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The recombinant proregion of transforming growth factor β1 (Latency-associated peptide) inhibits active transforming growth factor β1 in transgenic mice

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ABSTRACT **All three isoforms of transforming growth factors β (TGF-β1, TGF-β2, and TGF-β3) are secreted as latent complexes and activated extracellularly, leading to the release of the mature cytokines from their noncovalently associated proregions, also known as latency-associated peptides (LAPs). The LAP region of TGF-β1 was expressed in a baculovirus expression system and purified to homogeneity. *In vitro* assays of growth inhibition and gene induction mediated by TGF-β demonstrate that recombinant TGF-β1 LAP is a potent inhibitor of the activities of TGF-β1, -β2, and -β3. Effective dosages of LAP for 50% neutralization of TGF-β activities range from 4.7- to 80-fold molar excess depending on the TGF-β isoform and activity examined. Using ^{125}I-labeled LAP, we show that the intraperitoneal application route is effective for systemic administration of LAP. Comparison of concentrations of LAP in tissues shows a homogenous pattern in most organs with the exception of heart and muscle, in which levels of LAP are 4- to 8-fold lower. In transgenic mice with elevated hepatic levels of bioactive TGF-β1, treatment with recombinant LAP completely reverses suppression of the early proliferative response induced by TGF-β1 in remnant livers after partial hepatectomy. The results suggest that recombinant LAP is a potent inhibitor of bioactive TGF-β both *in vitro* and *in vivo*, after intraperitoneal administration. Recombinant LAP should be a useful tool for novel approaches to study and therapeutically modulate pathophysiological processes mediated by TGF-β.**

The transforming growth factors β (TGF-β) are multifunctional cytokines with diverse effects on cell growth, differentiation, and function (1). Experiments in animal model systems and transgenic mice, as well as studies of human diseases, have implicated TGF-β in the regulation of important physiological and pathophysiological processes (2–4).

Latent forms of TGF-β purified from platelets and secreted by a number of cell types are high molecular weight complexes (5, 6) in which homodimeric mature TGF-β is noncovalently associated with a dimer of its proregion, which itself can be disulfide-bonded to structurally and genetically unrelated latent TGF-β binding proteins (7–10). In contrast, recombinant cell systems and certain cell types secrete a "small latent complex" consisting only of mature TGF-β and its proregion (11–13). The proregion of TGF-β1 [aa 30–278 (14)], when independently expressed, associates noncovalently with mature TGF-β, inactivating its biological activities *in vitro* (15). Therefore, latency of TGF-β is a function conferred by its proregion, which is also termed latency-associated peptide. Before TGF-β can exert its biological effects, latent complexes must be activated, presumably resulting in the release of mature TGF-β (16–19). The structural changes of latency-associated peptide required for release of active TGF-β and its fate after completion of the activation process are not known. Although certain types of *in vitro* activation are reversible (12), it is not known whether the intact proregion and active TGF-β can reassociate *in vivo*.

Elevated endogenous TGF-β levels in diseased organs have been associated with the pathogenesis of fibrosis in experimental animal models and human diseases (4), and elevated circulating TGF-β1 is associated with the development of conditions characterized by fibrosis in liver and lungs in humans (20). Approaches to prevent matrix accumulation in experimental animal models using systemic antagonists of TGF-β have been promising (21–24). Thus, decreasing the activity of TGF-β1 has enormous clinical potential in conditions with excessive scarring.

Liver regeneration following partial hepatectomy is a well-characterized model system for evaluating the effects of growth factors on hepatocellular proliferation *in vivo* (25–27). Intravenously administered active TGF-β reversibly inhibits the early synchronized proliferative wave after partial hepatectomy in rats and mice (28, 29). Furthermore, Factor *et al.* (unpublished data) find a severely suppressed early proliferative response after partial hepatectomy in transgenic mice with persistently elevated levels of active TGF-β in the liver. These transgenic mice have severe fibrosis of the liver and elevated circulating TGF-β1 levels, resulting in sclerosis and inflammation in several distant organs (30).

In the present work, we have asked whether systemic administration of recombinant TGF-β1 latency-associated peptide (LAP) is feasible, and whether it would decrease the activity of excess active TGF-β1 *in vivo*. Our results suggest that LAP forms a small latent complex with TGF-β1, remains intact in tissues and circulation after intraperitoneal (i.p.) administration, and is a potent TGF-β1-antagonist *in vitro* and *in vivo*.

