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Apolipoprotein E Activates Akt Pathway in Neuro-2a in an Isoform-Specific Manner

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Apolipoprotein E (apoE) is a ligand for members of the low density lipoprotein (LDL) receptor family, receptors highly expressed in neurons. A study of one of the mechanisms by which apoE might affect neuronal cell metabolism is reported herein. ApoE can induce Akt/protein kinase B phosphorylation in Neuro-2a via two different pathways. Both pathways are mediated by phosphatidylinositol 3-kinase and cAMP-dependent protein kinase. The first pathway is stimulated by apoE3 and E4, but not by E2, after a 1-h incubation. The process requires the binding of apoE to the heparan sulfate proteoglycan/LDL receptor-related protein complex. The second pathway is activated after a 2-h incubation of the cells, in another isoform-dependent manner (E2 = E3 ≫ E4) and is mediated by calcium. Our results suggest that apoE might affect cell metabolism and survival in neurons in an isoform-specific manner by inducing novel signaling pathways.

Key Words: **apolipoprotein E; Akt; low density lipoprotein receptor-related protein; heparan sulfate proteoglycan; Neuro-2a.**

Apolipoprotein E (apoE) is a key-protein in lipid transport in plasma and the brain (1). Plasma apoE is mainly expressed in the liver and the apoE found in CSF is produced by astrocytes in the brain (2–4). ApoE is polymorphic and it is known that apoE3 represents the most common isoform. ApoE2 and E4 are the other main variants and are the result of a single amino acid mutation (1). ApoE is a ligand for members of the low density lipoprotein receptor (LDL-R) family including the low density lipoprotein receptor-related protein (LRP). The localization of these receptors on the surface of neuronal cells suggests that apoE might affect neuronal cell metabolism.

ApoE has been shown to be involved in apoptosis, a programmed process for cell death in neurons. This neurotoxic effect of apoE is isoform-specific, with E4 being more toxic than E3 and might involve the proteolysis of apoE (5). Apoptosis is apoE-receptor mediated (6) and requires calcium influx (5). Thus the possibility that apoE neurotoxicity might involve multiple signaling pathways cannot be excluded.

Recently, apoE receptors have been shown to play a role in signal transduction (7, 8) and apoE, in addition to its role in lipid metabolism, has been shown to be involved in cell signaling (9): apoE inhibits platelet aggregation (10) and vascular smooth muscle cell proliferation via the activation of nitric oxide synthase (11), and regulates inflammatory gene expression following stimulation with lipopolysaccharides (12). More recently, apoE4 has been shown to modulate ERK phosphorylation in rat hypocampal cells suggesting an isoform-specific effect in neurons (13).

The serine/threonine kinase which is referred to as Akt or protein kinase B (PKB) is a critical mediator of cell survival in response to growth factor stimulation. The mechanism for this activation requires the translocation of Akt to the membrane and its subsequent binding to the PI3K-generated 3-phosphoinositides. For full activation, Akt must be phosphorylated at both positions Thr308 and Ser473. Caspase-9, and Bad, a member of the pro-apoptotic Bcl-2 family, and glycogen synthase kinase 3β (GSK3β) have been shown to be

Abbreviations used: Akt/PKB, protein kinase B; ApoE, apolipoprotein E; BAPTA-AM, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester; ERK, extracellular signal-regulated kinase; GSK, glycogen synthase kinase; HSPG, heparan sulfate proteoglycan, LDL-R, low density lipoprotein receptor; LRP, low density lipoprotein receptor-related protein; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; PKA, cAMP-dependent protein kinase; RAP, receptor-associated protein.

