N-terminal Cleavage of GSK-3 by Calpain

A NEW FORM OF GSK-3 REGULATION*

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Although GSK-3 activity can be regulated by phosphorylation and through interaction with GSK-3-binding proteins, here we describe N-terminal proteolysis as a novel way to regulate GSK-3. When brain extracts were exposed to calcium, GSK-3 was truncated, generating two fragments of ~40 and 30 kDa, a proteolytic process that was inhibited by specific calpain inhibitors. Interestingly, instead of inhibiting this enzyme, GSK-3 truncation augmented its kinase activity. When we digested recombinant GSK-3α and GSK-3β protein with calpain, each isoform was cleaved differently, yet the truncated GSK-3 isoforms were still active kinases. We also found that lithium, a GSK-3 inhibitor, inhibits full-length and cleaved GSK-3 isoforms with the same IC50 value. Calpain removed the N-terminal ends of His-tagged GSK-3 isoenzymes, and exposing cultured cortical neurons with ionomycin, glutamate, or N-methyl-D-aspartate led to the truncation of GSK-3. This truncation was blocked by the calpain inhibitor calpeptin, at the same concentration at which it inhibits calpain-mediated cleavage of NMDAR-2B and of p35 (the regulatory subunit of CDK5). Together, our data demonstrate that calpain activation produces a truncation of GSK-3 that removes an N-terminal inhibitory domain. Furthermore, we show that GSK-3α and GSK-3β isoenzymes have a different susceptibility to this cleavage, suggesting a means to specifically regulate these isoenzymes. These data provide the first direct evidence that calpain promotes GSK-3 truncation in a way that has implications in signal transduction, and probably in pathological disorders such as Alzheimer disease.

The GSK-3 protein (EC 2.7.11.26) is a proline-directed serine/threonine protein kinase that was originally identified and named for its ability to phosphorylate the enzyme glycogen synthase (1). Since its discovery, GSK-3 has been postulated to be involved in many physiological processes, and it plays important roles in embryonic development, cell differentiation, microtubule dynamics, cell cycle division, cell adhesion, glucose metabolism, and apoptosis (2, 3). Deregulation of GSK-3 activity is believed to play a key role in the pathogenesis of chronic central nervous system disorders such as Alzheimer disease (AD),2 bipolar disorder, and Huntington disease (3, 4), as well as of metabolic disorders such as type II diabetes (5). In AD, GSK-3 has been shown to phosphorylate Tau at most of the hyperphosphorylated serine and threonine residues in paired helical filament Tau, both in cells (6) and in vivo (7–10). Furthermore, GSK-3 modulates β-amyloid production from its precursor amyloid precursor protein (11) and its accumulation in the cytoplasm of pre-tangle neurons (12, 13).

In mammals, two GSK-3 isoenzymes (α and β) that share 95% amino acid identity have been described (14), although there is weaker homology at the N- and C-terminal ends than the central portions. Both isoenzymes are the products of two independent genes (mapped to chromosome 19q12.3 and 3q13.3, respectively (15, 16)). Meanwhile, the GSK-3β gene encodes a 47-kDa protein. Recently, a new alternative splice isoform of GSK-3β with an additional 13-amino-acid insert in the catalytic domain has also been described (17). Furthermore, the GSK-3β gene has been analyzed (18) and its promoter studied to identify variations that could be associated with abnormal function (19).

GSK-3 can phosphorylate a variety of cytoplasmic and nuclear proteins, and its substrates include cytoskeletal proteins, transcription factors, and metabolic regulators. Thus, GSK-3 plays a prominent role in establishing and maintaining cell architecture, gene expression, and apoptosis (3). Many GSK-3 substrates require prior phosphorylation by a priming kinase on a Ser or Thr residue, and its catalytic domain is roughly cut at the GSK-3 target residue. Indeed, the crystal structure of human GSK-3β has provided a model for the binding of prephosphorylated substrates to the kinase (Protein Data Bank ID are 1109 (20) and 1H8F (21)). Thus, the primed Ser/Thr is recognized by a positively charged “binding pocket” that facilitates the binding of primed substrates. Identifying the priming kinase is clearly of interest, and kinases such as cdk-5 (22–24), PAR-1 (25), casein kinase I (26), or protein kinase A (27) could act as priming kinases for GSK-3 phosphorylation.

