The K Protein Domain That Recruits the Interleukin 1-responsive K Protein Kinase Lies Adjacent to a Cluster of c-Src and Vav SH3-binding Sites

IMPLICATIONS THAT K PROTEIN ACTS AS A DOCKING PLATFORM*

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The heterogeneous ribonucleoprotein particle (hnRNP) K protein interacts with multiple molecular partners including DNA, RNA, serine/threonine, and tyrosine kinases and the product of the proto-oncogene, Vav. The K protein is phosphorylated in vivo and in vitro on serine/threonine residues by an interleukin 1 (IL-1)-responsive kinase with which it forms a complex. In this study we set out to map the K protein domains that bind kinases. We demonstrate that the K protein contains a cluster of at least three SH3-binding sites (P1, PPGRGGRPPPSRR, amino acids 265–278; P2, PRRGPPPPPPGRG, 285–297; and P3, RARNLPLPPPPPRGG, 303–318) and that each one of these sites is capable of selectively recruiting c-Src and Vav SH3 domains but not SH3 domains of Abi, p85 phosphatidylinositol 3-kinase, Grb-2, and Csk. We demonstrate that the K protein domain that recruits and is phosphorylated in an RNA-dependent manner by the IL-1-responsive kinase, designated KPK for K protein kinase, is contained within the 338–425-amino acid stretch and thus is contiguous but does not include the cluster of the SH3-binding sites. K protein and KPK co-immunoprecipitate from cell extracts with either c-Src or Vav, suggesting that K protein-KPK-c-Src and K protein-KPK-Vav complexes exist in vivo. Furthermore, in the context of K protein, c-Src can reactivate KPK in vitro. The succession of kinase-binding sites contained within the K protein that allow it to form multienzyme complexes and facilitate kinase cross-talk suggest that K protein may serve as a docking platform that promotes molecular interactions occurring during signal transduction.

The heterogeneous ribonucleoprotein particle (hnRNP) K protein is endowed with diverse biochemical properties, and, as a result, the K protein is a highly interactive molecule. K protein was first discovered as a component of hnRNP, where it exists bound to RNA (1, 2). There are several isoforms of K protein that represent alternatively spliced products of a gene that in humans has been mapped to chromosome 9 (3). K protein binds preferentially to poly(C) compared to other ribonucleic acid homopolymers (1, 2). As a component of the hnRNP, K protein may participate in processing and transport of pre-mRNA (4). The hnRNP contains at least 20 major proteins that have different RNA binding specificities. It is already clear that the differences between K protein and the other hnRNP proteins go far beyond differences in RNA binding properties. K protein also binds single- and double-stranded DNA in a sequence-selective manner and can activate gene promoters that contain cognate DNA motifs (5–7). In these two respects, the K protein resembles the Xenopus transcription factor TFIIIA; TFIIIA is both a positive transcription factor and serves to store and transport 5 S RNA to the cytoplasm (8, 9).

The functional diversity of the K protein appears to be even greater than that described for TFIIIA. The K protein was recently discovered to bind the SH3 domains of the protein-tyrosine kinases, Src, Fyn, and Lyn (10, 11). Although these observations suggested that K protein can potentially engage these protein-tyrosine kinases, it is not itself tyrosine phosphorylated (10, 12). Nonetheless, the potential interaction with these tyrosine kinases suggests that the K protein is involved not only in the regulation of gene expression but also in signal transduction. A link to the signal transduction cascade is further illustrated by recent reports that K protein interacts in vivo and in vitro with the proto-oncogene product, Vav (13, 14). The interaction of K protein with Vav is mediated via SH3 domains, another typical feature of signal transduction processes (10, 11, 13, 14). K protein can potentially interact with c-Src and its ability to bind RNA resembles the property of another protein, p68/p62 or Sam68, that is a target for c-Src and also binds RNA (11, 15, 16).

K protein is phosphorylated in vivo and in vitro on serine and threonine residues (12). At least, in part, this phosphorylation is mediated by an associated kinase which can respond to treatment of cells with IL-1 and other agents (12, 17). This IL-1-responsive kinase adds yet another factor to the list of K protein molecular partners. The identity of this kinase is not known (12, 17). This enzyme is activated by phosphorylation (17) and might, therefore, be linked to one of the kinase cascades (18). Phosphorylation of K protein by K protein kinase (KPK) is facilitated by either the cδ enhancer or poly(C) RNA motifs, both of which can bind the K protein (6, 12, 17). Whether other DNA enhancer motifs that recognize K protein (5, 7) influence its phosphorylation is not known. Because K
protein interacts with some of its molecular partners via SH3 interactions (10, 11, 13, 14), and the fact that serine/threonine kinases that contain SH3 domains have been identified (19), we set out to map the K protein SH3-binding domains and determine whether or not these sites participate in the recruitment of KPK to K protein.

MATERIALS AND METHODS

Cell Line—The murine thymoma EL-4 6.1 C10 (20) cell line was grown in suspension. Cells were grown at 37°C in complete RPMI 1640 medium supplemented with 5% fetal calf serum or Fetal Cline, 2 mM glutamine, 50 μg/ml ampicillin, and 100 units/ml streptomycin (0.01%) and humidified with 7–9% CO2/air gas mixture. The reaction was stopped by washing the beads twice with 800 μl of 270°C 32P]ATP was added with or without 1 mg/ml poly(C) RNA, and the phosphorylation reaction was carried out for 30 min at 30°C. After resuspending the beads in 270 μl of binding buffer containing 30 μl of NaCl, the K protein was eluted from the bottom module with elution buffer containing 400 mM NaCl. The DEAE-Sephadex eluate was diluted with 40 μl of 1 binding buffer and further purified on the tandem IgG-affinity column (24). The top two modules contained Affi-Gel 10 beads bearing anti-kinase No. 54 IgG, and the bottom module contained Affi-Gel 10 beads bearing anti-K protein No. 54 IgG. After washing the modules with 1 binding buffer containing 175 mM NaCl, the K protein was eluted from the bottom module with elution buffer containing 400 mM NaCl.

