JOURNAL OF CELLULAR PHYSIOLOGY 199:67–76 (2004)

Latent TGF-β1 Activation by Platelets

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Platelets are a major source of transforming growth factor-β1 (TGF-β1) in the circulation as they release latent growth factor in response to activation. We report here that human platelets, when stimulated with thrombin, activated a significant proportion of the latent TGF-β released. Latent TGF-β activation was independent of cytokine release, since activation was delayed compared to platelet degranulation. Activation occured in releasates and did not require the continuous presence of platelets. Classical mechanisms of latent TGF-β activation were not involved, since activation was not affected by gene deletion and/or inhibitors of the known TGF-β activators/co-factors, thrombospondin-1 (TSP-1), mannose 6-phosphate/insulin-like growth factor-II receptor (M6P/IGF-IIR), plasminogen/plasmin, or several other candidate proteases. In contrast, latent TGF-β activation was significantly inhibited by the furin inhibitors, decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone and L-hexaarginine. We show that platelets contain a furin-like enzyme which is released upon platelet activation. We conclude that, following activation, platelets release and activate latent TGF-β1 via mechanisms involving the release and activity of a furin-like proprotein convertase. This novel mechanism of latent TGF-β activation might represent an important mediator and therapeutic target of platelet TGF-β1 functions, for example, in early wound repair, fibrosis, or arteriosclerosis. J. Cell. Physiol. 199: 67–76, 2004.

Platelet activation and aggregation are crucial events in normal hemostasis to prevent excessive bleeding and enable wound repair. Upon thrombin activation of platelets, shape changes and aggregation occur. These are accompanied by the release of the α-granule contents, including transforming growth factor-β (TGF-β, Assoian and Sporn, 1986).

TGF-β released from activated platelets can initiate early stages of wound repair, since TGF-β1 promotes inflammatory responses and angiogenesis as well as the healing of acute and chronic wounds (Mustoe et al., 1987; Pierce et al., 1989; Yang and Moses, 1990, Chen and Wahl, 1999). Furthermore, platelets are thought to be involved in various other pathologic conditions such as fibrosis, arteriosclerosis, and tumor progression (Baumgartner and Hosang, 1988; Männel and Grau, 1997). The specific pathogenic role of platelet activation in these conditions might, in part, be ascribed to the release of TGF-β.

Mammalian cells express three TGF-β isoforms (1, 2, and 3) which are synthesized as "small latent complexes" consisting of disulfide-bonded TGF-β homodimers noncovalently associated with homodimers of the TGF-β latency-associated peptide (LAP) (Munger et al., 1997; Annes et al., 2003). The small latent TGF-β complex can be disulfide-linked to latent TGF-β binding proteins to form the "large latent complex," resulting in targeting of latent TGF-β to specific sites in the extracellular matrix (Taipale et al., 1994). Release of the biologically active TGF-β homodimer from these latent complexes is a major regulatory step in TGF-β bioavailability and activity, and several enzymatic and non-enzymatic mechanisms of latent TGF-β activation have been described (Munger et al., 1997; Annes et al., 2003).

Although platelets are a major source of latent TGF-β1 in the circulation, little is known regarding their ability to generate active TGF-β. We have shown previously, using a rat incisional wound healing model, that latent TGF-β is activated immediately following wounding, suggesting the involvement of activated

Robert Blakytny, Anna Ludlow, and Gail E.M. Martin contributed equally to this work.

Contract grant sponsor: Deutsche Forschungsgemeinschaft (DFG); Contract grant sponsor: Biotechnology and Biological Sciences Research Council (BBSRC); Contract grant sponsor: Johnson & Johnson Medical Ltd.; Contract grant number: BR 1125/2-1.

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Received 23 May 2003; Accepted 4 September 2003

DOI: 10.1002/jcp.10454

platelets (Yang et al., 1999). This hypothesis is supported by the finding that induction of plasminogen activator inhibitor-1 gene expression in endothelial cells by thrombin-stimulated bovine platelets is dependent on active TGF-β (Slivka and Loskutoff, 1991). Using a bioassay as well as ELISA, we have shown that thrombin-stimulated murine platelets activate latent TGF-β and that activation does not require thrombospondin-1 (TSP-1, Abdelouahed et al., 2000).

In this article, we demonstrate that activation of human platelets produces active TGF-β1 in a time-dependent fashion, independent of latent TGF-β release. Latent TGF-β activation occured in platelet-free releasates and did not require TSP-1, plasmin, or the mannose 6-phosphate/insulin-like growth factor-II receptor (M6P/IGF-IIR) receptor, proteins known to be essential for activation in other cell systems. We show that activated platelets release a furin-like proprotein convertase which is involved in the extracellular activation of platelet latent TGF-β1.

MATERIALS AND METHODS

Reagents

Tumor tissue cryosections of human 293 renal sarcoma clone B9 (producing latent TGF-β) and clone C19 (producing predominantly active TGF-β) were kindly provided by Dr. Mary Helen Barcellos-Hoff (University of California, Berkeley, CA) (Barcellos-Hoff et al., 1995). The peptide, LSKL, which is derived from the TGF-β LAP and the scrambled control peptide, SLLK, were gifts from Dr. Joanne Murphy-Ullrich (University of Alabama, Birmingham, AL) (Ribeirio et al., 1999).

Human recombinant TGF-β1 and 3 and porcine recombinant TGF-β2 were purchased from R&D Systems (Wiesbaden-Nordenstadt, Germany). Luciferin was obtained from Biosynth AG (Staad, Switzerland), and luciferase lysis buffer from BD Biosciences (Heidelberg, Germany). DME medium, fetal calf serum, low-endotoxin BSA, and geneticin (G418) were provided by PAA Laboratories (Cölbe, Germany). Aprotinin, leupeptin, Pefabloc, and pepstatin were from Roche Diagnostics (Mannheim, Germany), human thrombin, calpain inhibitor peptide, M6P, bis-benzimide (Hoechst stain No. 33258), and 1,2-diazabicyclo(2,2,2) octane (Dabco) from Sigma (Munich, Germany), calpain inhibitor II from Serva (Heidelberg, Germany), caspase-3 inhibitor I and α_1-antitrypsin Portland (α_1-PDX) furin inhibitor from Calbiochem (Schwalbach, Germany), and the furin inhibitors, decanoyl-Arg-Val-Lys-Arg-chloromethylketone (dec-RVKR-cmk) and hexa-L-arginine, as well as the fluorogenic furin substrate, L-pyroglutamyl-Arg-Thr-Lys-Arg-7-amido-4-methylcoumarin (pyr-RTKR-amc), from Bachem (Heidelberg, Germany). Vinyl alcohol-25 (gelvatol) was obtained from Fisher Scientific (Nidderau, Germany).

