P24, a glycogen synthase kinase 3 (GSK 3) inhibitor

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Abstract

A heat resistant glycogen synthase kinase 3 (GSK 3) binding protein, p24, that inhibits its kinase activity at a low magnesium concentration (in a way similar to that of lithium) was found in microtubules from adult rat brains. This protein associates with GSK 3 in microtubules and corresponds to one previously described in the literature as p25, although it has a relative molecular weight of 23,472. p24 is a poor substrate for GSK 3 but it could be phosphorylated by other protein kinases such as cAMP dependent protein kinase and cdk 5. Since p24 could form complexes with GSK 3, it may not only regulate GSK 3 activity but also it might act as an anchoring protein for the kinase. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Glycogen synthase kinase 3 (GSK 3) is a serine/threonine kinase that was first described by its ability to phosphorylate glycogen synthase and therefore control glycogen metabolism. However, additional roles for GSK 3 in cell growth and development have been indicated [1,2]. Furthermore, GSK 3 may also be involved in Alzheimer’s disease (AD) [3] through the phosphorylation of microtubule associated tau [4] by the kinase [5]. Hyperphosphorylated tau is the main component of neurofibrillary tangles [6] that are found in brains of AD patients [7].

GSK 3 is expressed as a single isoform in invertebrates but in vertebrates two isoforms have been described [8]. The activity of GSK 3 can be regulated through its interaction with proteins such as GSK 3 binding protein (GBP) [9], FRAT1 [10], and axin [11].

GSK 3 is also known as tau protein kinase 1 (TPK1), since it phosphorylates tau protein [5]. GSK 3 has been isolated from brain microtubules [12]. We have isolated GSK 3 from newborn and adult rat brain microtubules and have detected a protein with an electrophoretic mobility corresponding to 24 kDa that copurifies with GSK 3. This protein is present in the microtubule fraction from adult rat brain but not from newborn rat brain. We report the isolation and characterization of this novel GBP, which has previously been identified by other authors [13,14]. We have studied the phosphorylation of p24 by GSK 3 and other kinases and discuss the consequences of this phosphorylation in a similar region to that found in the protein tau [13,14].
2. Materials and methods

2.1. Materials

\[^{32}\text{P}]\text{ATP}\) was purchased from Amersham. Mouse monoclonal antibody to GSK 3\(\beta\)/TPK1 was purchased from Transduction Laboratories (Lexington, KY, USA). ATP was purchased from Boehringer-Mannheim. DEAE-cellulose and phosphocellulose were from Whatman. The recombinant GSK 3\(\beta\), containing histidine tails (catalog number G 1663), and the catalytic subunit of cAMP dependent protein kinase (PKA, P 2645) were purchased from Sigma (St. Louis, MO, USA). According to the supplier’s specifications, the kinases were purified to greater than 90% homogeneity from \textit{Escherichia coli} and from bovine heart to a specific activity of 30 and 50 U/\(\mu\)g protein, respectively. The activity of GSK 3\(\beta\) using glycogen synthase 1 (GS-1) as substrate was 175 pmol/min/\(\mu\)g protein. The activity of PKA using p24 as substrate was 20 pmol/min/\(\mu\)g protein. Phenylmethylsulfonyl fluoride (PMSF), benzamidine, EDTA, EGTA, heparin-acrylic beads, MES and Tris were obtained from Sigma. Alkaline phosphatase was obtained from Roche (catalog number 713023, Germany).