MATERIALS AND METHODS

Expression and Iodination of LAP. The LAP portion of TGF-β1 was isolated by polymerase chain reaction from human TGF-β1 cDNA, in which site-directed mutagenesis

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Abbreviations: TGF-β, transforming growth factor β; LAP, recombinant TGF-β1 latency-associated peptide; TCA, trichloroacetic acid; PAI-1, plasminogen activator inhibitor 1.

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(Promega) was used to replace Cys33 with serine. This mutation was introduced to prevent the formation of spurious disulfide bonds between this cysteine and cysteines in the mature TGF-β (9, 14, 31). The LAP portion was subcloned into a transfer vector and expressed with a Baculovirus expression system (Clontech). Serum-free conditioned media was collected 48 h after high multiplicity of infections infections of insect cells, followed by adjustment to pH 8.0 and clarification by centrifugation. The conditioned media (5 liters) was diluted 4-fold to lower the conductivity before loading onto a Q Sepharose FF column (200 ml) and an S Sepharose FF column (200 ml) connected in series. The columns were previously equilibrated with 20 mM Tris·HCl. After washing the columns with equilibration buffer to remove unbound proteins, the LAP was eluted with a salt gradient from 0 to 1 M NaCl at 15 ml/min. The pooled LAP was dialyzed overnight at 4°C against 20 vol of 20 mM citrate-PBS (pH 6.5). The sample was then loaded on a HEMA SB (Alltech Associates) cation exchange column equilibrated with the dialysis buffer and eluted with a salt gradient from 0 to 1 M NaCl at 2 ml/min. The pooled LAP was diluted 2-fold with 10 mM PBS (pH 6.5) and loaded onto a Mono S (10/10) column (Pharmacia). The column was washed with 10 mM PBS (pH 6.5) and LAP was eluted with a salt gradient from 0 to 1 M NaCl. The purity of the protein preparation was verified using N terminus analysis and SDS/PAGE visualized by silver staining (R & D Systems). LAP was labeled with ^{125}I following a modified chloramine T method (32). Iodinated LAP was purified by PD10 sephadex column fractionation (Pharmacia). Specific activity of ^{125}I-labeled LAP was ≈100 μCi/μg (1 Ci = 37 GBq), and its purity was verified by SDS/PAGE and autoradiography.

Bioassays in Mv1Lu Cells. Growth inhibition was monitored in Mv1Lu cells (American Type Culture Collection) as described (33). Equipotent concentrations of porcine TGF-β1 (8 pM), porcine TGF-β2 (6 pM), and recombinant human TGF-β3 (2 pM) (all from R & D Systems), mediating 90% inhibition of [^{3}H]thymidine incorporation (DuPont/NEN), were added to 0.5×10^5 Mv1Lu cells in 24-well dishes together with increasing concentrations of LAP. Induction of the plasminogen activator inhibitor 1 (PAI-1) promoter by TGF-β1 was measured in Mv1Lu cells stably transfected with a PAI-1 promoter fused to a luciferase reporter gene (34).

Primary Hepatocyte Isolation and Proliferation Assay. Hepatocytes were isolated from 8-week-old male FVB/N mice by two-step collagenase perfusion of the liver followed by isodensity centrifugation in Percoll (viability >95%) (35, 36). Hepatocytes were plated at a density of 0.3×10^6 cells per collagen-coated 35-mm dish in DMEM/F12 (GIBCO/BRL) supplemented with 18 mM Hepes (pH 7.4), 5 mM sodium pyruvate, 6 mM sodium bicarbonate, 1 mg/ml galactose, 30 μg/ml L-proline, 2 mM glutamine, 1% ITS+ (Collaborative Biomedical Products, Bedford, MA), 0.1% gentamicin, and 10% fetal bovine serum (HyClone). After cell attachment, media were changed to serum-free media containing supplements as listed above and 10 ng/ml epidermal growth factor (Upstate Biotechnology, Lake Placid, NY). DNA synthesis in primary hepatocyte cultures was measured after incubation of cells with [^{3}H]thymidine between 48 and 72 h after plating, followed by trichloroacetic acid (TCA) precipitation of cell extracts as described (25). [^{3}H]Thymidine incorporation was measured in a liquid scintillation counter and normalized for DNA content per plate. LAP and TGF-β1 were added together with [^{3}H]thymidine without preincubation.

