Isoform Differences in Substrate Recognition by Glycogen Synthase Kinases 3α and 3β in the Phosphorylation of Phosphatase Inhibitor 2†

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ABSTRACT: Phosphorylation of inhibitor 2, the regulatory subunit of the ATP–Mg-dependent protein phosphatase, by glycogen synthase kinase 3 (GSK-3) causes activation of the phosphatase. Prior phosphorylation by casein kinase II has been shown to enhance both phosphorylation and activation of the phosphatase by GSK-3 (DePaoli-Roach, A. A. (1984) *J. Biol. Chem. 259*, 12144–12152). Reported here is a comparison of the phosphorylation of inhibitor 2 by two defined isoforms of GSK-3, GSK-3α and GSK-3β. GSK-3β was a significantly better inhibitor 2 kinase than was GSK-3α. The V_{max}/K_m value for GSK-3β was ~10-fold higher than that for GSK-3α. GSK-3β phosphorylated inhibitor 2 to a stoichiometry of ~1.0 mol of phosphate/mol of inhibitor 2. The phosphorylation by GSK-3β was determined to be exclusively at Thr-72 on the basis of the inability of the enzyme to modify a mutant inhibitor 2 in which Thr-72 was changed to alanine. Prior phosphorylation by casein kinase II promoted the action of GSK-3α in keeping with earlier reports using undefined GSK-3 preparations. Phosphorylation by GSK-3β, in contrast, was unaffected by the previous action of casein kinase II. These results suggest that there can be important differences in substrate recognition by different isoforms of the same protein kinase and may help explain why some reported GSK-3 substrates require prior phosphorylation whereas others do not. It is proposed that substrate recognition by GSK-3 involves multiple determinants, the relative importance of each depending on both the substrate and the GSK-3 isoform in question.

Glycogen synthase kinase 3 (GSK-3)[1] is a monomeric second-messenger-independent protein kinase that was first discovered through its ability to activate the ATP–Mg-dependent form of type 1 protein phosphatase (F_A activity) and to phosphorylate glycogen synthase (Embi et al., 1980; Rylatt et al., 1980; Vandenheede et al., 1980). It is now apparent that there are multiple isoforms of GSK-3. Two rat brain cDNAs encoding GSK-3 were isolated that corresponded to isoforms designated GSK-3α and GSK-3β, with apparent M_rs of 51 000 and 47 000, respectively (Woodgett, 1990). The zeste-white3/shaggy (*zw3*^{sgg}) gene of *Drosophila* is involved in developmental programs and also encodes a protein kinase (Bourious et al., 1990; Siegfried et al., 1990) with significant homology to mammalian GSK-3 (Woodgett, 1991). Mammalian GSK-3β can substitute functionally for *zw3*^{sgg}, suggesting that it is indeed a homolog of the insect kinase (Ruel et al., 1993; Siegfried et al., 1993). In the budding yeast *Saccharomyces cerevisiae*, the *MCK1* and *MDS1*[2] genes encode proteins with 44 and 58% identity, respectively, to the kinase domain of GSK-3, and both gene products are capable of phosphorylating substrates characteristic of those recognized by mammalian GSK-3 (J. Puziss, T. A. Hardy, R. Johnson, P. J. Roach, and P. Hieter, unpublished data).

