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# DIPLOMARBEIT

Titel der Diplomarbeit

„A novel interaction partner of plasminogen in regulation of fibrinolysis“

Verfasser

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# 1. Abstract:

Fibrinolysis represents an essential mechanism for maintenance of the haemostatic balance. Herein, in the process regulated by a multitude of enzymes, the activation of inactive protease plasminogen to the active plasmin describes a crucial step. By resolving blood clots (thrombi), plasmin counteracts blood coagulation. Plasmin degrades fibrin polymers, which represent the main components of thrombi. In addition to clot resolving, plasmin is commonly used by cells, e.g. immune and endothelial cells, to facilitate migration. Based on its broad spectrum of activity, plasmin and its regulatory molecules not only play an important role in various physiological but also in pathological processes. In case of Alzheimer's disease, plasminogen levels as well as plasmin activity in the brain are reduced, which could play a role in development or progression of this disease. On the other hand, plasmin facilitates metastasis of cancer cells by degrading extracellular matrix components, thereby allowing tumor cells to invade tissues, evade the immune system or local nutrient limitations. In the following work a novel regulator of the fibrinolytic system is characterized. Its binding to plasminogen and functional consequences are investigated by the use of molecular biological, biochemical and cell biological methods. Ultimately, a synthetic peptide derived from this regulatory protein is presented, which might provide a therapeutic tool.

# 2. Introduction:

## 2.1. The plasminogen/plasmin system:

Plasmin (Plm) is a serine protease with broad range specificity involved in many physiological processes like fibrinolysis or re-organization of the extracellular matrix, hence defining the integrity of our connective tissues. Plm is synthesized as the inactive zymogen plasminogen (Plg) in the liver (Raum et al., 1980), and is released into the bloodstream where it is present at a concentration of about 2 µmol/L. (Cederholm-Williams, 1981) Plg can be proteolytically converted to active Plm (Robbins et al., 1967) by a variety of enzymes. For instance urokinase-type plasminogen activator (urokinase, uPA) or tissue plasminogen activator (tPA) are able to catalyze the proteolytic conversion to active Plm. Both, uPa and tPA, can be inhibited by plasminogen activator inhibitors (PAI) 1 and 2. (Schaller and Gerber) While tPA can perform its action directly, uPA has to be produced by proteolytic cleavage from its zymogen pro- (or single-chain) urokinase (pro-uPA, sc-uPA), which has only little intrinsic activity. (Pannell and Gurewich, 1987) The conversion of pro-uPA to uPA is accomplished by Plm thereby generating a positive feedback loop. (Blasi et al., 1987; Petersen et al., 1988) Since uPA is bound to the urokinase-type plasminogen activator receptor (uPAR, PLAUR, CD87) which is tethered to the cell membrane via a GPI anchor, Plg conversion by uPA is restricted to the cell surface. Moreover, free Plm is blocked by alpha 2-antiplasmin (α2AP) and alpha 2-macroglobulin, two protease inhibitors repressing its action. (Schaller and Gerber) Consequently, Plm can only be active when it is bound to cells or matrix. In addition to tPA and uPA, kallikrein (Colman, 1969), factor XIa and XIIa (Mandle and Kaplan, 1979) can directly elicit the conversion of Plg to active Plm. Upon Plg cleavage not only Plm is formed, but in addition the angiogenesis inhibitor called angiostatin is produced. (Castellino and Ploplis, 2005) Plm and its main regulators are illustrated in figure 1.

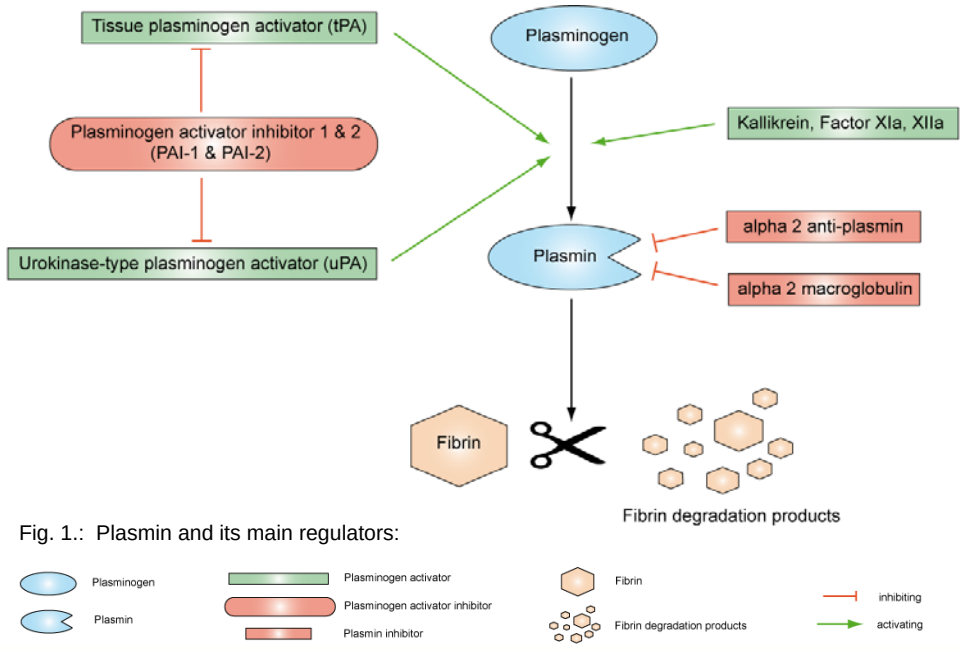



Fig. 1.: Plasmin and its main regulators:

In terms of fibrinolysis, Plm resolves blood clots formed by coagulation and therefore is the key enzyme of this central physiological event. (Ratnoff, 1948) The process of coagulation is based on a multitude of zymogens circulating in the blood stream which get activated upon blood vessel damage. (Macfarlane, 1964) Injured vessel walls trigger the release of several factors; the most prominent von Willebrand factor (vWF) binds collagen, thereby facilitating platelet adhesion. (Nyman, 1980) Von Willebrand factor in addition binds several clotting factors which trigger an amplifying cascade resulting in active thrombin which in turn converts fibrinogen to fibrin. Latter one forms a tight meshwork by polymerization, hence provoking the formation of blood clots. Consequently, the bleeding stops and wound healing is initiated. Plm on the other hand counteracts coagulation by degrading these thrombi. (Francis and Marder, 1982) Since hypo- as well as hyper-function of the fibrinolytic system might pose serious health risks, it needs to be tightly regulated.

Furthermore, Plm can trigger extracellular matrix modulation by activating metalloproteases (MMPs). (Quigley et al., 1990) These findings underline the important role Plm plays in natural cell migration and wound healing. (Chan et al., 2001; Li et al., 2003) Apart from modifying the pericellular microenvironment, the Plm system directly regulates cellular adhesion via uPAR which binds to integrins (Simon et al., 2000) and intercedes with

integrin signaling cascades. (Aguirre Ghiso et al., 1999; Wei et al., 1999) The Plg/Plm system with its major interaction partners is schematically depicted in figure 2.

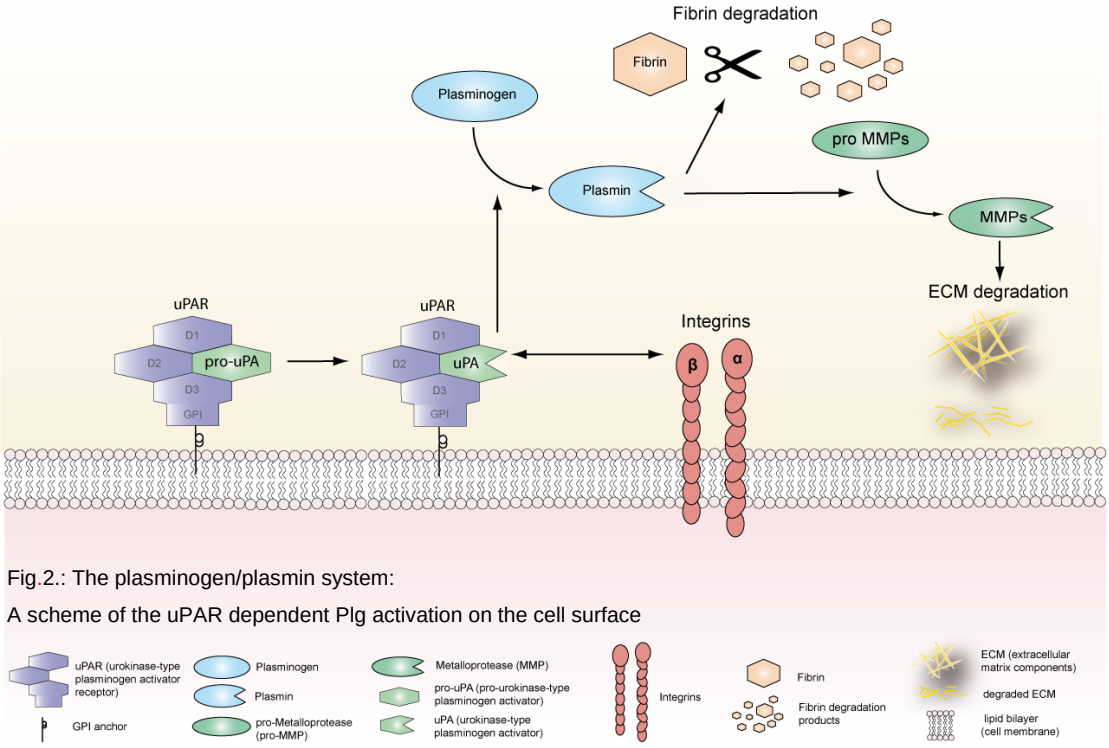



Fig.2.: The plasminogen/plasmin system:
A scheme of the uPAR dependent Plg activation on the cell surface

Evidences for engagement of the fibrinolytic system in several pathological processes evolved during the last decades. Hitch-hiking the fibrinolytic system is a prevalent feature amongst a number of bacteria (Kitano et al., 1981; Lewis et al., 1949) and viruses (Howett et al., 1978; Lazarowitz et al., 1973) which exploit the cellular machinery in order to invade host tissues. For instance, staphylococcal staphylokinase (Kowalska-Loth and Zakrzewski, 1975) and streptococcal streptokinase (Troll and Sherry, 1955) are able to activate Plg, and consequently the Plm triggered remodeling of the extracellular matrix enables bacterial migration (Eberhard et al., 1999).

Moreover, Plm is mediating inflammation either directly or through its cleavage products. For instance, fibrin degradation products are able to influence inflammation by regulating vascular permeability. (Sueishi et al., 1981; Triantaphyllopoulos, 1976)

Additionally, Plm is cleaving amyloid beta precursor protein (APP) and degrading amyloid beta peptide (Aβ), which is responsible for the formation of Alzheimer's disease related plaques in the brain. (Ledesma et al., 2000) In

fact, uPA and Plg levels decrease in an age-related manner, leading to reduced activity of brain Plm. (Aoyagi et al., 1994)

The Plm system also plays a fundamental role in cancer. Tumor cells utilize the fibrinolytic system to escape spatial nutrient supply limitations and flee from local opponents belonging to the immune system. Notably, increased Plm and uPA activity favours metastasis due to exaggerated breakdown of extracellular matrix components, thereby enabling enhanced mobilization of tumor cells. (Goldberg et al., 1990; Hearing et al., 1988) In addition, cancer cells empower themselves to metastasize from local tissues to distinct parts of the body by using components of the Plm system which interact with integrins (Simon et al., 2000) and facilitate angiogenesis (Yasunaga et al., 1989). Since Plg activation requires regulation by uPA and uPAR, the whole fibrinolytic system seems to be involved in cancer progression and metastasis. (Dass et al., 2008) In fact, the expression of some of its components (e.g. uPA or uPAR) is modulated by growth factors and cytokines. Both are over-expressed by tumor cells and can be used as prognostic tumor markers. (Hildenbrand et al., 2009; Kwaan and McMahon, 2009)

In a nutshell, the regulation of Plg conversion to active Plm plays an important role in a wide variety of physiological as well as pathological processes. Therefore, it is absolutely necessary to investigate molecules implicated in the fibrinolytic system in order to get a better understanding of the human body and its dysfunctions.

### 2.2. The mannose 6 phosphate / insulin like growth factor 2 receptor:

The cation independent mannose 6-phosphate or insulin like growth factor 2 receptor (CI-MPR, M6P/IGF2R or CD222) is a multifunctional protein, which is considered a tumor suppressor. (Gary-Bobo et al., 2007) The ubiquitously expressed 250 kDa type I transmembrane protein M6P/IGF2R binds many distinct and structurally, as well as functionally, different ligands and thus is capable of accomplishing numerous diverse tasks like protein trafficking and internalization. Over 90% of the total amount of receptor molecules is located inside the cell within cellular substructures such as vesicles, early and late endosomes and especially the Golgi apparatus, whereas only 5 to 10% are on

the cell surface. (Scott and Firth, 2004) Its major physiological role known so far is sorting proteins from the trans Golgi network, in particular targeting newly synthesized M6P-tagged enzymes to lysosomes and recycling membrane vesicles. (Ni et al., 2006) Indeed, M6P/IGF2R seems to be more efficient in endocytosis and trafficking proteins intracellularly rather than exporting them to the plasma membrane. (Hille-Rehfeld, 1995) Furthermore, M6P/IGF2R exhibits the capacity of suppressing tumor growth on the one hand by internalization, and thereby facilitating degradation of cellular growth factors, like insulin-like growth factor 2 (IGF2) (Mathieu et al., 1990), and on the other hand by activation of e.g. latent transforming growth factor beta (TGF-β) (Godar et al., 1999), therefore regulating cell proliferation and growth. (Leksa et al., 2005) In addition, some of M6P/IGF2Rs ligands like granzyme B (Motyka et al., 2000) or retinoic acid (Kang et al., 1997) were shown to induce apoptosis, thus generating strong evidence for the participation of M6P/IGF2R in the process of programmed cell death.

According to previously published data of our group, M6P/IGF2R is responsible for the elevated cleavage of uPAR. Upon silencing of M6P/IGF2R, molecules like uPA, Plg and also αVβ3 integrins accumulate at the cell surface. Hence, pericellular Plg activation is increased, which results in augmented cellular motility. (Schiller et al., 2009) Indeed, M6P/IGF2R is also involved in cell migration. (Leksa et al., 2002) Moreover, M6P/IGF2R is considered as a tumor suppressor due to its ability to control αV integrin homeostasis which is closely linked to metastasis. (Felding-Habermann et al., 2001) Another tumor suppressive feature of M6P/IGF2R is regulating the turnover of enzymes participating in degradation of extracellular matrix components. (Martin-Kleiner and Gall Troselj, 2010; Sengupta et al., 2001)

### 2.3. Lactoferrin:

Human lactoferrin (hLF, LF) is an 80 kDa glycoprotein abundantly present in secretions of all mammals and is officially denoted as lactotransferrin (LTF). It was also described as lactosiderophilin (Montreuil and Mullet, 1960) or red milk protein when discovered. (Blanc and Isliker, 1961) A recombinant form of LF is currently used in clinical trials with the commercial name talactoferrin.

