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Research Article

Transforming growth factor beta 1 (TGF-beta 1) is a key regulator of cell growth and differentiation. Under normal physiological conditions, it is made as a biologically latent complex whose significance is unknown. Previous work has indicated that active TGF-beta 1 has a very short plasma half-life in rats (Coffey, R. J., L. J. Kost, R. M. Lyons, H. L. Moses, and N. F. La-Russo. 1987. J. Clin. Invest. 80:750-757). We have investigated the possibility that latent complex formation may extend the plasma half-life of TGF-beta 1 and alter its organ distribution. Radiolabeled latent TGF-beta 1 was formed by noncovalent association of 125I-TGF-beta 1 with the TGF-beta 1 precursor "pro" region from recombinant sources. TGF-beta 1 in this latent complex had a greatly extended plasma half-life (greater than 100 min) in rats compared with active TGF-beta 1 (2-3 min). Whereas active TGF-beta 1 was rapidly taken up by the liver, kidneys, lungs, and spleen and degraded, TGF-beta 1 in the latent complex was largely confined to the circulation, and was less than 5% degraded after 90 min. The pharmacokinetics of TGF-beta 1 in the latent complex were shown to be critically dependent on the degree of sialylation of the complex. The results suggest that formation of latent complexes may switch endogenous TGF-beta 1 from an autocrine/paracrine mode of action to a […]

Recombinant Latent Transforming Growth Factor β1 Has a Longer Plasma Half-Life in Rats than Active Transforming Growth Factor β1, and a Different Tissue Distribution

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Abstract

Transforming growth factor β1 (TGF-β1) is a key regulator of cell growth and differentiation. Under normal physiological conditions, it is made as a biologically latent complex whose significance is unknown. Previous work has indicated that active TGF-β1 has a very short plasma half-life in rats (Coffey, R. J., L. J. Kost, R. M. Lyons, H. L. Moses, and N. F. LaRusso. 1987. *J. Clin. Invest.* 80:750–757). We have investigated the possibility that latent complex formation may extend the plasma half-life of TGF-β1 and alter its organ distribution. Radiolabeled latent TGF-β1 was formed by noncovalent association of ^{125}I–TGF-β1 with the TGF-β1 precursor "pro" region from recombinant sources. TGF-β1 in this latent complex had a greatly extended plasma half-life (> 100 min) in rats compared with active TGF-β1 (2–3 min). Whereas active TGF-β1 was rapidly taken up by the liver, kidneys, lungs, and spleen and degraded, TGF-β1 in the latent complex was largely confined to the circulation, and was < 5% degraded after 90 min. The pharmacokinetics of TGF-β1 in the latent complex were shown to be critically dependent on the degree of sialylation of the complex. The results suggest that formation of latent complexes may switch endogenous TGF-β1 from an autocrine/paracrine mode of action to a more endocrine mode involving target organs distant from the site of synthesis. (*J. Clin. Invest.* 1990. 86:1976–1984.) Key words: transforming growth factor β1 • latent complex formation • plasma half-life

Introduction

Transforming growth factor-β1 (TGF-β1)[1] is the prototype of a family of proteins that is intimately involved in the regulation of virtually every physiological process (for review see reference 1). Experiments in animal model systems have indicated that TGF-β1 has great clinical potential in the areas of wound healing (2), immunosuppression and myeloprotection (3), and tumor ablation (4). Human clinical trials for TGF-β1 in some of these areas are anticipated in the coming 5 yr.

TGF-β1 differs from the majority of growth regulatory factors in that it is generally synthesized and secreted in a biologically latent form, and this must be activated before TGF-β1 can exert its biological effects on target cells (5, 6). The nature of the activation mechanism in vivo is unclear, but may involve proteases, and in some instances may be dependent on cell–cell interactions (7–9). The major latent form of TGF-β1 that occurs naturally is a high molecular weight complex, in which the homodimeric active TGF-β1 is noncovalently associated with a dimer of the remainder of its precursor "pro" region, and this in turn is disulfide-bonded to a third, structurally unrelated protein of 135 kD (10, 11).

Recombinant systems expressing the entire coding region of the TGF-β1 gene also make TGF-β1 in a latent form (12, 13). However, in this case the latent complex consists only of active TGF-β1, noncovalently associated with the precursor "pro" region. This indicates that the precursor "pro" sequence alone is sufficient to confer latency on TGF-β1, and the 135-kD protein of the natural latent complex must have a different function. The dimeric form of the TGF-β1 precursor "pro" region is now termed the TGF-β1 latency-associated peptide (LAP).

The in vivo significance of the latent form of TGF-β1 is unclear. However, since it appears to be the predominant naturally occurring form of the molecule, it may be a more appropriate form of TGF-β1 to use in many clinical settings than the active molecule. Given the pleiotropic effects of active TGF-β1 on virtually every organ system, systemic administration of active TGF-β1 may bypass normal regulatory mechanisms that operate on latent TGF-β1 to restrict its activity spatially and temporally, and could result in undesirable responses in non–target organs. Furthermore, recent data have indicated that active TGF-β1 has an extremely short plasma half-life in rats (14). In the present work we have asked whether one role of latent complex formation might be to extend the plasma half-life of TGF-β1, and to alter its organ distribution. To do this, we have compared in rats the relative pharmacokinetics of active TGF-β1, and latent TGF-β1 complexes, formed by recombining ^{125}I–TGF-β1 with TGF-β1 LAP from recombinant sources.

Methods

Assays for TGF-β1 and LAP

Radioreceptor assays. Porcine platelet TGF-β1 was obtained from R&D Systems Inc. (Minneapolis, MN), and iodinated to a specific activity of ~ 80 μCi/μg by the modified chloramine T method (15). The latent TGF-β1 complex was quantitated by radioreceptor assay

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1. *Abbreviations used in this paper:* α_2M, α_2-macroglobulin; BS^3, bis-sulfosuccinimidyl suberate; DHFR, dihydrofolate reductase; LAP, latency-associated peptide; MTX, methotrexate; TGF-β1, transforming growth factor β1; S-LAP, sialylated LAP; U-LAP, undersialylated LAP.

