Plasminogen-dependent internalization of soluble melanotransferrin involves the low-density lipoprotein receptor-related protein and annexin II

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Abstract

We investigated the effect of plasminogen (Plg) on the internalization of recombinant soluble melanotransferrin (sMTf) using U87 human glioblastoma cells and murine embryonic fibroblasts (MEF) deficient in the low-density lipoprotein receptor-related protein (LRP). Using biospecific interaction analysis, both Glu- and Lys-Plg were shown to interact with immobilized sMTf. The binding of sMTf at the cell surface increased in the presence of both forms of Plg in control and in LRP-deficient MEF cells, whereas the uptake was strongly stimulated only by Lys-Plg in control MEF and U87 cells. In addition, in the presence of Lys-Plg, the internalization of sMTf was a saturable process, sensitive to temperature and dependent on the integrity of lysine residues. The addition of the receptor-associated protein, lactoferrin and aprotinin, as well as a monoclonal antibody (mAb) directed against LRP, inhibited the Lys-Plg-dependent uptake of sMTf. These results suggest an important role for LRP in this process. In addition, using binding and uptake assays in the presence of anti-annexin II mAb, we showed that annexin II might be responsible for the initial binding of sMTf in the presence of Plg. Our results suggest a Plg-mediated internalization mechanism for the clearance of sMTf via annexin II and LRP.

Keywords: annexin II; endocytosis; low-density lipoprotein receptor-related protein; plasminogen; soluble melanotransferrin.

Introduction

For several decades, it has been assumed that plasmin generation is fundamental both to fibrinolysis and to many other biological processes involving the remodeling of basement membranes and of the extracellular matrix (Castellino and Ploplis, 2005). Plasminogen activators (PAs), both urokinase-type (u-PA) and tissue-type (t-PA), catalyze conversion of the inactive zymogen plasminogen (Plg) to the active serine proteinase plasmin, which facilitates cancer cell invasion into surrounding tissues

(Dano et al., 2005). Various cell receptors play significant roles in the regulation of Plg activation at the cell surface. These receptors can be classified on a functional basis into two groups: activator receptors and clearance receptors (Hajjar, 1995). It is likely that integrated actions of both classes of receptors are essential for the homeostatic regulation of plasmin generation at the cell surface (Hajjar, 1995).

The activator receptors serve to localize Plg on the cell surface and, in some cases, to potentiate Plg activation into plasmin by PAs (Hajjar, 1995). These receptors are expressed by several cell types, including endothelial and tumor cells (Hajjar, 1995). Among them, the annexin II receptor specifically binds to circulating N-terminal glutamic acid Plg (Glu-Plg) and mediates the conversion of Glu-Plg to its truncated, non-circulating form, N-terminal lysine Plg (Lys-Plg), through proteolytic release by a plasmin-dependent mechanism of a 76-aa preactivation peptide (Hajjar et al., 1994; Hajjar and Krishnan, 1999; Miles et al., 2003). This process enhances the efficiency of Plg activation by t-PA (Hajjar and Krishnan, 1999; Miles et al., 2003). Another family, the clearance receptors, has been shown to promote physiological clearance of plasmin, Plg, PAs and PAs complexed with their inhibitors, i.e., PAI (Hajjar, 1995). They are found primarily in hepatocytes, fibroblasts, tissue macrophages and tumor cells such as glioblastoma (Zheng et al., 1994; Maletinska et al., 2000). For example, the low-density lipoprotein receptor-related protein (LRP) serves as a crucial regulator of extracellular proteolytic activity by stimulating the clearance from blood of Plg and of PAs such as the u-PA:PAI-1 and the t-PA:PAI-1 complexes (Nykjaer et al., 1992; Herz and Strickland, 2001; Hussain, 2001).

Melanotransferrin (MTf) is slightly expressed in normal tissues, but is present at much larger amounts in neoplastic cells and fetal tissues (Brown et al., 1981). Two forms of MTf have been described to date: a soluble MTf (sMTf) and a glycosyl phosphatidylinositol (GPI)-anchor membrane-bound MTf (mMTf) (Food et al., 2002). MTf possesses a high level of sequence similarity (37%–39%) with human serum transferrin (Tf), human lactoferrin (Lf) and chicken Tf (Brown et al., 1982). A study from our laboratory demonstrated that sMTf transcytosis across an *in vitro* model of the blood-brain barrier (BBB) could involve LRP (Demeule et al., 2002).

Recently, we demonstrated that sMTf binds to Plg (Demeule et al., 2003) and inhibits *in vitro* cell invasion (Michaud-Levesque et al., 2005a), *in vitro* endothelial cell (EC) migration and tubulogenesis (Demeule et al., 2003; Michaud-Levesque et al., 2005b), as well as *in vivo* angiogenesis (Michaud-Levesque et al., 2007). We also observed that sMTf decreased the capacity of the EC surface to activate Plg into plasmin (Demeule et al.,

2003). In the present work, we investigated the internal-ization of sMTf with regard to its interaction with Plg. Our results indicate that annexin II and LRP cooperate to internalize sMTf in the presence of Plg.

