CHARACTERIZATION OF PROTEASOME ASSOCIATED PROTEINS

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Abstract

To maintain the protein homeostasis and thus ensure a normal running of the molecular process in the cell, there is a constant turnover of the proteins. The proteins are constantly synthesized and degraded. The half-life of proteins goes from some minutes to months. One of the main paths of the degradation in cells is the UPS (ubiquitin proteasome system) which agent of degradation is the proteasome. The proteasome is responsible for the degradation of around 80% of intracellular proteins. The proteasome is a multi-subunit complex composed of the barrel shaped core particle, site of the enzymatic activity, associated at its extremities by two regulatory particles, the 19S (PA700). The subunits of the core particle are α1–α7 (outer ring) and β1–β7 (inner ring). The 19S consists of ATPase-subunits (Rpt1–6) and non-ATPase subunits (Rpn1–3, 5–12). Due to its functions, the proteasome is involved in cell cycle control, cell stress response, immune response and various degenerative diseases like Parkinson' disease. Because of its importance the understanding of the dynamics and regulation of the proteasome is critical. A first step toward this understanding is to know its components and their functions. Already, the subunits of the 20S and 19S are well known. A couple of regulatory proteins associated to the proteasome had also been well characterized. The most common regulatory proteins are PA28α/β, PA28γ, PA200, and ECM29. However, the proteasome is a dynamic complex which can change its composition according to the needs of the cells. For example, the β1, β2, and β5 subunits are substituted by β1i, β2i, and β5i during the immune response. In addition to those well-known regulatory particles, it is accepted that the proteasome binds with proteins in sub-stoichiometric amounts in a more or less loose way. Furthermore, in our laboratory, single or multiple KO mice for those particles results in viable mice with seemingly normal life and only few are showing an interesting phenotype like the PA28γ mice which present a retardation of growth. This thesis represents an

attempt to find the new proteins associated to the proteasome, which allows it to maintain a function in those conditions. By using the single or multiple KO mice for PA28α/PA28β, PA28γ, PA200, and/or ECM29, I changed the normal condition of the proteasome, which allowed new proteins involved to be more visible and thus easier to identify. From the liver of those mice, I was able to purify proteasomes after improvement of a gentle affinity method retaining more proteins associated. This method allowed me to identify by Mass Spectrometry the proteins, which present a variation on the different proteasome's profile. Among them I identified HSP90, a heat shock protein involved in the folding of proteins. I then showed that HSP90 in the absence of ECM29 binds in a greater quantity to the proteasome and helps it to maintain its 26S structure, qualifying HSP90 as a new proteasome's regulatory protein associated. Because the proteasome is not a constant single unified complex through time, and binds constantly to various proteins, I had to use a very gentle method of purification. Overall my work more than identifying HSP90 as a new regulatory particle for the structure of the proteasome, provides the material (mice strains) and the experimental protocol for the study of the proteasome in its various forms, in various organs and during different stress conditions.

Abbreviations

ATP	: adenosine triphosphate
CBB	: coomassie brilliant blue
CP	: core particle
DMEM	: dubelcco's modified eagle's medium
DMSO	: dimethyl sulfoxide
DNA	: deoxyribonucleic acid
DTT	: dithiothreitol
GA	: geldanamycin
GDGC	: glycerol density gradient centrifugation
HRP	: horseradish peroxidase
IFN-γ	: interferon γ
KDa	: kilo Dalton
KO	: knock out
LPS	: lipopolysaccharide
MALDI-TOF	: matrix assisted laser desorption/ionization-time of flight
MEF	: mouse embryonic fibroblast
MHC-I	: major histocompatibility complex-I
MS	: mass spectrometry
PAGE	: poly acrylamide gel electrophoresis
PBS	: phosphate buffered saline
PMF	: peptide mass fingerprint
PVDF	: polyvinylidene fluoride

