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Studies on Coactosin-Like Protein Interaction with 5-Lipoxygenase

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If the doors of perception were cleansed, everything would appear to man as it is...

William Blake, The Marriage of Heaven and Hell, 1793

Abstract

Leukotrienes (LTs) are proinflammatory lipid mediators derived from polyunsaturated arachidonic acid, which is ester-bound at the *sn*-2 position of glycerol in cellular phospholipid bilayers. In LTs biosynthesis, 5-lipoxygenase (5-LO) represents the key enzyme, which catalyzes two initial steps. The aim of present thesis was to investigate the effects of a small 5-LO binding protein named Coactosin-like Protein (CLP) on the 5-LO activation and stability in order to define the potential role of CLP in mechanisms involved in formation of LTs.

First, we found that NMR structure of human CLP is composed of a five-stranded β-sheet surrounded by four α-helices. The structure also revealed high flexibility of the C-terminus of CLP molecule and the loop connecting $\beta 3$ and $\beta 4$. Surface-exposed Lys-75, a critical binding residue for F-actin, and Lys-131, essential residue for CLP binding to 5-LO are shown to be close to each other, precluding simultaneous binding of F-actin and 5-LO to CLP.

The effects of CLP on the 5-LO activation and modulation of its enzymatic product profile were assessed in 5-LO activity assays *in vitro*. We have found three major CLP-stimulated effects on the 5-LO catalytic reaction. First, CLP upregulates Ca^{2+}/Mg^{2+}-induced 5-LO activity in the absence of phosphatidylcholine (membrane). In such incubations, formation of 5-HETE is found to be highly increased, while the production of leukotriene A_4 (LTA_4) is minute. These data suggest that CLP can function as a scaffold for 5-LO similar to membranes. Second, CLP increases the amount of LTA_4 formed, by approximately 3-fold, when present together with phosphatidylcholine. Third, CLP promotes formation of 5-hydroxyeicosatetraenoic acid (5-HETE) by substantial reduction of 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which results in an increased ratio of 5-HETE/5-HPETE independently of the presence of a membrane.

CLP also stabilizes 5-LO and prevents its non-turnover inactivation, which together with the effects on the 5-LO activity, suggests that CLP might be functioning as a chaperone of 5-LO.

Trp-102, positioned in the β-sandwich of the 5-LO structure, is required for binding of CLP to 5-LO and for all the above mentioned CLP-induced effects on 5-LO activity and its structural stabilization. Therefore, the β-sandwich appears to be the essential part of the CLP binding surface on the 5-LO molecule.

The same pattern of subcellular trafficking (nuclear/nonnuclear association) was found for 5-LO and CLP upon activation of human polymorphonuclear leukocytes and Mono Mac 6 cells induced by different cell stimuli. This finding indicates an association between 5-LO and CLP in the cell, and combined with the results from the 5-LO activity assays, it also implies an important role of CLP in upregulation of LT biosynthesis at the nuclear membrane.

Hyperforin, an anti-inflammatory compound isolated from St. John's wort and previously reported to inhibit 5-LO activity both *in vitro* and in cellular systems, successfully interrupted formation of functional 5-LO:CLP complex. Hyperforin reduced the binding of 5-LO to CLP at concentrations shown to suppress synthesis of the 5-LO products, implying its mode of action by interfering with the 5-LO β-sandwich.

Taken together, the findings presented in this thesis have pinpointed CLP as a novel player in the 5-LO signaling pathway, capable of activating 5-LO enzyme and modulating its product pattern. Thus, these results have also potentially opened an interesting window for therapeutic intervention on LT biosynthesis.

Contents

Preface **iv**
List of Publications iv
Acknowledgements v
Abbreviations x

1 Introduction **1**
1.1 Inflammation - A tale of two roles 1
1.2 Eicosanoids 2
1.2.1 Arachidonic acid a membrane bound precursor of eicosanoids 2
1.2.2 Formation of Eicosanoids - in time and space 4
1.2.3 Biological actions of Leukotrienes 4
1.3 Leukotriene biosynthesis in the cell 6
1.3.1 Calcium as an inducer of cellular formation of Leukotrienes 6
1.3.2 Enzymes and Proteins in LT Biosynthesis 9
1.3.3 5-Lipoxygenase 10
1.3.4 Coactosin-Like Protein as a 5-LO- and actin-binding protein 17
1.3.5 5-lipoxygenase activating protein (FLAP) 20
1.3.6 Leukotriene A_4 Hydrolase 21
1.3.7 Leukotriene C_4 synthase 22
1.3.8 Leukotriene receptors 23
1.4 Activation of 5-LO enzyme *in vitro* 25
1.4.1 Stimulatory factors 25

2 Aims & Methods **30**
2.1 Aims 30
2.2 Methods 30

3 Results **31**
3.1 Structural determination of CLP by NMR 31
3.2 Characterization of 5-LO interaction with CLP *in vitro* 33
3.2.1 CLP up-regulates Ca^{2+}-induced 5-LO activity in the absence of PC 34
3.2.2 CLP up-regulates formation of LTA_4 in the presence of PC 34
3.2.3 CLP promotes formation of 5-HETE 35
3.2.4 CLP supports Mg^{2+}-induced 5-LO activity 36
3.2.5 Molar stoichiometry of the functional 5-LO:CLP complex 36
3.2.6 CLP stabilizes and protects 5-LO against non-turnover inactivation 37
3.2.7 Importance of Trp residues (13/75/102) 38
3.3 5-LO interaction with CLP in the cell 40
3.3.1 Subcellular localization and trafficking of 5-LO and CLP 40
3.3.2 Hyperforin, a novel type of 5-LO inhibitor 42

4 Discussion **44**

5 Concluding Remarks **47**
5.1 Summary 47
5.2 Future perspective 48
5.2.1 Chemoresistance: Ways to avert it 49
5.2.2 Inflammation and cancer: back to Virchow 49

Bibliography **51**

Preface

List of Publications

The thesis has been written to fulfill the requirements of the Doctorate of Medicine at the Karolinska Institute. The presented work was carried out at the Department of Medical Biochemistry and Biophysics.
The thesis is based on the following four publications, which will be refferred to in the remainder of the thesis by their roman numerals.

I. *NMR structure of human coactosin-like protein*, J Biomol NMR. 2004 Nov;30(3):353-6.,
Liepinsh E, **Rakonjac M**, Boissonneault V, Provost P, Samuelsson B, Rådmark O, Otting G

II. *Coactosin-like protein supports 5-lipoxygenase enzyme activity and up-regulates leukotriene A4 production*, Proc Natl Acad Sci U S A. 2006 Aug 29;103(35):13150-5. Epub 2006 Aug 21.
Rakonjac M, Fischer L, Provost P, Werz O, Steinhilber D, Samuelsson B, Rådmark O

III. *Coactosin-like protein functions as a stabilizing chaperone for 5-lipoxygenase: role of tryptophan 102* (accepted for publication in *Biochem. J.*)
Julia Esser*, **Marija Rakonjac***, Bettina Hofmann, Lutz Fischer, Patrick Provost, Gisbert Schneider, Dieter Steinhilber, Bengt Samuelsson, and Olof Rådmark
* denotes equal contribution

IV. *Hyperforin is a novel type of 5-lipoxygenase inhibitor with high efficacy in vivo*, Cell Mol Life Sci. 2009 Aug;66(16):2759-71. Epub 2009 Jul 5.,
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Abbreviations

5-LO	5-lipoxygenase
5-HETE	5(S)-hydroxy-8,11,14-*cis*-6-*trans*-eicosatetraenoic acid
5-HPETE	5(S)-hydroperoxy-8,11,14-*cis*-6-*trans*-eicosatetraenoic acid
13-HPODE	13-Hydroperoxyoctadeca-9,11-dienoic acid
AA	Arachidonic acid, 5,8,11,14-eicosatetraenoic acid
ATP	Adenosine-5'-triphosphate
CLP	coactosin-like protein
$cPLA_2$	Cytosolic phospholipase A_2
CYP450	Cytochrome P450
FLAP	5-lipoxygenase activating protein
LT	Leukotriene
LTA_4	Leukotriene A_4
LTA_4H	Leukotriene LTA_4 hydrolase
LTB_4	Leukotriene B_4
MM6	Mono Mac 6 cells
PC	Phosphatidylcholine
PMA	Phorbol-12-myristate-13-acetate
PMNL	Polymorphonuclear leukocytes
TGFβ	Transforming growth factorβ

Chapter 1

Introduction

The specific intracellular events that govern cellular responses to stimulatory extracellular signals, are of wide interest in order to understand pivotal intracellular pathways in diseases characterized by enhanced inflammatory responses. In that matter, protein-protein interactions, which often serve as key regulatory points for signal propagation in cell response to different stimuli, apart from representing a common mechanism responsible for the functioning of numerous physiological processes in the cell, may also have an essential role in pathogenesis of different diseases. The interaction between 5-lipoxygenase, as the key enzyme in formation of leukotrienes, a family of potent pro-inflammatory lipid mediators derived from cell membrane phospholipids, with the small cytosolic protein named coactosin-like protein (CLP) is the main theme of this thesis.

1.1 Inflammation - A tale of two roles

Infection and injuries, whether physical, chemical or infectious, always trigger a sequence of events that constitute the inflammatory response as an essential process for maintenance of homeostasis and recovery from tissue injuries (pathogens).

The hallmarks of inflammation are classically known as redness (*rubor*), swelling (*tumor*), heat (*calor*), pain (*dolor*) and loss of function (*functio laesa*), the first four of which were originally described around 40 A.D. by Celsius, a Roman physician and medical writer. These signs are consequences of vasodilation, extravasation of plasma and infiltration of leukocytes into the inflammatory site, with the objective of eradication of the irritant and repair of surrounding tissue. For the survival of the host, inflammation is a necessary and beneficial process that constitutes the part of normal host

defence. However, excessive and inappropriate inflammation contributes to a range of acute and chronic human diseases, including cardiovascular and neurodegenerative conditions, autoimmune disorders, and cancer.

The development, maintenance and resolution, as stages of the natural course of inflammation, are controlled by a complex network of plasma factors and chemical mediators released from cells. Among these are the eicosanoids, a family of structurally related autocrine and paracrine mediators derived from the oxidative metabolism of fatty acids containing 20 carbon atoms, in particular arachidonic acid. Eicosanoids produce a wide range of biological effects on specific cellular responses of importance for inflammation and immunity, but they have also been implicated in pathogenesis of a number of proinflammatory diseases.

1.2 Eicosanoids

1.2.1 Arachidonic acid a membrane bound precursor of eicosanoids

Our understanding of the roles that lipids play in the cell has changed dramatically since, in addition to their accepted structural importance as the building blocks of cellular membranes, an extensive body of evidence has demonstrated that fatty acids, glycero- and phospholipids, ceramides and many other lipid-derived compounds participate as hormon-like mediators in a variety of signaling cascades, including cell differentiation, replication and apoptosis. The arachidonic acid signaling pathway serves as a premier illustration of the role that lipid-derived mediators play in the normal cell function and also under pathological conditions. One and the same signaling lipid can provoke different cellular responses, depending on the cell type and underlying signaling network present in the target cell. Arachidonic acid (AA), also known as 5,8,11,14-eicosatetraenoic acid belongs to a group of polyunsaturated fatty acids with twenty carbons and four *cis* double bonds, with the final at the ω-6 position (20:4, ω-6). As a constituent of biological membranes in the resting cells, AA is ester-bound at *sn-2* position of glycerol in cellular phospholipid bilayers. Only trace levels of free AA may be found in the cells, since the steady state of free AA is small, prohibiting any storage of preformed molecules [120]. It can be obtained directly from a diet or synthesized endogenously from the essential linoleic acid (18:2, ω-6).

Upon cellular stimulation, activated cytosolic phospholipase A_2 translocates towards perinuclear region, where it cleaves AA from the membrane phospholipids. Once liberated AA can be oxidized by three major metabolic

pathways:

1. cyclooxygenase (COX) pathway resulting in production of various prostaglandins (PGs), prostacyclin (PGI_2) and thromboxanes (TXA_2 and TXB_2)
2. the lipoxygenase (LO) pathway, which forms leukotrienes (LTs), hydroxytetraenoic acids (5-, 12-, and 15-HETEs) and lipoxins (LXs)
3. the cytochrome P450 pathway, which generates epoxy-eicosatrienoic acids (EETs)

The compounds synthesized from these metabolic pathways comprise the major part of a family of structurally related lipid mediators that are collectively referred to as eicosanoids.

Brief history of the eicosanoid field

Early work at the Karolinska Institute showed that prostaglandins were formed by oxygenation and further transformation of AA and other polyunsaturated fatty acids [12]. In 1974, the first intermediate in prostaglandin biosynthesis was identified and the enzyme *cyclooxygenase* was termed [97]. The mode of action of non-steroidal antiinflammatory drugs (NSAIDs), was shown to be inhibition of prostaglandin biosynthesis [268]. In 1975, steroids were proposed to inhibit prostaglandin formation by reducing the availability of AA, by blocking its release from intracellular stores [90], i.e. distinct from the action of NSAIDs. This discovery led to the search for a COX-independent biosynthesis route of proinflammatory metabolites of AA, which resulted in identification of leukotrienes [237].

The first identified lipoxygenase product was 12-hydroxyeicosatetraenoic acid (12-HETE), formed by the action of 12-LO in platelets [96]. In the following year, 12-LO was shown to exhibit weak chemotactic properties [263]. In 1976, the 5-LO pathway of AA metabolism was discovered by Borgeat, Hamberg and Samuelsson [14], and a few years later several AA derivatives, such as LTB_4 and the pivotal epoxide intermediate LTA_4, were identified [16, 15]. In addition, Slow Reacting Substance of Anaphylaxis (SRS-A), first described by Feldberg and Kellaway in 1938 [70], and further investigated by Brocklehurst [23], was identified and structurally determined as a group of cysteinyl-containing LTs, i.e. LTC_4, LTD_4 and LTE_4, derived from the same intermediate as LTB_4 [102, 175]. In 1980, LTB_4 was found to act as a strong chemokinetic and aggregating compound for neutrophils *in vitro* [77] and to induce neutrophil-dependent increased microvascular permeability *in vivo* [273].

1.2.2 Formation of Eicosanoids - in time and space

Eicosanoids (as a common term used for derivatives of 20-carbon polyunsaturated fatty acids), when *de novo* synthesized from phospholipids upon stimulation, act locally in the cells in which they are formed or in adjacent cells, exerting a wide range of biological effects through receptor mediated G-protein linked signaling pathways. They can also be subjected to transcellular metabolism i.e. secreted from one and further metabolized in another cell [235, 76].

Apart from AA, other 20-carbon fatty acids, including dihomo-γ-linolenic (20:3, ω-6) and eicosapentaenoic acid (20:5, ω-3), can also serve as precursor of eicosanoids. Fatty acids with double bond beyond position 9 of the carbon backbone can not be endogenously synthesized in human cells, and will therefore depend on the dietary supply of their precursors linoleic and linolenic acids. Differences in the intake of these essential lipids can thus determine the profile of the eicosanoids formed, with consequences for the biological activity of the final compounds.

