This study characterizes the insulin-activated serine/threonine protein kinases in H4 hepatoma cells active on a 37-residue synthetic peptide (called the SKAIPS peptide) corresponding to a putative autoinhibitory domain in the carboxyl-terminal tail of the p70 S6 kinase as well as on recombinant p70 S6 kinase. Three peaks of insulin-stimulated protein kinase active on both these substrates are identified as two (possibly three) isoforms of the 40–45-kDa erk/microtubule-associated protein (MAP)-2 kinase family and a 150-kDa form of cdc2. Although distinguishable in their substrate specificity, these protein kinases together with the p54 MAP-2 kinase share a major common specificity determinant reflected in the SKAIPS peptide: the requirement for a proline residue immediately carboxyl-terminal to the site of Ser/Thr phosphorylation. In addition, however, at least one peak of insulin-stimulated protein kinase active on recombinant p70, but not on the SKAIPS peptide, is present although not yet identified.

MPF/cdc2 phosphorylates both rat liver p70 S6 kinase and recombinant p70 S6 kinase exclusively at a set of Ser/Thr residues within the putative autoinhibitory (SKAIPS peptide) domain. erk/MAP kinase does not phosphorylate rat liver p70 S6 kinase, but readily phosphorylates recombinant p70 S6 kinase at sites both within and in addition to those encompassed by the SKAIPS peptide sequences. Although the tryptic 32P-peptides bearing the cdc2 and erk/MAP kinase phosphorylation sites co-migrate with a subset of the sites phosphorylated in situ in insulin-stimulated cells, phosphorylation of the p70 S6 kinase by these proline-directed protein kinases in vitro does not reproducibly activate p70 S6 kinase activity.

Thus, one or more erk/MAP kinases and cdc2 are likely to participate in the insulin-induced phosphorylation of the p70 S6 kinase. In addition to these kinases, however, phosphorylation of the p70 S6 kinase by other as yet unidentified protein kinases is necessary to recapitulate the multisite phosphorylation required for activation of the p70 S6 kinase.

The signal transduction pathways that mediate the mitogenic and metabolic responses to insulin receptor activation are incompletely understood (1). Physiological substrates for the insulin receptor kinase have not yet been identified, although a set of 160–180-kDa polypeptides undergo insulin-stimulated tyrosine phosphorylation in many cell types (2). In addition, phosphatidylinositol 3-kinase activity precipitability by anti-phosphotyrosine antibodies increases substantially after insulin receptor activation (3, 4).

Insulin receptor tyrosine kinase activation results in increased Ser/Thr phosphorylation of many cellular proteins to an extent that greatly exceeds the abundance of phosphotyrosine (5). This suggests that one mechanism for amplifying and transmitting the signals generated at the receptor is by recruitment of intracellular serine/threonine protein kinases. In fact, a growing number of insulin-stimulated serine/threonine protein kinases have been characterized at a molecular level, including the p70 S6 kinase (6), the p85 S6 kinase (8, 9) (also known as p90 rsk), casein kinase II (10, 11), c-raf (12, 13), a 42-kDa MAP-2 kinase (14), and related enzymes designated as erk (15, 16); a number of other, less fully characterized insulin-regulated serine/threonine protein kinases have also been described. Several of these serine kinases (e.g. c-raf1 and the S6 kinases) are themselves activated by Ser/Thr phosphorylation catalyzed by protein kinases situated upstream. The 42-kDa MAP-2 kinase is probably one such upstream activator inasmuch as this enzyme, partially purified from insulin-stimulated 3T3-L1 adipocytes, has been shown to phosphorylate and partially (–30%) reactivate the phosphatase 2A-deactivated Xenopus (p85) S6 kinase II (17). Thus, the p42 MAP-2 kinase and p85 S6 kinase II appear to be sequential elements in an insulin/mitogen-activated protein kinase cascade. The insulin-stimulated p42 MAP-2 kinase, first detected in 3T3-L1 adipocytes, is now known to be one of a set of 40–45-kDa polypeptides that undergo tyrosine phosphorylation in response to serum platelet-derived growth factor, EGF, and active phorbol esters in many cultured cells (14, 18, 19). A regulatory role for the phosphotyrosine residues on the p42 MAP-2 kinase has been established by the demonstration that treatment of the p42 MAP-2 kinase with the tyrosine phosphatase CD45 leads to deactivation of its serine/threonine protein kinase activity (20). Mitogen-activated protein kinases of 40–45 kDa that phosphorylate MAP-2 and myelin basic protein (MBP) and are inactivated by treatment with tyrosine and Ser/Thr phosphatase have now been described in several systems (21–26).
The p70 S6 kinase isolated from rat liver, although overlapping in substrate specificity with the p85 S6 kinase II (27) and also activated in response to insulin by Ser/Thr phosphorylation at multiple sites on the enzyme polypeptide (6, 28), is not phosphorylated or activated in vitro by the p42 MAP-2 kinase under conditions that give extensive phosphorylation and partial activation of the Xenopus (p85) S6 kinase II (6, 29, 30). In attempting to understand the regulation of the p70 S6 kinase, the noncatalytic sequences of rat liver p70 were scrutinized for potential regulatory motifs; a segment immediately carboxyl-terminal to the extended catalytic domain was observed to contain sequences resembling (28% identity over 25 residues) the region on S6 that contains all of the 5–6 serine residues phosphorylated by the p70 S6 kinase. Based on this similarity, a model was developed (7) wherein this basic Ser/Thr/Pro-rich region of the p70 S6 kinase was proposed to function as an autoinhibitory pseudosubstrate site, which bound to and occluded the substrate site, producing the low activity of the enzyme in the basal state. According to this model, insulin/mitogen activation of the p70 S6 kinase reflected a disinhibition of the enzyme catalyzed by mitogen/insulin-activated serine/threonine protein kinases situated upstream, which phosphorylate the multiple Ser residues in the putative p70 pseudosubstrate domain, thereby dislocating it from the substrate-binding site.

