Peptide Microarray Analysis of Substrate Specificity of the Transmembrane Ser/Thr Kinase KPI-2 Reveals Reactivity with Cystic Fibrosis Transmembrane Conductance Regulator and Phosphorylase*

Hong Wang and David L. Brautigan‡

Human lemur (Lmr) kinases are predicted to be Tyr kinases based on sequences and are related to neurotrophin receptor Trk kinases. This study used homogeneous recombinant KPI-2 (Lmr2, LMTK2, Cprk, brain-enriched protein kinase) kinase domain and a library of 1,154 peptides on a microarray to analyze substrate specificity. We found that KPI-2 is strictly a Ser/Thr kinase that reacts with Ser either preceded by or followed by Pro residues but unlike other Pro-directed kinases does not strictly require an adjacent Pro residue. The most reactive peptide in the library corresponds to Ser-737 of cystic fibrosis transmembrane conductance regulator, and the recombinant R domain of cystic fibrosis transmembrane conductance regulator was a preferred substrate. Furthermore the KPI-2 kinase phosphorylated peptides corresponding to the single site in phosphorylase and purified phosphorylase b, making this only the second known phosphorylase b kinase. Phosphorylase was used as a specific substrate to show that KPI-2 is inhibited in living cells by addition of nerve growth factor or serum. The results demonstrate the utility of the peptide library to probe specificity and discover kinase substrates and offer a specific assay that reveals hormonal regulation of the activity of this unusual transmembrane kinase. Molecular & Cellular Proteomics 5:2124–2130, 2006.

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the Lmr group of kinases have also been called “apoptosis-associated protein Tyr kinases” (AATYK) (7, 11–17) because there is induction of KIAA1079 (mouse homologue of Lmr1) in cerebellar granule cells induced to undergo apoptosis by low potassium levels, and overexpression of AATYK1 in these cells increased the number of apoptotic cells (12). The expression level of AATYK1 was increased in myeloid cells during growth arrest or differentiation (17). This family of kinases has not been found in zebrafish, Drosophila, or Caenorhabditis elegans, suggesting that they are exclusively mammalian kinases.

The Lmr2 kinase has been discovered independently by three approaches. We found KIAA1079 (Lmr2) as yeast two-hybrid prey from a mouse embryonic day 9.5 CDNA library using phosphatase inhibitor-2 as bait and demonstrated PP1 binding, so we named it KPI-2 (1). A separate yeast two-hybrid screen of a human brain cDNA library isolated KIAA1079 using the p35 subunit of CDK5 as bait. The kinase was named Cprk for cyclin-dependent kinase 5/p35-regulated kinase (8). The third approach used a database search for novel kinases in brain that revealed KIAA1079, and its mRNA was found primarily in brain by Northern hybridization and RT-PCR. This group therefore named KIAA1079 as brain-enriched protein kinase (BREK) (9). All three groups have reported autophosphorylation of the kinase, and either phosphoamino acid analysis or immunoblotting with phosphospecific antibodies showed only Ser(P) and Thr(P). This raised the issue that the biochemical properties were different from those predicted based on sequence similarities to various Tyr kinases. Co-expression of KIAA1079 with CDK5/p35 was reported to inhibit autophosphorylation possibly due to inhibitory CDK phosphorylation (8). Overexpressed BREK was phosphorylated upon stimulation of PC-12 cells with NGF, but it was not determined whether this was Tyr or Ser/Thr phosphorylation. Transfection of kinase-defective BREK enhanced neurite outgrowth in PC12 cells indicating that BREK might inhibit neurite outgrowth by phosphorylation (9). KIAA1079 is also called AATYK2 because its sequence is related to AA-TYK1 and AATYK3, but there are no reports about involvement of this kinase in apoptosis.

The cellular substrates of any of the Lmr kinases are unknown. To examine the specificity of KPI-2 kinase against a wide selection of possible peptide substrates we performed a PepChip kinase assay with an array of >1,100 peptides. We found phosphorylation of Ser and Thr but not Tyr peptides. Based on the peptide sequences we identified two unusual possible protein substrates, cystic fibrosis transmembrane conductance regulator (CFTR) (18) and glycosen phosphorylase (phos-b) (19). We show that these proteins are substrates for KPI-2 kinase, and phos-b was used as a highly specific KPI-2 substrate to demonstrate that NGF signaling in living cells reduces the activity of KPI-2 kinase.

