Molecular evolution of a C5aR antagonist against inflammatory disease

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Abstract

New anti-inflammatory drugs with fewer adverse effects than existing drugs may prove to be useful in the treatment of several inflammatory disorders.

A critical step in acute inflammation is the infiltration of neutrophils into tissues. Therefore, molecules that target and inhibit this early inflammatory event are attractive to engineer to suit medical needs. The Chemotaxis Inhibitory Protein of *Staphylococcus aureus* (CHIPS) binds and blocks the C5a receptor (C5aR) and formylated peptide receptor (FPR) and is therefore a potent inhibitor of activation and migration of neutrophils. However, as the majority of the human population has been exposed to *S. aureus*, pre-existing antibodies against CHIPS will be present in human sera.

This thesis is based on four original papers, with the overall aim to decrease the interaction of CHIPS with pre-existing human IgG in order to tailor it for pharmaceutical purposes. Specific IgG epitopes on the CHIPS surface were mapped by the use of phage displayed random peptide libraries. This study showed that polyclonal CHIPS specific IgGs mainly recognize conformational epitopes exposed on the surface. In addition, amino acid residues in CHIPS that are involved in this interaction, as well as in C5aR inhibition were identified.

Directed evolution is a process commonly used to improve certain protein properties without the need for detailed prior knowledge of the protein structure. This can be performed by the use of *in vitro* DNA recombination, a procedure by which beneficial mutations from a randomly mutated library can be recombined to generate new protein variants. In this thesis, the DNA recombination technology Fragment INduced Diversity® (FIND®), was applied in combination with molecular modeling and site-directed mutagenesis to generate CHIPS variants with low interaction with human IgG and retained ability to inhibit the C5aR. One CHIPS variant, designated ADC-1004, was selected for further studies. This new CHIPS variant, has high affinity for the C5aR and inhibits it efficiently, despite the truncation and seven mutations that mediate low interaction with pre-existing human IgG.

Key words

Antagonist, C5a receptor, Chemotaxis Inhibitory Protein of *Staphylococcus aureus*, directed evolution, epitope, IgG, immunogenicity, inflammation, molecular evolution, purification

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Contents

Papers I-IV

Original papers

This thesis is based on the following original papers:

I. **Gustafsson E*, Haas P-J*, Walse B, Hijnen M, Furebring C, Ohlin M, van Strijp JAG and van Kessel KPM**
Identification of conformational epitopes for human IgG on Chemotaxis Inhibitory Protein of *Staphylococcus aureus.*
(BMC Immunology (2009), 10:13)

II. **Gustafsson E, Rosén A, Barchan K, van Kessel KPM, Haraldsson K, Lindman S, Forsberg C, Ljung L, Bryder K, Walse B, Haas P-J, van Strijp JAG and Furebring C**
Directed Evolution of Chemotaxis Inhibitory Protein of *Staphylococcus aureus* generates biologically functional variants with reduced interaction with human antibodies.
(Submitted)

III. **Gustafsson E, Forsberg C, Haraldsson K, Lindman S, Ljung L and Furebring C**
Purification of truncated and mutated Chemotaxis Inhibitory Protein of *Staphylococcus aureus* - an anti-inflammatory protein.
(Protein Expression and Purification (2009), 63, 95–101)

IV. **Gustafsson E, Rosén A, van Kessel KPM, Haraldsson K, Ljung L, Forsberg C and Furebring C**
The Chemotaxis Inhibitory Protein of *Staphylococcus aureus* variant ADC-1004 is a potent C5aR antagonist with reduced unwanted immunoreactivity.
(Manuscript)

* Authors contributed equally to this work.

Abbreviations

ADAs	Anti-drug antibodies
C5aR	C5a receptor
C5L2	C5a-like receptor 2
CD	Circular dichroism
dsDNA	Double-stranded DNA
CHIPS	Chemotaxis Inhibitory Protein of *Staphylococcus aureus*
DAG	Diacylglycerol
EAP	Extracellular adherence protein
Exo	Exonuclease
FcγR	Fcγ receptor
FIND®	Fragment INduced Diversity®
fMLP	N-formyl peptide
FPR	Formylated peptide receptor
HLA	Human leukocyte antigen
IP_3	Inositol triphosphate
I/R	Ischemia/Reperfusion
MHC	Major histocompatibility complex
NAbs/NNAbs	Neutralizing antibodies/Non-neutralizing antibodies
NMR	Nuclear magnetic resonance
pIII	phage-coat protein III
PAF	Platelet-activating factor
SSL	Staphylococcal superantigen-like protein
ssDNA	Single-stranded DNA
SLE	Systemic lupus erythematosus
TLR	Toll-like receptor
Wt	Wild-type

1. Introduction

Molecular evolution, the evolution of organisms at the DNA level, is a process distinguished by selection and adaptation in nature. The development of recombinant DNA technology has made it possible to study evolution and to evolve proteins by mimicking and accelerating the natural process *in vitro*. The technique 'directed evolution' is commonly used for the engineering of proteins to display new or improved properties, without the need for prior knowledge of the protein structure. Usually this is achieved by random mutagenesis and recombination of genes, followed by selection or screening for the properties of interest. Directed evolution has been applied for improving for example the affinity, stability or activity of proteins (1). Several recombinant proteins are currently approved for pharmaceutical use and directed evolution is considered a powerful tool to optimize properties of proteins to better suit medical needs and formulation requirements (2-4).

The immune system is a mechanism aimed at recognizing and eliminating pathogens and abnormal cells. This tightly controlled organization of cells and proteins is able to distinguish between self and non-self structures in order to minimize damage to host tissues. The innate immune response is commonly referred to as the 'first line of defence'. It is composed of myeloid cells, anti-microbial peptides and complement proteins. The innate response relies on germline-encoded pattern recognition receptors specific for conserved motifs on pathogens (5). Adaptive immunity is a later response, dependent on a diverse repertoire of receptors on B- and T-lymphocytes that are able to recognize an

enormous variety of foreign structures. Activation of these cells subsequently leads to the development of immunological memory.

Inflammation is a complex process which is mediated by soluble factors and cells, triggered early after tissue injury. Normally, this process resolves and leads to tissue repair and healing, but it can be excessive and improperly regulated and thereby cause substantial tissue destruction (6). Anti-inflammatory drugs are therefore sometimes required for control of unwanted or excessive inflammatory responses. Non-specific anti-inflammatory agents such as glucocorticoids, have powerful anti-inflammatory activity but their use is associated with severe adverse effects (7). Therefore, the development of more specific anti-inflammatory drugs is attractive. Unresponsiveness among certain groups of patients or the development of drug-resistance, as in anti-TNF-α treatment (8), is further increasing the demand for alternative anti-inflammatory drugs to be developed.

Evolution has adapted organisms to function well in their respective environments. For example, bacteria have evolved many strategies to evade the immune system upon colonization of a host. These strategies can be studied and potentially utilized in the design of for example new anti-inflammatory agents. This thesis, based on four original papers, describes the molecular evolution of a bacterial protein with anti-inflammatory properties. The Chemotaxis Inhibitory Protein of *Staphylococcus aureus* (CHIPS), which binds and blocks the C5a receptor (C5aR) and formylated peptide receptor (FPR) is a potent inhibitor of activation and migration of neutrophils (9), an early and key event in acute inflammation. *S. aureus* is a bacterium that commonly infects humans and CHIPS is expressed by the majority of clinical isolates. Hence, *S. aureus* infected humans have specific circulating antibodies towards CHIPS (10). Therefore, the aim of this study was to decrease the interaction of CHIPS with pre-existing human IgG in order to make it suitable for pharmaceutical purposes. The design of such a molecule requires reduction of epitopes targeted by human IgG, which could bind the CHIPS molecule and potentially cause negative effects such as neutralization of the anti-

inflammatory activity of CHIPS or induction of immune complex formation. The first papers of this thesis (Paper I and II) describes two different approaches of molecular evolution to design an improved CHIPS molecule. The approach in Paper I was aimed at studying epitopes for human IgG on the CHIPS surface by the use of phage displayed peptide libraries. The goal was to identify the epitopes involved in the interaction with human IgG, and subsequently eliminate these by mutagenesis. Peptide libraries were screened for binding of human affinity purified anti-CHIPS IgG and this way, we were able to select peptides that could be mapped to form seven potential conformational antibody epitopes on the CHIPS surface. Mutagenesis of selected amino acids in the mapped epitopes confirmed the involvement of these in the interaction with human IgG.

The second approach (Paper II) was initiated in parallel with the first study with the aim to decrease antibody binding without prior knowledge of which amino acids in CHIPS are relevant in the binding to human IgG. To achieve this, we designed CHIPS libraries by directed evolution (random mutagenesis and DNA recombination by Fragment INduced Diversity, FIND®) as well as computational analysis and rational design. Repeated rounds of mutagenesis, recombination, selection and screening were applied and this way we were able to identify significantly improved CHIPS variants with retained biological function. The final papers of this thesis describe different properties of a selected new improved CHIPS variant, termed ADC-1004. In Paper III, we describe a new protocol for purification of ADC-1004 and in Paper IV, this variant was shown to display very low IgG titer (lower than the titer for Streptokinase, an existing pharmaceutical of bacterial origin) and to be a poor inducer of the complement system, compared to the wild-type (wt) CHIPS molecule. ADC-1004 was furthermore shown to be specific for the C5aR and the ability to inhibit the C5aR was retained. In addition, by estimating the T_H-cell epitope content and comparing it to wt CHIPS, approved protein drugs and drugs under development, ADC-1004 may be considered to be of moderate immunogenicity.