In support of this model, a 37-mer synthetic peptide, based on the putative pseudosubstrate sequences (called the S6 Kinase Autoinhibitory Pseudosubstrate peptide (SKAIPS peptide), was found to act as a competitive inhibitor of the p70 S6 kinase, without itself being phosphorylated by this kinase (31). Moreover, although the SKAIPS peptide was also not a substrate for casein kinase II or protein kinase C or A, it was phosphorylated by an array of insulin-stimulated serine/threonine protein kinases, which were activated in H4 hepatoma cells by insulin more rapidly than the p70 S6 kinase itself. These insulin-activated SKAIPS peptide kinases are strong candidates to be among the upstream activators of the p70 S6 kinase. This study demonstrates that the three major peaks of the insulin-stimulated SKAIPS peptide kinase resolved on Mono Q anion-exchange chromatography contain enzymes closely related to the erk/p42 MAP kinases in size, regulatory properties, immunochemical cross-reactivity, and substrate specificity. In addition, one peak of the insulin-stimulated SKAIPS peptide kinase corresponds to a form of the cdc2 protein kinase. The substrate specificity of these SKAIPS peptide kinases shares an important common feature: a marked dependence on the presence of a proline residue carboxyl-terminal to the site of Ser/Thr phosphorylation. In addition, secondary specificity determinants distinguish cdc2 from the erk/MAP kinases and p54 MAP-2 kinase (29), another proline-directed serine/threonine protein kinase. cdc2 and erk/MAP kinase each phosphorylate a recombinant p70 S6 kinase at distinct sites, which correspond to a subset of the sites on p70 phosphorylated in insulin-stimulated cells. Activation of recombinant p70 S6 kinase after phosphorylation in vitro, however, was not observed. Employing recombinant p70 S6 kinase as substrate in parallel to the SKAIPS peptide, at least one major chromatographic peak of insulin-stimulated p70 S6 kinase is observed that is not detected with the SKAIPS peptide substrate. These data indicate that one or more erk/MAP kinases and cdc2 are likely to participate in the insulin-stimulated phosphorylation of the p70 S6 kinase; in addition, however, other unidentified protein kinases, acting at sites on p70 outside of those re-
subjected to gel filtration on Superose 12. Mono Q Peak 1, although broad, eluted as a single symmetrical peak at ~35 kDa (Figs. 2 and 3); Mono Q Peak 2, however, was resolved on gel filtration into two peaks (A and B). Peak 2A was ~150 kDa, whereas Peak 2B was ~50 kDa (Figs. 2, 3, and 5). Mono Q Peak 3, the elution pattern of which on Mono Q was most complex, exhibited a broad profile on gel filtration (data not shown). The leading and trailing halves of Mono Q Peak 3 were pooled separately and subjected to gel filtration, and each also showed a similar broad elution pattern. Mono Q Peak 3 was not examined further by gel filtration; Peaks 1, 2A, and 2B of the SKAIPS kinase were taken for further characterization.

Based on the similarities in apparent molecular mass, substrate specificity, susceptibility to deactivation by phosphotyrosine phosphatase and Ser/Thr protein phosphatase 2A, and immunochemical cross-reactivity (as shown in the Miniprint), we conclude that Peak 1, 2B, and much of Peak 3 SKAIPS kinases are closely related and probably identical to the insulin/mitogen-activated erk/MAP kinases described previously; by comparison to the results described by Boulton et al. (16), the p45 polypeptide coeluting with Peak 1 probably corresponds to erk-1 and the p41 polypeptide to erk-2. The 45-kDa erk-like polypeptides that compose Peak 2B and Mono Q Peak 3 SKAIPS peptide kinases may also be erk-1 or (more likely) one or more additional erk isoforms.

**Identification of Peak 2A SKAIPS Peptide Kinase as Insulin-activated cdc2**—The selective coelution of histone H1 kinase activity with Peak 2A SKAIPS peptide kinase (Fig. 5) prompted the consideration of the possibility that Peak 2A was related to cdc2; consequently, direct evidence as to the presence of cdc2 in Peak 2A was sought. p13 Suc-1, the product of the fission yeast Suc-1 gene, binds tightly and selectively to the cyclin-cdc2 complex of yeast (40) and Xenopus (41). Suc-1 immobilized covalently to Sepharose adsorbed the kinase activity in Peak 2A, but did not bind Peak 1 or 2B kinase; Sepharose 4B alone or Sepharose/bovine serum albumin gave no adsorption. Essentially all of the SKAIPS kinase in Peak 2A could be depleted by adsorption to Suc-1-Sepharose beads, and 40% of the original Peak 2A kinase activity could be recovered by assay of the Suc-1-Sepharose beads. These findings indicate strongly that cdc2 itself or a closely related protein kinase capable of binding p13 Suc-1 is the dominant component of Superose Peak 2A SKAIPS peptide kinase. Evidence that cdc2 itself is present in Peak 2A is provided by the demonstration that an antisera to a synthetic peptide corresponding to the carboxy-terminal heptapeptide of human cdc2 (34) selectively immunoprecipitates a portion of the MBP/SKAIPS kinase activity corresponding to Peak 2A.

The identification of cdc2 among the cytoplasmic insulin-activated SKAIPS peptide kinases was unexpected and prompted a specific examination of whether cdc2 activity is actually increased after insulin treatment of serum-starved H4 hepatoma cells. In each of the five experiments, we observed a 50–100% increase in cytosolic (Suc-1-precipitable) H1 kinase activity at 30 min after insulin treatment of serum-deprived H4 hepatoma cells; by comparison, a 2–7-fold increase in total cytosolic SKAIPS peptide kinase activity was observed. On Mono Q chromatography of extracts from serum-starved and insulin-treated cells (Fig. 6), total H1 kinase eluted over a broad zone in a complex pattern that overlapped with but did not correspond closely to Peaks 2 and 3 of SKAIPS peptide kinase activity; the major peak of H1 kinase activity eluted at the leading edge of Peak 3 SKAIPS peptide kinase and contained ~2-fold more H1 kinase activity after insulin treatment. Suc-1-precipitable H1 kinase paralleled total H1 kinase and was also increased ~2-fold by 30 min after insulin treatment. These data indicate that cytoplasmic cdc2 activity is modestly increased after insulin treatment and contributes to but does not account for a major component of the insulin-stimulated SKAIPS peptide kinase activity in Peak 2 (e.g. as in Figs. 2 and 3) and probably Peak 3 as well.

