M6P/IGFII-receptor complexes urokinase receptor and plasminogen for activation of transforming growth factor-β1

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Transforming growth factor-β1 (TGF-β1) is a critical cytokine for cell proliferation and differentiation. It is secreted by many cells in a latent pro-form (LTGF-β1) from which biologically active TGF-β1 is released by an *in vivo* mechanism that is not known. Here we show that the mannose-6-phosphate/insulin-like growth factor II-receptor (M6P/IGFII-R), which binds LTGF-β1, complexes with urokinase (plasminogen activator)-receptor (uPA-R) on the surface of human monocytes and directly binds plasminogen (Plg). Plasmin generated from Plg in the complex mediates release of TGF-β1 when M6P/IGFII-R is associated with uPA-R. Thus, this interaction of M6P/IGFII-R and uPA-R suggests a potential mechanism for the generation of TGF-β1 by cells.

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1 Introduction

TGF-β1 is a pluripotent cytokine involved in many biological processes such as immunomodulation, wound healing, extracellular matrix synthesis, proteolysis and embryogenesis by regulating growth, differentiation and development of many cell types [1–3]. The biological importance is dramatically demonstrated by studies on TGF-β1 null mice which exhibit a disastrous phenotype including multiorgan inflammatory syndrome, lack of Langerhans cells, progressive growth retardation and death by 4 weeks of age [4–6]. TGF-β1 is produced by many mammalian cells [7] and almost all cell types and tissues express high-affinity receptors [8–12]. Because of the numerous cells producing TGF-β1 and its broad biological action, its activity must be tightly controlled. The basis for regulation of activity seems to be the secretion of TGF-β1 as a complex with latency-associated peptide (LAP) forming latent TGF-β1 (LTGF-β1), which is biologically inactive [13]. To elicit a biological response via the interaction with specific cell surface receptors, TGF-β1 must be released from LTGF-β1 [14, 15].

In vitro, denaturing treatments like extremes of pH or heat [16] can release active TGF-β1 from the non-covalent association with LAP. Proteolytic activation has been shown by plasmin, cathepsin D [17, 18] and calpain [19]. Furthermore, LTGF-β1 activation has been described through thrombospondin-1, which induces TGF-β1 release probably by a conformational change upon interaction with LTGF-β1 [20]. Recently it has been shown that histological abnormalities in lung and pancreas of thrombospondin-1 and TGF-β1 null mice are similar. However, early death and the autoimmune destruction seen in the latter animals are not displayed by the thrombospondin-1 null mice [21]. This indicates that several pathways for LTGF-β1 activation do exist

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Abbreviations: AP: α2-antiplasmin **BB:** Binding buffer **BS:** Binding solution **GPI:** Glycosylphosphatidylinositol **LAP:** Latency-associated peptide **LTGF-β1:** Latent transforming growth factor-β1 **M1P:** Mannose-1-phosphate **M6P/IGFII-R:** Mannose-6-phosphate/insulin-like growth factor II receptor **Plg:** Plasminogen **Plm:** Plasmin **PTK:** Protein tyrosine kinase **TA:** Tranexamic acid **uPA:** Urokinase plasminogen activator **uPA-R:** Urokinase plasminogen activator-receptor **suPA-R:** Soluble recombinant uPA-R

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and, in particular, that those underlying LTGF-β1 conversion for down-regulation of the immune system may not require thrombospondin-1.

One of these further pathways for LTGF-β1 activation seems to involve the mannose-6-phosphate/insulin-like growth factor II receptor (M6P/IGFII-R), a multifunctional cell surface receptor [22]. M6P residues present in LAP mediate binding of LTGF-β1 to M6P/IGFII-R [23, 24], and inhibition of this binding by M6P or Ab to M6P/IGFII-R prevented TGF-β1 formation in a cell culture system [25]. Besides M6P/IGFII-R, the fibrinolytic system appears to be also of critical importance for LTGF-β1 activation. Plasmin (Plm) treatment of supernatants of transfectants secreting LTGF-β1 resulted in appearance of TGF-β1 [18]. Furthermore, TGF-β1 formation correlated with the expression level of plasminogen (Plg), urokinase plasminogen activator (uPA) or uPA-R on various cells [26–28], and this activation could be inhibited by M6P [26, 27]. Taken together, these studies imply a connection between M6P/IGFII-R and the fibrinolytic system for LTGF-β1 activation by cells.

