Rapid Flux in Transforming Growth Factor-\(\beta\) Receptors on Bone Cells*

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The proportion of transforming growth factor-\(\beta\) (TGF-\(\beta\)) binding among conventional membrane receptors on bone cells can vary with hormone or growth factor treatment or with the state of osteoblast-like activity and appears to determine the nature of its biological effects. Therefore, functional TGF-\(\beta\) receptor stability could be an important aspect of regulation. Suppression of protein synthesis reduced TGF-\(\beta\) binding to types I and II receptors with \(t_{1/2}\) of 2 h and to betaglycan with \(t_{1/2}\) of 6 h. In contrast, suppression of mRNA transcription reduced TGF-\(\beta\) binding at least 3-fold more slowly at each receptor site. Preexposure to TGF-\(\beta\) decreased its binding at all three sites within 4 h in osteoblast-enriched cultures. This effect was transient with lower TGF-\(\beta\) concentrations, where the receptor profile was nearly fully restored within 24–48 h. In contrast, less differentiated bone cells were less sensitive to betaglycan receptor down-regulation. Agents that alter protein kinase and phosphatase activity also modified the TGF-\(\beta\) binding profile in specific ways. Together, these results indicate that cell surface TGF-\(\beta\) receptors turn over rapidly by ligand-independent and ligand-dependent mechanisms, demonstrate that the binding capacity of TGF-\(\beta\) receptors is less stable than their mRNAs, and that functional receptor levels may be determined in part by post-transcriptional events.

Transforming growth factor type \(\beta\)s (TGF-\(\beta\)s) are potent, ubiquitous growth regulators that are abundant in skeletal tissue. Their expression is controlled in temporal and spatial ways during skeletal tissue development, and they produce complex stimulatory and inhibitory changes in osteoblast function in vitro. The presence of TGF-\(\beta\)s in bone and their importance as local skeletal growth regulators were first described in fetal rat calvariae, and cells derived from various bone tissues have provided much information about TGF-\(\beta\) activity and receptor expression during osteogenesis (reviewed in Ref. 1). Similar to cells found in many tissues, fetal rat bone cells express type I (53 kDa) and type II (73 kDa) glycoprotein receptors, as well as type III proteoglycans (\(~\sim\) 250 betaglycans) that could directly or indirectly influence TGF-\(\beta\) binding to the signaling types I and II sites (2–5). In this model, the association of TGF-\(\beta\) with each receptor varies with the basal state of bone cell differentiation, and relative mRNA abundance levels generally reflect the cell surface binding profiles found in untreated cells (5). Rapid changes in the proportion of TGF-\(\beta\) binding among receptors, induced by certain growth factors and hormones, are consistent with specific changes in its biological effects (5–8). For example, glucocorticoid decreases TGF-\(\beta\) binding to types III sites, and reduces its overall effectiveness (6). In contrast, bone morphogenetic protein (BMP)-2 reduces TGF-\(\beta\) binding to type II receptors and betaglycan, whereas it enhances binding to type I receptors. After BMP-2 treatment, the mitogenic effect of TGF-\(\beta\) declines in osteoblast-enriched cultures, whereas its influence on collagen synthesis and alkaline phosphatase increase synergistically (5). The differences found in these situations correspond well to variations in TGF-\(\beta\) binding and function that occur during early phases of expression of the osteoblast phenotype and emphasize the usefulness of this model to understand normal bone cell function. Importantly, the rate at which hormone- and growth factor-induced changes in TGF-\(\beta\) binding occur predict rapid reorganization of TGF-\(\beta\) receptors on osteoblasts.

We now report striking ligand-independent and ligand-dependent TGF-\(\beta\) receptor flux on fetal rat bone cells. Although transcriptional effects are important, constitutive protein expression appears to predominate. In addition, changes in TGF-\(\beta\) binding also result from phosphorylation-dependent events. In total, our studies reveal surprisingly fast and independent variations in the presence or the binding capacity of the three major TGF-\(\beta\) binding sites on bone cells, consistent with post-transcriptional regulatory events. These changes could contribute to the overall TGF-\(\beta\) binding profiles that occur in response to systemic, local, or developmental events and could alter how TGF-\(\beta\) functions during various phases of skeletal organogenesis.