**Microarray Analysis of KPI-2 Kinase Substrate Specificity**

**Experimental Procedures**

Constrasts—pCMV-KPI-2—Full length was constructed by inserting KPI-2 full length into pCMV C-terminal c-Myc-tagged vector, and pFastBac-His-pKPI-2 kinase was constructed by inserting KPI-2 fragment (residues 94–600) into pFastBac HTb vector as described before (1). This construct was chosen as kinase to include the minimal kinase fold (predicted as residues 137–407) plus an additional C-terminal segment that is unique to Lmr proteins and might be involved in regulation and/or substrate recognition. The solubility, stability, activity, and relative resistance to proteolytic pruning during purification suggest that this kinase has a globular native structure. A plasmid encoding the R domain of CFTR (residues 589–830) fused to GST was a kind gift from Dr. William Reenstra, University of Pennsylvania.

Expression and Purification of Recombinant KPI-2 Kinase Protein—For protein expression, S97 cells were infected with recombinant baculoviruses, and cells were harvested 60 h later. The recombinant proteins were purified by IMAC as described before (1). The fractions containing KPI-2 kinase were pooled and loaded onto a HiTrap™ Q XL anion exchange column (Amersham Biosciences). Proteins were eluted by linear gradient of NaCl concentration from 0.2 to 0.7 M. Fractions were subjected to 9% SDS-PAGE and analyzed by GelCode Blue staining (Pierce). Fractions containing KPI-2 kinase were concentrated using Centricon YM-50 (Millipore) and then loaded to Ultralag AcA64 column (1.5 × 43 cm). Blue dextran 2000 was added to the loading sample to determine void volume of the column. The proteins were eluted at 10 ml/h with the buffer (20 mM Hepes, pH 7.4, 10 mM MgCl2, 1 mM dithiothreitol, 0.1% β-mercaptoethanol, 10% glycerol, 100 mM NaCl, and 20 µg/ml ovalbumin), and 80 fractions of 1 ml each were collected. The fractions containing protein were pooled, and the concentrations were measured by Bio-Rad protein assay.

**PepChip Array Kinase Assay and Analysis**—PepChip kinase array was purchased from Pepscan Systems. The size of PepChip kinase slides is 25 × 75 mm. The full slide contains two duplicate arrays of 1,176 peptides each. For kinase assay, 50 μl of kinase reaction solution containing 500 ng/ml purified recombinant KPI-2 kinase (fractions 24 and 25 from AcA54, Fig. 1D, box), 2× kinase reaction buffer, 10 μM ATP, and 300 μCi/ml [32P]ATP was added to the PepChip slide and incubated for 2 h at 30 °C. The 2× kinase reaction buffer includes 100 mM Hepes, pH 7.4, 40 mM MgCl2, 20% glycerol, 0.02 mg/ml BSA, and 0.02% Brij 35. After incubation, the slide was washed once with PBS-TX (1% Triton X-100, PBS), twice with NaCl-TX (1% Triton X-100, 2 M NaCl), and twice with distilled H2O and then air-dried. The phosphorylation was detected by PhosphorImager (GE Healthcare). The result was quantitated and analyzed by Image-quant software.

**Cell Culture, Transfection, Immunoprecipitation, and Western Blot**—HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn calf serum. Cells were transfected using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen). After 48 h, the cells were incubated in the medium without serum for 3 h followed by treatment with 100 ng/ml NGF for 30 min. Cells were lysed with 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM Na3VO4, 1 mM Microcystin-LR, plus protease inhibitors). For immunoprecipitation, equal amounts of proteins were incubated with anti-c-Myc agarose (Sigma) for 2 h at 4 °C. The beads were washed three times with lysis buffer and once with kinase buffer and then subjected to SDS-PAGE for Western blot or used as kinase source for kinase assay.