To explore the cellular mechanism of TGF-β1 formation, we therefore analyzed a possible physical and functional association of M6P/IGFII-R and the fibrinolytic system. The cell surface-associated fibrinolytic system is primed by uPA-R, a glycosylphosphatidylinositol (GPI)-anchored membrane protein, also termed CD87. Upon binding, its ligand pro-uPA is proteolytically cleaved to uPA which proteolytically converts Plg into the serine protease Plm. Plm can directly degrade matrix proteins and activates a variety of biologically potent substances [29, 30]. Previously, we found that uPA-R and other GPI-anchored proteins are physically and functionally associated with protein tyrosine kinases (PTK) of the Src family [31, 32]. In human monocytes, which among immune cells represent an important source of TGF-β1 [33], the uPA-R – Src-PTK association is organized as a large multicomponent membrane microdomain, termed uPA-R complex, which also contains β2-integrins and additional unidentified molecules [32, 34]. Now we can report that M6P/IGFII-R is one of these previously unidentified components of the uPA-R complex. Furthermore, we found that M6P/IGFII-R directly interacts with uPA-R and also binds Plg. Moreover, the M6P/IGFII-R – uPA-R complex can simultaneously recruit the ligands uPA, Plg and LTGF-β1, and controls uPA-mediated activation of Plg to Plm for TGF-β1 release.

2 Results and discussion

2.1 M6P/IGFII-R is part of the uPA-R complex

Monocytes were lysed in a solution of 1 % detergent Brij-58, and the lysate was size fractionated on a Sepharose 4B column, which has an exclusion limit of tens of millions of daltons. Essentially all uPA-R and M6P/IGFII-R were eluted in fractions close to the void volume of the column, indicating that not only is uPA-R a component of a large membrane complex as reported previously by us [32], but also M6P/IGFII-R (Fig. 1A). In contrast, the transmembrane protein CD147 [35] and the cytoplasmic molecule annexin II, a known Plg receptor [36], were detected only in fractions corresponding to smaller complexes or to uncomplexed molecules. The common β-chain of β2-integrins, CD18, was distributed in all fractions (Fig. 1A). These results indicated that most of M6P/IGFII-R is contained in complexes of similar size as uPA-R. To analyze whether M6P/IGFII-R and uPA-R are physically linked to a common complex, the uPA-R complex was immunoprecipitated from surface-biotinylated monocytes and then dissociated with a more stringent detergent (see Sect. 4.3). M6P/IGFII-R was specifically reprecipitated from these samples with a rabbit antiserum to M6P/IGFII-R, but neither with rabbit polyclonal anti-CD18 Ab nor rabbit pre-immune serum which were used as controls (Fig. 1B). Thus, we found that M6P/IGFII-R is associated with uPA-R within a large plasma membrane complex.

2.2 Stability of the association of uPA-R and M6P/IGFII-R

To determine more details of the structural relationship between uPA-R and M6P/IGFII-R in the complex, surface-biotinylated monocytes were solubilized under progressively more stringent detergent conditions to systematically disrupt the complex. uPA-R was immunoprecipitated and co-isolated biotinylated proteins were visualized with streptavidin-peroxidase on a blot. Interestingly, the uPA-R precipitate obtained from the lysate using 1 % NP40 plus 0.2 % deoxycholate contained only two biotinylated components: a 35–60-kDa zone corresponding to uPA-R, and a 250-kDa zone corresponding in size to M6P/IGFII-R (Fig. 2). Indeed, by a similar reprecipitation approach as shown before (Fig. 1B), the 250-kDa band was identified as M6P/IGFII-R. This indicates that M6P/IGFII-R is the surface protein most strongly associated with uPA-R in monocytes and that both receptors may represent the “core” of the uPA-R complex.

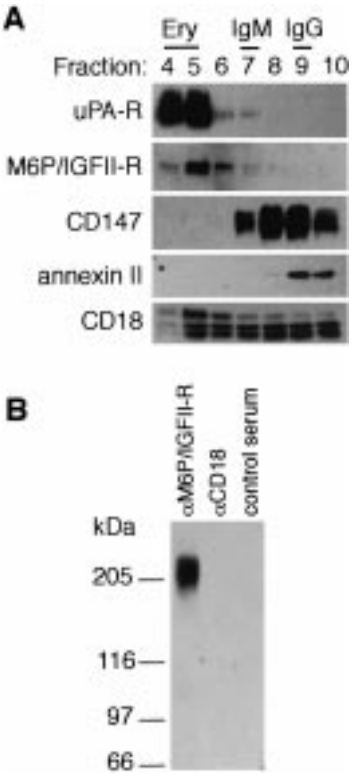

Figure 1. Co-localization of uPA-R and M6P/IGFII-R in monocytes. (A) Distribution of uPA-R and M6P/IGFII-R in size-fractionated monocyte lysate. The lysate was gel filtrated and the individual fractions were analyzed for presence of the indicated molecules by immunoblotting with mAb H2 to uPA-R, rabbit antiserum to M6P/IGFII-R, mAb MEM-M6/2 to CD147, mAb to annexin II and mAb MEM-48 to CD18. Fraction numbers are shown on top, as well as the elution volume of erythrocytes (Ery), IgM- and IgG-Ab that were used for calibration of the column. (B) Co-isolation of M6P/IGFII-R and uPA-R. Immunoprecipitates from lysates of biotinylated monocytes obtained by anti-uPA-R mAb H2 were subjected to reprecipitation using the indicated rabbit antisera. Samples were analyzed by SDS-PAGE using a 4 % gel followed by transfer to a membrane and detection of biotinylated proteins using a streptavidin-peroxidase conjugate and chemiluminescence.