Antibodies

The neutralizing mouse mAb to human plasmin (PG19) was kindly provided by Dr. Michael Kramer (University of Heidelberg, Germany) and has been characterized previously (Meissauer et al., 1992).

The neutralizing antibodies, chicken anti-human TGF-β1 (AF-101-NA and AB-101-NA), goat anti-porcine TGF-β2 (AB-112-NA), and goat anti-human TGF-β3 (AB-244-NA) were purchased from R&D Systems. The neutralizing ability of these antibodies was validated and their cross-reactivity with other TGF-β isoforms determined using recombinant TGF-β1, 2, and 3. With the exception of the anti-TGF-β3 antibody (approximately 93% neutralization and 57% cross-reactivity with TGF-β2 at 10 μg/ml), the anti-TGF-β1 (5 μg/ml) and anti-TGF-β 2 antibodies (10 μg/ml) proved to be completely neutralizing without cross-reacting with other TGF-β isoforms.

Rabbit anti-human TGF-β1 (SC-146) was from Santa Cruz Biotechnology (Wiltshire, UK), and rat mAb to CD 41 (glycoprotein α_{IIb} chain) from BD Biosciences.

The secondary antibodies, FITC-conjugated donkey anti-chicken IgY $F(ab')_2$ and TRITC-goat anti-rat IgG, as well as FITC-conjugated streptavidin were obtained from Jackson ImmunoResearch Laboratories/Dianova (Hamburg, Germany). Biotinylated donkey anti-rabbit IgG was from Amersham Pharmacia (Buckinghamshire, UK).

E_{max} TGF-β1 ImmunoAssay kits were from Promega (Mannheim, Germany). Asserachrom platelet factor 4 ELISA kits were obtained from Shield Diagnostics (Dundee, UK) and Roche Diagnostics (Mannheim, Germany).

Transgenic mice

The generation of mice deficient in TSP-1, plasminogen, or double-deficient in IGF-II and M6P/IGF-II receptor has been described previously (Bugge et al., 1995; Ludwig et al., 1996; Lawler et al., 1998).

Cell culture

Mink lung epithelial cells transfected with the plasminogen activator inhibitor-1/luciferase (PAI/L) DNA construct (Abe et al., 1994) were a gift from Dr. Daniel B. Rifkin (New York University Medical Center, NY). Cells were cultured in high glucose (4,500 mg/l) DME medium supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, and 250 μg/ml of geneticin and passaged with trypsin/EDTA (0.25%/0.02%, w/v). Cells were used for the PAI/L bioassay for TGF-β between passages 8 and 30.

Preparation of blood fractions

Peripheral venous blood samples from healthy adult volunteers (aged between 21 and 50) were taken into 20-gauge S-Monovettes (Sarstedt, Nümbrecht, Germany) following informed consent. Anti-coagulants used were acid-citrate-dextrose (ACD) for plasma preparation and EDTA for platelet preparation.

Blood samples were centrifuged at 300*g* for 10 min at room temperature in a swing-out rotor, and the supernatant (platelet-rich plasma) was harvested. Platelet-rich plasma was centrifuged at 2,500*g* for 15 min, resulting in the platelet fraction (pellet) and the platelet-poor plasma (supernatant). During blood fractionation, temperature changes or mechanical stress were avoided in order to prevent premature platelet activation. Platelet-poor plasma samples normally contained 1×10^3 IU/ml of platelet factor-4 (as compared to 34.8×10^3 IU/ml in platelet releasates) indicating that only approximately 2.9% of the platelets had been

activated during preparation. Platelet releasates were prepared by re-suspending the platelet fraction in the original volume of serum-free DME medium supplemented with 2 mM L-glutamine, 10 mM HEPES, and 0.1% low-endotoxin BSA (DMEM-BSA), followed by stimulation with 0.1 U/ml of human thrombin for 30 min at 37°C. In some experiments, platelets were incubated on a shaker which appeared to enhance activation. Platelet activation was terminated by centrifugation at 2,500*g* for 10 min at room temperature and harvesting the supernatant (platelet releasate).

Alternatively, blood was collected in the absence of an anti-coagulant and allowed to clot for 1–2 h at 37°C. The resulting serum was harvested and cleared by two centrifugation steps, first at 300*g* for 10 min followed by 2,000*g* for 5 min.

Plasma, serum, and platelet releasates were analyzed immediately for TGF-β in the PAI/L assay or for furin enzyme activity.

PAI/L bioassay for TGF-β

The PAI/L assay using mink lung epithelial cells transfected with a luciferase reporter construct under the control of a TGF-β-specific, truncated PAI-1 promoter was performed as described previously (Abe et al., 1994). Blood fractions were assayed at 2–3 dilutions in the linear range of the assay. Total TGF-β was determined following dilution and heat activation of latent TGF-β by incubation at 80°C for 10 min. Recombinant human TGF-β1 (2 μg/ml in 4 mM HCl) was used as a standard. Luciferase activity in cell lysates was determined using a MLX microtiter plate luminometer (Dynex, Chantilly, VA) and luciferin as a substrate.

Enzyme-linked immunosorbent assay (ELISA)

Platelet factor-4 content of platelet-poor plasma and platelet releasates was determined using ELISA. The assay was performed according to the manufacturer's instructions.

Immunofluorescence staining

Cryosections of 293 renal sarcoma (4 μm) or murine liver tissue (7 μm) on poly-L-lysine coated glass slides were fixed and permeabilized by incubation for 6 min at −20°C in methanol:acetone (1:1) (Barcellos-Hoff et al., 1995). Sections were air dried and free protein binding sites blocked by incubation for 1 h at room temperature with 0.5% (w/v) casein in PBS. Following incubation with the primary antibodies overnight at 4°C in a humid chamber, sections were washed and incubated with the secondary antibodies for 1 h at room temperature. When indicated, sections were washed and incubated with FITC-streptavidin for another 40 min at room temperature. In some experiments, cell nuclei were counterstained by including 0.25 μg/ml of Hoechst stain 33258 in the last incubation step.