**Substrate Specificity of SKAIPS Peptide Kinases**—Although both MBP and MAP-2 are capable of being phosphorylated by many protein kinases, the coincident profiles of SKAIPS kinase activity, MBP kinase, and MAP-2 kinase indicated (Fig. 1 and 2) that most and perhaps all of the insulin-stimulated serine(threonine) protein kinases in H4 hepatoma cells active on MBP and MAP-2 are also detected by their phosphorylation of the SKAIPS peptide, and vice versa. This observation suggested that the major determinants that specified the phosphorylation of MAP-2 and MBP by these insulin-stimulated kinases are represented in the SKAIPS peptide. A novel feature of the amino acid sequence of the SKAIPS peptide is that 5 of the 6 Ser/Thr residues are
immediately amino-terminal to proline: KEKSFEPKIR-SPPRFIFSPRTPVSPVFKFSGDFWGR (7, 31). Several protein kinases have been shown to phosphorylate preferentially Ser/Thr residues followed by proline, including MPF/cdc2 (42) and a mitogen-activated tyrosine hydroxylase-kinase (43). In addition, the insulin-stimulated p42 MAP-2 kinase from 3T3-L1 cells (44) and a p44 MBP protein kinase from sea star (45) each phosphorylates preferentially a sequence in MBP corresponding to VTPRTPPQSGK. Moreover, the EGFR is phosphorylated at Thr693 (GVEPLTPSGEAPNQ) by an EGF-activated serine/threonine protein kinase (46), which copurifies through several steps with the 3T3-L1 cell MAP kinase activated by EGF (47). We therefore examined the ability of Peak 1, 2A, and 2B SKAIPS peptide kinases, in comparison with MPF, sea star p44 MBP kinase, and the p54 MAP-2 kinase, to phosphorylate two sets of synthetic peptides: one based on EGFR sequences surrounding Thr693 and a second based on sequences from MBP surrounding Thr97 (Table I). The peptides were each screened as substrates at 1 mM and compared to the SKAIPS peptide at 50 μM, near its $K_m$ for these kinases. Relative to their ability to phosphorylate the SKAIPS peptide, Peak 1 and 2B SKAIPS kinases, the p44 MBP kinase, and the p54 MAP-2 kinase avidly phosphorylated the EGFR receptor Thr693 peptide, whereas MPF and Peak 2A SKAIPS kinase exhibited more modest activity. Replacement of the proline at position +1 (i.e., immediately carboxyl-terminal to the phosphorylation site) by alanine reduced phosphorylation by Peak 1 and 2B SKAIPS kinases and sea star p44 MBP kinase by ≥85% and effectively abolished phosphorylation by the p54 MAP-2 kinase; phosphorylation by Peak 2A SKAIPS kinase and MPF was reduced by two-thirds, but the lower phosphorylation of the wild-type EGFR peptide by these two kinases limited interpretation. Replacement of the proline at position −2 by alanine in the EGFR peptide also diminished phosphorylation catalyzed by Peak 1 and 2B SKAIPS kinases and the p44 MBP kinase by ≥85%, but actually stimulated slightly phosphorylation by the p54 MAP-2 kinase; the negative impact on Peak 2A SKAIPS kinase- and MPF-catalyzed phosphorylation was also substantial; but here again, the lower phosphorylation of the parent peptide prohibited a confident interpretation. Replacement of prolines at both positions −2 and +1 by alanines in the EGFR peptide virtually eliminated phosphorylation by all the kinases tested.

A synthetic peptide (i.e., APRTFGGRR) modified (48) from the sequence in MBP surrounding the site phosphorylated by the p44 MAP kinase (TPRTTPPSQ) was phosphorylated much more vigorously by Peak 2A SKAIPS kinase and MPF than was the EGFR peptide, and significant phosphorylation of the modified MBP sequence by Peak SKAIPS kinases 1 and 2B was also observed; in contrast, the MBP peptide was poorly phosphorylated by the p54 MAP-2 kinase, as was MBP itself (29). Replacement of the proline at position +1 by alanine in the MBP peptide resulted in a profound decrease in peptide phosphorylation, catalyzed by all SKAIPS kinases as well as by MPF, the p54 MAP-2 kinase, and the p44 MBP kinase; peptide phosphorylation was not restored by insertion of a proline at position +2. These findings confirm the crucial nature of the requirement for a proline at position +1 for each of these protein kinases. Replacement of the proline at position −2 by alanine in the modified MBP peptide yielded results that more clearly distinguish classes of specificity among these kinases. The p44 MBP kinase and Peak 1 and 2B SKAIPS kinases showed a 80–90% decrease in peptide phosphorylation, as observed for the parallel modification of the EGFR Thr693 peptides. Peak 2A SKAIPS kinase and MPF, by contrast, exhibited a more modest 40–50% decrease in their ability to phosphorylate the MBP peptide in which proline at position −2 had been replaced by alanine; reinsertion of the proline at position −1 or −3 did not restore peptide phosphorylation to that seen with proline at position −2. Phosphorylation of the MBP peptide by the p64 MAP-2 kinase was too low for valid comparison.

A second feature that distinguishes among these proline-directed protein kinases is illustrated by the effect of replacement of the arginine at position −1 by alanine, glutamic acid, phenylalanine, leucine, or glutamine in the MBP peptides; Peak 2A SKAIPS kinase phosphorylation of the MBP peptides that lack arginine at position −1 was half that seen for the peptide with arginine at position −1, a pattern also seen with MPF. In contrast, Peak 1 and 2B SKAIPS kinases as well as the p44 MBP kinase and p54 MAP-2 kinase exhibited no preference for an arginine at position −1, although a mild negative effect of glutamic acid at position −1 was evident.

Thus, each of the three peaks of the SKAIPS kinase exhibited a marked, nearly total dependence on the presence of a proline immediately carboxyl-terminal to the Ser/Thr pro-
phorylation site, a feature shared by the p44 MBP kinase, the p54 MAP-2 kinase (especially), and MPF. Beyond this common feature, the SKAIPS kinases can be separated into two subgroups: Peak 1 and 2B SKAIPS kinases also exhibited a 80-80% decline in peptide phosphorylation if the proline at position -2 in both the EGFR and MBP peptides was substituted by alanine, but no preference for phosphorylation of MBP peptides with an arginine at position -1. These specificity features are shared with sea star p44 MBP kinase. Peak 2A SKAIPS kinase exhibited a more modest 50% decline in peptide phosphorylation if the proline at position -2 was substituted by alanine in the EGFR and MBP peptides and a comparable 50% decline if the arginine at position -1 in the MBP peptide was replaced by another residue; both features are shared with MPF. Finally, peptide phosphorylation by the p54 MAP-2 kinase was completely dependent on a proline at position +1, but was not affected adversely by omission of the proline at position -2; the identity of the other crucial determinant for this kinase, presumably present in the EGFR peptide but lacking in the MBP peptide, remain to be identified.

