Assembly of the 26S Proteasome Is Regulated by Phosphorylation of the p45/Rpt6 ATPase Subunit†

Kazuki Satoh, Hitoshi Sasajima, Ken-ichi Nyoumura, Hideyoshi Yokosawa, and Hitoshi Sawada*

Department of Biochemistry, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan

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ABSTRACT: We investigated whether the assembly/disassembly of the 26S proteasome is regulated by phosphorylation/dephosphorylation. The regulatory complex disassembled from the 26S proteasome was capable of phosphorylating the p45/Sug1/Rpt6 subunit, suggesting that the protein kinase is activated upon dissociation of the 26S proteasome or that the phosphorylation site of p45 becomes susceptible to the protein kinase. In addition, the p45-phosphorylated regulatory complex was found to be incorporated into the 26S proteasome. When the 26S proteasome was treated with alkaline phosphatase, it was dissociated into the 20S proteasome and the regulatory complex. Furthermore, the p45 subunit and the C3/α2 subunit were cross-linked with DTBP, whereas these subunits were not cross-linked by dephosphorylating the 26S proteasome. These results indicate that the 26S proteasome is disassembled into the constituent subcomplexes by dephosphorylation and that it is assembled by phosphorylation of p45 by a protein kinase, which is tightly associated with the regulatory complex. It was also revealed that the p45 subunit is directly associated with the 20S proteasome α-subunit C3 in a phosphorylation-dependent manner.

Intracellular proteins, including short-lived proteins such as cyclin, Mos, Myc, p53, NF-κB, and IκB, are degraded by the ubiquitin−proteasome system (*1*−*12*). In this system, the substrate proteins are tagged with multi-ubiquitin chains and are then degraded by the 26S proteasome (*1*−*12*). ATP hydrolysis is required for both ubiquitination and proteolysis (*1*−*12*). The 26S proteasome (a 2 MDa complex) is made up of two subcomplexes: the 20S proteasome and the regulatory complex. The former is a 700 kDa cylindrical protease complex consisting of four stacks of heptameric rings with 28 subunits (i.e., α7β7β7α7) with molecular masses of about 20−35 kDa, whereas the latter is a 700−1000 kDa complex consisting of at least 18 subunits with molecular masses of 28−110 kDa (*1*−*14*), including 6 putative ATPases (Rpt1−Rpt6) and 12 non-ATPase subunits (Rpn1−12) (for nomenclature, see ref *15*).

It is well-known that the 26S proteasome requires ATP hydrolysis for ATP-dependent protein degradation of ubiquitinated substrates and for the 26S proteasome assembly. However, the roles of ATP hydrolysis in protein degradation and also in the assembly of the 26S proteasome are still unclear. We previously reported that the highly purified 26S proteasome contains a protein kinase activity that is able to phosphorylate the regulatory subunits, including p45/Sug1/Rpt6 and substrate protein casein, and that this kinase may play a key role in ATP-dependent proteolysis because a good correlation exists between the inhibition pattern of protein kinase inhibitors against the phosphorylation of p45 and that against the ATP-dependent proteolytic activity (*16*).

In this study, we investigated whether phosphorylation of the p45 subunit by a protein kinase (we tentatively call p45-kinase) associated with the 26S proteasome is responsible for the assembly of the 26S proteasome and also whether the dephosphorylation of the 26S proteasome leads to its dissociation into the 20S proteasome and the regulatory complex. Since it has been reported that a *base* subcomplex of the regulatory particle, which contains S1, S2, and six putative ATPases (*14*), faces the α-subunits of the 20S proteasome, we speculated that the p45 subunit may directly interact with the α-subunit of the 20S proteasome. In this context, we attempted to identify the α-subunit of the 20S proteasome, which directly interacts with the p45 subunit.

