Purification and Characterization of Transforming Growth Factor-β2.3 and -β1.2 Heterodimers from Bovine Bone*

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A unique form of transforming growth factor-β (TGF-β), TGF-β2.3 heterodimer, has been purified from bovine bone extract. TGF-β2.3 migrated as a single 25-kDa band by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, whereas under reducing conditions it migrated as a 12.5 kDa band. The TGF-β2.3 reacted positively with anti-TGF-β2 and anti-TGF-β3 antibodies on immunoblots. Equal levels of TGF-β2 and TGF-β3 sequences were detected by N-terminal sequencing. TGF-β2.3 eluted as a single sharp peak by reverse-phase high performance liquid chromatography. However, prior reduction of the protein with dithiothreitol resulted in the protein eluting in two peaks, one containing predominantly TGF-β3 and the other containing predominantly TGF-β2. TGF-β2.3 inhibited proliferation of mink lung epithelial cells and promoted the formation of colonies of normal rat kidney fibroblasts in culture with specific biological activity similar to those of TGF-β1 and TGF-β2. These results demonstrate that the protein is TGF-β2.3 heterodimer, consisting of one polypeptide chain each of TGF-β2 and TGF-β3 linked by one or more disulfide bonds. In addition, TGF-β1.2 heterodimer, previously found only in porcine platelets, has also been purified from bovine bone extract.

Transforming growth factor-β (TGF-β),1 a multifunctional regulator of cellular proliferation and differentiation, is a member of a supergene family of structurally related proteins (1). TGF-βs are homodimeric proteins with molecular weights of approximately 25,000. TGF-β1.2, consisting of one polypeptide chain each of TGF-β1 and TGF-β2, has been purified from porcine platelets and is the only TGF-β heterodimer thus far reported (2). Although TGF-β1, TGF-β2, and TGF-β3 have been purified from natural sources (3–5), the existence of TGF-β3 heterodimer, consisting of one polypeptide chain each of TGF-β2 and TGF-β3 linked by one or more disulfide bonds, and the ability of TGF-β to inhibit proliferation of mink lung epithelial cells (14, 15) have been demonstrated (12, 13). The synthetic peptide was conjugated to keyhole limpet hemocyanin prior to immunization (16).

**Experimental Procedures**

Bovine Bone Extract—Extract of bovine bone in guanidine HCl was prepared from 400 pieces of fresh metatarsal and metacarpal bones and fractionated essentially as previously described (3, 9). The extract was concentrated by ultrafiltration through a sheet of Amicon YM-10 membrane and fractionated by Sephacryl S-200 gel filtration column chromatography. However, prior reduction of the protein with dithiothreitol resulted in the protein eluting in two peaks, one containing predominantly TGF-β3 and the other containing predominantly TGF-β2. TGF-β2.3 inhibited proliferation of mink lung epithelial cells and promoted the formation of colonies of normal rat kidney fibroblasts in culture with specific biological activity similar to those of TGF-β1 and TGF-β2. These results demonstrate that the protein is TGF-β2.3 heterodimer, consisting of one polypeptide chain each of TGF-β2 and TGF-β3 linked by one or more disulfide bonds. In addition, TGF-β1.2 heterodimer, previously found only in porcine platelets, has also been purified from bovine bone extract.

Purification of TGF-β2.3 Heterodimer—The TGF-β2.3 fractions from carboxymethylcellulose chromatography were pooled and chromatographed by C18 RP-HPLC. The bound proteins were eluted with a linear acetonitrile gradient in 0.1% trifluoroacetic acid (Fig. 1A). TGF-β2 eluted as the major peak. A peak of TGF-β1 eluted 3.6 min before TGF-β2. SDS-PAGE revealed that fraction 5, containing the proteins eluting slightly before the TGF-β2 peak, contained a major 25 kDa band that migrated slightly slower than TGF-β1 and TGF-β2 on the SDS-polyacrylamide gel (Fig. 1B).