Fibronectin Secretion Assays. Fibronectin production by Mv1Lu cells was measured as described (37). LAP, TGF-β1, and neutralizing polyclonal turkey anti-TGF-β1 antibodies (38) were added for an overnight incubation. Fibronectin band intensities were quantified by densitometry and relative amounts expressed as *x*-fold difference compared with fibronectin secreted by untreated cells.

Tissue and Plasma Distribution Studies. Nine-week-old (C57BL/6J × CBA) F_1 mice were injected intraperitoneally with 5 μCi (≈50 ng) of ^{125}I-labeled LAP in 0.5 ml PBS. Blood samples and organs were harvested from individual mice at 10, 30, and 120 min after LAP injection. Blood samples were collected in heparin tubes, and plasma was prepared by centrifugation in a microcentrifuge for 5 min. Whole organs (liver, lungs, heart, kidneys, and spleen) and tissue fragments (duodenum and muscle) were excised and immediately weighed. Radioactivity in 100 μl of plasma and in whole organs was counted in a gamma counter. The extent of degradation of the ^{125}I-labeled LAP was assessed in plasma and in homogenates of representative tissues in lysis buffer by determining the fraction of label that was precipitable by 20% TCA. Lysis buffer consisted of 150 mM NaCl, 50 mM Tris·HCl (pH 8.0), 1% Triton X-100, 0.5% sodium deoxycholate, 0.25% SDS, and the protease inhibitors AEBSF, aprotinin, pepstatin, leupeptin, and antipain.

Immunoprecipitations of LAP. Whole liver was homogenized in 4 ml of cold lysis buffer followed by centrifugation at 8000 rpm for 20 min at 4°C. Homogenate (1 ml) was incubated with 6 μl of anti-human LAP antibody (AB-246-NA; R & D Systems) overnight at 4°C, followed by precipitation with protein-A sepharose (Pharmacia) and five washes. Plasma (200 μl) were diluted in 200 μl of 2× lysis buffer (as described above, except NaCl concentration of 1×) and incubated at 4°C overnight with 3 μl of anti-human LAP antibody, followed by procedures as described above. All samples were eluted by boiling for 3 min in sample buffer and run on 4–12% SDS/PAGE in parallel with native ^{125}I-labeled LAP. Gels were dried and exposed to autoradiography.

Liver Regeneration After Partial Hepatectomy. The Alb/TGF-β1 transgenic mice were generated in a (C57BL/6J × CBA) F_1 genetic background as described (30). Control mice were age- and sex-matched nontransgenic (C57BL/6J × CBA) F_1 mice. The animal study protocol was in accordance with the National Institutes of Health guidelines for animal care. Male mice of 8 weeks of age were subjected to a standard 70% hepatectomy under metofane anesthesia using the technique of Higgins and Andersen (39). Osmotic minipumps (Alzet 2001D; Alza) were loaded with either LAP or PBS and inserted into the abdominal cavity after partial hepatectomy. Pumps were loaded with various dilutions of LAP (50 μg, 12.5 μg, or 3 μg) in PBS (220 μl total volume), or PBS alone, followed by either i.p. LAP (14 μg, 3.5 μg, or 1 μg) or PBS bolus injections, respectively, at the end of pump delivery (≈24 h after insertion). To monitor the kinetics of DNA synthesis, all animals were given i.p. injections of BrdUrd (Boehringer Mannheim) at a dose of 150 mg/kg of body weight 1 h before sacrifice. Mice were killed 36 h after surgery. Tissues were fixed in Bouins' fixative (Polysciences). Proliferation of hepatocytes was assessed as described (30). After counterstaining with hematoxylin, the nuclear DNA labeling index was determined by counting of BrdUrd-positive nuclei per 3000 hepatocyte nuclei and expressed as percent per 100 nuclei.

Statistical Analysis. Differences in nuclear labeling indeces between the treatment groups were tested for statistical significance with the Wilcoxon signed rank test. All *in vitro* assay data are presented as mean ± SD; all *in vivo* assay data are presented as mean ± SEM.

