Regulation of binding of lamin B receptor to chromatin by SR protein kinase and cdc2 kinase in *Xenopus* egg extracts*

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Running title: Regulation of binding of LBR to chromatin

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Participation of multiple kinases in regulation of the binding of lamin B receptor (LBR) to chromatin was previously suggested (Takano et al. 2002. Eur. J. Biochem. 269, 943-953). To identify these kinases, regulation of the binding of the nucleoplasmic region (NK, amino acid residues 1-211) of LBR to sperm chromatin was studied using a cell-cycle dependent *Xenopus* egg extract *in vitro*. The binding was stimulated on specific phosphorylation of the NK-fragment by an S-phase egg extract. Protein depletion with beads bearing SF2/ASF, which binds SR protein kinases, abolished this stimulation, suggesting that an SR protein kinase(s) is responsible for the activation of LBR. This was confirmed by direct phosphorylation and activation with recombinant SR protein-specific kinase 1. The binding of the NK-fragment to chromatin pretreated with an S-phase extract was suppressed by incubation with an M-phase extract. Enzyme inhibitor experiments revealed that multiple kinases participate in the suppression. One of these kinases was shown to be cdc2 kinase using a specific inhibitor, roscovitine, and protein depletion with beads bearing p13, which specifically binds cdc2 kinase. Experiments involving a mutant NK-fragment showed that the phosphorylation of serine 71 by cdc2 kinase is responsible for the suppression.

Key words: cdc2, lamin B receptor, LBR, nuclear envelope, SRPK.

[1]The abbreviations used are: DMAP, 6-dimethylaminopurine; GST, glutathione-S-transferase; GST-NK, GST-fused fragment comprising amino acid residues 1-211 of

human LBR; GST-RS, GST-fused fragment comprising amino acid residues 53-89 of human LBR; LAP, lamina-associated polypeptide; LBR, lamin B receptor; MC, M-phase *Xenopus* egg cytosol fraction; PKC, protein kinase C; SC, S-phase *Xenopus* egg cytosol fraction; SR protein, serine/arginine-rich protein; SRPK, SR protein-specific kinase.

The nuclear envelope separates the nucleoplasm from the cytoplasm and thereby organizes the nuclear architecture. The nuclear envelope consists of two lipid bilayers (inner and outer), nuclear pore complexes and the nuclear lamina, and undergoes repeated dynamic assembly and disassembly in every cell cycle in higher eukaryotes. Studies on the molecular mechanisms underlying nuclear envelope assembly and disassembly are important to understand the mechanisms underlying cell division and prenucleus formation. Studies on the organization of the nuclear architecture by the nuclear envelope during the cell cycle are also important to understand how mutants of nuclear envelope proteins, *i.e.* lamin A/C (1), emerin (2), and lamin B receptor (LBR) (3), cause genetic diseases (4).

The assembly and disassembly of the nuclear envelope in the cell cycle accompany association and dissociation of the inner nuclear membrane with and from chromatin, respectively. All major inner nuclear membrane proteins such as the lamina-associated polypeptide 2 (LAP2) family, LBR, and emerin, are known to bind to chromatin *in vitro* (5, 6), and to accumulate on the surface of chromatin during the anaphase-terophase transition *in vivo* (7). Therefore, the binding of these proteins to chromatin is thought to support the binding of the inner nuclear membrane to chromatin. Indeed, LBR and LAP2 have been demonstrated to play functional roles in the interaction of the nuclear envelope and chromatin through *in vitro* experiments, as follows. Depletion of LBR from nuclear envelope precursor vesicles suppresses nuclear assembly (8), neutralization

of LBR by antibodies in a sea urchin cell-free system inhibits targeting of nuclear envelope precursor vesicles to the surface of chromatin (9), and the addition of an amino-terminal fragment of LAP2 inhibits nuclear envelope formation (10).

The binding of these proteins to chromatin has to be regulated precisely throughout the cell cycle. The binding of LAP2 to chromatin was shown to be regulated by phosphorylation using HeLa cell extracts (5). LAP2β is phosphorylated by PKC (11). We demonstrated that the binding of LBR to chromatin is regulated by phosphorylation of LBR in the nucleoplasmic region by cell cycle-specific *Xenopus* egg cytosol fractions (6). It is also known that LBR is phosphorylated by cdc2 kinase (12, 13), SR protein-specific kinase (SRPK) (14), protein kinase A (12), calmodulin-dependent protein kinase II (12), and casein kinase II (12). However, in the cases of all inner nuclear membrane proteins, the protein kinase responsible for the regulation of the binding to chromatin has not yet been identified.

In this study, we analyzed kinases responsible for regulation of the binding of LBR to chromatin using cell cycle-specific *Xenopus* egg cytosol fractions. It was shown that the stimulation of the binding of LBR to chromatin by an S-phase cytosol fraction (SC) was caused by phosphorylation by SRPK. On the other hand, suppression of the binding of LBR to chromatin by an M-phase cytosol fraction (MC) was caused by multiple kinases, one of which was identified as cdc2 kinase. Based on these findings and others, regulation of the binding of LBR to chromatin is discussed.

MATERIALS AND METHODS

Materials—Okadaic acid and a phosphatase inhibitor, I-2, were purchased from Wako

(Osaka, Japan) and Biomol Research Laboratories Inc. (Plymouth Meeting, PA), respectively. Protein kinase inhibitors, *i.e.* A3, K-252b and Calphostin C, were obtained from Calbiochem-NovaBiochem (San Diego, CA). A protein kinase A inhibitor (PKI, P0300), apyrase, staurosporine, 6-dimethylaminopurine (DMAP), and DNA-cellulose (double-stranded) were from Sigma Chemicals Co. (St. Louis, MO). Cdc2 kinase was affinity-purified from a *Xenopus* egg extract using p13 resin (15) or purchased from Calbiochem-NovaBiochem. Protein kinase C (PKC), trypsin, and glutathione-Sepharose 4B were from Molecular Probes (Eugene, OR), Promega Co. (Madison, WI), and Amaersham Bioscience Co. (Piscataway, NJ), respectively.

Buffer—The extraction buffer comprised 50 mM Hepes-KOH (pH 7.7), 250 mM sucrose, 50 mM KCl, and 2.5 mM $MgCl_2$; and the PP1 reaction buffer comprised 50 mM Tris-HCl (pH 7.0), 0.1 mM EGTA, 0.5 mM dithiothreitol, and 1 mg/ml BSA.

Preparation of demembranated Xenopus sperm chromatin and Xenopus egg cytosol fractions— S-phase and M-phase *Xenopus* egg cytosol fractions (SC and MC, respectively), and demembranated *Xenopus* sperm chromatin were prepared as previously described (6).

Chromatin binding assay—The nucleoplasmic region of human LBR (NK, amino acid residues 1-211) and the arginine-serine repeat-containing region within NK (RS, amino acid residues 53-89) were expressed in *Escherichia coli* as glutathione-S-transferase (GST) fusion proteins, and then bound to glutathione-Sepharose beads as previously described (6). In some experiments, a mutant GST-NK, GST-NK(S71A), was used. In this mutant, serine 71 is substituted by alanine. Using the thus prepared beads, the chromatin binding assay was carried out as previously described (6). Briefly, *Xenopus* sperm

chromatin, which was demembranated with lysolecithin and subsequently decondensed with heated *Xenopus* egg cytosol (6), in 20 μl of extraction buffer (7500 per μl) was added to 4 μg of GST-fused protein bound to 2 μl of gulutathione-Sepharose 4B beads suspended in 10 μl of extraction buffer. After incubation at 4 °C for 10 min, the binding reaction was stopped by pipetting 15 μl samples onto glass slides spotted with 12 μl of a fixing solution (3% formaldehyde, 6 μg/ml Hoechst dye 33342, 80 mM KCl, 15 mM NaCl, 50% glycerol, and 15 mM Pipes, pH 7.2). The fixed samples were observed by phase-contrast and fluorescence microscopy. One hundred to two hundred beads were observed for every sample and "the percentage of beads with bound chromatin" was calculated. This value was used as an index of the affinity of beads bearing LBR fragments and chromatin. The values in the figures are shown after subtraction of a blank value. The blank value was determined in each experiment using Sepharose beads with GST bound instead of GST-LBR fragments. In the preceding study, we compared this method and an established in vitro binding method involving soluble protein and chromatin, and showed that this bead method gives the same results as the established method (6). Therefore, we used this method to determine the affinity of protein fragments to chromatin in this study.

