Review

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Functional aspects of extracellular cyclophilins

Abstract: The cyclophilin family of peptidyl prolyl *cis/trans* isomerases includes several isoforms found to be secreted in response to different stimuli, thus existing both in the interior and the exterior of cells. The extracellular fractions of the cyclophilins CypA and CypB are involved in the control of cell-cell communication. By binding to the cell membrane receptor CD147 and cell surface heparans they elicit a variety of intracellular signaling cascades involved in inflammatory processes. Increased levels of cyclophilins in inflammatory tissues and body fluids are considered as an inflammatory response to injury. Thus, the extracellular portion of cyclophilins probably plays an important role in human diseases associated with acute or chronic inflammation like rheumatoid arthritis, sepsis, asthma and cardiovascular diseases. Specific inhibition of the cyclophilins in the extracellular space may open an effective therapeutic approach for treating inflammatory diseases.

Keywords: CD147; CypA; CypB; inflammation; prolyl *cis/trans* isomerization; signal transduction.

DOI 10.1515/hsz-2014-0125
Received February 13, 2014; accepted March 27, 2014; previously published online April 4, 2014

Introduction

Cyclophilins belong to the enzyme class of peptidyl prolyl *cis/trans* isomerases (PPIases, EC 5.2.1.8), which additionally comprises two other subfamilies, the FK506-binding proteins (FKBP) and the parvulins. These enzymes are characterized by powerfully catalyzing the reversible *cis*/*trans* interconversion of the two energetically preferred conformers of the peptide bond preceding proline (Figure 1) and can thus accelerate slow steps in the folding and restructuring of proteins. This type of biocatalysis has been found in all folding states of a polypeptide chain. These enzymes are thus termed 'foldases'. Cyclophilins are highly conserved across species. At present, 18 cyclophilin isoenzymes of different molecular masses have been described in human tissues. Seven of these are formed by the prototypic cyclophilin domain of about 160 amino acids devoid of additional N- or C-terminal domains. The most abundant member of the cyclophilin family is the archetypical human cyclophilin A (CypA), which was the first proline-directed foldase that was discovered (Fischer et al., 1984). The presently available results suggest that three mammalian cyclophilin isoforms, CypA, cyclophilin B (CypB) and cyclophilin C (CypC), can shuttle between the interior and exterior of cells, but with more sensitive methods other cyclophilins will probably be added to this list.

The CypA level is high in many mammalian cells, comprising as much as 0.4% of the total cytosolic protein fraction in T cells (Koletsky et al., 1986). In *Saccharomyces cervisiae* this level translates to 86 000 molecules/cell (West et al., 2010). Human CypB was the second cyclophilin identified (Price et al., 1991), and mainly differs from CypA by the presence of a cleavable N-terminal signal sequence that directs the protein to the endoplasmic reticulum (ER).

The immunosuppressive drug cyclosporin A (CsA) acts as nanomolar, reversible inhibitor for most cyclophilins (Fanghanel and Fischer, 2004). In addition to their role as protein folding catalysts, several cyclophilins were found to elicit multiple effects on various client proteins *in vitro*. To exert immunosuppression, CsA by gain-of-function forms a heterooligomeric complex with host cell CypA that inhibits the protein phosphatase calcineurin (Liu et al., 1991). However, by the use of non-immunosuppressive inhibitors and PPIase activity-reduced enzyme variants it became increasingly clear that a great number of biological effects of cyclophilins is determined by their catalytic interaction with prolyl bonds, and thus mediated by the PPIase activity.

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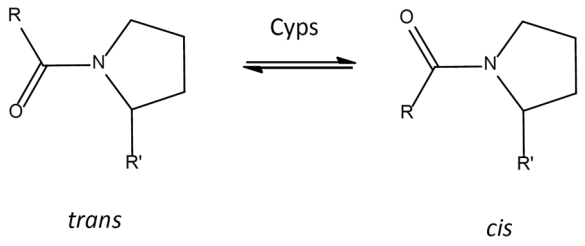

Figure 1 *cis/trans* isomerization of a peptidyl prolyl bond catalyzed by cyclophilins.

Cyclophilins are implicated in a variety of processes crucial for cellular life. Biochemical studies have shown *in vivo* and *in vitro* that a diverse set of proteins can functionally interact with CypA or CypB. For example, CypA participates in the nuclear translocation of the apoptosis-inducing factor AIF in neurons after cerebral hypoxia-ischemia (Zhu et al., 2007). It was also shown to interact with P53 and STAT3 implicated in transcription regulation (Bauer et al., 2009; Baum et al., 2009) and it is necessary for the CXCR4-mediated nuclear export of the heterogeneous nuclear ribonucleoprotein A2, a spliceosomal RNA-binding protein (Pan et al., 2008). CypA also controls the function of the T-cell-specific tyrosine kinase ITK following T-cell receptor stimulation (Colgan et al., 2004).

Induction of transcription is achieved by interaction of STAT5 with an intranuclear complex of CypB and prolactin, thus enhancing prolactin-induced, STAT5-mediated gene expression (Rycyzyn and Clevenger, 2002). In the ER, CypB forms a hetero-trimeric complex with prolyl 3-hydroxylase 1 and cartilage-associated protein, which 3-hydroxylates proline residues of collagen chains (Marini et al., 2007; Ishikawa et al., 2009).

Both CypA and CypB have been found to have important functions in the replication and infectivity of several viruses, such as human immunodeficiency virus (HIV), hepatitis C virus (HCV), measles virus and influenca A virus (Zhou et al., 2012; Frausto et al., 2013). Additionally, CypA is also able to regulate host IFN-I response to viral infections (Hopkins et al., 2012).

