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The *Saccharomyces cerevisiae* ubiquitin E3 ligase Asr1p targets calmodulin for ubiquitylation

Thomas Fries [a], Ronald Frank [b], Susanne M. Bailer [a,*]

[a] Max-von-Pettenkofer-Institut, Pettenkoferstr. 9a, Ludwig-Maximilian-Universität München, 80336 München, Germany
[b] Department of Chemical Biology, Helmholtz Centre for Infection Research, Braunschweig, Germany

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ABSTRACT

Yeast calmodulin known to be ubiquitylated *in vivo* in a Ca^{2+} dependent manner has long remained an orphan substrate. Here we identify *Saccharomyces cerevisiae* Asr1p as an ubiquitin E3 ligase for yeast calmodulin, a protein involved in calcium signaling. A short region within Asr1p-C harboring two putative calmodulin-binding motifs is sufficient and necessary for interaction with calmodulin. The interaction is direct, occurs *in vivo* and depends on physiological concentrations of Ca^{2+}. A minimal set of purified proteins including Asr1p E3 ligase was sufficient for *in vitro* ubiquitylation of calmodulin, a reaction that required a functional Asr1p Ring domain. We propose a role of the Asr1p E3 ligase activity in coping with stress.

1. Introduction

Ubiquitylation of proteins is a versatile way to regulate multiple processes in the cell [1]. Ubiquitin, an essential globular polypeptide composed of 76 residues, can be linked to proteins in a monomeric form or as a chain of multiple ubiquitins. This conjugation occurs via the ubiquitin C-terminally exposed glycin (G76). Proteins carrying ubiquitin chains linked by lysin 48 are generally targeted for degradation by the 26S proteasome. Single ubiquitins or chains linked to other lysins often result in alteration of protein function, binding or activity [2]. Protein ubiquitylation requires three basic enzymes. The E1 ubiquitin activating enzyme binds to ubiquitin, thereby generating an active E1-ubiquitin complex. The E2 ubiquitin conjugating enzymes are acceptor proteins for the activated ubiquitin. In cooperation with substrate specific E3 ubiquitin ligases they mediate the transfer of ubiquitin to a target protein [3].

Asr1p is a nonessential Ring/PHD finger protein of baker's yeast that constitutively shuttles between nucleus and cytoplasm and accumulates in the nucleus during stress [4–6]. Recently, Asr1p was characterized as a novel E3 ubiquitin ligase specific for the RNA polymerase II (RNAPII) subunit Rpb1p ([7]; Fries and Bailer, unpublished data). Here we identify calmodulin as a novel substrate ubiquitylated by Asr1p. Calmodulin is a small calcium binding protein that is able to transduce an influx of calcium ions to effector proteins. Thus, it is involved in myriads of cellular processes including mitosis, bud growth, actin organization and many more. An increase in calcium concentration to about 10^{-5} M results in binding of three calcium ions to yeast calmodulin (Cmd1p; [8]). Concomitantly, its conformation is changed, enabling Cmd1p to bind to effector proteins [9]. We identified two putative calmodulin-binding motifs (residues 243–280) within Asr1p-C that are sufficient and necessary for interaction with calmodulin. The interaction is direct, occurs *in vivo* and depends on physiological concentrations of Ca^{2+}. A minimal set of proteins consisting of the purified Asr1p E3 ligase, the E1 enzyme and the E2 enzyme UbcH5a was sufficient for *in vitro* ubiquitylation of calmodulin, a reaction that requires a functional Asr1p Ring domain.

2. Materials and methods

2.1. Yeast strains, plasmids, and microbiological techniques

Yeast strains, their manipulation, and growth, cloning and mutagenesis, purification of ProtA-fusion proteins from yeast, SDS–PAGE, as well as Western blotting, were done essentially as described [4,10,11]. The yeast plasmids used are pRS425-pNOP1-ProtA fused to the ASR1 coding region or fragments thereof encoding Asr1p, Asr1p-N (residues 2–211), or Asr1p-C (residues 212–310). The plasmids pET9d-GST-ASR1, pET9d-GST, pMal-ASR1 (Asr1p wildtype, Ring, and PHD mutant), pMAL-ASR1 residues

Abbreviations: GST, glutathione *S*-transferase; MBP, maltose-binding protein; Ring, really interesting new gene; PHD, plant homeodomain; NES, nuclear export sequence.

