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Enzymatic Activity of Protein Kinase LOSK: Possible Regulatory Role of the Structural Domain

E. S. Potekhina[1]*, L. A. Zinovkina[1], and E. S. Nadezhdina[2]

[1]Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Moscow, 119992 Russia; fax: (095) 939-3181; E-mail: skel@genebee.msu.su
[2]Institute of Protein Resarch, Russian Academy of Sciences, Pushchino, Moscow Region, 142292 Russia; fax: (095) 924-0493

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Abstract—LOSK (LOng Ste20-like Kinase) protein kinases of mammals belong to a recently identified family of GCK kinases which are involved in the induction of apoptosis. LOSK have an N-terminal acidic catalytic domain and a long C-terminal basic structural domain which is cleaved off in cells by caspases during apoptosis. To study the LOSK enzymatic activity and its dependence on the structural domain, two preparations of this protein kinase were prepared: a natural full-length protein immunoprecipitated from CHO-K1 cultured cells and a recombinant N-terminal catalytic fragment synthesized in *E. coli*. Both preparations displayed the ability for autophosphorylation and the ability for phosphorylation of MBP and of H1 histone, and their activities were comparable. H1 histone was a better substrate for LOSK than casein and ATP was a better substrate than other nucleotides. The pH dependence of the activity of the immunoprecipitated protein was more pronounced than the pH dependence of its recombinant fragment deprived of the C-terminal domain. The catalytic and the structural domains of LOSK can interact through electrostatic forces; therefore, effects were studied of various polyions at the concentration of 0.1 mg/ml on the activity. Heparin, protamine sulfate, and poly(L-Lys) decreased tenfold the ability of the full-length kinase to phosphorylate H1 histone. Heparin did not affect the activity of the recombinant fragment, whereas protamine sulfate and poly(L-Lys) had a slight effect. Moreover, protamine increased fourfold the autophosphorylation of the immunoprecipitated protein kinase. These data suggest that the structural C-terminal domain of LOSK should be involved in the regulation of its protein kinase activity: the LOSK protein kinase with C-terminal domain cleaved off could significantly less depend on conditions in the cell than the full-size enzyme.

Key words: protein kinase, LOSK, hSLK, Ste20, phosphorylation, antibodies, immunoprecipitation, protamine, histone H1, heparin, polyions

The supposed protein kinase p3D2 (hereafter called LOSK/p3D2) associated with microtubules and the centrosome in cell cultures was identified by us in the Chinese hamster cell culture CHO-K1 [1], and the question of its functions in the cell was established. To determine the functions of the protein kinase in the cell, it was necessary to reveal its substrates, to determine the effect of phosphorylation on the substrate functions, and to elucidate how the activity of the protein kinase itself was regulated by the cell and how hyperfunction or inhibition of the protein kinase activity affected the cell. To identify substrates of LOSK/p3D2, it was necessary first to determine the optimal conditions for the phosphotransferase reaction of this enzyme.

Several tens of animal cell protein kinases clearly homological to LOSK/p3D2 have been described by different groups of authors during recent years. At present, these protein kinases are combined in a family of so-called GCK [2] from the name of the first member of this family that was identified (the Germinal Center Kinase) [3]. GCK kinases are assigned to the superfamily of Ste20-like protein kinases. The "forefather" of this superfamily is the yeast protein kinase Ste20 (Sterile 20) which is involved in the cell response to pheromones. In addition to the GCK protein kinase, the family of GCK kinases includes GCKR (GCK-Related kinase), GLK (GCK-Like kinase), HPK1 (Haemopoetic Progenitor kinase-1), NIK (Nck-Interacting kinase), SOK1 (Ste20 Oxidant stress-activated kinase-1), Krs (Kinase Responsive to Stress), MST 1/2/3/4 (Mammalian Sterile Twenty-like-1/2/3/4), LOK (Lymphocyte-Oriented kinase), severine kinase, TNIK (Traf2- and Nck-interacting kinase), xPlkk1 (kinase of Polo-like kinase), etc. [2, 4, 5]. The closest to LOSK/p3D2 are protein kinases hSLK, mSLK, and some others, and their features are presented in Table 1. The catalytic domain of GCK kinases located

* To whom correspondence should be addressed.