Different types of cells produce different types of eicosanoids, reflecting specific expression pattern of synthesizing or metabolizing enzymes among diverse cell types. Typically, human inflammatory cells contain high proportions of the ω-6 PUFA such as AA and low proportions of ω-3 PUFA, eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) [32]. The significance of this difference is that AA is the precursor of the 2-series PGs and the 4-series LTs, which are highly active mediators of inflammation, while EPA gives arise to PGs of the 3-series and LTs of the 5-series known to have much less potent activities than their AA-derived counterparts e.i. LTB_4 and TXA_2 [181, 140]. This thesis is focusing on 5-lipoxygenase enzyme, as a key determinant in formation of a potent, proinflammatory group of eicosanoids named Leukotrienes.

1.2.3 Biological actions of Leukotrienes

LTB_4 has long been known as a strong chimoattractant in the innate immune response, active on neutrophils, monocytes/macrophages, and eosinophils [249, 33, 158]. LTB_4 recruits leukocytes to site of inflammation by directing their migration and converting leukocytes from rolling on endothelium to firm adhesion, but also promoting transendothelial migration [53, 258]. Recently, it has been shown that LTB_4 also functions as a potent chemoattractant for dendritic cells [245] and activated $CD4^+$ and $CD8^+$ T lymphocytes, mediating their early recruitment to inflammed tissues [257, 86, 195], therefore providing an essential link between the innate and adaptive effector responses

to inflammation. In addition to its chemotactic effects, LTB_4 also stimulates leukocyte activation and variety of effector functions, such as generation of reactive oxygen species, ROS [256], release of granular enzymes [218], and phagocytosis of bacteria [154]. LTB_4 has also been demonstrated to play a critical role in anti-microbial defense, since it augments phagocyte microbial killing by stimulating lysosomal enzyme release, and generation of defensins, ROS and nitric oxide [206]. Summary of the LTB_4 biological actions is presented on Figure1.1.

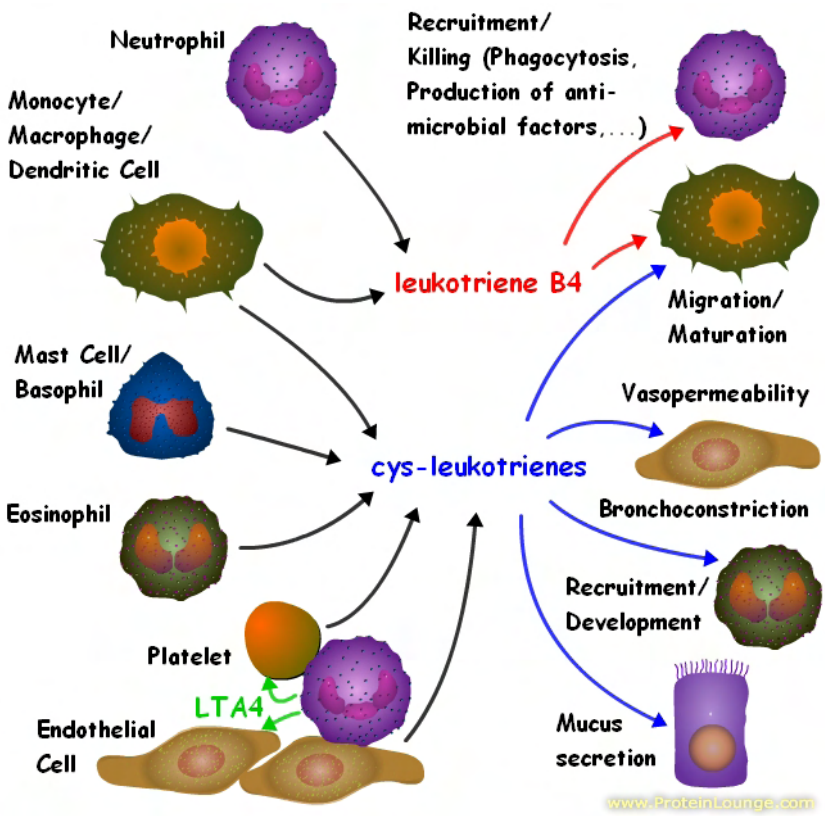

Figure 1.1: Cellular sources of leukotrienes and summary of their effects on airway and inflammatory cells.

On the other hand, LTB_4 has also an important role in the pathogenesis of many inflammatory and immune diseases. Elevated levels of LTB_4 have been detected in sputum, bronchoalveolar lavage fluid (BAL), exhaled breath condensate from patients with asthma [170], cystic fibrosis [134], and chronic obstructive pulmonary disease [51]. In addition, LTB_4 has been suggested to be involved in development of rheumatoid arthritis [3], inflammatory bowel disease [241], psoriasis [63], multiple sclerosis [182] and atherosclerosis [300,

214].

The cysteinyl-LTs (cys-LTs) are potent broncho- and vascularconstrictors, active at nanomolar concetrations. In human subjects, cys-LTs were shown to be approximately 1000-fold more potent than histamine in eliciting airway constriction[10, 274]. They also increase microvascular permeability that allows extravasation of inflammatory cells and leakage of plasma components which for the consequence has development of edema [129, 53, 249]. In addition, cys-LTs enhance mucous secretion, eosinophil infiltration in human airways, as well as bronchial smooth muscle cell proliferation [157, 137, 65]. They also promote recruitment of neutrophils, T lymphocytes, maturation and migration of dendritic cells [206]. Cys-LTs were also shown to enhance production of Th2 cytokines (e.g. IL-4, -5 and -13), which in turn stimulate the production of cys-LTs themselves [206, 192]

1.3 Leukotriene biosynthesis in the cell

The first two reactions in biosynthesis of proinflammatory LTs are catalyzed by the 5-LO enzyme, which represents a dioxygenase with two enzymatic activities (oxygenase and LTA_4 synthase activity) [236]. In both activities, stereo-selective abstraction of hydrogen is an early step (Figure 1.3). Free AA is first oxygenated by the action of 5-LO into the 5-hydroperoxy eicosatetraenoic acid (5-HPETE), and subsequently dehydrated into the allylic unstable epoxide intermediate LTA_4 [230]. Depending on a cell type and the enzymes present, LTA_4 can be either metabolized to LTB_4 by the action of LTA_4 hydrolase (LTA_4H) or conjugated with glutathione to form LTC_4 by the action of LTC_4 synthase (LTC_4S), as shown in Figure 1.2 A. Release of LTC_4 to the extracellular microenvironment and its further metabolism by sequential peptide cleavage by γ-glutamyl transpeptidase and the membrane bound dipeptidase yields in formation of LTD_4 and LTE_4, respectively [238].

Upon cell activation with different cell stimuli and in response to an increased intracellular concentration of Ca^{2+}, 5-LO translocates to the nuclear envelope where it functionally associates to 5-LO-activating protein (FLAP) (Figure 1.2 B), which presence appears to be required for cellular LT biosynthesis from endogenous AA [229, 288].

1.3.1 Calcium as an inducer of cellular formation of Leukotrienes

The concentration of cytosolic free calcium is critically important for the control of many cellular responses, including biosynthesis of leukotrienes [236].

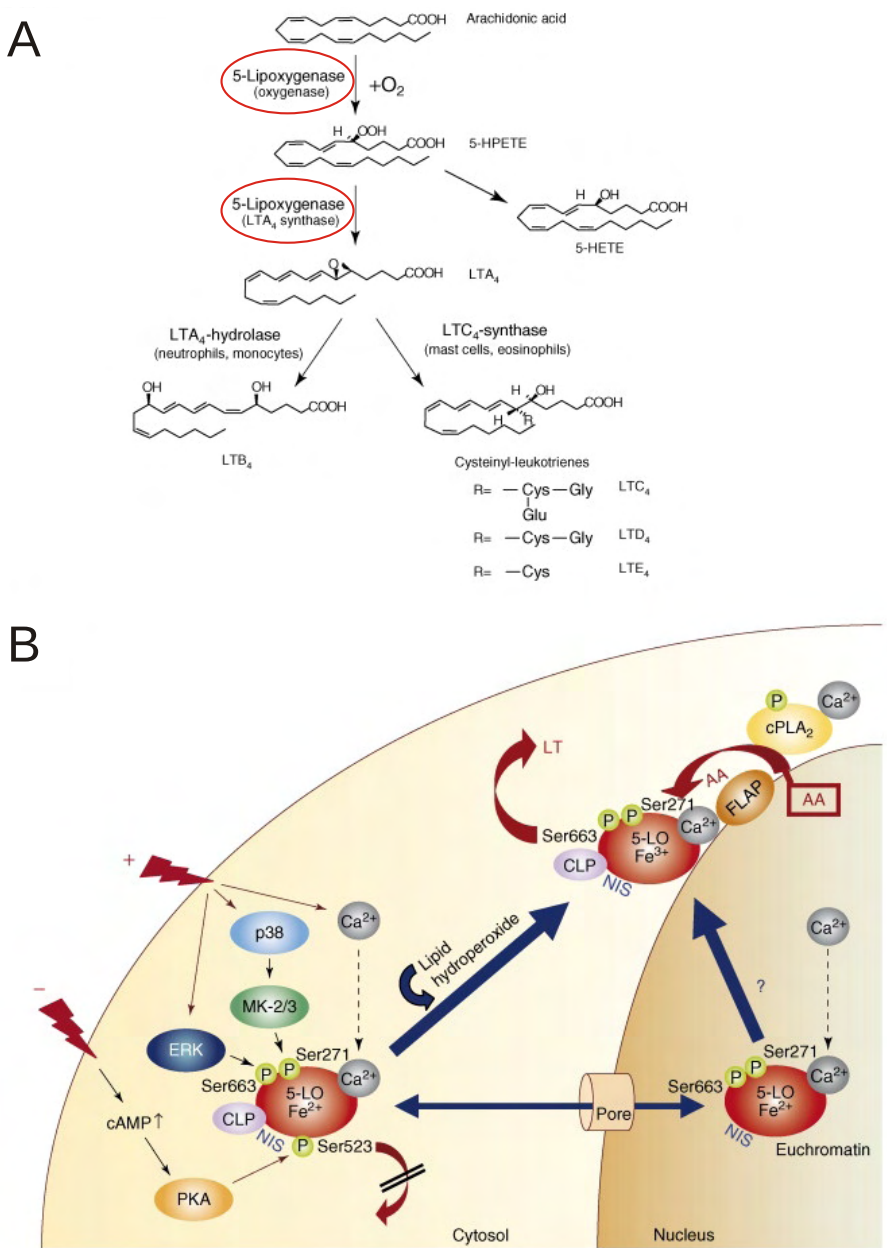

Figure 1.2: 5-LO reaction and activation in the cell. **A**. 5-LO catalyzed reaction. **B**. Activation of the 5-LO pathway in the cell.

In the resting cells, intracellular Ca^{2+} is maintained at about 10^{-7} M, and can increase to $5x10^{-6}$ M upon stimulation. Two pathways responsible for the increase in intracellular Ca^{2+} have been indentified. In electrically active cells, such as neurons, voltage-gated Ca^{2+}-channels are opened upon

membrane depolarization by the action potential, allowing diffusion of extracellular Ca^{2+} into the cell.

Another, more ubiquitous pathway common to almost all cells is mediated by the binding of extracellular signaling molecules to their respective membrane-bound receptors, which consequently leads to a release of Ca^{2+} from the intracellular stores, located primarily in the endoplasmic/sarcoplasmic reticulum compartments. Signaling further activates phosphoinositide-specific phospholipase C (PLC) in either G-protein dependent or independent manner, resulting in production of inositol-triphosphate (IP3) and diacylglycerol (DAG).

Binding of IP3 to IP3 receptor on the cytoplasmic side of ER/SR opens Ca^{2+} channels in the membrane, leading to transient increase in intracellular Ca^{2+}, since IP3 is rapidly dephosphorylated by a specific kinase in parallel with Ca^{2+} being rapidly pumped out of the cell. This pump mechanism is inhibited by ionomycin, which is usually used to increase intracellular Ca^{2+} in cellular experimental settings.

On the other side, DAG exerts quite different effect. It can either be cleaved to yield AA for eicosanoid synthesis or, it activates Ca^{2+}-dependent protein kinase C (PKC), which phosphorylates specific Ser and Thr residues of targeted proteins. The effects of DAG can be mimicked by phorbol esters, plant products that bind and activate PKC directly.

For the purpose of experiments carried out in cells, Ca^{2+}-influx can be stimulated by ionophores, which are either mobile ion *carriers* within membranes (e.g. A23187) capable of forming cage-like structures around ions shielding their charge from the surrounding environment and thus facilitating crossing through hydrophobic interior of the lipid membrane, or *channels*, which form hydrophilic pores through which ions diffuse. Rather low concentrations of calcium (200nM) seem sufficient for the 5-LO activation in intact cell [239, 72]. In the biosynthesis of LTs, Ca^{2+} regulates activities of two enzymes, cytosolic phospholipase A_2 ($cPLA_2$) and 5-lipoxygenase, inducing translocation of both enzymes in parallel from cytosol towards the perinuclear region. Higher concentration was reported to be required for activation of $cPLA_2$ compared to 5-LO [239].

Interestingly, liberated AA and one of its metabolites, LTB_4, seem to modulate intracellular Ca^{2+} levels in an opposing manner. The IP3 receptor is inhibited by AA, while the ryanodine receptor (another type of calcium release channel) was found to be unaffected. In contrast to this, LTB_4 (100nM) activated the ryanodine receptor, without affecting IP3 receptor imply that AA and LTB_4 modulate intracellular calcium levels, possibly by acting on different Ca^{2+} stores [255].

1.3.2 Enzymes and Proteins in LT Biosynthesis

Cytosolic Phospholipase A_2

Mammalian cells contain structurally different forms of PLA_2 including cytosolic (c), secreted (s), calcium-independent (i), the platelet activating factor (PAF) acetylhydrolases and the lysosomal PLA_2 [26]. These enzymes are characterized by their ability to specifically hydrolyze the *sn-2* ester bond of phospholipids and are subdivided into groups based on sequence, molecular weight, disulfide bonding patterns and requirement of Ca^{2+}. The products of hydrolysis of the *sn-2* ester bond of a phospholipid are a free fatty acid and a lysophospholipid. Both products represent the first step in generating second messengers that play important physiological roles. Among the PLA_2s, the 85 kDa cytosolic PLA_2 (α isoform) preferentially catalyzes the release of AA from the *sn-2* position of phospholipid and is therefore believed to play a pivotal role in providing this free polyunsaturated fatty acid for eicosanoid biosynthesis [109, 143]. In addition to liberating AA, $cPLA_2$ also exhibits Ca^{2+}-independent lysophospholipase activity and weak transacylase activity [104, 142, 222]. The coordinated release of *sn-2* AA followed by the *sn-1*, usually saturated, fatty acid of diacylphospholipids provides an efficient mechanism for controlling the levels of potentially cytotoxic lysophospholipids in the cell.

Cellular distribution. $cPLA_2$ is widely distributed at a relatively constant level in almost all human tissues. This is in line with the features of the promotor region, typical of a house-keeping gene with no TATA or CAAT box, although atypical in not being GC rich and lacking Sp1 binding sites [289, 242].

Activity. Stimulation of the cells with agents that mobilize intracellular calcium and/or promote the phosphorylation of $cPLA_2$-α leads to translocation of the enzyme from cytosol to endoplasmatic reticulum, Golgi apparatus and perinuclear membranes, and also induces changes in configuration of $cPLA_2$ which increases the phospholipid binding affinity and arachidonic acid release [27, 186]. The Ca^{2+}-dependancy of the $cPLA_2$ activity originates in the presence of a calcium-dependent phospholipid binding domain, similar to C2 domains in enzymes such as protein kinase C (PKC) and PLC-γ [46].