(Digumarti et al., 2011) LF is especially enriched in milk, tears, saliva and the secretory granules of neutrophil granulocytes. Actually, the highest concentration of LF (up to 7 g/L) can be found in the first milk of human mothers, the colostrum. (Rodriguez-Franco et al., 2005) As a major compound of the human colostrum, LF provides a quickly established first line defense of the newborn's immune system, which serves to protect the infant from dangerous pathogens and fight against infections. (Goldman, 1977) However, LF levels progressively decrease within the first few days after birth until they even out at average concentrations between 1 – 2 g/L in mature milk after 5 months. (Reddy et al., 1977) The concentrations of LF in other body fluids range from approximately 8 mg/L in saliva, 1.4 g/L in tears to 0.5 g/L in seminal plasma. (Haupt and Baudner, 1973) In secretory granules of neutrophils LF is present to an extent of 15 µg/$10^6$ cells. (Bennett and Kokocinski, 1978)

Human LF is composed of 711 amino acids and consists of two symmetrical iron-binding lobes. (Anderson et al., 1987) It belongs to the family of transferrins and is closely related to human serum transferrin (TF) which in turn is mainly responsible for iron ($Fe^{3+}$) binding and transport within the human body. However, LF is not only able to bind $Fe^{2+}$ and $Fe^{3+}$ ions, but also other metallic cations like $Zn^{2+}$, $Cu^{2+}$ or $Mn^{2+}$. (van der Strate et al., 2001) The most prominent form, which can be found in milk and leukocytes, is positively charged and has a more basic isoelectric point (pI) of 8.7 (Moguilevsky et al., 1985) in comparison to mono- and diferric TF with pI of 5.6 and 5.2 respectively. (Hovanessian and Awdeh, 1976) If LF is loaded with two iron ions, it is called holo-lactoferrin (depicted in figure 3). Although the iron-free form of human LF called apo-lactoferrin

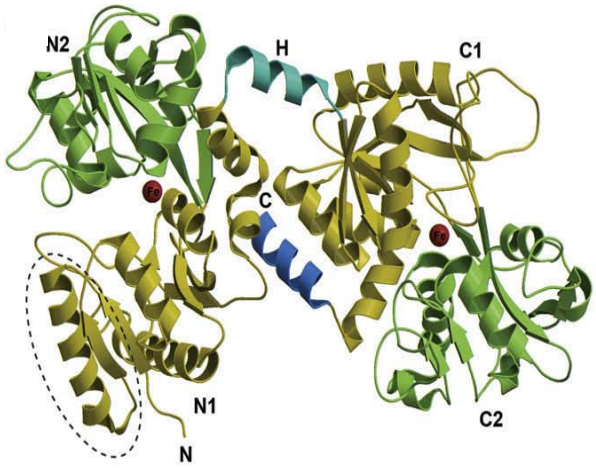



Fig. 3.: Holo-lactoferrin polypeptide fold, taken from (Baker and Baker, 2009) – N and C termini are labelled with N and C respectively. N and C terminal lobes are subdivided in 2 domains each, denoted as N1, N2 and C1, C2. The helix linker region is indicated in turquoise (H) and iron ions are depicted in red.

has a higher affinity for iron than serum TF (Ainscough et al., 1980), the latter one seems to be sufficient for iron transport since LF knockout mice show completely normal iron homeostasis. (Ward et al., 2003) Concerning the pH stability of iron-TF and iron-LF complexes, the former is less stable. TF is releasing its two iron molecules between pH 7.0 (Moguilevsky et al., 1985) and 5.5 (Mazurier and Spik, 1980), while LF is rejecting its first $Fe^{3+}$ around pH 5.0 and its second at pH 3.5. (Moguilevsky et al., 1985)

According to basic local sequence alignment algorithm (NCBI - BLAST) not only are LF and TF highly conserved among many species, but in addition human LF is nearly identical to human TF on the protein level. Moreover, the two iron-binding lobes of LF are also virtually identical to each other. These data strongly suggest that LF arose from a gene duplication, which was already evidenced. (Park et al., 1985)

The gene expression of LF is estrogen responsive, indicated by the upstream steroid factor 1 (SFRE) and estrogen response elements (ERE). Since estrogen plays a critical role throughout female's life, LF levels are reasonably regulated in this way. (Teng et al., 2002) Hormone dependent regulation of LF gene expression is additionally reflected by the fact that contraceptives influence breast milk LF levels. (Lonnerdal et al., 1980) Moreover, the expression of the LF gene has been shown to be stimulated by the innate immune system. (Li et al., 2009) Besides, another shorter isoform of LF can be generated by alternative transcription. It is called delta lactoferrin (ΔLF) and lacks the first exon which contains the 5'-untranslated region and the signal sequence which is comprised of amino acids 1-19. (Siebert and Huang, 1997) Consequently, ΔLF demonstrates different localization patterns than LF. (Goldberg et al., 2005) Moreover, its expression is altered in different breast cancer cells (Benaissa et al., 2005) probably resulting from the fact that ΔLF was shown to play a role in regulation of cell cycle progression. (Breton et al., 2004)

Once LF is ingested it is ultimately processed by several proteases within the digestive system, predominantly pepsin, trypsin and chymotrypsin. Upon proteolysis by trypsin or chymotrypsin the two iron binding lobes can be separated. (Bluard-Deconinck et al., 1978) If LF is digested by pepsin, a small bioactive fragment called lactoferricin (LFcin) is released (Kuwata et al.,

1998), which holds true for pepsin-mediated hydrolysis of bovine (Bellamy et al., 1992a) and human (Yamauchi et al., 1993) LF. LFcin (depicted in figure 4) consists of the first 45 - 50 amino acid residues cleaved from the N-terminus of LF. (Gifford et al., 2005) Yet, the actual length of LFcin is controversial throughout literature, meaning that it ranges between amino acids 1-49, 1-45, 20-47, 19-36, 20-38 of the full-length protein. Nevertheless the functionality of this bioactive peptide(s) is indisputable, which is prevalently its anti-pathogenic activity against a wide variety of bacteria, fungi and viruses. (Chapple et al., 1998; Wakabayashi et al., 2003) Indeed, LFcin seems to be even more effective than full-length LF concerning anti-pathogenicity. (Farnaud et al., 2004)

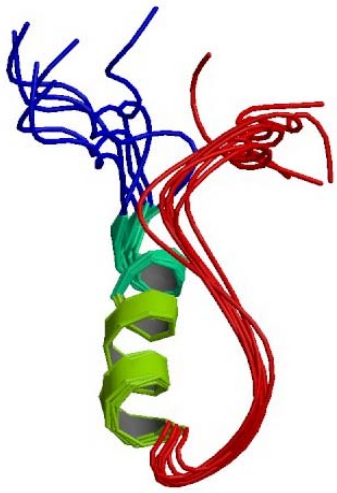


Fig. 4.: Biological assembly of human LFcin, taken from (Hunter et al., 2005) – Overlay of lowest energy conformations of human LFcin obtained from NMR spectroscopy data.

Both LF and LFcin bear potent antimicrobial activity towards various kinds of bacteria, in particular gram-negative ones like *Escherichia coli*, *Pseudomonas aeruginosa*, *Samonella typhimurium*, or *Yersinia enterocolitica*, but as well gram-positive ones like *Staphylococcus aureus*, *Streptococcus mutans, Listeria monocytogenes* or *Clostridium difficile*. (Bellamy et al., 1992a) It is a widely accepted fact that full-length LF is inhibiting the bacterial growth by means of iron sequestration (Oram and Reiter, 1968), nonetheless other studies implicated additional mechanisms. (Arnold et al., 1982) LFcin is able to effectively neutralize the major endotoxin of gram-negative bacteria, namely lipopolysaccharide (LPS). In particular, the first six amino acids (GRRRRS) are most crucial in inhibiting LPS action. (Zhang et al., 1999) Another mechanism how LFcin is exerting its antimicrobial action is impelling the permeabilization of bacterial membranes. (Aguilera et al., 1999) Recent publications revealed that an additional bioactive peptide derived from the N-terminal half of LF contains a LFcin-like structure which imparts antibacterial activity. It is called lactoferrampin (LFampin) and is comprised of sequences between amino acids 260-285 which are similar to amino acids 21-49 of the full-length protein. (Adao et al., 2011; Haney et al., 2009)

Furthermore, LF displays antiviral activity against several RNA and DNA based viruses (van der Strate et al., 2001) including Herpes Simplex Virus (HSV) (Andersen et al., 2003), Cytomegalie Virus (CMV) (Beljaars et al., 2004), human Papilloma Virus (HPV) (Mistry et al., 2007) and human Immunodeficiency Virus (HIV). (Zuccotti et al., 2007) LF prevents initial binding of the virus to host cells by competing for the docking receptors used by viruses in order to enter a cell. Consequently, these adsorbance molecules e.g. heparan sulfate or glycosaminoglycans are blocked and no longer accessible for the virus. (Andersen et al., 2003; Andersen et al., 2001) Additionally, LF and LFcin can directly bind Hepatitis C Virus (HCV). In case of rotaviruses, LF might inhibit viral replication inside host cells. (Superti et al., 1997)

Regarding antifungal activity, LF is known to inhibit the growth of e.g. Candida albicans by sequestration of iron. (Kirkpatrick et al., 1971) Obviously, LF in addition upregulates the host defense by activating neutrophils which in turn combat the fungal infection. (Ueta et al., 2001) Besides, LFcin can directly target and kill fungi via the membrane interaction as it was shown for bacteria. (Bellamy et al., 1993)

Moreover, LF is inhibiting the parasitic growth by means of iron depletion but also the iron-saturated form or N-terminal derived peptides of LF demonstrate antiparasitic activity, suggesting additional mechanisms. (Fritsch et al., 1987; Turchany et al., 1995)

Asides the antimicrobial effect, LF and LFcin demonstrate anti-tumor activity. (Yoo et al., 1997) For instance, it has been shown that LF inhibits tumor growth (Bezault et al., 1994) and tumor-induced angiogenesis. (Shimamura et al., 2004) Additionally, human LF attracts anti-carcinogenic NK cells and $CD8^+$ T lymphocytes. (Wang et al., 2000) Combined with conventional chemotherapy LF enhances positive effects on patients' treatment. (Varadhachary et al., 2004) In case of LFcin, it was shown to force tumor cells into apoptosis. (Mader et al., 2005) In general, LFcin is thought to be especially effective in accomplishing its antimicrobial and anti-tumor tasks because of its highly positive net charge. (Yang et al., 2004)

# 3. Objectives:

The aim of the study was to characterize the binding of human LF to human Plg. Via mass spectrometric analysis LF was initially revealed as a novel interaction partner of M6P/IGF2R. Since M6P/IGF2R was known to cooperate with components of the fibrinolytic system from previous studies (Leksa et al., 2002), Plg was discovered as an additional interaction partner of LF. Therefore, the identification of molecular determinants responsible for specific binding of LF to Plg obtained priority in the following work. One key mechanism how Plg and respectively Plm is binding its partners is via C-terminal lysines of target proteins. (Holvoet et al., 1986) Since LF provides a C-terminal lysine, our main focus was directed on the interaction of Plg and LF's C-terminus. Ultimately, functional consequences resulting from the interaction of the two molecules were examined.

# 4. Materials and Methods:

## 4.1. Materials:

Tris, glycine, NaCl, $NaHCO_3$, 32% HCl, 2-propanol, glycerol, acrylamid Rotiphorese Gel 30, LB-agar (Luria/Miller), BSA were purchased from Roth, Karlsruhe, Germany. Agarose LE was bought from Biozym Scientific, Hessisch Oldendorf, Germany. Protease inhibitor cocktail tablets were obtained from Roche Applied Science, Penzberg, Germany. The chromogenic Plg substrate S-2251 was obtained from Coachrom Diagnostica, Vienna, Austria. Aminocaproic acid (6-ACA), imidazole, tricine, hydroxylamine hydrochloride, methanol, acetic acid, tetramethylethylenediamine (TEMED), 10 % ammonium persulfate (APS), RPMI 1640, LF from human milk L0520 were purchased from Sigma-Aldrich, St.-Louis, MO, USA. Ethidiumbromide as well as all DNA modifying enzymes and related buffers were purchased from Fermentas (ThermoFisher Scientific), Waltham, MA, USA. The LF derived peptides were synthesized by Peptide 2.0, Chantilly, VA, USA. Plg (purified Glu-Plg) was obtained from Technoclone, Vienna, Austria. Biotin was obtained from Pierce and trypsin-EDTA, L-glutamine, penicillin and streptomycin from Invitrogen, Carlsbad, CA, USA. The PVDF membranes were purchased from Millipore, Billerica, MA, USA. Human LF cDNA NM_002343.3 (SC118685) in the pCMV-XL5 vector was obtained from OriGene, Rockville, MD, USA

## 4.2. Buffers:

LB medium: 40 g/L LB agar – autoclave!
50x TAE buffer: 2 M Tris.HCl, 0.05 M EDTA + 57.1 ml Glacial acetic acid / 1 L
1x TAE buffer: 100 ml 50x TAE + 900 ml $dH_2O$
5x KCM buffer: 0.5 M KCl, 0.15 M $CaCl_2$, 0.25 M $MgCl_2$
Miniprep buffer P1: 50 mM Glucose, 25 mM Tris.HCl, 10 mM EDTA, pH 8.0
Miniprep buffer P2: 0.2 M NaOH, 1% (w/v) SDS
Miniprep buffer P3: 5 M Potassium acetate (60ml), 11.5ml Glacial acetic acid ad 100 ml with $dH_2O$, pH 5.5

Lysis buffer: 20 mM Tris.HCl, 140 mM NaCl, pH 8.2, 1% Nonidet P-40, add 1 protease inhibitor tablet (Roche cOmplete Protease Inhibitor Cocktail) per 30-50 ml

Anode buffer for BN-PAGE: 25 mM Imidazole.HCl pH 7.0

Cathode buffer (CB) for BN-PAGE: 50 mM Tricine, 7.5 mM Imidazole.HCl pH 7.0

Deep blue cathode buffer for BN-PAGE: CB + 0.02% CBB G250

Slightly blue cathode buffer for BN-PAGE: CB + 0.002% CBB G250

Sample buffer for BN-PAGE: 5 % Coomassie Brilliant Blue in 0.5 M 6-ACA

10x running buffer for BN-PAGE: 250 mM Tris.HCl, 1920 mM Glycine

10x Western blotting buffer for BN-PAGE: 250 mM Tris.HCl, 1920 mM Glycine

Coupling buffer: 0.1 M $NaHCO_3$, 0.5 M NaCl

Washing buffer: 20 mM Tris.HCl pH 9.3

Elution buffer: 20 mM Tris.HCl pH 11.7

Running gel buffer: 1 M Tris.HCl, pH 8.8, 0.27 % SDS

Stacking gel buffer: 1 M Tris.HCl, pH 6.8, 0.8 % SDS

10x running buffer (10x ELFO): 250 mM Tris.HCl, 1920 mM Glycine, 0.1 % SDS - don’t adjust pH, it will be 8.3 after dilution!

1x running buffer (1x ELFO): 100 ml 10x ELFO + 900 ml $dH_2O$

10x Western blotting (WB) buffer: 250 mM Tris.HCl, 1920 mM Glycine.

1x WB buffer: 100 ml 10x WB buffer + 200 ml methanol ad 1 L with $dH_2O$

10x TBS: 20 mM Tris.HCl, 1.38 M NaCl, pH 7.6

TBST: 100 ml 10x TBS ad 1 L with $dH_2O$ + 1 ml Tween 20®

FACS buffer: 1x PBS, 1 % BSA, 0.02 % $NaN_3$

Solutions for silver staining:

- Fixing solution: 30% ethanol, 10% acetic acid
- Stop solution: 5% acetic acid
- Ethanol wash: 10% ethanol

Biotin labeling buffer: 0.1 M NaHCO3, 0.1 M NaCl pH 8.4

### 4.3. Antibodies:

The biotinylated rabbit anti-human LF polyclonal antibody (LFb, ab25811) was purchased from Abcam and mouse anti-human LF monoclonal antibodies

(mAbs) 1D5, 4C5, 3D5 and 4E2 (each used 1:1000) were kindly provided by Dr. Otto Majdic from the Institute of Immunology, Centre for Pathophysiology, Infectiology and Immunology, Medical University Vienna. The mouse anti-human Plg mAb (clone 7Pg) was obtained from Technoclone, Vienna. Anti-mouse IgG antibody conjugated to horseradish peroxidase (HRP) produced in rabbit (used 1:50000) was bought from Sigma Aldrich. The Streptavidin-biotinylated horseradish peroxidase complex (used 1:3000) was purchased from GE Healthcare, UK.

### 4.4. Cell culture:

The human monocytic cell line THP-1 from ATCC and the human kidney epithelial tumor cell line TCL-598 were a gift from the Novartis Research Institute, Vienna, Austria. The cells were cultivated in RPMI 1640 medium with 10% heat inactivated fetal calf serum (FCS), supplemented with 2 mM L-glutamine, 100 units/ml penicillin and 100 units/ml streptomycin. For the Plg activation assay the cells were seeded in serum-free medium in order to avoid bovine serum Plg contamination. The cells were maintained at 37°C with 5 % $CO_2$ and passaged twice a week using trypsin-EDTA.

### 4.5. Cloning of human LF:

Bacterial cells:

*E.Coli* DH5$\alpha$: fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17

Cloning strategy:

The cDNA of human LF NM_002343.3 (SC118685) in the pCMV-XL5 vector was obtained from OriGene, Rockville, MD, USA. The sequencing reactions and synthesis of primers were accomplished by Eurofins MWG, Ebersberg, Germany. Three forward primers (fw) were designed to introduce a HindIII restriction enzyme cleavage site on the N-terminal end of the generated PCR products. Forward primer 3 was used to produce ΔLF, another isoform of LF which lacks the first 44 amino acids of the full length protein. Five reverse

primers (rev) were designed to introduce a NotI cleavage site at the C-terminal end, and one reverse primer (rev2) to introduce a point mutation and thus changed the C-terminal amino acid from lysine to threonine. Three of the reverse primers should have generated truncated versions of the protein. The primer sequences are highlighted in appendix 1 (section 8.1. DNA sequence of human LF) and listed in table 1 with restriction sites underlined. The cDNA after PCR amplification were to be subcloned into pGEMT (or pBKS-) and afterwards ligated into the pBMN-Z vector backbone (pBMN), which is suitable for expression in mammalian cells.