MATERIALS AND METHODS

Suc-Leu-Leu-Val-Tyr-MCA[1] and 7-amino-4-methylcoumarin were purchased from the Peptide Institute. [γ-^{32}P]ATP, cold ATP (A2383), and DTBP were from ICN, Sigma, and Pierce, respectively. The FPLC (and ÄKTA FPLC) system equipped with Superose 6 HR10/30 and Mono Q HR5/5 is a product of Pharmacia-LKB. Calf intestinal alkaline phosphatase was obtained from Nippon Gene. Antibodies raised against the p45/S8/Sug1/Rpt6, Mss1/S7/Rpt1, p42/S10b/Rpt4, Tbp1/S6′/Rpt5, α-subunits (HC2, HC3, HC8, XAPC7, zeta, iota), HC3/α2, XAPC7/α4, and zeta/α5 were from Affiniti Research Products Ltd. A monoclonal antibody (IB5) specific to the p27 (iota/α1) subunit is a kind gift from Dr. Klaus Scherrer (*17*).

The 26S proteasome was purified from porcine cardiac muscles as described previously (*16*). SDS−PAGE, nonde-

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* To whom correspondence should be addressed. Tel: +81-11-706-3720. Fax: +81-11-706-4989. E-mail: hswd@pharm.hokudai.ac.jp.

[1] Abbreviations: DTBP, dimethyl 3,3′-dithiobispropionimidate; DTT, dithiothreitol; MCA, 4-methylcoumaryl-7-amide; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Suc, succinyl; 2D PAGE, two-dimensional PAGE.

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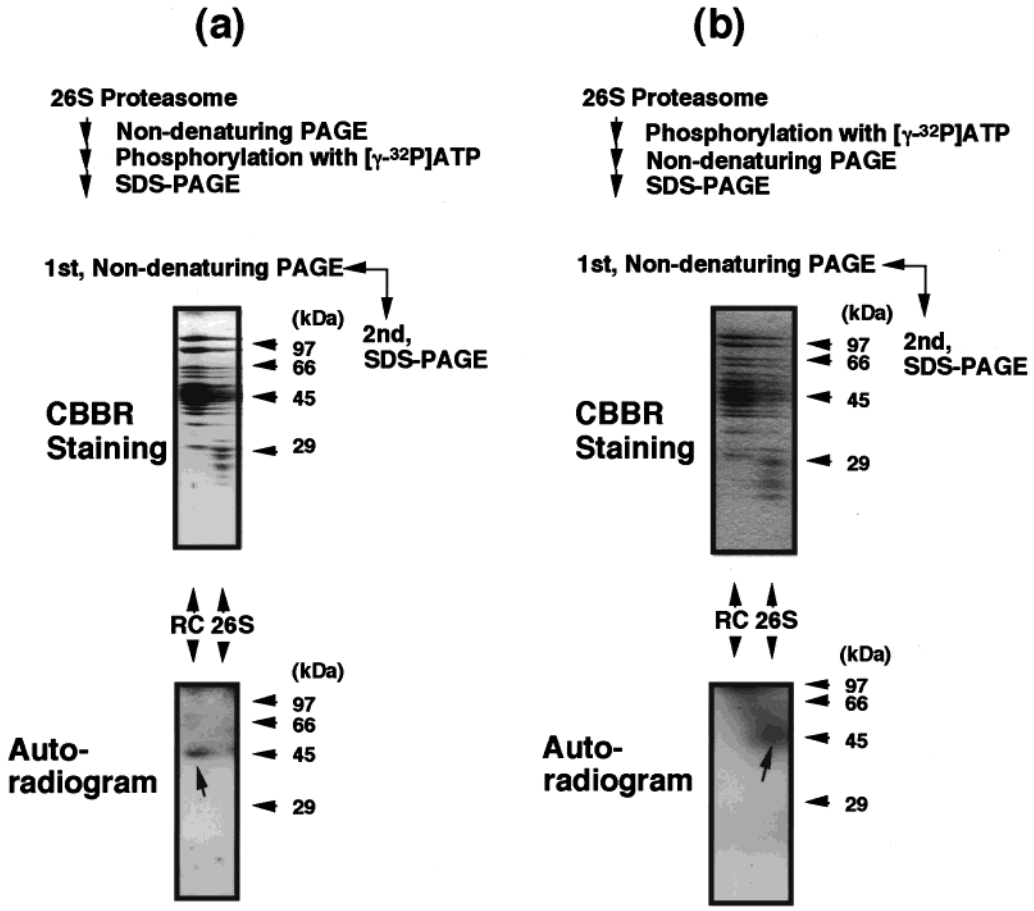