2. Inflammation

Inflammation is generally described as the tissue response to injury, which can for example arise from infection, trauma, toxins, autoimmunity or ischemia. The word 'inflammation' is Latin for "set on fire" and its basic signs, rubor (redness), calor (heat), dolor (pain) and tumor (swelling) were first described by the Roman physician and medical writer Cornelius Celsus during the first century.

The inflammatory process is mediated by a network of soluble molecules and cells that kill pathogens, remove injured structures and cells and initiate healing by supporting cell and matrix repair and replacement, eventually leading to re-established tissue function (11).

Inflammation can be acute or chronic, depending on the duration of the response and the cells involved. Acute inflammation starts within seconds to minutes after tissue injury and is a short-lived response (minutes to days) with neutrophils as its main cellular mediators. When inflammation persists for more than a week, it is referred to as chronic inflammation, which is characterized by infiltration of macrophages and lymphocytes as well as tissue destruction, fibrosis and angiogenesis. Continuous, and sometimes permanent tissue damage is caused by granulomas; immune cells trapped by the formation of a surrounding fibrotic wall (12, 13).

The focus of this thesis is on the inhibition of acute inflammation; hence chronic inflammation will not be further discussed.

2.1 The acute inflammatory response

Acute inflammation is initiated through activation of endothelial cells of local blood vessels. The process is mediated by several soluble inflammatory factors, such as plasma proteases, lipid mediators, peptides/amines, acute-phase reactants and pro-inflammatory cytokines, which are released upon tissue damage.

The physiological changes associated with inflammation include locally increased blood flow due to vasodilation (which gives rise to the redness and heat), increased leaking of fluid and plasma proteins into interstitial spaces due to increased vascular permeability (which gives rise to the pain and swelling) and leukocyte (e.g. neutrophil) adhesion, mediated through the alterations in blood flow and expression of cell-adhesion molecules on the activated endothelium. Furthermore, clotting is induced in blood vessels at the site of the inflammation to prevent spreading of pathogens if the inflammation was caused by an infection.

Neutrophils are leukocytes that are crucial to the inflammatory response, as well as to immunity. These cells can be activated by many agents, such as C5a, CXCL8, fMLP leukotriene B4 and PAF (14). Upon activation, neutrophils are able to adhere to endothelial cells by first binding selectins via carbohydrates and secondly to cell adhesion molecules (e.g. ICAMs) via integrins (15, 16) that are expressed on the surface of activated neutrophils. Then, they migrate over the endothelium and respond to chemotactic agents in a process called chemotaxis (17-19). Phagocytosis, the ingestion of foreign or damaged particles, is another distinct feature of the activated neutrophil (20). Especially opsonized particles are ingested efficiently and subsequently destroyed by e.g. oxygen-dependent killing mechanisms. Activated neutrophils generate toxic oxygen metabolites (21) and release granulae that in turn contain effector proteins such as defensin, elastase and lysozyme. The life-span of neutrophils is very short, limited to hours. This short life-span is necessary for resolution of acute inflammation and neutrophil death commonly occurs through apoptosis and subsequent phagocytosis (22).

Products of the complement system are involved in the recruitment of neutrophils to inflammatory sites, for example, by mediating activation of the endothelium and by functioning as chemotactic agents.

2.2 Complement and acute inflammation

The human complement system is a network consisting of over 30 circulating or membrane-bound plasma proteins. Its primary function is to identify and kill microorganisms, mediate inflammation and to clear immune complexes and apoptotic cells (23). In addition, the complement system is a regulator for innate and adaptive immunity (24, 25).

Complement activation is induced by antigen-antibody complexes, recognition and binding of pathogen-associated molecular patterns by pattern recognition receptors or by spontaneous hydrolysis of complement component C3. Three pathways (the classical, lectin and alternative pathways) all give rise to C3 convertases, which enzymatically cleave the central protein C3 into C3a and C3b (23). C5 convertases are then assembled through binding of C3b to the C3 convertase. The resulting products of the complement cascade include C3a, C4a and C5a, known collectively as the anaphylatoxins due to their ability to sensitize/trigger degranulation from for example mast cells and neutrophils. C5a is the most potent anaphylatoxin with 100 times higher biological activity than C3a, which in turn has higher activity than C4a (26, 27). C3b amplifies complement activation and functions as an opsonin, marking foreign surfaces to be recognized by complement receptors on phagocytes. The membrane attack complex (C5b-9) forms a pore in bacterial cell membranes, which leads to cell lysis and death. Furthermore, the complement system is under control of several regulatory proteins, which function at different levels in the cascade to inhibit non-specific complement activity from causing damage to normal cells and tissues (23).

Anaphylatoxins modulate the inflammatory response by mediating the activation of macrophages to secrete pro-inflammatory cytokines such as IL-1β and TNF-α (28, 29). These cytokines activate the endothelium, leukocytes and fibroblasts (30, 31). Moreover, the anaphylatoxins activate mast cells, neutrophils and monocytes to release factors such as histamine, proteases, chemokines and eicosanoids that mediate vasodilation and vascular permeability and act as chemotactic factors that mediate leukocyte recruitment (23, 32-34). Anaphylatoxins, for the most part C5a, also enhance the recruitment of leukocytes by facilitating adhesion of leukocytes to endothelial vessels through upregulation of adhesion molecules on the endothelium as well as on the leukocytes (35-37).

Even though the aim of the inflammatory response is to initiate resolution and healing of damaged cells and tissues, this process can give rise to destructive effects. Neutrophils are normally involved in the defence against bacteria and parasites, but their powerful effects generated by degranulation, phagocytosis and release of free oxygen species, can lead to tissue damage in for example ischemia/reperfusion (I/R) injury if dysregulated (38-40). Many clinical conditions, such as acute myocardial infarction, acute respiratory distress syndrome, stroke, hemorrhagic shock, sepsis and organ transplantation may be associated with I/R injury, reviewed in (41). A massive inflammatory response is triggered upon the restoration of blood supply to an organ or tissue (reperfusion), which by complement activation and neutrophil infiltration further enhances the injury caused by the ischemia (42). Complement is thought to be a major contributor to inflammation in association with I/R injury (43) and several experimental models have demonstrated reduction of I/R injury through inhibition of complement (44-48).

The numerous mediators and pathways described to trigger and maintain inflammation reflect the complexity of the inflammatory response. Hence, there are several levels to target in an effort to control inflammatory disorders. Targeting complement to achieve this is likely to be a good strategy, since excessive

complement activity has been associated with several pathological conditions apart from conditions associated with I/R injury. Such conditions include for example systemic lupus erythematosus (SLE), rheumatoid arthritis, asthma, psoriasis, inflammatory bowel disease and sepsis (49). However, many attempts to control complement activity in a clinical setting have failed. One explanation for this may be the difficulty to affect large protein-protein interactions with small molecule drugs (49, 50). The development of recombinant proteins for clinical use may resolve such issues associated with small molecule drugs.

This thesis describes the development of a protein derived C5aR antagonist, which was developed with the aim to efficiently inhibit the actions of C5a, known to be the most potent pro-inflammatory peptide of the complement system (51).

3. C5a, receptors and antagonists

3.1 C5a

The anaphylatoxin C5a is a polypeptide released upon cleavage of C5 by the C5 convertase (23) or locally by proteolytic enzymes such as kallikrein (52) and thrombin (53). It is composed of 74 amino acids, folded into an anti-parallel four-helix bundle (54-56), stabilized by three disulfide bonds and connected by three loops (Figure 1).

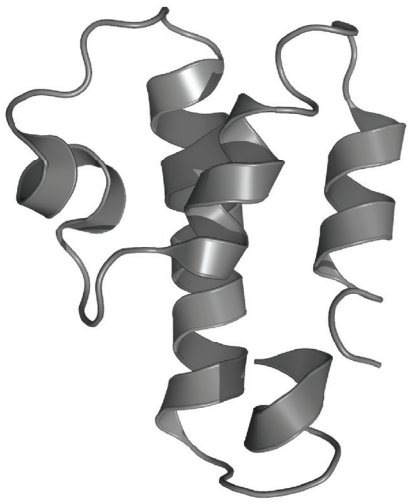

Figure 1. Ribbon diagram of C5a, based on the NMR structure, PDB code: 1KJS (54).

C5a is rapidly cleaved by carboxypeptidases in the circulation (57); the C-terminal arginine is cleaved off, and the remaining product is referred to as 'C5a

desArg', which binds the C5aR with lower affinity than C5a and is generally less active than C5a, but displays retained chemotactic properties (58, 59).

C5a is a potent chemoattractant for neutrophils, monocytes and macrophages (58), it causes oxidative burst, increases phagocytosis, granulation, cytokine release as well as increases vascular permeability, induces smooth muscle contraction and vasodilation (34, 60-64). Furthermore, C5a enhances the expression of neutrophil adhesion molecules (36, 65), causes reduced neutrophil apoptosis (66), increased thymocyte apoptosis (67, 68) and activates the coagulation pathway (69). C5a has in addition been shown to interfere with danger transmission signals from toll-like receptors (TLRs) (70) as well as to be involved in angiogenesis (35) and tumor growth (71).

