The catalytic subunit of cAMP-dependent protein kinase radiolabeled with [35S]methionine in wild-type S49 mouse lymphoma cells was degraded with half-lives of ~9.2 h in unstimulated cells and ~4.5 h in cells stimulated with a membrane-permeable cAMP analog. Turnover in kinase-negative mutant cells was about three times faster than in stimulated wild-type cells and appeared to involve a unique 47-kDa intermediate. Levels of catalytic subunit protein revealed by Western immunoblotting were consistent with the measured differences in turnover, but whereas the protein was mostly soluble in wild-type cell extracts, it was almost entirely insoluble in the mutant cell extracts. A substantial fraction of the catalytic subunit labeled in a 5-min pulse was soluble in kinase-negative cell extracts, but most of this material was rendered insoluble by incubating the cells for an additional 30 min before extraction. Degradation of the catalytic subunit in kinase-negative, but not in wild-type, cells was inhibited strongly by two specific peptide aldehyde inhibitors of the proteasomal chymotrypsin-like activity. An inhibitor of the proteasomal protease that prefers branched-chain amino acids had less of an effect on catalytic subunit degradation in the mutant cells.

Kinase-negative (kin−)1 mutants of S49 mouse lymphoma cells are defective in post-translational maturation and/or accumulation of the catalytic (C) subunit of cAMP-dependent protein kinase with the result that the cells have no detectable C subunit activity and little or no C subunit protein (1–4). Because the kin− phenotype is fully dominant in somatic cell hybrids between wild-type and kinase-negative (kin−) mutants of S49 mouse lymphoma cells and can be reverted to give cells that are temperature-dependent for C subunit activity and little or no C subunit protein (1–4), we decided to use kin− cells and can be reverted to give cells that are temperature-dependent for C subunit expression, it would appear that the underlying mutation is in a protein-encoding regulatory gene that is in some way critical for expression of a functional C subunit (1, 2). Expression of mRNAs for the two major isoforms of the C subunit (Cα and Cβ) is normal in kin− cells, as is synthesis of the C subunit proteins (3, 4). The coding region of the Cα subunit gene amplified from kin− cell cDNA can direct expression of a functional C subunit when introduced into a suitable host cell via a mammalian expression plasmid (3), and the coding sequences of Cα subunit cDNAs from wild-type and kin− cells are identical.2

As noted by Orellana and McKnight (3), the normal rate of C subunit production in kin− cells implies that the C subunit deficiency results from enhanced C subunit degradation. Such accelerated turnover could be either the immediate cause of the mutant phenotype or a consequence of the production of defective C subunit protein. At least two post-translational maturation steps appear to precede the appearance of a functional C subunit in wild-type S49 cells: the first results in solubilization of the newly synthesized protein, and the second is phosphorylation at Thr-197, which is required for efficient catalysis (4, 5). In kin− cells, the majority of newly synthesized C subunit remains insoluble (4). From this observation, we have hypothesized that the primary defect in kin− cells involves a failure to fold properly the newly synthesized C subunit protein (4). In this view, enhanced C subunit turnover might reflect the activity of a pathway specific for clearance of aberrant cell proteins.

For further analysis of the kin− phenotype, we decided to measure rates of C subunit turnover in wild-type and kin− S49 cells. Although several studies have reported enhanced degradation of the C subunit in cells treated chronically with cAMP (6–9), there are no published data on the rates of C subunit turnover in mammalian cells. We report here that cAMP-dependent kinase activation enhances turnover in wild-type cells by ~2-fold. In kin− cells, not only is C subunit turnover faster than in wild-type cells, but also the pathway for C subunit turnover appears to involve a unique intermediate.