Assaying of cell cycle-dependent binding of LBR fragments to sperm chromatin—GST-fused proteins attached to glutathione-Sepharose beads (4 μg protein / 2 μl beads) were pretreated with 20 μl of either SC or MC at 23 °C for 1 h. After washing twice with extraction buffer, the beads were used for the chromatin binding assay. The addition of 1

M NaCl to the washing buffer to remove possible bound proteins from the gel beads had no effect on the binding of chromatin to the beads. In some experiments, protein kinase inhibitors and other reagents were added to SC and MC prior to the addition of GST-fused protein beads. Pretreatment of a cytosol fraction with apyrase was carried out at 30 °C for 30 min. For DNA binding assaying and phosphopeptide mapping, beads bearing GST-fused proteins were pretreated by the same procedures.

Depletion of SRPK and cdc2 kinase from Xenopus egg extracts—Depletion of SRPK from SC was carried out with GST-SF2/ASF beads according to the procedure reported previously (16). GST-SF2/ASF was expressed in *E. coli* BL21 (DE3) and then bound to glutathione-Sepharose 4B beads. The thus prepared beads were pretreated with 3 % BSA in phosphate-buffered saline at 4 °C for 30 min to reduce nonspecific adsorption. To deplete SRPK from SC, 5 μl of SF2/ASF-bearing beads (0.5 μg GST-SF2/ASF /μl of beads) was added to 20 μl of SC, followed by incubation at 4 °C for 1h and centrifugation at 3,000 rpm for 1 min. The supernatant was used as SRPK-depleted SC.

To deplete cdc2 kinase from MC, p13-beads, which specifically bind cdc2 kinase (15), were used, as follows. Plasmid pRK172, containing the fission yeast suc1+ gene (kindly provided by Dr. T. Kishimoto, Tokyo Institute of Technology), was transformed into *E. coli* BL21 (DE3). The $p13^{suc1}$ protein was expressed, purified and immobilized on CNBr-activated Sepharose 4B as described by Kusubata *et al.* (15). The thus prepared beads were pretreated with 3 % BSA in phosphate-buffered saline at 4 °C for 30 min to reduce nonspecific adsorption. To deplete cdc2 kinase from MC, 5 μl of p13-bearing

beads (5 μg $p13^{suc1}$ /μl of beads) was added to 20 μl of MC, followed by incubation at 4 °C for 30 min and centrifugation at 3,000 rpm for 1 min. The supernatant was used as cdc2 kinase-depleted MC.

Analysis of LBR phosphorylation—Phosphopeptide mapping was performed essentially as previously described (6). Briefly, GST-NK and GST-NK(S71A) phosphorylated with [γ-^{32}P] ATP *in vitro* were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane. The protein bands were excised, soaked in 0.5% poly(vinyl pyrrolidone) K-30 (Wako, Tokyo) in 100 mM acetic acid for 30 min at 37 °C, and then washed five times with water and two times with 50 mM NH_4HCO_3. These proteins were digested with trypsin in 50 mM NH_4HCO_3 at 37 °C for 15 h. The released peptides were loaded onto a cellulose thin layer chromatography plate of 20 cm x 20 cm (Funacell; Funakoshi Co., Tokyo). Electrophoresis in the first dimension was performed at pH 8.9 (1% ammonium carbonate) for 20 min at 1000V, and ascending chromatography in the second dimension was performed using a solvent system of 37.5% 1-butanol, 25% pyridine, and 7.5 % acetic acid in water (v/v). The dried plate was exposed to Fiji X-ray film with intensifying screens.

Binding assay of LBR fragments to DNA-cellulose—The purified GST and GST fused LBR fragments (2 μg proteins) were incubated with 30 μl of a 10% suspension of double-stranded DNA-cellulose in 500 μl of DNA binding buffer, 20 mM Hepes-KOH (pH 7.6), 0.2 M KCl, 0.5 mM EDTA, 0.1 % Triton X-100, 0.1 mM phenylmethanesulfonyl fluoride,

1 μg/ml aprotinine, 20 % glycerol, and 1 mg/ml BSA, at 4 °C for 6 h with rotation. Then, the DNA-cellulose was washed three times with the binding buffer and once with the binding buffer without BSA. LBR fragments bound to the cellulose were eluted with a 2% SDS solution, separated by SDS-PAGE, and then analyzed by Western blotting with polyclonal antibodies against GST as previously described (17).

Statistical analysis—The significance of the pretreatment of beads bearing LBR fragments with various reagents in the chromatin binding assay was evaluated by means of Student's t-test (n=3).

RESULTS

We previously showed that the binding of GST-NK to chromatin is stimulated by phosphorylation of GST-NK in the RS region by a *Xenopus* egg S-phase cytosol (SC) fraction (6, and Fig. 1). To identify the kinase responsible for this stimulation, we first examined protein kinase A and showed that it could cause the stimulation (6). However, an inhibitor specific for the kinase could not suppress the stimulation by the cytosol (6). Then we examined cdc2 kinase, calmodulin-dependent kinase II, and casein kinase II, which have been shown to phosphorylate LBR (12). However, they did not stimulate the binding (data not shown). It has been reported that a nuclear envelope –associated protein kinase phosphorylates LBR (18) and that its substrate specificity is identical to that of SR protein-specific kinase 1 (SRPK1) (14). Then, we treated GST-NK (amino acid residues 1-211 of LBR) with SRPK1 and found that the enzyme clearly stimulates the binding of GST-NK to chromatin (Fig. 1, column 6). Stimulation of the binding by pretreatment with SC and SRPK1 was similarly inhibited by kinase inhibitors having broad specificities such as A3, K232b (Fig. 1), and staurosporine (data not shown). The 50 % inhibition value for both stimulating kinases, *i.e.* the kinase in SC and SRPK1,

with A3 was 0.1 mM (data not shown). These results suggested that the kinase responsible for the stimulation of the binding of GST-NK to chromatin by SC is SRPK. When GST-RS (amino acid residues 53-89 of LBR) was used instead of GST-NK to narrow the phosphorylation site, similar stimulation on treatment with SC and SRPK1 was observed (Fig. 1, columns 10 to 13). These results suggested that the stimulation is caused by phosphorylation of residues in the RS region. To verify the participation of SRPK in SC in the stimulation, affinity-depletion experiments on SRPK were carried out. When SC was affinity-depleted of SRPK using SF2/ASF beads, the SC lost the ability to stimulate the binding of GST-NK to chromatin (Fig. 2, column 5). Subsequent addition of purified SRPK1 to the SRPK-depleted SC restored its ability to stimulate the binding (Fig. 2, column 6). These data clearly indicate that the stimulation of the binding of GST-NK to chromatin is caused by SRPK in SC. The phosphorylation sites of GST-NK treated with SC, SC depleted of SRPK, and SRPK1 itself were compared by phosphopeptide mapping. As can be seen in Fig. 3, the two maps obtained for GST-NK treated with SC and SRPK1 were very similar to each other. Moreover, all spots except No.1 disappeared on depletion of SRPK from SC (Fig. 3, Dep.SC). A part of the complex patterns of the phosphopeptide maps of (SC) and (SRPK) was possibly caused by alternative phosphorylation of serines within RS motifs (arginine-serine repeats), as suggested previously for chicken LBR (18, 13). Incomplete digestion by trypsin may also have been involved. GST itself was not phosphorylated at all (data not shown). Thus, we concluded that SRPK in SC phosphorylates GST-NK and stimulates the binding to chromatin.