Beside these intracellular functions, the extracellular role of cyclophilins in inflammation has prompted a growing interest. Therefore, this review will focus on: i) how cyclophilins get out of cells; ii) what cyclophilins will do in the extracellular space; iii) what are the signals that are triggered by the extracellular cyclophilins; iv) how extracellular cyclophilins are involved in pathophysiological processes; and finally, v) how extracellular cyclophilins can be inhibited specifically.

Secretion of cyclophilins

Although cyclophilins have been shown to exhibit a large variety of functions requiring an intracellular protein pool (Fischer and Aumuller, 2003), it becomes increasingly clear that cells are able to secrete single-domain cyclophilins (Sherry et al., 1992; Price et al., 1994). Once secreted, the extracellular fraction of the cyclophilins (eCyps) is thought to contribute to various regulatory mechanisms that are able to control cell-cell communication. Cyclophilin isoforms potentially prone to be secreted, such as CypA, CypB and CypC, differ in their intracellular localization and thus also in the secretory pathways and the nature and intensity of stimuli causing protein release (Figure 2).

CypB is an ER-resident protein, which contains a C-terminal ER retention motif that differs from the classical KDEL ER retention signal. Proteolytic removal of the C-teminal five amino acids results in secretion of the truncated CypB found in human milk (Mariller et al., 1996). Upon administration of the competitive small molecule inhibitor CsA, full-length CypB is specifically and rapidly secreted into the extracellular space via the constitutive secretory pathway (Price et al., 1994; Fearon et al., 2011). It is likely that CsA competes with substrate proteins in the ER for binding to CypB, thereby greatly changing the molecular mass and the chemical properties of the resulting CypB complex (Price et al., 1994). The CsA-induced CypB secretion can be found in a variety of epithelial cell types (Table 1). In fact, active site dependent binding of newly synthesized proteins to CypB is considered to contribute to the retention of CypB in the ER (Price et al.,

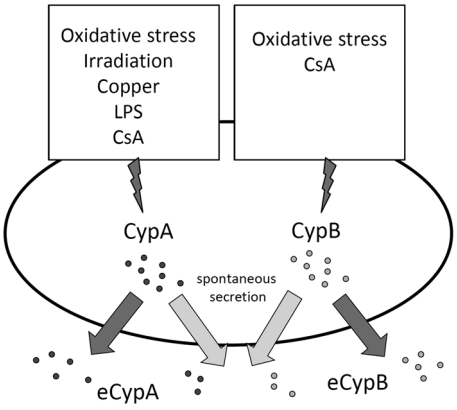

Figure 2 Secretion of CypA and CypB is induced by several stimuli. Oxidative stress and treatment with CsA, but also irradiation and application of copper ions or lipopolysaccharides can induce release of CypA or CypB, respectively. Both cyclophilins are also secreted spontaneously.

Table 1 Secretion of cyclophilins by different cell types.

Cyclophilin	Cell type	Stimulus	References
CypA	Macrophages	LPS	Sherry et al., 1992
	Macrophages	LPS	Sherry et al., 1992
	Endothelial cells	LPS	Kim et al., 2004
	Fibroblast-like synoviocytes	Spontaneous; LPS	Nishioku et al., 2012
	HNSCC cells	Spontaneous	Ralhan et al., 2011
	Kidney intercalated cells	Spontaneous	Peng et al., 2009
	Vascular smooth muscle cells	Oxidative stress	Jin et al., 2000
	Cardiac myocytes	Hypoxia/reoxygenation	Seko et al., 2004
	Epithelial breast cancer cells	Irradiation	Chevalier et al., 2012
	Hepatoma cells	SHB expression	Tian et al., 2010
	Neurons	Copper	Spisni et al., 2009
	Trophoblasts/endometrial cells	Coculture	Tien et al., 2012
	Embryonic kidney cells	CsA	Lamoureux et al., 2012
	Platelets	Thrombin	Coppinger et al., 2004
	Progenitor cells	Coculture with platelets	Seizer et al., 2010
	Adipocytes	Adipogenic cocktail	Wang et al., 2004
CypB	Epithelial cervix carcinoma cells	CsA	Price et al., 1994
	Renal epithelial cells	CsA	Wilmes et al., 2013
	Embryonic kidney cells	CsA	Lamoureux et al., 2012
	Epidermal keratinocytes	CsA	Fearon et al., 2011
	Chondrocytes	Spontaneous; csa	De Ceuninck et al., 2003
	Pancreatic cancer cells	Spontaneous	Mauri et al., 2005
	Vascular smooth muscle cells	Oxidative stress	Liao et al., 2000
	Pituitary cells	Estradiol, insulin, EGF	Lee et al., 2000
CypC	Leptomeningeal cells	Spontaneous	Ohe et al., 1996
	Adipocytes	Adipogenic cocktail	Wang et al., 2004

HNSCC, head and neck squamous cell carcinomas; SHB, HBV small surface protein.

1994; Meunier et al., 2002). Although showing a dramatic increase of eCypB after exposure with CsA, the secretome of HEK-293 cells was found to be altered for many proteins. It remained undetermined whether these non-cyclophilin proteins might belong to the CsA binding molecules (Lamoureux et al., 2012).

Unlike CypB, the amino acid sequence of CypA does not indicate a secretion signal and this protein is thus preferentially found in the cytoplasm and the nucleus. Surprisingly, CypA is substantially released from renal cells upon CsA application (Lamoureux et al., 2012). In the absence of CsA, CypA release was frequently found in response to different types of stress, such as oxidative stress (ROS), irradiation or systemic stress by lipopolysaccharide (LPS) application. For a large variety of cells under stress conditions, such as macrophages, neuronal, cardiac and epithelial cells, CypA secretion was reported as a characteristic finding (Sherry et al., 1992; Kim et al., 2004; Seko et al., 2004; Spisni et al., 2009) (Table 1). Secretory proteins not transported through the classical ER-Golgi secretory pathway are secreted through unconventional mechanisms in the absence of an ER signal sequence (Nickel and Rabouille, 2009). A vesicular transport mechanism for CypA secretion was revealed in vascular smooth muscle cells (VSMC) by its colocalization with vesicle-associated membrane protein at the plasma membrane (Suzuki et al., 2006). This pathway involves RhoA, Cdc42 and Rho kinase signaling events, eliciting myosin II activation and actin remodeling. ROS-induced CypA secretion in VSMC is inhibited by statins.

