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Isolation of a yeast protein kinase that is activated by the protein encoded by *SRP1* (Srp1p) and phosphorylates Srp1p complexed with nuclear localization signal peptides

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ABSTRACT **Srp1p, the protein encoded by *SRP1* of *Saccharomyces cerevisiae*, is a nuclear-pore-associated protein. Its *Xenopus* homolog, importin, was recently shown to be an essential component required for nuclear localization signal (NLS)-dependent binding of karyophilic proteins to the nuclear envelope [Gorlich, D., Prehn, S., Laskey, R. A. & Hartman, E. (1994) *Cell* 79, 767–778]. We have discovered a protein kinase whose activity is stimulated by Srp1p (Srp1p fused to glutathione *S*-transferase and expressed in *Escherichia coli*) and is detected by phosphorylation of Srp1p and of a 36-kDa protein, a component of the protein kinase complex. The enzyme, called Srp1p kinase, is a protein-serine kinase and was found in extracts in two related complexes of ≈180 kDa and 220 kDa. The second complex, when purified, contained four protein components including the 36-kDa protein. We observed that, upon purification of the kinase, phosphorylation of Srp1p became very weak, while activation of phosphorylation of the 36-kDa protein by Srp1p remained unaltered. Significantly, NLS peptides and the nuclear proteins we have tested greatly stimulated phosphorylation of Srp1p, suggesting that Srp1p, complexed with karyophilic proteins carrying an NLS, is the *in vivo* substrate of this protein kinase.**

SRP1, the gene for a nuclear-pore-associated protein (Srp1p), was originally identified as a suppressor of certain temperature-sensitive mutations of RNA polymerase I in *Saccharomyces cerevisiae* (1). Srp1p is mainly localized to the nuclear pore as judged by immunofluorescence microscopy (1) and by its direct interaction with the products of *NUP1* and *NUP2* (Nup1p and Nup2p, respectively), two nuclear pore proteins (2). *SRP1* is an essential gene, and its mutation or depletion of the gene product leads to pleiotropic phenotypes (3). Because of the association of Srp1p with the nuclear pore, the possibility of direct participation of Srp1p in nuclear transport processes was considered, which would explain the observed mutational effects as indirect consequences of the primary defects in the transport process (3). The alternative interpretation was that Srp1p functions as a regulatory element localized at the pore, controlling various nuclear structures and activities, perhaps in response to nuclear–cytoplasmic transport (3). Gorlich *et al.* (4) have now established an essential role of a *Xenopus* homolog of Srp1p, importin, in nuclear protein transport.

Previous studies have demonstrated that nuclear transport of proteins involves recognition of a nuclear localization signal (NLS) and that the transport can be divided into two stages: binding of proteins to the nuclear pore complex, which is temperature- and energy-independent, and subsequent temperature- and energy-dependent translocation of the proteins through the nuclear pore complex (for reviews, see refs. 5–7). Importin/Srp1p was required in the first binding step in the *Xenopus in vitro* protein import system (4). Furthermore, the mammalian 54/56-kDa protein purified as the protein responsible for NLS recognition ("NLS receptor"; ref. 8) has turned out to be a homolog of importin/Srp1p (S. Adam, unpublished experiments cited in ref. 9). We have also observed that a glutathione *S*-transferase (GST)–Srp1p fusion protein binds NLS peptides specifically, confirming the postulated NLS receptor function of Srp1p (see below). These developments appear to have clarified the initial step in nuclear import; proteins carrying an NLS first interact with importin/Srp1p (or Srp1p–p97 complex; for p97, see refs. 9 and 10) and then bind to the nuclear pore complex, perhaps through the interaction of Srp1p with Nup1, Nup2, and/or other pore components. However, the question of how the second energy-dependent translocation step takes place is unanswered except for the identification of some "cytoplasmic" soluble factors, such as the RAN–TC4-B2 complex (11–13) and Hsc70 (14, 15), that somehow participate in this process (for a review, see ref. 9).

In this paper, we describe the discovery of a protein kinase in yeast cell extracts, whose activity is stimulated by Srp1p fused to GST and is detected by phosphorylation of Srp1p and a 36-kDa protein, a component of the protein kinase complex. We have purified this kinase, which we call Srp1p kinase, and have found that phosphorylation of Srp1p by the purified enzyme is greatly stimulated by NLS peptides. We discuss these findings in connection with the possible functions of Srp1p subsequent to the initial energy-independent nuclear-pore-binding step in nuclear transport.

