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Regulation of transformation-sensitive secreted phosphoprotein (SPPI/osteopontin) expression by transforming growth factor-β

Comparisons with expression of SPARC (secreted acidic cysteine-rich protein)

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Secreted phosphoprotein I (SPPI; osteopontin), a highly phosphorylated form of which has been associated with cell transformation, is one of the major phosphorylated proteins in bone. Populations of rat bone cells derived from fetal calvariae, neonatal parietal bone and a rat osteosarcoma cell line (ROS 17/2.8) produce several forms of the protein, the major forms having apparent molecular masses of 55 and 44 kDa by SDS/PAGE on 15% (w/v) cross-linked gels and of 60 and 56 kDa on 10% gels. Northern blot analysis of SPPI mRNA using total cellular RNA revealed a single 1.5 kb mRNA species, indicating that the nascent protein chains of these phosphoproteins are identical. On treatment of the cells with transforming growth factor-β (TGF-β; 1 ng/ml), the levels of SPPI mRNA and the synthesis of the 55 kDa phosphoprotein, but not of the 44 kDa phosphoprotein, were increased by 1.8–4.5-fold in the normal osteoblastic cells, the stimulation first being evident at 3 h and reaching a maximum at 12 h. In the transformed ROS 17/2.8 cells, TGF-β did not alter significantly the SPPI mRNA level or the synthesis of either the 55 kDa or the 44 kDa SPPI over the 24 h period studied. By comparison, neither the steady-state levels of SPARC (secreted protein, acidic, rich in cysteine) mRNA nor the synthesis of SPARC protein were affected significantly by the addition of TGF-β to any of the osteoblastic bone cells. The half-lives for SPPI and SPARC mRNAs in the osteoblastic calvarial cells were calculated to be 18 h and > 50 h respectively, in both the presence and the absence of TGF-β. Since the stability of the mRNA was unchanged by TGF-β and the increased expression of SPPI mRNA could be blocked by cycloheximide, TGF-β appears to increase transcription of the *Sppl* gene indirectly by stimulating the synthesis of a protein that promotes transcription. These results demonstrate that several forms of SPPI are synthesized constitutively by bone cells, and that there are clear differences in the regulation of *Sppl* gene expression by TGF-β in normal bone cells compared with the tumorigenic ROS 17/2.8 cells. The differential responses of normal osteoblastic cells to TGF-β in the expression of SPPI and the selective stimulation of specific forms of the SPPI protein may be important in bone repair and remodelling.

INTRODUCTION

One of the major phosphoproteins in rat bone has been identified as a 41.5 kDa phosphorylated glycoprotein that is characteristically rich in acidic amino acids and serine and contains 12 phosphoserine residues, one phosphothreonine residue and five or six *O*-linked and one *N*-linked oligosaccharide chains (Prince *et al.*, 1986). The amino acid sequence of this protein, derived from cDNA nucleotide sequence, has revealed a Gly-Arg-Gly-Asp-Ser cell-binding sequence, and consequently the protein has been given the name osteopontin (Oldberg *et al.*, 1986). Although tissue-extracted osteopontin can mediate the adhesion of various bone cells, including rat osteosarcoma (ROS 17/2.8) cells (Oldberg *et al.*, 1986), it also mediates the attachment of fibroblastic cells (Somerman *et al.*, 1987, 1989). Immunolocalization of the protein has shown a tissue distribution that is largely restricted to bone tissues, including osteoid, and to predentine and otoconia (inner ear), but its presence has also been demonstrated in neurosensory epithelial cells of the inner ear, neural cells of the inner ear and brain, and hypertrophic chondrocytes (Mark *et al.*, 1987, 1988*a*,*b*). A possible relationship between osteopontin and calcium metabolism is indicated by the tissue distribution of the protein and by the regulation of osteopontin gene expression by 1,25-dihydroxyvitamin D_3 (Prince & Butler, 1987; Yoon *et al.*, 1987), and by its presence in milk (Senger *et al.*, 1989). However, the osteopontin gene can also be induced by tumour promoters and growth factors in various cell types in culture (Smith & Denhardt, 1987), and studies of developmental expression have revealed osteopontin mRNA in several epithelial tissues and in the bone-marrow-derived metrial gland cells (Nomura *et al.*, 1988). More recently osteopontin has been shown to be similar to a transformation-related phosphoprotein, known in rat systems as pp69 (Craig *et al.*, 1988, 1989; Nemir *et al.*, 1989), the expression of which by cells and levels in serum have been correlated with tumorigenicity (Senger *et al.*, 1988). Since neither the physiological function of

Abbreviations used: SPPI, secreted phosphoprotein I; *Sppl*, gene for secreted phosphoprotein I; SPARC, secreted protein, acidic, rich in cysteine; *Sparc*, gene for SPARC; TGF-β, transforming growth factor-β1; RC cells, rat calvarial cells; ROS 17/2.8 cells, rat osteosarcoma 17/2.8 cells; FBS, fetal bovine serum; α-MEM, α-minimal essential medium; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole.

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this protein nor its role in carcinogenesis is known, the term 'secreted phosphoprotein I' (SPPI) has been suggested (Craig *et al.*, 1989; Fet *et al.*, 1989) and will be used here.

Transforming growth factor-β (TGF-β) is a 25 kDa homodimeric protein that can influence the growth, differentiation and activity of various cell types (Sporn *et al.*, 1987). It is relatively abundant in bone tissue (Seyedin *et al.*, 1987) and its synthesis by bone cells appears to be hormonally regulated (Petkovich *et al.*, 1987; Pfeilschifter & Mundy, 1987; Eriksen *et al.*, 1988; Komm *et al.*, 1988). While it is clear that TGF-β stimulates synthesis of collagen (Centrella *et al.*, 1986, 1987) and matrix protein (Wrana *et al.*, 1988; Overall *et al.*, 1989) by bone cells, its effects on the proliferation and differentiation of these cells are more complex. The preferential stimulation of type I collagen in rat calvarial (RC) cells (Wrana *et al.*, 1988) and the stimulation of alkaline phosphatase in rat osteosarcoma cells (Noda & Rodan, 1987) indicate that TGF-β may promote bone formation. However, other effects, including changes in morphology to a more fibroblastic shape, stimulation of fibronectin synthesis, a decrease in alkaline phosphatase (Wrana *et al.*, 1988), and the inhibition of mineralized tissue formation by fetal RC cells cultured in the presence of TGF-β (Antosz *et al.*, 1989), indicate a suppression of the osteoblastic phenotype. A recent report on the regulation of the synthesis of osteopontin (SPPI) and other matrix proteins by TGF-β in ROS 17/2.8 cells (Noda *et al.*, 1988) indicates that there may be fundamental differences in the response of transformed cells to TGF-β compared with their normal counterparts.

