Formation of Protein Kinase Recognition Sites by Covalent Modification of the Substrate

MOLECULAR MECHANISM FOR THE SYNERGISTIC ACTION OF CASEIN KINASE II AND GLYCOGEN SYNTHASE KINASE 3*

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The mechanism for synergistic phosphorylation by glycogen synthase kinase 3 (GSK-3) and casein kinase II was studied using a synthetic peptide which contains the sequence of a potentially important proline-serine-rich regulatory region of rabbit muscle glycogen synthase. The peptide, Ac-PRPAS(3a)VPPS(3b)PSLS(3c)RHSS(4)PHQS(5)EDDEEP-amide, has five known phosphorylation sites of the native enzyme designated sites 3a, 3b, 3c, 4, and 5, which are spaced every fourth residue. The peptide was phosphorylated specifically at site 5 by casein kinase II with an apparent $K_m$ of 2 $\mu$m, but it was not phosphorylated by GSK-3. However, after initial phosphorylation of site 5 by casein kinase II, the peptide became an effective substrate for GSK-3 with an apparent $K_m$ of 2 $\mu$m. GSK-3 introduced up to four phosphates and appeared to catalyze the sequential modification of sites 4, 3c, 3b, and 3a, respectively. The results can be explained if GSK-3 recognizes the sequence -SXXXX(X)P. Phosphorylation of site 5 by casein kinase II creates this recognition site. Thereafter, each successive phosphorylation introduced by GSK-3 generates a new recognition site. The results provide a molecular basis to explain the synergistic action of casein kinase II and GSK-3 that is also observed with native glycogen synthase. In addition, this investigation emphasizes how protein recognition sites in some cellular targets may have to be formed post-translationally.

Reversible phosphorylation of regulatory proteins is a widespread mechanism for the control of cellular processes (1). A critical aspect of this control is the ability of a given protein kinase to modify only selected target proteins at specific functionally relevant amino acid residues. Even though multiple phosphorylation of target proteins is common, there is a high degree of discrimination in the recognition of the residues modified. In several instances, the importance of the local primary structure in determining kinase specificity has been established. In the well studied case of cyclic AMP-dependent protein kinase, the best recognition sites have the sequence -Arg-Arg-X-Ser- (2). For the enzyme casein kinase II, acidic residues COOH-terminal to the modified residue are of special importance (3). It is also acknowledged that such sequence determinants, though required, are not necessarily sufficient for effective phosphorylation and, in native proteins, higher order structures can influence recognition (4). The necessary amino acid residues must be accessible, presumably at the surface of the protein, in an appropriate configuration for interaction with the protein kinase.

Ordinarily, a protein kinase recognition site should exist once the protein substrate has been synthesized. In this investigation, we explored a somewhat different type of protein kinase action in which the generation of the substrate recognition site required a prior phosphorylation reaction. This work addresses synergistic phosphorylation involving two distinct protein kinases (casein kinase II and glycogen synthase kinase 3) as has been observed with several protein substrates including glycogen synthase (5, 6), phosphatase inhibitor 2 (7), and the regulatory (RII) subunit of cyclic AMP-dependent protein kinase (8).

The original observation, made independently in this laboratory (6) and that of Cohen (5), was that phosphorylation of native glycogen synthase by casein kinase II, itself without effect on enzyme activity, potentiated the ability of GSK-3* to phosphorylate and inactivate glycogen synthase. This synergism is associated with phosphorylation of a particular proline- and serine-rich segment of the COOH-terminal CNBr fragment (CB-2) of rabbit muscle glycogen synthase that is believed to be critically involved in the hormonal control of this enzyme (9–11). Contained in this region of glycogen synthase are several known phosphorylation sites (Fig. 1). Casein kinase II modifies a single serine, designated as site 5 (12). GSK-3 was reported to phosphorylate sites 3a, 3b, and 3c (13). Another site, site 4, was identified as a weak recognition site for cyclic AMP-dependent protein kinase under conditions that promoted high levels of phosphorylation (14). Woodgett and Cohen (15), using a chymotryptic fragment of glycogen synthase, have shown that the synergistic phosphorylation by casein kinase II and GSK-3 involves intramolecular interactions within this region of glycogen synthase. In this report, we show that a 27-residue synthetic peptide based on the Pro/Ser sequence of glycogen synthase undergoes similar synergistic phosphorylation. A molecular basis for substrate recognition and a mechanism for multisite phosphorylation by GSK-3 are proposed.