A number of *in vitro* substrates for GSK-3 have been described, and the sites modified have been identified in several cases (Plyte et al., 1992). One feature of substrate recognition by GSK-3 is that, in some substrates, there is close to an absolute requirement for prior phosphorylation. GSK-3-catalyzed phosphorylation of these substrates, which include glycogen synthase (DePaoli-Roach et al., 1983; Picton et al., 1982), type 1 phosphatase R_{GI} subunit (Fiol et al., 1988; Dent et al., 1989), ATP–citrate lyase (Ramakrishna et al., 1990), and the transcriptional factor CREB (Fiol et al., 1992), can be explained by recognition of the sequence motif -S-X-X-X-S(P)- where the phosphate is introduced by a separate protein kinase [for review, see Roach (1991)]. A second group of substrates appear not to require prior phosphorylation in order to act as effective GSK-3 substrates. Included here are the c-Jun (Boyle et al., 1991) and c-Myc (Saksela et al., 1992) transcriptional factors. Some members of this group of substrates, such as phosphatase inhibitor 2 and the RII subunit of cyclic AMP-dependent protein kinase, are phosphorylated by GSK-3, but the rate is significantly enhanced by prior COOH-terminal phosphorylation. Inhibitor 2 is in fact a regulatory subunit of the cytosolic ATP–Mg-dependent form of type 1 phosphatase (Yang et al., 1981), and its phosphorylation by GSK-3 results in the activation of the phosphatase (Hemmings et al., 1982; Ballou et al., 1983), as noted above. Activation of the phosphatase by GSK-3 is enhanced if inhibitor 2 has previously been phosphorylated by casein kinase II (DePaoli-Roach, 1984). The GSK-3 site in inhibitor 2 is Thr-72 (Aitken et al., 1984), and the casein kinase II site involved in the synergism has recently been identified as Ser-86 (Park & DePaoli-Roach, 1993).

The basis for these different modalities of substrate recognition by ostensibly the same protein kinase has been neither well understood nor particularly carefully studied. In the present paper, we report on differences in the ability of GSK-3α and GSK-3β to phosphorylate inhibitor 2. First, GSK-3β is a significantly better inhibitor 2 kinase compared with GSK-3α. Second, while GSK-3α action is enhanced by

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[1] Abbreviations: GSK-3, glycogen synthase kinase 3; CREB, cyclic AMP responsive element binding protein.

[2] *MDS1* (*MCK1* Dosage Suppressor) has been previously designated *SMC10*.

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prior phosphorylation by casein kinase II, GSK-3β activity is unaffected. Therefore, these different forms of the same protein kinase exhibit potentially important differences in substrate recognition characteristics.

EXPERIMENTAL PROCEDURES

Protein Kinases. The GSK-3α isoform was isolated from rabbit skeletal muscle as previously described (Fiol et al., 1990). The GSK-3β isoform was a recombinant protein expressed in *Escherichia coli*. Cloning of a cDNA encoding rabbit skeletal muscle GSK-3β and details of its expression will be described elsewhere (Q. M. Wang and P. J. Roach, unpublished data); briefly, expression was achieved by inserting the coding sequence of GSK-3β in the pET-3c vector. *E. coli* cells expressing the protein kinase were broken with a French press. After a negative treatment with DEAE-cellulose, the enzyme was purified by chromatography on columns of phosphocellulose, Cibacron Blue, and S-Sepharose. Analysis of the resulting enzyme by SDS–PAGE indicated that approximately 50% of the protein was present as a species of apparent M_r 46 000 corresponding to GSK-3β. One unit of GSK-3 activity is defined as the amount of enzyme that, at 30 °C, transfers 1 nmol of phosphate per minute into the phospho-CREB peptide as described below. One unit of casein kinase II activity is defined as the amount of enzyme that catalyzes 1 nmol of phosphate incorporation per minute into the peptide substrate (RRRDDDSDDD) at 30 °C. Homogeneous bovine testis casein kinase II and bovine cardiac muscle cAMP-dependent protein kinase catalytic subunit were kindly provided by Dr. D. Litchfield (Manitoba Institute of Cell Biology) and Dr. E. G. Krebs (University of Washington), respectively.

CREB Peptide Phosphorylation. We have adopted the phosphorylation of a synthetic peptide, phospho-CREB peptide, based on the sequence of the CREB protein as a routine substrate for assays of GSK-3 activity (Fiol et al., 1992). The 13-residue peptide, KRREILSRRPSYR, is an effective GSK-3 substrate once it has been phosphorylated by cyclic AMP-dependent protein kinase. The peptide was phosphorylated by the catalytic subunit of cAMP-dependent protein kinase at 30 °C for 15 h in a reaction mixture containing 50 mM Tris, pH 7.4, 1.5 mM ATP, 10 mM $MgCl_2$, and 1.2% 2-mercaptoethanol to a stoichiometry of 0.85 mol of phosphate/mol of CREB peptide. The phosphorylated CREB peptide (KRREILSRRPS(P)YR) was adsorbed to a Sep-Pak C18 cartridge (Millipore) to remove unreacted ATP and other reaction components, eluted with 50% acetonitrile and 0.1% trifluoroacetic acid, dried, and resuspended in water. GSK-3 phosphorylation of the phospho-CREB peptide was performed at 30 °C in a reaction mixture (15 μL) containing 50 μM phospho-CREB peptide, 100 μM [γ-^{32}P]ATP (500–2000 cpm/pmol), 10 mM $MgCl_2$, 1 mM dithiothreitol, 5% glycerol, 0.1 mg/mL BSA, and 25 mM Tris-HCl, pH 7.4. The ^{32}P-labeled peptides were recovered on Whatman p81 phosphocellulose paper, washed four times with 75 mM phosphoric acid for a total of 20 min, and quantitated by liquid scintillation counting once the paper was dry.