Kinase Assays on Beads—All phosphorylation reactions were carried out directly on beads as described previously (6, 17). Briefly, after loading with the kinase, beads were washed with 5 mM of 1 binding buffer containing 175 mM NaCl plus the full complement of phosphate inhibitors (30 mM p-nitrophenyl phosphate, 10 mM NaF, 1 mM Na3VO4, 0.1 mM Na2MoO4, and 10 mM β-glycerophosphate). Protein content was measured using the BCA method (Pierce).

Preparation and Binding of Beads Bearing Synthetic K Peptides—4 to 5 mg of each peptide (P1, MPRGPRGRGPRGPRSGGC; P2, MPRGRGGPPPPPPRSGGC; P3, GSRARLPPPLPPPRPGGC; P4, CMETEPQEFPPN; P5, CQSKNAGAVIGK) dissolved in 50 mM Tris-HCl buffer, pH 8.5, containing 5 mM EDTA was used for coupling to SulfoLink Gel (Pierce) as per the manufacturer’s protocol. Peptides all contained free sulfhydryl groups in their end to allow coupling (tauric acid). 50 μl of beads were used for binding experiments. Prior to the incubation with the fraction of interest, beads were saturated overnight at 4°C with 125 μg of BSA in 500 μl of T binding buffer. The excess of BSA was washed 6 times with 1 ml of T binding buffer (5 mM Tris-HCl, pH 7.5, 25 mM NaCl, 1.0 mM EDTA, and 0.05% Nonidet P-40), then 2 μg of GST-Src SH3 or GST-Csk SH3 fusion proteins were added and incubated for 30 min at 4°C. Beads were then spun down, and the supernatant was saved and precipitated overnight at −70°C with 1 ml of cold acetone. Beads were washed 4 times with 1 ml of T binding buffer containing 0.2% deoxycholate and 0.1% Triton X-100. 30 μl of SDS loading buffer was added, beads were boiled, and released proteins were loaded onto a 13% SDS gel. After transfer onto a polyvinylidene difluoride membrane, proteins were immunostained as described below.

Far Western Blot Analysis—GST-K deletion mutants or purified natural K protein were run on a 13% SDS gel, transferred onto a polyvinylidene difluoride membrane, and immunostained as follows. The membrane was incubated overnight at 4°C in renaturation buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.01% Nonidet P-40) and then incubated for 2 h at room temperature in TTBS (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20), 2.5% BSA (w/v). The membrane was then rinsed three times for 5 min with TTBS and incubated in TTBS, 1% BSA containing a 2 mg/ml concentration of a GST SH3 domain protein for 2 h at room temperature. The membrane was then rinsed three times for 5 min with TTBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20). The first antibody (either monoclonal anti-Src SH3 domain (10) or anti-GST) was then added for 1 h at room temperature, at the concentration of 1/1,000 and 1/1,000, respectively. The membrane was then washed three times for 5 min at room temperature with TTBST and incubated for 30 min with alkaline phosphatase-conjugated goat anti-mouse IgGs (Promega). The staining was developed by incubating the membrane in 17.5 ml of substrate buffer (0.1 mM Tris-HCl, pH 9.5, 0.05 mM NaCl, 0.025 mM MgCl2) containing 77 μl of nitro blue tetrazolium and 58 μl of 5-bromo-4-chloro-3-indoly phosphate (Life Technologies, Inc.). The GST SH3 domains used were: GST-Src SH3, GST-Csk SH3, GST-Abl SH3, GST-GB2 NT SH3, GST-Grb2 COOH SH3, and GST-P85 P13 SH3. To obtain GST-Src SH3 protein labeled with 32P, Src SH3 fragment was cloned into pGEX-2T vector (Pharmacia, Uppsala, Sweden) (23) using BamHI and PstI sites. After transformation of BL21(DE3)pLyS3 cells with this plasmid, GST-Src SH3 protein was expressed as described above. After binding to glutathione beads, labeling was done directly on the beads using γ-32P-ATP and protein kinase A as per manufacturer’s protocol (Pharmacia). 32P-Labeled GST-Src SH3 protein was eluted from the beads with 5 ml reduced glutathione in 50 mM Tris-HCl, pH 8.0.

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To purify the K protein and the associated Kinase KPK—KPK was co-purified with K protein by tandem IgG-affinity column using a previously described approach (24). Briefly, nuclear extracts were chromatographed on a DEAE-Sephacel column, and after a wash with 1× binding buffer containing 100 mM NaCl, proteins were eluted with 30 mM elution buffer (20 mM HEPES-NaOH, pH 7.5, 2 mM EDTA, and 2 mM EDTA) containing 200 mM NaCl. The DEAE-Sephadex eluate was diluted with 40 μl of 1× binding buffer and further purified on the tandem IgG-affinity column (24). The top two modules contained Affi-Gel 10 (Bio-Rad) beads bearing immunoreactive No. 54 IgG, and the bottom module contained Affi-Gel 10 beads bearing anti-K protein No. 54 IgG. After washing the modules with 1× binding buffer containing 175 mM NaCl, the K protein was eluted from the bottom module with elution buffer containing 400 mM NaCl.

Kinase Assays on Beads—All phosphorylation reactions were carried out directly on beads as described previously (6, 17). Briefly, after loading with the kinase, beads were washed with 5 ml of 1× binding buffer containing 175 mM NaCl plus the full complement of phosphate inhibitors (30 mM p-nitrophenyl phosphate, 10 mM NaF, 0.1 mM Na3VO4, 0.1 mM Na2MoO4, and 10 mM β-glycerophosphate), once with 1 ml of 1× binding buffer without phosphate inhibitors, and finally once with 1 ml of 1× kinase buffer (20 mM HEPES-NaOH, pH 7.5, 10 mM MgCl2, 5 mM dithiothreitol). After resuspending the beads in 270 μl of binding buffer, 30 μl of 1× kinase buffer containing 5 μCi of [γ-32P]ATP was added with or without 1 mg/ml poly(C) RNA, and the phosphorylation reaction was carried out for 30 min at 30°C. The reaction was stopped by washing the beads twice with 800 μl of binding buffer. Proteins were eluted from the beads by boiling in 100 μl of 1× SDS loading buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol) (25), loaded on a 10% SDS gel, and autoradiographed.