The sections were mounted in gelvatol/Dabco and examined using a Leica DRMB microscope. Microphotographs were taken onto Kodak ASA 400 film, and the slides were scanned using a Nikon LS-1000 scanner. The resulting TIFF files were assembled in Microsoft PowerPoint.

Furin enzyme assay

Furin-like enzyme activity was assayed in black 96-well plates using the fluorogenic substrate, pyr-RTKR-amc, for 19 h at 30°C (Molloy et al., 1992). Enzyme activity was determined as the release of the fluorescent AMC moiety within the assay period.

RESULTS

Latent TGF-β1 activation by platelets

It is known that platelets represent a major source of latent TGF-β1 in the circulation (Assoian et al., 1983; Grainger et al., 2000) and that activation of this growth factor reservoir might regulate wound repair and scarring, fibrosis, arteriosclerosis, and tumor progression (Shah et al., 1994; Grainger et al., 1995; Roberts, 1995; Blobe et al., 2000). To investigate whether platelets activate the latent TGF-β1 that they release, human platelets were isolated by differential centrifugation. Following platelet activation the levels and isoforms of latent and active TGF-β in releasates were determined using the PAI/L bioassay.

Platelet activation with human thrombin triggered significant latent TGF-β release and activation (Table 1). The data obtained by bioassay or ELISA were comparable. Supernatants of resting platelets contained small amounts of latent as well as active TGF-β, which were probably generated by partial spontaneous activation during platelet preparation and incubation. Shaking the platelet suspension during thrombin stimulation greatly increased active TGF-β production (to 896 pg/ml, representing 1.8% of the total TGF-β), possibly by enhancing platelet activation. The active TGF-β levels observed with platelets are in the range of other latent TGF-β activation systems and are biologically significant, considering that the detection limit for PAI-1 promoter induction is approximately 5 pg/ml of TGF-β, and saturation of the response is reached at 250–500 pg/ml.

Pan-specific neutralizing anti-TGF-β antibodies completely abolished the signal obtained in the PAI/L assay,

TABLE 1. Latent transforming growth factor-β (TGF-β) activation by human platelets

Blood fraction	Active TGF-β[a] (pg/ml)	Total TGF-β[a] (ng/ml)
Platelet-poor plasma (n = 8)	n.d.[b]	2.7 ± 0.4[c]
Releasates from resting platelets (n = 11)	13 ± 7	13.6 ± 3.9
Releasates from activated platelets[d] (n = 37)	159 ± 27	60.2 ± 6.8
Serum (n = 10)	175 ± 24	57.2 ± 4.7

[a]Active and total (following heat activation) TGF-β in platelet releasates were determined at two to three dilutions in triplicate in the linear range of the plasminogen activator inhibitor-1/luciferase (PAI/L) bioassay using recombinant TGF-β1 as a standard. Note that active TGF-β is expressed in pg/ml and total TGF-β in ng/ml.
[b]Not detectable (<25 pg/ml).
[c]Data represent mean values ± SEM.
[d]Platelets were activated with 0.1 U/ml of human thrombin for 30 min at 37°C (without agitation).

verifying that active TGF-β was measured (data not shown). Latent TGF-β activation was platelet-mediated, since exogenous thrombin added to platelet releasates or recombinant small latent TGF-β1 complex failed to activate latent TGF-β directly (data not shown). Antibody inhibition experiments revealed that platelets express exclusively the TGF-β1 isoform.

Active and total TGF-β levels in human serum were comparable to those in platelet releasates (Table 1) and also represented TGF-β1 (data not shown), supporting the view that platelets are a major source of TGF-β in blood coagulation. While platelet-poor human plasma contained latent TGF-β, no active TGF-β was detected.

Latent TGF-β activation by thrombin-stimulated platelets was time-dependent but independent of TGF-β release (Fig. 1). The release of latent TGF-β was rapid with the bulk of this α-granule protein being released within approximately 15 min following thrombin stimulation (half-maximal release within 9.5 min). The kinetics of latent TGF-β activation, however, were comparatively delayed with active TGF-β levels increasing over a time period of at least 40 min (half-maximal activation required more than 15.5 min).

Transgenic analysis of the mechanism of latent TGF-β activation

A number of enzymatic and non-enzymatic mechanisms of latent TGF-β activation have been identified, some of which appear to be cell-type specific (Munger et al., 1997; Annes et al., 2003). In order to identify the mechanism operating in platelets, we analyzed murine platelets deficient in single known activators/regulators of latent TGF-β, such as TSP-1, plasmin, or M6P/IGF-II receptor, for their ability to produce active TGF-β in vivo and in vitro.

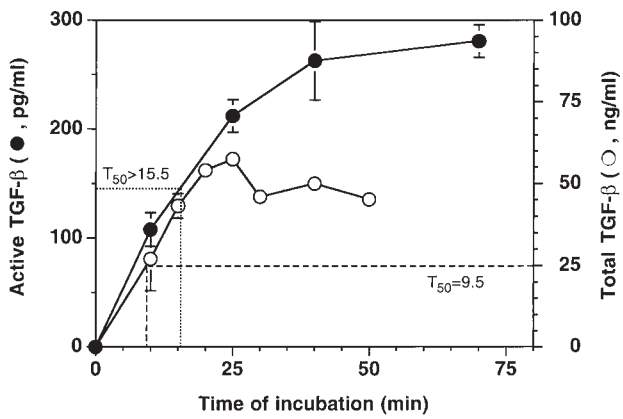

Fig. 1. Latent transforming growth factor-β (TGF-β) activation by thrombin-stimulated platelets is delayed compared to TGF-β release. Human platelets were isolated by differential centrifugation and activated by the addition (at time zero) of 0.1 U/ml of human thrombin. Following incubation at 37°C (without agitation), platelet suspensions were centrifuged at 2,500*g* for 10 min at room temperature and the supernatants (platelet releasates) harvested at the time points indicated. Releasates were analyzed for total (following heat activation) (○) and active TGF-β (●) using the plasminogen activator inhibitor-1/luciferase (PAI/L) bioassay. Note that total TGF-β is in ng/ml and active TGF-β in pg/ml. The graphs indicate the mean values ± SEM of pooled data from at least four separate experiments assayed in triplicate. The time period (T_{50}) required for half-maximal latent TGF-β release or activation is indicated.