RESULTS

Effects of LAP on TGF-βs Activities *in Vitro*. We tested the ability of LAP to bind and neutralize TGF-βs in several *in vitro* assays. Using chemical cross-linking, we confirmed that LAP, generated in a baculovirus expression system, can associate with mature TGF-β1 in a noncovalent complex with electro-

phoretic characteristics of the small latent complex (data not shown). In a standard bioassay using Mv1Lu cells, TGF-β1 LAP effectively neutralized the growth inhibitory activity of equipotent concentrations of all three mammalian isoforms of TGF-β (Fig. 1*A*). Effective dosages for 50% neutralization (ED_{50}) of TGF-β activity were approximately 58-fold, 20-fold, and 4.7-fold molar excess for TGF-β1, TGF-β2, and TGF-β3, respectively. When corrected for differences in TGF-β isoform concentrations, TGF-β3 and TGF-β2 activities were inhibited by TGF-β1 LAP approximately 3-fold and 2.3-fold more effectively than TGF-β1 activity, respectively. LAP also neutralized the antiproliferative activities of autocrine TGF-β in a dose-dependent manner as indicated by increased DNA synthesis in cells incubated with LAP alone (see Fig. 1*A*, solid line). PBS solution itself, the vehicle for LAP, had no effect on thymidine incorporation. To assess the effects of LAP on hepatocyte growth *in vitro*, we incubated primary hepatocyte cultures with LAP in the absence or presence of exogenous TGF-β1 (Fig. 1*B*). LAP effectively neutralized TGF-β1-mediated inhibition of hepatocyte growth, as assessed by [^{3}H]thymidine incorporation. We also examined the effects of LAP on genes induced by TGF-β. LAP blocked activation of the PAI-1 promoter induced by TGF-β in Mv1Lu cells stably transfected with a PAI-1/luciferase reporter gene construct with an ED_{50} similar to that demonstrated in the Mv1Lu growth inhibition assays (Fig. 1*C*). LAP also antagonized the induction of fibronectin in Mv1Lu cells resulting from exogenously added TGF-β1 (Fig. 1*D*, lane 3), as well as from autocrine TGF-βs (Fig. 1*D*, lane 4). Neutralization of autocrine TGF-βs by LAP at a concentration of 5 nM was as effective as a neutralizing turkey antiserum to TGF-β1 (33) used at 1:200 dilution (Fig. 1*D*, lanes 4 and 5). LAP and antiserum together had no additive effect (Fig. 1*D*, lane 6). These results show that LAP is a potent and specific antagonist for growth inhibitory and gene inducing activities of exogenously added active TGF-β, as well as for TGF-β secreted and activated by cells in autocrine loops *in vitro*.

Tissue Distribution of Radioactivity After Intraperitoneal Injection of ^{125}I-labeled LAP in Mice. We examined the feasibility of i.p. administration of LAP and the associated tissue distribution profile in mice. After i.p. bolus injections, significantly more radioactivity was recovered from the liver and kidneys when compared with lungs, heart, and spleen (Fig. 2*A*). Of the recovered radioactivity, 40–55% was TCA-precipitable at 30 and 120 min after injection in selected organs (liver and kidney), suggesting that a substantial amount of the injected ^{125}I-labeled LAP is still intact at these later time points (data not shown). Normalization of recovered radioactivity to organ wet weight revealed that the concentrations of ^{125}I-labeled LAP were comparable within a 2-fold range in kidneys, lungs, liver, spleen, and duodenum, whereas cardiac and skeletal muscle had 4-to 8-fold lower concentrations of radioactivity (Fig. 2*B*). Therefore, the tissue distribution of ^{125}I-labeled LAP closely resembles that observed for the reconstituted small latent complex containing ^{125}I-labeled TGF-β1 injected intravenously in rats, and is substantially different from the tissue distribution of ^{125}I-labeled TGF-β1 (40, 41). In all organs examined except for the spleen, there was a consis-