**MPF/cdc2 Phosphorylates p70 S6 Kinase within Segment Corresponding to SKAIPS Peptide**—The identification of erk/MAP kinases and cdc2 as the major insulin-stimulated SKAIPS peptide kinase in H4 hepatoma cells led us to examine the ability of these protein kinases to phosphorylate and activate/reactivate purified rat liver p70 S6 kinase.

MPF/cdc2, purified from Xenopus oocytes, phosphorylated rat liver p70 S6 kinase, with incorporation of up to 0.3-0.5 mol of phosphate/p70 subunit (estimated from the specific activity of purified rat liver p70 S6 kinase). Tryptic peptide maps of rat liver p70 phosphorylated by MPF exhibited seven 32P-peptides (Fig. 8), four of which (designated a-d) corresponded to 32P-peptides generated by p70 autophosphorylation in vitro (in the absence of another protein kinase). The remaining three 32P-peptides (designated 1-3), which all migrated to the cathode on thin-layer electrophoresis at pH 3.5, corresponded closely to the 32P-peptides observed in a tryptic map of the SKAIPS peptide phosphorylated by MPF. Thus, MPF catalyzed phosphorylation of rat liver p70 (as well as COS recombinant p70 S6 kinase; see below and Fig. 9) exclusively at sites that were encompassed in the p70 sequence corresponding to the SKAIPS peptide. Tryptic maps of the p70 S6 kinase purified from 32P-labeled insulin-stimulated H4 hepatoma cells exhibited numerous 32P-peptides, greatly exceeding in complexity the pattern generated from p70 phosphorylated in vitro by MPF. Nevertheless, the digests of p70 32P-labeled in situ exhibited a set of basically charged 32P-peptides similar in mobility to those bearing the sites of MPF-catalyzed phosphorylation in vitro. These results indicate that cdc2 or a protein kinase with very similar substrate specificity (i.e. one that requires a proline carboxyl-terminal to Ser/Thr in situ as well as multiple basic residues nearby) participates in the insulin-stimulated phosphorylation of p70 in H4 hepatoma cells.

Phosphorylation of rat liver p70 S6 kinase by MPF did not reproducibly increase p70 kinase activity toward 40 S subunits. Prior deactivation/dephosphorylation of p70 with protein phosphatase 2A did not improve the response to MPF; in fact, dephosphorylation of p70 by phosphatase 2A led to a
leisure incorporation of $^{32}$P catalyzed by MPF than was observed with untreated purified rat liver p70 S6 kinase as substrate.

*erk/MAP Kinase Phosphorylates Recombinant p70 S6 Kinase*—In the rapid phosphorylation of rat liver p70 S6 kinase by MPF/cdc2, a partially purified but highly active preparation of H4 hepatoma MAP kinase corresponding to Peak 1 SKAIPS peptide kinase did not phosphorylate rat liver p70 S6 kinase at all, whether or not prior treatment with phosphatase 2A had been carried out. This result recapitulated our previous findings using the insulin-stimulated H4 hepatoma cells (6, 29) as well as those reported by Ballou et al. (30). Nevertheless, the ability of erk/MAP kinases to phosphorylate the SKAIPS peptide and the evidence that these sequences on rat liver p70 S6 kinase were available for phosphorylation by MPF/cdc2 indicated that the failure of erk/MAP kinase to phosphorylate rat liver p70 S6 kinase was due to improper conditions or an inappropriate substrate. Recent studies examining the expression of p70 cDNA in COS cells (49) have shown that the recombinant p70 polypeptides are expressed as a mixture of phosphorylated, but catalytically inactive polypeptide together with a small proportion of more highly phosphorylated, catalytically active enzyme. These studies indicated that only the most highly phosphorylated p70 S6 kinase polypeptides acquire S6 kinase activity.

Extrapolating from these observations, the inability of erk/
kinase (i.e. autophosphorylation) or with the addition of MPF/cdc2 or erk/MAP kinase (Fig. 8A). In contrast to the inability of erk/MAP kinase to phosphorylate rat liver p70 S6 kinase, both erk/MAP kinase and MPF phosphorylated recombinant epitope-tagged p70 S6 kinase (Fig. 9A). Activation of the S6 kinase activity of the recombinant epitope-tagged p70 polypeptide has not, however, been reproducibly observed consequent to MPF- or erk/MAP kinase-catalyzed p70 phosphorylation.

Tryptic peptide maps of epitope-tagged p70 phosphorylated by MPF and erk/MAP kinase were compared to those derived from the SKAIPS peptide phosphorylated by each of these kinases. The tryptic 32P-peptide generated from recombinant epitope-tagged p70 S6 kinase phosphorylated by MPF (Fig. 9B) exhibited three major, basically charged tryptic 32P-peptides (designated 1-3 in Fig. 9) that corresponded closely to the tryptic 32P-peptides derived from the SKAIPS peptide phosphorylated by MPF (Figs. 8 and 9) and in turn to the peptide maps generated from purified rat liver p70 S6 kinase phosphorylated by MPF (Fig. 8). Thus, as with the rat liver enzyme, the sites on recombinant epitope-tagged p70 S6 kinase that were phosphorylated by MPF were located entirely within the sequences corresponding to the SKAIPS peptide.

The peptide map of the SKAIPS peptide phosphorylated by erk/MAP kinase also exhibited three major, basically charged tryptic 32P-peptides, each of which co-migrated with the tryptic 32P-peptides bearing the sites of MPF-catalyzed SKAIPS peptide phosphorylation (designated 1-3 in Fig. 9, compare D to E). The relative preference for these sites, however, was markedly different for the two proline-directed kinases: MPF phosphorylated the SKAIPS peptide preferentially at Peptide 1, the most basic tryptic 32P-peptide, with detectable but much less phosphorylation at Peptides 2 and 3; erk/MAP kinase, by contrast, phosphorylated peptide 2 preferentially, with detectable but minor phosphorylation at peptides 1 and 3. Thus, as with the synthetic peptide studies in Table 1, MPF and erk/MAP kinase exhibited overlapping, but clearly distinguishable substrate specificities for the array of sites available on the SKAIPS peptide.