In order to be able to demonstrate active TGF-β in platelets, we screened anti-TGF-β antibodies for their ability to specifically immunostain the "active" form of TGF-β1. While antibody binding, per definition, cannot identify the biological activity of a growth factor, we screened for antibodies that specifically recognize free TGF-β (refered to as active TGF-β) and do not cross-react with latent TGF-β complexes. Screening was done by immunostaining tumors produced by human 293 renal sarcoma cell clones genetically engineered to overproduce latent (clone B9) or active TGF-β1 (clone C19) (Barcellos-Hoff et al., 1995). We found that, in contrast to the majority of anti-TGF-β antibodies, the neutralizing chicken anti-TGF-β1 IgY (AF-101-NA) exclusively stained C19 tissue containing active TGF-β1 (Fig. 2A compared to C), whereas rabbit anti-TGF-β1 IgG (SC-146) predominantly stained B9 tissue containing latent TGF-β1, although weak staining was also observed with C19 tissue (Fig. 2D,B). The lack of B9 tissue staining by the chicken anti-TGF-β IgY indicated that the antibody specifically recognized active TGF-β1. This antibody was used for the transgenic analysis described below. A similar specificity for active TGF-β has been reported previously for antibody LC(1-30) (Barcellos-Hoff et al., 1995).

TSP-1 has been shown to act as a major activator of latent TGF-β in vivo, particularly in the lung and the pancreas (Crawford et al., 1998). Platelets contain large amounts of TSP-1 which are released together with latent TGF-β1 from α-granules in response to activation. We have shown previously, however, that murine platelets deficient in TSP-1 maintain their ability to generate active TGF-β in vitro (Abdelouahed et al., 2000). To confirm that active TGF-β is associated with TSP-1-deficient platelets in vivo, we immunostained liver tissue from TSP-1-deficient mice using the above antibody. Platelets were identified by positive staining for the platelet marker, CD41 (glycoprotein α_{IIb} chain; red label), and by the absence of a nucleus (blue label) (Fig. 3). Active TGF-β1 (green label) co-localized with platelets (resulting in yellow labeling) and was associated with wild-type as well as TSP-1-deficient platelets (parts A–C and D–F).

Similarly, platelets from mice deficient in plasminogen or M6P/IGF-II receptor stained positively for active TGF-β (parts G–I and K–M). Although the precise molecular mechanism of action and the physiological significance of these proteins in latent TGF-β activation in vivo have not yet been defined, conversion of plasminogen to plasmin and ligand binding to the M6P receptor are required for latent TGF-β activation in certain cell models, for example, in co-cultures of pericytes or smooth muscle cells with endothelial cells (Antonelli-Orlidge et al., 1989; Sato and Rifkin, 1989; Dennis and Rifkin, 1991) or in activated macrophages (Nunes et al., 1995). Similar to our findings with TSP-1-deficient platelets (Abdelouahed et al., 2000), latent TGF-β activation in releasates of M6P/IGF-II-receptor deficient platelets was not decreased compared to wild type platelets (Fig. 4), which is consistent with the above immunostaining data.

In summary, our data indicate that TSP-1, plasmin, and M6P/IGF-II receptor, although required for latent TGF-β activation in other cell systems, are not

