Lactoferrin is a natural inhibitor of plasminogen activation

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ABSTRACT

The plasminogen system is essential for dissolution of fibrin clots, and in addition, it is involved in a wide variety of other physiological processes, including proteolytic activation of growth factors, cell migration, and removal of protein aggregates. On the other hand, uncontrolled plasminogen activation contributes to many pathological processes, e.g. tumor cells’ invasion in cancer progression. Moreover, some virulent bacterial species, e.g. Streptococci or Borrelia, bind human plasminogen and hijack the host’s plasminogen system to penetrate tissue barriers. Thus, the conversion of plasminogen to the active serine protease plasmin must be tightly regulated. Here, we show that human lactoferrin, an iron-binding milk glycoprotein, blocks plasminogen activation on the cell surface by direct binding to human plasminogen. We mapped the mutual binding sites to the N-terminal region of lactoferrin, encompassed also in the bioactive peptide lactoferricin, and kringle 5 of plasminogen. Finally, lactoferrin blocked tumor cell invasion in vitro and also plasminogen activation driven by Borrelia. Our results explain many diverse biological properties of lactoferrin, and also suggest that lactoferrin

may be useful as a potential tool for therapeutic interventions to prevent both invasive malignant cells and virulent bacteria from penetrating host tissues.

INTRODUCTION

The plasminogen activation system plays a fundamental role in dissolution of fibrin clots. However, the active serine protease plasmin, a central player of the system, is in addition employed in a plethora of physiological processes, such as the proteolytic activation of growth factors and pro-metalloproteinases; cell migration; removal of protein aggregates. Moreover, a variety of human pathologies are associated with the imbalanced plasminogen activation, e.g. cancer cells’ dissemination (1); neurodegeneration (2); various inflammatory disorders (3).

The conversion of plasminogen (Plg) to plasmin is catalyzed by several enzymes. First, the tissue-type plasminogen-activator (tPA) is responsible for proteolytic activation of Plg on the extracellular matrix required for the resolution of blood clots. Second, the urokinase-type plasminogen activator (uPA) is the central serine protease for Plg activation by migratory cells, such as activated leukocytes, endothelial cell, fibroblasts, but also tumor cells. Upon binding to the urokinase-type plasminogen
activator receptor (uPAR, CD87), inactive pro-urokinase is processed to active uPA, which in turn specifically converts cell-bound Plg to plasmin. The uPA/Plg system might be also hijacked by bacterial species, e.g. *Borrelia burgdorferi*, which contributes to their virulence (4,5). In addition, *Streptococcus pyogenes* secretes streptokinase (SK) capable of binding one Plg molecule to convert another Plg molecule to plasmin independently of host’s uPA (6).

On the other hand, there are several ways to keep Plg activation under control. First, uPA is susceptible to the inhibition by plasminogen activator inhibitors (e.g. PAI-1); second, direct plasmin inhibitors (e.g. α2-antiplasmin) are present in the plasma and rapidly inactivate unbound plasmin; and third, Plg interacts, typically via lysine-binding sites located in its kringle domains, with lysines encompassed in receptors, which restricts Plg activation on the cell surface.

Here we identify the milk immunomodulatory glycoprotein lactoferrin (LF), also known as lactotransferrin, as a natural and specific inhibitor of the uPA-mediated plasminogen activation. LF is an iron-binding glycoprotein from the family of transferrins. The main source of human LF is human milk, secondary granules of neutrophils, and it is present also in serum and exocrine secretions. Antimicrobial, antitumor, and immunomodulatory activities have been attributed to LF (7,8). Here, we demonstrate a so far unknown function of LF in regulation of fibrinolysis, and unravel the molecular determinants and mechanism whereby lactoferrin blocks plasminogen activation.

**RESULTS**

Our previous studies demonstrated that the mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R, CD222) binds and internalizes Plg (9-12). In order to get a better insight into the underlying molecular mechanisms, we sought for further ligands of M6P/IGF2R. We purified M6P/IGF2R (molecular weight ~300 kDa) by affinity chromatography from the lysate of human monocytic THP-1 cells and co-isolated a ~80 kDa protein (Fig. 1A). A trypsin in-gel digestion followed by nano-electrospray mass spectrometry analysis revealed 25 peptide sequences identical to human LF (Table 1). Besides LF, other proteins with the molecular weight ranging from 70 to 90 kDa were identified (Table 2) including Plg and N-acetylgalcosamine-6-sulphatase, both known ligands of M6P/IGF2R (9,13).

The Western blot analysis confirmed the specificity of LF (Fig. 1B). We verified the interaction further by co-immunoprecipitating LF with M6P/IGF2R from human granulocytes, which are known to produce high amounts of LF (Fig. 1C). Mannose 6-phosphate (M6P), which was found in LF (14), did not significantly influence the interaction between LF and M6P/IGF2R, suggesting that the M6P-binding sites in M6P/IGF2R were not required for the interaction (Fig. 1C). These findings were proven by an *in vitro* binding assay wherein M6P did not block the interaction, similarly to soluble uPAR (Fig. 2A). In contrast, the presence of Plg, a M6P/IGF2R ligand (9), enhanced the weak interaction between LF and M6P/IGF2R (Fig. 2A). Based on the latter finding, we tested the possibility of a direct LF-Plg binding. Indeed, an *in vitro* binding assay revealed the direct binding of LF and Plg (Fig. 2B).

In order to map the determinants responsible for the LF-Plg binding, we designed three peptides: pLF1 derived from the N-terminal end of LF and encompassed within lactoferricin (LFC), a small bioactive fragment with anti-pathogenic activity (15); pLF2 derived from the helix linker region of LF encompassing a stretch of lactoferramin, another bioactive peptide (16); and pLF3 made up of the C-terminal part containing a C-terminal lysine (Table 3), since C-terminal lysines are generally known to be involved in Plg binding (17). We found that pLF1 blocked the binding of LF and Plg with the half maximum concentration (IC₅₀) of about 6 µg/mL (2.8 µM) (Fig. 2 C-E). The lysine analogue tranexamic acid (TA), which is known to block the lysine-dependent Plg binding, did not have significant effects on the binding as well as the other peptides. This indicates that the Plg-binding site is encompassed within the amino-terminal part of LF.

To map the determinants responsible for binding of Plg to LF, we applied fragments derived from
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Plg generated as previously described (18): angiostatin, consisting of kringle domains 1-4; mini-Plg containing kringle 5 and the proteolytic domain; and micro-Plg encompassing just the proteolytic domain. LF bound strongest to mini-Plg, although we detected a weak binding to all fragments (Fig. 2 F, G). Upon a higher stringency, i.e. in the presence of 0.1% detergent Triton X-100, LF bound only to mini-Plg (Fig. 2F). These findings indicate that the major LF-binding site is encompassed within kringle 5 of Plg.

In the next steps, we quantified the binding of LF and Plg by surface plasmon resonance (SPR). To preserve the surface of LF as much as possible, we opted for its immobilization on a streptavidin sensor chip, which facilitated a flexible attachment following biotinylation of the oligosaccharides present at Asn147 and Asn478 in the N- and C-terminal lobe of LF, respectively (19). Kinetic analysis revealed that LF and Plg formed a high affinity complex with the dissociation constant equal to 69 ± 24 nM (Table 4, Fig. 3A).

