Activation of an S6/H4 Kinase (PAK 65) from Human Placenta by Intramolecular and Intermolecular Autophosphorylation*

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The S6/H4 kinase purified from human placenta catalyzes phosphorylation of the S6 ribosomal protein, histone H4, and myelin basic protein. In vitro activation of the p60 S6/H4 kinase requires removal of an autoinhibitory domain by mild trypsin digestion and autophosphorylation of the catalytic domain (p40 S6/H4 kinase). The two autophosphorylation/autoactivation sites contain the sequences SSMGVTPY (site 1) and SVIDPVPAPVGDSHDGAAK (site 2). These sequences identify S6/H4 kinase as the rac-activated PAK65 (Martin, G. A., Bollag, G., McCormick, F. and Abo, A. (1995) EMBO J. 14, 1971-1978). Site 1 phosphorylation is most rapid, but activation does not occur until site 2 is autophosphorylated. The site 1 phosphorylation occurs by an intramolecular mechanism whereas site 2 autophosphorylation occurs by an intermolecular mechanism. A model is proposed in which phosphorylation of sites 1 and 2 occurs sequentially. The model proposes that trypsin treatment of the inactive holoenzyme removes an inhibitory rac-binding domain which blocks MgATP access to the catalytic site. The pseudosubstrate domain at site 1 is autophosphorylated and subsequent bimolecular autophosphorylation at site 2 fully opens the catalytic site. Phosphorylation by a regulatory protein kinase may occur at site 2 in vivo.

Cellular activation of serine/threonine protein kinases occurs as a result of conformational changes induced by binding of small ligands, such as cyclic AMP or calcium, binding of regulatory proteins, such as Ras, Rho, or cyclin, or phosphorylation by upstream protein kinases (reviewed in Refs. 1, 2). Although the events which trigger activation of serine/threonine protein kinases are quite variable, a common molecular mechanism by which the enzyme inhibition is relieved has been proposed (3). Intrastereic activation, the conformational changes at the active site which result in increased accessibility to MgATP, protein substrate, or both, has been proposed as a common feature of protein kinase activation. Data obtained by x-ray crystallography demonstrate that in the inactive enzyme conformation, auto-inhibitory or pseudosubstrate domains of the protein kinase bind at the protein substrate-binding site and restrict access of substrates to the catalytic center (4-8). As demonstrated in studies of both the cyclic AMP-dependent protein kinase and the twitchin kinase, the autoinhibitory or pseudosubstrate domain competes effectively with exogenous protein substrates for enzyme binding and may also block binding of MgATP in the catalytic cleft (4, 5, 9). Displacement of the autoinhibitory domain from the active site occurs when conformational changes initiated by the regulatory ligand binding decrease the binding affinity of the pseudosubstrate domain.

In some protein kinases, phosphorylation of the autoinhibitory domain may be required for displacement from the catalytic center and subsequent activation. Most protein kinases catalyze autophosphorylation although for many serine/threonine protein kinases, this self-modification does not alter catalytic function. However, recently several protein kinases which require autophosphorylation for activation have been reported (10-14). In this laboratory we have demonstrated that the catalytic domain of an S6/H4 kinase from human placenta undergoes rapid autophosphorylation concomitant with activation, and inhibition of autophosphorylation is correlated with inhibition of phosphotransferase activity (10). Similar results have been observed in studies of a pig liver glycogen synthase kinase (11), a bovine kidney phosphatase 2A kinase (12), an interferon-induced, RNA-dependent protein kinase (13), and a rac-activated brain enzyme which has sequence identity to the STE20 yeast gene product involved in pheromone signaling (14). The diversity of these enzymes with respect to physical and enzymatic properties suggests that a requirement for autophosphorylation may be a widespread occurrence within the serine/threonine protein kinase family.

In this report we have investigated the mechanism by which autophosphorylation of the S6/H4 kinase occurs. By analogy to the pseudosubstrate domain hypothesis, we propose that a regulatory domain in the S6/H4 kinase binds at the protein substrate-binding site. However, the data suggest that in the S6/H4 kinase, this domain contains a serine which must become phosphorylated in order for it to be displaced from the catalytic cleft and permit binding of exogenous substrates. In addition, maximum activation of the S6/H4 kinase appears to require a second phosphorylation on the surface of the enzyme.