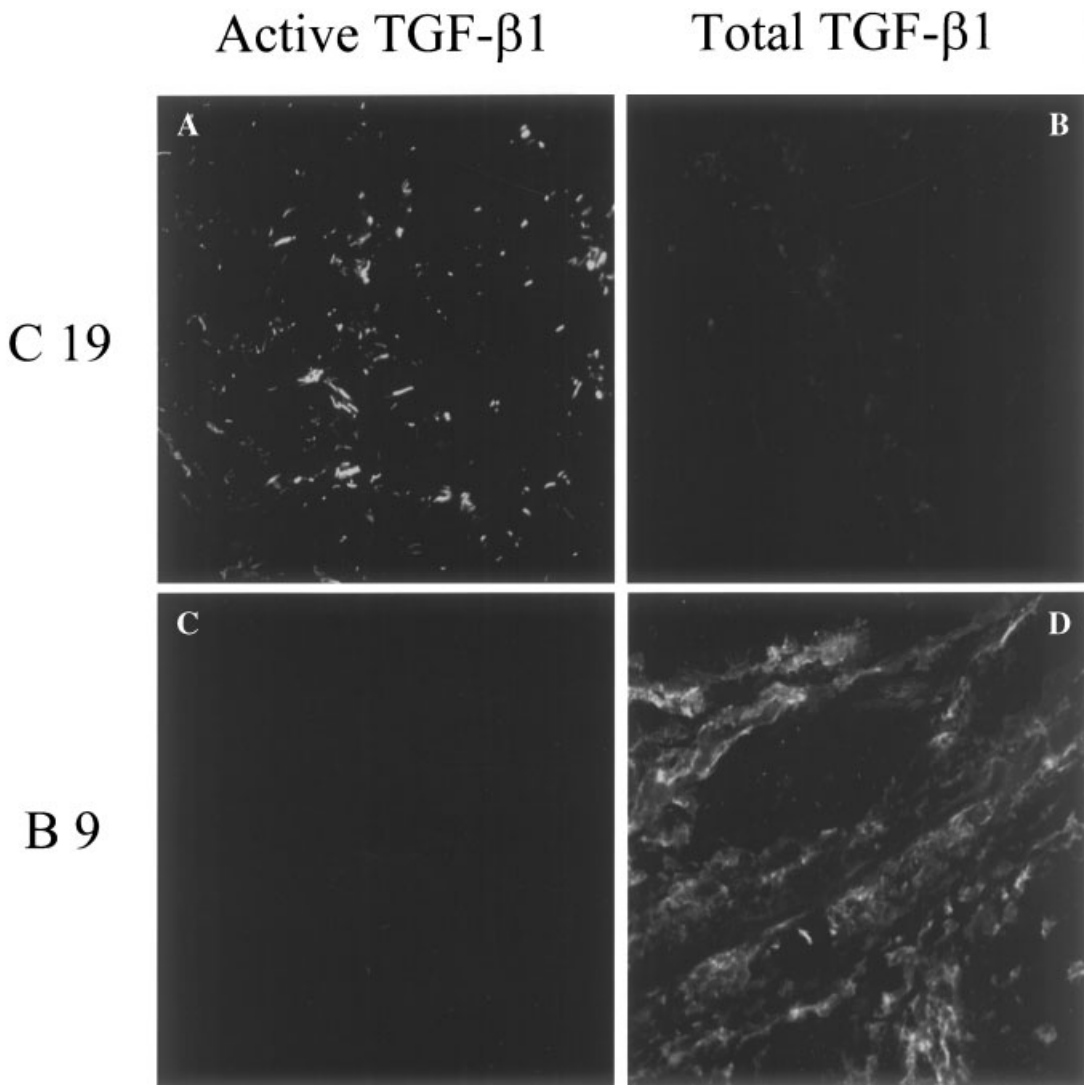

Fig. 2. Demonstration of antibody specificity for active TGF-β1. Cryosections (4 μm thick) of tumor tissue were fixed in methanol/acetone and immunostained with neutralizing chicken anti-TGF-β1 IgY (AF-101-NA; 5 μg/ml; parts A and C) or with rabbit anti-TGF-β1 (SC-146; 1 μg/ml; parts B and D). Antibody binding was visualized by incubation with FITC-conjugated donkey anti-chicken IgY (15 μg/ml) or by incubation steps with biotinylated donkey anti-rabbit IgG (1:200) and FITC-conjugated streptavidin (4 μg/ml). C19 tumor, genetically engineered to constitutively produce active TGF-β1; B9 tumor, producing latent TGF-β1 (Barcellos-Hoff et al., 1995). Controls using irrelevant chicken IgY or rabbit IgG did not show significant staining.

essential for the platelet-mediated generation of active TGF-β1.

Inhibitor analysis of the mechanism of latent TGF-β activation

Gene deletions involving activators of latent TGF-β might induce an up-regulation of compensatory mechanisms of activation during development, thus concealing a potential physiological role of the deleted activators. In fact, platelets from mice deficient in TSP-1 or M6P/IGF-II receptor appeared to activate higher levels of latent TGF-β1 upon thrombin stimulation than the corresponding wild types (Fig. 4 and Abdelouahed et al., 2000), possibly indicating compensation for a potential lack of activation in other tissues. Furthermore, the mechanisms of latent TGF-β activation might be species-specific, i.e., distinct mechanisms might be operating in human versus murine platelets. We, therefore, attempted to block latent TGF-β activation in human platelets by utilizing inhibitors of the above activators (TSP-1, plasmin, M6P/IGF-II receptor) as well as of additional potential activators of latent TGF-β such as other serine proteinases, cysteine proteinases, calpain I and II, caspase-3, and furin-like enzymes.

Human platelets were pre-incubated with the inhibitors prior to stimulation with thrombin, and active and total TGF-β levels were determined in platelet releasates. The results confirmed the above transgenic analysis of platelets from mutant mice, i.e., latent TGF-β activation was not significantly affected by inhibitors specific for TSP-1 (LSKL peptide (Ribeirio et al., 1999)), plasmin (neutralizing monoclonal antibody, PG19