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(13). The LAP was assayed by its ability to inhibit the binding of ^{125}I-TGF-β1 to its receptor on A549 cells after preincubation of samples with ^{125}I-TGF-β1 for 2 h at 20°C to allow complex formation to occur. A unit of LAP activity was defined as the amount of LAP required to cause 50% reversal of the specific binding of 250 pg ^{125}I-TGF-β1 to receptors on A549 cells. 40 U LAP was sufficient to neutralize completely 1 ng TGF-β1.

Native agarose gel electrophoresis. Latent complexes formed between ^{125}I-TGF-β1 and LAP proteins were visualized by native agarose electrophoresis in uncharged, 0.5% composite agarose-galactomannan gels (Isogel; FMC Bioproducts Corp., Rockland, ME) essentially according to McCaffrey et al. (16). Gels were prepared with 50 mM Tris-acetate buffer, pH 7.0, and run at 100 V for ~ 6 h with cooling. Dried gels were exposed overnight to G-Mat film (Eastman Kodak Co., Rochester, NY).

Chemical cross-linking and SDS-PAGE analysis. Latent complex formation was also analyzed by denaturing gel electrophoresis under nonreducing conditions, using 3–15% polyacrylamide gradient gels. The noncovalent latent complex was stabilized before electrophoresis by chemical cross-linking of samples with 2 mM bis-sulfosuccinimidyl suberate (BS^3; Pierce Chemical Co., Rockford, IL) for 30 min at 4°C. In the absence of cross-linking, the noncovalent complex is dissociated by the SDS in the electrophoresis sample buffer. Cross-linking analysis allows the molecular weight of the latent complex to be determined. However, since the cross-linking reaction is relatively low efficiency (~ 25%), it does not indicate what fraction of total TGF-β is in the latent form. Dried gels were exposed to Kodak X-Mat film for 36 h to visualize bands.

Transfections and selections

A vector encoding TGF-β1 (pSBβ, reference 13) was cotransfected into dihydrofolate reductase–negative Chinese hamster ovary ($DHFR^-$ CHO) cells (17) with the DHFR vector pFD11 (18). Cells expressing DHFR were selected as described (18). $DHFR^+$ cells were screened for TGF-β1 expression by metabolic labeling and RIA (13). Cells positive for TGF-β1 expression were treated with increasing concentrations of methotrexate (MTX) to select derivatives having amplified copies of the transfected plasmids. Two clones exhibiting elevated levels of TGF-β1 expression were subjected to further analysis: clone MJβ1, selected in 100 nM MTX; and clone C3/12A, selected in 1 μM MTX. Subsequent analysis (see Results) revealed that clone MJβ1 represented a variant CHO cell (19) that produced a form of latent TGF-β1 deficient in sialic acid.

Medium collection

Cells were seeded into eight 2-liter roller bottles at a density of 3.3 × 10^5 cells per bottle. The medium was a modified mixture of selective Ham's F-12 and DME with additions including either 100 nM (clone MJβ1) or 1 μM (clone 3/12A) MTX and 2% extensively diafiltered serum. After 5 d, the cells were grown in a serum-free mixture of Ham's F-12 and DME. Cell supernatants were harvested after 7 d and concentrated from an initial volume of 2.5 liters to ~ 200 ml by ultrafiltration in a Stir Cell (Amicon Corp., Danvers, MA) (30-kD cutoff).

Purification of LAP

All initial clearance experiments were performed on LAP purified from the MJβ1 clone. The conditioned medium was dialyzed against 20 mM Tris-Cl, pH 7.5, and fractionated on a Mono Q HR16/10 anion-exchange column. All chromatography steps were performed at room temperature using a fast protein liquid chromatography system (Pharmacia-LKB Biotechnologies Inc., Piscataway, NJ.) The latent TGF-β1 complex eluted in the flow-through, and was then dialyzed against 20 mM sodium phosphate, pH 7.0, containing 8 M urea to dissociate the latent complex. The dialyzed material was fractionated on a Mono S HR5/5 cation exchange column, developed with a 70 ml gradient of 0–0.3 M NaCl, in the presence of 8 M urea. The TGF-β1 LAP eluted at 0.08–0.09 M NaCl, whereas free TGF-β1 eluted at 0.13 M NaCl. Pooled peak fractions were concentrated by ultrafiltration using a Centricon 10 device, and further purified by gel filtration on a Superose 12 column with PBS containing 10% glycerol as the elution buffer. The LAP eluted with a retention volume of 14 ml. The final material was 80–90% pure as judged by SDS polyacrylamide gel electrophoresis. Since subsequent analysis (see Results) revealed this material was undersialylated, it is referred to as U-LAP.

For purification of TGF-β1 LAP from the C3/12A clone, the conditioned medium was dialyzed and loaded on a Mono Q anion-exchange column as above. Since the latent complex produced by this culture system was anionic, due to more extensive sialylation of the complex than occurred with the MJβ1 clone (see Results), it bound to the column and was eluted at 0.05–0.1 M NaCl. Pooled peak fractions were dialyzed against 20 mM Tris-Cl, pH 7.5, containing 6 M urea, and fractionated on a Mono Q HR5/5 column, using a 0–0.3 M NaCl gradient in the presence of 6 M urea. The LAP eluted at 0.11–0.14 M NaCl, well separated from free TGF-β1 that eluted in the flow through, and from the bulk of contaminating protein that eluted at lower ionic strength. Pooled peak fractions were further purified on Superose 12 as above. SDS-PAGE analysis indicated the LAP was 40–50% pure. Because of the more extensive sialylation of this species, this material is referred to as S-LAP.

Neuraminidase digestion

The sialic acid content of the LAP samples was examined by comparing the molecular weight of the reduced LAP species on Western blots, before and after digestion of conditioned media or partially purified LAP protein overnight at 37°C with 14 U/ml soluble or immobilized neuraminidase (Type XA; Sigma Chemical Co., St. Louis, MO) in 100 mM sodium acetate, pH 5.6, containing 1 μg/ml leupeptin and pepstatin, and 2 μg/ml aprotinin. Immunoblots of the conditioned media samples were probed with a polyclonal antibody against a synthetic peptide corresponding to residues 46–56 of the TGF-β1 precursor to visualize the LAP, as described (13).