RESULTS

Mapping of K Protein SH3-binding Domain and Its Specificity as a c-Src SH3 Domain Ligand—We have previously shown that K protein forms a complex with an SH3 domain that is phosphorylated by an 1L-1-responsive serine/threonine kinase (6, 12, 17). K protein contains a proline-rich stretch (2, 6) that could function as a SH3 ligand (10, 11, 13, 14). Given the fact that kinases can interact with their substrates via SH3 binding (19), we set out to identify the K protein SH3-binding site(s) and determine

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whether or not KPK binds to K protein via SH3 interactions. The purified full-length GST-K fusion protein and a number of GST-K protein deletion mutants were separated by SDSPAGE and transferred to Immobilon-P membrane. Blotted proteins were either immunostained with anti-GST monoclonal antibody (immunoblot, A) or probed with $^{32}$P-labeled GST-SrcSH3 fusion protein (autoradiograph, B). The GST-fusion protein constructs and the localization of the K protein stretch that contains the Src SH3-binding domain (Src SH3-BD) is illustrated in the bottom panel. Molecular mass markers are shown in kDa. Predicted sizes (in kDa) are: GST-K, 91.0; GST-K1, 30.4; GST-K2, 53.9; GST-K3, 42.2; GST-K5, 56.2; GST-K6, 39.8; GST-K7, 42.3; GST-K10, 33.1; GST-K13, 60.1.

![Fig. 1. Far Western mapping of the Src SH3-binding domain in K protein.](image)

Far Western blot analysis of K protein probed with different SH3 domains. Purified nuclear K protein was electrophoresed on SDS-PAGE and transferred to Immobilon-P membrane. Each lane contained the same amount of K protein. K protein in lane 1 was identified with anti-K antibody (antibody No. 54 (17) at 1:10,000 dilution). Proteins in lanes 2–8 were probed with a panel of GST SH3 domains (2 mg/ml) from phosphatidylinositol 3-kinase-associated p85 subunit (26) (lane 2), Ras-GAP (27) (lane 3), Grb-2 (28) (lane 4), NH$_2$ terminus SH3, and lane 5, COOH terminus SH3), Abl (29) (lane 6), c-Src kinase (Csk) (lane 7) (11), and c-Src (Src) (lane 8). The GST fusion proteins were detected with anti-GST monodonal antibody (0.1 mg/ml). Molecular mass markers are shown in kDa. The GST-Src SH3 domain bound to K protein when directly used as a probe (lanes 8). In contrast, SH3 domains from the phosphatidylinositol 3-kinase-associated p85 subunit (26) (lane 2), Ras-GAP (27) (lane 3), Grb-2 (28) (lane 4), NH$_2$ terminus SH3, and lane 5, COOH terminus SH3), Abl (29) (lane 6), and c-Src kinase (Csk) (lane 7) (11) did not bind to the K protein. These results show that the ability of K protein to bind SH3 domains is selective for the c-Src SH3 domain.

Each One of the Three Proline-rich Sites Can Independently Bind c-Src and Vav SH3 Domains—Inspection of the murine and the human K protein 209–318-amino acid stretch (2, 6) revealed three proline-rich sites that could serve as potential SH3-binding ligands (29, 30): PPGPRGPRPPSRR (amino acids 265–278), SPRGRGPPPPGPRG (284–297), and SRARNLPLPPPLPPRG (302–318). Affinity beads bearing synthetic peptides, each representing one of the three K protein proline-rich residues were used to determine which, if any, of these sites bind directly and specifically the c-Src SH3 domain. To do that, these peptide beads were mixed with buffer containing either GST-Src or GST-Csk SH3 fusion proteins. After centrifugation, proteins on the beads and in the supernatants were analyzed by SDS-PAGE and immunoblotting with an anti-GST antibody (Fig. 3). As expected from above experiments (Fig. 2), the GST-Csk SH3 fusion protein did not bind to beads bearing P1 (aa 264–278), P2 (aa 283–297), or P3 (aa 301–318) synthetic peptides (Fig. 3A, lanes 7–9), and, as a consequence, all of GST-Csk SH3 protein was recovered in the supernatants (Fig. 3A, lanes 1–3). In contrast, the GST-Src SH3 fusion protein bound in a very efficient manner to the P1, P2, and P3 beads (Fig. 3A, lanes 10–12), while only trace amounts remained in the supernatant of P1 and P2 (Fig. 3A, lanes 5 and 6). The specificity of the binding was assessed by using beads bearing P4 (aa 1–12) and P5 (aa 50–60) peptide which represent sequences outside of the SH3-BD (see bottom panel). As illustrated in B, there was no binding of GST-SrcSH3 to P4 (lane 6) or P5 (lane 5) compared to P3 peptide (lane 4). Once again, the GST-Csk SH3 domain did not bind to any of these peptides (lanes 1–3). These results demonstrate that (i) each one of the three K protein proline-rich sites can engage Src SH3 inde-
pendently of the other two, and (ii) although the amino acid sequences of these three K protein sites are different, these interactions are specific for the SH3 domain of c-Src.