Many of the properties displayed by C5a are pro-inflammatory, hence C5a must be tightly balanced in order to prevent uncontrolled inflammation or improper immune responses from occurring (72-74). The effects of C5a are suggested to play important roles in the pathogenesis of many inflammatory diseases. These include SLE, rheumatoid arthritis, asthma, psoriasis, inflammatory bowel disease, I/R injury and sepsis (75-84). C5a is a key molecule in the massive recruitment of neutrophils in acute inflammation, since it participates in endothelial activation (35), affects the expression of adhesion molecules on leukocytes (36, 37) as well as on the endothelium (35) and is a strong chemoattractant for neutrophils (58). The C5aR has been shown to be upregulated over time on cardiomyocytes in a mouse model of myocardial I/R injury. Blockade of the C5aR both inhibited leukocyte adhesion to the coronary artery endothelium and reduced microvascular permeability in the ischemic area (85). However, C5aR antagonists have shown beneficial effects on renal I/R injury even in neutrophil-depleted mice (86), so the role of C5a in I/R injury can be both neutrophil dependent and neutrophil independent.

Immune complexes, formed upon antigen recognition by antibodies, can induce inflammatory tissue injury if deposited in certain blood vessels or organs

and result in diseases such as SLE (87). Immune complexes activate the complement cascade and interact with several complement receptors and Fcγ receptors (FcγRs). C5a has been shown to stimulate the expression of the activating FcγRIII and inhibit the expression of the inhibitory FcγRIIB. Thereby, C5a plays a regulatory role, enabling efficient response to immune complexes (88).

Another example of the involvement of C5a in the pathogenesis of inflammatory disease is its ability to modulate the release of cytokines (74, 89). In addition, C5a can affect the coagulation pathway by promoting the formation of fibrin clots through tissue factor and CXCL8 upregulation (90-92). Furthermore, C5a mediates increased apoptosis of lymphocytes, which has been shown to be associated with poor outcome of sepsis (93).

C5a mediates its effects through two different receptors; the C5aR (CD88) and the C5a-like receptor 2 (C5L2). The C5aR was cloned by two different groups in 1991 (94, 95), whereas C5L2 was described for the first time in 2000 (96).

3.2 Receptors for C5a

3.2.1 C5aR (CD88)

The C5aR is a G protein coupled seven transmembrane receptor belonging to the rhodopsin family of transmembrane receptors (94, 95). The C5aR is widely expressed on myeloid (95, 97, 98) and non-myeloid cells, for example in the lung and liver (99, 100). The expression level on neutrophils is high, approximately 200,000 receptors per cell (101).

C5a binds with nanomolar affinity to the C5aR (58) and a two-site binding model has been described for this interaction. The basic core of C5a initially interacts with the acidic residues in the N-terminus of the C5aR, whereas the C-terminal end of C5a binds at the transmembrane region of the receptor (102-105). The receptor is coupled to heterotrimeric G proteins (mainly of subclass Gi2) (106,

107) and undergoes a conformational change upon binding of C5a, which mediates the interaction with the Gi2 protein. The α-subunit exchanges GDP to GTP and dissociates from the βγ-subunit which in turn activates phospholipase C and MAP kinase, leading to the accumulation of diacylglycerol (DAG) and inositol triphosphate (IP_3). DAG and IP_3 trigger the release of calcium from intracellular stores and activate protein kinase C isoforms, which subsequently activate downstream second messenger pathways and effector systems that regulate gene expression and alter cellular responses, reviewed in (108).

The C5aR is phosphorylated after activation, which promotes binding of arrestins, preventing further binding to G proteins. This initiates the internalization of the receptor into clathrin coated pits. Then the receptor faces different fates; it can either be dephosphorylated and recycled to the plasma membrane, or degraded in lysosomes (109).

3.2.2 C5L2

A second receptor for C5a, C5L2, was discovered some years ago by Ohno and colleagues (96) and has since been the subject for several studies. C5L2 is widely expressed on a number of different cell types (96, 110, 111), for example on granulocytes, and has been shown not to couple via G proteins upon interaction with C5a (112). The functional role of C5L2 is still under investigation. It has been reported to be involved in both anti-inflammatory and pro-inflammatory responses. For example, $C5L2^{-/-}$ mice have shown enhanced responses to C5a and C5a desArg (113). Furthermore, C5L2 was found to be down-regulated on neutrophils in sepsis, which correlated with lethality (114). Since C5L2 is uncoupled from G proteins, it has been suggested that this receptor acts as a decoy receptor that regulates the activity of C5a and C5a desArg (112, 115, 116). However, a recent study on experimental sepsis describes a pro-inflammatory role for C5L2, which contributes to the release of mediators in the inflammatory response (84).

3.3 Antagonists against the C5aR

3.3.1 CHIPS

CHIPS is short for Chemotaxis Inhibitory Protein of *Staphylococcus aureus*. This 14.1 kDa protein was first discovered in the supernate of growing staphylococci (9, 117) and is produced by >60 % of clinical *S. aureus* isolates (9). Since its discovery, CHIPS has been characterized with respect to structure, receptor binding and inhibition, as well as further interaction with the human immune system. The solution structure of the N-terminally truncated CHIPS variant $CHIPS_{31\text{-}121}$ has been determined by multi-dimensional NMR spectroscopy. CHIPS is folded into a N-terminal amphipathic α-helix and a four-stranded anti-parallel β-sheet (Figure 2) and shows structural homology with the C-terminal domain of staphylococcal superantigen-like proteins (SSLs) 5 and 7, staphylococcal extracellular adherence protein (EAP) as well as the superantigens TSST-1 and SPE-C of staphylococci and streptococci, respectively (118).

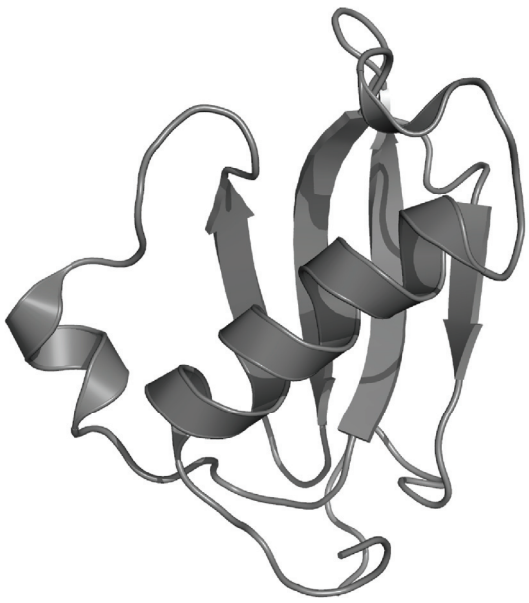

Figure 2. Ribbon diagram based on the NMR structure of $CHIPS_{31\text{-}121}$ (PDB code: 1XEE) (118).

CHIPS binds directly to the human C5aR and FPR with a K_D of 1.1 (±0.2) and 35.5 (±7.7) nM, respectively, and was found to be specific for the human C5aR and FPR in a comparative study with 37 different receptors (119). By binding these receptors, CHIPS inhibits C5a and fMLP induced calcium mobilization in neutrophils and stably transfected U937 cells (9, 119). Another study showed that different sites in CHIPS are involved in blocking of the C5aR and FPR and that the N-terminal residues of CHIPS are crucial for maintaining the FPR blocking activity (120). So far, the regions comprised of residues 43-63 and 95-112 (10), particularly the loop between the α-helix and strand β1 (residues 52-59) (121) as well as the arginine at position 44 and the lysine at position 95 (118) in the CHIPS molecule have been suggested to play an important role in the interaction with the C5aR. In Paper I, we speculate that the β3 sheet (amino acids 94-100) is important for the C5aR inhibiting activity of CHIPS, since we showed that especially substitutions in positions 95 and 97 decreased the C5aR blocking activity of C-terminally truncated CHIPS. A recent study by Ippel *et al* investigated the effect of substitutions Y48A, T53A, Q58A, Y97A, S106A and S107A on CHIPS C5aR inhibiting activity. The most substantial decrease in activity was seen for mutants Y97A and S106A, which further strengthens previous data that Y97 is important for the interaction of CHIPS with the C5aR (121).

CHIPS is structurally different from C5a and has been found to interact with the N-terminus and not with the transmembrane region of the C5aR (9, 122). It interacts specifically with the aspartic acid residues at positions 10, 15 and 18 and the glycine at position 12 in the N-terminal region of the C5aR (122).

Wright *et al* have characterized CHIPS further with respect to C5L2 binding. CHIPS was found to bind weakly to C5L2 and C5a binding to this receptor could not be inhibited by CHIPS. However, binding of C5a desArg to C5L2 could be inhibited by addition of CHIPS, but the IC_{50} of this inhibition was 274 nM; more than 40 times higher than the IC_{50} of the CHIPS mediated inhibition of C5a

binding to the C5aR. Therefore, Wright *et al* speculate that CHIPS mediated inhibition of C5a desArg/C5L2 is unlikely to occur *in vivo* (10).