Results

Glu- and Lys-Plg interact with sMTf

We first evaluated the interaction between human recom-binant sMTf and both forms of Plg, i.e., Glu- and Lys-type Plg, using real-time biological interaction analysis (Figure 1A). For this analytical approach, sMTf was first immobilized on the surface of a sensor chip by standard NHS/EDC coupling procedures. When Glu- and Lys-Plg (0.05 μg/μl) were subsequently injected over immobi-lized sMTf, protein interaction occurred between both forms of Plg and sMTf (Figure 1A; left panel). However, a stronger interaction could be observed when Glu-Plg was injected over sMTf compared to Lys-Plg. In fact, the net surface plasmon resonance (SPR) signals generated by the interaction of immobilized sMTf with Glu- and Lys-Plg were approximately 450 and 150 relative units (RU), respectively (Figure 1A; right panel). The kinetic data obtained from the binding of both Plgs to immobilized sMTf biosensor surfaces were evaluated using 1:1 Lang-muir binding model. The dissociation constants (K_D) obtained are presented in Table 1. These results suggest that Lys-Plg has lower affinity for immobilized sMTf com-

Table 1   Dissociation constants (K_D) for the inter-action between Lys- and Glu-Plg and immobilized sMTf.

Immobilized protein	Ligand	K_D ($\times 10^{-6}$ M)
sMTf	Glu-Plg	0.22
	Lys-Plg	1.2

The dissociation constant (K_D) was calculated from the apparent association (K_a) and dissocia-tion (K_d) constants.

pared to Glu-Plg. Furthermore, we previously showed that plasmin and angiostatine (K1-4), two Plg fragments, do not interact with immobilized sMTf (Demeule et al., 2003). Taken together, these results demonstrate that sMTf interaction is specific to the native Plg molecule.

Both Glu- and Lys-Plg increase sMTf binding, whereas only Lys-Plg stimulates sMTf uptake on U87 cell monolayers

Based on the observation that both types of Plg could interact with sMTf, we then evaluated the impact of unla-beled sMTf, as well as Glu- and Lys-Plg (150 nM), on the binding (4°C) and uptake (37°C) of human recombinant [125I]-sMTf by U87 cell monolayers (Figure 1B). Although both Glu- and Lys-Plg similarly increased [125I]-sMTf binding to the U87 cell surface in the binding assay, only Lys-Plg strongly stimulated the uptake of [125I]-sMTf. The similar levels for both binding and uptake of [125I]-sMTf in the presence or absence of Glu-Plg indicate that sMTf uptake is unaffected by Glu-Plg. In addition, binding of [125I]-sMTf in the presence of Lys-Plg was clearly lower than its uptake (Figure 1B). However, in the absence of Plg (basal control condition), sMTf uptake was much low-er and was unsaturable, since the internalization of [125I]-sMTf could not be inhibited by a 125-fold molar excess of unlabeled sMTf (2 μM). This result suggests that sMTf alone is not internalized by a specific mechanism in U87 cells. In addition, we evaluated uptake of [125I]-sMTf in the presence of various concentrations of both Glu- and Lys-Plg (Figure 1C). Uptake of [125I]-sMTf was strongly stimulated in a dose-dependent manner in the presence of Lys-Plg, in contrast to Glu-Plg, which had a minor effect on this process. The apparent half-saturation con-stant [K_m(app)] and maximum velocity [V_{max}(app)] for Glu- and Lys-Plg-dependent sMTf uptake were estimated from these curves (Table 2). The results indicate that Glu- and Lys-Plg-dependent sMTf uptake demonstrate similar affinity, whereas Lys-Plg increased the maximum sMTf uptake velocity by at least three-fold compared to Glu-Plg. Taken together, these results suggest that sMTf
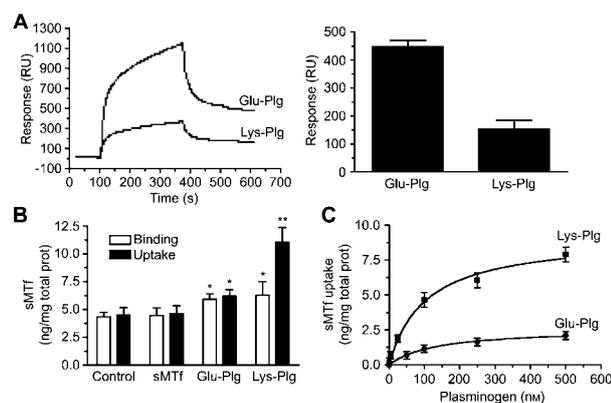

Figure 1   Glu- and Lys-plasminogen interact with sMTf and modulate its binding and uptake.
(A) Biospecific molecular interaction analysis of Plg with sMTf using BIAcore technology. Glu-Plg and Lys-Plg (0.05 μg/ml), diluted in Ringer/HEPES, were injected at a flow rate of 5 μl/min onto sMTf immobilized on a sensor chip. The SPR response for these proteins was plotted in RU as a function of time (left panel) and the net SPR signal was quantified and expressed in RU for both forms of Plg (right panel). Results obtained from a representative experiment are shown (n=3). (B) Binding and uptake of [125I]-sMTf (16 nM) were measured using U87 cell monolayers in the presence or absence of unlabeled sMTf (2 μM), Glu- and Lys-Plg (150 nM), as described in mate-rials and methods. *$p<0.05$, **$p<0.01$ significant difference com-pared to the corresponding control (n=3). (C) Uptake of [125I]-sMTf (16 nM) was measured using U87 cell monolayers in the presence of various concentrations of Glu- and Lys-Plg, as described under materials and methods. Results are expressed as specific sMTf uptake by subtracting the background sMTf uptake occurring without Plg (n=6).