Pharmacokinetic studies

Labeled latent complex for the pharmacokinetic studies was made by incubating ^{125}I-TGF-β1 with an excess of LAP (≥ 40 U LAP/ng TGF-β1), in PBS, overnight at 4°C. Each animal received the equivalent of 20–160 ng ^{125}I-TGF-β1, corresponding to 1–8 μCi, in a volume of 0.5 ml. No difference in tissue distribution or plasma half-life was observed over this dose range. All experiments were conducted with male Sprague-Dawley rats weighing between 225 and 295 g. Rats were anesthetized with intraperitoneal pentobarbital (50 mg/kg). A PE10 catheter was introduced into the right iliac artery, and animals were heparinized by injection of ~ 45 U of heparin in 0.1 ml PBS. Samples were injected into the contralateral femoral vein in a total volume of 0.5 ml. Blood samples of 0.15–0.2 ml were withdrawn from the iliac artery at timed intervals after sample injection. 10 U heparin was immediately added and plasma was prepared by spinning out the blood cells for 2 min in a microfuge. Both plasma and blood cell fractions were counted for radioactivity. Essentially no radioactivity was recovered in the blood cell fraction (data not shown). The extent of degradation of the labeled material in the plasma was assessed by determining the fraction of label that was precipitable by 20% TCA. Plasma half-lives for the various labeled species were determined from the rate of decay of TCA precipitable counts in the plasma with time. The distribution volumes were determined by extrapolation of plasma radioactivity to t = 0 min, using the initial linear portion of the decay curve on a log plot. At the end of the time course, the animals were killed by pentobarbital overdose and organs were excised, weighed; and counted for ^{125}I in a gamma counter. In some experiments the organs were then fixed in neutral buffered formalin for autoradiography. In others, the radioactive material in the organs was recovered by acid-ethanol extraction. Briefly, organs were minced, and 4 ml/g tissue of an ice-cold solution consisting of 93% EtOH, 230 mM HCl, 1 μg/ml PMSF, and 5 μg/ml pepstatin was added. The tissue was immediately homogenized in a tissuemizer. Homogenates were then rocked over-

night at 4°C and centrifuged for 30 min at 3,000 *g* to remove insoluble material. The extent of degradation of the labeled material in each organ was then assessed by determining the fraction of extracted ^{125}I that was precipitable by 20% TCA.

Since heparin has been shown to affect the association of active TGF-β and α-2-macroglobulin in vitro (16), one experiment was performed in which the clearance of active TGF-β was monitored in an unheparinized animal. Although blood clotting in the catheter prevented the construction of a complete plasma clearance curve, the time points that were obtained were essentially superimposable on those obtained from heparinized animals, and the distribution of label between organs at the end of the experiment was identical in heparinized and nonheparinized animals (data not shown). All animals thereafter were heparinized.

Results

Formation of iodinated latent TGF-β1 complexes. Iodinated latent TGF-β1 complexes were formed by recombining partially purified recombinant LAP species with ^{125}I–TGF-β1 of high purity. This approach was chosen since TGF-β1 is relatively inaccessible in the latent complex and iodination of intact latent complex would be expected to label the LAP rather than the TGF-β1 (13). All recombinant LAP species caused a dose-dependent inhibition of the ability of ^{125}I–TGF-β1 to bind to its receptor on A549 cells, indicating that latent complexes had formed (Fig. 1). Treatments that are known to activate latent TGF-β1 complexes, such as transient acidification to pH 3, or heating to 80°C for 5 min (20), restored the ability of the ^{125}I–TGF-β1 to bind to its receptor, indicating that the reformed latent complex can be activated in the same way as native complexes. Agarose gel electrophoresis under nondenaturing conditions allowed visualization of complex formation. Iodinated TGF-β1 alone did not migrate from the origin under these conditions (Fig. 2 *A*, lanes *1* and *2*). However, when combined with U-LAP, the form of LAP made by the MJβ1 clone, a new species formed that migrated towards the cathode (Fig. 2 *A*, lane *3*). Heating the sample to 80°C for 5 min resulted in the disappearance of this species (Fig. 2 *A*, lane *4*). Similar results were obtained with S-LAP, the form of LAP that was made by the C3/12A clone. However, in this case the complex had a net negative charge and migrated in the opposite direction (Fig. 2 *A*, lanes *5* and *6*). Taken together, the data indicate that the partially purified LAP species are capable of recombining with iodinated TGF-β1 to form biologically latent complexes.

Pharmacokinetics of latent TGF-β1 in rat. The latent TGF-β1 complexes were injected into rats to determine the plasma half-lives, and the results are presented in Table I. Active TGF-β1 was cleared very rapidly from rat plasma with a half-life of 2–3 min, in agreement with previously published work (14). Initial experiments in which latent TGF-β1 was

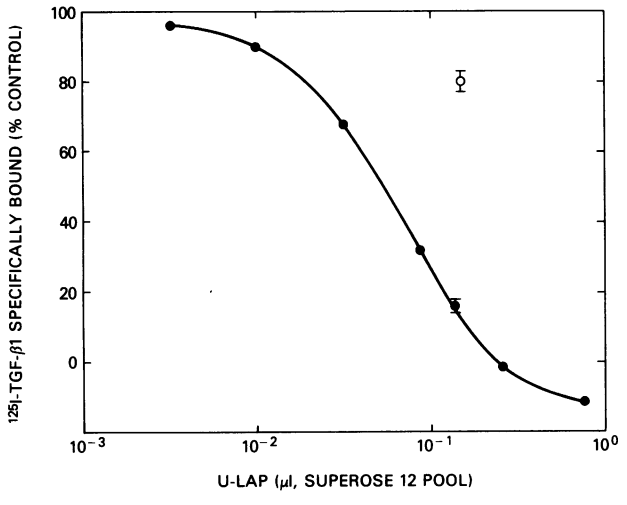

Figure 1. Inhibition of binding of ^{125}I–TGF-β1 to cell surface receptors due to complex formation with LAP species. Varying concentrations of U-LAP were preincubated with 50 pM ^{125}I–TGF-β1 to allow complex formation to occur, and uncomplexed TGF-β was assayed by its ability to bind to TGF-β receptors on A549 cells, as described in Methods. The sample volume/well was 200 μl, corresponding to 250 pg TGF-β, and control samples had no added LAP. In the absence of added LAP, total binding was 3,395±39 cpm/well, and the nonspecific binding, determined in the presence of 10 nM unlabeled TGF-β, was 489±49. A unit of LAP activity was defined as that amount of LAP required to give 50% inhibition of the specific binding of ^{125}I–TGF-β1 in this assay system. At high concentrations, LAP reversed nonspecific binding as well as specific binding of ^{125}I–TGF-β1. The open circle indicates binding observed when the U-LAP/^{125}I–TGF-β1 complex is activated by heating at 80°C for 5 min. Essentially identical results were obtained with preparations of S-LAP (not shown).