Brains were isolated from Wistar rats or mice of different ages and homogenized in buffers containing 0.32 M sucrose, 10 mM phosphate pH 7.0, 1 mM MgCl\(_2\), 2 mM EGTA, 1 mM PMSF and 1 mM benzamidine. Homogenates were centrifuged at 100 000 \(\times\) g for 1 h at 4\(^\circ\)C. The supernatants (brain cytosolic fractions) were collected. Subsequently p24 was purified from rat brain microtubules [13] following in part the procedure described by Woodgett [8] for GSK 3\(\beta\) including an extra boiling step, to separate GSK 3 from p24. The protein in buffer 10 mM Tris–HCl pH 7.4, 1 mM EGTA and 15 mM 2-mercaptoethanol (buffer A) was heated at 100\(^\circ\)C for 20 min. Denatured proteins were removed by centrifugation and the supernatant was chromatographed on DEAE-cellulose. The flow-through fractions in buffer A were applied to a heparin-acrylic column. p24 was eluted with a gradient of 100–500 mM NaCl. Fractions containing p24 were dialyzed against buffer A and chromatographed on S-Sepharose by eluting with buffer A+250 mM NaCl. The p24 fractions were diluted two-fold and applied to ATP-agarose. After washing with buffer A+125 mM NaCl, p24 was eluted with 0.125–1 M NaCl. p24 fractions were concentrated by ammonium sulfate precipitation and dialyzed against buffer A containing 30% glycerol and stored at \(-20\)^\circ\)C [8]. About 0.4 \(\mu\)g was obtained from 1 mg of initial protein (brain homogenate soluble protein). Cdk 5 and PKC were purified from rat brain as indicated by Takahashi et al. [13] and Yokozeki et al. [14], respectively.

The glycogen synthase peptides (GS-1/GS-1-P) YRRAVPPSPLSRSHPQ*EDEE containing serine 21 in phosphorylated or unphosphorylated form were synthesized on an automatic solid phase peptide synthesizer (type 430A, Applied Biosystems) and purified by reverse phase HPLC on a NovaPak C18 column. The glycogen synthase peptide containing phosphoserine 21 is a good substrate for GSK 3, whereas the same peptide containing unmodified serine is a poor substrate for the enzyme [15].

Recombinant stathmin [16] was purified from \textit{E. coli} transformed with bacterial expression plasmids encoding wild-type stathmin. Extraction and purification of recombinant stathmin was performed as follows: 2 h after induction by isopropyl thio-\(\beta\)-D-galactoside, the cells were pelleted by centrifugation at 4\(^\circ\)C and resuspended in buffer A, with 1 mM benzamidine and 1 mM PMSF. The cell suspension was sonicated five times for 60 s at 0\(^\circ\)C, and disrupted cells were centrifuged at 4\(^\circ\)C. The supernatant was purified as described by Belmont and Mitchinson [17] with some modifications. As an additional step in the purification, proteins were chromatographed on DEAE-cellulose, before the HPLC step.

2.2. Electrophoresis

SDS-PAGE was carried out by the procedure of Laemmli on 8%, 10% or 15% polyacrylamide gels [18]. Gels were either stained with Coomassie brilliant blue or subjected to Western blot analysis (see below). Densitometric analysis was performed on different Western blot samples and the data processed with an imaging densitometer (GS-6470 model, BioRad). Data were analyzed with Molecular Analyst software.
2.3. Antibodies and analysis by Western blots

Polyclonal antibodies against p24 were raised in rabbit by successive injections of 0.2 mg purified protein. The specific reaction of antibody against p24 was checked by dot-blot Western blot and competition assays as indicated by Ulloa et al. [19]. A standard curve to determine the reaction of the antibody with increasing amounts of p24 was performed.

To test for the interaction of p24 with GSK 3β, we employed a non-denaturing PAGE system. Accordingly, purified p24 (0.125 µg) was mixed with GSK 3β (0.015 µg) in buffer 50 mM Tris-HCl pH 7.5 containing 10 mM MgCl₂. The mixture was incubated at 37°C for 30 min to allow p24 binding to GSK 3β. At the end of incubation running 40 mM Tris-HCl, 200 mM glycine and 24 mM EDTA buffer (TGE buffer) was added and then loaded on to a 6% polyacrylamide gel and run at 100 V for 2 h. At the end of the run, the gels were electrotransferred to nitrocellulose filters to detect the mobility for p24, GSK 3 and the possible complex between both proteins.

Also, to test for the interaction of p24 with GSK 3β, co-immunoprecipitation analysis was carried out. Soluble protein from newborn and adult mouse brain homogenates were mixed with antibody raised against p24 protein and, afterwards, with protein A Sepharose (Transduction Laboratories, catalog number PS40041). The pelleted protein (after low speed centrifugation) was characterized by electrophoresis and Western blot using antibodies raised against GSK 3 and the possible complex between both proteins.