Table 2   Plg-dependent apparent half-saturation constant [K_m(app)] and maximum velocity [V_{max}(app)] for sMTf uptake on U87 cell monolayers.

Plg type	K_m(app) ($\times 10^{-9}$ M)	V_{max}(app) (ng/mg total protein×h)
Glu-Plg	133	1.3
Lys-Plg	103	4.6

internalization is due to a receptor-mediated active endocytosis involving Lys-Plg.

εACA, RAP and LRP ligands inhibit [^{125}I]-sMTf uptake in the presence of Lys-Plg

To further characterize the internalization of human recombinant sMTf, Lys-Plg-dependent uptake of [^{125}I]-sMTf was measured using U87 cell monolayers in the presence of unlabeled sMTf, εACA (a specific lysine-analog Plg-binding inhibitor) and α2-AP (a plasmin inhibitor) (Figure 2A). A 125-fold molar excess of unlabeled sMTf (2 μM), as well as 100 mM εACA, inhibited Lys-Plg-induced uptake of [^{125}I]-sMTf by approximately 90%. In addition, Lys-Plg-dependent uptake of [^{125}I]-sMTf was unaffected by the α2-AP inhibitor (300 nM) under these conditions. Moreover, α2-AP interacts with the plasmin(ogen) lysine-binding sites located on Kringle 1 and 4 (Frank et al., 2003; Wang et al., 2003; Coughlin, 2005) and also prevents plasmin formation and inhibits plasmin activity (Levi et al., 1993; Syrovets and Simmet, 2004; Rolland et al., 2006). Thus, these results indicate that for the α2-AP binding domain of plasmin(ogen), i.e., Kringle 1 and 4, neither the generation of plasmin from Plg nor

Figure 2 εACA, RAP and LRP ligands inhibit the uptake of sMTf in the presence of Lys-Plg.
(A) Uptake of [^{125}I]-sMTf (16 nM) was measured using U87 cell monolayers in the presence or absence of Lys-Plg (150 nM), unlabeled sMTf (2 μM), εACA (100 mM) and α2-AP (300 nM) as described in materials and methods (n=6). (B) Binding and uptake of [^{125}I]-sMTf (16 nM) were measured using U87 cell monolayers in the presence or absence of Lys-Plg (150 nM), as well as RAP (25 μg/ml) (n=3). (C) Uptake of [^{125}I]-sMTf (16 nM) was measured using U87 cell monolayers in the presence or absence of Lys-Plg (150 nM), as well as unlabeled human Lf, bovine aprotinin, human holo-Tf and BSA (2 μM) (n=6). *$p<0.05$, **$p<0.01$, significant difference compared to the corresponding control.

the plasmin activity itself are involved in this mechanism. Collectively, these results indicate that, in the presence of Lys-Plg, uptake of sMTf is a saturable process that is dependent on lysine residues.

It has been reported that sMTf transcytosis across BBB may involve LRP (Demeule et al., 2002) and that LRP could mediate Plg clearance in hepatocytes (Strickland et al., 1990; Hajjar, 1995). Thus, to investigate the possible involvement of LRP in the internalization of sMTf in the presence of Lys-Plg, binding and uptake experiments were performed on U87 cell monolayers in the presence of RAP, a protein chaperone that regulates LRP and other LDL receptor family members (Bu, 2001) (Figure 2B). Recombinant RAP (25 μg/ml) did not affect the binding and uptake of [^{125}I]-sMTf alone, whereas RAP completely abolished the increased uptake of [^{125}I]-sMTf induced by Lys-Plg without affecting the binding to U87 cell monolayers, suggesting that sMTf internalization might involve Lys-Plg and a member of the LDL-receptor family, potentially LRP. However, the initial binding of sMTf in the presence of Lys-Plg was unaffected by RAP.

To characterize the involvement of LRP in the Lys-Plg-dependent uptake of sMTf, we performed [^{125}I]-sMTf uptake experiments in the presence of Lys-Plg on U87 cell monolayers with or without a 125-fold molar excess of human Lf (a ligand of LRP; Hussain, 2001), bovine aprotinin (a polybasic drug that antagonizes ligand binding to LRP; Hussain, 2001), human holo-Tf and BSA (2 μM) (Figure 2C). In these uptake assays, both Lf and bovine aprotinin strongly inhibited [^{125}I]-sMTf uptake in the presence of Lys-Plg, suggesting that LRP might be involved in the Lys-Plg-dependent internalization of sMTf in U87 cells. This is further supported by the observation that BSA, which binds to LRP2 (megalin), another LDL-receptor member (Hussain, 2001), had no impact on Lys-Plg-dependent uptake of sMTf in U87 cells. Moreover, the Lys-Plg-dependent uptake of [^{125}I]-sMTf was unaffected by holo-Tf in U87 cell monolayers, indicating that the transferrin receptor is not required for the internalization process.
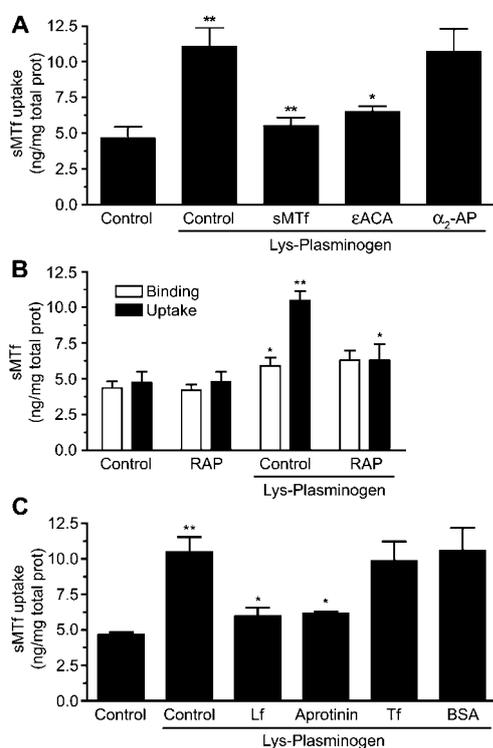
LRP is involved during [^{125}I]-sMTf uptake in the presence of Lys-Plg

