Vol. 213, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 4, 1995 Pages 7-14

PHOSPHORYLATION OF PROTEASOME SUBSTRATE BY A PROTEIN KINASE ASSOCIATED WITH THE 26 S PROTEASOME IS LINKED TO THE ATP-DEPENDENT PROTEOLYSIS OF THE 26 S PROTEASOME

Kazuki Satoh, Takahiro Nishikawa, Hideyoshi Yokosawa and Hitoshi Sawada*

Department of Biochemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan

Received June 24, 1995

A protein kinase phosphorylating the 45-kDa proteasome subunit was co-purified with the 26 S proteasome from the porcine heart. This kinase appears to be associated with the 26 S proteasome, since the kinase activity was co-eluted with the 26 S proteasome on Superose 6 FPLC and immunoprecipitated with anti-20 S proteasome antibody. This kinase also phosphorylated the casein. Furthermore, the phosphorylated casein was more efficiently hydrolyzed by the 26 S proteasome than the dephosphorylated casein without ATP. Inhibition patterns of kinase inhibitors against the 45 kDa subunit and casein were well in accord with the inhibition pattern against the ATP-dependent proteolysis of the 26 S proteasome, suggesting that the phosphorylation of casein by a protein kinase associated with the 26 S proteasome is linked to the ATP-dependent proteolysis of the 26 S proteasome.

Intracellular abnormal and short-lived proteins are rapidly degraded by the ubiquitin-proteasome system (1-4). In this system, the target proteins are tagged with multi-ubiquitin chains, the reaction of which is catalyzed by ubiquitin-activating enzyme, ubiquitin-conjugating enzyme and ubiquitin-ligase. These multi-ubiquitin tagged proteins are degraded by the ubiquitin/ATP-dependent protease complex (26 S proteasome) (1-4). Both ubiquitination and proteolysis require an ATP hydrolysis, since non-hydrolyzable ATP analogs do not stimulate these processes (1-4).

The 26 S proteasome is composed of at least two subcomplexes: one is a 700 kDa proteolytic core complex called the 20 S proteasome with 28 subunits (20-35 kDa) and the other is a 700-1000 kDa regulatory subunit complex made up of about 20 subunits (28-110 kDa) (5-10). These two subcomplexes are thought to reconstitute the 26 S proteasome in an ATP-dependent manner (5-10, see Reviews 2, 4).

Recent studies demonstrated that the 26 S proteasome has an ATPase activity linked to ATP-dependent proteolysis (11) and this activity exists in a regulatory subunit complex or an ATP-dependent activator (PA 700) toward the 20 S proteasome (12). In

*To whom correspondence should be addressed. Fax: 81-11-706-4989.

0006-291X/95 $12.00

addition, at least four regulatory subunits (S4 [or MTS2] (13, 14), S6 [or TBP7] (12, 15), S7 [or MSS1, CIM5] (14, 16, 17), and SUG1 [or CIM3] (12, 17)) of the 26 S proteasome have been proposed to belong to a new family of putative ATPase. All of these proteins have a consensus sequence to nucleotide binding motif and show high homology to some ATPases (18). From these results, it is currently proposed that several "ATPase subunits" in the 26 S proteasome play a key role in the ATP-dependent proteolysis of the 26 S proteasome. However, the molecular mechanisms concerning the role of ATP hydrolysis in protein degradation have not yet been elucidated. In addition, we can not rule out the possibility that apparent ATP hydrolysis by the 26 S proteasome is a result of a combination of phosphorylation and dephosphorylation.

Here, we describe that a protein kinase phosphorylating at least the 45-kDa proteasome subunit (a SUG1 homolog), which is referred to as the 45-kDa subunit-specific kinase in this report, is co-purified with the 26 S proteasome from porcine cardiac muscle, and that this protein kinase also phosphorylates a protein substrate (casein). In addition, we present evidence that the phosphorylation of casein is linked to the ATP-dependent degradation of the substrate by the 26 S proteasome.