* Corresponding author. Fax: +49 (0)89 5160 5292.
E-mail address: Bailer@mvp.uni-muenchen.de (S.M. Bailer).

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243–280 and pMAL were used for bacterial expression. The Ring-finger mutant (C45S/H47S) of Asr1p was generated using the QuikChange XL mutagenesis kit (Stratagene). Purification and subsequent binding assays with immobilized GST, MBP, GST- and MBP-fusion proteins were done as described [11].

2.2. Peptide spot binding assay

Peptides consisting of 18 residues of Asr1p starting at residue 218 and ending at residue 307 with an offset of 3 amino acids were synthesized as described previously [12] on Amino-PEG_{500}-UC

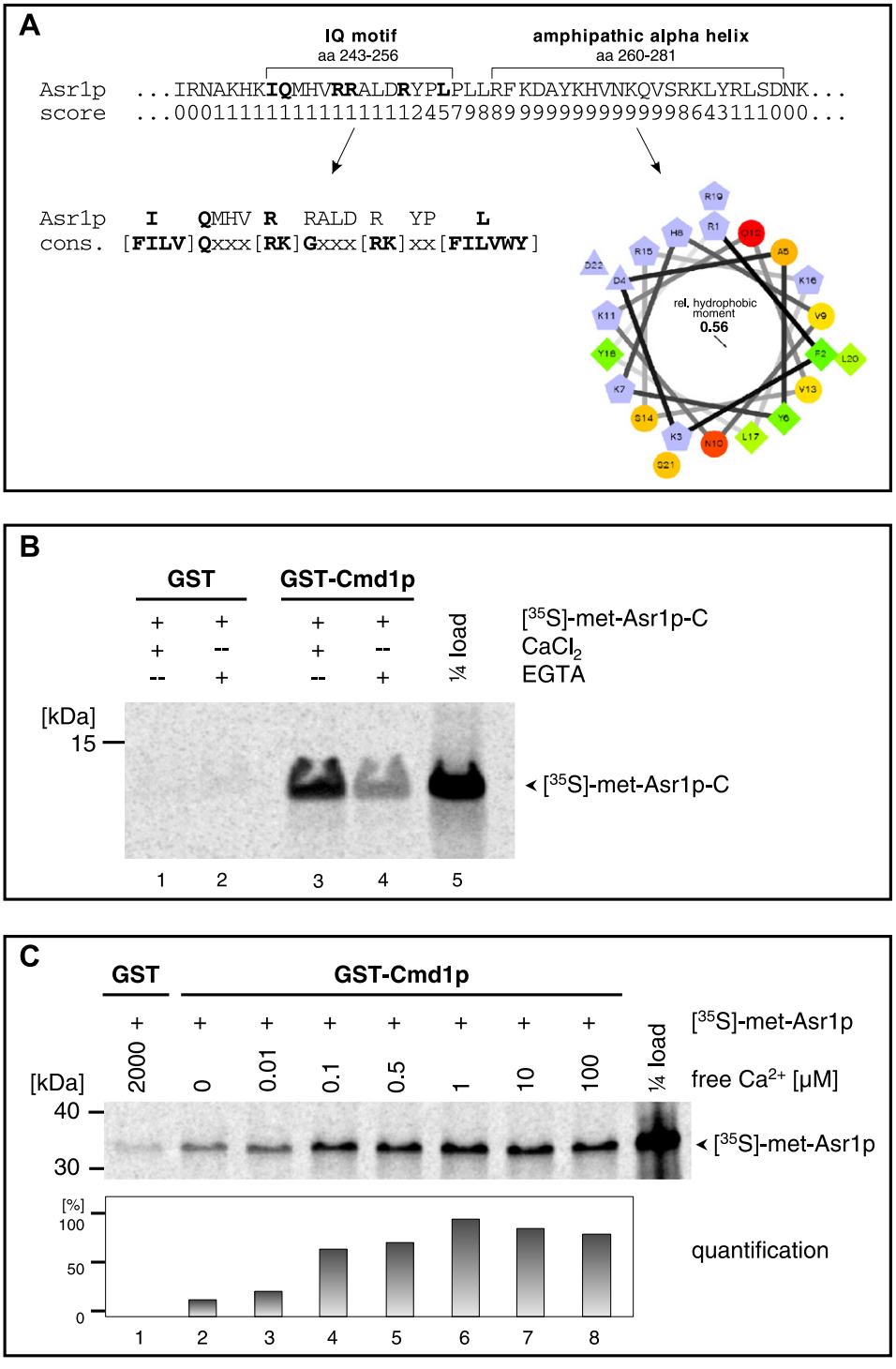

Fig. 1. The yeast ubiquitin E3 ligase Asr1p binds calmodulin. (A) Sequence analysis using the "calmodulin target database" (http://calcium.uhnres.utoronto.ca/ctdb/ctdb/home.html) revealed the existence of two calmodulin-binding motifs present in Asr1p, an IQ motif and an amphipathic alpha helix. The helical wheel projection was done by an online calculator (http://rzlab.ucr.edu/scripts/wheel/wheel.cgi). (B) GST-Cmd1p (lanes 3 and 4) or GST (lanes 1 and 2) purified from bacteria was immobilized on GSH–Sepharose prior to incubation with Asr1p-C (residues 212–310) synthesized in [^{35}S]-methionine containing rabbit reticulocyte lysate (lane 5). Incubation was done in presence of $CaCl_2$ (lanes 1 and 3) or EGTA (lanes 2 and 4). The bound proteins were subjected to SDS–PAGE and visualized by autoradiography. (C) Incubation of [^{35}S]-methionine labelled Asr1p with immobilized GST-Cmd1p or GST was done in presence of varying concentrations of free $CaCl_2$. The free calcium concentration in the buffer was calculated using the Ca-EGTA calculator v1.3 (http://www.stanford.edu/~cpatton/CaEGTA-TS.htm). For detection see Fig. 1B.