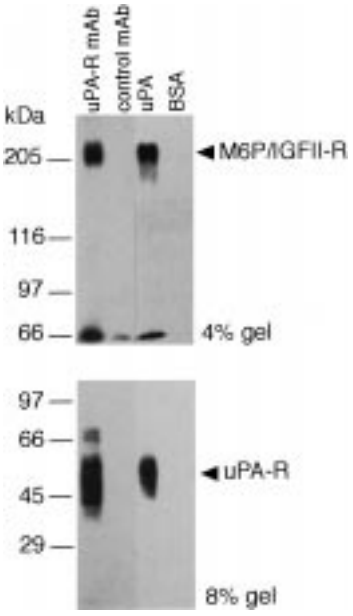

Figure 2. Stability of the uPA-R – M6P/IGFII-R association. Monocytes were surface biotinylated and lysed in lysis buffer containing 1 % NP40 plus 0.2 % deoxycholate. uPA-R was precipitated either by mAb H2 or by its natural ligand uPA. mAb AFP-01 and BSA were used as controls. For high resolution, immunoprecipitates were separated by SDS-PAGE both on a 4 % (top) and a 8 % (bottom) gel. After transfer to a membrane, biotinylated proteins were visualized by a streptavidin-peroxidase conjugate and chemiluminescence.

2.3 Direct interaction of uPA-R and M6P/IGFII-R

The detergent-resistant association between M6P/IGFII-R and uPA-R suggested a direct protein-protein interaction. To confirm this, we expressed a recombinant form of M6P/IGFII-R fused at the C terminus with a small tag, termed pTag, for convenient isolation and detection. The resulting construct, M6P/IGFII-R-pTag, bound in an *in vitro* binding assay natural GPI-linked uPA-R that was affinity purified from U937 cells. M6P/IGFII-R-pTag also interacted with a soluble recombinant uPA-R construct lacking the GPI anchor (suPA-R, amino acids 1-277) which was affinity isolated from supernatants of stably transfected Chinese hamster ovary (CHO) cells. However, it did not react with a variety of control proteins, including BSA and collagen (Fig. 3A). Neither form of uPA-R reacted with a pTagged construct of the cell surface protein CD147 [35] used as a further control (Fig. 3B). Thus, this experiment demonstrates that M6P/IGFII-R and uPA-R can directly interact, and indicates that this binding is independent of the GPI part of uPA-R. These results are in accordance with a recent report by Nykjaer et al. [37]. We investigated whether an interaction between putative M6P residues in the uPA-R and the M6P-binding site in the M6P/IGFII-R may be responsible for the binding of the two proteins. However, blocking the M6P-binding sites on M6P/IGFII-R by co-incubation of the receptor with M6P did not inhibit the binding to uPA-R (Fig. 3A).

Next, we examined whether ligation of M6P/IGFII-R or uPA-R by natural ligands modifies their interaction. Co-incubation of M6P/IGFII-R with either LTGF-β1 or IGFII, the classical ligand of M6P/IGFII-R, had no significant

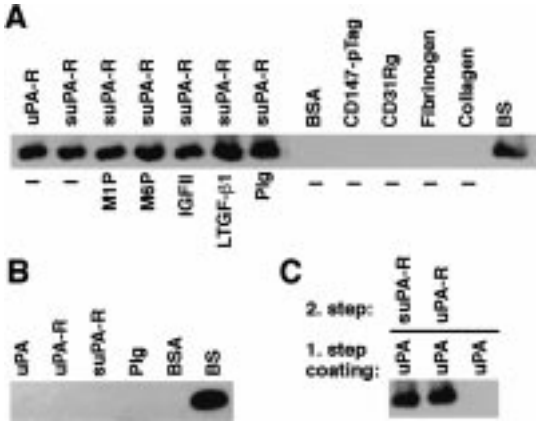

Figure 3. Analysis of the binding of uPA-R to M6P/IGFII-R. Wells of Falcon plates were coated with 10 μg/ml of the indicated molecules (A–C, upper rows). After blocking and washing, wells were preincubated either with the second ligand (C), or incubated for 1 h directly with BS that contained either 5 μg/ml of M6P/IGFII-R-pTag (A and C) or CD147-pTaq (B) and was further supplemented with or without the indicated molecules (bottom row). Binding of M6P/IGFII-R-pTag or CD147-pTag was analyzed by SDS-PAGE using a 4 % (A, C) or a 10 % gel (B), followed by immunoblotting using anti-pTag mAb H902. The quality and quantity of M6P/IGFII-R-pTag and CD147-pTag in the BS were checked by applying 20 μl of the respective BS directly to the gel (A, B).