Inhititor 2 Phosphorylation. The production of the recombinant wild-type inhibitor 2 and the Ala-72 mutant inhibitor 2 is to be described in detail elsewhere (Park et al., 1994). Briefly, the inhibitor 2 was expressed in *E. coli* using a construct based on the pET8d vector. The proteins were purified close to homogeneity by a variation of previously described methods (DePaoli-Roach, 1984). Inhibitor 2, at the indicated concentration, was phosphorylated by casein kinase II, GSK-3α, GSK-3β, or combinations of the protein kinases at 30 °C in a reaction mixture (20 μL) that contained 25 mM Tris-HCl, pH 7.4, 5% glycerol, 10 mM $MgCl_2$, and 0.2 mM ATP (1000–5000 cpm/pmol). The reaction was initiated by the addition of ATP and Mg^{2+} and terminated by the addition of 5× SDS–PAGE sample buffer (final conposition: 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.125% bromophenol blue, and 62.5 mM Tris-HCl, pH 6.8) followed by heating. Proteins were separated by SDS–PAGE using 12% polyacrylamide gels. Phosphoproteins were detected by autoradiography. For quantitation of the ^{32}P-labeled proteins, the corresponding gel regions were excised and subjected to liquid scintillation counting.

Other Materials and Methods. Protein concentration was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin as standard. [γ-^{32}P]ATP was from New England Nuclear. Chemicals for gel electrophoresis were from Bio-Rad.

RESULTS

Phosphorylation of Inhibitor 2 by GSK-3α and GSK-3β. Most previous work from our laboratories had used preparations of GSK-3 isolated from rabbit muscle prior to the discovery of distinct isoforms. Therefore, the specific isoform used was not formally defined. Western blotting of the current rabbit muscle GSK-3 preparation used for this work, however, indicated that essentially all of the immunoreactive material had an apparent M_r of 51 000 by SDS–PAGE, indicating that the purified protein corresponded to GSK-3α. GSK-3β has been reported to be present in muscle extracts, and it is possible that the purification scheme selected for the GSK-3α isoform (Woodgett, 1990). Certainly, the fact that we isolated a cDNA encoding GSK-3β from a rabbit skeletal muscle library suggests the existence of some level of message in this tissue. In any event, for the comparative studies described below, the GSK-3α and GSK-3β both had origins in rabbit muscle.

The CREB transcriptional factor is phosphorylated by both GSK-3α and GSK-3β in a reaction that requires prior phosphorylation by cyclic AMP-dependent protein kinase (C. J. Fiol, J. S. Williams, Q. M. Wang, P. J. Roach, and O. Andrisani, unpublished data). A synthetic peptide based on the CREB sequence has also been shown to be a substrate for GSK-3 (Fiol et al., 1992). Again, prior phosphorylation is a prerequisite and this is true for both GSK-3α and GSK-3β isoforms (Figure 1). The apparent K_m values for GSK-3α and GSK-3β were 140 and 200 μM, respectively. We have adopted the use of the phospho-CREB peptide as a routine assay for GSK-3 and GSK-3-like enzymes. In the present study, GSK-3α and GSK-3β levels were carefully calibrated so that the rates of phospho-CREB peptide phosphorylation by GSK-3α and GSK-3β were almost identical (Figure 2A). In parallel reactions, the rate of inhibitor 2 phosphorylation was determined (Figure 2B). GSK-3β phosphorylated inhibitor 2 11 times faster than GSK-3α under the conditions of the experiment. In the past, we had experienced difficulties in achieving significant stoichiometries of phosphorylation of inhibitor 2 by GSK-3 preparations (DePaoli-Roach, 1984), which we now infer were most likely to have contained GSK-3α. Other groups had also reported very low stoichiometries of inhibitor 2 phosphorylation by GSK-3 (Jurgensen et al., 1983; Hughes et al., 1991). With GSK-3β, however, phosphorylation to a stoichiometry close to 1 mol/mol of inhibitor 2 was readily attainable under the conditions of Figure 3. When the inhibitor 2 concentration was varied, simple