Posttranslational modifications might affect protein secretion (van Vliet et al., 2003). Recently, a complex pattern of posttranslational modifications of CypA including lysine acetylation has been demonstrated for eCypA secreted by irradiated breast cancer cells (Chevalier et al., 2012). In VSMC, acetylation of K^{82} and K^{125} were shown to be required for Ang II-mediated CypA secretion (Soe et al., 2013). Interestingly, eCypA citrullinated on Arg^{37} was detected in the inflamed joints of rheumatoid arthritis (RA) patients by proteomic analysis of albumin-depleted synovial fluid samples (van Beers et al., 2013).

In several cases, external stimuli are not necessary for cyclophilin secretion (Table 1). Constitutive secretion of CypA was found in fibroblast-like synoviocytes isolated from mice with collagen-induced arthritis, in head and neck/oral squamous cell carcinoma cells and in kidney

intercalated cells (Peng et al., 2009; Ralhan et al., 2011; Nishioku et al., 2012). CypB is spontaneously secreted by chondrocytes and pancreatic cancer cells (De Ceuninck et al., 2003; Mauri et al., 2005).

Additionally, necrotic cell death was shown to result in the release of CypA into the extracellular space (Christofferson and Yuan, 2010; Dear et al., 2011).

Only a few data exist for CypC secreted by rat leptomeningeal cells (Ohe et al., 1996) and murine fibroblasts during adipocyte differentiation (Wang et al., 2004).

Signaling by extracellular cyclophilins

A large amount of evidence has accumulated that CD147 forms the principal cell surface receptor for transmission of eCyp signals into target cells.

CD147 (Basigin; extracellular matrix metalloproteinase inducer, EMMPRIN) is a ubiquitously expressed type I transmembrane glycoprotein found to be involved in reproduction, neural function, inflammation, tumor invasion, and HIV infection (Muramatsu and Miyauchi, 2003). Originally, Pro^{180} and Gly^{181} in the extracellular domain of CD147 were found to be important for eCyp signaling (Yurchenko et al., 2002). The CD147 variant, where both the Pro^{180} and the Gly^{181} residue are replaced by alanine, was shown to be unable to transduce eCypA signals. A 15mer peptide containing amino acids 173-187 of CD147, when measured in the presence of CypB reveals an increased *cis/trans* isomerization rate of the Asp-Pro bond centered in the peptide (Hanoulle et al., 2007).

However, NMR analysis of the receptor extracellular domain $CD147^{94–214}$ demonstrated that CypA exclusively targets and catalyzes the CD147 Trp^{210}-Pro^{211} bond (Schlegel et al., 2009). This prolyl bond is the origin of conformational heterogeneity of the folded receptor protein in solution, in a *cis/trans* isomer ratio of 33:67. In the uncatalyzed case, the isomers interconvert slowly on the NMR time scale. NMR titration experiments showed that CypA binds to CD147 via its PPIase site, chemical shift changes localized in the PPIase active site of ^{15}N-CypA were obtained upon titration with the $CD147^{22–214}$ fragment, which contains the Trp^{210}-Pro^{211} bond. No interaction was detected using either $CD147^{94–205}$ or $CD147^{22–205}$ shown to exhibit correctly folded Ig-like domains (Schlegel et al., 2009). Contradictory results were reported in another study showing a direct interaction of CypA and $CD147^{22–205}$ (Song et al., 2011). As CypA is an enzyme that is targeted to prolyl bonds in peptide chains, an even minor portion of the CD147 fragment in a partially folded state might interact with CypA, thus reflecting the proline-directed peptide binding ability of CypA.

The affinity between CypA and CD147 is relatively weak as implied by the impossibility to complete NMR titration experiments to the point of near-saturation. Consistently, the interaction of CypA with the 10mer model peptide HLAALW**P**FLG reflecting the CD147 residues 206–214 exhibits a K_d of 4.2 mM (Schlegel et al., 2009). Low affinity substrate binding is a typical feature of highly evolved enzymes acting under reversible reaction conditions (Burbaum et al., 1989). The mode of action in signal transduction might be different when comparing classical protein ligand-receptor binding to the interaction of the biocatalytically active eCypA with CD147.

The necessity of the PPIase activity of CypA for signal transduction was shown by the use of CypA variants exhibiting strongly reduced PPIase activity (Yurchenko et al., 2002; Malesevic et al., 2013). CypA $F^{60}A$, $H^{126}A$ and $R^{55}A$ variants fail to initiate signaling events, whereas the almost fully active CypA $F^{113}A$ and $W^{121}A$ variants do not much deviate from the effects of wildtype CypA (Yurchenko et al., 2002; Malesevic et al., 2013). Similarly, the nearly PPIase inactive CypB $R^{62}A$ and $F^{67}A$ variants fail to initiate activation of signal transduction (Carpentier et al., 2002; Pakula et al., 2007). In contrast to these studies Song et al. found the CypA $R^{55}A$ variant capable of inducing signaling (Song et al., 2011). Differences in the purification protocols of recombinant CypA from *Escherichia coli* may result in LPS contaminations in preparations of CypA or CypA variants, which are able to simulate induction of signaling by CypA (Payeli et al., 2008). Thus, taken together the results of different groups using different cyclophilin variants of CypA as well as CypB, a PPIase activity dependent action of eCyps seems feasible.