EXPERIMENTAL PROCEDURES

Purification of S6/H4 Kinase and the Catalytic Domain of S6/H4 Kinase (p40 S6/H4 Kinase)—The S6/H4 kinase was purified from human placenta as described previously (10). The enzyme was obtained as an inactive protein kinase with M, 60,000. This holoenzyme autophosphorylated at a very slow rate (10). The catalytic domain of the holoen-

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1 The abbreviations used are: S6/H4 kinase, inactive holoenzyme; p40 S6/H4 kinase, catalytic domain of the S6/H4 kinase; TLE, thin layer electrophoresis; TLC, thin layer chromatography; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; dpm, disintegrations/minute.
zyme, designated p40 S6/H4 kinase, was generated from the holoenzyme as described previously (10) and purified on a Mono Q 5/5 column equilibrated with 20 mM Tris-Cl, pH 7.5, containing 2 mM EDTA, 10 mM 2-mercaptoethanol, 2 μM leupeptin, 100 μM phenylmethylsulfonyl fluoride, and 10% (v/v) glycerol (Buffer A). Proteins were eluted with a 50-ml linear gradient of 0-0.7 M NaCl in Buffer A. The purified p40 S6/H4 kinase was used in rapid autophosphorylation and activation. The enzyme was stable at -80 °C for at least 1 year.

Assay of S6/H4 Kinase Activity—S6/H4 kinase activity was quantitated by measuring the amount of [32P]P incorporated into protein or peptide substrates. All reactions were carried out at 30 °C. Aliquots of inactive S6/H4 kinase (10 μL) were subjected to limited proteolysis with 5 μg of diphenylcarbamyl chloride-treated trypsin (20). Trypsin stock solutions were prepared in 40 mM Tris-Cl, pH 8, 4 mM 2-mercaptoethanol, and 4 mg/ml bovine serum albumin. The reaction was stopped by the addition of 5 μl of soybean trypsin inhibitor at a final concentration of 6 μg/ml in 10 mM Tris-Cl, pH 7.5. p40 S6/H4 kinase did not require trypsin treatment for activation; however, 1 mg/ml bovine serum albumin was used in the assay to stabilize the protein. Both S6/H4 kinase and p40 S6/H4 kinase required incubation with MgATP to achieve full activation. The reaction mixture (20 μl) was incubated with 12 mM magnesium acetate and 125 μM [γ-32P]ATP in 0.04 mM Tris-Cl, pH 7.5. For assays with protein or peptide substrates, the ATP-specific activity was 100–300 dpm/pmol. For autophosphorylation studies, the ATP-specific activity was 2,000–20,000 dpm/pmol. Maximum autophosphorylation was observed after 10 min of incubation with MgATP. The time intervals used to investigate phenomena correlating to partial activation are noted in the individual experiments. Following activation, 10 μl of protein or peptide substrate was added for 10 min. The peptide substrate S6–21 (15) was added at a final concentration of 190 μM; the protein substrate Histone 4 was added to a final concentration of 1 mg/ml. Incubation with substrate was carried out for 10 min after which the reaction was stopped by pipetting 70% of the reaction mixture onto P81 paper for S6–21 assays (16) or ET31 paper for H4 assays (17). Papers were immediately immersed in ice-cold 3% acetic acid or 10% trichloroacetic acid, respectively, and washed as described previously (16, 17). Phosphate incorporation was measured by liquid scintillation counting of the dried papers.

For electrophoresis studies, the reaction was stopped after the MgATP incubation by the addition of SDS sample buffer, and the reactions products were analyzed by SDS-PAGE (18). The resolved proteins were transferred to nitrocellulose membranes in Towbin buffer (19) and the radiolabeled bands detected by autoradiography. Radioactivity was quantitated by excising the bands from the membrane, and counting the [32P]P incorporation by the Cerenkov method or in 10 ml of toluene-based scintillation fluid.

