CVAK104 Is a Novel Poly-L-lysine-stimulated Kinase That Targets the β2-Subunit of AP2*

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Isolated clathrin adaptor protein (AP) preparations are known to co-fractionate with endogenous kinase activities, including poly-L-lysine-stimulated kinases that target various constituents of the clathrin coat. We have identified CVAK104 (a coated vesicle-associated kinase of 104 kDa) using a mass spectroscopic analysis of adaptor protein preparations. CVAK104 is a novel serine/threonine kinase that belongs to the SCY1-like family of protein kinases, previously thought to be catalytically inactive. We found that CVAK104 co-fractionates with adaptor protein preparations extracted from clathrin-coated vesicles and directly binds to both clathrin and the plasma membrane adaptor, AP2. CVAK104 binds ATP, and kinase assays indicate that it functions in vitro as a poly-L-lysine-stimulated kinase that is capable of autophosphorylation and phosphorylating the β2-adap-
CVAK104 Cloning—To generate the full-length CVAK104 cDNA, a commercially available cDNA, CSODC0265707 in PMV-spor6 (Invitrogen) (Genbank accession number CB212890), containing the 5’ initiating methionine and additional CVAK104 coding sequence, was digested with KpnI. The isolated insert was ligated into the KpnI site of KIAA1360 (Kazusa Research Institute, Chiba, Japan). The resulting full-length cDNA was then used as a template for PCR reactions to integrate an N-terminal Myc tag and 5’-3’-EcoRI restriction sites for subcloning into a pVL1383-GST fusion vector (5) and baculovirus production (see “CVAK Antibody & Protein Production”). PCR primers included: 5’-GAATTCATGGAGCAGAAGCTGATCTCAGAGGAGGACCAGATGAGTTGATCCTAGTCCATGTTAATAAATGG-3’ and 5’-GAATTCCCATGAAAAGAAGTTCACAGGGC-3’, which was subsequently used as an antigen for antibody production in rabbit (number 6369) using established protocols (21). Antibody specificity was tested by immunoblot analysis with preimmune sera showing no immunoreactivity to bovine brain coat constituents (data not shown). The CVAK104-GST fusion construct in pVL1383-GST (see “CVAK104 Cloning”) was used for insect cell-mediated protein expression in the BaculoGold expression system following the manufacturer’s protocols (Pharmingen). After 48 h of infection with recombinant baculovirus and expression in TN5 cells, the cells were pelleted, processed, and fusion protein was isolated following the identical procedures as those followed for bacterially expressed GST fusion proteins, with one exception. Following GST fusion protein binding to glutathione beads, bound protein was washed three times with 20 bead volumes of PBST, and then eluted with the reduced glutathione.

Nucleotide Binding Assays—To assay nucleotide binding, 2 μg of isolated CVAK- or AAK1-GST fusion protein was incubated in kinase buffer (see “Kinase Assays”) and 50 μM AB11 (Affinity Labeling Technologies, Inc., Lexington, KY), a biotinylated, UV light, cross-linkable ATP analogue, in the absence or presence of unlabeled ATP at various concentrations. Following AB11 addition, samples were incubated for 5 min at room temperature and then exposed to UV light at 254 nm using a handheld UV lamp for 2.5 min. The binding assay was terminated by the addition of protein sample buffer and boiling. Samples were resolved by SDS-PAGE, transferred to nitrocellulose, probed for bound biotin using alkaline-phosphatase conjugated to avidin, and developed as described previously (22).

Kinase Assays—Kinase assays were performed essentially as described previously (5), with minor modification. Briefly, isolated baculovirus-expressed CVAK-GST was incubated in kinase buffer (see “Kinase Assays”) and 50 μM AB11 (Affinity Labeling Technologies, Inc., Lexington, KY), a biotinylated, UV light, cross-linkable ATP analogue, in the absence or presence of unlabeled ATP at various concentrations. Following AB11 addition, samples were incubated for 5 min at room temperature and then exposed to UV light at 254 nm using a handheld UV lamp for 2.5 min. The binding assay was terminated by the addition of protein sample buffer and boiling. Samples were resolved by SDS-PAGE, transferred to nitrocellulose, probed for bound biotin using alkaline-phosphatase conjugated to avidin, and developed as described previously (22).