Although SPPI is not expressed exclusively by bone cells, its synthesis appears to be an important characteristic of cells of the osteoblast lineage. Therefore we initiated studies on SPPI expression by various bone cell populations so that we could evaluate further the effects of TGF-β on bone metabolism. Comparisons were made with another protein present in bone, SPARC (secreted protein, acid, cysteine-rich)/osteonectin (Termine *et al.*, 1981; Mason *et al.*, 1986*a*; Domenicucci *et al.*, 1988), hereafter referred to as SPARC. Although this protein is not specific to bone (Wasi *et al.*, 1984; Tung *et al.*, 1985), its expression by osteoblastic cells is regulated by TGF-β in a characteristic manner (Wrana *et al.*, 1988). In the course of these studies, it became evident that several forms of SPPI are expressed by bone cells and that TGF-β has a selective effect on their synthesis.

MATERIALS AND METHODS

Isolation and culture of bone cells

Rat bone cells from fetal calvariae and neonatal (2-day-old) rat parietal bone were isolated using methods described previously (Rao *et al.*, 1977; Bellows *et al.*, 1986). Bones were cleaned of periosteal and loose soft connective tissue and the sutures were cut away. Approx. 40 calvariae or parietal bones were digested sequentially at 37 °C with 5 ml portions of a digestion mixture containing bacterial collagenase. Digestion times of 10, 20, 30, 50 and 70 min were used to prepare four or five separate populations (I–V), populations IV and V being combined when cell yields were low. The individual populations were filtered through a 200 mesh Nitex sieve to remove debris and the cells were collected by centrifugation at 400 *g* for 5 min. The cells were then resuspended in α-minimal essential medium (α-MEM) containing 15% (v/v) fetal bovine serum (FBS) and antibiotics, plated in T-75 tissue culture flasks and grown to confluence.

The clonal osteoblastic cell line RCA 11 was isolated from population IV fetal RC cells (Ber *et al.*, 1991) using the limiting dilution procedure described by Aubin *et al.* (1982). These cells were characterized by the production of unusually high amounts of extracellular matrix and by osteoblastic features that include polygonal morphology, a moderate cyclic AMP response to parathyroid hormone and moderate alkaline phosphatase levels. The well-characterized rat osteosarcoma cell line ROS 17/2.8 was generously provided by Dr. G. A. Rodan (Merck, Sharp and Dohme, West Point, PA, U.S.A.) and Dr. R. J. Majeska (University of Connecticut Health Science Center, Farmington, CT, U.S.A.).

To study the synthesis of SPPI, confluent cells were trypsin-treated and $(1–3) \times 10^4$ cells were seeded into 35 mm culture dishes in triplicate and grown to confluence in α-MEM containing 15% (v/v) FBS. To prepare total RNA, $(1–2) \times 10^5$ cells were seeded into 100 mm culture dishes and grown to confluence. At confluence, $(3.5–6.5) \times 10^6$ cells were synchronized by incubation for 24 h in 1% (v/v) FBS before being incubated with 1.0 ng of TGF-β/ml (in 4 mM-HCl containing 1 mg of Pentex BSA/ml as vehicle) in α-MEM, or with vehicle alone, for 24 h (or as specified in the Results section) in a humidified incubator at 37 °C. The level of TGF-β1 (kindly provided by Dr. M. B. Sporn and Dr. A. B. Roberts, NIH, Bethesda, MD, U.S.A.) used in these studies (1 ng/ml) was shown previously to be optimal for stimulating matrix protein synthesis in human fibroblasts (Wrana *et al.*, 1986; Overall *et al.*, 1989) and fetal RC cells (Wrana *et al.*, 1988), and from preliminary experiments it was also shown to be optimal for phosphoprotein synthesis.

Radiolabelling procedures

To study the biosynthesis of phosphoproteins, confluent cells were labelled with $Na_3{}^{32}PO_4$ (specific radioactivity 1000 mCi/mmol; ICN Radiochemicals, Irving, CA, U.S.A.). At 2 h before labelling, the medium of the confluent cells was replaced with phosphate-free Dulbecco's minimal essential medium containing 25 mM-Hepes buffer, 0.2% (v/v) FBS, 29 μg of glutamine/ml and 50 μg of sodium ascorbate/ml. After preincubation, the medium was replaced with fresh medium containing $Na_3{}^{32}PO_4$ (100 μCi/ml) and the incubation was continued for 4 h. To analyse the synthesis of proteins by the bone cell populations, confluent cells were pulse-labelled for 30 min with 50 μCi of L-[^{35}S]methionine/ml (specific radioactivity 1100 Ci/mmol, ICN) in methionine-free Dulbecco's minimal essential medium supplemented with sodium ascorbate (50 μg/ml), as described previously (Wrana *et al.*, 1988). After the pulse period, the cells were washed twice in α-MEM containing 0.2% (v/v) FBS, and then incubated for a further 4 h at 37 °C in the same medium.

Immunoprecipitation procedures

Radiolabelled phosphoproteins and SPARC were immunoprecipitated using specific antisera raised in rabbits against rat SPPI (Prince *et al.*, 1986) and pig SPARC (Domenicucci *et al.*, 1988). In some experiments, affinity purified antibodies (Mark *et al.*, 1988*a*) were used, as indicated in the Results section. The procedure used has been described in detail previously (Otsuka *et al.*, 1984). Briefly, freeze-dried portions (usually 0.5 ml) of radiolabelled culture medium were mixed with an equivalent volume of immunoprecipitation buffer [0.3% (v/v) Nonidet P-40, 0.3% (v/v) sodium deoxycholate, 0.15% (w/v) BSA in 10 mM-Tris/HCl/0.15 M-NaCl (pH 7.4)/0.02% (w/v) NaN_3], and incubated with 5 μl of pre-immune serum in 1–5 ml plastic micro-centrifuge tubes for 2 h at 4 °C. Pansorbin (100 μl; Sigma) was added, incubation was continued for 1 h and the non-specifically precipitated material was removed by centrifugation at 10000 *g* for 5 min in a Microfuge (Eppendorf). The supernatant was quantitatively transferred to a second tube containing specific antibodies bound to Protein A–Sepharose and incubated for 16 h at 4 °C with gentle shaking. The immune

complexes were collected by centrifugation and washed at least three times in immunoprecipitation buffer. In some experiments the supernatant was used for a second immunoprecipitation with different antibodies. The radiolabelled protein in the specific immunoprecipitate was dissolved in 20 μl of electrophoresis sample buffer containing dithiothreitol and heated for 5 min at 95 °C.