GSK-3 is regulated at the post-translational level by phosphorylation, and autophosphorylation at Tyr-216 of GSK-3β or Tyr-276 of GSK-3α is necessary for its activation (28, 29). From the crystal structure, it has been proposed that unphosphorylated Tyr-216/276 blocks the access of primed substrates. Indeed, the published structure of phosphorylated GSK-3β (30)

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2 The abbreviations used are: AD, Alzheimer disease; NMDA, N-methyl-D-aspartate; ALLN, acetyl-leucine, leucine, norleucinal; DIV, days in vitro.

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shows that phosphorylated Tyr-216 suffers a conformational change that allows the primed substrate to bind the enzyme. In Dictyostelium, the Zaphod kinase activates GSK-3 by Tyr-phosphorylation (31), whereas in mammals, Fyn tyrosine kinase (32) or some related tyrosine kinase may be involved in this process. However, it seems that phosphorylation of these residues may also be the result of autophosphorylation (29, 33).

Inhibition of the enzyme can be achieved by two different mechanisms (2). The first mechanism is triggered by insulin and growth factors, and it is mainly mediated by protein kinase B, which phosphorylates Ser-9 in GSK-3β and Ser-21 in GSK-3α. A model for this inhibition has been generated through structural studies (20, 21), and accordingly, the phosphorylated Ser-9/21 binds as a competitive pseudosubstrate to the primed-binding site, inhibiting the binding of the protein and its ensuing phosphorylation. The second mechanism of GSK-3 inhibition involves the wingless (Wnt) signaling pathway. GSK-3 contributes to a multiprotein complex formed by axin and adenomatous polyposis coli, in which it is able to phosphorylate β-catenin, targeting it for proteasome degradation (34). Wnt proteins bind to the Frizzled receptor, activating the Dishevelled protein, which in turn inhibits GSK-3 activity by disrupting this multiprotein complex. As a consequence, β-catenin accumulates and translocates into the nucleus, where it activates transcription by interacting with certain transcription factors.

In addition to these regulatory mechanisms, the mechanisms governing the turnover of GSK-3 may be critical in diseases in which GSK-3 activity is altered. The GSK-3β promoter is a TATA-less promoter with the characteristics of housekeeping genes, and with regard to Alzheimer disease, there are no TATA-less promoters with the characteristics of housekeeping genes from Calbiochem. The commercial antibodies used were: anti-GSK-3β/α (pS9/21) and anti-GSK-3β/α obtained from BioSource (Camarillo, CA); anti-p35/25 obtained from Santa Cruz (Santa Cruz, CA); anti-His and anti-α-tubulin obtained from Sigma; and anti-NR2C (35) obtained from Molecular Probes (Eugene, OR). Calpain was purchased from Calbiochem (catalogue number 208718), GSK-3α was from Upstate-Millipore (Chicago, IL; catalogue number 14-492), and GSK-3β was from Sigma (catalogue number G1663). Ionomycin, aprotinin, and pepstatin were all obtained from Sigma, whereas MK801, ALLN, and calpeptin were from Calbiochem.

**Calpain-mediated Truncation of GSK-3**

Cortical mouse brain extracts were homogenized in 50 mM Tris-HCl, pH 7.4, containing 1.0 mM EDTA. Samples were then incubated at 30 °C for several minutes with or without 5.0 mM CaCl2 and in the presence or absence of protease inhibitors. The samples were then mixed with Laemmli sample buffer, electrophoresed, and Western blotted to identify the products of proteolysis by probing with antibodies against GSK-3. Commercial GSK-3 isoforms (0.5 µg) were incubated in 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 5 mM β-mercaptoethanol and 0.2 units calpain/ml. Samples were then incubated at 30 °C for several minutes in the presence or absence of 5.0 mM CaCl2 and 10 µM calpeptin. Samples were then mixed with Laemmli sample buffer supplemented with 10 µM calpeptin and separated by electrophoresis.