EXPERIMENTAL PROCEDURES

Materials

Chemicals and Radiochemicals—Deoxyribonuclease I, Nonidet P-40, sodium deoxycholate, Triton X-100, Tween 20, spermidine, and spermine were from Sigma; ribonuclease A was from Promega (Madison, WI); and 8-(2-chlorophenylthio)-cAMP (CPT-cAMP) was from Boehringer Mannheim. Alkaline phosphatase-conjugated anti-goat immunoglobulin G (whole molecule) was from the Cappel Products Division of Organon Teknika Corp. (Durham, NC), and Pansorbin was from Calbiochem. [3H]Leucine and [35S]methionine were from DuPont NEN. Benzoyloxycarbonyl-Leu-Leu-phenylalaninalinal (Z-LLF-CHO), benzoyloxycarbonyl-Gly-Pro-Phe-leucinal; RIPA, radiolmmune precipitation assay; PAGE, polyacrylamide gel electrophoresis.

1 The abbreviations used are: kin−, kinase-negative; C subunit, catalytic subunit of cAMP-dependent protein kinase; CPT-cAMP, 8-(2-chlorophenylthio)-cAMP; Z-LLF-CHO, benzyloxycarbonyl-Leu-Leu-phenylalaninalinal; Z-GPFL-CHO, benzyloxycarbonyl-Gly-Pro-Phe-leucinal; Z-PP-CHO, benzoyloxycarbonyl-Pro-prolinal; MG-132, benzoyloxycarbonyl-Leu-Leu-leucinal; RIPA, radioimmunoprecipitation assay; PAGE, polyacrylamide gel electrophoresis.

2 S.-L. Lee and R. A. Steinberg, unpublished results.
Pro-proline (Z-PP-CHO) were generously provided by Dr. Marian Orłowski (Mount Sinai School of Medicine); benzylxoycarbonyl-Leu-Leu-Leu-methyl (MG-132) was supplied by ProScript, Inc. (formerly MyoGenics, Inc., Cambridge, MA). All other chemicals were reagent grade or better and used without further purification.

Medium and Buffer Solutions—Low methionine medium was prepared with either 2.5 or 5 μM L-methionine as described previously (10). Chase medium was prepared by sterilizing a 1:1 mixture of fresh growth medium and supernatant fractions from late log phase cultures of S49 cells (4). Phosphate-buffered saline contained 137 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM dibasic sodium phosphate, and 1.5 mM monobasic potassium phosphate. RIPA buffer contained 10 mM Tris-HCl (pH 7.4), 158 mM sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS. FB buffer contained 50 mM Tris-HCl (pH 7.4), 2 mM dithiothreitol, 0.1 mM EDTA, 2 mM L-methionine, 150 mM potassium chloride, 0.5 mM spermine, 0.15 mM sperminine, and 0.05% Nonidet P-40.

Methods

Culture and Radiolabeling of S49 Cells—Wild-type (subline 24.3.2) and kin− (subline 24.6.1) S49 mouse lymphoma cells were grown in suspension culture in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum, preincubated, and labeled with [35S]methionine in low methionine medium at 37°C as described previously (4, 10). The details of the labeling protocols are provided in the figure legends. For pulse-labeled samples, incorporation was stopped by adding 20 μl of the cell culture to 700 μl of phosphate-buffered saline containing 2 mM L-methionine, and cells were collected by centrifugation. Cell pellets were frozen on dry ice and either extracted immediately or stored at −70°C for later extraction. For pulse-chase experiments, labeled kin− cells were diluted 40-fold with fresh culture medium, and incubation was continued at 37°C. After various times of chase, cells from 800-μl cultures were harvested by centrifugation through 500-μl cushions of phosphate-buffered saline containing 10% glycerol. Because the residual isotopic toxin was toxic over the longer periods required for turnover experiments with wild-type cells (see Fig. 2 and Table I), these cells were centrifuged through 2 or more volumes of heat-inactivated horse serum before suspending with chase medium (with or without 100 μM CPT-CAMP). For the experiments of Fig. 6 and Table I, chase medium contained dimethyl sulfoxide-solubilized inhibitors at the concentrations indicated; the final concentration of dimethyl sulfoxide in all cultures was adjusted to 0.2%.

