Proteasomal interactors control activities as diverse as the cell cycle and glutaminergic neurotransmission

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Abstract

The six regulatory non-redundant ATPases in the base of the 19 S regulator of the 26 S proteasome belong to the AAA superfamily of ATPases. Yeast two-hybrid genetic screens, biochemical analyses and cell biological studies have identified and characterized new interactors of the human S6 (rpt3) and S8 (rpt6) ATPases of the 19 S regulator of the 26 S proteasome. The S6 ATPase interacts with gankyrin. This protein is found in purified human 26 S proteasomes and in a smaller complex(es) containing CDK4 and free S6 ATPase. Gankyrin overexpression causes the phosphorylation of the retinoblastoma protein (pRb) and the release of E2F transcription factor to trigger the expression of DNA synthesis genes. Gankyrin is oncogenic in nude mice and is overexpressed in hepatocellular carcinoma cells (HCCs). The S8 ATPase interacts with members of the large Homer-3 protein family. There are three Homer genes; the Homer 1 and 2 gene products control trafficking and calcium-store-related functions of metabotropic glutamate receptors (e.g. mGluR1α). Homer-3A11 by binding to the S8 ATPase brings mGluR1α to the 26 S proteasome for degradation. The degradation of mGluR1α is blocked by proteasomal inhibitors and by overexpression of the N-terminus of Homer which binds to the receptor. The S8 ATPase and mGluR1α are co-localized in Purkinje dendrites in rat cerebellum. The data are discussed in terms of the regulation of the cell cycle and glutaminergic receptor functions by the 26 S proteasome.

Introduction

Hexameric ATPase ring complexes control proteases in prokaryotes [1] and eukaryotes [2]. In prokaryotes the hexamers are made from six identical ATPases which combine substrate recognition with the capacity for protein substrate unfolding before feeding the proteins into the proteolytic catalytic chambers of interacting multisubunit proteases. In the eukaryotic 26 S proteasome six ATPases form part of the 'base' of the 19 S regulator on which is superimposed a multisubunit 'lid' to complete the structure of the regulator. Multi-ubiquitylated protein substrates bind to the 19 S regulator [3], followed by de-ubiquitylation by the rpn11 zinc metalloprotease in the lid [4] and unfolding of the proteins by the combined action of the hexameric ATPases [2]. The unfolded proteins are then fed into the internal chambers of the 20 S core of the 26 S proteasome for fragmentation into small peptides [5].

It might be expected that proteins in the regulator would interact with modulatory cellular proteins and pathogenic viral proteins. The ATPases may be particularly targeted since they have a key role in controlling proteasomal processing of proteins for degradation. The ATPases are non-identical and non-redundant (deletion of the gene for any of the ATPases in yeast is lethal). Therefore, it might be expected that each ATPase has specific cellular interactors to control the degradation of specific proteins or protein families in the cell. Several cellular and viral proteins have already been shown to bind to specific ATPases [6]. The cellular and viral proteins which bind to proteasomal ATPases have critical functions in processes such as transcriptional regulation. It is against this background that the search for new interactors of proteasomal ATPases was carried out based on yeast two-hybrid (Y2H) screens.

The cDNAs for the six human ATPases were subcloned into appropriate vectors for Y2H screens and into eukaryotic expression vectors for subsequent studies in human cells. The results of Y2H screens, biochemical analyses and cell biological studies with the S6 and S8 ATPases have progressed furthest.

Gankyrin, the S6 ATPase and the cell cycle

A Y2H screen with the S6 ATPase against a human brain cDNA library gave several clones including one that on DNA sequencing was found to code for a small protein (approx. 26 kDa) containing multiple ankyrin repeats. The interaction was confirmed biochemically by interaction of *in vitro*-translated S6 ATPase with glutathione S-transferase–gankyrin. The other ATPases did not interact with gankyrin.

Key words: gankyrin, Homer, metabotropic glutamate receptor, proteasomal ATPase.