As with c-Src, K protein has also recently been shown to engage Vav through SH3 interactions (13, 14). Vav contains two SH3 domains, but only the one present in the COOH terminus (Vav SH3C) binds K protein (13). We used the same approach with the peptide-bearing beads to determine which of the K protein proline-rich domains can engage Vav SH3 domain. Results of this experiment are shown in Fig. 4. As before (Fig. 3), proteins in the supernatant and proteins bound to the peptide beads were analyzed by SDS-PAGE and immunoblotting using an anti-GST antibody. The results showed that beads bearing each one of the proline-rich peptides (P1, P2, or P3) precipitated most of the GST-Vav SH3C fusion protein (compare beads (lanes 8–10) to supernatant (lanes 3–5)). The P3 peptide appeared most efficient since, after mixing with these beads, there was no GST-Vav SH3C protein remaining in the supernatant (compare lane 3 to lanes 4 and 5). In contrast to the beads bearing the proline-rich peptides, with beads bearing P4 (aa 1–12) and P5 (aa 50–60) peptides, most of GST-Vav SH3C remained in solution (lanes 1 and 2), with only a trace amount of the protein bound to the beads (lanes 6 and 7). No binding was observed with GST-Vav SH2 and the NH2 terminus GST-Vav SH3 domains to any of the peptide beads (data not shown). Therefore, as with c-Src SH3 domain (Fig. 3), each one of the K protein proline-rich sites can independently and specifically bind the Vav COOH terminus SH3 domain. Because there was significantly less GST-Vav SH3C protein remaining in the supernatant from beads bearing P3 peptide (compare lane 3 to lanes 4 and 5), the Vav SH3C domain exhibits preference for the third SH3-binding site, P3.

The Three K Protein SH3-binding Sites Show Different Affinities for the c-Src SH3 Domain—The above experiments suggested that the K protein proline-rich sites may bind SH3 domains with different affinities. To determine relative affinities of each of the SH3-binding sites, we compared the ability of synthetic peptides containing one of the K protein proline-rich sites to block SH3 interactions. To do that, Far Western blotting of GST-K protein was carried out using a 32P-labeled GST-Src SH3 probe incubated with increasing concentrations of the competitor peptides, P1, P2, and P3. Although each one of the peptides effectively competed with binding of the 32P signal is the weakest with the peptide P3 and the strongest with the peptide P1. To quantitate relative affinities of Src SH3 domain for the K protein P1, P2, and P3 proline-rich sites, radioactivity of each band, expressed as a percent of maximal counts, was plotted as
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![Fig. 5. Comparison of affinities of the K protein proline-rich sites for the c-Src SH3 domain.](image)

A, equal amounts of purified GST-K protein (1.5 µg/lane) were electrophoresed on SDS-PAGE and transferred to Immobilon-P membrane. After renaturation and blocking, the membranes were mounted in a manifold device (Hoefer, San Francisco, CA), and each lane was incubated for 2 h (25°C) with 2 mg/ml 32P-labeled GST-Src SH3 probe alone (lane 2) or in the presence (lanes 3-9) of increasing concentrations of either P1, P2, or P3 synthetic peptides. 750 µM P5 synthetic peptide was used as a negative control (lane 1). After binding, membranes were washed and autoradiographed. B, 32P-labeled bands were excised from the membranes and counted in a scintillation counter. Binding of the 32P-labeled GST-Src SH3 probe to GST-K is expressed as percent of maximal counts (100% = no peptide added, lane 2). C, amino acid sequences of the synthetic peptides (P1, P2, P3, and P5) and their location in the K protein.

The curves, the concentrations that yield 50% inhibition, IC50, for P1, P2, and P3 peptides are estimated to be around 0.2, 1, and 5 µM, respectively. This range is in good agreement with the concentrations of a peptide, identified by phage display selection (RLRPLPPLPPP), that also inhibited c-Src SH3 domain-K protein interaction (10). The inhibition of the binding of 32P-labeled GST-Src SH3 probe to GST-K by P1, P2, and P3 peptides was specific, since another peptide representing a different region of the K protein, P5, diminished the binding by only 13% even though it was used at the highest peptide concentration for this experiment (750 µM).

Two competitive inhibitors, the following relationship holds true, $IC_{50}/IC_{50} = K_i/K_j$, where $K_i$ is an inhibitor dissociation constant for an inhibitor (31). Also, the c-Src SH3 domain interactions with any of the two K protein proline-rich sites can be depicted by the following, $K_i/K_j = K_i/K_0$, where $K_0$ is the dissociation constant for a given site. Thus, using the IC50 ratios, we estimate that the relative affinity of the K protein P3 site for the Src SH3 domain is about 5-fold higher than the affinity of the P2 site and approximately 25-fold higher than the affinity of the P1 proline-rich site. Collectively, the above studies (Figs. 1-5) suggest that (i) the K protein contains at least three regions that bind the Src SH3 domain, (ii) each one of the regions binds c-Src SH3 domain with significantly different affinities, and (iii) the $K_0$ values of these interactions are in the micromolar range.

Mapping of the K Protein Domain That Recruits the KPK—Using a panel of GST-K deletion mutants, we next set out to define whether or not the region that recruits the serinethreonine K protein kinase includes the K protein SH3-binding domain. GST-K protein deletion mutants bound to glutathione beads were mixed with an eluate from an affinity anti-K IgG column containing the K protein-KPK complex (6). After washing the glutathione beads several times with increasing salt concentrations to remove nonspecific binding, the level of phosphorylation of the proteins bound to the beads was assessed with or without poly(C) RNA in the phosphorylation reaction as described previously (17). Fig. 6 illustrates an autoradiograph of 32P-labeled proteins eluted from the beads and separated by SDS-PAGE. As in the case of full-length GST-K protein (K, aa 1–464), the GST-K protein deletion mutants K4 (aa 84–464), K5 (aa 171–464), and K7 (aa 318–464) were phosphorylated in a poly(C) RNA-dependent manner, while K1 (aa 425–464), K2 (aa 84–337), and K3 (aa 171–337) mutants were not phosphorylated at all. Beads bearing the GST-K7 mutant do not bind to Immobilon-P membrane. After renaturation and blocking, the membranes were mounted in a manifold device (Hoefer, San Francisco, CA), and each lane was incubated for 2 h (25°C) with 2 mg/ml 32P-labeled GST-Src SH3 probe alone (lane 2) or in the presence (lanes 3-9) of increasing concentrations of either P1, P2, or P3 synthetic peptides. 750 µM P5 synthetic peptide was used as a negative control (lane 1). After binding, membranes were washed and autoradiographed. B, 32P-labeled bands were excised from the membranes and counted in a scintillation counter. Binding of the 32P-labeled GST-Src SH3 probe to GST-K is expressed as percent of maximal counts (100% = no peptide added, lane 2). C, amino acid sequences of the synthetic peptides (P1, P2, P3, and P5) and their location in the K protein.