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1 The abbreviations used are: GSK-3, glycogen synthase kinase 3; PI, isoelectric point; CNBr, cyanogen bromide; HPLC, high performance liquid chromatography.
Glycogen Synthase Kinase 3 Recognition Site

EXPERIMENTAL PROCEDURES

Synthesis and Characterization of Peptide—The 27-residue peptide amide was synthesized by the solid phase method in a Beckman 990 automated apparatus, using a methyl benzhydrolamine-polystyrene support, 0.2 mEq/g. Aspartic acid and glutamic acid side chains were protected by cyclohexyl esters, histidine and arginine by tosyl, and automated apparatus, using a methyl amide was synthesized by the solid phase method in a Beckman 990 support, 0.2 mEq/g. Aspartic acid and glutamic acid side chains were homogeneous by analytical HPLC were combined and lyophilized. Satisfactory amino acid analysis was performed using o-phthalaldialdehyde precolumn derivatization of the peptide hydrolyzates and HPLC analysis as previously described (17).

Phosphorylation Reactions—Phosphorylation reactions were performed with a slight modification of published conditions (7). The reaction mixture contained 42 mm Tris-HCl, pH 7.5; 1.4 mM [γ-32P]ATP (3000 cpm/pmol); 8.6 mM Mg(CH3COO)2; 15 μg/ml casein kinase II and/or 3 μg/ml GK-3; and substrates (peptide or glycogen synthase) at the indicated concentrations. Whenever a sequential phosphorylation was performed, the concentrations of buffer and cofactors were readjusted to the original values described above. The casein kinase II reaction was stopped by adding heparin to a final concentration of 6 μg/ml. The GSK-3 reaction was stopped by adding EIPA to a final concentration of 25 mM. Glycogen synthase phosphorylation was quantitated as previously described (Assay 1 of Ref. 18). Where initial rates were measured, peptide or glycogen synthase phosphorylation was determined at several time points and the rate estimated from the initial linear portion of the time course.

Isoelectric Focusing—Analytical isoelectric focusing of the different phosphopeptide forms was performed in thin layer (0.5 mm) polyacrylamide gels bonded to plastic support films (GelBond PAG) in an LKB 2117 Multipher system. The gel contained 4.8% (w/v) acrylamide, 0.15% (w/v) bisacrylamide, 10% (v/v) ureaprlme, 0.15% (w/v) Ampholine pH 2.5-4, 0.06% (w/v) Ampholine pH 3.5-4, 0.35% (w/v) Ampholine, pH 4-6, and 7 μl urea (19). The catholyte and anolyte were 1 M NaOH and 1 M H2PO4, respectively. The samples (5-10 μl) were loaded in preformed wells on the gel. All experiments were performed at 10 watts constant power and 15 °C. The voltage at equilibrium was usually 1100 V. The pI values of the phosphopeptides were determined by measuring the pH range of the gel; a strip of gel was cut into 0.5-cm pieces, the gel pieces were soaked in 1 ml of water to dissolve the Ampholines, and the pH of the solution was measured. This combination of Ampholines and electrolytes gave a linear pH range from pH 3.75 to 5.5. The pI values assigned to the different phosphopeptides were not corrected for the presence of urea (20). The gels were dried in a 60 °C oven. The labeled peptides were localized by autoradiography, and the radioactive gel pieces were excised and dissolved overnight in 30% hydrogen peroxide at 60 °C in glass counting vials. The liquid scintillation mixture for aqueous samples (Ready-Solv, Beckman) was added and the radioactivity quantitated in a liquid scintillation counter.

HPLC Purification of Phosphopeptides—Phosphopeptides were purified by reverse phase HPLC on a Synchropak RP-P C18 column with a Beckman gradient HPLC apparatus equipped with a flow-through radioactive monitor (Ramona D, Raytstat). Solvent A was 50 mM potassium phosphate, pH 2.1, and solvent B: 50 mM potassium phosphate, 60% acetonitrile (v/v). The gradient was 0-10 min, 0% B; 10-14 min, 0-3% B; 14-70 min, 3-24% B; 70-80 min, 24-60% B; 80-95 min, 60% B. The collected fractions were diluted 3-fold and loaded on a SEF-PAX C18 cartridge. The cartridge was washed with water to remove the nonphosphate and the peptide eluted with a buffer containing 0.25 M ammonium bicarbonate and 30% acetonitrile. This buffer was subsequently removed in a centrifugal evaporator (Speedvac).