Tryptic Digestion of S6/H4 Kinase—S6/H4 kinase samples were incubated with Mg[γ-32P]ATP, 2,500–20,000 dpm/pmol for designated time intervals. The autophosphorylation reaction was stopped with SDS-PAGE sample buffer, and the proteins were analyzed by SDS-PAGE (18). The resolved proteins were transferred to nitrocellulose in Towbin buffer (19) and the autophosphorylated proteins were detected by autoradiography. The S6/H4 kinase bands of interest (p60 holoenzyme or p40 catalytic domain) were excised and digested with trypsin according to the procedure of Luo et al. (20). Optimal digestion parameters were found to be 10 μg of diphenylcarbamyl chloride-treated trypsin for 2 h in 150–200 μl of 0.05 M ammonium bicarbonate, pH 7.2, at 37 °C, followed by addition of 10 μg of trypsin and incubation for an additional 2 h. The reactions were stopped by the addition of approximately 1 ml of water. The sample was microfuged to remove the membrane pieces, and the supernatant was vacuum centrifuged to dryness. In some experiments trypsin peptides were subjected to oxidation by hydrogen peroxide in formic acid (21) prior to analysis.

For sequencing experiments, S6/H4 kinase (1 mg/ml) was incubated with 5% (w/w) diphenylcarbamyl chloride-treated trypsin in 0.1 M ammonium bicarbonate, pH 7.8, for 24 h at 37 °C. Half of the trypsin was present for the first 12 incubations; the remaining trypsin was added after 12 and 18 h of incubation. Phosphoamino Acid Analysis—Purified [32P]P40 S6/H4 kinase was hydrolyzed with 6 N HCl in vacuo at 110°C for 1 h. The sample was analyzed by two-dimensional TLC on microcrystalline cellulose at pH 1.9 for 2 h at 800 V and pH 3.5 at 1 h at 800 V. Standard phosphoserine, phosphothreonine, and phosphoarginine (7 μg each) were run in each dimension. Standards were visualized by spraying plates with 10% triethylamine in dichloromethane followed by 0.6 mg/ml fluorescamine to develop a fluorescent image. Fluorescent spots were detected with 254 nm ultraviolet light. Radiolabeled phosphoamino acids were detected by autoradiography.

Gel Electrophoresis—Samples were applied to 4% polyacrylamide isofocusing tube gels cast in the presence of 2.0% Triton X-100, 2.4% Bio-Lyte 5/7 and 0.6% Bio-Lyte 3/10 (Bio-Rad), and 9.5 M urea. Sample were focused between 100 mM NaOH and 10 mM H3PO4 for 6 h at 750 V with no pre-electrophoresis. After focusing, gels were extruded and applied to a 12% polyacrylamide slab gel. SDS-PAGE was carried out according to Laemmli (18).

Peptide Mapping of Trypsin-digested S6/H4 Kinase by Thin Layer Electrophoresis and Thin Layer Chromatography—Peptide mapping was carried out according to the procedure of Boyle et al. (21). Trypsin-digested samples (2 μl) were applied in 0.5-μl aliquots onto 160-μm microcrystalline cellulose plates. For the first dimension analysis, plates were electrophoresed at 1000 V for 1 h at 4 °C in thin layer electrophoresis buffer containing 2% formic acid and 8% acetic acid, pH 1.9. Plates were air dried and chromatographed in 37.5% n-butanol, 25% pyridine, and 7.5% acetic acid until the mobile phase was approximatley 2.5 cm from the top of the plate. Phosphopeptides were detected by autoradiography of the dried plates. In some experiments several samples were analyzed on a single plate by thin layer electrophoresis only.

Purification and Sequence Analysis of S6/H4 Kinase Phosphopeptides—Peptides obtained from trypsin digestion of soluble p40 [32P]S6/H4 kinase were purified by chromatography on a Mono S HR5/5 column equilibrated with 10 mM H3PO4, pH 2, and eluted with a linear gradient of 50 ml of 10 mM H3PO4, pH 2, containing 0-6 M NaCl. Phosphopeptides were detected by liquid scintillation counting and the radioactive peaks were pooled individually, dried, and analyzed by reverse phase HPLC according to the method of Juhl and Soderling (22). For phosphopeptides 1 and 2 a symmetrical peak of radioactivity was eluted from the reverse phase column. Purified peptides were sequenced by the Baylor College of Medicine Core Facility on an Applied Biosystems model 473A sequencer using amino-terminal Edman degradation chemistry. Amounts of eluted PTH-derivatives were calculated from pre-run standards.