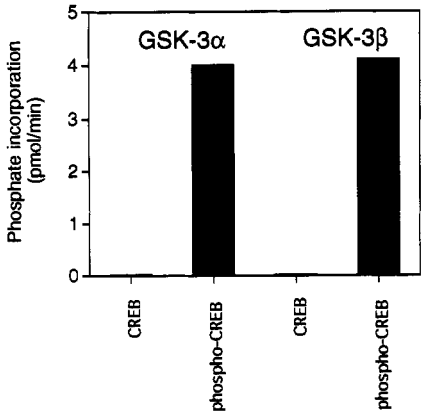

FIGURE 1: Requirement of phosphorylation of CREB peptide by GSK-3α and GSK-3β for prior phosphorylation. CREB or phospho-CREB peptide (50 μM) was incubated with 4 munits of either GSK-3α or GSK-3β, and the phosphorylation of the peptide after 5 min was quantitated as described under Experimental Procedures.

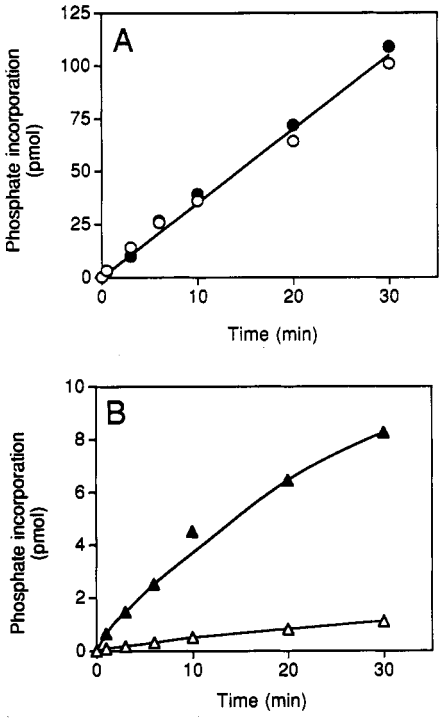

FIGURE 2: Phosphorylation of phospho-CREB peptide and inhibitor 2 by GSK-3α and GSK-3β. Phospho-CREB peptide (50 μM; panel A) or inhibitor 2 (10 μM; panel B) was incubated with 4 munits of GSK-3α (○, △) or 4 munits of GSK-3β (●, ▲), and the phosphorylation of the peptide and protein was quantitated, at different times, as described under Experimental Procedures.

hyperbolic kinetics defining a K_m of 15 μM were observed (Figure 4). With GSK-3α, the low rate of phosphorylation was essentially proportional to inhibitor 2 concentration up to 25 μM, making it impossible to define an apparent K_m. This dependence on inhibitor 2 concentration defines an apparent first-order rate constant 9-fold lower than the V_{max}/K_m value for GSK-3β, consistent with the results in Figure 2.

Synergistic Phosphorylation of Inhibitor 2 by GSK-3 and Casein Kinase II. In previous studies, prior phosphorylation of inhibitor 2 by casein kinase II was shown to potentiate phosphorylation by GSK-3 (DePaoli-Roach, 1984). Similar results are reported here using a defined GSK-3α (Figure 5). However, when the same experiment was performed using GSK-3β, no synergism was observed. Similar results were obtained over a range of GSK-3β concentrations (not shown).