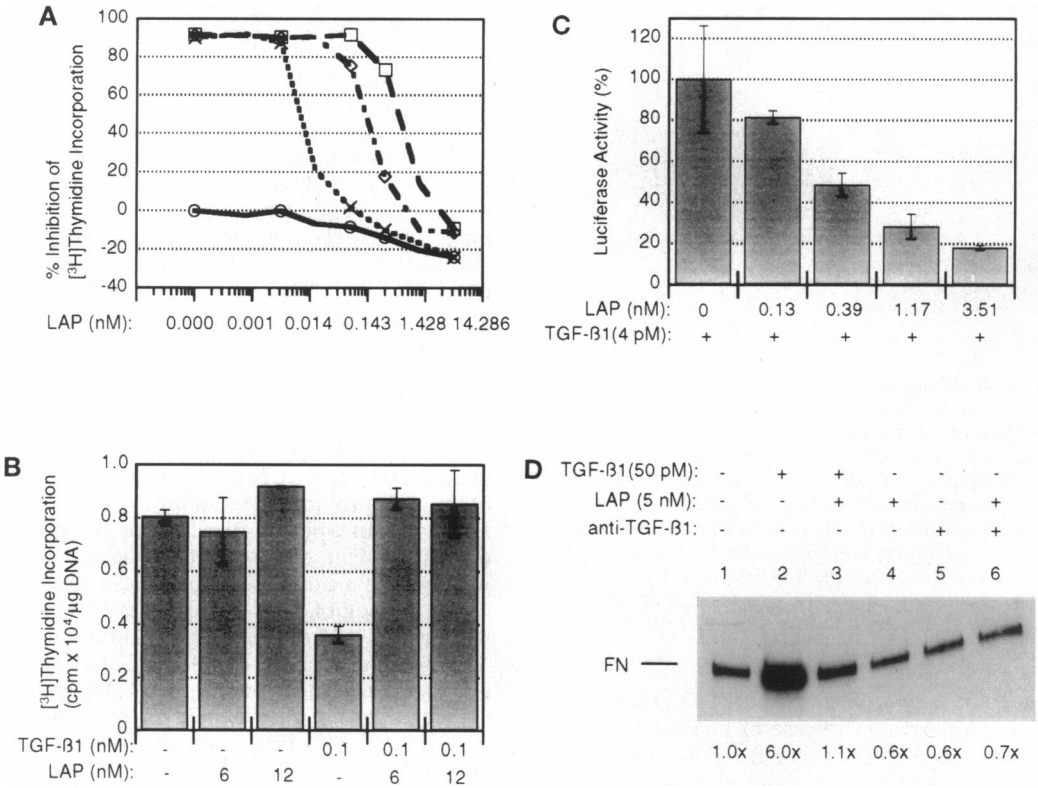

FIG. 1. LAP effects on TGF-β activities *in vitro*. (*A*) Increasing concentrations of LAP were added to Mv1Lu cells alone (solid line) or together with equipotent concentrations of TGF-β1 (8 pM; dashed line), -β2 (6 pM; dashed-dotted line), and -β3 (2 pM; dotted line), respectively. Results are expressed as % inhibition of [^{3}H]thymidine incorporation in a representative triplicate experiment. (*B*) Primary hepatocytes in culture were either treated with LAP alone or LAP together with TGF-β1. [^{3}H]Thymidine incorporation was quantified as cpm/μg DNA and compared to untreated cells and TGF-β1 treated cells. Results from a representative triplicate experiment are shown. (*C*) MvLu-1 cells stably transfected with a PAI-1/luciferase reporter gene construct (34) were treated with TGF-β1 alone or TGF-β1 together with increasing concentrations of LAP. Induction of PAI-1 was quantified as luciferase activity and the amount of luciferase activity in cells treated with TGF-β1 alone (0.06 relative light units) was assigned 100%. A representative triplicate experiment is shown. (*D*) Secretion of fibronectin by metabolically labeled Mv1Lu cells was visualized by autoradiography. Band intensities were quantified by densitometry and levels of expression denoted as fold above baseline expression (1.0×) in untreated cells (lane 1).