Expression and Labeling of Recombinant C Subunit—Recombinant murine Cα subunit was expressed in Escherichia coli BL21(DE3) and purified as described previously (5). A carboxyl-terminally truncated, 33-kDa fragment of the Cα subunit was expressed and labeled with [3H]leucine as described (11) for use as an internal standard in immunoadsorptions.

Immunoadsorption—Pellets of radiolabeled cells were thawed on ice, extracted by suspension in 20 μM RIPA buffer containing 10,000 acid-precipitable cpm of the [3H]leucine-labeled 33-kDa C subunit fragment, and centrifuged for 12 min at 178,000 × g in a Beckman Airfuge. The supernatant fractions were then immunoadsorbed with an affinity-purified goat anti-C subunit antibody using a two-cycle procedure described elsewhere (11). In brief, radiolabeled extracts were preclarified with Pansorbin and then immunoadsorbed using 3.2 μg of anti-C subunit antibody and Pansorbin. After washing the Pansorbin-bound complexes several times with RIPA buffer, they were dissociated by incubation for 10 min at 0°C in a 1% (w/v) solution of SDS containing 1 mM 2-mercaptoethanol. The Pansorbin was removed by centrifugation, and the supernatant fractions were diluted and read老虎机r with fresh antibody and Pansorbin. For antibody blocking (see Fig. 1, lane d), the antibody was preincubated with 20 μg of recombinant C subunit for 15 min on ice before the second immunoadsorption cycle. Immunocomplexes were solubilized with SDS gel sample buffer (12), Pansorbin was removed by centrifugation, and supernatant fractions were saved for scintillation counting and electrophoresis. Incorporation of [35S]methionine in extracts and cell fractions was measured by scintillation counting after acid precipitation and filtration (10). For label-chase and/or cell fractionation experiments, equal amounts of tritium radioactivity from each sample were loaded onto gel lanes.

For fractionation experiments (see Fig. 5), radiolabeled cells harvested as described above were extracted directly with 200 μl of FB buffer (without freezing). Pellet fractions from low and high speed centrifugations (details in the figure legend) were dissolved with RIPA buffer, and the final supernatant fractions were diluted 2-fold with twice concentrated RIPA buffer. ~10,000 cpm of the [3H]leucine-labeled 33-kDa C subunit fragment were added to each fraction, and C subunits were immunoadsorbed as described above.

Western Immunoblots—For immunoblots, cells were harvested by centrifugation, washed once with phosphate-buffered saline, and extracted directly into ice-cold FB buffer at a density of 5 × 10⁷ cells/ml. Dexyribonuclease I and ribonuclease A were added to concentrations of 250 μg/ml, and samples were incubated for 2 h on ice with occasional gentle shaking. Portions of the extracts were diluted directly with an equal volume of twice concentrated SDS gel sample buffer for analysis, and the remainder was centrifuged for 10 min at 10,000 × g. Supernatant fractions were diluted 2-fold with twice concentrated SDS gel sample buffer, and pellet fractions were dissolved with SDS gel sample buffer. Protein concentrations were determined by the method of Lowry et al. (13) using bovine serum albumin as a standard. After fractionation by SDS-polyacrylamide gel electrophoresis (PAGE), proteins were transferred electrophoretically to Immobilon-P membranes (Millipore Corp., Bedford, MA), and C subunits were visualized using affinity-purified goat anti-C subunit antibody as the primary antibody, alkaline phosphatase-coupled anti-goat immunoglobulin in G as the secondary antibody, and Radi-free Lumi-Phos 530 chemiluminescent substrate sheets (Schleicher & Schuell) as described (11).

Gel Electrophoresis, Fluorography, and Quantitation of Radioactivity or Chemiluminescence—Gel Patterns—SDS-PAGE was carried out as described by Laemmli (14) using 10% polyacrylamide gels. For fluorography, gels were impregnated with 2,5-diphenyl oxazole in dimethyl sulfoxide and dried as described by Bonner and Laskey (15). X-ray films from fluorograms or Western immunoblots were scanned with a Molecular Dynamics Model 300A computing densitometer, and integrated absorbances corresponding to the various C subunit species were determined as described (11). Absorbances for the 39-kDa C subunit species and for the putative 47-kDa C subunit conjugate were normalized to that in the 33-kDa marker protein.