2.4. Enzymatic assays

GSK 3 assays were carried out as previously described [20]. It was also done at different Mg²⁺ concentrations (1–10 mM). Our results indicated that a similar activity was found at 3.3 and 10 mM Mg²⁺ whereas at 1 mM Mg²⁺ activity was less than 40% of that seen with 3.3 mM Mg²⁺. The phosphorylation of stathmin by PKA was performed as previously described by Moreno et al. [21]. Phosphorylation by PKC was measured as described by Correas et al. [22]. Phosphorylation by cdk 5 was assayed as described by Takahashi et al. [13]. Alkaline phosphatase treatment was done as described by Ulloa et al. [19].

2.5. ‘In gel’ digestion of proteins, extraction of peptides and analysis by mass spectrometry

SDS-PAGE gels were stained with Coomassie blue, and the bands of interest excised and subjected to in situ digestion with trypsin as described Svechenko et al. [23]. Gel extracts were pooled, dried down, resuspended in 10 µl of 0.1% trifluoroacetic acid and subjected to an automated desalting procedure [24] with slight modification. Briefly, the resuspended extracts were injected onto a RP-HPLC nanocolumn (Vydac C18, 300 µm i.d.) at a flow of 3–5 µl/min using a Smart HPLC instrument with automatic fraction collection (Pharmacia Biotech, Uppsala, Sweden) equipped with a flowsplitter and working at about 100 µl/min. Peptides were eluted in a single step with methanol/water (7:3) containing 0.1% formic acid, and fractions of about 5 µl were collected. The fraction containing the peptide pool was used for mass spectrometry analysis, using an ion trap mass spectrometer model LCQ (Finnigan, ThermoQuest, San Jose, CA, USA) equipped with a nanospray interface, as described previously [25]. MS/MS spectra from selected peptides were used for database search using Sequest program (Finnigan ThermoQuest), in the comprehensive non-redundant protein sequence database nr.FASTA (NCBI Resources, NIH, Bethesda, MD, USA).

2.6. EDC cross-linking

Purified p24 (0.48 mg/ml) was incubated in 50 mM Tris–HCl pH 7.4, 1 mM EGTA, 1 mM EDTA, 10 mM MgCl₂ and cross-linked with freshly prepared 1 mM 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDC) in the dark [26]. The reaction was stopped after 30 min by the addition of glycine and 2-mercaptoethanol at final concentrations of 50 mM.
and 10%, respectively, and the cross-linked proteins characterized by gel electrophoresis and Western blot.

3. Results

3.1. Presence of a 24 kDa protein in adult rat brain microtubule fraction

Since GSK 3 has been shown to be associated with microtubules [5], microtubules were isolated by in vitro polymerization as the first purification step for the kinase. Since one of the GSK 3 isoforms (GSK 3β) is developmentally regulated in brain [27], microtubules were obtained from newborn and adult (6 month) rat brain extracts. Since differences in the composition of the high molecular weight microtubule associated proteins (MAPs) have been extensively described before [28], we focused on differences in the low molecular weight MAPs. We observed a protein with an electrophoretic mobility corresponding to p24 kDa in the MAP fraction only from adult rat brain (Fig. 1). A decrease in the amount of GSK 3 (49%) in adult brain microtubules was also observed compared to that of newborn brain microtubules (as determined by Western blot analysis). To compare the relative amounts of p24 protein, a Western blot analysis was carried out by testing samples from different brain developmental stages together with a known amount of purified p24. From the data of Fig. 1C we calculate that in a 21 day old rat there is 0.4 μg of p24/mg total protein.

3.2. Purification and characterization of the p24 protein

Based on our initial observation that p24 is a heat resistant protein, we performed, on isolated microtubule protein, a preliminary purification step involving heat denaturation (see Section 2). Subsequently, chromatography with DEAE-cellulose, heparin-acrylic and ATP-agarose were performed to yield an essentially homogeneous protein (Fig. 2A).