MATERIALS AND METHODS

Preparation of GST–Srp1p and GST–ΔN-Srp1p. GST–Srp1p is a fusion protein consisting of GST and Srp1p and was prepared by expressing the fusion gene in *Escherichia coli* from plasmid pNOY3198. This plasmid was prepared as follows: the *SRP1* gene carrying a 5′ *Bam*HI site was prepared by PCR with pNOY3152 (a pUC19 derivative carrying *SRP1*) as a template and two oligonucleotide primers, 5′-TTTGGATCCATGGATAATGGTACAGATTC-3′ and 5′-GCGGATAACAATTTCACACAGGA-3′. The product was digested with *Bam*HI and then inserted between the *Bam*HI and *Sma* I sites of pGEX-2T (Pharmacia). GST–ΔN-Srp1p is a deletion mutant of GST–Srp1p that lacks the N-terminal 216 amino acids of the 542-amino acid Srp1p segment and was prepared by expressing the mutant gene from pNOY3199. Plasmid pNOY3198 carries two *Nco* I sites, one just preceding the *SRP1* coding region and the other within the coding region. Digestion of pNOY3198 with *Nco* I followed by ligation fused the C-terminal *SRP1* coding for 326 amino acids to the upstream *GST* gene in-frame, yielding pNOY3199. For preparation of the proteins, a 1-liter culture of *E. coli* DH5α carrying pNOY3198 (or pNOY-

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Abbreviations: DTT, dithiothreitol; GST, glutathione *S*-transferase; NLS, nuclear localization signal; Srp1p, protein encoded by *SRP1*; SV40, simian virus 40; TAg, tumor antigen.

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3199) was treated with isopropyl β-D-thiogalactoside (200 μg/ml) at 37°C for 4 h. Cells were collected and disrupted by sonication in 20 ml of 150 mM NaCl/20 mM sodium phosphate, pH 7.3/1% Triton X-100/1 mM phenylmethyl-sulfonyl fluoride. Cell extracts obtained after centrifugation were applied to 1 ml of glutathione-agarose beads (Sigma) suspended in buffer A [50 mM Tris·HCl, pH 8.0/1 mM dithiothreitol (DTT)/1 mM EDTA/5% (vol/vol) glycerol/50 mM $(NH_4)_2SO_4$], and GST–Srp1p (or GST–ΔN-Srp1p) bound was eluted with buffer A containing 5 mM glutathione (16). The eluate was next applied to 0.5 ml of Q-Sepharose (Pharmacia) equilibrated with buffer A, and after washing the column, GST–Srp1p (or GST–ΔN-Srp1p) was eluted with 1 ml of buffer A containing 200 mM $(NH_4)_2SO_4$. GST was prepared similarly by using *E. coli* DH5α carrying pGEX-2T.

Purification of Srp1p Kinase. *S. cerevisiae* JHR20-2CΔ1 (*Mata his3-Δ200 ura3-52 leu2-3,112 pep4-Δ::URA3*; ref. 17) was used. Cells (100 g) were suspended in 200 ml of buffer B (20 mM Hepes, pH 7.4/1 mM EDTA/1 mM DTT/5% glycerol/1 mM phenylmethylsulfonyl fluoride) containing a 0.1% volume of the protease inhibitor mixture [chymostatin (100 μg/ml)/aprotinin (100 μg/ml)/pepstatin (100 μg/ml)/leupeptin (100 μg/ml)/antipain (100 μg/ml)/bepstatin (20 μg/ml)/aminobenzamidine (2 mg/ml)] and disrupted by a Bead-beater (Biospec Products, Bartlesville, OK) as described (18). The homogenate was centrifuged at 100,000 × *g* at 4°C for 30 min and the pellets were extracted with buffer B containing 0.1% Tween 20, 200 mM $(NH_4)_2SO_4$, and 0.1% of the protease inhibitor mixture, followed by centrifugation at 100,000 × *g* at 4°C for 60 min. The supernatant was applied to a Q-Sepharose column (30 ml) equilibrated with buffer B containing 200 mM $(NH_4)_2SO_4$, followed by elution with a 200-ml linear gradient of 200–800 mM $(NH_4)_2SO_4$ in buffer B. Fractions showing Srp1p kinase activity that were eluted at ≈380 mM $(NH_4)_2SO_4$ were pooled, diluted with buffer B to make the $(NH_4)_2SO_4$ concentration 200 mM, and applied to a Mono Q (Pharmacia) 0.5 × 5 cm column, followed by elution with a 15-ml linear gradient of 200–800 mM $(NH_4)_2SO_4$ in buffer B. Fractions with the kinase activity were eluted at 400 mM $(NH_4)_2SO_4$. They were pooled, dialyzed against buffer B containing 25 mM $(NH_4)_2SO_4$, and applied to a 5-ml SP-Sepharose column (Pharmacia). A 40-ml linear gradient of 25–800 mM $(NH_4)_2SO_4$ in buffer B was used for elution. Two peaks of Srp1p kinase activity were observed corresponding to 180 mM and 250 mM $(NH_4)_2SO_4$ (called Srp1p kinase I and II, respectively). Kinase I and II fractions were pooled separately, and each was applied to a HiLoad (Pharmacia) 1.6 × 60 cm gel filtration column. Form I kinase was eluted at a position corresponding to 180 kDa, and form II was eluted at 220 kDa. Proteins contained in kinase fractions were analyzed by SDS/PAGE followed by silver staining (19).