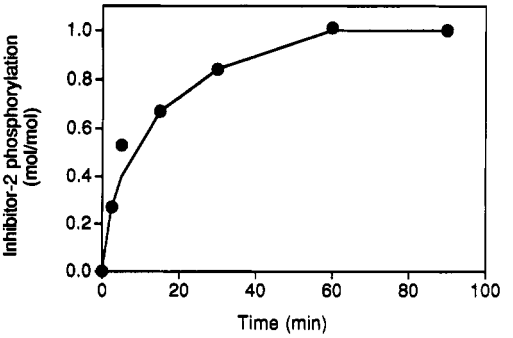

FIGURE 3: Stoichiometry of inhibitor 2 phosphorylation by GSK-3β. Inhibitor 2 (3.5 μM) was incubated with GSK-3β (64 munits) as described under Experimental Procedures. At the indicated times, an aliquot of the reaction mixture was removed for SDS–PAGE and quantitation of the ^{32}P-labeled inhibitor 2.

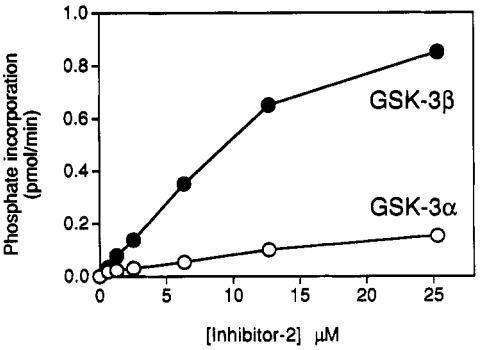

FIGURE 4: Substrate dependence of inhibitor 2 phosphorylation by GSK-3α and GSK-3β. Inhibitor 2 phosphorylation by GSK-3α and GSK-3β was determined as a function of inhibitor 2 concentration. The assay was as described under Experimental Procedures except that 125 μM [γ-^{32}P]ATP was present. The reaction time was 6 min so as to give an estimate of the initial rate.

Note that phosphorylation of inhibitor 2 by GSK-3β or GSK-3α acting in concert with casein kinase II caused a reduction in mobility on SDS–PAGE (Figure 5B), as has been reported previously (DePaoli-Roach, 1984). It has been shown, using GSK-3 of undefined isoform composition, that the residue in inhibitor 2 modified by GSK-3 was Thr-72 (Aitken et al., 1984). Therefore, a mutant form of inhibitor 2, in which Thr-72 was mutated to alanine, was also tested as a substrate. Neither GSK-3α nor GSK-3β was able to phosphorylate the mutant inhibitor 2 even though it was a substrate for casein kinase II (Figure 5). These data demonstrate that Thr-72 was the sole site of GSK-3β action.

Ionic strength has been reported to influence the degree of synergism observed between casein kinase II and GSK-3 for the activation of the ATP–Mg-dependent protein phosphatase and, by inference, for the phosphorylation of inhibitor 2 (Henry & Killilea, 1993). Increasing the NaCl concentration accentuated the extent to which GSK-3 action was dependent on prior phosphorylation. Therefore, the experiments described above were repeated in the presence of 100 mM NaCl to determine whether GSK-3β action now required prior phosphorylation of inhibitor 2 (Figure 6). Although GSK-3β activity toward inhibitor 2 was reduced by NaCl (half-maximal at 30 mM NaCl; data not shown), the presence of salt did not induce synergism between GSK-3β and casein kinase II (compare Figures 5 and 6). In contrast, the synergism with GSK-3α was increased by elevated salt, as had been reported by Henry and Killilea for the activation of the ATP–Mg-dependent protein phosphatase purified from porcine heart (Henry & Killilea, 1993). In other words, for GSK-3α, phosphorylation of inhibitor 2 became more dependent on