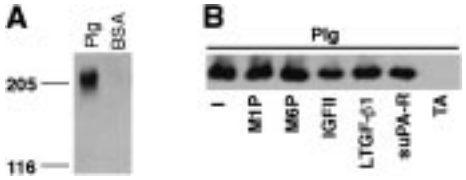

Figure 4. Analysis of the binding of Plg to M6P/IGFII-R. (A) Reprecipitation of M6P/IGFII-R by Plg. Monocytes were biotinylated and solubilized using 1 % NP40 plus 0.2 % deoxycholate. Immunoprecipitates obtained by anti-uPA-R mAb H2 were subjected to reprecipitation using Plg or BSA. After SDS-PAGE and transfer to a membrane, biotinylated proteins were visualized by a streptavidin-peroxidase conjugate and chemiluminescence. (B) M6P/IGFII-R binds Plg *in vitro*. Falcon plates were coated with 10 μg/ml Plg. After blocking and washing, wells were incubated for 1 h with BS that contained 5 μg/ml of M6P/IGFII-R-pTag and was further supplemented with or without the indicated molecules (bottom row). Binding of M6P/IGFII-R-pTag was analyzed by SDS-PAGE using a 4 % gel followed by immunoblotting using anti-pTag mAb H902. Controls for this assay are shown in Fig. 3.

influence on the binding of M6P/IGFII-R to uPA-R (Fig. 3A). When uPA-R or suPA-R were engaged by uPA that was coated on a plate, M6P/IGFII-R-pTag was able to bind. uPA alone did not interact with M6P/IGFII-R-pTag, demonstrating that the binding of M6P/IGFII-R to uPA-R is neither mediated nor inhibited by uPA (Fig. 3C). Together, these experiments indicate that neither ligation of M6P/IGFII-R nor ligation of uPA-R prevents their mutual interaction.

2.4 M6P/IGFII-R binds Plg and controls conversion of Plg to Plm

uPA-R-bound uPA converts Plg into Plm [38], but the underlying molecular mechanism at the cell surface is unknown. Since M6P/IGFII-R is a multifunctional receptor [22], we tested whether it also acts as a receptor for Plg and controls its activation. Indeed, M6P/IGFII-R co-isolated through uPA-R from monocyte lysates was reprecipitated by Plg (Fig. 4A). Plg reacted also with the recombinant M6P/IGFII-R-pTag in an *in vitro* binding assay. The interaction was not significantly modified by mannose-1-phosphate (M1P), M6P, IGFII, LTGF-β1 or suPA-R, but was inhibited by tranexamic acid (TA), a lysine analog, suggesting that lysine residues are involved in the binding (Fig. 4B).

To study the function of M6P/IGFII-R in Plg activation, we allowed biotinylated Plg to bind to M6P/IGFII-R, and analyzed conversion of Plg by uPA using SDS-PAGE (uPA converts Plg to Plm by proteolytic cleavage at amino acid Arg_{560}-Val_{561}, which can be detected on a gel). We found that M6P/IGFII-R-bound Plg was activated, and that the generated Plm remained associated with M6P/IGFII-R (Fig. 5A). Under physiological conditions, Plm must be protected against plasma proteases to prevent its immediate inactivation. Plm bound to M6P/IGFII-R was protected against a fast-acting natural inhibitor α2-antiplasmin (AP), and was not released from the receptor (Fig. 5B, complex and supernatant). Under the same conditions, Plm in solution was immediately inhibited by complex formation with AP (Fig. 5B, control). Thus, M6P/IGFII-R binds Plg and seems to control the conversion to and activity of the generated Plm.

2.5 Release of active TGF-β1 from the uPA-R-M6P/IGFII-R complex

Finally, we analyzed the involvement of the identified uPA-R – M6P/IGFII-R complex on release of TGF-β1 from LTGF-β1. TGF-β1 was formed when we treated a protein complex composed of immobilized suPA-R, M6P/IGFII-R, Plg and LTGF-β1 with uPA (Fig. 6A). LTGF-