Adaptor Protein and Clathrin Interaction Tests—Isolated adaptor protein and clathrin preparations co-fractionate with endogenous kinase activities that target various subunits of the AP2 complex. We previously identified AAK1, a μ2 kinase present in these preparations, using a phage display protein interaction screen (5). To identify other endogenous kinase activities, isolated adaptor protein preparations derived from bovine brain (19) were analyzed using mass spectroscopy. This analysis revealed the presence of AAK1, as expected, and led to the identification of a previously uncharacterized kinase encoded by human cDNA KIAA1360 (GenBank™ accession number AB037781). Sequence analysis indicated that KIAA1360 lacks an initiating methionine, whereas domain analysis using SMART (24) suggested the presence of a kinase domain.

To identify the full-length cDNA, the 5’ nucleotide sequence of KIAA1360 was used to search the human expressed sequence tag data base (NCBI). This resulted in the identification of the initiating methionine and full-length cDNA (other full-length sequences have now been identified, see accession number NP_060658) that encodes a kinase we now call CVAK104. CVAK104 has a predicted molecular mass of ~104 kDa and belongs to a class of phosphotransferases, whose specificity for serine/threonine or tyrosine is ambiguous (Fig. 1). Interestingly, although CVAK104 has other kinase signature motifs, it lacks the highly conserved aspartic acid residue used for nucleotide catalysis in other kinases. Thus, CVAK104 belongs to the SCY1-like family of protein kinases (referred to as SCY1-L) (25) that are thought to be catalytically inactive. CVAK104 contains two weak clathrin binding motifs, DLL (26) and an NPF motif shown to support interaction with EPS15 homology domain-containing proteins, such as eps15 and intersectin (27).

Phylogenetic analysis indicates that CVAK104 is highly conserved in mammals, sharing >93% identity with the full-length amino acid sequences of rat (GenBank™ accession number XM_2350507) and mouse (accession number NM_198021) and 98% identity within their predicted kinase domains (not shown).

CVAK104 Co-fractionates with Adaptor Protein Preparations—To analyze the distribution of CVAK104, we raised polyclonal antibodies against the C-terminal two-thirds of the protein (see “Experimental Procedures”). In crude bovine brain lysates, CVAK antisera specifically recognized three protein species (104-, 116-, and 118-kDa) (Fig. 2A). Of these, the 104-kDa species had a size consistent with the predicted molecular weight of the full-length cDNA. This species was predominantly membrane-associated and co-fractionated with isolated clathrin-coated vesicles (Fig. 2A). The larger two CVAK species are found in both cytosolic and membrane fractions, but neither is enriched along with clathrin-coated vesicles. Whether they represent alternate splice forms of CVAK104, as we have observed for AAK1 (5), or another antigenically related protein remains to be determined.

Further fractionation of Tris-extracted clathrin coat protein preparations, resolved by gel filtration and hydroxyapatite chromatography (19, 20), revealed that CVAK104 elutes from the hydroxyapatite column at low salt in fractions enriched with AP1, as detected by immunoblot analysis using an anti-μ-adaptin antibody (Fig. 2B). However, we do not believe that this co-fractionation reflects a direct interaction between AP1 and CVAK104 (see next paragraph). Together, these results confirm our mass spectroscopy data and those of others (28), indi-
cating the presence of CVAK104 in AP preparations, and sug-
ger that CVAK104 may directly interact with clathrin coat
c constituents to regulate their function.