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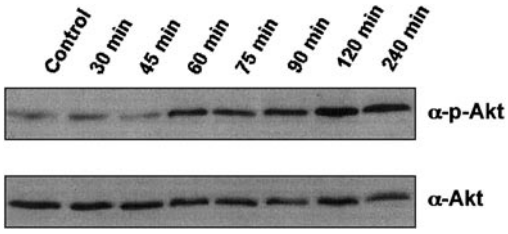

FIG. 1. Time-course analysis of Akt phosphorylation induced by apoE in Neuro-2a cells. Cells were treated with 1 μg/mL apoE for the indicated times. Whole cell lysates were subjected to 12.5% SDS–PAGE and transferred to a nitrocellulose membrane. The blots were probed with anti-phospho-Akt (α-p-Akt, upper panel) and anti-Akt (α-Akt, lower panel) antibodies as described under Experimental Procedures.

phosphorylated and inactivated by Akt. Akt regulates a variety of cellular processes including gene transcription, glucose metabolism and protein synthesis, and thus plays an important role in cell metabolism and survival (14–17).

To elucidate one of the mechanisms by which apoE might affect cellular events in neurons, we examined the effects of apoE isoforms on Akt phosphorylation, in murine neuroblastoma cells. Our findings show that apoE is able to induce Akt phosphorylation via two different pathways both of which are mediated by PI3K and PKA. These two pathways are independent of each other and are activated in an apoE isoform-dependent manner. Our results suggest that apoE may affect neuronal cell metabolism and survival in an isoform-specific manner.

EXPERIMENTAL PROCEDURES

Materials. Unless otherwise specified, the apoE used in this study was isolated and purified from human plasma very low density lipoproteins (Calbiochem, La Jolla, CA). Human recombinant apoE2, E3 and E4 isoforms were prepared as described previously (18). A tandem repeat dimer of apoE amino acids 141–155 (LRKLR KRLLR DADDL LRKLR KRLLR DADDL) was synthesized by the Peptide Institute (Osaka, Japan). Wortmannin and KT5720 were obtained from Calbiochem. Heparinase I was purchased from Sigma (St. Louis, MO). RAP was obtained from Progen Biotechnik (Heidelberg, Germany). BAPTA-AM was obtained from Molecular Probes (Leiden, The Netherlands).

Cell culture. Murine neuroblastoma cells (Neuro-2a) were a gift from Dr. Masaya Tohyama (Department of Anatomy, Osaka University Medical School). Cells were cultured in Dulbecco's modified Eagle medium which contained 10% (v/v) fetal calf serum, penicillin and streptomycin. Cells were grown to 60–80% percent confluence in 6-well tissue-culture plates before use in the experiments.

Western blotting. Cells were rinsed twice with ice-cold phosphate-buffered saline (PBS), harvested in lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% (w/v) Nonidet P-40, 10% (w/v) glycerol, 5 mM sodium pyrophosphate, 10 mM NaF, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/mL aprotinin, 5 μg/mL leupeptin and 1 mM dithiothreitol). Cell lysates were centrifuged at 15,000*g* for 10 min at 4°C, the supernatants were collected, and protein concentrations were determined using a protein assay CBB kit (Nacalai tesque, Kyoto, Japan). For Western blot analysis, whole cell lysates were subjected to 12.5% SDS–polyacrylamide gel electrophoresis (PAGE) and the resulting proteins were transferred to nitrocellulose membranes (Schleicher & Schuell). The blots were probed with anti-Akt or anti-phospho-serine 473 Akt antibodies (Cell Signal, Tokyo, Japan). After the blots had been incubated with peroxidase-conjugated secondary antibody, immunoreactive bands were visualized using an ECL kit (Amersham Pharmacia Biotech, UK).

RESULTS

ApoE Induces Akt Phosphorylation at Ser473 in Neuro-2a Cells

To determine whether apoE activates signaling pathways in Neuro-2a cells, Akt activation was examined after apoE treatment. One μg/mL of human apoE, purified from plasma very low density lipoproteins, was added to the cells for the indicated times and Western blotting of cell lysates was carried out followed by probing with anti-Akt and anti-phospho-serine 473 Akt antibodies. The results showed that apoE induced Akt phosphorylation in Neuro-2a cells in a time-dependent manner with 2 peaks at 1 and 2 h (Fig. 1). The first peak was attenuated within less than 1 h whereas the second peak persisted for more than 2 h.

