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論文内容の要旨

It is generally accepted that proteolysis plays an important role in the regulation of the eukaryotic cell cycle. Eukaryotic cells contain a nonlysosomal large protease called the proteasome (or the multicatalytic protease) which is found in all eukaryotes, from yeast to man. Although it has been suggested that proteasomes are involved in the ubiquitin-dependent proteolytic system, their exact role in intracellular protein breakdown is still uncertain. Recent studies using various protease inhibitors suggest that proteasomes are involved in meiotic maturation of animal oocytes. More recent studies have demonstrated that the termination of mitosis and meiosis, transition from metaphase to anaphase is induced by the degradation of cyclin B, a regulatory subunit of maturation-promoting factor or M-phase promoting factor (MPF). Furthermore, it has been suggested that cyclin B is degraded by an ubiquitin-dependent proteolytic pathway. Since proteasomes are known to be a component of the ubiquitin-dependent proteolytic system, it can be hypothesized that proteasomes play a role in cyclin degradation. However, there is no direct evidence for this hypothesis at present.

Fish oocytes provide an appropriate experimental system to investigate the above problems. In recent years, considerable progress has been made in identifying the factors responsible for the regulation of meiotic maturation of fish oocytes; these include the isolation and characterization of a fish maturation-inducing hormone (17α,20β-dihydroxy-4-pregnen-3-one, 17α,20β-DP) and the components of MPF ($p34^{cdc2}$, the catalytic subunit and cyclin, the regulatory subunit).

The present study was designed to investigate (1) the structure of proteasomes present in goldfish oocytes, and (2) the possible role of the enzymes in the regulation of oocyte maturation and egg activation. A particular emphasis was placed on the role of proteasomes in cyclin B degradation, the event which is crucial in exiting metaphase and entering the next interphase during the cell cycle. To this end, a SDS-dependent (latent) proteasome was purified and characterized from goldfish oocyte cytosol and raised monoclonal antibodies against this enzyme. The cytosol fraction (150,000 g supernatants) of goldfish ovary hydrolyzed a fluorogenic peptide, succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide, a well-known

substrate for proteasome, regardless of the addition of SDS to the reaction mixture. Four steps of column chromatography resulted in a 135-fold purification of proteasome from this supernatant. Both SDS-independent and SDS-dependent hydrolyzing activities co-migrated during purification. However, the SDS-independent activity was markedly reduced during purification. The purified SDS-dependent proteasome (latent proteasome) possessed weak hydrolyzing activity (chymotrypsin-, trypsin-, and V8 protease-like activities) even in the absence of SDS; the chymotrypsin- and V8 protease-like activities were significantly increased in the presence of SDS. Its molecular weight and sedimentation coefficient were estimated to be 620 kDa and 19.2 S, respectively. Three kinds of monoclonal antibodies were raised against the purified latent proteasome. Western blot analyses revealed that these antibodies recognized a single species of protein on native PAGE, but recognized several subunits ranging in molecular mass from 23.5 to 31.5 kDa on SDS-PAGE. Cytosol fractions containing the SDS-independent activity had a band which migrated slower than that of purified latent proteasome. The SDS-independent protease activity was depleted when the cytosol fraction was immunoprecipitated with the anti-latent proteasome antibody. From these structural and enzymatical properties it is concluded that the purified proteasome corresponds to 20 S proteasomes reported in other eukaryotic cells.

The preceding studies demonstrated that although goldfish oocyte cytosols contain an active form of proteasome which can hydrolyze its substrates in the absence of activators, this enzymatic activity markedly reduced during further purification. A new procedure involving the use of a step elution and ATP was developed to prevent the loss of activity during purification. Using this method, active proteasome was purified to homogeneity from ovarian cytosol using five steps of chromatography. The purified active proteasome had chymotrypsin-, trypsin-, and V8 protease-like activities even in the absence of SDS. The enzyme exhibited two bands on native PAGE. Electrophoresis and Western blot analyses showed that the enzyme consists of at least 15 protein components ranging in molecular mass from 35.5 to 140 kDa, as well as the multiple subunits of the latent proteasome (20 S proteasome) ranging in molecular mass from 23.5 to 31.5 kDa. The molecular weight and sedimentation coefficient of the active proteasome

were estimated to be 1,200 kDa and 29.4 S, respectively, both of which are larger than those of the latent proteasome of the same species. In electron micrographs, the active proteasome appeared as a dumbbell-like image. It is concluded that the active proteasome purified from goldfish oocyte cytosol is identical to the 26 S proteolytic complex reported in human and rabbit.

As a first step to understand the possible function of proteasomes during meiotic maturation of goldfish oocytes, changes in the protein levels and activity of protesomes during oocyte maturation and egg activation were examined. Proteasome activity was measured using the fluorogenic peptide. During oocyte maturation, the activity and protein levels of proteasome changed synchronously with two peaks, one prior to the migration of germinal vesicle and the other just after the completion of germinal vesicle breakdown. Upon egg activation, there was a two-fold increase in the activity of proteasome, followed by a sharp drop soon after cyclin B degradation. This drop in the enzyme activity was coincident with a significant decrease in the levels of immunoreactive proteasome components, except for one component which was weakly stained with one of the anti-proteasome monoclonal antibodies. These results suggest that active proteasome is involved in the regulation of oocyte maturation and egg activation, and further support the hypothesis that active proteasome may play an important role in the regulation of MPF inactivation, namely cyclin degradation, which occurs immediately after egg activation.