MATERIALS AND METHODS

Materials: β-Casein, dephosphorylated-β-casein, ATP, protein A-agarose, quercetin, myricetin and heparin were purchased from Sigma. [γ-^{32}P]ATP (10 mCi/ml) and [α-^{32}P]ATP (10 mCi/ml) were purchased from ICN. [^{14}C]HCHO was purchased from NEN. DEAE-cellulose (DE-32) and phosphocellulose (P-11) were obtained from Whatman. FPLC system is a product of Pharmacia-LKB. Centricon 10 was purchased from Amicon. H89 and CKI-7 were purchased from Seikagaku Kogyo. Genistein was obtained from Extrasynthese.

Assay for protease activity: Protease activity was determined as follows. The reaction mixture (200 μl) consisted of 50 mM Tris/HCl (pH 7.8), 2 mM ATP, 10 mM $MgCl_2$, 1 mM dithiothreitol and 5 μg of dephosphorylated [^{14}C]methyl-β-casein, which was prepared by reductive methylation with [^{14}C]HCHO (19). After incubation at 37°C for 2 h, the reaction was terminated by adding 25 μl of 10% (w/v) bovine serum albumin and 600 μl of 10% (w/v) trichloroacetic acid. The radioactivity in trichloroacetic acid-soluble materials was measured with a liquid scintillation counter.

Protein determination: Protein concentration was determined by the method of Bradford (20) with bovine serum albumin as a standard.

Assay for protein kinase activity of the 26 S proteasome: The reaction mixture which contained 50 mM Tris/HCl (pH 7.8), 2 mM ATP including [γ-^{32}P]ATP, 10 mM $MgCl_2$, 1 mM dithiothreitol and the 26 S proteasome was incubated at 37°C for an appropriate time in the presence and absence of 5 μg dephosphorylated β-casein. The phosphorylation reaction was terminated by the addition of the sample buffer for SDS-PAGE in the presence of 5 mM EDTA and 5% (v/v) 2-mercaptoethanol. These samples were subjected to SDS-PAGE and the gels were stained with Coomasie Brilliant Blue R-250 followed by autoradiography. Alternatively, the amount of the ^{32}P bound to each protein band was quantified by using a Fuji Imaging Analyzer BAS 2000.

Purification of 26 S proteasome from porcine cardiac muscle: The 26 S proteasome was purified from porcine cardiac muscles, which were obtained from Ebetsu abattoir, according to the method described previously (6) except for the conditions of ultracentrifugation (100,000 x g, 12 h). Approximately 3.6 mg of the purified enzyme was obtained from 3 individuals with a yield of 1.3% from the DEAE-cellulose adsorbed fraction. ATP dependency in the caseinolytic activity was 3-fold.

Electrophoresis: SDS-PAGE was performed as described in (21).

Western blotting and immunoprecipitation: Western blot analysis and immunoprecipitation were carried out as described in (22) and (23), respectively. A

monoclonal antibody (IgG) called GD6 raised against the duck erythroblast 20 S proteasome (prosome) was purified from mouse ascitic fluid (a gift from Dr. K. Scherrer (24)) by protein A-agarose affinity chromatography.

Determination of phosphoamino acid residue: Phosphoamino acid analysis was performed by the procedure described in (25).

Amino acid sequence analysis: The 26 S proteasome (1.67 mg) was applied on a Phenyl-5PW RP column (0.46 x 7.5 cm) equilibrated with 36% (v/v) acetonitrile in 0.05% trifluoroacetic acid. After washing with the equilibration solvent, the adsorbed materials were eluted by a 36-44% (v/v) linear gradient of acetonitrile (total volume, 45 ml) at a flow rate of 0.5 ml/min. A peak fraction which contained the [^{32}P]phosphorylated 45 kDa subunit eluted at about 37% acetonitrile was subjected to SDS-PAGE followed by blotting to the nitrocellulose membrane. The 45 kDa band was excised and then digested with lysyl endopeptidase according to the method of Matsudaira (26). The protein digest was applied to a C_{18} column (Applied Biosystems RP-18 5μ, 0.21 x 3 cm) equilibrated with 0.05 % trifluoroacetic acid and the peptide fragments were separated by a 0-80% (v/v) linear gradient of acetonitrile (total volume, 15 ml) in 0.05% trifluoroacetic acid at a flow rate of 0.25 ml/min. Peaks in this HPLC were subjected to amino acid sequence analyses.