MATERIALS AND METHODS

Cell Cultures—Parietal bones from 22-day-old rat fetuses (Crl: CD®/SD) were dissected free of suture and digested for 5 20-min intervals with collagenase. The first digestion releases less differentiated cells (population 1), the second digestion contains intermediate, less well characterized cells, and the last three digestions (population 3–5) are enriched with cells exhibiting differentiated osteoblast characteristics (3, 4). Primates from each group were plated at 6–9 \(\times\) 10\(^3\) cells/cm\(^2\) in Dulbecco's modified Eagle's medium containing 20 mM HEPES buffer, pH 7.2, 100 \(\mu\)g/ml ascorbic acid, penicillin, and streptomycin and 10% fetal bovine serum. At confluence (approximately 5–6 \(\times\) 10\(^3\) cells/cm\(^2\)) they were refed identical medium lacking fetal bovine serum. Twenty h later they were refed medium containing various agents for the times indicated in each experiment. Clonal osteosarcoma-derived osteoblast-like ROS 17/2.8 cultures (obtained from Drs. Masaki Noda and Gideon Rodan, Merck Sharp and Dohme Research Laboratories, West Point, PA) and NRK-
49F fetal rat fibroblasts (CRL 1570, obtained from the American Type Culture Collection) were cultured and treated by the same procedures (5, 7, 9).

Reagents—Cell culture reagents were obtained from Life Technologies, Inc. Cyclamethoxime, α-amanitin, N-[2-(methylamino)ethyl]-5-isquinolinesulfonylanilide (designated as H-8, N-[2-(3-(4-bromophenyl)-2-propanamino)-ethyl]-5-isquinolinesulfonylanilide (designated H-88), and staurosporine were obtained from Sigma. The TGF-β1 used in these studies was a recombinant simian preparation identical in amino acid sequence to human TGF-β1 (examined in collaboration with Bristol-Myers Squibb, Inc., Seattle, WA) and exhibited binding and biological characteristics indistinguishable from our earlier studies with other native or recombinant preparations (Fig. 1 in this paper, and Refs. 5–7, 9).

Cell Replication—DNA synthesis rate was measured by labeling with 5 μCi/ml [methyl-3H]thymidine (80 Ci/mmol) during the last 2 h of culture, lysing the cells in 0.1 n sodium dodecyl sulfate, 0.1 n NaOH, collecting the precipitate formed with 10% trichloroacetic acid, and scintillation counting (9).

Protein Synthesis— Cultures were pulsed with 12.5 μCi/ml [3H-2,3]proline (2.5 Ci/mmol) for the last 2 h of culture. Cell layers were lysed by freeze-thawing and extracted in 0.5% Triton X-100 (Sigma). The samples were precipitated with 10% trichloroacetic acid and chilled, and insoluble material was collected by centrifugation. Precipitates were acetic-acid-extracted, dried, redissolved in 0.5 n acetic acid, and neutralized with NaOH. Total protein synthesis was measured in the precipitates, and [3H]proline incorporation into collagen (collagenase-digestible gel) was measured using bacterial collagenase free of nonspecific protease activity (11, 12).

Alkaline Phosphatase Activity—Enzyme activity was assessed in cell extracts prepared by lysis in 0.5% Triton X-100 (~1% of total alkaline phosphatase is released to the medium).2 Hydrolysis of p-nitrophenyl phosphate was measured at 410 nm after a 30-min incubation at 37 °C (5, 13). Data are expressed as pmol of p-nitrophenol released per min per μg of cell protein, determined by the method of Bradford (40).

Binding Studies—TGF-β1 was radiiodinated with chloramine T to a specific activity of 4000–4500 Ci/mmol. Radioligand was separated from unincorporated 125I by gel filtration on Sephadex G-50 in a solution of 0.1 n acetic acid and 4 mg/ml bovine serum albumin. Binding was determined by incubation with serum-free medium containing 4 mg/ml bovine serum albumin and 50–150 pM 125I-TGF-β1 for 3 h at 4 °C. To visualize TGF-β1 binding complexes, cultures were rinsed with chilled binding medium, cross-linked with 0.2 mM disuccinimidyl suberate (Pierce), extracted, fractionated by electrophoresis on polyacrylamide gels, and examined by autoradiography as previously reported (5–7). Densitometry was assessed using a ScanMan densitometer and Sigma Gel® (Jandel, San Rafael, CA).