Trypsin Digestions—The purified phosphopeptide was redissolved in water. Reduced-carbodiimide acetic acid were used to maintain a basic pH: trypsin was added to give a trypsin ratio of 10:1, w/w, with a final concentration of trypsin of 0.02 mg/ml. The tryptic peptides were purified by HPLC at previously described.

Amino Acid Sequencing—Amino acid sequence determination was performed using a gas phase Sequencer (Applied Biosystems model 470A, programs 02hnbg and 02nrun). The phenylthiohydantoin derivatives were analyzed using a modification (21) of the method of Hunskeller et al. (22).

Identification of Phosphorylation Sites—The location of phosphorylated residues was determined by a modification of the procedure of Wang et al. (22, 23). A piece of the glass fiber sample filter was removed from the amino acid Sequencer at predetermined cycles to analyze the extent of inorganic phosphate release up to that cycle as follows: for monophospho-site(4+5) peptide, at cycles 1, 2, 3, 6, 7, and 13; for diphospho-site(4+5) peptide, at cycles 1, 2, 3, 5, and 13; for monophospho-site(3+4+5) peptide, at cycles 2, 3, 6, 8, 10, and 12; and for triphospho-site 3 peptide, at cycles 5, 6, 10, and 12. The inorganic phosphate and remaining shortened phosphopeptide were extracted three times from the filter by sonication in 200 μl of 50% formic acid. The recovery of 32P from the filter was greater than 95%. Inorganic phosphates was separated from the shortened phosphopeptide by thin layer electrophoresis at pH 3.5 for 1 h at 500 V. The solvent system used was 0.5% pyridine and 5% glacial acetic acid (v/v). After localization of the peptide and inorganic phosphate by autoradiography, the spots were scraped and radioactivity quantitated by liquid scintillation counting.

Other Methods—Glycogen synthase was purified from rabbit skeletal muscle by the method of Takeda et al. (24) as modified by Ahmad et al. (25). Casein kinase II (26, 27) and GK-3 (28) were purified by published procedures. The concentration of [32P]ATP was determined spectrophotometrically.

RESULTS

Phosphorylation of Site(3+4+5) Peptide by Casein Kinase II and GK-3—A 27-residue peptide, Ac-PRPASVPPSPSRHSSPQHSEDDEEP-amide, was synthesized to have the amino acid sequence of the Pro/Ser-rich region of rabbit muscle glycogen synthase (see Fig. 1) and was used as a protein kinase substrate. The peptide contains sites 3a, 3b, 3c, 4, and 5, as well as a series of 5 acidic residues COOH-terminal to site 5; it is referred to as the site(3+4+5) peptide. In initial experiments (Fig. 2), it was established that the peptide could be phosphorylated by casein kinase II (lane 2) but not by GK-3 (lane 3). Analysis by isoelectric focusing revealed a single phosphorylated species (pI = 5.3) produced by the action of casein kinase II. This phosphorylated peptide was a substrate for GK-3 and was converted into a series of four other discrete species (lane 4). The time course of the reaction (discussed below) indicated the successive appearance of peptides with progressively lower isoelectric points. The most obvious explanation, given that GK-3 modifies multiple serine residues in native glycogen synthase, is that the reaction products correspond to a series of multiply phosphorylated forms of the site(3+4+5) peptide. Much of the work described below was aimed at further characterization of these reaction products.