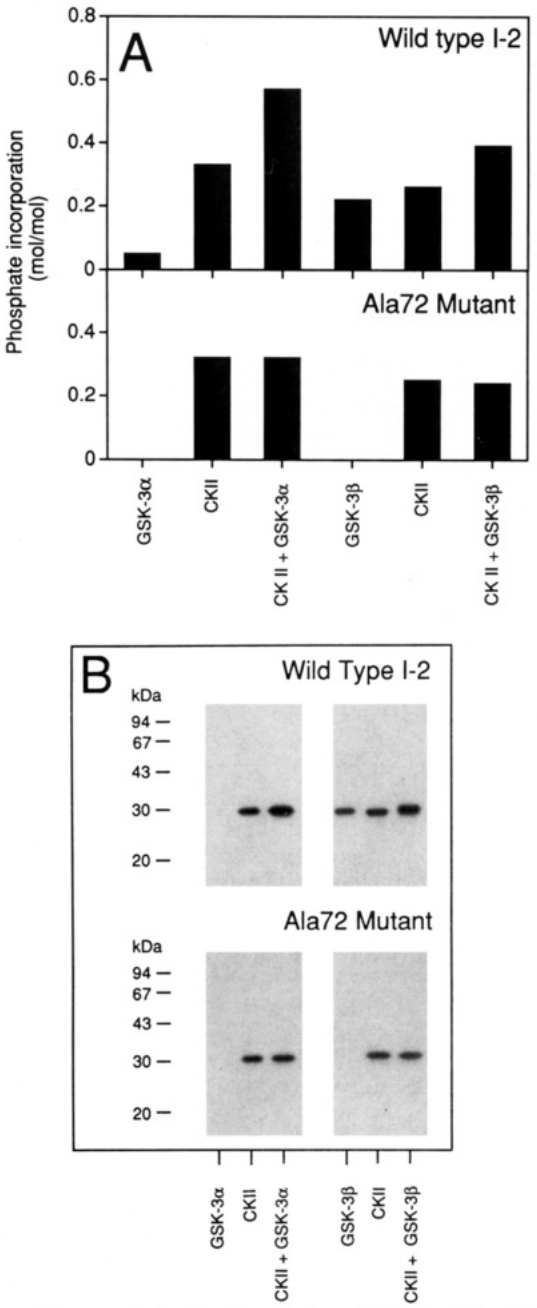

FIGURE 5: Synergistic phosphorylation of inhibitor 2 by GSK-3α but not GSK-3β. Phosphorylation of wild-type inhibitor 2 (1 μM) and the Ala-72 mutant inhibitor 2 (0.8 μM) by the indicated protein kinases was initiated by the addition of ATP and Mg^{2+}. The reaction was for 20 min. Reaction mixtures contained 4.5 munits of casein kinase II and 4 munits of GSK-3. Panel A: Quantitation of ^{32}P-labeled inhibitor 2 after SDS–PAGE. Panel B: Autoradiogram of SDS–PAGE.

prior phosphorylation at the higher salt concentration. Such was not true for GSK-3β, and so the differences in inhibitor 2 phosphorylation by GSK-3α and GSK-3β were not affected by changing the ionic strength.

DISCUSSION

As the discovery rate for protein kinases escalates, it is increasingly common to uncover the existence of multiple isoforms of closely related enzymes present in the same species and often the same tissue. Some fairly large subfamilies of protein Ser/Thr kinases are now known. Examples include protein kinase C (Nishizuka, 1989; Stabel & Parker, 1991), the cdc2 kinases (Sherr, 1993), and casein kinase I (Rowles et al., 1991; Graves et al., 1993). One of the challenges is to understand the differences which presumably confer functional uniqueness to some, if not all, members of protein kinase subfamilies. The GSK-3 family of protein kinases is still quite limited in number, and the two most studied mammalian forms are GSK-3α and GSK-3β. One example of isoform-specific differences is the observation that protein kinase C regulates GSK-3β but not GSK-3α (Goode et al., 1992). Another example is that GSK-3β, but not GSK-3α, is able to rescue the phenotype caused by the shaggy gene mutation in *Drosophila* (Ruel et al., 1993). The present investigation establishes that these two GSK-3 isoforms from rabbit muscle exhibit clear differences in substrate recognition. The main results are that (i) GSK-3β is a significantly better inhibitor 2 kinase than GSK-3α, and (ii) GSK-3β action, unlike that of GSK-3α, is not influenced by prior phosphorylation of the inhibitor 2.