RESULTS AND DISCUSSION

Purification and purity of the 26 S proteasome from porcine cardiac muscle: The 26 S proteasome was highly purified from porcine cardiac muscle as described previously (6). The purity of the proteasome preparation was examined by Superose 6 gel filtration. As shown in Fig. 1, the protein showed a single peak where all fractions (16-21) showed the same SDS-PAGE pattern characteristic of the 26 S proteasome (see the left panel in Fig. 2), and the protein peak corresponded to that of the ATP-dependent caseinolytic

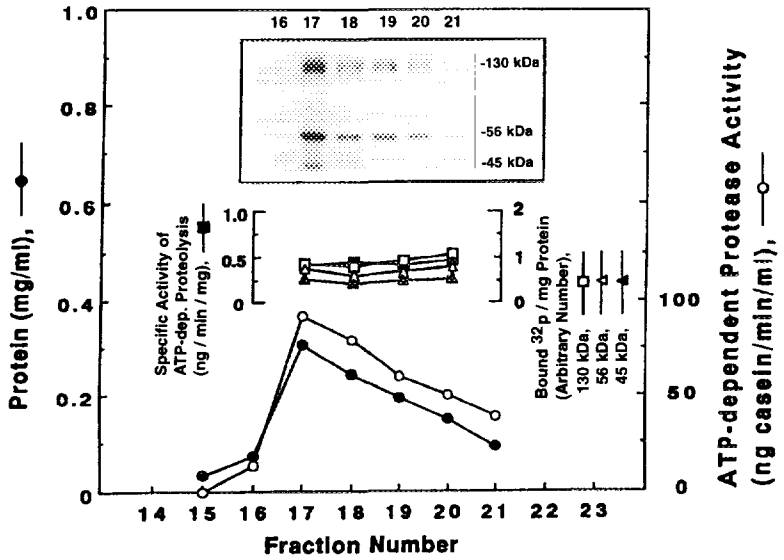

Fig. 1. Superose 6 gel filtration analysis of the 26 S proteasome. -●-, protein concentration (mg/ml); - ○ -, ATP-dependent protease activity toward casein (ng casein/min/ml); -■-, specific caseinolytic activity (ng casein/min/mg protein); specific activity of protein kinase toward 130 kDa subunit (- □ -), 56 kDa subunit (- △ -), and 45 kDa subunit (- ▲ -). *Inset*, autoradiography after SDS-PAGE of the 26 S proteasome incubated with [γ-^{32}P]ATP.

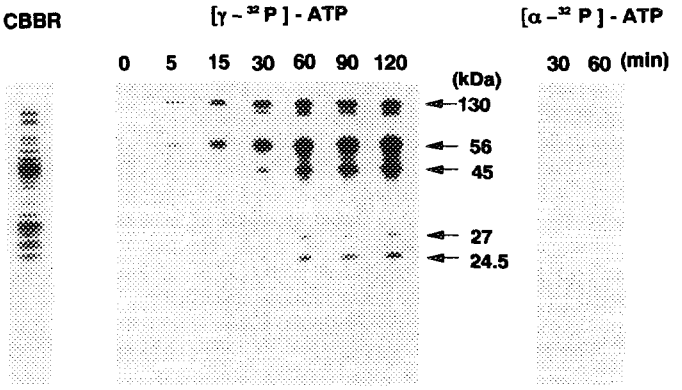

Fig. 2. Phosphorylation reaction of the 26 S proteasome. The 26 S proteasome preparation was incubated with [γ-^{32}P]ATP (middle panel) or [α-^{32}P]ATP (right panel) and then subjected to SDS-PAGE and autoradiography. The left panel shows the protein bands of the purified 26 S proteasome stained with Coomasie Brilliant Blue R-250.

activity. These results indicate that the 26 S proteasome preparation is highly pure and that the enzyme efficiently hydrolyzes casein without ubiquitination.