FIGURE 1: 2D PAGE of the 26S proteasome in the presence of [γ-^{32}P]ATP. (a) The 26S proteasome was subjected to nondenaturing PAGE followed by incubation with [γ-^{32}P]ATP/Mg for the in-gel kinase reaction and then second-dimensional SDS−PAGE. (b) The 26S proteasome was incubated with [γ-^{32}P]ATP/Mg for the kinase reaction and then subjected to 2D PAGE.

naturing PAGE, and 2D PAGE (first dimension, SDS−PAGE under nonreducing conditions; second dimension, SDS−PAGE under reducing conditions) were done as described in refs *13* and *18*).

After first-dimensional nondenaturing PAGE, the gel was immersed in 200 μL of the reaction mixture, which contained 50 mM Tris-HCl (pH 7.8), 2 mM ATP including [γ-^{32}P]ATP, 10 mM $MgCl_2$, and 1 mM DTT, and was incubated at 37 °C for 30 min. After addition of the sample buffer for SDS−PAGE, the gel was placed on the top of the slab gel for SDS−PAGE. After SDS−PAGE, proteins were stained with Coomassie Brilliant Blue R-250, and the phosphorylated bands were analyzed by autoradiography. Alternatively, the 26S proteasome (15 μg) was phosphorylated under the same conditions for the phosphorylation reaction and then subjected to 2D PAGE followed by autoradiography.

The purified 26S proteasomes (100 μg) were incubated with or without 1 unit of alkaline phosphatase for 1 h at 37 °C and were applied to a Superose 6 column equilibrated with 50 mM Tricine/NaOH (pH 7.8) containing 0.1 mM ATP, 10% glycerol, 1 mM DTT, and 0.1 M NaCl. Five hundred microliter fractions were collected at a flow rate of 0.25 mL/min.

Western blotting was performed according to ref *19*, by using antibodies to p45, Mss1, Tbp1, p42, C3, XAPC7, iota, zeta, and all the α-subunits (C2, C3, C8, C9, XAP7, zeta, iota).

RESULTS

We previously reported that the purified 26S proteasome dissociates into the 20S proteasome and the regulatory complex during nondenaturing PAGE and that the dissociation is inhibited by treatment with a bifunctional cross-linker (*13*, *16*, *18*). In addition, we detected a protein kinase activity in our highly purified 26S proteasome preparation (*16*). The fact that ATP hydrolysis is required for assembly of the 26S proteasome, together with our above-mentioned results, led us to propose the idea that the assembly of the 26S proteasome may be elicited by phosphorylation of the subunits via electrostatic interaction, located at the contact face between the 20S proteasome and the regulatory complex: the phosphorylated state might be labile, since the 26S proteasome easily dissociates into the 20S proteasome and the regulatory complex under ATP-depleted conditions or during nondenaturing PAGE without ATP.

In this context, we first attempted the following experiment. The purified 26S proteasome was subjected to nondenaturing PAGE to dissociate into two subcomplexes, and the gel was subsequently incubated with [γ-^{32}P]ATP in the buffer for the protein kinase assay in order to carry out the in-gel self-phosphorylation reaction. The gels thus incubated were rinsed repeatedly and subjected to second-dimensional SDS−PAGE. As shown in Figure 1a, only the dissociated regulatory complex, unlike the 26S proteasome, was able to label the p45 subunit. This result strongly suggests that self-phosphorylating activity toward the p45 subunit of the