In addition to an increase of intracellular calcium, activation of $cPLA_2$-α is also regulated by phosphorylation of Ser resides (Ser-505, Ser-515 and Ser-727) by the action of mitogen activated protein kinases (MAPKs), mitogen activated protein kinase interacting kinase (MNK1) and calmodulin kinase II (CamK II) respectively [17, 106, 177, 146]. Other phosphorylation sites have been reported at Ser-454 and Ser-437 in Sf cells [56], but there is currently

no information of the effects of phosphorylation at these residues. Additionally, ceramide-1-phospasphate (C1P) has also been shown to be an allosteric activator of cPLA$_2$-α, since down-regulation of ceramide kinase, the enzyme that forms C1P, with short RNAi blocked agonist-induced AA release and prostaglandin production [251].

Full activation of cPLA$_2$-α requires both increased cytosolic Ca^{2+} and phosphorylation of Ser residues of the enzyme, although the impact of phosphorylation appears to be dependent on Ca^{2+} concentration. Phosphorylation of cPLA$_2$-α on Ser-505 increased the phospholipid binding affinity at low concentration of calcium but was found to be less important at higher Ca^{2+} levels.

Structure. The structure of cPLA$_2$-α protein contains an N-terminal C2 domain, involved in calcium-dependent lipid binding and a catalytic domain. Both of these domains are required for full activity [115]. There are three regions in the C2 domain responsible for calcium binding of cPLA$_2$-α through Asp and Asn residues. This in turn promotes association of hydrophobic residues in the catalytic domain with membrane phosphatidylcholine, causing penetration of the enzyme into membrane bilayer and further release of AA [27].

Cellular regulation. Extracellular stimuli can alter the mRNA and protein levels of cPLA$_2$-α. Numerous cytokines such as interleukin-1α (IL-1α), tumor necrosis factor-α (TNF-α), and interferon-γ (INF-γ) have been reported to up-regulate gene expression of cPLA$_2$-α, thereby contributing to the cytokine stimulated AA release [46, 145, 291, 290].

cPLA$_2$ knock-out mice. Recent knock-out studies of cPLA$_2$-α in mice have proven the pivotal role of this enzyme in eicosanoid generation (i.e. stimulated peritoneal macrophages of these mice exhibited a marked decrease in the production of PGs and LTs [265] and related diseases, in particular chronic inflammation and intestinal tumorigenesis [244, 110].

1.3.3 5-Lipoxygenase

Cellular localization and subcellular distribution

In line with the function of LTs as mediators of inflammation, 5-LO protein expression under normal conditions is found to be largely restricted to cells of mainly myeloid lineage, including granulocytes, monocytes/macrophages, mast cells, dendritic cell and B lymphocytes. Strong expression of this enzyme was also found in Langerhans cells of epidermis, suggesting a role of 5-LO in early events of the immune response in the skin. Recently, 5-LO expression has also been confirmed in non-myeloid cells, such as endothe-

lial cells [299], fibroblasts [125] and smooth muscle cells, where human cytomegalovirus infection strongly induced expression of 5-LO protein [215]. In contrast, platelets, T lymphocytes and erythrocytes are devoid of 5-LO presence.

Subcellular localization of 5-LO in resting cells has been found to be specific. The 5-LO enzyme has cytosolic localization in neutrophils, eosinophils, monocytes and peritoneal macrophages. Intranuclear pools of 5-LO associated with chromatin are observed in cultured mast cells, Langerhans cells and rat basophilic leukemia cells [280], while dual localization of 5-LO, representing a unique example of 5-LO import into the nucleoplasm during differentiation, was found in resting alveolar macrophages [49]. These data imply that the possibility of migration through the nuclear envelope, presumably via the nuclear pore complex, does not have to be associated with the activation of 5-LO and subsequent LT synthesis.

Regulation of 5-LO activity in the cell

Effects of Ca^{2+} and AA. Alteration of the ability of the cells that express 5-LO to synthesize leukotrienes occurs by several mechanisms. As described above, elevation of intracellular concentration of Ca^{2+}, upon different cell stimuli e.g. fMLP, PAF, opsonized zymosan, LTB_4, C5a, IL-8 and ionophores, has a fundamental effect on the hydrophobicity of cytosolic PLA_2 and 5-LO and their parallel translocation to the perinuclear region that contains glycerophospolipids with esterified AA. However, recent evidence has suggested that the substrate, AA, may play an important role in altering the fundamental hydrophobic properties of cytosolic 5-LO separately from these Ca^{2+} effects. Binding of AA to cytosolic 5-LO, appears to substantially alter the affinity of 5-LO for the nuclear bilayer [74]. In addition to this, the amount of free AA available as a substrate for 5-LO [295] and its accessibility to 5-LO [149] were shown to be important determinants of leukotriene biosynthesis.

Effects of phosphorylation. Phosphorylation of 5-LO has also an essential role in regulating enzymatic activity. Three separate sites have been identified: a p38 MAPK-regulated MAPKAPK (mitogen-activated protein kinase-activated protein kinase)-2/3-dependent site at Ser-271[277, 278], an ERK2 (extracellular signal-regulated kinase 2)-dependent site at Ser-663 [281] and a cAMP/PKA (proetin kinase A)-dependent site at Ser-523 [150]. Phosphorylation at the first two Ser residues results in activation of 5-LO (only in the cellular systems), whereas phosphorylation of Ser-523 leads to inactivation of the enzyme and inhibition of LTs biosyntehsis (both *in vitro* and in the cell). Phosphorylation of Ser-523 has also been shown to prevent

nuclear localization of 5-LO by inhibiting the nuclear import function of a nuclear import sequence close to the kinase motif [148]. This inhibition seems to be the molecular basis for the suppressive effects on 5-LO of exogenous adenosine and increased cAMP, which activate PKA [75]. Interestingly, AA which promotes phosphorylation on Ser-273 and Ser-663, prevented cAMP-mediated inhibition of 5-LO translocation from cytosol towards perinuclear region, and therefore also product synthesis in activated neutrophils [74].

Effects of 5-LO interacting proteins. The localization of 5-LO within the nucleus introduces an interesting level of complexity to the 5-LO synthetic pathway, since it is clear that 5-LO must assemble with other proteins in particular FLAP, as well as encounter AA derived from from $cPLA_2$. Since the nuclear envelope is a dual-membrane bilayer separated by a luminal space, this has led to speculation that the assembly of the proteins might be different between the nuclear localization on the outer nuclear bilayer relative to the inner nuclear bilayer [21]. However, regardless of 5-LO localization in resting cell, in either cytoplasm or nucleoplasm, activation of the cell initiates translocation of 5-LO to the nuclear envelope, where presence of FLAP facilitates formation of LTs. FLAP appears to be absolutely required for the cellular biosynthesis of LTs from endogenous AA [59, 166].

Our study on CLP interaction with 5-LO, led to suggestion that CLP might act as a potential scaffold for 5-LO, on the basis of the profound increase in the quantity of LTA_4 formed by 5-LO in the presence of this small cytosolic protein, and its translocation from the cytosol to the perinuclear region of neutrophils upon stimulation [219].

Impact of the cellular redox status on 5-LO. Since catalysis of 5-LO requires oxidation of ferrous (Fe^{2+}) to the active ferric(Fe^{3+}) state by lipid hydroperoxides, the redox tone comes up as another important regulatory mechanism of cellular 5-LO activity. Therefore conditions that promote lipid peroxidation, such as formation of reactive oxygen species by: phorbol 12-myristate 13-acetate (PMA), addition of peroxides, inhibition of GPx enzymes and depletion of glutathione, upregulate 5-LO product synthesis, while reduction of peroxides by GPx-1 and GPx-4 suppresses formation of the 5-LO products[280]. Apart from promoting conversion of the iron into an active state, increased peroxide tone upon oxidative stress might also trigger activation of 5-LO in the cells by promoting phosphorylation, since oxidative stress was shown to activate p38 MAPK in B-lymphocytes [277, 282].

Cellular Degradation of 5-LO. Recently, an example of 5-LO regulation by caspase-mediated degradation has been reported. Splitting of the Epstein-Barr virus (EBV)-transformed B-lymphocyte cell line BL41-E95-A caused a pronounced, but transient reduction of functional 5-LO protein, accompanied by the appearance of a 62-kDa 5-LO cleavage product, in

parallel with activation of caspase-6 and caspase-8 [285]. Treatment with peptide-inhibitors of caspases prevented degradation of 5-LO. Furthermore, *in vitro* studies confirmed cleavage of purified 5-LO by caspase-6 after Asp-170 residue, which in a homology-based 3D model of 5-LO structure was found to be located on the enzyme periphery.

Inhibition of 5-LO activity by pharmacological intervention. Because of the importance of 5-LO-derived LTs in the pathogenesis of inflammatory diseases, there has been a great interest in identifying inhibitors of 5-LO activity. Compounds that directly interfere with the 5-LO enzyme are classified, according to their molecular mode of actions, into four groups: (1) redox inhibitors, which act by reducing active Fe^{3+} form to inactive Fe^{2+} state; (2) iron ligand inhibitors, with chelating ability of active iron site; (3) non-redox competitive inhibitors; (4) compounds that act on 5-LO by so far unrecognized mechanism. Hyperforin, a 5-LO inhibitor isolated from the plant *Hypericum perforatum*, used in our latest study (paper IV), has been characterized as a non-competitive inhibitor, devoid of reducing properties and with unique mode of action.

Structural and catalytic properties

As one of six human lipoxygenases, 5-LO is a soluble, monomeric enzyme of about 78 kDa [231]. It contains 673 amino acids. The structure of 5-LO has not been reported yet, even though it was first purified and cloned more than 20 years ago [161].

The amino acid sequence of human 5-LO exhibits high similarity to other mammalian 5-LOs, but also to other classes of LOs [60, 161]. A model structure of 5-LO, based on the three dimensional structure of rabbit reticulocyte 15-LO, as the first and the only determined crystal structure of mammalian LOs [84], consists of an N-terminal β-sandwich (residues 1-114) and the helical C-terminal catalytic domain (residues 121-673) containing a non-heme iron in the active site. Mutagenetic analysis has demonstrated that His-372 and His-550 are iron ligands, while the iron-binding function of His-367 remains uncertain [183, 298, 121, 297, 203]. From the 3D structure of soybean LO-1 [167], it was found that C-terminal isoleucine, Ile-673 in human 5-LO, also functions as an iron ligand [217, 101].

Electron paramagnetic resonance (EPR) studies have shown that the iron of purified recombinant, inactive enzyme is in the ferrous state (Fe^{2+}), and that the treatment with 5-HPETE and other fatty acid hydroperoxides (15-HPETE and 13-HPODE) could restore the active, ferric (Fe^{3+}) form of iron [37, 98].

Iron at the catalytic site is essential for 5-LO activity, since in the gen-

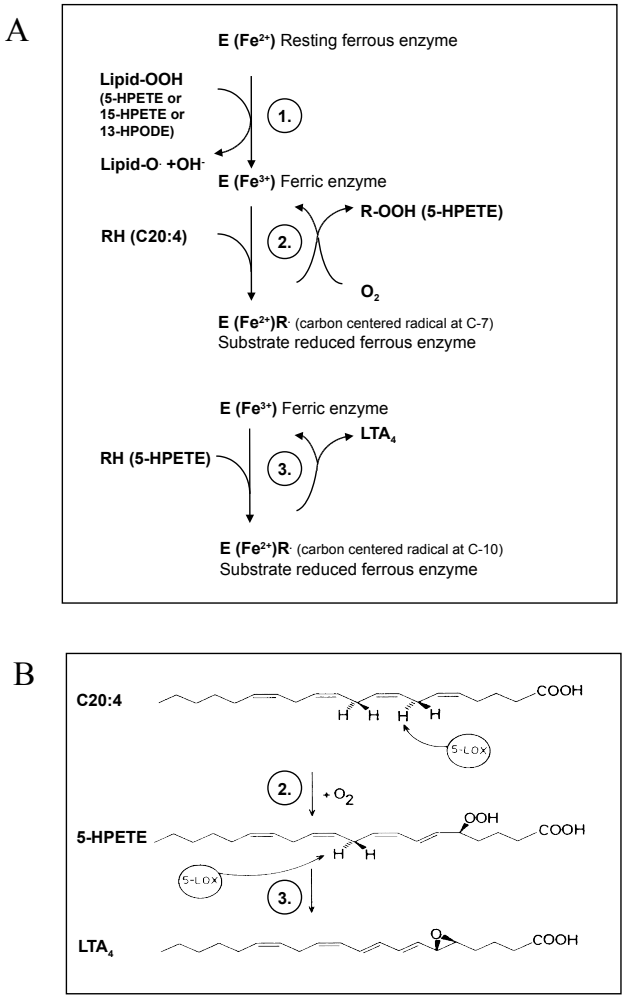

Figure 1.3: Catalytic cycle of 5-LO **A**. The role for iron in the generally accepted scheme for the lipoxygenase reaction (the radical reaction mechanism) **B**. Mechanism of oxygenase and LTA_4 activities of 5-LO

erally accepted scheme for the lipoxygenase reaction (the radical reaction mechanism, Figure 1.3 A), it acts as an electron acceptor or donor during hydrogen abstraction and peroxide formation [266]. When AA is converted to 5-HPETE, the pro-5S hydrogen is abstracted from C-7 leading to production of 5(S)-HPETE [135, 266]. Subsequent conversion of 5(S)-HPETE to LTA_4 involves the abstraction of the pro-R hydrogen from C-10 and allylic shift of

the radical to C-6, resulting in formation of the 5,6 epoxide [243], as shown in Figure 1.3 B. Alternatively, 5-HPETE is reduced to 5-HETE. The ratio of LTA_4 to 5-HPETE formed depends on the *in vitro* assay conditions, e.g. the relative concentrations of free AA, membrane associations, amount of 5-LO, but also the presence of FLAP in the cellular systems [287, 108, 227, 1].

The smaller N-terminal domain is a C2-like β-sandwich with typical ligand-binding loops. Residues in these loops of 5-LO have been shown to bind Ca^{2+} and cellular membranes [99], which consequently led to enzyme activation. Furthermore, residues 1-80 of the β-sandwich of 5-LO, directed an import of a green fluorescent protein (GFP) fusion protein to the nucleus of transfected HEK-293 cells [42], suggesting that nuclear localization signal is located in this region. Three nuclear import sequences have been identified for 5-LO, at Arg-112, Lys-158 and Arg-518 [127, 128]. The combined mutation of these three import sequences inhibits nuclear localizations and reduces LTB_4 synthesis by 90 %, demonstrating that 5-LO positioning within nucleus is crucial for LTs production upon activation [149].

The 5-LO gene and regulation of 5-LO protein expression

The gene encoding human 5-LO is located on chromosome 10 and consists of 14 exons and 13 introns encompassing over 82 kb of genomic DNA [81]. The promoter region is GC-rich and lacks typical TATA and CAAT boxes, resembling promoter regions of house-keeping genes. Naturally occurring mutations in the 5-LO promoter and variable numbers of binding sites for transcriptional factors Sp1/Erg-1 has been identified in the population. However, these mutations had varying effects on reporter-gene activity [118, 246]. Asthmatics with promoter variants mediating reduced 5-LO expression may not benefit from pharmacological anti-LT treatment [62]. By contrast, in relation to atherosclerosis, mutations of the tandem GC-boxes are connected with an increase in intima-media thickness and higher plasma level of C-reactive protein [64]. This observation seems to be compatible with an increased production of pro-inflammatory LTs, due to upregulated 5-LO expression. Apparently, the effect of mutations in this crucial part of the human 5-LO gene promoter is unclear and possibly it could differ between cell types.