Abbreviations used: Y2H, yeast two-hybrid; pRb, phosphorylation of the retinoblastoma protein; HA, haemagglutinin; mGluR, metabotropic glutamate receptor; ER, endoplasmic reticulum.

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The gankyrin sequence contains six ankyrin repeats and a putative retinoblastoma-binding motif. The protein was named gankyrin because Gann is the Japanese word for cancer and the corresponding gene is overexpressed at the mRNA and protein levels in all hepatocellular carcinoma cells (HCCs) studied [7]. Some gankyrin purifies with 26 S proteasomes from human red blood cells. Gankyrin is associated with 19 S particles after electrophoresis in non-denaturing conditions of partially purified 26 S proteasomes to separate 19 S and 20 S particles. This is expected since gankyrin binds to the S6 ATPase of the 19 S regulator. However, gankyrin is also found after non-denaturing electrophoresis in smaller complex(es) [8]. Transfected gankyrin increases the phosphorylation of the retinoblastoma protein (pRb) and consequently releases E2F transcription factor to trigger the expression of DNA synthesis genes [7]. Western analysis of immunoprecipitated haemagglutinin (HA)–gankyrin after transfection of HEK-293 cells reveals the cyclin-dependent kinase, CDK4. This, again, might be expected since pRb is the only known substrate of CDK4. Complexes containing immunoprecipitated CDK4 also contain HA–gankyrin. Glycerol gradient analyses of extracts of HEK-293 cells show that gankyrin is found with proteasomal particles but also in a smaller complex(es). Interestingly, the proportion of gankyrin with the proteasome or in the smaller complex(es) is dependent on the presence of deoxycholate in the cell homogenization buffer. In the absence of this detergent considerably more gankyrin is associated with proteasomal sub-particles. This may indicate that gankyrin is washed from 26 S particles in the process of homogenization in detergent-containing buffer. However, the fact that the bulk of gankyrin extracted from the cells in the presence or absence of detergent is in the small complex(es) may indicate that gankyrin is predominantly in non-proteasomal complex(es) in the cell. Western analyses indicate that free S6 ATPase is also in the smaller complexes [8].

Gankyrin is overexpressed in all HCCs so far studied at the mRNA and protein levels. Expression of gankyrin transforms NIH cells and is oncogenic in immunocompromised nude mice [7]. The complete mechanism of action of gankyrin to cause HCC is not known but overexpression of gankyrin is anti-apoptotic. One reason for this may be that gankyrin expression decreases p53 transcriptional activity by accelerating the degradation of p53 in the cell.

What are gankyrin's functions in the cell?

Gankyrin can be defined as a 'sometime' subunit of the 19 S regulator of the 26 S proteasome by specifically binding to the S6 ATPase. However, gankyrin is also found in smaller complex(es) in the cell with CDK4 and other unknown proteins. The S6 ATPase is also in these complexes. The latter observation is interesting since the HEC (highly expressed in cancer) protein is reported to be present with the S7 ATPase in non-proteasomal complexes and to regulate mitosis [9]. Gankyrin has orthologues in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Deletion of the gankyrin gene in *S. cerevisiae* has no obvious phenotype [8]. Genetic tagging of a proteasome subunit in *S. cerevisiae*, pull-down and proteomic analysis has shown that the yeast orthologue of gankyrin is bound to the 26 S proteasome in the absence of ATP but not in the presence of ATP [10]. However, the binding of *in vitro*-translated S6 to glutathione S-transferase–gankyrin is not influenced by ATP. This may imply that the binding of gankyrin to S6 becomes ATP-dependent when all six ATPases are in an intact proteasomal particle.

