Phosphorylation of Protein Kinase C-α on Serine 657 Controls the Accumulation of Active Enzyme and Contributes to Its Phosphatase-resistant State*

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Serine 657 in protein kinase C-α (PKCα) is a site of phosphorylation on expression of the recombinant protein in mammalian cells. To define the function of this phosphorylation, PKCα species with mutations of this site were investigated. The alanine mutant, S657A PKCα, displayed slow phosphate accumulation in pulse-chase experiments, indicating a rate-limiting role in the initial phase of phosphorylation. Consistent with this, the aspartic acid mutant, S657D PKCα, showed an increased rate of phosphate accumulation. Both the S657D and S657A PKCα mutants were slow to accumulate as fully phosphorylated forms during a second phase of phosphorylation. This latter property is shown to correlate with an increased phosphatase sensitivity and decreased protein kinase activity for these two PKCα mutants. It is further shown that once fully phosphorylated, the S657D PKCα mutant displays WT PKCα properties with respect to thermal stability and phosphatase sensitivity in vitro and in vivo; in contrast, the S657A PKCα mutant remains sensitive. The properties of the Ser-657 site PKCα mutants define functional roles for this phosphorylation in both the accumulation of phosphate on PKCα as well as in its agonist-induced dephosphorylation. These results are discussed in the context of a working model of PKCα behavior, providing insight into the workings of other kinases with equivalent sites of phosphorylation.

The phosphorylation of protein kinases has long been established in the field of protein phosphorylation. In fact, the first target of regulated phosphorylation to be elucidated (phosphorylase b) lies at the end of a protein kinase cascade, wherein the cAMP-dependent protein kinase phosphorylates and activates phosphorylase kinase (see Ref. 1). Such cascades are now relatively commonplace among the protein kinase class, and there are many examples of positive regulatory cascades (e.g. the Raf-MEK-MAPK cascade) (2) and some of negative regulatory domain (see Ref. 4). Within what has been termed the “activation loop” of the kinase, the aspartic acid mutant, S657D PKCα, showed an initial phase of phosphorylation. Consistent with this, the alanine mutant, S657A PKCα, displayed slow phosphate accumulation in pulse-chase experiments, indicating a rate-limiting role in the initial phase of phosphorylation. Consistent with this, the aspartic acid mutant, S657D PKCα, showed an increased rate of phosphate accumulation. Both the S657D and S657A PKCα mutants were slow to accumulate as fully phosphorylated forms during a second phase of phosphorylation. This latter property is shown to correlate with an increased phosphatase sensitivity and decreased protein kinase activity for these two PKCα mutants. It is further shown that once fully phosphorylated, the S657D PKCα mutant displays WT PKCα properties with respect to thermal stability and phosphatase sensitivity in vitro and in vivo; in contrast, the S657A PKCα mutant remains sensitive. The properties of the Ser-657 site PKCα mutants define functional roles for this phosphorylation in both the accumulation of phosphate on PKCα as well as in its agonist-induced dephosphorylation. These results are discussed in the context of a working model of PKCα behavior, providing insight into the workings of other kinases with equivalent sites of phosphorylation.

Contrary to the kinases referred to above, the cAMP-dependent protein kinase is regulated acutely through the cellular production of the second messenger cAMP (see Ref. 5, and references therein), and there is no evidence of acute regulation through modified phosphorylation within the activation loop. Nevertheless, it is evident that this region in its phosphorylated state plays a key role in aligning the substrate binding site for catalysis (6, 7). Members of the related protein kinase C (PKC) family are also second messenger-dependent protein kinases. For PKCa and PKCb, homologous sites in their activation loops have been shown to play an essential role (8–11). Thus, while these second messenger-dependent protein kinases may not be the subject of acute regulation through these phosphorylation sites, a similar requirement for phosphorylation persists.