Promoter analysis using reporter gene assays in HeLa cells (which do not express endogenous 5-LO) revealed two positive and two negative regulatory regions and a modest enhancement in transcriptional activity by phorbol ester [113]. More recent promoter analysis, using Mono Mac 6 cells (which express 5-LO), revealed a positive regulatory region that was not detected in HeLa cells. This region contains several vitamin D response elements (VDRE) and its binding of the vitamin D receptor was demonstrated by

several methods [248].

The 5-LO promoter activity is reduced by DNA methylation [264], and induced by histone-deacetylase inhibitors [132]. Interestingly, DNA metylation also regulates expression of the LTB_4 receptor BLT1, which has an expression profile similar to that of 5-LO [130]. It seems possible that DNA methylation is responsible for suppression of 5-LO expression in most of non-myeloid cell types, and that aberrant methylation could lead to upregulated 5-LO expression in tumor cells.

Upregulation of the 5-LO expression at the protein level has been shown during differentiation of leukemic cell lines of granulocytic or monocytic origin [11, 254, 47]. Differential inducers, such as retinoic acid, dimethyl sulfoxide (DMSO), vitamin D3 and transforming growth factor β (TGF-β) were observed to have a prominent effect on 5-LO mRNA and protein [47, 24, 25]. This increase is thought to reflect the upregulation of 5-LO expression during leukocytes differentiation in the bone marrow. Strong ($\sim$100 fold) induction of 5-LO mRNA, protein and activity was found after differentiation of HL-60 and human monocytic Mono Mac 6 (MM-6) cells upon treatment with TGF-β and vitamin D3. However, no induction of 5-LO promoter activity by the same stimuli, has been found in the reporter gene assays nor changes in 5-LO mRNA half-life have been observed, all together indicating that effect of vitamin D3 and TGF-β does not seem to be related to changes in transcription initiation, but rather posttranscriptional events, such as elongation and maturations [253]. Granulocyte/macrophage colony-stimulating factor (GM-CSF) has also been shown to increase 5-LO gene transcription and protein expression ($\sim$2-3 fold) in mature blood leukocytes, such as human neutrophils [252, 209]. The expression of 5-LO was upregulated by IL-3 in mouse mast cells [174]. IL-4 is shown to reduce 5-LO expression in monocytes and maturing dendritic cells (DC), but also upregulate 15-LO type I [250]. TGF-β counteracted this down-regulatory effect of IL-4 in maturing DCs, indicating discordant effects of TGF-β on maturing versus mature macrophages [79]. Interestingly, dexamethasone has been found to increase 5-LO expression in both monocytes and the acute monocytic leukemia cell line THP-1 [223]. Opposing data have appeared in the case of human mast cell line HMC-1 [8].

5-LO expression in cancer cells

Aberrant expression of 5-LO has been observed in variety of cancer cells [82, 284]. The untransformed cells of the respective healthy tissues usually do not express the same levels of 5-LO mRNA or protein. Prostate and esophageal cancers are good example of such cases, since 5-LO was found to

be overexpressed in malignant compared with matched benign tissues of the same origin [92], and the treatment with 5-LO inhibitors induced massive apoptosis in human breast, pancreatic, prostate and bladder cancer cells [111, 207, 185, 105].

It was also reported that addition of 5-LO products, particularly 5-HETE, prevents selenium-induced apoptosis in prostate cancer cell line LNCaP [83], and that treatment with LY293111, acting as an LTB_4 receptor antagonist, induces apoptosis of pancreatic cancer cells [261]. An interesting connection between 5-LO and p53 has been shown in a lung carcinoma-derived cell line A549 and malignant mesothelioma (MM) cells, where genotoxic stress induced by adriamycin caused upregulation of 5-LO, which in turn antagonized apoptosis by 5-LO-dependent altering of p53 nuclear trafficking [35]. In addition, 5-LO was described as a key regulator of MM cell proliferation and survival via a VEGF-related circuit [228].

Recently, the 5-LO gene has been identified as a critical regulator for leukemia stem cells (LSCs) in BCR-ABL-induced chronic myeloid leukemia, since the loss of 5-LO gene caused impairment of the function of LSCs but not normal hematopoietic stem cells and that consequently prevented development of chronic myeloid leukemia [43].

Cancer cells often have increased generation of reactive oxygen species and an altered redox status compared to normal cells [262]. Excessive levels of ROS stress may be relevant for activation of lipoxygenases in cancer cells, since it is known that LOs for their activity require oxidation of ferrous form of iron (Fe^{2+}) into ferric (Fe^{3+}).

5-LO knock-out mice

The 5-LO-deficient mice showed enhanced lethality from *Klebsiella pneumoniae* in association with decreased alveolar macrophage phagocytic and bacterial activities [8]. On the other hand, 5-LO knock-out mice exhibited reduced airway reactivity followed by the antigen exposure [119], and were more resistant lethal anaphylaxis induced by platelet-activating factor [87, 41]. Resistance to development of variety of inflammatory diseases in 5-LO KO mice was confirmed for different experimental settings.

1.3.4 Coactosin-Like Protein as a 5-LO- and actin-binding protein

CLP was first identified in immunoprecipitation assays where it was found to co-precipitate with an actin myosin complex. It was named in a view if

its similarity with coactosin, an actin binding protein isolated from the slime mold *Dictyostelium discoideum*.

Structure. Given the amino acid sequence similarity between CLP and coactosin (33% identity and 75% similarity), CLP likely represents the human homologue of coactosin, which belongs to the family of actin-depolymerizing factor/cofilin group of actin binding proteins [57]. In addition, CLP and coactosin show similarities to the N-terminal domains of two other members of this family, actin-binding protein 1 and drebrin [139, 122]. Several solution (NMR) and crystal structure of CLP have been determined. These results will be discussed in detail in *'Result and Discussion'* of the thesis.

Activity and interaction with other proteins. It is not known if CLP possesses any enzymatic activity. However, under physiological conditions, monomeric globular actin (G-actin) is in equilibrium with filamentous actin (F-actin), which forms the actin cytoskeleton and is responsible for maintaining and modifying cell shape in motility, phagocytosis, and cytokinesis. The actin cytoskeleton is regulated by numerous actin-binding proteins, which interact with actin and regulate the cytoskeleton in cells. Several actin binding proteins in the actin-depolymerizing factor/cofilin family have been demonstrated to associate with actin via electrostatic interactions involving basic and acidic amino acid residues [34, 165]. As shown for the other members of its group, CLP was also found to directly interact with the filamentous, F-actin but does not form a stable complex with globular, G-actin [211, 122, 139]. This interaction was pH-insensitive and Ca^{2+}-independent. In addition, CLP was found to bind actin filaments with a stoichiometry of 1:2 (CLP:actin subunits) and was shown to have no direct effect on actin poly- or depolymerization.

Site-direct mutagenesis revealed the involvement of Lys-75 of CLP in actin binding, a residue highly conserved in related proteins and exposed on the surface of the CLP protein [211, 144]. Also in transfected mammalian cells, CLP was found to co-localize with actin stress fibers [211].

In the yeast two-hybrid system when using 5-LO as bait, CLP was identified as one of three different 5-LO associating proteins [212]. Co-immunoprecipitation experiments using epitope-tagged 5-LO and CLP proteins transiently expressed in human embryonic kidney 293 cells, revealed the presence of CLP in 5-LO immunoprecipitates confirming binding between these two proteins. In reciprocal experiments, 5-LO was detected in CLP immunoprecipitates [211], indicating that 5-LO forms a complex with CLP in mammalian cells. Non-denaturing polyacrylamide gel electrophoresis and cross-linking experiments showed that 5-LO binds CLP in a 1:1 molar stoichiometry in a Ca^{2+}-independent manner. In addition, also mouse CLP was found to bind 5-LO with the same 1:1 stoichiometry [61].

Site-directed mutagenesis suggested an important role for surface-exposed Lys-131 of CLP in mediating 5-LO binding. In addition to binding, the importance of this CLP residue for 5-LO activation *in vitro* was confirmed in the 5-LO activity assays [219].

Considering the ability of CLP to bind 5-LO and F-actin, several studies have addressed the possibility of CLP being physically linked to 5-LO and actin filaments at the same time. However, formation of ternary complex of 5-LO-F-actin-CLP has not been found. Structural studies of CLP have indicated overlapping binding sites positioned spatially very close on the surface of CLP, which could explain why a ternary complex could not be experimentally validated [212, 144, 147]. Moreover, 5-LO was found to interfere with actin polymerization in co-sedimentation assays and cross-linking experiments. 5-LO-CLP and CLP-F-actin interactions appeared to be mutually exclusive and suggest a modulatory role for 5-LO in actin dynamics [212].

CLP gene and cellular distribution of CLP protein. The CLP gene, initially found as a sequence flanking a deletion on a human chromosome 17 characterizing the Smith-Magenis syndrome [38], encodes a protein composed of 142 amino acid residues with a molecular mass of 16 kDa. Endogenous CLP is localized in the cytosol of myeloid cells. CLP mRNA shows a wide tissue distribution. It is predominantly expressed in placenta, lung, kidney and peripheral blood leukocytes and low levels of CLP were detected in brain, liver, and pancreas [211].

CLP has recently been described as a human pancreatic antigen, as its mRNA is found to be overexpressed in pancreatic cancer cell lines, if compared to normal pancreatic tissues. Three epitopes of CLP have been recognized by HLA-A2 restricted and tumor-reactive cytotoxic T-lymphocytes and it has been suggested that peptides 1524 and 104113 of CLP could be appropriate candidates for humoral immunity [179, 180] and therefore appropriate vaccine candidates for peptide-based immunotherapy of HLA-A2(+) cancer patients. In addition, CLP has also been suggested as tumor antigen in the case of bladder cancers, since immunoscreening of urinary bladder cancer cDNA library revealed upregulation of CLP expression [39]. Treatment of the PaCa44 adenocarcinoma cell line with 5-aza-2'-deoxycytidine had been found to result in a 22-fold reduction in the expression of CLP protein [36]. CLP protein levels were found to increase during differentiation of 3T3-L1 fibroblasts into adipocytes [275]. In proteomic analysis of the neuroblastoma cell line N1E-115, CLP was detected upon differentiation of the cells with DMSO [193]. Recently, a tissue based comparative proteome study of healthy and diseased human substantia nigra has revealed that CLP was also differentially regulated in substantia nigra in Parkinson's disease, together with

annexin V and beta-tubulin cofactor A [276].

Upregulation of CLP has also been implicated in development and progression of autoimmune, inflammatory diseases, e.g. rheumatoid arthritis (RA). Jin and coworkers identified CLP as a rheumatoid arthritis (RA) candidate gene that is highly expressed in peripheral leukocytes of RA patients [126].

CLP knock-out mice. So far, there are no reports on generation of CLP knock-out mice.

1.3.5 5-lipoxygenase activating protein (FLAP)

The discovery of FLAP came as a result of the observation that the LT biosynthesis inhibitor MK-886 inhibits 5-LO activity in intact cells by binding to a membrane-associated protein, without affecting 5-LO, phospholipases or other non-selective mechanisms that could lead to leukotriene inhibition [172].

Structure. FLAP is an 18 kDa integral membrane protein which forms homotrimers within the nuclear membrane. Each monomer is composed of four transmembrane helices that are connected by two cytosolic loops and a luminal loop [166, 59, 71]. The FLAP trimer is cylinder-shaped, with a flattered cytosolic top and pointed luminal base. Recent studies have demonstrated that FLAP is present also as a monomer and a homodimer in human PMNL, and that organization of FLAP as a homodimer appears to be essential for LT biosynthesis [208]. It has also been reported that FLAP can form a heterodimer or trimer together with LTC_4 synthase [155].

This small membrane bound protein is a member of the MAPEG (**m**embrane-associated proteins in eicosanoid and glutathione metabolism) family of proteins. In contrast to other members of this group including LTC_2 synthase, microsomal PGE_2 synthase, and the microsomal glutathione-S-transferase [123], FLAP does not bind glutathione and does not express glutathione transferase activity, typical for the members of this group of proteins.

Activity. FLAP has no known enzymatic activity. It has been shown to be an AA binding protein. This interaction can be interrupted by the indol-class compound such as MK-886 or quinoline-indol based inhibitors such as MK-591 and ABT-080 compounds [152]. In addition, it was recognized that FLAP inhibitors also weakly inhibit LTC_4 synthase [117] and mPGES-1 [133]. Since evidence for physical association of FLAP to 5-LO is lacking, FLAP has been suggested to play a role as a substrate transfer protein presenting $cPLA_2$-liberated AA to 5-LO, and also enhancing the sequential oxygenation of AA to 5-HPETE and dehydration to leukotriene A_4 [1]. The requirement

of FLAP for cellular LT biosynthesis was first visualized in experiments with osteosarcoma cells transfected with 5-LO, where concurrent transfection with FLAP was required for A23187-induced LT production [59]. However, there is no absolute requirement for FLAP when cells are stimulated in the presence of exogenous AA [1] and MK-886 failed to suppress LT formation under such condition. Nevertheless, it was found that FLAP can also stimulate the utilization of exogenous AA by 5-LO [280] and there was a huge stimulatory effect on 5-LO catalyzed conversion of 12-HETE [153].

Cellular distribution. Expression of FLAP is consistent with the occurrence of 5-LO protein in myeloid cells like granulocytes, monocytes/macrophages and B-lymphocytes and up-regulation of FLAP often correlates with that of 5-LO [270].

FLAP-deficient mice. Activated macrophages from FLAP knock out mice were unable to produce LTs and they appeared to mimic 5-LO deficient mice regarding the blunted inflammatory response [30]. Moreover, severity of collagen-induced arthritis was substantially reduced if compared to wild type mice, demonstrating an essential role of FLAP in LTs biosynthesis involved in both the acute and chronic inflammatory response in mice [89].

1.3.6 Leukotriene A_4 Hydrolase

Cellular distribution. LTA_4H is a solubile zinc-containing enzyme, which catalyzes the conversion of the unstable epoxide LTA_4 into LTB_4. It is widely expressed in most tissues and cells. Among hematopoietic cells, LTA_4H was found in neutrophils [269], monocytes [85], lymphocytes [124], mast cells [78] and erythrocytes [164]. Eosinophis have low levels of this enzyme [269, 13], while basophils [272] and platelets [151] seem to be devoid of its expression. LTA_4H is a cytosolic enzyme, although nuclear localization has also been reported [20, 22]. In addition, LTA_4H activity has also been detected in blood plasma and bronchoalveolar lavage fluid [73, 173].

Structure. LTA_4H is a monomeric enzyme, which contains one Zn atom essential for the catalytic function [94]. The crystal structure of LTA_4H in complex with the inhibitor bestatin has been solved. The protein was found to be folded into three domains: N-terminal, catalytic and C-terminal, which together form a deep cleft harboring the zinc site [259].

