubation of cells pretreated with COOH-propeptide fragments with additional peptide at later times, with or without ascorbic acid, did not restimulate extracellular protein biosynthesis (data not shown). This indicates that peptide degradation or depletion of medium of vitamin C levels is not the cause for this unusual kinetics of response. Currently, we are at a loss for a good explanation for this effect. We speculate that an unknown autoregulatory mechanism or a down-regulation of a "receptor" may be involved in this process. The finding that these peptides only affect extracellular matrix protein biosynthesis in subconfluent cells (rapidly growing) is rather unprecedented. In general, studies with NH_2- and COOH-propeptides or their subfragments were previously done with confluent cell monolayers, or in cell-free systems, and it is possible, therefore, that this phenomenon may have been missed (Krieg, 1978; Wiestner et al., 1979; Paglia et al., 1979; Horlein et al., 1981; Goldenberg & Fine, 1985; Wu et al. 1986; Schlumberger et al., 1988; Aycock et al., 1986). This observation highlights the underlying differences in collagen metabolism between the rapidly growing vs confluent cells. Investigations are currently in progress to elucidate the molecular mechanisms by which these peptides stimulate extracellular matrix accumulation only in subconfluent fibroblasts.

The effect of COOH-propeptide fragments on extracellular matrix biosynthesis appears to be specific, since accumulation of only procollagen types I and III and fibronectin was altered and the peptide fragments did not alter type V collagen production in A204 cells (a rhabdomyosarcoma cell line; Choe et al., 1987) regardless of their state of confluency (data not shown). We propose, therefore, that the effect of R9 and R11 may be due to their unique sequence specificity. While COOH-propeptides of types I and III collagen α chains share strong similarities with each other, their similarities to type V procollagen are weak. However, such an explanation cannot account for the observed effect of the COOH-propeptides on fibronectin synthesis since the latter shares no sequence homology with these propeptides. Interesting, this effect is neither cell-specific nor species-restricted since biosynthesis of types I and III procollagen and fibronectin in rapidly growing rat dermal fibroblasts or Ito cells was also stimulated by R9 and R11 (data not shown). We believe that COOH-propeptides act by posttranscriptional mechanisms since the steady-state levels of mRNA encoding type I procollagen and fibronectin remained unchanged. This observation is consistent with our previous findings and is in apparent contrast with the data of Wu et al., who reported that intact NH_2- and COOH-propeptides of type I procollagen inhibited procollagen synthesis in IMR 90 fibroblasts at the transcriptional level (Wu et al., 1986). This discrepancy may be due to differences in the target cells, their state of confluency, or the conformational difference between the intact propeptide and their synthetic subfragments used. An additional and related property of these peptides was their ability to inhibit the serum-mediated burst of DNA synthesis of quiescent fibroblasts. A precise relationship between these two biological responses remains to be established. This is especially difficult in light of earlier findings which suggested that the rates of collagen production in growing versus quiescent cells were similar (Tolstoshev et al., 1986; Voss & Bornstein, 1986).

Finally, it is worth reemphasizing the specificity of this effect; peptides R1–R8 which represent more than half of the COOH-propeptide possessed no detectable biological activity. R9 stimulated collagen synthesis whereas R14 inhibited activity. Intriguingly, R11, which contained the COOH half of R9 and all of R14, gave the maximum stimulation despite the presence of the inhibitory sequence of R14 in its sequence. Thus, it seems that if both stimulatory and inhibitory activities are present in the same peptide, the capacity to stimulate extracellular protein synthesis must be able to override any inhibitory effects. Conceivably, the conformation of R11 may be such that the inhibitory sequences become masked or altered in such a way as to void their effect. In this respect, it is interesting to note that R10, which contains a five amino acid overlap with R9, and all of the R14 had no regulatory effect, suggesting that only five amino acids were sufficient to override the inhibitory action of R14. Finally, R12, which consists of the COOH half of R9, did elevate matrix production while R13 was inactive. The minimum sequence/conformation of R12 that is suffucient to elevate matrix production is currently unknown.

In the present report, we have provided unprecedented evidence suggesting that some COOH-propeptide subfragments of the collagen molecule can up-regulate the production of the

three important extracellular matrix proteins: types I and III procollagen and fibronectin. The COOH-propeptide subfragments apparently work at physiological concentrations expected in vivo (Rhode et al., 1976). With regard to the question of whether subfragments of propeptides actually regulate procollagen and fibronectin production in vivo, we speculate that they may do so in rapidly growing cells during postinflammatory regenerative processes. We envisage that depending upon the unique proteolytic milieu at the site of inflammation, a variety of NH_2- or COOH-propeptide subfragments may be generated to yield either stimulatory or inhibitory peptides, exhibiting either positive or negative feedback control. A detailed analysis of the mechanisms by which proteolytic fragmentation of terminal propeptides occurs may help understand the pathogenesis of several collagen production abnormalities which accompany defects in the processing of COOH-propeptides (Peltonen et al., 1980; Bateman et al., 1989).