![Fig. 6. Mapping of the K protein domains that bind and are phosphorylated by the K protein-associated kinase.](image)

50 µl of purified nuclear K protein/K protein-associated kinase preparation was mixed overnight (4°C) with glutathione beads (30-µl beads in 1 ml of binding buffer) bearing either the full-length or one of the deletion fragments of K protein fused to GST. After binding, beads were spun down and washed, and phosphorylation of the beads was done in 270 µl of 1 × binding buffer/30 µl of 10 × kinase buffer (200 µM HEPES, pH 7.5, 100 mM MgCl2, 50 µM dithiothreitol) containing 5 µCi of [γ-32P]ATP with (+) or without (−) poly(C) RNA. Phosphorylation was terminated by washing the beads twice with 800 µl of binding buffer. 32P-labeled proteins were eluted from the beads and were analyzed by SDS-PAGE and autoradiography. The lower panel illustrates the GST-K protein constructs used in the assay. Src SH3 BD, Src SH3-binding domain, and 32P/ATP/ binding designates the GST-K constructs that were phosphorylated by the bound kinase in an RNA-dependent manner. Molecular mass standards are shown in kDa. Predicted size (in kDa) for GST-K4 was 70 and for GST-K8, 37.5. (See Fig. 1 legend for the size of the other mutants.)
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Fig. 7. Mapping of the K protein domain that binds the K protein kinase, KPK. 30 μl of glutathione beads bearing plain GST, GST-K, or GST-K7 fusion proteins were mixed with purified nuclear K protein/K protein-associated kinases as in Fig. 6. After binding, the beads were spun down, washed, divided into 2 aliquots, and resuspended in phosphorylation buffer while the supernatants were mixed with 30 μl of glutathione beads containing the full-length GST-K fusion protein. After binding, GST-K protein beads were washed as before. Beads from the first (Beads) (left panel, lanes 1–6) and second (Supernatants) (right panel, lanes 1–6) round of binding were phosphorylated with (+) or without (–) poly(C) RNA as before (Fig. 6). After phosphorylation, proteins were eluted from the beads by boiling, separated by SDS-PAGE, stained with Coomassie Blue (upper panels, Coomassie) and autoradiographed (lower panels, 32P). Molecular mass standards are shown in kDa. The diagram shown below the gels illustrates the GST-K full-length and K deletion fusion proteins used in this experiment.

full-length K protein indicating that the 318–425 region is not a dimerization domain (data not shown). Thus, KPK recruitment by the K7 mutant is not mediated by the K protein dimerization site. From these experiments we can conclude that the domain which recruits KPK, denoted KPK-BD, is located within the 318–464-amino acid stretch (see the diagram in the lower part of Fig. 6). Interestingly, the K8 deletion fragment (aa residues 1–108), which represents another region of K protein, is also phosphorylated, but in this case the reaction is independent of poly(C) RNA. This observation suggests that there is a second kinase that binds to a site located in the NH2 terminus of K protein.

The fact that the GST-K7 deletion mutant (aa 318–464) was phosphorylated, while the GST-K1 deletion mutant (aa 425–464) was not, may indicate that either the K1 fragment does not engage KPK, that it does not contain suitable phosphorylation sites, or both. The next series of experiments was done to determine whether or not the K1 fragment can recruit KPK. Glutathione beads bearing plain GST, GST-K1, or GST-K7 fusion proteins were mixed with IgG-affinity-purified K protein/KPK preparation. Following binding, beads were spun down, and supernatants were then mixed with beads containing the full-length GST-K protein. Washed glutathione beads from the first and the second round of binding were phosphorylated as before with or without poly(C) RNA. Fig. 7 illustrates Coomassie Blue-stained (upper part) and autoradiographed SDS-PAGE (lower part) of proteins eluted from these beads (Beads, left panel). As in Fig. 6, GST-K7 (Beads, lanes 5 and 6) was phosphorylated in an RNA-dependent fashion, while plain GST (lanes 1 and 2) and GST-K1 (lanes 3 and 4) were not phosphorylated at all. The supernatants (right panel) from plain GST (lanes 1 and 2) and GST-K1 (lanes 3 and 4) beads contained a kinase activity that bound and phosphorylated the full-length GST-K protein to a far greater extent and in an RNA-dependent manner compared to the supernatant from the GST-K7 beads (lanes 5 and 6). These results demonstrate that while glutathione beads bearing the GST-K7 deletion mutant removed KPK from the preparation, the GST-K1 fragment containing the last 40 COOH terminus K protein amino acid residues did not, indicating that the 425–464 region does not contain the kinase-binding domain.

KPK is an IL-1-responsive kinase that binds to K protein independent of SH3 interactions. The in vivo phosphorylation of K protein is stimulated by treatment of cells with IL-1 (12). Similarly, in vitro phosphorylation of K protein by the associated kinase is enhanced when the kinase is purified from IL-1-treated compared to untreated cells (12, 17). Here we wished to test whether or not the KPK-BD that we mapped above (aa 318–425) engages the previously described IL-1-responsive kinase. To this end, the K protein–KPK complex was immunoprecipitated from nuclear extracts prepared from a time course of IL-1-treated EL-4 cells using protein A beads bearing anti-K protein IgG (Fig. 8). To eliminate binding of nonspecific kinases, all nuclear extracts samples were preclared with an excess of preimmune protein A–IgG beads as described previously (17). After immunoprecipitation, protein A beads bearing anti-K protein IgG were washed extensively and then were mixed overnight with glutathione beads bearing GST-K7 fusion protein to allow the transfer of the kinase from the natural K protein to the GST-K7 deletion mutant. The bead mixture was pelleted and washed, and again a phosphorylation reaction was performed with or without poly(C) RNA. 32P-labeled proteins were analyzed by SDS-PAGE, Coomassie-stained (not shown), and autoradiographed (Fig. 8A). The autoradiograph reveals a predominant band located below the 43-kDa marker, that represents the GST-K7 fusion protein (42.3 kDa). The level of GST-K7 phosphorylation was greater when poly(C) RNA was present in the phosphorylation reaction (lanes 7–11) compared to when it was absent (lanes 1–5). With poly(C) RNA in the kinase reaction, the level of phosphorylation of GST-K7 peaked with immunoprecipitates extracted after 15 min of IL-1 treatment (lane 8) and returned to baseline in nuclear extracts from cells treated with IL-1 for 2 h (compare lane 7 to 11). These results demonstrate that the GST-K7 deletion mutant (aa 318–464) is phosphorylated in a poly(C) RNA-dependent fashion by an IL-1-responsive kinase that coimmunoprecipitates with K protein. Moreover, phosphorylation of the immobilized GST-K7 fusion protein serves as evidence that the IL-1-responsive kinase was transferred from the immunoprecipitated native K protein and bound to the GST-K7 deletion fragment.