The tryptic 32P-peptide map of recombinant epitope-tagged p70 S6 kinase phosphorylated by erk/MAP kinase (Fig. 9C) encompassed the pattern seen with the erk-phosphorylated SKAIPS peptide (Fig. 9E) (i.e. Peptide 2 >> Peptides 3 and 1), but exhibited at least three additional tryptic 32P-peptides that were not seen at all in digests of the 32P-labeled SKAIPS peptide or in digests of epitope-tagged p70 S6 kinase phosphorylated by MPF. These erk/MAP kinase-specific sites of p70 phosphorylation (designated 4-6 in Fig. 9C) presumably represent erk/MAP kinase phosphorylation sites on p70 S6 kinase outside of the sequences corresponding to the SKAIPS peptide. The tryptic map of p70 purified from 32P-labeled, insulin-stimulated H4 hepatoma cells (Fig. 8) exhibited a set of 32P-peptides that migrated very similarly to Peptides 2-6; the clustering of these peptides, however, precluded a definitive conclusion as to which of the erk/MAP kinase sites designated 4-6 phosphorylated in vitro correspond to those labeled in situ. Finally, the superimposition of the peptide maps of p70 phosphorylated in vitro by both MPF and erk/MAP kinases still does not recreate entirely the array of tryptic 32P-peptides labeled in situ.

**Insulin-stimulated Recombinant p70 S6 Kinase—Kinases—**

The inability of the SKAIPS peptide kinases, i.e. MPF/cdc2 and erk/MAP kinase, to activate p70 in vitro together with the inability of these kinases to phosphorylate in vitro many of the sites on p70 that undergo phosphorylation in intact insulin-stimulated cells suggest that p70 kinase kinases, in addition to the SKAIPS peptide kinases (i.e. erk/MAP kinase and cdc2), were yet to be found. Consequently, the Mono Q column eluates were assayed for protein kinase activity using recombinant epitope-tagged 70 S6 kinase as well as the SKAIPS peptide as substrate. As shown in Fig. 6, four distinct peaks of p70 kinase-kinase were resolved on Mono Q chromatography. Three of these p70 kinase-kinase peaks corresponded closely in elution profile to the three peaks of SKAIPS peptide kinase characterized above; gel filtration (data not shown) confirmed this conclusion. In addition, however, a Mono Q peak of protein kinase acting on recombinant p70 S6 kinase, but not on the SKAIPS peptide, eluted between Mono Q Peak 2 and 3 SKAIPS peptide kinases. This additional peak p70 S6 kinase-kinase(s) thus joins the list of potential participants in the multisite phosphorylation of the p70 S6 kinase in vivo that underlies its activation by insulin.

**DISCUSSION**

The insulin/mitogen-activated p70 kinase is known to be activated in situ by an insulin-induced phosphorylation of multiple Ser/Thr residues on the enzyme polypeptide. The goal of this work was to identify the immediate upstream activators of this ubiquitous insulin/mitogen-activated p70 S6 kinase. Our initial strategy was based on a model that predicted that the mechanism for p70 activation involved phosphorylation of the enzyme on one or more Ser/Thr residues within an autoinhibitory pseudosubstrate domain, thereby dislodging this endogenous inhibitor from the protein substrate-binding site, disoccluding this site and disinhibiting the enzyme. Consistent with this model, a synthetic peptide substrate corresponding in sequence to a putative autoinhibitory pseudosubstrate domain on the p70 S6 kinase (SKAIPS peptide) (7) is phosphorylated by a set of serine/threonine protein kinases that are activated within minutes after insulin addition (31). The major protein kinase species, which account for nearly all of the three major peaks of insulin-activated SKAIPS peptide kinase activity detected on Mono Q chromatography, were identified as several isoforms of the erk/MAP kinase family; a modest contribution by a 150-kDa form of cdc2 was also documented.

Although these SKAIPS peptide kinases can phosphorylate recombinant p70 in vitro on tryptic peptides that resemble closely a subset of the sites phosphorylated on p70 in intact cells, we have not succeeded as yet in activating recombinant p70 S6 kinase by phosphorylation with these SKAIPS peptide kinases in vitro. We therefore continue to designate the SKAIPS peptide kinases as “candidate” upstream activators of the p70 S6 kinase. Our working hypothesis is that in addition to phosphorylation of p70 in the autoinhibitor domain, phosphorylation at one or more sites outside of this 37-amino acid sequence is also required to achieve activation of the p70 S6 kinase in situ. Employing recombinant p70 as substrate, we have detected at least one additional insulin-activated protein kinase that appears to phosphorylate p70 at sequences outside of these corresponding to the SKAIPS peptide; the identity of this additional candidate activator is not known.

The strongest evidence implicating cdc2 as one of the protein kinases acting on p70 S6 kinase in intact cells is the observation that the sites on rat liver and recombinant p70 S6 kinases phosphorylated by MPF in vitro are found on a segment of the p70 molecule likely to be crucial to the regulation of enzyme activity (i.e. the putative autoinhibitory pseudosubstrate domain) and correspond closely on thin-layer electrophoresis/TLC to a subset of the sites phosphorylated on p70 in intact cells. Conversely, several observations detract
from the strength of the evidence: the ability of MPF to phosphorylate "already" active rat liver p70 (as well or better than it phosphorylates p70 previously treated with phoshpase 2A) indicates that some portion of the MPF sites are unphosphorylated in already active rat liver p70 S6 kinase. The modest extent of insulin-induced activation of cdc2 and the relatively slow onset of this stimulation as compared to the erk/MAP kinase response indicate that cdc2 is actually a minor component of the insulin-activated SKAIPS peptide kinases. Nevertheless, each of these observations can be interpreted in several ways, some compatible with an important role for cdc2 in the phosphorylation of p70 in situ. Thus, the phosphatase treatment of rat liver p70 may yield a misfolded enzyme, incapable of being activated by MPF. The small activation of cdc2 may belie the operation of a specific subfamily of cdc2 molecules within the complex pattern of Suc-1-precipitable H1 kinase seen in Fig. 6. Finally, the activation of p70, which is considerably slower than that of erk/MAP kinases, may be slowed by a requirement for phosphorylation by cdc2 as well as erk/MAP kinase.