SDS/polyacrylamide-gel electrophoresis

Radiolabelled proteins after separation were analysed by SDS/PAGE carried out in Tris/glycine buffers using 10% (w/v) and 15% (w/v) cross-linked gels and a mini-slab gel apparatus (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). The details of the electrophoresis have been described in earlier studies (Overall *et al.*, 1989). After electrophoretic separation, radiolabelled proteins were vizualized either by autoradiography (for ^{32}P-labelled proteins) using Kodak X-OMAT AR X-ray film with one intensifying screen at −70 °C for 1–7 days, or by fluorography of 2,5-diphenyloxazole-impregnated gels on Kodak SB-5 X-ray film at −70 °C. Quantification of radioactive bands was achieved from multiple exposures so that scanning densitometry at 633 nm, using an LKB Ultrascan XL laser densitometer, could be conducted in the linear photographic range (Wrana *et al.*, 1988). Molecular masses were estimated using the following [^{14}C]methylated protein standards: myosin (200 kDa), β-galactosidase (116.5 kDa), phosphorylase *b* (92.5 kDa), BSA (66.6 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

Western blotting of proteins extracted from fetal and adult calvarial bones was performed as described previously (Domenicucci *et al.*, 1988). Proteins separated by SDS/PAGE on 15% (w/v) cross-linked gels were transferred electrophoretically on to a nitrocellulose membrane, beneath which a modified nylon membrane (Zeta-Probe, Bio-Rad) was placed.

Chromatography on f.p.l.c. Mono Q

Samples of ^{32}P-labelled proteins were diluted 1:2 (v/v) with start buffer (7 M-urea/50 mM-Tris/HCl, pH 7.4) and applied to a 0.5 cm × 5.0 cm column of f.p.l.c. Mono Q resin (Pharmacia). The proteins were eluted with a linear salt gradient (0–1.0 M-NaCl) at a flow rate of 1.0 ml/min and collected in 1.0 ml fractions. After dialysis and freeze-drying, portions were digested with 1 unit of thrombin (Sigma) for 30 min at 37 °C in 50 mM-Tris/HCl buffer, pH 8.0, and the digestions were analysed by SDS/PAGE on 15% (w/v) cross-linked gels.

Analysis of mRNA by Northern hybridization

The following mouse cDNA probes were used to measure SPPI and bone SPARC mRNA levels, respectively: 2ar (Smith & Denhardt, 1987), generously supplied for these studies by Dr. D. T. Denhardt and Dr. A. M. Craig, University of Western Ontario, London, Ontario, Canada, and SPARC (Mason *et al.*, 1986*b*), kindly provided by Dr. B. L. M. Hogan, Vanderbilt University, Nashville, TN, U.S.A. To quantify mRNA levels for SPPI and bone SPARC, mRNA was prepared as described previously (Overall *et al.*, 1989). Confluent cells in 100 mm dishes were trypsin-treated and pelleted by centrifugation at 1000 *g* for 7 min at 0 °C. The pellets were dissolved by vigorous vortex-mixing in lysis buffer [6 M-urea, 3 M-LiCl, 0.1% (v/v) SDS, 70 mM-β-mercaptoethanol and 10 mM-sodium acetate, pH 5.5]. After transfer of the solution to a silicone-coated Microfuge tube, the DNA was sheared by brief sonication and the sonicate was incubated for 16 h at 0 °C. The RNA was collected by centrifugation at 12000 *g* for 30 min and suspended in 200 μl of an RNAase-free solution of 0.1 M-sodium acetate/0.5% (w/v) SDS, pH 5.5. After brief vortex-mixing, successive extractions with equal volumes of phenol (pre-equilibrated with acetate buffer) and phenol/chloroform (1:1, v/v) were carried out and the resultant aqueous phase was precipitated overnight at −70 °C with 0.3 M-sodium acetate and 2.5 vol. of ethanol. The RNA was dissolved in 20 μl of RNAase-free water and 2.5 μl was used for absorbance measurements at 280 nm and 260 nm. Samples containing approx. 20 μg of total RNA were fractionated on 1.2% (w/v) agarose gels containing 2.2 M-formaldehyde and the RNA was transferred on to a BioTrans membrane (0.22 μm pore size; ICN, Costa Mesa, CA, U.S.A.), hybridized (Maniatis *et al.*, 1982) and the blot washed for 4 × 5 min at 22 °C in 2 × SSC (30 mM-sodium citrate/0.3 M-NaCl)/0.1% SDS, pH 7.0, followed by 2 × 20 min washes at 50 °C in 0.1 × SSC/0.1% SDS. Probes were labelled with [^{32}P]dCTP (> 3000 Ci/mmol, Amersham) using an oligolabelling kit (Pharmacia, Uppsala, Sweden). After drying, the blots were exposed to Kodak X-OMAT film at −70 °C using two intensifying screens. The radioactive bands were quantified by densitometry as described above and normalized to the actual amount of RNA loaded as measured from a transparency of the ethidium bromide-stained gel, photographed before transfer. For analysis by slot-blotting, 10 μg of total RNA in 100 μl of RNAase-free water was denatured by adding 300 μl of 50% (v/v) formaldehyde in 10 × SSC and heating at 68 °C for 10 min. After rapid cooling, the RNA from replicate cultures were loaded on to a slot-blot apparatus (Schleicher and Schuell, Keene, NH, U.S.A.) and, after air drying, immobilized to the membrane by a 4 min exposure to u.v. light from a transilluminator (Fotodyne). All subsequent hybridization, wash and detection procedures were as described above.

Where indicated, cycloheximide (from a stock solution of 10 mg/ml in water) was added to a final concentration of 100 μg/ml 15 min before addition of TGF-β. For studies on mRNA stability, cultures were incubated in the presence or absence of TGF-β (1.0 ng/ml) for 24 h, after which 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole [DRB; stock solution of 12 mM in 95% (v/v) ethanol] was added to a final concentration of 60 μM and incubations were continued until the indicated times, when total RNA was prepared as described above.

Quantification by densitometric scanning of fluorographs and autoradiographs was performed on an LKB Laser Densitometer (Pharmacia). Integration of the scans was performed on a Macintosh Plus computer (Apple Inc) using the Curves program developed by Dr. P. N. Lewis (Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada). Differences were analysed on replicate (≥ 3) samples using Student's *t* test.

RESULTS

Characteristics of the bone cell populations

The normal bone cell populations prepared from neonatal parietal bone were similar to the more extensively characterized fetal cell populations. The later-eluting populations were enriched with osteoblastic cells as indicated by polygonal morphology, a greater cyclic AMP response to parathyroid hormone and higher levels of alkaline phosphatase. In extended culture, these cells also formed nodules of tissue that mineralized in the presence of 5 mM-sodium β-glycerophosphate (T. Kubota, unpublished work). The clonal bone cell line RCA 11, derived from a population of cells isolated from fetal rat calvarial bone, displayed a pleiomorphic morphology, responded to parathyroid hormone with a 5–10-fold increase in cyclic AMP, had a moderate level of alkaline phosphatase activity and, most characteristically, produced unusually high amounts of extracellular matrix (Ber *et al.*, 1991). TGF-β (1.0 ng/ml) did not affect cell proliferation significantly in any of the cell populations over the 24 h period of

study, but did suppress alkaline phosphatase activity, except in ROS 17/2.8 cells in which alkaline phosphatase activity was markedly increased (results not shown), as described previously (Noda & Rodan, 1987).