RNA Analysis—Total cell RNA was extracted with guanidine mono-thiocyanate and collected by isopropanol alcohol precipitation (5). TGF-β1 receptor transcripts were assessed by RNase protection assay with probes prepared in our laboratory. For type I receptor, rat CDNA R4 (a gift from D. P. Donahoe, Massachusetts General Hospital, Boston; Ref. 15) was digested with EcoRI and PvuI to obtain a fragment comprising the first 402 base pairs. This clone, termed pT7-TbRII, was amplified in DH5α E. coli after ligation into pGEM®-4Z (Promega, Madison WI) previously digested with EcoRI and Smal. A type II receptor probe was cloned by reverse transcription polymerase chain reaction. Degenerate primers were synthesized based on a conserved intracellular amino acid sequence found in the human probe that we used in Northern analysis of bone cell cultures (5) and common to human and pig. Sense primer (5′-CGGAAATCTCGGAGGTTTCATCAATGC/C/GTC/GGGC/T/GGA/AG/CAG/CA-3′) encodes a 5′ EcoRI restriction site directly before nucleotides encoding the tryptophan residue in the FSEH (H; 210–215). Antisense primer (5′-CGGGTTCTCCTG/CT/GAG/GAGAGTTG/GTG/CTGGTTTCG/AGTG/CTG-3′) encodes a 5′ BamHI restriction site followed by the conserved motif QNTSEQ (amino acids 265–270; Ref. 16). First strand cDNA was synthesized from total RNA of fetal rat popliteal lymph node cultures (which express types I, II, and III receptors; Refs. 5–7) using the DNA Cycle kit (Stratagene, San Diego CA) and random primers. An aliquot of the product was used for polymerase chain reaction with Taq DNA polymerase (Promega). Four initial cycles (denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and 55 °C for 2 min, and extension at 72 °C for 2 min) preceded 25 cycles of 94 °C for 1 min, 55 °C for 0.5 min, and 72 °C for 1 min. A small aliquot of the product was re-amplified with the final 3-step program. The predicted 182 bp pair product was 2 M. Centrella and T. L. McCarthy, unpublished results.

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Rapid TGF-β Receptor Flux

![Fig. 1. TGF-β1 binding and potency in fetal rat bone cells. Confluent serum-deprived cultures were treated for 24 h with the amounts of TGF-β1 indicated. Panel A, DNA synthesis in osteoblast-enriched population 3–5 cultures treated with unlabeled (○) or radiiodinated (●) TGF-β1 (4500 Ci/mmol). DNA synthesis was measured in 0.32-cm² cultures by pulsing with 5 μCi/ml [3H]thymidine. Data are total isotope incorporation × 104 ± S.E. of four replicate cultures per condition. Less than 5% of isotope incorporation could be attributed to 125I at the highest concentration tested. Panel B, relative biological potencies of TGF-β1 in less differentiated population 1 and population 3–5 cultures. DNA synthesis was measured as in panel A, and collagen synthesis was measured in 2-cm² cultures by pulsing with 12.5 μCi/ml [3H]proline during the last 2 h of incubation. Alkaline phosphatase specific activity was determined in culture extracts. The recombinant TGF-β1 preparation produced analogous results in more than 20 separate experiments.

Characterization of Recombinant TGF-β Binding and Function—Osteoblast-enriched fetal rat bone cell cultures are particularly sensitive to TGF-β1-induced mitogenesis (5, 7, 9), and we used them to establish the molar amount of receptor-reactive material after radiiodination of the recombinant TGF-β1 used in these studies. Unlabeled and radiiodinated samples each induced rapid, biphasic stimulatory effects on DNA synthesis. Radiiodination and subsequent gel filtration purification appeared to alter activity only moderately (Fig. 1A). In addition to effects on DNA synthesis, this preparation of TGF-β1 increased collagen synthesis and inhibited alkaline phosphatase activity, analogous to results with several native and recombinant TGF-β preparations (5–7, 9). Each effect was more pronounced in osteoblast-enriched population 3–5, by comparison with the less differentiated population 1 (Fig. 1B).