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Table 1. Proteins of the LOSK group

Accession number	Biological species	Protein name	Homology of particular domains of the protein to the corresponding domain of the KIAA0204 protein (Q92603, *Homo sapiens*)*			Reference
			N-terminal	central	C-terminal	
O00211	*Homo sapiens*	hSLK	100	84	84	[24]
O54988	*Mus musculus*	mSLK STK2	100	59	75	[7, 25]
O08815	*Ratus norvegicus*	SK2	100	58	83	—
O55092	*Cavia porcellus*	SLK	100	57	75	[26]
O08986	*Cricetulus longicaudatus*	p3D2	— **	57	89	[1]

* By amino acid sequence predicted on the base of nucleotide sequence in cDNA.
** cDNA of this protein fragment has not been isolated.

in the N-terminal of the molecule is homologous to Ste20, and their long C-terminal structural domain is unique [5]. GCK kinases are widely distributed in cells of vertebrates but are minor cell proteins. Up to now, their functions are studied very poorly and the enzymatic activities are characterized insufficiently. GCK kinases are suggested to be involved in mitogen-activated protein kinase cascades (especially in the SAPK/JNK (stress-activated protein kinase/Jun N-terminal kinase) cascade) as kinases of kinases of kinases of mitogen-activated protein kinases. An actin-binding protein gelsoline/severine and a protein kinase Plk1 are described as substrates of GCK kinases [4, 6]; for the great majority of GCK kinases specific substrates are still unknown.

At least some GCK kinases, including the protein kinase mSLK closely homologous to the LOSK/p3D2, induce apoptosis if their cDNA is hyperexpressed in animal cells [7-9]. The activity of apoptosis-inducing protein kinases is under strict cell control. However, the mechanism of regulation of the GCK-kinase activity is not studied. Theoretically, based on the location of GCK-kinase in the cascade, they are certain to be regulated by minor GTPases, secondary messengers, or adaptor proteins. But neither GCK-kinase has structural motifs responsible for binding to minor GTPases or to second messengers. Some GCK-kinases have structural motifs for binding to proteins containing the SH3-domain and seem to be regulated by them. Such protein kinases are combined into the GCK I group. GCK II kinases (which, in particular, also include LOSK/p3D2) has no such motif, i.e., they cannot be regulated through the binding to the SH3-domains. However, it has been shown that the long C-terminal structural domain specific for GCK-kinases can inhibit the phosphotransferase activity of these enzymes. Thus, a mutant of the MST1/Krs2 protein kinase which lacked the C-terminal domain was ninefold more active than the wild type [10]. The protein kinases MST/Krs, MST1/Krs2, and mSLK could be activated *in vivo* during apoptosis by splitting off the C-terminal domain by caspase-3 [8, 11, 12]. It is unknown whether the interaction of the catalytic and structural domains resulting in changes in the protein kinase activities can be disturbed without the involvement of proteolysis.

Molecules of the LOSK/p3D2 and of the closely homologous protein kinases (Table 1) have three pronounced domains (Fig. 1). The conservative N-terminal domain (amino acids 1-326) has 11 signatures (specific amino acid sequences) specific for the catalytic domain of

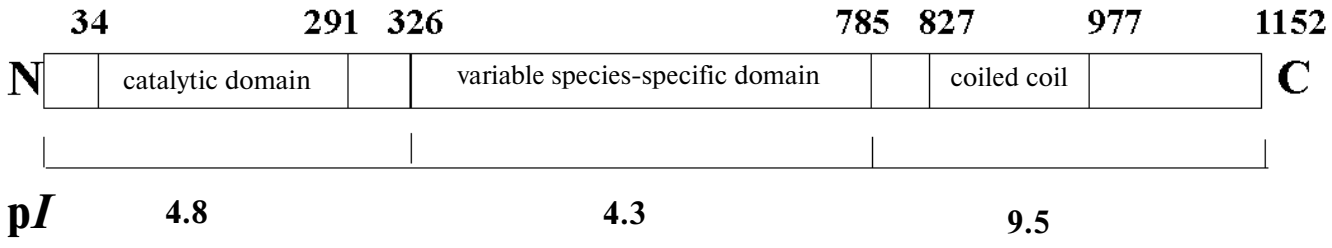

Fig. 1. Scheme of suggested domain structure of the LOSK protein kinase molecule. The figures above the rectangles indicate the numbers of amino acid residues.

serine-threonine protein kinases [13], i.e., this is a supposed catalytic domain. The central domain (amino acids 326-785) has no specific structural features. The C-terminal domain (beginning from amino acid 786) is enriched with glutamine residues and contains a region of 150 amino acids which with more than 80% probability determines the secondary structure of a coiled coil. Because the N-terminal domain of LOSK is acidic and the C-terminal domain is basic, they can interact by electrostatic forces, and polyionic substances are likely to influence the domain interaction in LOSK.

The goal of the present work was to characterize *in vitro* the enzymatic properties of the full-size native protein kinase LOSK/p3D2 isolated from the cell culture and to compare them to the properties of the recombinant fragment LOSK/hSLK expressed in *E. coli* (a similar human protein kinase) which lacked the C-terminal structural domain. The characteristics of the LOSK enzymatic properties suggested its assignment to histone or casein kinases, to ATP- or GTP-dependent kinases, and the determination of the pH optimum of its activity. Effects of various polyions (polyanion heparin and polycations poly(L-Lys), protamine, histone H1, spermine, spermidine, and putrescine) on the full-size enzyme and on the enzyme deprived of the C-terminal domain were also studied to establish whether polyions can regulate the activity of this protein kinase.