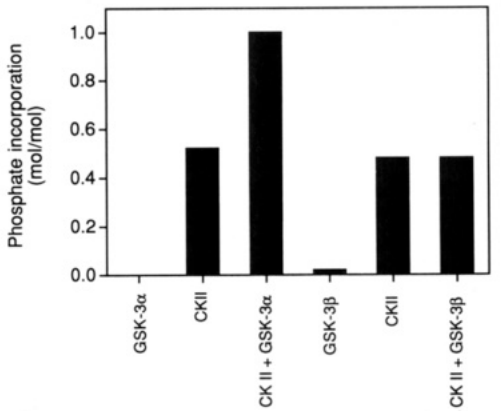

FIGURE 6: Effect of increased ionic strength on inhibitor 2 phosphorylation. Inhibitor 2 (1 μM) was phosphorylated under identical conditions to those described in the caption to Figure 4 except that the reaction mixture contained 100 mM NaCl.

Since isoforms of protein kinases are defined mostly on the basis of sequence similarities within the protein kinase domain, there is an obvious likelihood that some fundamental catalytic properties should be similar. Thus, within the GSK-3 family, mammalian GSK-3α and GSK-3β, as well as two related yeast kinases, MCK1 and MDS1, all phosphorylate the phospho-CREB peptide, and the reaction is completely dependent on the presence of the phosphate in the substrate. Similarly, the phosphorylation of recombinant mammalian glycogen synthase by both GSK-3α and GSK-3β was completely dependent on prior phosphorylation by casein kinase II (Q. M. Wang and P. J. Roach, unpublished results). Thus, there were no observed differences between GSK-3α and GSK-3β acting in this modality of substrate recognition. However, with other classes of substrate, more substantial differences can be observed. Such is true for inhibitor 2, as described in the present study. The only prior comparisons of inhibitor 2 phosphorylation by the different GSK-3 isoforms did not utilize recombinant substrate, leaving open the possibility that residual phosphate on the inhibitor 2 could have influenced the results (Hughes et al., 1991). Nonetheless, from assays at a single substrate concentration, it was reported that GSK-3β phosphorylated inhibitor 2 some 3 times faster than did GSK-3α. In the present study, we used recombinant inhibitor 2, thus removing any ambiguities about residual phosphate already present in the substrate, and have determined the K_m of GSK-3β for inhibitor 2 as 15 μM. Additionally, we have shown that GSK-3β action is completely independent of prior phosphoryation of the inhibitor 2. This observation raises the possibility that other GSK-3 substrates for which prior

phosphorylation is not an absolute requirement may also be more effectively phosphorylated by GSK-3β than by GSK-3α. For example, GSK-3β phosphorylates a c-Myc fusion protein many times faster than GSK-3α (Q. M. Wang, P. J. Roach, B. Lutterbach, and S. Hann, unpublished results). The earlier literature may also be complicated by a lack of certainty as to which isoform of GSK-3 was being used. These differences in substrate recognition between GSK-3α and GSK-3β are likely to be important for the selection of substrates *in vivo* and hence for physiological function.

The results also pose interesting questions as to the mechanism of substrate recognition by GSK-3. Since phosphorylation of the phospho-CREB peptide appears the same for the two mammalian isoforms as well as the more distant yeast enzymes MCK1 and MDS1, we propose that one modality of recognition, involving the minimal -S-X-X-X-S(P)- motif of Fiol et al. (1990), is due to conserved residues in the GSK-3 family. The other modality of action, in which the phosphoserine is not obligatory, must involve other features of the substrate that are not yet defined. This mode of action brings out differences between the mammalian isoforms, as exemplified for inhibitor 2 in this study. Within the protein kinase domain, the GSK-3α and GSK-3β sequences are 98% identical (Woodgett, 1990). Thus, it is likely that the NH_2- and/or COOH-terminal regions, which are much more divergent, contribute to these differences in substrate recognition. Essentially, this model simply states that there are multiple potential determinants for substrate recognition, for which the relative importance depends on both the specific substrate and the GSK-3 isoform involved. In fact, such a proposal is not inconsistent with the recent structural analysis of cyclic AMP-dependent protein kinase bound to a peptide inhibitor (Knighton et al., 1991). Here, this very tight interaction involves multiple contacts between several different residues of the inhibitor as well as the protein kinase, but adequate substrates can be defined by only a subset of those interactions.

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