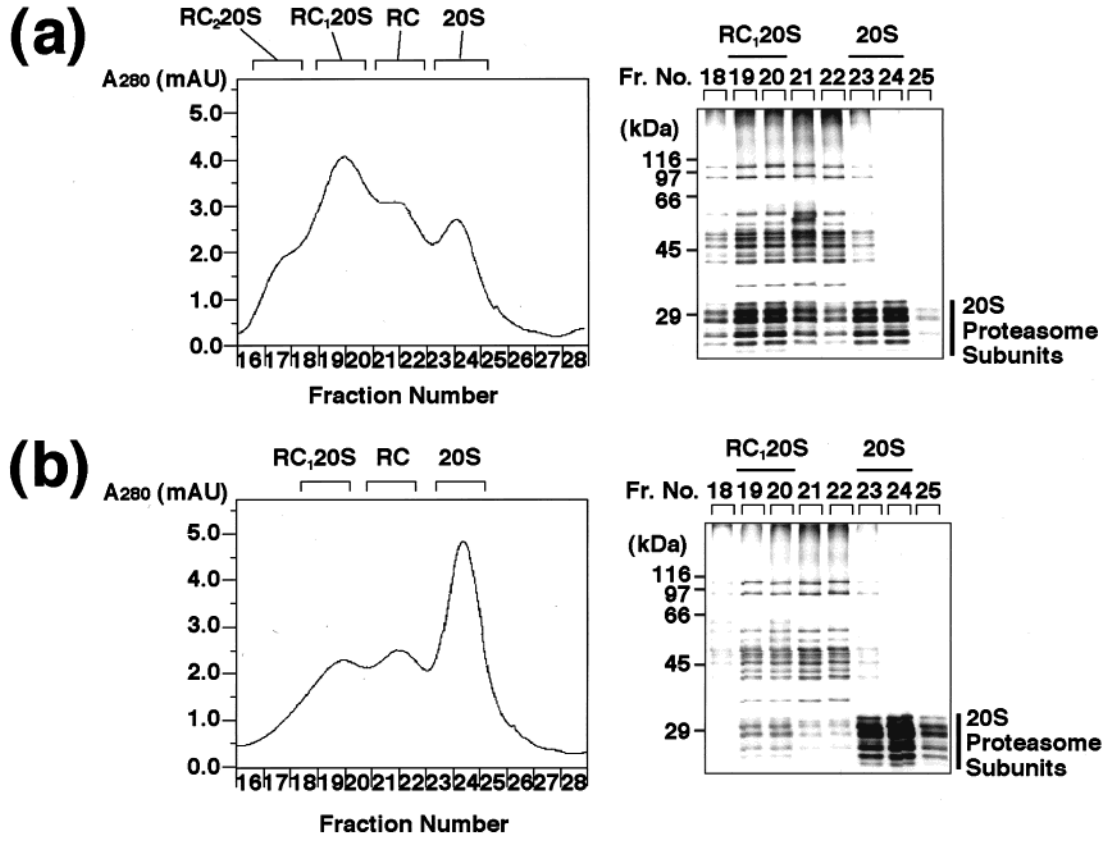

FIGURE 2: Dissociation of the 26S proteasome by phosphatase treatment. The 26S proteasome was incubated at 37 °C for 1 h in the absence (a) or presence (b) of alkaline phosphatase followed by Superose 6 gel filtration. $RC_2$20S, $RC_1$20S, RC, and 20S represent the elution positions of the 20S proteasome capped with two regulatory complexes, the 20S proteasome capped with one regulatory complex, the regulatory complex, and the 20S proteasome, respectively.

regulatory complex is activated upon dissociation of the 26S proteasome. Alternatively, the amino acid residue of p45, which is to be phosphorylated by p45 kinase, may be susceptible or exposed to p45 kinase upon dissociation. When we performed the same experiments at different gel concentrations, we could obtain the same results (data not shown). Since proteins are separated by nondenaturing PAGE depending on their size and charge, a protein kinase, if it is a contaminant, must be separated from the regulatory complex by electrophoresis at different gel concentrations (*20*). Therefore, our results clearly showed that the p45 subunit of the regulatory complex is phosphorylated by the kinase tightly associated with or contained in the regulatory complex.

Our next question was whether the phosphorylated regulatory complex, which is once dissociated from the 26S proteasome, is capable of assembling into the 26S proteasome. To assess this issue, the purified 26S proteasome was first incubated with [γ-^{32}P]ATP and was then subjected to 2D PAGE (first, nondenaturing PAGE; second, SDS−PAGE) (Figure 1b). The ^{32}P-labeled p45 was detected in the 26S proteasome but not in the regulatory complex. Taking into account that only the dissociated regulatory complex is capable of phosphorylating the p45 subunit (Figure 1a), this result strongly suggests that the once-dissociated regulatory complex containing the radiolabeled p45 associates with the 20S proteasome to make the 26S proteasome and that the extent of the 26S proteasome assembly is dependent on the phosphorylation of p45 in the dissociated regulatory complex by its intrinsic p45 kinase.