Interestingly, the SPR profile of Plg association and dissociation gives the best fit to a two-state reaction model (Fig. 3A). According to this model, the proteins initially form a metastable complex 1, which is rearranged on a millisecond time scale to a final, slowly-dissociating complex 2 (Table 4). To verify the two-state reaction model, we examined dissociation of the LF-Plg complex in two separate SPR experiments, comparing short (60 s) and prolonged (300 s) Plg association time. Indeed, in the experiment with prolonged association time we detected formation of a higher amount of the slowly-dissociating complex 2 (data not shown), which ultimately confirmed two-step interaction mechanism.

A freshly prepared LF sensor chip had a very low capacity to bind Plg and required activation by three consecutive six second pulses of 100 mM HCl. The sensor chip response decreased by 100 RU during the treatment, but the LF surface became fully competent to interact with Plg, and maintained an unchanged reactivity over one month of running experiments.

This surprising observation prompted us to investigate the underlying reasons. As the LF-bound iron might be depleted during activation with the acid (20), at first we tested whether the re-saturation of LF with iron could influence Plg binding. However, no change in complex formation was found, therefore, iron presence had no direct influence on the binding.

Secondly, we examined the oligomeric state of biotinylated LF used in the sensor chip preparation, as LF is able to form tightly bound dimers in diluted solutions at physiologic pH (21), which might influence the Plg binding. We measured molecular radius by the dynamic light scattering (DLS) and found that biotinylated LF preparation exhibited a higher average particle size than the initial protein (Table 5), with the weight-averaged molar mass of biotinylated LF corresponding prevalently to dimeric form (177 kDa versus 106 kDa of native LF). The amount of high-molecular weight aggregates was negligible. It suggests that the unresponsiveness of the fresh LF surface might reflect the LF dimerization during labelling, which had occluded surface patches needed for the interaction with Plg. The dimerization of LF occurs also at physiological conditions (22) and may represent a mechanism to control its interaction with Plg.

Each of the three proteins: sM6P/IGF2R, Plg and LF; had been detected in human serum (23-26). We found that LF co-immunoprecipitated with both sM6P/IGF2R and Plg in human serum (Fig. 3B). In addition, blue native electrophoresis analysis (BN-PAGE) implied the presence of several complexes associated with LF in serum (Fig. 3C): The low molecular weight species seemed to contain exclusively LF and very likely corresponded to dimers that did not bind Plg as described above; the intermediate diffused complex(es) smaller than 272 kDa contained apparently both LF and Plg; the intermediate diffused complex(es) of approximately 500 kDa contained apparently predominantly sM6P/IGF2R, as shown previously (12); and the high molecular weight complex(es) of more than 880 kDa appeared to contain Plg, LF and sM6P/IGF2R (Fig. 3C, arrowheads). These data suggest that LF may co-exist with Plg in serum in native complexes.

Next, we tested whether binding of LF to Plg could interfere with Plg activation. First, we found that pre-incubation with LF abrogated AF647-labelled Plg binding to THP-1 cells.
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(known to express high levels of uPAR and uPA (11)) in a concentration-dependent manner with the maximal inhibition (52%) at the concentration of 50 μg/mL (Fig. 4A). We analyzed only the binding to viable cells. Second, when the cells were subjected to a Plg activation assay, we found a concentration-dependent inhibitory effect of LF with maximal inhibition at the concentration of 10 μg/mL (Fig. 4B). This effect was independent of M6P/IGF2R, previously identified by us to bind and internalize Plg (9,11), since in both control and M6P/IGF2R-silenced THP-1 cells, generated previously by us (11), a similar effect was observed: 75% and 82% inhibition, respectively. However, M6P/IGF2R-silenced cells displayed a 30% higher capacity to activate Plg compared to control cells (Fig. 4B) which was in line with our previous works showing the M6P/IGF2R-dependent negative regulation of Plg activation by means of the stable genetic knock-out and knock-down experiments (10,11). Third, by a cell-free system, we found that LF hampered Plg activation on coated uPA in a concentration-dependent manner with an IC_{50} value of 3 μg/mL (43 nM), similarly to the plasmin inhibitor aprotinin (Fig. 4C). An in vitro binding assay revealed that LF completely blocked the binding of Plg to uPA at the concentration of 10 μg/mL (Fig. 4D), which is in agreement with its effect on Plg activation.

Finally, we performed an in vitro binding assay in the presence of mAbs specific to both LF and Plg, and we found that the anti-LF mAb 1D2 blocked the interaction between LF and Plg (Fig. 5A). A pre-incubation of LF with mAb 1D2 abrogated the inhibition of Plg activation by LF at the concentration of 20 μg/mL (Fig. 5B, C).

We also tested whether LF was able to block Plg activation by purified streptokinase (SK), a protein secreted by Streptococci that binds and activates Plg independently of uPA (27,28). We found that LF did not have any inhibitory effect on Plg activation by SK (Fig. 4E). Together, these results suggest that LF inhibits plasmin generation on the surface of cells through a blockade of Plg binding to uPA.

Although LF is structurally highly similar to transferrin (TF) (29), the superimposed structure comparison of LF and TF showed that their amino-terminal parts possessed several distinctions very likely caused by the presence of several positively charged amino acid residues within LF not present in TF, e.g. an amino-terminal stretch of arginines (Fig. 6A arrow, B, C.). Since we mapped the Plg-binding site into this amino-terminal part of LF (Fig. 2B-E), these differences might be decisive in a contrasting capability of LF and TF to block Plg activation. This finding further underlines the specificity of the LF-mediated inhibition of Plg activation.

In the last series of experiments, we tested the effect of LF on patho/physiological processes associated with the Plg system. The Plg system is utilized by migratory cells to penetrate tissues, e.g. endothelial cells during angiogenesis harness uPA-dependent Plg activation (30,31). However, also tumor cells activate Plg by uPA, which contributes to their dissemination (32). Accordingly, we performed in vitro matrigel cell invasion assays by using the kidney carcinoma cell line TCL-598 and primary human umbilical vein endothelial cells (HUVEC), both shown by us previously to migrate in a uPA/Plg-dependent manner (10,12). We found that LF reduced the invasiveness of both TCL-598 cells and HUVEC (Fig. 7A, B). Thus, LF can exert negative regulatory effects on cell migration via inhibition of Plg activation.

Also virulent bacteria hijack the Plg system to increase their invasiveness. For instance, Streptococci secret streptokinase (SK) which on the one hand binds one ‘enzyme’ Plg molecule and on the other hand activates another ‘substrate’ Plg molecule in a uPA-independent manner (27,33). In contrast, Borrelia do not produce any intrinsic Plg activator; instead they bind and employ components of the host’s fibrinolytic system, namely Plg and uPA, via outer surface proteins (4,5,34,35) to penetrate the host. Thus, we tested the ability of LF to inhibit Plg activation by Borrelia burgdorferi and Streptococcus pyogenes. When we incubated human serum with Borrelia burgdorferi and the plasmin specific chromogenic substrate, we observed a blockade of Plg activation by LF (Fig. 7C). In contrast, in
the presence of *Streptococcus pyogenes* LF did not reduce Plg activation (Fig. 7D). This observation was in line with the afore-described experiments with purified enzymes showing that LF was able to inhibit Plg activation by uPA but not SK (Fig. 4). Taken together, these results show that LF can modulate pathophysiological processes of Plg such as tumor cell and invasion of certain bacteria.

**DISCUSSION**

LF is known to display a plethora of antimicrobial properties and antitumor activities (7). Its iron binding ability and the unique positively charged N-terminal region are considered to explain most of its biological activities (8). Here, we identified a so far unknown function of LF: It binds Plg and blocks its activation by uPA but not by bacterial SK.