CHIPS is expressed by a majority of *S. aureus* strains (9). Since *S. aureus* commonly infects humans, anti-CHIPS antibodies are likely to be present in human serum. In a study with 32 donors, all individual sera were found to have anti-CHIPS antibody titers and half of the sera were shown to neutralize CHIPS binding to the C5aR (10). In another study with 168 donors, all individuals displayed detectable IgG titers against CHIPS (123). Furthermore, in a small pilot study of six healthy individuals, human sera were found to contain circulating anti-CHIPS antibodies, which potentially could have contributed to the adverse effects seen through formation of immune complexes (123). However, this was a small study and the results of the study were ambiguous and not fully conclusive.

The development of CHIPS into an effective therapeutic molecule is therefore dependent on reduction of the interaction with pre-existing IgG, which was the overall aim of the work that forms the basis of this thesis.

3.3.2 ADC-1004

Chapter 5 of this thesis describes the evolution of CHIPS into ADC-1004, which is a truncated CHIPS molecule with seven mutations and a calculated molecular weight of 9.8 kDa (Figure 3). This CHIPS variant is a potent C5aR antagonist with very low reactivity with pre-existing human IgG. ADC-1004 was generated in order to increase the feasibility of using a CHIPS derived molecule for therapeutic purposes. The low interaction with pre-existing IgG is hypothesized to make this CHIPS variant less susceptible for IgG mediated neutralization and less prone to form harmful immune complexes with circulating human IgG.

The work, on which this thesis is based, proposes that ADC-1004 is a promising C5aR antagonist for *in vivo* use, and in the near future, this molecule will be studied in a porcine model for I/R injury associated with myocardial infarction.

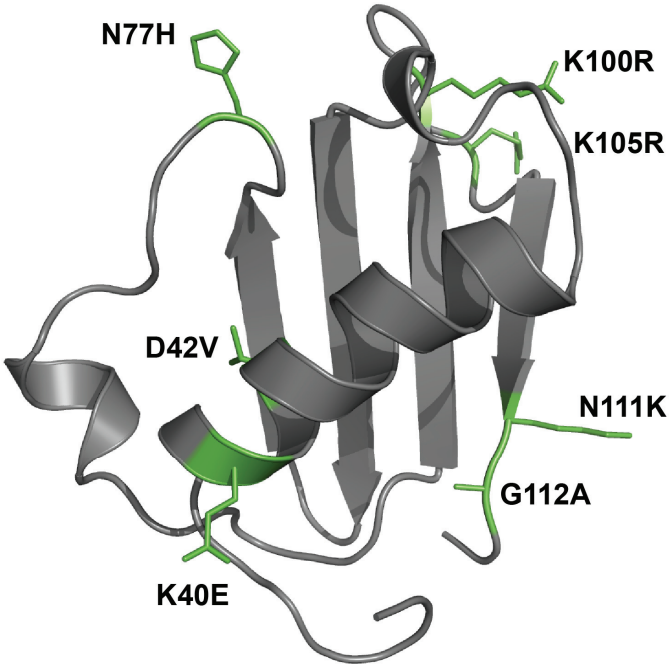

Figure 3. Ribbon diagram of ADC-1004, made from the NMR structure of $CHIPS_{31-121}$ (PDB code: 1XEE) (118), with mutations marked in the figure.

de Haas *et al* characterized the binding of CHIPS to neutrophils of different species. CHIPS was shown to bind human neutrophils the best among all species tested (rabbit, pig, mouse, guinea pig, rat and dog). CHIPS bound porcine neutrophils to 40 % of the binding towards human neutrophils and displayed less than 20 % binding to all other species tested (9). The activity of ADC-1004 has only been verified on human and porcine neutrophils (unpublished data). Based on these results and CHIPS data, a porcine model is the method of choice for *in vivo* studies of ADC-1004.

The half-life of wt CHIPS was estimated to 1.5 hours in the small pilot study on healthy volunteers (123). Furthermore, the half-life for ADC-1004 has recently been determined in kinetic studies in rat and mini pig (1 hour and 1.5 hours, respectively) (unpublished data). Based on this kinetic profile, ADC-1004 would most likely be suitable for short-term treatments of acute inflammatory disorders.

3.3.3 Additional antagonists

Several groups have attempted to target the C5aR and develop potent specific antagonists to block C5a mediated effects, reviewed in (59, 124, 125). The antagonists developed are of different types; for example short peptides (126, 127), cyclic peptides (128-131), non-peptide small molecules (132), C5a mutants (47, 133) and monoclonal antibodies (134-136).

Some C5aR antagonists have shown good results *in vivo*; for example the C5a mutant C5aRAM has been shown to inhibit C5a-induced neutropenia and dermal edema (133) and its dimeric form C5aRAD has been shown to inhibit neutrophil activation and to reduce the size of infarction in a revascularization model (137). Furthermore, the C5a mutant ΔpIII-A8 has been shown to inhibit neutrophil recruitment in immune complex mediated peritonitis and to reduce tissue damage in a model for I/R injury (47). In addition, the non-peptide antagonist W-54011 has been shown to inhibit C5a-induced neutropenia in gerbils (132) and the cyclic peptide F-[OpdChaWR] and its acetylated form AcF-[OpdChaWR] has shown promising results in several experimental disease models, such as I/R injury (82, 138), arthritis (139), inflammatory bowel disease (80) and sepsis (140).

A number of C5aR antagonists have reached pre-clinical or clinical development, such as AcF-OpdChaWR (PMX-53), which has been under development by Promics and later Arana Therapeutics for indications such as rheumatoid arthritis, osteoarthritis, psoriasis and acute macular degeneration. Jerini AG reports development of two peptide-based C5aR antagonists (JSM-1375 and JSM-7717) for treatment of acute macular degeneration and inflammatory disorders, Novo Nordisk A/S reports development of an anti-C5aR antibody for treatment of rheumatoid arthritis and SLE and Mitsubishi Pharma develops the small molecule antagonist (MP-435) for treatment of rheumatoid arthritis.

4. Immune responses to protein drugs

Recombinant proteins are becoming more and more attractive and important therapeutic options to chemical or synthetic small molecule drugs. However, to generate safe and effective therapeutics, attention needs to be paid to the immunogenicity issues associated with protein therapeutics. All proteins can be immunogenic to some extent. An immune reaction against a protein drug can cause unwanted effects such as allergic reactions or neutralization of efficacy by the presence of specific antibodies, reviewed in (141).

Therefore, substantial work has been carried out to determine factors that contribute to immunogenicity. Many different factors may have an influence; patient-related factors such as genetic background, underlying disease or immune status might predispose for an immune reaction to a certain protein pharmaceutical. Other factors related to the pharmaceutical, such as the source of protein, the manufacturing process or the formulation and stability characteristics are also known to have an impact on the induction of immunogenicity. In addition, the dose and dosing interval, the duration of treatment and route of administration are known to affect the immunogenicity of protein pharmaceuticals, reviewed in (142).

4.1 Anti-drug antibodies

Antibodies generated against protein therapeutics are generally referred to as anti-drug antibodies (ADAs). The binding of ADAs can have different consequences.

Non-neutralizing antibodies (NNAbs) do not negatively affect the efficacy of the drug, but can instead increase drug efficacy (143) or alter pharmacokinetics (144). NNAb dependent clinical complications include symptoms resembling serum-sickness and anaphylactic shock (145, 146).

Neutralizing antibodies (NAbs) bind the therapeutic protein in such way that the drug efficacy is reduced. This has been seen for proteins such as factor VIII (147) and Streptokinase (148). Worse consequences are seen when NAbs also inactivate the function of autologous proteins (149, 150).

In Paper II, we describe the generation of new CHIPS variants with reduced interaction with pre-existing human IgG. CHIPS has induced high antibody titers in humans and approximately half of these were shown to neutralize the biological function of CHIPS (10). We therefore speculated that wt CHIPS would not be an efficient C5aR blocking molecule *in vivo*. Furthermore, the risk for immune complex formation between CHIPS and antibodies, constituted a safety issue if CHIPS was to be administered *in vivo*. However, Streptokinase (151) that has been used for treatment of myocardial infarction for decades has also been shown to have induced antibody titers in humans (152). In Paper IV, we compared the titer towards wt CHIPS and ADC-1004 with the titer towards Streptokinase. The wt CHIPS titer was 12 times higher than the Streptokinase titer, which in turn was 15 times higher than the ADC-1004 titer. We therefore conclude that the remaining titer towards ADC-1004 is low. However, the influence of remaining IgG as well as IgA on the efficacy of ADC-1004 has to be analyzed in an *in vivo* model.

In Paper II we only addressed the problem with pre-existing IgG and not the risk for generation of a new immune response to variants of the CHIPS molecule. The production of ADAs of IgG class is generally dependent on T_H-cells that control class-switch and affinity maturation (153). Naïve B-cells require two signals to become antibody secreting plasma cells. Apart from recognizing epitopes on the protein drug via surface receptors, B-cells need stimulatory cytokines and CD40L (CD154) from T_H-cells to be activated. Such responses require T_H-cell recognition

of epitopes in the protein drug, which is achieved through recognition of peptide:MHC class II complexes displayed on antigen presenting cells. This second signal for B-cell activation is known as linked recognition, since B- and T_H-cells recognize the same antigen, although the epitopes do not need to be identical (154).

Immune responses against protein therapeutics are therefore dependent on both T_H- and B-cell epitopes. The correct prediction of immunogenicity is a challenge, but development of methods for the prediction of epitopes and the reduction of immunogenicity is important. To date, a number of such methods are available and several were reviewed recently by De Groot and Scott (141).