2 M. Centrella and T. L. McCarthy, unpublished results.
Fig. 2. **TGF-β1** binding site stability in fetal rat bone cells. Confluent serum-deprived population 3–5 cultures were treated with cycloheximide (2 μM) or α-amanitin (1 μg/ml) for the times indicated. Panel A, cultures were rinsed, incubated with 125I-TGF-β1, and cross-linked, and cell extracts were fractionated on a polyacrylamide gel and visualized by autoradiography. Panel B, cultures were scanned and analyzed by densitometry. Relative values for each receptor were determined by comparison to untreated cultures, denoted as 1.0 relative OD unit. Data represent means ± S.E. from a total of six separate experiments using 125I-TGF-β1 labeled on three separate occasions.

**Ligand-independent Receptor Turnover—**Fetal rat bone cells express conventional 125I-TGF-β-bound complexes at 65 kDa (type I receptor), 85 kDa (type II receptor), and >200 kDa (betaglycan) corresponding to those on many mesenchymal cells (2–7). To establish basal receptor longevity, cultures were treated with inhibitors of protein or mRNA synthesis before radioligand binding. Within 2 h of treatment with cycloheximide to inhibit new protein synthesis, 125I-TGF-β1 binding to type I and II receptors declined to approximately 50% of its initial levels, with negligible binding after 24 h. Cycloheximide also decreased binding to betaglycan, but this was less rapid, with a 50% reduction after 6 h. Treatment with α-amanitin to block type II RNA polymerase activity also reduced the levels of all TGF-β binding sites. In this case, 7 h were needed to achieve a decrease analogous to the effect of a 2-h cycloheximide treatment at type I receptor, while a 50% decrease in TGF-β binding to type II receptor and betaglycan was reached only after 17–18 h (Fig. 2 and Table 1). Although the initial proportions of TGF-β binding to each receptor site differed among the various cell models that we tested (see Ref. 5), essentially the same relative effects of each inhibitor occurred on the individual receptors that are found on less differentiated population 1, on fetal rat fibroblasts, and on rat osteosarcoma-derived ROS 17/2.8 cultures. For example, essentially only type I receptors are seen on ROS 17/2.8, and they were reduced ~4-fold more rapidly by cycloheximide than by α-amanitin treatment (data not shown).

**Ligand-dependent Variations in Cell Surface Receptors—**When population 3–5 cultures were preincubated for 4 h with as little as 4–40 pm unlabeled TGF-β1, 125I-TGF-β binding decreased specifically at all receptors, and a nearly complete absence of radioligand-reactive sites occurred with 400 pm TGF-β1. Virtually the full cell surface binding capacity returned after 24–48 h exposure to 4 or 40 pm TGF-β1, although the apparent M₀ of betaglycan was somewhat larger with 40 pm TGF-β1 treatment. In contrast, down-regulation persisted with 400 pm TGF-β1 (Fig. 3, left panel). Analogous to results from biological studies (Fig. 1B), lower concentrations of TGF-β1 were less effective in population 1 at any time point examined (Fig. 3, right panel), whereas down-regulation was evident with TGF-β1 at 400 pm. Furthermore, the relative M₀ of betaglycan in population 1 did not increase as noticeably with TGF-β1 treatment, but it was initially greater than that on population 3–5 (5).

**Receptor Transcripts—**Up to this point our findings indicated that functional TGF-β receptors turned over rapidly, that steady state binding site levels required continuous new translation and transcription, and that the binding capacity of all three cell surface components seemed less stable than their corresponding mRNAs. To confirm the possibility that protein stability had a more dominating role in receptor equilibrium, receptor transcript turnover was examined. RNase protection assays with receptor-specific cRNAs demonstrated discrete protected fragments corresponding to the length of each probe (type I receptor, 275 nucleotides; type II receptor, 182 nucleotides; betaglycan, 225 nucleotides, Fig. 4A). Smaller protected fragments of about 100 and 70 nucleotides were observed with type I receptor cDNA, and fragments of 130–140 nucleotides with the type II receptor cRNA, consistent with a homology among the large family of serine/threonine kinase receptor proteins presently reported (discussed in Refs. 2–4) and some not yet fully characterized. For type II receptor, this may relate to transcripts found in pituitary gland that differ from the probe we cloned from fetal rat osteoblasts by only 8% (19). Treatment with α-amanitin showed a decrease in the presence of pre-existing receptor transcripts over time (Fig. 4B). To ensure specificity for TGF-β receptors, only protected bands consistent with the entire length of the probes are shown, although the material present in the smaller protected fragments exhibited parallel decay curves (data not shown). The rates of transcript loss with transcription inhibitor were each compatible with the ligand binding studies shown in Fig. 2 and corroborate that receptor protein levels appeared to turn over more rapidly than steady state receptor transcripts (Table 1).