As the next step, we examined the change in the binding affinity of LBR to chromatin on the transition from the S-phase to the M-phase. As can be seen in Fig. 4, the stimulated binding of GST-NK to chromatin on treatment with SC was suppressed by

subsequent treatment with MC (compare columns 2 and 3 in Fig. 4). We previously suggested that the suppression is caused by phosphorylation (6). Protein phosphatase inhibitor okadaic acid had no effect on the suppression (data not shown). To clarify the kinase(s) participating in the suppression, we examined various kinase inhibitors. The kinase(s) responsible for the suppression was sensitive to inhibitors with broad specificity, *i.e.* staurosporine and DMAP (Fig. 4, columns 4 and 5). The 50 % inhibition value for the suppression by staurosporine was 5±2 nM (n=3, data not shown). Complete depletion of ATP from the reaction mixture by pretreatment with apyrase also inhibited the suppression (data not shown). When specific inhibitors of cdc2 kinase, protein kinase A and PKC were examined, a cdc2 kinase inhibitor, roscovitine, showed partial inhibition of the suppression, although the other inhibitors had no effect (Fig. 4, bars 6 to 8). An inhibitor of calmodulin-dependent protein kinase II, *i.e.* EGTA, had no effect on the suppression (data not shown). These results suggested that multiple kinases including cdc2 kinase participate in the suppression. Then, to further clarify the participation of cdc2 kinase in the suppression, a mutant GST-NK, GST-NK(S71A), of which the major phosphorylation site serine for cdc2 kinase (13) was substituted by alanine, was used instead of wild-type GST-NK. As can be seen in Fig. 4, columns 9 to 12, the binding of mutant protein GST-NK(S71A) was partially suppressed by MC-treatment and the suppression was insensitive to roscovitine (compare columns 11 and 12 in Fig. 4). These results supported the idea that phosphorylation of serine 71 by cdc2 kinase participates in the suppression of the binding of GST-NK to chromatin. To verify the participation of cdc2 kinase, affinity depletion experiments on cdc2 kinase were carried out. As can be seen in Fig. 5, when MC was affinity-depleted of cdc2 kinase using p13 beads, the SC partially lost the ability to suppress the binding of GST-NK to chromatin

(compare columns 3 and 6 in Fig. 5A). Subsequent addition of authentic cdc2 kinase to the cdc2 kinase-depleted MC restored its ability to suppress the binding (Fig. 5A, column 7). These results clearly indicate that cdc2 kinase is responsible for a part of the suppression of the binding of GST-NK to chromatin.

Phosphopeptide mapping was carried out to compare the phosphorylation sites of GST-NK after treatment with SC, MC, SC followed by MC, cdc2 kinase, and PKC (Fig. 6). When GST-NK was treated with cdc2 kinase (cdc2), two major spots (*c* and *e*) and three minor ones (*a, b* and *d*) were observed. On the other hand, when GST-NK(S71A) was phosphorylated with cdc2 kinase (cdc2/S71A), the two major spots observed for (cdc2) disappeared. Therefore, the two major spots that disappeared should represent tryptic peptides containing serine 71, and the serine should be phosphorylated. Based on the amino acid sequence of LBR, the relative positions of spots *c* and *e*, and the substrate specificity of trypsin, spots *c* and *e* could be considered to be peptides phosphorylated at serine 71 comprising amino acid residues 65-75, GGSTSSSPSRR, and 65-74, GGSTSSSPSR, of LBR, respectively. Furthermore, when autoradiography films of (cdc2) and (MC) obtained on the same day were superposed, *spots a, b, c, d* and *e* completely coincided with spots 1, 3, 4, 6 and 8, respectively (data not shown). Therefore, we concluded that these matching spots contain the same phosphopeptides. The phosphopeptide map of GST NK treated with PKC (PKC) showed a single spot, *f*, which overlapped spot 6 in Fig. 6 (MC). Phosphopeptide maps obtained for GST-NK treated with MC (MC), and with SC and subsequently MC (SC-MC) were very similar to each other, although the patterns were very complicated. Spot 7 irregularly appeared. When the patterns of (SC-MC) and (SC) were compared, spots 4, 6, 8, 10, 11 and 12 seemed to be relatively enhanced by the treatment with MC. For (SC-MC), spots 4 and 8, which correspond to spots *c* and *e* for (cdc2), indeed incorporated a little more ^{32}P than

in the case of (SC). Moreover, when (MC) and (SC-MC) were compared with (SC), MC and SC-MC treatments were seen to clearly stimulate ^{32}P incorporation into spots 12 and 10. When GST-NK was treated with MC in the presence of a specific inhibitor of cdc2 kinase (MC+Rosco), spots 12 and 10 disappeared. Moreover, when GST-NK(S71A) was treated with MC (MC/S71A), spot 12 disappeared and ^{32}P incorporation into spot 10 decreased to the level in (SC). These results suggested that the peptides corresponding to spots 12 and 10 contained serine 71 and were phosphorylated by cdc2 kinase in MC. The differences in the positions of major phosphopeptides observed on treatment with authentic cdc2 kinase, *i.e.* spots *c* and *e* for (cdc2), compared with those obtained with cdc2 kinase on MC treatment, *i.e.* spots 12 and 10 for (MC) and (SC-MC), could be explained as follows: the peptides in spots *c* and *e*, which contained phosphorylated serine 71, incorporated a second phosphate group, and thus these peptides shifted to spots 12 and 10, respectively.

From the results of the above experiments involving protein kinase inhibitors and mutant GST-NK, it was suggested that SRPK in SC and cdc2 kinase in MC participate in the stimulation and suppression of the binding of GST-NK to chromatin, respectively. Then we verified these results by reconstitution of the regulation system with the purified enzymes (Fig. 7). When GST-NK was treated with purified SRPK1 instead of SC, a similar increase in the chromatin binding was observed (compare columns 2 and 3 in Fig. 7). On the other hand, when GST-NK pretreated with SC and SRPK1 was subsequently treated with purified cdc2 kinase, a similar decrease in the chromatin binding was observed (compare columns 5 and 6 in Fig. 7). These results clearly show that the stimulation and a part of the suppression of the binding of GST-NK to chromatin by SC and SC-MC can be reproduced with purified SRPK1 and cdc2 kinase, respectively.

We previously showed that LBR binds to the DNA moiety of chromatin. Then,

we examined whether the binding of LBR to DNA is regulated in a cell cycle-dependent manner or not by the DNA-cellulose method (Fig. 8). Pretreatment of GST-NK with SC had little effect on the binding to DNA-cellulose (compare Fig. 8, A1 and A2). The results may show that the stimulation of the binding of GST-NK to chromatin by pretreatment with SC (Figs. 1 and 7) depends on a specific configuration of DNA, as in chromatin (discussed in more detail below). On the other hand, the DNA-binding of GST-NK was completely suppressed by SC-MC treatment, as in the case of chromatin binding (compare Fig. 8, A3 and A4). The results obtained in experiments involving a cdc2 kinase inhibitor, *i.e.* roscovitine, suggested that cdc2 kinase is responsible for a part of the suppression of the binding of GST-NK to DNA-cellulose (Fig. 8, A5-A9). Calphostin C, a PKC inhibitor, had no effect on the binding (compare Fig. 8, A4 and A8). Similar results were obtained using GST-RS (amino acid residues 53-89) instead of GST-NK (amino acid residues 1-211) (compare Fig. 8, A and B). These results suggested that the binding of NK to DNA through the RS region was regulated by MC treatment. When GST-NK(S71A) was used instead of wild type GST-NK, (SC-MC) treatment did not cause complete suppression of the binding to DNA-cellulose (Fig. 8, C4). All these results are consistent with the idea that the phosphorylation of serine 71 by cdc2 kinase in MC is responsible for a part of the suppression of the binding of GST-NK to DNA-cellulose.

DISCUSSION

It was suggested in this study that SRPK in SC is responsible for the stimulation of the binding of GST-NK to chromatin, as judged with affinity-depletion of SRPK from SC (Fig. 2), the addition of authentic SRPK to the SRPK-depleted SC (Fig. 2), and kinase inhibitors (Fig. 1). A specific inhibitor of SRPK also suppressed the stimulation

of the binding of GST-NK to chromatin and the incorporation of ^{32}P into GST-NK on treatment with SC (unpublished observations). Phosphorylation of GST-NK by SC and authentic SRPK caused similar stimulation of the binding and gave similar phosphopeptide maps (Figs.1, 3 and 7). From these results we concluded that the kinase in SC responsible for the stimulation of the binding of GST-NK to chromatin is SRPK. When the chicken LBR amino terminal region (cGST-wtNt), which corresponds to the human GST-NK used in this study, was treated with either SRPK1 or an SR protein kinase, a very similar phosphopeptide map to that of GST-NK treated with SC or SRPK1 was obtained (14). Phosphorylation of cGST-wtNt by SRPK1 and the SR protein kinase was completely suppressed when the RS repeat motifs in cGST-wtNt, which correspond to amino acid residues 79-86 (RSRSRSRS) of hLBR, were deleted (14). Furthermore, it was suggested that serine residues within the repeat motifs in cGST-wtNt were alternatively phosphorylated by the SR protein kinase (18). Therefore, in human LBR, the most possible site of phosphorylation by RS protein kinase in a cytosol fraction and SRPK1 is either 80Ser, 82Ser, 84Ser or 86Ser, and the phosphorylation of any of these residues may cause stimulation of the binding of GST-NK to chromatin.