We identified the interaction by means of mass spectrometry analysis of the proteins co-immunoprecipitated with M6P/IGF2R. This suggests that M6P/IGF2R, a Plg receptor, might be involved in effects of LF. In this respect it is of interest that M6P/IGF2R is required for *Herpes simplex* virus type 1 infection (36), and LF inhibits *Herpes simplex* infection and trafficking (37). Moreover, also nucleolin, another protein identified by us as a partner of M6P/IGF2R (Table 2), seems to be involved in *Herpes simplex* virus type 1 infection (38). The putative functional network among M6P/IGF2R, LF and *Herpes simplex* virus remains for future studies.

Here, we mapped the mutual binding sites of LF and Plg within the N-terminal region of LF and kringle 5 of Plg, respectively. We show that through this interaction LF controls Plg activation. We propose that LF inhibits Plg activation on cells via the blockade of the Plg-uPA interaction, which is in agreement with the observation that the majority of lysine-dependent Plg binding to the cell surface is dependent on the presence of uPA (39). However, it is also possible that LF via binding to Plg blocks a conformational conversion of Plg (40) required for its activation.

LF shares significant sequence and structural homology with TF, a further member of the iron-binding transferrin family. However, TF does not possess the inhibitory activity of LF towards Plg, which might be due to the different composition within their N-termini.

Plg is an inactive zymogen circulating in plasma at relatively high concentration (2 µM) (23,41). Plg binding to the matrix protein fibrinogen (K_D = 0.32 µM) is required for conversion of Plg to active plasmin during blood clots’ resolution (42). In addition, Plg binds with K_D values ranging from 0.1 to 2 µM also to various cell surface receptors, which is indispensable for cell migration (17,39,43). Both pathways, i.e. Plg activation on the clots and on the cellular surface, are mediated by Plg activators, tPA and uPA, respectively (32). Strikingly, Plg binds with higher affinity (K_D = 420 pM) to bacterial SK (44-46). SK is a Plg activator employed by invasive human pathogens to facilitate their spreading through host barriers (27,28). Plg activators are susceptible to inhibition by specific plasminogen activator inhibitors. Also, direct plasmin inhibitors are present in plasma and rapidly inactivate free unbound plasmin, e.g. α2-antiplasmin binds to various Plg types with K_D values ranging from 0.2 to 1.9 µM (47-50).

By using SPR we determined the dissociation constant of the Plg-LF interaction to be 69 ± 24 nM, and the IC_{50} of LF in inhibiting Plg activation to be 43 nM. These measures are similar, yet somewhat higher than the concentrations of LF in human serum that range from 5 to 30 nM in healthy donors ((51,52), and our unpublished data). This suggests that upon normal conditions, Plg activation would not be blocked by LF. However, LF concentrations might be upregulated in site-specific microenvironments, e.g. upon degranulation of neutrophils (7,53). Further, high levels of LF were reported to be secreted by apoptotic cells (54). In apoptosis, LF might be necessary to downregulate extracellular plasmin activity to avoid unwanted degradation of the surrounding tissue, inappropriate cell migration or activation of proenzymes. The serum levels of LF might further increase during pregnancy (55). Thus, an anti-fibrinolytic property of LF may provide an intriguing clue to the reported higher risk of thromboembolism during pregnancy (56,57).

The physiological relevance of the LF-Plg complex is indicated by the fact that we were able to coimmunoprecipitate both proteins from human serum. The SPR profile of the LF-Plg complex...
interaction suggests a two-state model with metastable initial complex 1 and stable complex 2. Time-dependent stabilization of the LF-Plg interaction may have important physiological consequences in situations where prolonged elevated levels of interacting proteins occur. Our data further suggest that the LF dimerization status, rather than its iron saturation, might modulate its capacity to interact with Plg. In summary, LF intrinsically inhibits Plg activation through blocking the interaction between Plg and uPA. Thus, LF represents a novel pathway and tool to regulate plasmin-dependent functions.

EXPERIMENTAL PROCEDURES

Materials
Ammonium persulfate, TEMED, sodium dodecyl sulfate (SDS), acrylamide, N,N'-methylenebis-acrylamide were purchased from SERVA (Heidelberg, Germany). Human uPA and Glu-Plg were products of Calbiochem (Darmstadt, Germany). BSA was from Carl Roth (Karlsruhe, Germany). Lactoferrin, streptokinase, aprotinin (inhibitor of serine proteases), alpha-2 antiplasmin (inhibitor of plasmin), polybrene, mannose 1-phosphate, mannose 6-phosphate, all protease inhibitors, puromycin, tranexamic acid (TA), monensin were from Sigma-Aldrich (St. Louis, MO). Beriglobin was from CSL Behring (King of Prussia, PA, USA). Nonidet P-40 and Triton X-100 were obtained from Pierce Biotechnology Inc. (Rockford, IL, USA). Plasmin-specific substrate S-2251 were products of CoaChrom Diagnostica (Vienna, Austria). The streptavidin HRP-conjugate was supplied by GE Healthcare (Uppsala, Sweden). The LF-derived peptides were synthesized by Peptide 2.0 (Chantilly, VA, USA). Human serum samples were obtained from healthy donors after informed consent that a part of the routine blood samples will be used for research purposes (approved by the Ethical Committee of the Medical University of Vienna). The study abides by the Declaration of Helsinki principles. All samples were stored at -20°C before use.

Antibodies
The monoclonal antibody (mAb) MEM-238 to M6P/IGF2R was generated by us, produced and purchased from EXBIO (Prague, Czech Republic); it was biotinylated or fluorescently conjugated by us. The AFP-01 mAb was generously provided by Dr. Václav Hořejší (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic). mAbs to human LF (LF5-1D2, LF65-3D5, LF95-4C5 and LF124-5E2) were generated in the laboratory of Otto Majdic. Both biotinylated and FITC-conjugated rabbit polyclonal Ab to LF were purchased from Abcam (Cambridge, UK), the mAbs to Plg (7Pg, 4Pg) from Technoclone (Vienna, Austria). The goat anti-mouse IgG secondary antibody, both unlabeled and HRP-conjugated were from Sigma-Aldrich. Goat anti-mouse IgG+IgM (H+L)-FITC conjugate was from An der Grub (Kaumberg, Austria); the anti-mouse IgG conjugates labelled fluorescently with Alexa-Fluor 488 (AF-488) or Alexa-Fluor 647 (AF-647) were from Molecular Probes (Invitrogen).

Cells
The human monocytic cell line THP-1 was from ATCC and the human kidney epithelial tumor cell line TCL-598 was obtained from the Novartis Research Institute (Vienna, Austria). The cell lines were cultured in RPMI 1640 medium (GIBCO/Invitrogen) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine and 10% heat inactivated FCS (Sigma). All the cells were grown in a humidified atmosphere at 37°C and 5% CO₂ and passaged suitably using trypsin-EDTA solution. Peripheral blood was separated into plasma, PBMCs and granulocytes with red blood cells using the Lymphoprep gradient centrifugation (Axis Shield, Oslo, Norway), the latter followed by red cell lysis using aqua test.