Materials—Human placenta for S6/H4 kinase purification was obtained from caesarean births and transported to the laboratory in ice. Tryptic type XI, soybean trypsin inhibitor type I-S, bovine serum albumin fraction V, and polyvinyl pyrrolidone 360 were purchased from Sigma. P81, ET31 papers were purchased from Whatman. Microcrystalline cellulose chromatography plates were obtained from Kodak. Histone 4 and the S6–21 peptide were prepared as described previously (15, 23).

RESULTS

Quantitation of S6/H4 Kinase Autophosphorylation Sites—Inactive S6/H4 kinase can be activated by mild trypsin digestion to yield the p40 S6/H4 kinase catalytic domain and MgATP incubation, which results in autophosphorylation. To determine the number of autophosphorylation sites in the enzyme, S6/H4 kinase was subjected to mild trypsin digestion, the reaction was stopped with trypsin inhibitor, and the mixture was incubated with Mg[γ-32P]ATP for 20 min in order to fully activate the enzyme. The reaction products were analyzed by SDS-PAGE, transferred to nitrocellulose, and the [32P]P40 S6/H4 kinase was digested with trypsin as described under “Experimental Procedures.” Four phosphopeptides were observed after TLC/TE peptide mapping of the [32P]P40 S6/H4 kinase tryptic digest (Fig. 1). All four peptides were resolved by TLE at pH 1.9, and the peptides were numbered according to migration toward the cathode. Phosphopeptide 1 was the fastest migrating and phosphopeptide 2 was the slowest migrating at pH 1.9. In the TLE analysis, phosphopeptides 1 and 3 and phosphopeptides 2 and 4 were not well resolved from each other (Fig. 1). Phosphopeptides 1 and 3 migrated almost twice
as far as phosphopeptides 2 and 4, suggesting that the latter are larger and/or more polar.

To determine that none of the observed peptides were generated by incomplete digestion with trypsin or by the occurrence of multiple oxidation states of a single peptide, [32P]p40 S6/H4 kinase immobilized on nitrocellulose was digested with trypsin for times ranging from 2 to 6 h, and the tryptic peptides were subjected to performic acid/hydrogen peroxide oxidation, as described under "Experimental Procedures." Neither of these treatments changed either the number of phosphopeptides observed on the TLE/TLC maps or the relative amounts of the peptides (data not shown).

The number of autophosphorylation sites in purified p40 S6/H4 kinase was also investigated. Peptides obtained from trypsin digestion of [32P]p40 S6/H4 kinase were analyzed by HPLC. In agreement with phosphopeptide maps obtained from in situ trypsin treatment and autophosphorylation (Fig. 1), four radioactive peptides were detected in the column eluate (data not shown). These data establish that there are at least two distinct phosphorylation sites in p40 S6/H4 kinase.

Since serine, threonine, and tyrosine residues occur in the phosphopeptide sequences, the identity of the phosphoamino acid in p40 S6/H4 kinase was determined. Only phosphoserine was detected (Fig. 3). The failure to detect phosphothreonine or phosphotyrosine and the observation that dehydroalanine was detected in the first rounds of phosphopeptides 1 and 2 sequencing supports the conclusion that the serine residues at the beginning of both peptides are the phosphorylation sites, and neither the threonine nor the tyrosine residues are autophosphorylated during the in vitro autophosphorylation and activation.

Time Course for Activation by Autophosphorylation—In previous studies which correlated p40 S6/H4 kinase activation with autophosphorylation, a slight lag time between autophosphorylation and activation was suggested (10). In agreement with previous results, a lag time for activation was consistently observed with either S6–21 (Fig. 4) or H4 (data not shown). In addition, when the activation time course was determined at several enzyme dilutions, the time required for activation increased at greater enzyme dilutions (Fig. 4). At 96 ng/μl p40 S6/H4 kinase, maximum activation was observed after 10 min of incubation with MgATP. When the enzyme concentration was decreased from 96 to 72 ng/μl, the enzyme was 88% activated in 10 min. However, when the enzyme concentration was decreased to 48 ng/μl, only 55% of the expected activation was observed after 10 min.

The lag time for activation was well correlated with a lag time for phosphorylation of site 2, but not site 1 (Fig. 5). At a concentration of p40 S6/H4 kinase which was activated less than 15% after 5 min of incubation with MgATP, phosphorylation of site 2 was barely detectable whereas phosphorylation of sites 1, 3, and 4 were nearly maximum. After 10 min of incubation with MgATP, both activation and autophosphorylation of site 2 were approximately 60% of maximum.