Although the activation loop phosphorylation sites in PKC and other protein kinases share a defined, necessary role in catalysis, phosphorylation of PKC sites outside this region plays a more subtle role in controlling function. In both PKCa and PKCb, phosphorylation of two C-terminal sites has been reported (11, 12). In PKCa, one of these, threonine 638 (Thr-638), has been shown to control the rate of agonist-induced dephosphorylation and inactivation of the protein in vivo but not to be required for catalytic activity (13). This property appears to be governed by interactions between the C-terminal region of the kinase and its catalytic core, with phosphorylation at Thr-638 and at the activation loop (Thr-497) being required to maintain the active, phosphatase-resistant, closed conformation of the kinase domain (13). Here, we have analyzed the role of the serine 657 (Ser-657) phosphorylation site in PKCa. It is established that phosphorylation of this site controls the accumulation of phosphate at other sites on PKCα, as well as contributing to the maintenance of the phosphatase-resistant conformation.

**EXPERIMENTAL PROCEDURES**

Mutagenesis—Bovine protein kinase C-α (14) was tagged with six histidine residues at the N terminus (His-tag PKCα) by synthesizing oligonucleotide cassettes for substitution insertion. It was mutagenized and sequenced using the Altered Sites in vitro mutagenesis system (Promega) and the Sequenase Version 2.0 DNA sequencing kit (U.S. Biochemical Corp.) and finally subcloned into the pKS1 vector according to a previously described procedure (13). The following mutagenic oligonucleotides, which are sense with respect to the PKCα cDNA, were used (changed bases are underlined; an additional point mutation was designed, introducing a snab 1 restriction site with no change at the protein level): S657A, CTGATTTTGAAGGCTTCGCCTACGTA-AACCCCCAGTTCG; S657D, CTGATTTTGAAGGCTTCGACTACGTA-AACCCCCAGTTCG; S657E, CTGATTTTGAAGGCTTCGATACGTA-AACCCCCAGTTCG; S657F, CTGATTTTGAAGGCTTCGATACGTA-AACCCCCAGTTCG.

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1 The abbreviations used are: PKC, protein kinase C; DMEM, Dulbecco’s modified Eagle’s medium; TPA, 12-O-tetradecanoylphorbol-13-acetate; PAGE, polyacrylamide gel electrophoresis; PP1cγ, γ isoform of human protein phosphatase 1; WT, wild-type.
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AACCCCGAGTTCG. The mutations at the 497 and 638 sites were prepared as described previously (13).

Transfection and Treatments of COS-1 Cells—COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 1000 units/ml penicillin, and 100 μg/ml streptomycin. All procedures were performed with DNA isolated using Qiagen's kit. DNA was introduced into 0.8 ml of 60–80% confluent COS-1 cells (5 × 10⁶ cells) by electroporation (0.45 kV for 8–10 msec). After 48 h of transient expression, the cells were lysed in 500 μl of an ice-cold lysis buffer containing 20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1% (v/v) Triton X-100, 2 mM Aprotinin, 5 mM benzamidine, 50 μg/ml leupeptin, 50 μg/ml aprotinin, 50 μg/ml trypsin inhibitor, 5 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM Na₂VO₄, 1 mM p-nitrophenyl phosphate, and 5 mM imidazole. The suspension was stroked 20 times in a Dounce homogenizer and centrifuged at 12,000 × g for 30 min at 4 °C. Recombinant mutant or WT PKCs in the Triton-soluble extract was purified with nickel-agarose (Qiagen) using a batch procedure described before (13) and subsequently eluted in lysis buffer (0.02% Triton X-100) supplemented with 100 mM imidazole.

For treatment with phorbol esters, the culture medium was changed after 30 h of transient expression and 500 mM 12-O-tetradecanoylphorbol-13-acetate (TPA) was added. At various time points, the incubation medium was removed, and cells were quickly rinsed in ice-cold phosphate-buffered saline and lysed in lysis buffer (as above but without imidazole) supplemented with 10 μM microcin. The lysate was passed twice through a 27-gauge needle and centrifuged for 20 min at 12,000 × g at 4 °C before analysis on SDS-PAGE.

For pulse-chase experiments, the culture medium was removed after 24 h of transient expression and replaced by DMEM supplemented with 10% fetal calf serum. After 1 h, cells were pulse-labeled for 15 min with 100 μCi/ml of [³⁵S]methionine (Amersham, Life Science) in DMEM plus 5% (v/v) dialyzed fetal calf serum. Chase was then started in DMEM supplemented with 10% fetal calf serum and 5 mM methionine and stopped after various time points. Cells were finally lysed, and PKC was purified with nickel-agarose as described above.