Bacteria
*Streptococcus pyogenes* type strain was obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ No. 20565) and grown in Brain-Heart infusion (BHI) broth (Carl Roth, Germany) at 37°C. Bacterial growth was observed by absorbance at 600 nm (A600) and was correlated to colony forming units per mL (CFU/mL) by plating on Columbia blood agar plates (bioMérieux, Austria). An overnight culture of the bacteria was inoculated to fresh BHI broth at an A600 of 0.05 and grown at 37°C until it reached an A600 of 0.47 (approx. 1x10⁸...
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CFU/mL). Bacteria were pelleted at 6,000 x g for 10 min at room temperature and resuspended in sterile phosphate buffered saline (PBS) for use in the experiment. *Borrelia burgdorferi* sensu stricto strain B31 was obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ No. 4680). *Borrelia* were grown in a modified BSK II medium as described (58). They were grown to logarithmic phase and enumerated using a counting chamber. The respective amount of culture was centrifuged at 6,000 x g for 10 min at room temperature and the resulting pellet was washed once with 1 ml of sterile PBS. Subsequently, the bacteria were brought up to a density of 4 x 10^7/µL in fresh PBS and 25 µL (containing 10^7 spirochetes) were added to each reaction.

**Flow cytometry and cell surface binding assay**

Cells were harvested, washed with PBS containing 1% BSA and 0.02% sodium azide, blocked with 2% beriglobin for 15 min on ice and afterwards incubated for 30 min on ice with specific mAb, either fluorescently labeled or unlabeled. For the latter, a second step staining was done with FITC-conjugated F(ab')2 anti-mouse IgG+IgM antibodies. Prior to analysis, the cells were washed again. Apoptotic and necrotic cells were discriminated by staining with 7-amino-actinomycin D (7AAD) and the annexin V-PE conjugate (Becton Dickinson, San Jose, CA). Then the analysis was carried on as described above. Flow cytometry was performed with a LSRII flow cytometer (Becton Dickinson). Data acquisition was executed with the FACS DIVA software. Data analysis was accomplished with the FlowJo software (Treestar Inc., Ashland, OR).

To measure Plg binding to cells, the Plg molecule labeled with either AF-488 or AF-647 (10 µg/mL), prepared in our laboratory, was pre-incubated on ice for 30 min with or without LF in PBS at the indicated concentrations. In parallel, THP-1 cells were harvested, washed and then incubated on ice for 20 min with the Plg preparations at the indicated concentrations. Afterwards, the cells were washed with PBS and cell-surface-bound Plg was analyzed by flow cytometry.

**RNA interference**

The stable knock down for M6P/IGF2R in THP-1 was generated by the delivery of a short hairpin RNA expression cassette as described in detail (10,11).

**Trypsin in-gel digestion and LC-MS/MS analysis**

The 80 kDa Coomassie-stained gel band containing proteins co-purified with M6P/IGF2R was cut and destained with a mixture of methanol and 50 mM ammonium-hydrogen carbonate. Proteins were reduced by dithiothreitol, alkylated by iodoacetamide and treated with trypsin in cleavage buffer (100 mM Tris-HCl, pH 8.0, 1 mM CaCl_2, 10% acetonitrile). The digestion was carried out overnight at 37°C and stopped by formic acid (1%). Peptide extraction was performed using trifluoroacetic acid (0.1%) for the analysis. The HPLC system used was from UltiMate™ system (Dionex Corporation; Sunnyvale, CA) equipped with a PepMap C18 purification column (300 µm x 5 mm) and a 75 µm x 150 mm analytical column of the same material. The peptides were loaded on LC Packings “Switchos” module (Dionex, Inc., Sunnyvale, CA) and a linear gradient (5% - 80%) of acetonitrile in formic acid (0.1%) was used for the elution. The LC-MS/MS analyses were carried out with the UltiMate™ system interfaced to an LTQ linear ion trap mass spectrometer (Thermo; San Jose, CA). The nanospray source of Proxeon (Odense, Denmark) was used with the distal coated silica capillaries of New Objective (Woburn, MA, USA) at voltage of to 1500V. Peptide spectra were recorded over the mass range of m/z 450-1600, MS/MS spectra were recorded in information dependent data acquisition over the mass range of m/z 200-2000. One full spectrum was recorded followed by 4 MS/MS spectra, automatic gain control was applied and the collision energy was set to the arbitrary value of 35 as collision gas. MS/MS spectra were interpreted by Mascot (Matrix Science Ltd, London, UK), peptide tolerance was set to +/- 2 Da and MS/MS tolerance was set to +/- 0.8 Da, by using the nr protein database of NIH (NCBI Resources, NIH, Bethesda, MD, USA). Proteins identified by the MASCOT search were inspected to create a protein list. The whole analysis was performed by the Mass
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Spectrometry Facility, Medical University of Vienna, Vienna, Austria.

**Protein purification**

M6P/IGF2R was isolated from THP-1 cells on cyanogen bromide-activated Sepharose 4B (Uppsala, Sweden) conjugated with the anti-M6P/IGF2R mAb MEM-238. The cells were lysed in lysis buffer containing 20 mM Tris-HCl (pH 8.2), 140 mM NaCl, 1% Nonidet P-40 detergent and a mixture of protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 100 nM Na-p-tosyl-L-lysine chloromethylketone, 100 nM N-CBZ-L-phenylalanine chloromethyl ketone, 1 mM peptstatine A) for 30 min on ice. The lysate was clarified by centrifugation at 2,000 g for 30 s at 4°C and loaded onto the column. The column was washed with lysis buffer (3 x vol). The elution was performed using the lysis buffer under stepwise increase of the pH (9.0, 10.0 and 11.7). After elution, the samples were immediately neutralized by adjusting the pH to 7.0 using HCl. Afterward, the fractions were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting or Coomassie blue gel staining. By using a uPA-conjugated agarose column soluble uPAR was isolated as described previously (59) from the conditioned medium of CHO cells expressing recombinant soluble uPAR. Purified proteins were optionally used in *in vitro* binding assays.

**In vitro binding assay**

For the binding assay, various molecules solubilized in PBS (pH 8.7) at a concentration of ~5 μg/mL were coated on wells of a 96-well BD Falcon™ plate for 2 h at 37°C. Then the wells were blocked with 1% BSA in PBS for 1 h at room temperature and washed two times with binding buffer (20 mM Tris-HCl, 140 mM NaCl, pH 7.5). The wells were incubated for 4 h on ice with binding buffer supplemented with purified assayed proteins (~5 μg/mL) in the absence or presence of additional molecules, and afterwards washed two times with ice-cold binding buffer. Bound material was analyzed by SDS-PAGE and immunoblotting.

**Immunoprecipitation**

For immunoprecipitation, a 96-well BD Falcon™ plastic U-bottom plate was coated with goat anti-mouse IgG (5 μg/mL in PBS, pH 8.7) for 2 hours at 37°C. The plate was washed twice with PBS, incubated with specific antibodies (10 μg/mL in PBS) for 2 hours at 37°C, blocked with 1% BSA in PBS for 1 hour at 37°C, and washed twice with cold lysis buffer. Granulocytes were lysed for 30 min on ice with 1% Triton X-100 together with 0.5% dodecylmaltoside in 20 mM sodium phosphate buffer (pH 7.4) with a mixture of protease inhibitors and benzonase (Merck; Darmstadt, Germany; 50 U/mL). Afterwards, the lysate was passed twice through a needle (25G). The lysate was clarified by centrifugation at 2,000 g for 30 s at 4°C. The supernatant, or in some experiments human serum, was loaded into the wells (50 μL/well) and incubated 4 hours at 4°C. Then, the wells were washed 4 times with cold lysis buffer and the precipitates were collected with sample buffer and analyzed using SDS-PAGE followed by immunoblotting.