The evidence that one or more erk/MAP kinases participate in the phosphorylation of p70 in situ is stronger overall, but still incomplete. Positive points are the speed and magnitude of erk/MAP kinase activation in response to insulin and the observation that the sites of erk phosphorylation on p70 are inaccessible when the protein substrate employed is the already active p70 S6 kinase as purified from rat liver, but are accessible when the substrate employed is a recombinant p70 S6 kinase, isolated largely in an unactivated form from COS cells. Finally, the tryptic phosphopeptides phosphorylated on recombinant p70 S6 kinase by erk/MAP kinase correspond, at least in part, to peptides phosphorylated in situ. A definitive conclusion as to the role of cdc2 and MAP kinase must await the demonstration that p70 can be reactivated by these or any other protein kinases, and efforts in this direction are ongoing.

SKAIPS Peptide Substrate Monitors a Broad Array of Insulin/Mitogen-activated, Proline-directed Protein Kinases—Studies with two sets of synthetic peptide substrates, based on sequences from the EGFR and MBP, established that the ability of the partially purified SKAIPS kinases as well as highly purified MPF/cdc2, sea star p44 MBP kinase, and the p54 MAP-2 kinase to phosphorylate peptide substrates is strongly dependent on the presence of a proline residue immediately carboxyl-terminal to the Ser/Thr phosphorylation site. The detection of a set of insulin-activated kinases that share such a novel and relatively restricted specificity is, to a considerable extent, predeterminated by the use of the SKAIPS peptide, inasmuch as 5 of the 6 Ser/Thr residues in this peptide are followed directly by a proline. Thus, protein kinases A and C and insulin/mitogen-activated serine/threonine protein kinases of different specificity, e.g. such as the S6 kinases themselves, and casein kinase II do not phosphorylate the SKAIPS peptide at all (31) and are not monitored by assay with this peptide substrate (the SKAIPS peptide also lacks tyrosine residues). Conversely, the selectivity of the SKAIPS peptide has enabled the clear-cut demonstration that among the protein kinases activated by insulin is a family of enzymes that share a major specificity determinant, i.e. the requirement for a proline immediately carboxyl-terminal to the site of phosphorylation. It should be emphasized that apart from the demonstration of the requirement for proline at position +1, this study does not establish the consensus recognition motif for these protein kinases. The relatively high KN of the parent EGFR Thr peptides (~0.4 mM) and MBP peptides (near 1 mM) for the SKAIPS peptide kinases and sea star p44 MBP kinase makes it unlikely that all of the important structural features that specify a high affinity substrate are represented in these peptides. Nevertheless, the differential effect of systematic variations in peptide structure on peptide phosphorylation by each kinase does allow the identification of distinguishable subclasses of protein kinase based on specificity. Among the six proline-directed kinases examined, three subgroups of specificity can be defined: Peak 1 and 2B SKAIPS kinases and sea star p44 MBP kinase appear to prefer the motif P-X-S/T-P from among the peptides examined. Nevertheless, an indication that as yet undefined determinants, more influential than the presence of proline at position −2, specify the phosphorylation of native substrates by Peak 1 and 2B SKAIPS kinases is the fact that Xenopus rax α (9), which is avidly phosphorylated by Peak 1 SKAIPS kinase (see Fig. 7), has 10 SP/TP pairs in its coding region, but lacks entirely a P-X-S/T-P sequence. MPF/cdc2 and Peak 2A SKAIPS kinase/cdc2 exhibit a modest preference for proline at position −2 and arginine at position −1; previous studies also indicated a strongly positive effect of a basic residue at position +2 and proposed a consensus cdc2 recognition motif: (R/K)-T/S-P-X-R/K. Finally, the p54 MAP-2 kinase (29), which is not among the insulin-stimulated H4 hepatoma kinases identified thus far, is indifferent to the presence of proline at position −2 or arginine at position −1; its preferred motif other than S/T-P remains to be defined.

We endorse the term "proline-directed protein kinase" to denote a family of enzymes united by their requirement for a proline situated immediately carboxyl-terminal to the site of Ser/Thr phosphorylation (43). The enzymes include cdc2, the p40-45 erk/MAP kinases, and the p54 MAP-2 kinase. These enzymes are also "SKAIPS peptide kinases" and share, in addition to substrate specificity, critical regulatory features, notably the regulation of their kinase activity through both Ser/Thr- and Tyr-specific phosphorylation. Whether the structurally closely related yeast protein kinases KSS-1 (51) and FUS-3 (52) as well as the newly cloned protein kinases Ctk/STY (53, 54), Spk1 (55) and MCK1 (56) also exhibit a proline-directed substrate specificity is not yet known.

Identification of Peak 2A SKAIPS kinase as Insulin-activated cdc2—The finding that insulin activates cdc2 in serum-deprived H4 hepatoma cells merits discussion. The ability of insulin to promote the G1/S transition and to act as a mitogen through its unique tyrosine kinase receptor is rather modest, but has now been consistently observed in stable transformants that overexpress recombinant insulin receptor (57-59). In such cells, the insulin receptor is <10% as efficient in generating a mitogenic signal as compared to an equal number of highly homologous type 1 insulin-like growth factor receptors expressed in the same line, although other aspects of the cellular response to activation of the two receptors are achieved with comparable efficiency (59). Previous studies have also established the mitogenic capacity of insulin acting through its own receptor in H4 hepatoma cells (60, 61). These cells spontaneously overexpress insulin receptors, but lack entirely expression of the type 1 insulin-like growth factor receptor as well as EGF/transforming growth factor receptors. Serum deprivation of H4 hepatoma cells arrests these cells in early G1 phase (61), and addition of insulin alone will permit cell division. Studies in fission yeast have established that cdc2 activity is rate-limiting to normal cell cycle progression at two distinct points: during the G2/M transition and at some point in G1 so as to achieve transition into S phase (reviewed in Refs. 62 and 63). Thus, the ability of insulin to stimulate cdc2 activity in serum-deprived H4 cells within 30 min leads us to speculate that this insulin-activated cdc2...
activity may play a crucial role in the transition from G1 to S phase. On the other hand, the magnitude of the increase in cdc2 activity observed after insulin is relatively modest; the 1.5–2-fold increase in Suc-1-precipitable H1 kinase activity seen at 30 min should be contrasted with the 2–7-fold increase in erk/MAP kinase activity seen at 5 min. We have not ascertained directly the extent to which the serum starvation has induced the cells to a quiescent state; the presence of a significant fraction of cells that continue to cycle may account for a relatively high base-line activity of cdc2. In addition, the Mono Q elution pattern of Suc-1-precipitable H1 kinase activity is very complex, perhaps reflecting the contributions of multiple molecular forms of cdc2, differing in regulation and subunit composition. Thus, the significance of the modest increase in cdc2 activity induced by insulin as well as the biochemical mechanisms that underlie this response are not known. It will be of interest to determine whether the ability of insulin to activate cdc2 is restricted to permanent or transformed cell lines, such as H4 hepatoma, or is also a component of the action in its "physiological" target cells such as adipocytes and hepatocytes, which are permanently presumed to be in G3 phase. The function/regulation of cdc2 in such cells has received little attention; treatment of sheep platelets with thrombin and platelet-activating factor has recently been shown to activate p34 cdc2 (64).