**Protein Kinase-related Effects—**Each result predicted that cell surface TGF-β receptors were likely to be regulated by transcriptional and post-transcriptional events. Sequence analysis first revealed that type II and type I TGF-β receptors contain serine/threonine kinase domains necessary for ligand-dependent receptor transphosphorylation and TGF-β-mediated signal transduction, respectively. However, receptor phosphorylation has also been observed prior to, and therefore independently of, ligand engagement (20–23). Consequently, TGF-β receptors also appear to be substrates for other cellular kinases. Little else about ligand-independent TGF-β receptor

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**Table 1**

| Inhibitor | Binding cycloheximide | Binding α-amanitin | Transcripts α-amanitin |
|-----------|------------------------|-------------------|-----------------------|
| Type I receptor half-life | 2 h | 7 h | 6 h |
| Type II receptor half-life | 2 h | 17 h | 18 h |
| Betaglycan half-life | 6 h | 18 h | 20 h |

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3 V. Rosen, personal communication, Genetics Institute, Cambridge, MA.
phosphorylation is known, and we hypothesized that changes in kinase or phosphatase activity might influence functional receptor stability. To initiate studies in this regard, osteoblast-enriched population 3–5 cultures were used to examine the effect of changes in kinase and phosphatase activity on overall $^{125}$I-TGF-$\beta$-binding capacity. Treatment for 24 h with either H-8 or staurosporine to inhibit endogenous cellular protein kinase activity revealed striking alterations in the $^{125}$I-TGF-$\beta$-1 binding profile. H-8 at 3–30 $\mu$m and staurosporine at 3–30 $\mu$m dose-dependently enhanced cell surface betaglycan binding capacity. Both agents also enhanced $^{125}$I-TGF-$\beta$-1 binding to type II receptors, whereas binding to type I receptors peaked after treatment with lower doses and declined at higher concentrations of protein kinase inhibitor. In the latter instance, disproportional high levels of TGF-$\beta$-binding to betaglycan coincided with a decrease in its binding at type I receptors (Fig. 5A).

Both protein kinase inhibitors induced similar changes in population 1 cultures and also enhanced $^{125}$I-TGF-$\beta$-1 binding within an uncharacterized 50-kDa complex that was less obvious in population 3–5 (data not shown). The inhibitor concentrations at which these effects occurred suggested the importance of kinases other than protein kinase A (24–26). To examine this possibility, cells were treated with H-89, which at low concentrations (50 $\mu$m) specifically reduces protein kinase A activity (27). Unlike the effects of H-8 and staurosporine, no significant increases in TGF-$\beta$-binding site occupancy occurred with 50 $\mu$m H-89 for various time intervals up to 24 h (Fig. 5B) or with H-89 concentrations up to 5 $\mu$m (data not shown). Treatment with 10 $\mu$m H-8 or 10 $\mu$m staurosporine, which effectively increased TGF-$\beta$-binding, did not have similar stimulatory effects on cellular protein content or another cell membrane-associated protein, alkaline phosphatase, and inhibited new protein synthesis rates by 20–30%. Moreover, these agents also inhibited new DNA synthesis rates by 40–50% (Fig. 6). These results indicate that the increased levels of TGF-$\beta$-binding that were observed with these agents did not result from nonspecific membrane effects or from increases in new protein or DNA synthesis.

Finally, the influence of endogenous phosphatase activity on TGF-$\beta$-binding was examined. Treatment with 1 $\mu$m of the phosphatase inhibitor okadaic acid reduced cell attachment within 3 h in all cell culture models that we examined. However, cells remained bound during 24 h treatment with lower concentrations (0.1–0.3 $\mu$m) of okadaic acid or with two other phosphatase inhibitors, vanadate and fluoride. In contrast to effects produced by kinase inhibitors, these phosphatase inhibitors caused a striking decrease in binding to all three sites (e.g. 24 h treatment with 0.1–1 $\mu$m vanadate or 1–3 $\mu$m fluoride; Fig. 5C). No amounts of fluoride that we tested significantly reduced cellular protein content, alkaline phosphatase activity, or protein synthesis within this time interval, whereas at 1 $\mu$m, DNA synthesis was stimulated 3-fold. With regard to vanadate, even at 0.3 $\mu$m, where TGF-$\beta$-binding was potently suppressed, total cellular protein content was not significantly affected, alkaline phosphatase activity was at 80%, and the rate of protein synthesis was at 40% of the control level. In contrast to the stimulatory effect of fluoride, only new DNA synthesis was potently suppressed to 20% of control by vanadate treatment (Fig. 6). Therefore, the reduced levels of TGF-$\beta$-binding that occurred with phosphatase inhibitors could not be directly attributed to consistent, proportional, or in all cases parallel changes in cell recovery or viability.