At intermediate times of incubation with GK-3 (e.g. 50 min with the conditions used), there was close to quantitative conversion of the starting substrate, pI 5.3, to the species of pI 4.4. Only at longer times was the latter species appreciably converted to lower pI forms. From quantitation of the 32P associated with the species separated by isoelectric focusing, it was determined that the pI 4.4 species had approximately three times the 32P content as the starting pI 5.3 species.

site(3+4+5) peptide

\[ \text{Ac-PRPASVPPSPSRHSSPQHSEDDEEP-amide} \]

\[ \text{trypsin} \]

site 3 peptide

site(4+5) peptide

\[ \text{Ac-PRPASVPPSPS} \]

\[ \text{HRHSSPQHSEDDEEP-amide} \]

Fig. 1. Amino acid sequence of the site(3+4+5) synthetic peptide. The synthetic peptide had the amino acid sequence of the Pro/Ser-rich region of glycogen synthase and contained sites 3a, 3b, 3c, 4, and 5. The tryptic digestion of this peptide resulted in three fragments, a dipeptide, the site 3 peptide, and the site(4+5) peptide.
Phosphorylated to 0.6-0.7 mol to a final stoichiometry of 2.2-2.5 mol peptide phosphorylated sequentially by casein kinase I1 and GSK-3 peptide was an excellent substrate for GSK-3, comparable to phosphorylated with casein kinase I1. Kinetic analysis of this phosphorylation of glycogen synthase which had been previously phosphorylated with casein kinase I1, inhibition by heparin, followed by incubation with GSK-3. Lane 2 corresponds to peptide incubated with casein kinase II only, phosphorylated to 0.6-0.7 mol of P/mol of substrate; lane 3 corresponds to peptide incubated with GSK-3 alone; and lane 4 corresponds to peptide phosphorylated sequentially by casein kinase II and GSK-3 to a final stoichiometry of 2.2-2.5 mol of P/mol. Shown next to each phosphopeptide band is its isoelectric point.

Since phosphorylation has to involve discrete stages, we can thus assign the PI 4.4 species as triphospho-site(3+4+5) peptide, and we propose that the peptide with intermediate PI, 4.7, is diphospho-site(3+4+5) peptide. The results described below are also consistent with the species of PI 4.4 being tetraphospho-site(3+4+5) peptide and that with PI 3.8 being pentaphospho-site(3+4+5) peptide.

Comparison of the initial rate of phosphorylation of the peptide with that for purified rabbit skeletal muscle glycogen synthase (Fig. 3) indicated that the peptide was a relatively good substrate for both casein kinase II and GSK-3. At low concentrations (<12 μM), the rate of phosphorylation of the peptide by casein kinase II (Fig. 3A) was approximately half the rate of phosphorylation of glycogen synthase. At higher concentrations (>23 μM), the peptide was phosphorylated as efficiently as the enzyme. Casein kinase II had an apparent \( K_m \) of 23 μM for the peptide. High concentrations of glycogen synthase in the assay inhibited phosphorylation whereas this phenomenon was not observed with the peptide. The synthetic monophosphopeptide was phosphorylated very efficiently by GSK-3 (Fig. 3B). The rate of incorporation of phosphate into this peptide was similar to the rate of phosphorylation of glycogen synthase which had been previously phosphorylated with casein kinase II. Kinetic analysis of this reaction is complex, but these data show that the synthetic peptide was an excellent substrate for GSK-3, comparable to the native enzyme, with an apparent \( K_m \) of 2 μM.

Tryptic Mapping of Phosphorylation Sites—Peptide phosphorylated by casein kinase II ran as a single discrete species on reverse phase HPLC (T44; Fig. 4A). After further phosphorylation of the peptide by GSK-3, HPLC analysis indicated that the phosphopeptide peak was broadened (Fig. 4C). This broadening correlated with the generation of multiple phosphorylated species as judged by isoelectric focusing (Fig. 2). Tryptic cleavage of the site(3+4+5) peptide should yield two smaller peptides, designated site 3 peptide and site(4+5) peptide (Fig. 1). Analysis of the tryptic digest of peptide phosphorylated by casein kinase II and purified by HPLC revealed a single \( ^{32}P \)-labeled species both by HPLC (T26, eluting at 26 min, Fig. 4B) and by IEF (pI = 4.6, lane 1 in Fig. 5). The retention time of 26 min for this peptide is consistent with the behavior of the corresponding tryptic peptide obtained from glycogen synthase phosphorylated in vitro by casein kinase II (not shown). The identity of this species as the site(4+5) peptide was further confirmed by amino acid sequencing. Similar HPLC analysis of the tryptic digest of peptide phosphorylated sequentially by casein kinase II and GSK-3 resolved three tryptic phosphopeptides (Fig. 4D), T26 as in the previous analysis, and two others, T34 and T38, with retention times of 34-35 min. The identities of these tryptic peptides were established by amino acid sequence analysis (data not shown). The T26 species was the more hydrophilic site(4+5) tryptic peptide, as expected. The \( ^{32}P \) content of this peptide was doubled by the action of GSK-3 (compare Fig. 4, B and D). Thus, unexpectedly, GSK-3 also