Since association of the regulatory complex with the 20S proteasome appears to be induced by phosphorylation of the p45 subunit within the regulatory complex, we next investigated whether dephosphorylation of the 26S proteasome results in dissociation of the 26S proteasome into the 20S proteasome and the regulatory complex. The purified 26S proteasome was incubated at 37 °C for 1 h in the presence or absence of alkaline phosphatase and was then subjected to Superose 6 gel filtration (Figure 2). By phosphatase treatment, a protein shoulder (fraction 17/18), which corresponds to the 20S proteasome capped with two regulatory complexes ($RC_2$20S), and a protein peak (fraction 19/20), which corresponds to the 20S proteasome capped with one regulatory complex ($RC_1$20S), were significantly decreased, while the peak of the 20S proteasome was markedly increased (comparison between panels a and b of Figure 2). The amount of regulatory complex was not markedly increased by phosphatase treatment. This may be explicable by the fact that the dissociated regulatory complex is apt to partially dissociate into the smaller subcomplexes such as lid and base subcomplexes (*14*) during nondenaturing PAGE and Superose 6 FPLC (data not shown). Alternatively, a small amount of the 20S proteasome may be included in the purified 26S proteasome preparation. On SDS−PAGE, the relative amount of the 20S proteasome subunits (20−35 kDa) in fractions 23/24 to that in fractions 19/20 was dramatically increased by phosphatase treatment. These results clearly indicate that the 26S proteasome is dissociated into the constituent subcomplexes by dephosphorylation.