Srp1p Kinase Assay. Reactions were carried out in 40 μl of 20 mM Hepes, pH 7.4/1 mM EDTA/1 mM DTT/5 mM $MgCl_2$/1 μCi of [γ-^{32}P]ATP (4.2 nM; 1 Ci = 37 GBq) containing 2 μg of GST–Srp1p (0.58 μM) (or 2 μg of GST or GST-ΔN-Srp1p as control) and a sample (usually 5 μl) containing kinase. After incubation at room temperature (23°C) for 25 min, two methods were used to determine the degree of phosphorylation. For the filter binding assay, 500 μl of stop buffer (20 mM Hepes, pH 7.4/0.1 mM EDTA/1 mM DTT/5 mM $MgCl_2$/50 mM NaCl/1 mM sodium pyrophosphate) was added, and the mixture was passed through a nitrocellulose filter. After washing with 10 ml of stop buffer twice, filters were dried and the amounts of ^{32}P-labeled proteins retained were determined. Values obtained for control (GST or GST–ΔN-Srp1p) were then subtracted from experimental values obtained with GST–Srp1p. For SDS/PAGE assay, 15 μl of SDS buffer [150 mM Tris·HCL, pH 6.8/300 mM DTT/6% (wt/vol) SDS/0.3% bromophenol blue/30% glycerol] was added, and the mixture was boiled for 5 min at 95°C. Samples were then analyzed by SDS/PAGE, followed by autoradiography.

NLS Peptide Binding Assay. The NLS binding assay was adapted from the method used by Adam *et al.* (20). Synthetic NLS and control peptides were obtained from Bio-synthesis (Lewisville, TX) or from Harumi Kasamatsu (Department of Biology, University of California, Los Angeles). Peptide labeling (21) was carried out using Iodo-Beads (Pierce) by the manufacturer's instructions. For the assay; 2 μg of GST–Srp1p was mixed with ^{125}I-labeled NLS peptide (1 μM) with or without a competitor peptide in 40 μl of 150 mM NaCl/20 mM sodium phosphate, pH 7.3, and incubated at room temperature for 20 min. Five microliters of 5 mM disuccinimidyl suberate in dimethyl sulfoxide was then added, followed by incubation at room temperature for 20 min (22). The ^{125}I-labeled peptide cross-linked to GST–Srp1p was separated from the free peptide by SDS/PAGE and analyzed by autoradiography.

Other Methods. Phosphoamino acids were analyzed after hydrolysis of ^{32}P-labeled proteins in 6 M HCl at 110°C for 1 h (23). Samples were mixed with reference phosphoserine, phosphothreonine, and phosphotyrosine and subjected to electrophoresis on a TLC plate at pH 3.5, followed by ninhydrin reaction and autoradiography.