p54 MAP-2 Kinase Is Proline-directed Protein Kinase of Entirely Novel Specificity—The p54 MAP-2 kinase was detected as the MAP-2 kinase activated in the livers of rats treated with cycloheximide (29). The kinase activity of this 54-kDa polypeptide can be completely abolished by treatment of enzyme with either recombinant rat brain phosphotyrosine phosphatase or Ser/Thr phosphatases (phosphatase 2A > 1) (29, 38). These regulatory features are closely related to the protein kinases of the p42 MAP kinase/erk Peak 1 and 2B SKAIPS kinase family. Earlier studies showed that the p54 MAP-2 kinase exhibits very poor MBP phosphorylation as compared to the p42 MAP kinase, and p54 fails to phosphorylate/reactivate Xenopus (p85) S6 kinase II and rat liver p70 S6 kinase (29). The present results establish that the p54 MAP kinase is essentially completely dependent on the presence of a proline at position +1 in its potential substrates and further show that the p54 MAP-2 kinase represents a third subclass of proline-directed protein kinase distinguishable in its specificity from cdc2 and the 40–45-kDa MAP/MBP/SKAIPS kinases. Amino acid sequences of several pp54 tryptic peptides can be aligned to the catalytic domains of either erk-1 or FUS-3 with ~50% identity, and we have recently isolated partial cDNA corresponding to these sequences. The molecular structure, downstream targets, and role in signal transduction of this novel protein kinase remain to be defined.

In conclusion, these studies have identified a family of insulin-activated, proline-directed protein kinases that share common features both in regard to their regulation and their selection of downstream targets. In regard to regulation, the catalytic subunit of each of these enzymes is subject to phosphorylation at both tyrosine and Ser/Thr residues, and the phosphorylation of both residues must be in the appropriate state (dephosphorylation for cdc2; phosphorylation for Peak 1 and 2B SKAIPS peptide kinases) for kinase activity to be expressed.

The second common feature in this extended protein kinase family is the proline-directed substrate specificity, in particular, the requirement for a proline immediately carboxy-terminal to the Ser/Thr residue. Three subgroups within this specific activity have been identified, of which cdc2 has been most extensively (although not yet fully) defined. It seems certain that most or all of the native substrates for this kinase family will be crucial in the positive and negative regulation of cell division. The mechanisms by which the insulin receptor activates these proline-directed serine(threonine) protein kinases as well as the identity of the native targets of this array of protein kinases, each with a unique and distinguishable proline-directed substrate specificity, will be of considerable interest.

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REFERENCES

1. Rosen, O. M. (1987) Science 237, 1452–1458
2. White, M. P., Maron, R., and Kahn, C. R. (1985) Nature 318, 183–186
3. Ruderman, N., Kapeller, R., White, M. E., and Cantley, L. C. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1411–1415
4. Endeman, G., Yonezawa, K., and Roth, R. A. (1990) J. Biol. Chem. 265, 396–400
5. Avruch, J., Tornqvist, H. E., Gunalsius, J. R., Yurkov, E. J., Kyriakis, J. M., and Price, D. J. (1990) in Handbook of Experimental Pharmacology (Cuesta, R., and Jacobs, S., eds) Vol. 92, pp. 313–366, Springer-Verlag, Federal Republic of Germany
6. Price, D. J., Gunalsius, J. R., and Avruch, J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 7944–7948
7. Banerjee, P., Ahmad, M. F., Grove, J. R., Koslosky, C., Price, D. J., and Avruch, J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8550–8554
8. Erikson, E., and Maller, J. L. (1989) J. Biol. Chem. 264, 13711–13717
9. Jones, S. W., Erikson, E., Bennis, J., Maller, J. L., and Erikson, R. L. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 3377–3381
10. Sommecorn, J., Mulligan, J. A., Lozeman, F. J., and Krebs, E. G. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 8834–8838
11. Klarlund, J. K., and Czech, M. P. (1988) J. Biol. Chem. 263, 15872–15875
12. Kovacina, K. S., Yonezawa, K., Brautigan, D. L., Tonks, N. K., Rapp, U. R., and Roth, R. A. (1990) J. Biol. Chem. 265, 12115–12118
13. Blackshear, P. J., Haupt, D. M., App, H., and Rapp, U. R. (1990) J. Biol. Chem. 265, 12131–12134
14. Ray, L. B., and Sturgill, T. W. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1502–1506
15. Boulton, T. G., Yancopoulos, G. P., Gregory, J. S., Slaughter, C., Moomaw, C., Hsu, J., and Cobb, M. H. (1990) Science 249, 64–67
16. Boulton, T. G., Nyosh, N., Robbins, P. J., Ip, N. Y., Radziejewski, E., Morgenbesser, S. D., DePinto, R. A., Panayotatos, N., Cobb, M., and Yancopoulos G. C. (1991) Cell 65, 663–675
17. Sturgill, T. W., Ray, L. B., Erikson, E., and Maller, J. L. (1988) Nature 334, 715–718
18. Cooper, J. A., and Hunter, T. (1985) Mol. Cell. Biol. 5, 3304–3309
19. Rossmann, A. J., Payne, D. M., Weber, M. J., and Sturgill, T. W. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 6940–6943
20. Anderson, N. G., Maller, J. L., Tonks, N. J., and Sturgill, T. W. (1990) Nature 343, 651–653
21. Sanger, J. S., Padton, H. B., Bader, S. A., and Pelcle, S. L. (1990) J. Biol. Chem. 265, 52–57
22. Ahn, N. G., Seger, R., Bratton, R. L., Diltz, C. D., Tonks, N. K., and Krebs, E. G. (1991) J. Biol. Chem. 266, 4220–4227
23. Ahn, N. G., and Krebs, E. G. (1990) J. Biol. Chem. 265, 11495–11501
24. Ferrell, J. E., and Martin, E. G. (1990) Mol. Cell. Biol. 10, 3020–3026
25. Gotoh, Y., Nishida, E., Matsuda, S., Shin, N., Josako, H., Shiokoma, K., Akiyama, T., Ohta, K., and Sakai, H. (1991) Nature 349, 251–254
An Array of kinase-activated. Protein-directed Iser/Thr Protein Kinases Phosphorylate the p70 S6 Kinase*