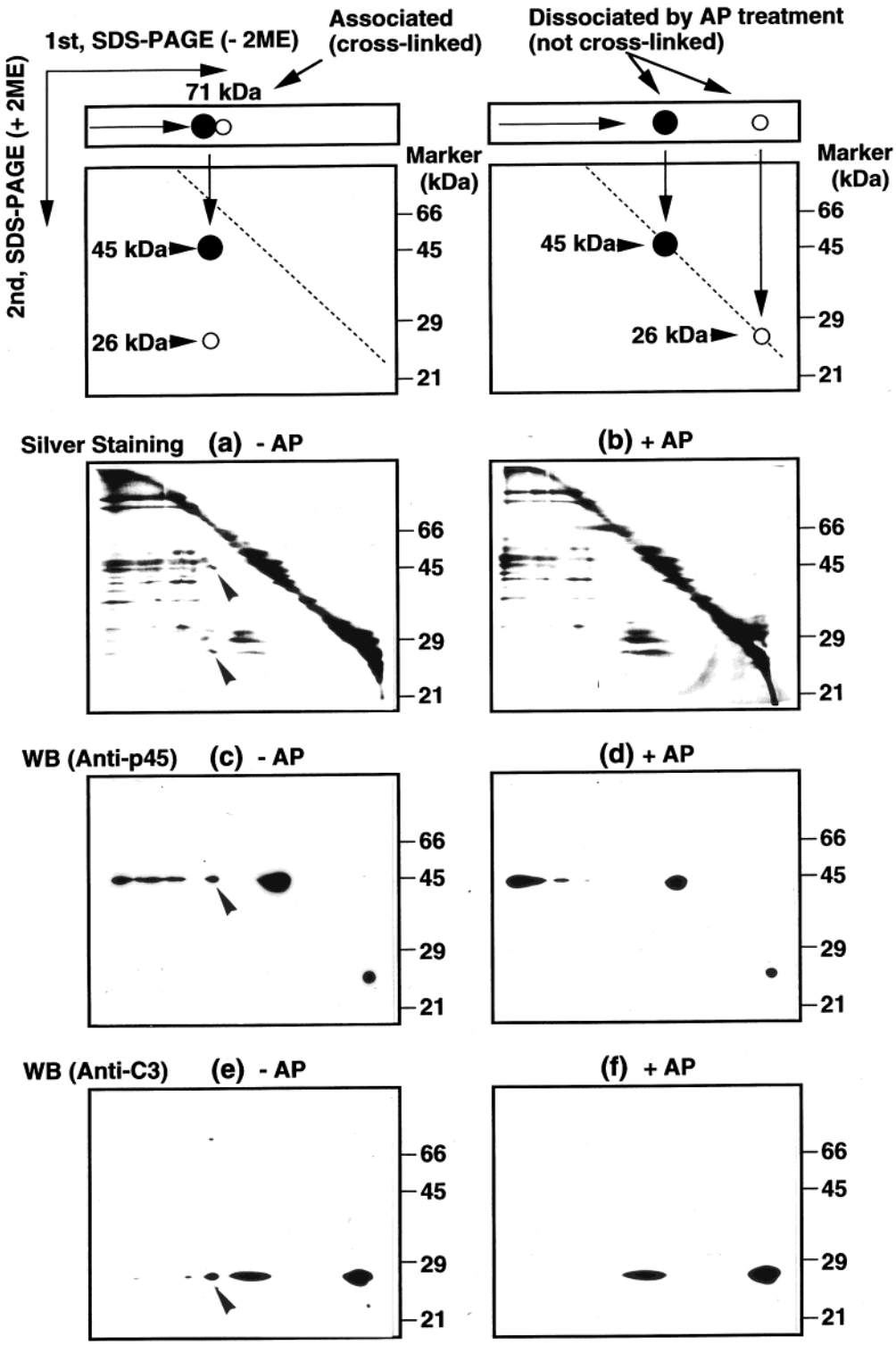

FIGURE 3: Interaction between the p45 ATPase subunit and the 20S proteasomal α-subunit C3. The 26S proteasome was incubated in the absence (a, c, e) or presence (b, d, f) of alkaline phosphatase at 37 °C for 30 min, followed by cross-linking with DTBP as described (*18*). These samples were subjected to SDS−PAGE under nonreducing (first) and reducing (second) conditions. After 2D PAGE, silver staining (a, b) and Western blottings with an anti-p45 ATPase subunit (c, d) and with anti-α-subunit C3 (e, f) were carried out. The upper panels illustrate the expected 2D PAGE patterns, in which the 45 kDa protein (●) associated with the 26 kDa protein (○) are dissociated by phosphatase treatment. Arrowheads in (c) and (e) represent the p45/Rpt6 and C3/α2 subunits, respectively.

Although the above results indicate that the p45 subunit is involved in the assembly of the 26S proteasome, it is unclear whether the p45 subunit directly interacts with the 20S proteasome complex. To assess this issue, the 26S proteasome was incubated in the absence or presence of alkaline phosphatase, followed by cross-linking with a bifunctional cross-linking reagent, DTBP (dimethyl 3,3′-dithiobispropionimidate), that possesses a single disulfide bond in its molecule. The cross-linked 26S proteasome was subjected to first-dimensional SDS−PAGE under nonreducing conditions followed by second-dimensional SDS−PAGE under reducing conditions (Figure 3). By this experiment, the silver staining pattern of the gel revealed that two subunits (45 and 26 kDa) are cross-linked but are not cross-linked

after treatment with phosphatase (see Figure 3). Since the molecular mass of the cross-linked protein was estimated to be about 71 kDa on first-dimensional SDS−PAGE, it is evident that the 45 and 26 kDa subunits are directly associated with each other. The present results indicate that the 45 and 26 kDa subunits are nearest neighbor subunits and that this interaction is maintained by phosphorylation of the 26S proteasome. Western blot analysis was performed to identify the 45 and 26 kDa subunits. Since the above results suggested the participation of the phosphorylated p45 subunit in the interaction between the regulatory complex and the 20S proteasome, we first tested whether the 45 kDa subunit in question is identical to the p45 ATPase subunit by Western blot analysis using the p45-specific antibody. The 45 kDa subunit was reacted with the anti-p45 antibody (Figure 3), but it was not reacted with the anti-Mss1, anti-p42, or anti-Tbp1 antibody (data not shown). Furthermore, the shape of the spot of p45 visualized by Western blotting was identical to the shape of the spot on the membrane visualized by protein staining with colloidal gold (data not shown). These results clearly show that the 45 kDa subunit, which is cross-linked with the 26 kDa subunit, is the p45/Rpt6 ATPase subunit (Figure 3).