In order to identify p24, it was separated by SDS-PAGE, subjected to ‘in gel’ digestion with trypsin, and the resulting peptides analyzed by nanospray ion trap mass spectrometry as described in Section 2. Two of the peptides produced clear MS/MS spec-
tra (Fig. 2B), which were used for database searching. These two peptides, VDLVDESGYVPGYK and TITFEQFK, were positively identified. These peptides corresponded to a protein of 25 kDa previously described [29] and known as p25. Since ‘p25’ is also used to refer to another protein that interacts with tau protein kinase II (cdk 5), a kinase that also modifies tau protein [30], we will henceforth continue to refer to the protein of this study as p24 (since this corresponds to its molecular weight as determined by its amino acid composition [29].

Further characterization of p24 by gel filtration (not shown) indicated that it could behave as a dimer. To test this hypothesis experiments using EDC as crosslinker were performed. Fig. 3 shows that p24 could form dimers as determined by gel electrophoresis. At higher EDC concentrations larger protein aggregates were also observed at the top of the gel (not shown).

![Fig. 2. p24 protein purification and sequencing. (A) p24 was isolated from an adult brain microtubule preparation through different purification steps: heat treatment and DEAE-cellulose, heparin-acrylic and ATP-agarose chromatographies. A protein purified almost to homogeneity was isolated. (B) Identification by mass spectrometry of one tryptic peptide from p24. The band corresponding to p24 was digested with trypsin, the extracted peptide pool subjected to microdesalting and analyzed by nanospray ion trap mass spectrometry. Shown is the MS/MS spectrum obtained from one of the peptides. The spectrum was subjected to database searching using Sequest software, and a clear match was found with the sequence depicted. The identity of the fragment peaks has been assigned in the spectrum using the nomenclature of [38].]

![Fig. 3. p24 could form dimers. Purified p24 (0.48 mg/ml) was incubated in the presence of 1 mM EDC for 10 and 20 min at 37°C. The treated protein was fractionated by gel electrophoresis. The electrophoretic mobilities for p24, p24 dimers and molecular weight markers is shown. (a) p24 in the absence of EDC; (b) p24 after 10 min incubation in the presence of EDC; (c) p24 after 20 min incubation in the presence of EDC.]

\[\text{p24} \rightarrow \text{p24 dimer}\]
3.3. Binding of GSK 3 to p24

Since we used a microtubule associated fraction as a source to obtain GSK 3 and p24 we tried to develop a protocol to fractionate these proteins. However, we observed that GSK 3 (measured by its activity) copurifies with p24 (when the heat resistant step was omitted) through DEAE-cellulose, heparin-acrylic and ATP-agarose chromatographies. Contaminating GSK 3 was undetectable by Coomassie blue staining after gel electrophoresis, and its presence could only be measured either by enzymatic activity or by Western blot. We observed that purified recombinant GSK 3 binds to purified p24 (free of GSK 3 after the heat resistant step) as determined by native gel electrophoresis followed by Western blot (Fig. 4A). Different amounts of p24 from 0.01 to 3 µg/10 µl

![Image of gel electrophoresis](Fig. 4. Binding of GSK 3 to p24. (A) Purified p24 (0.125 µg in 10 µl) was incubated in the absence (a) or in the presence of recombinant GSK 3 (0.015 µg in 10 µl) (a’) and incubated for 30 min at 37°C. p24 (a) and the resulting complex (a’) were identified by native electrophoresis, in 6% acrylamide gels in the presence of TGE buffer by Western blot using an antibody raised against p24. Additionally, the electrophoretic mobilities of GSK 3 (b) and that of the complex GSK 3–p24 (b’) were identified by Western blot using an antibody raised against GSK 3. (B) Co-immunoprecipitation analysis of newborn rat brain protein (Po) and 6 month old rat brain protein (Pm) using an antibody against p24 (+) or a preimmune serum (−). The Western blots using GSK 3 antibody (to react with the immune precipitate (P) or supernatant (SN) fractions) or p24 antibody are shown. (C) Interaction of p24 with GSK 3 was mixed in the presence (a) or absence (b) of 0.5 µg of GSK 3 bearing histidine tails and the protein bound to nickel beads was identified by its reaction with both antibodies raised against p24 and GSK 3.