LRP Is Involved in Akt Phosphorylation Induced after 1-h but Not after 2-h Treatment with ApoE

To determine the receptor that is involved in modulation of Akt phosphorylation by apoE, heparinase which prevents apoE from binding to the heparan sulfate proteoglycan (HSPG)/LRP complex, and RAP which blocks the binding of apoE to all the LDL-R family members were used (19). Heparinase caused a decrease, and RAP a total suppression of the phosphorylation of Akt induced after a 1-h treatment with apoE but these 2 reagents had no effect on the 2-h phosphorylation peak (Fig. 2). We assumed from these results that the first Akt phosphorylation peak requires the

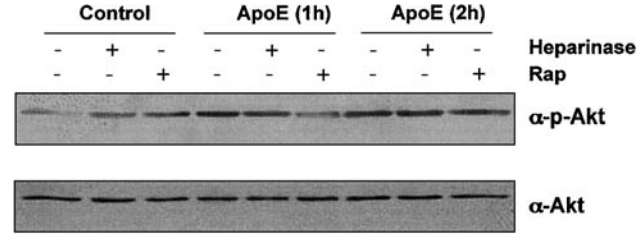

FIG. 2. Identification of receptor(s) involved in Akt phosphorylation induced by apoE. Cells were preincubated with RAP (1 μM) or heparinase (10 units/mL) for 2 h and then treated with 1 μg/mL apoE for 1 or 2 h. Whole cell lysates were subjected to 12.5% SDS–PAGE and transferred to a nitrocellulose membrane. The blots were probed with anti-phospho-Akt (α-p-Akt, upper panel) and anti-Akt (α-Akt, lower panel) antibodies as described under Experimental Procedures.

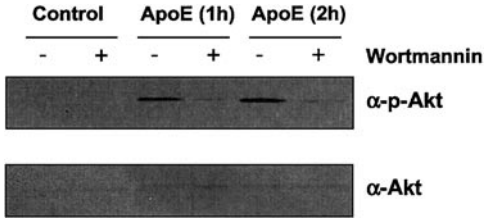

FIG. 3. Role of PI3K in Akt phosphorylation induced by apoE. Cells were pre-incubated with wortmannin (100 nM) for 60 min and then treated with 1 μg/mL apoE for 1 or 2 h. Whole cell lysates were subjected to 12.5% SDS–PAGE and transferred to a nitrocellulose membrane. The blots were probed with anti-phospho-Akt (α-p-Akt, upper panel) and anti-Akt (α-Akt, lower panel) antibodies as described under Experimental Procedures.

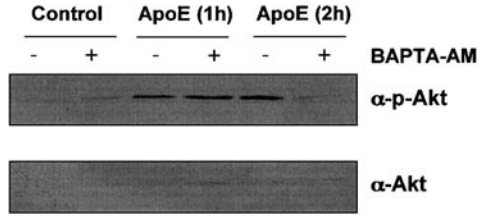

FIG. 5. Examination of the role of calcium in Akt phosphorylation induced by apoE. Cells were pre-incubated with BAPTA-AM (10 mMμ for 45 min and then treated with 1 μg/mL apoE for 1 or 2 h. Whole cell lysates were subjected to 12.5% SDS–PAGE and transferred to a nitrocellulose membrane. The blots were probed with anti-phospho-Akt (α-p-Akt, upper panel) and anti-Akt (α-Akt, lower panel) antibodies as described under Experimental Procedures.

binding of apoE to the HSPG/LRP complex while the second peak does not. Therefore, we hypothesized that the 1- and the 2-h Akt phosphorylation peaks were induced by different mechanisms.

