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The Proteasome Is an Essential Mediator of the Activation of Pre-MPF during Starfish Oocyte Maturation

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Starfish oocyte maturation was blocked by the addition of 100 μM MG115, a potent proteasome inhibitor, whereas no inhibition was observed by membrane permeable cysteine protease inhibitor, E-64-d. The inhibition by MG115 was diminished by adding at a time corresponding to the half time required for germinal vesicle breakdown. Potent inhibition of germinal vesicle breakdown was also observed by microinjection of anti-proteasome-a-subunit antibodies. The antibody-injected oocytes failed to activate pre-maturation promoting factor (pre-MPF), since the dephosphorylation of phospho-Tyr15 in cdc2 kinase was not observed even in the presence of 1-methyadenine, a maturation-inducing hormone. These results indicate that the proteasome triggers the activation of pre-MPF via the dephosphorylation of cdc2 kinase in the signal transduction pathway in response to the hormonal stimulus during starfish oocyte maturation.

Intracellular abnormal and short-lived proteins are tagged with multi-ubiquitin chains and then degraded by the 26S proteasome in an ATP-dependent manner (see reviews 1-3). This 26S proteasome (1500-2000-kDa complex) is dissociated into two subcomplexes in the absence of ATP: the 20S proteasome (650-700-kDa protease core complex) and the regulatory complex (700-1000-kDa complex containing the ATPase subunits). The 20S proteasome is a cylindrical protein complex comprising of 4 stacked 7-membered rings: two inner rings consisting of b-subunits and outer rings consisting of a-subunits.

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Abbreviations used: GVBD, germinal vesicle breakdown; MCA, 4-methylcoumaryl-7-amide; MPF, maturation-promoting factor; Suc, succinyl; Z, benzyloxycarbonyl.

Recent studies revealed that intracellular proteins involved in cell cycle or mitosis such as cyclins (4), p53 (5), Mos (6), cyclin-dependent kinase inhibitors (7, 8), and nuclear oncoproteins (9) including Myc, Fos, Jun and E1A are degraded by ubiquitin-proteasome pathway (see also reviews: 3, 10). However, the role of proteasome in meiosis has not been studied well (11-13).

Starfish oocytes are arrested at the first meiotic prophase and the meiosis is reinitiated by 1-methyladenine, a maturation-inducing hormone, secreted from the follicle cells (14). Addition of 1-methyladenine to oocytes activates pre-maturation-promoting factor (pre-MPF) to maturation-promoting factor (MPF), which leads to germinal vesicle breakdown (GVBD), chromosome condensation, extrusion of the first and second polar bodies, and formation of a female pronucleus. In our previous paper (12), we proposed the chymotrypsin-like activity in proteasome may involved in starfish oocyte maturation, since the inhibitor spectra of leupeptin analogs toward the chymotrypsin-like activity of the 650-kDa protease in oocyte extracts coincided well with the inhibitor spectrum toward GVBD.

In this paper, we show that the proteasome triggers the activation of pre-MPF via the dephosphorylation of phospho-Tyr15 of cdc2 in the signal transduction pathway in response to a maturation-inducing hormone.

MATERIALS AND METHODS

Chemicals. Suc-Leu-Leu-Val-Tyr-MCA, 7-amino-4-methylcoumarin, MG115 (Z-Leu-Leu-norvalinal), and E-64-d ([L-3-*trans*-ethoxycarbonyloxirane-2-carbonyl]-L-leucine (3-methylbutyl)amide) were purchased from Peptide Institute, Inc. (Osaka, Japan). FPLC system equipped with Superose 6 HR10/30 is a product of Pharmacia-LKB. Polyclonal antibody specific to Tyr15-phosphorylated cdc2 were purchased from New England Biolabs., Inc.

Animals. The starfish, *Asterina pectinifera,* were collected at Mutsu Bay near Asamushi (Aomori Prefecture), Japan.

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Assay of GVBD. Immature oocytes were preincubated at 20°C for 30 min with or without inhibitors followed by the addition of 250 nM 1-methyladenine. After incubation for 1h at 20°C, the ratios of GVBD were counted under a microscope(12). Dimethylsulfoxide at a concentration of 2% was used in all the assays to dissolve the inhibitor. This concentration of dimethylsulfoxide shows no inhibitory effect on GVBD (15).