CVAK104 Specifically Interacts with Endocytic Vesicle Coat Components—We postulated that, because CVAK104 co-fracti-
ates with clathrin-coated vesicles, CVAK104 might also
directly interact with at least one of the major coat proteins. To
directly test this possibility, baculovirus-expressed CVAK104-GST
fusion protein was immobilized on glutathione beads and incu-
bated with the major coat proteins, AP1, AP2, and clathrin.
CVAK104-GST specifically bound clathrin triskelia and iso-
lated AP2 complexes (Fig. 3), whereas no interaction was ob-
served following incubation of these coat protein complexes
with immobilized GST. In contrast, we were unable to detect
interaction between CVAK104 and AP1 under our experimen-
tal conditions (Fig. 3), although minor contaminating amounts
of AP2 in our AP1 preparations were detected and found to
interact with CVAK104. These data would suggest that
CVAK104 might be preferentially associated with clathrin-
coated vesicles derived from the plasma membrane rather than
from the trans-Golgi network or endosomes. However, we were
unable to test this prediction, because our antibodies were not
effective reagents for immunofluorescence localization studies.

The C-terminal ear domains of α- and β-adaptin are regions
of the AP2 complex known to support interaction with a variety
of endocytic accessory factors. However, binding studies (5) indi-
cate that the AP2-CVAK104 interaction is not supported through
the ear domain of α-adaptin; interaction between the isolated
α-adaptin ear domain and CVAK104-GST fusion protein was not
detectable under conditions known to support binding to other
factors, such as AAK1 (data not shown). Attempts to directly test
a potential interaction between the ear domain of β-adaptin and
CVAK104 proved inconclusive, as both proteins were found to
nonspecifically bind to agarose beads (data not shown). These
results suggest that CVAK104 binds specifically to AP2 at a site
distinct from the appendage domain and by a means distinct
from other AP2-interacting proteins. This is consistent with the
lack of AP2-interacting motifs in its sequence. These AP2-
CVAK104 interactions are likely disrupted by the 0.5 M Tris used
to extract coated vesicles, and the co-elution of CVAK104 with
AP1 during hydroxyapatite chromatography most likely reflects
its interactions with hydroxyapatite medium rather than direct
interactions with AP1.

CVAK104 Is a Serine/Threonine Kinase That Phosphorylates
the β2-Adaptin Subunit of AP2—We have shown that
CVAK104 encodes a putative kinase that is present in AP

FIG. 1. The predicted amino acid se-
quence of human coated vesicle-assoc-
iated kinase, CVAK104. The N-termi-
nal serine/threonine kinase domain of
CVAK104 is shaded, whereas a predicted
coiled-coil (C–C) domain is underlined
(amo acids 664–699). The arrowhead
indicates the position of the asparagine in
CVAK104 that, in other kinases, is an
aspartic acid used for nucleotide cataly-
sis. CVAK104 also contains two conserved
protein interaction motifs (boxed) that are
thought to support binding to clathrin
(DLL) and EH domain-containing pro-
teins (NPF). Amino acids 145–933 were
used for the production of polyclonal an-
tibodies (see “Experimental Procedures”).

FIG. 2. CVAK104 co-fractionates with clathrin-coated vesicles.
A, immunoblot analysis of fractionated bovine brain reveals three
CVK species (115-, 116-, and 104-kDa), with the 104-kDa species
being associated with clathrin-coated vesicles (CCVs). B, coat proteins
extracted from bovine brain clathrin-coated vesicles were fractionated
by gel filtration and hydroxyapatite chromatography (see “Experimen-
tal Procedures”) and analyzed for the presence of CVAK104. The mono-
clonal antibodies 100/2 and 100/3 that specifically recognize α- and
γ-adaptin, respectively, and polyclonal antibodies against CVAK104
were used for immunoblot analysis. CHC, clathrin heavy chain; CL,
clothin, α and c, α-adaptin isoforms.
preparations, which contain endogenous kinase activities. Therefore, we tested the ability of CVAK104 to function as a kinase. Initially, we analyzed the ability of baculovirus-expressed CVAK104-GST fusion protein to bind ATP. As a positive control, we used the AAK1-GST fusion protein that, as expected, was capable of binding a UV light cross-linkable, biotinylated ATP analogue. This binding was effectively competed by incubation with unmodified ATP (Fig. 4). Similarly, we found that CVAK104 bound the ATP analogue, which also could be competed with unlabeled ATP. The identity of the nucleotide binding species was confirmed by immunoblot analysis. Basic polypeptide chains, such as poly-L-lysine, stimulate endogenous kinases associated with clathrin-coated vesicles; therefore, we tested their effect on CVAK104 nucleotide binding. However, the presence or absence of poly-L-lysine did not alter the ability of CVAK104 to bind nucleotide (Fig. 4).