**GSK-3 Activity Assay**—Cortical mouse brain extracts were homogenized in 50 mM Tris-HCl, pH 7.4, containing 1.0 mM EDTA. Samples were then incubated at 30 °C for several minutes in the presence or absence of 5.0 mM CaCl2 and 10 µM calpeptin. Reactions were terminated by adding a mixture of peptidase inhibitors (Roche Applied Science, Basel, Switzerland) supplemented with 20 µM calpeptin. Extracts were centrifuged at 14,000 × g for 15 min, and the supernatants were collected to assay GSK-3 activity. The GS1 peptide (YRRAAVPSPSLSRHSSHQS*DEE) in which Ser-21 is phosphorylated was used as a substrate as described previously (36). Samples were incubated at 30 °C with 30 µM GSK-3 peptide in the presence of 50 µM ATP (1,000 cpm/pmol of [γ-32P]ATP, PerkinElmer Life Sciences) in 25 mM Tris, pH 7.5, 1 mM dithiothreitol, 10 mM MgCl2, and either 10 mM NaCl or 10 mM LiCl. The assays were stopped by spotting aliquots onto P81 phosphocellulose paper, and the filters were processed as described previously (36). GSK-3 activity was calculated as the difference between the activity in the presence of 10 mM NaCl and the activity in the presence of 10 mM LiCl. The results were expressed as the percentage of activity with respect to the wild-type extracts. Commercial GSK-3 isoforms (0.5 µg) were incubated in 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM β-mercaptoethanol, and 0.2 units calpain/ml. Samples were then incubated at 30 °C for several minutes in the presence or absence of 5.0 mM CaCl2. The reactions were stopped with calpeptin 20 µM and a mixture of peptidase inhibitors (Roche Applied Science). GSK-3 activity was then measured as described above in the presence or absence of LiCl at different concentrations (0.01–20 mM). GSK-3 activity was normalized to the total GSK-3 protein levels measured after SDS-PAGE and immunoblotting.

**EXPERIMENTAL PROCEDURES**

**Antibodies, Enzymes, and Reactives**—The commercial antibodies used were: anti-GSK-3β/α (pS9/21) and anti-GSK-3β/α obtained from BioSource (Camarillo, CA); anti-p35/25 obtained from Santa Cruz (Santa Cruz, CA); anti-His and anti-α-tubulin obtained from Sigma; and anti-NR2C (35) obtained from Molecular Probes (Eugene, OR). Calpain was purchased from Calbiochem (catalogue number 208718), GSK-3α was from Upstate-Millipore (Chicago, IL; catalogue number 14-492), and GSK-3β was from Sigma (catalogue number G1663). Ionomycin, aprotinin, and pepstatin were all obtained from Sigma, whereas MK801, ALLN, and calpeptin were from Calbiochem.

**Dephosphorylation Experiments**—Commercial GSK-3 isoenzymes were dephosphorylated with A-phosphatase (BioLabs, IZASA SA, Barcelona, Spain) in 50 mM Tris, pH 7.8, 300 mM NaCl, 10% glycerol, 0.5 mM MnCl2, 5 mM dithiothreitol, and a mixture of peptidase inhibitors (Roche Applied Science) for 0.5 h at 30 °C to remove the adventitious phosphorylation that occurs during expression. The dephosphorylation of the protein was confirmed by Western blotting using the antibody anti-GSK-3β/α (pS9/21) and the anti-GSK-3β/α antibody as loading control.
glutamate. Experiments were performed in the presence of glycine 100 μM.

**RESULTS**

**GSK-3 Is Cleaved by Calpain in Brain Extracts**—We have previously observed that when immunobots probed with some anti-GSK-3 antibodies are overexposed, additional bands smaller than the full-length GSK-3 isoforms (about 40–30 kDa) can be detected, suggesting the presence of proteolytic fragments. To test this possibility, we analyzed by Western blotting the pattern of GSK-3 bands and fragments in mouse brain tissue homogenized in the presence of Tris/EDTA buffer. As shown in Fig. 1A, GSK-3 extracts obtained with an anti-GSK-3 antibody to correct for any deviation in protein loading or with respect to the values obtained with anti-tubulin antibody to correct for total protein content.