Activity. Apart from epoxide hydrolase activity, LTA_4H possesses an aminopeptidase activity, which cleaves the N-terminal aginine moiety of tripeptides such as Arg-Gly-Asp, Arg-Gly-Gly and Arg-His-Phe [95]. However, no natural substrate for the peptidase activity has been identified [88, 187]. Biochemical studies and mutational analysis suggest that the two enzyme activities of LTA_4H are exerted via distinct and yet overlapping ac-

tive sites, with the zinc atom necessary for both catalyses. Both enzyme activities of LTA_4H are subject to covalent modification and inactivation by LTA_4, LTA_4 methyl ester and the structural isomers LTA_3 and LTA_5, a process commonly referred to as suicide inactivation [66, 194]. Tyr-378 has been identified as the LTA_4-binding amino acid during inactivation since the mutation of this residue abolished suicide inhibition [171]. The peptidase activity of LTA_4H is stimulated by albumin and several monovalent anions, while the epoxide hydrolase activity remained unaffected [286]. In addition, phosphorylation of Ser-415 has been reported to inhibit the epoxide activity but not the aminopeptidase activity [233, 232].

Cellular regulation of LTA_4H expression. There are only a few reports on the cellular regulation of LTA_4H. IL-4 and IL-13 have been shown to increase mRNA expression and protein synthesis of LTA_4H in human PMNL [294]. In contrast to this, another study demonstrated that INF-γ increased LTA_4H mRNA expression, whereas IL-4 and IL-13 reduced LTA_4H mRNA in monocytes [169].

LTA_4H knock-out mouse. LTA_4H deficient mice develop normally and are healthy. A comparison of the phenotype of these mice with 5-LO knock out mice allowed the respective roles of LTB_4 and cysteinyl-LTs to be ascribed to various parts of the inflammatory responses. LTB_4 causes the influx of neutrophils accompanying topical AA administration and contributes to the vascular changes seen in this model. In contrast, LTB_4 influences only the cellular components of zymosan A-induced peritonitis whereas LTC_4 appears to be responsible for plasma protein extravasation [31].

1.3.7 Leukotriene C_4 synthase

Cellular distribution. LTC_4S is an 18kDa integral membrane protein, located in the outer nuclear membrane and peripheral endoplasmatic reticulum of the cell [44]. LTC_4S is found in various cells of hematopoietic origin, specially eosinophils, basophils, mast cells, monocytes/macrophages and platelets, as well as in non-hematopoietic cells and tissues lacking 5-LO, in which LTC_4 is formed form LTA_4 via transcellular metabolism [93, 199].

Structure. As a MAPEG protein, LTC_4S exhibits 31% sequence identity to FLAP at the amino acid level and it was previously suggested that LTC_4S can be organized as a heterodimer together with FLAP [155].

Two years ago, the crystal structure of the human LTC_4S was presented in its apo- and GSH-complexed forms to 2.00 and 2.15 resolution, respectively [159]. The structure reveals a homotrimer, where each monomer is composed of four transmembrane segments. The structure of the enzyme in complex with substrate shows that the active site enforces a horseshoe-shaped con-

figuration on GSH, and effectively positions the thiol group for activation by a nearby arginine at the membrane-enzyme interface. In addition, the structure provides a model for how the ω-end of the lipophilic co-substrate is pinned at one end of a hydrophobic cleft, providing the molecular 'ruler' to align the reactive epoxide at the thiol of glutathione [159]. This provides new insights into the mechanism of LTC_4S formation and also suggests that the observed binding and activation of GSH might be common for a family of homologous proteins important for inflammatory and detoxification responses.

Activity. LTC_4S catalyzes the committed step in cysteinyl-LT formation by conjugation of reduced glutathione to LTA_4. LTC_4 is transported out of the cell by the multidrug resistance-associated protein, and is subsequently converted into LTD_4 (through cleavage of glutamic acid) and further to LTE_4 through cleavage of glycine [80, 236].

LTC_4S activity is stimulated by phosphotidylcholine and divalent cations ($Mg^{2+} > Ca^{2+}$), whereas glutathione is essential for enzyme stability [184, 200]. LTC_4S contains two potential PKC phosphorylation sites, the phosphorylation of which appears cell-specific and reduces LTC_4S activity [6, 91]. The FLAP inhibitor MK-886 was shown to inhibit LTC_4S as well [138].

Cellular regulation of LTC_4S expression. Increased expression of LTC_4S, as determined by immunostaining, was observed in bronchial biopsies from patients with aspirin-intolerant asthma [50]. Regulatory effects of cytokines on the LTC_4 expression have been in several cellular studies [240, 224, 114]. IL-4 triggered a profound induction of LTC_4S in human mast cells [114]. TGF-β increased LTC_4S expression at the transcriptional level in the monocyte-like cell line, THP-1 [224].

LTC_4S knock-out mice. These knock out mice develop normally and are fertile. The bone marrow-derived mast cells from LTC_4S knock out mice produce no LTC_4 in response to IgE-dependent activation, whereas the production of LTB_4, PGD_2 and 5-HETE is normal. In *in vivo* inflammatory models, LTC_4S knock out mice exhibited markedly attenuated antigen-induced pulmonary inflammation with reduced IgE secretion and Th2 cytokine production, suggesting a role for cys-LTs in regulating the Th2 cell-dependent pulmonary inflammation [131]). Congenital LTC_4S deficiency has been reported to be linked to a fatal neurometabolic disorder [163, 162].

1.3.8 Leukotriene receptors

All LT receptors are seven-transmembrane spanning G-protein couple receptors (GPCRs) and the LTs either activate the Gq subtype (resulting in an

increased intracellular calcium concentration) and/or the Gi subtype (resting in decreased intracellular cyclic adenosine monophosphate, cAMP) [260, 67], as presented in Figure 1.4.

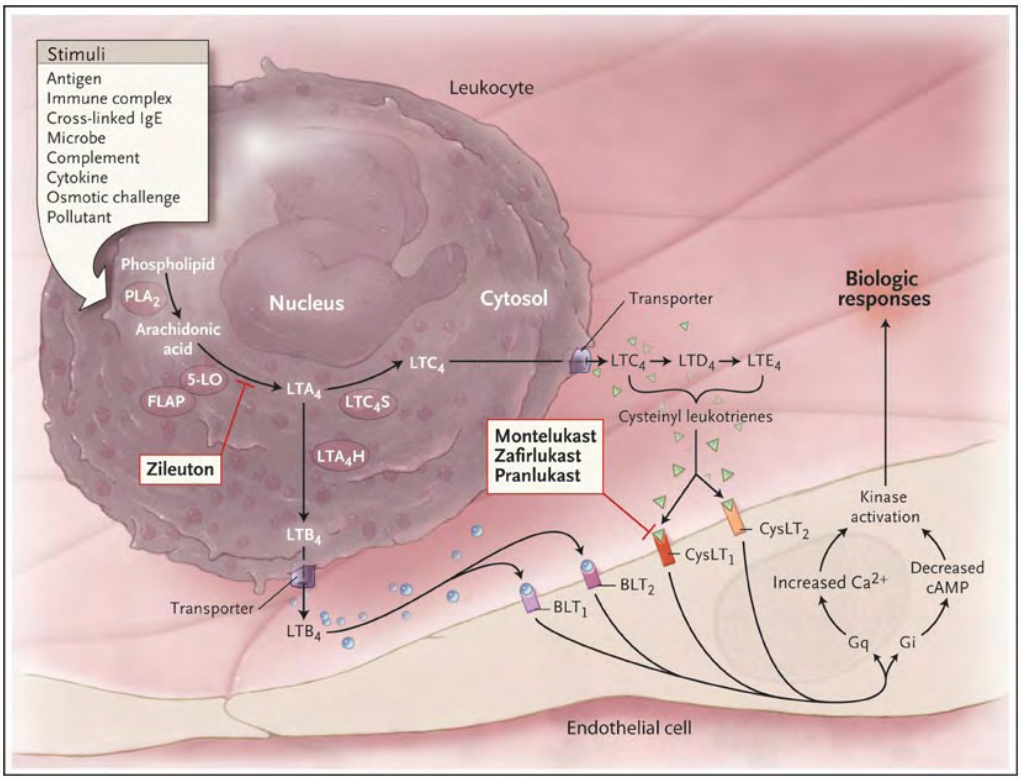

Figure 1.4: Leukotriene receptors and signaling with sites of action of anti leukotriene drugs. Adopted from Marc Peters-Golden, The New Engl. J. of Med. 2007.

LTB_4 exerts its effects by binding to two receptors, termed B leukotriene receptor 1 (BLT_1, high affinity receptor) and B leukotriene receptor 2 (BLT_2, low affinity receptor) [260] and PPAR-α, the peroxisome proliferator-activated receptor alpha.

LTB_4 has been shown to be a natural ligand and activator of PPAR-α. Activation of PPAR-α results in the induction of genes involved in the fatty acid oxidation pathways that degrade fatty acids and fatty acid derivatives like LTB_4 [58]. Direct interaction between PPAR-α and LTB_4, which results in induction of enzymes for catabolism of LTB_4, may represent a feedback mechanism that controls the duration of inflammatory responses.

BLT_1 is primarily expressed on leukocytes, and mediates most of the

LTB_4-induced effects. BLT_2 is more ubiquitously expressed and it binds LTB_4 with significantly less specificity than BLT_1. Several eicosanoids other than LTB_4 including 12-HETE, 12-HPETE and 15-HETE were found to compete with LTB_4 binding in a dose dependent manner to membrane fractions of chinese hamster ovarian (CHO) cells expressing human BLT_2, but not human BLT_1 [292]. Although its functional role is less clear, a recent study showed that LTB_4 mediates dendritic cell chemotaxis via BLT_2 [245].

Cys-LTs act via two receptors named cysteinyl-leukotrienes receptor type 1 and type 2 (cys-LT_1 and cys-LT_2), as shown in Figure 1.4. Cys-LT_1 is mainly expressed in the spleen, peripheral blood leukocytes, and less expressed in the lung (smooth muscle cells and interstitial macrophages), small intestine and placenta. The cys-LT_1 receptor antagonists montelukast, zafirlukast and pranlukast are registered drugs for the treatment of asthma and allergic rhinitis [206]. The cys-LT_2 receptor is mainly found in the heart, adrenal medulla, peripheral blood leukocytes, spleen and lymph nodes with weaker expression throughout the CNS [190, 116]. Recently, the orphan receptor GPR17 was identified as a new dual uracil nucleotides/cysteinyl-leukotrienes receptor [45], which was found to be highly expressed in the organs typically undergoing ischemic damage, such as brain, heart and kidney.

1.4 Activation of 5-LO enzyme *in vitro*

1.4.1 Stimulatory factors

The induction of 5-LO activity *in vitro* has extensively been studied through years and shown to involve the following factors.

Ca^{2+}

Although other lipoxygenases (LOs) do not require Ca^{2+} for catalysis, Ca^{2+} can strongly stimulate 5-LO activation *in vitro*. However, the extent of activation by various stimuli, including Ca^{2+}, depends strongly on the lipid:arachidonate ratio [221, 227]. Nevertheless, under conditions optimized to give maximal activity, Ca^{2+} was shown to be the most potent stimulus. For purified 5-LO, half-maximal excitatory activation (EC_{50}) is obtained in presence of 1-2μM calcium, whereas concentrations of 4-10μM of Ca^{2+} caused maximal activation of the enzyme [189, 202].

5-LO binds Ca^{2+} in a reversible manner. For the intact enzyme, a dissociation constant (Kd) of 6 μM has been determined by equilibrium dialysis

and the stoichiometry of maximum binding has been shown to average approximately two Ca^{2+} ions per molecule of 5-LO [100]. Similar results (two Ca^{2+} per 5-LO; Kd 7-9 μM) have been obtained for the His-tagged C2-like domain (residues 1-115). Mutations of Asn-43, As-p44 and Glu-46 in the ligand binding loop of C2-like domain of 5-LO have been indicated to be essential for 5-LO binding to Ca^{2+} and Ca^{2+}-stimulated enzyme activity [99]. The C2-like domain of 5-LO is negatively charged, and binding of Ca^{2+} is thought to neutralize it [136].

Ca^{2+}-stimulated activation of 5-LO requires the presence of phosphatidylcholine (PC) or coactosin like protein (CLP), and therefore Ca^{2+} might induce a productive binding to these scaffold factors. Ca^{2+} also lowers the Michaelis constant (Km) of 5-LO for arachidonic acid (AA). It also changes the kinetics of the reaction leading to substrate inhibition at high concentrations of AA [221].

Ca^{2+} also appears to modify the affinity of 5-LO for lipid hydroperoxides, since in the presence of this divalent cation, glutathione peroxidase-1 (GPx-1) is less efficient in inhibiting 5-LO activity. This effect of Ca^{2+} is drastically diminished for the 5-LO loop-2 mutant with decreased Ca^{2+} affinity [28]. For the reduction of 5-HPETE into 5-HETE, a pseudoperoxidase activity of 5-LO has also been accounted to have Ca^{2+}-dependent character [226]. Interestingly, in addition to Ca^{2+} also Mg^{2+}, at millimolar concentrations present in cells, can activate 5-LO *in vitro* [221]. In contract, Zn^{2+}, Co^{2+} and Cu^{2+} had been shown to inhibit 5-LO activation in *in vitro* assays [202]. Due to appearance of some basal 5-LO activity in the absence of Ca^{2+} and Mg^{2+}, the divalent cations appear not to be part of the catalytic mechanism.

Phosphatidylcholine

During purification of 5-LO from human leukocytes, it was observed that the enzyme activity depended on microsomal membranes [231]. Among the different phospholipids that have been tested, only PC promoted 5-LO activity, although 5-LO could associate also with phosphatidylethanolamine and phosphatidylserine [213, 189]. Synthetic PC vesicles could replace the cellular membrane and thus function as a stimulatory factor. Binding of PC to 5-LO has been described in the presence as well as the absence of Ca^{2+}, however stimulatory effects of PC on 5-LO activity *in vitro* were significantly more pronounced upon addition of this divalent cation [221, 197].

Most lipids were shown to have inhibitory effect on 5-LO activity, while cationic lipids had stimulatory effect. The complex pattern of dependence of the 5-LO activity on various lipids indicates that the negative surface charge of membranes is inhibitory especially in the absence of Ca^{2+}, but is required

for Ca^{2+}-mediated membrane binding and activation of 5-LO. Furthermore, Ca^{2+}-induced binding to PC vesicles was shown to stabilize structure of both 5-LO protein and the membrane [221, 196]. Ca^{2+}, as well as Mg^{2+}, increased the hydrophobicity of 5-LO in a phase partition assay [100, 221].

Binding of 5-LO to PC has been shown to involve three surface-exposed Trp residues, positioned in the 5-LO β-sandwich [136]. Ca^{2+} binding at the C2-like domain appears to change orientation of these three tryptophan side chains, so as to maximize membrane insertion potential, resulting in an increased enzyme affinity for certain membrane phospholipids, specifically binding to phosphatidylcholine molecular species typically found within the nuclear membrane [197]. Such phospholipid classes are not particularly abundant on the inner leaflet of the cellular plasma membrane, which has more anionic bulk character due to higher abundance of glycerophosphoserine and glycerphosphoinositol [176].

In line with this, the isolated 5-LO C2-like β-sandwich containing the three previously mentioned Trp residues, was also shown to have a higher affinity for zwitterionic PC vesicles than for anionic (phosphatidylserine and phosphatidylglycerol). It was suggested that the PC selectivity directs 5-LO to the nuclear envelope [136]. This data is in accordance with the requirement of the β-sandwich for translocation of GFP-5-LO constructs to the nuclear membrane in ionophore-stimulated HEK 293 cells [40].

Increased membrane fluidity favored 5-LO association, and it was argued that this should be the factor directing 5-LO to the AA enriched nuclear envelope [197]. Interestingly, addition of cholesterol to a membrane preparation *in vitro* reduced 5-LO activity by half [197], and cholesterol sulfate could also inhibit 5-LO in intact cells [5].