MATERIALS AND METHODS

Preparation of recombinant N-terminal domain of LOSK/hSLK kinase. The complementary DNA of the N-terminal catalytic domain (1-342 amino acids) of LOSK/hSLK protein kinase (Accession number Q92603 in GenBank) was isolated from the cDNA clonotheque of human brain using the polymerase chain reaction; the primers 5′-GCAGGAGCTCATGTCCTTCTTCAGTTTCC-3′ (the direct one) and 5′-GATACTAAGGTCAGAAGATGCAC-3′ (the reverse one). This cDNA was cloned in a pUC18 vector (Amersham Pharmacia Biotech, Great Britain), and its identity with hSLK was confirmed by sequencing. Then the cDNA was recloned into a pQE30 vector (Quiagen, Germany) by SacI and KpnI sites and into a pGEX2T vector (Amersham Pharmacia Biotech) by BamHI and SmaI sites. The cloning was performed using enzymes from MBI Fermentas (Lithuania). To hyperproduce a protein fused with glutathione-S-transferase (GST), *E. coli* BL21 strain (Amersham Pharmacia Biotech) was used. The recombinant protein was purified by affinity chromatography on glutathione-agarose (Sigma, USA). To hyperproduce a protein fused with 6 His the M15 strain was used, and the protein was purified on Ni-NTA-agarose (Quiagen).

Antibodies, proteins, polyions. Polyclonal rabbit antibodies pol3D2 and polKIA purified by affinity were used. The pol3D2 antibodies [1] were prepared to the LOSK/p3D2 fragment including the C-terminal and central domains of the protein and fused with 6 His. The polKIA antibodies were prepared to the above mentioned N-terminal domain of LOSK/hSLK fused with 6 His. Goat antibodies to rabbit IgG conjugated with alkaline phosphatase and also histone H1, MBP (myelin basic protein), heparin, protamine sulfate, spermine, spermidine, poly(L-Lys) (M_r 70,000), and putrescine were from Sigma. A dephosphorylated casein was kindly provided by Dr. S. M. Elizarov.

Cell cultures. Cell culture CHO-K1 (fibroblast-like cells of Chinese hamster ovary) was grown on F12 medium (Fisher, USA) supplemented with 10% bovine serum (Biolot, Russia) and gentamicin (100 mg/liter) (Sigma) in 100-mm Petri dishes at 37°C in the presence of 5% carbon dioxide.

Electrophoresis and immunoblotting. The proteins were separated by stepwise gradient SDS-polyacrylamide gel electrophoresis (PAGE) [14]. 6-12% gradient polyacrylamide gel of 8 × 6 cm in size was used. For immunoblotting, the proteins were transferred from the gel onto a Hybond C nitrocellulose filters (Amersham Pharmacia Biotech) by semi-dry electrotransfer [15]. The filter was blocked with 2% Tween-20 in TBS (100 mM Tris, pH 7.5, 0.9% NaCl) for 2 min. Alkaline phosphatase conjugated with antibodies was developed in a BCIP/NBT solution (Sigma).

Immunoprecipitation of LOSK. Petri dishes 100 mm in diameter with a monolayer of CHO-K1 cells were rapidly washed twice with PBS solution, and then homogenized in 300 μl of lysing buffer (10 mM Tris, pH 7.6, 100 mM KCl, 1 mM EDTA, 1% Nonidet P-40 (Sigma), 1 mM dithiothreitol (DTT, Serva, Germany)) which contained 0.5 mM sodium vanadate (Sigma) and inhibitors of proteases: 1 mM phenylmethylsulfonyl fluoride (PMSF, Serva), 10 μg/ml leupeptin (Sigma), 0.1 mM pepstatin (Sigma), 10 μg/ml TAME (Sigma), 10 μg/ml aprotinin (Sigma), and 10 μg/ml soybean trypsin inhibitor (Sigma). The resulting homogenate was incubated for 20 min at 4°C and then centrifuged for 45 min at 33,000 rpm in a 50 Ti rotor of a Spinco L8 centrifuge (Beckman, USA). The supernatant (cell extract) was used for immunoprecipitation of the LOSK/p3D2 protein kinase. The immunoprecipitation was performed using protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) suspended in 0.1 M solution of boric acid containing 75 mM NaCl (pH 8.4) with pol3D2 antibodies fused at the ratio of 5-10 mg antibodies per 1 ml of the resin suspension. The reaction of resin and antibody fusion was performed for 1 h with constant rotation of the tube at room temperature. Afterwards the resin with the antibodies bound was washed thrice with PBS containing 5-10 mg/ml BSA and thrice with a K-buffer which was used to perform the kinase reaction (50 mM HEPES (Serva), or MES

(Serva), or Tris, 100 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, and 1 mM DTT) at the pH required for a particular experiment (pH 7.6 if not otherwise stated). Then the resin was incubated with the CHO-K1 cell extract for 1 h at 4°C with careful stirring and precipitated using a desktop centrifuge. The resulting immunoprecipitated preparation (IPP) was washed thrice with P-buffer (K-buffer supplemented with 1 M KCl and 1% NP-40) and twice with K-buffer. In some experiments the P-buffer was supplemented not with 1 M but with 0.1-0.5 M KCl.