**Statistical Analysis**—Statistical analysis of the data were performed using one-way analysis of variance.
Interestingly, the Ca\(^{2+}\) concentration was higher in the absence of calcium or in the presence of calcium and calpeptin. All samples were incubated with calpain.

Supernatants of the GSK-3 isoforms are expressed in terms of the percentage of the total enzyme present in each treatment with calpain. The GSK-3 isoforms are different proteases. Neither aprotinin nor pepstatin, serine, and aspartic protease inhibitors, respectively, significantly inhibited the Ca\(^{2+}\)-induced degradation of GSK-3 (Fig. 1, A, C, and D), excluding the involvement of these types of proteases in the GSK-3 proteolysis. Taken together, these results indicated that elevated Ca\(^{2+}\) most probably activates calpain, which in turn cleaves GSK-3 (38).

We then investigated whether the truncation of GSK-3 affects its kinase activity. GSK-3 activity was assessed in the same manner as analyzed previously in brain extracts (Fig. 1A). Interestingly, the Ca\(^{2+}\)-treated extracts showed a significant increase (p < 0.02, 51.8 ± 6%) in GSK-3 activity (Fig. 1E) when compared with control extracts. Furthermore, the Ca\(^{2+}\)-induced increase in GSK-3 activity was inhibited by calpeptin.

**Experimental Procedures**

**Calpain Cleaves GSK-3a and GSK-3b at Their N-terminal End**—To assess whether both GSK-3 isoenzymes were substrates for calpain, recombinant GSK-3a and GSK-3b were incubated with the protease. Both isoforms were cleaved by calpain, producing two calpain-derived fragments (Fig. 2A). When these fragments were analyzed in the same gel (Fig. 2C), we observed that both isoforms produce a fragment of apparent molecular mass of 30 kDa (fragment II), whereas fragment I of GSK-3a had a slightly higher apparent molecular mass (42 kDa) than that from the β-isofom (40 kDa). Quantification of these proteolytic products (Fig. 2B) showed that both full-length isoforms were rapidly digested by calpain (Fig. 2B, open circles). However, fragment I generated from the β-isofom is a better substrate for calpain than the one generated from the α-isofom since after 5 min of calpain digestion, fragment I generated from GSK-3β represented about 25% of the total GSK-3β, whereas in the same experimental conditions, the equivalent fragment represented the 60% of the GSK-3α protein.

The recombinant GSK-3 isoenzymes were used as His-tagged at their N-terminal ends, and when assayed as above and probed with an antibody against the His tag, the breakdown products generated from both GSK-3 isoforms were not detected, indicating that the N-terminal end was that removed by calpain (Fig. 2D). Furthermore, this confirms that calpain digests both full-length isoforms rapidly to generate fragment I. Indeed, the antibody that recognizes the His-tagged epitope is unable to detect the full-length GSK-3 isoforms 2 min after exposure to calpain.

**Truncated GSK-3 Isoenzymes Are Active Kinases**—To determine whether calpain-mediated GSK-3 proteolysis alters its kinase activity, the effects of calpain on recombinant GSK-3 isoenzymes were studied. The study was performed with GSK-3 isoforms treated with calpain for 0.5–5 min. Fig. 3A shows that the GSK-3β kinase activity increased over incubation time. Thus, calpain-mediated proteolysis of recombinant GSK-3β showed a significant (p < 0.01) increase in GSK-3 activity (330.1 ± 7.3% at 5 min) when compared with the untreated enzyme. When the same experiment was carried out with the GSK-3α isoform, a similar increase in kinase activity was not observed. These data demonstrate that the GSK-3 fragments are catalytic and suggest that calpain-mediated proteolysis selectively augments the kinase activity of the β isoform.
A well established mechanism that increases GSK-3 activity involves dephosphorylating the Ser-21/9 present in the N-terminal end of GSK-3. To validate the differential effect of calpain on each isoform, it was important to investigate whether the recombinant enzymes were similarly phosphorylated at Ser-21/9. We found that although recombinant GSK-3\(_{\beta}\) was phosphorylated at Ser-9, there was very little phosphorylation of Ser-21 in the \(\alpha\)-isoform (Fig. 3B). These results were confirmed by treating GSK-3 isoforms with phosphatase, which abolished the binding of the anti-P-Ser 21/9-GSK-3\(_{\beta}\) antibody without altering the total amount of protein (Fig. 3B). Thus, the differential effect on the \(\alpha\)-isoform can be explained by the removal of its inhibitory domain, leading to increased kinase activity. On the contrary, the \(\beta\)-isoform was not phosphorylated at Ser-21, and therefore, its cleavage did not affect the kinase activity.