### 4.6. Transformation of chemically competent bacteria:

Heat shock transformation:

- Mix 10-100 ng of plasmid with 100 µl of $RbCl_2$ competent DH5$\alpha$
- Incubate 30' on ice
- Heatshock for 90 sec. on 42°C and put back on ice for 2'
- For recovery, add 300 µl fresh LB and incubate for 1 h at 37°C while shaking
- Plate on LB + amp (100 µg/ml) & incubate o/n at 37°C
- Inoculate a single colony in 3-5 ml LB + amp o/n at 37°C while shaking

KCM transformation:

- Mix 80 µl KCM competent bacteria + 20 µl 5x KCM Buffer + 1-10 ng plasmid
- Incubate 10' on ice
- Incubate 10' at RT
- Add 1 ml LB and incubate 1 h at 37°C while shaking
- Plate 100 µl on LB + amp (100 µg/ml) and incubate o/n at 37°C

### 4.7. Plasmid preparation (mini prep):

- Inoculate a single colony in 3-5 ml LB + amp (100 µg/ml) o/n at 37°C constantly shaking
- Spin down bacteria for 2' at 14.000 rpm

- Add 200 µl buffer P1 + 20 µg/ml RNase A and resuspend
- Add 200 µl buffer P2, mix by inverting the tube 4-6x & incubate 5' at RT
- Add 200 µl buffer P3, mix by inverting the tube 4-6x
- Spin 15' at 14.000 rpm
- Transfer the supernatant to a new tube
- Add 500 µl isopropanol and mix by inverting the tube 4-6x
- Spin 20' at 14.000 rpm and decant supernatant
- Wash pellet with 70% EtOH
- Spin 3' at 14.000 rpm & remove supernatant
- Air-dry and resuspend in 30-50 µl $dH_2O$

### 4.8. Maxi prep:

Dilute overnight culture 1:100 and grow at 37°C o/n while shaking. Then follow NucleoBond® Xtra (Macherey-Nagel) protocol.

### 4.9. PCR reactions:

Phusion® polymerase protocol:

| | |
|---|---|
| $dH_2O$ | 11.8 µl |
| 5x HF-buffer | 4 µl |
| 10 mM dNTPs | 0.4 µl |
| 10 µM primer fw | 1 µl |
| 10 µM primer rev | 1 µl |
| template DNA (20 ng/µl) | 1 µl |
| Phu polymerase (2 u/µl) | 0.2 µl |
| DMSO | 0.6 µl |

| | | | |
|---|---|---|---|
| denaturating | 98°C | 30'' | |
| denaturating | 98°C | 10'' | 35 cycles |
| annealing | 64°C | 30'' | |
| elongation | 72°C | 35'' | |
| final extension | 72°C | 7' | |

[DMSO is added to reduce unspecific binding and can be omitted]

Taq polymerase protocol:

| | |
|---|---|
| 10x buffer | 5 µl |
| 10 mM dNTPs | 1 µl |
| 10 µM primer fw | 2.5 µl |
| 10 µM primer rev | 2.5 µl |
| template DNA (270 ng/µl) | 1 µl |
| Taq polymerase (5 u/µl) | 0.5 µl |
| $MgCl_2$ (25 mM) | 4 µl |
| $H_2O$ | 33.5 µl |

| | | | |
|---|---|---|---|
| denaturating | 98°C | 2' | |
| denaturating | 98°C | 30'' | 35 cycles |
| annealing | 64°C | 30'' | |
| elongation | 72°C | 2.5" | |
| final extension | 72°C | 10' | |

Touchdown PCR protocol:

Reaction mix is identical to Phusion® polymerase protocol. Annealing temperature is decreased by 1°C every 3 cycles for 12 cycles, and then run 25 cycles with 66°C.

| | | |
|---|---|---|
| denaturating | 98°C | 30'' |
| denaturating | 98°C | 10'' |
| annealing | 70 - 67°C | 30'' |
| elongation | 72°C | 35'' |
| final extension | 72°C | 7' |

### 4.10. Agarose gel electrophoresis:

For the analysis of DNA, 0.8% agarose gels were used in the 'PerfectBlue Gelsystem Mini M' apparatus from Peqlab, Germany.

- Mix 0.64 g agarose + 80 ml 1x TAE buffer
- Heat 2-3 min in the microwave
- Let cool down, add 3 µl EtBr (10 mg/ml) and pour the gel

The gel was run constantly at 120 V for 20-45 min, depending on the size of observed fragments.

### 4.11. Elution of PCR products from an agarose gel:

According to instructions of PROMEGA Wizard SV gel & PCR clean-up kit. The principle of the kit is based on the DNA binding capacity of membranes within special tubes. Centrifugation removes melted agarose and other contaminating molecules like proteins for example, while DNA remains bound to the membrane and can be eluted as a final step.

### 4.12. Ligation reactions:

Calculate amount of insert:

$$\text{ng of insert} = \frac{\text{ng vector} \times \text{bp insert}}{\text{bp vector}}$$

x = (50 ng pBKS x 2430 bp insert) /2950 bp pBKS = 41 ng
Use a 10:1 ratio (recommended 1:10 to 10:1)

Reaction mix:

| | |
|---|---|
| 5x ligation buffer | 4 µl |
| vector | 50 ng |
| insert | x ng |
| T4 ligase (5 u/µl) | 1 µl |
| $dH_2O$ | ad 20 µl |

- Put on 4°C (recommended 12 - 16°C) o/n
- Transform DH5α with 5 or 10 µl ligation mixture
- Blue/white screening for positive clones:
    - add 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-gal per plate ½ h before plating the bacteria, incubate plate o/n at 37°C
- Pick white colonies & inoculate in 5 ml LB+amp o/n at 37°C while shaking

### 4.13. Restriction enzyme digestion:

Incubate the following reaction mix for 2 h at 37°C:

| | |
|---|---|
| 10x buffer R | 2 µl |
| DNA | 1 µg |
| 10 u/µl restriction enzyme 1 (HindIII ) | 0.5 µl |
| 10 u/µl restriction enzyme 2 (NotI ) | 2 µl |
| $dH_2O$ | ad 20 µl |

The amount of restriction enzyme used was calculated by the DoubleDigest™ tool on the Fermentas homepage. The samples were directly loaded on an agarose gel and the corresponding bands were cut out and eluted.

### 4.14. In vitro proteolysis of human LF:

Coupling of proteases to beads:

- Incubate CNBr-activated sepharose beads (1 ml) in 3 mM HCl (10 ml) for 15 min. at RT
- Wash 2-3 times with 1x Coupling buffer (~20 ml)
- Incubate beads in a 2 ml Eppendorf tube with 1 ml protease (2 mg/ml) in 1x Coupling buffer for 2 h at RT permanently shaking
- Stop the coupling reaction by blocking remaining active groups with 50 mM glycine for 1 h at RT constantly shaking
- The beads can be stored in PBS or in FACS buffer (long-term storage)

Enzymatic digest:

- Add 50 µg LF to 100µl (protease coupled) beads and add 850 µl of 0.035 M NaCl or PBS in a 2 ml Eppendorf tube
- Adjust pH to enzymes' optimum (pepsin: pH ~2-3, trypsin: pH~8)
- Incubate at 37°C while constantly shaking
- Take aliquots at indicated time points, readjust pH and store at -20°C
- Add 10 µl 4x non-reducing SDS sample buffer to 30 µl of aliquots and use for SDS-PAGE

### 4.15. Design of LF-derived peptides:

I designed 4 peptides derived from human LF. Peptide #1 (pLF1) is designed in order to mimic human LFcin and therefore it is comprised of the amino acids 20-38 of human LF. The peptide #2 (pLF2) is built up from the amino acids 283-301 of human LF. It contains a C-terminal lysine which could possibly be exposed upon trypsin digestion of LF. Furthermore, peptide #2 covers parts of human LFampin. The peptide #3 (pLF3) is created to encompass the very C-terminus of human LF, so it is made up of the last 19 amino acids – 693-711. Peptide #4 (pLF4) is a scrambled peptide which serves as control. It is comprised of the same amino acids as peptide #3 but randomly arranged. The peptides were created in such a way that they show a similar secondary structure. All the peptides were subjected to the consensus secondary structure prediction server NPS@ of the Institute of biology and chemistry of proteins (ICBP), Lyon, France and the PEP-FOLD de novo peptide structure prediction server. The peptides 1-3 are highlighted in appendix 2 (section 8.2. protein sequence of human LF) and listed in table 2.

### 4.16. Cell lysis:

- Lysis works best with approximately $1.5x10^6$ cells per ml
- Wash cells with PBS (centrifuge suspension cells at 2000 rpm for 5 min)
- Lyse cells in 75 µl lysis buffer 20 min on ice
- Remove debris by spinning down the lysate at 13000 rpm for 2 min
- Transfer the supernatant to a new tube and store at –20°C or use directly

### 4.17. Purification of M6P/IGF2R from cell supernatants:

- Anti M6P/IGF2R antibody (MEM238 or MEM240) was coupled to sepharose CNBr beads as described for proteases in 4.14, which were afterwards filled in a column
- Wash column 2x with PBS
- Load the lysate (see 4.16.) of THP-1 cells 3x serially

- Apply 1 ml washing buffer (pH 9.3)
- Apply 2x 1 ml elution buffer (pH 11.7)
- Apply 1 ml elution buffer the 3rd time + resuspend column with a cut tip
- Apply 2 ml elution buffer
- Wash once with PBS and once with FACS buffer
- Store the column in FACS buffer and freeze the fractions

Aliquots (75 µl) were taken for electrophoresis after each washing or elution step.

### 4.18. Binding assays:

- Dilute all molecules in PBS
- Coat Falcon® flexible 96-well PVC plates (Becton Dickinson) with 250 ng (50 µl of 5 µg/ml) protein at 37°C for 2 h
- Add 50 µl 2 % BSA to yield a final concentration of 1 % BSA in order to block free binding sites of the wells for 1 h at RT
- Wash the wells 4 - 5 times with 1x PBS
- [In competition binding assays: dilute assayed molecules in PBS with 0.1% Triton X-100 and optionally pre-incubate additional (competing) molecules for 30 min on ice]
- Add 1 µg (50 µl of 20 µg/ml) or indicated concentrations of assayed molecules to the wells and incubate from 2 h to overnight at 4°C
- Wash the wells 4 - 5 times with chilled 1x PBS
- To release the bound molecules, add 10 µl of 1x non-reducing SDS-PAGE sample buffer to the wells
- Use either directly for SDS-PAGE or store at -20°C

### 4.19. SDS polyacrylamide gel electrophoresis (SDS-PAGE):

Electrophoresis was performed with the ‘PerfectBlue Dual Gel System Twin’ apparatus from Peqlab, Germany.

- Mix the ingredients according to the table below in order to yield the desired quantity and percentage of running gels
- Add 10% APS and TEMED in the end to start the polymerization reaction

| | | | | | |
|---|---|---|---|---|---|
| Total volume | 10.00 | 20.00 | 30.00 | 40.00 | ml |
| 1% | 1 gel | 2 gels | 3 gels | 4 gels | |
| Acrylamid | 0.33 | 0.66 | 0.99 | 1.32 | ml |
| Tris buffer 8.8 | 3.75 | 7.50 | 11.25 | 15.00 | ml |
| H20 | 5.92 | 11.84 | 17.76 | 23.68 | ml |
| APS 10% | 100.00 | 200.00 | 300.00 | 400.00 | µl |
| TEMED | 8.00 | 16.00 | 24.00 | 32.00 | µl |

6%

| | | | | | |
|---|---|---|---|---|---|
| Acrylamid | 1.98 | 3.96 | 5.94 | 7.92 | ml |
| Tris buffer 8.8 | 3.75 | 7.50 | 11.25 | 15.00 | ml |
| H20 | 4.27 | 8.54 | 12.81 | 17.08 | ml |
| APS 10% | 100.00 | 200.00 | 300.00 | 400.00 | µl |
| TEMED | 8.00 | 16.00 | 24.00 | 32.00 | µl |

7.5%

| | | | | | |
|---|---|---|---|---|---|
| Acrylamid | 2.48 | 4.95 | 7.43 | 9.90 | ml |
| Tris buffer 8.8 | 3.75 | 7.50 | 11.25 | 15.00 | ml |
| H20 | 3.78 | 7.55 | 11.33 | 15.10 | ml |
| APS 10% | 100.00 | 200.00 | 300.00 | 400.00 | µl |
| TEMED | 8.00 | 16.00 | 24.00 | 32.00 | µl |

10%

| | | | | | |
|---|---|---|---|---|---|
| Acrylamid | 3.30 | 6.60 | 9.90 | 13.20 | ml |
| Tris buffer 8.8 | 3.75 | 7.50 | 11.25 | 15.00 | ml |
| H20 | 2.95 | 5.90 | 8.85 | 11.80 | ml |
| APS 10% | 100.00 | 200.00 | 300.00 | 400.00 | µl |
| TEMED | 8.00 | 16.00 | 24.00 | 32.00 | µl |

12.5%

| | | | | | |
|---|---|---|---|---|---|
| Acrylamid | 4.13 | 8.25 | 12.38 | 16.50 | ml |
| Tris buffer 8.8 | 3.75 | 7.50 | 11.25 | 15.00 | ml |
| H20 | 2.13 | 4.25 | 6.38 | 8.50 | ml |
| APS 10% | 100.00 | 200.00 | 300.00 | 400.00 | µl |
| TEMED | 8.00 | 16.00 | 24.00 | 32.00 | µl |

15%

| | | | | | |
|---|---|---|---|---|---|
| Acrylamid | 4.95 | 9.90 | 14.85 | 19.80 | ml |
| Tris buffer 8.8 | 3.75 | 7.50 | 11.25 | 15.00 | ml |
| H20 | 1.30 | 2.60 | 3.90 | 5.20 | ml |
| APS 10% | 100.00 | 200.00 | 300.00 | 400.00 | µl |
| TEMED | 8.00 | 16.00 | 24.00 | 32.00 | µl |

17.5%

| | | | | | |
|---|---|---|---|---|---|
| Acrylamid | 5.78 | 11.55 | 17.33 | 23.10 | ml |
| Tris buffer 8.8 | 3.75 | 7.50 | 11.25 | 15.00 | ml |
| H20 | 0.48 | 0.95 | 1.43 | 1.90 | ml |
| APS 10% | 100.00 | 200.00 | 300.00 | 400.00 | µl |
| TEMED | 8.00 | 16.00 | 24.00 | 32.00 | µl |

- Pour the solution in the casting chamber
- Overlay the gel with some drops isopropanol
- Let polymerize for 20 min
- Pour off isopropanol and get rid of remaining alcohol with a thin filter paper
- Prepare the stacking gel solution by mixing: (recipe for 1 gel)
    - 0.66 ml acrylamid,
    - 0.63 ml stacking gel buffer
    - 3.66 ml $dH_2O$

  (can be done in parallel with mixing the running gel)
- Then add 50 µl 10 % APS and 5 µl TEMED per stacking gel

- Pour the stacking gel onto the running gel and insert a comb with the appropriate number of wells while avoiding formation of air bubbles underneath the comb
- Let polymerize for 20 min, then remove the comb and clean the wells properly
- Place the casting chambers into the running device and fill up with 1x ELFO buffer, then the gel is ready for loading
- Boil the samples for 2 - 5 min at 95°C before loading

Run the gel constantly at 90 V, until the samples reach the running gel (approximately 20 - 30 min), then increase the voltage to 140 V until the running front reaches the end of the gel

### 4.20. Blue native polyacrylamide gel electrophoresis (BN-PAGE):

BN-PAGE was executed according to (Schagger and von Jagow, 1991).

Mix the ingredients according to the recipes depicted below:

| | Stacking Gel | Gradient - Running Gel | |
|---|---|---|---|
| | 3% | 3% | 13% |
| AA 30% | 1.2 ml | 1.93 ml | 8.73 ml |
| 3x gelbuffer | 4 ml | 6.43 ml | 5.23 ml |
| Glycerol | - | - | 3.18 g |
| $H_2O$ | 6.65 ml | 11.5 ml | 2 ml |
| APS 10% | 110 µl | 107 µl | 78.6 µl |
| Temed | 10 µl | 10.8 µl | 7.86 µl |

Glycerol is added to the 13% gel-mixture to establish the gradient. It guarantees a better blending of the two gel-mixtures within the gradient mixer. The 3-13% gradient gel was prepared with a gradient mixer (lower percentage in back, higher percentage in front chamber), a 3% stacking gel was poured on top. After polymerization, it was placed in the running apparatus and equilibrated at 4°C in the cold room. Anode buffer was poured into the lower chamber and any remaining air bubbles were removed with the help of a syringe. Then the slots were slightly filled with (colorless) cathode buffer and

samples were loaded in the cold room. The upper chamber was filled with deep blue cathode buffer and electrophoresis was conducted at 80 V for 4 h until the samples had reached the running gel at least. Then the voltage was set to 120 V overnight. In the next morning, deep blue cathode buffer was exchanged to slightly blue cathode buffer until the run was completed (another 2-4 h). The individual lanes were cut out and either directly used in a 2nd dimension SDS-PAGE or frozen. As marker, ferritin (dimer at 880 kDa and monomer at 440 kDa) and jack bean urease (trimer at 272 kDa) were used. In case of 2nd dimension SDS-PAGE (see figure 5), the corresponding lane was placed on top of a common SDS-PAGE gel with a specially modified comb without single slots and the gel was run as described in section 4.19.