Analysis of secreted phosphoproteins

Two major forms of SPPI, with molecular masses of 55 and 44 kDa by SDS/PAGE on 15% (w/v) cross-linked gels (Fig. 1), and of 60 and 56 kDa on 10% (w/v) cross-linked gels (results not shown), were immunoprecipitated from ^{32}P-labelled RC cell populations and ROS 17/2.8 culture media with affinity purified antibodies to rat bone SPPI. However, only a 55 kDa (60 kDa) band was detected in the RCA 11 cells. The 55 kDa SPPI was the predominant band in the RC cell populations (Fig. 1*a*), whereas the 44 kDa SPPI was the major radiolabelled form in the ROS 17/2.8 cells. These phosphoproteins accounted for essentially all of the original radiolabelled protein in the 43–67 kDa region and were higher in intensity in the more osteoblast-like cell populations. Pulse–chase studies carried out on both RCA 11 and ROS 17/2.8 cells over a 24 h period showed that the phosphoproteins were stable, with no evidence of any interconversion between these two forms (results not shown).

The 55 kDa and 44 kDa SPPIs from the RCA 11 and ROS 17/2.8 cells were characterized further by fractionation on a 0.5 cm × 5.0 cm column of Mono Q anion-exchange resin by f.p.l.c. Both phosphoproteins bound to the resin in the equilibration buffer and were eluted with a linear gradient of 0–1.0 M-NaCl. The 55 kDa protein from both the RCA 11 and the ROS 17/2.8 cells was eluted at 0.32–0.36 M-NaCl (Fig. 2*a*), whereas the 44 kDa phosphoprotein prominent in the ROS 17/2.8 cells was eluted at 0.35–0.38 M-NaCl (Fig. 2*b*), indicating stronger binding of the 44 kDa phosphoprotein to the cationic resin. The gradual transition in migration between the two forms on SDS/PAGE indicated the possibility of additional, intermediate species.

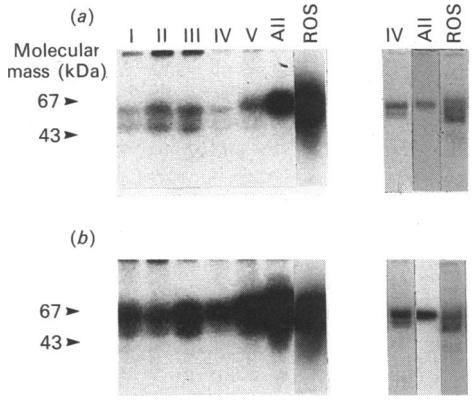

Fig. 1. Specific immunoprecipitation with anti-SPPI antibodies of ^{32}P-labelled proteins secreted by rat bone cells

Equal portions of culture media containing the ^{32}P-labelled proteins were immunoprecipitated with antisera to SPPI and the specific immunoprecipitates were analysed by SDS/PAGE on 15% (w/v) cross-linked mini-gels. Samples from the control cultures (*a*) and cultures treated with TGF-β (1.0 ng/ml) (*b*) are shown for parietal bone cell populations I–IV, RCA 11 cells and ROS 17/2.8 cells. The positions of the 67 kDa and 43 kDa marker proteins are indicated. Portions of immunoprecipitated proteins from RC IV, RCA 11 and ROS 17/2.8 cells, adjusted to contain similar amounts of radioactivity within each group, are shown on the extreme right. These radiolabelled bands were resolved using single-sided Kodak SB-5 X-ray film without an intensifying screen.

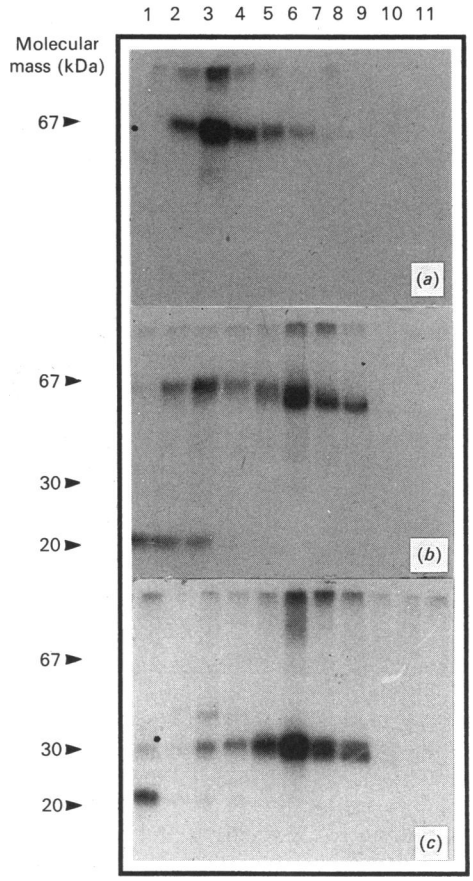

Fig. 2. F.p.l.c. chromatography of phosphoproteins on Mono Q

Samples of culture medium (3 ml) from RCA 11 and ROS 17/2.8 cells containing ^{32}P-labelled proteins were applied to a Mono Q column equilibrated with 50 mM-Tris/HCl buffer, pH 7.4, containing 6 M-urea. Bound material was eluted with a linear gradient of 0–1.0 M-NaCl and fractions (1.0 ml) containing radioactivity (43–53, lanes 1–11) were analysed by SDS/PAGE on 15% (w/v) cross-linked gels. The salt gradient across these fractions increased linearly from 0.31 M-NaCl (fraction 43) to 0.41 M-NaCl (fraction 53). (*a*) Phosphoproteins synthesized by RCA 11 cells; (*b*) phosphoproteins synthesized by ROS 17/2.8 cells. In both cases the 55 kDa phosphoprotein was eluted at 0.32–0.36 M-NaCl, whereas the 44 kDa phosphoprotein from the ROS 17/2.8 cells was eluted at 0.35–0.38 M-NaCl. (*c*) Thrombin digest of the 55 kDa and 44 kDa SPPI phosphoproteins shown in (*b*). Note the common 30 kDa band and the additional 28 kDa band in the fractions corresponding to the 44 kDa SPPI.