CVAK104 directly interacts with AP2, is present in enriched adaptor protein preparations, and binds nucleotide. Thus, we tested its ability to function as a phosphotransferase and to target adaptor proteins. The activity of endogenous kinases that co-fractionate with adaptor proteins from bovine brain was readily visualized following the incubation of samples with [γ-32P]ATP (Fig. 5A, lanes labeled APs). The major phosphorylated product, which results from the action of AAK1 (5), was the μ2-subunit of AP2. The addition of poly-L-lysine to these kinase reactions resulted in the stimulation of an endogenous kinase(s) leading to an increase in the phosphorylation of the large adaptor protein subunits, the adaptins. When isolated CVAK104 was incubated with [γ-32P]ATP, we did not observe any significant kinase activity. However, when reactions were supplemented with poly-L-lysine, CVAK104 was capable of autophosphorylation (Fig. 5A, lanes labeled CVAK). To assay the ability of CVAK104 to target adaptor proteins, we inactivated endogenous kinases by pre-incubation with the ATP analogue FSBA, which irreversibly binds the active site and inhibits kinase activity (5, 12). The addition of baculovirus-expressed CVAK104-GST fusion protein and poly-L-lysine to FSBA-inactivated AP1 samples did not reveal any significant CVAK104-mediated phosphorylation beyond CVAK104 autophosphorylation (Fig. 5A, lanes labeled FSBA-AP1), consistent with the inability of CVAK104 to directly interact with AP1 (Fig. 3). In contrast, incubation of CVAK104 and poly-L-lysine with AP2 resulted in the phosphorylation of proteins at a molecular weight consistent with that of the large adaptin subunits (Fig. 5A, lanes labeled FSBA-AP2). As was observed for autophosphorylation, CVAK104-mediated phosphorylation of the AP2-associated adaptins required the presence of poly-L-lysine.

![Fig. 3. CVAK104 binds endocytic vesicle coat components.](image)

![Fig. 4. CVAK104 binds ATP in a poly-L-lysine-independent manner.](image)

![Fig. 5. CVAK104 is a poly-L-lysine-stimulated kinase that targets the β2-subunit of AP2.](image)
The system in the first dimension, followed by standard SDS-PAGE in the second dimension. This approach, developed by Ahle et al. (23), separates the large adaptin subunits, as these proteins fail to resolve under isoelectric focusing conditions (23, 29). When kinase reactions containing CVAK104, poly-L-lysine, and AP2 were resolved under this system, immunoblot analysis indicated that the β2-subunit of AP2 was selectively phosphorylated (Fig. 5, D and E) with no detectable phosphorylation of the α-adaptin subunit (Fig. 5, F and G).

In addition to assessing CVAK104 activity toward isolated adaptor proteins, other potential substrates were tested. Poly-L-lysine-stimulated CVAK104 was also found to phosphorylate an ~60 kDa protein from FSBA-inactivated cytosol (Fig. 6). Interestingly, when CVAK104 is incubated with Tris-extracted proteins from isolated microsomes that contain clathrin coat proteins, we observed a phosphorylated band at ~90 kDa and no detectable phosphorylation of the β2-subunit of AP2. Additionally, we tested CVAK104 activity toward polyGlu:Tyr (4:1) peptides, a general substrate for protein tyrosine kinases. However, the only phosphorylated product detected was CVAK autoposphorilated (Fig. 6). Collectively, these observations demonstrate that CVAK104, like many kinases, has multiple targets in different cellular locations.