![Image of Western blot](Fig. 5. Action of GSK 3 on p24. To test if p24 is a suitable substrate for GSK 3, p24 (4 µM, about 0.08 mg/ml) was incubated with GSK 3 (0.5 U, about 1.3 µg/ml) in the presence of increasing amounts of ATP. Phosphate incorporation (less than 0.01 mol phosphate/mol protein at 100 µM ATP) was measured (▲) (see right ordinate). In parallel the phosphate incorporation into GS-1 peptide (15 µM) (●) was measured (see left ordinate) yielding an incorporation of 1.5 mol of phosphate incorporated/mol of GS-1 peptide at 100 µM ATP. (B) In a similar way to that described in panel A the phosphorylation of p24 by PKA and cdk 5 was determined at an ATP concentration of 100 µM.)
were mixed with a fixed amount of GSK 3 (0.02 μg/10 μl) and native gel electrophoresis (followed by western blot) was carried out to look for complex formation. Our results indicate that 0.02 μg of GSK 3 and 0.05 μg of p24 mixed in a final volume of 10 μl is the minimal amount required for complex formation. It suggests that one molecule of GSK 3 could bind to around five molecules of p24. Since p24 could form aggregates (F. Moreno, unpublished observations) it is possible to suggest that GSK 3 mainly binds to p24 aggregates.

To further study the binding of GSK 3 to p24, co-immunoprecipitation (using p24 antibody) was tested. Fig. 4B shows that p24 is essentially absent in newborn rat and, therefore, no GSK 3β was found in the immunoprecipitate, whereas in mature rat there is co-immunoprecipitation of p24 and GSK 3. No immunoprecipitation was observed in either case with a preimmune serum (Fig. 4B).

Additionally, the binding of p24 to GSK 3 was measured by using a GSK 3 protein bearing histidine tails (which binds to nickel beads) and by testing the binding of p24 to those beads, in the presence or absence of the previously indicated type of GSK 3 protein. The results in Fig. 4C support the hypothesis that p24 forms complexes with GSK 3, since p24 only binds to nickel beads in the presence of GSK 3.

3.4. Effect of p24 on protein kinase activities

We tested if p24 is a substrate for GSK 3, and found that it is a poor substrate for the kinase (see Fig. 5). We next tested how p24 affects GSK 3 activity as measured by phosphorylation of a GS-1 peptide and the protein tau. Fig. 6 shows that in the presence of p24 a decrease in kinase activity was observed. As controls we tested the effect of p24 on other protein kinase activities. We found that it has no effect on PKC activity (not shown), whereas it slightly increases PKA activity (measured on stathmin as substrate). We also found that p24 is a good substrate for PKA. In this case, an incorporation of 0.7 mol phosphate/mol p24 was found by
increasing ATP concentration, in a way similar to that shown in Fig. 5. We also observed that p24 is modified by cdk 5 (tau protein kinase II) yielding about 0.9 mol phosphate incorporated per mol p24 (see also [13]). Since p24 is a good substrate for PKA, we did the phosphorylation of p24 with PKA under conditions in which about one mol of phosphate was incorporated per mol of protein and we tested if that modification affects the inhibitory effect of p24 on GSK 3β activity. Fig. 7 shows a decrease in this inhibitory effect when phospho-p24 was tested (Fig. 7A). On the other hand, alkaline phosphatase treatment on purified p24 did not affect GSK 3 activity (Fig. 7B).

3.5. p24 inhibition of GSK 3 is dependent on magnesium concentration

An inhibitory action of lithium of the specific GSK 3 inhibitor has been found at low Mg2+ concentrations [31]. More recently it has been shown that lithium is a competitive inhibitor of GSK 3 with respect to Mg2+, but not to substrate or ATP [32]. We tested if a similar situation holds for the p24 inhibitory effect on GSK 3. Fig. 8 shows that the inhibitory effect of p24 on GSK 3 is indeed maximal at low magnesium concentration (Fig. 8A), as observed with LiCl (Fig. 8B). This result suggests a possible common mechanism for p24 and LiCl for inhibition of GSK 3.