Makoto M. Kurahashi, Daniel J. Pintch, John T. Myirambo, Steven Pelcsh, and Joseph A. Vasil

EXPERIMENTAL PROCEDURES

Cell Culture - H4 hepatoma cells were grown to confluence in Swain S7 medium (Sigma) with 10% horse serum and the final volume was 1:1 crude extracts 10.35 ml.

Chromatography of Cell Extracts - Mono: C DEAE chromatography: 100 ml DEAE buffer (pH 7.4) containing 100 mM NaCl, 0.1 mM DTT, 2 mM EDTA, 2 mM EGTA, 100 mM MgCl2, 200 mM NaCl, 2 mM threonin, 2 mM vanadate, 0.1% Triton X-100 and 0.1% BSA. The column was washed with 100 ml of buffer A, and 100 ml of buffer B were collected. The phosphorylation values were determined by analysis of phosphorylation at 32P.

Immunoblotting - Proteins separated on 9% polyacrylamide SDS-Polyacrylamide gels were transferred onto Immobilon P (Millipore). Transfers were blocked in 5% milk in 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2 mM EGTA, 2 mM DTT, 0.1% Tween 20 (IBT), washed in TBS (Tris-buffered saline), washed in TBS-T (Tris-buffered saline containing 0.01% Tween 20), and transferred to Immobilon membranes. Filters were washed in TBS-T, and incubated with an antibody to the carboxyterminal heptapeptide (Incstar, Stillwater, MN) at 1:1000 dilution for 1 h at room temperature. Filters were washed in TBS-T, and incubated with a peroxidase-labeled secondary antibody (1:2000 dilution) for 1 h at room temperature. Filters were washed in TBS-T, and incubated with 3,3'-diaminobenzidine tetrahydrochloride (Sigma) at 1:500 dilution in 0.05 M Tris-HCl, pH 7.4.

Protein Kinase Assays - Extracts, column fractions and immunoprecipitates were assayed for phosphotransferase of various protein and peptide substrates at concentrations indicated in the text. Reactions mixtures contained 50 mM NPPS, pH 7.4, 10 mM MgCl2, 1 mM DTT, 5 PM MY-51 peptide, and 100 mM NaCl. Reactions were incubated at 30'C for 15 min. After stopping the reactions by addition of 100 mM EDTA, the phosphorylation values were determined by analysis of phosphorylation at 32P.

Supplemental Material

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RESULTS

Identification of Peaks 1, 2A, and 2 SKAIPS Peptide Kinase as Insulin-activated erk/MAP Kinases

The Mono Q and subsequent Superose gel filtration eluates were assayed for protein kinase activity toward MAP-2 and MBP, the profiles of SKAIPS peptide kinase activity. MMP kinase and MAP-3 kinase were coincident, and nearly identical on both columns (Fig. 1). This evidence suggested that at least some of the SKAIPS peptide kinase corresponded to protein kinases previously characterized using these protein substrates (14,15,21,26). This appeared especially likely for Peaks 1 and 2B, which exhibited an gel filtration an elution near ovalbumin, similar to that previously observed for the insulin/nitrogen-stimulated p42 MAP kinase and star p44 MBP kinase. A characteristic property of this 40-45 kDa group of nitrogen-activated MAP/MBP protein is their susceptibility to deactivation by both PTPase and the Ser/Thr phosphatase-2A (20). In fact, Peaks 1 and 2B SKAIPS kinase are potentially and specifically inactivated by recombinant rat brain protein tyrosine phosphatase as well as by protein phosphatase-2A, whereas Peak 2A SKAIPS kinase is not reproducibly altered by these phosphatases (Fig. 3).

The Mono Q chromatography of Mono Q peaks 1 and 2 SKAIPS peptide kinase activity resolved on Mono Q chromatography (see Fig. 1) were pooled and assayed for protein kinase activity toward MAP-2 and MBP as described in Methods. Fractions (0.5 ml) were assayed for SKAIPS peptide (e), myelin basic protein (O) or MAP-2 (W) kinase.

Immunoblot analysis of SKAIPS peptide kinase activity in cell extracts revealed that the enzymatic activity observed in the Mono Q chromatography (see Fig. 1) was pooled and assayed for protein kinase activity toward MAP-2 and MBP as described in Methods. Fractions (0.5 ml) were assayed for SKAIPS peptide (e), myelin basic protein (O) or MAP-2 (W) kinase.

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Fig. 3. Deactivation of SKAIPS peptide kinases by tyrosine or Ser/Thr-specific phosphatase.

Alignment of Superose-12 fractions corresponding to Peaks 1 and 2 coincident to those shown in Fig. 2 (Panel A: Peak 1; Panel B: Peak 2, 3B) were treated with upper panels, A and B) or 0.5 ml brain cortical synaptic protein tyrosine phosphatase 1 (rat PTPase-1) alone (O), or PTPase 1 plus 1 mg VC (b) or left untrated (h), and then assayed for SKAIPS peptide phosphorylation as described in Methods. Panel C (Bottom) shows the phosphorylation of SKAIPS peptide phosphorylation as described in Methods. Panel D shows the phosphorylation of SKAIPS peptide phosphorylation as described in Methods.

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