PI3K is Involved in Akt Phosphorylation Induced by ApoE

Akt is an important effector of survival signaling downstream of PI3K. However, PI3K-independent activation of Akt has been reported (20). To further investigate this, the effect of wortmannin, a specific inhibitor of PI3K, on the role of PI3K in the phosphorylation of Akt was examined. Wortmannin was found to inhibit the Akt phosphorylation induced after the 1- and 2-h treatments with apoE (Fig. 3). These results indicate that apoE activates Akt through PI3K-dependent mechanisms.

PKA Activation Is Required for Akt Phosphorylation Induced by ApoE

To elucidate the pathways involved in Akt phosphorylation, cells were pre-incubated with KT5720, a PKA inhibitor. KT5720 inhibited the formation of both the 1- and 2-h Akt phosphorylation peaks (Fig. 4). This indicates that PKA is involved in the Akt phosphorylation that is observed following apoE treatment.

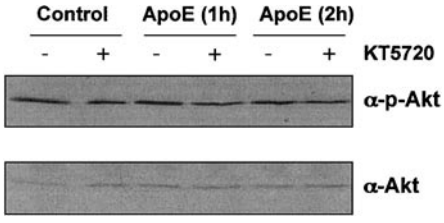

FIG. 4. Examination of the role of PKA in Akt phosphorylation induced by apoE. Cells were preincubated with KT5720 (5 nM) for 60 min and then treated with 1 μg/mL apoE for 1 or 2 h. Whole cell lysates were subjected to 12.5% SDS–PAGE and transferred to a nitrocellulose membrane. The blots were probed with anti-phospho-Akt (α-p-Akt, upper panel) and anti-Akt (α-Akt, lower panel) antibodies as described under Experimental Procedures.

Calcium Is Required for Akt Phosphorylation Induced after 2-h Treatment with ApoE

ApoE is known to induce an increase in intracellular calcium levels in neuronal cells either via calcium release from the intracellular stores or via an influx of extracellular calcium (21). To determine whether or not calcium is required for the Akt phosphorylation induced by apoE treatment, cells were incubated for 40 min with BAPTA-AM, a chelator of intracellular calcium. As shown in Fig. 5, BAPTA-AM prevented the Akt induced phosphorylation after the 2-h but not after the 1-h treatment with apoE. These results show that calcium is required for the Akt induced phosphorylation after the 2-h treatment with apoE.

ApoE Induces Akt Phosphorylation in an Isoform-Dependent Manner

To assess the effect of apoE polymorphism on the Akt phosphorylation, 1 μg/mL of recombinant apoE2, E3 or E4 was incubated with cells for up to 4 h. All 3 apoE isoforms were found to induce Akt phosphorylation after a 2-h treatment. This activation was isoform dependent: E2 = E3 $\gg$ E4, but, only apoE3 and E4 were able to stimulate Akt phosphorylation after the 1-h treatment (Fig. 6).

An Apolipoprotein E Peptide Induces Akt Phosphorylation in Neuro-2a

In this experiment, a peptide of apoE consisting of a tandem repeat of amino acids 141–155 was used. This apoE peptide, which contains the region responsible for the binding of apoE to its receptors, was examined for its ability to induce Akt phosphorylation in Neuro-2a. The apoE peptide (2 μM) was added to cells for the indicated times. Figure 7 shows that the apoE peptide induced Akt phosphorylation, after 30 min and later than 1 h.

