Development of a DUB-selective fluorogenic substrate†

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Ubiquitination is a post-translational modification that is involved in a plethora of cellular processes. Target proteins can be specifically modified with a single ubiquitin (Ub) molecule or with complex chains. In recent years, research has focused on deubiquitinating enzymes (DUBs) as potential therapeutic candidates in various diseases. USP16 is an emerging target due to its involvement in mitosis and stem cell self-renewal. Generally, activity-based probes (ABPs) used to study DUBs are based on the ubiquitin scaffold, thus lacking target selectivity. To overcome this issue, we designed a Ub-based activity probe bearing specific mutations to achieve selectivity for USP16, by combining structural modelling and analysis and mutational calculation predictions. We develop a fluorogenic substrate, the first of its kind, that is processed exclusively by USP16, which allows us to monitor USP16 activity in complex samples.

Introduction

Ubiquitin (Ub) is a small protein that plays an essential role in cellular processes and is highly conserved in eukaryotic cells.1 This protein is covalently attached via an isopeptide bond to an amino group (lysine residue or at the N-terminal site) of a target protein through an enzymatic cascade involving three enzymes. Different combinations of these enzymes can result in various mono- or polymeric ubiquitin adducts.2 Deubiquitinas (DUBs) catalyse the release of ubiquitin from the substrate and degradation of polyubiquitin chains.3 This class of enzymes regulates many biological processes that are under Ub control.4 DUBs are considered important drug targets due to their involvement in various diseases.5 Therefore, more insight is needed into their biological function. Different probes are currently available to study DUB-activity, but they are generally based on the ubiquitin wild-type sequence, making it difficult to achieve selectivity. The most common tools used by the scientific community are activity-based probes (ABP) and fluorogenic substrates.4–8 An ABP reacts in a covalent fashion with DUBs and is generally used to detect or isolate active enzymes. Fluorogenic substrates for DUBs are usually ubiquitin-based reagents, modified with reporter groups at the C-terminus of ubiquitin.

Ubiquitin-specific proteases (USPs) are the largest class within the DUB family, with 56 proteins out of approximately 100 known human DUBs. USP16, a member of the USP family, could be considered a multifaceted therapeutic target and the elucidation of the full extent of its biological role is therefore of great importance. This DUB deubiquitinitates histone H2A and is essential in cell cycle progression.13 In addition, USP16 was shown to be a key regulator of hematopoietic stem cell differentiation.18 It has previously been reported that downregulation of USP16 has contributed to hepatocellular carcinoma pathogenesis.17 Furthermore, during DNA damage, there is an increase of ubiquitinated DNA-damage foci, that is negatively regulated by USP16.18 USP16 is one of the genes located on...
chromosome 21, of which partial or full trisomy results in Down syndrome (DS). USP16 is known to decrease the self-renewal ability of somatic stem cells that contributes to DS pathogenesis. It has been shown that by negatively regulating this enzyme it is possible to rescue proliferation anomalies in human DS fibroblasts and alleviate some of the pathologies associated with Down’s syndrome. For these reasons USP16 is a target of interest for the development of inhibitors and as such it is important to have good tools to better understand its function. In order to shed light on the activity of USP16 and elucidate possible upregulation/downregulation mechanisms essential for its function, we aimed to generate a USP16-selective activity-based probe (ABP) and a matching selective fluorogenic substrate.

We have previously reported an ABP highly selective for USP7, modified at the N-terminus with a fluorescent tag, rhodamine (Rh) and at the C-terminus with a reactive alkyne functionality (PA). Mass spectrometry experiments showed that this probe reacts to a minor extent with two other USPs, USP15 and USP16. We hypothesized that this covalent reagent can be fine-tuned to only react with USP16 by modulating the primary structure of ubiquitin. In order to disturb specific interactions between the Ub structure and USP7 and USP15, we incorporated mutations in the sequence of this mutant, (M6).