**Electrophoresis and immunoblotting**

Samples obtained from cell lysis, protein purification, *in vitro* binding assays, *in vitro* proteolysis assay or immunoprecipitation analysis were analyzed by electrophoresis on an appropriate SDS-polyacrylamide gel (SDS-PAGE) followed by immunoblotting or Coomassie blue gel staining. By using a uPA-conjugated agarose column soluble uPAR was isolated as described previously (59) from the conditioned medium of CHO cells expressing recombinant soluble uPAR. Purified proteins were optionally used in *in vitro* binding assays.

**Blue-native electrophoresis (BN-PAGE)**

As described in detail elsewhere (12), in the first dimension a native separation gel was used. Ferritin (monomer 440 kD, dimer 880 kD), jack bean urease (monomer 91 kD, trimer 272 kD, hexamer 545 kD) and BSA (all from Sigma) were used as markers. The serum samples were loaded as described (12), and electrophoresis was performed at 80 V and room temperature overnight. The following day, the lanes were cut from the gel, put on the top of a second-dimension SDS-PAGE gel, and run at room temperature overnight. Afterwards, the gel was blotted onto a Immobilon polyvinylidene difluoride membrane, the membrane was blocked with 4% milk, and then incubated with specific antibodies for visualization.
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**Plasminogen activation assay**

For the analysis of Plg activation in a cell-free system, uPA or SK (10 nM) in PBS (pH 8.7) was coated on a 96-well Falcon plate for 2 h at 37°C. The wells were blocked with 1% BSA in PBS for 1 h at room temperature, washed two times with PBS and afterwards incubated at 37°C in PBS with human Glu-Plg (50 nM) and the chromogenic plasmin substrate (S-2251; 0.8 mM). For the analysis of Plg activation at the cell surface, the cells were washed twice with serum-free RPMI 1640 medium and afterwards incubated at 37°C in serum-free medium together with human Glu-Plg (50 nM) and S-2251 in a 96 well plate (5x10⁷ cells per well). The absorbance change at 405 nm was monitored at different time points by using an enzyme-linked immunosorbent assay reader (SpectraMax M5, Molecular Devices). In order to block the activity of unbound plasmin and to study Plg activation at the cell surface only, the assays were performed in the presence of alpha-2 antiplasmin (2.5 µg/mL). Optionally, various substances were added to the reaction.

For the analysis of Plg activation by bacteria, a 2x mastermix containing BSA (final conc. 2 µg/mL), Plg (50 nM), and the chromogenic substrate S-2251 (0.8 mM), diluted in PBS was prepared. Bacteria were added to the mastermix (5x10⁷ CFU/ml in case of *S. pyogenes* and 1x10⁷ CFU/ml for *Borrelia*). Aliquots without bacteria were used as controls. Finally, the reaction volume was made up to 100 µL with PBS. Tubes were shaken at 37°C overnight and the color change was measured in triplicates using an EL 808 microplate reader (BioTek, Germany). The basal level of Plg activation measured only with human serum without bacteria was subtracted.

**Cell invasion assay**

The invasion of both TCL-598 cells and HUVECs was measured by an assay based on the Boyden chamber principle with filter inserts containing 8 µm pore size polycarbonate membranes coated with basement membrane matrigel matrix (Becton Dickinson, San Jose, CA). Optionally, the matrigel was pre-incubated with inhibitors (LF or aprotinin) diluted in serum-free medium for 1 h at 37°C. Either EGF (10 ng/mL) or VEGF (50 ng/mL) in RPMI 1640 medium (10% FCS) were added into the lower chambers to induce migration of the TCL-598 cells or HUVECs, respectively. The cells were seeded into the upper part of the filter (1x10⁵ cells/filter) and incubated 8 h at 37°C. Then, the filters were removed, the upper surface of the membrane was scraped free of cells and the number of cells invaded onto the lower side of the filters was evaluated after fixing and staining with crystal violet by counting.

**Structure comparisons**

All three-dimensional structures were retrieved from Protein Data Bank (PDB) (www.rcsb.org) (60). Comparison of structures was carried out by Multiprot (61) and the structures were visualized by WebLabViewerLite (Molecular Simulations, Inc.). Superimposed structure of LF (real structure; PDB code: 1LFG (19)) as compared with TF (real structure; PDB: 3QYT (62)). The superimposed part covered 441 Ca-atoms with a 1.2 Å room-mean square deviation. The amino-terminal part of LF corresponding to the antimicrobial peptide lactoferricin - LFC (partial structure; PDB code: 1LFG (19) residues Gly1-Ala48) was compared with consonant part of transferrin (partial structure; PDB: 3QYT (62); residues Val1-Ala51). The superimposed part covers 45 Ca-atoms with a 0.88 Å room-mean square deviation.

**Determination of binding by surface plasmon resonance (SPR)**

A Biacore 3000 instrument with a streptavidin Sensor Chip SA (GE Healthcare Bio-Sciences, Uppsala, Sweden) was used. All experiments were performed at 25°C in PBS (10 mM phosphate buffer pH 7.2, 150 mM NaCl) with 0.005% of Tween-20 as running buffer. For biotinylation, iron-saturated recombinant human LF (Sigma, L1294), 1.8 mg/ml in 0.1 M sodium acetate pH 5.5 was treated with a 10 mM sodium meta-periodate for 15 min on ice to introduce carbonyl groups on LF’s glycans. Oxidized LF was buffer-exchanged using a desalting column equilibrated with PBS, giving 0.7 mg/ml LF solution. A DMSO stock solution of EZ-link hydrazide biotin (Thermo Scientific, 21339) was added to 5 mM final concentration and the mixture was incubated for 45 min at 25°C. Unreacted ligand was removed on a desalting column equilibrated with PBS. Biotinylated LF, approximately 1 µg/ml, was immobilized on the
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SA sensor chip to a final level of 1400 response units (RU). After sensor chip pretreatment with 100 mM HCl (see results for details), serially diluted plasminogen (10nM – 160 nM) was injected at a 30 µl/min flow rate, allowing 60 s and 120 s the association and dissociation time, respectively. Regeneration of the LF surface was accomplished by a 13 s injection of 10 mM HCl. Binding data were double-referenced (63) and the kinetic constants and affinity were derived from fitting a 1:1, two-state reaction model as implemented in BIAevaluation software v. 4.1.1. Rate constants were approximated globally, maximal responses were fitted locally and the bulk response was set to zero. The influence of the LF iron content on the binding of plasminogen was tested after iron re-saturation with 10 µM iron(III) sulphate in 100 µM imidazole pH 7.0 and 10 µM sodium bicarbonate.

**Light scattering measurements**

LF solutions were centrifuged at 10,000 g for 10 min, transferred into a 1 µl cuvette and measured in a Dynapro NanoStar instrument controlled by Dynamics software v. 7.7.0.125 (Wyatt Technology). Both dynamic light scattering (DLS) and static light scattering (SLS) data were collected from sample. Evaluation of data was performed by the Dynamics software. Adopting a globular protein model, DLS autocorrelation data were analyzed by a cumulants analysis with polydispersity calculation and a regularization analysis by Dynals algorithm for characterization of average particle radius and high molecular weight species, respectively. SLS averaged intensity was used for molar mass calculation using sphere model. To cull the acquisitions influenced by dust or irregular particles, an automatic filtering of autocorrelation functions was applied with a baseline threshold 0.997-1.003 and a maximal allowed sum-of-squares (SOS) error for cumulants fit equal 10.