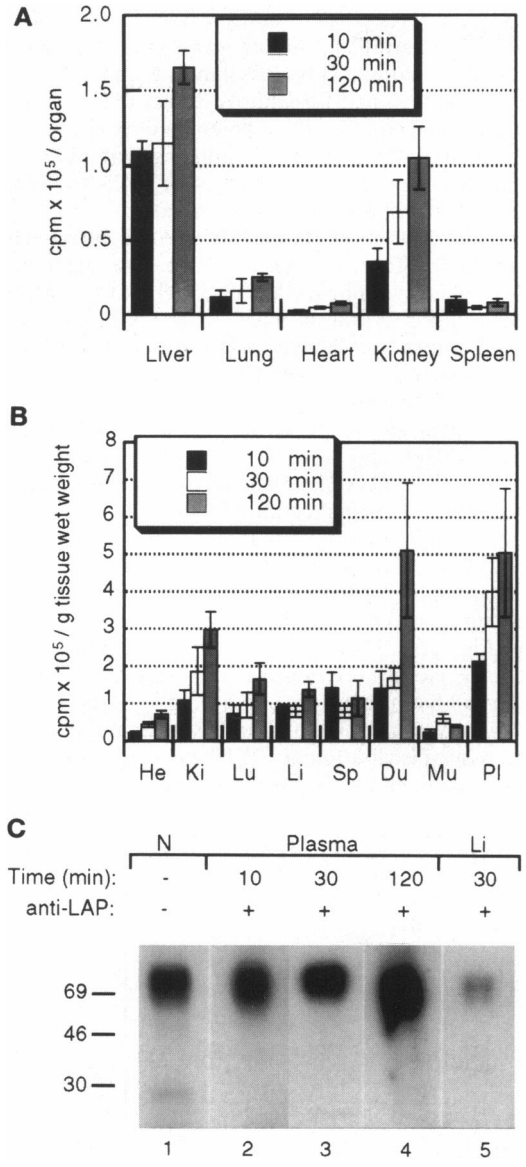

FIG. 2. Tissue distribution and plasma appearance of ^{125}I-labeled LAP after systemic administration. Tissue distribution of radioactivity in cpm in whole organs (*A*) and tissue distribution of radioactivity in cpm per gram tissue wet weight (*B*) at 10, 30, and 120 min after i.p. bolus injection of ^{125}I-labeled LAP, respectively. He, heart; Ki, kidney; Lu, lung; Li, liver; Sp, spleen; Du, duodenum; Mu, muscle; Pl, plasma. Bars represent means ± SEM of results obtained in three mice per time point. (*C*) Immunoprecipitation of ^{125}I-labeled LAP from plasma using a polyclonal anti-LAP antibody (R & D Systems) at 10, 30, and 120 min after i.p. bolus injection, respectively (lanes 2, 3, and 4), and from liver at 30 min after i.p. bolus injection (lane 5). Lane 1, native ^{125}I-labeled LAP.

tent increase of radioactivity between 10 min and 120 min after injection, indicating a persistent release of injected radioactivity from the peritoneum (Fig. 2 *A* and *B*). To determine whether ^{125}I-labeled LAP recovered from tissues was intact, a polyclonal anti-human LAP antiserum was used to detect ^{125}I-labeled LAP in liver homogenates prepared 30 min after injection. As shown in Fig. 2*C*, the ^{125}I-labeled LAP immunoprecipitated 30 min after injection (lane 5) was indistinguishable from native ^{125}I-labeled LAP (lane 1), suggesting that a easily detectable amount of LAP remains unaltered *in vivo*.

^{125}I-Labeled LAP in Plasma After Intraperitoneal Injection in Mice. Radioactivity in plasma was recovered at 10, 30, and 120 min after injection of ^{125}I-labeled LAP and quantified as counts per min (cpm) per ml plasma. We found a constant increase in levels of radioactivity per ml plasma (Fig. 2*B*, Pl column), representing 2.4%, 4.8%, and 6.0% of the total amount of the injected radioactivity at these time points, respectively. The specific activity of ^{125}I-labeled LAP injected was 100 μCi/μg. Considering an estimated plasma volume of 1 ml in mice of 23 g total body weight, the plasma concentration of ^{125}I-labeled LAP 2 h after i.p. injection of 50 ng (5 μCi) was calculated to be ≈2.8 ng/ml (40 pM). Taken together (see Fig. 2), these findings suggest that ^{125}I-labeled LAP is slowly absorbed from the peritoneal cavity into the portal and systemic circulation. This prohibits determination of plasma half life and rates of degradation of ^{125}I-labeled LAP within the examined time frame. Immunoprecipitations of ^{125}I-labeled LAP from plasma using polyclonal anti-LAP antiserum showed that the absorbed ^{125}I-labeled LAP remained intact (Fig. 2*C*, lanes 1–4). Furthermore, 55%, 48%, and 41% (values not significantly different) of the radioactivity were found in the TCA precipitable fractions from plasma collected at 10, 30, and 120 min, respectively. Taken together, these data indicate that stable systemic levels of intact ^{125}I-labeled LAP can be achieved after i.p. administration in mice.