Fraction 5 from two lots of 400 bones was combined and chromatographed on Mono-S FPLC (Fig. 2). Bound proteins were eluted with a pH 4.6–6.7 gradient, followed by a pH 6.7–9.0 gradient and a linear 10–300 mM NaCl gradient at pH 9.0. Some proteins eluted during the pH 4.6–5.7 gradient (Fig. 2).
but the majority of the proteins eluted during the pH 6.7–9.0 gradient. SDS-PAGE of the fractions from the pH 4.6–6.7 gradient revealed that fraction 4 contained predominantly a 25-kDa protein, which migrated at $M_r = 12,500$ when reduced (result not shown), as would be expected for TGF-$\beta$. However, in a separate study, we observed TGF-$\beta_2$ and TGF-$\beta_1$ to elute during the pH 6.7–9.0 gradient and the salt gradient at pH 9.0, respectively (Fig. 7).

Fraction 4 from the pH 4.6–6.7 gradient was chromatographed by analytical C18 RP-HPLC (Fig. 3). The 25-kDa protein eluted as a major peak (peak 1), followed by a minor peak (peak 2). The yield of the 25-kDa protein in peak 1 was approximately 0.5 µg/kg of bone powder.

Characterizations—SDS-PAGE revealed that both the major (peak 1) and minor (peak 2) peaks contained a single 25 kDa band under nonreducing conditions and a 12.5 kDa band under reducing conditions (Fig. 4).

N-terminal sequencing, performed on the 25-kDa protein from peak 1, revealed the presence of TGF-$\beta_2$ and TGF-$\beta_3$ sequences at nearly equal levels, in particular at sequence cycles 11, 13, 17, and 33 (Fig. 5). However, the data suggest that the sample contained somewhat more TGF-$\beta_2$ polypeptide chain than TGF-$\beta_3$. No TGF-$\beta_1$ sequence was detected. The sequence data demonstrate that the 25-kDa protein is TGF-$\beta_2.3$ heterodimer.

In addition, the results of immunoblot analysis showed that the 25-kDa protein in both peaks 1 and 2 contained TGF-$\beta_2$ and TGF-$\beta_3$ epitopes. The 25-kDa protein reacted positively with anti-TGF-$\beta_3$ polyclonal and 3C7.14 anti-TGF-$\beta_2$ monoclonal (12) antibodies (result not shown).
TGF-β1, TGF-β2, and TGF-β3 sequences are shown as a reference (1). Numbers in parentheses correspond to picomoles of the amino acid residue that was detected during sequencing of 200 pmol of TGF-β1.2 and 1200 pmol of TGF-β2.3. Cys residues were not detected and are designated Xxx.

**TABLE 1**

Relative potencies of TGF-β1.2 and TGF-β2.3 on cell growth

| TGF-β  | ED<sub>50</sub> (ng/ml) | MvLu<sup>a</sup> | NRK-49P<sup>b</sup> |
|--------|------------------------|----------------|-----------------
| TGF-β1.2 | 0.049                  |               |                 |
| TGF-β2.3, peak 1 | 0.018              |               |                 |
| TGF-β2.3, peak 2 | 0.022              |               |                 |
| TGF-β1 | 0.013                  |               |                 |
| TGF-β2 | 0.024                  |               |                 |

<sup>a</sup> Mink lung epithelial cells.
<sup>b</sup> Rat kidney fibroblasts.

TGF-β1 elutes before TGF-β2, whereas TGF-β3 elutes after TGF-β2 by C18 RP-HPLC performed with a linear acetonitrile gradient in 0.1% trifluoroacetic acid (8). In contrast, the TGF-β2.3 (peak 1) eluted as a single sharp peak slightly before TGF-β2, but after TGF-β1, by C18 RP-HPLC (result not shown). When TGF-β2.3 was reduced with dithiothreitol prior to HPLC, the protein eluted in two peaks (Fig. 6). The position of the later eluting peak coincided with the elution position of TGF-β2 when similarly reduced. Immunoblotting performed with B1/29 anti-TGF-β2 (15) and anti-TGF-β3 polyclonal antibodies revealed that the later eluting peak contained predominantly TGF-β2, whereas the earlier eluting peak contained predominantly TGF-β3 (not shown). N-terminal sequencing revealed that the earlier eluting peak contained approximately 70% TGF-β3 and 30% TGF-β2 sequences.