Gankyrin binds directly to *Baculovirus*-expressed CDK4 but not to *in vitro*-translated CDK4, which suggests that CDK4 may need to be modified, e.g. by phosphorylation in the insect cells in order to directly interact with gankyrin. The discovery of a new ankyrin-repeat-containing protein which increases the phosphorylation of cellular pRb immediately suggests that gankyrin may compete with a member of the inhibitor of kinase (pINK) family of cyclin-dependent kinase inhibitors which also contain similar numbers of ankyrin repeats to gankyrin. Recently, it has been shown that this is the case and that gankyrin competes with pINK16s to control the activity of CDK4 [11]. There is now an additional player in the control of the G_1/S phase of the cell cycle. The pINKs inhibit the activity of CDK4 whereas gankyrin enhances the activity of CDK4 by competition with pINKs. The regulation of pRb phosphorylation is now in the hands of gankyrin as well as the pINKs p21 and p27.

Homer, the S8 ATPase and metabotropic glutamate receptors (mGluRs)

A Y2H screen with the human S8 ATPase against a HeLa cell cDNA library showed interaction with a Homer-3A_{11} protein. This was confirmed biochemically by the interaction of *in vitro*-translated proteins. There are three Homer genes with 17 alternative transcripts [12]. Each set of gene products includes long and short versions of the proteins. All the proteins contain N-terminal EVH-like domains (which bind to a PPXXFX motif in the cytosolic tails of mGluRs) and the long Homers contain C-terminal coiled-coil domains through which long Homers can form homo- and heterodimers [13]. Analysis of the interactions of the S8 ATPase with members of the three generic types of Homer proteins biochemically and in the Y2H screen shows that the S8 ATPase only binds to long Homer-3 proteins. The N-terminus of the ATPase interacts with the C-terminus of the long Homer proteins. Neither short Homer-3 proteins nor Homer-2 and Homer-1 proteins interact with the S8 ATPase. Glycerol gradient analyses of extracts of cells previously transfected with FLAG-tagged mGluR (FLAG-mGluR1α) and HA–Homer-3A_{11} showed by Western analyses that there was a heterogeneous distribution of the proteins on the gradients. However, a proportion of both of the proteins was in dense fractions containing subunits of the 26 S proteasome. As expected, the receptor was detected on the Western blots as a high-molecular-mass (150–220 kDa) smear of N-glycosylated forms. Immunoprecipitation of 26 S proteasomes with a monoclonal antibody to a core α-subunit

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(α2) followed by Western analyses revealed transfected HA-tagged and endogenous Homer-3 proteins, species of transfected FLAG–mGluR1α together with proteasomal subunits including the S8 ATPase. However, the size range of mGluR1α species was approx. 65–90 kDa and not the mature 150–220 kDa glycoforms. Deletion of a region of mGluR1α containing the PPXXFX motif necessary for binding to the EVH-like domain in the N-terminus of Homer prevents binding of receptor fragments to the 26 S proteasome.

Transfected mGluR1α in HEK-293 cells is degraded with a half-life of approx. 2 h (protein synthesis inhibited with emetine). Degradation is blocked by the proteasome inhibitors MG132 and lactacystin. The degradation of the receptor is also blocked by the transfected N-terminus of Homer in HEK-293 cells transiently transfected with mGluR1α and BHK cells stably transfected with mGluR1α.

Confocal microscopy shows that mGluR1α and the S8 ATPase are co-localized in Purkinje dendritic spines. The proteasomal S8 ATPase is also found in the nuclei of nerve cells in the absence of mGluR1α, as expected for a plasma membrane protein.

What is the relationship between Homer, 26 S proteasomes and mGluRs?