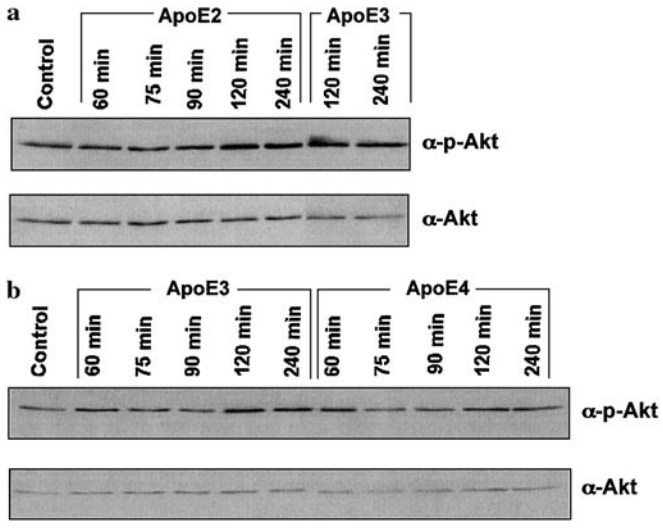

FIG. 6. Akt phosphorylation by apoE: Comparison of apoE isoforms. Cells were treated with 1 μg/mL of recombinant apoE2 (a), E3 (a, b) and E4 (b) for the indicated times. Whole cell lysates were subjected to 12.5% SDS–PAGE and transferred to nitrocellulose membranes. The blots were probed with anti-phospho-Akt (α-p-Akt, upper panel) and anti-Akt (α-Akt, lower panel) antibodies as described under Experimental Procedures.

DISCUSSION

We report herein on a study of the effect of apoE on Akt phosphorylation in Neuro-2a. Akt phosphorylation was significantly increased after 1- and 2-h incubations with apoE at a concentration of 1 μg/mL. To better understand the mechanisms by which Akt was phosphorylated, inhibition experiments were performed. Pre-treatment with wortmannin, a specific inhibitor of PI3K, was found to prevent the formation of both the 1- and 2-h Akt phosphorylation peaks. This suggested that the phosphorylation of Akt induced by apoE was PI3K-dependent. We further showed that Akt phosphorylation after both a 1- and 2-h incubation with apoE was inhibited by KT5720. In contrast to recent reports which showed that cAMP or PKA stimulates Akt via a PI3K-independent pathway (20, 22), the findings here indicate that the activation of Akt by apoE requires both PI3K and PKA activation. Li *et al.* (23) have shown that Akt can be activated in primary rat hepatocytes by cAMP level-elevating reagents. This cyclic-nucleotide mediated activation is blocked by both PI3K and PKA inhibitors. Others (24) have reported that a cAMP reagent directly activates PI3K in sympathetic neurons. However, the mechanism of activation of Akt by apoE in Neuro-2a remains to be investigated.

ApoE binds to all the LDL-R family members. Our data demonstrate that the LDL-R family members are not involved in the 2-h phosphorylation peak whereas formation of the 1-h phosphorylation peak may require the binding of apoE to the HSPG/LRP complex. Li *et al.* (25) have shown that LRP tail is phosphorylated by PKA and that this phosphorylation facilitates the endocytosis of the LRP. Thus the possibility that apoE binding to LRP regulates signal transduction via an enhanced phosphorylation of the receptor by PKA cannot be excluded at this time.

ApoE2, E3 and E4 isoforms differ in primary structure at 2 sites: residue 112 and 158. ApoE3 contains cysteine at residue 112 and arginine at residue 158 while in the case of E2 cysteine is present at both sites and E4 has arginine residues at these sites. We found that, unlike apoE2, apoE3 and E4 induce Akt phosphorylation after a 1-h incubation of the cells. Ohkubo *et al.* (13) reported that the apoE4 and apoE peptide induce ERK activation in rat sympathetic neurons after 1-h and 15-min incubations of the cells, respectively, via a mechanism which involves a member of the LDL-R family. The authors suggested that this time difference was due to the proteolysis of full-length apoE in the medium, and that the activation of the signaling pathway was linked to the apoptotic effect of apoE4. Our results are consistent with their work in terms of the delayed signaling which suggests that activation of the first pathway might be a consequence of apoE proteolysis.

