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Ubiquitin is a versatile scaffold protein for the generation of molecules with *de novo* binding and advantageous drug-like properties

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ABSTRACT

In the search for effective therapeutic strategies, protein-based biologicals are under intense development. While monoclonal antibodies represent the majority of these drugs, other innovative approaches are exploring the use of scaffold proteins for the creation of binding molecules with tailor-made properties. Ubiquitin is especially suited for this strategy due to several key characteristics. Ubiquitin is a natural serum protein, 100% conserved across the mammalian class and possesses high thermal, structural and proteolytic stability. Because of its small size and lack of posttranslational modifications, it can be easily produced in *Escherichia coli*. In this work we provide evidence that ubiquitin is safe as tested experimentally *in vivo*. In contrast to previously published results, we show that, in our hands, ubiquitin does not act as a functional ligand of the chemokine receptor CXCR4. Cellular assays based on different signaling pathways of the receptor were conducted with the natural agonist SDF-1 as a benchmark. In none of the assays could a response to ubiquitin treatment be elicited. Furthermore, intravenous application to mice at high concentrations did not induce any detectable effect on cytokine levels or hematological parameters.

1. Introduction

1.1. Scaffolds

Targeted intervention at protein structures on cells or in serum is the principle of therapeutic approaches using specifically developed or designed binders like monoclonal antibodies, soluble receptor molecules and the new class of scaffold proteins. The latter are based on natural or artificial protein molecules which are engineered by randomizing surface exposed amino acid positions to generate new binding properties to selected targets. In this field several concepts are pursued. They range from small protein domains such as the protein A domain (Affibody, [1]), PDZ domains [2] and ankyrin repeat proteins (Darpins, [3]), through small full-length proteins, such as the commonly used thioredoxin scaffold [4,5] to higher-molecular-weight beta-barrels and Ig-like structures such as lipocalins (Anticalins, [6]), green fluorescent protein (GFP, [7]) and the T-cell receptor complex [8]. One particularly well-suited scaffold for therapeutic and other applications is ubiquitin, a protein naturally occurring intracellular as well as in serum [9].

1.2. Ubiquitin

Ubiquitin possesses unique features with respect to protein characteristics, production and safety aspects. It has favorable biochemical properties like a stable structure over a wide pH range, thermal shifts or proteolytic degradation [10–12]. As a human serum protein ubiquitin exhibits low immunogenic potential when applied in humans. Its sequence is fully conserved in mammals, enabling fast development tracks due to dispensability of species-specific surrogate molecules during preclinical development. Ubiquitin can be

Abbreviations: CXCR4, CXC motif chemokine receptor 4; SDF-1, stromal cell-derived factor 1; PBS, phosphate buffered saline; ^{125}I-SIB, N-succinimidyl 3-(^{125}iodo)-benzoate; ID, injected dose; MCV, mean cell volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration

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easily produced as soluble protein in high yields in the cytoplasm of *Escherichia coli* [12]. Dimeric head-to-tail fusions of two derivatized ubiquitin molecules are the basis for so called Affilin® compounds currently under development [9].

Ubiquitin is present within the cell at concentrations in the low μM range as free monomer as well as in conjugated form [13]. The different states are mainly balanced by conjugation and deubiquitinylation [14]. Intracellular ubiquitin has a half-life of several hours. Its turnover is counter steered in the cell mainly by modulation of intracellular ubiquitin pools and *de novo* synthesis [15,16]. Cellular ubiquitin is involved in several functions in the cytoplasm and the nucleus respectively [17]. In the cytoplasm it is an important player in processes like autophagy, proteasome mediated proteolysis, endocytosis of activated transmembrane proteins, cargo sorting [18], innate immunity [19] and endoplasmic reticulum associated degradation (ERAD, [20]). In the nucleus ubiquitin is a key component for mRNA-transport, transcriptional control, DNA damage tolerance and DNA repair [21]. The interactions by which ubiquitin assists in the above mentioned processes are manifold and include covalent as well as non-covalent binding of ubiquitin to numerous cellular proteins. Non-covalent interactions between ubiquitin and target proteins are of low affinity with K_D values mostly in the high μM range [22,23]. With respect to these interactions, several partially overlapping epitopes on the surface of ubiquitin have been identified [24]. Covalent conjugation of ubiquitin to several proteins and to other ubiquitin entities is of importance in proteolytic and non-proteolytic processes of the cell. The initial step in this event is the activation of ubiquitin and its transfer to the indicated protein. In this process which is driven by a complex enzyme machinery (E1, E2 and E3 enzymes) the two C-terminal glycine residues at positions 75 and 76 of ubiquitin play a key role [25]. Modification of ubiquitin at these positions via amino acid exchange completely abolishes the transfer of ubiquitin to proteins and the formation of polyubiquitin chains [26]. In addition to conjugation to other proteins ubiquitin molecules are activated and covalently attached to distinct lysine residues of other ubiquitin entities. That way, polyubiquitinylated proteins are formed. Currently, at least eight different types of ubiquitin chains are known, with each individual linkage affecting distinct cellular processes [27–31].