It was shown in this study that the phosphorylation of the RS region (amino acid residues 53-89) of LBR by SRPK in SC stimulates the binding of LBR to chromatin (Fig. 1). However, the binding to DNA-cellulose was not stimulated by the phosphorylation (Fig. 8). It is known that the amino terminal domain of LBR recognizes DNA curvature and supercoiling, and binds to the structures with enhanced affinity (19). Therefore, a specific configuration of DNA, as in chromatin, may be required for the stimulated binding of SC-treated GST-NK. However, the participation of the protein moieties of chromatin directly in the enhanced binding can not be excluded.

It has been reported that *in vivo* phosphorylation of splicing factors having SR repeats by SRPK in HeLa cells is cell cycle-dependent, and that these factors were phosphorylated 3 to 5-fold more in M-phase than I-phase cells (20). However, in the case of the RS protein, LBR, in chicken hepatoma cells, DU249, the level of phosphorylation was similar in I-phase and M-phase cells *in vivo* (13). In our cell-free system involving *Xenopus* egg cytosol fractions, the level of phosphorylation of LBR by SRPK was similar in S-phase and M-phase extracts (data not shown). Therefore, in the case of LBR, stimulation of the binding to chromatin by SRPK seemed to be cell cycle-independent.

It has been shown that overexpression of SRPK in mammalian cells causes disassembly of nuclear speckles (21). It is also known that the formation of a complex between an SR-protein, SF2/ASF, and SRPK, which is influenced by the phosphorylation state of SF2/ASF, plays regulatory roles in the assembly and localization of the splicing factor (16, 22). In the case of LBR, phosphorylation of LBR by an LBR-associated RS kinase in turkey erythrocyte nuclear envelopes abolishes the binding of p32/p34 (18). On the other hand, phosphorylation by a similar SRPK in a *Xenopus* egg extract stimulated the binding of LBR to chromatin (Figs. 1 and 2). Therefore, phosphorylation of LBR by SRPK may regulate or maintain interactions among components in the LBR complex containing chromatin, *in vivo*.

Phosphorylation of GST-NK by cdc2 kinase in MC caused partial suppression of the binding to chromatin (Figs. 4 and 5). Direct phosphorylation of serine 71 in LBR by cdc2 kinase in MC could be explained by the phosphopeptide maps, although the patterns were complicated (Fig. 6). Phosphorylation of GST-NK by SRPK in MC was also observed (compare Figs. 3 and 6). Suppression of the binding of LBR to chromatin on treatment with MC could not be fully explained by cdc2 kinase, suggesting the presence

of another kinase(s) that participates in the suppression (Figs. 4 and 5). Which spot in the phophopeptide maps in Fig. 6 corresponds to the phosphorylation by the kinase is not clear. However, one possible explanation is as follows: spots 10 and 12 contain peptides having two molar phosphate groups per molar peptide, and their phosphate groups are transferred by cdc2 kinase and the other kinase. The kinase of interest is sensitive to staurosporine and DMAP (Fig. 4). Pfaller and Newport (23) reported that both cdc2 kinase and "membrane release kinase" are necessary to release nuclear membrane vesicles from the chromatin surface in a *Xenopus* egg extract system. They partially purified the kinase (23). The membrane release kinase phosphorylates an about 60 kDa protein in a *Xenopus* egg membrane fraction (23). The kinase is inhibited by DMAP but not by specific inhibitors for known kinases such as protein kinase A, PKC and calmodulin-dependent protein kinase (23). All these characteristics are the same as those of our kinase, that is, our kinase phosphorylates 60 kDa nuclear envelope protein LBR and is inhibited by DMAP (Fig. 4), but not by specific inhibitors for protein kinase A, PKC and calmodulin-dependent kinase (Fig. 4). Moreover, the membrane release kinase acts with cdc2 kinase in vesicle release (23), and our kinase suppresses the binding of GST-NK to chromatin with cdc2 kinase. Therefore, the kinase working with cdc2 kinase for suppression of the binding of GST-NK to chromatin is very likely the same enzyme as this membrane release kinase.

In living cells, LBR, LAP2, emerin, and other inner nuclear membrane proteins have to dissociate from chromatin in the prometaphase to early anaphase of the cell cycle. It was suggested in this study that the binding of LBR to chromatin is suppressed by the phosphorylation of serine 71 by cdc2 kinase and some other residue by a kinase similar to "membrane release kinase" in MC. However, the kinases responsible for the regulation of the binding of other inner nuclear membrane proteins to chromatin are not yet known.

Identification of these kinases is very important for understanding the molecular mechanisms underlying nuclear envelope disassembly.

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LEGENDS FOR FIGURES

Fig. 1. **Stimulation of the binding of GST-NK to chromatin by SC.** A GST fusion protein comprising the amino-terminal domain of LBR (GST-NK, amino

acid residues 1-211) attached to glutathione-Sepharose beads was pretreated with SC or other media, as indicated below. The thus treated GST-NK beads were incubated with decondensed sperm chromatin, and then observed by fluorescence microscopy after staining of DNA to determine "% of beads with bound chromatin" values as described under MATERIALS AND METHODS. In the cases of columns 10 to 13, GST-RS (amino acid residues 53-89) was used instead of GST-NK. Treatments: control buffer (1), SC (2), SC containing 10 nM staurosporine (3), SC containing 1 mM A3 (4), SC containing 1 μM K252b (5), SRPK1 with an ATP regenerating system (6), SRPK1 without the ATP regenerating system (7), SRPK1 with the ATP regenerating system and 1 mM A3 (8), SRPK1 with the ATP regenerating system and 1 μM K252b (9), control buffer (10), SC (11), SRPK1 with the ATP regenerating system (12), and SRPK1 without the ATP regenerating system (13). Each column shows the mean ± S.D. (vertical bar) for three experiments. * indicates a significant difference from the respective control ($P < 0.05$). These significant differences were reproducibly observed regardless of the cytosol and chromatin preparations, although the "% of beads with bound chromatin" value changed a little from preparation to preparation.

Fig. 2. **Effect of affinity-depletion of SRPK from SC on its ability to stimulate the binding of GST-NK to chromatin**. To remove SRPK from SC, 20 μl of SC was pretreated with 5 μl of SF2 beads at 4 °C for 1h. GST-NK beads were incubated with extraction buffer (1), SC (2), SC diluted with extraction buffer 1.25-fold (3), SC pretreated with blank GST beads (4) or SF2 beads (5), and SC pretreated with SF2 beads and subsequently mixed with 0.1 μg of SRPK1 (6). Then,

the binding of chromatin was examined as in Fig. 1. Each column shows the mean ± S.D. (vertical bar) for three experiments. * indicates a significant difference from the respective control ($P < 0.05$).

Fig. 3. **Tryptic phosphopeptide mapping of GST-NK treated with SC, SC depleted of SRPK, and SRPK1.** Beads bearing 20 μg GST-NK were incubated with 20 μl of SC (SC), SC depleted of SRPK (Dep.SC), or 0.1 μg of SRPK1in extraction buffer (SRPK1) supplemented with 2 μl of 3.3 μM γ ^{32}P-ATP (110 TBq/m mole) at 23℃ for 1 h. The thus treated proteins were subjected to phosphopeptide mapping as described under MATERIALS AND METHODS. Peptides were separated by electrophoresis (horizontal direction; cathode to the left) and by ascending chromatography. The points of sample application are near the bottom-left corners.