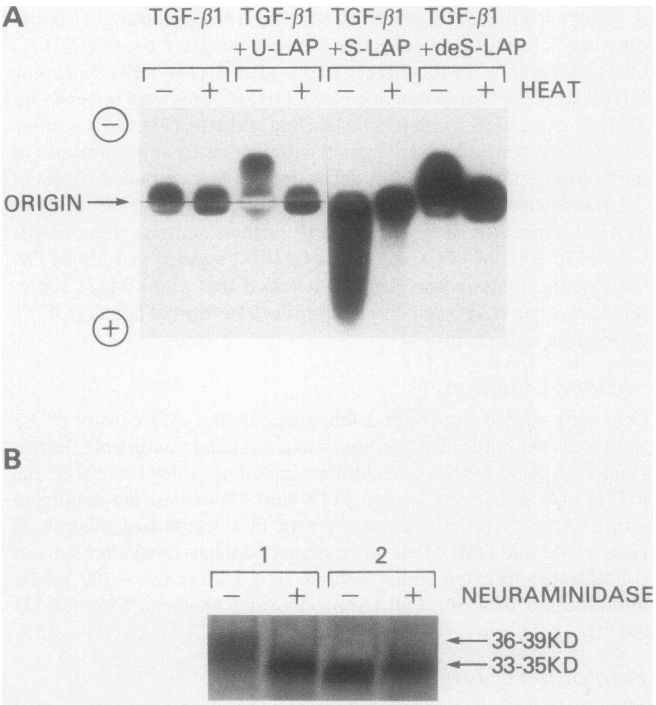

Figure 2. (*A*) Native agarose gels of recombinant latent TGF-β1 complexes. Complexes were formed between ^{125}I–TGF-β1 and recombinant LAP species, and analyzed on agarose gels, with and without activation of the complex, by heating to 80°C for 5 min, as described in Methods. U-LAP, LAP from the MJβ1 clone; S-LAP, LAP from the C3/12A clone; deS-LAP: S-LAP desialylated with neuraminidase. (*B*) Immunoblot analysis of extent of sialylation of LAP. LAP species in serum-free conditioned medium from C3/12A clone (sample *1:* lanes 1 and 2) or MJβ1 clone (sample *2:* lanes 3 and 4) were compared in immunoblots before and after removal of sialic acid with neuraminidase as described in Methods.

Table I. Effect of Latent Complex Formation on Plasma Half-Life and Distribution Volume of TGF-β1 in Rat

Form of TGF-β	Plasma $T_{1/2}$	V_d
	min	*ml*
Active TGF-β1		
TGF-β1	2.7±0.4	81.5±26.5
Latent TGF-β1		
TGF-β + U-LAP	1.2±0.3	40.0±5.6
TGF-β1 + U-LAP + asialofetuin	9.2±1.4	15.2±2.7
TGF-β1 + S-LAP	108.6±8.2	17.1±2.3
TGF-β1 + deS-LAP	1.2	27.4

^{125}I-TGF-β1 was incorporated into a latent complex with different forms of LAP, and injected into the femoral veins of rats. The plasma half-lives were determined from the decrease in TCA-precipitable radioactivity in the plasma with time. For active TGF-β1, and latent complexes formed with U-LAP and deS-LAP, the decrease in radioactivity was biphasic and half-life determinations were made for the initial rapid phase, only using time points up to 5 min after injection. The distribution volumes were determined by extrapolation to $t = 0$ of the initial linear portion of the decay curve in a log plot. Data are the mean±SD of three determinations, except for TGF-β1 alone ($n = 6$) and TGF-β1 + deS-LAP ($n = 1$).

formed from ^{125}I-TGF-β1 and U-LAP gave the unexpected result that the latent complex was cleared even more rapidly than the active TGF-β1. Since the labeled material went almost exclusively to the liver (data not shown), and the latent complex was cationic, we reasoned that the complex might be undersialylated and be cleared through the asialoglycoprotein receptor in the liver. This receptor is known to scavenge aged desialylated plasma proteins (21). Co-injection of an excess of asialofetuin to saturate the asialoglycoprotein receptor resulted in a greater than sevenfold increase in the half-life of the latent complex, consistent with its clearance via this route (Table I). Western blot comparison of the LAP protein in conditioned media from the MJβ1 and C3/12A clones, before and after treatment with neuraminidase, indicated that the LAP made by the MJβ1 clone (U-LAP) was undersialylated compared with that from the C3/12A clone (S-LAP) (Fig. 2 *B*). The molecular weight of the reduced S-LAP (36–39 kD) was similar to that observed for LAP derived from human platelet latent TGF-β (10). Latent complex formed with S-LAP had a greatly extended plasma half-life of > 100 min (Table I). Removal of sialic acid from S-LAP by neuraminidase digestion resulted in formation of a latent complex with the same very short half-life as complexes formed with U-LAP. Representative plasma decay curves for ^{125}I-TGF-β1 with and without S-LAP are shown in Fig. 3.