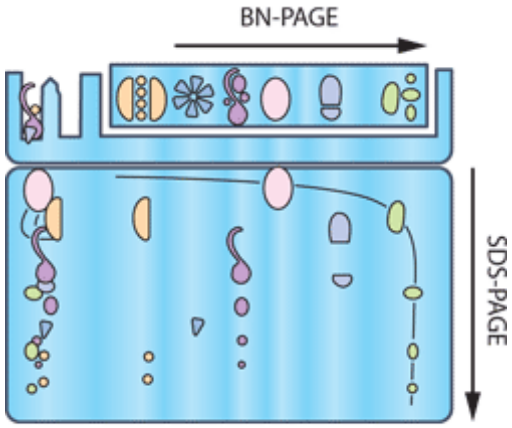



Fig. 5. BN-PAGE 2nd dimension scheme, taken from Science Signaling, 25 July 2006 Vol 2006, Issue 345 – Individual BN-PAGE gel stripes were cut out, rotated 90° counterclockwise and placed on top of the 2nd dimension SDS-PAGE gel, which separates individual proteins from their conserved protein complexes.

### 4.21. Western blot:

Western blotting was performed with the 'PerfectBlue Semi-Dry Electro Blotter' from Peqlab, Germany and the Immobilon-P™ PVDF Membranes from Millipore, USA.

- Soak the PVDF membrane in methanol (MeOH) for 1 min
- Pre-wet the membrane and 2 pieces of 3 mm Whatman filter papers in 1x WB buffer
- Assemble blotting sandwich in the following manner (anode - cathode):
    - 3 mm Whatman filter paper (anode)
    - PVDF membrane
    - Gel
    - 3 mm Whatman filter paper (cathode)
- Wipe out air bubbles carefully by rolling a plastic pipette

- Close the apparatus, tighten the screws evenly moderate and run with 20 V limited to 400 mA for 1 h
- Block free binding sites on the membrane with 5% non-fat dry milk for 1 h at RT while constantly shaking
- Rinse the membrane once and wash it 2 - 3 times with TBST for 5 min

Immunodetection:

- Incubate the membrane with an adequate primary antibody for 1 h at RT while constantly shaking
- Wash the membrane 3 times for at least 5 min with TBST
- Incubate the membrane with a secondary antibody HRP-conjugate suitable to the primary antibody for 1 h at RT while constantly shaking
- Wash the membrane 3 times for at least 5 min with TBST
- Place the membrane within a plastic foil or bag, add a 1:1 mix of substrate and luminol enhancer from the 'ChemiGlow® West Chemiluminescence Substrate Kit' (from Cell Biosciences - Santa Clara, California)
- Distribute the solution well all over the membrane and get rid of excess liquid and any air bubbles by wiping the foil carefully with a paper towel
- Analyze the Western blot with the Fujifilm ImageQuant LAS-4000 CCD camera system and Multi-Gauge software

### 4.22. Silverstaining of SDS-PAGE gels:

(According to Pierce® Silver Stain Kit for Mass Spectrometry)

- Wash a gel twice with $dH_2O$ water for 5 min
- Decant water and incubate the gel twice for 15 min at RT in the fixing solution
- [The gel may remain in fixing solution overnight without affecting the stain performance]
- Wash the gel twice with ethanol and twice with $dH_2O$ for 5 min each
- Just before use, prepare sensitizer working solution (1 part Silver Stain Sensitizer + 500 parts $dH_2O$)

- Incubate the gel in sensitizer working solution for exactly 1 min, then wash twice with $dH_2O$ for 1 min
- Mix 1 part Silver Stain Enhancer with 100 parts Silver Stain, immediately add it to the gel and incubate the gel for 5 min
- Quickly wash the gel twice with $dH_2O$ for 20 seconds
- Immediately add developer working solution (1 part Silver Stain Enhancer with 100 parts Silver Stain Developer) and incubate until protein bands appear (optimal signal vs. background 2 - 3 min)
- When the desired band intensity is reached, replace developer working solution with stop solution. Wash the gel briefly, then replace acetic acid with $dH_2O$ and incubate for 10 min

### 4.23. Dot far Western blot:

- Soak a piece of PVDF membrane for several seconds in MeOH
- Rinse membrane with 1x WB buffer
- Assemble the dot blotting sandwich in the following manner:
    - PVDF membrane (top)
    - 1 mm Whatman paper
    - 3 mm Whatman paper
    - paper towel (bottom)
- Directly apply samples in a small volume (0.5 - 2 µl) with a pipette
- Let the membrane bind the protein/peptide samples for 5 - 15 min but do not let the membrane dry completely.
- Block remaining binding sites with 5 % non-fat dry milk for 1 h at RT while constantly shaking
- Wash the membrane 3 times for 5 min at least with TBST

[Skip the following 2 steps when performing a Western dot blot]

- Incubate the membrane with the assay molecule solution (1 µg/ml in TBST) for 1 – 2 h or o/n at 4°C while constantly shaking
- Wash the membrane 3 times for at least 5 min with TBST
- Continue with immunodetection (of bound assay molecules) as described in 4.21. Western blot

### 4.24. Biotin-labeling of antibodies:

- Dialyze 1 mg purified antibody using biotin labeling buffer at 4°C o/n
- Add 100 µg biotin (dissolved in DMSO) per mg of antibody
- Incubate 1.5 h at RT while rotating
- Stop coupling reaction by addition of 10% (of total volume) 1.5 M hydroxylamine pH 8.5
- Remove unbound biotin by dialysis in PBS at 4°C o/n
- Add a preservative like 0.02% $NaN_3$ (inhibits HRP) or 0.01% thimerosal

### 4.25. Plg activation assay:

- Seed 50.000 TCL cells/well in serum-free RPMI 1640 in a 96-well plate 24h before the assay
- Incubate 100 nM Plg, 5 µg/ml α2AP, 1.6 mM chromogenic Plg substrate (S-2251) and indicated assayed molecules (10 nM TA, 20 µg/ml LF, pLFs) together for 30 min on ice
- Wash the cells 2x with serum-free medium
- Add 50 µl of serum-free medium and 50 µl of the incubated mix to each well (avoid air bubbles!)
- Measure absorption at 405 nm every hour using an enzyme-linked immunosorbent assay reader (Mithras LB 940 platereader from Berthold, Bad Wildbad, Germany)

[Mithras platereader can heat up to 37°C, thus keep the plate inside while measuring every hour, 20 mM HEPES has to be included in medium]

### 4.26. Quantification of Western blotting membranes:

Densitometric quantification of Western blots was done with the Fujifilm Multigauge software. Rectangles of exactly same size were drawn close to the boarders of individual bands. In addition, one rectangle per lane was used for detection of background signal. The corresponding background signal was subtracted from individual bands. From this value the negative control bands (always BSA), if present, were subtracted to yield the final quantity value. The

amount of emitted chemiluminescence signals was measured in arbitrary units (AU) which represent the relative density value of a band.

### 4.27. Statistical analysis:

All experiments were performed at least three times≥(n3). Statistical analysis was done in Microsoft Excel. For the detection of outliers I used 2 widely approved tests: first, the Grubbs outlier test, and second, the Dean and Dixons Q-test. Both tests considered the same data points as outliers, which therefore have been excluded from further calculations. The data are expressed as mean values ± S.E.M. (standard error of the mean). Significance was assessed via one-way ANOVA followed by Tukeys HSD test. P values less than * 0.05, ** 0.01, *** 0.001 were considered as significant or highly significant, respectively.

# 5. Results:

## 5.1. Preliminary data:

Preliminary data of our group identified LF as a binding partner of M6P/IGF2R. Additionally, they indicated that human LF bound to Plg but the exact binding site remained unknown. Since Plg is very well known for preferably binding C-terminal lysines, we assumed that the attachment to LF which offers a lysine at its C-terminus, probably occurred in an analogous manner.

## 5.2. Cloning of LF mutant forms:

In order to localize a decisive Plg binding region in LF, a first step was to clone a C-terminal lysine to threonine mutant and several truncated forms of LF, which were supposed to be subsequently analyzed by biochemical methods. Plg binds many of its known binding partners via interaction with their C-terminal lysines. Should it be with LF as well, the mutation changing LF's C-terminal amino acid lysine to threonine would delete the corresponding interaction platform. Since the C-terminal lysine of LF is encoded by AAG, a single point mutation, introduced by PCR site-directed mutagenesis, to ACG was sufficient to change lysine (K) to threonine (T). If the C-terminal lysine was not responsible for interaction with Plg, truncated LF variants would be a backup to narrow down the Plg binding region. Albeit I was able to clone the desired PCR products (~2200 bp for recombinant full-length LF and LF C-terminal K to T mutant; ~1400bp for truncated LF) into subcloning vectors e.g. pGEMT-easy (see Fig. 5.2.), I could rarely detect colonies after plating of bacteria transformed with LF variants ligated into the pBMN expression vector backbone. Even if colonies were growing, I could not extract any mammalian expression vector (pBMN) containing LF DNA of correct corresponding size. Since bacterial promoters might be leaky, functional LF which is able to suppress bacterial growth might have been produced, thereby killing positive clones. In order to cope with this problem and compensate for one of LF's major antibacterial mechanisms, namely the sequestration of iron, I generated

agar plates providing this essential nutrient in excess. However, no colonies were detectable.

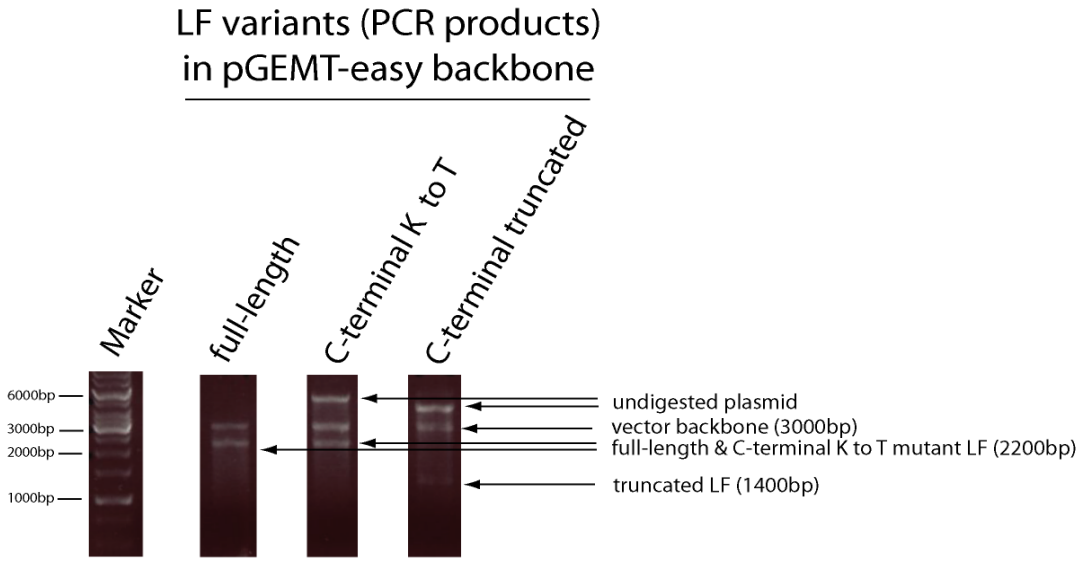



**Figure 5.2. Digest of LF variants ligated into pGEMT-easy backbone:**
Double restriction enzyme digest (HindIII + NotI) of plasmids derived from positively selected clones after transformation of *E.Coli DH5α* with LF PCR products ligated into pGEMT-easy vector backbone (~3000 bp).

### 5.3. Natural in vitro proteolysis of human milk derived LF:

Because I was not able to produce any LF variant by molecular cloning, I had to focus on the alternative aside the cloning approach. Namely, the proteolytic generation of LF fragments to determine the Plg binding site of LF in subsequent biochemical assays. I subjected LF derived from human milk to in vitro proteolytic cleavage by pepsin and trypsin. According to literature, trypsin would yield the 2 iron- binding lobes, the N- and C-terminal halves of the molecule respectively, whereas pepsin is supposed to release the bioactive peptide LFcin. The two proteases were coupled to sepharose beads according to section 4.14. in order to separate them from digestion products after proteolysis. LF and enzyme coupled beads were incubated together at 37°C at enzymes' optimal pH while constantly shaking. Aliquots were taken at indicated time points and pH was readjusted with 1 M NaOH. The aliquots were used for SDS-PAGE and the gel was stained with Pierce® Silver Stain Kit in order to visualize all proteins and protein derived fragments. Pepsin

rapidly digested LF to smaller fragments, predominantly a 50 kDa fragment and a peptide smaller than 10 kDa. Latter might be the bioactive peptide LFcin that has the calculated molecular weight around 4-5 kDa. Trypsin digestion yielded 3 major fragments with molecular weights ranging from 30 to 45 kDa, which could represent the iron-binding lobes. (see Fig. 5.3.)

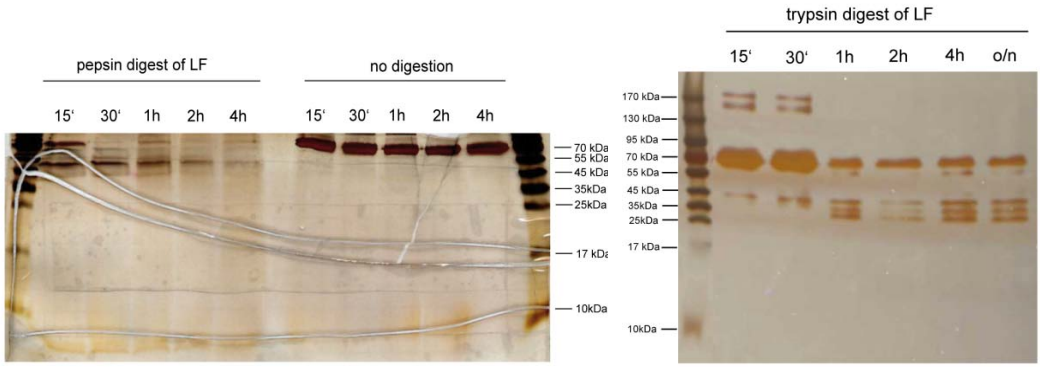



**Figure 5.3. Natural in vitro proteolysis of human milk derived LF:**
LF (50 µg) derived from human milk (L0520 from Sigma) and enzyme coupled beads (100 µl) were incubated together in 0.035 M NaCl (1 ml total volume) adjusted to the enzymes optimal pH (for pepsin pH 2.5; for trypsin pH 8) and temperature (37°C) while constantly shaking. Aliquots were taken at indicated time points and pH was readjusted to 7 with 1 M NaOH. SDS-PAGE was run and the gel was silver-stained by Pierce® Silver Stain Kit.

## 5.4. Binding assay with LF hydrolysates:

In order to narrow down the binding region of LF to Plg, I used LF hydrolysates generated by pepsin (P) and trypsin (T) cleavage together with undigested LF (ctrl) in a binding assay. The idea was to confine the Plg binding region on LF by identifying corresponding LF fragments, which bound to Plg. To do so, I coated Plg, M6P/IGF2R or BSA (as a negative control) on wells of a 96-well plate at 37°C for 2 h. Then the wells were blocked 1 h at RT with 1% BSA, washed with PBS and afterwards incubated overnight on ice with hydrolysates. Next, unbound molecules were washed away with PBS and subsequently, bound molecules were released by addition of 10 µl 1x NR sample buffer. The individual samples were analyzed by means of SDS-PAGE and Western blotting. Herewith, LF fragments bound to Plg or BSA respectively were to be visualized. The binding was performed under the low stringency (overnight binding, without detergent) conditions in order to obtain even weakly binding fragments; therefore the background binding of

undigested LF to the negative control (BSA) was strongly pronounced. I was able to identify one 50 kDa LF derived fragment of pepsin cleavage which was specifically binding to Plg and M6P/IGF2R. Yet, only little amounts could be detected, indicating that the 50 kDa fragment might not be the most determining part of LF in binding Plg. Moreover, no trypsin cleavage product was able to bind to Plg nor M6P/IGF2R and neither BSA. Consequently, it seems that upon cleavage of the full-length protein, the specific binding site either got lost or could not be detected by the polyclonal antibody used. (see Fig. 5.4.)