Fig. 4. **Effect of treatment with MC on the chromatin-binding of LBR fragments pretreated with SC.** GST-NK beads (1-8) or GST-NK(S71A) beads (9-12) were pretreated with extraction buffer (1 and 9) or SC (2-8 and 10-12). These beads were subsequently treated with extraction buffer (1), SC (2), MC (3), MC + 10 μM staurosporine (4), MC + 3 mM DMAP (5), MC + 1 mM roscovitine (6), MC + 50 μg/ml PKI (7), MC + 0.5 μM calphostin C (8), extraction buffer (9), SC (10), MC (11), and MC + 1 mM roscovitine (12). The thus treated beads were incubated with chromatin and the "% of beads with bound chromatin" values were determined as in Fig. 1. Each column shows the mean ± S.D. (vertical bar) for three experiments. * and

** indicate a significant difference from the respective control at $P < 0.05$ and $P < 0.1$, respectively.

Fig. 5. **Effect of treatment with cdc2 kinase-depleted MC on the chromatin-binding of GST-NK pretreated with SC.** *A*. GST-NK beads were pretreated with extraction buffer (1) or SC (2-7). These beads were subsequently treated with extraction buffer (1), SC (2), MC (3), MC + 1 mM roscovitine (4), MC treated with blank beads (5), MC treated with p13 beads to deplete cdc2 kinase (6), and MC treated with p13 beads and subsequently mixed with cdc2 kinase (7). Then, the binding to chromatin was examined as in Fig. 1. Each column shows the mean ± S.D. (vertical bar) for three experiments. * indicates a significant difference from the respective control ($P < 0.05$). *B*. Confirmation of depletion of cdc2 kinase from MC with p13 beads. MC was treated with p13 beads as shown under MATERIALS AND METHODS. Then, the proteins bound to beads were analyzed by SDS-PAGE followed by silver staining (First/p13). The p13 bead-treatment was repeated once more for the thus treated MC, and then the proteins bound to beads were analyzed (Second/p13). For moc-treatment, blank Sepharose beads were used instead of p13 beads (First/Moc and Second/Moc). The cdc2 kinase band was identified by co-electrophoresis of authentic cdc2 kinase. These results show that the cdc2 kinase in MC was fully removed by the first treatment with p13 beads.

Fig. 6. **Tryptic phosphopeptide mapping of LBR fragments treated with SC, MC, SC-MC, cdc2 kinase and PKC.** Beads bearing 20 μg GST-NK were incubated with 20 μl of SC (SC), MC (MC), MC containing 1 mM roscovitine

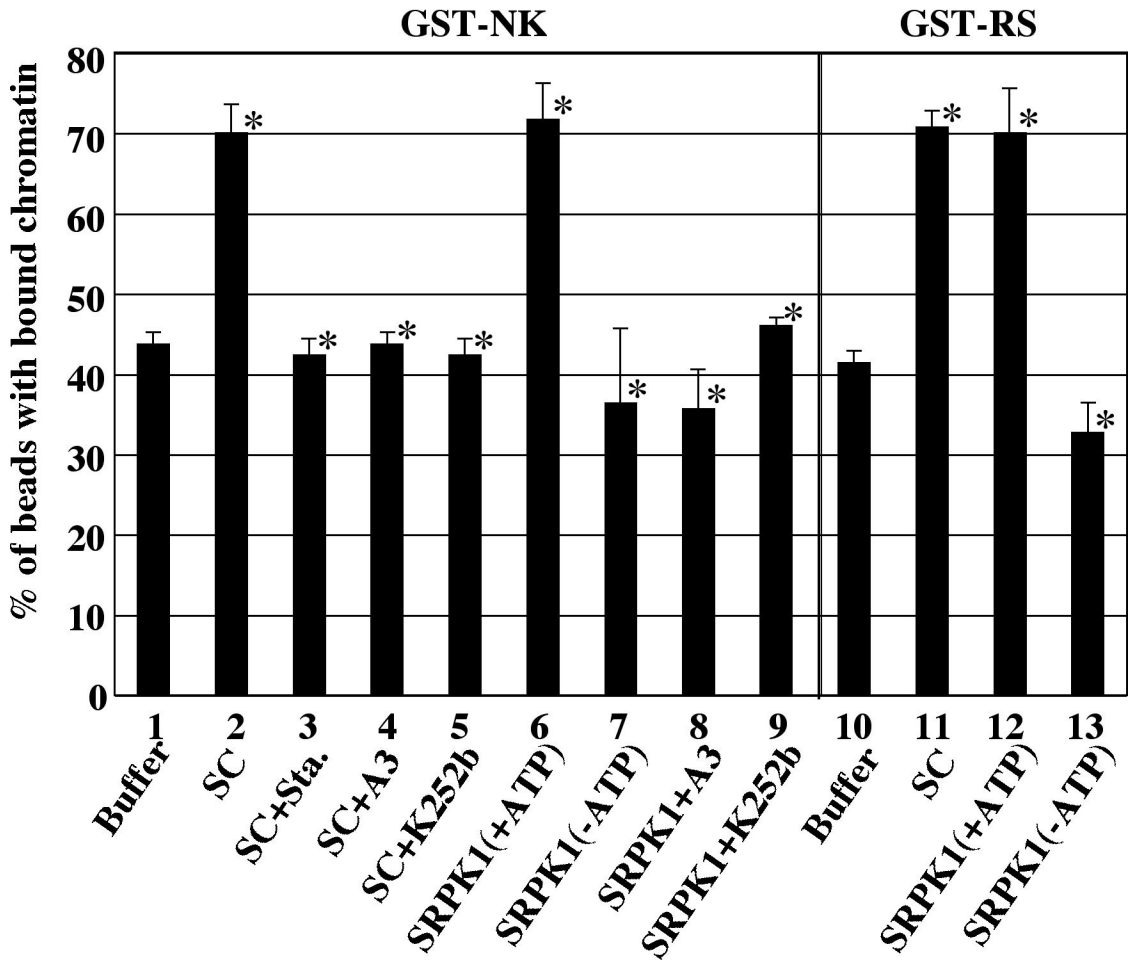
(MC+Rosco), 4 U cdc2 kinase (cdc2), or 0.03 μg PKC in extraction buffer (PKC) supplemented with 2 μl of 3.3 μM γ ^{32}P-ATP (110 TBq/m mole) at 23°C for 1 h. Beads bearing 20 μg GST-NK(S71A) were treated with cdc2 kinase (cdc2/S71A) or MC (MC/S71A) under the same conditions. In the case of (SC-MC), the beads were preincubated with SC in the absence of radioactive ATP and subsequently treated with MC in the presence of radioactive ATP as above. The thus prepared samples were subjected to tryptic phosphopeptide mapping as in Fig. 3. Spots *a, b, c, d* and *e* in (cdc2) correspond to spots 1, 3, 4, 6 and 8 in (MC), respectively.

Fig. 7. **Regulation of the binding of GST-NK to chromatin by purified kinases.** GST-NK beads were pretreated with 25 μl of extraction buffer (1), SC (2, 4 and 5), and 0.025 μg SRPK1-containing extraction buffer (3 and 6) in the presence of an ATP regenerating system at 23 °C for 1h. After washing, these beads were treated with extraction buffer (1), SC (2), 0.025 μg SRPK1 (3), MC (4), and 4 U cdc 2 kinase (5 and 6) under the same conditions. Then the binding to chromatin was examined as in Fig. 1. Each column shows the mean ± S.D. (vertical bar) for three experiments. * indicates a significant difference from the respective control ($P < 0.05$).