The possibility that some of the phosphoprotein might represent bone sialoprotein, which is abundant in rat bone, was ruled out for the following reasons. The phosphoproteins eluted from Mono Q resin at a lower salt concentration than did bone sialoprotein extracted from bone (Zhang *et al.*, 1990). Further, no bone sialoprotein expression could be detected in confluent rat bone cell cultures when analysed by either Northern blotting or by using a monoclonal antibody to the protein. To confirm that the 55 kDa and 44 kDa phosphoproteins were SPPIs, portions from each fraction were digested with thrombin, which specifically cleaves the transformation-related SPPI (Senger *et al.*, 1988). As shown in Fig. 2(*c*), fragments of ~ 30 kDa were generated from the complete digestion of both the 55 kDa and the 44 kDa phosphoproteins. In addition, a faster migrating ~ 28 kDa fragment with radioactivity similar to that of the 30 kDa fragment was obtained from the 44 kDa SPPI. Together, these data confirm the identity of the ^{32}P-labelled phospho-

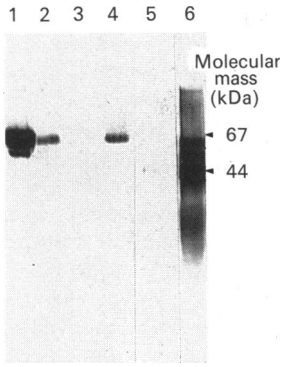

Fig. 3. Immunotransfer of proteins extracted from rat bone

Proteins extracted with 0.5 M-EDTA from fetal and adult rat calvarial bone, previously extracted with 4 M-guanidinium hydrochloride, were separated by SDS/PAGE on 15% (w/v) cross-linked gels and then electrophoretically transferred to a nitrocellulose membrane beneath which was placed a Zeta-Probe membrane. The membranes were incubated with affinity purified antibodies to rat SPPI and protein-bound antibodies were revealed as purple bands after incubation with peroxidase-linked second antibodies and 4-chloro-1-naphthol as substrate. Lanes 1–4 are results from the Zeta-Probe membrane showing proteins extracted from adult rat long bone, adult calvariae, purified 44 kDa SPPI and proteins from fetal rat calvariae respectively; lane 5, reverse side of Zeta-Probe membrane for 44 kDa SPPI showing some immunoreactivity, lane 6, nitrocellulose membrane with SPPIs eluted from a Mono Q ion exchange column showing stronger staining for the 44 kDa SPPI, reflecting the superior binding of this form of SPPI to nitrocellulose.

proteins synthesized by cultures of rat bone cells as distinct forms of SPPIs.

Since SPPI isolated from rat bone has a molecular mass of approx. 44 kDa on SDS/15%-PAGE gels (Prince *et al.*, 1986), proteins were extracted from fetal and adult rat calvariae to determine if the 55 kDa form of SPPI synthesized by the bone cells was also present in bone tissues. Proteins extracted from adult calvariae were also separated on Mono Q resin and protein samples were analysed by Western blotting with affinity purified antibodies to SPPI. Immunoreactive SPPI migrating just below the 67 kDa marker could be clearly observed in extracts of the total proteins that were bound to Zeta-Probe membrane (Fig. 3). In contrast, the 44 kDa form observed previously (Prince *et al.*, 1986) bound poorly to Zeta-Probe and could be observed only on the reverse side of the membrane. However, on the nitrocellulose membrane the 44 kDa SPPI bound well compared with the slower-migrating form of SPPI (Fig. 3, lane 6). To confirm the identity of SPPIs extracted from rat bone, total proteins were fractionated on Mono Q resin, which can clearly resolve SPPI (eluting at 0.32–0.38 M-NaCl) from bone sialoprotein (eluting at 0.5 M-NaCl) and bone acidic glycoprotein 75 (eluting at higher salt concns.) (Gorski & Shimizu, 1988). Both immunoreactive forms of SPPI eluted from Mono Q resin in the same fractions (Fig. 3, lane 6) and both were selectively degraded by thrombin (results not shown), thus confirming their identity as SPPIs.

Effects of TGF-β on SPPI and SPARC synthesis

After a 24 h exposure of the neonatal (or fetal) RC cells to TGF-β, a strong 1.8–4.5-fold increase in the intensity of the ^{32}P-labelled 55 kDa SPPI was observed (Fig. 1*b*). In RCA 11 cells, the 55 kDa SPPI was increased 2-fold by TGF-β, but no significant changes were evident for the 44 kDa SPPI in either the normal bone cells or in the ROS 17/2.8 cells.

To determine if the increase in ^{32}P-labelled protein stimulated by TGF-β was due to increased protein synthesis or to increased

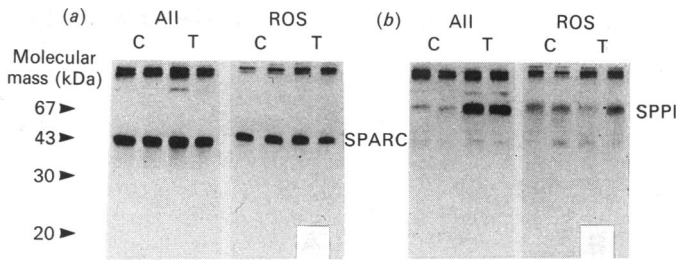

Fig. 4. Specific immunoprecipitation of [^{35}S]methionine-labelled proteins secreted by rat bone cells

Portions of chase medium containing [^{35}S]methionine-labelled proteins were specifically immunoprecipitated with anti-SPARC antibodies and affinity purified SPPI antibodies and analysed by SDS/PAGE on 15% (w/v) cross-linked mini-gels under reducing conditions. Results are shown for both control (C) and TGF-β-stimulated (T) cultures. (*a*) Immunoprecipitation of SPARC from 250 μl samples of medium from clonal bone cell populations RCA 11 and ROS 17/2.8; (*b*) immunoprecipitation of SPPI from 1.0 ml samples of medium from clonal bone cell populations RCA 11 and ROS 17/2.8. The radiolabelled material at the top of the gels was non-specifically bound to the Protein A–Sepharose. Fluorographs were exposed for 3 days (*a*) or for 10 days (*b*). The predominance of SPARC provides an indication of the relative amounts of these proteins synthesized by the bone cells.

Table 1. SPPI and SPARC expression by rat bone cell populations in the presence of TGF-β

Hybridization of SPPI and SPARC cDNA to 10 μg of total RNA from triplicate cultures of each bone cell population was measured on slot-blots. Quantification was obtained from densitometric scans of radioautographs exposed to provide a response in the linear range, and expressed in arbitrary units. Differences in hybridization between control (C) and TGF-β-treated (T) cultures are expressed as a ratio (T/C) and compared with corresponding ratios (in parentheses) from measurements of the respective [^{35}S]methionine-labelled proteins secreted into the culture medium of the same cells. Significant increases in both mRNA and protein ($P < 0.01$) are indicated by *. Increases in [^{35}S]methionine-labelled SPPI in RC cell populations could not be measured because the protein was not detectable in control cultures.