RESULTS

Detection and Purification of Srp1p Kinase. Srp1p kinase was first detected by incubating yeast cell extracts with [γ-^{32}P]ATP with and without a recombinant GST–Srp1p fusion protein expressed in *E. coli*. Phosphorylation of two proteins was prominent in the presence of GST–Srp1p relative to control; one was a protein of ≈36 kDa (called p36 hereafter) and the other was ≈90 kDa, which corresponds to GST–Srp1p (see Fig. 1, lanes 1–6, for a partially purified kinase preparation). The phosphorylation activity appeared to be most abundant in a fraction obtained by extracting an insoluble particulate fraction using a buffer containing salt [200 mM $(NH_4)_2SO_4$] and detergent (0.1% Tween 20). We purified a protein kinase responsible for the observed phosphorylation ≈8000-fold from this fraction (estimated by the filter binding assay) by a combination of several column chromatographic procedures (Figs. 2 and 3). Upon chromatography on SP-Sepharose, the activity was separated into two peaks (Fig. 2) and final preparations were obtained from each of these two peaks. Estimates of the size of the enzyme and analyses of the protein components indicated that Srp1p kinase exists in two complexes: One (Srp1p kinase I) is ≈180 kDa in size, and the

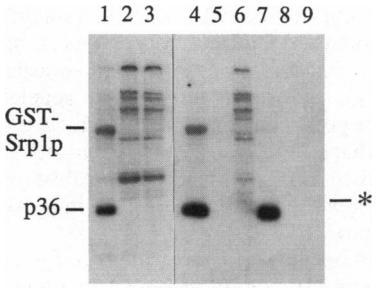

FIG. 1. Phosphorylation of GST–Srp1p and p36. Phosphorylation was carried out with a partially purified Srp1p kinase preparation (0.7 μg of protein, after Q-Sepharose; lanes 1–6) or purified Srp1p kinase II (5 ng of protein, after HiLoad; lanes 7–9). Lanes 1–3 are from one experiment and lanes 4–9 are from another experiment. Lanes: 1, kinase + GST–Srp1p; 2, kinase + GST; 3, kinase only; 4, kinase + GST–Srp1p; 5, kinase + GST–ΔN-Srp1p; 6, kinase only; 7, kinase + GST–Srp1p; 8, kinase + GST–ΔN-Srp1p; 9, kinase only. The position of the very weak band seen in lane 9 (indicated by an asterisk) corresponds to that of p38 (see the text). Autoradiograms of SDS/PAGE gels of phosphorylated proteins are shown.

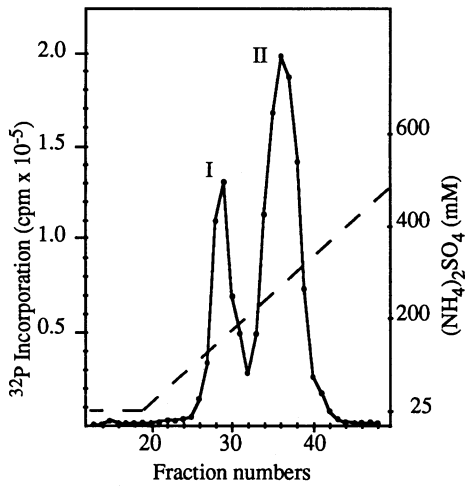

FIG. 2. SP-Sepharose chromatography of Srp1p kinase. A linear gradient of 25–800 mM $(NH_4)_2SO_4$ was used for elution. Aliquots of fractions were used to assay Srp1p kinase in the filter binding assay (solid line) and for conductivity measurement to estimate $(NH_4)_2SO_4$ concentrations (broken line). Positions of two enzyme complexes, Srp1p kinase I and II, are indicated as I and II, respectively.

other (Srp1p kinase II) is ≈220 kDa in size, as estimated by comparison with the elution pattern of marker proteins [β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), and bovine serum albumin (67 kDa)]. As can be seen from the data for Srp1p kinase II (Fig. 3), elution patterns of four proteins (38, 36, 32.5, and 31 kDa) and of kinase activity coincided, suggesting that this enzyme complex is largely homogeneous and these four proteins represent components of the enzyme. The phosphorylated protein, p36, recognized by autoradiograms (Fig. 3*B*) coincided with the second largest protein band recognized by silver staining. Similar data (not shown) indicated that Srp1p kinase I contains three (38, 36, and 31 kDa) of these protein components but does not contain the 32.5-kDa protein. So far, we have not found a significant difference in protein phosphorylation reactions between the two enzyme preparations. The data for purified kinase shown below are from experiments using the purified preparation of Srp1p kinase II.