Li selectively inhibits GSK-3, which may explain some of its pharmacological effects (39). Although its binding site has not been identified, Li is a competitive inhibitor of magnesium with respect to GSK-3 (40). We investigated the effects of Li on truncated GSK-3 activity using commercial GSK-3 isoenzymes (Fig. 3C). Li inhibited GSK-3\(_{\beta}\) and GSK-3\(_{\alpha}\) with IC\(_{50}\) values of 3.91 ± 1.25 and 2.63 ± 1.13 mM, respectively, reducing kinase activity by 90% at 20 mM LiCl. Interestingly, lithium inhibited the truncated GSK-3 isoforms generated after 0.5 and 5 min of calpain treatment with a similar IC\(_{50}\). Therefore, full-length and truncated GSK-3\(_{\alpha}\) and GSK-3\(_{\beta}\) are inhibited by Li in the same way, suggesting that GSK-3 cleavage does not affect the Li-binding site.

**Calcium Influx in Cultured Cortical Neurons Induces GSK-3 Proteolysis through Calpain Activation**—Treatment of cultured cortical neurons with the calcium ionophore ionomycin in the presence of extracellular calcium induced GSK-3 truncation in a time-dependent manner (Fig. 4). This GSK-3 truncation was prevented by the addition of the calpain inhibitor calpeptin to the culture. Thus, we addressed whether the GSK-3 truncation by calpain could also be observed after stimulation of ionotropic NMDA receptors, which upon activation, increase intracellular Ca\(^{2+}\) concentrations. We first analyzed the cleavage of NMDA subunits by calpain in primary neuronal cultures, a well characterized system where calpain activation occurs (35, 41). Activation of the NMDA receptor by NMDA or glutamate led to calpain-specific proteolysis of the NR2B subunit (Fig. 5A).
This activation of NMDA receptors increased the levels of a 115-kDa product recognized by a specific antibody (35), whereas a parallel decrease of the 170-kDa full-length NR2B subunit was observed. The effect of NMDA was mediated by calpain as calpeptin and ALLN prevented the formation of the 115-kDa product (Fig. 5B). MK-801 preincubation blocked both the decrease of the full-length NR2B subunit and the increase of the low molecular weight form. We then analyzed the status of GSK-3 in Western blots of the same samples, and we detected the additional low molecular weight isoforms of GSK-3 (Fig. 5A). This effect was time-dependent (Fig. 5B) and mediated by NMDA receptors as MK-801 abolished GSK-3 truncation (Fig. 5A). Glutamate, the physiological ligand of NMDA receptors, induced the same response in cultured cortical neurons (DIV12) as MK801 abolished GSK-3 activity after a 30 min incubation with NMDA (Fig. 5A). The same samples were immunoblotted with the anti-p35/p25 antibody and the antibody that recognizes the 170-kDa full-length NR2B subunit (Fig. 5A). This activation of NMDA receptors increased the levels of a 115-kDa product recognized by a specific antibody (35), whereas a parallel decrease of the 170-kDa full-length NR2B subunit was observed. The effect of NMDA was mediated by calpain as calpeptin and ALLN prevented the formation of the 115-kDa product (Fig. 5B). MK-801 preincubation blocked both the decrease of the full-length NR2B subunit and the increase of the low molecular weight form. We then analyzed the status of GSK-3 in Western blots of the same samples, and we detected the additional low molecular weight isoforms of GSK-3 (Fig. 5A). This effect was time-dependent (Fig. 5B) and mediated by NMDA receptors as MK-801 abolished GSK-3 truncation (Fig. 5A). Glutamate, the physiological ligand of NMDA receptors, induced the same response in cultured cortical neurons. Moreover, since MK-801 prevented both effects (GSK-3 and NR2B subunit truncation, Fig. 5A), the effect of glutamate was mediated by NMDA receptors. Thus, glutamate induces calpain-mediated truncation of GSK-3 by acting on NMDA receptors, which can be inhibited by calpeptin in a dose-dependent manner (Fig. 5C), confirming the involvement of calpains. Although fragment I was prominent in these experiments (Fig. 5), fragment II was only detected in overexposed immunobLOTS.