sheets (Abimed) by means of the SPOT method [13]. For blotting, the membranes were moistened with ethanol, and blocked with MBS buffer (2× blocking buffer (Sigma–Aldrich) in T-TBS buffer (137 mM NaCl, 2.7 mM KCl, 25 mM Tris–HCl, pH 7.4)) over night at 4 °C. Three identical membrane stripes each containing the 25 different Asr1p peptide spots were incubated in parallel with 5 μg/ml of recombinantly expressed and purified GST-Cmd1p or GST in 2 ml MBS buffer with 2 mM $CaCl_2$ or 5 mM EGTA for 4 h at room temperature. After washing, all stripes were incubated with mouse anti-GST antibodies (Sigma) over night at 4 °C followed by anti-mouse antibodies conjugated to alkaline phosphatase (Bio-Rad) for 2 h at RT. The Immun-Star GAM-AP chemiluminescence kit (Bio-Rad) was used for detection.

2.3. Ubiquitylation assays

The reaction mixture containing 40 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM ATP, 2 mM dithiothreitol (DTT), 300 μg/ml bovine ubiquitin (Sigma), 25 μM proteasome inhibitor MG132 (Sigma), 5% rabbit reticulocyte lysate (as a source of E1 and E2) and 12 ng/μl MBP or MBP-Asr1p was incubated at 30 °C for 0 h or 3 h. For MBP pull-down assays 25 μl of amylose resin and 140 μl column buffer (20 mM Tris/HCl pH 7.4, 200 mM NaCl, 100 μM $ZnSO_4$) were added to the reaction and incubated at 4 °C for 45 min. Protein eluted from the resin after washing was subjected to immunoblotting with an anti-MBP antibody (New England BioLabs) or an anti-ubiquitin antibody (Sigma). For *in vitro* reconstitution of Asr1p E3 ligase activity, 3 ng/μl human E1 (Boston Biochem) and 13 ng/μl human E2 (Boston Biochem) in presence of 1× Mg–ATP solution (Boston Biochem) were used as enzyme source.

3. Results

3.1. Asr1p binds Cmd1p in a Ca^{2+} dependent manner

Sequence analysis of Asr1p revealed the existence of an IQ-motif followed by an amphipathic alpha helix between residues 243 and 281, two features typically involved in calmodulin recognition (Fig. 1A, [14,15]; http://calcium.uhnres.utoronto.ca/ctdb/ctdb/home.html). To test whether Asr1p is indeed able to bind calmodulin, GST-Cmd1p or GST were expressed in bacteria, purified to homogeneity and incubated with GSH–Sepharose. The Asr1p C-terminal region (residues 212–310) was synthesized in rabbit reticulocyte lysate in presence of [35S]-methionine and subsequently applied to immobilized GST-Cmd1p and GST. As determined by autoradiography, strong binding of Asr1p-C to GST-Cmd1p