Tissue distribution of latent and active TGF-β1. The distribution volume (V_d) of active TGF-β1 was ~ 80 ml, suggesting that active TGF-β1 leaves the circulation and distributes into

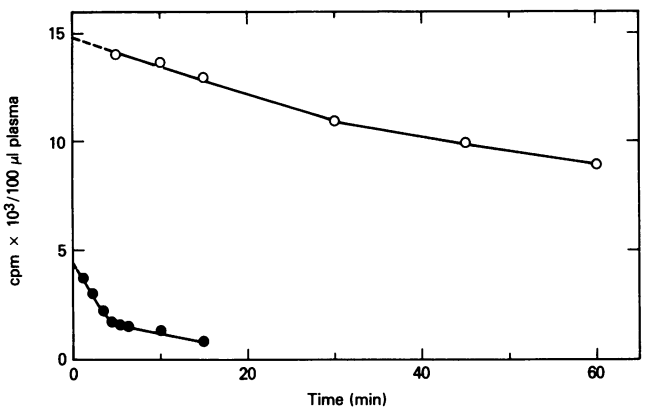

Figure 3. Effect of latent complex formation on the disappearance of circulating ^{125}I-TGF-β1 from rat plasma. ^{125}I-TGF-β1, alone or complexed with S-LAP, was injected into the femoral artery and the TCA precipitable counts in the plasma were determined at various times after injection, as described in Methods. Data points are the TCA precipitable cpm in 100 μl plasma, normalized to 5×10^6 cpm injected. In this experiment, the plasma half-life for the active TGF-β was 3.1 min, and that for the latent TGF-β was 110.0 min. Data collection for the latent complex was continued up to $t = 90$ min to allow more accurate determination of the half-life. Active TGF-β (●), latent TGF-β (TGF-β + S-LAP) (○).

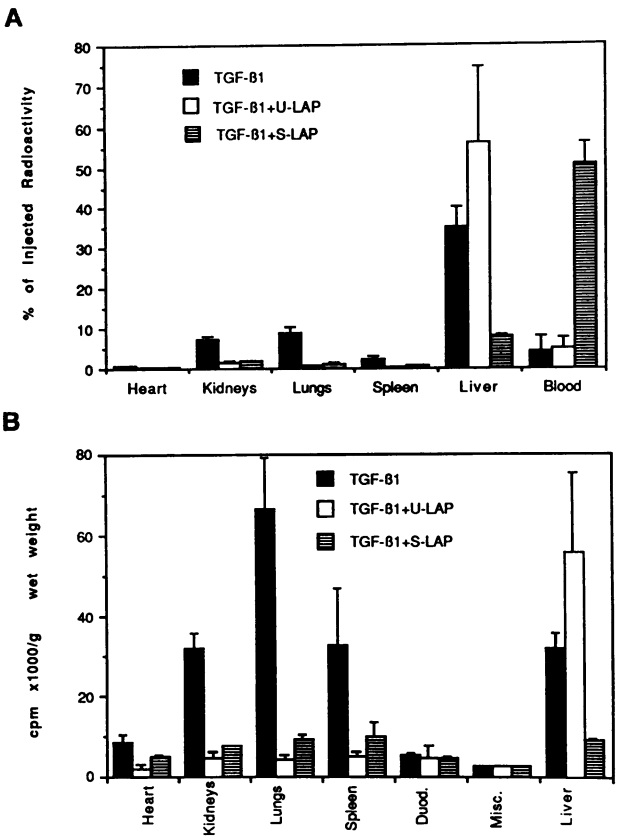

Figure 4. Tissue distribution of radioactivity after intrafemoral administration of ^{125}I-TGF-β1 with or without added LAP to form latent TGF-β1 complexes. (*A*) Results are expressed as the percent injected radioactivity. (*B*) Recovered radioactivity is normalized for tissue wet weight to give a measure of the concentration of radioactivity in the tissue. The miscellaneous category includes thymus, fat, and muscle. Results are the mean±SD for four rats (except S-LAP group, where $n = 3$). Tissues were taken at ~ 90 min after injection for samples with S-LAP, and ~ 30 min after injection for the others. The tissue distribution of S-LAP containing samples was similar at 30 and 90 min, but with a slightly higher concentration of label in the blood at the earlier time points.