In the above type of an experiment (Fig. 8A) one cannot rule out a possibility that during the phosphorylation reaction the IL-1-responsive KPK merely comes off the immunoprecipitated native K protein, then stays in solution and phosphorylates the GST-K7 fusion protein without meaningful binding to it. To demonstrate, as it was done in Fig. 6, that the GST-K7 deletion mutant actually binds the IL-1-responsive KPK, crude nuclear extracts, rather than immunoprecipitates (Fig. 8A), from the
same IL-1 time course were applied to glutathione beads bearing the GST-K7 fusion protein (Fig. 8B). To ensure that the kinase binding is specific for the KPK-BD (aa 318–425), nuclear extracts, which may contain other K protein partners, were first precleared by overnight mixing with glutathione beads containing excess amounts of a mixture made of GST-K13 (aa 1–337) and GST-K1 (aa 425–464) fusion proteins. Supernatants from these beads were then reapplied to plain glutathione beads to clear residual GST-K13 and GST-K1 fusion proteins and, in turn, this supernatant was applied to beads bearing the GST-K7 deletion mutant. After several hours of incubation, the GST-K7 beads were phosphorylated with or without poly(C) RNA as before. An autoradiograph from this experiment shows an IL-1-responsive, poly(C) RNA-facilitated, GST-K7 phosphorylation that was nearly identical with the pattern obtained using K protein/KPK immunoprecipitates (Fig. 8, compare A to B). These two experiments demonstrate that the KPK-BD mapped to the amino acids 318–425 (Figs. 6 and 7) recruits a kinase that is indeed IL-1-responsive, and that phosphorylation of K protein by this enzyme is facilitated by poly(C) RNA. Because the GST-K13 mutant (aa 1–337) does not retain the KPK (Fig. 8B), we can further narrow down the KPK-binding domain to the amino acids 337–425. KPK may bind to the K protein directly, or the binding may be mediated by another K protein molecular partner. This issue cannot be unequivocally resolved until KPK is cloned and recombinant protein becomes available.

Evidence That c-Src and Vav Associate with K Protein and KPK in Vivo—The above series of experiments demonstrated that the SH3 domains of c-Src and Vav bind K protein in vitro (Figs. 1–5). If in cells c-Src and Vav exist in a complex with K protein, then it ought to be possible to immunoprecipitate K protein from cell extracts using antibodies directed against either c-Src or Vav. To test that and to determine if in vivo the binding is mediated through SH3 interactions, c-Src and Vav were immunoprecipitated from EL-4 cytoplasmic extracts in the presence or absence of an excess of the most effective SH3-binding site competitive inhibitor peptide P3 (Fig. 5). Western blot of Vav and c-Src immunoprecipitates probed with the anti-K antibody (Fig. 9A) demonstrates that K protein co-immunoprecipitated with either c-Src (lane 1) or Vav (lane 3). When the P3 competitor peptide was present during immunoprecipitation (compare lanes 2 to 1, and lanes 4 to 3), anti-c-Src and anti-Vav antibodies failed to precipitate K protein, indicating that the P3 peptide dissociated the K protein from K protein-c-Src and K protein-Vav complexes. The effect was specific since immunoprecipitation of K protein by anti-K antibody was not blocked in the presence of the P3 peptide (lane 6). This experiment suggests that K protein-c-Src and K protein-Vav complexes exist in vivo and that these interactions are mediated via K protein SH3-binding sites. The level of K protein in both c-Src and Vav immunoprecipitates was consistently lower compared to the amounts of K protein immunoprecipitated directly with the anti-K protein serum (compare lanes 1 and 3 to lane 5). This suggests that not all of the K protein pool in the cytoplasmic extracts is complexed with Vav and c-Src.
Interaction of K Protein with Protein Kinases

K Protein and KPK Can Immunoprecipitate with c-Src and Vav from EL-4 cell extracts. A, 50 μg of cytoplasmic protein extracts from EL-4 cells were incubated with 0.5 μl of either anti-pp60^{src} monoclonal antibody (lanes 1 and 2), anti-Vav C-14 polyclonal antibody (lanes 3 and 4), or anti-K protein serum No. 54 (lanes 5 and 6) with lanes 2, 4, and 6 without (lanes 1, 3, and 5) 750 μM P3 synthetic peptide (see Fig. 5). The immunocomplexes were recovered with 20 μl of protein A beads (Pharmacia, Uppsala, Sweden) and centrifugation (IP). Beads were washed once with 1 ml of binding buffer, twice with 1 ml of binding buffer containing 175 mM NaCl, and one more time with 1 ml of binding buffer. After the washes, beads were boiled in loading buffer, and eluted proteins were resolved on a 10% SDS-PAGE, electrotransferred to PVDF membranes, and immunostained with isotype control (lanes 1, 2, 3, and 4) or anti-pp60^{src} monoclonal antibody (lanes 5 and 6) (17) or with 1 mg/ml poly(C) RNA. The reaction was stopped by washing the beads twice with binding buffer. The immunocomplexes were recovered with 20 μl of protein A beads and centrifugation (IP). Beads were washed once with 1 ml of binding buffer, twice with 1 ml of binding buffer containing 175 mM NaCl, and one more time with 1 ml of binding buffer. After washing, protein A beads were resuspended in binding buffer to which 25 μl of glutathione beads bearing the GST-K7 fusion protein were added. The beads suspension was mixed for 8 h, then divided into two equal aliquots, and the phosphorylation reaction was carried out as before (Fig. 8) with or without 1 mg/ml poly(C) RNA. The reaction was stopped by washing the beads twice with binding buffer. 32P-labeled proteins were eluted from the beads by boiling and were analyzed by SDS-PAGE and autoradiography. Molecular mass standards are shown in kDa.