**KPI-2 Kinase Assays**—Kinase assays were performed by using either purified KPI-2 kinase or full-length KPI-2 kinase immunoprecipitated from HEK293T cells by c-Myc tag. KPI-2 kinases were precipitated from HEK293T cells by c-Myc tag. KPI-2 kinases were precipitated from HEK293T cells by c-Myc tag. KPI-2 kinases were precipitated from HEK293T cells by c-Myc tag. KPI-2 kinases were precipitated from HEK293T cells by c-Myc tag.
incubated in a total reaction volume of 20 μl containing 10 mM MgCl₂, 5 mg/ml different substrates, 100 μM [³²P]ATP (10 μCi). For immunoprecipitated KPI-2 kinase, 200 μg Na₃VO₄, 20 nM Microcystin-LR, and 1 mM EGTA (final concentrations) were added to the kinase reaction. The phosphorylation was detected by PhosphorImager (GE Healthcare). The results were quantitated and analyzed by ImageQuant software.

RESULTS

Peptide Microarray Assay and Analysis—The KPI-2 kinase domain (Fig. 1A; residues 94–600) was produced by a baculovirus expression system and purified in three steps. First KPI-2 kinase was purified from the soluble extract by IMAC, and one major protein was recovered (Fig. 1B, box). This was the only radiolabeled protein observed during autophosphorylation with [³²P]ATP (not shown). However, we suspected there were multiple kinases present at this level of purification because in kinase assays the preparation phosphorylated Ser/Thr residues in different substrates and also phosphorylated the synthetic polymer poly(Glu-Tyr). Proteins were eluted by stepwise increase in concentration of imidazole-HCl (Im-HCl), pH 7, as indicated. C, fast protein liquid chromatography anion exchange chromatography. Fractions 5–10 from nickel-nitrilotriacetic acid column (B in box) were combined and loaded onto a HiTrap Q XL column. Proteins were eluted by linear gradient of NaCl concentration from 0.2 to 0.7 M. D, size exclusion chromatography. Fractions 12–25 from HiTrap Q XL column (C in box) were pooled and concentrated using Centricon YM-50. Blue dextran 2000 was added to the concentrated sample to determine void volume of column. The sample was loaded onto an Ultragel AcA54 column (1.5 × 43 cm), and 80 fractions of 1 ml each were collected. 10 μl of each fraction from above columns were subjected to 9% SDS-PAGE and analyzed by GelCode staining. The lower bands of D are ovalbumin added in the elution buffer to prevent loss of protein due to absorption to surfaces.

FIG. 1. Purification of recombinant KPI-2 kinase. The recombinant baculovirus of KPI-2 kinase (residues 94–600) was made using Bac-to-Bac baculovirus expression system. Recombinant proteins were purified by IMAC followed by ion exchange and size exclusion chromatography. A, constructs of KPI-2 used in this study. a, pCMV-KPI-2-Full length. TM stands for transmembrane domain. KD is kinase domain. VTF is PP1C binding motif. b, pFastBac-His₆-KPI-2 kinase used in Sf9 cells. B, IMAC. The lysates from infected Sf9 were incubated with nickel-nitrilotriacetic acid resin for 2 h, and proteins were eluted by stepwise increase in concentration of imidazole-HCl (Im-HCl), pH 7, as indicated. C, fast protein liquid chromatography anion exchange chromatography. Fractions 5–10 from nickel-nitrilotriacetic acid column (B in box) were combined and loaded onto a HiTrap Q XL column. Proteins were eluted by linear gradient of NaCl concentration from 0.2 to 0.7 M. D, size exclusion chromatography. Fractions 12–25 from HiTrap Q XL column (C in box) were pooled and concentrated using Centricon YM-50. Blue dextran 2000 was added to the concentrated sample to determine void volume of column. The sample was loaded onto an Ultragel AcA54 column (1.5 × 43 cm), and 80 fractions of 1 ml each were collected. 10 μl of each fraction from above columns were subjected to 9% SDS-PAGE and analyzed by GelCode staining. The lower bands of D are ovalbumin added in the elution buffer to prevent loss of protein due to absorption to surfaces.
been removed. For final and complete purification the KPI-2 was concentrated and loaded onto an Ultradel AcA54 column where it was separated from other proteins and eluted as a monomer (Fig. 1D, box) that was used as the purified kinase.