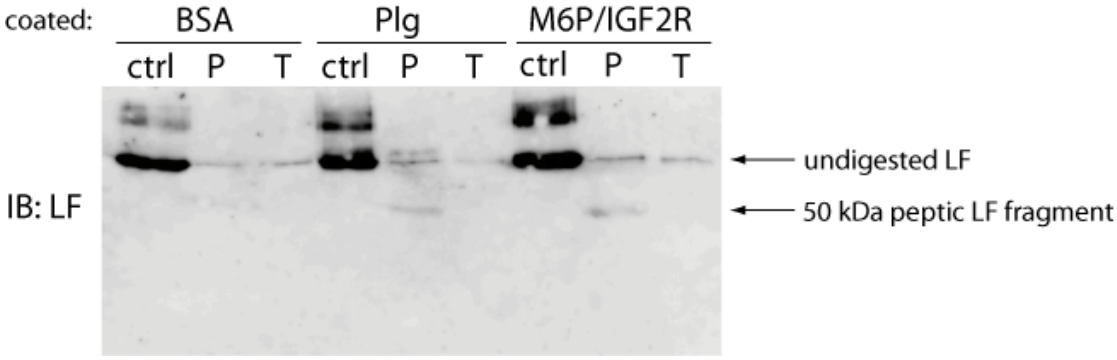



**Figure 5.4. Binding assay with LF hydrolysates**

The wells of a 96-well plate were coated for 2h at 37°C with 250 ng Plg (50 µl of 5 µg/ml Plg ), 50 µl purified M6P/IGF2R (estimated concentration 5-10 µg/ml) or 1% BSA as a control. After blocking with 1% BSA and washing with PBS, the wells were incubated overnight on ice with pepsin and trypsin hydrolysates or undigested LF as a control. Bound LF fragments were detected by SDS-PAGE followed by Western blotting. Immunoblot (IB) was performed with a polyclonal biotinylated human anti-LF antibody.

## 5.5. Design of LF derived peptides

Since binding assays with pepsin and trypsin LF hydrolysates did not reveal specific binding fragments, the next option was to design synthetic LF derived peptides and use them to determine the binding sites in blocking assays. I designed four peptides, denoted pLF1, pLF2, pLF3 and pLF4. Again the main focus was directed on the C-terminal lysine of LF, therefore one of the peptides, pLF3, was made up of the C-terminal 19 amino acids of human LF encompassing the terminal lysine. Peptide pLF4 was designed to serve as a control, hence it comprised the same amino acids as pLF3, but randomly

arranged. The peptide pLF2 was derived from the N-terminal part of LF near the helix linker region to emcompass the LFampin's sequence and also featured a C-terminal lysine that might have got exposed upon trypsin cleavage of full-length LF. Finally, pLF1 was derived from LF's N-terminal end and was designed to cover parts of LFcin. (see Fig. 5.5.) In particular, pLF1 comprised the most relevant amino acids concerning LPS neutralizing activity (Zhang et al., 1999). When designing the peptides I attached great importance to the secondary structure to avoid any effects merely caused due to conformational differences. Therefore I subjected the peptide sequences to two independent structure prediction servers (see section 4.15.). Both servers yielded that all peptides share a common secondary structure, illustrated with indicated positions within the full-length LF in figure 5.5.: a coiled-coil region in the beginning and a helix motif followed by another short coiled-coil region. The peptides sequences are presented in Table 2 and highlighted in the protein sequence of LF in Appendix 8.2.

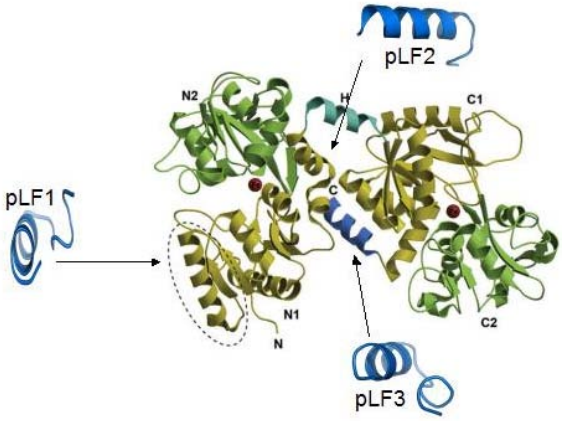
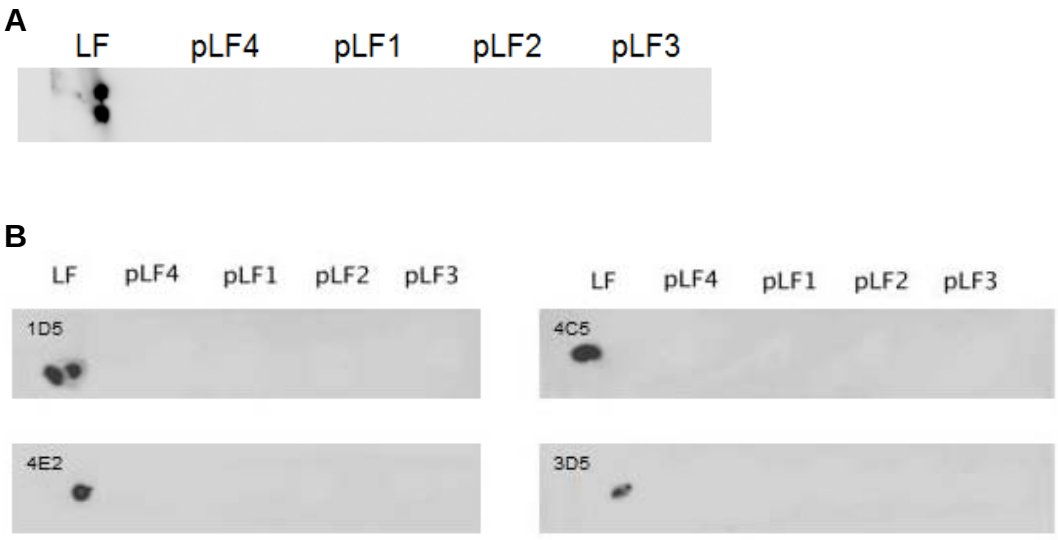



Fig. 5.5. Peptide structures with indicated positions, modified from (Baker and Baker, 2009) – pLF1,2,3 and the C-terminus are illustrated in blue. N and C termini are labelled with N and C respectively. N and C terminal lobes are subdivided in 2 domains each, denoted as N1, N2 and C1, C2. The helix linker region (H) is indicated in turquoise and iron ions are depicted in red.

## 5.6. Peptide mapping of antibodies:

I first tested whether the synthetic LF derived peptides could be recognized by the available anti-human LF antibodies. Our polyclonal mouse anti-human LF antibody did not recognize any of the four LF-derived peptides I had designed. (see Figure 5.6.A) Then, I scanned four different monoclonal mouse anti-human LF antibodies. By means of dot Western blot method I found that all monoclonal antibodies bound the full-length protein comparably well, except monoclonal antibody clone 3D5, which exerted only a moderate binding.

However, none of the antibodies was able to detect any of the spotted LF-derived peptides. (see Fig. 5.6.B)




**5.6. Peptide mapping of antibodies:**

LF and LF-derived peptides (1 µg each) were spotted onto PVDF membranes, which were washed, blocked and subsequently incubated with anti LF antibodies. In A, the membrane was incubated with the biotinylated polyclonal LF antibody ab25811. Bound primary antibody was detected by streptavidin-biotinylated horseradish peroxidase complex. In B, the membrane was incubated with different monoclonal antibodies (1D5, 4C5, 4E2, 3D5). Bound primary antibodies were detected by secondary rabbit anti-mouse IgG conjugated to horseradish peroxidase.

## 5.7. Competition binding assay:

Because pLFs could not be detected by antibodies and peptides in general display low coating efficiency on simple plastic plates, competition binding assays were the most convenient method to discover the definite binding site of LF. Hence, a binding assay in a cell-free system was performed in the absence or presence of indicated peptides. The underlying principle was that molecules containing the putative binding site would bind to coated Plg and therefore hinder the binding of LF to Plg. The competition of indicated peptides for LF binding to Plg was analyzed by means of a binding assay followed by SDS-PAGE and Western blotting. In these competition binding assays I increased the stringency (binding for 2h and use of 0.1% Triton X-100) in order to see only specific effects (compare with Fig. 5.4.). Here I found

that pLF1, a synthetic peptide comprised of a 19 amino acid sequence stretch identical to human LFcin, interfered with the binding of full-length LF to coated Plg and therefore seems to be a compulsory region of the LF molecule when interacting with Plg. (see Fig. 5.7.1.)

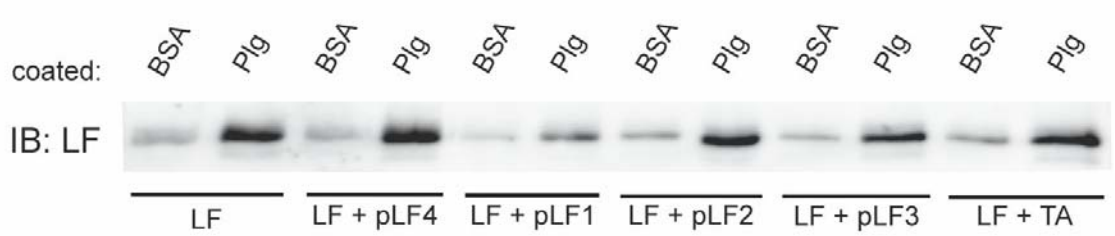



**Figure 5.7.1. Representative WB analysis of competition binding assay - LF binding to Plg:**

The wells of a 96-well plate first were coated for 2 h at 37°C with 5 µg/ml Plg or 1% BSA (negative control) then blocked 1 h at RT with 1% BSA and incubated with or without 20 µg/ml of LF peptides (pLF1-4) or 5 mmol/L tranexamic acid (TA) for 2 h on ice. After 4-5x washing with 1x PBS, 20 µg/ml LF were added to each well and the binding in presence of assay molecules was analyzed by Western blotting. Immunoblot (IB) was done with mouse anti-human LF 4E2 mAb.

Densitometric quantification and statistical analysis of western blots revealed highly significant inhibition of the LF/Plg interaction in the presence of pLF1. Here, the binding to BSA was subtracted from Plg binding lanes and in addition a background correction for each individual lane was applied. Conversely, the lysine analogue tranexamic acid (TA) as well as the terminal lysine containing peptides pLF2 and pLF3 did not have a significant effect on the binding of LF to Plg. However, in case of co-administration of pLF3 the amount of LF bound to coated Plg probably was slightly yet not significantly (n.s.) reduced. (see Fig. 5.7.2.)

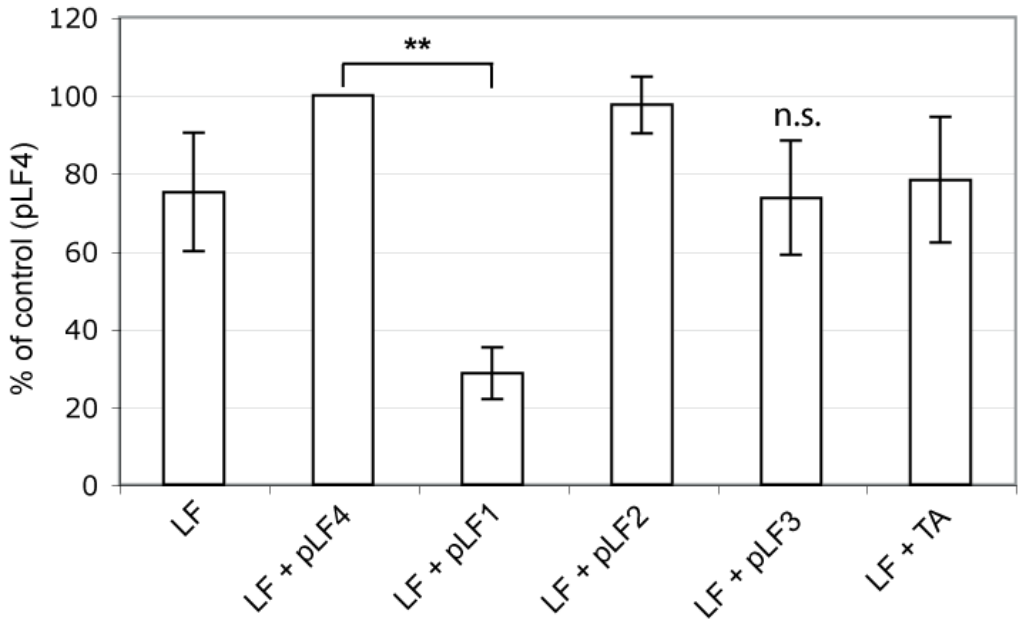



**Figure 5.7.2. Densitometric WB quantification of competition binding assay - LF binding to Plg:**

The individual bars show the relative means with standard errors or the means of at least three independent experiments. The scrambled peptide pLF4 served as control and therefore was set as relative maximum of 100%. The immunoblots were quantified with the Fuji MultiGauge software as described in section 4.26. BSA lanes were subtracted from Plg lanes and in addition a background correction for each individual lane was applied.

## 5.8. Inhibition of LF binding to Plg by pLF1 is concentration dependent:

In order to get a better insight into the dynamics of the inhibitory action of pLF1, I conducted competition binding assays in the presence of different concentrations of LF peptide pLF1 and as a negative control the scrambled peptide pLF4. Herewith, I was able to show that the peptide pLF1 was a potent competitor for LF in binding Plg. Even pLF1's concentrations as little as 5 µg/ml considerably impaired the binding of LF to Plg. Upon addition of 40 µg/ml of pLF1 the ability of LF to bind Plg was almost completely abolished (5.8.1.A), whereas in the control (pLF4) it was more or less unimpeded (5.8.1.B).

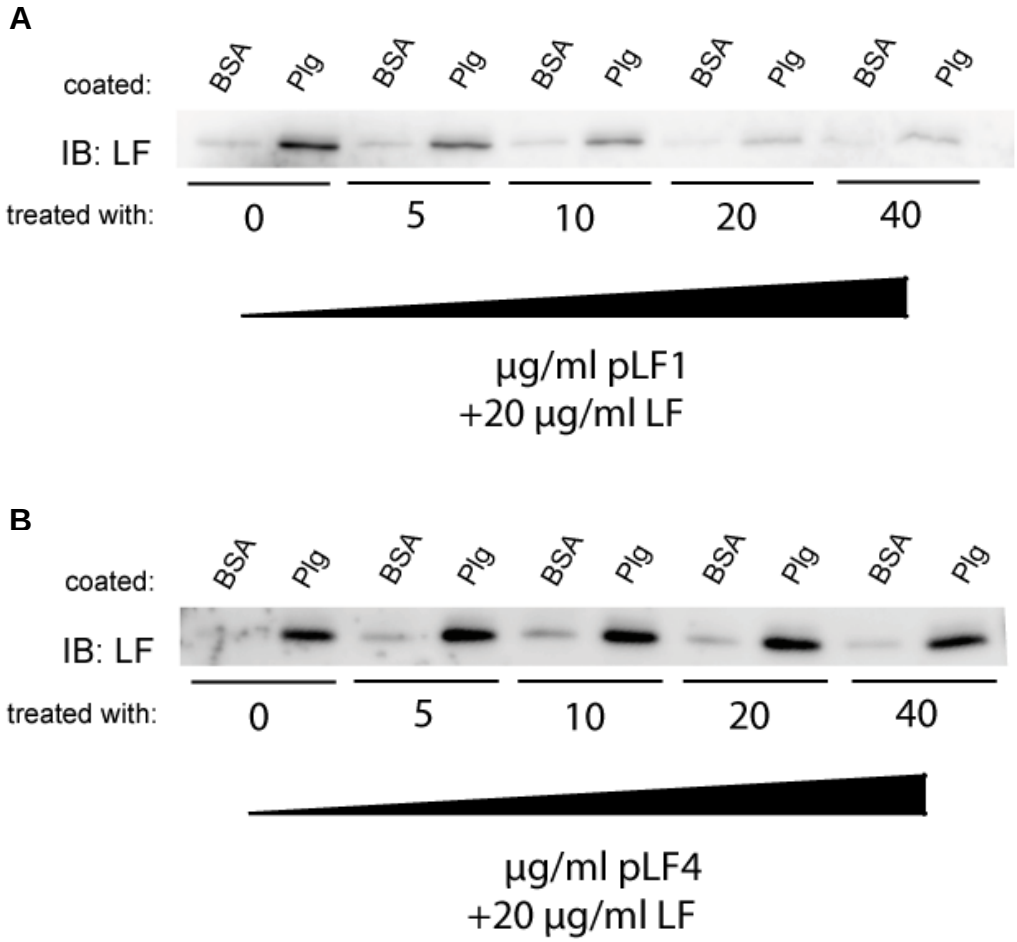



**Figure 5.8.1. WB analysis of binding assay with different peptide concentrations:**
The wells of a 96-well plate were coated 2 h on 37°C with 5 µg/ml Plg or 1% BSA (negative control) then blocked 1 h with 1% BSA at RT and incubated 2 h on ice with increasing concentrations (0, 5, 10, 20 and 40 µg/ml) of either the LF resembling peptide pLF1 (Fig. 5.8.1. A) or the scrambled peptide pLF4 (Fig. 5.8.1. B), which served as a control. After 4-5x washing with 1x PBS, LF (20 µg/ml) was added to each well and the binding in presence of peptides was analyzed by Western blotting. Immunoblot (IB) was done with the primary mouse anti-human LF 1C5 mAb.

