Insulin-activated Protein Kinases Phosphorylate a Pseudosubstrate Synthetic Peptide Inhibitor of the p70 S6 Kinase*

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p70 S6 kinase, a major insulin-mitogen-activated ribosomal S6 protein kinase in mammalian cells, is activated by phosphorylation of multiple Ser/Thr residues on the enzyme polypeptide. A synthetic peptide, corresponding to a 37-residue segment from the carboxyl-terminal tail of the kinase which resembles the sequence phosphorylated in S6, acts as a competitive inhibitor of p70 S6 kinase without itself being phosphorylated by the enzyme. This synthetic peptide is phosphorylated by an array of protein kinases which are rapidly activated by insulin. Thus, these sequences of p70 S6 kinase constitute a potential autoinhibitory pseudosubstrate site, whose phosphorylation is catalyzed by candidate upstream-activating protein kinases.

The initial intracellular steps which transmit, diversify, and amplify the signals generated at the insulin receptor are poorly understood. The functional identity of the physiologically relevant substrates of the insulin receptor tyrosine kinase are not yet known (1), with the possible exception of a phosphotyrosine kinase (2, 3). The rapid increase in Ser/Thr phosphorylation detected in response to insulin suggested that insulin-activated Ser/Thr protein kinases probably served as critical signaling intermediates, and an understanding of the regulation of these enzymes might permit elucidation of the earlier steps in this pathway (4–7). Progress along this line has been achieved recently through study of the ubiquitous insulin-mitogen-activated S6 protein (Ser/Thr) kinases. The S6 kinases are expressed as two distinct types in most higher eukaryotic cells: a family of 85–90-kDa polypeptides, corresponding to the enzymes first purified (8) and cloned (9) from Xenopus eggs, and 70-kDa polypeptides, the quantitatively dominant activity toward 40 S subunits in cultured mammalian cells (10, 11) and liver (12). Both types of S6 kinase are activated through mitogen-induced Ser/Thr phosphorylation of the enzyme polypeptide (13–15). The p85 S6 kinase, after dephosphorylation by protein phosphatase 2A, can be phosphorylated and partially (30%) reactivated by a 42-kDa protein kinase active against microtubule-associated protein 2, which was initially detected and partially purified from insulin-stimulated 3T3-L1 cells (16). The p70 S6 kinase purified from rat liver, by contrast, does not appear to be a substrate for this microtubule-associated protein 2 kinase (15, 17).

Among the important questions concerning the regulation of p70 S6 kinase are: the basis for its low activity in the resting state and the mechanism by which phosphorylation increases p70 kinase activity; the most pressing question is the identity of the activating protein kinases.

A clue to these questions is provided by the primary sequence of the p70 S6 kinase (18, 19); a segment in the carboxyl-terminal tail of the kinase is 28% identical in amino acid sequence (over 25 residues) to the region at the carboxyl terminus of rat liver S6 (20), which bears all of the multiple sites of S6 phosphorylation (21) (Fig. 1A). Based on this similarity, we proposed a model for the regulation of p70 S6 kinase (18) which hypothesizes that this segment of the S6 kinase acts as an autoinhibitory pseudosubstrate domain. The model predicts that the low activity of the p70 S6 kinase in unstimulated cells is due to the binding of this autoinhibitory segment to the protein substrate binding site which is thereby occluded. Mitogen activation of the enzyme represents relief of this inhibition, caused by the phosphorylation of multiple Ser/Thr residues on the autoinhibitory segment by upstream mitogen-activated kinases, and resultant release of the pseudosubstrate segment from the substrate-binding site. To evaluate these ideas, we synthesized a 37-amino acid peptide which encompasses the putative autoinhibitory sequences in p70 S6 kinase and employed this peptide to test several predictions generated from this hypothesis. The results provide considerable evidence in support of the model and moreover have enabled the detection of a set of insulin-activated protein kinases which are strong candidates for the upstream activators of p70 S6 kinase.

MATERIALS AND METHODS

Rat liver p70 S6 kinase was purified as described in Ref. 12. Protein kinase A catalytic subunit was purchased from Sigma and protein kinase C from Lipidex. Kinase M was prepared by incubation of purified kinase C with trypsin (0.1 μg/ml) for 30 min at 37 °C; the reaction was terminated by the addition of a 5-fold excess of soybean trypsin inhibitor, and the phospholipid-independent histones H1 kinase activity was separated by anion-exchange chromatography on a Mono Q HR 5/5 column.