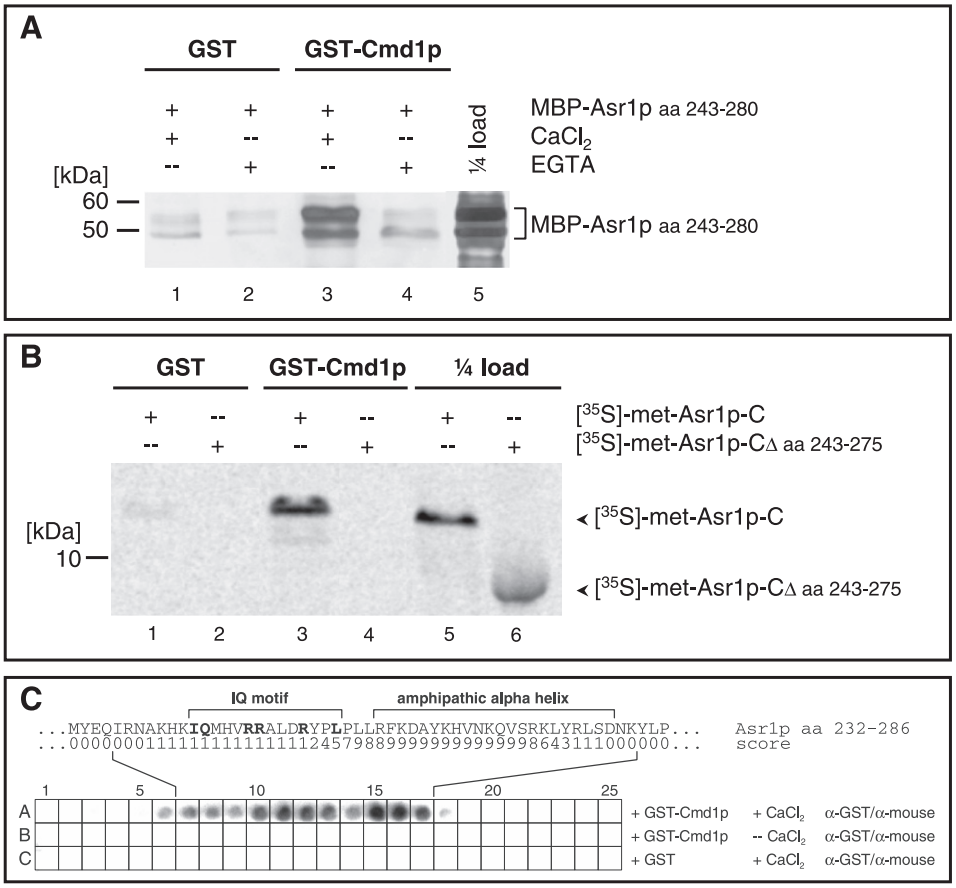

Fig. 2. Asr1p (residues 243–280) are sufficient and required for direct interaction with Cmd1p. (A) GST-Cmd1p (lanes 3 and 4) or GST (lanes 1 and 2) purified from bacteria were immobilized on GSH–Sepharose and subsequently incubated with recombinantly expressed and purified MBP-Asr1p (residues 243–280), either in presence of $CaCl_2$ (lanes 1 and 3) or EGTA (lanes 2 and 4). The bound proteins were subjected to immunoblotting using anti-MBP antibodies followed by secondary reagents and visualized by ECL. (B) GST-Cmd1p (lanes 3 and 4) or GST (lanes 1 and 2) were immobilized on GSH–Sepharose prior to incubation with Asr1p-C or Asr1p-C lacking residues 243–275 synthesized in rabbit reticulocyte lysate containing [^{35}S]-methionine (lanes 5 and 6). For detection see Fig. 1B. (C) Asr1p 18mer peptides spanning the putative calmodulin binding region (residues 243–281) were synthesized by the SPOT method, immobilized onto amino-PEG_{500}–UC sheets and incubated with purified GST-Cmd1p in presence of $CaCl_2$ (row A), EGTA (row B) or GST (row C). Binding was detected by mouse anti-GST antibodies followed by alkaline phosphatase conjugated anti-mouse antibodies and visualized by Immun Star GAM-AP.