The role of CD147 in eCyp-induced signaling was demonstrated by signal abolishment using anti-CD147 antibody or a CD147 antagonistic peptide (Pushkarsky et al., 2001; Yurchenko et al., 2001; Zhu et al., 2005). Using CD147 deficient mice and selective eCyp inhibitors, we could show that the functional capacity of eCyps during inflammation in a mouse model of experimentally induced peritonitis and in delayed-type hypersensitivity reaction is entirely based on its interaction with CD147 (Malesevic et al., 2013). Our findings suggest that exclusively targeting the fraction of eCyps by selective eCyp inhibitors might be considered as an effective therapy for reducing cell invasion to the site of inflammation in response to inflammatory stimuli and the subsequent release of leucocyte regulatory and proinflammatory molecules.

In addition, the functional CD147-eCyp interaction can be promoted by binding of eCypA and eCypB to the complex polysaccharide chains exposed on the cell surface adjacent to the relatively low dense CD147 molecules. In fact, CypB was described to bind to heparan sulfate moieties of cell surface proteoglycans with the binding site in its N-terminal extension. Mutations in the ^{3}KKK^{5} and ^{15}YFD^{17} motifs of CypB abolished heparan sulfate binding (Denys et al., 1998; Carpentier et al., 1999). NOE NMR data confirm that at least the N-terminal KKK motif is involved in direct physical interaction with the sugar moiety. An octasaccharide was found to be the minimal saccharide length necessary for efficient binding of eCypB (Vanpouille et al., 2004). Specifically, the transmembrane heparan sulfate proteoglycan syndecan-1 was found to form a functional coreceptor for eCypB, acting in cooperation with CD147 (Pakula et al., 2007). Because of the heparan sulfate binding, secreted CypB is able to stay bound to the surface of cells surrounded by a matrix containing highly sulfated proteoglycans, as can be found in chondrocytes. Matrix metalloproteinases are able to release eCypB from the cell surface (De Ceuninck et al., 2003). Possibly, the N-terminal extension of CypB is generally involved in anchoring the enzyme next to its substrate: thus, interactions of CypB with the P-domain of calnexin/calreticulin and the lysyl hydroxylase 1 are abolished by the G6R mutation of the N-terminal extension of CypB in the American Quarter Horse, leading to hereditary equine regional dermal asthenia (Ishikawa et al., 2012).

Despite lacking the N-terminal extension typical to CypB, eCypA was also shown to bind to cell surface heparans. Heparinase treatment, which removes heparan sulfate moieties, prevents eCypA binding to HeLa cells. An *in vitro* ELISA-based binding assay revealed that CypA interacts with heparin. Binding is mediated by the four basic residues in the C-terminal region of CypA Arg^{148}, Lys^{151}, Lys^{154}, and Lys^{155}, complete substitution of these residues for alanine abolishes heparin binding as well as cell surface heparan binding (Saphire et al., 1999).

Cell surface heparan sulfate proteoglycan binding of eCyps might assist an efficient functional interaction with CD147 because heparinase treatment of human neutrophils (Yurchenko et al., 2002) or resting T lymphocytes (Allain et al., 2002) impedes eCyp-induced signaling to a large extent. Interestingly, eCyp-induced signaling of activated T cells does not require the assisting interaction with heparan sulfate.

eCypA and eCypB both induces extracellular signal-regulated kinase (ERK 1/2), IκB-α phosphorylation, NF-κB activation and increased Ca^{2+} mobilization in different cell types (Yurchenko et al., 2001, 2002; Seko et al., 2004; Pakula et al., 2007; Yang et al., 2007; Yuan et al., 2010; Trachtenberg et al., 2011; Bahmed et al., 2012; Kim et al., 2012). In addition, eCypA stimulates p38 MAPK and JNK activation in endothelial cells (Jin et al., 2004), whereas eCypB is unable to activate p38 MAPK and JNK in macrophages (Marcant et al., 2012). Stress-activated protein kinases (SAPKs), Akt and the JAK/STAT (Janus kinases/signal transducers and activators of transcription) pathway are activated by CypA application in cardiac myocytes or VSMC, respectively (Jin et al., 2000; Seko et al., 2004).

eCypA itself is considered a proinflammatory cytokine. It promotes activation of matrix metalloproteinases (MMPs), especially MMP-1 and MMP-9 and induces the expression of MMP-2 and MMP-9. The production of proinflammatory cytokines IL-6, IL-8, IL-1β, MCP-1 and TNF-α as well as adhesion molecules including E-selectin and vascular cell adhesion molecule in monocytes and endothelial cells, respectively, is stimulated when eCypA is externally administered (Jin et al., 2004; Kim et al., 2005; Yuan et al., 2010). Interestingly, only external application of truncated versions of CypA found in brains following scrapie infection is able to stimulate cytokine release from murine brain microglia and astroglia (Tribouillard-Tanvier et al., 2012).

In VSMC, lysine-acetylated eCypA was proved to be more effective than its nonacetylated congener in the induction of ERK1/2 phosphorylation, MMP-2 activation, and ROS production (Soe et al., 2013).

In contrast, eCypB, which also acts in a cytokine-like manner on its own, was shown to be unable to induce the production of proinflammatory cytokines (Marcant et al., 2012). Pretreatment of macrophages with eCypB rather attenuates the expression of inflammatory mediators induced by LPS stimulation. eCypB induces the expression of B cell CLL/lymphoma 3 (BCL-3), which results in inhibition of initiation of TNF-α transcription (Marcant et al., 2012).