Because the sites that engage KPK and the SH3 domains do not overlap, K protein could simultaneously bind KPK and c-Src or Vav. If so, K protein would serve to promote multienzyme complex formation. To test whether KPK exists in a complex with either c-Src or Vav in vivo, c-Src, Vav, and K immunoprecipitates were prepared from EL-4 cell cytoplasmic extracts that were first preincubated with an excess of protein A beads bearing preimmune IgG. As before (Fig. 8A), following an extensive wash, the immunoprecipitates were incubated with a suspension of beads bearing GST-K7 fusion protein to transfer immunoprecipitated kinases from the native K protein to the K7 deletion mutant. The mixed beads were washed several times, and phosphorylation reaction of the mixed beads was carried out with or without poly(C) RNA (Fig. 9B). Similar to the results with the immunoprecipitates obtained with the anti-K protein antibody (lanes 1 and 2), the GST-K7 fusion protein bound and was phosphorylated in a poly(C) RNA-dependent fashion by a kinase that co-immunoprecipitated with either Vav (lanes 3 and 4) or c-Src (lanes 5 and 6). These results suggest that KPK can exist in a complex with c-Src and Vav in vivo, and that this association is likely mediated by K protein (Fig. 9A). The higher level of GST-K7 phosphorylation seen with the immunoprecipitates obtained with the anti-K protein antibody is to be expected since with the anti-K antibody more K protein was immunoprecipitated compared to the anti-c-Src and anti-Vav antibodies (Fig. 9A). Also, with the anti-K protein immunoprecipitates, not all KPK was transferred to the K7 mutant, accounting for the 32P-labeled band that represents the RNA-dependent phosphorylation of the native immunoprecipitated K protein (Fig. 9B, lane 2).

KPK Can Be Activated in Vitro by c-Src That Is Concurrently Bound to the K Protein—Treatment of cells with IL-1 can result in the activation of protein-tyrosine kinases (32–34). We have previously provided evidence that KPK is activated by phosphorylation (17). Because K protein can simultaneously engage KPK and c-Src (Fig. 9), and because the K protein domain that binds c-Src is contiguous with the domain that recruits KPK (Figs. 1 and 7), we wished to test whether or not the coupling of the two enzymes by K protein reflects the ability of c-Src to activate KPK. Although we used a recombinant and purified human c-Src kinase preparation from insect cells, we wished to ensure that contaminating insect enzymes cannot directly phosphorylate the K protein. To this end, we tested which if any of the GST-K fusion constructs can be directly phosphorylated by the c-Src preparation. The results of these experiments showed that while plain GST, GST-K1, GST-K7, and GST-K8 deletion mutants were not phosphorylated, the full-length GST-K, GST-K3, and GST-K13 fusion proteins were. However, with each of these fusion proteins, the observed phosphorylation was low and independent of poly(C) RNA (data not shown). These experiments, therefore, demonstrated that neither recombinant c-Src nor any possible contaminating insect kinases mimic KPK binding and phosphorylation activities toward K protein.

In order to attribute activation of KPK to the recombinant c-Src, we carried out the following experiment (Fig. 10) taking advantage of the fact that c-Src can bind to the GST-K protein SH3-binding domains. The recombinant c-Src preparation was mixed with glutathione beads bearing either GST-K7 protein or beads bearing a mixture of GST-K13/GST-K1 deletion mutants. (The GST-K13 mutant contains all three Src SH3-binding sites while GST-K7 contains none (Fig. 1)). Concurrently, inactivated KPK (17) was co-immunoprecipitated with K protein from crude nuclear extracts using protein A beads as described above (Figs. 8 and 9). The glutathione GST-K7 and GST-K13/K1 beads were then spun down, and the respective supernatants were incubated with K protein/KPK immunoprecipitates to allow binding of K-Src. After washing extensively, a phosphorylation reaction was performed on protein A beads in c-Src kinase buffer with or without poly(C) RNA. An autoradiograph of the 32P-labeled proteins eluted from the beads by boiling and were analyzed by SDS-PAGE and autoradiography. Molecular mass standards are shown in kDa.

Because the sites that engage KPK and the SH3 domains do not overlap, K protein could simultaneously bind KPK and c-Src or Vav. If so, K protein would serve to promote multienzyme complex formation. To test whether KPK exists in a complex with either c-Src or Vav in vivo, c-Src, Vav, and K immunoprecipitates were prepared from EL-4 cell cytoplasmic extracts that were first preincubated with an excess of protein A beads bearing preimmune IgG. As before (Fig. 8A), following an extensive wash, the immunoprecipitates were incubated with a suspension of beads bearing GST-K7 fusion protein to transfer immunoprecipitated kinases from the native K protein to the K7 deletion mutant. The mixed beads were washed several times, and phosphorylation reaction of the mixed beads was carried out with or without poly(C) RNA (Fig. 9B). Similar to the results with the immunoprecipitates obtained with the anti-K protein antibody (lanes 1 and 2), the GST-K7 fusion protein bound and was phosphorylated in a poly(C) RNA-dependent fashion by a kinase that co-immunoprecipitated with either Vav (lanes 3 and 4) or c-Src (lanes 5 and 6). These results suggest that KPK can exist in a complex with c-Src and Vav in vivo, and that this association is likely mediated by K protein (Fig. 9A). The higher level of GST-K7 phosphorylation seen with the immunoprecipitates obtained with the anti-K protein antibody is to be expected since with the anti-K antibody more K protein was immunoprecipitated compared to the anti-c-Src and anti-Vav antibodies (Fig. 9A). Also, with the anti-K protein immunoprecipitates, not all KPK was transferred to the K7 mutant, accounting for the 32P-labeled band that represents the RNA-dependent phosphorylation of the native immunoprecipitated K protein (Fig. 9B, lane 2).
Interaction of K Protein with Protein Kinases