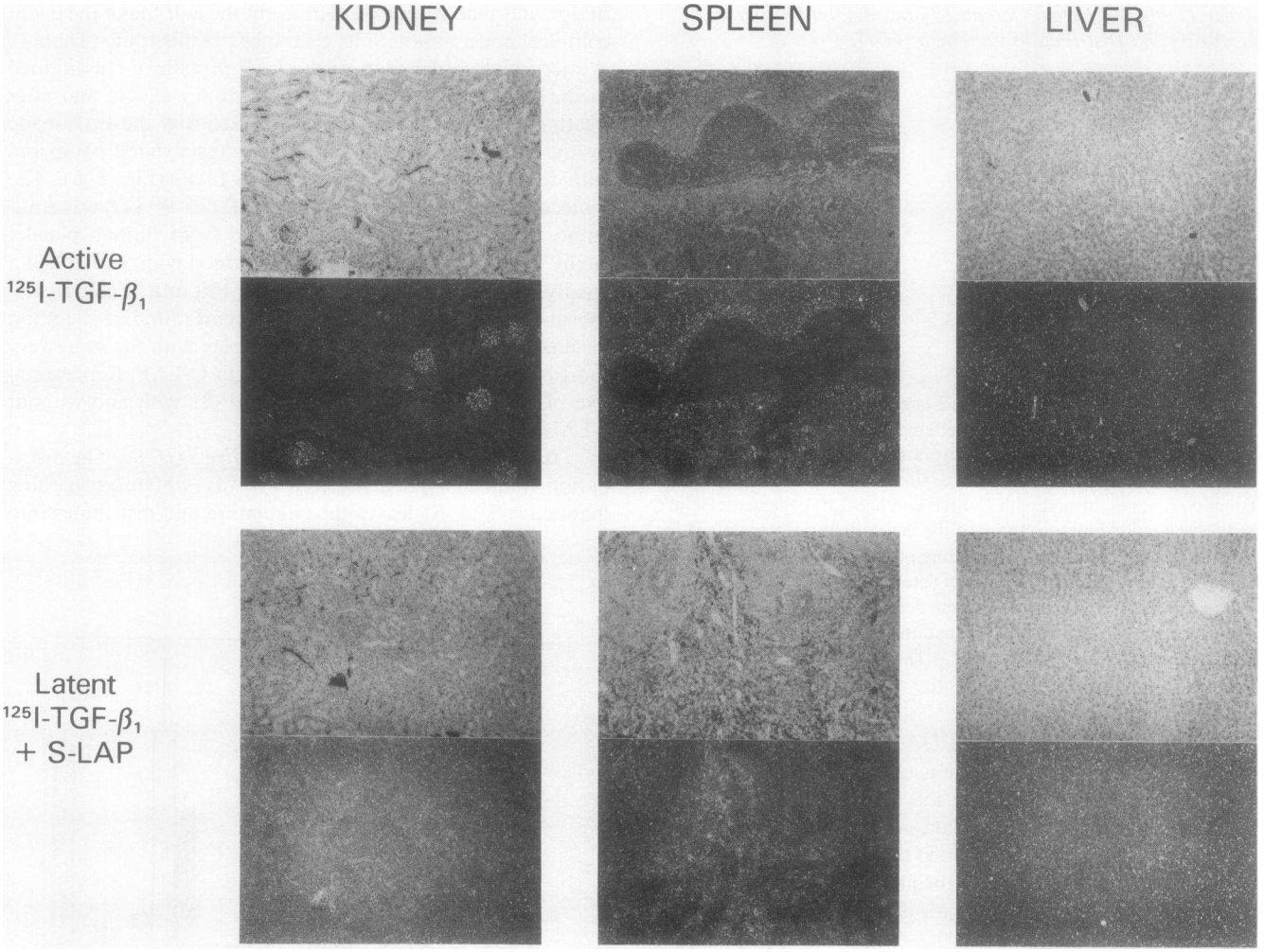

Figure 5. Autoradiography of tissue sections from rats injected with ^{125}I–TGF-β1 alone or complexed with S-LAP. 5-μm sections were rehydrated through xylene and graded alcohols, and then dipped in Kodak NTB2 emulsion. After 5 wk, slides were developed and stained with hematoxylin & eosin. Brightfield and darkfield photographs were taken with a Zeiss Axioplan microscope at ×100. For details, see text. Higher magnification photographs show that the refractility seen in the spleen of animals treated with latent TGF-β is due to blood cells and not to ^{125}I (not shown). In all other instances, the refractile material represents silver grains indicating the presence of radioiodinated material.

the extracellular fluid or some organ sink. In contrast, the V_d for latent TGF-β1 formed with S-LAP was ~ 17 ml, suggesting that the latent complex is largely confined to the circulatory system (Table I). This was confirmed by analysis of the recovery of radioactivity in the various organs upon killing the animal. For active TGF-β1, more radioactivity was recovered in the liver than in any other organ, and only ~ 4% was recovered in the plasma at 20 min after injection (Fig. 4 *A*). By contrast, for latent TGF-β1 formed with S-LAP, only 8% of the radioactivity was recovered in the liver as late as 90 min after injection, and all other organs had ≤ 2%, whereas 62.9±6.1% was recovered in the plasma. The recovery of active TGF-β1 in the liver is consistent with previously published data suggesting that active TGF-β1 is cleared by this organ (14). Interestingly, while the liver had the largest amount of TGF-β, normalization of recovered radioactivity to organ wet weight indicated that ^{125}I–TGF-β1 was actually most concentrated in the lungs. The concentration in kidneys, spleen, and liver were comparable to each other and approximately half that in the lungs (Fig. 4 *B*). By contrast, very little of the ^{125}I–TGF-β1 in the latent complex formed with S-LAP was found in any organ, as long as 90 min after injection, confirming that the majority of this complex does not pass out of the circulation (Fig. 4 *B*). As expected, the latent complex formed with U-LAP or with desialylated S-LAP was recovered almost exclusively in the liver.

Localization of iodinated material within organs. The ^{125}I in the various organs was further localized by autoradiography of organ slices, and the results are shown in Figs. 5 and 6. For animals injected with active TGF-β (^{125}I–TGF-β alone), the pattern of grains in the liver was fairly uniform, suggesting association of the TGF-β1 with the hepatocytes, consistent with the proposal of Coffey et al. that hepatocytes, but not nonparenchymal cells, can take up ^{125}I–TGF-β1 (14). In the kidney, labeled material was concentrated in the glomeruli, and in the lung it was found throughout the interstitium. The ^{125}I in the spleen localized to an area surrounding the white pulp, but was largely excluded from the red pulp and the center of the white pulp. By contrast, in animals that had been injected with the fully sialylated latent TGF-β1, the majority of