Thus, eCyps lead to a wide range of cellular responses in various cell types (Figure 3). Stimulation of the different pathways by eCypA or eCypB culminates in chemotaxis of inflammatory cells. Concomitantly, eCypA stimulates the adhesion of human monocytes to endothelium under arterial shear conditions (Seizer et al., 2011). Similarly, THP-1 cells used as a model mimicking the function and regulation of monocytes and macrophages reveal increased cell adhesion to the extracellular matrix in the presence of eCypA (Yang et al., 2008). eCypB enhance integrin-mediated adhesion of T cells mainly of the CD4+/CD45RO+ phenotype to the extracellular matrix (Pakula et al., 2007). *In vitro*, peripheral blood T lymphocytes can be activated for adhesion to immobilized fibronectin, a component of the

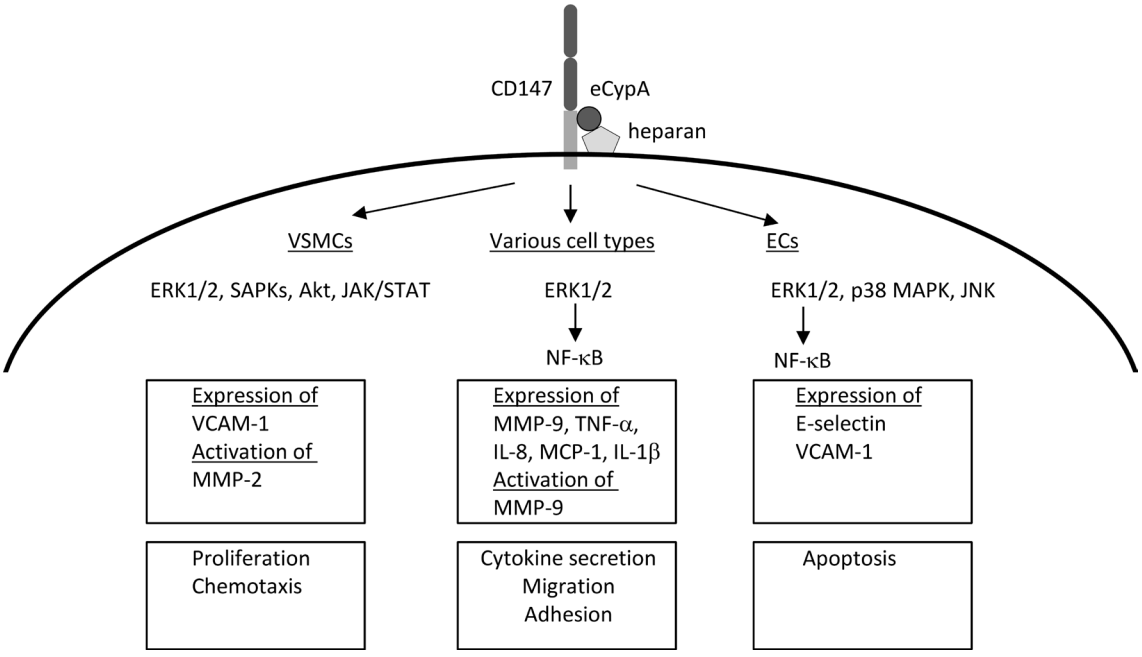

Figure 3 Extracellular cyclophilin (eCyp) binding to CD147 induces different cellular signaling pathways including ERK 1/2 and NF-κB activation and the JAK/STAT pathway.
Various cellular responses like proliferation, migration, adhesion or apoptosis are mediated, dependeing on the cellular context.

extracellular matrix, using eCypB (Carpentier et al., 2002). Platelet adhesion to collagen can be augmented by eCypB (Allain et al., 1999).

eCypA-induced signaling mediates the protection of neurons from oxidative stress and *in vitro* ischemia (Boulos et al., 2007; Ge et al., 2009), the increase of cell proliferation (Yang, 2005) and prevention of apoptosis of VSMC (Jin et al., 2000). Application of eCypB to the growth medium enhances the prolactin-driven cellular proliferation (Rycyzyn and Clevenger, 2000).

Taken together, the eCyp/CD147 interaction initiates a variety of signaling cascades involved in inflammatory processes. In this respect, CypA and CypB appear to share some similar functional properties. However, only direct comparison of the two isoforms would allow evaluation of the effect of cellular context on the differences of their functional properties.

Although the eCyps are found to exhibit cytokine-like activity, some of their properties differ from the classical picture of cytokines. Generally, many cytokines are expressed and released in an inducible manner in response to cell stimulation (Arai et al., 1990). In contrast, the constitutive level of both cyclophilins is rather high, with the CypA concentration around 10-fold higher than that of CypB (Koletsky et al., 1986; Allain et al., 1995). It remains unclear as to what extent an increased expression of cyclophilins is associated with its enhanced secretion. In addition, the affinity to the eCyp cellular receptor CD147 is low (Yurchenko et al., 2006; Schlegel et al., 2009), whereas the cytokine interactions with their binding receptors are found to be strong (Schreiber and Walter, 2010). Importantly, because CD147 forms a weakly binding substrate of eCyps, implicating an unconventional mode of ligand/receptor interaction, pharmacological interference by small molecule inhibitors with receptor mediated signaling appears feasible.

Extracellular cyclophilins under pathophysiological conditions

Increase of eCyp levels in inflammatory tissues and body fluids are well documented and can be considered as an inflammatory response to injury. Thus, eCyps are assumed to play an important role for key processes in acute and chronic inflammation.

Rheumatoid arthritis

RA is an autoimmune inflammatory disease that primarily affects the joints and leads to their progressive destruction. Several studies suggest that the eCypA/CD147 pathway is implicated in RA pathogenesis.

Initially, increased eCypA levels were found in the synovial fluid of RA patients but not in patients with osteoarthritis (Billich et al., 1997). Macrophages of the synovial lining layer were identified as origin of eCypA in the RA synovium (Kim et al., 2005). Fibroblast-like synoviocytes isolated from mice with collagen-induced arthritis (CIA mice) were found to secrete CypA upon LPS stimulation (Nishioku et al., 2012).