**Statistical analysis**

All experiments were performed at least three times in at least triplicates. The data were expressed as mean values with standard deviations. Statistical significance was evaluated by using a Student’s t-test; values of p*<0.05, and p**<0.005 (as indicated) were considered to be significant or highly significant, respectively.

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This work is dedicated to Professor Otto Majdic, who passed away on February 9, 2018.

**AUTHOR INFORMATION**

The authors declare no competing financial interests.

**AUTHORSHIP CONTRIBUTIONS**

V.L. conceived and designed the experiments. V.L., A. Z., M.R., M.G., R.S., O.C., performed experiments and analyzed data. R.S. contributed to the design of SPR and DLS experiments. H.S., A.O.-R., R.S., E.K. and O.M. contributed with ideas, comments and materials. V.L. wrote the paper. All authors read and corrected the manuscript.
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**Abbreviations:** BN-PAGE, blue native polyacrylamide gel electrophoresis; DLS, dynamic light scattering; K_D, dissociation constant; LF, lactoferrin; LFC, lactoferricin; M6P/IGF2R, mannose 6-phosphate/insulin-like growth factor 2 receptor; mAb, monoclonal antibody; Plg, plasminogen; shRNA, short hairpin RNA; SK, streptokinase; SLS, static light scattering; SPR, surface plasmon resonance; TA, tranexamic acid; TF, transferrin; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor
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Table 1 | MS analysis of the 80 kDa protein band co-purified with M6P/IGF2R: Peptides matched to LF (Mascot protein score = 613).

| LF residues | Peptide                  | Observed mass (Da) | Predicted mass (Da) |
|-------------|--------------------------|--------------------|---------------------|
| 23-39       | RRSVQWCAVSQPEATKC        | 1745.85            | 1747.13             |
| 93-107      | KLRPVAEAVYGTERQ          | 1459.78            | 1460.41             |
| 108-120     | RTHYYAVAVVKK             | 1149.62            | 1149.36             |
| 119-134     | KGGSFQNLQGLKSCHTGLRR     | 2088.02            | 2086.91             |
| 171-184     | RFFSASCVPGADKG           | 1284.58            | 1284.88             |
| 220-231     | RDGAGDVAFIRE             | 1019.5             | 1019.56             |
| 269-279     | RVPSHAVVARS              | 934.53             | 935.02              |
| 283-293     | KEDAIWNLRRQ              | 1128.59            | 1128.95             |
| 364-377     | RVVWCAGGEQELRK           | 1444.71            | 1445.24             |
| 406-425     | KGEADAMSLDDGYVYTAGKC     | 1803.8             | 1803.2              |
| 424-437     | KCGLVPVLAENYKS           | 1361.7             | 1362.15             |
| 462-475     | RRSSTSLLTWNSVKG          | 1392.7             | 1393.28             |
| 504-521     | KFDEYSQSCAPGSDPRS        | 1861.76            | 1862.15             |
| 520-537     | RSNLCAICIDEQGENKC        | 1806.79            | 1807.25             |
| 544-554     | RYYGYTGAFRC              | 1096.5             | 1096.98             |
| 553-567     | RCLAENAGDVAFVKD          | 1392.67            | 1392.68             |
| 566-585     | KDVTVLQNTDGNNEAWAKD      | 1987.92            | 1989.08             |
| 587-600     | KLADFALLCLDGKR           | 1334.69            | 1335.19             |
| 607-623     | RSHCMLAANPHAVSVM         | 1664.79            | 1665.55             |
| 628-640     | RLRQVLLHQQAKF            | 1304.79            | 1305.38             |
| 642-660     | RNGSDCPDKCLFQSETKN       | 2031.87            | 2032.26             |
| 658-673     | KNLLFNDNTECLARL          | 1578.75            | 1579.54             |
| 681-696     | KYLGPOQVAGITNLKK         | 1535.83            | 1536.33             |
| 681-697     | KYLGPOQVAGITNLKCV       | 1663.93            | 1664.75             |

M6P/IGF2R was purified with the anti-M6P/IGF2R mAb MEM-240 from lysates of THP-1 cells. The 80 kDa band co-purified with M6P/IGF2R was excised from the gel for MS/MS analysis. Identification was performed by MASCOT and the NCBI non-redundant protein database. Peptides identified to match to LF are listed.
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Table 2 | MS analysis of the 80 kDa protein band co-purified with M6P/IGF2R: Protein hits.

| Protein name                                      | NCBI GI number | Predicted mass (kDa) | Mascot score | Queries matched |
|---------------------------------------------------|----------------|----------------------|--------------|-----------------|
| Lactoferrin                                       | 18490850       | 80                   | 613          | 41              |
| ATP-dependent DNA helicase II                     | 4503841        | 70                   | 448          | 26              |
| Hydroxysteroid (17-beta) dehydrogenase 4          | 4504505        | 80                   | 329          | 12              |
| RNase L inhibitor                                 | 3273417        | 68                   | 266          | 13              |
| Gastrin-binding protein                           | 595267         | 84 (78*)             | 172          | 14              |
| Plasminogen**                                     | 27806815       | 94                   | 147          | 12              |
| Replication protein A1                            | 4506583        | 69                   | 108          | 4               |
| Heat shock protein 70                             | 386785         | 70                   | 107          | 4               |
| Lamin B1                                          | 5031877        | 66                   | 91           | 9               |
| N-acetylglucosamine-6-sulfatase precursor (Glucosamine-6-sulfatase) | 4504061 | 63 (100*) | 88 | 4 |
| Nucleolin                                         | 189306         | 76 (105*)            | 70           | 2               |
| Moesin                                            | 4505257        | 68                   | 57           | 3               |

M6P/IGF2R was purified with the anti-M6P/IGF2R mAb MEM-240 from lysates of THP-1 cells. The 80 kDa band co-purified with M6P/IGF2R was excised from the gel for MS/MS analysis. Identification was performed by MASCOT and the NCBI non-redundant protein database. Analysis revealed several proteins with the predicted molecular mass ranging from 70 to 90 kDa.

*The apparent molecular mass in SDS-PAGE;

**Species of all proteins were identified as Homo sapiens with exception of plasminogen (Bos taurus) indicating that the plasminogen molecule co-purified with M6P/IGF2R was derived from the fetal calf serum used for supplementing the culture medium.
Table 3 | Sequences of the LF derived peptides.

| Name | Location      | Sequence                  | Length       |
|------|---------------|---------------------------|--------------|
| pLF1 | N-terminal    | GRRRSVQWCAVSQPEATKC       | 19 amino acids |
| pLF2 | middle        | EDAIWNLLRQAQEKFHKDK       | 19 amino acids |
| pLF3 | C-terminal    | NLKKCSTSPLEACEFLRK        | 19 amino acids |
| pSCR | scrambled     | NFRTKSCPLEAKELKLCSC       | 19 amino acids |
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Table 4 | Kinetics and affinity of LF-Plg binding.

| $k_{ON}$ (M$^{-1}$s$^{-1}$) | $k_{OFF1}$ (s$^{-1}$) | $k_{ONconf}$ (s$^{-1}$) | $k_{OFF2}$ (s$^{-1}$) | $K_D$ (M) |
|--------------------------|---------------------|------------------------|---------------------|----------|
| (8.6 ± 1.5) x10$^5$     | 0.15 ± 0.02         | (9.6 ± 0.5) x10$^{-3}$ | (6.2 ± 1.4) x10$^{-3}$ | (6.9 ± 2.4) x10$^{-3}$ |