4.2 Prediction and reduction of immunogenicity

Protein pharmaceuticals based on recombinant human proteins are usually immunogenic due to impurities, aggregate formation or protein degradation (155-157). Immunogenicity of non-human proteins as well as modified human proteins, on the other hand, depends on the low sequence identity with human proteins. Therefore, B- and T_H-cell epitope analysis is a good strategy to apply when predicting if immune responses are likely to occur upon administration of such a protein to humans.

ADAs can be detected by direct binding assays and the effect of NAbs can be studied by performing functional assays in the presence of purified ADAs or patient sera (158, 159). Mapping of antibody epitopes has mostly relied on peptide-based technologies. Geysen introduced the technique for studying antibody binding to synthesized overlapping peptides (160) and related techniques followed (161, 162). In Paper I, we utilized peptide phage display to map conformational antibody epitopes on the surface of the CHIPS molecule. This technique (163, 164), allows for the selection of peptides that might represent 3-dimensional recognition sites for antibodies and thus the identification of conformational epitopes as well as linear epitopes is facilitated, reviewed by Rowley *et al* (165).

Several *in silico* models have been developed for the prediction of B-cell epitopes, but binding of antibodies is often dependent on the 3-dimensional structure of antigens, which makes the prediction of B-cell epitopes more difficult than the prediction of T_H-cell epitopes (described below) (166).

T_H-cell responses can be investigated *in vivo, in vitro* or *in silico*. If *in vivo* models are utilized for evaluation of immunogenicity, it is important to consider the differences in animal and human major histocompatibility complex (MHC). The best alternative for *in vivo* studies of immunogenicity is therefore to utilize transgenic animals that express the human MHC, human leukocyte antigen (HLA) (167, 168). Several *in vitro* methods have been developed for immunogenicity screening, for example mass spectrometry (169), binding (170) and activation assays (171). These are useful tools, but the high polymorphism in HLA class II and the different types of HLA class II molecules result in that these assays require analysis of cells from numerous donors (172). HLA can present a diverse array of peptides since they are encoded by several genes with high polymorphism. A HLA class II molecule is built up of two chains, α and β (A and B) which form six HLA class II types; DR1, DQ, DP, DR3, DR4 and DR5. Not all types occur in all haplotypes and a maximum of 12 HLA class II molecules can be expressed simultaneously (2 DR1 types, 2 DR3/4/5 types, 4 DQ types and 4 DP types) (173). *In silico* T_H-cell epitope prediction mediates a convenient analysis of several HLAs and is a therefore a good complement to *in vitro* assays (174). Several methods for T_H-cell epitope mapping have been described (173) and results from such methods have also been shown to correlate well with *in vitro* data (175, 176). In Paper IV, we utilized the Epibase® technology (175, 177) to estimate the number of T_H-cell epitopes in ADC-1004 and compare it to wt CHIPS. We could conclude that the truncation and mutagenesis of CHIPS, that we applied to develop ADC-1004, not only affected the interaction of the protein with pre-existing IgG, but also altered predicted T_H-cell epitopes, even though this property was not screened for during the evolution of CHIPS. ADC-1004 was found to contain fewer predicted T_H-cell

epitopes than wt CHIPS and several of the epitopes in ADC-1004 were different from those in wt CHIPS. The number of epitopes was found to be reduced both due to truncation of the molecule and due to mutagenesis. These findings were considered important and we speculate that potential T_H-cell responses against ADC-1004 will most likely be non-memory based responses upon a single administration of the protein.

A number of strategies have been described to facilitate reduction in immunogenicity of protein therapeutics. There are however risks associated with these methods. Functional activity of the protein might be decreased or new immunogenic epitopes may arise (178). The biological activity of CHIPS variants with decreased IgG binding was indeed affected, as described in Paper II. A decrease in biological activity was observed for the truncated CHIPS molecule and the different CHIPS variants were affected to varying extent both by this truncation and by the different mutations introduced.

Efforts in optimizing production, purification and formulation can be made to decrease the risk for inducing immune reactions against protein therapeutics (144, 179). Another way of reducing immunogenicity is by chemically modifying the protein by for example PEGylation (the addition of polyethylene glycol moieties), that may sterically block antibody binding (180). In addition, PEGylation has been shown to decrease immunogenicity by increasing protein solubility and modifying pharmacokinetics (181). For instance, IgG responses against IFN-β1b have been significantly reduced by PEGylation (182).

Sequence modifications, such as those made in ADC-1004, have previously been shown to decrease the interaction of Streptokinase with ADAs (183). Another very good example on how sequence modifications alter immunogenicity are the different generations of chimeric and humanized antibodies, engineered to reduce the immunogenicity of monoclonal antibodies of mouse origin (184, 185). Taken together, the combined profile of low interaction with pre-existing IgG and

moderate immunogenicity makes ADC-1004 a more attractive candidate protein therapeutic than wt CHIPS.

5. Molecular evolution of CHIPS

Laboratory molecular evolution enables researchers to confer desired properties on proteins to be used in for example industrial processes, as diagnostic tools or pharmaceuticals. Directed evolution, often performed by randomly mutating or recombining gene segments, is a procedure that allows for the evolution of proteins without prior detailed knowledge of the protein structure, and is described as an efficient way to rapidly accumulate beneficial mutations (2). This method differs from rational design, which is generally referred to as structure-based protein design, by which point mutations are introduced into the gene sequence by site-directed mutagenesis based on predictionsmade by analyzing the 3D structure of the protein. Although rational design has proven successful in protein optimization studies (186, 187), the method is time-consuming and it is not always possible to predict hot-spots for the mutagenesis by structural analysis (188, 189).

A commonly used approach for protein optimization is based on the design of diverse protein libraries that contain many protein variants with different properties. The variants that display improved properties can then be isolated by selection and screening and then further characterized.

Directed evolution is commonly performed in 3-5 rounds of mutagenesis and recombinations to accumulate favourable mutations. This has proven to be a successful strategy, and to further enhance the yield of improved proteins, directed evolution is now often combined with computational analyses and rational design to identify multiple mutations needed for the improvement of certain protein characteristics as well as to introduce novel functions (190-192) (Figure 4).

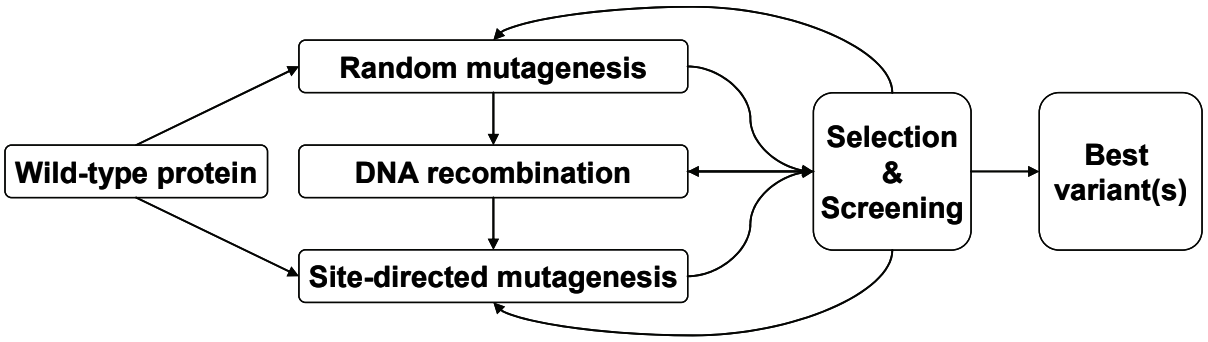

Figure 4. A generalized scheme for directed evolution and site-directed mutagenesis. Library design and selection/screening are often performed in several rounds, to accumulate beneficial mutations.

Using this approach, the CHIPS molecule was improved to display significantly decreased interaction with specific human IgG (Paper II). Directed evolution (random mutagenesis and FIND®) generated CHIPS variants with only 2.5 % residual binding to specific human IgG compared to wt CHIPS. Further decrease in binding to 0.5 % of wt was subsequently achieved through molecular modeling and site-directed mutagenesis.

In this chapter, the techniques applied for library design, selection, screening and characterization of new CHIPS variants are outlined and discussed.

5.1 Library design

The design of a diverse library is the initial step in directed evolution of new protein variants. The quality of a library determines much of the success rate of the directed evolution process. Prior knowledge of the protein should therefore be taken into consideration when available. CHIPS single mutants K61A, K69A and K100A had previously shown decreased binding to human IgG (unpublished data). To improve the quality of the CHIPS libraries created in Paper II, CHIPS

sequences mutated to generate these substitutions were consequently included in the randomly mutated libraries of CHIPS variants.