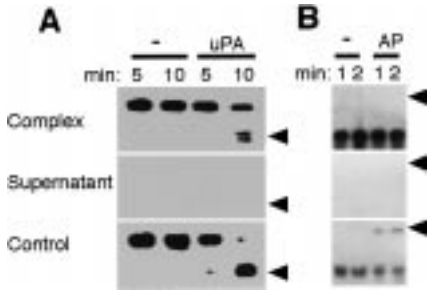

Figure 5. Functional cooperation between M6P/IGFII-R and Plg/Plm. (A) M6P/IGFII-R bound Plg is converted to Plm. Wells coated with M6P/IGFII-R-pTag were incubated with biotinylated Plg. After washing, the samples were treated with or without 5 μg/ml uPA for indicated times at 37 °C. Plg both complexed to M6P/IGFII-R-pTag and released in the supernatant was analyzed by SDS-PAGE under reducing conditions using a 8 % gel followed by blotting and staining with streptavidin-peroxidase. The control sample shows conversion of Plg (1 μg/ml) by uPA in solution. The positions of Plg proteolytically converted to Plm are indicated by the arrowheads. (B) M6P/IGFII-R-bound Plm is protected against inhibition by complex formation with AP. Wells coated with M6P/IGFII-R-pTag were incubated with biotinylated Plm. After washing, the samples were treated with or without 10 μg/ml AP for the indicated times at 37 °C. Plm both complexed to M6P/IGFII-R-pTag and released in the supernatant was analyzed by SDS-PAGE under nonreducing conditions using a 6 % gel. The control sample shows the inhibition of free Plm in solution. The positions of the Plm-AP complex are indicated by arrowheads.

β1 was also activated in the presence of AP which confirms the protective role of M6P/IGFII-R for bound Plm against its natural inhibitor and indicates that LTGF-β1 activation can occur at this complex at physiological conditions. Furthermore, this experiment suggests that Plm bound to M6P/IGFII-R and not free Plm is responsible for activation of LTGF-β1. This assumption was supported when we omitted uPA and Plg and treated the complex of LTGF-β1 and M6P/IGFII-R on immobilized suPA-R with Plm alone. Only when we used amounts of soluble Plm that exceeded at least ten times the amount of Plm generated from Plg at M6P/IGFII-R under assistance of uPA, was TGF-β1 released. This TGF-β1 activation, however, made only approximately 50 % the level seen in the presence of uPA and Plg, and was completely inhibitable by AP (Fig. 6A). Since we found that Plm can degrade M6P/IGFII-R (data not shown), we suggest that under this condition M6P/IGFII-R is more easily degraded, which may result in the loss of its protective role for LTGF-β1 and subsequent activation of LTGF-β1 by free Plm. This is in agreement with previous findings describing activation of LTGF-β1 by Plm in conditioned medium [18] and our own observation

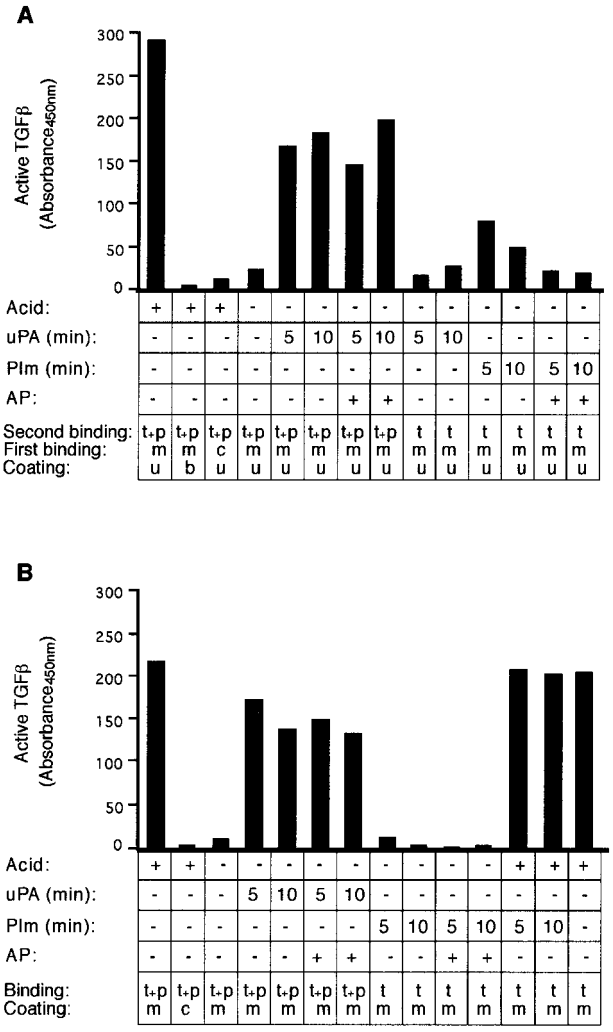

Figure 6. Release of active TGF-β1 by the uPA-R – M6P/IGFII-R complex. Protein complexes were built up on 96-well plates by coating the first molecule followed by blocking, washing and incubation with 10 μg/ml of the next molecule. Abbreviations are: b, BSA; c, CD147-pTag; u, suPA-R; m, M6P/IGFII-R-pTag; p, Plg; t, LTGF-β1 (500 ng/ml). After the last washing, samples were equilibrated with BB or AP (10 μg/ml) and incubated with 5 μg/ml uPA or 2 μg/ml Plm at 37 °C for the indicated times. As control, some of the samples were subjected to acid treatment. For detection of formation of biologically active LTGF-β1, an ELISA based on recombinant TGF-β1 type II receptor (R&D Systems) was used.

of LTGF-β1 activation by Plm in solution (data not shown).