RESULTS

Fig. 1 shows SDS-PAGE patterns of [35S]methionine-labeled proteins immunoadsorbed from wild-type and kin− S49 cells with an anti-C subunit antibody. The patterns from both cell lines had a cluster of bands of ~39 kDa (C) that correspond to phosphorylated and nonphosphorylated forms of Cα and Cβ subunits (4, 16). An additional species of ~47 kDa (CJ) was seen only in the patterns from kin− cells. The 33-kDa band (M) seen in Fig. 1 (lanes a and b) is a tritium-labeled marker.
protein added to cell extracts to monitor recoveries. Immunoadsorption of both the 39-kDa C subunit forms and the 47-kDa species seen in kin- samples was blocked by preincubation of the antibody with purified recombinant C subunit (Fig. 1, lane d). The minor species that ran slightly slower than the 47-kDa species in some wild-type patterns (e.g. Fig. 1, lane a) was not blocked by the C subunit (data not shown), but several minor higher molecular mass species in the kin- samples were blocked (Fig. 1, lanes c and d; and data not shown).

Figs. 2 and 3 show results from label-chase experiments designed to monitor the intracellular degradation of the C subunit in wild-type and kin- cells. In wild-type cells, the C subunit label decreased exponentially after ~1 h of chase with half-lives of ~9.2 h in untreated cells and 4.5 h in cells treated with sufficient CPT-CAMP to activate fully the endogenous kinase holoenzyme (Fig. 2). The C subunit label in kin- cells disappeared with a half-life of ~1.5 h, and the 47-kDa species was degraded at a similar rate (Fig. 3). There were no apparent differences in turnover of total radiolabeled protein among the mutant, drug-free wild-type, and CPT-CAMP-treated wild-type cells (data not shown).

Fig. 4 shows Western immunoblot patterns that compare levels of C subunit protein in extracts of wild-type and kin- cells. Consistent with the radiolabeling studies, a cluster of bands corresponding to the various forms of the C subunit was detected in extracts from both cell lines, and an extra 47-kDa species was detected only in the mutant preparation. C subunit protein was mostly soluble in wild-type extracts, but almost entirely particulate in kin- extracts. In additional experiments, various amounts of extracts from the two cell lines were analyzed, and the proportions of protein in the C subunit were estimated by densitometry using a standard curve of purified recombinant C subunit for reference (11). The wild-type cell extracts had approximately five times as much C subunit/unit protein as did the kin- cell samples (data not shown).

Fig. 5 shows the results of experiments comparing the distribution of the C subunit and total protein label in extracts from wild-type and mutant cells. Centrifugation conditions were chosen to fractionate the extracts roughly into nuclei and large particulates (fraction P1), polysomes (fraction P2), protein complexes larger than ~900 kDa but smaller than polysomes (fraction P3), and soluble proteins (fraction S3). In extracts of pulse-labeled wild-type cells, ~15–30% of the total protein label was found in each fraction, but the majority of the C subunit label was in the soluble fraction (Fig. 5A). After a 30-min chase, there was little change in the distribution of either the C subunit or total protein label except for a decrease of the total label in the polysome fraction with corresponding increases in fractions P1 and S3 (Fig. 5B). The behavior of the total protein label in kin- extracts was similar to that in the wild-type extracts, but that of the C subunit label was distinct. Although ~50–60% of the C subunit label was soluble in extracts of kin- cells pulse-labeled for 5 min (Fig. 5C), <30% remained soluble by 30 min of chase (Fig. 5D). The 47-kDa species (here treated as an alternative form of the C subunit) was seen only after the chase and accounted for ~40–50% of the total C subunit radioactivity in each fraction (Fig. 5D).