Protein Kinase C Treatment of Purified PKC—The γ isoform of human protein phosphatase 1 (PP1γ) (15) was obtained as a recombinant protein after introduction of the pCW PP1 plasmid into competent Escherichia coli DH5α. Expression and purification were carried out as described in Refs. 13 and 16. Purified recombinant PKCα (eluted from Ni-agarose in the absence of NaF, Na₂VO₄, and p-nitrophenyl phosphate) was treated at 22 °C for 30 min with purified recombinant PP1γ in the presence of 1 mM MnCl₂. Reactions were stopped by adding 10 μM microcin.

Protein Assays and Analysis—Protein kinase C activity was assayed as described earlier (13) using Histone III-S as a substrate. Protein concentration was determined by the bicinchoninic acid method (Pierce) using bovine serum albumin as the standard. High resolution SDS-PAGE was performed on samples diluted twice in 5 × Laemmli buffer (17) using 12-cm long running gels containing 7.5% acrylamide and 0.06% bisacrylamide. Gels were transferred onto nitrocellulose and subjected to immunoblotting using a polyclonal antibody raised against the C terminus of PKCα (18). Reproductions of autoradiographs were obtained as computer-scanned images.

RESULTS AND DISCUSSION

Aberrant Phosphorylation of S657A and S657D PKCα Mutants—Previously, it has been shown that PKCs is phosphorylated at Ser-657 (11). Using β8 protease digestion, high pressure liquid chromatography purification, and mass spectrometry analysis, serine 657 was found to be phosphorylated also in recombinant ³²P-labeled PKCα expressed in COS cells.¹ In order to study the role of this phosphorylation, substitution mutants were made to prevent (S657A) or partially mimic (S657D) phosphorylation at this site. Both Ser-657 site PKCα mutants were expressed in COS cells, fully recovered in the detergent-soluble fraction after lysis, and could be purified on Ni-agarose (Fig. 1). Unlike the WT PKCα molecule that migrates as a single 80-kDa band, a doublet was reproducibly detected for both the 657 site mutants. It would therefore appear that mutating the 657 position in PKCα perturbs...

¹ F. Boroncin, D. Rahman, D. J. C. Pappin, and P. J. Parker, unpublished data.
sequent sections provide evidence for three components to Ser-657 phosphorylation: (i) the phosphorylation of Ser-657 is rate-limiting for the first phase of PKCα phosphorylation; (ii) Ser-657 phosphorylation is required for efficient second phase phosphorylation of PKCα; and (iii) phosphorylation of Ser-657 contributes to “locking” fully phosphorylated PKCα in a closed, phosphatase-resistant conformation.

The conclusions to be drawn from these analyses are entirely consistent with the steady-state pattern of S657D PKCα expression, which displays both fully phosphorylated and partially phosphorylated species (see Fig. 2B; i.e. processing can go to completion for this PKCα mutant, but it is incomplete in the steady state). By contrast, for the WT PKCα, although fast migrating forms can be detected by pulse labeling techniques (Fig. 2A), these forms are rapidly phosphorylated such that they do not contribute significantly to the steady-state level of WT PKCα. Thus, fast migrating forms are poorly detected by Western blotting for WT PKCα (Fig. 2B).

The S657D PKCα Mutant Displays Functional Heterogeneity—The slow rate of phase 2 phosphorylation of the S657D PKCα mutant indicated that the partially phosphorylated form may be functionally compromised. In order to assess the relative specific activity and protein phosphatase sensitivity of the S657D PKCα mutant, WT and S657D PKCα were purified from COS cells as above and treated in vitro with PP1cy. As shown in Fig. 3A, the faster migrating S657D PKCα species (78 kDa) was sensitive to PP1cy while the slower migrating (80 kDa) species was not; the 80-kDa species behaved exactly as WT PKCα (80 kDa). There was very little change in activity associated with the dephosphorylation of the 78-kDa S657D PKCα (Fig. 3A), implying that this species does not contribute significantly to the determined activity. This is consistent with calculation of the specific activity of the S657D mutant; accounting for only the 80-kDa species yielded a specific activity for S657D PKCα which was 103% of that of WT PKCα.