Bone cell population	mRNA expression (units)					
	SPPI			SPARC		
	C	T	T/C	C	T	T/C
RC I	0.5	1.5	3.0*	3.9	6.7	1.7 (1.5)*
RC II	1.5	2.7	1.8	21.3	20.3	1.0
RC III	1.9	8.5	4.5*	12.8	6.9	1.3
RC IV	2.7	8.9	3.5*	17.0	14.9	0.9 (0.8)
RCA 11	23.9	48.0	2.0 (3.0)*	14.5	15.1	1.0 (1.1)
ROS 17/2.8	13.0	15.1	1.2 (1.0)	2.7	3.2	1.2 (1.0)

phosphorylation, cells were pulse-labelled with [^{35}S]methionine for 30 min and media proteins from a 4 h chase were analysed by SDS/PAGE and fluorography after immunoprecipitation with antibodies to SPPI and SPARC (Fig. 4). Immunoprecipitation with anti-SPARC antiserum revealed a single radiolabelled protein at 40 kDa on SDS/PAGE run under reducing conditions. As found previously (Wrana *et al.*, 1988) for fetal RC cells, SPARC synthesis was increased by TGF-β in the fibroblastic RC I populations, but in the more osteoblastic RC II–IV populations, which expressed higher constitutive levels of SPARC, TGF-β was without effect (Table 1). Although the constitutive levels of

SPARC expression in RCA 11 cells and especially in the transformed ROS 17/2.8 cells were lower that in the RC II–IV populations, TGF-β did not alter SPARC synthesis in these cells either (Fig. 4*a* and Table 1).

From immunoprecipitations with affinity purified SPPI antibodies a [^{35}S]methionine-labelled 55 kDa SPPI was detectable in control cultures of RCA 11 and ROS 17/2.8 cells (Fig. 4*b*). The intensity of this band was increased approx. 3-fold in the RCA 11 cells incubated with TGF-β but was not significantly altered in the ROS 17/2.8 cells (Table 1). After TGF-β stimulation, a radiolabelled band corresponding to the 55 kDa SPPI was evident in osteoblast-enriched RC IV cells, but the degree of stimulation could not be measured because the protein could not be detected in the control cultures. Moreover, despite the presence of two methionine residues in the rat bone SPPI, a [^{35}S]methionine-labelled protein band corresponding to the 44 kDa protein could not be identified definitively in any of the cultures, even after long exposures of fluorographs, or when cells were continuously labelled for 24 h with [^{35}S]methionine. Since SPPI can undergo proteolytic processing *in vivo* (Zhang *et al.*, 1990), we attempted to detect synthesis of the 44 kDa form by radiolabelling with [^{14}C]aspartic acid and [^{14}C]glycine. In neither case could radiolabelled 44 kDa protein be detected. These data indicate that little synthesis of the 44 kDa form of SPPI occurs in confluent bone cell cultures.

Effects of TGF-β on SPPI and SPARC mRNAs

To determine whether the effects of TGF-β on the synthesis of SPPI were related to alterations in mRNA levels, total RNA from control and TGF-β-treated cells were used for Northern and slot-blot hybridization analysis. Using the 2ar probe (Smith & Denhardt, 1987) for mouse SPPI (Craig *et al.*, 1988), specific hybridization to a 1.5 kb mRNA was observed in each of the bone cell populations (Fig. 5). Higher constitutive levels of SPPI mRNA were evident in the more osteoblast-like RC IV cells, with greater amounts in the ROS 17/2.8 cells and especially high levels in RCA 11 cells. In the presence of TGF-β, SPPI mRNA levels in each of the RC I–IV cell populations and in RCA 11 cells were increased markedly, but only a slight, insignificant, increase was detected in the ROS 17/2.8 cells (Table 1).

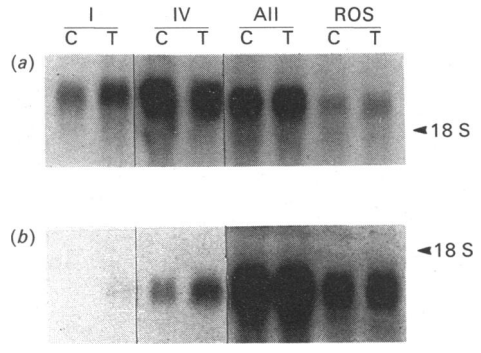

Fig. 5. Northern blot analysis of SPPI and SPARC mRNA

Total RNA (20 μg) extracted from bone cell populations I and IV, RCA 11 and ROS 17/2.8 cells cultured for 24 h in the presence of 1.0 ng of TGF-β/ml (T) or vehicle alone (C) was separated on 1.2% (w/v) agarose gels containing 2.2 M-formaldehyde and transferred to a BioTrans membrane. Transfers were probed with ^{32}P-labelled cDNAs for SPARC (*a*) and for SPPI (*b*) using the mouse 2ar probe, and hybridization was revealed by autoradiography. The sizes of the SPARC and SPPI mRNAs were estimated as 2.0 and 1.5 kb, respectively.

Using a cDNA probe for mouse SPARC, hybridization to a 2.0 kb mRNA species corresponding to that of rat SPARC (Mason *et al.*, 1986*b*) was observed (Fig. 5). The highest amounts of SPARC mRNA were present in the more osteoblast-like RC IV cell populations and RCA 11 cells, with a lower amount in the RC I populations and the lowest amount in ROS 17/2.8 cells. Except in the RC I cells, in which there was a 1.7-fold increase in the 2.0 kb mRNA, TGF-β did not alter SPARC mRNA levels significantly in the other cell populations. When analysed on a series of slot-blots, the SPPI and SPARC mRNA levels in the various cell populations, and the changes induced by TGF-β, correlated quite closely with the observed differences and changes in SPPI and SPARC protein synthesis measured by radiolabelling (Table 1).