\[^2\text{Kinetic analysis is particularly difficult when the substrate is native glycogen synthase since it is not known with certainty what proportion of the glycogen synthase is available for GS}\text{K-3 phosphorylation. Thus, the glycogen synthase concentration was based on the moles of subunit phosphorylated by casein kinase II. In addition, since there are multiple reaction products (with both glycogen synthase and peptide as substrates) the observed initial rates may be a complex function of the rates for individual steps.}^2\]
reverse phase HPLC analysis of phosphopeptides. In A, an analysis of monophospho-site(3+4+5) peptide generated by casein kinase II (CK II) is shown. An analysis of a tryptic digest of this peptide is shown in B. In C, an analysis of multiply phosphorylated site(3+4+5) peptide generated by sequential phosphorylation by casein kinase II and GSK-3 is shown. Analysis of the tryptic digestion of the multiphospho-site(3+4+5) peptide forms is shown in D. Equal amounts of peptide were loaded in B and D. The phosphopeptides were applied to the Synchropak C18 column, eluted with a 50 mM potassium phosphate/acetonitrile gradient and analyzed by an online radioactivity monitor as described under "Experimental Procedures."

Isoelectric focusing analysis of tryptic phosphopeptides. The tryptic phosphopeptides were purified by HPLC as shown in Fig. 4 and analyzed by isoelectric focusing as described under "Experimental Procedures." T26 (2000 cpm) produced by casein kinase II was run in lane 1. Phosphopeptides generated by the combined action of CK-II and GSK-3 were run as follows: T26 (900 cpm) in lane 5; T34 (4000 cpm) in lanes 6–8; and T38 (4000 cpm) in lane 9. The numbers in parentheses are the isoelectric points for each peptide. The samples that ran in lanes 2, 3, and 4 are not pertinent.

Labeled the site(4+5) peptide. Isoelectric focusing of the purified T26 peptides (lanes 1 and 5 in Fig. 5) showed that the site(4+5) peptide generated by GSK-3 had a more acidic isoelectric point (pI = 4.3) compared to the T26 (pI = 4.6) generated by casein kinase II, consistent with the presence of an additional phosphate. The quantitative conversion to the lower pI species and the doubling of the 32P content in this peptide indicated that a diphospho-site(4+5) peptide had been generated by GSK-3.

The T34 and T38 phosphopeptides both corresponded to the site 3 peptide based on the amino acid sequence analysis. Isoelectric focusing showed that the reverse phase HPLC achieved partial separation of peptides with different levels of phosphorylation.3 The T38 peptide had a pI = 5.2 (lane 9 of Fig. 5), and we infer that this corresponds to a monophospho-species. The T34 peptide had a pI = 2.5 (lane 6 in Fig. 5) and is inferred to be a triphospho-site 3 peptide (see below).

Site Specificity of Phosphorylation—Automatic Edman degradation of the tryptic peptides was used to analyze the sites modified by the protein kinases. In the analysis of the site(4+5) peptide generated by tryptic digestion of the phosphopeptide produced by casein kinase II (Table I), there was no phosphate released until cycle seven, at which significant release of phosphate occurred. The rest of the phosphate release occurred over the next five cycles as a result of carry-over. There was no evidence for any phosphate release at cycle 2 or cycle 3, the cycles which correspond to the other serines in the site(4+5) peptide. Thus, casein kinase II was specific for site 5 phosphorylation in the synthetic peptide. Similar analysis of the diphospho-site(4+5) peptide generated after GSK-3 action (Table I) indicated no phosphate release at cycle 2 which corresponded to a serine residue. The first significant phosphate release was at the next cycle, the serine corresponding to site 4. There was significant carry-over, recovered in the filter for the next three cycles. The subsequent 7 cycles yielded phosphate that was primarily associated with site 5. Site 4 was the predominant phosphorylation site recognized by GSK-3 in the site(4+5) peptide.