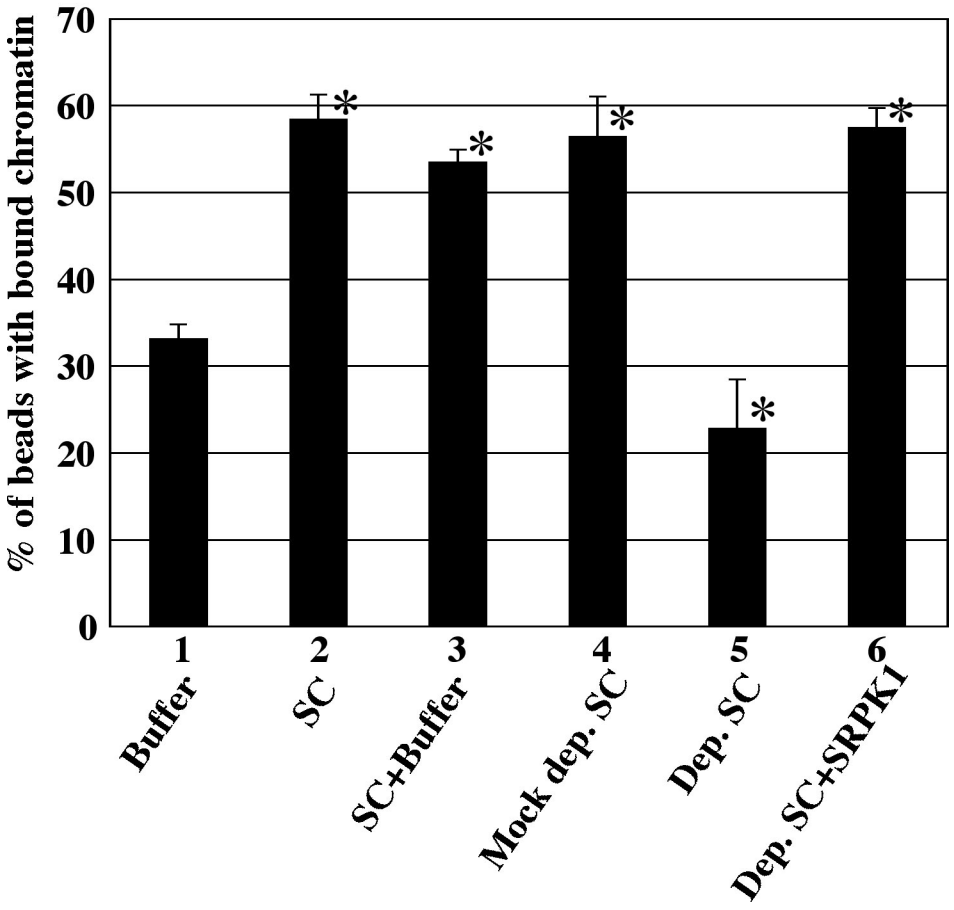
Fig. 8. **Binding of LBR fragments to DNA-cellulose.** Beads bearing LBR fragments GST-NK (A), GST-RS (B), and GST-NK(S71A) (C), or control GST (D) were treated with extraction buffer (1) and SC (2-9) at 23 °C for 1 h. The beads were subsequently treated with MC (4), MC + 14 U/ml apyrase (5), MC + 10 μM

staurosporine (6), MC + 1 mM roscovitine (7), MC + 15 μM calphostin C (8), and MC + 15 μM calphostin C + 1 mM roscovitine (9) as above. The thus treated LBR fragments were eluted from the beads with glutathione and then incubated with DNA-cellulose. LBR fragments bound to the DNA-cellulose were eluted, electrophoresed, transferred to a nitro-cellulose membrane, and detected with anti-GST antibodies. For other details, see MATERIALS AND METHODS.

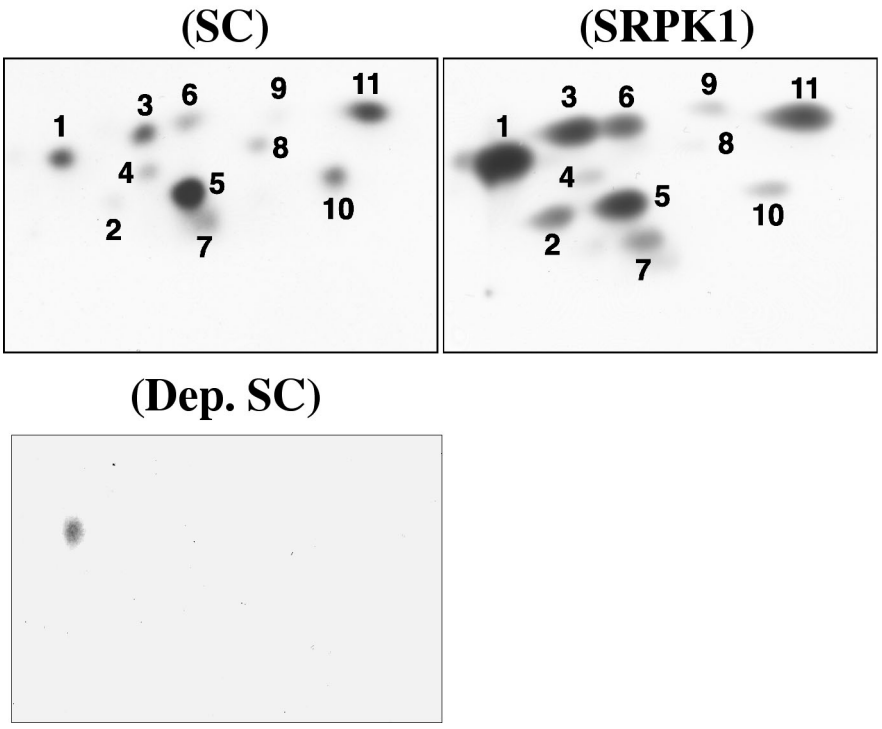
(Fig. 1)


GST-NK
GST-RS
% of beads with bound chromatin
80
70
60
50
40
30
20
10
0
1 Buffer
2 SC
3 SC+Sta.
4 SC+A3
5 SC+K252b
6 SRPK1(+ATP)
7 SRPK1(-ATP)
8 SRPK1+A3
9 SRPK1+K252b
10 Buffer
11 SC
12 SRPK1(+ATP)
13 SRPK1(-ATP)

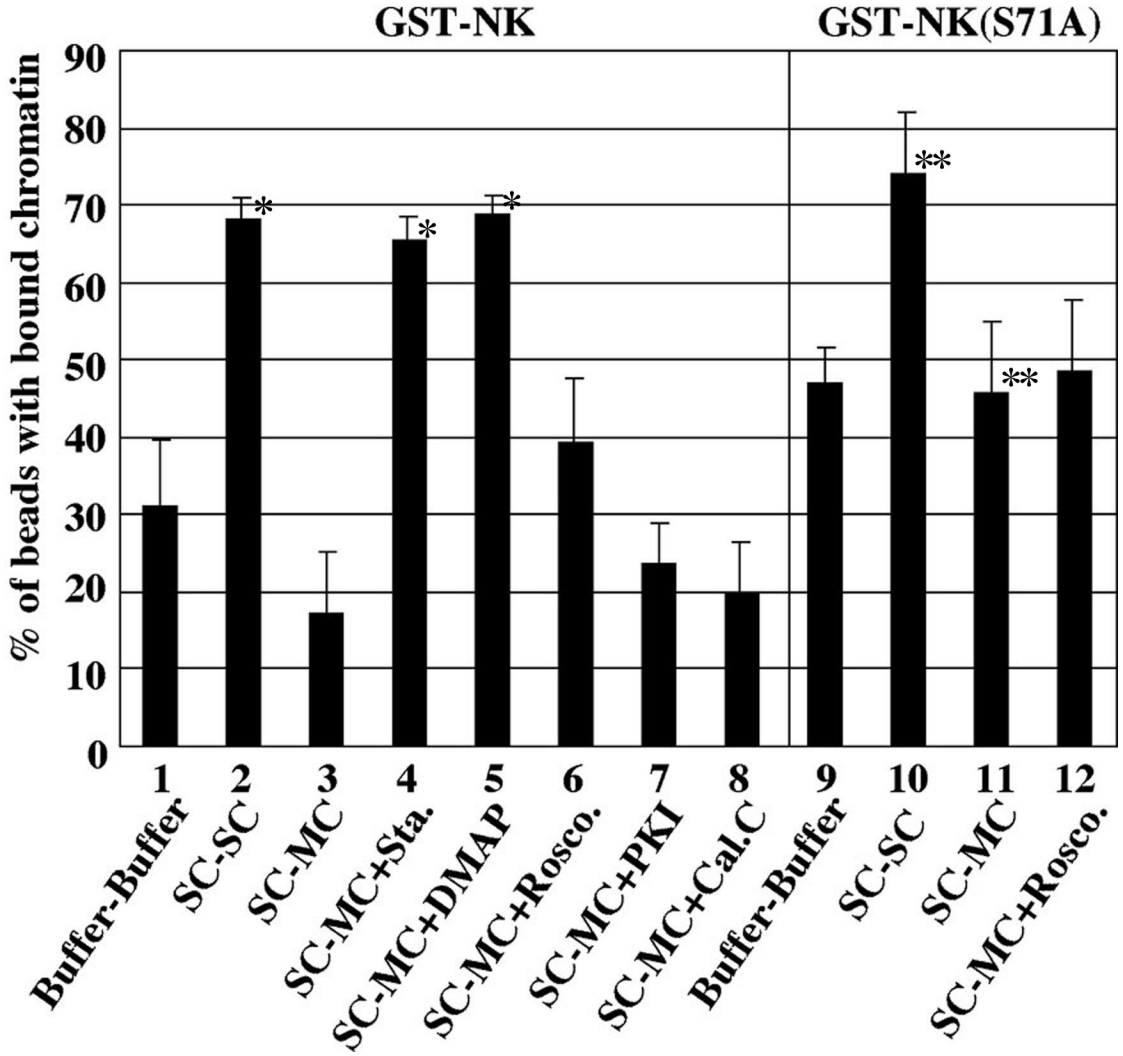
(Fig. 2)