**DISCUSSION**

The abundance of TGF-$\beta$s in many tissues often may exceed the amounts needed for cell function. Some control could exist at the level of TGF-$\beta$-activation from latent complexes, but this may not fully explain dissimilar effects by TGF-$\beta$s in separate tissues or even in different cells within the same tissue (1, 28). For example, fetal rat skin and kidney fibroblasts and less differentiated and osteoblast-like bone cells respond quantitatively and qualitatively in very different ways to TGF-$\beta$, yet all exhibit essentially the same three TGF-$\beta$-binding sites (5). We therefore speculated that, at least in bone, variations in cellular effects might result from different intracellular response systems, from changes in the proportions of particular TGF-$\beta$ receptors on individual cells or from some combination of both. In this way, specificity would be focused at the responding cell without obstructing requirements by other cells within the same tissue. In this respect, changes in TGF-$\beta$-binding may have been associated with development and cellular differentiation, with neoplastic transformation, and in response to hormones and other growth factors (reviewed in Ref. 1). Nonetheless, little information is available about the biochemical events that control the selective gain, loss, or stability of TGF-$\beta$ receptors.

In the present study we wished to define basal receptor...
stability. To do so, we examined the longevity of pre-existing TGF-β binding sites in the presence of agents that suppressed new protein or mRNA expression. Inhibition of protein synthesis quickly depleted the amounts of signal transducing type I and type II receptors, and these changes were 3–9-fold more rapid than the effects that occurred when mRNA transcription was inhibited. The more rapid rate of receptor depletion when protein synthesis was disrupted indicated that the levels of ligand-reactive receptor protein were less stable than their corresponding mRNAs, an observation confirmed by assessment of receptor transcript turnover.

Decreased binding of radioligand in inhibitor-treated cultures occurred without added TGF-β, indicating that functional receptors turned over in the absence of previous engagement of ligand, and are constantly replaced by translational and transcriptional events. However, incubation with unlabeled TGF-β also rapidly reduced subsequent binding of radioligand. With lower amounts of TGF-β, the decrease in binding capacity was transient. Our latter findings contrast with earlier studies suggesting that TGF-β receptors on fetal rat fibroblasts do not down-regulate appreciably (29, 30). Those studies evaluated receptor down-regulation after up to a 2-h treatment with unlabeled TGF-β followed by a 2–2.5-h recovery period before radioligand binding, while we examined specifically bound and cross-linked receptors immediately after treatment periods of 4–48 h. Consequently, these differences could be accounted for by the shorter periods of receptor engagement in the previous studies or the inclusion of a “washout” period during which the rapid re-expression of cell surface receptors that we report may have occurred. Similar to results from biological studies, more differentiated bone cell cultures are more sensitive to low concentrations of TGF-β, although even in these cells down-regulation persisted after treatment with higher concentrations of growth factor. In total, the rapid loss of binding sites for TGF-β in cultures where protein synthesis was inhibited and the swift recovery from down-regulation induced by low to moderate concentrations of TGF-β are both consistent with short receptor half-lives and steady state binding profiles that occur in the
presence of a rapid flux in cell surface receptors. To our knowledge, these are the first studies that directly evaluate TGF-β receptor stability in these ways and reveal the importance of post-transcriptional events on receptor expression and ligand binding.