Determination of protein kinase activity of LOSK. The protein kinase activity of LOSK was determined using IPP washed and suspended in K-buffer with appropriate pH or the recombinant catalytic domain of LOSK dialyzed against the same buffer. The reaction mixture (20 μl) was supplemented with 36 nM (20,000 cpm/pM) [γ-^{32}P]ATP (FEI Research Center, Obninsk, Russia). Polyions were used at the concentration of 0.1 mM. The reaction was performed for 1 h at 25°C and stopped by addition of a triple buffer to samples for SDS-PAGE or by freezing. The preparations were separated by SDS-PAGE, from the gel stained with Coomassie R250 strips were cut which corresponded to proteins under study, and their radioactivity was counted with a Tracor Analytic Delta 300. In some experiments the gels were dried, and their radioautographs were prepared.

A search in the banks of nucleotide and protein sequences and the analysis of protein primary structure were performed using a Genebee program packet [16] placed in the Internet site of the Belozersky Institute of Physico-Chemical Biology, Moscow State University at the address of http:/www.genebee.msu.su/genebee.html.

RESULTS AND DISCUSSION

Identification of a group of proteins similar to the supposed protein kinase p3D2. How widely is the supposed protein kinase p3D2 distributed and does it have close homologs? We have earlier shown that antibodies to p3D2 recognize a polypeptide of the same molecular weight as the p3D2 in cell cultures from various species and from different rat organs [17]. In 2001 banks of nucleotide and amino acid sequences contained data on six proteins from vertebrates which were more than 65% homologous to p3D2. Data on these proteins are summarized in Table 1. The N-terminal domains of the proteins presented in Table 1 are nearly 100% homologous. The substrate specificity of protein kinases is determined by the catalytic domain; consequently, the protein kinases listed in Table 1 are bound to have the same substrates. The homology of the central domains of these protein kinases is only 57-59%, whereas the homology between the C-terminal domains is more than 80%. Based on these data, we combined protein kinases hSLK, SMAK (mSLK), SK2, p3D2, STK2, and SLK into a special group of closely related protein kinases. It is not improbable that all these protein kinases are the same protein with a species-specific central domain if each mammal is confirmed to have only a single gene for this protein. In fact, only a single gene has been found in humans. The protein kinase group identified we have named LOSK after LOng Ste20-related Kinase.

The isoelectric points predicted for the N-terminal and central domains are acidic, and for the C-terminal domain it is alkaline (Fig. 1). Therefore, it was suggested that at neutral pH molecules of the LOSK protein kinase in the cells should be rolled and closed such that the C-terminal can interact with the N-terminal of the molecule. Such an interaction suggested the involvement of the C-terminal of the molecule in the regulation of its activity. The inactive form of protein kinase C has a similar closed conformation [13].

Preparation of enzymatically active natural protein kinase LOSK and of its recombinant catalytic domain. To study the enzymatic activity of LOSK/p3D2, its pure preparations were required. To isolate it, immunoprecipitation was chosen, which is often used for isolation of protein kinases. But this approach is usually used for isolation of proteins hyperproduced in cells and fused with peptide tag and then these proteins are "drawn" from cell extracts with antibodies to the tag. We decided to immunoprecipitate the native protein kinase with antibodies pol3D2 to the structural domain of the molecule. During immunoblotting of the CHO-K1 cell homogenates both antibodies pol3D2 and polKIA (to the catalytic domain of the molecule) recognized the 210-kD polypeptide (Fig. 2a).

If protein A-Sepharose with antibodies pol3D2 fused was incubated with the cell extract and then washed with the P-buffer containing 1 M KCl, the Coomassie-stained electrophoregram of this IPP (Fig. 2b, lane *2*) presented only the 210-kD polypeptide (the supposed protein kinase LOSK/p3D2) and a 50-kD polypeptide with electrophoretic mobility corresponding to that of IgG heavy chain (Fig. 2b, lane *1*). There were no other polypeptides in the Coomassie-stained electrophoregram. During immunoblotting, both antibodies pol3D2 and polKIA recognized only the 210-kD polypeptide (Fig. 2b, lanes *3*, *4*). Thus, by immunoprecipitation we succeeded in the isolation of protein kinase LOSK/p3D2 from the CHO-K1 cell culture. To study the enzymatic activity, the IPP was incubated with [γ-^{32}P]ATP, subjected to SDS-PAGE, and then the radioautograph of the gel was obtained. In the radioautograph only one radioactive band was evident; it corresponded to the LOSK/p3D2 (Fig. 2c, lane *1*), and this suggested that LOSK/p3D2 should really be an active protein kinase. Moreover, it seemed that the IPP washed with 1 M KCl contained only one active protein kinase: other protein kinases if present in the preparations would have displayed themselves by autophosphorylation.