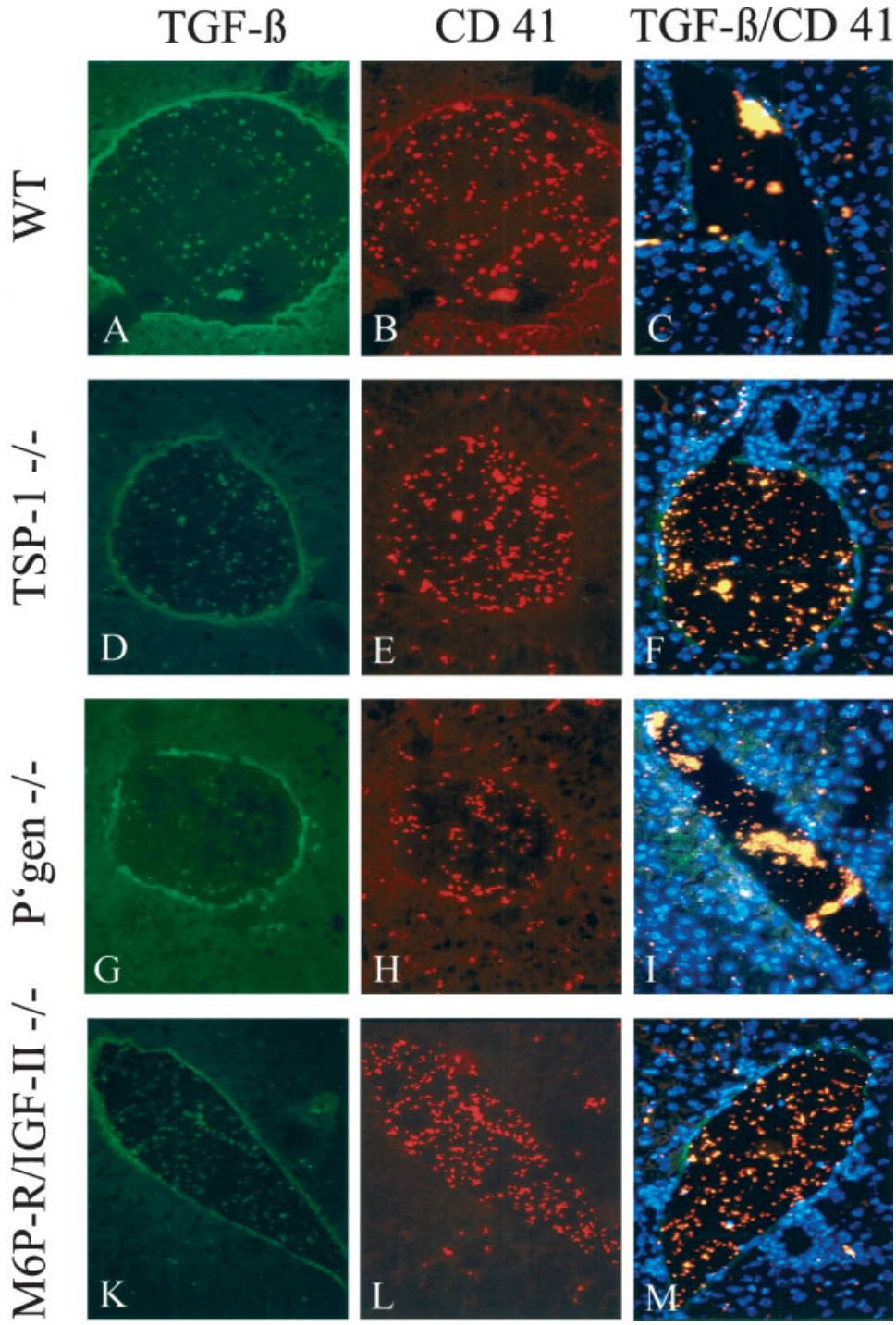

Fig. 3. Platelet-associated active TGF-β1 in mice deficient in proteins known to be involved in latent TGF-β activation. Liver cryosections (7 μm thick) were fixed in methanol/acetone and double-immunostained for active TGF-β (green label) and the platelet marker, CD41 (red label), using chicken anti-TGF-β1 IgY (5 μg/ml) and rat anti-CD41 (5 μg/ml), followed by incubation with FITC-conjugated donkey anti-chicken IgY (15 μg/ml) and TRITC-conjugated goat anti-rat IgG (7.5 μg/ml). Yellow color indicates co-localization of both proteins to platelets. Double-stained sections (TGF-β/CD41) were counterstained with Hoechst stain 33258 (0.25 μg/ml) to visualize the nuclei of tissue cells and confirm platelet identity. Staining was analyzed by standard fluorescence microscopy as described in "Materials and Methods" section. Note that the staining intensity is influenced by the number and aggregation state of the platelets. Similar results were obtained in three separate experiments.

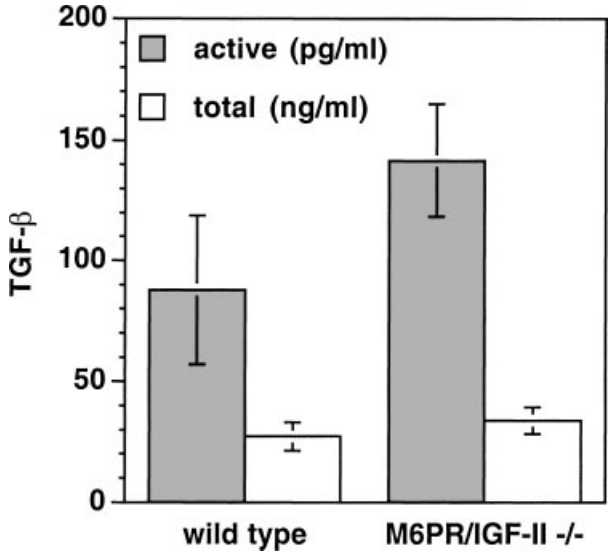

Fig. 4. M6P/IGF-II receptor expression is not required for platelet-mediated latent TGF-β activation. Platelets were isolated from M6P/IGF-II-receptor deficient or wild-type mice by differential centrifugation and activated by incubation with 0.1 U/ml of thrombin for 30 min at 37°C. Active and total (following heat activation) TGF-β present in the platelet releasates was determined using the PAI/L bioassay. Data represent the mean values ± SEM of platelet releasates from three to four mice assayed in triplicate.

(Meissauer et al., 1992), and aprotinin), or M6P/IGF-II receptor (M6P (Dennis and Rifkin, 1991)). Moreover, a number of inhibitors of other proteinases potentially involved in latent TGF-β activation (Pefabloc, α_1-antitrypsin, E-64, pepstatin, leupeptin, caspase-3 inhibitor, calpain inhibitor (ALLM)) also proved to be ineffective.

Involvement of a furin-like enzyme in latent TGF-β activation

Incubation of thrombin-stimulated platelets with the membrane-permeable protease inhibitor, dec-RVKR-cmk, considerably reduced the generation of active TGF-β in releasates (Fig. 5A). Dec-RVKR-cmk is a specific and potent inhibitor of subtilisin/Kex2p-like proprotein convertases, with its peptide sequence being based on the substrate recognition sequence of these enzymes (Nakayama, 1997). The most prominent and ubiquitously expressed member of this endoprotease family is furin, which typically cleaves at the consensus sequence motif R-X-(K/R)-R (Stieneke-Gröber et al., 1992). The membrane-impermeable furin inhibitor, hexa-L-arginine (Cameron et al., 2000), also significantly reduced active TGF-β levels in releasates (Fig. 5A) indicating that activation occured extracellularly following latent TGF-β release. Both inhibitors also inhibited latent TGF-β activation, when platelets were stimulated with type I collagen instead of thrombin (data not shown). In contrast, the bioengineered α_1-antitrypsin variant, α_1-PDX (Jean et al., 1998), a more selective inhibitor of the furin family members, furin and PC5/PC6, did not significantly affect activation (up to a concentration of 100 nM; data not shown). Furin is known to be involved in pro-TGF-β processing, resulting in heat-activatable latent TGF-β complex (Dubois et al., 1995). However, the furin inhibitors used in these studies did not affect