To further characterize CVAK104 activity, we analyzed its sensitivity to an array of kinase inhibitors. In contrast, CVAK104 was relatively insensitive to the cAMP-dependent kinase inhibitor H89 (100 μM), heparin (5 μg/ml), a casein kinase II inhibitor, cAMP-dependent protein kinase inhibitor peptides (600 nm), or protein kinase C/calmodulin kinase inhibitor mixtures (Fig. 7A; see “Experimental Procedures”). Moreover, CVAK104 activity was not perturbed by incubation with genistein (10 μM) at concentrations known to inhibit a range of protein tyrosine kinases, consistent with its lack of activity toward tyrosine kinase substrates (polyGlu:Tyr) peptides (Fig. 6). Treatment of synaptosomes with reagents that inhibit cdk5 activity alters clathrin-mediated synaptic vesicle recycling, although with differentially reported effects (30, 31). Given that CVAK104 targets the β2-subunit of AP2, a core component of the endocytic machinery, we examined CVAK104 sensitivity to the cdk5 inhibitors olomoucine and roscovitine. Olomoucine treatment had little effect on CVAK104 activity toward β2-adaptin (Fig. 7B), whereas roscovitine showed an inhibitory effect at 10 μM, a concentration 10-fold greater than that required to inhibit cdk5. The differential activities of roscovitine versus olomoucine may, in part, account for their differential effects on synaptic vesicle recycling in neurons (30, 31). Collectively, these observations suggest that CVAK104 is a poly-L-lysine-stimulated serine/threonine kinase whose activity is responsible for the phosphorylation of the β2-adaptin subunit of AP2 in isolated adaptor protein preparations.

**DISCUSSION**

We have identified a novel coated vesicle-associated kinase, CVAK104, which belongs to the SCY1-like family of protein kinases, previously thought to be catalytically inactive, as they lack the highly conserved catalytic aspartic acid (25). Although we do not currently understand the mechanism of catalysis, here we provide evidence that CVAK104 is a poly-L-lysine-stimulated serine/threonine kinase that has autokatalytic activity and targets the large β2-subunit AP2. The isolated CVAK104-GST fusion protein used in our studies derives from insect cell overexpression; thus, we cannot eliminate the possibility that our protein samples are contaminated with trace amounts of another tightly associated kinase. However, we do not favor this scenario for several reasons. 1) It is unlikely that a contaminating baculovirus-derived kinase would share the same properties as that of the endogenous activity found in adapter protein preparations. CVAK104 is not only present in adapter protein preparations, its activity, similar to that of the endogenous activity found in adapter protein preparations.

![Fig. 6. CVAK104 has multiple substrates.](Image)

Isolated cell fractions were treated with FSBA to inactivate endogenous kinase activities. Samples were then incubated with 50 μg/ml poly-L-lysine and [γ-32P]ATP in the presence or absence of isolated CVAK-GST fusion protein as indicated. PolyGlu:Tyr (4:1) peptides were also analyzed as a potential substrate for CVAK-GST. CVAK-GST-specific phosphorylated protein products are indicated by the arrows. Phosphorylated proteins were visualized by autoradiography using a phosphorimaging plate following SDS-PAGE.

![Fig. 7. CVAK104 is sensitive to a general serine/threonine kinase inhibitor.](Image)

CVAK104 sensitivity to kinase inhibitors was assayed by incubating CVAK104-GST fusion protein, 50 μg/ml poly-L-lysine, and [γ-32P]ATP with isolated AP2, in the presence or absence of the indicated inhibitor (A, upper panel). Immunoblot analysis was performed to verify equal gel loading using polyclonal antibodies against CVAK104 and the 100/1 monoclonal antibody that recognizes β2-adaptin (A, lower panel). B, the serine/threonine kinase activity of CVAK was further explored by testing CVAK sensitivity to olomoucine and roscovitine (cyclic-dependend kinase inhibitors) at the indicated concentrations. All samples were resolved by SDS-PAGE and phosphorylated proteins were detected by phosphorimaging; PKA, cAMP-dependent protein kinase; PKC, protein kinase C. CamK, Calmodulin kinase.
CVAK104 Phosphorylation of β2-Adaptin

cles along with AAK1 and GAK, μ2-adaptin kinases, and phosphofructokinase (28, 32). However, in neither our analysis nor that of others were any other kinases identified. Thus, it is likely that CVAK104 corresponds to the previously unidentified poly-L-lysine-stimulated β-adaptin kinase associated with clathrin-coated vesicles and coat protein preparations.