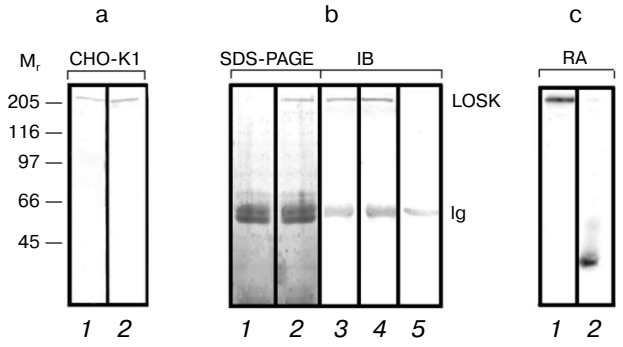

Fig. 2. Isolation of protein kinase LOSK/p3D2 from cell culture by immunoprecipitation and enzymatic activity of the preparation. a) Specificity of antibodies to the protein kinase LOSK. Immunoblotting of the CHO-K1 cell homogenate with antibodies pol3D2 (*1*) and polKIA (*2*); b) characteristics of the immunoprecipitated preparation (IPP) of protein kinase LOSK/p3D2 (*1*, *2*); SDS-PAGE: *1*) antibodies pol3D2 conjugated with protein A-Sepharose and not incubated with the cell extract; *2*) IPP washed with 1 M KCl; *3-5*) immunoblotting (IB) of preparation shown in lane (*2*) with antibodies polKIA (*3*), pol3D2 (*4*), and without addition of the first antibodies (*5*); c) phosphotransferase activity of LOSK/p3D2 IPP. Radioautograph (RA) of preparations incubated with [γ-^{32}P]ATP: *1*) autophosphorylation of the preparation shown in (b) (lane *2*); *2*) phosphorylation of histone H1 added to IPP.

Unfortunately, on an attempt to elute the LOSK from protein A-Sepharose with glycine buffer (pH 2.7) the enzyme became completely inactive, and in further studies on the phosphotransferase activity we had to use the IPP conjugated with antibodies and a carrier.

Similarly to the immunoprecipitated full-size kinase, the recombinant N-terminal domain of LOSK (kipGEX2T) purified by affinity on glutathione-agarose and fused with GST had capability for autophosphorylation (Fig. 3). That suggested the presence of autophosphorylation sites in the N-terminal domain of LOSK. Both the IPP of LOSK/p3D2 and kipGEX2T phosphorylated MBP and histone H1, which are nonspecific substrates of many serine-threonine protein kinases (Fig. 2c, lane *2*; Fig. 3, lanes *3*, *3′*). Note that the activity of the "tailless" enzyme was not increased compared to that of the full-size enzyme. In fact, kipGEX2T was synthesized in a heterologous bacterial system and this could somewhat decrease its activity. An addition of the histone as a substrate usually decreased the LOSK autophosphorylation. The phenomenon of substrate-induced inhibition of autophosphorylation has been described for various protein kinases.

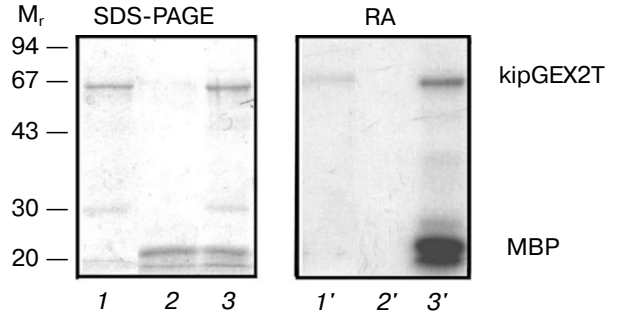

Fig. 3. Phosphotransferase activity of recombinant N-terminal fragment kipGEX2T of LOSK/hSLK synthesized in *E. coli*. *1-3*) SDS-PAGE; *1′-3′*) radioautograph (RA). The proteins were incubated with [γ-^{32}P]ATP; *1*, *1′*) kipGEX2T preparation; *2*, *2′*) MBP preparation; *3*, *3′*) MBP added to kipGEX2T.

Determination of the substrate specificity of LOSK. The cAMP-independent kinases are usually clearly different in their ability to phosphorylate either casein (casein protein kinases) or histones (histone protein kinases). Because cAMP itself failed to noticeably influence the enzymatic activity of the IPP (data not presented), we studied the LOSK *in vitro* ability to phosphorylate casein and histone. The phosphorylation of H1 histone with the immunoprecipitated protein kinase LOSK/p3D2 was several times higher than the incorporation of [γ-^{32}P]ATP into casein at the same concentrations in the reaction medium of the substrates under study (Fig. 4a). Therefore, LOSK was classified as a histone type protein kinase. However, the label incorporation into casein was never zero, i.e., in this case the substrate specificity of the protein kinase LOSK was not absolute.