Preparation of antibodies. The trideca peptide (FSPEGRLYQVEYA, 99.2 % pure) which corresponds to the first consensus sequence of the α-subunit of 20S proteasome was synthesized and purified by Hokudo Co. Ltd. (Sapporo). The peptide was cross-linked to keyhole limpet hemocyanin with glutaraldehyde, and immunized 5 times to two JW rabbits. The anti-α-subunit peptide antibodies (IgG) were purified by POROS Protein A Consep LC (Millipore). The non-immune rabbit IgG was similarly purified and used as a control IgG. The above purified antibodies supplied by Hokudo, were dialyzed against Milli Q water, and were then concentrated by a Taitec Centrifugal Concentrator. The IgG solutions were adequately diluted with 10-times concentrated PBS and Milli Q water up to a final concentration of 100 mg/ml IgG in PBS.

Microinjection of antibodies. Each antibody was microinjected into the cytoplasm of immature oocytes with a sharp glass needle by the pressure injection method using a wedge-shaped egg holder (16). The final concentration of each antibody in the oocyte was adjusted to be 2 mg/ml. These oocytes, after microinjection or without injection, were collected in a dish filled with natural seawater and incubated for 15 min before the application of 1-methyladenine. The meiosis was reinitiated by the addition of 1-methyladenine into the surrounding seawater. The percentage of GVBD was monitored under a microscope after incubation for 40 min and 60 min at 20°C following the addition of 1-methyladenine. The breakdown of germinal vesicle occurred at around 30 min in control oocytes from the same animals.

Western blotting. The secondary antibody, a horseradish peroxidase-conjugated anti-rabbit IgG goat antibody, was pretreated with PBS containing 0.1% Tween 20 and 3% skimmed milk, and then with the primary antibody in the blocking solution, since the secondary antibody strongly reacted to the microinjected rabbit IgG on the nitrocellulose membrane. The excess amount of the secondary antibody was reacted to the rabbit antiserum (anti-20Sα). The complex of primary and secondary antibodies was used in this study. Detection was performed by the Enhanced ChemiLuminescence system (Amersham) according to the manufacturer's protocol.

RESULTS

Effects of proteasome inhibitors on GVBD. Although we proposed that the Z-Phe-Ser-argininal-susceptible protease involved in the starfish oocyte maturation is the chymotrypsin-like protease contained in the proteasome complex by examining their effects on GVBD and proteasome activities in our previous study, we could not rule out the possibility that peptidyl-argininals may affect some undetectable trypsin-like enzyme(s) potentially involved in the starfish oocyte maturation rather than the proteasome. We examined, therefore, the effect on GVBD of Z-Leu-Leu-norvalinal (MG115), which is a potent inhibitor against proteasomes (17) but not against typical trypsin-like proteases on the structural basis. As shown in Fig. 1, MG115 inhibited GVBD by 40% at 50 μM and 90% at 100 μM. As MG115 is reported to inhibit cathepsin B and calpain activities in addition to the proteasome activity (17), the effect of E-64-d on GVBD was also investigated. E-64-d, a membrane permeable potent inhibitor against cysteine proteinases including cathepsins and calpain, showed no inhibitory effect on GVBD at 100 μM. These results suggest that proteasome, but neither cathepsins nor calpain, plays a key role in the starfish oocyte maturation.

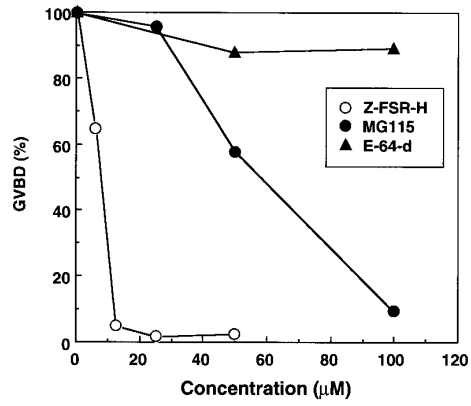

FIG. 1. Effect of protease inhibitors on GVBD. The starfish oocytes were suspended in artificial seawater containing the respective inhibitor, and treated with 1-methyladenine at a final concentration of 250 nM. - ○ -, Z-Phe-Ser-argininal; - ● -, MG115 (Z-Leu-Leu-norvalinal); - ▲ -, E-64-d.

Timing of the function of proteasome in 1-methyladenine -induced oocyte maturation. The timing of the function of the proteasome was investigated by adding MG115 at various times after 1-methyladenine treatment (Fig. 2). Inhibitory potency of MG115 was diminished at about 10 min, which is half the time required for GVBD. The timing of the function was similar to the chymostatin-susceptible protease (18). This also supports our previous assumption that the chymostatin-sensitive protease is the proteasome.