To further determine whether sMTf internalization in the presence of Lys-Plg is mediated via LRP, we used MEF cells that were either positive or deficient for LRP (LRP$^{+/+}$ and LRP$^{-/-}$), as well as U87 cells. LRP expression in these cell lines was characterized by Western blotting (Figure 3A). Under non-reducing and denaturing conditions, LRP was immunodetected at high levels in the U87 and LRP$^{+/+}$ cell lysates, whereas it was not detected in LRP$^{-/-}$ cells (Figure 3A), indicating that these cells represent a suitable cellular model for studying the impact of LRP expression on the Plg-dependent internalization of sMTf. We first used these cells to confirm whether LRP is involved in sMTf internalization in the presence of Lys-Plg. As shown in Figure 3B,C, in the absence of Plg, the binding and uptake of [^{125}I]-sMTf was not affected by a 125-fold molar excess of unlabeled sMTf (2 μM). This suggests that sMTf internalization without Plg is not saturable and is not mediated by a specific mechanism in both LRP$^{+/+}$ and LRP$^{-/-}$ MEF cells. However, [^{125}I]-sMTf
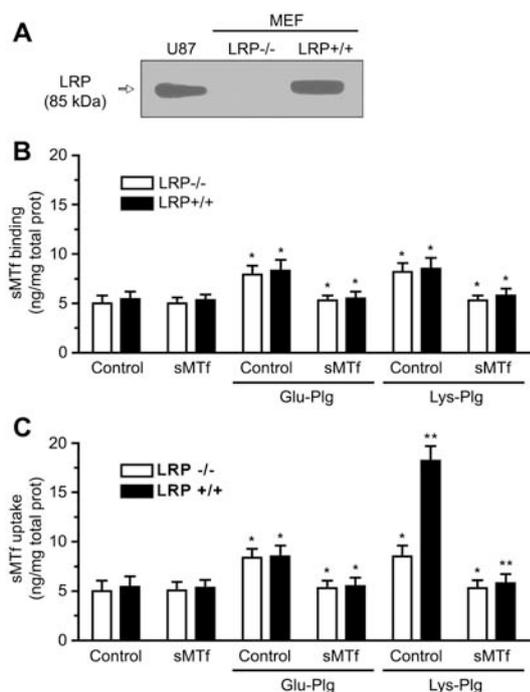
Figure 3 LRP is involved in sMTf uptake in the presence of Lys-Plg.
(A) LRP was immunodetected in cell lysates (10 µg protein) from U87, LRP$^{-/-}$ and LRP$^{+/+}$ cells using a mAb directed against LRP. (B) Binding and (C) uptake of [^{125}I]-sMTf (16 nM) were performed using LRP$^{-/-}$ and LRP$^{+/+}$ cell monolayers in the presence or absence of Glu- and Lys-Plg (150 nM), as well as unlabeled sMTf (2 µM) (n=3). *$p<0.05$, **$p<0.01$, significant difference compared to the corresponding control.

binding increased to a similar extent in the presence of either Glu- or Lys-Plg in both LRP$^{-/-}$ and LRP$^{+/+}$ cell monolayers. This binding was saturable, since a 125-fold molar excess of unlabelled sMTf (2 µM) reduced the binding of [^{125}I]-sMTf in the presence of either Glu- or Lys-Plg (Figure 3B). We next measured the uptake of [^{125}I]-sMTf in the presence of Plg by LRP$^{-/-}$ and LRP$^{+/+}$ cell monolayers (Figure 3C). In these assays, Lys-Plg strongly stimulated the uptake of [^{125}I]-sMTf in LRP$^{+/+}$ compared to LRP$^{-/-}$ cells (Figure 3C). In contrast, Glu-Plg-dependent binding and uptake of [^{125}I]-sMTf was equivalent in LRP$^{-/-}$ and LRP$^{+/+}$ cells. Taken together, these experiments demonstrate that, after initial binding to another cell surface receptor, LRP internalizes sMTf only in the presence of Lys-Plg.