The specificity of LOSK for nucleotides was also interesting because some protein kinases in addition to ATP can also use GTP as a substrate. To study the nucleotide specificity of LOSK/p3D2, we tried to inhibit the autophosphorylation by addition into the reaction mixture of excess non-radioactive nucleotides together with [γ-^{32}P]ATP. A 100-fold excess of radioactive ATP compared to the labeled ATP (3.6 μM cold nucleotide) inhibited tenfold the incorporation of [γ-^{32}P]ATP into the enzyme (Fig. 4b). GTP and CTP, even at 1000-fold excess, inhibited the autophosphorylation only twofold, whereas UTP and GTP had no effect. Thus, GTP was a significantly weaker substrate for the LOSK than ATP.

LOSK seems to be a constituent of protein complexes influencing its activity. Various protein kinases, especially those involved in intracellular signal transmission, are constituents of protein complexes which organize possible cascades [18-20]. To identify proteins which could bind to LOSK, we washed IPP with P-buffer containing 0.1-0.2 M instead of 0.5-1.0 M KCl. Apart from the LOSK band, in the radioautograph of the IPP washed with 0.1-0.2 M KCl there were many additional radioactive bands (Fig. 5a). These bands probably correspond to proteins (protein kinases?) which could bind to LOSK and/or to substrates of LOSK and of these additional pro-

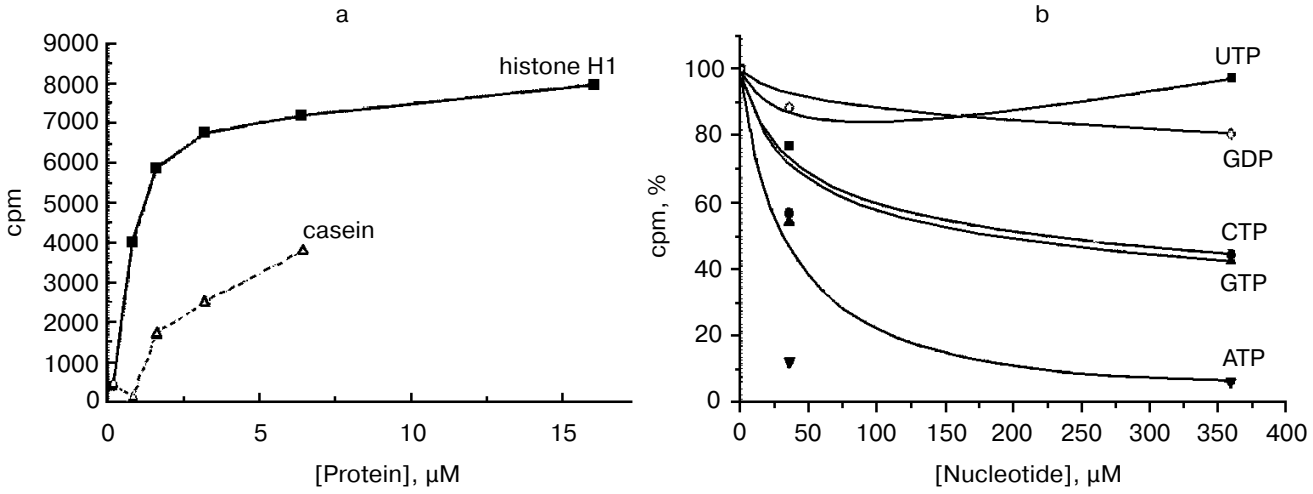

Fig. 4. Substrate specificity of LOSK/p3D2. a) Incorporation of ^{32}P into H1 histone (*1*) and into dephosphorylated casein (*2*) during their incubation at varied concentrations with LOSK/p3D2 IPP and [γ-^{32}P]ATP; b) effect of non-radioactive nucleotides on autophosphorylation of LOSK/p3D2 IPP (incorporation of ^{32}P from [γ-^{32}P]ATP into LOSK/p3D2). The value of 10,500 cpm is taken as 100%.

tein kinases. The number of radioactive bands decreased with an increase in the concentration of KCl on the washing of IPP. Moreover, the intensity of the band corresponding to LOSK was noticeably higher after the IPP had been washed with P-buffer containing 0.1 M instead of 0.5-1.0 M KCl. However, the amount of LOSK protein kinase in the IPP revealed by immunoblotting did not depend on the concentration of KCl during the washing (Fig. 5b). Based on these findings, it was suggested that either the IPP washed with 0.2 M KCl contained another active protein kinase with the same relative molecular weight as that of LOSK or (more likely) proteins which were able to activate LOSK were washed off the IPP with an increase in the ionic strength. This IPP washed with 0.2 M KCl could be used further for studies on proteins which could bind to LOSK. SAPK/JNK protein kinases are sometimes activated with GCK family protein kinases and are bound to them, but we failed to detect the JNK2 protein kinase in the IPP washed with 0.2 M KCl (data not shown). There was also no microtubular tubulin with which the LOSK was associated in the cells (data not shown).