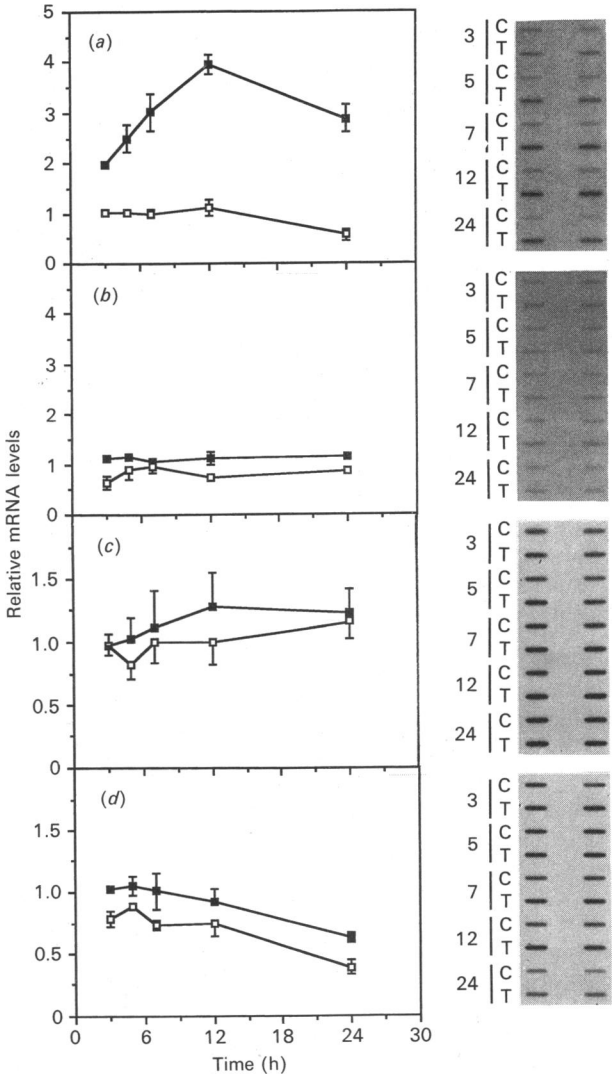

Fig. 6. Temporal effects of TGF-β on SPPI (*a*, *b*) and SPARC (*c*, *d*) mRNA

Confluent quiescent cultures of RC bone cells (population IV) were incubated for the indicated times in the presence (■, T) or absence (□, C) of TGF-β (1.0 ng/ml) and in the presence (*b*, *d*) or absence (*a*, *c*) of cycloheximide (100 μg/ml). The cells were then collected by trypsin treatment and total cellular RNA was extracted, blotted and probed with SPPI (*a*, *b*) or SPARC (*c*, *d*) cDNAs as described in the Materials and methods section. Representative autoradiographs are presented to the right of each graph with quantification of hybridization obtained by laser densitometry of autoradiographs. All data are expressed as the mean signal intensity ± S.D. expressed relative to control cell levels (in the absence of TGF-β and cycloheximide).

Effects of TGF-β on SPPI and SPARC expression

To determine how TGF-β might regulate SPPI and SPARC gene expression, confluent RC IV cells stimulated with TGF-β (1.0 ng/ml) were cultured for various times in the presence or absence of cycloheximide (100 μg/ml) and the mRNA levels of both SPPI and SPARC were measured by hybridization on slot-blots (Fig. 6). In control cultures, the expression of SPPI mRNA was increased by TGF-β, beginning at 3 h and reaching a maximum 4-fold increase at 12 h (Fig. 6*a*). In the presence of cycloheximide the TGF-β-mediated stimulation of SPPI mRNA was completely blocked. In contrast with SPPI, the SPARC mRNA level was not changed significantly by TGF-β (Fig. 6*b*). However, in the presence of cycloheximide the decrease in SPARC mRNA observed in control cells at 24 h was partially blocked by TGF-β, indicating that TGF-β may affect the stability of a transcription factor that is otherwise degraded, or not synthesized, in the presence of cycloheximide.

When RC IV cells were cultured in the presence of DRB to block transcription (Zandomeni *et al.*, 1983), the half-life of SPPI mRNA measured by hybridization of slot-blots (Fig. 7) was determined to be 18 h, compared with a half-life of > 50 h for the SPARC mRNA. Similar half-lives were obtained for the mRNA of both proteins in RC IV cells stimulated with TGF-β (1.0 ng/ml), demonstrating that TGF-β did not affect the stability of the mRNA significantly. To determine if the rate of transcription in RC cell populations was affected by TGF-β, nuclear run-on transcriptions were carried out using nuclei isolated from control cells and cells incubated for 24 h with TGF-β. In triplicate analyses, hybridization of radiolabelled *Spp1* transcripts from TGF-β-stimulated cells to SPPI cDNA was variable, but in each case hybridization was increased > 4-fold when compared with controls.

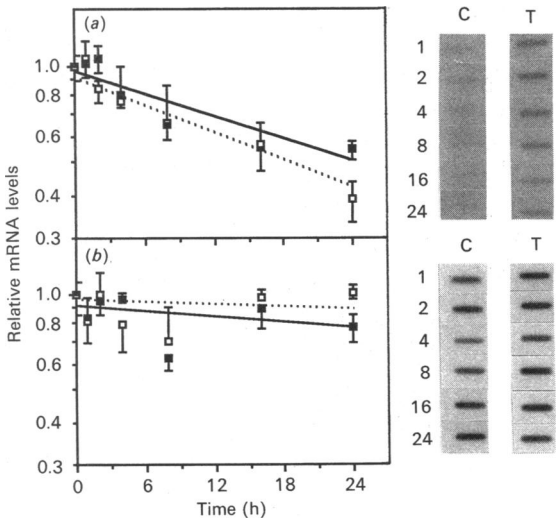

Fig. 7. Effects of TGF-β on the stability of SPPI (*a*) and SPARC (*b*) mRNAs

After a 24 h incubation in the presence (■, T) or absence (□, C) of TGF-β (1.0 ng/ml) confluent quiescent cultures of RC bone cells (population IV) were treated with 60 μM-DRB at 0 h and further incubated until the indicated times, after which total cellular RNA was extracted, blotted and probed as described in the Materials and methods section. Representative slot-blots are displayed to the right of each graph. Data have been plotted on semi-log scale as the mean intensity ± S.D. relative to the 0 h level for each treatment. Half-lives were estimated from best-fit lines drawn for control (·····) and TGF-β-treated (——) cultures.

DISCUSSION

Our studies on the synthesis of SPPI by bone cells in culture have revealed the presence of two major ^{32}P-labelled proteins that migrate at 55 kDa and 44 kDa on 15% (w/v) cross-linked SDS/PAGE gels. The recognition of these proteins by affinity purified antibodies to rat SPPI (Fig. 1), and the pattern of fragments produced by thrombin (Fig. 2), provide strong evidence that these are two distinct forms of SPPI (Kubota *et al.*, 1989). Studies on an established rat kidney fibroblastic cell line (NRK-49F) have similarly identified distinct forms of SPPI. An SPPI species, known earlier as transformation-related protein and designated pp69 (Laverdure *et al.*, 1987), may correspond to the 55 kDa form synthesized by bone cells, since it co-migrates at 55 kDa on SDS/15%-PAGE gels. The second 44 kDa form of SPPI that we have identified, which is the major ^{32}P-labelled phosphoprotein synthesized by the tumorigenic ROS 17/2.8 cells, may correspond to a 60–62 kDa phosphoprotein preferentially stimulated by tumour promoters (Laverdure *et al.*, 1987; Craig *et al.*, 1988), since they co-migrate on SDS/PAGE and neither protein could be labelled readily with [^{35}S]methionine. We have also been unable to metabolically label the 44 kDa form using radioactive aspartic acid or glycine, further supporting conclusions that this form of SPPI is highly phosphorylated but is synthesized at minor levels relative to the 55 kDa form. This may also explain the faster electrophoretic migration of the 44 kDa SPPI relative to the 55 kDa form. Interestingly, more recent studies have identified a non-phosphorylated form of pp69, which is regulated independently of the phosphorylated form (Nemir *et al.*, 1989). Since there is no evidence of alternative splicing of rodent SPPI mRNA, differences between distinct forms of SPPI would appear to result from differences in post-translational modifications (Craig *et al.*, 1989).