Quantification of at least three independent experiments is shown in Figure 5.8.2. Statistical analysis revealed that merely 5 µg/ml of peptide pLF1 was sufficient to significantly reduce the binding of LF to Plg ($p < 0.05$). Upon addition of gradually increasing concentrations of pLF1 the binding was progressively debilitated ($p < 0.01$ for 10 µg/ml, $p < 0.001$ for 20 µg/ml and 40 µg/ml) and reached a plateau at concentrations around 20-40 µg/ml. The calculated relative half maximum inhibitory concentration (relative IC50) of peptide pLF1, which was determined by a four-parameter logistic (4PL)

nonlinear regression curve fit, is 6.038 µg/ml. The corresponding molar concentration is 2.8 µM.

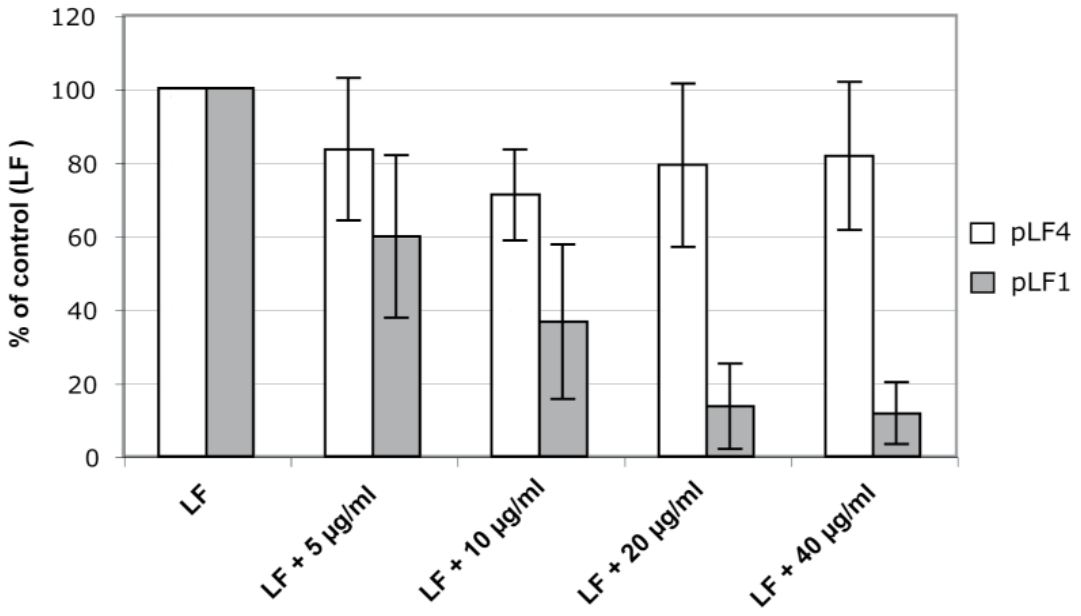



**Figure 5.8.2. WB quantification of binding assay with different peptide concentrations:** The individual bars show the relative means with standard errors or the means of at least three independent experiments. LF without any peptide was set at 100%. The immunoblots were quantified with the Fuji MultiGauge software. BSA lanes were subtracted from Plg lanes and 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.ncgc.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.

## 5.9. Dot far Western blot:

In competition binding assays I was able to show that pLF1 interfered with the binding of LF to Plg. However, the exact molecule on which pLF1 was acting, namely LF or Plg, had not been determined with certainty. To examine if pLF1 did bind to Plg or LF, dot far Western blots were performed as follows: LF and derived peptides (1 µg each) were spotted (directly pipetting 1 µl of a 1 mg/ml solution) onto a PVDF membrane, which then was blocked with 5% non-fat dry milk for 1 h and incubated with Plg (Fig. 5.9.A) or LF (Fig. 5.9.B) (each 1

µg/ml in TBST) for 1 h. Bound Plg or LF was detected by antibodies directed against Plg or LF, respectively. By means of dot far Western blotting I was able to confirm that Plg not only bound LF but in addition peptide pLF1 (5.9.A), the very one competing with LF for the binding to Plg (see Fig.5.7.1. - 5.7.2.). Furthermore, dot far Western blot revealed that LF was binding to pLF1 (5.9.B) as well.

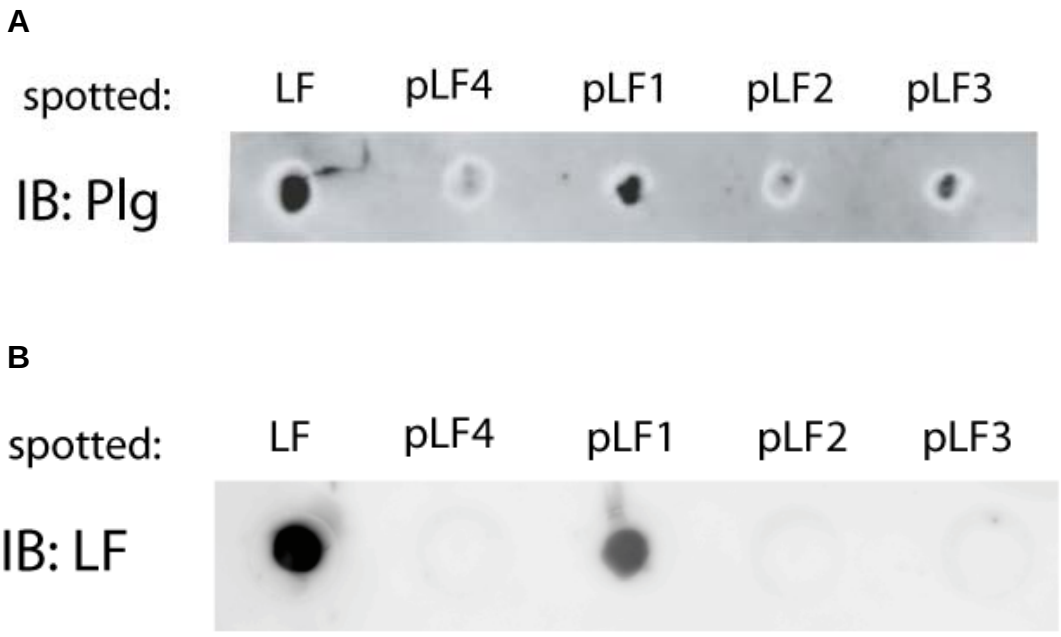



**Figure 5.9. Dot far Western blot of LF and derived peptides**

LF and derived peptides (1 µg/ml each) were directly spotted on a PVDF membrane, which was afterwards blocked in 5 % non-fat dry milk and incubated with 1 µg/ml Plg (A) or LF (B) in TBST. The membrane was immunostained with mouse anti-human Plg (7Pg) in A and with biotinylated mouse anti-human-LF (LFb) in B.

## 5.10. Cell surface activation of Plg in the presence of LF peptides:

Next, I wanted to examine the impact of LF and derived peptides on the fibrinolytic system within a cellular environment. Therefore, Plg activation on the human epithelial kidney tumor cell line (TCL-598) was performed in the presence of LF, LF derived peptides (pLFs) or TA, the lysine analogue, which is known to effectively inhibit the conversion of Plg to Plm on the cell surface. Plg is supposed to bind to uPA, which is bound to its receptor uPAR on the surface of TCL cells, which were shown to express high amounts of both uPA

and uPAR. (Koshelnick et al., 1997) Upon binding to uPAR, pro-uPA gets activated to uPA, which in turn triggers the conversion of Plg to active Plm (see Fig. 2). The chromogenic substrate S-2251 was used to monitor Plm activity. The substrate was hydrolyzed by active Plm and the cleavage product emitted light at a wavelength of 405 nm, which was measured every hour. In order to avoid non-specific Plm activity, the cells were seeded in serum-free medium and potentially free Plm was inhibited by addition of α2AP. Hence, only the effect of cell-surface activated Plm should have been perceived. Therefore, here one could directly read out cell-surface mediated Plg conversion to active Plm. The activation of Plg in the presence of LF was reduced to a level comparable to the anti-fibrinolytic drug TA, whereas none of the lactoferrin-derived peptides had a direct influence on Plg activation. (see Fig. 5.10.1)

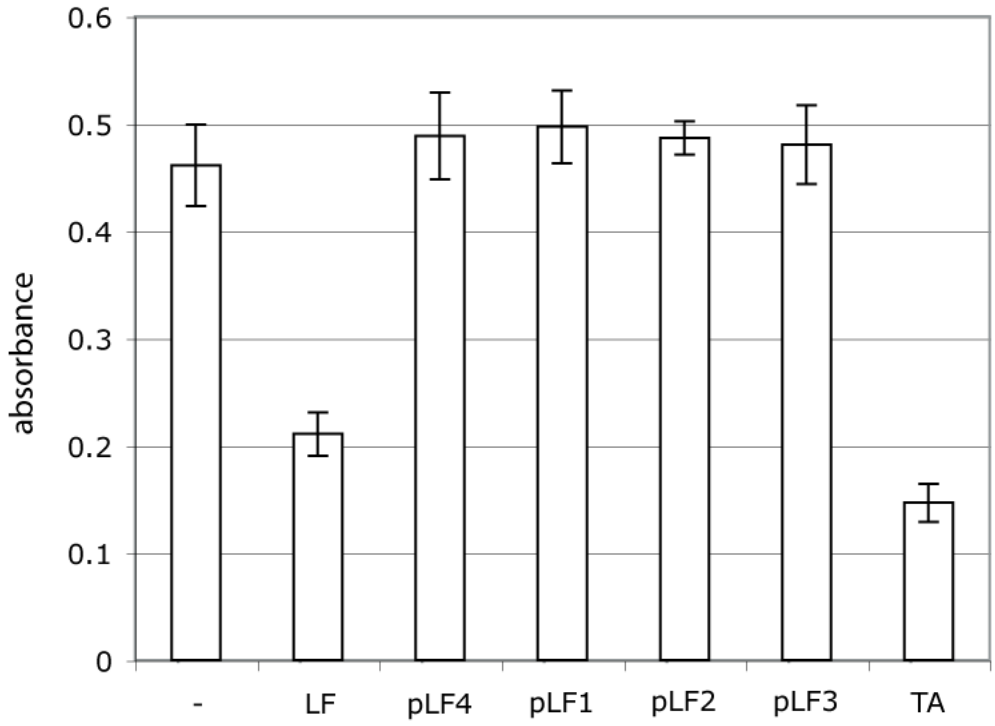



**Figure 5.10.1 Surface activation of Plg in the presence of LF peptides:**
TCL cells (50.000/well) were seeded in serum-free RPMI 1640 medium in a 96-well plate 24 h before the assay. Plg (100 nM), the chromogenic Plm substrate S-2251 (1.6 nM) and α2AP (5 µg/ml) were incubated together with assay molecules (20 µg/ml LF, pLFs or 10 nM TA ) in serum-free RPMI 1640 medium for 30 min on ice. Then the mixture was added to the cells and absorbance was measured every hour. The individual bars represent mean absorbance

levels, with standard deviations. Absorbance was generated by the Plm cleavage product of the chromogenic Plm substrate S-2251 (emits light at 405 nm).

Since competition binding assays revealed that pLF1 obstructed the LF/Plg binding in a cell-free system, it might as well interfere with the LF induced inhibition of Plg activation in a cell system. Indeed, upon co-administration of pLF1 the decreased activation of Plg to Plm elicited by LF could be partly restored. (see Fig. 5.10.2.)

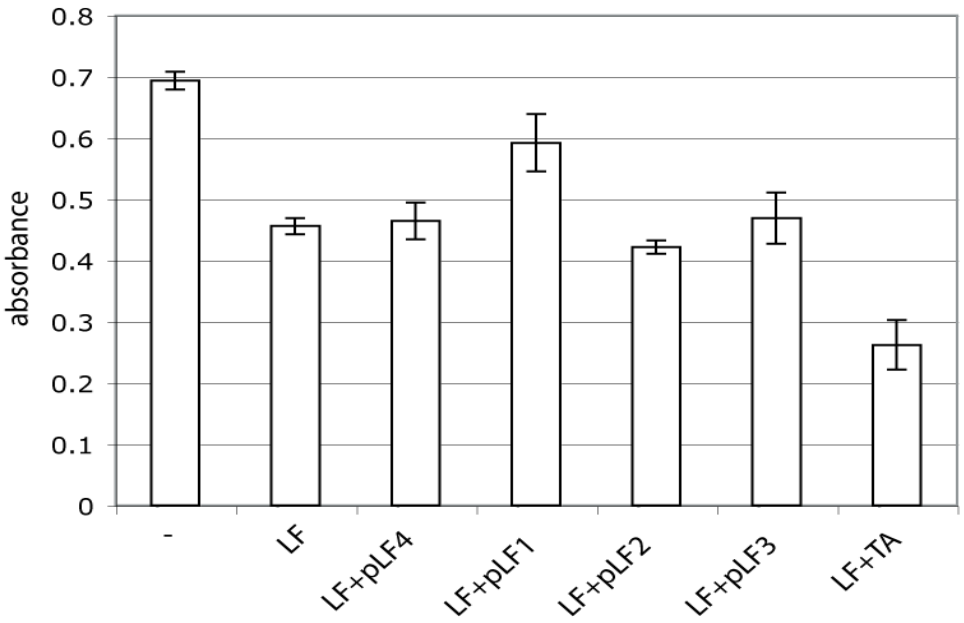



**Figure 5.10.2. Reconstitution of Plg surface activation**

TCL cells (50.000/well) were seeded in serum-free RPMI 1640 medium in a 96-well plate 24 h before the assay. Plg (100 nM) , the chromogenic Plm substrate S-2251 (1.6 nM) and α2AP (5 µg/ml) were incubated together with assayed molecules (20 µg/ml pLFs or 10 nM TA ) in serum-free RPMI 1640 medium for 30 min on ice. Then LF (20 µg/ml) was added to all wells except the control (-). The mixture was added to the cells and absorbance was measured every hour. The individual bars represent mean absorbance levels, with standard deviations. Absorbance was generated by the Plm cleavage product of the chromogenic Plm substrate S-2251 (emits light at 405 nm).

## 5.11. Determination of natural LF – Plg interaction by blue native polyacrylamide gel electrophoresis:

In order to study whether LF and Plg were actually interacting under physiological conditions, I examined human serum samples with blue native polyacrylamide gel electrophoresis (BN-PAGE). BN-PAGE is a method to isolate native protein complexes, maintaining their physiological context. Hence, BN-PAGE allows analysis of intact and functional native protein-protein interactions. Protein complexes are dissected in the 1st dimension by blue native run and individual proteins are separated from their physiological complexes in the 2nd dimension by SDS-PAGE gel run. Analysis of human serum by this method revealed partial colocalization of LF and Plg in the high (left) and moderately high (middle) molecular complexes around 880 kDa and 440 kDa, respectively. This human serum sample from a patient with liver disorder was used in former studies of our group (approved by the Ethical Committee of the Medical University of Vienna). Therein, it was reported that soluble M6P/IGF2R and Plg appeared in human serum within a high (above 880 kDa) and a moderately high (around 440 kDa) molecular complex, similar to those described here. (Leksa et al., 2011) Consequently, LF, Plg and soluble M6P/IGF2R might be part of the same physiological protein complexes. The lower (right) molecular weight bands, which are smaller than 272 kDa, might represent individual molecules and/or homo-oligomers. Actually, these low molecular weight bands could probably mirror interacting LF and Plg molecules. (see Fig. 5.11.)

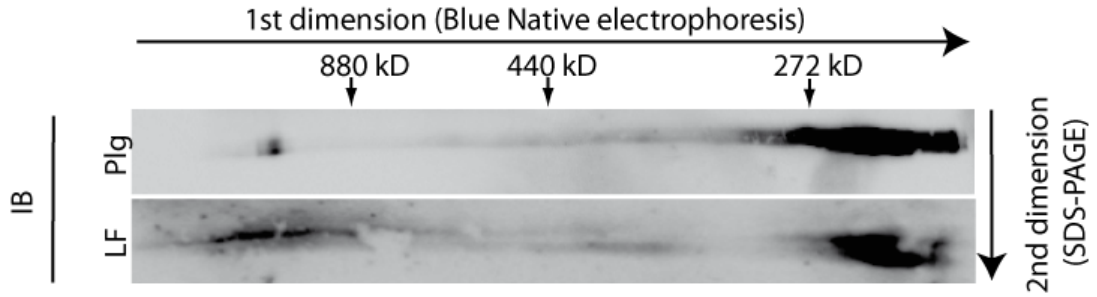



**Figure 5.11. Blue native polyacrylamide gel electrophoresis:**

Serum samples were loaded on a BN-PAGE gel. The gel was run at 4°C at 80 V for 4 h. Then voltage was increased to 120 V o/n. The next day the cathode buffer was exchanged to slightly blue cathode buffer and the gel was run 2 h. The gel was cut into individual sample

stripes, which were used for the 2nd dimension (SDS-PAGE). Afterwards the gel was blotted followed by staining with either the mouse anti-human Plg mAb 7Pg or biotinylated mouse anti-human LFb antibody. Ferritin and jack bean urease were used as markers.