Since the 47-kDa immunoreactive protein was not seen in samples from kin- cells labeled in a short pulse (Fig. 5), we undertook experiments to monitor the appearance of this spe-
cies. Fig. 6 shows that the 47-kDa species accounted for [35S]methionine. Over the next 20 min, the label in the 47-kDa of the immunoadsorbed label from cells pulsed for 10 min with kin

was recovered in control or Z-PP-CHO chase samples, presence of Z-GPFL-CHO and Z-PP-CHO. None of these inhibitors affected the recovery of acid-precipitable radioactivity in total extracts of the chase samples (data not shown).

In light of a report that inhibitors of the proteasomal proteases could stabilize long-lived proteins that might be degraded by ubiquitin-independent pathways (22), we attempted to assess the effects of the inhibitors used above on turnover of the C subunit in wild-type cells. Analysis of inhibitor effects on C subunit turnover in unstimulated wild-type cells was precluded by the toxicity of the drugs. Judging by the release of acid-precipitable radioactivity from [35S]methionine-labeled cells, both MG-132 and Z-LLF-CHO caused apparent cell lysis after treatments in excess of 7 h.2 Table I shows data from an experiment testing the effects of proteasomal protease inhibitors on the enhanced C subunit turnover observed in cells treated with CPT-cAMP. None of the inhibitors appeared to stabilize the C subunit significantly in this or three related experiments. (The apparent destabilizing effect of Z-GPFL-CHO in the experiment of Table I was not reproducible.)
Catalytic Subunit Turnover in S49 Mouse Lymphoma Cells

TABLE I

| Sample         | Initial valuea | Final valuea | Fractional decay |
|----------------|----------------|--------------|------------------|
| No inhibitor   | 0.235          | 0.113        | 0.52             |
| MG-132         | 0.260          | 0.151        | 0.42             |
| Z-LLF-CHO      | 0.325          | 0.202        | 0.38             |
| Z-GPF-CHO +    | 0.385          | 0.0985       | 0.74             |
| Z-PP-CHO       | 0.368          | 0.170        | 0.54             |

a C subunit radioactivity expressed as the ratio of absorbance in the [3H]leucine-labeled 33-kDa C subunit marker protein.

DISCUSSION

This study reports, for the first time, quantitative data on the intracellular turnover of the C subunit of cAMP-dependent protein kinase. The protein was degraded with a half-life of ~9.2 h in unstimulated cells and about twice as quickly in cells stimulated with CPT-cAMP. This difference in turnover rates accounts quite well for the 2-3-fold reductions in C subunit activity and/or protein reported in previous studies on a number of different cell systems treated chronically with either agents that elevate intracellular cAMP or cAMP analogs (6–9).

We suspect that the enhanced turnover of free C subunit results from greater accessibility to degradative enzymes and represents a general mechanism for down-regulation of the enzyme under persistent activation conditions.

C subunit turnover in kin− cells was three times faster than that in wild-type cells activated with CPT-cAMP and about six times faster than that in unstimulated wild-type cells. The 5-fold difference in C subunit protein levels found in extracts of wild-type and kin− cells by Western immunoblotting was roughly consistent with the observed differences in turnover rates. On the other hand, the differences in C subunit turnover were not significant to account for the 50-100-fold reductions in C subunit activity found in kin− cells (1). Consistent with our previous observation that newly synthesized [35S]methionine-labeled 39-kDa C subunit species to that in the [3H]leucine-labeled 33-kDa C subunit marker protein.

Stabilization of the 47-kDa species by MG-132 and Z-LLF-CHO implicates the chymotrypsin-like proteasome protease in turnover of the conjugate (21, 22). That higher molecular mass conjugates were not observed in cells treated with the proteasome protease inhibitors argues against polyubiquitination of the C subunit in the kin− cells, but it is also possible that polyubiquitinated forms did not accumulate because of the activity of isopeptidases selective for such species (28). Turnover of the C subunit in wild-type cells neither involved a protein conjugate nor was affected appreciably by inhibitors of the proteasome proteases. It appears, therefore, that C subunit turnover pathways in wild-type and mutant cell lines are distinct. The overall turnover of [35S]methionine-labeled proteins was equivalent in wild-type and kin− cells, and the proteasome protease inhibitors had no apparent effect on turnover of acid-precipitable label from either cell type. It thus seems unlikely that kin− cells have an unusually active ubiquitin-dependent proteolysis system.