% of beads with bound chromatin
70
60
50
40
30
20
10
0
*
*
*
*
*
1
Buffer
2
SC
3
SC+Buffer
4
Mock dep. SC
5
Dep. SC
6
Dep. SC+SRPK1

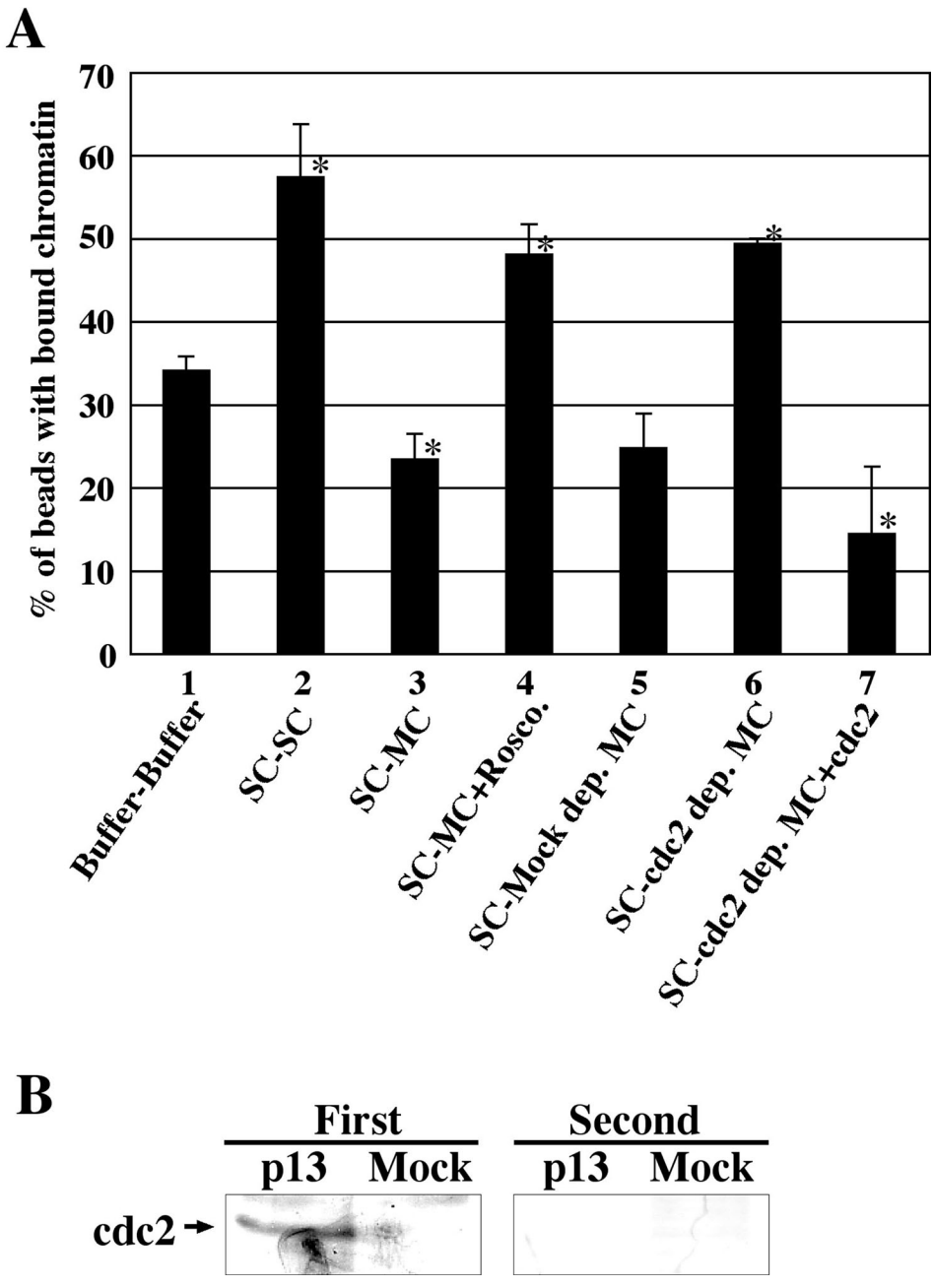
(Fig. 3)



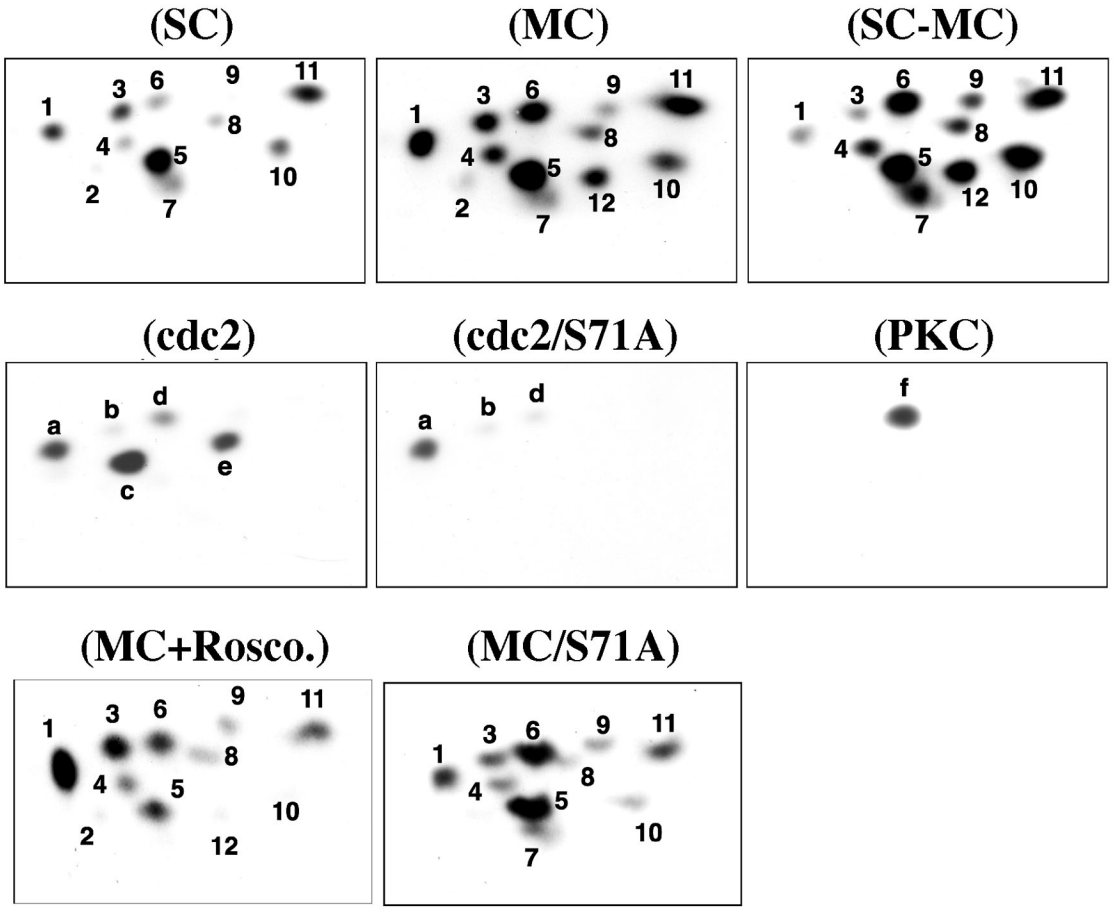
(Fig. 4)


GST-NK
GST-NK(S71A)
% of beads with bound chromatin
0
10
20
30
40
50
60
70
80
90
*
*
*
**
**
1 Buffer-Buffer
2 SC-SC
3 SC-MC
4 SC-MC+Sta.
5 SC-MC+DMAP
6 SC-MC+Rosco.
7 SC-MC+PKI
8 SC-MC+Cal.C
9 Buffer-Buffer
10 SC-SC
11 SC-MC
12 SC-MC+Rosco.