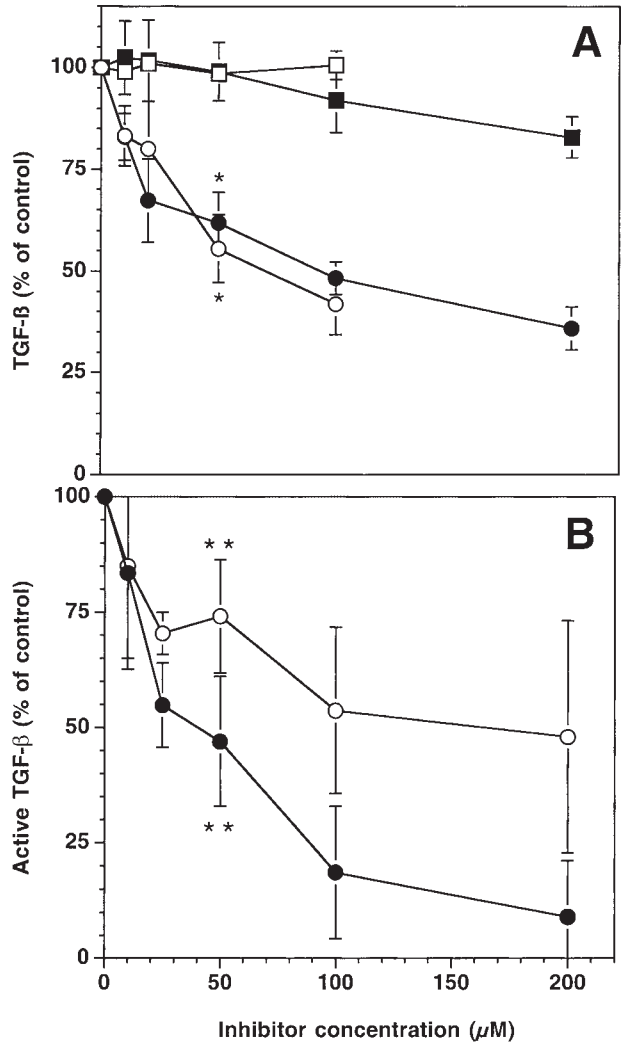

Fig. 5. A furin-like enzyme is involved in platelet-mediated latent TGF-β activation. Platelets were activated with thrombin in the absence or presence of furin inhibitors (**A**). Platelets were pre-incubated with hexaarginine (■,○), whereas dec-RVKR-cmk (□,○) was added 5 min after thrombin addition because of interference with platelet activation at higher concentrations. Active (●,○) and total TGF-β (□,■) in releasates were determined in the PAI/L bioassay. Active TGF-β levels in the controls were 82 pg/ml (dec-RVKR-cmk data) and 61 pg/ml (hexaarginine data), total TGF-β levels were 33.4 ng/ml (dec-RVKR-cmk data) and 39.5 ng/ml (hexaarginine data). Alternatively, furin inhibitors were added to platelet-free releasates of activated platelets (**B**), and activation was allowed to continue in the absence of platelets for 30 min at 37°C. ●, dec-RVKR-cmk; ○, hexaarginine Active TGF-β levels in the controls were 77 pg/ml (dec-RVKR-cmk data) and 104 pg/ml (hexaarginine data). Incubation on ice reduced activation in the controls to approximately 56% (data not shown). Data represent the mean values ± SEM of three (A) or four (B) independent experiments assayed in triplicate. Statistical analysis was done using the *t*-test. Asterisks indicate the lowest inhibitor concentration resulting in significant inhibition ($^{*}P \leq 0.04$; $^{**}P \leq 0.004$) compared to control.

latent TGF-β levels in platelets (data not shown) or releasates (Fig. 5A), indicating that potential platelet pro-TGF-β processing or platelet activation itself were not affected.

Latent TGF-β activation appeared to be enzymatic and independent of the continuous presence of platelets,

since incubation of platelet-free releasates on ice (as compared to 37°C) reduced active TGF-β levels to approximately 56%. As observed for platelet suspensions, activation in releasates was inhibited, in a dose-dependent fashion, by the furin inhibitors, dec-RVKR-cmk, and hexa-L-arginine (Fig. 5B) but not by α_1-PDX (data not shown). This indicates that the furin-like enzyme involved in latent TGF-β activation is released from activated platelets.

Expression of furin-like enzymes in platelets has not been reported so far. We, therefore analyzed hypotonic lysates as well as releasates of human platelets for the presence of furin-like enzyme activity using the fluorogenic furin substrate, pyr-RTKR-amc. Platelet lysates contained a furin-like enzyme activity, part of which (approximately 15%) was released upon thrombin stimulation. Enzyme activity was inhibited by dec-RVKR-cmk and hexa-L-arginine (Fig. 6), but not by α_1-PDX (data not shown) confirming above data. Hexa-L-arginine appeared to inhibit released enzyme less efficiently than enzyme present in hypotonic cell lysates, which might be due to potential interference of other molecules present in releasates with the positively charged inhibitor.

DISCUSSION

Platelets activate latent TGF-β1 independently of its release

Activated platelets release large amounts of TGF-β1 and therefore provide a reservoir of latent growth factor (Assoian et al., 1983) for the highly regulated generation of active TGF-β. In contrast to paracrine mechanisms of latent TGF-β activation described in other TGF-β activation systems, which involve extracellular matrix interactions, proteolytic processing events, and/or receptor interactions requiring more than one cell type (Munger et al., 1997; Annes et al., 2003), we found that platelets generate significant amounts of active TGF-β1 via autocrine mechanisms. Activation of human platelets with thrombin triggered rapid release of latent TGF-β followed by delayed, time-dependent generation of active TGF-β. This suggests that latent TGF-β release by platelet degranulation and subsequent extracellular activation in platelet releasates are independent events.

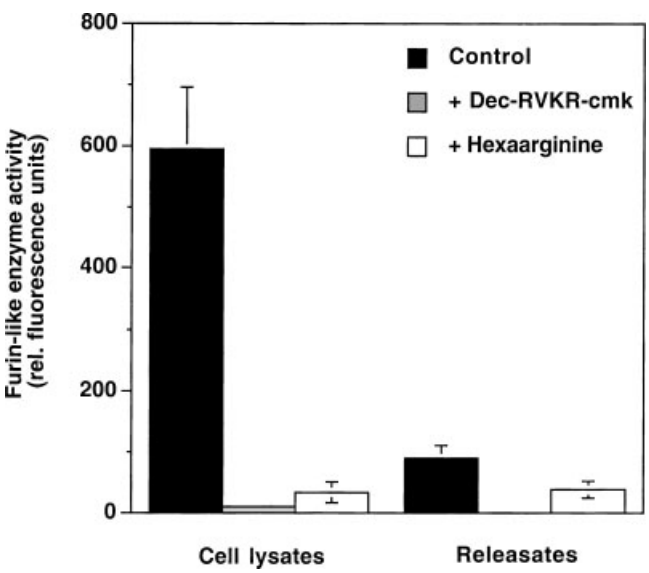

Fig. 6. Platelets contain and release furin-like enzyme activity. Releasates or hypotonic lysates of activated platelets (final dilution 1:2) were assayed using the furin substrate, pyr-RTKR-amc in the absence or presence of the furin inhibitors, hexaarginine (200 μM) or dec-RVKR-cmk (150 μM). Values were corrected for substrate-independent endogenous fluorescence (control without substrate) as well as for spontaneous substrate hydrolysis (buffer control). Mean values ± SEM of three to four separate experiments asssayed in duplicate are shown.