(Fig. 1B, lane 3) but not to GST (lane 1) was observed in presence of $CaCl_2$. Addition of EGTA to chelate Ca^{2+} present in the rabbit reticulocyte lysate led to a strong reduction of Asr1p bound to GST-Cmd1p (lane 4).

The same experimental setting was applied to determine the concentration of free Ca^{2+} required for efficient interaction between Cmd1p and Asr1p. The concentration of free Ca^{2+} in the binding buffer was calculated using the Ca-EGTA calculator v1.3 (http://www.stanford.edu/~cpatton/CaEGTA-TS.htm). Residual binding of Asr1p to GST-Cmd1p observed in nonsupplemented rabbit reticulocyte lysate steadily increased upon addition of calcium and reached a plateau at 100–500 nM $CaCl_2$ (Fig. 1C). We thus conclude that the E3 ligase Asr1p C-terminal domain interacts with Cmd1p in presence of physiologically relevant Ca^{2+} concentrations.

3.2. Asr1p residues 243–280 are sufficient for direct interaction with Cmd1p

To probe for interaction of GST-Cmd1p with the predicted calmodulin-interacting motifs of Asr1p, Asr1p residues 243–280 fused to the C-terminus of maltose-binding protein (MBP-Asr1p residues 243–280) was recombinantly expressed, purified and applied to immobilized GST-Cmd1p and GST. MBP-Asr1p 243–280 efficiently bound to GST-Cmd1p but not to GST (Fig. 2A). Consistent with the results shown above (Fig. 1B), interaction of Cmd1p and Asr1p (residues 243–280) was dependent on Ca^{2+}. Thus, residues 243–280 of Asr1p are sufficient for direct and Ca^{2+}-dependent interaction with Cmd1p. To determine whether this region of Asr1p is necessary for Cmd1p binding, Asr1p-C and Asr1p-C lacking residues 243–275 were radiolabelled during synthesis in rabbit reticulocyte lysate (Fig. 2B, lanes 5 and 6) and applied to GST-Cmd1p (lanes 3 and 4) or GST (lanes 1 and 2). While synthesis of both proteins was comparable (lanes 5 and 6), only Asr1p-C could bind GST-Cmd1p further supporting the notion that Cmd1p binding to Asr1p crucially involves residues 243–280. To map the interaction of Cmd1p with the predicted binding regions within Asr1p-C, 18mer peptides of Asr1p residues 218–307 with an offset of three residues were synthesized by the SPOT method and analyzed for interaction with purified GST-Cmd1p in presence of $CaCl_2$ or EGTA (Fig. 2C). Binding was monitored by incubation of the peptide stripes with GST-specific mouse antibodies followed by mouse-specific alkaline phosphatase coupled secondary reagents. Moderate binding of Cmd1p was observed with peptides 10–13 spanning the IQ motif where the VRR motif seemed to be of particular importance. Cmd1p strongly bound to the amphipathic alpha helix formed by peptides 15–17 and binding to this region was independent of the IQ motif. Binding of GST-Cmd1p occured in presence of $CaCl_2$ and was completely abrogated upon addition of the Ca^{2+} chelator EGTA. In summary, Cmd1p directly interacts with both Cmd1p recognition motifs located in the C-terminal region of Asr1p.

3.3. The ubiquitin E3 ligase Asr1p modifies calmodulin

Previous experiments support the existence of an ubiquityl-calmodulin synthetase in the yeast cell lysate however so far the enzyme responsible for this activity remained unidentified [16]. To analyze whether Asr1p is able to ubiquitylate calmodulin, an *in vitro* ubiquitylation assay was set up using defined components. Purified MBP-Asr1p, the human E1 and the E2 enzyme UbcH5a (Boston Biochem) were incubated with purified His_6-Cmd1p in presence of ubiquitin and ATP. Subsequent to immobilization on Ni–NTA resin, His_6-Cmd1p was analyzed by SDS–PAGE and Western blotting using rabbit anti-Cmd1p antibodies and rabbit-specific secondary reagents conjugated to peroxidase (Fig. 3A). A modified form of His_6-Cmd1p migrating at around 25 kDa was readily detected after 3 h of incubation with wildtype Asr1p (Fig. 3A, lane 2) while the unmodified His_6-tagged calmodulin migrated around 17 kDa (Fig. 3A, lanes 1–4). Upon use of the MBP-tagged Asr1p Ring-finger mutant previously shown to be impaired in E3 ligase activity [7] instead of the wildtype MBP-Asr1p no shift in gel mobility was observed (lane 4). The change in migration is consistent with the Asr1p mediated conjugation of a single ubiquitin to Cmd1p. Thus we conclude that Asr1p is a yeast ubiquitin E3 ligase that specifically ubiquitylates calmodulin.