To discover the mutations that are necessary to obtain the desired selectivity, we took advantage of data from structural analysis of these DUBs in complex with ubiquitin and FoldX-based mutational predictions. Various point mutation combinations were applied during the design and screening process to find selective ABPs for USP16. From almost 40 new synthetic Ub mutants that we synthesized, the best mutant proved to be mutant M20 (Rh-M20-PA), showing high reactivity and selectivity for the target USP16. In addition, we synthesized an AMC fluorogenic substrate based on the sequence of this ubiquitin mutant. Here, we report a USP16-selective ABP and a matching fluorogenic substrate which is selectively recognized and processed only by USP16.

Results and discussion

Rationale behind library design
To study and evaluate the interactions between USP16/USP7/USP15 and ubiquitin, crystal structures of these DUBs are desirable. The structures of USP16 and USP15, however, were not available at the time. We therefore used in silico modelling to generate models of these USPs. Swiss-Model is one of several tools used to build three-dimensional protein structures based on structural homology to closely related proteins. After structurally evaluating the models obtained with Swiss-Model, we selected the model generated using the template of USP4 (PDB ID: 2Y6E) as a structural model for USP15. USP4 is the closest related member from the USP family to USP15 (57% sequence identity). For USP16 we chose the model based on the USP2–Ub complex (PDB ID: 2HD5), which has a sequence identity of 34%. Subsequently, we aligned the structures of USP7, USP15, and USP16 using UCSF Chimera (Fig. 1).

The modelled USP-Ub structures were then used to identify the residues that are involved in the interaction between these three DUBs and Ub. From almost 40 new synthetic Ub mutants that we synthesized, the best mutant proved to be mutant M20 (Rh-M20-PA), showing high reactivity and selectivity for the target USP16.
deduce that upon binding to Ub, structural changes were necessary to accommodate ubiquitin close to the catalytic site (Fig. S4b†). To our knowledge, so far USP7 and USP15 are the only DUBs with a misaligned catalytic triad, in a non-active conformation. We hypothesized that we could insert mutations in specific regions of the Ub structure that would either lead to steric hindrance, resulting in disturbance of the interactions between Ub and these two DUBs, or prevent the necessary conformational changes within the USP15 structure. Structural analysis led to the identification of possible favourable positions (e.g. glutamate 16 and lysine 33) in the ubiquitin sequence that might increase the affinity of the reagents towards USP16 (Fig. 1).

We recently successfully used the FoldX software package to perform structure-based computational predictions to identify possible mutations, which would alter the binding affinity between Ub and USP7.4 FoldX-based calculations were performed also on the structures modelled here, whereby we selected possible mutations which would enhance the affinity for USP16 and negatively affect the affinity for either USP7 or USP15 or both (ESI data 1†).

By combining the information acquired from the approaches described above, we selected residues of Ub in positions 12, 15, 16, 33 and 62–65 (Fig. 1). Our rationale for selecting these residues and their mutations is as follows: E16 in the Ub structure does not interact with any amino acids present in the structure of USP16 and by mutating it into a bulky group (e.g. lysine–biotin) we might be able to introduce steric hindrance or interrupt possible conformational changes, which are crucial for USP15 (Fig. 1 and S4†). K33 is part of the interaction site between ubiquitin and both USP15 and USP7. The mutation K33E, as shown in Fig. S5a,† would lead to the loss of the hydrogen bonds formed between this lysine and aspartate (D376) in case of USP7 and possibly an electrostatic repulsion. A similar effect can be hypothesized for USP15, once conformational changes take place upon ubiquitin binding (E804 in USP15, Fig. S5b†). The mutation of E64 for a phenylalanine or histidine residue was based on FoldX calculations. And finally, we also considered randomly mutating the C-terminus of ubiquitin which is generally known to be an important part in the recognition and activity of DUBs. All these data together were applied in the library design.

Developing a USP16-selective ABP reagent
Our main objective was to generate a selective fluorogenic substrate for a single DUB. As a proof of principle, we first synthesized an ABP mutant library and tested this library using a previously developed method.4 Such an ABP reagent, having a rhodamine moiety attached at the N-terminus, enables a quick assay read-out, while the propargyl moiety reacts covalently and traps the targeted DUBs.24 With this convenient screening process, we could determine the preference in labeling specific DUBs for each synthesized mutant probe. This information can subsequently be used in the selection step and applied in case new mutants are needed to be designed with increased affinity for the desired target. Moreover, using this
Labeling experiment with the Rh-Ub-PA and Rh-M20-PA. Wild-type and catalytically inactive versions of Flag-tagged USP5, USP7, USP15 and USP16 were overexpressed in HeLa cells. Flag antibody is used to follow the levels of labeling. β-actin is used as a loading control.