This data indicates that the fast migrating species is a low activity form that is sensitive to dephosphorylation. Previous studies on the PKCα Thr-638 site mutants have correlated phosphatase hypersensitivity in vitro and in vivo (13). A similar correlation was found for the S657D PKCα mutant. Thus, on transient expression of S657D PKCα in COS cells, TPA treatment induced a rapid (~15 min) dephosphorylation of the fast migrating form but not the slow migrating form (Fig. 3B). As observed in vitro (Fig. 3A), the slow migrating form behaved essentially as WT PKCα (not shown) while the fast migrating form was hypersensitive to phosphatases. Notably, the S657D PKCα doublet was entirely Triton X-100-soluble in transfected cells, and only following TPA treatment did antigen accumulate in the Triton X-100-insoluble fraction.

In PKCα Thr-638 site mutants, phosphatase sensitivity parallels thermal instability (13). This appears to be due to an alteration in the way the C terminus (V5 domain) interacts with the catalytic core (C3/4 domain), producing an “open” conformation. For the S657D mutant, kinase activity is largely accounted for by a slowly migrating form that is phosphatase-resistant, i.e. behaves like WT PKCα. It can be predicted that this mutant would resemble the WT protein with respect to thermal inactivation, and this is, in fact, the case. Incubation at 25 °C for 30 min induced a 5% loss of activity precisely that observed for WT PKCα (Fig. 4). By way of comparison under these same conditions, the Thr-638 mutants (that do not accumulate as partially phosphorylated forms, see Fig. 1) displayed a much greater degree of thermal inactivation. The S657A mutant displayed an intermediate loss of activity (see below).

The properties of the two differentially phosphorylated S657D species indicate that once fully phosphorylated, the aspartic acid substitution is sufficient to mimic any requirement for phosphorylation at this site. This is reflected in the full activity, phosphatase resistance (in vitro and in vivo), and thermal stability of the kinase activity. However, it is equally evident that the partially phosphorylated (fast migrating) form is of low activity and is phosphatase-sensitive. It is these latter properties that would account for the slow second phase of S657D PKCα phosphorylation as discussed above.
S657A PKCα Reveals a Stabilizing Role for the 657 Phosphorylation Site—Contrary to the S657D substitution, S657A PKCα could be dephosphorylated and inactivated in vitro by PP1cγ (Fig. 5A). However, the extent of inactivation correlated with the dephosphorylation of the slower migrating form, indicating that, as for S657D PKCα, the faster migrating form contributes little to the overall perceived activity. Accounting for only the upper slow migrating S657A PKCα species reveals a specific activity for this protein of 109% of the WT PKCα. The results demonstrate that phosphorylation of this site is not essential for catalysis but affects phosphatase sensitivity in vitro.

Phosphatase hypersensitivity of S657A PKCα was observed also in vivo (Fig. 5B). Thus, on TPA treatment of S657A PKCα transfected COS cells, the mutant was dephosphorylated with virtually complete loss of the most highly phosphorylated form by 15 min. As described for the S657D mutant, the dephosphorylated form accumulated in the Triton X-100-insoluble fraction; prior to TPA treatment, the S657A PKCα forms were fully Triton X-100 extractable (discussed below). The observed increased sensitivity to phosphatases for S657A PKCα, redistribution to the cytoskeleton, and the loss of activity observed on dephosphorylation in vitro suggests that phosphorylation at the 657 site (or an aspartic acid) normally contributes to the “closed” conformation (V5-C3/4 interacting; see Ref. 13) of the protein. Consistent with this view was the moderate thermal instability of the S657A PKCα kinase activity noted above (see Fig. 4). Thus, it would seem that phosphorylation of the 657 site not only influences the accumulation of phosphate as described above but also contributes to maintaining the active closed conformation (i.e. effective V5-C3/4 interactions). Evidence that this is the case is suggested by studies with PKCα mutants altered at the Thr-497 or Thr-497 and Thr-638 sites. The T497E/T638E mutant retains reduced protein kinase activity, but this mutant can still be inactivated by PP1cγ similar to the T497E mutant (Fig. 6). While not necessarily the only other regulatory phosphorylation site on PKCα, the phosphatase-dependent inactivation of the T497E/T638E mutant is consistent with Ser-657 dephosphorylation. This conclusion is further supported by the finding that an T497E/T638E/S657E mutant is both inactive and, by migration, insensitive to TPA-induced dephosphorylation in vitro (data not shown).