The p70 S6 Kinase AutoInhibitory PseudoSubstrate (SKAIPS) peptide (Fig. 1A), the S6 kinase peptide (12), and the cassette kinase II substrate, RRREEETEEE, were synthesized on an ABI model 430 peptide synthesizer, cleaved, deprotected, desalted, and purified on a Waters preparative high pressure liquid chromatography (Delta System) using Vydc C18 columns and elution with a gradient of acetonitrile in 0.1% trifluoroacetic acid. Peptide mass and structure were verified by amino acid analysis and automated Edman degradation on an ABI gas-phase sequenator. SKAIPS peptides representing several independent cleavages and purifications of two synthseses were examined in the studies presented. LRRASLG (Kemptide) was purchased from Peninsula Laboratories, Inc.

Protein kinase assays employing peptide substrate were carried out as described in Ref. 12, employing adsorption to P81 phosphocellulose paper. Details are provided in the figure legends.
RESULTS AND DISCUSSION

Functional Characterization of Potential Autoinhibitory Pseudosubstrate Sequences in p70 S6 Kinase—An important prediction of the model proposed for the regulation of p70 S6 kinase is that the sequences encompassing the putative autoinhibitory domain of the enzyme will act as an inhibitor, but not a substrate, of the p70 S6 kinase (otherwise the enzyme would autoactivate). The ability of the rat liver S6 kinase to phosphorylate the 37-mer S6 kinase peptide was compared with the phosphorylation of a synthetic peptide modeled on the carboxyl-terminal 32 residues of rat liver S6 (12) which encompasses all S6 phosphorylation sites (Fig. 1A). The p70 S6 kinase, purified from rat liver in an activated state (12), avidly phosphorylates this 32-mer S6 peptide with a $K_m$ of 5–10 $\mu$M; S6 peptide is also phosphorylated by protein kinase A catalytic subunit, protein kinase C, and protein kinase M (Ref. 12 and Fig. 1B), the phospholipid-independent form of protein kinase C generated by light tryptic digestion. In contrast, the 37-mer S6 kinase peptide, which shares 28% identity over a region of 25 residues, as well as predominantly basic charge and a similar number of Ser and Thr residues, is not phosphorylated at all by p70 S6 protein kinase, protein kinase A, protein kinase C, or protein kinase M (Fig. 1B). As will be shown subsequently, however, this S6 kinase peptide is capable of being rapidly phosphorylated by other protein kinases.

Despite the inability to serve as a substrate for the p70 S6 kinase, the 37-mer S6 kinase peptide is a potent inhibitor of the p70 S6 kinase, a property not exhibited by other "nonsubstrate" peptides such as the protein kinase A inhibitor, PKI(1–3–1) (22), the casein kinase II substrate RRREEEEEE, and the protein kinase A substrate, Kemptide (Fig. 1C). Inhibition of p70 S6 kinase by the 37-mer S6 kinase peptide is observed whether the enzyme is assayed using the synthetic 32-mer S6 peptide (Fig. 1C, solid lines) or 40 S ribosomal subunits (Fig. 1C, dashed lines); the IC$_{50}$ at 5 $\mu$M S6 peptide, is 20 ± $\mu$M ($n = 6$) (Fig. 1B). In fact, the 37-mer S6 kinase peptide is equipotent to the high affinity 32-mer S6 peptide substrate as an inhibitor of 40 S subunit phosphorylation. Kinetic analysis indicates that the 37-mer S6 kinase peptide inhibits p70 S6 kinase through a competitive mechanism, as illustrated by the double-reciprocal plots (Fig. 2). The secondary plot of $K/V$ is nonlinear in a reproducibly parabolic fashion (Fig. 2, inset). Maneuvers aimed at minimizing peptide loss caused by adsorption (such as addition of carrier proteins and/or detergents) did not alter the shape of these curves or the IC$_{50}$ (20 ± 2 $\mu$M). The parabolic nonlinearity may reflect the binding of a second molecule of SKAIPS peptide to the enzyme; of note, high concentrations of the 32-mer S6 peptide substrate itself are autoinhibitory (Fig. 1A). Similar parabolic competitive inhibition has been reported for synthetic peptides corresponding to the pseudosubstrate domains of smooth