Next, the possible role of active proteasome in the regulation of cyclin B degradation was investigated, for the first time, using *E. coli* produced goldfish cyclin B and purified goldfish active proteasome. It was found that active proteasome can digest the wild cyclin B, producing an intermediate cyclin B protein (42 kDa). In contrast, cyclin B mutants lacking the first 42, 68, and 96 N-terminal amino acids were not digested with active proteasome, suggesting that the N-terminal amino acids are necessary for cyclin B degradation. Amino acid sequence analysis of the 42 kDa intermediate protein revealed that active proteasome cuts the C-terminal peptide bond of lysine 57. Experiments using various protease inhibitors suggest that trypsin-like activity may be responsible for cyclin B degradation. Full-length goldfish cyclin B was also degraded in *Xenopus* egg extracts after activation by the addition of Ca^{2+}. Taken together, these results provide the first evidence to indicate that proteasomes are involved in the regulation of cyclin B

degradation.

Ubiquitin was purified from the cytosol fraction of goldfish ovaries containing full-grown postvitellogenic oocytes using four steps of column chromatagraphy. It was also shown that a large amount of ubiquitin occurs as a free polypeptide in immature oocytes of goldfish. Furthermore, approximately the same amount of proteasome as ubiquitin was present in immature oocytes. These findings are consistent with the notion that both proteasome and ubiquitin are involved in cyclin B degradation during the meiotic cell cycle of goldfish oocytes and eggs.

Based on the data presented in this study, together with those of other studies, a hypothetical model for the possible participation of proteasomes in the regulation of cyclin B degradation is presented as follows. Upon fertilization (egg activation), an increase in Ca^{2+} activates a Ca^{2+}/calmodulin-dependent protein kinase. This serine/threonine kinase then either directly activates proteasome or makes cyclin B a better substrate for the proteasome through an unknown mechanism. Proteasome then cuts the N-terminus (Lys 57) of cyclin B and expose an ubiquitinating site of cyclin B, thus enabling cyclin B to interact with ubiquitinating enzymes. After ubiquitination, cyclin B-ubiquitin complexes are degraded by active proteasome to small peptides, leading to the inactivation of MPF.

論文の審査結果の要旨

卵成熟促進因子(maturation-promoting factor, MPF)は動物卵に減数分裂を再開させ受精能を獲得させる成熟因子であるばかりでなく、細胞分裂一般においてもG2期からM期へ移行させるM期促進因子でもある。MPFは触媒ユニットのcdc2キナーゼと調節ユニットのサイクリンBからなり、その活性はサイクリンBの合成と分解によるcdc2キナーゼのリン酸化、脱リン酸化によって制御されている。このうちMPF活性化の機構については、cdc2キナーゼのリン酸化部位、リン酸、脱リン酸化酵素などの実体も明らかにされつつある。しかし、MPF不活性化の機構に関しては、サイクリンBの分解によることが知られているものの、その分解機構は不明である。申請者の徳元俊伸は、このMPFに不活性化をもたらすサイクリンBの分解機構を解明することを目的として、キンギョ(Carassius auratus)卵からATP依存性プロテアーゼ複合体であるプロテアゾームを精製し、その化学構造を明らかにするとともにサイクリン分解に果たす役割を調べた。

キンギョの未成熟卵の150,000g遠心上清より不活性型(20S)と活性型(26S)プロテアゾームを精製しその化学構造を決定した。キンギョの26Sプロテアゾームは分子量20,000-140,000の25種類のサブユニットからなり、中心部に20Sプロテアゾームを有するダンベル状構造を示すことが明らかになった。またキンギョプロテアゾームに対する数種のモノクロナル抗体を作製した。つぎに、こらら精製26Sプロテアゾーム、モノクロナル抗体、及び大腸菌で発現されたキンギョの種々の変異サイクリンBを用いて、プロテアゾームのサイクリン分解に果たす役割をin vitroの実験系で解析した。その結果、キンギョのサイクリンBの分解は、26Sプロテアゾームによるリジン残基（N末端57番目）C末端の切断を引き金として起こることがはじめて明らかになった。また、同じサイクリンB中間体はキンギョ卵のふ活（受精）時にみられるMPFの不活性化の際にも認められた。

申請者の研究は、MPF不活性化をもたらすサイクリンBの分解が、プロテアゾームによる限定分解を初期反応とした一連の蛋白質分解カスケードによるものであることを示した最初の研究であり、動物卵の成熟機構の研究のみならず細胞周期一般の調節機構の研究に大きく貢献したと判断され、審査委員会は本博士論文に対して、合格の判定を下した。