We then investigated whether NMDA-mediated GSK-3 proteolysis alters its kinase activity (Fig. 5A and B). Interestingly, the NMDA-treated cultures showed a significant increase in GSK-3 activity when compared with control cultures (43.5 ± 17.8% over basal levels, p < 0.01). Furthermore, the NMDA-induced increase in GSK-3 activity was inhibited by 10 μM calpeptin (Fig. 5D).

Cdk5 is another proline-directed Ser/Thr protein kinase that is expressed in neurons together with its regulatory subunit p35. The N-terminal truncation of p35 to p25 by calpain results in deregulation of Cdk5 and contributes to the neuronal cell death associated with several neurodegenerative diseases (42–44). We tested whether the breakdown of p35 to p25 also occurs in our experimental conditions. When primary cultures were probed with an antibody that recognizes the p35/p25 protein, the p25 fragment was generated in parallel with the truncated GSK-3 isoforms (Fig. 5B) and with a similar sensitivity to the increase in the concentration of the calpain inhibitor calpeptin (Fig. 5C).

DISCUSSION

GSK-3 is a constitutively active kinase that is inactivated by phosphorylation at its N-terminal end. In this study, we show that GSK-3 activity can be also regulated by calpain-induced proteolysis of the N terminus, based on the fact that: (i) GSK-3 is truncated by calpain in vitro; (ii) this truncation eliminates the regulatory N-terminal domain; (iii) truncated GSK-3 forms are active kinases that are inhibited by Li+ with an IC50 value similar to that of the full-length isoforms; and (iv) ionomycin-, glutamate-, and NMDA-stimulated neurons induce GSK-3 truncation by activating calpain. Furthermore, our data show a different susceptibility of GSK-3α and GSK-3β isoenzymes, suggesting that this may be a means to differentially regulate calpain activity.
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both isoenzymes. Collectively, these data identify a novel role for calpain in the regulation of GSK-3 activity and describe a new way of regulating GSK-3.

Calcium influx into cortical neurons caused by an ionophore increased GSK-3 truncation in a time-dependent manner. This increase was prevented by calpain inhibitors, indicating that calpains were required to mediate the effect of intracellular calcium on GSK-3 truncation. Incubation of cortical neurons with glutamate or NMDA caused the same calpain-dependent GSK-3 truncation. Interestingly, this proteolytic event correlates with an increase in GSK-3 activity (Fig. 5D). As an indicator of calpain activation, we measured the degradation of the NMDA subunit NR2B, a well-characterized calpain-dependent process (45), as well as the truncation of the p35 regulatory subunit of CDK-5 into the p25 truncated form. Our data show that the proteolytic fragment generated by NMDA/glutamate stimulation is not accumulated. This suggests that calpain cleaves the regulatory N-terminal end, yielding a short-lived, constitutively active form of the enzyme. It is interesting to note that in vitro assays with recombinant GSK-3 isoenzymes, the truncated fragment I does not accumulate, and it is the 30-kDa fragment that accumulates. When similar studies were performed on primary neuronal cultures and on brain tissue, only the 40-kDa form accumulates. A possible explanation for this is that when GSK-3 is sequentially truncated by calpain first into the fragment I and then into fragment II, this latter product is rapidly degraded, and it is, therefore, not easily detected.