By using a partially purified fraction, we examined substrate specificity of the enzyme in the presence of GST–Srp1p. Ten commercially available peptide substrates for known protein kinases were examined. [These included substrates of Src tyrosine protein kinase (Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly), protein kinase A (Arg-Arg-Lys-Ala-Ser-Gly-Pro, Gly-Arg-Gly-Leu-Ser-Leu-Ser-Arg, Leu-Arg-Arg-Ala-Ser-Leu-Gly, and Leu-Arg-Arg-Ala-Ser-Val-Ala), protein kinase C (Gln-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu, Lys-Arg-Thr-Leu-Arg-Arg, and Val-Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu), and calmodulin-dependent kinase (Pro-Leu-Ala-Arg-Thr-Leu-Ser-Val-Ala-Gly-Leu-Pro-Gly-Lys-Lys, and Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys).] None of these was found to be phosphorylated. Proteins such as casein, protamine, and histone were also not phosphorylated by the Srp1p kinase. Although there might be other yeast proteins that could be phosphorylated by Srp1p kinase (see below and *Discussion*), we have not identified any other than p36 and Srp1p. We analyzed amino acids labeled in p36 and GST–Srp1p and identified phosphoserine, but no phosphothreonine or phosphotyrosine, in these two proteins after the phosphorylation reaction (data not shown). We also demonstrated the presence of phosphorylated Srp1p *in vivo* by metabolically labeling cells with ^{32}P, followed by immunoprecipitation of Srp1p, and observed the presence of phosphoserine, but not the other two phosphoamino acids (unpublished experiments).

A unique feature of the phosphorylation reaction observed with Srp1p kinase is its dependency on GST–Srp1p. It should be noted that GST–Srp1p is an activator and not an autocatalytic protein kinase. Incubation without an Srp1p kinase preparation did not cause any phosphorylation of GST–Srp1p (see Fig. 5, lanes 4, 5, 11, and 12), whereas a weak phosphorylation of one or more proteins was observed with purified Srp1p kinase in the absence of GST–Srp1p (Fig. 1, lane 9, and see Fig. 5*A*, lanes 13 and 14; indicated by an asterisk). Thus, we conclude that the purified protein complex is the kinase. As described above, p36, which is phosphorylated strongly upon mixing kinase preparations with GST–Srp1p, is a genuine component of Srp1p kinase. However, p36 is different from the protein in the purified kinase preparations that was phosphorylated in the absence of GST–Srp1p. The latter appears to be the p38 component. The significance of phosphorylation of these proteins in relation to that of Srp1p must await further studies. We also note that in all of the experiments described in this paper, we used the GST–Srp1p fusion protein and compared with control GST or GST–Srp1p carrying a large N-terminal deletion (GST–ΔN-Srp1p). Neither GST (Fig. 1, lane 2) nor GST–ΔN-Srp1p (Fig. 1, lanes 5 and 8) caused stimulation of p36 phosphorylation and failed to undergo phosphorylation by the kinase. Thus, the results obtained with the GST–Srp1p fusion protein will almost certainly apply for Srp1p. [We noticed that GST–Srp1p, in addition to causing specific phosphorylation of p36 and GST–Srp1p, inhibited phosphorylation of proteins in (partially purified) kinase preparations (Fig. 1; compare lane 1 with lane 3 and lane 4 with lane 6). Such inhibition was also observed with GST–ΔN-Srp1p (Fig. 1; compare lanes 5 and 6), but not with GST (Fig. 1, compare lanes 2 and 3). It appears that the deletion in GST–ΔN-Srp1p abolishes the ability to stimulate phosphorylation of p36 and its own phosphorylation but retains the ability