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**FIG. 1. Specificity of phosphorylation and inhibition of kinases by SKAIPS peptide.** A, the amino acid sequence of p70 SKAIPS synthetic peptide is compared with the amino acid sequence of the carboxyl-terminal segment of rat liver S6 (20) which encompasses all known phosphorylation sites on S6 (21) (arrows). B, phosphorylation of S6 peptide (continuous lines) and SKAIPS peptide (dashed lines) by various protein kinases. The S6 peptide is a 32-residue synthetic peptide described in Ref. 12, modeled on the carboxyl-terminal segment of rat liver S6 shown in A. Rates of phosphorylation catalyzed by each kinase are normalized to the maximal rate of phosphorylation of the S6 peptide catalyzed by that kinase. Peptide phosphorylation by each kinase was assayed as described in Ref. 12. •, p70 S6 kinase, 100% = 2.56 pmol/min; □, protein kinase A, catalytic subunit, 100% = 74.9 pmol/min; C, protein kinase C, 100% = 28.5 pmol/min; Δ, protein kinase M, 100% = 8.94 pmol/min. C, inhibition of p70 S6 kinase by various peptides. p70 S6 kinase was assayed with 5 $\mu$M S6 peptide (solid lines), giving (100%) activity of 2.7 pmol/min, or 40 S ribosomes 10 A$_{260}$ units/ml (dashed lines), giving (100%) activity of 0.13 pmol/min. The kinase activity was inhibited with added Kemptide (▲), RRREEEEEE (●), SKAIPS peptide (○), or S6 peptide (□). D, inhibition of various protein kinases by SKAIPS peptide. □, p70 S6 kinase assayed with 4.2 $\mu$M S6 peptide; ○, protein kinase M assayed with 4.2 $\mu$M S6 peptide; ▲, casein kinase II assayed with 167 $\mu$M RRREEEEEE peptide; Δ, protein kinase A catalytic subunit assayed with 4.2 $\mu$M S6 peptide; □, protein kinase A catalytic subunit assayed with 10 $\mu$M Kemptide. [γ-32P]ATP was 83 $\mu$M in all assays. 100% values for enzyme activities were: protein kinase M, 4.09 pmol/min; p70 S6 kinase, 2.06 pmol/min; protein kinase A, 4.61 pmol/min (S6 peptide), 21.90 pmol/min (Kemptide); casein kinase II, 3.9 pmol/min.
muscle myosin light chain kinase (23) and a 14-residue peptide from phosphorylase b kinase (24). The 37-mer S6 kinase peptide also inhibits p70 S6 kinase with respect to ATP, but with mixed kinetics (data not shown). These data suggest that the 37-mer S6 kinase peptide, despite its inability to be phosphorylated by p70 S6 kinase, competes for the same binding site on p70 S6 kinase as does the 32-mer S6 peptide substrate. These features are consistent with those predicted for an autoinhibitory pseudosubstrate domain (25). Consequently, this 37-mer peptide will henceforth be referred to by the acronym, SKAIPS, to avoid confusion with the various synthetic peptides based on the sequence of S6.

The specificity of SKAIPS peptide inhibition of p70 S6 kinase was evaluated by examining the ability of SKAIPS peptide to inhibit other protein kinases, each assayed with various synthetic peptides based on the sequence of S6. Casein kinase II, like p70 S6 kinase, protein kinase A, and protein kinase M, is also able to phosphorylate SKAIPS peptide (data not shown); casein kinase II phosphorylation of the peptide, RRREETEE (at 0.17 mM), is not significantly inhibited by concentrations of SKAIPS peptide up to 0.2 mM (Fig. 1D). Protein kinase A, however, is modestly inhibited by SKAIPS peptide (IC50 ~0.2 mM), whether assayed with Kemptide (10 mM) or S6 peptide (5 mM). Interestingly, protein kinase M (assayed with S6 peptide at 5 mM) is inhibited by SKAIPS peptide with a potency indistinguishable from that observed for the inhibition of p70 S6 kinase. Thus, inhibition of p70 S6 kinase by SKAIPS peptide is relatively but not absolutely specific, consistent with the behavior of autoinhibitory domain peptides modeled on other Ser/Thr protein kinases (26). In summary, the kinetic properties and relative specificity of the inhibition of p70 S6 kinase by the 37-mer synthetic peptide, modeled on the p70 S6 kinase sequence, provides strong support for the designation of this segment as an autoinhibitory pseudosubstrate site which serves to maintain the kinase in its basal, inactive state.

Insulin-stimulated SKAIPS Peptide Kinases—A distinctive aspect of the regulatory model proposed for the p70 S6 kinase is the idea that disinhibition/activation is accomplished not through binding of an allosteric ligand to a regulatory domain or subunit, as occurs with the messenger-activated protein kinases (25), but through the direct phosphorylation of Ser/Thr residues in the pseudosubstrate domain, catalyzed by mitogen-activated protein kinase(s) situated upstream. If true, then the SKAIPS peptide will be a substrate for such upstream protein kinases.