Coactosin-like protein (CLP)

CLP was initially found to bind 5-LO in the yeast 2-hybrid system [212]. The binding of purified proteins were later confirmed in numerous *in vitro* assays. Non-denaturing polyacrylamide gel electrophoresis and cross-linking experiments showed that 5-LO binds CLP in a 1:1 molar stoichiometry, in a Ca^{2+}- independent manner [211]. The effect of CLP on the 5-LO activation and modulation of the enzyme product profile will be in detail discussed in *'Result and Discussion'* of the thesis.

ATP

5-LO catalytic activity has been shown to be stimulated by ATP and to lesser extent by the other nucleotides, including ADP, AMP, cAMP, CTP and UTP

[191, 188, 68]. Among LOs, it appears that only 5-LO can bind ATP and be activated by nucleotides. However, database analysis revealed no consensus on ATP- or nucleotide-binding site in the amino acid sequence.

The extent of 5-LO stimulation at 0.1-2 mM ATP is in the range of 2- to 6-fold, and Ka values for ATP were determined as 30-100 μM. ATP was first shown to stimulate crude 5-LO [191] and it was long thought that the ATP stimulation requires Ca^{2+} presence. Additional studies, however, have demonstrated that ATP triggers activity of 5-LO in the absence of Ca^{2+} [247], although combination of Ca^{2+} and ATP gives the best stimulatory effect [221].

Since the non-hydrolysable analogue γ-S-ATP caused comparable effects, ATP hydrolysis appeared not to be required for stimulation. In stead, it seems that ATP acts by having an allosteric and/or stabilizing effect on the enzyme.

In vivo, the intracellular concentration is assumed to be in the millimolar range, and MgATP2- (the major form of ATP inside the cell) rather than ATP might be the physiological stimulus [221].

Lipid Hydroperoxides

Activation of resting 5-LO requires the oxygenation of the iron to the ferric Fe^{3+} state, therefore the redox environment appears to be of importance for 5-LO activity in cellular but also in vitro systems.

Electron paramagnetic resonance (EPR) studies showed that the iron of purified recombinant 5-LO is ferrous (Fe^{2+}) [37, 98], and the treatment with 5-HPETE as well as other lipid hydroperoxides (12-HPETE and 13-HPODE) gave ferric form of 5-LO. In accordance with EPR data, 5-LO in crude leukocyte homogenates was shown to be stimulated by lipid hydroperoxides but not by the presence of hydrogen peroxide.

Lipid hydroperoxides can shorten the lag phase of 5-LO, which is usually observed after addition of substrate to crude 5-LO in cell homogenates or to a solution of the purified enzyme. In addition, conditions that promote lipid peroxidation stimulate 5-LO activity in leukocytes [225]. Glutathione peroxidases (GPx) reduce lipid hydroperoxides, and when added to *in vitro* 5-LO activity assays, GPx-1 was shown to inhibit 5-LO product formation. GPx-1 and also catalase can stabilize 5-LO enzyme *in vitro* by scavenging ROS formation [296].

Glycerides

Various glycerides have been found to activate 5-LO. Of the compounds tested, 1-oleoyl-2-acetyl-sn-glycerol (OAG) was found to be the most potent [112]. This activation occurs in the absence of Ca^{2+}. However, in the presence of Ca^{2+}, OAG caused no stimulation of 5-LO. Phospholipids or cellular membranes also abolished the stimulatory effects of OAG.

The same tryptophan residues that mediate binding to phosphatidylcholine [136] and are essential for binding to CLP [219], appear to be involved in binding to OAG, since 5-LO triple mutant lacking tryptophan residues (W13A/W75A/W102A) was not stimulated with OAG [112]. In addition, phospholipids or cellular membranes abolished the effects of OAG. As previously found for Ca^{2+}, OAG protected 5-LO against the inhibitory effect of GPx-1 [112].

Chapter 2

Aims & Methods

2.1 Aims

The aim of this thesis was to functionally characterize interaction of CLP (a human F-actin binding protein) with 5-lipoxygenase and decipher its role in biosynthesis of leukotrienes, with the intent to increase our understanding of mechanisms involved in leukotriene formation and also enable more efficient pharmacological treatment of diseases where these pro-inflammatory compounds are implemented.

2.2 Methods

Methodologies used in this thesis are established in the field of biochemistry, immunology and molecular biology. For experimental details, see the Materials and Methods sections in papers I-IV.

Chapter 3

Results

3.1 Structural determination of CLP by NMR

In the half-century since the first glimpse of a protein structure at atomic resolution, structural biology has had a fundamental role in advancing our understanding of biological function at a molecular level. Yet, although it is generally agreed that proteins must have some flexibility in order to perform their roles, uncertainty remains about how well static structural models capture the essential features underlying function. A picture may be worth a thousand words, but does it always tell the entire story?

In order to gain the knowledge on the CLP movements (rather than obtaining a static crystal structure of the protein), which could enable determination of ensembles of structures that represent the conformational fluctuations of the protein, and advance our understanding of CLP interactions with actin filaments and 5-LO enzyme, we performed NMR spectroscopy, as a powerful tool for studying the structure and dynamics of small proteins.

For the purpose of the experiment, CLP was isotopically ^{15}N-labelled and expressed in *E. coli*. It was previously reported that human CLP could exist both as a monomer and a dimer [211]. During purification, we also observed that CLP showed two bands with molecular masses corresponding to the monomer and the dimer on SDS-PAGE. However, CLP analyzed by mass spectroscopy (MALDI) gave a sharp single peak with the monomer mass (unpublished data).

The structure featured a five-stranded β-sheet with two α-helices on either side, Figure 3.1.

The superposition of our NMR structure of CLP with another solution CLP structure, determined almost at the same time [54], and crystal struc-

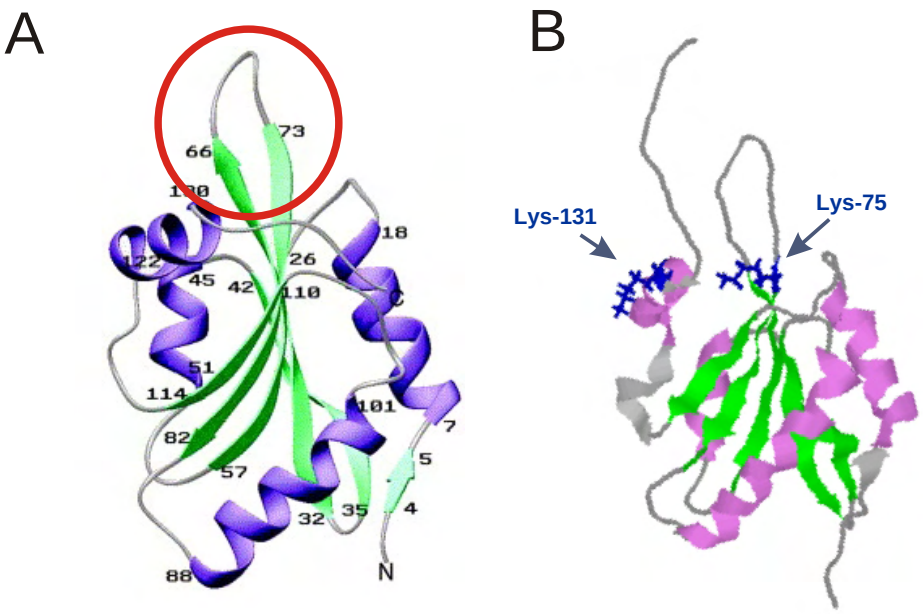

Figure 3.1: Ribbon diagram of human CLP. The five-stranded β-sheet is highlighted in green and the connecting loops are in grey. Four helices are highlighted in purple. In **A**, the mobile loop connecting $\beta 3$ and $\beta 4$ is indicated. In **B**, the Lys residues binding 5-LO (Lys131) and F-actin (Lys75)are indicated

ture of CLP [147] in backbone shows that the loop (residues 66-75) between β_3 and β_4 and the C-terminus (residues 136-142) is flexible (encircled on Figure 3.1 A), while the other regions are kept highly similar and rigid.

Polar residues cover most areas of the CLP surface, with a few hydrophobic residues exposed, suggesting that CLP binds to other proteins with hydrogen bonds and/or salt bridges. Previous reports from our laboratory indicated that the critical binding residues for F-actin and 5-LO are Lys-75 and Lys-131, respectively. Mutation of Arg-73 has also been reported to affect CLP binding to β-actin [210].

In the CLP structure, Lys-75 is located on the surface of the molecule, which together with Lys-72 and Arg-73 creates a positively charged microenvironment that favors an electrostatic bond with an acidic site of the surface of actin. In that respect actin subdomain 1, in particular the acidic N-terminus of actin, is of interest. Actin subdomains 1 and 2, as shown before,

are favored targets for F-actin binding proteins [165], since several proteins were found to cross-link to this area of actin [267]. The complementary interfaces in these proteins were regions rich in basic amino acid residues, suggesting that the N-terminus of actin, which protrudes from F-actin as a highly mobile unit [107], acts as a fishing-rod in attracting positively charged surfaces of actin binding proteins, including CLP.

Our NMR structure also revealed that Lys-131, involved in CLP binding to 5-LO, is positioned on the surface of helix 4, which is followed by the mobile C-terminus.

The binding of mouse CLP to F-actin was favored at low salt conditions (in agreement with electrostatic interactions), while binding of CLP to 5-LO was favored at high salt concentrations, indicating a hydrophobic interaction [61].

The Cα atoms of the functionally important residues, Lys-75 and Lys-131, are found to be separated by about 14 Å. Therefore, it appears plausible that these two residues overlap, making the observation of this ternary complex experimentally impossible. We attempted to obtain information on CLP residues binding to 5-LO by addition of purified 5-LO to ^{15}N-labelled CLP. However no striking difference in the CLP NMR-spectrum before and after the addition of 5-LO could be seen.

Despite great expectations from this experiment, the NMR structure of CLP turned out to be a story on mute, since it did not reveal much in regard to directions of future experiments. However, the CLP structure has been used to model the CLP-5-LO complex (paper III in this thesis).

3.2 Characterization of 5-LO interaction with CLP *in vitro*

Although papers that came out from our laboratory have demonstrated direct interaction between 5-LO and CLP by several experimental approaches, the physiological significance of this interaction was missing. In this study, we investigated if presence of an equimolar concentration (1:1) of CLP in the solutions with purified 5-LO, under different experimental conditions, could effect the 5-LO enzymatic activity and modulate its product profile *in vitro*. Formation of 5(S)-HPETE, 5(S)-HETE, and LTA_4 (measured as the nonenzymatic hydrolysis products 6-*trans*-LTB_4 and 12-*epi*-6-*trans*-LTB_4) was monitored at 235nm and 270nm, respectively, by using reverse-phase high pressure liquid chromatography (HPLC).

As is often the case in science, this study benefited from a fortuitous

accident.

3.2.1 CLP up-regulates Ca^{2+}-induced 5-LO activity in the absence of PC

Early on it was determined that 5-LO activity (as well as a degree of activation) is different at various combinations of arachidonic acid (AA) and PC [221], and the concentrations of 100μM of AA and 25μg/ml of PC were chosen as our standard conditions *in vitro* activity assays. Analysis of AA metabolites, 5(S)-HETE and 5(S)-HPETE, in samples where 5-LO was pre-exposed to an equimolar amount of CLP, without presence of PC, serendipitously revealed that Ca^{2+}-induced activation of this enzyme was up-regulated by about 3-fold, compared to controls.

CLP-induced up-regulation of the 5-LO activity in the absence of PC was further investigated and confirmed by the spectrophotometric cuvette assay, where this 16kDa small protein strikingly increased initial velocity of the 5-LO catalysis, even under modified conditions due to method limitations (as explained in paper II).

These data were surprising, since it had been postulated that the presence of a membrane preparation, usually PC vesicles, is required in addition to Ca^{2+}, for the 5-LO enzymatic activity *in vitro*, as it is supposed to mimic the membrane interfaces at which 5-LO catalysis *in vivo* is occurring.

To ascertain that this effect of CLP on 5-LO activity *in vitro* mirrored the specific 5-LO-CLP interaction, 5-LO was incubated with either of two CLP mutants. Unlike the wild type, a mutated CLP (K131A) protein (with reduced binding ability to 5-LO, [210]) had no effect on the 5-LO activity and failed to trigger the formation of 5-HPETE and 5-HETE in the absence of PC.

On the other hand, the K75A mutation which abolished the actin-binding properties of CLP without interfering with binding to 5-LO [211], caused equally strong activation of 5-LO enzyme as the wild type protein, confirming previous finding which indicated the importance of Lys-131 of CLP in 5-LO binding.

3.2.2 CLP up-regulates formation of LTA_4 in the presence of PC

Addition of CLP-wt or its K75A mutant to the standard 5-LO reaction mixture, including PC, was furthermore found to cause elevated formation of LTA_4 by about 3- up to 5-fold compared with the control samples. This up-

regulation of LTA_4 was maximal at the highest concentration of PC tested (25μg/ml).

Interestingly, by decreasing the amounts of membrane preparation in the incubating mixture (from 25μg/ml, 10μg/ml, 2.5μg/ml to zero), CLP gradually elevated the formation of 5-HETE with a simultaneous reciprocal decrease of LTA_4 synthesis, which was completely abolished in samples lacking PC. In those samples 5-HETE appeared as a main and practically the only 5-LO product formed, considering that formation of LTA_4 metabolites was barely detectable (shown in Fig. 1A, paper II).

In a parallel set of experiments, presence of CLP in 5-LO incubations at increasing amounts of PC, gradually led to an up-regulation of 5-LO-catalyzed formation of LTA_4, which appeared as a result of diminished 5-HPETE reduction. These findings implied that type of the 5-LO products as well as the degree of their up-regulation by CLP appears to be highly dependent on the amount of PC present in the assay. In addition, the highest yield of LTA_4 was obtained in samples when an equimolar 5-LO:CLP complex was incubated at the highest concentration of PC, thereby suggesting that CLP and PC do not compete, but rather function together to improve the capacity of 5-LO for leukotriene biosynthesis. Based on this, we concluded that CLP in the presence of PC promotes formation of LTA_4.

3.2.3 CLP promotes formation of 5-HETE

Variations in the amounts of PC in samples, as described in paper II, have clearly revealed a few prominent features of CLP in connection to 5-LO catalysis, among which is pronounced reduction of lipid-hydroperoxides, particularly 5-HPETE.

Presence of an equimolar amount of CLP (1:1), that could replace the membrane preparation, and have such a strong impact on the 5-LO activation *in vitro*, has additionally received our attention for its apparent peroxidase activity and clear promotion of 5-HETE formation. Inclusion of CLP in the incubation mixture shifted the formation of 5-LO products monitored at 235nm, from 5-HPETE to 5-HETE (Fig. 1A, paper II). This was particularly clear in the absence of PC if compared with the control samples.

To rule out the possibility that the conversion of 5-HPETE, as the main 5-LO metabolite formed under standard assay condition, is due to a presence of reducing agents (or some unspecific interaction of CLP with one of the 5-LO cofactors or even a substrate itself rather than with 5-LO), experiment was performed in presence of only Ca^{2+} and 13-HPODE.

Also under this condition, the degree of 5-HPETE conversion was not changed and it was found that interacting 5-LO:CLP complex, rather than

CLP by itself, is capable of drastically reducing 5-HPETE to 5-HETE.

As shown in Fig.4 (paper II), in the same manner, even exogenously added 5-HPETE was highly converted into 5-HETE, as only 28% of starting amount remained unchanged.