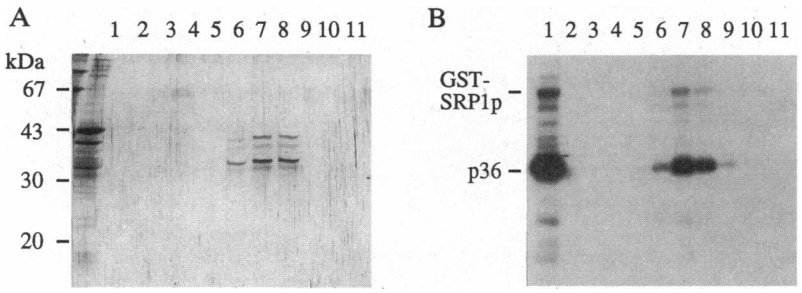

FIG. 3. HiLoad gel filtration chromatography of Srp1p kinase II. Kinase fraction II obtained after SP-Sepharose column (peak II in Fig. 2) was subjected to gel filtration chromatography. Samples (lanes 2–11, representing fractions 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24) and the input sample (lane 1) were analyzed for protein (a silver-stained SDS/PAGE gel shown in *A*) and for kinase activity (an autoradiogram of SDS/PAGE gel of reaction products shown in *B*). The sizes of the four protein components in the kinase peak were estimated by comparing their positions with those of molecular mass standards shown on the left in *A*.

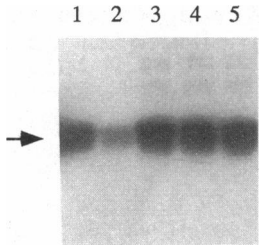

FIG. 4. Binding of a ^{125}I-labeled SV40 TAg NLS peptide to GST–Srp1p in the presence of competitors. Binding reactions were carried out by using 1 μM ^{125}I-labeled wild-type SV40 TAg NLS peptide (for the sequence, see the text) in the presence of competitor nonradioactive wild-type (lanes 1 and 2) or mutant (reverse NLS; see the text) peptide (lanes 4 and 5). Concentration of competitors: 0 (lane 3); 6.3 μM (lanes 1 and 4); 31 μM (lanes 2 and 5). An autoradiogram of an SDS/PAGE gel is shown. The position of GST–Srp1p carrying the cross-linked ^{125}I-labeled NLS is shown by an arrow.

to interact with the kinase, leading to inhibition of phosphorylation of other proteins in the preparations. The significance of this inhibitory activity of Srp1p is unknown.]

Another feature we observed in the course of kinase purification was that phosphorylation of GST–Srp1p relative to that of p36 was strong when the kinase preparations were relatively crude, and it became much weaker when the kinase was purified (see Fig. 1; compare lanes 1 and 4 with lane 7). We thought that there were some proteins in the crude kinase preparations that stimulated phosphorylation of Srp1p by Srp1p kinase and considered the possibility that they might be proteins carrying an NLS and interacting with Srp1p. Thus, we first examined the proposition that Srp1p is an NLS receptor (see Introduction).

Srp1p Is an NLS Receptor. To examine the postulated interaction between Srp1p and NLS, we followed the method of Adam and Gerace (8) using a synthetic peptide containing simian virus 40 (SV40) large tumor antigen (TAg) NLS, Gly-Tyr-Gly-Pro-Lys-Lys-Lys-Arg-Lys-Val-Glu-Asp, and a control nonfunctional "reverse NLS" peptide, Gly-Tyr-Gly-Asp-Glu-Val-Lys-Arg-Lys-Lys-Lys-Pro (reversed parts underlined; see refs. 4 and 20). The SV40 NLS peptide functions in yeast (24) and, hence, was used in this experiment. The NLS peptide was labeled with ^{125}I, incubated with GST–Srp1p in the presence or absence of competitor peptides, and treated with disuccinimidyl suberate, and its cross-linking to GST–Srp1p was analyzed by SDS/PAGE. As shown in Fig. 4, interaction of the ^{125}I-labeled NLS peptide with Srp1p was in fact demonstrated, and this interaction was competed by excess nonradioactive NLS peptide but not by the reverse NLS peptide. The results confirm the unpublished experiments by S. Adam (cited in ref. 8) that the rat 54/56 NLS receptor is probably a homolog of Srp1p.