Mechanism of Autoactivation by Autophosphorylation—The lag time for site 2 autophosphorylation and enzyme activation suggested that autophosphorylation and activation required some degree of cooperativity and/or a bimolecular reaction. In order to correlate specific site autophosphorylation with activation, the activation kinetics and the specific site phosphorylation of [32P]p40 S6/H4 kinase was determined at several enzyme dilutions. If activation is determined by intramolecular autophosphorylation only, the enzyme activation and specific site phosphorylation should vary in direct proportion to enzyme dilution. If activation is dependent on intermolecular autophosphorylation, enzyme activation and specific site phosphorylation will not vary in direct proportion to enzyme dilution, rather a cooperative or sigmoidal relationship between these parameters should be observed.

The activation kinetics of p40 S6/H4 kinase were determined by incubating various dilutions of p40 S6/H4 kinase with MgATP for 2, 4, or 10 min prior to assay with H4 as the protein substrate (Fig. 6). At all three activation times, no direct proportionality between enzyme activity and enzyme dilution was observed. In control experiments in which activation was carried out for 10 min and the time course for the assay with H4 was monitored, linear incorporation of phosphate into H4 was observed, demonstrating that the nonlinearity of activity with respect to dilution reflected cooperativity in the activation kinetics, and not in the H4 assay (data not shown).
When the enzyme concentration was increased 2-fold from 10.3 to 20.6 ng/ml, and the enzyme was activated for 2 min with MgATP, the activity of the enzyme with the H4 substrate increased 4.2-fold from 1.9 to 7.9 pmol/min. Activation of the same enzyme concentrations for 4 min resulted in a 5.5-fold activity change from 4.8 to 26 pmol/min. Linear regression analysis of the data in Fig. 6 indicated that for all of the MgATP activation times studied, the change in enzyme activity with respect to enzyme dilution was better fit to a curve than to a straight line. For the 10-min activation time, the correlation coefficient was 0.993 for the curved line but 0.866 for a straight line. Since the data fit the curved line plot better, activation of the S6/H4 kinase by an intermolecular autophosphorylation is suggested. These data do not eliminate the possibility that an intramolecular autophosphorylation is also involved in the activation mechanism.

Mechanism of Site 1 and Site 2 Autophosphorylations—To correlate activation with phosphorylation of specific sites, both
the effect of activation time and enzyme dilution on specific site phosphorylation of p40 S6/H4 kinase was examined. The phosphorylation kinetics of site 2 were analyzed using the three lowest concentrations of enzyme studied in Fig. 6 and a 10-min activation time. The autophosphorylation kinetics of site 2 were nonlinear with respect to enzyme dilution (Fig. 7). At lower enzyme concentrations, autophosphorylation of site 2 could not be reliably determined even at 10 min of activation. When the enzyme concentration was doubled from 10 to 20 ng/ml, a 3.5-fold increase in site 2 autophosphorylation was observed. The lag time observed in site 2 autophosphorylation and the nonlinearity of site 2 autophosphorylation with enzyme dilution indicate that site 2 is probably modified by an intermolecular phosphorylation and site 2 autophosphorylation is closely correlated both temporally and mechanistically with enzyme activation.

Site 1 phosphorylation was linear with respect to enzyme dilution, suggesting that this modification occurs intramolecularly (Fig. 7). In this experiment, phosphorylation of phosphopeptide 3 was too low to determine a mechanism reliably. However, the relative amount of phosphopeptide 1 and phosphopeptide 3 label varied somewhat from experiment to experiment, and other data indicate that site 3 autophosphorylation exhibits linear kinetics (data not shown). The stoichiometry of site 4 phosphorylation was so low in most experiments that no firm conclusion regarding its kinetics could be drawn. These data suggest that site 1 and 3 autophosphorylation are intramolecular reactions, with site 1 being the major modification and site 3 a minor modification and that both of these phosphorylations occur with no demonstratable time lag.