The 2-h phosphorylation peak requires calcium as shown in the experiment with BAPTA-AM. Since BAPTA-AM completely inhibits the 2-h peak but has no effect on the 1-h peak, we conclude that the 1-h Akt phosphorylation peak is attenuated within less than 1 h whereas the 2-h phosphorylation peak persists for more than 2 h. This result is particularly relevant, considering the fact that apoE isoforms have distinct effects on these 2 peaks. The activation of the 2-h phosphorylation peak by apoE4 is weak. Therefore, the activation of the 1-h Akt phosphorylation by apoE4 might be related to its apoptotic effect, which is responsible for the refractory relationship to central nervous system injury and disease. In addition, the apoE2-inducing effect on the 2-h pathway only could account for this isoform-specific "protective" effect (26–28). Our

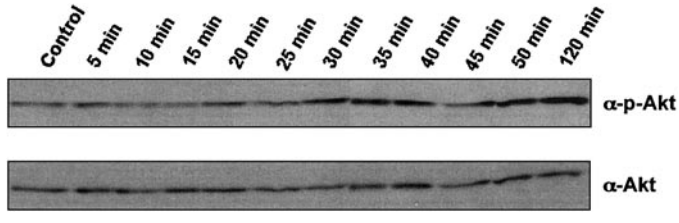

FIG. 7. Time-course analysis of Akt phosphorylation induced by apoE peptide. Cells were treated with 2 μM of apoE peptide for the indicated times. Whole cell lysates were subjected to 12.5% SDS–PAGE and transferred to a nitrocellulose membrane. The blots were probed with anti-phospho-Akt (α-p-Akt, upper panel) and anti-Akt (α-Akt, lower panel) antibodies as described under Experimental Procedures.

findings provide a possible explanation for the reported beneficial effect of apoE in terms of neuronal degeneration. ApoE expression is increased following injury to the brain (29–31) and apoE $\epsilon 4$ allele is associated with a poor prognosis following traumatic injury (32) and with many brain disorders.

We were able to confirm that the induction of the phosphorylation of Akt leads to its activation: our finding show that GSK3β, which is a known substrate of Akt, was phosphorylated in a PI3K-dependent manner after incubation of cells with apoE for 1- or 2-h (unpublished data). It is interesting to note that GSK3β substrates include protein bound to microtubules (33) since apoE has been reported to regulate Tau phosphorylation (34).