Next, to identify the 26 kDa subunit, which directly interacts with the p45 subunit, Western blot analysis was carried out by using several monoclonal antibodies specific to the α-subunits of the 20S proteasome. The 26 kDa subunit was recognized by the antibody raised against the human C3 subunit (α2 subunit) (see Figure 3e) as well as with the antibody against all of the α-subunits (C2/α6, C3/α2, C8/α7, C9/α3, zeta/α5, iota/α1, XAPC7/α4 subunits) (data not shown), but it was not recognized by three monoclonal antibodies against XAPC7 (α4 subunit), zeta (α5 subunit), or iota (α1 subunit) (data not shown). The results of silver staining (a, b) and Western blotting with anti-p45 (c, d) and anti-C3 (e, f) in the absence or presence of alkaline phosphatase treatment are shown in Figure 3 together with illustrations (upper panels) of the predicted electrophoretic patterns of the 45 and 26 kDa proteins cross-linked with DTBP.

The results depicted in Figure 3 clearly show that the regulatory complex and the 20S proteasome are interconnected at least via the direct interaction between p45 and C3 subunits. Since the alkaline phosphatase treatment markedly diminished the cross-linking efficiency between p45 and C3 subunits, phosphorylation of the 26S proteasome subunit(s), most probably the p45 subunit itself, is thought to be indispensable for interaction between p45 and C3 subunits.

DISCUSSION

We have presented several lines of evidence for the first time showing that the 26S proteasome assembly is regulated by phosphorylation and dephosphorylation: The 26S proteasome assembly is closely linked to the phosphorylation of the p45 subunit in the dissociated regulatory complex, catalyzed by the regulatory complex-associated kinase (p45-kinase), that is activated upon dissociation of the 26S proteasome (see Figure 4). The results of the present study gave us an answer to the question "why is ATP hydrolysis required for the assembly of the 26S proteasome?". Since the assembly and disassembly of the 26S proteasomes are

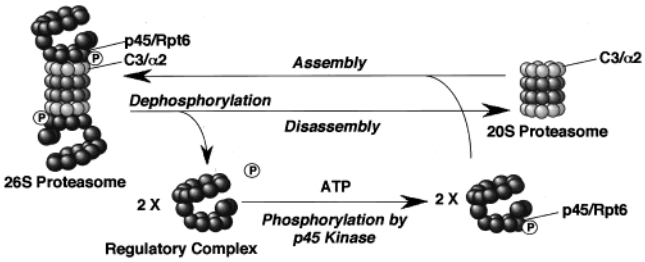

FIGURE 4: Hypothetical model on the regulation of the 26S proteasome assembly. Autophosphorylation activity of the dissociated regulatory complex is activated, or the phosphorylation site is exposed after dissociation from the 26S proteasome. When the p45 subunit of the regulatory complex is phosphorylated, the regulatory complex is capable of associating with the 20S proteasome, resulting in assembly of the 26S proteasome. The phosphorylated p45/Rpt6 directly interacts with the 20S proteasome α-subunit C3/α2.

reported to be regulated during the cell division cycle or by hormonal stimuli (*21*−*24*), and also since a protein kinase inhibitor (W-7) is capable of preventing the assembly of the 26S proteasome as well as egg activation in ascidians (*21*), it seems likely that the proteasomal activity is regulated also in vivo by phosphorylation-dependent assembly of the 26S proteasome.

Our present results indicated that dephosphorylation of the 26S proteasome elicits the dissociation of the 26S proteasome into the regulatory complex and the 20S proteasome. However, we cannot exclude the possibility that dephosphorylation of the regulatory subunits other than the p45 subunit or dephosphorylation of the 20S proteasomal subunits may also be involved in its dissociation, since several ATPase subunits and the 20S subunits are known to be phosphorylated within the cells (*4*, *25*).

