PROTEOLYSIS

Cut in the middle by you

If you arrive late at the theatre, getting to your seat can be a problem, especially if it's in the middle of a row that's already full. Wouldn't it be convenient if you could access your seat without having to make the arduous journey from the end of the row? In the present model for the action of the 26S proteasome, it makes a similar onerous journey, digesting unfolded substrates processively from their termini. However, this model cannot explain all proteasome-catalysed proteolytic events, and now, in *Science Express*, Liu and colleagues show that some proteasome substrates can be cut in the middle.

The authors began by comparing the degradation of two 'natively disordered', physiological substrates — cyclin-dependent kinase inhibitor $p21^{cip1}$ (p21) and α-synuclein — and the stable green fluorescent protein (GFP) by the latent/closed 20S proteasome (the proteasome core) and the active/open 26S proteasome (the 20S core plus the PA700 regulatory cap).

They found that p21 and α-synuclein were efficiently degraded in the presence of either the 20S or 26S proteasome, but that GFP was not degraded in either case. The 20S proteasome cannot degrade short peptide substrates, so the fact that it can efficiently degrade p21 and α-synuclein indicates that "...unfolded proteins themselves could open the gate which controls access to the otherwise occluded catalytic sites, thereby initiating a process similar to that employed by the proteasome regulators PA700 and PA28".

Next, the authors studied the degradation of fusions between GFP and p21 or α-synuclein. When GFP was fused to either the amino or the carboxy termini of p21 or α-synuclein, they found that the 20S and 26S proteasomes efficiently degraded the p21 or α-synuclein domains, but left the GFP domain intact. They obtained the same result when they fused GFP to both termini of p21 or α-synuclein, which indicates that the 20S and 26S proteasomes are capable of endoproteolytic cleavage.

DNA REPLICATION

The fragility of DNA

DNA is not unlike the finest bone china — subject it to stress and it will break. One particular stress is caused by conditions that partially inhibit DNA replication (folate deficiency, for example, or treatment with aphidicolin), and in this case the breaks occur preferentially at specific loci called common fragile sites. These sites — which form breaks and gaps on metaphase chromosomes — are rearranged in many tumours, hence the interest in knowing how their stability is regulated.

Reporting in *Cell*, Thomas Glover, graduate student Anne Casper and colleagues show that the replication checkpoint kinase ATR (ataxia-telangiectasia and Rad3-related) is a crucial factor in fragile-site stability. The authors were led to look at this kinase because ATR — along with the related ATM (ataxia-telangiectasia, mutated) — is implicated in the response to DNA damage during cellular replication.

To test the idea that ATM and ATR might be involved in fragile-site stability, Glover and co-workers first looked at the effects of 2-aminopurine (2-AP), which inhibits the kinase activities of both proteins. Although fragile-site gaps and breaks were seen when human lymphocytes were treated with aphidicolin alone, the addition of 2-AP led to a dramatic rise, with over 90% of these gaps occurring at known fragile sites.

Glover and colleagues next asked whether both ATM and ATR are involved. To do this they first compared fragile-site stability in control cell lines versus lymphoblast cell lines with truncating mutations in ATM. There was little difference in the stability of fragile sites in the normal and mutant cell lines, even after treatment with aphidicolin, suggesting that ATM is not involved in fragile-site stability.

ATR, by contrast, is crucial, as shown by several experiments. One difficulty is that *ATR*$^{-}$ animals are not viable, so the authors first used a dominant-negative approach. They tested the effects of inducing fragile sites with aphidicolin in human osteosarcoma cells stably transfected either with wild-type ATR or with a kinase-dead form (ATR-kd). They found that, after the addition of aphidicolin, the number of chromosome breaks and gaps increased more than 20-fold in the cells expressing ATR-kd compared with those expressing wild-type ATR. Glover and colleagues confirmed this finding using two other approaches — Cre/*lox*-mediated inactivation of ATR, and RNA interference (RNAi) against ATR. In both cases, the appearance of gaps and breaks increased in response to aphidicolin in the ATR-deficient cells.

These results indicate that, during DNA replication, stalling of the replication fork at fragile sites could be involved in the instability of these sites, and the known replication-checkpoint function of ATR is consistent with a role for this protein. So, could stalling at fragile sites be a normal occurrence during cellular replication? The authors reasoned that, if this were the case, they might observe fragile-site instability in ATR-deficient cells in culture, without the need for aphidicolin. When they tested this over five days, they found up to a 30-fold increase in chromosome breaks and gaps in the ATR-deficient cells compared with wild type.

The authors propose, then, that fragile sites represent unreplicated single-stranded chromosomal regions, which result from stalled replication forks that escape the ATR-dependent replication checkpoint. They also predict that tumours with alterations in the replication checkpoint, or in the homologous-recombination machinery that compensates for such problems, might show increased chromosome rearrangements at fragile sites.