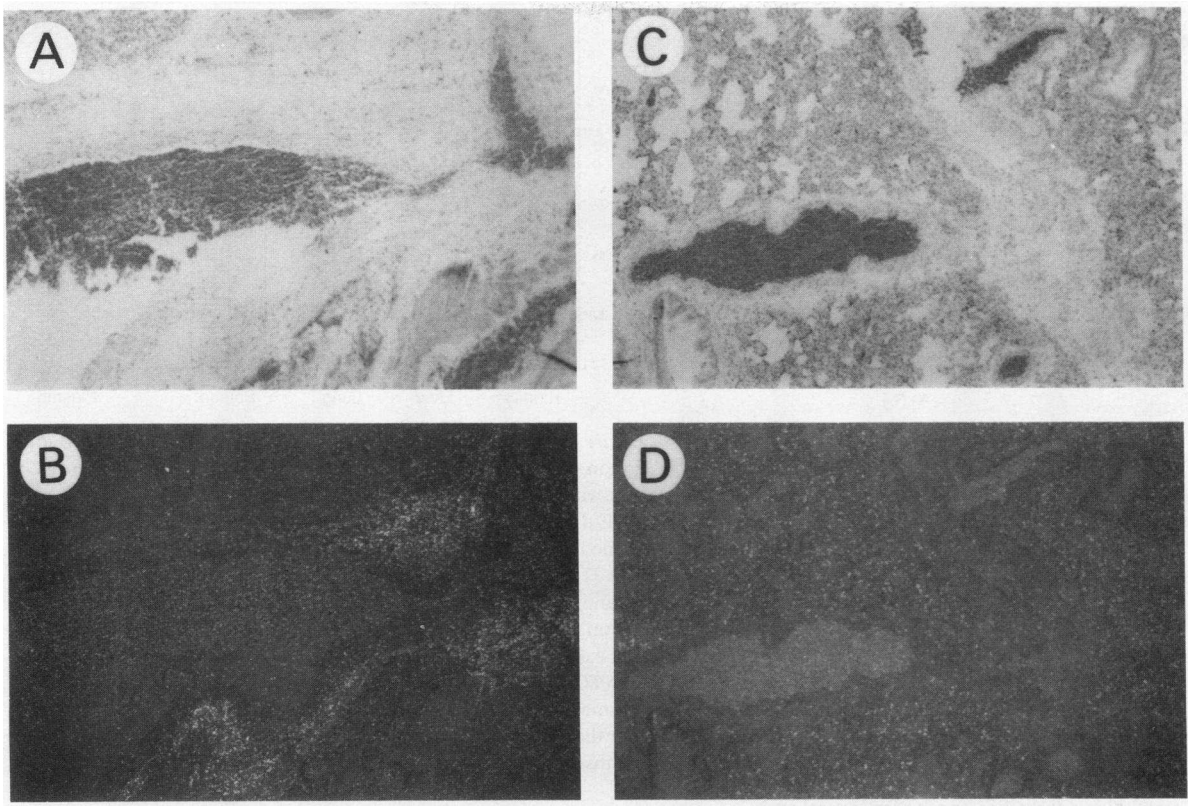

Figure 6. Autoradiography of lung sections from rats injected with ^{125}I–TGF-β alone or complexed with S-LAP. Sections were treated as for Fig. 5. *A* and *B* are from animals injected with latent TGF-β (^{125}I–TGF-β + S-LAP), and *C* and *D* are from animals injected with active TGF-β (^{125}I–TGF-β alone). *A* and *C* are brightfield, and *B* and *D* the corresponding darkfield exposures.

the iodinated material was associated with the blood, and there was little labeling above background in any organ. The only significant concentration of label was observed in the wall of a large blood vessel in the lung (Fig. 6 *B*). Since no sections from the other organs contained large blood vessels, it is not clear if this is a feature specific to the lung. However, it is apparent that in addition to distributing at low levels to most organs (see Fig. 4), the latent complex may concentrate in certain sites outside the circulatory system.

Degradation of circulating TGF-β1. By 15 min after injection of active ^{125}I–TGF-β1, only 53±17% of the radioactivity recovered from the plasma was precipitatable by TCA (Fig. 7). This indicates that active TGF-β1 is rapidly degraded and that some of the degradation products either remain in or reenter the circulation. By contrast, the ^{125}I–TGF-β1 in the latent complex formed with S-LAP appeared to be protected from this degradation process, with < 5% of the circulating material degraded, as long as 90 min after injection.

Analysis by SDS-PAGE of the iodinated material circulating in the plasma 30 min after injection of the animals with latent TGF-β (^{125}I–TGF-β + S-LAP), confirmed that there is little degradation of the ^{125}I–TGF-β in the complex, as indicated by the absence of bands below the band corresponding to intact TGF-β at 25 kD (Fig. 8, lane *3*). Chemical cross-linking of the labeled material in the plasma before electrophoresis gives a band at 100 kD (Fig. 8, lanes *2* and *4*), characteristic of the recombinant latent complex (Fig. 8, lane *8*). This suggests that the latent complex is circulating in the form in which it was injected. Note that chemical cross-linking reactions of this type are low efficiency, so only a fraction of the complex is crosslinked and stable to the denaturing conditions of electrophoresis. In the case of animals injected with active TGF-β (^{125}I–TGF-β alone), the very rapid clearance results in a low ratio of radioactivity to total plasma protein which precludes analysis of these samples by SDS-PAGE.

Extent of degradation of ^{125}I–TGF-β in tissues. To determine whether the radioactivity seen in the tissue autoradio-

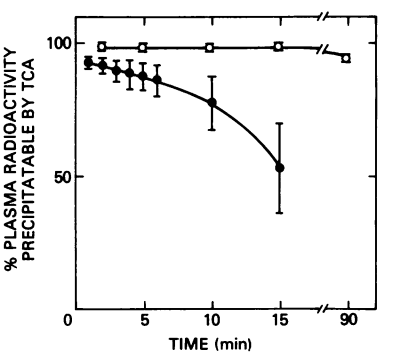

Figure 7. Degradation of circulating ^{125}I–TGF-β1. Degradation of circulating ^{125}I–TGF-β1 alone, or in latent complexes with LAP species, was monitored by the change in TCA precipitability of the radioactivity in the plasma with time. Data are mean±SD for three determinations in each group. Active TGF-β (●); latent TGF-β (TGF-β + S-LAP) (○).

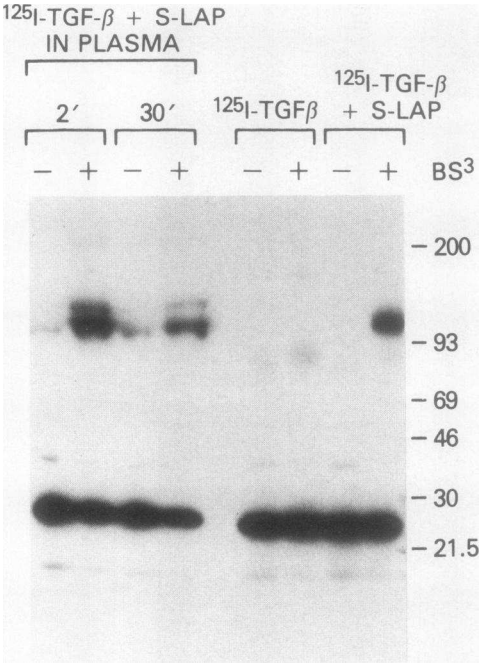

Figure 8. SDS-PAGE analysis of iodinated material recovered from rat plasma after injection with latent TGF-β. Plasma was prepared from rats 2 and 30 min after injection with latent TGF-β (^{125}I–TGF-β + S-LAP). After a 3× dilution with PBS, the plasma samples were treated with the crosslinking agent BS^3 or a buffer control as described in Methods, and samples were analyzed on 3–15% gradient gels under nonreducing conditions. Radioactive bands were visualized by autoradiography. Lanes *4–8* show the expected bands obtained on crosslinking an equivalent amount of purified active or latent TGF-β.