Iterative mutagenesis and screening of low mutation libraries is a common approach in directed evolution to stepwise accumulate beneficial mutations and generate improved variants in a manner that mimics natural evolution (193). The mutation rate of a library has been demonstrated to affect functionality of protein variants selected from randomly mutated libraries (194). A mutation frequency of 1-4 amino acid substitutions per gene is usually regarded as an adequate frequency, but the outcome also depends on the protein, the mutagenesis protocol and the method used for selection and screening (195). Higher mutation frequencies can be deleterious, but sometimes beneficial. The likelihood of finding completely new protein functions is increased with a higher mutation frequency and functional mutants have indeed been isolated from such libraries (194, 196, 197). In Paper II, we decided to create randomly mutated libraries with different mutation frequencies to increase the likelihood to find improved functional protein variants. The lowest mutation frequency of the CHIPS libraries was 1.1 amino acids per sequence and the highest was 3.6 amino acid changes per sequence, which is rather high, since the truncated CHIPS molecule subjected to the mutagenesis is a small protein composed of only 83 amino acids. However, the high and low mutation frequency libraries were pooled directly after phage selections and therefore the library origin of clones selected from these libraries could not be determined.

5.1.1 Mutagenesis

Diversity can be introduced into a DNA sequence in several ways. In Paper II, the mutagenesis was both random and rational site-directed mutagenesis, which was based on predictions from structural analyses. The technique for site-directed mutagenesis was first introduced by Hutchison in 1978 (198) and since then, several different protocols for site-specific introduction of point mutations have been published (199-202). The mutagenesis is commonly performed by PCR with

oligonucleotides carrying the desired base change. In Paper II, this mutagenesis was carried out by the use of the Quikchange site-directed mutagenesis kit from Stratagene, by which mutations are introduced through amplification of an entire plasmid (carrying the gene of interest) with mutagenic primers and a proofreading DNA polymerase. This generates nicked circular DNA, which is then treated with a restriction enzyme (DpnI) specific for methylated parental DNA (203). As a result, only the mutated DNA is left undigested.

Random mutations can be introduced into a sequence by several different strategies. These are generally divided into synthetic chemistry methods; e.g. by DNA modifying chemicals, (204), whole cell methods; e.g. mutator host strains (205) and enzyme based methods, like error-prone PCR, which is a PCR performed under suboptimal conditions for the DNA polymerase. The mutation frequency generated by this method is dependent on several factors that can reduce the fidelity of the DNA polymerase; such as the addition of Mn^{2+} to the reaction, the amount of Mg^{2+}, dNTPs and template as well as the type of polymerase used (206, 207). The DNA polymerase from *Thermus aquaticus* (*Taq* polymerase) is widely used for error-prone PCR since it lacks 3'-5' proofreading activity (208), and thus has a high intrinsic error rate, but the drawback with this enzyme is that it is biased for certain mutations (AT → GC transitions and AT → TA transversions) (208, 209), depending on the reaction conditions. To avoid or reduce such biases, libraries can be designed by the use of different enzymes or by combining different methods for random mutagenesis (210). In Paper II, error-prone PCR was used for introducing mutations at different frequencies in two libraries. These were combined with two additional libraries, designed by the use of a mutagenesis kit from Stratagene that utilizes the highly error-prone polymerase, Mutazyme. This enzyme has been shown to have a different mutational spectrum (GC → AT transitions and GC → TA transversions), which allows for G and C residues to be mutated at a higher rate (211-213).

5.1.2 In vitro DNA recombination

Recombinations occur in many natural processes for generation of diversity, such as the crossovers between the variable regions of immunoglobulins (214). *In vitro*, beneficial mutations from a randomly mutated library can be recombined in new combinations and thus generate changed protein functions. One *in vitro* DNA recombination method, called DNA shuffling, was described by Stemmer in 1994 and is based on recombination of DNA fragments generated through fragmentation of double-stranded DNA (dsDNA). Fragmentation is typically performed through enzymatic digestion by an endonuclease, DNaseI, and the subsequent reassembly of the fragments is achieved in a primer less PCR (215, 216). This method has been used to for example change substrate specificity (217), enhance enzyme activity (217), improve protein folding (218) and increase the specific activity of a human cytokine (219). Besides recombining mutations from randomly mutated sequences, DNA shuffling can be utilized to combine genetic material between homologous genes (called family shuffling) (220). Several additional methods for *in vitro* DNA recombination have been developed and many of these are summarized in a review by Yuan *et al* (2).

In Paper II, DNA recombination was performed by the use of a method called Fragment INduced Diversity (FIND®), which is schematically outlined in Figure 5. This method differs from DNA shuffling in that it utilizes single-stranded DNA (ssDNA), which is fragmented by exonucleases (Exo I (221), Exo V (222, 223) and/or Exo VII (224, 225). Resulting fragments are reassembled in a primer less PCR and then amplified by the use of primers to generate full-length recombined genes. FIND® has been used to optimize different properties of proteins. Carboxypeptidase U was previously optimized towards higher thermo stability (226) and IL-1 receptor antagonists were increased in activity by the use of FIND® (227).

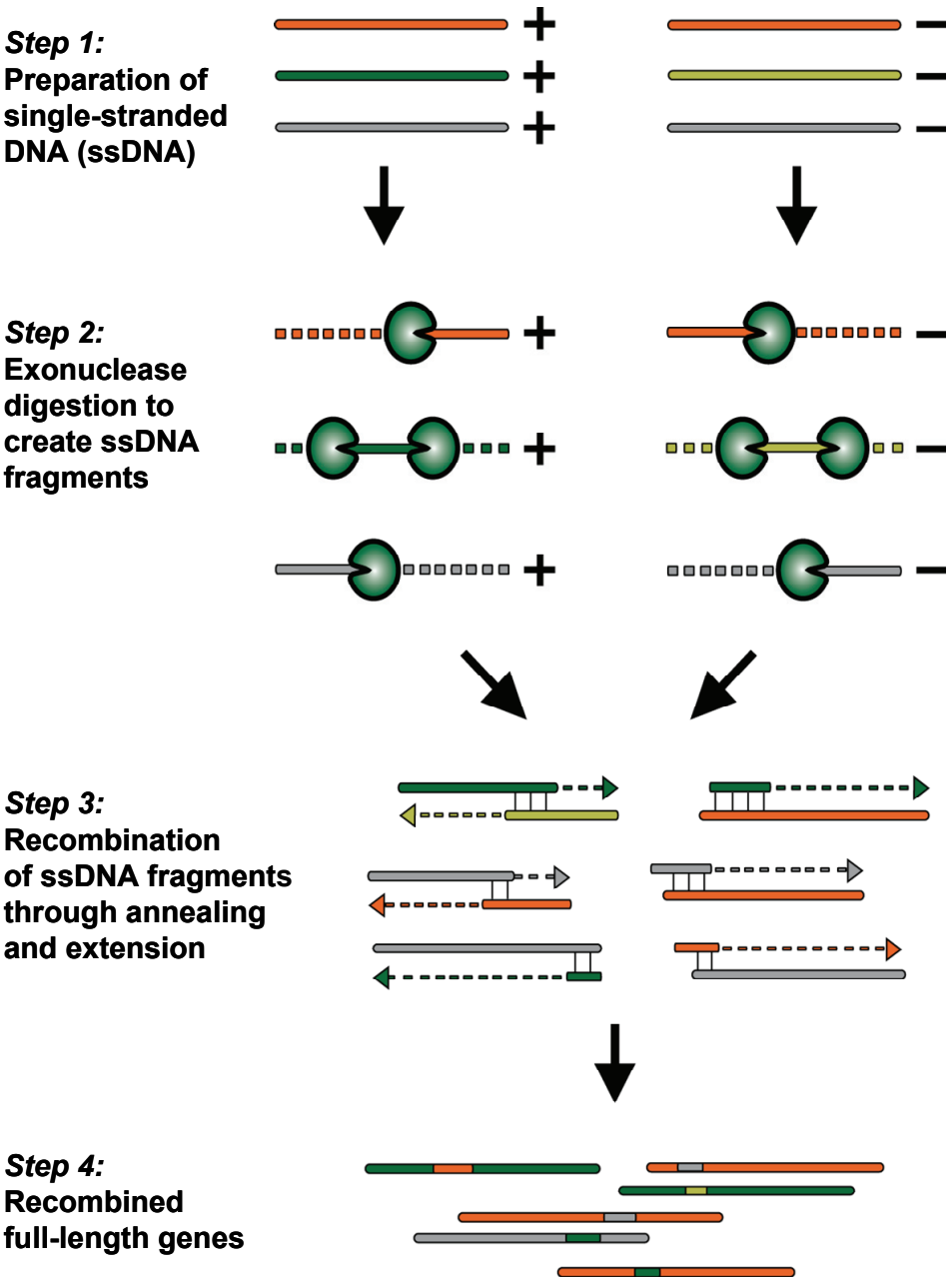

Figure 5. Schematic depiction of the different steps of Fragment INduced Diversity® (FIND®).

One disadvantage that has been recognized for the DNA shuffling method, is the strong predisposition for forming unshuffled parental genes (228, 229). Kikuchi *et al* introduced the concept of using ssDNA as starting material for DNA recombination to achieve a higher recombination frequency (230, 231). DNA recombination utilizing either ssDNA or dsDNA as starting material were compared in optimization of cathecol 2,3-dioxygenase temperature stability and the

recombination frequency obtained was 14 % for the ssDNA and less than 1 % for the dsDNA libraries (231).