CD147 expression is increased on monocytes of peripheral blood and synovial fluid, and on neutrophils in RA (Zhu et al., 2005; Wang et al., 2011). CD147 is also highly expressed in the joints of CIA mice (Nishioku et al., 2012). Anti-CD147 monoclonal antibody application results in strong reduction of arthritis in CIA mice (Damsker et al., 2009). *In vitro*, treatment with anti-CD147 monoclonal antibody results in the loss of the ability of neutrophils, monocytes and activated CD4(+) T cells to migrate in response to eCypA (Damsker et al., 2009). Thus, the eCypA/CD147 interactions might promote the recruitment of leucocytes into joint tissues (Damsker et al., 2009; Wang et al., 2011).

eCypA mediates the upregulation of MMP-9 and MMP-2 expression and secretion in peripheral blood monocytes and macrophages from RA patients and THP-1 cells and the increase of the invasive potential of the cells (Yang et al., 2008; Wang et al., 2010). The blockade of eCypA by an anti-CypA antibody significantly decreases arthritis severity and joint damage in the murine CIA model. In the SCID-HuRAg model of RA treatment with the anti-CypA antibody reduces cartilage erosion, inflammatory cell numbers and MMP-9 production (Wang et al., 2013).

Taken together, data suggest eCypA/CD147-induced MMP secretion and cell invasion may contribute to the cartilage invasion and bone destruction of RA.

Sepsis

Sepsis as systemic inflammatory response to infection was found to be associated with elevated levels of eCyps. The eCypA-related PPIase activity was reported to be significantly increased in serum of patients with severe sepsis compared to healthy subjects and this increase was found to be associated with high mortality rates (Tegeder et al., 1997). A cecal ligation and puncture mouse model of polymicrobial sepsis has a higher abundance of CypA in the liver. A role of eCypA in sepsis is implicated by the importance of its receptor CD147 in sepsis-induced renal injury. Administration of anti-CD147 blocking monoclonal antibodies results in reduction of TNF-α, IL-6, and IL-10 cytokines and attenuation of sepsis-induced renal dysfunction and pancreatic injury (Dear et al., 2007).

Lipoteichoic acid from *Staphylococcus aureus*, described as contributing to the pathogenesis of *S. aureus* sepsis, significantly upregulates CypA expression in THP-1 cells (Zeng et al., 2010). In contrast, in an animal model of burn sepsis, CypA was found to be downregulated compared to sham-treated animals (Zhang et al., 2010).

Asthma

In a mouse model of acute asthmatic inflammation it was shown that eCypA levels are elevated in the mice airways (Gwinn et al., 2006). *In vitro*, eCypA induces CD147-dependent chemotaxis of activated CD4+ T cells, a cell type known to play a prominent role in asthmatic inflammation. Treatment of the mice with allergic airway inflammation using either anti-CD147 monoclonal antibodies (Gwinn et al., 2006) or the non-immunosuppressive CsA analogue NIM811 (Arora et al., 2005; Balsley et al., 2010) significantly reduces the production of cytokines and the influx of inflammatory cells into lung tissues during inflammation.

Both treatments were shown to reduce airway epithelial mucin production and bronchial hyperresponsiveness, two central features of asthma (Gwinn et al., 2006; Balsley et al., 2010). Even in the chronic phase of asthma, elevated concentrations of eCypA have been detected, whereas classical chemokines typical of the acute phase asthma response are not found. Here, inhibition of the PPIase activity of cyclophilins by the non-immunosuppressive CsA analogue NIM811 reduced the number of persisting leukocytes, a hallmark feature of chronic human asthma. This treatment also reduced asthma reactivation after allergen challenge (Stemmy et al., 2011a). An increased level of eCypB compared to classical chemokines was found in the nasal wash samples of asthma patients in the chronic phase of the disease relative to nonasthmatic controls. This suggests an eCypB contribution to the persistent recruitment of proinflammatory leukocytes during chronic asthma (Stemmy et al., 2011a). Interestingly, eCypA was not found to be increased in the nasal wash samples, however, its level correlated with parameters of asthma severity (Stemmy et al., 2011b).

Cardiovascular diseases

Atherosclerosis

Atherosclerosis is an inflammatory disease characterized by formation of atherosclerotic plaques containing VSMC,

endothelial cells, leukocytes, and foam cells. Oxidative stress is considered to be a causative agent in vascular inflammation. ROS elicit signaling pathways to mediate the initiation of endothelial cell activation, fatty streak development and lesion progression to plaque rupture as key components of the pathogenesis of atherosclerosis.

CypA is found to be highly expressed in atherosclerotic plaques in apolipoprotein E deficient mice as a model characterized by impaired clearing of plasma lipoproteins and development of atherosclerosis in a short time (Jin et al., 2004). Combined deficiencies in *Apoe*$^{-/-}$ *Ppia*$^{-/-}$ mice result in less severe atherosclerosis (Nigro et al., 2011). Furthermore, CypA RNAi treatment of apolipoprotein E deficient mice induces regression of existing atherosclerotic plaques and reduces lipid accumulations (Yan et al., 2013). Cyclophilins are found to be secreted from several cell types involved in atherosclerosis. Both, eCypA and eCypB were identified to be oxidative stress-induced factors secreted from VSMC (Jin et al., 2000; Liao et al., 2000). Stimulation of human umbilical vein endothelial cells with lipopolysaccharide induces secretion of CypA (Kim et al., 2004). eCypA is also released during the differentiation of CD34(+) progenitor cells to foam cells, the hallmark of atherosclerosis (Seizer et al., 2010). eCypA is described as follows as involved in several molecular mechanisms of the pathogenesis of atherosclerosis. (i) eCypA stimulates VSMC proliferation and intimal thickening (Jin et al., 2000; Satoh et al., 2008). (ii) eCypA increases endothelial cell activation and induces expression of adhesion molecules including E-selectin and vascular cell adhesion molecule-1, which facilitates monocyte adhesion (Jin et al., 2004). (iii) Like the proapoptotic TNF-α, eCypA induces apoptosis of endothelial cells with blocked protein synthesis (Jin et al., 2004). Conversely, application of eCypA to human umbilical vein endothelial cells upregulates the expression of the antiapoptotic protein BCL-2 (Wei et al., 2013). (iv) eCypA induces the recruitment of monocytes and the expression of MMP-9, IL-6 and TNF-α (Payeli et al., 2008; Yuan et al., 2010).