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REFERENCES

1. Mahley, R. W., and Stanley, C. R. Jr. (2000) *Ann. Rev. Genomics Hum. Genet.* **01,** 507–537.
2. Elshourbagy, N. A., Liao, W. S., Mahley, R. W., and Taylor, J. M. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 203–207.
3. Boyles, J. K., Pitas, R. E., Wilson, E., Mahley, R. W., and Taylor, J. M. (1985) *J. Clin. Invest.* **76,** 1501–1513.
4. Pitas, R. E., Boyles, J. K., Lee, S. H., Foss, D., and Mahley, R. W. (1987) *Biochim. Biophys. Acta* **917,** 148–161.
5. Tolar, M., Keller, J. N., Chan, S., Mattson, M. P., Marques, M. A., and Crutcher, K. A. (1999) *J. Neurosci.* **19,** 7100–7110.
6. Tolar, M., Marques, M. A., Harmony, J. A., and Crutcher, K. A. (1997) *J. Neurosci.* **17,** 5678–5686.
7. Nimpf, J., and Schneider, W. J. (2000) *Biochim. Biophys. Acta* **1529,** 287–298.
8. Herz, J. (2001) *Trends Neurosci.* **24,** 193–195.
9. Swertfeger, D. K., and Hui, D. Y. (2001) *Front. Biosc.* **6,** D526–535.
10. Riddell, D. R., Graham, A., and Owen, J. S. (1997) *J. Biol. Chem.* **272,** 89–95.
11. Ishigami, M., Swertfeger, D. K., Hui, M. S., Granholm, N. A., and Hui, D. Y. (2000) *Arterioscler. Thromb. Vasc. Biol.* **20,** 1020–1026.
12. Lynch, J. R., Morgan, D., Mance, J., Matthew, W. D., and Laskowitz, D. T. (2001) *J. Neuroimmunol.* **114,** 107–113.
13. Ohkubo, N., Mitsuda, N., Tamatani, M., Yamaguchi, A., Lee, Y. D., Ogihara,T., Vitek, M. P., and Tohyama, M. (2001) *J. Biol. Chem.* **276,** 3046–3053.
14. Hisamoto, K., Ohmichi, M., Kanda, Y., Adachi, K., Nishio, Y., Hayakawa, J., Mabuchi, S., Takahashi, K., Tasaka, K., Miyamoto, Y., Taniguchi, N., and Murata, Y. (2001) *J. Biol. Chem.*, in press.
15. Hisamoto, K., Ohmichi, M., Kurachi, H., Hayakawa, J., Kanda, Y., Nishio, Y., Adachi, K., Tasaka, K., Miyoshi, E., Fujiwara, N., Taniguchi, N., and Murata, Y. (2001) *J. Biol. Chem.* **276,** 3459–3467.
16. Kandel, E. S., and Hay, N. (1999) *Exp. Cell. Res.* **253,** 210–229.
17. Blume-Jensen, P., and Hunter, T. (2001) *Nature* **411,** 355–365.
18. Barbier, A., Visvikis, A., Mathieu, F., Diez, L., Havekes, L. M., and Siest, G. (1997) *Eur. J. Clin. Chem. Clin. Biochem.* **35,** 581–589.
19. Hussain, M. M. (2001) *Front. Biosci.* **6,** D417–428.
20. Sable, C. L., Filippa, N., Hemmings, B., and Van Obberghen E. (1997) *FEBS Lett.* **409,** 253–257.
21. Wang, X. S., and Gruenstein, E. (1997) *J. Cell. Physiol.* **173,** 73–83.
22. Filippa, N., Sable, C. L., Filloux, C., Hemmings, B., and Van Obberghen, E. (1999) *Mol. Cell. Biol.* **19,** 4989–5000.
23. Li, J., Yang, S., and Billiar, T. R. (2000) *J. Biol. Chem.* **275,** 13026–13034.
24. Crowder, R. J., and Freeman, R. S. (1999) *J. Neurochem.* **73,** 466–475.
25. Li, Y., van Kerkhof, P., Marzolo, M. P., Strous, G. J., and Bu, G. (2001) *Mol. Cell. Biol.* **21,** 1185–1195.
26. Corder, E. H., Saunders, A. M., Risch, N. J., Strittmatter, W. J., Schmechel, D. E., Gaskell, P. C., Jr., Rimmler, J. B., Locke, P. A., Conneally, P. M., Schmader, K. E., *et al.* (1994) *Nat. Genet.* **7,** 180–184.
27. Bedlack, R. S., Strittmatter, W. J., and Morgenlander, J. C. (2000) *Arch. Neurol.* **57,** 1561–1565.
28. Siest, G., Bertrand, P., Herbeth, B., Vincent-Viry, M., Schiele, F., Sass, C., and Visvikis, S. (2000) *Clin. Chem. Lab. Med.* **38,** 841–852.
29. Poirier, J. (1994) *Trends Neurosci.* **17,** 525–530.
30. Messmer-Joudrier, S., Sagot, Y., Mattenberger, L., James, R. W., and Kato, A. C. (1996) *Eur. J. Neurosci.* **8,** 2652–2661.
31. Zarow, C., and Victoroff, J. (1998) *Exp. Neurol.* **149,** 79–86.
32. Teasdale, G. M., Nicoll, J. A., Murray, G., and Fiddes, M. (1997) *Lancet* **350,** 1069–1071.
33. Frame, S., and Cohen, P. (2001) *Biochem. J.* **359,** 1–16.
34. Wang, X., Luebbe, P., Gruenstein, E., and Zemlan, F. (1998) *J. Neurosci. Res.* **51,** 658–665.