The changes we observe could depend on TGF-β receptor synthesis or on other proteins that shuttle receptors between the membrane and a cytosplasmic pool. For example, recent histochemical studies suggest hormone-dependent changes in membrane and cytosplasmic type II receptor in ovarian cells (31). Alternatively, they could require the presence of components that maintain receptors in a state of binding “competence.” To address the latter possibility, we considered the involvement of protein phosphorylation. This was based on two sets of observations. First, type I and type II TGF-β receptors are themselves phosphorylated prior to TGF-β engagement or by in vitro kinase assays in the absence of ligand (21–23). Second, their appearance on bone cells is altered by BMPs (5) that are thought to activate their own receptor kinases (32). We therefore hypothesized that changes in other cellular kinases or in phosphatase activity might influence functional TGF-β binding site stability. Indeed, certain inhibitors of protein kinase activity induced distinctive and pronounced effects. TGF-β binding to type I and II receptors and betaglycan all increased after treatment with specific kinase inhibitors. However, the increased level of TGF-β binding to type I receptor declined with higher inhibitor concentrations, similar to earlier studies in bone cells where large increases in binding to betaglycan coincide with a loss at type I receptors (6). The substrate specificities of inhibitors needed to enhance TGF-β binding suggested the involvement of kinases other than protein kinase A (24, 25, 27). Consistent with results from kinase inhibitor studies, inhibitors of cellular phosphatase activity rapidly reduced TGF-β binding. The enzymes and the substrates responsible for these changes are not yet defined. However, they do not seem to be nonspecific effects since another cell membrane-associated protein, alkaline phosphatase, was not similarly affected by these enzyme inhibitors, and changes in other parameters of cellular activity were not influenced in parallel or consistent ways. Only new DNA synthesis was suppressed to an extent that approached the decrease in TGF-β binding, but this change reflects new rounds and replication and not cell number. Moreover, our previous studies (9) demonstrated that new protein synthesis rates in unstimulated or TGF-β-induced cultures were not significantly affected when DNA synthesis was blocked with hydroxyurea. These results thus uncouple TGF-β receptors themselves and the effect of this factor on protein synthesis from new rounds of cell replication. In addition to the effects on TGF-β receptors that we note, other evidence reveals that changes in the balance between kinase and phosphatase activity could be an important component of recycling of receptors for matrix proteins on isolated neutrophils, although the phosphoproteins involved in this event also have not yet been identified (33). Our results provide the only evidence that we know suggesting that variations like these might affect functional cell surface TGF-β receptor longevity or activity in any tissue-derived cell.

Differences in individual binding sites may help to focus TGF-β to specific receptors or courses of events. In several cell models, type II TGF-β receptor phosphatases and activates type I receptor after ligand binding (21–23, 28, 32, 34, 35). In this regard, mutation of several amino acids in or near the GS domain of type I receptor reduces its phosphorylation and ligand-mediated signal transduction. In contrast, mutation of a single threonine just prior to the kinase domain of the type I receptor to a negatively charged amino acid, an effect that mimics its phosphorylation, induces type II receptor-independent cellular activities similar to those in TGF-β-treated cultures (20–23). There is no in vivo evidence for an analogous type I receptor phosphorylation, even after cells are treated with TGF-β. Nonetheless, it might result from the action of kinases other than the type II receptor itself. Our prior studies in less and more differentiated bones cells or in response to BMP-2 (5) and in endothelial cells in response to changes in matrix organization (36) suggest that changes in the ratio of TGF-β binding to type I and type II receptors can dissociate effects that depend on both TGF-β receptors from those mediated by type I receptor predominantly. Consequently, certain independent downstream events in response to TGF-β may accompany upward or downward variations in one or another TGF-β receptor, mediated by complex transcriptional and post-transcriptional events.

In total, our current results indicate surprisingly swift TGF-β receptor flux that contrasts with the relatively longer half-lives of platelet-derived growth factor and insulin-like growth factor receptors (37) previously observed on fetal rat bone cells under identical conditions. TGF-β have potent effects on bone cell replication and phenotype expression that vary with differentiation, hormone, and growth factor treatment, and perhaps with age (1). Therefore, rapid changes in TGF-β receptor gene expression, protein synthesis, cell surface half-life, and ligand recognition could provide many opportunities for reversible changes in its function according to the immediate or progressive needs or constraints placed on skeletal cells. Of these, the most significant may be changes in ligand recognition, imposed on overall receptor protein expression. Detailed evidence for the importance of each process under specific physiological or pathological circumstances becomes possible when the basic mechanisms of receptor expression and ligand recognition have been defined. Evidence from our present studies could provide an initial step to understand how these changes occur during osteogenesis.

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