Moreover, when we directly bound M6P/IGFII-R to the plastic and loaded it with LTGF-β1, treatment with 2 μg/ml Plm did not release measurable TGF-β1 (Fig. 6B); even 10 μg/ml Plm did not show any effect (data not

shown). However, TGF-β1 was released from this complex when Plm was generated from Plg under the assistance of uPA, and the release was not inhibited by AP. This shows that direct plastic coating of M6P/IGFII-R does not abrogate LTGF-β1 activation; though it seems to provide a higher resistance for M6P/IGFII-R towards digestion by free Plm. One could argue that incubation with Plm alone did not result in detection of active TGF-β1 because Plm in solution is activating and immediately digesting the released TGF-β1. However, we could exclude this possibility by demonstrating that LTGF-β1 is not degraded in Plm-incubated samples as virtually the same quantity of TGF-β1 was released from these as compared to untreated ones upon standard *in vitro* LTGF-β1 activation by acid treatment (Fig. 6B). In this experiment we could further show that LTGF-β1 activation on M6P/IGFII-R was independent of the presence of uPA-R, suggesting that the role of uPA-R in this complex is restricted to the cell surface for directing uPA to M6P/IGFII-R bound Plg (Fig. 6B). Together, these results indicate that M6P/IGFII-R protects LTGF-β1 against soluble Plm and, furthermore, that activation of LTGF-β1 requires controlled conversion of Plg to Plm by uPA on M6P/IGFII-R.

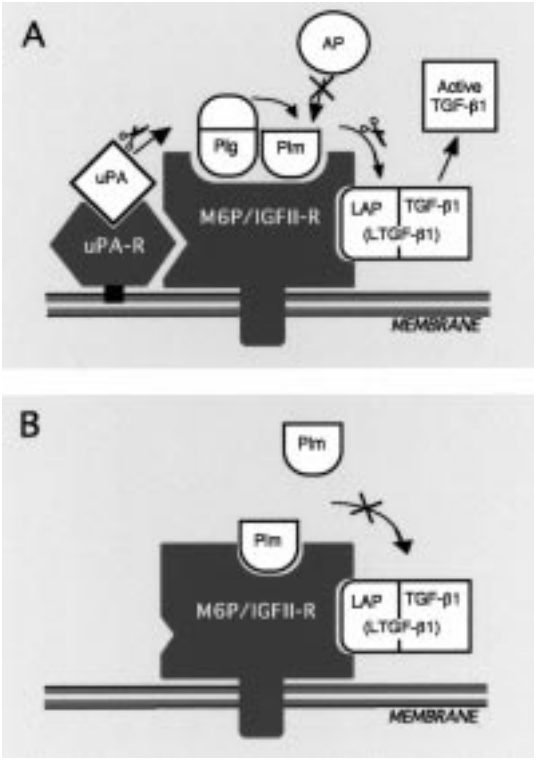

Figure 7. Schematic model of TGF-β1 activation by the uPA-R – M6P/IGFII-R complex. (A) uPA-R with its ligand uPA can directly interact with M6P/IGFII-R. M6P/IGFII-R binds via a distinct site Plg and enables uPA-mediated conversion of Plg to Plm. Plm generated at the complex is protected against inhibition by the fast-acting inhibitor AP and mediates release of active TGF-β1 from LTGF-β1. (B) Treatment of LTGF-β1 bound to M6P/IGFII-R by Plm does not result in the release of active TGF-β1. This suggests that the M6P/IGFII-R controls generation of Plm from Plg by uPA for conversion of LTGF-β1 and protects LTGF-β1 from uncontrolled activation by external Plm.