The impact of a mAb directed against LRP on Lys-Plg-induced [^{125}I]-sMTf uptake was examined to determine whether LRP could be involved (Figure 4A,B). In U87 cells, Lys-Plg-dependent uptake of [^{125}I]-sMTf was reduced by approximately 75% by the anti-LRP mAb (5 µg/assay), while non-specific IgG had no significant effect (Figure 4A). These results strongly suggest that LRP is involved in the Lys-Plg-dependent internalization of sMTf in U87 cells. The effect of the mAb directed against LRP on the Lys-Plg-dependent uptake of [^{125}I]-sMTf was also measured in LRP$^{-/-}$ and LRP$^{+/+}$ cell (Figure 4B). Addition of the anti-LRP mAb reduced the Lys-Plg-induced increase in uptake of [^{125}I]-sMTf by LRP$^{+/+}$ cell monolayers by approximately 75%, without affecting the uptake observed in LRP$^{-/-}$ cells (Figure 4B). Our data for
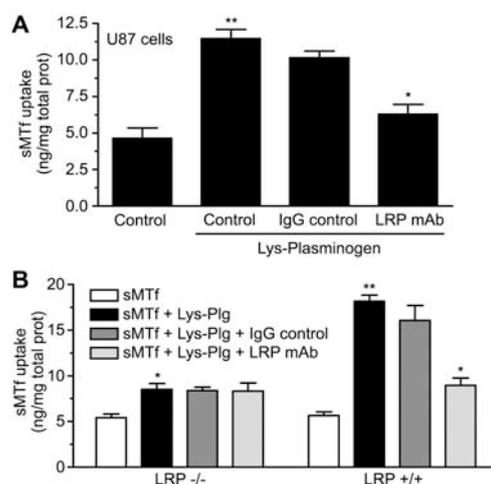

Figure 4 LRP is involved in the internalization of sMTf in the presence of Lys-Plg.
(A) Uptake of [^{125}I]-sMTf (16 nM) was performed using U87 cell monolayers in the presence or absence of Lys-Plg (150 nM), as well as a mAb directed against LRP (5 µg/assay) or non-specific control IgG (5 µg/assay) (n=6). (B) Uptake of [^{125}I]-sMTf (16 nM) was measured on LRP$^{-/-}$ and LRP$^{+/+}$ cell monolayers in the presence or absence of Lys-Plg (150 nM), a mAb directed against LRP (5 µg/assay) or non-specific control IgG (5 µg/assay) as described under materials and methods (n=3). *$p<0.05$, **$p<0.01$, significant difference compared to the corresponding control.

the anti-LRP mAb and MEF-deficient cells strongly support the fact that LRP is involved in sMTf internalization in the presence of Lys-Plg. However, in LRP-deficient cells, we found that: (1) sMTf binding was increased by Glu- and Lys-Plg to a similar extent; (2) binding (4°C) and uptake (37°C) of sMTf in the presence of Lys-Plg were equal, indicating that the uptake observed rather corresponds to cell surface binding; and (3) sMTf uptake in the presence of Lys-Plg was observed in spite of the presence of the anti-LRP mAb. In addition, Lys-Plg-dependent sMTf binding was not affected by RAP in U87 cells. Overall, these results confirm that initial binding of sMTf in the presence of Plg involved a cell-surface receptor other than LRP.

Annexin II is involved in the initial binding of sMTf in the presence of Plg

Annexin II is one of the best-characterized receptors involved in Plg binding and activation at the cell surface (Hajjar, 1995). In fact, upon binding to annexin II, Glu-Plg is converted to Lys-Plg by a plasmin-dependent mechanism that promotes efficient generation of plasmin by PAs (Hajjar, 1995; Hajjar and Krishnan, 1999; Miles et al., 2003). To determine whether this receptor is involved in initial binding of sMTf in the presence of Plg, we first analyzed annexin II expression levels in U87 and MEF (LRP$^{-/-}$ and LRP$^{+/+}$) cells by Western blotting (Figure 5A). Under non-reducing and denaturing conditions, annexin II migrated as a 36-kDa protein and was immunodetected in U87 cells, as well as in both control and LRP-deficient MEF cell lysates (Figure 5A). Using MEF (LRP$^{-/-}$ and LRP$^{+/+}$) cell monolayers, we next measured the impact of a mAb directed against annexin II on the binding of [^{125}I]-sMTf in the presence of Glu-Plg (Figure 5B) and Lys-
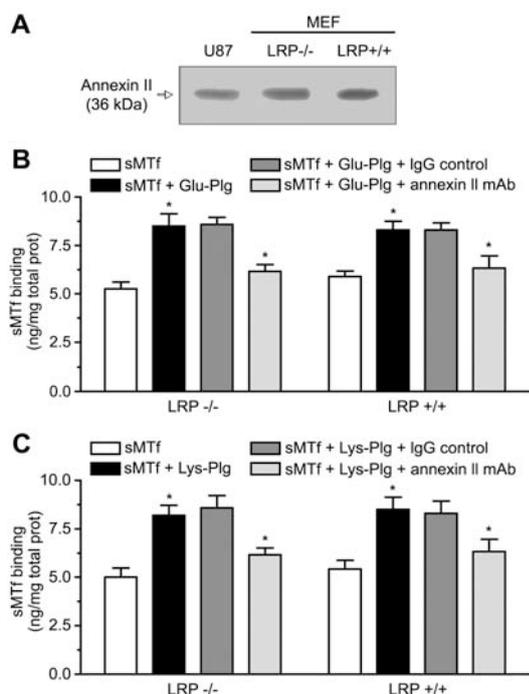
Figure 5   Annexin II is involved in the initial binding of sMTf in the presence of Plg.
(A) Annexin II was immunodetected in cell lysates (10 μg protein) from U87, LRP^{-/-} and LRP^{+/+} cells using a mAb specific for the protein. The binding of [^{125}I]-sMTf (16 nM) to LRP^{-/-} and LRP^{+/+} cell monolayers was measured in the presence of (B) Glu-Plg (150 nM) or (C) Lys-Plg (150 nM), as well as a mAb directed against annexin II (5 μg/assay) or a non-specific control IgG (5 μg/assay) (n=3). *$p<0.05$, significant difference compared to the corresponding control.