Although a number of normal proteins are degraded by the pathway involving ubiquitination and the 26 S proteasome, the pathway was identified initially by its involvement in the rapid turnover of denatured proteins or proteins containing amino acid analogs (29–31). Selective use of this pathway for C subunit degradation in kin− cells reinforces the notion that the folded structures of C subunits in this cell line are in some way abnormal despite their having wild-type amino acid sequences (3). The rapid apparent aggregation of newly synthesized C subunits into insoluble complexes suggests that hydrophobic regions of the protein might be unusually exposed after synthesis in the mutant cell line. Both 39- and 47-kDa C subunit forms were found in and insoluble fractions of the kin− cell extracts.
cells, so the aggregation and conjugation processes are apparently independent of one another. We had thought previously that phosphorylation of the C subunit at Thr-197, which is required for full activity, was limited to wild-type cells. Using conditions that prevent dephosphorylation of the C subunit, we found recently that at least some of the C subunit synthesized in kin-2 cells is phosphorylated at Thr-197. 2 This observation argues that lack of phosphorylation is not the critical factor preventing accumulation of soluble C subunit in the mutant cells.

The hypothesis that the kin-2 mutation prevents proper folding of newly synthesized C subunit is problematical in view of both the dominance of the phenotype and its apparent specificity for the C subunit. The dominance of the kin-2 phenotype in somatic cell hybrids between wild-type and kin-2 cells would imply either that the mutation alters the function of an unknown cellular component so that it actively prevents proper C subunit folding or that the mutation alters the structure of a normal component of the protein folding machinery in such a way that it can inhibit proper folding of the C subunit even when it is present with an excess of wild-type copies of the same component. The latter situation seems plausible for the chaperonin proteins, which function in higher order oligomeric ring structures (32–34), but it is difficult to imagine that C subunit folding requires a specific chaperonin species that is not also essential for proper folding of many other cellular proteins. Specificity cofactors have been demonstrated for folding of tubulin by the chaperonin complex containing t complex polypeptide 1 (35), but mutations in such accessory factors would be expected to produce recessive enzyme deficiency phenotypes. Assuming that different proteins require different levels of chaperonin function for efficient folding, an alternative model could explain the apparent specificity of the kin-2 mutation for the C subunit. For proteins like the C subunit that apparently cannot fold properly without chaperonins, 2 thresholds of chaperonin activity might exist below which folding essentially fails. If the level of chaperonin activity (or, perhaps, activity of some particular chaperonin complex with selectivity for the C subunit) were just sufficient in S49 cells for C subunit folding to keep pace with expression, a moderate reduction in activity might have a disproportionate effect on C subunit folding. The defect would appear to be specific for the C subunit if other proteins using the same chaperonin complex(es) had lower critical thresholds.

Acknowledgments—We thank Robert Cauthron for advice and assistance in preparation of purified recombinant C subunit. We also thank Drs. Marian Orlowski and frans Stassen (ProScript, Inc.) for generously providing selective inhibitors of the proteasomal proteases, Drs. Alexander Vinitsky and Christopher Cardozo (Mount Sinai School of Medicine) and Ross Stein (ProScript, Inc.) for providing helpful suggestions about the use of these inhibitors, and Dr. Arthur Haas (Medical College of Wisconsin) for providing affinity-purified antibodies to ubiquitin.