Presence of protein kinase in the purified 26 S proteasome preparation: By using the purified 26 S proteasome preparation, we first tested whether it has a protein kinase activity toward the proteasome subunits when incubated with [γ-^{32}P]ATP. The autoradiogram showed that several bands including 45 kDa, 56 kDa and 130 kDa bands were radiolabeled (Fig. 1 inset). A few subunits (24.5 and 27 kDa) characteristic of the 20 S core complex were also weakly labeled after prolonged incubation (Fig. 2). No radiolabeling was observed with [α-^{32}P]ATP (Fig. 2), indicating that this radiolabeling is derived from the protein phosphorylation. The specific protein kinase activities toward the above three bands and also the specific caseinolytic activity were practically identical across the 26 S proteasome-containing fractions (see Fig. 1). These results strongly suggest that the protein kinase is co-purified by our purification procedure.

Among the above phosphorylated bands, at least the 45 kDa protein is considered to be a 26 S proteasome subunit, since the 45-kDa protein was one of the main components of the 26 S proteasome on the basis of SDS-PAGE analysis (Fig. 2) and of amino acid sequence analysis of this protein (see below). Thus, the protein kinase co-purified with the 26 S proteasome is referred to as the 45-kDa proteasome subunit-specific kinase in this report.

Next, we examined whether the co-purified protein kinase is tightly associated with the 26 S proteasome by immunoprecipitation using a monoclonal antibody (GD6) raised against the 20 S proteasome. This antibody specifically recognized the 33 kDa subunit of the porcine heart 26 S proteasome by Western blotting (the left panel in Fig. 3). Three bands with molecular masses of 45 kDa, 56 kDa and 130 kDa were again found to be phosphorylated by the immunoprecipitates (the right panel in Fig. 3), whereas no appreciable phosphorylation was observed in the control experiment using the Protein A-

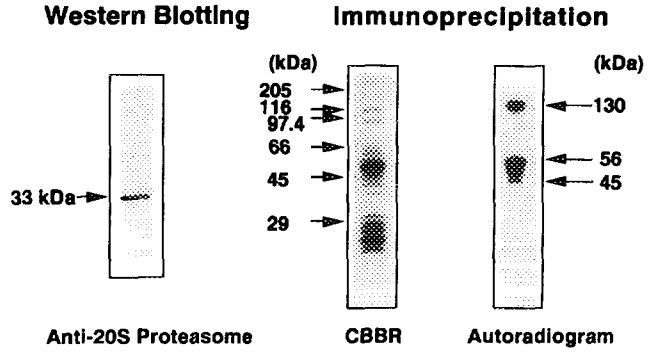

Fig. 3. Western blotting and immunoprecipitation of the 26 S proteasome. The purified 26 S proteasome was subjected to SDS-PAGE followed by Western blotting with anti-20 S proteasome monoclonal IgG (GD6) (left panel). The immunoprecipitates of the 26 S proteasome preparation with anti-20 S proteasome monoclonal antibody (GD6) and Protein A-agarose beads were repetitively washed and then incubated with [γ-^{32}P]ATP in a reaction mixture for protein kinase assay. The sample thus obtained was subjected to SDS-PAGE (middle panel) and autoradiography (right panel).

agarose beads instead of anti-20 S proteasome monoclonal antibody-coupled Protein A-agarose beads (data not shown). These results indicate that the 45-kDa proteasome subunit-specific protein kinase is tightly associated with the 26 S proteasome.