A Model for the Phosphorylation Control of PKCα—The properties and phosphorylation of the S657A and S657D PKCα mutants are summarized in Fig. 7, alongside those predicted for the WT PKCα. The presence of phosphate in the Thr-497 site in the low/partial activity form resulting from “phase 1” is based upon mapping of 32P-labeled peptides from the fast migrating form of the S657D mutant (data not shown). Phosphorylation of Thr-638 in the second phase is predicted from

![Figure 4. Thermal stability of the PKCα C-terminal phosphorylation mutants.](image)

![Figure 5. Lack of phosphate on Ser-657 correlates with increased phosphatase sensitivity.](image)

![Figure 6. Phosphates on Ser-657 and Thr-638 cooperate to maintain kinase activity.](image)
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Ser-657 phosphorylation appears to play a role alongside that of Thr-638 in forming this closed conformation although the Thr-638 site is dominant in this regard. This dominance of the Thr-638 site is evidenced by the greater phosphatase sensitivity (13) and thermal instability of the T638A mutant compared with the S657A mutant.

The partially phosphorylated forms of both the S657A and S657D mutants are of low activity, and this is consistent with the slow progress of phase 2 compared with WT PKCα. It can be concluded then that the conformation/activity of the partially phosphorylated WT PKCα is more favorable for completion of phase 2. The attainment of this partially phosphorylated state is also dependent upon Ser-657 phosphorylation, as evidenced by the pulse-chase experiments with the 657 site mutants. These reveal a rate-limiting role for Ser-657 phosphorylation in phase 1. While this phase appears to lead to phosphorylation of the Ser-657 and Thr-497 sites in WT PKCα, the fact that Ser-657 phosphorylation is rate-limiting does not define the order of these events.

A unifying hypothesis derived from these studies and those on Thr-638 site mutants (13) is that the phosphorylated Ser-657 site contributes to contact between the C-terminal (V5) domain and the catalytic core (C3/4). In the partially phosphorylated state (no Thr-638 phosphorylation), phosphate at Ser-657 acts alone to generate the appropriate conformation, and aspartic acid substitution is an insufficient surrogate. In the fully phosphorylated state, this conformation is contributed by both the Thr-638 and Ser-657 sites; here, aspartic acid substitution is sufficient to act in synergy with phosphate at the Thr-638 site. Contrary to previous conclusions (11, 20), these priming phosphorylations are not involved in solubilizing the primary translation product as evidenced by the complete solubility of the mutants described here. In fact, the detergent-insoluble form that can accumulate in cells is associated with dephosphorylation of the activated enzyme. This property of the dephosphorylated enzyme distinguishes it from the primary translation product that is soluble and maintain solubility and permit appropriate folding/phosphorylation. In view of the poor solubility of PKCα expressed even at low levels in E. coli, the existence of a mammalian chaperone would seem a probable mechanism. This conclusion is further supported by the finding that dephosphorylation of WT PKCα in vitro leads to aggregation and insolubility of the protein.

The results presented here and previously (13) on PKCα define the processes involved in generating and maintaining an active kinase. The implications of these findings are broad. There are a number of protein kinases that, like PKCα, have conserved threonine and serine sites in locations equivalent to Thr-638 and Ser-657. For example, of particular current interest is the control of the PKB/akt/RAC kinase. This protein has recently been shown to be fully activated in an agonist-dependent fashion through phosphorylation in both its catalytic core (activation loop) site (residue T308) and in a C-terminal site equivalent to Ser-657 in PKCα (PKB residue S473) (21). The manner of operation of these sites in PKB is likely to be as described here for PKCα.