Since 13-HPODE also was reduced to a similar extent, this indicated that CLP might be able to promote reduction of lipid-OOH bound to a proposed second fatty acid binding site in 5-LO [234, 168].

3.2.4 CLP supports Mg^{2+}-induced 5-LO activity

The pronounced stimulatory effect of Ca^{2+} is regarded to be specific. However, some other divalent cations have also been shown to stimulate 5-LO activity *in vitro*, although less effectively [202, 221]. Compared with the effect of Ca^{2+} on the 5-LO product synthesis, mediated via ligand-binding loops in the 5-LO C2-like domain [99], the concentration of free Mg^{2+} required to achieve maximal activity response in the presence of PC was approximately 50-fold higher.

Based of these earlier studies and considering high concentrations of free Mg^{2+} in the cell (up to 1mM), we investigated if 5-LO catalysis would be affected in the same way as it had been in the case with Ca^{2+}. We added an equimolar amount of CLP, only in the presence of Mg^{2+} as a stimulatory cation. Our experiments revealed that all three, previously described, CLP-induced effects on 5-LO activation and modulation of its product profile have also been consistently observed with Mg^{2+} as a stimulatory co-factor.

These data were confirmed in additional experiments, carried out by spectrophotometric assays, where time courses of 5-LO activity in the presence of CLP and Mg^{2+} had the same general appearance as with CLP and Ca^{2+}, with a high initial velocity of the reaction that leveled out after 1-2 min (Fig. 3A and B, paper II). Due to method limitation, cuvette assays were performed at lower concentrations of AA than in standard incubation conditions, showing that even below the critical micellar concentrations CLP is efficient.

3.2.5 Molar stoichiometry of the functional 5-LO:CLP complex

It is noteworthy that when 5-LO activity was assayed with CLP in molar stoichiometries 1:2, 1:5, and 1:10, no additional impact on the observed CLP-induced effects on 5-LO catalysis has been noticed. On the other hand, the reduced amount of CLP (stoichiometry 1:0.1) gave no up-regulation of the 5-LO activity neither did it appear to have any modulation effect on the 5-LO

product profile. These results dovetail with the previously reported study [210] where non-denaturing PAGE experiment together with chemical cross-linking assays showed that interacting covalently linked 5-LO:CLP complex can only have 1:1 molar stoichiometry. Heat-treated CLP, probably with deranged structure, was not functional.

It is of importance to mention, that apart from an equimolar amount of CLP present in the 5-LO reaction mixture, separate experiments have shown that presence of Ca^{2+} or Mg^{2+} is absolutely required for all the effects of CLP and/or PC we had observed. We find this to be a puzzling disconnect from a report on 5-LO:CLP binding which was shown to be Ca^{2+}-independent [210]. On the other hand, as it was suggested that 5-LO can bind to membranes in the productive/nonproductive modes (i.e. membrane binding *per se* may not confer 5-LO activity), it also appears possible that the binding mode of 5-LO to CLP could depend on the presence of Ca^{2+} in that way resembling the productive/nonproductive modes of membrane binding [196].

3.2.6 CLP stabilizes and protects 5-LO against non-turnover inactivation

In addition to supporting Ca^{2+}-induced enzyme activity, we have found that CLP could also successfully prevent inactivation of 5-LO over time. Solutions of purified 5-LO kept at RT (on the lab-bench) for up to five days in sealed Eppendorf tubes and under normal atmosphere, were found to be gradually inactivated with the half of activity remaining after 24h, and about 20% after 120h. However, in presence of CLP (molar stoichiometry 1:1), 5-LO enzyme activity was preserved, considering that about 2/3 of its starting activity remained after 120 h (Fig. 4A, paper III). Since no substrate was present during this time frame, experimental set implied the conclusion that CLP could protect 5-LO also against non-turnover inactivation caused by exposure to oxygen which has previously been shown to inactivate 5-LO due to loss of the prosthetic iron [201, 55].

Similarly, CLP provided protection of 5-LO also against high temperature, as the degree of heat-induced inactivation of 5-LO appeared to be significantly reduced compared to the heat exposed 5-LO samples in which CLP was not added. In contrast to this, CLP failed to protect 5-LO against turnover related inactivation that had earlier been shown to appear as a consequence of the enzyme interaction with its own products 5-HPETE and LTA_4 [2, 141]. As described in the result section of paper III, repeated addition of the substrate into reaction mixture with an equimolar 5-LO:CLP complex, did not result in significantly increased formation of 5-LO products.

Specificity of the above described effects, were again proved to be strictly dependent on the binding between CLP and 5-LO, since CLP K131A mutation lead to a complete absence of each of them.

3.2.7 Importance of Trp residues (13/75/102)

Three surface-exposed tryptophan residues (Trp-13, Trp-75, and Trp-102), have previously been reported to be involved in the membrane binding, in particular the PC selectivity of the 5-LO C2like domain, playing a dual role of accelerating the membrane association and prolonging the membrane residence time [136].

Since much of the above discussion has been focused on the similarity of the CLP-induced effects on 5-LO activity in relation to those of PC, we have further investigated whether those properties of CLP could be blocked by a specific cadre of 5-LO mutants generated by single-point, and triple mutations of three triptophan residues 13, 75 and 102 positioned in the ligand binding loops of the 5-LO C2-like domain, also referred to as β-sandwich.

GST-pull down assays showed that the triple 5-LO (W13/75/102A) mutant was not able to associate physically with CLP, which in turn abolished all previously seen CLP-dependent effect of 5-LO catalysis (Fig. 2B and 1B, paper II). Yet more remarkable is the fact that three Trp residues appeared not to be essential for the effects of PC on Ca^{2+}-induced 5-LO activation *in vitro* since at standard incubation condition, with the highest concentration of PC tested, 5-LO activity of the triple mutant was even higher than for the wild type enzyme (Fig 2B, paper II).

However, lower concentrations of PC resulted in a reduced activity of triple 5-LO (W13/75/102A) mutant, which was even more pronounced when concentration of AA was also lowered (Table 2, paper II). Although these three Trp residues were previously identified as crucial for binding of the isolated 5-LO β-sandwich to PC [136], they appear not to be overtly essential for membrane binding of the intact enzyme.

It is possible that these Trp residues are not the only residues relevant for PC-supported 5-LO activity. Nonetheless, it is clear that they can appreciably contribute to the Ca^{2+}-stimulated enzyme activation, particularly at the low concentrations of PC and AA (below micelle formation levels), in this way supporting the concept that other residues also in the catalytic domain of 5-LO may contribute to membrane association (as described for 12/15-LO, [271]), and in accordance with other protein containing a C2-like domain such as cytosolic phospholipase A2 [216].

However, the tryptophan residues were shown to be essential for the binding of CLP and required for CLP scaffolding of Ca^{2+}-induced 5-LO activation

(as shown in paper II). Additionally, single-point mutation of W13, W75 and W102 has particularly hinted the importance of W102 in that matter. The 5-LO (W102A) mutant had drastically reduced binding ability to CLP (Fig. 3, paper III), which was consequently followed by the absence of all CLP-induced effects on 5-LO stability and activation (Fig. 1A and B; Fig. 5A and 5B, paper III), that have earlier been described in the text. Therefore activity of 5-LO (W102A) mutant could not be supported by CLP, in the same way as CLP-K131A could not support activation of wild type 5-LO.

In an attempt to further elucidate the relevance of two amino acid resides W102 of 5-LO and K131 within CLP structure, for their mutual interaction, we performed protein-protein docking of CLP and 5-LO by using the ClusPro server [48] and applying the DOT algorithm [156, 7].

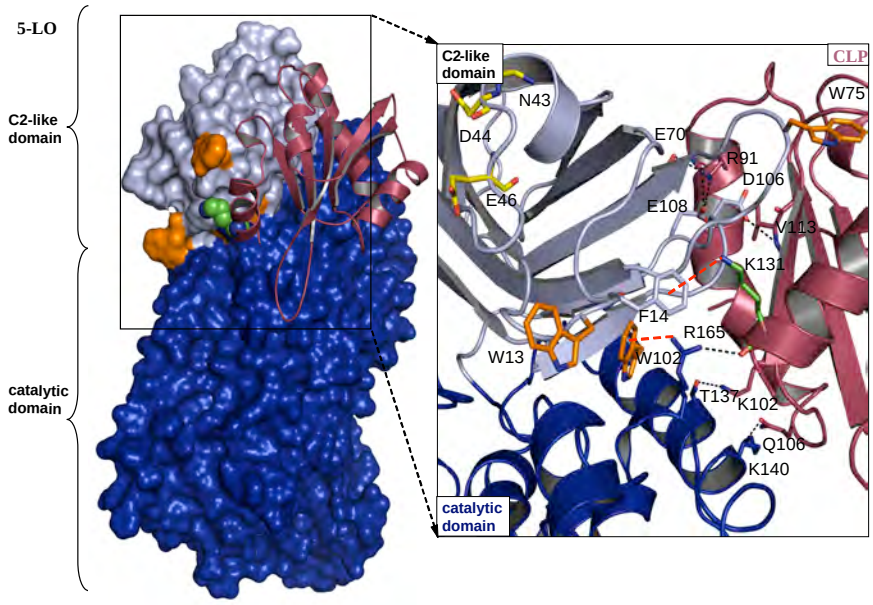

Figure 3.2: 5-LO:CLP model structure.

Our model of the CLP-5-LO complex (Figure 3.2) suggests a direct pi-cation interaction of CLP-K131 (with 5-LO-F14), confirming the importance of K131 for binding to 5-LO but also for the CLP-supported effects on 5-LO activation [219] and enzyme stability (Fig. 6, paper III). On the other hand, 5-LO-W102 appeared to influence CLP binding via 5-LO-R165. Such an indirect role of W102 is compatible with the 5-LO model structure, in which

W102 is partially hidden in the cleft between the two domains and not easily accessible for a binding partner. This is also analogous to the corresponding residue W100 [103] in the 12/15-LO structure, used as the basis for our 5-LO model as its structure has remained unsolved up to date.

Furthermore, the 5-LO:CLP model complex indicates five additional hydrogen bonds between 5-LO and CLP, two of which involve residues in the 5-LO catalytic domain, while the other three involve residues in the β-sandwich. Therefore binding of CLP to 5-LO may influence the relative positions of the two domains in 5-LO.

In this context, it is noteworthy that CLP could protect 5-LO against thermolysin-catalyzed proteolysis (paper III) which was drastically delayed in the presence of CLP. This thermo-stable protease from *B. thermoproteolyticus*, which preferentially cleaves sites with bulky and aromatic residues, is usually used in a method called pulse proteolysis for determination of structural stability of proteins. Our results highlight the possibility of CLP hindering access of thermolysin-preferred cleavage sites on 5-LO, thus delaying proteolytic degradation of 5-LO.

3.3 5-LO interaction with CLP in the cell

Several studies have suggested that cellular 5-LO activity and distribution is regulated by interaction with other proteins. In that perspective an association of 5-LO with CLP *in vivo*, could have important implications for translocation and modulation of cellular 5-LO activity.

3.3.1 Subcellular localization and trafficking of 5-LO and CLP

The subcellular distribution of 5-LO differs among cell types and changes in response to various stimuli. 5-LO is mainly localized in the cytosol, although presence in the soluble compartment of the nucleus have been reported for a few cell types (see *Introduction*). Upon cell activation, both cytosolic and nuclear 5-LO translocate in parallel with cytosolic PLA_2 to the nuclear envelope [205].

As expected, in unstimulated human PMNL (mostly neutrophils), 5-LO was localized in the cytosolic (non-nuclear) fraction, while stimulation of the cells with ionophore for 3, 10 and 25 min. led to an increase in the amounts of 5-LO that reached the nuclear region (Fig. 5, paper II). Interestingly, CLP showed the same pattern of translocation. Treatment of the cells with Ca^{2+}-ionophore A23187 led to redistribution of cytosolic CLP from nonnuclear to

nuclear fractions. We estimated the amounts of 5-LO and CLP by Western blot analyses of a 10.000 x g supernatants obtained from sonicated human leukocytes with known amounts of purified proteins loaded on membrane as control. The soluble protein fraction from 1 million cells contained ∼20-50 ng of 5-LO and ∼20-50 ng of CLP. Considering Mr values (78kDa for 5-LO and 16 kDa for CLP), CLP would be approximately five times more abundant than 5-LO, on molar basis.

The apparent 5-LO-dependency of CLP translocation found in human leukocytes was also tested in Mono Mac 6 cells (MM6), known to express substantial levels of 5-LO only upon induction with transforming growth factor-β (TGF-β) and calcitriol (vitD_3) [25]. That allowed us to employ a few new experimental settings that were essential for the purpose of our investigation. In undifferentiated MM6 cells, expression of CLP but not 5-LO was detected. Regardless of stimulation, in these undifferentiated cells almost all CLP was found to be located in non-nuclear fraction as shown in Fig. 8A (paper III).

On the other hand, differentiated MM6 cells (expressing 5-LO) required priming with PMA, in order for 5-LO to translocate from cytosol and associate with the nuclear membrane in response to ionophore [279]. 5-LO was primarily found in non-nuclear fractions upon cellular stimulation with ionophore alone. The same pattern of cellular distribution appeared for CLP. However, priming with PMA (PKC activator) followed by the subsequent addition of A23187 with or without AA, resulted in 5-LO activation that consequently led to translocation towards nuclear envelope, as large amounts of 5-LO were found in the nuclear fractions (Fig. 8B, paper III). In differentiated MM6 cells, CLP migrated in the same manner as 5-LO (Fig. 8B, paper III).

Our data on the 5-LO-dependent trafficking of CLP was further supported by the recent study on a gender difference in regard to subcellular localization of 5-LO [204]. It was shown that in neutrophils from males a substantial amount of 5-LO associated with the nucleus already in resting cells. Upon ionomycin stimulation, the compartmentalization of 5-LO was not significantly altered. In contrast to this but in accordance with already well established model for 5-LO activation, translocation of 5-LO to the nucleus in neutrophils from females was detected only upon cell challenge. Interestingly, the subcellular distribution of CLP was also shown to be gender-dependent, as it consistently co-localized with 5-LO in female and in male neutrophils.

These experiments strongly indicated that CLP translocation has 5-LO-dependent character. It tightly follows changes in the 5-LO migration, independently on the type of cell stimuli, further supporting our prevailing

hypothesis of CLP functioning as a chaperone of 5-LO.

3.3.2 Hyperforin, a novel type of 5-LO inhibitor

Hyperforin, a natural compound isolated from St. Johns wort (*Hypericum perforatum*), has previously been reported to act as a dual inhibitor of COX-1 and 5-LO enzyme [4]. In relation to the 5-LO pathway, hyperforin has been shown to abrogate the 5-LO nuclear membrane translocation and inhibit 5-LO product synthesis induced by different cell stimuli.

We have recently found that this compound possesses a novel and unique pharmacological profile as a 5-LO inhibitor with high efficacy *in vivo*. Hyperforin significantly suppressed formation of LTB_4 in pleural exudates of carrageenan-treated rats, which was earlier described and used as a suitable model for studying anti-inflammatory efficacy of 5-LO inhibitors [220, 52]. By contrast, the COX1/2 inhibitor indomethacin, similarly efficient in reducing exudate volume and number of inflammatory cells as hyperforin, failed to significantly suppress formation of LTB_4 (Fig. 1, paper IV), emphasizing that hyperforin-induced effects on 5-LO pathway are specific, directly related to interference with 5-LO activity and not due to general impairment of inflammatory response.