The [^{32}P]phosphorylated amino acid residues was identified as threonine by TLC after tryptic digestion and mild acid hydrolysis of the ^{32}P-labeled 26 S proteasome. This result, together with the fact that a class P-ATPase including Na^+-K^+ ATPase and Ca^{2+}ATPase is transiently autophosphorylated as an intermediate via aspartic acid residue (27), strongly suggests that the 45-kDa subunit-specific protein kinase belongs to threonine kinase and that the apparent phosphorylation of the 26 S proteasome is not a result of the reaction intermediate of the class P-ATPase.

Phosphorylation of casein by the 45-kDa subunit-specific protein kinase is linked to the ATP-dependent casein hydrolysis by the 26 S proteasome: It was found that our 26 S proteasome preparation not only phosphorylated the 45-kDa subunit, but also phosphorylated the protein substrate, casein (Fig. 4). It is inferred that phosphorylation of the protein substrate could stimulate the unfolding of the protein, which makes the protein more susceptible to the hydrolysis by the 26 S proteasome. Very interestingly, we found that the phosphorylated casein degraded 1.4 times more rapidly (1.39 ± 0.07 (SEM, n=3)) by the 26 S proteasome in the absence of ATP than the dephosphorylated casein. In contrast, no significant difference was observed between the activities toward the phosphorylated casein and the dephosphorylated casein in the presence of ATP (1.05 ± 0.05 (SEM, n=3)). These results strongly suggest that phosphorylation of the substrate protein may play a key role in ATP-dependent proteolysis of the 26 S proteasome.

During these experiments, we noticed that the phosphorylation of the 45 kDa subunit, but not of the 56 kDa and 130 kDa bands, was inversely related to

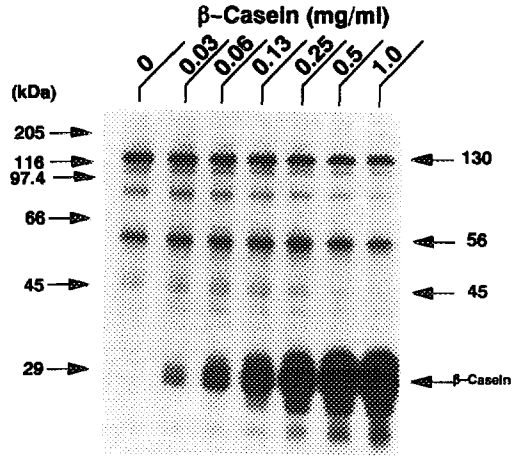

Fig. 4. Phosphorylation of casein by the 26 S proteasome. Increasing amounts of the dephosphorylated β-casein were mixed with the 26 S proteasome and then incubated with [γ-^{32}P]ATP. After incubation, the sample was subjected to SDS-PAGE and autoradiography.

phosphorylation of casein by increasing the amount of casein (Fig. 4). Thus, it is thought that the protein kinase phosphorylates the 45-kDa proteasome subunit when the substrate is absent in the reaction mixture, while it phosphorylates the protein substrate when the protein substrate is present. In the latter case, casein may mask the phosphorylation site of 45-kDa proteasome subunit.

Since phosphorylations of both the 45 kDa subunit and casein appeared to be closely related, we investigated the effects of protein kinase inhibitors on phosphorylation of these two proteins and also on ATP-dependent casein hydrolysis. As shown in Table 1, phosphorylation of both the 45 kDa subunit and casein was substantially inhibited by 100 μM quercetin and myricetin, both of which are good inhibitors for the ATP-dependent proteolysis. In addition, inhibitory potencies of these two compounds toward the phosphorylation were comparable with those toward the ATP-dependent protease activity. In contrast, H89, genistein, CKI-7 and heparin showed less or no inhibition. Phosphorylation of both the 56 kDa and 130 kDa bands was not correlated to the phosphorylation of casein or ATP-dependent proteolysis. These results also suggest that the protein kinases mediating the phosphorylation of the 45 kDa subunit and casein are the same, and that the phosphorylation of casein is linked to the ATP-dependent protease activity of the 26 S proteasome.