Requirement for Site 1 and 2 Autophosphorylation in S6/H4 Kinase Activation—Site 2 autophosphorylation of p40 S6/H4 kinase is required for enzyme activation since it is the only site which exhibits autophosphorylation kinetics consistent with the activation kinetics of S6/H4 kinase. However, these data do not exclude a role for site 1 phosphorylation in the activation mechanism. To investigate if site 1 phosphorylation was essential for activation, an experiment was designed in which the S6/H4 kinase was phosphorylated only at site 2 and the activity of the enzyme determined. Holoenzyme (p60 S6/H4 kinase, 19.4 μg) was incubated for 10 min with catalytic concentrations of preactivated p40 S6/H4 kinase (1.4 μg) and MgATP and the phosphorylation and activation of the p60 protein was determined (Fig. 8, lane C). The control reactions to measure autophosphorylation of p40 S6/H4 kinase or S6/H4 kinase alone were stopped by addition of SDS-PAGE sample buffer (Fig. 8, lanes A and B). Autophosphorylation of all samples was determined by SDS-PAGE and autoradiography (Fig. 8); activity was determined by assaying with H4 histone. In addition, as a
control experiment, the total activity of p60 S6/H4 kinase was determined by trypsin and MgATP activation (10 min) prior to addition of H4.

p40 S6/H4 kinase was autophosphorylated after the 10-min incubation with MgATP, and this autophosphorylation was increased when the MgATP incubation time was extended to 20 min (Fig. 8, lanes A and C). Little autophosphorylation was detected when p60 S6/H4 kinase alone was incubated with MgATP (Fig. 8, lane B). However, when p60 S6/H4 kinase was incubated with p40 S6/H4 kinase (Fig. 8, lane C), an increase in p60 phosphorylation was detected.

When phosphopeptides from the immobilized p60 bands were analyzed by trypsin digestion and HPLC analysis, an increase in site 2 phosphorylation, but not in site 1 or 3 phosphorylation, was observed (data not shown). In control experiments where an equivalent amount of holoenzyme was activated by trypsin and MgATP, sites 1 and 2 were both actively phosphorylated. These results confirm the hypothesis that site 2 phosphorylation occurs by a bimolecular mechanism and activated p40 S6/H4 kinase can catalyze this reaction.

To correlate p60 S6/H4 kinase site 2 phosphorylation with activation, the change in activity after phosphorylation by p40 S6/H4 kinase was determined. This experiment was designed so that as little as 5% activation of the p60 S6/H4 kinase holoenzyme could be detected in the presence of the catalytic amounts of p40 S6/H4 kinase used. In control samples, p60 S6/H4 kinase (2.5 μg) and p40 S6/H4 kinase (0.5 μg) were activated fully by trypsin and MgATP incubation and assayed in the presence of H4. The combined total activity of the two enzymes was 12.8 pmol/min; the activity of p40 S6/H4 kinase alone incubated with MgATP for 10 min was 1.1 pmol transferred/min. When p60 S6/H4 kinase was incubated with MgATP alone, the observed activity was 0.22 pmol transferred/min. The observed activity of p60 S6/H4 kinase preincubated with MgATP and p40 S6/H4 kinase was 1.5 pmol transferred/min; based on the amount of phosphate incorporated into site 2, an activity of 3.5 pmol/min would be predicted if site 2 alone was sufficient for activation. Since p60 S6/H4 kinase is phosphorylated at site 2 by p40 S6/H4 kinase, and no increase in activity over that predicted for the p40 S6/H4 kinase alone was detected, these data indicate that site 2 phosphorylation of p60 S6/H4 kinase is necessary, but not sufficient to activate the holoenzyme.

The results from the previous experiment do not exclude the possibility that site 2 autophosphorylation is sufficient to activate the catalytic domain (p40 S6/H4 kinase) of the enzyme. To test if site 2 phosphorylation was sufficient to activate p40 S6/H4 kinase, p60 S6/H4 kinase was phosphorylated with p40 S6/H4 kinase as described above, and the unreacted MgATP was removed by passing the enzyme mixture over a superfine G25 column (1.5 ml) equilibrated in 0.1 M Tris-Cl, pH 7.5, containing 2 mM EDTA, 2 mM EGTA, 10 mM 2-mercaptoethanol, 2 μM leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 0.2 M NaCl. Protein-containing fractions were detected by spot Bradford analysis, and Mgγ-32P]ATP-containing fractions were detected by liquid scintillation counting. The peak protein-containing fraction which did not overlap the Mgγ-32P]ATP elution was used to test for activation of the enzyme by site 2 phosphorylation. To determine the activity of p40 S6/H4 kinase with site 2 phosphorylated, the S6/H4 kinase obtained from the Sephadex G-25 chromatography was assayed in the presence and absence of trypsin and MgATP-dependent activation.