**Fig. 2** Labelling experiment with the Rh-Ub-PA and Rh-M20-PA. Wild-type and catalytically inactive versions of Flag-tagged USP5, USP7, USP15 and USP16 were overexpressed in HeLa cells. Flag antibody is used to follow the levels of labeling. β-actin is used as a loading control.
binding to the catalytic site. It would be very interesting to see if we can maintain the selectivity by designing a synthetic AMC fluorogenic substrate based on the sequence of mutant M20. These reagents were produced following a previously published procedure. Ubiquitin WT or the M20 mutant (residues 1–75) were synthesized using Fmoc-based solid phase peptide synthesis, followed by cleavage from the trityl resin using a mixture of 20% (v/v) hexafluoropropanol in methylene chloride. GlyAMC was coupled at the C-terminus using a standard peptide coupling procedure. Finally, M20-AMC and Ub-AMC were obtained after global deprotection followed by HPLC purification. We tested these reagents using fluorescent activity assays, first with purified recombinant DUBs, followed by HAP1 lysate experiments. Following the assay we can monitor in time the catalytic activity of the DUBs. The fluorescent signal is due to the release of free AMC upon hydrolysis of the substrate. If the substrate is not being processed by the enzymes, no increase in the fluorescent signal will be measured.

Both reagents were tested towards USP5, USP7, USP15, and USP16 (Fig. 5). All these enzymes were highly reactive towards Ub-AMC, shown by the fluorescence increment. On the other hand, USP16 was the only USP to process M20-AMC, proving that the selectivity could be preserved between different types of reagents. Furthermore, similar experiments were performed with HAP1 WT and HAP1 USP16KO lysate (Fig. 6). Ub-AMC was processed at the same rate in both cases, while M20-AMC showed an increase in fluorescent signal, due to the release of...
For these two reagents, we also determined affinity ($K_M$) and catalytic turnover ($k_{cat}$) using the Michaelis–Menten equation (Fig. 7 and S9†). DUBs were incubated with different substrate concentrations. The slope (initial rates) for each of the concentrations was plotted and fitted using the Michaelis–Menten equation. USP16 demonstrated a higher affinity (two-fold) for the wild-type substrate compared to M20. USP7 and USP15 did not process M20-AMC even at high substrate concentration, no $K_M$ could be determined. We hypothesize that the selectivity of the reagent for USP16 can possibly be explained by affinity differences towards other DUBs. In contrast, we observed a four-fold increase in catalytic turnover for M20 compared to the wild-type AMC reagent. The difference in $k_{cat}$ might be due to a slightly altered active site placement of the C-terminal Ub tail making the hydrolysis mechanistically more favourable.

**Conclusions**

In summary, through an elaborate design and screening process, we developed an ABP and a fluorogenic substrate highly selective for the DUB USP16. This study illustrates the possibility of designing reagents for a target protein with an unknown structure by combining modelling, structural analysis, and computational prediction. Here, we reported the first example of a DUB-selective fluorogenic substrate. The activity-based probe facilitates the detection of active levels of USP16. Rh-M20-PA is a double-functionalized reagent which enables for a quick assay read-out using the fluorescent tag. Secondly, the biotin handle can be used for pull-down experiments (co-immunoprecipitation) followed by mass spectrometry analysis. The USP16-selective ubiquitin-based probe and fluorogenic substrate can be valuable tools for the development of small molecules for therapeutic purposes. This work contributes to offering the necessary tools to study USP16 and related biology.

**Conflicts of interest**

H. O. is shareholder of the company UbiQ.

**Acknowledgements**

We would like to thank Robbert Q. Kim for discussions and protein production. We would like to thank Dris El Atmioui for peptide synthesis and Patrick Celie for protein production. We would also like to thank Jimmy J. Akkermans for USP7 constructs. This work was financially supported by a VICI grant from the Netherlands Organization for Scientific Research N.W.O. (724.013.002).

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