A second poly-L-lysine-stimulated, casein kinase II-like activity also associates with clathrin-coated vesicles, and following stimulation, it phosphorylates a broad range of coat protein constituents, including the clathrin light chain (16). The activity we observed following CVAK104 addition to kinase assays cannot result from casein kinase II contamination, because unlike casein kinase II, which is potently inhibited by heparin (33), CVAK104 activity is heparin-insensitive. Therefore, we believe that CVAK104 is indeed a catalytically active kinase, although future studies incorporating site-directed mutagenesis will be needed to definitively demonstrate its activity and define the catalytic mechanism.

Although β2-adaptin appears to be the only substrate targeted in coated vesicle extracts, CVAK104 can phosphorylate a 60-kDa protein in cytosolic fractions and a 90-kDa protein extracted from microsomal membranes. The identity of these other substrates remains to be determined, but these data suggest that CVAK104, similar to other kinases, may control multiple protein activities in vivo. Whether these other substrates are also involved in vesicular trafficking or function in other biological processes also remains to be determined.

What is the functional consequence of CVAK104-mediated phosphorylation of β2-adaptin? The β2-subunit is known to support interaction with AP180, eps15, epsin, amphiphysin, and clathrin (6, 34). These protein interactions occur over a relatively small region of the protein, and thus it is unlikely that β2 can support the simultaneous interaction with each protein. Thus, it is feasible that phosphorylation may regulate the ability of β2 to interact with other endocytic factors. Previous observations suggest that the poly-L-lysine-dependent phosphorylation of β2, at multiple locations, prevents its recruitment to and sedimentation with preassembled clathrin cages (13). However, our attempts to repeat this observation were unsuccessful; the addition of poly-L-lysine, which is required for β2-adaptin kinase activity, cross-links clathrin coat constituents (35). Hence, in our hands, β2-adaptin was consistently found associated with clathrin cages, following the addition of poly-L-lysine and high speed centrifugation, independent of its phosphorylation state (not shown).

β2-adaptin phosphorylation may also influence AP2 localization. Previous studies (11) have established that β2 is phosphorylated in vivo by a stauroporine-sensitive kinase whose function is balanced by the constitutive activity of protein phosphatase 2A. Lauritsen et al. (11) observed that treatment of Jurkat cells with agents that block protein phosphatase 2A function perturb AP2 localization at the plasma membrane and disrupt transferrin internalization. Whether CVAK104 is responsible for this phosphorylation reaction in vivo or whether β2-adaptins are also substrates for other kinases, remains to be determined.

In summary, we have identified CVAK104 as an endogenous poly-L-lysine-stimulated kinase that phosphorylates the β2-subunit of the AP2 complex. To gain a better understanding of CVAK104 function, it will be important to identify other interacting partners and isolate and characterize its other substrates.