The p60 S6/H4 kinase which was phosphorylated by p40 S6/H4 kinase at site 2 could be fully activated by subsequent incubation with trypsin and MgATP, but no activation was observed in the absence of trypsin treatment either with or without incubation with MgATP (65 pmol phosphate transferred/min versus 3.2 pmol phosphate transferred/min). When the sample was treated with trypsin, but no preincubation with MgATP, the observed activity was 29% of the total activity elicited by trypsin and MgATP treatment. This amount of activity did not differ from that observed when a comparable amount of p60 S6/H4 kinase and p40 S6/H4 kinase were treated with trypsin and assayed with H4 histone without activation by autophosphorylation. It is likely that amount of activation occurs in the assay as a result of incomplete inhibition of autophosphorylation by H4 in the presence of these relatively high concentrations of p40 S6/H4 kinase. These data indicate that site 2 phosphorylation is not sufficient to activate either p60 or p40 S6/H4 kinase and that intramolecular site 1 auto- phosphorylation is also required for enzyme activation.

Both site 1 and 2 phosphorylation appear to be required for S6/H4 kinase activation, but the data presented do not establish that both modifications occur on the same enzyme molecule. To exclude the possibility that site 1 and 2 phosphorylation were mutually exclusive, phosphorylated p40 S6/H4 kinase was analyzed by two-dimensional SDS-PAGE and isoelectric focusing (Fig. 9). Two prominent 32P-labeled enzyme forms were observed when samples incubated with...
Mg\(^{32}\text{P}\)ATP for 3 and 15 min were subjected to isoelectric focusing in a pH 5–7 polyacrylamide gel. At the shorter time, the amount of radiolabel in the two spots was approximately equal. Since the more acidic form has at least two phosphates, the data predict that approximately 66% of the \(^{32}\text{P}\)-labeled enzyme is monophosphorylated at this time point. After maximum activation by incubation for 15 min with Mg\(^{32}\text{P}\)ATP, the more acidic form, i.e. more highly phosphorylated form, predominated. These data establish that after maximum phosphorylation, the major portion of the enzyme contains two modified sites. After SDS-PAGE in the second dimension, all enzyme isoforms migrated with \(M_r\) 40,000; the retardation of migration rate observed with many phosphorylated proteins was not observed with the S6/H4 kinase.

**DISCUSSION**

Although the conserved domains of many protein kinases and the crystal structure of a small number of protein kinases suggest that this large enzyme family shares a common molecular organization in the catalytic core, the molecular mechanisms by which inhibitory domains are displaced from the catalytic cleft during activation may vary substantially. In the cyclic AMP-dependent protein kinase, the binding energy of the second messenger causes a conformational change in the regulatory subunit sufficient to break key bonds which anchor an inhibitory domain in the protein-binding groove of the catalytic site (4, 5). In contrast, the mechanism by which the inhibitory domain of twitchin, a homologue of a myosin light chain kinase, blocks catalysis appears more global (3, 6). In the case of this enzyme, the inhibitory domain sterically interferes with MgATP binding and blocks key catalytic residues in addition to binding in the protein substrate-binding groove. The activation of both MAP kinase and cdk requires phosphorylation by an exogenous protein kinase (2). Crystal structures reveal that these modifications are not in the substrate-binding groove, but rather modulate the accessibility of key residues required for high affinity MgATP binding (7, 8).

Activation of the S6/H4 kinase appears to require removal of an inhibitory domain which can be accomplished by mild trypsin digestion in vitro (10, 23). Since the trypsin-activated enzyme quickly binds MgATP and autophosphorylates, the holoenzyme appears to be inhibited as the result of a blocked MgATP substrate-binding site. Removal of the 20-kDa trypsin-sensitive regulatory domain rac-binding domain (24) is not sufficient to permit productive binding of MgATP and protein substrate at the active site. Autophosphorylation is required to remove the pseudosubstrate domain from the active site and generate a fully active enzyme.