**Purification and Characterization of TGF-β1.2 Heterodimer**—The fraction 4 from C18 RP-HPLC (Fig. 1A) was rechromatographed by C18 RP-HPLC, followed by Mono-S FPLC. The bound proteins were eluted from the Mono-S column with pH 4.6-6.7 and pH 6.7-9.0 gradients, followed by a linear 10–300 mM NaCl gradient at pH 9.0 (Fig. 7). SDS-PAGE revealed that fractions 6 and 7 contained a 25-kDa protein (result not shown). In contrast, TGF-β1 and TGF-β2 homodimers eluted elsewhere (Fig. 7). Fractions 6 and 7 were pooled, and the 25-kDa protein was purified by C18 RP-HPLC.

The results of SDS-PAGE (Fig. 4), immunoblot analysis, C18 RP-HPLC, N-terminal sequencing (Fig. 5), and cell culture assay (Table 1) demonstrated that the 25-kDa protein is TGF-β1.2 heterodimer. The yield was approximately 1 μg/kg of bone powder.

**DISCUSSION**

TGF-βs have been isolated from a number of tissues. However, bone is an especially rich source of TGF-βs (9). This is consistent with the known roles of these factors in chondrogenesis and osteogenesis (18, 19). Although TGF-β1.2 and TGF-β2.3 are present in relatively very low levels in the bone, it is possible that these heterodimeric forms of TGF-β have unique biological activities and potencies differing from those of TGF-β1 and TGF-β2. TGF-β3 homodimer and heterodi-

![Graph](image)

**Fig. 5.** N-terminal sequences of TGF-β2.3 and TGF-β1.2 heterodimers. TGF-β1, TGF-β2, and TGF-β3 sequences are shown as a reference (1). Numbers in parentheses correspond to picomoles of the amino acid residue that was detected during sequencing of 200 pmol of TGF-β1.2 and 1200 pmol of TGF-β2.3. Cys residues were not detected and are designated Xxx.

**Fig. 6.** C18 RP-HPLC of TGF-β2.3 under reducing conditions. TGF-β2 (20 μg) and TGF-β2.3 (25 μg) were reduced in the presence of 25 mM dithiothreitol and chromatographed by C18 RP-HPLC (0.2 x 25 cm, Vydac, 218TP52) using a linear acetonitrile gradient in 0.1% trifluoroacetic acid. TGF-β2.3 (peaks 1 and 2) inhibited proliferation of mink lung epithelial cells half-maximally (ED<sub>50</sub>) at 0.018 and 0.023 ng/ml, respectively (Table 1). In addition, peak 1 stimulated formation of colonies of normal rat kidney fibroblasts with the ED<sub>50</sub> of 0.06 ng/ml (Table 1). These specific biological activities of TGF-β2.3 are comparable with those of TGF-β1 and TGF-β2.
meric forms of TGF-β are not commonly found in the natural sources.

Since the dimerization of monomeric TGF-β precursor polypeptide chains occurs intracellularly (20), a majority of the cells in bone probably express and secrete either TGF-β1 or TGF-β2, whereas very few cells co-express both TGF-β2 and TGF-β3 genes, leading to production of TGF-β2.3 heterodimer. However, it is also possible for one type of cells to produce two forms of TGF-β homodimers simultaneously. A cell line of African green monkey kidney epithelial cells (BSC-1 cells) has been shown to produce both TGF-β1 and TGF-β2 homodimers (21). These cells have not been shown to secrete TGF-β1.2. Although we have discovered TGF-β2.3 heterodimer, we have not yet detected the TGF-β3 homodimer in the bone extract.

REFERENCES