Calpains can regulate protein kinases involved in several signal transduction pathways (38). Thus, calpains cleave the regulatory domain of protein kinase C, yielding a short-lived, constitutively active form of the enzyme (46, 47). Cdk5 is also altered by the calpain-mediated generation of two cdk5 activators, p25 and p29, from their respective p35/p39 precursors (42–44). Calmodulin kinase II is degraded by calpain to an active fragment, which is not regulated by calmodulin (48). We add here GSK-3 as a new protein kinase whose activity is upregulated by proteolysis. Together, these data suggest a common pattern that connects calpain and different protein kinases. Thus, calpains release kinase from the inhibitory mechanisms that ensure the normal repression of catalytic domains.

The generation of a similar GSK-3 fragment with a higher specific activity than the intact form has been reported previously (49). The generation of such a proteolytic fragment was described as an aberrant process thought to take place during the purification procedure. Our data demonstrate that this fragment is not merely due to aberrant proteolysis but that it reflects a regulated mechanism with physiological implications. More precisely, N-terminal truncation eliminates the regulatory domain that inhibits the catalytic activity and offers a new way to regulate GSK-3 that is independent of the protein kinase/protein phosphatase equilibrium.

The present results have clear implications in all the transduction pathways in which GSK-3 is involved and especially in neurodegenerative processes such as AD. Thus, it is well established that aberrant Tau phosphorylation in AD is due to GSK-3 and/or CDK5 activation, although the connection and the relative contribution of each protein remains unclear. Taking into account that calpains induce p35 truncation (42, 43) and that calpain also induces GSK-3 activation through N-terminal truncation (present data), it is possible that the deregulation of calcium homeostasis may contribute to AD neuropathology through the calpain/GSK-3/CDK5 system. Thus, our data support the view that calpains may provide a link between both kinases (Fig. 6).

In summary, the present study has demonstrated for the first time that calpain promotes N-terminal GSK-3 truncation. This has implications in signal transduction pathways such as those activated through glutamatergic stimulation of NMDA receptors. Furthermore, GSK-3 truncation may have important consequences in pathological disorders such as AD, in which this kinase has been implicated. Further studies will be required to fully elucidate the role of calpain-mediated GSK-3 truncation and to evaluate how this process affects transduction pathways in which GSK-3 is involved.

REFERENCES
1. Cohen, P., and Frame, S. (2001) Nat. Rev. Mol. Cell Biol. 2, 769–776
2. Frame, S., and Cohen, P. (2001) Biochem. J. 359, 1–16
3. Jope, R. S., and Johnson, G. V. (2004) Trends Biochem. Sci. 29, 95–102
4. Avila, J., Lucas, J. J., Perez, M., and Hernandez, F. (2004) Physiol. Rev. 84, 361–384
5. Eldar-Finkelman, H. (2002) Trends Mol. Med. 8, 126–132
6. Lovestone, S., Reynolds, C. H., Latimer, D., Davis, D. R., Anderton, B. H., Gallo, J. M., Hanger, D., Mulot, S., Marquardt, B., Stabel, S., Woodgett, J. R., and Miller, C. C. J. (1994) Curr. Biol. 4, 1077–1086
7. Hong, M., Chen, D. C., Klein, P. S., and Lee, V. M. (1997) J. Biol. Chem. 272, 52526–52532
8. MunozMontano, J. R., Moreno, F. J., Avila, J., and DiazNido, J. (1997) FEBS Lett. 411, 183–188
9. Spittaels, K., Van den Haute, C., Van Dorpe, J., Geerts, H., Mercken, M., Bruinsseels, K., Lasrado, R., Vandezande, K., Laenen, I., Boon, T., Van Lint, J., Vandenhende, J., Moechars, D., Loos, R., and Van Leuven, F. (2000) J. Biol. Chem. 275, 41340–41349
10. Lucas, J. J., Hernandez, F., Gomez-Ramos, P., Moran, M. A., Hen, R., and Avila, J. (2001) EMBO J. 20, 27–39

FIGURE 6. Diagram showing how altered calcium homeostasis following NMDA receptor activation may contribute to the physiology and eventually to neuropathological activation of the calpain/GSK-3/CDK5 pathway.