Since DTBP is unable to cross-link the amino groups in neighboring subunits located at a distance of more than 11.9 Å, we cannot rule out the possibility that several subunits other than p45 or C3 are also involved in interconnection between the regulatory complex and the 20S proteasome. It is proposed that the p45/Sug1 subunit is associated with the α1/SCL1 subunit of the 20S proteasome, since the SCL1 mutant is able to suppress the p45-defective mutant in yeast (*4*, *26*). Since the α1 and α2 subunits are contiguous subunits, it is plausible that the p45/Rpt6 subunit contacts with the α1 subunit as well as the α2 subunit. The reason p45 and α1 subunits were not cross-linked in our present experiments is probably due to the long distance (more than 11.9 Å) between the amino groups in these contiguous subunits. Topological positions between the regulatory subunits may not be changed by dephosphorylation, since the cross-linking pattern within the regulatory subunits (30−110 kDa) was scarcely changed with or without phosphatase treatment. These results suggest that the interaction between the regulatory complex and the 20S proteasome must be specifically regulated by phosphorylation by p45-kinase.

We previously reported that the purified 26S proteasome as well as the immunoprecipitated 26S proteasome by a monoclonal antibody against the proteasome are able to radiolabel the 45, 56, and 130 kDa subunits with [γ-^{32}P]-ATP (*16*). In contrast, our present results showed that only the p45-subunit is phosphorylated among 20−110 kDa bands. These results suggest that phosphorylation of the 56 kDa (and 130 kDa) bands does not take place in the

dissociated regulatory complex. The above three bands of the purified 26S proteasome were definitely radiolabeled with [γ-^{32}P]ATP when incubated before nondenaturing PAGE. However, we noticed that the 56 kDa/130 kDa-phosphorylated 26S proteasome generates a self-associating complex (Sawada et al., unpublished results). Since the inhibition pattern of kinase inhibitors against phosphorylation of the 45 kDa band (p45), but not of the 56 or 130 kDa bands, correlated to the inhibition pattern against the ATP-dependent proteolysis of the 26S proteasome, only the p45 phosphorylation is thought to be important for the 26S assembly. It is evident that the radiolabeled 45 kDa band is a p45/Rpt6 subunit itself from the results of direct protein sequencing of their lysylendopeptidase-digested fragments (*16*).

As previously reported (*13*), it is very difficult to reassemble the 26S proteasome from the regulatory complex once dissociated from the 26S proteasome and the 20S proteasome. The regulatory complex dissociated from the 26S proteasome appears to be very unstable (*13*) and is apt to form the homodimer and homotrimer under the conditions of the phosphorylation reaction in the presence of ATP (*13*). It is also reported that several subunits other than the p45 subunit are phosphorylated (*25*), although the physiological significance has not been studied well. From these intrinsic features of the regulatory complex, it appears to be very difficult to selectively isolate the p45-phosphorylated regulatory complex, which is able to reassociate with the 20S proteasome. Furthermore, PA700 and the regulatory complex once dissociated from the 26S proteasome appear to be different from each other on the basis of 2D PAGE: the former and the latter contain characteristic 25 and 43 kDa subunits, respectively (*27*). Some auxiliary factors may be necessary for the efficient assembly of the 26S proteasome (*28*), or several subunits may be separated from the regulatory complex once dissociated from the 26S proteasome by electrophoresis or gel filtration. In any case, it should be noted that the p45-phosphorylated regulatory complex and the 20S proteasome were able to make up the 26S proteasome under a certain equilibrium state of the purified 26S proteasome preparation.

The results of Figure 3c indicate that p45/Rpt6 subunit interacts only with the C3/α2 subunit among seven α-subunits under our experimental conditions. We first imagined that the regulatory complex might be a rotary motor like a mitochondrial F_oF_1ATPase (*29*), since the basic structure of six putative ATPase subunits may construct a hexameric ring such as NSF, an AAA family protein (*30*). However, our present study revealed the existence of a fixed partner of the p45 subunit among seven α-subunits. From these results, we propose the idea that the regulatory complex (or putative hexameric ATPase ring-shaped subunits of the base subcomplex) may not be an ATP-driven rotary motor moving on the heptameric α-subunit ring of the 20S proteasome.

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