Insulin treatment of H4 hepatoma cells increases the SKAIPS peptide-phosphorylating activity of cytosolic extracts (Fig. 3A); the increase in SKAIPS peptide kinase activity is detectable within 1 min of hormone addition, peaking initially at 5 min, 4-fold over basal levels, with a secondary peak commencing at 15 min, increasing to 8-fold over basal levels by 60 min. The rate of rise in SKAIPS peptide kinase is more rapid than the increase in cytosolic 40 S S6 kinase activity (known to be attributable to the p70 S6 kinase (15)) but slower than the insulin-stimulated tyrosine phosphorylation of an endogenous 180,000-dalton polypeptide, a ubiquitous insulin receptor kinase substrate, whose tyrosine phosphorylation is already maximal at 1 min (Fig. 3A). The concentration of insulin giving half-maximal activation of SKAIPS peptide kinase activity (3 × 10−9 M) is similar to that of insulin-stimulated tyrosine phosphorylation of the endogenous 180,000-dalton peptide (and receptor tyrosine autophosphorylation, not shown) but much higher than that required for half-maximal activation of 40 S S6 kinase (Fig. 3B). The more rapid activation and right-shifted dose response of insulin-stimulated SKAIPS peptide kinase as compared with 40 S S6 kinase are consistent with the interposition of the SKAIPS peptide kinase activity between the insulin receptor and the p70 S6 kinase. If the SKAIPS kinase(s) are among the immediate upstream activators of the p70 S6 kinase, the large difference in apparent sensitivity to insulin would indicate that small activations of SKAIPS kinase can catalyze substantial activation of p70 S6 kinase, i.e. that a large amplification of the signal occurs at this step.

Anion exchange chromatography of H4 hepatoma cytosolic extracts demonstrates that insulin activates multiple peaks of SKAIPS peptide kinase activities; at least three zones of stimulated peptide kinase activity are reproducibly detected (data not shown). The same peaks are observed in the initial (5 min) and secondary (30 min) phases. Thus, one, several, or some combination of these peaks of insulin-activated protein kinase are strong candidates to be among the upstream activators of the p70 S6 kinase; direct evidence for such a role will require the demonstration that these SKAIPS peptide kinases can phosphorylate and activate a suitable preparation of the p70 S6 kinase itself.

Although the present data are strongly supportive, a variety of additional studies will ultimately be required to determine the validity of the proposed model for mitogen regulation of p70 S6 kinase. Such studies include identification of the sites on p70 S6 kinase phosphorylated after mitogen stimulation and an examination of the properties of recombinant p70 molecules, altered by mutation within, or truncation of the putative autoinhibitory pseudosubstrate domain.

In addition the specific determinants of S6 peptide and SKAIPS peptide binding to the p70 S6 kinase remain to be defined as does the effect of SKAIPS peptide phosphorylation at some or all of the six (Ser/Thr) residues.

The model for p70 S6 kinase regulation makes two further predictions concerning the structural features of the pseudosubstrate segment (18). Five of the six Ser/Thr residues found in this segment are followed by a proline immediately car-
FIG. 3. Insulin-stimulated SKAIPS peptide kinases in H4 hepatoma cells. A, time course of insulin-stimulated protein kinase activities and tyrosine phosphorylation. H4 hepatoma cells were grown as described in Ref. 15 and deprived of serum for 24 h prior to insulin (10^{-7} M) addition. At the times indicated, the plates were rapidly rinsed in ice-cold phosphate-buffered saline and scraped into a buffer containing 10 mM MOPS (4-morpholineethanesulfonic acid), pH 7.4, 2 mM EDTA, 5 mM EGTA ([ethylenbis(oxyethylenenitrilo)]tetraacetic acid), 2 mM dithiothreitol, 2 mM NaVO_{3}, 25 mM β-glycerol phosphate, 0.1% digitonin, 2 mM leupeptin, 2 μM pepstatin, 10 units/ml Trasylol (aprotinin), and 0.2 mM phenylmethylsulfonyl fluoride. After homogenization, the extracts were centrifuged at 100,000 × g for 45 min. For extraction of the p180 polypeptide and insulin receptor subunit, 0.1% Triton X-100 was substituted for digitonin. Assay of S6 kinase (■) was carried out using 40 S ribosomes. In a reciprocal manner, we predicted that the activating boxyl-terminal (Fig. 1A). We proposed that the inability of p70 S6 kinase to autoactivate by intramolecular autophosphorylation is due to these proline residues, which act as a negative determinant of p70 S6 kinase substrate specificity. In a reciprocal manner, we predicted that the activating kinase activities situated upstream in the signal transduction pathway probably require such an SP/TP motif as part of their substrate specificity. Preliminary studies indicate that each peak of insulin-stimulated SKAIPS peptide kinase activity exhibits a strong dependence on the presence of a proximal immediately carboxyl-terminal to the Ser/Thr site of phosphorylation.

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