Effects of Recombinant LAP During Liver Regeneration After Partial Hepatectomy in TGF-β1 Transgenic Mice. A synchronized wave of DNA synthesis occurs in the remaining liver of mice between 32 and 42 h after resection of two thirds of the liver (partial hepatectomy). A peak of mitosis follows between 42 and 48 h (42, 43). In Alb/TGF-β1 transgenic mice with high levels of biologically active TGF-β1 expressed in the liver, the early proliferative response measured at 36 h after partial hepatectomy by nuclear BrdUrd labeling index was 5-fold lower when compared with nontransgenic control mice (V.M.F., *et al.*, unpublished work). We hypothesized that neutralization of TGF-β1 activity by LAP should result in a restoration of the normal early proliferative response after partial hepatectomy in Alb/TGF-β1 mice. The levels of TGF-β1 protein as detected immunologically by the anti-LC(1–30-1) antibody (44) were consistently elevated in 8-week-old male Alb/TGF-β1 mice, when compared with male age-matched nontransgenic control mice (30). Such Alb/TGF-β1 and control mice underwent a two-thirds partial hepatectomy, followed immediately by insertion of osmotic minipumps with or without LAP into the abdominal cavity. The rate of DNA synthesis as measured by nuclear BrdUrd labeling was 4.7-fold lower in Alb/TGF-β1 transgenic hepatectomized mice treated with PBS (1.0% ± 0.3%) when compared with hepatectomized nontransgenic control mice treated with PBS (4.7% ± 1.7%). In contrast, LAP treatment of Alb/TGF-β1 mice resulted in a nearly complete restoration of the early proliferative response (4.5% ± 1.7%) (Fig. 3*D*; see Fig. 3 *A–C* for typical BrdUrd staining). This effect of LAP was dose-dependent, as it was only observed at a total dose of 64 μg. The labeling index in Alb/TGF-β1 mice treated with either 16 μg or 4 μg of LAP was 0.4% ± 0.2% and 0.7% ± 0.3%, respectively, and not significantly different from that of Alb/TGF-β1 mice treated with PBS (Fig. 3*D*). In nontransgenic control mice treated with 64 μg of LAP, the early proliferative response was not significantly different from that of control mice treated with PBS (data not shown). In summary, these data indicate that LAP effectively reverses the antiproliferative activity of elevated TGF-β1 during liver regeneration in transgenic mice.

DISCUSSION

Using a transgenic mouse model with overexpression of bioactive TGF-β1 in the liver as an *in vivo* assay system, we have shown that LAP can successfully block the antiproliferative effects of active TGF-β1 during liver regeneration. To our knowledge, our data represent the first demonstration of