Alison Mitchell

References and links

ORIGINAL RESEARCH PAPER Casper, A. M. *et al.* ATR regulates fragile site stability. *Cell* **111**, 779–789 (2002)

Liu and co-workers concluded by showing that the 20S and 26S proteasomes can effectively degrade circular α-synuclein and GFP–p21 substrates (in the latter case, the GFP moiety was left intact). This work has therefore shown that proteasomes can endoproteolytically cleave proteins and has provided a mechanism for the "...regulated release of transcription factors from inactive precursors as well as a means of accessing internal folding defects of misfolded multi-domain proteins". It has also highlighted a potential physiological role for the 20S proteasome in the absence of its regulatory proteins.

Rachel Smallridge

References and links

ORIGINAL RESEARCH PAPER Liu, C. W. *et al.* Endoproteolytic activity of the proteasome. *Science Express* 2002 December 12 (DOI: 10.1126/science.1079293)

FURTHER READING Weissman, A. M. Ubiquitin and proteasomes: themes and variations on ubiquitylation. *Nature Rev. Mol. Cell Biol.* **2**, 169–178 (2001)

WEB SITES

Philip Thomas' laboratory: http://www2.utsouthwestern.edu/thomaslab/

George DeMartino's laboratory: http://www2.utsouthwestern.edu/demartinolab/

MEMBRANE TRAFFIC

Sort and transport

When moving house, we have to sort our belongings into boxes and transport these boxes to our new abode — a function that the GGA (Golgi-localized, γ-ear-containing, ADP-ribosylation factor (Arf)-binding) proteins seem to have in the cell. GGAs associate with the cytosolic face of the *trans*-Golgi network (TGN) and sort mannose 6-phosphate receptors (MPRs) into clathrin- and GGA-coated carriers, which are then transported to endosomes. The interactions that deliver these carriers to endosomes have remained unclear, but now, reporting in *The EMBO Journal*, Bonifacino and colleagues provide new insights.

The monomeric GGA proteins contain four domains: a VHS domain (which binds MPRs); a GAT domain (which binds activated Arf-family GTP-binding proteins); a hinge region (which binds clathrin); and a GAE domain, which is thought to bind accessory factors that can regulate the function of GGA-containing coats or GGA-coated carriers.

Bonifacino and co-workers began by looking at the interaction of the human GGAs (GGA1, GGA2 and GGA3) with Rabaptin-5, a protein that is important in endosome fusion. Although previous studies had shown that GGA-GAE–Rabaptin-5 interactions were weak, and therefore probably not physiologically significant, the authors decided to investigate these interactions further because of their possible implications.

By carrying out pull-down experiments using various glutathione-*S*-transferase (GST)–GGA domains and Rabaptin-5 from bovine brain cytosol, the authors found that GGA-GAE domains interact with Rabaptin-5 in the Rabaptin-5–Rabex-5 complex (a complex that regulates endosome fusion), and that these interactions are stronger than was previously thought. They confirmed that the GGA–Rabaptin-5 interaction occurs *in vivo* by immunoprecipitating endogenous proteins.

The authors then used the yeast two-hybrid system to analyse the structural determinants of the GGA–Rabaptin-5 interaction. They showed that residues 428–455 of Rabaptin-5, which are in a predicted random coil, contain the minimal sequence needed for interactions with the GAE domains of GGA1–3. In addition, they showed that the GGA–Rabaptin-5 interaction is bipartite for GGA1 and GGA2, as the carboxy-terminal coiled-coils of Rabaptin-5 interact with the GAT domains of these GGAs.

Using alanine-scanning mutagenesis, Bonifacino and colleagues were able to further define the putative GGA-GAE-binding motif in Rabaptin-5. This motif is FGXLV from residues 439–443, where X is any amino acid.

So, what happens when Rabaptin-5 binds GGAs? The authors studied the effect of His_6-tagged Rabaptin-5 fragments on the binding of clathrin to GST–GGA-hinge+GAE constructs *in vitro*, and found that Rabaptin-5 interferes with clathrin–GGA interactions.

Finally, using immunofluorescence microscopy, the authors studied the change in the localization of GGA1 and its associated MPR cargo in HeLa cells after these cells had been transfected with green fluorescent protein–Rabaptin-5. Before transfection, they found that GGA1 and its cargo were localized to the TGN, whereas after transfection, endogenous GGA1 and MPR were localized to Rabaptin-5-stabilized large endosomes.

These data have revealed "…a functional link between proteins regulating TGN cargo export and endosomal tethering/fusion events". They have also allowed Bonifacino and co-workers to suggest that GGA–Rabaptin-5–Rabex-5 interactions cause clathrin to be released from GGA-coated intermediates or prevent clathrin re-binding, which might allow TGN-derived carriers to fuse with endosomes and deliver their MPR cargo.

Rachel Smallridge

References and links

ORIGINAL RESEARCH PAPER Mattera, R. *et al.* Divalent interaction of the GGAs with the Rabaptin-5–Rabex-5 complex. *EMBO J.* **22**, 78–88 (2003)