Figure 6   Initial binding to annexin II of sMTf in the presence of Plg is required for its subsequent internalization by LRP.
Uptake of [^{125}I]-sMTf (16 nM) was measured using (A) LRP^{+/+} or (B) U87 monolayers in the presence or absence of Lys-Plg (150 nM), a mAb directed against annexin II (5 μg/assay) or non-specific control IgG (5 μg/assay) (n=3). **$p<0.01$, significant difference compared to the corresponding control.

Plg (Figure 5C). Compared to non-specific control IgG, addition of an anti-annexin II mAb during the assay completely abolished Glu-Plg-dependent (Figure 5B) and Lys-Plg-dependent binding of [^{125}I]-sMTf (Figure 5C) on both LRP^{-/-} and LRP^{+/+} cell monolayers. These results strongly suggest the involvement of annexin II during initial binding of sMTf in the presence of Plg.

Initial binding to annexin II of sMTf in the presence of Plg is required for its subsequent LRP internalization

Based on the observation that annexin II seems to play a significant role during Plg-dependent initial binding of sMTf to the cell surface, we next determined if this initial binding is required for subsequent sMTf internalization by LRP by monitoring the effect of the annexin II mAb on [^{125}I]-sMTf uptake in the presence of Lys-Plg by LRP^{+/+} (Figure 6A) and U87 cell monolayers (Figure 6B). Addition of the annexin II mAb during the assays completely abolished [^{125}I]-sMTf uptake in the presence of Lys-Plg on both LRP^{+/+} (Figure 6A) and U87 (Figure 6B) cell monolayers. These results thus suggest that sMTf internalization by LRP in the presence of Plg is dependent on initial binding to annexin II.

Discussion

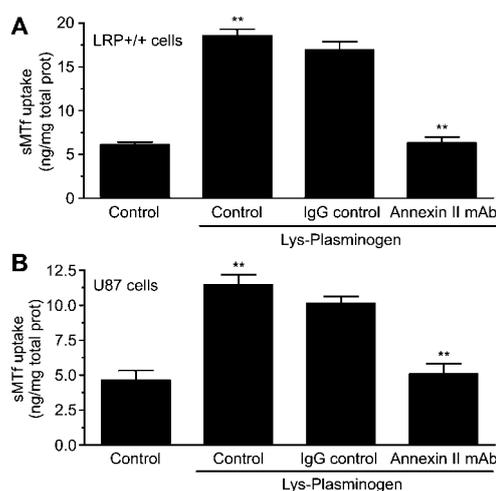Many known cell receptors have the ability to localize and/or potentiate Plg activation at the cell surface or to mediate the physiological clearance of plasmin(ogen) and PAs (Hajjar, 1995). These regulators of plasmin generation are fundamental to many physiological and pathological processes, such as embryonic development, wound healing, reproductive cycles, diabetic retinopathy, chronic inflammation, angiogenesis, tumor growth and metastasis (Castellino and Ploplis, 2005). Previous studies in our laboratory have shown that regulation of the PAs-dependent plasminolytic system by sMTf inhibits cell migration and invasion (Demeule et al., 2003; Michaud-Levesque et al., 2005a), as well as EC tubulogenesis in vitro (Michaud-Levesque et al., 2005b) and angiogenesis in vivo (Michaud-Levesque et al., 2007). Here, we have demonstrated for the first time that Plg-induced sMTf internalization by LRP requires initial binding to annexin II.

Based on our previous findings that sMTf binds to Plg (Demeule et al., 2003; Michaud-Levesque et al., 2005a), we provide here the first evidence that binding of sMTf to the cell surface is increased in the presence of both Glu-Plg and Lys-Plg, but that only Lys-Plg has the ability to stimulate the uptake of sMTf. In fact, Lys-Plg strongly increased the maximum velocity [V_{max}(app)] of sMTf uptake compared to Glu-Plg. However, in the absence of Plg, sMTf internalization was much lower and was not saturable, indicating that sMTf is not internalized by a specific mechanism in U87 and MEF cells. The present result is in agreement with a recent study showing that sMTf is internalized by a non-specific process involving adsorptive pinocytosis followed by lysosomal degradation (Food et al., 2002). Our results raise the interesting possibility that, in the presence of Plg, sMTf could bind to the cell surface and undergo receptor-mediated endocytosis into cells.