Per definition, exonucleases are enzymes that digest DNA or RNA and cleave off nucleotides sequentially from the ends of a sequence by catalyzing the hydrolysis of phosphodiester bonds. By terminating the enzymatic reaction at various time points, fragments of varying sizes can be generated. In a simplified way, one could say that short times of digestion yield long fragments, while long digestion times consequently yield shorter fragments. However, different exonucleases have different kinetics and specific mechanisms for this digestion. Some exonucleases digest DNA from only one end, while some have activity from both ends. Exo I has 3' activity, while Exo V and Exo VII have activity from both ends (5' and 3') (232, 233). The size of the gene to be fragmented determines the exonuclease digestion time required for recombinations to be distributed over the entire sequence. The possibility to use different exonucleases, to stop the exonuclease digestions at different time points and to change temperature or buffer conditions are all factors that add to the diversity of libraries created by FIND®.

In Paper II, diversity was first introduced into the CHIPS sequence by random mutagenesis. FIND® was then applied in three rounds on the selected or screened top clones of each round. Different approaches were used for the design of the FIND® libraries in order to increase the mutation frequency or recombination frequency of the libraries. Two libraries were designed in the first round of FIND®. The starting material was the same in both libraries, namely the top clones from the randomly mutated library. However, one library was designed by FIND® under error-prone conditions in both the primer less PCR and the subsequent PCR step to increase the diversity in the entire CHIPS sequence. In the second library, additional mutations were introduced in the C-terminal end of CHIPS by adding a randomized oligonucleotide, corresponding to this part of the protein, in the primer less PCR step in one FIND® library. This was performed

since mutations in this part of the protein were found to contribute to decreased interaction with human IgG during the screening of the randomly mutated library.

Two different libraries were designed in the third round of FIND® as well. A different number of clones from the previous round were used as starting material in these libraries, but FIND® was performed likewise. An additional FIND® reaction was applied directly after the first in these libraries, which generated a very high frequency of recombined sequences (92 %).

5.2 Selection and screening of protein libraries

After diversification of the genetic material and expression of corresponding protein variants, it is necessary to apply a powerful method for assaying the resulting protein libraries in order to find improved variants. Libraries can be analyzed by different methods, but two major approaches can be distinguished; (i) selection, which is a method for analyzing all variants at the same time, and (ii) screening, which is individual analysis of single clones.

All selection or screening procedures are required to maintain the link between genotype and phenotype. In screening, this can be achieved by physical separation in microtiter plate format, however several methods for maintaining this link without separating the library members (selection methods) have been described (234). These are commonly based on display technologies and the most widely used method is phage display (235-237), which was first described by Smith in 1985 (238). Individual library members are displayed on the surface of filamentous bacteriophage particles, of which M13 is the most widely used (234). The gene encoding the protein to be displayed can be introduced by cloning in fusion with the gene for a phage coat protein in a phagemid or phage vector. The libraries of CHIPS variants designed in Paper II were cloned in a phagemid vector in fusion with the minor phage-coat protein III (pIII). This protein is displayed on the phage surface in 3-5 copies, and is the most frequently used coat protein to

display for example antibody fragments (239). The phagemid does not contain any phage genes apart from the fusion gene and co-infection with a helper phage is required to produce phage particles. The phagemid carries an origin of replication for double-stranded DNA as well as an antibiotic resistance gene and can thus be propagated in
E. coli. Another origin of replication mediates the production of single-stranded vector and subsequently the formation of phage particles after co-infection with helper phages. Vectors based on the natural phage sequence, which contain all genes necessary for production of phage particles, can also be utilized for phage display. Such a vector was used in Paper I for display of two random 7-mer peptide libraries fused to pIII of phage M13.

In common selection procedures, the binding target is usually immobilized on plastic surfaces (240) or magnetic beads (241) (Paper I and II). The binding step is followed by extensive washing to remove unbound phages. Bound phages can then be eluted through for example competitive elution with a soluble antigen (Paper I), incubation at extreme pH (Paper I and II) or proteolytic cleavage (Paper II). The eluted phages are subsequently allowed to infect *E. coli* and produce new phage particles with selected library genes. Usually 3-5 cycles of selections are performed to allow enrichment of improved binders. Several parameters can be varied to achieve enrichment of improved binders (242). Higher stringency can for example be achieved through the use of limited amounts of antigen, by extensive washing or by competition with soluble antigen (241). In Paper I, phages bound to human anti-CHIPS IgG on magnetic beads were eluted by incubation with the soluble wt CHIPS molecule to further increase the specificity of the phage selection.

In Paper II, selections were performed against two different antigens. In the so called positive selection, phages that bound an N-terminal peptide of the C5aR (known to contain the CHIPS binding site (122)) were eluted at low pH. Then, a negative selection was performed against anti-CHIPS IgG coupled to magnetic beads. The supernatant, which was expected to only contain phages with low

affinity for human IgG, was collected. This way, the biological activity of the CHIPS variants was monitored in parallel with evolving CHIPS towards decreased binding to affinity purified human IgG. The continuous selection for C5aR peptide binding was most likely very important for the possibility to finally select clones with low IgG binding, but with retained biological function.

Phage selection is a convenient and widely used method, by which the link between genotype and phenotype is easy to maintain. This method is typically applied for selection for improved affinity or specificity, even though properties such as thermal stability can be improved by applying a suitable selection pressure (243). Conversely, screening systems can be utilized for a wider array of purposes. For example, screening can be used to find clones with certain improved enzymatic or cellular activities. In addition, the expression system for such assays is not limited to prokaryotic expression, thus facilitating the correct folding and post-translational modification of many human derived proteins.

The success in finding binders has been reported to be directly coupled to the size of a phage display library (244, 245), and it is regarded more efficient to screen a low number of clones in a large library than to screen a smaller library more thoroughly (246). A large number of clones can be screened individually by the use of high throughput assays. Even though these allow for large libraries to be screened, it is not always possible to maintain a high accuracy of such assays. One way of screening for an improved protein property is to first apply a rough primary assay, by which a high number of clones can be screened and a few selected for more detailed further screening or characterization (247). For example, phage display or membrane/agar plate based screening (248) can be utilized as primary assays, followed by microtiter based assays in a secondary screen and subsequent characterization in for example cell based assays to determine biological functionality.

In Paper II, phage display was used as a primary selection method in the screening of the randomly mutated libraries and the first FIND® library. Selected

clones were then expressed in *E. coli* and screened in ELISA for decreased specific IgG binding and retained C5aR peptide binding. In the second and third round of FIND®, ELISA screening of maximum 9600 clones was applied directly without any prior phage selections. The reason for this was that we observed a bias for certain amino acid substitutions during the previous round of phage selection. To reduce the risk for further biases during the screening rounds, we utilized different ELISA set-ups to measure specific IgG binding.

5.3 Characterization of improved protein variants

Careful characterization is usually performed on a small number of selected protein variants, to be able to finally pick the most promising clone(s) for future studies. Depending on the protein, methods applied for such characterization can be for example variants of methods used during the screening process, which now can be performed more carefully on a small number of clones, or complex biological assays and methods applied for studying biophysical parameters of the protein variants.

A common way of verifying clones selected by phage display is by applying a phage ELISA on single clones, with the target coated in a microtiter plate. This method was utilized in Paper I to verify binding of selected phages to affinity purified human IgG.

In Paper II, seven CHIPS variants were selected for further characterizations of IgG binding in pooled human serum, since this better resembles *in vivo* conditions than affinity purified IgG. The seven CHIPS variants were compared to truncated and full-length wt CHIPS and all clones showed decreased binding, which confirmed the results from the selection and screening. In Paper IV, serum IgG binding of one selected CHIPS clone (ADC-1004) was compared to wt and truncated CHIPS as well as to two existing protein pharmaceuticals (Streptokinase and Anakinra) to gain an even better understanding of the level of IgG binding.

ADC-1004 displayed the lowest IgG titer among the CHIPS variants and Streptokinase. The median value of 28 donors was 400 times lower for ADC-1004 than the median value for wt CHIPS. Only Anakinra, which is a recombinant human protein, displayed lower titer. Furthermore, potential IgG mediated effects were characterized through analysis of complement activation in ELISA using pooled and individual human sera. ADC-1004 was shown to induce complement activation to a lower extent than wt and truncated CHIPS. The median value of 28 donors was shown to be 15 times lower for ADC-1004 than the median value for wt CHIPS.

Other methods for characterization can be more protein specific, like the methods used in Paper II and IV to verify the biological function of the selected clones. These were expressed in higher amounts and purified and several cell based assays were applied. Even though binding towards a C5aR peptide was monitored during selection and screening, the specificity in binding to the entire C5aR was necessary to assess. Binding to the more complex structure of a large receptor is very different from a short peptide. Also, sometimes proteins show higher binding in plate format (ELISA) than in solution due to avidity effects. In Paper IV, binding to the C5aR and three other complement/chemokine receptors was characterized by flow cytometry on both transiently and stably transfected cells. ADC-1004 was shown to bind the C5aR with high affinity (2.9 nM), but did not bind other receptors tested, which was in agreement with previously published data on the wt CHIPS protein (119, 120).

For further analysis of biological activity, ADC-1004 was purified in high amounts from inclusion bodies with a protocol described in Paper III. The CHIPS mediated inhibition of the C5aR was studied by two different methods (Paper II and IV). C5a is chemotactic on neutrophils through activation of the C5aR, which is distinguished by for example the intracellular release of Ca^{2+}. CHIPS mediated inhibition of these properties was studied on human neutrophils in a transwell system and by flow cytometry, respectively. ADC-1004 was shown to inhibit Ca^{2+}

release with an IC_{50} value of 0.4 nM, twice that of wt CHIPS. The inhibition of neutrophil chemotaxis was also retained in ADC-1004. The IC_{50} of ADC-1004 was approximately six times lower than the IC_{50} of wt CHIPS (49 nM and 8.2 nM, respectively).