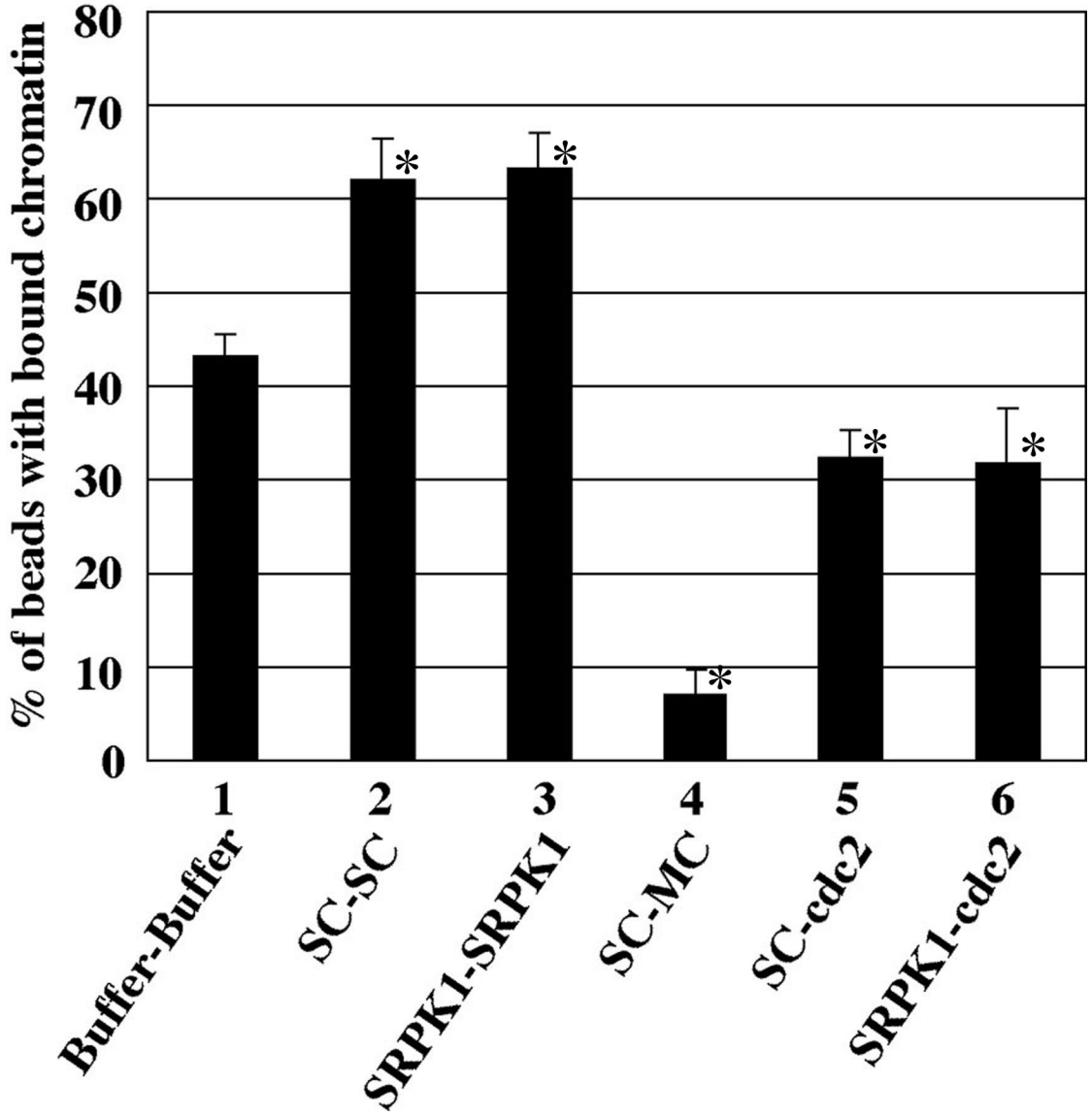
(Fig. 5)


A
% of beads with bound chromatin
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
Buffer-Buffer
SC-SC
SC-MC
SC-MC+Rosco.
SC-Mock dep. MC
SC-cdc2 dep. MC
SC-cdc2 dep. MC+cdc2
B
First
Second
p13
Mock
p13
Mock
cdc2

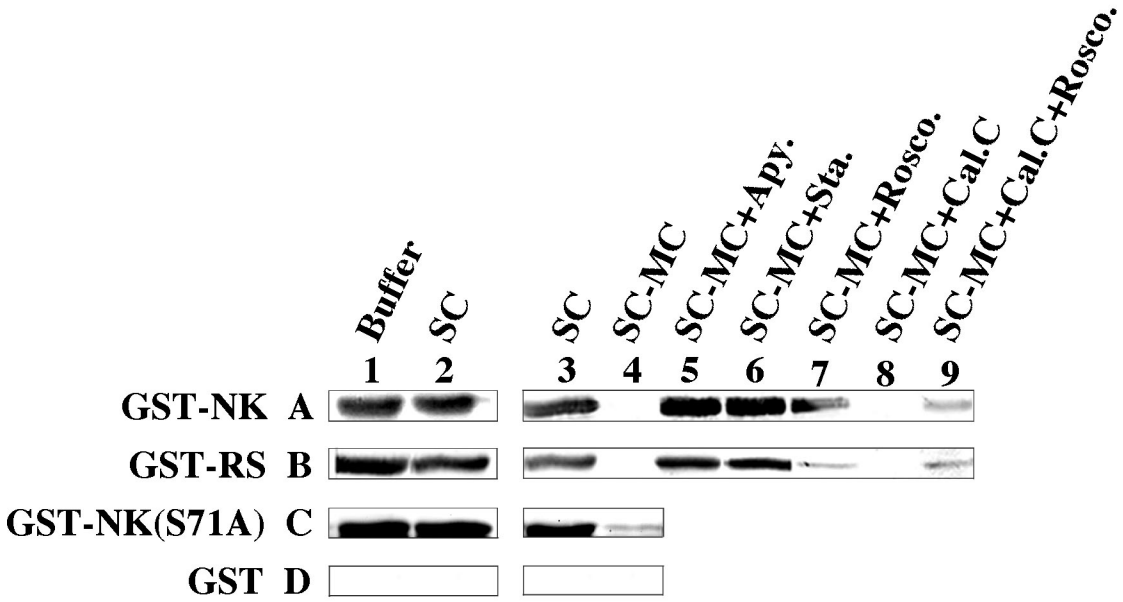
(Fig. 6)



(Fig. 7)


% of beads with bound chromatin
80
70
60
50
40
30
20
10
0
*
*
*
*
*
1
2
3
4
5
6
Buffer-Buffer
SC-SC
SRPK1-SRPK1
SC-MC
SC-cdc2
SRPK1-cdc2

(Fig. 8)


Buffer
SC
SC
SC-MC
SC-MC+Apy.
SC-MC+Sta.
SC-MC+Rosco.
SC-MC+Cal.C
SC-MC+Cal.C+Rosco.
1 2 3 4 5 6 7 8 9
GST-NK A
GST-RS B
GST-NK(S71A) C
GST D