Effect of pH on the enzymatic activity. The phosphorylation of substrates with full-size protein kinase LOSK/p3D2 and with kipGEX2T differently depended on pH (Fig. 6). A wide pH optimum for phosphorylation of H1 histone with kipGEX2T was in the range from 7 to 9 and was shifted to alkalinity as compared to the activity peak of the full-size kinase which was in the pH range of 7.0-7.5 (Fig. 6b). The autophosphorylation (in the absence of histone) of kipGEX2T was much more inhibited at pH below 7.0 than the autophosphorylation of the

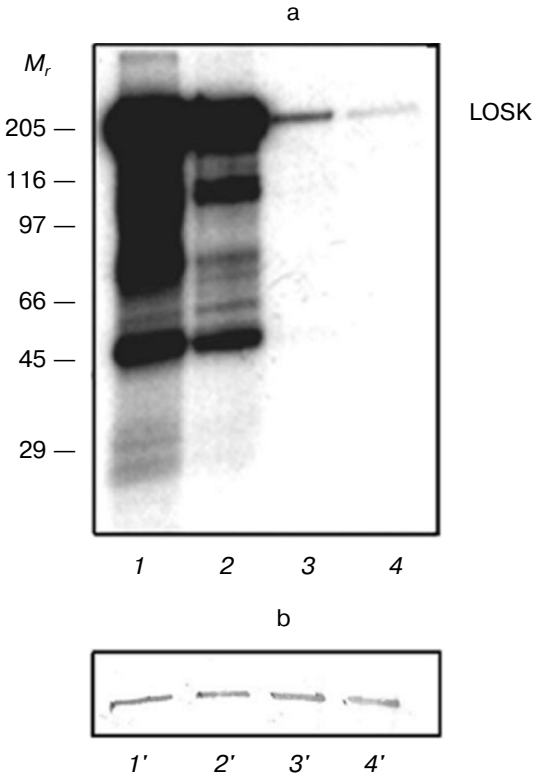

Fig. 5. Changes in the phosphotransferase activity of immunoprecipitated preparation of LOSK/p3D2 depending on its washing with varied concentrations of KCl. a) Radioautograph of the LOSK/p3D2 IPP; b) immunoblotting of IPP with antibodies pol3D2. The IPP was washed with P-buffer containing the following concentrations of KCl: *1*, *1'*) 100 mM; *2*, *2'*) 300 mM; *3*, *3'*) 500 mM; *4*, *4'*) 1000 mM.

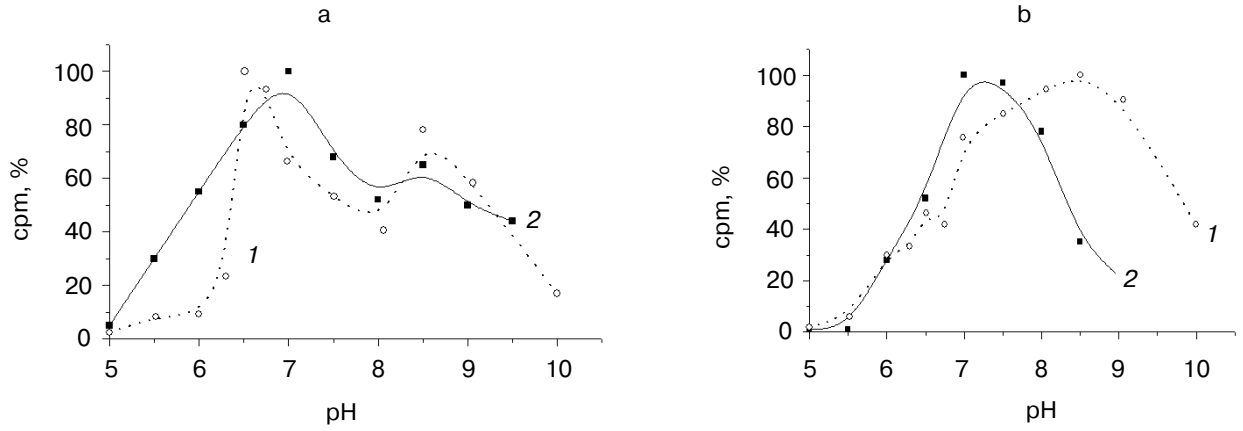

Fig. 6. The pH dependences of phosphorylation with immunoprecipitated protein kinase LOSK/p3D2 (*2*) and with recombinant catalytic fragment kipGEX2T (*1*). a) Autophosphorylation; b) phosphorylation of histone H1. The highest activity of the enzyme in each case is taken as 100% (2300 cpm for curve *1* and 28,000 cpm for curve *2* in Fig. 6a; 4800 cpm for curve *1* and 3600 cpm for curve *2* in Fig. 6b). Each curve corresponds to data obtained in one of three similar experiments.

full-size protein (Fig. 6a). Based on these findings, it was suggested that the C-terminal structural domain should be involved in the fine regulation of the activity of the enzyme. The removal of the C-terminal fragment of LOSK by proteolysis (e.g., with caspase during apoptosis [8]) seemed to change the activity of the enzyme just at certain intracellular pH which occurred under some particular cell conditions.