Effects of microinjection of anti-20S proteasome a-subunit antibody on GVBD. In order to obtain further evidence for the participation of the proteasome in GVBD, we examine the effects on GVBD of the microinjection of antibodies raised against the first consensus trideca peptide sequence (FSPEGRLYQVEYA) of the 20S proteasome α-subunit. This immunogenic peptide moiety corresponds to the H0 α-helix of the α-subunit of the 20S proteasome (19) and is considered to be involved in interaction of the α-subunit with the regulatory subunit complex. The anti-proteasomal α-subunit antibodies were capable of immunoprecipitating the proteasome (data not shown).

Two batches of the anti-proteasome antibodies substantially inhibited, rather than delayed, 1-methyladenine -induced GVBD (Fig. 3). In the control non-injected oocytes, GVBD was completed within 40 min after the addition of 1-methyladenine. At 60 min, the protrusion of the first polar body took place. In the experiment, after injection of the antibody, a germinal

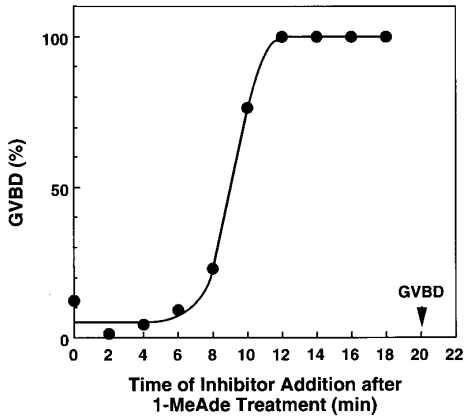

FIG. 2. Timing of the function of proteasome in oocyte maturation. The starfish oocytes were treated with 250 nM 1-methyladenine, and MG115 was added into the surrounding seawater at a final concentration of 100 μM at the indicated time. GVBD was monitored under a microscope 60 min after the addition of 1-methyladenine.

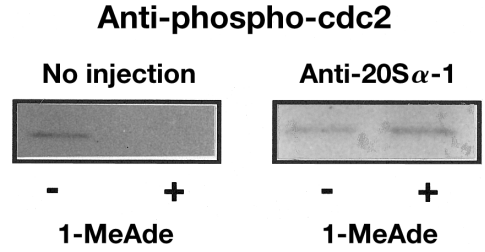

FIG. 4. Inhibition of dephosphorylation of phospho-Tyr15 of cdc2 kinase by microinjection of anti-20S proteasome α-subunit antibody was revealed by Western blotting with phospho-cdc2 (Tyr15) specific antibody as a primary antibody. Thirty oocytes, after microinjection or without injection of the antibodies, were collected in a dish filled with natural seawater and treated with 100 nM 1-methyladenine. After 40 min, the oocytes were collected and frozen. After thawing, the oocytes were homogenized with a Teflon homogenizer in 40 μl PBS, and a portion (20 μl) of the extract was subjected to SDS-PAGE and Western blotting with anti-phospho-cdc2 (Tyr15) specific antibody as a primary antibody. Note that anti-20S proteasome antibody potently inhibits the 1-methyladenine -induced dephosphorylation of Tyr15 of cdc2 kinase.

vesicle remained intact or a transparent part of the cytoplasm which has derived from the nucleoplasm, remained in most of oocytes at 60 min after the application of 1-methyladenine. No appreciable inhibition was observed toward GVBD by non-immune antibodies. These results clearly demonstrate the participation of proteasome in the starfish oocyte maturation.

Proteasome triggers dephosphorylation of phospho-Tyr15 of cdc2 kinase in the signal transduction under the influence of 1-methyladenine. Although the participation of the proteasome in GVBD was indicated by the above experiments, the molecular mechanisms of its function in the maturing process is still unclear. Since pre-MPF is activated by dephosphorylation of phospho-Tyr15 of cdc2 kinase, which is catalyzed by cdc25 protein phosphatase, we next investigated whether the antibody against the 20S proteasome a-subunit prevents the dephosphorylation of phospho-Tyr15 of cdc2 kinase under the influence of 1-methyladenine, using a specific antibody against the phospho-cdc2 kinase. As indicated in Fig. 4, the microinjected anti-20S proteasome α-subunit antibody potently blocked the dephosphorylation of phospho-Tyr15 of cdc2 kinase, which is induced by 1-methyladenine. These results indicate that the proteasome is involved in a process which triggers the dephosphorylation of phospho-Tyr15 in cdc2 kinase in the signal transduction pathway in response to 1-methyladenine stimulus.