RP : regulatory particle

RPM : rotation per minute

RT : room temperature

SDS : sodium dodecyl sulfate

TEV : tobacco etch virus

TNFα : tumor necrosis factor α

TRIS : tris (hydroxymethyl) aminomethane

Ub : ubiquitin

UPS : ubiquitin Proteasome System

WB : western blot

WST : water soluble tetrazolium salts

Introduction

Overview

The degradation of proteins has for a long time been a neglected subject, indeed scientists were mainly focusing on the DNA and the transmission of the information through RNA to the proteins. Because the formation of proteins consumes a great amount of energy, it was believed that proteins were a very stable entity, rarely undergoing degradation [folin]. It is only from 1940 with the works of Schoenheimer et al using isotopically labeled compounds that the idea of the proteins turn over emerged [105]. Later, the discovery of the lysosome, a membrane enclosed vacuole containing various enzymes strengthen this theory. However, the functioning of the lysosome which degrades bulk of proteins without selectivity couldn't account for the difference in proteins' life time [103, 38] or for the energy dependent degradation observed. In the late seventies, multiple works done on the reticulocytes, a terminally differentiating red blood cell that do not contain lysosomes [31, 50] offered a breakthrough for the characterization of the enzymes involved in the non-lysosome degradation. These works lead to the identification of the ubiquitin (Ub) [22, 23] as a part of a lysosome independent system of degradation. Ub is a small well conserved protein of 8.5 KDa binding to a protein, and thus tagging it for degradation. Next was the identification of three enzymes involved in the cascade of reactions for the binding of the Ub [21, 48, 49]. Finally, in the late eighties, Hough et al partially purified and characterized the downstream protease that specifically recognize ubiquitinated substrates; the 26S proteasome [53]. From then, more evidence led to the acceptance of the Ubiquitin Proteasome system (UPS) as the non-lysosome system of degradation. Studies from the last three decades showed that this selective, ATP dependent system is responsible for the degradation of 80% of the intracellular proteins [Rock et al, Lee and Goldberg (66, 67)]. It is composed of 3 main elements. The Ub that tags proteins for degradation, the enzymes E1 (Ubiquitin activating enzyme), E2 (Ubiquitin conjugating enzyme)

and E3 (ubiquitin ligase) that bind the Ub to the targeted proteins, and the proteasome, a multi subunit complex responsible for the degradation.

The UPS has been involved in major cellular process such as cell division, protein quality control, transcription, DNA repair, immune response and so on [51, 102, 37, 20]. Due to the importance of the UPS, the understanding of its dynamics and regulation is critical. One segment of this understanding is the characterization of the proteasome, by the identification of its components and their functions. Already, the subunits of the 20S (CP) particle and 19S (RP) particle that bind to form the 26S proteasome are well known. A couple of proteins associated to the proteasome and regulating its activity have also been identified. Among them, the well-known proteins are PA28α/β, PA28γ, PA200, and ECM29. However, the increasing number of interacting proteins identified suggests that the proteasomes, as they exist in the cell, are more diverse in composition than previously assumed.

The common method to study the protein associated to the proteasome are based on the purification of the proteasome and the identification of the proteins co-purified with it. The challenge in this strategy lies in the purification step. Indeed, the proteins associated to the proteasome bind to it in the more or less loose way and risk to be dissociated and lost during the various step of the purification [99]. Conventional purification of the 26S proteasome involves multiple chromatographic steps with an exposure to high salt concentrations during ion exchange which tends to strip away the more loosely associated proteins. Recently, more gentle affinity methods were developed and led to the identification of more proteins associated to the proteasome [122, 68, 99, 10].

In our laboratory, single or multiple KO mutant mice for the well-known regulatory particle of the proteasome (PA28α/β, PA28γ, PA200, and/or ECM29) were generated and maintained.

Interestingly, the resulting mice were viable and few were showing an aberrant phenotype. This thesis represents an attempt to find the new proteins associated to the proteasome that permit the maintenance of the proteasome activity in the KO conditions. I used a gentle affinity purification method deemed more efficient [68] to purify the proteasome directly from mice organs and analyze its components by Mass Spectrometry. I identified more than 60 proteins co-purified with the proteasome. Among them, I identified Hsp90, a heat shock protein involved in the folding of proteins. I then showed that Hsp90 in the absence of ECM29 helps the proteasome to maintain its 26S structure, qualifying Hsp90 as a new proteasome's regulatory protein associated. This thesis is the first study coupling the use of KO mice (single or multiple) for the regulatory particles of the proteasome and the purification method from Leggett et al [68] developed in yeast, to conduct a broad study of the proteasome components in mice' organs.