From the results of isoelectric focusing, we had inferred that the T34 peptide was a triphospho-peptide, while the T38 peptide was primarily a monophospho-peptide. During sequencing of the triphospho-species, T34 (Table II), phosphate release was observed in correspondence to cleavage of serines at sites 3a, 3b, and 3c. At cycles 9+10, there was significant phosphate release that could be explained either as carry-over from site 3b or as some phosphorylation of the serine at cycle 9. However, the amount released is too low to correspond to stoichiometrical phosphorylation of the serine at cycle 9, and we conclude that site 3b was the major site of phosphorylation. On this basis, then, the phosphate recoveries from sites 3a, 3b, and 3c were in the proportion 1:0.05:0.3. A perfect analysis of the triphospho-peptide would result in a 1:1:1 distribution of phosphate. The deviation from this ratio reflects incomplete coupling and cleavage during the Edman degradations, a particular problem in this proline-rich sequence (29). This was corroborated by a high level of carry-over of phenylthio-

3 The ability of reverse phase HPLC to resolve different phosphorylation states of the same peptide, in our experience, depends greatly on the peptide involved as well as the chromatographic conditions (columns, solvents, gradient). The results show clearly that, under our conditions, mono- and diphospho-site(4+5) peptide had indistinguishable elution times whereas phosphorylation had a much greater effect on the retention of the site 3 peptide.
TABLE II

Location of phosphorylated residues in the site 3 phosphopeptide

| Cycles | Site   | T34 | T38 |
|--------|--------|-----|-----|
| 1-6    | 3a     | 455 | 54  |
| 7-8    | 3b     | 135 | 16  |
| 9-10   | 3c     | 80  | 16* |
| 11-12  | 3c     | 130 | 40  |

* Values were corrected for the sample removed from the Sequencer during the run.
+ Ascribed to carry over from site 3b.

Fig. 6. Time course of phosphorylation of the synthetic peptide by GSK-3. The site(3+4+5) peptide was phosphorylated by casein kinase II as described in Fig. 2 to generate the monophospho-site(3+4+5) peptide which served as initial substrate for GSK-3. GSK-3 (3 μg/ml) was then added to the reaction. Aliquots (4 μl) of the GSK-3 phosphorylation reaction were taken at the indicated times and diluted 4-fold with buffer containing 25 mM EDTA. One-half volume (8 μl) of each diluted sample was exposed to trypsin (0.75 mg/ml) for 24 h at 30 °C. Aliquots (4 μl) of both trypticized and untreated samples were analyzed by isoelectric focusing and the radioactivity associated with each peptide band quantitated as described under "Experimental Procedures." In A, the appearance with time of the various phosphorylated forms of the site(3+4+5) peptide (no trypsin added) is indicated as follows: 1, monophospho-site(3+4+5) peptide; O, diphospho-site(3+4+5) peptide; △, triphospho-site(3+4+5) peptide; Δ, tetraphospho-site(3+4+5) peptide; and ■, pentaphospho-site(3+4+5) peptide. In B, the appearance of the phospho-species generated from the tryptic digest (site 3 and site(4+5) phosphopeptides) is indicated as follows: 1, monophospho-site(4+5) peptide; A, diphospho-site(4+5) peptide; C, monophospho-site 3 peptide; O, diphospho-site 3 peptide. Note that the monophospho-site 3 peptide was not quantitated since it was not separated from the ATP.

DISCUSSION

The results of this investigation demonstrate that a synthetic 27-amino acid peptide with the sequence of the Pro/Ser region of rabbit muscle glycogen synthase (residues 27-52 of CB 2 (11)) has the minimal structural requirements for specific recognition of site 5 by casein kinase II as well as for synergistic phosphorylation by GSK-3 and casein kinase II. This work provides a molecular basis for the synergism and confirms that the interactions among phosphorylation sites are intramolecular in nature. Upon incorporation of phosphate at site 5 by casein kinase II, phosphorylation of sites 4, 3a, 3b, 3c, and 5. We hypothesize that the tetraphospho-peptide is likely to lack phosphate at site 3a, but this has not been proven. It is notable that the tetraphospho-peptide and dihydrophospho-peptide do not accumulate in high amounts (see Fig. 2). From the above results, we infer that GSK-3 action involves initial phosphorylation of site 4 and then site 3c. Subsequent phosphorylation leads to modification of sites 3b and 3a, probably in that order.