Ubiquitin is detected in serum of healthy humans in a concentration of less than 100 ng/ml (<10 nM) [32]. An obvious source might be the passive release from cells undergoing physiological turnover [33], but there are also reports about ubiquitin release from intact cells [34–36]. On the other hand uptake of ubiquitin into cells of the hematopoietic system has been shown [35,37,38]. The exact function of extracellular ubiquitin is still under debate. A role in modulation of immune responses [39,40] and conditions of inflammation is discussed [41,42], for a comprehensive review see Majetschak [43]. Experimental findings suggest an influence on the ratios of the cellular components of the blood [35] and recently on myocardial remodeling [44–46]. In this context CXC motif chemokine receptor 4 (CXCR4) has been suggested as a putative receptor for ubiquitin with an affinity in the medium nM range [47–50]. Signaling and function are described as being similar but not equal to the hitherto known and well characterized CXCR4 ligand stromal cell-derived factor 1 (SDF-1) [51–53].

1.3. CXCR4

CXCR4 is a typical class A G-protein coupled receptor broadly expressed in the organism. It can be found especially in hematopoietic cells but also many other cell types for example of the central nervous system and the gastrointestinal tract. The biological functions of CXCR4 are crucial during development and hematopoiesis [54–56]. It plays pleiotropic yet not fully understood roles in the immune system and during tissue repair processes [57–60]. Particular interest for CXCR4 as a drug target is based on its role in HIV infection and metastatic diseases [61–66]. The receptor is bound by its natural ligand SDF-1 (or chemokine (C–X–C motif) ligand 12) and a non-cognate ligand MIF, migration inhibitory factor [67]. Receptor activation by SDF-1 leads to anti-inflammatory and organ protective effects in various disease models [68–71].

Because of these similarities in the overall effects to those of ubiquitin [72–75], CXCR4 was discussed as a possible receptor for ubiquitin in the literature [43,76,77]. In this paper we challenge this hypothesis and present significant results elucidating the signaling competence of ubiquitin via different branches of the complex CXCR4 downstream interaction network compared to the natural ligand SDF-1. Neither in cells naturally expressing CXCR4 nor in cells transfected with the CXCR4 gene ubiquitin dependent CXCR4 activity could be demonstrated. Furthermore comprehensive data concerning the *in vivo* application of ubiquitin are discussed.

2. Material and methods

2.1. Production and purification of ubiquitin proteins

Human ubiquitin for biodistribution and toxicity studies was produced with an F45W substitution [12] in *E. coli*. The cDNA of ubiquitin was subcloned into pSCIL008 expression vector and expressed in *E. coli* JM83 cells (DSMZ). After cell harvest and disruption via ultrasonication, cell lysate was heat denatured for 5 min at 75 °C in a water bath. Precipitated protein was removed and the supernatant was diluted in 50 mM sodium acetate pH 5.0 and loaded onto a SP-Sepharose FF column. Elution was performed by a sodium chloride gradient in 50 mM sodium acetate pH 5.0. Fractions of interest were pooled and purified via a Q Sepharose FF. The flow through was applied onto a SP Sepharose HP column and protein of interest was eluted by sodium chloride gradient in 50 mM sodium acetate pH 5.0.

A pharmacokinetic study of ubiquitin was realized using commercially available protein from R&D Systems (U-100H).

The genetic construct of di-ubiquitin was obtained via head to tail fusion of the DNA fragments of two F45W ubiquitin monomers. Di-ubiquitin was expressed from the expression vector pET20b (Novagen) in *E. coli* Nova Blue (DE3) cells. After cell disruption, solid $(NH_4)_2SO_4$ was added to 40% saturation. Precipitated protein was removed and the supernatant was applied to a Phenyl Sepharose HP column. Elution was performed by $(NH_4)_2SO_4$ gradient in 50 mM Tris/HCl, 1 mM EDTA, pH 7.5. Fractions containing the target protein were pooled and applied to a SP Sepharose HP column after cross-flow filtration in 50 mM acetic acid/NaOH, 1 mM EDTA, pH 5.5. Fractions of di-ubiquitin eluted in a sodium chloride gradient were concentrated and applied to a Superdex 75 prep grade column equilibrated in phosphate-buffered saline (PBS) for removal of monomeric ubiquitin fragments. Fractions containing the homogeneous target protein were concentrated, applied to a Q Sepharose FF anion exchange column and collected in the flow through.

Purified preparations of ubiquitin and di-ubiquitin were filtered through sterile 0.2 μm polyethersulfone disc filters (Millipore) and stored at −80 °C.

2.2. Protein analytics

Analytical size exclusion chromatography (SE-HPLC) was carried out on a Superdex 75 Tricorn 10/300 column (GE Healthcare) coupled to an Ultimate 3000 SD chromatographic system (Dionex GmbH, Idstein, Germany). PBS containing 0.05% sodium azide as