# 6. Discussion:

In preliminary studies LF was detected as interaction partner of M6P/IGF2R, which in turn formerly was identified to bind Plg. Furthermore, also LF and Plg demonstrated direct binding in preliminary experiments. Many of its known interactions can be attributed to Plg's affinity for binding C-terminal lysines. Since LF's amino acid sequence displays a C-terminal lysine, I put high priority on manipulation of LF's C-terminus.

I considered two initial approaches: first, the generation of LF C-terminal mutants and truncations by molecular cloning, and second, the enzymatic fragmentation of LF. Concerning the generation of recombinant LF variants, I cloned several LF-derived mutants into subcloning vectors (pGEM-T easy and pBluescript KS-), but I was unable to produce any mammalian expression vector (pBMN) containing either the full-length protein, the C-terminal lysine to threonine mutant or any truncated form of LF. My hypothesis on this issue is that retroviral promoters which are generally used in mammalian expression vector systems are somewhat leaky in bacteria (Kashanchi and Wood, 1989; Lewin et al., 2005), thereby resulting in protein expression which in this particular case might lead to bacteriostatic action of LF and its mutant forms. By supplementing agar plates with iron, I intended to compensate in particular LF's iron sequestrating effect. Although bacteria in general were able to survive these high-iron conditions, I could not detect any clone transformed with LF variants. However, the depletion of iron is not the only bactericidal mechanism of LF. One of the truncated forms of LF represents ΔLF and therefore lacks the potent anti-microbial domain LFcin. Nevertheless, even bacteria transformed with ΔLF constructs did not grow. Consequently, LF and its truncated forms might be able to act in an antimicrobial way through a combination of the iron sequestration, the action of the very potent N-terminus (LFcin), LFampin's action and maybe another unknown mechanism. Other research groups might have experienced similar difficulties in cloning human LF, since they circumvented this issue by introducing an intron which could be spliced in eukaryotic cells only. When this construct is expressed in bacterial cells, the intron disrupts LF's inherent antimicrobial activity, thereby allowing

survival of bacteria. As soon as the expression vector containing LF is transferred into mammalian cells, the intron is spliced out and a fully functional LF is expressed. (Bissonnette et al., 2006) However, we have decided to apply different strategies.

The first alternative step was to imitate natural proteolytic digest of LF as it would occur within the human digestive system and identify LF fragments that probably bind Plg. When proteolytically digested with pepsin, LF is rapidly degraded to smaller fragments. (see fig. 5.3.) In addition to a 50 kDa fragment also a peptide smaller than 10 kDa can be detected, which may be LFcin (4-5 kDa). In case of trypsin digest, three major fragments with molecular weights of approximately 30-35, 35-40 and 45 kDa were generated. These results are diverging from literature where the trypsin digest is supposed to yield two iron binding lobes (Brock et al., 1976). However, two of the three fragments observed by us still may represent the two lobes and the third one might represent an incompletely cleaved fragment. Additionally, LF cleavage might be different in dependence on its iron-status or other general digestion affecting parameters like pH for instance.

In vitro binding assays with the pepsin and trypsin LF hydrolysates revealed that the approximately 50 kDa fragment, generated by pepsin cleavage, bound to Plg and M6P/IGF2R. (see fig. 5.4.) We further see in figure 5.4. that LF appears on SDS-PAGE gels as a double band that probably depends on its glycosylation status (Spik et al., 1994). The very low staining of this 50 kDa fragment is propably due to the fact that upon digestion of the full-length molecule the most decisive and specific binding region is either destroyed or not recognized by our antibodies.

Because cloning as well as in vitro proteolysis concepts did not provide LF fragments which permit mapping of the definite Plg binding region, the next approach was to design synthetic peptides derived from LF, and use them in subsequent biochemical experiments to characterize the Plg interaction site. Via in vitro binding assays I was able to demonstrate not only that LF bound to Plg, but in addition that a small synthetic peptide derived from the bioactive LF peptide LFcin could obstruct the aforementioned interaction. Figure 5.7.1. illustrates that the LFcin-like peptide pLF1 hampers binding of LF to Plg. In contrast to the scrambled peptide pLF4 or C-terminal lysine containing

peptides pLF2 and pLF3, the amount of LF bound to Plg is significantly reduced (fig. 5.7.2.) in the presence of peptide pLF1 when compared with the control (LF without additional molecules). Moreover, TA which is a lysine derivative commonly used as a potent anti-fibrinolytic drug does not affect the binding of LF to Plg, which provides a strong evidence that Plg's affinity for C-terminal lysines in case of LF is not responsible for the establishment of this specific interaction. The four peptides have been synthesized so that they mimic defined parts of LF (see Table 2). Moreover, the peptides were designed in such a way that they all resemble each other as well as their originating location within full-length LF in terms of secondary structure (see Fig. 5.5.). Thus, potential effects provoked by structural peculiarities can be largely excluded. The structure of the peptides was determined by structure prediction algorithms as described in 4.15. However, these algorithms are not able to compute whether the peptides are circular. Since naturally occurring LFcin is described to be circular (Bellamy et al., 1992b), the physiological relevance of pLF1 has to be verified. In theory, pLF1 might form an intramolecular bond based on its two cysteine residues, although these two cysteines are not interacting in the natural LFcin. Instead, they are elements of two discrete cysteine bonds formed between two additional cysteines which occur in LFcin (Hunter et al., 2005) but not in the shorter synthetic peptide pLF1. Hence, pLF1 might be linear due to steric hindrance entailed by the close proximity of the two cysteines. Anyway, the circumstance that pLF1 might not be comparable to natural LFcin has to be taken into account before transforming results shown here to physiological situations.

To study the effectiveness of the inhibitory peptide pLF1, binding assays were repeated with increasing concentrations of pLF1 and the control peptide pLF4. Herein I was able to show that upon addition of increasing concentrations of pLF1 the binding of LF to Plg was progressively decreased, (see Fig. 5.8.1.A) whereas the scrambled control peptide pLF4 did not affect the binding. (see Fig. 5.8.1.B) The calculated relative half inhibitory concentration (IC50) is 6.038 µg/ml, demonstrating that even molar concentrations as low as 2.8 µM of pLF1 (M = 2162.48 g/mol) are sufficient to significantly inhibit interaction of LF and Plg. (see Fig. 5.8.2.) A very weak background binding to BSA (control) was observed, which I subtracted. A slight decrease in BSA binding can be

observed in case of pLF1. On account of this fact one might argue that pLF1 is not only specifically weakening the binding of LF to Plg but also to BSA or probably proteins in general. Indeed, the lower the stringency is the more LF tends to bind to BSA (see figure 5.4.). If the stringency is increased, as it was the case in competition binding assays, BSA binding is generally reduced (see figure 5.7.). To further limit the influence of dissimilar background BSA binding, I subtracted band density values of BSA binding from Plg binding, thereby generating a net Plg binding value.

Yet, the question whether pLF1 is specifically interacting with Plg cannot be answered by competition binding assays in this vein. The inhibitory peptide pLF1 might inhibit the LF/Plg interaction by binding to Plg as well as by binding to LF. Even though the assay molecules, among pLF1, were pre-incubated for half an hour on ice with coated Plg on the plate, one could not entirely exclude that they still bind to LF. Thus, I performed a dot far Western blot, to provide evidence for the direct interaction of the inhibitory peptide pLF1 and Plg. Dot far Western blot is derived from a method called far Western blot, wherein prey proteins transferred onto a membrane are incubated with another protein, the bait, which is either tagged itself or detected by a primary antibody directed against it. Hereby, I proved that Plg was directly binding to LF and to the inhibitory peptide pLF1. In addition, Plg binds to a minor extent to pLF3 in dot far Western blots (see fig. 5.9.1.A), which nicely mirrors plate binding assays: In plate binding assays pLF3 was also minimally reducing the binding. (see fig. 5.7.1. & 5.7.2.) Furthermore, dot far Western blots disclose that LF is directly binding to pLF1 (see fig. 5.9.1.B). Accordingly, pLF1 might interfere with the LF/Plg interaction on both molecules.

The fact that the Plg binding peptide pLF1 is not detectable by antibodies (see figure 5.6.) might explain why I was not able to recognize any specific Plg binding fragment in binding assays with LF hydrolysates (see figure 5.4.). Since pLF1 covers parts of LFcin, which according to literature is produced upon proteolytic processing of LF by pepsin, LFcin is supposed to bind Plg too. However, it might as well not be recognized by antibodies, therefore it could not be seen in binding assays with pepsin hydrolysates.

To investigate the effect of LF or LF derived peptides on the fibrinolytic system at the cellular level, Plg activation was studied in a cellular model. A chromogenic Plm substrate allows studying the conversion of Plg to active Plm. In addition, working with serum-free media and blocking potentially free Plm with α2AP, a serine protease inhibitor, ensures measurement of the cell surface-bound Plg conversion only. TCL-598 cells were used because they are known to express sufficient levels of both the Plg activating enzyme uPA and its receptor uPAR. (Koshelnick et al., 1997) I demonstrate that LF is effectively restraining the conversion of Plg to Plm. Actually, LF is comparably potent to the widely used anti-fibrinolytic drug TA (see fig. 5.10.1.), whereas none of the peptides directly altered the activation of Plg. However, upon co-administration of LF and the individual peptides, the LFcin-like peptide pLF1 tended to compensate for the impairment of Plg activation by LF (see fig. 5.10.2.), which suggests that by binding of pLF1 to Plg alone the Plg activation is not affected. Instead, pLF1 seems to obstruct the binding of LF to Plg, thereby restricting the inhibition of Plg activation by LF described above. Obviously, the full-length LF protein is able to limit the conversion of Plg to Plm, whereas pLF1 does not interfere with its activation, although it is binding to Plg. Since pLF1 is a small synthetic peptide comprised of only 19 amino acids, it might be simply too small to deter Plg activation, consequently suggesting that it needs the whole LF molecule to either mask a binding site of Plg for its converting enzyme uPA or sterically inhibit the proteolytic processing of Plg to active Plm. In turn, by blocking the binding site of LF on Plg, pLF1 seems to be able to confine LF's Plg activation diminishing action, thereby promoting fibrinolytic function and probably providing a basis for treatment of diseases with underlying fibrinolytic dysfunctions.

It would be highly interesting if Plm can cleave LF, thereby yielding a natural LFcin-like peptide, which acts comparably to pLF1. If this were the case, Plm would feature the ability of generating a feedback loop to protect its activation from inhibition by LF. As described (Aslam and Hurley, 1997; Dalsgaard et al., 2008), Plm is indeed able to cleave LF but these authors could not identify any peptide generated by the Plm cleavage akin to LFcin. Furthermore, it would be interesting if the naturally occurring LFcin produced by pepsin cleavage behaves analogously to the synthetic peptide pLF1. Undoubtedly, if

these scenarios were true they would in principle question the physiological relevance of the inhibition of Plg activation by LF, because the counteracting peptide LFcin might always be present under physiological circumstances. However, BN-PAGE analysis suggests the interaction in some way. Since BN-PAGE conserves physiological protein complexes, which can be further separated by a 2$^{nd}$ dimension SDS-PAGE, it is possible to shed light on natural protein-protein interactions with this method. Analysis of human serum by BN-PAGE demonstrated that LF and Plg might occur within the same molecular protein complexes in circulation. This provides a hint for possible physiological interaction of LF and Plg.

Adequate Plm activity seems to be an integral factor for accurate regulation of immune function. By degradation of the extracellular matrix it enables the migration of immune cells, e.g. neutrophils (Herren et al., 2001). LF might decrease the motility of immune cells by limiting Plg activation resulting in reduced ECM degradation. Consequently, LF may not only act synergistically to immune responses, e.g. via mediating T and B cell maturation (Zimecki et al., 1991; Zimecki et al., 1995), but in addition as an indirect immunosuppressant to avoid the overshooting immune reactions. As already mentioned, certain bacteria use the Plg system to invade tissues. In these cases, LF might be able to limit bacterial invasion by inhibiting Plg activation. Increasing evidences evolved during the last decade for Plm playing a crucial role in development and progression of Alzheimers disease. For instance, it was shown that brain Plm enhanced the cleavage of APP and degrades Aβ. Furthermore, Plm activity is reduced in Alzheimer dementia patients' brains, hence endorsing its role in Alzheimers disease (Ledesma et al., 2000; Tucker et al., 2000). Besides, LF was shown to accumulate in Alzheimers disease patients' brains (Kawamata et al., 1993). Moreover, Alzheimer dementia mouse models provide indications of LF being involved in the disease by detecting LF depositions in brains of Alzheimers disease mouse models. (Wang et al., 2010) These records suggest a possible interplay of Plg and LF in this disease. Due to the accumulation of LF, Plg activation might be decreased, consequently promoting the formation of senile plaques by reduced Aβ cleavage. Since the synthetic peptide pLF1 is shown here to be a potent blocker of the LF/Plg inhibitory interaction, pLF1 represents a new

promising candidate for therapeutic approaches in treatment of Alzheimers disease. Probably even the naturally occurring LFcin or other LFcin-derived peptides might be used as therapeutics in treatment of Alzheimers disease.