Abdominal aortic aneurysm (AAA)

AAA is the localized dilatation of the abdominal aorta. Initially, CsA was shown to reduce formation of AAAs in rats subjected to elastase infusion (Yamaguchi et al., 2000) but the mechanism of action has remained unclear.

Subsequently, CypA was described to be an essential mediator of AAA formation by analyzing the impact of CypA deficiency in a murine AAA model. Formation of AAAs promoted by infusion of angiotensin II (Ang II) in apolipoprotein E deficient mice is prevented by an additional CypA deficiency of the mice. Mice lacking CypA exhibit reduced aortic inflammation, oxidative stress, matrix degradation, recruitment of CD45+ inflammatory cells and aortic rupture. Involvement of CypA localized in the extracellular space in AAA formation is suggested by the Ang II-stimulated CypA secretion in mouse aortic VSMC. eCypA was also shown to promote activation of MMPs in VSCMs. Bone marrow transplantation experiments implicate that VSMC secret the fraction of eCypA involved in AAA formation (Satoh et al., 2009).

Cardiac diseases

Heart diseases like myocarditis, coronary artery disease, myocardial ischemia and hypertrophy are connected to inflammation. Several data suggest the involvement of eCypA in these diseases. Thus, it was found that expression of CypA and its receptor CD147 is upregulated in patients who died of acute myocardial infarction. In *Ppia*$^{-/-}$ mice, the infarct size and monocyte and neutrophil recruitment were reduced. An involvement of eCypA is implicated by the dependence of the CypA effects on CD147 and its chemotactic properties (Seizer et al., 2011). Also, the proinflammatory role of CypA in myocarditis is attributed to eCypA, mainly because of its role in monocyte migration (Seizer et al., 2012). Additionally, eCypA was described to be important for Ang II-mediated cardiac hypertrophy in apolipoprotein E deficient mice. Ang II-treated cardiac fibroblasts of these mice substantially secrete CypA. eCypA is considered to promote the hypertrophic response, because eCypA application upregulates markers of cardiac hypertrophy and increases protein synthesis in cardiac myocytes (Satoh et al., 2011). The eCypA level may form a marker for severity of acute coronary syndrome. The serum eCypA concentration is reported to be increased in patients with unstable angina and acute myocardial infarction compared to patients with stable angina and healthy controls (Yan et al., 2012). Accordingly, plasma eCypA levels are associated with an increased risk of coronary artery disease as judged by traditional cardiovascular risk factors and correlate with the severity of the disease (Satoh et al., 2013).

Periodontitis

Periodontitis, an inflammatory disease characterized by the destruction of periodontal tissues, is associated with the abundance of eCypA (Liu et al., 2013). CypA was found

to be overexpressed in connective tissues of the inflamed gingiva from patients with periodontitis in the infiltrating cells as well as in the extracellular matrix. eCypA induces a stronger migration of peripheral blood mononuclear cells/neutrophils from patients with periodontitis than of cells from healthy donors (Liu et al., 2013).

Diabetes

Inflammation participates in the pathogenesis of type 2 diabetes. Increased levels of eCypA were detected in the plasma of patients with type 2 diabetes and even more increased in those with additional coronary artery disease (Ramachandran and Kartha, 2012).

Viral infections

CypA and CypB have been found to interact with the HIV-1 Gag polyprotein (Luban et al., 1993). *In vivo*, CypA was shown to promote HIV-1 replication in the early phase after viral entry in human cells (Franke et al., 1994; Thali et al., 1994; Braaten et al., 1996; Franke and Luban, 1996). Incorporation of CypA into the newly produced virion through interaction with the capsid domain in the Gag polyprotein is required for infectivity (Franke et al., 1994; Thali et al., 1994). Besides these intracellular functions, CypA in the extracellular space was described to contribute to HIV-1 infectivity. During virus budding, CypA incorporated in the virus particle leaves the cytosol. Initially, CypA is incorporated into the interior of the virus particles, but its localization changes to the viral surface during maturation (Saphire et al., 1999). It is likely that HIV-1-associated CypA mediates virus entry via interaction with CD147 and heparans on target cells, thus significantly enhancing infection by HIV-1 (Sherry et al., 1998). Heparans are discussed to facilitate the CypA-CD147 interaction by initial binding of CypA followed by its presentation to CD147 (Saphire et al., 1999, 2002; Pushkarsky et al., 2001). CypA is also encapsidated within the influenca A virus and the vaccinia virus particle (Castro et al., 2003; Liu et al., 2009) suggesting a role of CypA analogous to that in HIV-1 in these cases. Interestingly, eCypB localized at the cell surface facilitates the infectivity of the oncogenic human papillomavirus types 16 and 18 by triggering the infectious internalization of viral particles (Bienkowska-Haba et al., 2009). Measles virus infection has been found to occur via CD147 and virion-associated eCypB (Watanabe et al., 2010).

Interestingly, the expression of the small surface protein of hepatitis B virus in hepatoma cells elicits CypA secretion. Consistently, increased levels of eCypA were found in the serum of chronic hepatitis B patients compared to healthy individuals (Tian et al., 2010). However, the anti-HBV effect of CsA caused by the prevention of virus entry into hepatocytes does not involve binding to a cyclophilin (Watashi et al., 2013).