ACKNOWLEDGMENTS

These studies were initiated by Dr. Richard Aycock, whose enthusiasm propelled us to undertake a systematic analysis of COOH-propeptides. We acknowledge Dr. Hitoshi Katai and Kuniaki Terato for many helpful discussions. We also thank Kuldeep Patel for her expert technical assistance and Kathleen Pearson for the excellent secretarial help.

REFERENCES

Armendariz-Borunda, J., Seyer, J. M., Kang, A. H., & Raghow, R. (1990) *FASEB J. 4*, 215–221.

Aycock, R. S., Raghow, R., Stricklin, G. P., Seyer, J. M., & Kang, A. H. (1986) *J. Biol. Chem. 261*, 14355–14360.

Bateman, J. F., Lamande, S. R., Dahl, H. H. M., Chan, D., Mascara, T., & Cole, W. G. (1989) *J. Biol. Chem. 264*, 10960–10964.

Bornstein, P., & Sage, H. (1989) *Prog. Nucleic Acid Res. Mol. Biol. 37*, 67–106.

Brenner, D. A., & Chojkier, M. (1987) *J. Biol. Chem. 262*, 17690–17696.

Choe, I., Aycock, R. S., Raghow, R., Myers, J. C., Seyer, J. M., & Kang, A. H. (1987) *J. Biol. Chem. 262*, 5408–5413.

Fine, A., & Goldstein, R. H. (1987) *J. Biol. Chem. 262*, 3897–3902.

Goldenberg, R., & Fine, R. E. (1985) *Biochim. Biophys. Acta 826*, 101–107.

Goldring, M. B., & Krane, S. M. (1987) *J. Biol. Chem. 262*, 16724–16729.

Horlein, D., McPherson, J., Goh, S. H., & Bornstein, P. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6163–6167.

Ignotz, R. A., & Massague, J. (1986) *J. Biol. Chem. 261*, 4337–4345.

Krieg, T., Horlein, D., Wiestner, M., & Muller, P. K. (1978) *Arch. Dermatol. Res. 263*, 171–180.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Paglia, L. M., Wilezek, J., Diaz de Leon, L., Martin, G. R., Horlein, D., & Muller, P. (1979) *Biochemistry 18*, 5030–5034.

Peltonen, L., Palotie, A., & Prockop, D. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6179–6183.

Penttinen, R. P., Kobayashi, S., & Bornstein, P. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1105–1108.

Postlethwaite, A. E., Raghow, R., Stricklin, G. P., Poppleton, H., Seyer, J. M., & Kang, A. H. (1988) *J. Cell Biol. 106*, 311–318.

Raghow, R., & Thompson, J. P. (1989) *Mol. Cell. Biochem. 86*, 5–18.

Raghow, R., Gossage, D., Seyer, J. M., & Kang, A. H. (1984) *J. Biol. Chem. 259*, 12718–12723.

Raghow, R., Gossage, D., & Kang, A. H. (1986) *J. Biol. Chem. 261*, 4677–4684.

Raghow, R., Postlethwaite, A. E., Keski-Oja, J., Moses, H. L., & Kang, A. H. (1987) *J. Clin. Invest. 79*, 1285–1288.

Rhode, H., Nowack, H., Becker, U., & Timpl, R. (1976) *J. Immunol. Methods 11*, 135–145.

Rossi, P. Karsenty, G., Roberts, A. B., Roche, N. S., Sporn, M. G., & de Crombrugghe, B. (1988) *Cell 52*, 405–414.

Sandmeyer, S., Gallis, B., & Bornstein P. (1981) *J. Biol. Chem. 256*, 5022–5028.

Schlumberger, W., Thie, M., Volmer, H., Rauterberg, J., & Robenek, H. (1988) *Eur. J. Cell Biol. 46*, 244–252.

Solis-Herruzo, J. A., Brenner, D. A., & Chojkier, M. (1988) *J. Biol. Chem. 263*, 5841–5845.

Sykes, B., Puddle, B., Francis, M., & Smith, R. (1976) *Biochem. Biophys. Res. Commun. 72*, 1472–1480.

Thomas, P. S. (1983) *Methods Enzymol. 100*, 255–266.

Tolstoshev, P., Berg, R. A., Rennard, S. I., Bradley, K. H., Trapnell, B. C., & Crystal, R. G. (1981) *J. Biol. Chem. 256*, 3135–3140.

Tyagi, J. S., Hirano, H., Merlino, G. T., & Pastan, I. (1982) *J. Biol. Chem. 258*, 5787–5793.

Voss, T., & Bornstein, P. (1986) *Eur. J. Biochem. 157*, 433–439.

Wiestner, M., Krieg, T., Horlein, D., Glanville, R. W., Fietzek, P., & Muller, P. K. (1979) *J. Biol. Chem. 254*, 7016–7023.

Wu, C. H., Donovan, C. B., & Wu, G. Y. (1986) *J. Biol. Chem. 261*, 10482–10484.

Wu, C. H., Walton, C. M., & Wu, G. Y. (1991) *J. Biol. Chem. 266*, 2783–2987.