3 Concluding remarks

We have shown in this study that M6P/IGFII-R and uPA-R are associated at the surface of human monocytes, and that this complex can simultaneously bind LTGF-β1, Plg and uPA. Furthermore, we found that the controlled generation of Plm from Plg in this complex mediates release of active TGF-β1 from LTGF-β1 (for illustration see Fig. 7). Whether activity of Plm generated by this complex is restricted to LTGF-β1 or also targets other Plm substrates is not known yet. It is evident from the thrombospondin-1 null mice that several LTGF-β1 activation mechanisms seem to exist [21] which may partially substitute each other. This may be the reason why Plg-deficient mice exhibit only delayed wound healing [39] and wasting syndrome [40] as significant failure of TGF-β1-related functions. Our data are supported by cellular assays demonstrating that uPA- and uPA-R-dependent Plg activation has strong impact on LTGF-β1 activation with macrophages [27], smooth muscle cells [28] and endothelial cells [41]. Furthermore, agents known to increase the expression of uPA, uPA-R or M6P/IGFII-R such as IFN-γ and LPS [20, 27, 42] can also increase activation of LTGF-β1 in cultures of monocytes/macrophages [39, 43, 44]. It has been proposed earlier that TGF-β1 may negatively regulate its own activity [45, 46]; TGF-β1 induces expression of Plg activator inhibitor-1 [47], which inactivates uPA-R-bound uPA [30]. Thus, the M6P/IGFII-R – uPA-R complex may have not only the potential for activation but also for feedback inhibition of TGF-β1 activity. Specific targeting of the individual mechanisms of LTGF-β1 activation as well as inducing pathological situations in knockout/transgenic mice will bring us closer to understand the individual types of LTGF-β1 activation mechanisms for particular function and to develop strategies to specifically control LTGF-β1 activation for therapeutic purposes.

4 Materials and methods

4.1 Antibodies

The following mAb were prepared in our laboratories: H2 (IgG1) to uPA-R [32], MEM-48 (IgG1) to CD18, MEM-M6/2 (IgG1) to CD147 [35], and isotype control mAb AFP-01 (IgG1) to α-fetoprotein. The mAb to annexin II (IgG1) was from Transduction Laboratories (Lexington, KY). Rabbit polyclonal antiserum to M6P/IGFII-R was a kind gift of Dr. P. Lobel (Center for Advanced Biotechnology and Medicine, Piscataway, NJ). Rabbit polyclonal Ab to CD18 was provided by G. Boonen (La Jolla Cancer Research Foundation, La Jolla, CA). The anti-pTag mAb H902-producing hybridoma cell line is reagent no. 521 from the NIH AIDS Research and Reference Program.

4.2 Size fractionation of monocyte lysate

Human PBMC were isolated from blood of healthy individuals by density gradient centrifugation over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Monocytes were separated by 1 h adhesion to plastic and solubilized as described [48]. Briefly, cells (2×10^7/ml) were lysed for 30 min at 4 °C in lysis buffer (20 mM Tris-HCl, pH 8.2, 140 mM NaCl) that contained 1 % Brij-58 (Pierce, Rockford, IL) as detergent, and a mixture of protease inhibitors [5 mM iodoacetamide, aprotinin and leupeptin (both 10 μg/ml), 1 mM phenylmethylsulfonyl fluoride, 0.1 mM quercetin, 0.1 mM N-tosyl-L-phenylalanine chlormethyl ketone, 0.1 mM N_α-*p*-tosyl-L-lysine chlormethyl ketone, 0.1 M N-CBZ-L-Phenylalanine chlormethyl ketone, 1 μM pepstatin A] (all from Sigma Chemical Co., St. Louis, MO). The lysate was centrifuged for 5 min at 10 000 × g and the supernatant was size fractionated on a Sepharose-4B column as described [49]. Resulting fractions were separated by SDS-PAGE followed by a transfer to Immobilon P (Millipore Corporation, Bedford, MA) polyvinylidene difluoride membranes. Membranes were blocked by using 2 % nonfat milk and immunostained. For visualization of proteins, the chemiluminescence system from Boehringer Mannheim (Mannheim, Germany) was used.

4.3 Immunoprecipitation and reprecipitation

Monocytes prepared as described above were surface biotinylated by using 0.5 mg/ml sulfo-NHS-LC-biotin (Pierce) in PBS. After a 1-h incubation at 4 °C, the reaction was stopped with 20 mM Tris-HCl (pH 8.2). Cells (2×10^7/ml) were lysed for 30 min at 4 °C in lysis buffer (see above) containing 1 % NP40 (Pierce) as detergent. The lysate was centrifuged for 5 min at 10 000 × g. The supernatant was divided and the resulting samples were treated with increasing concentrations of deoxycholate to further disrupt molecular interactions. Then, samples were precleared with IgG1-Sepharose (irrelevant IgG1 mAb coupled to CNBr-Sepharose) and subjected to immunoprecipitation using mAb- or ligand-coated Sepharose beads. Immunoprecipitates were washed four times with lysis buffer containing 1 % NP40 and analyzed by immunoblotting. Membranes were blocked with 5 % BSA in Tris-buffered saline, and biotinylated proteins were visualized on the blot by a streptavidin-peroxidase conjugate (Amersham, Aylesbury, GB) and chemiluminescence.