Cyclophilins have been shown to be important factors in the life cycle of a variety of other viruses including HCV, West Nile virus, arterivirus, rotavirus, human cytomegalovirus and vesicular stomatitis virus. However, in these cases a contribution of cyclophilins localized outside cells is not discussed.

Cyclophilins as allergens

Cyclophilins from different species have been described to form a pan allergen family able to elicit IgE-mediated hypersensitivity reactions in humans (Glaser et al., 2006). Plant allergens include serum IgE-reactive cyclophilins from birch pollen (Cadot et al., 2000), carrot (Fujita et al., 2001), raspberry (Marzban et al., 2008), ryegrass (*Lolium perenne*) (De Canio et al., 2009) and the *Platanus orientalis* trees (Pazouki et al., 2009). Cyclophilins from the basidiomycete *Psilocybe cubensis* (Horner et al., 1995), from the pathogenic mould *Aspergillus fumigatus* (Fluckiger et al., 2002) and from the skin-colonizing yeast *Malassezia furfur* (Lindborg et al., 1999) have also been isolated as serum IgE binding proteins.

Inhibition of extracellular cyclophilins

Selective inhibitors of eCyps have been introduced only recently and are based on the CsA warhead (Malesevic et al., 2010, 2013; Prell et al., 2013). The canonical cyclophilin inhibitor CsA inhibits the PPIase activity of CypA and CypB in the one-digit nanomolar concentration range. However, CsA is a cell permeable compound exhibiting the potential to target both intra- and extracellular cyclophilins. Furthermore, the CypA/CsA complex is able to prevent the dephosphorylation of the serine/threonine-specific protein phosphatase 2B (calcineurin) substrates by gain-of-function, thus mediating the immunosuppressive effects of CsA. Therefore, the physiological reactions observed after the administration of CsA are caused by the compulsory combination of both PPIase inhibition of

intra- and extracellular cyclophilins and gain-of-function. When restricted to eCyp binding, CsA derivatives are no longer able to allosterically inhibit intracellular calcineurin, and are thus *per se* non-immunosuppressive.

Nevertheless, CsA treatment influences a variety of inflammatory processes ascribed to the function of extracellular cyclophilins. Thus, CsA in combination with methotrexate has shown a substantial benefit in clinical practice to control aggressive rheumatoid arthritis (Gremese and Ferraccioli, 2004). CsA has also been shown to block the allergen-induced late asthmatic reaction, which is associated with mucosal inflammation (Sihra et al., 1997). The *in vitro* chemotactic activity of eCypA on monocytes and eCyPA-induced ERK1/2 activation in VSCMs is inhibited by CsA (Sherry et al., 1992; Jin et al., 2000; Payeli et al., 2008).

For inhibition of cyclophilins without targeting calcineurin a variety of non-immunosuppressive CsA derivatives, such as NIM811, Debio-025 and SCY-635 have been developed. These non-immunosuppressive CsA derivatives have been shown to inhibit several cyclophilin-mediated inflammatory processes. NIM811 has been shown to diminish neutrophil infiltration into lung tissues in murine models of acute lung injury as well as of chronic allergic asthma (Arora et al., 2005; Stemmy et al., 2011a). NIM811-treatment of mice with coxsackievirus B3 (CVB3)-induced myocarditis resulted in the reduction of myocardial fibrosis (Seizer et al., 2012). However, administration of these non-immunosuppressive CsA derivatives fails to distinguish between intra- and extracellular signaling pathways because of their cell permeability.

Therapeutic application of cell penetrating CsA derivatives must ultimately lead to the formation of cyclophilin/CsA complexes within cells and a subsequent amplification of cyclophilin secretion. The pathway of secretion enhancement should involve the release of the already assembled drug complexes from the cell. The question arises whether the proinflammatory properties of the CsA-induced fraction of eCyps are comparable to those of eCyps secreted in the absence of CsA. Obviously, the CsA concentration in the extracellular space is pivotal for the fate of the eCyp/CsA complex. Taking into account the generally high stability of the cyclosporin/cyclophilin complexes, even low extracellular concentrations of CsA could keep the complexes dominating and thus the eCyps inhibited in their ability to mediate inflammation. However, lowering the affinity of the complex by post-translational modifications of the cyclophilins like acetylation (Lammers et al., 2010) would make dissociation of the complex after arrival in the extracellular space less unlikely.

Specific targeting of extracellular cyclophilins by cell-impermeable CsA derivatives are hypothesized to elicit fewer side effects than a global cyclophilin inhibitor and would not get dispersed into a cellular sink when sequestered by off-target cyclophilins.

Initially, the cell-impermeable cyclophilin inhibitor MM218 has been synthesized that contains a 6-mer D-glutamic acid moiety and 5(6)-carboxytetramethylrhodamine as a fluorescence probe attached to side chain-modified [D-Ser]^{8}-CsA (Malesevic et al., 2010). MM218 is highly effective at inhibiting leukocyte recruitment, it reduces airway mucus and T_H2 cytokine levels and leads to improved lung function in a murine model of acute allergic asthma (Balsley et al., 2010). A structurally simplified cell-impermeable eCyp inhibitor was shown to be highly effective at inhibiting *in vitro* leukocyte migration towards CypA and in the recruitment of leukocytes during inflammation in a mouse model of experimental induced peritonitis and delayed-type-hypersensitivity reaction (Malesevic et al., 2013).

The strong anti-inflammatory impact of inhibition of extracellular Cyps in various *in vivo* inflammatory model systems suggests that eCyps are potential prime movers of a cytokine cascade. The collective findings suggest that CsA is chemically tractable to allow selective eCyp inhibition leading to effective drug candidates for reducing inflammatory diseases associated with leukocyte recruitment.

Acknowledgments: The work was supported by the Deutsche Forschungsgemeinschaft GRK 1026.

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