Released latent TGF-β was present in large excess over the active TGF-β produced, supporting the view that TGF-β action is regulated at the level of extracellular activation of the latent precursor (Munger et al., 1997; Annes et al., 2003). Platelet latent TGF-β1 is released as large latent complex, possibly serving a dual function. Part of it is activated to initiate immediate TGF-β signaling, for example, to promote early stages of wound repair such as inflammation and angiogenesis. The majority of TGF-β remains in the latent form possibly being deposited into the extracellular matrix, thus potentially serving as a reservoir for growth factor mobilization in later stages of wound repair.

Active TGF-β was also present in serum, and the levels were comparable to those in platelet releasates suggesting that platelet activation accounts for the bulk of active and latent TGF-β found in serum.

Latent TGF-β1 activation involves a furin-like platelet enzyme

Interaction with the platelet and extracellular matrix protein, TSP-1, represents the first mechanism of latent TGF-β activation shown to operate in vivo (Crawford et al., 1998). Therefore, TSP-1 interaction with latent TGF-β1 was likely to represent the mechanism of activation operating in activated platelets, particularly since TSP-1 is expressed in platelets in excess over TGF-β (Murphy-Ullrich and Poczatek, 2000), and both proteins are stored in and co-released from α-granules upon platelet activation. Whereas this protein interaction appears to occur in vivo (Murphy-Ullrich et al., 1992), it is not essential for platelet-mediated latent TGF-β activation. Thus, we have demonstrated previously that TSP-1-deficient murine platelets maintain their ability to activate latent TGF-β in vitro (Abdelouahed et al., 2000). In support of these findings, we show here that active TGF-β1 is associated with TSP-1-deficient murine platelets in vivo. Similarly, TSP-1 is not involved in latent TGF-β activation by human platelets arguing against the possibility that alternative mechanisms might have been up-regulated in TSP-1-deficient murine platelets, as a consequence of gene deletion, compensating for a potential deficiency in active TGF-β generation. Our findings are consistent with a recent report that TSP-1 is unable to activate latent TGF-β directly, neither in a chemically defined system nor in smooth muscle cell cultures, suggesting that additional factors might be required (Grainger and Frow, 2000).

Similarly, platelets from mice deficient in plasmin generation or M6P/IGF-II receptor expression maintained their ability to activate latent TGF-β in response to thrombin. Plasmin and M6P/IGF-II receptor are required for latent TGF-β activation in co-cultures of pericytes or smooth muscle cells with endothelial cells

(Antonelli-Orlidge et al., 1989; Sato and Rifkin, 1989; Dennis and Rifkin, 1991) as well as for activation by macrophages (Nunes et al., 1995). These proteins, however, are not essential for the generation of active TGF-β by platelets.

The extracellular generation of active TGF-β by thrombin-stimulated human platelets was significantly reduced in the presence of inhibitors of furin-like proprotein convertases. The residual TGF-β activity (approximately 35–40%) might have been generated prematurely during platelet preparation or incubation prior to the addition of the inhibitors. Alternatively, it is possible that more than one mechanism is involved in platelet-mediated latent TGF-β activation. Furthermore, the efficiency of the inhibitors in platelet suspensions could have been reduced by non-specific binding to the cell surface or uptake (e.g., of the membrane-permeable dec-RVKR-cmk) into the platelets. The latter might explain the lower efficiency of dec-RVKR-cmk in platelet suspensions as compared to releasates. In releasates, dec-RVKR-cmk was more potent than hexaarginine which is consistent with its lower K_i for furin family members (Jean et al., 1998; Cameron et al., 2000).

In certain cell types, furin-like proprotein convertases catalyze the maturation of pro-TGF-β precursor to heat-activatable latent growth factor complex (Dubois et al., 1995). Our data indicate, however, that platelet α-granules contain and release mature, heat-activatable latent TGF-β and that the levels are not affected by furin inhibitors. Thus, pro-TGF-β processing in the megakaryocytic lineage probably occurs in the megakaryocytes. Our data therefore identify a novel function of furin-like enzymes, namely involvement in the extracellular activation of platelet large latent TGF-β1 complex under physiological conditions.

The furin family currently encompasses seven members (Nakayama, 1997). We show here, for the first time, that platelets contain furin-like enzyme activity. The molecular nature of the enzyme present in platelets as well as the regulation of its release and activity are presently unknown. The pattern of inhibitor sensitivity observed with this enzyme (inhibited by dec-RVKR-cmk and hexa-L-arginine but not by α_1-PDX) is characteristic for several of the furin family members (Jean et al., 1998). However, only one of them, PACE4, is a secretory enzyme with broad tissue expression including megakaryoblastic cell lines (Bando et al., 2002). Further studies are required to identify the furin family member(s) involved in pro-TGF-β maturation versus latent TGF-β activation in the megakaryocytic lineage.

In summary, we found that platelets are not only major storage sites for latent TGF-β1 but also activate part of it following degranulation. While the mechanism of activation does not require any of the well-characterized activators, TSP-1, M6P/IGF-II receptor, or plasmin, the platelet latent TGF-β complex appears to be activated via a sequence of events involving protein processing by a furin-like proprotein convertase released by the platelets. Following release in vivo, this enzyme could continue to operate, independently of the presence of platelets, in the surrounding tissue (e.g., the wound area), possibly leading to the activation of extracellular-matrix associated latent TGF-β complex. Therefore, this novel mechanism of activation might represent a promising target to modulate TGF-β activity in pathologic conditions involving platelet degranulation, such as wound repair, fibrosis, arteriosclerosis, and cancer.

ACKNOWLEDGMENTS

We thank Dr. Jack Lawler (Harvard Medical School, Boston, MA) for providing liver tissue from TSP-1-deficient mice, Dr. Mary Helen Barcellos-Hoff (University of California, Berkeley, CA) for providing B9 and C19 renal sarcoma tumor tissue, Dr. Daniel B. Rifkin for the gift of mink lung epithelial cells transfected with the PAI/L DNA construct, Dr. Michael Kramer for supplying the neutralizing mAb to human plasmin (PG-19), and Dr. Joanne Murphy-Ullrich (University of Birmingham, Alabama) for the gift of LSKL and SLLK peptides. We also thank Dr. Sharon O'Kane (University of Manchester, UK) for explanting the livers from plasminogen-deficient mice, Tony Wade (University of Manchester, UK) for helping with confocal microscopy, Manuel Ruthenbürger (University of Münster, Germany) for helping with fluorimetry, our colleagues at the Fachklinik Hornheide for the blood donations, and the German Red Cross Institute in Münster for providing outdated platelet preparations for these studies.

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