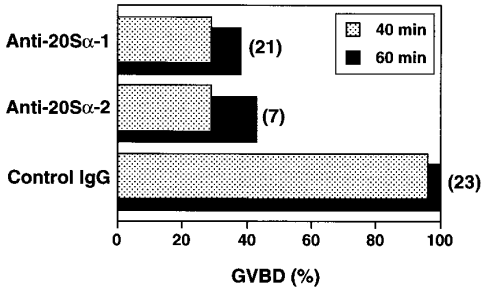

FIG. 3. Effect of microinjection of anti-20S proteasome α-subunit peptide antibodies on GVBD. Two lots (anti-20Sα-1, anti-20Sα-2) of anti-20S proteasome α-subunit-peptide antibody (IgG), and non-immune IgG dissolved in PBS were used. Each IgG (100 mg/ml) was microinjected at a final concentration of 2% of the volume of oocytes. The oocytes, after injection or without injection, were placed on separate dishes, and maturation was induced by adding 500 nM 1-methyladenine. All the oocytes without injection underwent GVBD within 40 min. The stippled and closed bars indicate the ratio of GVBD 40 min and 60 min after the addition of 1-methyladenine, respectively. The number of microinjected oocytes is indicated in parentheses.

DISCUSSION

It is currently believed that an intracellular protease is involved in the starfish oocyte maturation, especially in the process of activation of pre-maturation promoting factor (pre-MPF), since GVBD in 1-methyladenine-induced starfish oocyte is inhibited by trypsin inhibitors including leupeptin (acetyl-Leu-Leu-argininal) (18, 20, 21), but the MPF-microinjected oocytes undergo GVBD even in the presence of leupeptin (21). The entity of pre-MPF in immature starfish oocytes is proposed to be a complex of Tyr15-phosphorylated cdc2 and dephosphorylated cyclin B (22). In the present study, we have demonstrated that the proteasome is definitively involved in the starfish oocyte maturation. It is well known that the proteasome plays a key role in mitotic cell cycle, especially in the degradation of cyclins (4), and of cyclin-dependent kinase inhibitors including $p27^{Kip}$ (7) and $p40^{SIC1}$ (8). However, the biological role of the proteasomes in meiosis is only poorly

understood. The present study revealed that the proteasome is an essential mediator in the signal transduction pathway induced by 1-methyladenine, which triggers a process(es) leading to the dephosphorylation of Tyr15-phosphorylated cdc2 kinase.

The IC_{50} value of MG115 toward GVBD appears to be much higher than the Ki values for the Suc-Leu-Leu-Val-Tyr-MCA-hydrolyzing activity of the proteasome (17). However, it is unlikely that the inhibitory effect of MG115 is due to the possible side effect on GVBD, since GVBD occurs normally in the presence of MG115 when added at about 15 min after the addition of 1-methyladenine. In addition, it has been reported that a relatively high concentration (about 100 μM) of MG115 is required for inhibition against the caseinolytic activity or the ubiquitin-conjugate-degrading activity of the 26S proteasome in contrast to the Suc-Leu-Leu-Val-Tyr-MCA-hydrolyzing activity (17).

The inhibitory ability of MG115 against GVBD largely depends on the batches of oocytes: 100 μM MG115 potently inhibited GVBD in some cases, while it only weakly inhibited (about 10-20% inhibition) in the other cases. This variance may be at least in part due to the degree of maturation of oocytes in the ovaries. Since the sensitivity of GVBD of immature oocytes to microbial protease inhibitors depends on the concentration of 1-methyladenine (unpublished results), it is not surprising that the concentration dependency of protease inhibitors on GVBD is variable depending on the quantity and quality of the hormone receptors and the components comprising the signal transduction pathway.

Based on the crystallographic analysis (19), the peptide sequence used for immunogen corresponds to the external region of the α-subunit, that faces the regulatory subunit complex. Since the 20S proteasome may be newly incorporated into the higher molecular mass complex in response to 1-methyladenine during the oocyte maturation (unpublished results), it seems likely that the antibody may interrupt the interaction between the 20S proteasome and the regulatory subunit complex. Alternatively, this antibody may even dissociate the preformed complex of the 20S proteasome and the regulatory subunit complex.

In starfish, dephosphorylation of phospho-Tyr15 of cdc2 kinase leads to the activation of cdc2 kinase, which corresponds to the activation of pre-MPF to MPF. This dephosphorylation is catalyzed by cdc25 phosphatase, which is known to be phosphorylated by the cdc25-activating protein kinase (23). In immature starfish oocytes, wee1 kinase protects the activation of pre-MPF by phosphorylating Thr14 and Tyr15 residues of cdc2 kinase (23). The wee1-suppresser is proposed to be activated in response to 1-methyladenine, which triggers the activation of pre-MPF in concert with cdc25 phosphatase (23). This suppresser may be a potential wee1-inhibitory kinase (23). Based on our present results, it is likely that the proteasome triggers the activation of cdc25 or the deactivation of wee1 via the possible phosphorylation cascade and protein degradation pathway.

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