Homer-1a protein was discovered as an immediate-early gene product expressed in the brain during electroconvulsive shock [13]. Subsequently, three Homer genes were discovered. Current functions described for Homer proteins include the trafficking of mGluRs and controlling the release of calcium from intracellular stores. The latter function is mediated by Homer dimers which are thought to directly link the cytosolic tails of plasma membrane mGluRs and the cytosolic tails of inositol phosphate receptors or ryanodine receptors in smooth endoplasmic reticulum (ER). Receptor stimulation results in the release of calcium from the intracellular stores. Long Homer-3 proteins have a new function which is to act as adaptors through which mGluR1α can be attached to the proteasome. Deletion of a region of mGluR1α including the PPXXFX motif necessary for Homer binding to the mGluRs abrogates the binding of the receptor to the proteasome. Similarly, overexpression of the N-terminus of Homer-3A_{11} containing the EVH-like domain prevents the degradation of transfected mGluR1α, presumably by binding to receptors' tails and preventing the binding of endogenous cellular Homer-3 proteins. Endogenous mGluR1α is co-localized with the S8 ATPase in rat cerebellar Purkinje dendrites. The S6 and S7 ATPases are more generally distributed. Other proteasomal antibodies did not detect the corresponding rat cerebellar antigens. Again, therefore, it is formally possible that the S8 ATPase is in some non-proteasomal complex with the receptor. However, the fact that the receptor is degraded in a Homer- and proteasome-dependent manner suggests that whole proteasomes are in the dendrites adjacent to the mGluR1α receptors.

The fragments of mGluR1α immunoprecipitated with 26 S proteasomes appear not to be glycosylated since they are much smaller than the heterogeneous mixture of glycoforms and are not detected by Western analysis as glycoprotein smears. This can be explained if the receptors have been removed by long Homer-3s from the ER for proteasomal degradation. The ER-associated degradation system (ERAD) is well characterized and involves chaperone-assisted removal of proteins through the Sec61 channel (through which proteins are inserted into the ER), ubiquitylation, binding to cdc48/p97 adaptors [14] and ferrying to the 26 S proteasome [15]. A proteasomal N-glycanase can remove N-linked glycans from the ER proteins before de-ubiquitylation and degradation [16].

Since the fragments of transfected mGluR1α associated with the 26 S proteasome appear not glycosylated it is likely that the receptor is withdrawn from the ER for degradation. This interpretation is supported by two observations. First, there is good evidence that a large proportion of mgluR1α is found in the ER in different cell types including neuron-like cells [17]. The receptor shuttles in and out of the plasma membrane in a ligand-dependent manner. Secondly, there is currently no accepted mechanism by which transmembrane proteins can be removed from the plasma membrane and delivered to the 26 S proteasome. Indeed, there is growing new evidence that mono-ubiquitylation is one of the signals that links receptors to clathrin-coated pits and subsequent endocytosis to deliver membrane proteins to the endosome/lysosomes for degradation [18]. It is still formally possible that proteasomes are involved in this process but there is no clear evidence to support this proposal. Currently, there is no evidence that mGluR1α is ubiquitylated. If this is true, then Homer-3 proteins would offer a direct link to deliver mGluRs from the ER to the 26 S proteasome independent of protein ubiquitylation. The number and complexity of proteins, including mGluRs, in the post-synaptic density may have led to a ubiquitin-independent delivery of proteins to the 26 S proteasome in the course of neuronal evolution [19]. Homer-3 proteins may have a general role in linking transmembrane proteins to the 26 S proteasome since the Homer EVH-like binding motif PPXXFX is found in many proteins including the ER inositol phosphate receptor and ryanodine receptor, ER cytochrome P450, the Homer-associated Shank proteins and P/A-type voltage-gated calcium channels [13].

However, it should be noted that there is good evidence for ubiquitylated proteins in synapses [19]. Furthermore, the ubiquitin pathway has a pivotal role in synaptogenesis [20], the formation of neuromuscular junctions [21] and neurotransmitter receptor functions. The stability of the inhibitory γ-aminobutyric acid (GABA) A receptor [22], α1 glycine receptor [23], β_2-adrenergic receptor [24] and opiate receptors [25] are regulated by the ubiquitin-proteasome system. Clearly, the ubiquitin-proteasome system has a major role in neuronal development and homoeostasis. The realization of the importance of synaptic dynamics for synaptic plasticity and long-term potentiation and depression [26] suggests that there will be many more roles for the ubiquitin-proteasome system in controlling synapse functions.

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