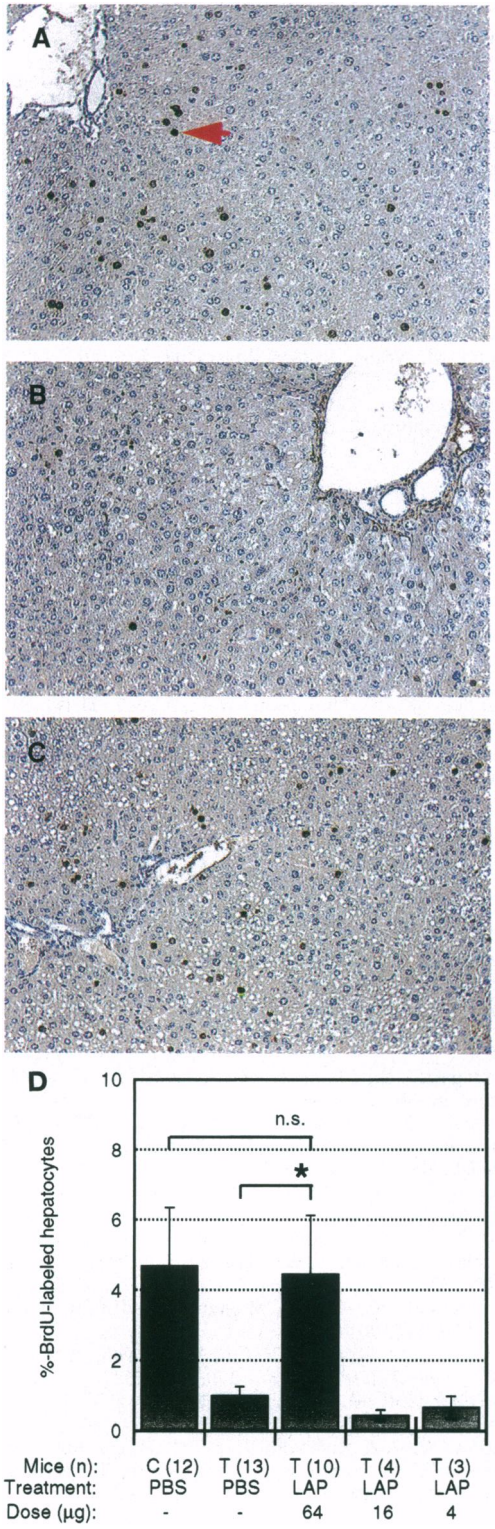

FIG. 3. Effect of LAP on the early proliferative response after partial hepatectomy in nontransgenic control and Alb/TGFβ1 transgenic mouse liver. (*A–C*) Anti-BrdUrd immunostaining of liver sections representing typical nuclear labeling (see red arrow) in various treatment groups, respectively: (*A*) PBS-treated nontransgenic control mouse, (*B*) PBS-treated Alb/TGF-β1 transgenic mouse, (*C*) Alb/TGF-β1 transgenic mouse treated with LAP (64 μg). (*D*) Histogram showing means ± SEM of nuclear labeling indeces in nontransgenic control (C) and Alb/TGF-β1 transgenic (T) mice at 36 h after partial hepatectomy and treatment with either PBS or LAP at varying doses.

functional inactivation of active TGF-β1 *in vivo* by its systemically administered proregion. After i.p. injection, LAP concentrations in plasma increase for at least 2 h, indicating that LAP is slowly released from the peritoneal cavity into the systemic circulation, resulting in significant systemic levels of LAP. This observation is consistent with the pattern of serum levels of ^{125}I-labeled TGF-β1 after i.p. injection (45). Our results demonstrate that i.p. administration of LAP is a feasible route of administration in mice.

Gentry and Nash (15) showed that TGF-β1 LAP expressed in a CHO-cell expression system can neutralize the growth inhibitory function of both TGF-β1 and TGF-β2 *in vitro*. LAP used in those studies differs from the recombinant LAP used here in its amino acid sequence by the deletion of Arg278, as well as the conservation of the Cys33. We have confirmed and extended *in vitro* results of Gentry and Nash (15) by demonstrating that LAP is a potent inhibitor of all three TGF-β isoforms as estimated by comparison of the ED_{50} in the Mv1Lu growth inhibition assay. Recently, the equilibrium dissociation constant (K_d) for ligand binding of LAP was found to be 15-fold lower when compared with a soluble extracellular domain of the TGF-β receptor type II (8 nM versus 120 nM, respectively), indicating superior TGF-β binding characteristics of LAP *in vitro* (46).

The ability of recombinant LAP to neutralize active TGF-β1 in our *in vivo* system has important physiological and clinical implications. First, active TGF-β1, as it occurs in the *in vivo* milieu in this transgenic system, likely must be accessible for latent complex formation with pharmacologically applied LAP. This implies that many potential interactions of active forms of TGF-β with relatively abundant matrix proteins, including type IV collagen, fibronectin, and the proteoglycans decorin, betaglycan, and endoglin, as demonstrated *in vitro* (47–51), do not appear to prevent reconstitution of latent TGF-β complexes *in vivo*. In this context, the presence of enhanced extracellular matrix in livers of Alb/TGF-β1 transgenic mice might represent a good model of the extracellular environment as found in many fibrotic diseases (30). Second, previous *in vitro* observations suggested that LAP might target latent TGF-β complexes to cell surface receptors, such as the mannose-6-phosphate/insulin-like growth factor receptor, that have been implied in activation of latent TGF-β *in vitro* (52–54); thus, LAP could potentially contribute to enhanced TGF-β activity. However, we observed inhibition of TGF-β1 activity suggesting that the *in vivo* reconstituted latent complex is not targeted to activation sites by LAP. Furthermore, the question whether reconstituted latent TGF-β *in vivo* is subject to previously described *in vitro* mechanisms of activation remains unanswered (16–18, 55). It is possible that such mechanisms apply to reconstituted latent TGF-β, but that they cannot overcome the effects of pharmacological doses of LAP.

In situ differentiation of active TGF-β from latent TGF-β has not been possible until recently, when methods for selective detection and quantitation of activated TGF-β *in situ* have been developed (56, 57). It should soon be possible to define localization and levels of active TGF-β *in situ* in experimental animal models and human diseases. This will greatly facilitate *in vivo* investigation of inhibitors of TGF-β with therapeutical potential.

Use of antagonists of TGF-β may form the basis of important novel approaches in the future for the treatment of a large spectrum of serious chronic conditions in which excessive TGF-β action appears to be responsible for tissue damage caused by scarring (4, 58). Indeed, in previous studies, antiserum to TGF-β or the proteoglycan, decorin, have been shown to protect against scarring in experimental skin lesions and kidney disease (22, 59). LAP has excellent potential to become a potent and specific inhibitor of TGF-β for the prevention of fibrotic conditions. In this context, it will be important to examine whether the promising short term activity of LAP, as shown in the present study, can be sustained in more chronic disease models.

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