Effects of polyions on enzymatic activity of LOSK and kipGEX2T. We suggested that the interaction of the N-terminal and C-terminal domains of LOSK should be influenced by heavily charged proteins and their effects could be experimentally modeled with polyions. To study the effects of polyions on the enzymatic activity of the catalytic domain kipGEX2T of LOSK and on the full-size kinase, the reaction mixture during the testing for the enzymatic activity was supplemented with various polyionic substances at the concentration of 0.1 mg/ml.

Protamine sulfate increased fourfold the autophosphorylation of the immunoprecipitated LOSK/p3D2, whereas other polyions studied did not influence the autophosphorylation (Table 2). But protamine sulfate,

Table 2. Activity dependence of immunoprecipitated protein kinase LOSK/p3D2 and of its recombinant fragment kipGEX2T on various polyions

Experiment conditions	Immunoprecipitated protein kinase LOSK/p3D2		Recombinant fragment kipGEX2T of the protein kinase LOSK/hSLK	
	autophosphorylation, %	phosphorylation of histone H1, %	autophosphorylation, %	phosphorylation of histone H1, %
Without polyions	100	100	100	100
Heparin	148 ± 26.9 (2)	7.4 ± 1.97* (2)	86 ± 61.6 (4)	108 ± 63.2 (4)
Poly(L-Lys)	172 ± 68.5 (4)	10.5 ± 4.96* (2)	88 ± 67.7 (4)	30.8 ± 9.87* (4)
Putrescine	144 ± 14.2 (2)	90 ± 15.5 (2)	—	—
Spermine	145 ± 45.2 (2)	52 ± 9.8 (2)	—	—
Spermidine	77 (1)	52 ± 16.9 (2)	—	—
Protamine	444 ± 61.5* (4)	10 ± 4.1* (4)	148.6 ± 62 (4)	40.6 ± 4.14* (4)

Note: Radioactivity (cpm) of LOSK/p3D2, kipGEX2T, or histone H1 in control samples in each experimental series was taken as 100%. In parentheses the number of independent experiments is indicated. During studies on LOSK autophosphorylation histone H1 was not added into the reaction mixture. The sign "—" means the absence of such an experiment; *, difference from the control is significant, $p < 0.05$.

poly(L-Lys), and heparin inhibited nearly tenfold the ability of the LOSK/p3D2 to phosphorylate histone H1. Note that not all polyions influenced the enzyme activity, i.e., not only their polyionic nature was important but some specific features of particular molecules. Similar molecules were also suggested to be involved in the *in vivo* regulation of the enzyme.

Polyions virtually failed to influence the kinase activity during autophosphorylation of the recombinant catalytic domain of LOSK KipGEX2T. Similarly to poly(L-Lys), protamine sulfate only 2-2.5-fold inhibited the phosphorylation of histone H1, whereas heparin had no effect on this phosphorylation. Thus, the C-terminal structural domain was important for the regulation of the catalytic activity of LOSK with polyions. Similar data have been published for some other protein kinases. Activities of the tyrosine protein kinase EGF-R and of its 42-kD fragment cleaved with protease were compared, and the full-size enzyme was found significantly more dependent on poly(Lys), protamine, and spermidine [21]. The removal of the regulatory domain of the protein kinase C prevented its stimulation with heparin [22].

Note that polycations and polyanions usually had opposite effects on the activity of protein kinases. Thus, the protein kinase Cμ was inhibited with alkaline polypeptides and activated with heparin and dextran sulfate [23]. In our case the effects of both heparin and poly(L-Lys) on the LOSK/p3D2 activity were virtually the same. It was suggested that in the regulation of the LOSK activity both the alkaline and the acidic part of the structural domain could be involved, including, for example, the cluster 315-328 of acidic amino acids. It should be also noted that the LOSK activity was not stimulated either with polycations or polyanions; we recorded only an inhibition (not considering the stimulation of the autophosphorylation with protamine sulfate).

Our work has shown that the full-size LOSK is fully active similarly to its fragment containing the catalytic domain. It seems that the reason for removal of this domain with caspase is to spare LOSK the inhibiting effect of some cellular factor that has to increase its *in vivo* activity. We have earlier shown that protein kinase LOSK interacts with microtubules. It also seems possible that tubulin of microtubules or their other proteins can also regulate the activity of full-size LOSK.

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