To determine whether Asr1p interacts with calmodulin *in vivo*, an *asr1Δ* strain expressing ProtA-Asr1p-C was subjected to affinity purification, and the isolated proteins were analyzed by SDS–PAGE and Western blotting (Fig. 3B). A prominent protein migrating at 14 kDa was specifically recognized in yeast cell homogenates by rabbit anti-calmodulin antibodies (Fig. 3B, lane 1). A protein of the same molecular weight was detected upon analysis of affinity-purified ProtA-Asr1p-C and co-isolated proteins (lane 2). A control rabbit anti-ProtA antibody readily decorated ProtA-Asr1p-C before (lane 4) and after affinity purification (lane 3) while no reaction with the 14 kDa band was observed (Fig. 3B, compare

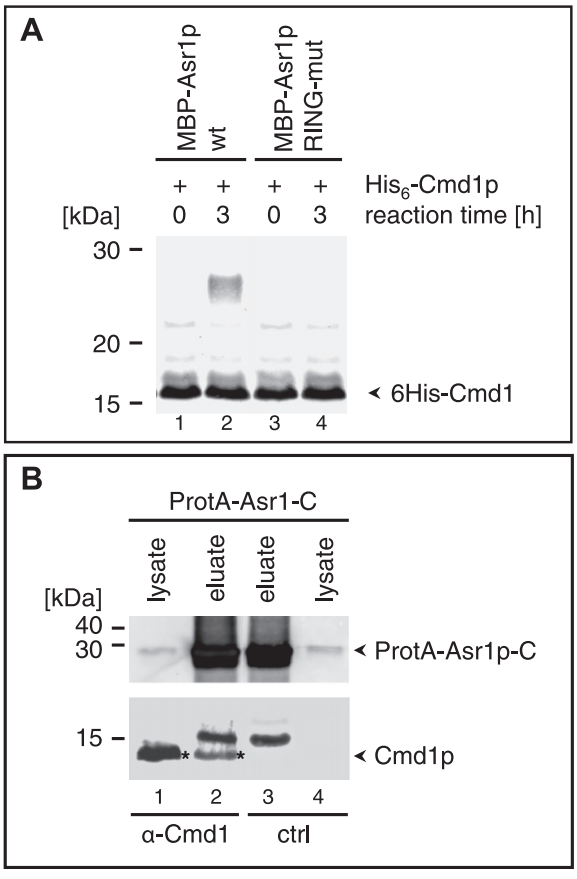

Fig. 3. Asr1p ubiquitylates calmodulin. (A) To reconstitute ubiquitylation of calmodulin by Asr1p *in vitro*, the recombinantly expressed and purified components MBP-Asr1p or the Ring-finger mutant MBP-Asr1p (C45S/H47S), His_6-Cmd1p, and the human E1 and E2-UbcH5a enzymes were incubated for the indicated times in presence of ubiquitin, $CaCl_2$ and ATP. Following pulldown of His_6-Cmd1p on Ni–NTA the bound proteins were subjected to SDS–PAGE and Western blotting and detected using anti-calmodulin antibodies. (B) To determine whether calmodulin interacts with Asr1p *in vivo*, *asr1* cells expressing ProtA-Asr1p-C were subjected to affinity-purification on IgG–Sepharose. Calmodulin present in the yeast lysate (lane 1) and co-isolated with ProtA-Asr1p-C (lane 2) analyzed by SDS–PAGE and Western blotting using rabbit anti-calmodulin antibodies followed by peroxidase coupled goat-anti rabbit antibodies and ECL. Unrelated rabbit antibodies were used for control (ctrl, lanes 3 and 4).

lanes 2 and 3). Our data are thus consistent with the *in vivo* interaction of Asr1p and Cmd1p and an *in vivo* function of Asr1p in ubiquitylation of calmodulin.