The data are averaged from four independent experiments and expressed as mean ± standard deviation. $k_{ON}$ – association rate constant of the initial complex formation; $k_{OFF1}$ – dissociation rate constant of the initial complex 1 break-up; $k_{ONconf}$ – rearrangement rate constant; $k_{OFF2}$ – dissociation rate constant of the final complex 2; $K_D$ – equilibrium dissociation constant.
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**Table 5 | LF size analysis by light scattering.**

|                      | Native LF | Biotinylated LF |
|----------------------|-----------|-----------------|
| c (mg/ml)            | 9         | 0.1 – 0.2       |
| MW-S (kDa)           | 89.1 ± 1.2| N.A.            |
| hydrodynamic radius (nm) | 4.37 ± 0.06 | 5.44 ± 0.19   |
| polydispersity (%)   | 11.9 ± 3.9| > 50 (multimodal)|
| MW-R (kDa)           | 106.0 ± 3.2| 177 ± 15       |
| aggregates > 50 nm (mass %) | N.D. | 0.8 ± 0.2 |

The data are expressed as mean ± standard deviation. MW-S – molecular mass derived from static light scattering of sample; MW-R – weight-averaged hydrodynamic molar mass derived from autocorrelation function by cumulants analysis; N.A. – not available; N.D. – not detected.
Figure Legends

Figure 1 | LF interacts with M6P/IGF2R. A, The fractions of M6P/IGF2R purified by affinity chromatography were analyzed by SDS-PAGE. The ~80 kDa Coomassie-stained gel band co-purified with M6P/IGF2R was cut for LC-MS/MS analysis. B, The fractions were analyzed by immunoblotting (IB) using mAbs to M6P/IGF2R (MEM-238), LF (4C5) and uPAR (H2). C, The human granulocyte cell lysate, obtained with 1% Triton X-100 together with 0.5% dodecylmaltoside as detergents, was subjected to immunoprecipitation on a 96-well plastic plate (50 µL/well) coated with goat anti-mouse IgG (5 µg/ml) and specific antibodies to M6P/IGF2R – mAb clone MEM-238, LF – mAb clone 4C5, uPAR – mAb clone H2; as isotype control mAb AFP-01 was used – ctr (all 5 µg/ml). The incubation was performed for 4 h at 4°C in the presence or absence of mannose 1-phosphate and mannose 6-phosphate (M1P, M6P; both 10 mmol/l). The precipitates were analyzed by SDS-PAGE followed by immunoblotting (IB) using the specific mAb to LF (LF95-4C5).

Figure 2 | LF interacts with Plg. A, LF, Plg or BSA (as a negative control), coated on wells of a plastic plate (5 µg/ml) were incubated for 4 h on ice with purified M6P/IGF2R (5 µg/ml) in the absence or presence of additional molecules: M1P and M6P (both 10 mmol/l), soluble uPAR (5 µg/ml), Plg (50 and 100 nmol/l) or LF (20 and 40 µg/ml). Bound material was analyzed by IB using the specific mAb MEM-238 to M6P/IGF2R. B, Coated Plg or BSA (5 µg/mL) were incubated 4 h on ice with LF (20 µg/ml). Before LF was added, some wells were pre-incubated for 2 h on ice with 20 µg/ml LF-derived peptides (pLF1, pLF2 or pLF3) and scrambled peptide (pSCR), or 5 mmol/l tranexamic acid (TA). Bound material was analyzed by IB using human LF specific mAb 4E2. C, The binding assay was performed as in B) but with the pre-incubation with increasing concentrations of pLF1 or scrambled peptide pSCR as indicated. D, E, Densitometric quantifications of LF binding assays. Representative immunoblot is shown in B) and C-D), respectively. Bars show the relative means with standard deviations of at least three independent experiments. The scrambled peptide pSCR served as a control and the corresponding bands were set as relative maximum of 100%. Immunoblotts were quantified with the Fuji MultiGauge software. Bands corresponding to LF binding to BSA were subtracted from the bands of LF binding to Plg bands; in addition a background correction for each individual lane was applied. The relative IC50 value was determined by a 4PL nonlinear regression curve fit, in accordance with National Institutes of Health (NIH) assay guidance. (Assay Guidance Manual Version 5.0, 2008, Eli Lilly and Company and NIH Chemical Genomics Center. Available online at: http://www.negc.nih.gov/guidance/manual_toc.html - last accessed 15.06.2011). Curve fitting was performed with MasterPlex ReaderFit, published by the MiraiBio Group of Hitachi Software Engineering America, Ltd. F, The binding assay was performed as in b) but Plg and the Plg fragments angioatin, mini-Plg and micro-Plg, or BSA, were coated onto plastic wells. Optionally, the binding assay was performed in the presence of Triton X-100 (0.1%). G, Densitometric quantification was performed as in D) and E). A representative immunoblot is shown in F) – without the detergent; p*<0.05, p**<0.005.

Figure 3 | Biochemical analysis of the LF – Plg complex. A, SPR analysis of LF – Plg binding. Duplicates of serially diluted Plg at indicated concentrations were injected over immobilized LF and the SPR response was fitted to a 1:1, two-state reaction model. Black traces – experimental response; red traces – fitted response. B, LF complexes with Plg and sM6P/IGF2R in serum. To analyze human serum, samples were diluted 20 times with PBS and subjected to immunoprecipitation on a 96-well plastic plate via goat anti-mouse IgG and specific antibodies to M6P/IGF2R (MEM-238), LF (LF95-4C5), Plg (7Pg); as an isotype control mAb AFP-01 was used (CTR). The precipitates were analyzed by SDS-PAGE followed by immunoblotting (IB) using biotinylated polyclonal Ab to LF and streptavidine HRP-conjugate. C, Analysis of LF-Plg complexes in serum. A serum sample (10 x diluted) was analyzed by BN-PAGE in the 1st dimension followed by SDS-PAGE in the 2nd dimension as described in Methods. Then, the immunoprecipitates were analysed by IB using biotinylated mAb to M6P/IGF2R (MEM-238), biotinylated polyclonal Ab to LF or mAb 7Pg to Plg followed by streptavidine HRP-conjugate or HRP-
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conjugated goat anti-mouse IgG secondary antibody. The arrows indicate the molecular weight standards. The open, grey and closed arrowheads indicate the high, intermediate and low molecular species of LF complexes.

Figure 4 | LF blocks Plg activation by uPA. A, To measure Plg binding to cells, Plg-AF647 (10 µg/ml) was pre-incubated on ice for 30 min with or without LF at the indicated concentrations. Then, THP-1 cells were incubated on ice for 20 min with the Plg preparations and the cell-surface-bound Plg was analyzed by flow cytometry. Histograms are representative of three experiments. The values show the percentage of differential mean fluorescence intensity. The anti-mouse IgG-AF647 conjugate was used as a negative control. B, Both control and M6P/IGF2R-silenced THP-1 cells were incubated at 37°C in serum free medium together with Plg (50 nmol/l) and the chromogenic plasmin substrate S-2251 (0.8 mmol/l in a 96 well plate (5x10^5 cells per well)). The reactions were performed with or without LF or aprotinin at the indicated concentrations. The absorbance change at 405 nm was monitored by using an ELISA reader. C, In a cell-free system, coated uPA (20 nmol/l) was incubated at 37°C with Plg (50 nmol/l) and substrate S-2251 (0.8 mmol/l) in a 96 well plate. The reactions were performed as in b). The experiments in b) and c) were performed at least in triplicates and results are representative for two independent experiments. D, uPA, coated as in c) was incubated with purified Plg (50 nmol) in the presence of aprotinin (2.5 µg/ml) and the indicated concentrations of LF. Bound material was analyzed by IB. Results are representative for three independent experiments. E, The Plg activation assays were performed as in c) but SK was coated instead uPA (20 nmol/l). The reactions were performed in the presence of LF as indicated. F, The Plg activation assays were performed as in c) in the in the presence of TF at the indicated concentrations. The experiments were performed at least in triplicates and results are representative for three independent experiments; p*<0.05, p**<0.005.