**DISCUSSION**

In this study we demonstrate that the K protein contains SH3-binding sites. This is in agreement with previous reports that demonstrated in vitro interaction of K protein with the protein-tyrosine kinases Src, Fyn, and Lyn (10, 11) SH3 domains and in vitro and in vivo SH3 interaction of K protein with the proto-oncogene Vav (13, 14). In the present study we have extended these observations and showed that the K protein, in fact, contains a cluster of at least three SH3-binding domains (Figs. 3–5), and that K protein may serve as a docking platform for multiple enzymes that bind to the adjacent domains. As such, the K protein contains consensus sequences for both class I and II types of SH3 domains, and specifically engage c-Src and Vav (Fig. 9). The results from the studies presented in this report are consistent with the notion that the K protein can simultaneously recruit kinases to contiguous domains thus facilitating cross-talk. One example of such an interaction is protein-tyrosine kinase (PTK)-mediated phosphorylation of the K protein which is then activated, and in turn, phosphorylates the K protein.

**KPK engaged by the K protein can be activated through phosphorylation mediated by a tyrosine kinase that is recruited to the K protein by the contiguous cluster of SH3-binding sites, SH3-BD (Fig. 11).**

The competition experiments using synthetic peptides containing these motifs indicate that these domains engage c-Src SH3 with affinities that differ by more than an order of magnitude. For example, we estimated that the affinity of the P1 region (aa 264–278) for the c-Src SH3 domain is 25-fold lower than that of the P3 region (aa 301–318 stretch) (Fig. 5). These differences suggest that in vivo the P1 region is less likely to engage c-Src than the other two proline-rich sites. Nonetheless, these results demonstrate that K protein contains several clustered SH3-binding sites which could simultaneously engage several of the same or different SH3 domain-containing proteins. Moreover, because the K protein contains consensus sequences for both class I and II types of sites, it could engage SH3 domains in opposite orientations (37).

Although the linear structure of the most proximal SH3-binding site (aa 264–278) is different from the other two (aa 283–297 and 301–318) proline-rich domains, all three SH3-binding sites exhibited similar selectivity toward Src SH3 and Vav SH3 (Figs. 2–4). The structural basis for the shared binding specificity of disparate SH3-ligand sequences (29, 37, 38) will have to await three dimensional studies. It is interesting to note that all three K proline-rich regions have an adjacent RGG motif, a site that is methylated by a specific methyltransferase (39). It has been suggested that methylation of these residues might regulate SH3 interactions (40). Because K protein is arginine-methylated in vivo (39), this modification may be one way by which the interaction of K protein with its many molecular partners is regulated.

Each of the three SH3-binding domains can independently and specifically engage c-Src and Vav SH3 domains (Figs. 2–5). These domains are contiguous with a domain that recruits an IL-1-responsive kinase that phosphorylates K protein in an poly(C) RNA-dependent manner (Figs. 6–8). The functional significance of the succession of several SH3-ligand sites follows by a kinase binding domain may reflect the ability of the K protein to bind simultaneously two or more proteins that contain SH3 domains, for example c-Src and Vav, in addition to other enzymes that bind to the adjacent domains. As such, the K protein may serve as a docking platform for multiple enzymes. Such a model is supported by the ability of the K protein to simultaneously engage in vivo KPK and c-Src or Vav (Fig. 9). Moreover, the ability of c-Src, once recruited to the K protein, to activate K protein-bound KPK (Fig. 10), indicates that the K protein not only provides docking sites but in fact may facilitate intermolecular communication (Fig. 11). As such, the function of K protein would be analogous to the role of the insulin receptor substrate (IRS-1) which recruits p85, Grb-2, and the protein-tyrosine phosphatase, Syp (41), and to the yeast Ste5 which facilitates interactions for kinases in the MAP kinase (shown in italics) (35). The competition experiments using synthetic peptides containing these motifs indicate that these domains engage c-Src SH3 with affinities that differ by more than an order of magnitude. For example, we estimated that the affinity of the P1 region (aa 264–278) for the c-Src SH3 domain is 25-fold lower than that of the P3 region (aa 301–318 stretch) (Fig. 5). These differences suggest that in vivo the P1 region is less likely to engage c-Src than the other two proline-rich sites. Nonetheless, these results demonstrate that K protein contains several clustered SH3-binding sites which could simultaneously engage several of the same or different SH3 domain-containing proteins. Moreover, because the K protein contains consensus sequences for both class I and II types of sites, it could engage SH3 domains in opposite orientations (37).

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cascade (42, 43). Notably, in the case of the K protein, phosphorylation of the COOH terminus region is modulated by RNA (Figs. 6–8); therefore, the cross-talk among the multiple proteins that are docked on the K protein might be regulated by cognate nucleic acid motifs.

The molecular mechanisms responsible for the IL-1-responsive phosphorylation of K protein (12, 17) remain to be defined. However, given the observation that KPK appears to be activated by phosphorylation (17), recruitment of c-Src or a related tyrosine kinase to K protein may serve to facilitate phosphorylation and thus activation of KPK (Figs. 10–11). Given the fact that IL-1 does activate protein-tyrosine kinases (33, 34), it is possible that the K protein may provide a mechanism by which KPK (17), which is engaged in a series of SH3-binding sites that is contiguous with a domain that may be engaged in a contiguous domain, is activated in response to IL-1 (Fig. 11).

In summary, this study shows that the K protein contains a series of SH3-binding sites that is contiguous with a domain that binds an IL-1-responsive kinase. K protein can simultaneously engage proteins and nucleic acids (Fig. 8) and appears to be engaged in a series of SH3-binding domains that may provide a mechanism by which KPK (17), which is engaged by a contiguous domain, is activated in response to IL-1 (Fig. 11).

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