4. Discussion

A novel GBP, p24, has been reported. Since this protein binds to GSK 3 we tested if p24 can be modified by the kinase or if GSK 3 activity can be modified by p24. Even at very high concentrations of GSK 3 phosphorylation of p24 is negligible and thus probably has little physiological relevance since the incorporation of mol phosphate per mol of protein is below 0.01 at high ATP concentration.

Despite the fact that p24 is not a good substrate for GSK 3, p24 protein can modify GSK 3 activity. Thus, p24 could be considered a new GBP that regulates its activity (see [9]). The regulation of GSK 3 activity by p24 is suppressed at high magnesium concentration and it appears that p24 could, in same way, mimic the action of lithium on GSK 3 [31,32], since they shared this feature. Since lithium binds to acidic residues of GSK 3, it should be further studied if basic residues of p24 are also involved in the binding to GSK 3.

Another role for p24 could be to act as a scaffold protein, to which GSK 3 and possibly cdk 5 (see [13]) could bind, forming a complex similar to those of other kinases bound to other scaffold proteins [33,34].

p24 can be modified by PKC (see [14]), and, as shown in this work also by PKA and cdk 5 (or other
cdks), and several substrate sequence motifs for these kinases are present in p24 (see Fig. 9A). The cdk target motif introduction of a p24 sequence, TPKSP (12–17 amino acids in p24), is shared with the MAP tau [13]. This sequence is phosphorylated by cdk in tau [35] and it is a putative target for the modification of p24 by that kinase [13].

p24 is expressed late in brain development [27] whereas GSK 3β decreases its expression during development [27]. Thus, a possible role for p24 could be to increase the downregulation of remaining GSK 3 activity in late development. The presence of p24 could inhibit phosphorylation of tau and other cellular substrates of GSK 3 (Fig. 9B,a). A further decrease in the phosphorylation of substrates, such as tau, by GSK 3 may occur due to competition of substrates since the sequence TPKSP is present in p24 [13] and the MAP tau (Fig. 9B,b). As a result of such competition, further phosphorylation of tau by GSK 3, which is promoted by its prior modification by cdk proteins [36], will not take place. Thus, p24 could inhibit the phosphorylation of substrates such as tau in at least two ways.

This competitive role of p24 and tau for common proteins could be related to the onset of neuropathological disorders such as AD as explained in the following way (Fig. 9B,c). It is known that tau protein can be phosphorylated by a cdk-type kinase at the TPKSP peptide thereby changing its conformation. This conformational change could be reversed by binding to Pin1, a chaperone protein, thus allowing further dephosphorylation by PP2A [35,37], thus recovering microtubule binding activity. Thus, if in AD there is an overexpression of kinases like cdc2 or other cdk-type kinases, as previously proposed [35], both tau and p24 could be phosphorylated and both phosphorylated proteins could compete for a protein like Pin1 (or PP2A), decreasing its activity and resulting in an aberrant hyperphosphorylation of tau protein, a feature that occurs in AD [6]. This possible model for the action of p24 in the modification of substrates like tau protein suggests that the presence of p24 should result in a decrease in the modification of proteins like tau by GSK 3, a point particularly relevant for AD pathology. From our experiments we can calculate a concentration of p24 in brain (if uniform) of about 0.4 μg/ml. This concentration is probably below that needed for GSK 3 binding. Thus, probably p24 should be more concentrated in some brain region (or cells) to allow that interaction.

On the other hand, modification of p24 by phosphorylation or dephosphorylation may affect its ef-
fect on GSK 3 activity. In this way our results indicate that phosphorylation by PKA decreases the effect of p24 on GSK 3 activity whereas no effect on GSK 3 activity was observed for alkaline phosphatase treated p24 compared with that of untreated p24. Then, changes in the level of phosphorylation of p24 could also play a role in p24 function.

In summary, we have described a novel GBP that could regulate the activity of this protein kinase and could also act as a competitor for tau.

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