Figure 5 | Reconstituting effect of the anti-LF mAb on Plg activation. A, The binding assay was performed as in Fig. 2B) but in the presence of various anti-LF mAbs (3D5, 4C5, 5E2, 1D2) and anti-Plg mAbs (4Pg, 7Pg); all at 10 µg/mL. As an isotype control (CTR) mAb we used AFP-fetoprotein (10 µg/mL). B, C, Plasminogen activation assays were performed as in Fig. 4B) but Plg was pre incubated for 30 min on ice with or without indicated concentrations of anti-LF mAb.

Figure 6 | Structure comparison of LF and TF. A, Superimposed structure of LF (red; real structure; PDB code: 1LFG (19)) with TF (blue; real structure; PDB: 3QYT (62)). The superimposed part covers 441 Ca-atoms with a 1.2 Å root-mean square deviation. The arrow indicates distinctions within aminoterminal parts. B, Comparison of the amino-terminal part of LF corresponding to the antimicrobial peptide lactoferricin - LFC (red; partial structure; PDB code: 1LFG (19) residues Gly1-Ala48) with consonant part of transferrin (blue; partial structure; PDB: 3QYT (62); residues Val1-Ala51). The superimposed part covers 45 Ca-atoms with a 0,88 Å root-mean square deviation. C, Sequence comparison of the amino-terminal parts of human LF and TF. The boxed residues are identical, the green residues are positively charged.

Figure 7 | The effect of LF on patho/physiological functions of the Plg system. A, Matrigel-coated membranes of Boyden chambers were pre-incubated or not with the indicated concentrations of LF or aprotinin. Either EGF (10 ng/ml) or VEGF (10 nmol/l) were added into the lower chambers to induce migration of the TCL-598 cells or HUVEC, respectively. The cells were seeded into the upper part of the filter (1 x 10^5 cells/filter) and incubated 8 h at 37°C. The cells that invaded onto the lower side of the filters were fixed and stained with crystal violet. Representative phase contrast pictures of crystal violet stained cells are shown. B, The numbers of cells that invades onto the lower side of the filters in a were evaluated. C, Borrelia burgdorferi sensu stricto B31 were incubated in human serum (40 x diluted in PBS) in the presence of plasmin substrate S-2251 and LF. D, Streptococcus pyogenes were incubated in human serum (40 x diluted in PBS) in the presence S-2251 and LF. In c) and d) the basal level of Plg activation measured only with human serum without bacteria was subtracted. The experiments in c) and
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d) were performed at least in triplicates and results are representative for three independent experiments; p*<0.05, p**<0.005.
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Fig. 1

A

B

C

IB: M6P/IGF2R
LF
uPAR

IB: LF

Cell lysate
unbound
pH 8.2
pH 9
pH 10
pH 11.7
elution
-
-230 kD
-
-130 kD
-
-95 kD
-
-72 kD
-
-55 kD

IP: ctr
M6P/IGF2R
LF
uPAR

Ib: LF

Treated:
-
M6P
M1P
M6P
M1P

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Fig. 2

A

IB: M6P/IGF2R
- M1P M6P uPAR
- Plg LF
- 230 kD

coated
BSA LF

IB: M6P/IGF2R
- M1P M6P uPAR
- Plg LF
- 230 kD

coated
BSA LF

B

coated: BSA Plg BSA Plg BSA Plg BSA Plg BSA Plg BSA Plg BSA Plg
IB: LF
- pSCR pLF1 pLF2 pLF3 TA
- 72 kD

coated: BSA Plg BSA Plg BSA Plg BSA Plg BSA Plg BSA Plg
IB: LF
- pSCR pLF1 pLF2 pLF3 TA
- 72 kD

C

coated: BSA Plg BSA Plg BSA Plg BSA Plg BSA Plg BSA Plg BSA Plg
IB: LF
- pLF1 (μg/ml)
- 72 kD

treated:
5 10 20 40

coated: BSA Plg BSA Plg BSA Plg BSA Plg BSA Plg BSA Plg BSA Plg
IB: LF
- pSCR (μg/ml)
- 72 kD

treated:
5 10 20 40

D

LF binding to Plg (% of binding without SCR)

| treated | pSCR | pLF1 | pLF2 | pLF3 | TA |
|---------|------|------|------|------|-----|
| 20 μg/mL| 5    | 10   | 20   | 40   |

E

LF binding to Plg (% of binding)

| treated | pLF1 (μg/ml) | pSCR (μg/ml) |
|---------|--------------|--------------|
| 20 μg/mL| 5            | 10           |
| 40 μg/mL|              |              |

F

coated: BSA Plg Ang MiniPlg μPlg
IB: LF
- 72 kD

coated: BSA Plg Ang MiniPlg μPlg
IB: LF
- 72 kD
+ 0.1% Triton X-100
Lactoferrin controls plasminogen activation

Fig. 3

A

Relative Response (RU)

0 20 40 60

Time (s) 0 60 120 180

160 nM 80 nM 40 nM 20 nM 10 nM

B

IP: ctr Plg LF

IB: LF

M6P/IGF2R - 72 kD

C

1st dimension (Blue Native electrophoresis)

880 kD 440 kD 272 kD

IB

M6P/IGF2R

Plg - 230 kD

LF - 95 kD

- 72 kD

2nd dimension (SDS-PAGE)
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Fig. 5

A

Lactoferrin controls plasminogen activation

B

Cells: THP-1

C

Cells: THP-1
Lactoferrin controls plasminogen activation

Fig. 6

A

B

C

human LF: GRRRSTVQWCASQPETKCFQWQRNMRKV
human TF: VPDKTVRWCAVESHEATKCGSFRDHMKSV
Lactoferrin controls plasminogen activation

Fig. 7

A

TCL-598
no LF + LF (20 μg/ml)

HUVEC
no LF + LF (20 μg/ml)

B

| LF (μg/ml) | HUVEC | TCL-598 |
|------------|-------|---------|
| -          | 120   | 100     |
| 5          | 80    | 60      |
| 10         | 60    | 40      |
| 20         | 40    | 20      |

Aprotinin (5 μg/ml)

C

+ Borrelia burgdorferi

Plasmin activity (OD405 nm)

D

+ Streptococcus pyogenes

Plasmin activity (OD405 nm)
Lactoferrin is a natural inhibitor of plasminogen activation
Alexander Zwirzitz, Michael Reiter, Rostislav Skrabana, Anna Ohradanova-Repic, Otto Majdic, Marianna Gutekova, Ondrej Cehlar, Eva Petrovciková, Eva Kutejova, Gerold Stanek, Hannes Stockinger and Vladimir Leksa

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