NLS Peptides Stimulate Phosphorylation of GST–Srp1p by Srp1p Kinase. GST–Srp1p phosphorylation by purified Srp1p kinase was carried out in the presence of the TAg NLS peptide or the reverse NLS peptide described above. As can be seen in Fig. 5*A*, a large (6.0-fold; ^{32}P radioactivity in pertinent gel slices) stimulation was observed for the TAg NLS peptide (lane 2 compared with lane 1), but not for the control reverse peptide (20% stimulation; lane 3 compared with lane 1; see also Fig. 5*B*, lanes 1–4). We also examined the stimulatory activity with another pair of peptides derived from the NLS of SV40 Vp2/3 capsids: Cys-Gly-Tyr-Gly-Pro-Asn-Lys-Lys-Lys-Arg-Lys-Leu-Gly-Gly and its Lys → Val mutant, Cys-Gly-Tyr-Gly-Pro-Asn-Lys-Val-Lys-Arg-Lys-Leu-Gly-Gly (mutated positions underlined) (25). The wild-type sequence contains the NLS for Vp2 (and Vp3), and the Lys → Val mutation abolishes the NLS activity of the peptide as judged by microinjection experiments using (nonnuclear) porter proteins conjugated to these peptides (25). As can be seen in Fig. 5*A* (lanes 1, 8, and 9), a large (12-fold) stimulation of GST–Srp1p phosphorylation was again observed with this wild-type (Vp2/3) NLS peptide, but only a small (2.8-fold) stimulation was observed with the mutant NLS peptide. Several other non-NLS peptides were also tested, and no stimulation was observed (an example is shown in Fig. 5*A*, lane 10). Thus, it appears that NLS peptides that interact with Srp1p stimulate phosphorylation of Srp1p. We then examined directly whether some nuclear proteins cause the same stimulatory effects as those observed with NLS peptides. As can be seen in Fig. 5*B*, two nuclear proteins, histone and protamine, in fact caused great stimulation of GST–Srp1p phosphorylation, whereas three control nonnuclear proteins, casein, alcohol dehydrogenase, and GST, did not show such effects. It is likely that Srp1p may take a specific conformation(s) upon interaction with NLS of karyophilic proteins and this conformation(s) is required for efficient phosphorylation by Srp1p kinase.

DISCUSSION

We have discovered a protein kinase, Srp1p kinase, in yeast extracts whose activity is greatly stimulated by Srp1p. The

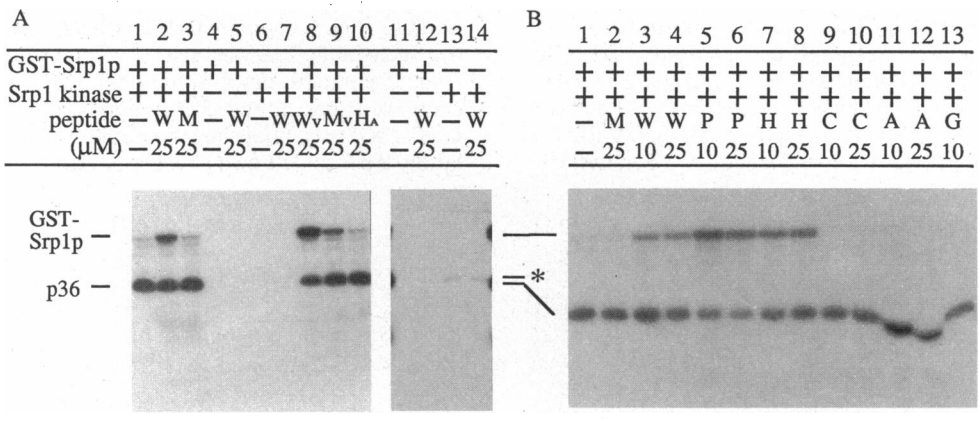

FIG. 5. Effects of NLS peptides and nuclear proteins on Srp1p kinase reactions. Kinase reactions were carried out with purified Srp1p kinase II and GST–Srp1p in the presence of various peptides or proteins (10 or 25 μM as indicated): W, TAg NLS; M, the reverse NLS; W_v, Vp2/3 capsid NLS peptide; M_v, the mutant form of W_v (see the text); H_A, control HA1 epitope peptide from influenza virus, Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala; P, protamine (from salmon, Sigma); H, histone (from calf thymus, Sigma); C, casein (from bovine milk, Sigma); A, alcohol dehydrogenase (from baker's yeast, Sigma); G, GST. The portion of lanes 1–10 in *A* is an autoradiogram of the gel after a 10-min exposure. Lanes 11–14 correspond to lanes 4–7 of the same gel after a 350-min exposure, showing a weak phosphorylation of the 38-kDa component of the enzyme (indicated by an asterisk). In the experiment shown in *B*, the molecular mass of alcohol dehydrogenase monomer (37 kDa) is very close to that of p36, which caused an anomaly in the position of p36 in lanes 11 and 12.