# 8. Appendices:

8.1. Appendix 1 - DNA Sequence of human LF (NCBI reference sequence: NM_002343.3): The primers described in 4.5. are depicted.

```
AGAACCAGGACAGGTGAGGTGCAGGCTGGCTTTCCTCTCGCAGCGCGGTG          50
TGGAGTCCTGTCCTGCCTCAGGGCTTTTCGGAGCCTGGATCCTCAAGGAA         100
CAAGTAGACCTGGCCGCGGGGAGTGGGGAGGGAAGGGGTGTCTATTGGGC         150
AACAGGGCGGGGCAAAGCCCTGAATAAAGGGGCGCAGGGCAGGCGCAAGT         200
GGCAGAGCCTTCGTTTGCCAAGTCGCCTCCAGACCGCAGACATGAAACTT         250
GTCTTCCTCGTCCTGCTGTTCCTCGGGGCCCTCGGACTGTGTCTGGCTGG         300
CCGTAGGAGGAGTGTTCAGTGGTGCGCCGTATCCCAACCCGAGGCCACAA         350
AATGCTTCCAATGGCAAAGGAATATGAGAAAAGTGCGTGGCCCTCCTGTC         400
AGCTGCATAAAGAGAGACTCCCCCATCCAGTGTATCCAGGCCATTGCGGA         450
AAACAGGGCCGATGCTGTGACCCTTGATGGTGGTTTCATATACGAGGCAG         500
GCCTGGCCCCCTACAAACTGCGACCTGTAGCGGCGGAAGTCTACGGGACC         550
GAAAGACAGCCACGAACTCACTATTATGCCGTGGCTGTGGTGAAGAAGGG         600
CGGCAGCTTTCAGCTGAACGAACTGCAAGGTCTGAAGTCCTGCCACACAG         650
GCCTTCGCAGGACCGCTGGATGGAATGTCCCTATAGGGACACTTCGTCCA         700
TTCTTGAATTGGACGGGTCCACCTGAGCCCATTGAGGCAGCTGTGGCCAG         750
GTTCTTCTCAGCCAGCTGTGTTCCCGGTGCAGATAAAGGACAGTTCCCCA         800
ACCTGTGTCGCCTGTGTGCGGGGACAGGGGAAAACAAATGTGCCTTCTCC         850
TCCCAGGAACCGTACTTCAGCTACTCTGGTGCCTTCAAGTGTCTGAGAGA         900
CGGGGCTGGAGACGTGGCTTTTATCAGAGAGAGCACAGTGTTTGAGGACC         950
TGTCAGACGAGGCTGAAAGGGACGAGTATGAGTTACTCTGCCCAGACAAC        1000
ACTCGGAAGCCAGTGGACAAGTTCAAAGACTGCCATCTGGCCCGGGTCCC        1050
TTCTCATGCCGTTGTGGCACGAAGTGTGAATGGCAAGGAGGATGCCATCT        1100
GGAATCTTCTCCGCCAGGCACAGGAAAAGTTTGGAAAGGACAAGTCACCG        1150
AAATTCCAGCTCTTTGGCTCCCCTAGTGGGCAGAAAGATCTGCTGTTCAA        1200
GGACTCTGCCATTGGGTTTTCGAGGGTGCCCCCGAGGATAGATTCTGGGC        1250
TGTACCTTGGCTCCGGCTACTTCACTGCCATCCAGAACTTGAGGAAAAGT        1300
GAGGAGGAAGTGGCTGCCCGGCGTGCGCGGGTCGTGTGGTGTGCGGTGGG        1350
CGAGCAGGAGCTGCGCAAGTGTAACCAGTGGAGTGGCTTGAGCGAAGGCA        1400
GCGTGACCTGCTCCTCGGCCTCCACCACAGAGGACTGCATCGCCCTGGTG        1450
CTGAAAGGAGAAGCTGATGCCATGAGTTTGGATGGAGGATATGTGTACAC        1500
TGCAGGCAAATGTGGTTTGGTGCCTGTCCTGGCAGAGAACTACAAATCCC        1550
AACAAAGCAGTGACCCTGATCCTAACTGTGTGGATAGACCTGTGGAAGGA        1600
TATCTTGCTGTGGCGGTGGTTAGGAGATCAGACACTAGCCTTACCTGGAA        1650
CTCTGTGAAAGGCAAGAAGTCCTGCCACACCGCCGTGGACAGGACTGCAG        1700
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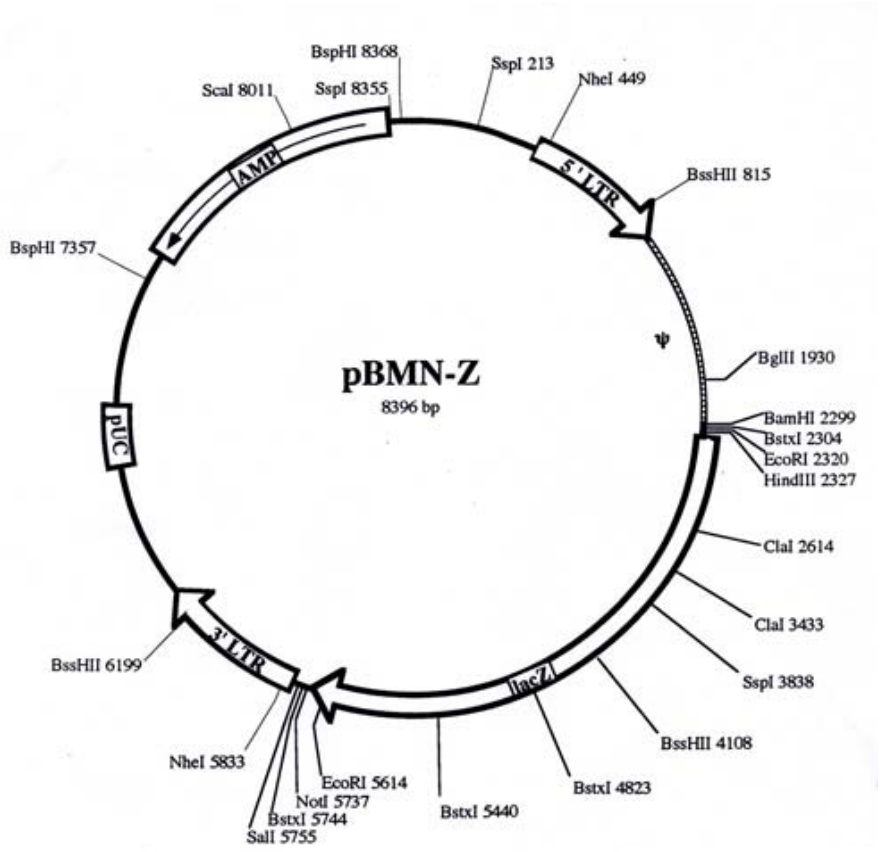
GCTGGAATATCCCCATGGGCCTGCTCTTCAACCAGACGGGCTCCTGCAAA 1750
TTTGATGAATATTTCAGTCAAAGCTGTGCCCCTGGGTCTGACCCGAGATC 1800
TAATCTCTGTGCTCTGTGTATTGGCGACGAGCAGGGTGAGAATAAGTGCG 1850
TGCCCAACAGCAACGAGAGATACTACGGCTACACTGGGGCTTTCCGGTGC 1900
CTGGCTGAGAATGCTGGAGACGTTGCATTTGTGAAAGATGTCACTGTCTT 1950
GCAGAACACTGATGGAAATAACAATGAGGCATGGGCTAAGGATTTGAAGC 2000
TGGCAGACTTTGCGCTGCTGTGCCTCGATGGCAAACGGAAGCCTGTGACT 2050
GAGGCTAGAAGCTGCCATCTTGCCATGGCCCCGAATCATGCCGTGGTGTC 2100
TCGGATGGATAAGGTGGAACGCCTGAAACAGGTGTTGCTCCACCAACAGG 2150
CTAAATTTGGGAGAAATGGATCTGACTGCCCGGACAAGTTTTGCTTATTC 2200
CAGTCTGAAACCAAAAACCTTCTGTTCAATGACAACACTGAGTGTCTGGC 2250
CAGACTCCATGGCAAAACAACATATGAAAAATATTTGGGACCACAGTATG 2300
TCGCAGGCATTACTAATCTGAAAAAGTGCTCAACCTCCCCCCTCCTGGAA 2350
GCCTGTGAATTCCTCAGGAAGTAAAACCGAAGAAGATGGCCCAGCTCCCC 2400
AAGAAAGCCTCAGCCATTCACTGCCCCCAGCTCTTCTCCCCAGGTGTGTT 2450
GGGGCCTTGGCCTCCCCTGCTGAAGGTGGGGATTGCCCATCCATCTGCTT 2500
ACAATTCCCTGCTGTCGTCTTAGCAAGAAGTAAAATGAGAAATTTTGTTG 2550
ATATTCTCTCCTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 2593

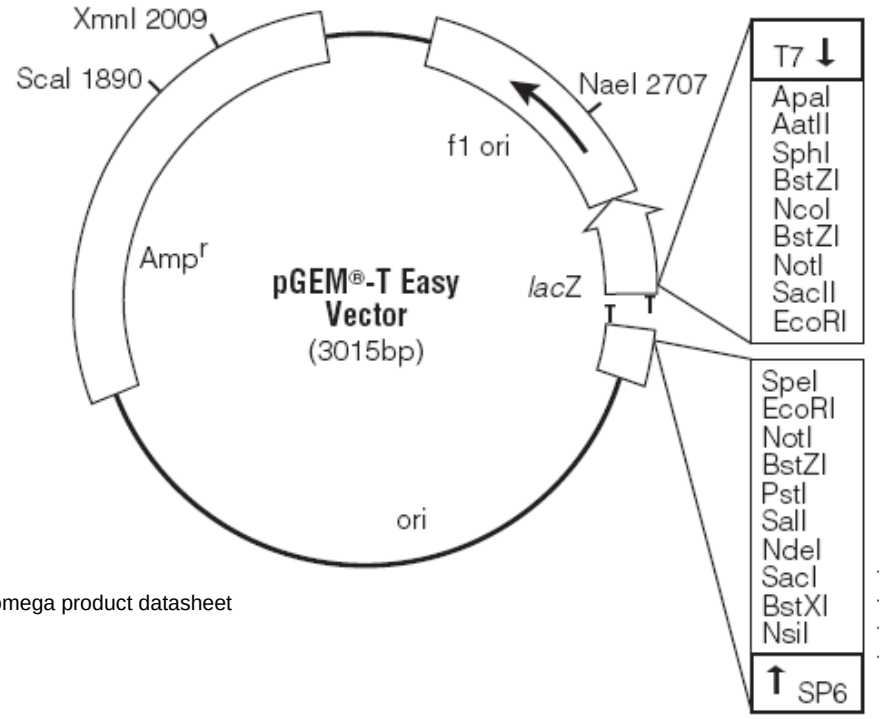
## 8.2. Appendix 2 - Protein sequence of human LF (NCBI reference sequence ID: NP_002334.2): The peptides described in 4.15. are depicted.

MKLVFLVLLFLGALGLCLAGRRRSVQWCAVSQPEATKCFQWQRNMRKVRG 50
PPVSCIKRDSPIQCIQAIAENRADAVTLDGGFIYEAGLAPYKLRPVAAEV 100
YGTERQPRTHYYAVAVVKKGGSFQLNELQGLKSCHTGLRRTAGWNVPIGT 150
LRPFLNWTGPPEPIEAAVARFFSASCVPGADKGQFPNLCRLCAGTGENKC 200
AFSSQEPYFSYSGAFKCLRDGAGDVAFIRESTVFEDLSDEAERDEYELLC 250
PDNTRKPVDKFKDCHLARVPSHAVVARSVNGKEDAIWNLLRQAQEKFGKD 300
KSPKFQLFGSPSGQKDLLFKDSAIGFSRVPPRIDSGLYLGSGYFTAIQNL 350
RKSEEEVAARRARVVWCAVGEQELRKCNQWSGLSEGSVTCSSASTTEDCI 400
ALVLKGEADAMSLDGGYVYTAGKCGLVPVLAENYKSQQSSDPDPNCVDRP 450
VEGYLAVAVVRRSDTSLTWNSVKGKKSCHTAVDRTAGWNIPMGLLFNQTG 500
SCKFDEYFSQSCAPGSDPRSNLCALCIGDEQGENKCVPNSNERYYGYTGA 550
FRCLAENAGDVAFVKDVTVLQNTDGNNNEAWAKDLKLADFALLCLDGKRK 600
PVTEARSCHLAMAPNHAVVSRMDKVERLKQVLLHQQAKFGRNGSDCPDKF 650
CLFQSETKNLLFNDNTECLARLHGKTTYEKYLGPQYVAGITNLKKCSTSP 700
LLEACEFLRK 710

## 8.3. Appendix 3 - Cloning vector maps:




Taken from OrbiGen product datasheet




Taken from Promega product datasheet

Table 1 - Primer sequences:

| | | |
|---|---|---|
| Fw1: | 5′ – GTAATACGACTCACTAAAGCTTGGCCGCG – 3′ | in vector |
| Fw2: | 5′ – AGAAGCTTCGTTTGCCAAGTCGCCTCCAG – 3′ | in 5’ UTR |
| Fw3: | 5′ – GTAAGCTTATGAGAAAAGTGCGTGGCCCT - 3′ | at ~400 bp |
| Rev1: | 5′ – TTGCGGCCGCTTACTTCCTGAGGAATTC – 3′ | full-length |
| Rev2: | 5′ – TTGCGGCCGCTTACGTCCTGAGGAATTC – 3′ | C-term. K-T |
| Rev3: | 5′ – GCGCGGCCGCGCCTCAGTCACAGGCTTCC – 3′ | at ~2000 bp |
| Rev4: | 5′ – AGGCGGCCGCAGTCCTCTGTGGTGGAGGC – 3′ | at ~1400 bp |
| Rev5: | 5′ – CTGCGGCCGCGCAGCTGACAGGAGGGC – 3′ | at ~400 bp |

Table 2 - Peptide sequences:

| name | location | sequence | length |
|---|---|---|---|
| pLF1 | N-terminal | GRRRSVQWCAVSQPEATKC | 19 amino acids |
| pLF2 | middle | EDAIWNLLRQAQEKFGKDK | 19 amino acids |
| pLF3 | C-terminal | NLKKCSTSPLLEACEFLRK | 19 amino acids |
| pLF4 | scrambled | NFRTKSCPLELAKELKLCS | 19 amino acids |

# 9. List of symbols, acronyms and abbreviations:

| | |
|---|---|
| ΔLF | delta lactoferrin |
| µ | micro |
| 4PL | four parameter logistic |
| Aβ | amyloid beta |
| amp | ampicillin |
| APP | amyloid precursor protein |
| APS | ammonium persulfate |
| BN-PAGE | blue native polyacrylamid gel electrophoresis |
| BSA | bovine serum albumin fraction V |
| CB | coupling buffer |
| CNBr | cyanogen bromide |
| DMSO | dimethylsufloxide |
| EDTA | ethylenediaminetetraacetic acid |
| EtBr | ethidiumbromide |
| EtOH | ethanol |
| FCS | fetal calf serum |
| g | gram |
| h | hour |
| hLF | human lactoferrin |
| HRP | horseradish peroxidase |
| IB | immunoblot |
| IC50 | half maximum inhibitory concentration |
| IPTG | Isopropyl-β-D-thiogalactopyranosid |
| kDa | kilo Dalton |
| L | liter |
| LB | lysogeny broth |
| LF | lactoferrin |
| LFampin | lactoferrampin |
| LFcin | lactoferricin |
| m | milli |
| M | molar |
| M6P | mannose 6-phosphate |
| M6P/IGF2R | mannose 6-phosphate/insulin-like growth factor 2 receptor |
| mAb | monoclonal antibody |
| MeOH | methanol |
| min or ‘ | minute |
| n | nano |
| o/n | overnight |
| PAI | Plasminogen activator inhibitor |
| PBS | phosphate buffered saline |

| | |
|---|---|
| pLF | lactoferrin derived peptide |
| Plg | plasminogen |
| PVDF | polyvinylidenfluoride |
| RT | room temperature |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| sec. or ‘’ | second |
| TAE | tris-acetate-EDTA buffer |
| TBS | tris buffered saline |
| TBST | tris buffered saline + 0.1% Tween 20® |
| TEMED | tetramethylethylenediamine |
| tPA | tissue plasminogen activator |
| u | unit |
| uPA | urokinase-type plasminogen activator |
| uPAR | urokinase-type plasminogen activator receptor |
| w/o | without |
| WB | western blot |
| X-Gal | bromo-chloro-indolyl-galactopyranoside |
| vWF | van Willebrand factor |

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# 11. Zusammenfassung:

Die Fibrinolyse stellt einen lebenswichtigen Mechanismus zur Aufrechterhaltung des hämostatischen Gleichgewichtes dar. Durch die Auflösung von Blutgerinnseln (Thromben) mit Hilfe des Enzyms Plasmin wirkt es der Koagulation entgegen. Plasmin baut Fibrinpolymere ab welche den Hauptbestandteil von Thromben darstellen. Dabei stellt die Aktivierung von inaktivem Plasminogen zu aktivem Plasmin einen wesentlichen Schritt dieses durch eine Vielzahl an Enzymen regulierten Prozesses dar. Zusätzlich ermöglicht Plasmin die Migration von z.B. Immun- und Endothelzellen. Aufgrund seines breiten Wirkungsspektrums spielt Plasmin samt seinen regulierenden Molekülen nicht nur in physiologischen, sondern auch in pathologischen Prozessen eine wichtige Rolle. Im Fall von Morbus Alzheimer sind sowohl der Gehalt an Plasminogen als auch die Aktivität von Plasmin im Gehirn vermindert. Weil Plasmin das Peptid Amyloid-beta abbaut, welches die für Alzheimer Dementia charakteristischen senilen Plaques bildet, könnte es eine zentrale Rolle in der Entstehung oder im Verlauf dieser Krankheit spielen. Andererseits ermöglicht Plasmin die Metastasierung von Krebszellen durch den Abbau von Komponenten der extrazellulären Matrix. So können Tumorzellen dem Immunsystem oder lokalen Nähstoff-Beschränkungen entkommen. In der vorliegenden Arbeit wird ein neuer Regulator des fibrinolytischen Systems charakterisiert. Die Bindung an Plasminogen und die daraus resultierenden Konsequenzen werden mithilfe von molekularbiologischen, biochemischen und zellbiologischen Methoden untersucht. Schließlich wird ein synthetisches, von diesem regulatorischen Protein abgeleitetes Peptid vorgestellt, welches einen therapeutischen Ansatz darstellen könnte.

# 12. Curriculum Vitae:

### Personal details

| | |
|---|---|
| Last name | Zwirzitz |
| First name | Alexander |
| Date of Birth | 04.01.1986 |
| Place of Birth | Rohrbach |
| Nationality | Austrian |
| Marital status | not married |

### Education

| | |
|---|---|
| 2009 | BSc in biology, University of Vienna |
| Since 2005 | molecular biology studies, University of Vienna |
| 2004 - 2005 | management science studies, WU Vienna |
| 1996 - 2004 | secondary school, Wels |
| 1992 - 1996 | elementary school, Wels |

### Professional experiences

| | |
|---|---|
| April 2010 - July 2011 | practical course and diploma thesis - molecular immunology unit (lab Hannes Stockinger), Medical University of Vienna |
| 2009 | 2 month practical course - lab Emanuelle Charpentier, MFPL Vienna |
| 2008 | 1 month internship - Bacteriology & Microbiology unit of general hospital Wels |
| 2001 - 2007 | summer jobs in food industry (Diamant, Wels ) |

### Additional skills

| | |
|---|---|
| mother tongue | German |
| other languages | English (fluent in spoken and written) |
| | French (basic) |
| | Spanish (basic) |
| computer knowledge | MS Word, Excel, Powerpoint |
| | basics in Adobe Photoshop & Illustrator |

| | |
|---|---|
| Personal interests | mountainbiking, skiing/snowboarding, trekking/hiking, travelling, music and cooking |