It has been reported previously that the major ^{32}P-labelled protein synthesized by tumorigenic ROS 17/2.8 cells exhibits similar behaviour to the bone-derived 44 kDa SPPI (Noda *et al.*, 1988). However, we have clearly demonstrated in this study that the major form of SPPI synthesized by normal bone cells is the 55 kDa form. Moreover, as discussed above, the highly phosphorylated 44 kDa SPPI appears to be related to cellular transformation, and the similarity of the migration of this form to that of the bone-derived 44 kDa SPPI may be a characteristic shared by two different forms of the protein. Notably, the phosphorylation of fibronectin, another cell attachment protein, is also increased in transformed cells (Ali & Hunter, 1981).

To determine whether the major 55 kDa form of SPPI that we identified in bone cell cultures could be correlated to distinct forms of SPPI found in rat bone, we extracted mineral-binding proteins from both fetal and adult rat bone. SPPI extracted from rat bone has been characterized previously as a single 44 kDa phosphoprotein species (Prince *et al.*, 1986). However, using affinity purified antibodies we have demonstrated that, in addition to the 44 kDa phosphoprotein, a 67 kDa SPPI is also present in adult and fetal rat bone. The 67 kDa form of rat SPPI that we have identified displays electrophoretic characteristics comparable with those of the 67 kDa SPPIs found in human (Fisher *et al.*, 1987), bovine (Fisher *et al.*, 1983) and pig (Zhang *et al.*, 1990) bones. It is of note that a 44 kDa SPPI does not appear to be present in these species. The 67 kDa rat SPPI extracted from bone migrates more slowly than the major 55 kDa form produced by normal rat bone cells in culture, the difference possibly reflecting differential post-translational modifications or processing of these two forms. Indeed, we have recently demonstrated that bone-derived SPPIs are sulphated, whereas incorporation of $^{35}SO_4$ into SPPIs produced by confluent cultures of bone cells is barely detectable (Nagata *et al.*, 1990). Consequently,

stimulation of the synthesis of the 55 kDa form of SPPI by TGF-β in bone cell cultures may reflect increased expression of the protein, but not necessarily in the form synthesized during bone formation.

The stimulation of *SppI* gene expression by growth factors was first observed in epithelial cells, leading to the isolation of the 2ar cDNA probe (Craig *et al.*, 1988). The effect of TGF-β on SPPI expression has been studied in established rat kidney NRK-49F cells (Laverdure *et al.*, 1987) and tumorigenic ROS 17/2.8 cells (Noda *et al.*, 1988). TGF-β alone was observed to decrease the synthesis of SPPI (pp69) in the kidney fibroblasts, whereas SPPI synthesis and mRNA levels were stimulated in ROS 17/2.8 cells but, importantly, not until 48 h after the addition of growth factor. Moreover, the two forms of SPPI that we have observed were not resolved in the study of the ROS 17/2.8 cells. In our studies, TGF-β induced a strong early stimulation of SPPI synthesis in normal RC cell populations, in contrast with its effects on the ROS 17/2.8 cells. In each case it was the 55 kDa phosphoprotein that was selectively stimulated, and this stimulation reflected a corresponding increase in SPPI mRNA. From the analysis of mRNA levels, the stimulation was first evident at 3 h and increased gradually to 12 h, slowly declining thereafter. In the presence of the transcription inhibitor DRB, the half-life of SPPI mRNA, estimated at 18 h, was not affected by TGF-β, indicating that TGF-β was increasing the steady-state levels of SPPI mRNA, possibly through an increase in transcription of the *SppI* gene as indicated by the nuclear run-on analyses. Since cycloheximide blocked the increase in SPPI mRNA, it would appear that TGF-β stimulates the synthesis of a transcription factor that enhances transcription of the *SppI* gene. Because TGF-β did not increase SPARC mRNA in osteoblastic cells, the effect on gene transcription is clearly selective. However, whereas some effects of TGF-β can be attributed to regulation at the transcriptional level, the results also provide evidence for post-translational regulation that directs the production of the 55 kDa form of SPPI, rather than the 44 kDa form.

In previous studies we have shown that TGF-β stimulates the synthesis of matrix proteins including collagen, fibronectin and plasminogen activator inhibitor, but suppresses alkaline phosphatase activity and has no significant effect on SPARC synthesis by osteoblastic RC cells (Wrana *et al.*, 1988). Similar results were obtained with the neonatal RC cells in this study. Whereas the selective stimulation of type I collagen synthesis (Wrana *et al.*, 1988) is consistent with the formation of a mineralizable bone-like matrix, the stimulation of fibronectin is not. Further, the decrease in alkaline phosphatase activity, which has also been reported for the MC1 3T3E1 osteoblastic cell line (Noda & Rodan, 1987), and the loss of polygonal morphology indicate a suppression of the osteoblastic phenotype. These effects of TGF-β appear to conflict with the stimulation of alkaline phosphatase and SPARC (Noda & Rodan, 1987) and the suppression of fibronectin gene expression (Noda *et al.*, 1988) in ROS 17/2.8 cells. However, the effects on matrix proteins, including SPPI, in ROS 17/2.8 cells are delayed and unlikely to be primary effects of TGF-β. Furthermore, there are fundamental differences in the response of normal versus established and transformed cells to TGF-β. Thus differences in the response of normal bone cells when compared with tumorigenic cells (ROS 17/2.8) and established cells (NRK fibroblasts) may relate to the abnormal behaviour of transformed and established cells towards growth factors.

In summary, this study has shown that normal RC cells express SPPI constitutively and that TGF-β stimulates selective expression of a 55 kDa form of SPPI. Furthermore, the 55 kDa SPPI regulated by TGF-β in normal bone cell cultures may correspond to the newly described 67 kDa SPPI that we have identified in rat bone matrix, with the differences in mobility possibly being due to sulphation of the matrix SPPI (Nagata *et al.*, 1990). As the function of SPPI in bone tissues is not known, the significance of the stimulation of the 55 kDa form by TGF-β in bone cells is currently unclear. However, since the 55 kDa protein is likely to share the cell-adhesion and mineral-binding properties of the previously characterized 44 kDa osteopontin, the effects may be important in the formation and remodelling of bone tissues.

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