The competition of [^{125}I]-sMTf Lys-Plg-dependent uptake with unlabeled sMTf in U87 and MEF cells confirmed the presence of a receptor for the sMTf:Lys-Plg complex. Because sMTf shares many properties with human Tf and because the Tf receptor has been detected

in the same tissues that express MTf, it has been postulated that sMTf is an alternate ligand for the Tf receptor (Rothenberger et al., 1996). Since human holo-Tf had no effect on the Lys-Plg-dependent uptake of [^{125}I]-sMTf, our results strongly indicate that the Tf receptor is not involved in this process. In addition, BSA, an LRP2 (megalin) ligand, had no significant inhibitory effect in the uptake assays, indicating that LRP2 is not implicated in Lys-Plg-dependent internalization of sMTf. Using RAP, a protein chaperone that is known to block the binding and internalization of LRP and other LDL receptor ligands (Bu, 2001), we showed that a member of the LDL-receptor family is involved in the Lys-Plg-dependent uptake of sMTf. We observed that Lf and aprotinin competed efficiently with [^{125}I]-sMTf Lys-Plg-dependent uptake, suggesting that these two proteins share the same receptor used by sMTf in the presence of Plg. LRP, a member of the large LDL-receptor family (Hussain, 2001), is the receptor for Lf and aprotinin in various cell types (Hussain, 2001). The involvement of this protein in the internalization of sMTf is strongly suggested by the inhibitory effect of a mAb against LRP and the absence of uptake observed in LRP-deficient MEF cells. Although LRP could internalize sMTf in the presence of Lys-Plg, this receptor was not involved in initial binding of the protein to the cell surface, since RAP did not block this process. Rather, we observed that annexin II, a well-characterized cell surface receptor for Plg (Hajjar, 1995; Hajjar and Krishnan, 1999), is most likely involved in binding of sMTf to the cell surface in the presence of both forms of Plg, independent of LRP. Since the annexin II mAb also inhibited the increased uptake of sMTf observed with Lys-Plg compared to Glu-Plg, this further suggests that annexin II initially binds sMTf in the presence of Plg before its subsequent internalization by LRP. Collectively, our data thus indicate that annexin II and LRP cooperate in order to internalize sMTf in the presence of Plg.

A cooperative mechanism has been proposed for endocytosis of the u-PA:PAI-1 complex by LRP (Herz et al., 1992; Nykjaer et al., 1992). It has been reported that u-PA complexed with PAI-1 was endocytosed by LRP on human monocytes *in vitro* and during mouse embryo implantation *in vivo*. However, initial binding of the u-PA:PAI-1 complex to the cells appears to require u-PAR, since RAP did not block the initial binding. To explain such results, it was proposed that the ligand (u-PA:PAI-1) was transferred from the u-PA receptor to LRP prior to internalization. Another study by Schwartz and co-workers showed that two receptor mechanisms are involved in the clearance of tissue factor pathway inhibitor (TFPI) by LRP *in vivo* (Narita et al., 1995; Warshawsky et al., 1996). In this study, LRP did not appear to be the major cell-surface receptor, since TFPI binding at 4°C was not inhibited by RAP. This result is consistent with the presence of an additional receptor. Using protamin inhibitor, the group identified heparan sulfate proteoglycans as the second receptor involved in TFPI binding. These results clearly demonstrate that two receptor systems are involved in the clearance of TFPI. Here, we postulate a similar cooperative receptor system involving LRP and annexin II for the clearance of sMTf in the presence of Plg. We propose that sMTf first interacts with

circulating Glu-Plg, forming a complex that could then be recognized by annexin II at the cell surface, via the Plg molecule. Since annexin II lacks a transmembrane domain or a cytoplasmic tail, endocytosis is not an intrinsic characteristic of this receptor (Hajjar, 1995). Thus, annexin II could bind the sMTf:Plg complex and facilitate the conversion of Glu-Plg to Lys-Plg. Our results clearly demonstrate that: (1) the affinity of sMTf for Glu-Plg is higher than that measured for Lys-Plg; (2) the initial binding of sMTf:Plg complexes involves annexin II; and (3) the uptake of sMTf in higher in the presence of Lys-Plg. Taken together, these results suggest that after initial binding of the sMTf:Glu-Plg complex to annexin II, the conversion of Glu-Plg to Lys-Plg favors complex recruitment and internalization by LRP.

This cooperative mechanism also involves lysine residues, as evidenced by the loss of uptake induced by εACA. Lysine derivatives with free α-carboxyl groups, such as εACA, or peptides with carboxy-terminal lysine residues, are effective inhibitors of Plg interaction (Sun et al., 2002). Since sMTf does not possess a carboxy-terminal lysine (Yang et al., 2004) and the interaction of Plg with annexin II involves a carboxy-terminal lysine (Hajjar, 1995), we hypothesize that the action of the lysine analog εACA occurs via its binding to plasminogen, which possesses high-affinity lysine binding sites (Hajjar, 1995; Sun et al., 2002). Thus, the inhibition of Lys-Plg-dependent sMTf uptake by εACA may be related to competition between sMTf and annexin II or εACA for the lysine-binding sites located on Plg.

In conclusion, these are the first *in vitro* results indicating that Plg may regulate cell-surface sMTf binding on annexin II and subsequent internalization by LRP. Previous investigations have shown that endogenous sMTf is observed in human serum at a low concentration (between 0.017 and 0.046 nM) (Brown et al., 1981; Kennard et al., 1996; Kim et al., 2001; Ujiie et al., 2002). In addition, human serum concentrations of Plg are much higher (approx. 2 mM) (Miyashita et al., 1988). Based on these observations, our results suggest that when sMTf is released into the circulation or the extracellular space, sMTf may interact with Plg and be internalized into cells via a cooperative mechanism involving annexin II and LRP. Thus, this Plg-induced clearance of sMTf could explain the low concentration of sMTf observed in human serum. In addition, since membrane-bound MTf binds and stimulates Plg activation by PAs (Michaud-Levesque et al., 2005a), our results suggest that sMTf could, in cooperation with annexin II and LRP, regulate plasmin generation by PAs at the cell surface. Further studies are under way to elucidate the molecular events involved in the intracellular trafficking of MTf following its internalization by LRP.