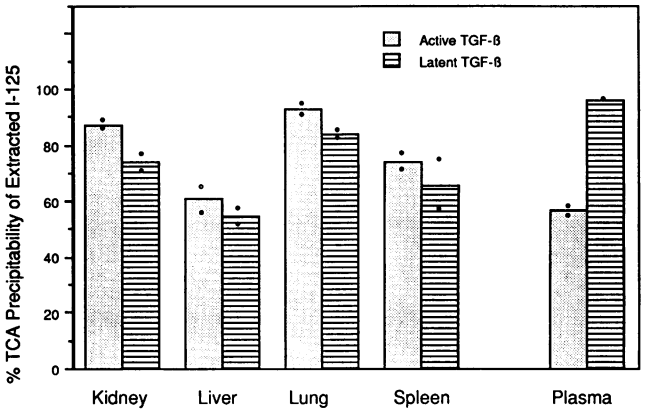

Figure 9. Effect of latent complex formation on the extent of degradation of TGF-β recovered from different organs. 30 min after injection with either latent TGF-β (^{125}I–TGF-β + S-LAP) or active TGF-β (^{125}I–TGF-β alone), animals were killed and the organs were immediately extracted with acid ethanol as described in Methods. The TCA precipitability of the radioactivity recovered from each organ was determined and used as a measure of the extent of degradation of TGF-β in that tissue. Bars represent the mean of two separate experiments, with the individual results indicated by the dots. The TCA precipitability of the iodinated material remaining in the plasma 30 min after injection is also given as a control. This shows that the extent of degradation of iodinated material in the various organs is not simply a passive reflection of that in the plasma.

graphs corresponded to intact or degraded ^{125}I–TGF-β, animals were killed after 30 min and organs were immediately extracted with acid-ethanol. The fraction of the extract that was precipitable by TCA was then determined. The results shown in Fig. 9 indicate that 70–90% of the ^{125}I recovered from lung and kidney was TCA precipitable, suggesting relatively low rates of degradation in these organs. More degradation was evident in the spleen, and particularly in the liver, where only 50–60% of the recovered ^{125}I was TCA precipitable. The TCA-soluble material probably represents iodotyrosine, small iodinated peptides, and possibly some free iodine released by the action of tissue dehalogenases. The high protein content of these extracts relative to the amount of radioactivity prevented SDS-PAGE analysis of the extracted radioactivity.

Although much less radioactivity distributed to the tissues of animals injected with latent TGF-β (^{125}I–TGF-β + S-LAP), the fraction that was TCA precipitable in each organ was very similar to that observed when active TGF-β (^{125}I–TGF-β alone) was injected. This suggests that the various tissues may process the two forms of TGF-β in a similar manner once it is delivered to them. Alternatively, it is possible that the latent TGF-β becomes activated in some way on leaving the circulation. The observation that active, not latent, TGF-β is recovered from the conditioned medium of endothelial cell and pericyte cocultures is interesting in this context (8). Since no antibodies are available for immunoprecipitation of the latent complex or S-LAP, it is not yet possible to determine whether latent TGF-β is taken up by organs as the complex or in the activated form. However, it is clear that regardless of whether the TGF-β is circulating in the active or the latent form, once it is taken up by the liver and spleen, it is degraded at a similar rate.

Discussion

The data show that recombinant latent TGF-β1 has a greatly extended plasma half-life, when compared with active TGF-β1, and a radically different tissue distribution. The rapid clearance of active TGF-β1 and its targeting predominantly to the liver, agree with a previous report (14). We have additionally shown a significant uptake of active TGF-β by lungs, spleen, and kidneys. The overall distribution pattern is similar to that observed for clearance of α-2-macroglobulin (α_2M) (22, 23) and is consistent with the proposal that active TGF-β1 may complex with α_2M and be cleared in this form (24). We have previously shown that latent TGF-β1, unlike active TGF-β1, is unable to bind to α_2M (10). Thus our demonstration of a much longer half-life for latent TGF-β1 suggests that an important role of the TGF-β1 LAP may be to protect TGF-β1 from being scavenged by circulating α_2M.

The extended plasma half-life of latent TGF-β1 has important physiological and clinical implications. From a physiological standpoint, the active TGF-β1 would be expected to act locally, close to its site of synthesis, since on leaving the cell it would be rapidly bound either by the ubiquitous TGF-β1 cell surface binding proteins, or by α_2M in the interstitial fluid. However, latent TGF-β1, which is unable to bind to the

TGF-β1 receptors or to α_2M, would be able to diffuse away from its site of synthesis and circulate to more distant target organs. Thus, whereas active TGF-β1 would have an autocrine/paracrine action, latent TGF-β1 might have a more endocrine mode of action. It is interesting to note that while most cells secrete TGF-β1 in the latent form, in a few instances, TGF-β1 is secreted in an active form (25, 26). In this way cells may be able to exert some control over the range of action of the TGF-β1 that they secrete. Normal human plasma contains significant levels of TGF-β from non-platelet sources (1.5±0.6 ng/ml (n = 10); Wakefield, L. M., unpublished data). The plasma TGF-β is latent, and while the lack of sensitive reagents for detecting latent TGF-β complexes so far precludes identification of the type of latent complex, it is clear that latent TGF-β does circulate in normal individuals and may play an important role in mediating regulatory interactions between organs.

In a clinical setting, the longer half-life of the latent TGF-β1 complex could be advantageous in greatly extending the duration of action of a single injected dose of the factor. Furthermore, the demonstration that systemically administered active TGF-β1 becomes concentrated in the lungs, kidneys, and spleen, as well as the liver, is potentially problematic in relation to the known ability of TGF-β1 to cause fibrosis in vivo (27). It will be important to determine whether the TGF-β1 delivered to these sites can exert any biological effect. In contrast, correctly sialylated latent TGF-β1 did not accumulate appreciably in any one organ, but seemed to distribute at low levels among them all, with the only significant concentration of latent TGF-β1 in any organ being to the wall of a large blood vessel in the lung.

It is not yet known which cell types are capable of activating latent TGF-β1 in vivo, but clearly this will determine the clinical targets for the latent complex, since it appears to distribute at low levels to most perfused organs. When large quantities of purified recombinant latent TGF-β1 become available, it should be possible to infuse animals with the material and look for effects on different organ systems. We have shown that the degree of sialylation of the latent complex is critical in determining both half-life and organ distribution. Since recombinant expression systems are variable in the degree to which they sialylate proteins (19), and all clinical work is likely to be done with recombinant material, it will be vital for investigators using this material to confirm that it has an adequate degree of sialylation. Furthermore, while native platelet and recombinant latent TGF-β1 are very similar in many in vitro properties (13, 20), it will be important to compare these in vivo. This may provide clues as to the role of the additional 135-kD protein in the natural latent TGF-β1, and will allow assessment of the degree to which the recombinant form will mimic or improve upon the natural complex in a clinical setting.

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