In respect of the Ser-657 site itself, this site lies within an FSF/Y motif. In p70S6kinase, the phosphorylation of the equivalent site within the same motif is sensitive to rapamycin (22); no such sensitivity is observed for PKCα, implying a distinct mechanism of control. Interestingly, in PKCζ and in the PRK family, this equivalent site is substituted by an acidic residue (23–25). Based upon the mutants described here, this would not affect the fully phosphorylated form, but it may preclude the existence of low activity forms of these proteins that are subject to control by acute phosphorylation and activation. Evidence that this may be the case exists for PRK1(PKN) (26). The implications of the pattern and consequence of PKCα phosphorylation thus impact on a number of related kinases, and it will be of great interest to see how well conserved these properties indeed are and how they integrate into the distinct modes of regulation of these kinases.

3 F. Bornancin, and P. J. Parker, unpublished data.
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REFERENCES
1. Heilmeyer, L. (1991) Biochim. Biophys. Acta 1094, 168–174
2. Cobb, M. H., and Goldsmith, E. J. (1995) J. Biol. Chem. 270, 14843–14846
3. Cross, D. A. E., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995) Nature 376, 785–789
4. Johnson, L. N., Noble, M. E., and Owen, D. J. (1996) Cell 85, 149–158
5. Taylor, S. S., Buechler, J. A., and Xuong, N.-H., Taylor, S. S., and Sowadski, J. M. (1991) Science 253, 414–420
6. Knighton, D. R., Zheng, J., Ten-Eyck, L. F., Xuong, N.-H., Taylor, S. S., and Sowadski, J. M. (1991) Science 253, 414–420
7. Knighton, D., Zheng, J., Ten-Eyck, L., Ashford, V., Xuong, N., Taylor, S., and Sowadski, J. (1991) Science 253, 407–414
8. Cazaubon, S., Bornancin, F., and Parker, P. J. (1994) Biochem. J. 301, 443–448
9. Cazaubon, S. M., and Parker, P. J. (1993) J. Biol. Chem. 268, 17559–17563
10. Orr, J. W., and Newton, A. C. (1994) J. Biol. Chem. 269, 27715–27718
11. Keranen, L. M., Dutil, E. M., and Newton, A. C. (1995) Curr. Biol. 5, 1394–1403
12. Tsutakawa, S. E., Medzirdradzky, K. F., Flint, A. J., Burlingame, A. L., and Koshland, D. E., Jr., (1995) J. Biol. Chem. 270, 26807–26812
13. Bernance, F. P., and Parker, P. J. (1996) Curr. Biol. 6, 1114–1123
14. Parker, P. J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M. D., and Ullrich, A. (1986) Science 233, 853–859
15. Barker, H. M., Brewis, N. D., Street, A. L., Spurr, N. K., and Cohen, P. T. W. (1994) Biochim. Biophys. Acta 1230, 212–218
16. Alessi, D. R., Street, A. J., Cohen, P., and Cohen, P. T. W. (1993) Eur. J. Biochem. 213, 1055–1066
17. Laemmli, U. K. (1970) Nature 227, 680–685
18. Marais, R. M., and Parker, P. J. (1989) Eur. J. Biochem. 182, 129–137
19. Pears, C., Stabel, S., Cazaubon, S., and Parker, P. J. (1992) Biochem. J. 283, 515–519
20. Newton, A. C. (1995) J. Biol. Chem. 270, 28495–28498
21. Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B. A. H. (1996) EMBO J., in press
22. Pearson, R. B., Dennis, P. B., Han, J. W., Williamson, N. A., Kozma, S. C., Wettenhall, R. E. H., and Thomas, G. (1995) EMBO J. 14, 5279–5287
23. Ono, Y., Fujii, T., Opta, K., Kikkawa, U., Igarashi, K., and Nishizuka, Y. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3099–3103
24. Mukai, H., and Ono, Y. (1994) Biochem. Biophys. Res. Commun. 199, 897–904
25. Palmer, R. H., Ridden, J., and Parker, P. J. (1995) Eur. J. Biochem. 227, 344–351
26. Amado, M., Mukai, H., Ono, Y., Chihara, K., Matsu, T., Hamajima, Y., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996) Science 271, 648–650