Materials and methods

Chemicals and reagents

Truncated human recombinant sMTf, which is produced by introducing a stop codon following the glycine residue at position 711 of the full-length MTf cDNA (27-aa C-terminal deletion), was kindly provided by Biomarin Pharmaceutical (Novato, CA,

USA). Glu-Plg and Lys-Plg were from Calbiochem (La Jolla, CA, USA). CM5 sensor chips were from BIAcore (Piscataway, NJ, USA). Monoclonal antibodies (mAbs) directed against LRP and annexin II were from Research Diagnostics Inc. (Flander, NJ, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Non-specific IgG antibodies and other biochemical reagents were from Sigma-Aldrich (Oakville, ON, Canada).

BIAcore analysis

sMTf was covalently coupled to a CM5 sensor chip via primary amine groups using N-hydroxysuccinimide (NHS)/N-ethyl-N'-(dimethylaminopropyl)carbodiimide (EDC) coupling agents, as previously described (Jonsson et al., 1991). Briefly, carboxy-methylated dextran was first activated with 50 μl of NHS/EDC (50 mM/200 mM) at a flow rate of 5 μl/min. sMTf (5 μg) in 20 mM acetate buffer, pH 4.0, was then injected and the unreacted NHS-esters were deactivated with 35 μl of 1 M ethanolamine hydrochloride, pH 8.5. Approximately 8000–10 000 RU of sMTf was immobilized on the sensor chip surface. Ringer's solution or 50 mM Tris/HCl buffer (pH 7.5) containing 150 mM NaCl and 50 mM CaCl$_2$ was used as the eluent. Glu- and Lys-Plg were diluted in the corresponding eluent and injected onto the sensor chip surface. Protein interactions were analyzed using the Langmuir binding model, which is the simplest model for 1:1 interaction between ligand and immobilized analyte.

Cell culture

Human glioblastoma cells (U87), and murine embryonic fibroblast (MEF, LRP$^{+/+}$) and LRP-deficient MEF (LRP$^{-/-}$) cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). U87 cells were cultured in modified Eagle's medium (MEM) from Invitrogen (Burlington, ON, Canada) containing 1 mM sodium pyruvate and 10% calf bovine serum. MEF cells were cultured in Dulbecco's modified Eagle's medium (DMEM) from Invitrogen containing 10% fetal bovine serum.

[^{125}I]-sMTf binding and uptake assays in the presence of Plg

sMTf was radioiodinated by standard procedures using Na-[I^{125}] (Amersham Pharmacia Biotech, Baie d'Urfé, QC, Canada) and an iodo-beads kit from Pierce Chemical Co. (Rockford, IL, USA). U87 and MEF cells were plated onto 12-well culture plates. Binding experiments were performed at 4°C to limit internalization during the procedure, whereas uptake experiments were performed at 37°C. First, [^{125}I]-sMTf (16 nM) was mixed with various Plg concentrations at room temperature for 15 min. After incubation, binding or uptake assays were initiated by adding 16 nM [^{125}I]-sMTf with or without Plg in Ringer/HEPES solution (150 mM NaCl, 5.2 mM KCl, 2.2 mM CaCl$_2$, 0.2 mM MgCl$_2$, 6.0 mM NaHCO$_3$, 2.8 mM glucose, 5 mM HEPES, pH 7.4) to cell monolayers. Assays were also conducted in the presence of receptor-associated protein (RAP) (25 μg/ml), ε-amino-n-caproic acid (εACA; 100 mM), α2-antiplasmin (α2-AP; 300 nM), a 125-fold molar excess (2 μM) of unlabelled sMTf, human holo-Tf, human Lf, bovine aprotinin or bovine serum albumin (BSA), as well as in the presence of mAbs (5 μg/assay) directed against LRP, annexin II or non-specific IgG (mouse IgG1 for LRP; rabbit IgG1 for annexin II) as controls. After 2-h incubation, cells were washed three times with Ringer/HEPES containing 0.1% ovalbumin and lysed in NaOH (0.3 M). After precipitation with trichloroacetic acid, cell-associated radioactivity was quantified using a gamma counter.

Western blot analysis

Near-confluent U87 and MEF (LRP$^{-/-}$ and LRP$^{+/+}$) cells were exposed to serum-free cell culture medium. After 6-h incubation, conditioned media were removed and the cells were solubilized in lysis buffer (1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 10 mM Tris, 2% N-octylglucoside, 1 mM orthovanadate, pH 7.5). Cell lysates (10 μg) were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) and separated proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Perkin Elmer Life Sciences, Boston, MA, USA). Following transfer, immunodetection was performed using mAbs directed against LRP or annexin II.

Data analysis

Data are expressed as mean±SD. Statistical analyses were performed with Student's t-test when one group was compared with the control group. To compare two or more groups with the control group, one-way analysis of variance (ANOVA) with Dunnett's post hoc test were used. All statistical analyses were performed using GraphPad Prism version 4.0c for Macintosh (GraphPad Software, San Diego, CA, USA; www.graphpad.com). Significance was assumed for p values less than 0.05.

Acknowledgments

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