4. Discussion

Calmodulin participates in numerous cellular processes thereby transfering the calcium influx to a diverse set of effectors. Due to a wide range of regulatory tasks, the localization, activity and stability of calmodulin requires intimate regulation. Ubiquitylation represents a versatile way to regulate abundance and activity of proteins. Indeed, ubiquitylation of calmodulin notedly repressed its capacity to activate phosphorylase kinase [17], suggesting that ubiquitylation of calmodulin is physiologically relevant however the enzyme involved remained elusive [18]. With *Saccharomyces cerevisiae* Asr1p we have identified the first E3 ubiquitin ligase that specifically ubiquitylates calmodulin in a Ca^{2+}-dependent manner involving orthologous proteins of the Ubc4/5 family. Asr1p is non-essential under normal growth conditions, while a strain deleted for ASR1 shows an alcohol-induced growth defect. We thus believe that ubiquitylation of Cmd1p involving Asr1p is triggered by specific external stimuli.

Our data and previous reports indicating that *in vivo* calmodulin is exclusively ubiquitylated at lysine 22 [18] are consistent with monoubiquitylation of calmodulin by Asr1p. *In vitro* however, substitution of several predicted lysins of calmodulin by alanines was unable to abrogate ubiquitylation suggesting that several acceptor lysines can be modified redundantly (Fries and Bailer, unpublished results). This is in line with the prediction of four ubiquitylation sites (K22, K91, K95 and K107) by UbiPred (http://iclab.life.nctu.edu.tw/ubipred/). Thus, the absence of K22 may promote ubiquitylation of other predicted acceptor lysines suggesting that calmodulin can potentially be ubiquitylated in a complex manner.

Asr1p directly interacts with calmodulin via a C-terminal sequence (residues 243–280) harboring both an IQ motif as well as a basic, amphipathic α-helix composed of 18 residues. In the peptide spot binding assay, Asr1p strongly bound to peptides able to form amphipathic α-helices in a Ca^{2+}-dependent manner. Weaker binding was observed between Asr1p and the IQ motif, unexpectantly however, this binding is abrogated upon Ca^{2+}-chelation. Both the stable interaction of calmodulin with Asr1p which is Ca^{2+} dependent, and the peptide spot assay suggests that in the context of calcium signaling the interaction of Cmd1p with Asr1p is primarily mediated by the amphipathic α-helix. A contribution of the Asr1p IQ motif to binding of Cmd1p under low concentrations of Ca^{2+} suggests that regulation of Cmd1p ubiquitylation contains multiple layers of complexity.

Previously we showed that the region within Asr1p-C responsible for interaction with calmodulin represents the minimal sequence required for nuclear import of Asr1p and directly interacts with import factors in a Ran-GTP dependent fashion [11]. In addition, an overlapping region mediates binding of Asr1p to the C-terminal domain of Rpb1p, the largest subunit of the RNA polymerase II ([7]; Fries and Bailer, unpublished data). At present we do not know how these diverse protein interactions can be accommodated by this rather short region of Asr1p. Each of these proteins could potentially represent a target of the E3 ubiquitin ligase activity and thus interact independently with Asr1p. Alternatively, the Asr1p-C could represent a scaffolding region where several of these proteins are brought together. In this context it is interesting to note that apart from the Ran-GTP dependent nuclear import a calmodulin-dependent one was recently identified [19]. Therefore, by interacting with classical nuclear import factors and calmodulin, Asr1p could operate between these two transport modes where stress-induced Ca^{2+}-influx triggers the switch.

Asr1p constitutively shuttles between cytoplasm and nucleus suggesting it is continuously involved in both nuclear and cytoplasmic functions. Indeed, we found that Asr1p associates with both Cmd1p and RNAP II even in absence of any stress (this analysis, Fries and Bailer, unpublished results). Upon alcohol stress, Asr1p rapidly accumulates in the nucleus [4–6] indicative of stress-induced alterations. These could include changes in conformation, posttranslational modifications as well as protein complex formation. Interestingly, functional nuclear export signals (NES) are nested within the Ring/PHD-fingers of Asr1p. Thus stress-induced association of proteins with the Asr1p Ring/PHD-fingers to execute or regulate its E3 ligase activity could mask the NES and lead to nuclear retention of Asr1p. Future experiments aim at analyzing the cellular processes where Asr1p is involved in general and more specifically the ubiquitylation of Cmd1p.

Acknowledgments

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