In this experiment, we used excess amounts (the concentrations used were more than 10 times the Ki or IC_{50} values (28)) of the above inhibitors to inhibit the protein kinase so far identified. Nevertheless, no or only weak inhibition was observed toward the 45-kDa subunit specific kinase activity, suggesting that this kinase may belong to a novel threonine kinase. It has been previously reported that the cAMP-dependent

Table 1. Effects of inhibitors on protein kinase activity and ATP-dependent protease activity of the 26 S proteasome

Inhibitor [a]	Concentration	Phosphorylation		Proteolysis [b]
		45 kDa subunit of 26 S proteasome	β-Casein [c]	
		% Inhibition		
Quercetin	100 μM	45	53	40
Myricetin	100 μM	34	42	53
H89	100 μM	12	17	5
Genistein	100 μM	8	8	<5
CKI-7	100 μM	<5	<5	<5
Heparin	100 nM	<5	8	8

[a] The concentrations used are more than 10 times the IC_{50} or Ki values for the respective protein kinases. The specificity of the inhibitors is as follows (28): quercetin (tyrosine kinase, phosphorylase kinase), H89 (cAMP-dependent protein kinase, cGMP-dependent protein kinase), genistein (tyrosine kinase), CKI-7 (casein kinase I), and heparin (casein kinase II). Concentration of heparin was defined by assuming that the mean molecular weight is 5000.

[b] The protease activity was determined by using radiolabeled dephosphorylated β-casein as a substrate in the presence of 2 mM ATP.

[c] 0.25 mg/ml dephosphorylated β-casein.

protein kinase (29) and the casein kinase II (30) are "contaminated" in purified preparations of the 20 S proteasome. These protein kinases have been reported not to be tightly associated with the proteasome. Furthermore, the fact that cAMP-dependent kinase inhibitor (H89) and casein kinase II inhibitor (heparin) scarcely inhibited the 45-kDa subunit specific kinase clearly indicates that our protein kinase does not belong to such kinases.

Identification of the 45-kDa phosphorylated subunit of the 26 S proteasome: Finally, we attempted to identify the phosphorylated 45 kDa protein band. It was found that the lysyl endopeptidase-digested three fragments had the sequences of Phe-Val-Val-Asp-Val-Asp-Lys (nonconserved region), Glu-Val-Ile-Glu-Leu-Pro-Val-Lys (relatively conserved region), and Gly-Val-Leu-Leu-Tyr-Gly-Pro-Pro-Gly-Thr (nucleotide binding motif). These sequences are identical to those of proteasome subunit p45 (12, 31), a homolog of the yeast SUG1. The results indicate that the 45 kDa phosphorylated subunit of the 26 S proteasome is a SUG1 homolog, p45.

Identification of the protein kinase associated with the 26 S proteasome is now in progress in our laboratory.

Acknowledgments: This research was supported in part by grants-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, Akiyama Foundation, Novo Nordisk Research Foundation, and Itoh Memorial Foundation. We are indebted to Dr. Kraus Scherrer of Institut Jacques Monod Université Paris for providing us a monoclonal antibody (GD6) raised against the 20 S proteasome/prosome. We are grateful to Dr. T. Kumazaki and Mr. Y. Abe of Hokkaido University for helpful comments in protein sequence analysis.