REFERENCES
1. Steinberg, R. A., van Daalen Wetters, T., and Coffino, P. (1978) Cell 15, 1251–1261.
2. van Daalen Wetters, T., Murtaugh, M. P., and Coffino, P. (1983) Cell 35, 311–320.
3. Orellana, S. A., and McKnight, G. S. (1990) J. Biol. Chem. 265, 3048–3053.
4. Steinberg, R. A. (1991) Mol. Cell. Biol. 11, 705–712.
5. Steinberg, R. A., Cauthorn, R. D., Symcox, M. M., and Shuntoh, H. (1993) Mol. Cell. Biol. 13, 2332–2341.
6. Venerinos, A. (1986) FEBS Lett. 196, 126–130.
7. Hove, G., Vintermyr, O. K., and Døskeland, S. O. (1990) Mol. Endocrinol. 4, 481–488.
8. Richardson, J. M., Howard, P., Massa, J. S., and Maurer, R. A. (1990) J. Biol. Chem. 265, 13635–13640.
9. Taskén, K., Andersson, K. B., Skålhegg, B. S., Taskén, K. A., Hansson, V., Jåhnsen, T., and Blomhoff, H. K. (1993) J. Biol. Chem. 268, 23483–23489.
10. Steinberg, R. A. (1983) Methods Enzymol. 99, 233–243.
11. Lee, S.-L., Gorman, K. B., and Steinberg, R. A. (1996) Mol. Cell. Endocrinol. 116, 233–241.
12. O’Farrell, P. H. (1975) J. Biol. Chem. 250, 4007–4021.
13. Lowry, O. H., Rosenthal, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
14. Laemmli, U. K. (1970) Nature 227, 680–685.
15. Bonner, W. M., and Laskey, R. A. (1974) Eur. J. Biochem. 46, 83–88.
16. Olsen, S. R., and Uhler, M. D. (1989) J. Biol. Chem. 264, 18662–18666.
17. Orlowski, M. (1990) Biochemistry 29, 10289–10297.
18. Goldberg, A. L. (1992) Eur. J. Biochem. 203, 9–23.
19. Hershko, A., and Ciechanover, A. (1992) Annu. Rev. Biochem. 61, 761–807.
20. Rechtesteiner, M., Hoffmann, L., and Dubel, W. (1993) J. Biol. Chem. 268, 13510–13515.
21. Vinitsky, A., Michaud, C., Powers, J. C., and Orlowski, M. (1992) Biochemistry 31, 9421–9428.
22. Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A. L. (1992) J. Biol. Chem. 267, 761–766.
23. Vinitsky, A., Cardozo, C., Sopp-aboutino, L., Michaud, C., and Orlowski, M. (1994) J. Biol. Chem. 269, 29860–29866.
24. Ziv, O., Scharfman, D. B., Engram, C., and Fisher, M. C. (1943) J. Gen. Physiol. 26, 325–333.
25. Jentsch, S. (1992) Annu. Rev. Genet. 26, 179–207.
26. Shaffer, J. R., and Kania, M. A. (1995) Biochemistry 34, 4015–4021.
27. Haas, A. L., and Bright, P. M. (1985) J. Biol. Chem. 260, 12464–12473.
28. Hadari, T., Warms, J. V., Rose, R. A., and Hershko, A. (1992) J. Biol. Chem. 267, 719–727.
29. Chin, D. T., Kuehl, R., and Rechtesteiner, M. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 5857–5861.
30. Hershko, A., Eytan, E., Ciechanover, A., and Haas, A. L. (1982) J. Biol. Chem. 257, 13964–13970.
31. Ciechanover, A., Finley, D., and Varshavsky, A. (1984) J. Biol. Chem. 259, 23483–23489.
32. Gold, Y., Thomas, J. O., Chow, R. L., Lee, G.-H., and Cowan, N. J. (1992) J. Biol. Chem. 267, 719–727.
33. Lewis, V. A., Hynes, G. M., Zheng, D., Saitoli, H., and Willison, K. (1992) Nature 358, 249–252.
34. Hendrick, J. P., and Harti, F.-U. (1993) Annu. Rev. Biochem. 62, 349–384.
35. Gao, Y., Vainberg, I. E., Chow, R. L., and Cowan, N. J. (1993) Mol. Cell. Biol. 13, 2478–2485.