Two autophosphorylation reactions occur quickly after MgATP is added to the p40 S6/H4 kinase form of the enzyme. First, phosphorylation of the enzyme at site 1 occurs. Analysis of the kinetics of this reaction with enzyme dilution and the MgATP activation time course establish that this reaction occurs by an intramolecular mechanism and precedes activation of the enzyme. At MgATP incubation time intervals where no activation of the enzyme is detected, site 1 is phosphorylated to 30–60% of the maximum observed at full activation. However, this reaction appears to be essential for activation since neither p60 nor p40 S6/H4 kinase which is phosphorylated at only site 2 is active. Since the substrate-binding site is blocked in the nonactivated p40 S6/H4 kinase and since this reaction is intramolecular, a logical prediction is that this phosphorylation occurs at a pseudosubstrate inhibitory domain which is bound at the active site. After autophosphorylation, the phosphorylated pseudosubstrate domain likely behaves as reaction product and binds in the substrate-binding groove with less affinity. This model is supported by data published after submittal of this report (24). Both site 1 and 2 sequences are predicted by the cDNA clone of a serine kinase which is activated by the GTP-binding protein rac and autophosphorylation. The site 1 sequence (amino acids 384–391) is adjacent to the APE sequence (amino acids 394–396) which positions the sequence in the catalytic cleft, consistent with the hypothesis that this is a pseudosubstrate domain.

The importance of the intermolecular phosphorylation at site 2 is more speculative. This modification is necessary, but not sufficient for activation. One possible role which might be predicted for this reaction is that the site 2 domain displaces the phosphorylated site 1 from the substrate-binding groove. If the autoinhibitory site 1 domain does not fully dissociate from the catalytic center after phosphorylation, such a mechanism might be required to attain the fully active conformation in which both MgATP and protein substrate bind with high affinity. This mechanism would predict that any exogenous substrate which binds with affinity high enough to compete with site 1 binding could fulfill the requirements for the second site phosphorylation in the activation mechanism. None of the current data exclude this possibility.

A second hypothesis for the role of site 2 phosphorylation addresses the in vivo mechanism for S6/H4 kinase activation. Although p40 S6/H4 kinase can catalyze phosphorylation of p60, this reaction is much slower than phosphorylation of substrates S6–21 and H4 by fully activated enzyme. This observation suggests that site 2 may be phosphorylated by a cellular kinase other than the S6/H4 kinase, i.e. an upstream regulatory kinase. In this case the role of the site 2 domain may be to alter the substrate-binding groove conformation sufficiently to facilitate the dissociation of phosphorylated site 1 from the catalytic site. In addition, activation by rac binding may promote a conformational change which alters the requirement for site 2 autophosphorylation.

Collectively, the data suggest that only sites 1 and 2 phosphorylations are required for activation of the trypsin-treated kinase. However, in some experiments phosphopeptide 3 is modified to nearly the same extent as phosphopeptide 1, and the kinetics of site 3 phosphorylation always parallel the kinetics of site 1 phosphorylation. In addition, the pl of p40 S6/H4 kinase is 6.3 whereas the putative mono- and diphosphorylated forms exhibited pl of 5.9 and 5.8, respectively. These data suggest that three or more phosphorylation reactions may occur. Since the amount of endogenous phosphorylation in each enzyme preparation may vary, a role for site 3 or site 4 cannot be completely excluded. Alternatively, site 3 may represent an alternate trypsin cleavage of site 1. Previous studies have demonstrated that the S6/H4 kinase requires a dibasic sequence, preferably K-R, amino-terminal to the modified serine (25). Depending on the enzyme/trypsin ratios and extent of endogenous phosphorylation, site 3 may be generated by trypsin cleavage between the K and R, as opposed to cleavage at the carboxyl side of R for site 1. This hypothesis is consistent with the observation that phosphopeptide 3 exhibits TLC properties similar to phosphopeptide 1, but is more basic at neutral pH. This prediction would also be consistent with the observation that site 3 phosphorylation kinetics mimic site 1 phosphorylation kinetics. Consistent with the hypothesis that phosphopeptides 1 and 3 may reflect phosphorylation of the same serine, the predicted amino acid sequence (24) confirms that there are both a lysine and an arginine amino-terminal to the autophosphorylation site.

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