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REFERENCES

1. Conner, S. D., and Schmid, S. L. (2003) Nature 422, 37–44
2. Slepen, V. I., and De Camilli, P. (2000) Nat. Rev. Neurosci. 1, 161–172
3. Brodsky, F. M., Chen, C. Y., Knuehl, C., Towler, M. C., and Wakeham, D. E. (2001) Annu. Rev. Cell Dev. Biol. 17, 517–568
4. Traub, L. M., Downs, M. A., Westrich, J. L., and Fremont, D. H. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 8907–8912
5. Conner, S. D., and Schmid, S. L. (2000) J. Cell Biol. 150, 921–929
6. Owen, D. J., Vallis, Y., Pearson, B. M., McMahon, H. T., and Evans, P. R. (2000) EMBO J. 19, 4216–4227
7. Turner, K. M., Burgeyone, R. D., and Morgan, A. (1999) Trends Neurosci. 22, 459–464
8. Slepen, V. I., Ochoa, G. C., Butler, M. H., Grabs, D., and Camilli, P. D. (1998) Science 281, 821–824
9. Robinson, P. J., Liu, J. P., Powell, K. A., Fykse, E. M., and Sudhof, T. C. (1994) Trends Neurosci. 17, 348–353
10. Wilde, A., Beattie, E. C., Lem, L., Rietho, D. A., Liu, S. H., Mobley, W. C., Soriano, P., and Brodsky, F. M. (1999) Cell 96, 677–678
11. Lauritsen, J. P., Menne, C., Kastrup, J., Dietrich, J., Odum, N., and Geisler, C. (2000) Biochim. Biophys. Acta 1497, 297–307
12. Olsanay, A., Andrews, P. D., Swedlow, J. R., and Smythe, E. (2001) Curr. Biol. 11, 896–900
13. Wilde, A., and Brodsky, F. M. (1996) J. Cell Biol. 135, 635–645
14. Cousin, M. A., and Robinson, P. J. (2001) Trends Neurosci. 24, 659–665
15. Bar-Ziv, D., and Branton, D. (1986) J. Biol. Chem. 261, 9614–9621
16. Korshikov, V. I., and Banting, G. (2002) Traffic 3, 429–439
17. Greener, T., Zhao, X., Nojima, H., Eisenberg, E., and Greene, L. E. (2000) J. Biol. Chem. 275, 1365–1370
18. Ball, C. L., Hunt, S. P., and Robinson, M. S. (1995) J. Cell Sci. 108, 2855–2875
19. Manfredi, J. J., and Banting, G. (1997) J. Biol. Chem. 272, 12182–12188
20. Smythe, E., Carter, L. L., and Schmid, S. L. (1992) J. Cell Biol. 119, 1163–1171
21. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
22. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
23. Ahle, S., Mann, A., Eichelsbacher, U., and Ungewickell, E. (1988) EMBO J. 7, 919–929
24. Schultz, J., Milpitz, F., Bork, P., and Ponting, C. P. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5857–5864
25. Manning, O., Whyte, D. B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002) Science 298, 1912–1918
26. Morgan, J. R., Prasad, K., Hao, W., Augustine, G. J., and Lafer, E. M. (2000) J. Neurosci. 20, 8667–8676
27. Celzign, A. E., Confalonieri, S., Iorio, M., Santolini, E., Tassi, E., Minenkova, O., Cesareni, G., Pelici, G. P., and Di Fiore, P. P. (1997) Genes Dev. 11, 2239–2249
28. Wasiak, S., Legendre-Guillemin, V., Puertollano, R., Blondeau, F., Girard, M., de Heuvel, E., Boismenu, D., Bell, A. W., Bonifacino, J. S., and McPherson, P. S. (2002) J. Cell Biol. 158, 855–862
29. Keen, J. H., and Black, M. M. (1986) J. Cell Biol. 102, 1325–1333
30. Tomizawa, K., Sunada, S., Lu, Y. F., Oda, Y., Kinuta, M., Ohshima, T., Saito, T., Wei, F. Y., Matsushita, M., Li, S. T., Tsutsui, K., Hisanaga, S., Mikoshiba, K., Takei, K., and Matsu, H. (2003) J. Cell Biol. 163, 813–824
31. Tan, T. C., Valva, V. A., Malladi, C. S., Graham, M. E., Berven, L. A., Jupp, O. J., Hanan, G., McCleure, S. J., Sarrevic, B., Boule, R. A., Larsen, M. R., Cousin, M. A., and Robinson, P. J. (2003) Nat. Cell Biol. 5, 701–710
32. Blondeau, F., Ritter, B., Allaire, P. D., Wasiak, S., Girard, M., Hussain, N. K., Angers, A., Legendre-Guillemin, V., Boy, L., Boisemenu, D., Kearney, R. E., Bell, A. W., Bergeron, J. L., and McPherson, P. S. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 3833–3838
33. Allende, J. E., and Allende, C. C. (1995) FASEB J. 9, 313–323
34. Hao, W., Luo, Z., Zheng, L., Prasad, K., and Lafer, E. M. (1999) J. Biol. Chem. 274, 22785–22794
35. Georgieva-Hanson, V., Schook, W. J., and Puszkin, S. (1988) J. Neurochem. 50, 307–315