For reprecipitation, immunoprecipitates were eluted with lysis buffer containing 1 % deoxycholate as a detergent. Afterwards, samples were diluted in lysis buffer containing 1 % NP40 to a final concentration of 0.05 % deoxycholic acid, and subjected to reprecipitation by using Ab or ligands coupled to Sepharose beads. Beads were washed twice with lysis buffer containing 1 % NP40. Analysis was performed by using SDS-PAGE followed by blotting and detection of proteins by chemiluminescence.

4.4 Construction of p-tagged receptor proteins

The pTag DNA [50] was fused to the 3'-end of the cDNA encoding M6P/IGFII-R (American Type Culture Collection, Rockville, MD), or CD147 [35] used as control. This resulted in the C-terminal addition of the amino acid sequence VDA-AMAHHHHHHGSRIQRGPGRAFVTIGKLEAAA to the natural protein sequence. RIQRGPGRAFVTIGK is the universal epitope for mAb H902. The DNA constructs were ligated into eucaryotic expression vectors, and the resulting plasmids were used to transiently transfect COS cells. The expressed proteins were termed M6P/IGFII-R-pTag and CD147-pTag, respectively.

4.5 Purification of receptor proteins

M6P/IGFII-R-pTag and CD147-pTag were purified to homogeneity by affinity chromatography using anti-pTag mAb H902 coupled to Sepharose. The natural form of uPA-R was purified from lysates of the monocytic cell line U937 by affinity chromatography using mAb H2. suPA-R (amino acids 1-277) was constructed and purified from supernatants of stably transfected CHO cells as described [51].

4.6 *In vitro* binding assay

Various molecules (see Figs. 3 and 4) were coated at a concentration of 10 μg/ml on 96-well Falcon plates in binding buffer (BB) (20 mM Tris-HCl, pH 7.5, 140 mM NaCl). Wells were blocked with 1 % BSA, washed and preincubated either with a second step molecule, or incubated for 1 h directly with binding solution (BS; BB supplemented with 0.2 % NP40 and 2 μg/ml aprotinin) that contained either 5 μg/ml of M6P/IGFII-R-pTag or CD147-pTag in the absence or presence of the following molecules: M1P and M6P (4 mM), LTGF-β1 (10 μg/ml) (R&D Systems, Minneapolis, MN), IGFII (5 μg/ml) (Calbiochem-Novabiochem, La Jolla,

CA), suPA-R and uPA-R (10 μg/ml), Plg (10 μg/ml) (American Diagnostica, Greenwich, CT), TA (1 mM) (Sigma Chem. Co.). Wells were washed four times with BB, and binding of M6P/IGFII-R-pTag or CD147-pTag was analyzed by SDS-PAGE followed by immunoblotting using anti-pTag mAb H902.

4.7 Plg activation and Plm protection assay

Plg (1 mg/ml) (Technoclone, Vienna, Austria) was biotinylated with 30 μg/ml biotin-X-NHS (Calbiochem) in 200 mM Na_2CO_3 (pH 9.0) for 1 h at room temperature. Biotinylation was quenched with 50 mM Tris-HCl (pH 9.0), and free biotin was removed by exhaustive dialysis against BB.

For the Plg activation assay, 96-well Falcon plates were coated with 10 μg/ml M6P/IGFII-R-pTag and blocked with 1 % BSA. Afterwards, wells were incubated on ice for 1 h with biotinylated Plg (10 μg/ml) in BB and washed four times. Samples were then incubated with or without 5 μg/ml uPA for various times at 37 °C. For the Plm protection assay, biotinylated Plg was converted to Plm by incubation with uPA (100:1 molar ratio) for 1 h at 37 °C. Afterwards, M6P/IGFII-R-pTag-coated wells were incubated for 1 h on ice with 10 μg/ml biotinylated Plm in BB containing 0.1 % BSA, washed four times, and incubated in the absence or presence of 10 μg/ml AP (Technoclone) at 37 °C for various times. Both the complex and the supernatant of each assay were analyzed by SDS-PAGE followed by blotting and staining with streptavidin-peroxidase.

4.8 TGF-β1 activation assay

Protein complexes were built up on 96-well plates by coating the first molecule (10 μg/ml) in BB for 1 h on ice followed by blocking with 1 % BSA, washing and incubation with 10 μg/ml of the next molecule. After the last washing, samples were equilibrated with BB with or without AP (10 μg/ml) and treated either by uPA (5 μg/ml) or Plm (2 μg/ml) for various times at 37 °C. Proteolytic treatment was stopped using 0.5 % BSA and 10 μg/ml aprotinin. Acid treatment was performed as described [18]. Active TGF-β1 formation was detected using an ELISA based on recombinant TGF-β1 type II receptor (R&D Systems).

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