enzyme was purified in two different but related complexes, each of which is a multisubunit complex containing the p36 subunit that becomes phosphorylated in the presence of Srp1p. The two complexes differ in the presence of an additional protein component, p32.5, that is in one complex and not in the other. Thus, p32.5 in complex II is not a catalytic subunit. The significance of the presence of the two different complexes in the extracts is not clear, but the possibility cannot be excluded that this might be created during cell disruption and enzyme extraction from the insoluble membrane/particulate fraction. By using purified preparations, we have demonstrated that Srp1p causes a large stimulation of phosphorylation of p36 and serves as a substrate to be phosphorylated by the enzyme as well. We have also demonstrated that Srp1p is in fact a yeast NLS receptor as suspected from the work using *Xenopus* and mammalian nuclear transport systems (4, 9). Thus, Srp1p appears to be a multifunctional protein with at least two functions that can be assayed *in vitro*, i.e., binding of NLS peptides and stimulation of Srp1p kinase phosphorylation.

Srp1p kinase appears to be a distinct protein kinase. Its phosphorylation activity as judged by p36 phosphorylation is greatly stimulated by Srp1p; that is, Srp1p is an activator of the enzyme, and NLS peptides are not required for this activation. In contrast, the efficient phosphorylation of Srp1p, the only substrate other than p36 identified so far, requires the presence of NLS peptides or proteins, such as histone and protamine, that presumably carry an NLS. Thus, the physiologically relevant form of the substrate for the kinase is probably Srp1p complexed with proteins carrying an NLS (discussed further below). An important question is whether Srp1p kinase has any other protein substrates beside Srp1p and p36, but we have to await further studies to answer this question.

The biological significance of activation of Srp1p kinase by Srp1p, Srp1p phosphorylation by the kinase, and the functional role of the kinase in yeast cells have not been precisely determined. However, since participation of Srp1p in nuclear transport as an NLS receptor appears to be established, it is very likely that Srp1p kinase may also be involved in nuclear transport. Silver and coworkers have identified a 70-kDa NLS binding protein from yeast (26) and demonstrated that phosphatase treatment abolished its NLS binding activity (27). Although it is not known whether this NLS binding protein is identical to Srp1p, Srp1p may also require phosphorylation for NLS binding. However, it should be emphasized that phosphorylation of Srp1p by Srp1p kinase described in this study is different from the phosphorylation discussed by Silver and coworkers (27). Our GST–Srp1p, purified as a recombinant protein expressed in *E. coli*, has the ability to interact with NLS peptides. As discussed above, phosphorylation of Srp1p by Srp1p kinase requires the presence of NLS peptide, and hence, this phosphorylation must take place *after* the binding of NLS proteins to Srp1p. Thus, if activation of Srp1p kinase by Srp1p followed by phosphorylation of Srp1p (Srp1p–NLS protein complexes) is in fact obligatorily involved in nuclear protein transport, as it seems likely, the known ATP requirement for the later step(s) of transport could be at least partly accounted for by the ATP requirement for Srp1p kinase reactions. However, it is not clear whether phosphorylation of Srp1p itself, phosphorylation of p36, phosphorylation of some other unidentified protein(s), or a combination of these reactions would be required for NLS-containing proteins to achieve translocation through nuclear pores.

Another possibility regarding the role of Srp1p kinase is that Srp1p functions as a regulatory element controlling various nuclear structures and activities perhaps in response to nuclear–cytoplasmic transport or independently of its transport function, as discussed (2, 3). Srp1p kinase might then participate in such hypothetical regulatory functions as an element of a signal transduction system. In this regard, it should be noted that RAN/TC4 (Gsp1p in yeast) is another protein identified as one of the "cytoplasmic" factors required for nuclear transport (11, 12, 28), and this protein, together with its guanine nucleotide exchange factor, RCC1 (Prp20p in yeast), participates in various nuclear functions (for review, see ref. 29). These nuclear functions of RAN/TC4 are independent of its role in nuclear transport (30, 31). Therefore, we cannot exclude the possibility that Srp1p might participate in nuclear functions independently of its established role as a NLS receptor. Cloning of the genes for the kinase subunit proteins and subsequent genetic analysis should clarify functional roles of Srp1p kinase and phosphorylation of Srp1p in cellular physiology, and nuclear protein transport in particular.

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