Note that if phosphorylation occurred randomly, tryptic digestion of the triphospho-site(3+4+5) peptide (30-min time point) should have yielded many more species: a monophospho-site 3a, a monophospho-site 3b, a monophospho-site(4+5) peptide, and various forms of a diphospho-site 3 peptide, in addition to the monophospho-site 3c peptide and diphospho-site(4+5) peptide. Also, the amount of these last two peptides would have been significantly less than observed.
of phosphate into sites 3b and 3a occurred only later, after a lag period. On the basis of the kinetic and sequencing data presented, we propose a mechanism in which GSK-3 catalyzes the sequential phosphorylation of sites 4, 3c, 3b, and 3a. With each successive phosphorylation, a new recognition site is generated.

![Fig. 7. Sequential model for substrate phosphorylation by GSK-3. In this model, a recognition site -SXXXS(P)- for GSK-3 is initially generated by phosphorylation of site 5 by casein kinase II (CK II). Afterward, GSK-3 introduces phosphates sequentially at sites 4, 3c, 3b, and 3a. With each successive phosphorylation, a new recognition site is generated.](image)

Our findings, however, suggest that the introduction of phosphate into site 5 is necessary and sufficient for recognition and efficient phosphorylation by GSK-3. Furthermore, phosphorylation of site 5 has resulted in initial recognition by GSK-3 specifically of site 4. Although this phosphopeptide contains additional serines which could be phosphorylated by GSK-3, our sequencing data suggest that phosphorylation is restricted to those serines which form part of the following sequence: XXXSXXXSXXXSXXXSXXXSXXXSXXXXXX. The sequential modification as well as the periodicity of the serines phosphorylated by GSK-3 can be explained if the recognition site for GSK-3 is a serine in the following pattern: -SXXXS(P)-. Thus, with each successive phosphorylation, a new recognition site is generated in the substrate as long as there is the indicated spacing of serines in the amino acid sequence. This hypothesis, in fact, would predict an obligate sequentiality of phosphorylation by GSK-3. Our results further indicate that the rate of phosphorylation of site 3c is significantly faster than the rate of phosphorylation of site 4, and phosphorylation of site 3a faster than that of site 3b, since neither diphospho- nor tetraphospho-peptide tend to accumulate (30). Also, the overall rate of phosphorylation of the NH$_2$-terminal pair of sites (3a and 3b) is slower than the rate of phosphorylation of the COOH-terminal pair (3c and 4). For each pair of sites, there appears to be a mechanism, by which introduction of the second phosphate by GSK-3 is faster, i.e., $k_1 < k_2 < k_3 < k_4$ in the model of Fig. 7. Further study is needed to refine the kinetic details of this model.

It is interesting to speculate about the structural features of the unusual amino acid sequence of the Pro/Ser-rich region that determine GSK-3 recognition. The periodicity of the target serines suggests some common and repeated structural element involved in interactions with the protein kinase. Indeed, it is not unreasonable to expect that the catalytic site of GSK-3 dictates a fixed distance between the hydroxyl of the serine to be modified and the COOH-terminal phosphoserine that is inferred to be important for substrate binding. The exact nature of the structural unit is a matter of speculation, but it is interesting that the relevant sequence of the Pro/Ser region contains a high proportion of residues frequently found in $\beta$-turns (31). One possibility is that the repeated structural element is based on some form of reverse turn. Reverse turns are generally characterized in terms of 4 amino acid residues, are located at the surface of proteins, and have been implicated as frequent sites of covalent modification (30). Though the peptide in solution may or may not favor such a structure, it is possible that a more ordered structure is stabilized upon binding to GSK-3. Obviously, this question requires further exploration.

The results of this study raise the interesting possibility that the physiological substrates for GSK-3 must be generated post-translationally, through the action of a second protein kinase. In the present example, this second enzyme is casein kinase II, but there is no a priori reason why other protein kinases might not serve in this ancillary role. As far as is known, substrate recognition by other protein kinases does not require prior modification of amino acids coded by the gene for the substrate. In this regard, GSK-3 appears unique thus far. However, there may well be other systems in which post-translational events are a prerequisite to protein kinase action, and the phenomenon may be more widespread.

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