REFERENCES

1. Rechsteiner, M. (1991) Cell **66**, 615-618
2. Goldberg, A. L. (1992) Eur. J. Biochem. **203**, 9-23
3. Goldberg, A. L. and Rock, K. L.(1992) Nature (London) **357**, 375-379
4. Hershko, A. and Ciechanover, A. (1992) Annu. Rev. Biochem. **61**, 761-807
5. Hoffman, L., Pratt, G. and Rechsteiner, M. (1992) J. Biol. Chem. **267**, 22362-22368
6. Sawada, H., Muto, K., Fujimuro, M., Akaishi, T., Takagi Sawada, M., Yokosawa, H. and Goldberg, A. L. (1993) FEBS Lett. **335**, 207-212
7. Udvardy, A. (1993) J. Biol. Chem. **268**, 9055-9062
8. Peters, J. -M., Franke, W. W. and Kleinschmidt, J. A. (1994) J. Biol. Chem. **269**, 7709-7718
9. Chu-Ping, M., Vu, J. H., Proske, R. J., Slaughter, C. A. and DeMartino, G. N. (1994) J. Biol. Chem. **269**, 3539-3547
10. Yoshimura, T., Kameyama, K., Takagi, T., Ikai, A., Tokunaga, F., Koide, T., Tanahashi, N., Tamura, T., Cejka, Z., Baumeister, W., Tanaka, K. and Ichihara, A. (1993) J. Struct. Biol. **111**, 200-211
11. Kanayama, H., Tamura, T., Ugai, S., Kagawa, S., Tanahashi, N., Yoshimura, T., Tanaka, K. and Ichihara, H. (1992) Eur. J. Biochem. **206**, 567-578
12. DeMartino, G. N., Moomaw, C. R., Zagnitko, O. P., Proske, R. J., Chu-Ping, M., Afendis, S. J., Swaffield, J. C. and Slaughter, C. A. (1994) J. Biol. Chem. **269**, 20878-20884
13. Dubiel, W., Ferrell, K., Pratt, G. and Rechsteiner, M. (1992) J. Biol. Chem. **267**, 22699-22702
14. Gordon, C., McGurk, G., Dillon, P., Rosen, C. and Hastie, N. D. (1993) Nature (London) **366**, 355-357
15. Dubiel, W., Ferrell, K. and Rechsteiner, M. (1994) Biol. Chem. Hoppe-Seyler **375**, 237-240
16. Dubiel, W., Ferrell, K. and Rechsteiner, M. (1993) FEBS Lett. **323**, 276-278
17. Ghislain, M., Udvardy, A. and Mann, C. (1993) Nature (London) **366**, 358-362
18. Rechsteiner, M., Hoffman, L. and Dubiel, W. (1993). J. Biol. Chem. **268**, 6065-6068
19. Dottavio-Martin, D. and Ravel, J. M. (1978) Anal. Biochem. **87**, 562-565
20. Bradford, M. M. (1976) Anal. Biochem. **72**, 248-254
21. Laemmli, U. K. (1970) Nature (London) **227**, 680-685
22. Fujimuro, M., Sawada, H. and Yokosawa, H. (1994) FEBS Lett. **349**, 173-180
23. Toratani, S., Sekine, N., Katada, T. and Yokosawa, H. (1993) FEBS Lett. **324**, 353-357
24. Olink-Coux, M. Huesca, M. and Scherrer, K. (1992) Eur. J. Cell Biol. **59**, 148-159
25. Neufeld, E., Goren, H. J. and Boland, D. (1989) Anal. Biochem. **177**, 138-143
26. Matsudaira, P. (1989) A practical Guide to Protein and Peptide Purification for Microsequencing, Academic Press, New York
27. Forgac, M. (1989) Physiol. Rev. **69**, 765-796
28. Hidaka, H. and Kobayashi, R. (1993) in Protein Phosphorylation. A Practical Approach (Hardie, D. G., ed) pp. 87-107, Oxford Univ. Press, Oxford
29. Pereira, M. E. and Wilk, S. (1990) Arch. Biochem. Biophys. **283**, 68-74
30. Ludemann, R., Lerea, K. M. and Etlinger, J. D. (1993) J. Biol. Chem. **268**, 17413-17417
31. Akiyama, K., Yokota, K. Kagawa, S., Shimbara, N., DeMartino, G. N., Slaughter, C. A., Noda, C., and Tanaka, K. (1995) FEBS Lett. **363**, 151-156