Circular Dichroism (CD) spectroscopy is a method for studying protein structure and is a sensitive method for determining alterations in conformation, resulting from changes in e.g. pH, temperature or ionic strength. The far-UV CD region (below 250 nm) reflects the secondary structure of a protein and such measurements give low-resolution information about the protein conformation (249). This method was applied in Paper I, II and III to study temperature stability and reveal any potential major structural changes caused by the mutagenesis, DNA recombination or purification protocol. Several of the clones described in Paper II displayed higher T_m values than the truncated wt CHIPS molecule, but the thermal unfolding, which was a reversible process in the truncated wt protein, was changed into an irreversible process in the selected seven clones.

The clones characterized with respect to protein folding in Paper I all displayed a preserved overall global fold, despite the introduced mutations that negatively affected their C5aR inhibiting activity. In Paper III, solubly and insolubly produced truncated CHIPS were also shown to have the same overall global fold, independent on the purification procedure.

6. Concluding remarks

In this thesis, molecular evolution of the staphylococcal protein CHIPS has generated protein variants with improved characteristics concerning unwanted immunoreactivity. A significant decrease in IgG binding was achieved and the sequence modifications mediating this did not abrogate the anti-inflammatory properties of the mutated CHIPS molecule. The unique inherent capacity to inhibit C5aR signaling was retained and the new selected CHIPS variant ADC-1004 is therefore a C5aR antagonist with potential to eventually be used *in vivo*. Certain properties distinguish this C5aR antagonist from other molecules under development for inhibition of the C5aR. ADC-1004 is a high-affinity, protein derived molecule with an estimated short half-life in the human circulation. Even though ADC-1004 is of bacterial origin, it is not predicted to display unwanted immunoreactivity *in vivo*. This is a result of the decreased interaction with pre-existing IgG, achieved by molecular evolution as well as the reduced number of T_H-cell epitopes, to a large part changed compared to the wt CHIPS molecule.

In Paper I, we identified potential epitopes for human IgG on the CHIPS surface by phage selection of peptide libraries, molecular modeling and subsequent mutagenesis. In addition to describing epitopes involved in IgG binding, this work gave further insight into amino acids in CHIPS important for the interaction with the C5aR, which was in agreement with previously published data (10, 120).

A different approach was taken in Paper II to reduce the interaction of CHIPS with pre-existing human IgG. Seven CHIPS variants with decreased IgG binding and retained biological activity were generated by applying directed

evolution (random mutagenesis and FIND®) and subsequent site-directed mutagenesis. This approach was initiated in parallel with the approach described in Paper I, but some conclusions were drawn from the results in Paper I when designing the final amino acid substitutions by site-directed mutagenesis in Paper II. One position, K100, was mutated in a number of the variants selected in Paper II. Even though a single mutant with the substitution K100A showed reduced C5aR blocking activity in Paper I, ADC-1004 (clone 376) described in Paper II, was shown to have completely preserved biological activity compared to truncated wt CHIPS, despite the substitution K100R. These data demonstrate that by applying an appropriate selection pressure, more beneficial mutations can be accumulated by a randomized approach, than by rational design. One such example from the literature is the tuning of enzyme substrate specificity, which has been successfully modified by directed evolution, even though rational design approaches previously resulted in limited success (250).

Nevertheless, some of the amino acids mutated in the final clones in Paper II correlate well with amino acids shown to be of importance in the IgG epitopes described in Paper I. Taken together, both approaches generated important information for the evolution of an improved CHIPS molecule. Directed evolution was however the method that produced mutants with the lowest interaction with pre-existing human IgG. Molecular modeling and site-directed mutagenesis proved to be good tools for further protein optimization after key amino acids were identified by directed evolution.

In molecular evolution, you get what you select for, while other protein properties might be affected by the process of mutagenesis and recombinations. In Paper II, libraries were selected and screened for both decreased IgG binding and retained C5aR binding, which was a very good approach to facilitate the selection of biologically functional clones with low IgG binding. The inclusion of screening for C5aR binding was most likely crucial for the biological activity to be retained.

However, it is not possible to monitor all potential properties that might be altered during protein evolution. In Paper II, libraries were for example not screened for preserved high soluble expression. The level of protein expression was never an issue before larger amounts of protein were necessary to express for downstream characterizations. Paper III describes a protocol for purification of ADC-1004 from inclusion bodies in *E. coli*, which was necessary to develop since expression of CHIPS shifted from soluble to insoluble expression upon truncation and mutagenesis. ADC-1004 and truncated wt CHIPS purified from inclusion bodies were shown to have the same biological activity and overall global structure as solubly expressed truncated wt CHIPS. This protocol therefore facilitated the further characterization of ADC-1004 described in Paper IV.

ADC-1004 was shown to display a very low IgG titer in a study on human sera, lower than the titer for Streptokinase (utilized for treatment of myocardial infarction) and was found to be a poor inducer of the complement system, as compared to the wt CHIPS molecule. These findings suggest that ADC-1004 is unlikely to induce a major IgG dependent activation of the human complement system *in vivo*, which is important for ADC-1004 to be a safe and well tolerated drug candidate.

Wt CHIPS was previously shown to be selective for the C5aR and FPR in comparison to 35 additional receptors (119). ADC-1004 binds and blocks the C5aR with high affinity and was shown to be specific for this receptor, when comparing receptor binding to the FPR, CXCR2 and C5L2. The lack of FPR binding confirmed previously published results, showing that the N-terminal end of CHIPS is essential for FPR signaling inhibition (120). The selectivity of ADC-1004 might be advantageous, since the specific activity of ADC-1004 most likely will be focused to the C5aR *in vivo* and not be consumed by other receptors, with the risk for adverse physiological responses. The biological effects of ADC-1004 will be further investigated in an animal model in the near future.

Populärvetenskaplig sammanfattning

Immunförsvaret är en försvarsmekanism, bestående av celler och molekyler som känner igen kroppsfrämmande strukturer. Vid en infektion eller skada uppstår vanligtvis en inflammation som är kroppens sätt att starta en läkningsprocess där bl.a. immunförsvarets celler rekryteras. Inflammationen syftar till att ta bort orsaken till skadan eller infektionen samt skadade celler och vävnad. Ibland blir dock inflammationen överdriven eller missriktad och kan i sig skada vävnader i kroppen. Då är det viktigt att kunna behandla denna reaktion med antiinflammatoriska läkemedel.

Den här avhandlingen beskriver utveckling av en molekyl, kallad Chemotaxis Inhibitory Protein of *Staphylococcus aureus* (CHIPS), som är en effektiv hämmare av inflammation genom att den hindrar tillströmningen av immunceller till den del av kroppen där en inflammation uppstått. CHIPS är ett bakteriellt protein som den vanliga hudbakterien *S. aureus* använder för att avväpna vårt immunförsvar så att den lättare kan infektera människor. CHIPS fungerar genom att binda och blockera en receptor, C5a-receptorn, genom vilken den inflammatoriska molekylen C5a utövar sin verkan. CHIPS är på så sätt en antiinflammatorisk molekyl som möjligtvis skulle kunna användas för att behandla inflammationer hos människor, men eftersom CHIPS är kroppsfrämmande, känns det igen av kroppens immunförsvar och därför har många människor producerat antikroppar som binder och hämmar CHIPS verkan.

I den här avhandlingen har de antiinflammatoriska egenskaperna hos CHIPS tagits tillvara, samtidigt som arbetet har syftat till att göra CHIPS mindre igenkänt av antikroppar. Två tillvägagångssätt har använts för att åstadkomma detta. I den ena studien testades CHIPS-specifika antikroppars bindning till en mängd korta aminosyrasekvenser (peptider) för att se om dessa kunde ge ledtrådar om vilka delar av CHIPS-molekylen som känns igen av dessa antikroppar. Därefter analyserades den tredimensionella proteinstrukturen av CHIPS. Vissa av de

framselekterade peptiderna kunde kartläggas på CHIPS-ytan och på så sätt kunde slutsatser dras av vilka delar av proteinet som binder antikroppar.

I den andra studien förändrades CHIPS gensekvens (DNA) och många olika CHIPS-varianter skapades. Genförändringarna gjordes genom att mutera CHIPS-sekvensen och genom användning av en rekombineringsteknik som kallas Fragment INduced Diversity® (FIND®). Vid applicering av FIND®, separeras DNAt så att det blir enkelsträngat, ”tuggas” upp med enzymer och de små genfragmenten som bildas, fogas sedan ihop i olika kombinationer till nya gener.

Stora ”molekylbibliotek” av CHIPS-varianter analyserades sedan för önskade egenskaper och de varianter som band antikroppar sämre, men som fortfarande band C5a-receptorn bra, valdes ut och karaktäriserades sedan ytterligare med avseende på hur de interagerade med antikroppar och hur väl de blockerade C5a-receptorn. Slutligen kunde en CHIPS-variant väljas ut som hade avsevärt förbättrade egenskaper och som troligen inte kan kännas igen av det immunförsvaret till samma grad som ursprungsproteinet.

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