Inhibition of 5-LO catalysis in cell-free system by hyperforin, but not by the iron-ligand type of 5-LO inhibitor BWA4C or the non-redox type such as ZM230487, was selectively abolished in the presence of PC (but not other phospholipids; Fig. 4A, paper IV) and strongly reduced by mutations of three triptophan residues at 13, 75 and 102 positions in the C2-like domain of 5-LO. Taken together, these results indicated the specific physical binding between hyperforin and ligand-binding loops of 5-LO C2-like domain (Fig. 4-5, paper IV).

In that regard, bearing in mind the importance of tryptophan residues also for the 5-LO binding to CLP (papers II and III), it was particularly interesting to further investigate whether this novel 5-LO inhibitor would also be efficient in disrupting functional interaction of 5-LO with CLP.

As shown in GST pull-down assay (Fig. 7, paper IV), hyperforin successfully interrupted formation of functional 5-LO:CLP complex, as it reduced the binding of 5-LO to CLP at the concentrations that inhibited 5-LO product synthesis (*in vitro* and in cellular systems). Moreover, the ability of CLP to support Ca^{2+}-induced activation of 5-LO upon treatment with ionophore A23187, was severely compromised in presence of hyperforin. Particularly interesting was the finding that this pharmacologically active constituent of St. Johns wort could suppress CLP-mediated upregulation of LTA_4 synthesis (expressed as LTB_4 isomers) much more efficiently than 5-HPETE

(Table 2), in this way confirming functional interference with the putative CLP/PL-binding site within C2-like domain of 5-LO. At the same time these data further supported our prevailing hypothesis of LT synthesis being favored by the formation of a three-partner complex consisting of 5-LO, CLP and PC (papers II and III). 5-LO and CLP as novel molecular targets for hyperforin, were also investigated in intact cells. In activated human neutrophils, hyperforin (but not BWA4C or ZM230487) prevented translocation and nuclear-membrane association of 5-LO (Fig. 8A and B, paper IV), consequently leading to inhibition of the 5-LO product synthesis (Fig. 2A-B-C, paper IV).

Hyperforin failed to block Ca^{2+} mobilization in neutrophils evoked by ionomycin [69], in that way excluding the possibility that inhibition in 5-LO redistribution occurred as a result of hyperforin-induced repression of Ca^{2+} mobilization. Since 5-LO product formation also from exogenous AA (20μM) was suppressed by hyperforin in human PMNL and platelets [4] without a significant loss of efficacy, a possible inhibitory effect of hyperforin on cytosolic PLA_2, which could consequently lead to limiting endogenous AA supply, was by this excluded.

Upon the treatment of activated neutrophils with hyperforin, translocation of CLP was found to be disrupted in the same manner as for 5-LO (unpublished data). The concentration which blocked redistribution of CLP and 5-LO toward the nuclear envelope was within the range of concentrations at which significant drop in synthesis of the 5-LO products in human PMNL and in *in vitro* assays had been observed previously. Based on previous results and this study, it is tempting to speculate that CLP might be functioning as a small chaperone of 5-LO capable of following 5-LO translocation and supporting the formation of LTs at the nuclear membrane as a site for eicosanoid biosynthesis. In addition, despite resemblance with permanently membrane-bound FLAP in regard to stimulatory effect on LT production, no sequence similarity between CLP and FLAP has been found.

Chapter 4

Discussion

Opposite to this LT-prone story of 5-LO interaction with CLP, which takes place at the nuclear membrane (option 1. in Figure 4.1), the cellular play with 'the same actors involved' might be having a quite different scenario and an interesting twist, leading to surprisingly different location and outcome of the play from the ones that have already been postulated and widely accepted. Given the finding above that CLP could replace PC and function as a scaffold for Ca^{2+}-induced 5-LO activity *in vitro*, it appears possible that 5-LO can be active in the cytosol of the cell without prerequisite association with the nuclear envelope (option 2. in Figure 4.1).

Reports supporting this concept have already appeared. In human PMNL and differentiated MM6 cells (both abundantly expressing 5-LO and CLP) subjected to hypertonic buffer conditions, which prevented migration of 5-LO towards nucleus, presence of exogenous AA successfully initiated 5-LO product formation [29]. In Epstein-Barr virus-transformed B-lymphocytic cell line, BL41-E95-A, 5-LO activity was found to be high upon addition of exogenous AA, whereas association with the nucleus was undetectable [277]. Cellular distribution of CLP stayed unchanged upon the same stimulus, showing no migration toward nucleus (unpublished data).

Localization of 5-LO and CLP were also found to be cytoplasmic in a few cancer cell lines, upon stimulation regardless on the type of cell challenge (unpublished data). In that respect and in a view of high Mg^{2+} requirements in rapidly metabolizing cancer cells [198], our results on CLP-supporting effects of 5-LO activity in the presence of high Mg^{2+} and ATP concentrations but not PC, appear particularly tempting for hypothesizing the essential role of CLP in providing the basis for active cytosolic 5-LO, which could produce 5-HETE. Additionally, nonnuclear 5-LO activity may also involve a lipid scaffold. Cytoplasmic lipid bodies of eosinophils were suggested as a site for eicosanoid biosynthesis [18].

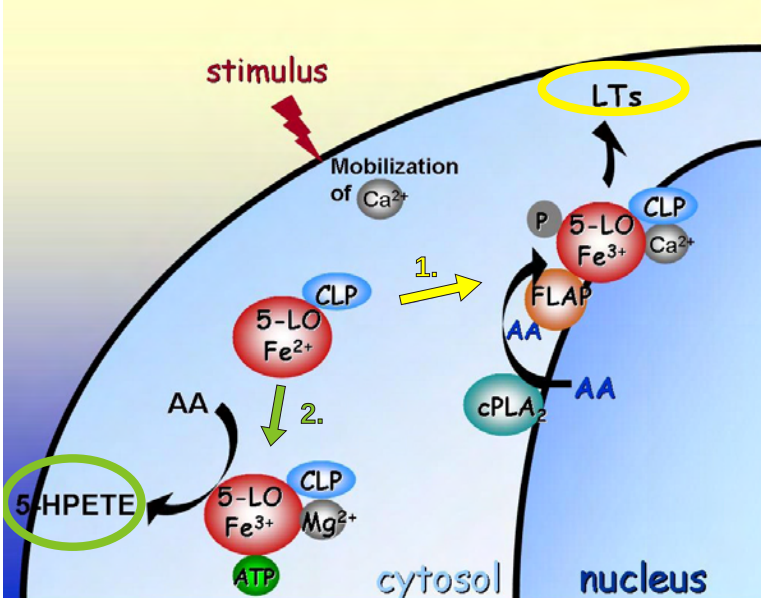

Figure 4.1: Subcellular localization of 5-LO activity. **Arrow 1**. Activity of 5-LO at the nuclear membrane. **Arrow 2**. Cytosolic activity of 5-LO.

A putative cytosolic 5-LO activity should depend on transcellular supply of AA or it should be provided from another cellular compartment. The S100A8/A9 complex (abundant in PMNL for instance), can be relevant in that matter, as it was suggested to function as an intermediate reservoir of AA, capable of delivering AA to metabolizing enzymes hours after phospholipid hydrolysis [178].

Evaluation of any of these concepts in cells related to cytosolic activity of 5-LO (devoid of nuclear membrane association), however, has yet to be proved. The final outcome of these studies will for sure further complicate but also challenge our already difficult search for the pharmaceutical strategies that could intervene with production of LTs.

Despite the large number of therapeutic indications and the strong need for efficient and safe drugs that target the 5-LO pathway, no 5-LO inhibitor is available on the market for the treatment of LT-related diseases of human subject, mostly due to either their ineffectiveness *in vivo* or poor track record for safety. Novel molecular strategies for intervention of 5-LO activity are needed for step forward in anti-LT therapy.

Up to now, no data demonstrating pharmacological inhibition of 5-LO by interfering with the C2-like domain were available. Most 5-LO inhibitors, synthetic ones as well as those isolated from natural sources (polyphenols, coumarins, quinones), act at the catalytic domain by reducing or chelating

the active iron site, by reduction of activating lipid hydroperoxides, or simply by scavenging electrons participating in the redox cycle of the iron [280, 283]. Given the above, hyperforin appears as the first compound shown to suppress activity of 5-LO by directly interfering with the N-terminal β-sandwich.

Lipophilic nature of hyperforin allows this molecule to cross the plasma membrane of the cells. However, major concern for hyperforin pharmacological use is in its potential interference with the other drugs through its activating effect on various CYP450 enzymes, particularly CYP3A4, which is involved in oxidative metabolism of more than 50 % of all drugs used. On the other hand, this modulation could be beneficial in some instance, inasmuch as hyperforin is a potent inhibitor of carcinogen formation by human CYP1A1 (a major human procarcinogen-activating enzyme), hyperforin acting as a competitive inhibitor. Therefore hyperforin may be worthy of further evaluation for cancer chemo-preventive potential.

However, the design of hyperforin analogs, retaining the pharmacological properties of the parental compound, but unable to stimulate expression of CYP3A4 will be a challenge for pharmaco-chemists in the near future. Taking into account also the antitumor activity of hyperforin that has recently been highlighted [293, 160], this molecule certainly deserves thorough investigation and it already got our full attention.

The core machinery of the 5-LO pathway has been delineated, at least in broad strokes. However, many conundrums still remain. Indeed, the results emerging from our plunge into bewildering complexity of cell signaling networks suggest that we may grasp their logic soon. Many questions and many principles still need to be established, but problems that have long worried 'eicosanologists', particularly those fans of the 5-LO pathways have begun to be unraveled.

Chapter 5

Concluding Remarks

5.1 Summary

The major conclusions from the studies described in this thesis are as follows:

1. The NMR structure of human CLP is composed of a five-stranded β-sheet surrounded by four α-helices. The loop connecting $\beta 3$ and $\beta 4$, and the C-terminus are flexible. Surface-exposed Lys-75 (a critical binding residue for F-actin), and Lys-131 (essential residue for CLP binding to 5-LO), are shown to be close to each other, precluding simultaneous binding of F-actin and 5-LO to CLP.

2. CLP supports Ca^{2+}/Mg^{2+}-induced 5-LO activity and modulates the 5-LO product profile. We have found and described three major CLP-dependent effects on the 5-LO catalytic reaction *in vitro*.

- CLP upregulates Ca^{2+}-induced 5-LO activity in absence of phosphatidylcholine (membrane). Apparently, CLP can function as a scaffold for 5-LO, similar to the effect of membranes on 5-LO catalysis. In such incubations, formation of 5-HETE was highly upregulated, while formation of LTA_4 was minimal.

- CLP gives significant 3- up to 5-fold increase in the amount of LTA_4 formed by 5-LO when present together with phosphatidylcholine, indicating the possible involvement in LTs biosynthesis at the nuclear membrane.

- CLP increases the ratio of 5-HETE/5-HPETE by substantially reducing 5-HPETE into 5-HETE. The reduction of 13-HPODE into 13-HOPE was also observed in the presence of CLP.

3. CLP stabilizes 5-LO and prevents its non-turnover inactivation *in vitro* induced by high temperature and appearing over time. Together with

the effects on 5-LO activity, this finding suggests that CLP functions as a chaperone for 5-LO.

4. Trp-102 in the β-sandwich of 5-LO is required for binding of CLP to 5-LO, and for all the above mentioned CLP-induced effects on 5-LO activity and stability. This indicates that the β-sandwich is part of the CLP binding surface on the 5-LO molecule.

5. Similar changes in subcellular localization of CLP and 5-LO were found in human polymorphonuclear leukocytes and Mono Mac 6 cells upon activation with different cell stimuli, which indicated a possible direct interaction of 5-LO and CLP also in the cell.

6. Hyperforin, an anti-inflammatory compound isolated from St. John's wort and previosuly reported to inhibit 5-LO activity both, *in vitro* and in cellular systems, successfully interrupted formation of functional 5-LO:CLP complex by imparing direct binding between CLP and 5-LO. This finding indicated that hyperforin, as a new type of 5-LO inhibitor, acts by interferring with the 5-LO β-sandwich.

5.2 Future perspective

A few reports have recently implied a possible role of the 5-LO pathway also in cancer cell chemoresistance. These data appeared to be particularly intriguing in the light of our findings on 5-LO interaction with CLP, since our studies (as mentioned earlier in this thesis), has hinted at a tumor-prone character of the functional 5-LO:CLP complex due to upregulation of LTA_4 and 5-HETE in particular, compounds previously described to be important for cancer cell survival and escape from apoptotic machinery. Understanding the molecular and cellular mechanisms that contribute to tumor formation, progression and restistance to chemotherapeutic agents has been a major challenge in cancer research. For many years, progress in this field has been dominated by the concepts and methods of molecular genetics, but advances in technologies for protein analysis over the last years have accelerated studies on cancer biology at the protein level. Having emphasized that, possible CLP-dependent alterations in levels and groups of 5-LO products formed (in cancer versus normal cells), have particularly challenged me to direct my lines of research along two fronts:

1. Possible importance of 5-LO interaction with CLP in cancer cell survival and chemoresistance

2. The role of 5-LO pathway as a pro-inflammatory signaling cascade in cancer progression

5.2.1 Chemoresistance: Ways to avert it

To paraphrase Tolstoy in the opening lines of *Anna Karenina*, normal cells are all alike in their response to drugs, but cancer cells each respond in their own way. Brushing up on literature leaves no doubt that there are many studies on drug resistance in cancer cells, but comparatively little is known about the underlying mechanisms. Combining previously reported data with our recent studies on interacting 5-LO:CLP complex, we have designed several ongoing projects, with the aim to elucidate the possible role of 5-LO and its small chaperone CLP in survival of cancer cells, specifically focusing on the possible role of their interaction in resistance to different anticancer drugs. For this purpose, as an ideal cell model, we chose two human, prostate cancer cell lines: DU145 and PC3 (with relatively high expression level of CLP) as they are both highly metastatic, androgen-receptor negative but still genetically different in regard to p53 gene status, and expression levels of pro- and anti-apoptotic members of the Bcl-2 family. Particularly intriguing is the fact that 5-LO expression is inducible in both cell lines upon drug treatments.

We hope that data from these experimental sets, will give us more detailed insight into the role of 5-LO pathway in the mechanisms of cancer cell chemo-resistance and potentially open an interesting window for therapeutic intervention.

5.2.2 Inflammation and cancer: back to Virchow

If formal proof that cancer can be initiated by inflammation in the absence of exogenous carcinogens is still lacking, there is abundant evidence that the inflammatory response can play a central role in modulating tumor growth and progression. As early as 1863, Rudolf Virchow recorded the presence of leukocytes in neoplastic lesions and hypothesized that malignant transformation originates from chronically inflamed tissues [9], providing the first conceptual framework for linking inflammation and cancer. Clinical and experimental observations of the past two decades increasingly support the notion that immune cells recruited to and activated within the tumor microenvironment play a strong supporting role in tumor progression [19].

In most cases, tumor cells hijack stromal cells' functions for their own benefit and ultimately dictate the rules of engagement to the host tissue microenvironment. How helpful the 5-LO pathway would be in that matter? Which of its pro-inflammatory products would be the most beneficial in promoting relentless expansion of tumor cells? Would expression of CLP appear to be important in that regard?

These are just some of the questions we addressed in our ongoing studies.

From the data obtained so far, the forthcoming years in 5-LO and CLP research promise to be very exciting.

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