We incubated KPI-2 kinase with radioactive ATP and a PepChip microarray (Pepscan Systems), which contains duplicate sets of 1,152 different peptides with a median length of nine residues, based on known phosphorylation sites in the PhosphoBase database (phospho.elm.eu.org). Reaction conditions were optimized with a test array prior to assay with the complete array. The results in the two duplicate arrays on the same slide (Fig. 2) matched one another. We quantitated the radioactivity to get relative phosphorylation levels of the peptides. We attributed this activity to a trace contaminant of a Tyr kinase, most of which was removed during anion exchange chromatography. Of the positives only about 1% of the total peptides (14 of 1,152 total) showed strong phosphorylation +++ to ++++++ (Table II). This attested to the purity and specificity of the KPI-2 kinase and the discrimination of the assay. No consensus sequence for substrate specificity could be discerned from the group of KPI-2 substrate peptides. However, the KPI-2 kinase did show preference for sites with basic residues, similar to PKA. KPI-2 also efficiently phosphorylated Ser/Thr residues adjacent to Pro as well as those not next to Pro, showing it has unique reactivity and substrate specificity distinct from PKA and other kinases.

Our attention was drawn to the most intensely phosphorylated peptide (position X21 Y2; see Fig. 2 for peptide positions), which is derived from CFTR (20–22) and contains the Ser-737 phosphorylation site (Fig. 3A). We identified four other peptides in the microarray that correspond to phosphorylation sites in CFTR. These peptides were much less intensely phosphorylated by KPI-2 kinase (Fig. 3A). We also noticed peptides corresponding to the single Ser phosphorylation site in phos-b (19, 23) (Fig. 3A). There were six different variants of the phos-b peptide in the array. The phosphorylation intensity of these peptides was not in the highest 1%, but we recognized phos-b as an example of a protein substrate that is exceptionally specific. There is only one kinase known to react with phos-b, namely phosphorylase b kinase (PhK), a Ca\(^{2+}\)- and calmodulin-activated kinase (24–26). This raised a possibility for us to use phos-b as substrate to

### TABLE I

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<th>Intensity</th>
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<tr>
<td>Total</td>
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### TABLE II

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<td>RKRSRKA</td>
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<tr>
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<td>X28 Y27</td>
<td>RTKRSGSV</td>
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<td>X2 Y28</td>
<td>KRPTQRAKY</td>
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measure KPI-2 kinase activity in the presence of EGTA.

**KPI-2 Phosphorylation of CFTR and Phosphorylase b**—To extend the results of the PepChip array assay, we performed KPI-2 kinase assays using different purified and recombinant proteins as substrates. These included recombinant GST-CFTR R domain (residues 589–830) expressed in bacteria, phosphorylase b purified from rabbit muscle (27), and histone H1. As shown in Fig. 3B, all these substrates were phosphorylated by KPI-2 with histone H1 most intensely phosphorylated and phos-b and GST-CFTR phosphorylated about equally. GST was not phosphorylated as expected for the negative control. The results show that the peptide phosphorylation in the microarray assay was predictive of phosphorylation of the intact proteins. As an additional control the same protein substrates were incubated in parallel with PKA, a known kinase for CFTR (28). As shown in Fig. 3B, GST-CFTR was phosphorylated by PKA, consistent with previous reports that the R domain in CFTR has multiple sites of PKA phosphorylation (29, 30).

**Assay of KPI-2 Kinase with Phosphorylase b as Specific Substrate**—Our results indicated that we could use phos-b as specific substrate to measure KPI-2 kinase activity in reactions performed with EGTA to chelate calcium. KPI-2 has been implicated in NGF signaling in PC12 cells based on indirect evidence of cell shape changes in response to NGF after cells were stably transfected with active or inactive kinase (9). To directly test whether KPI-2 kinase activity changes in response to NGF we expressed full-length c-Myc-tagged KPI-2 in HEK293T cells, treated the cells with NGF, and compared them with an untreated control. KPI-2 was immunoprecipitated with anti-c-Myc-agarose (Sigma). Kinase reaction was carried out by incubating KPI-2 kinase with different substrates and [³²P]ATP. PKA was used as a control kinase for comparison.

**DISCUSSION**
Among the 518 human protein kinases about 90 are classified as Tyr-specific based on sequence similarities in the catalytic domain (10). This study used a peptide microarray to
test the substrate specificity of one such putative protein Tyr
kinase of the Lmr branch of the kinase family, KPI-2 (1) (also
known as AATYK2, Lmr2, LMTK2, Cprk, and BREK). Based
on reaction with 1,152 peptides on the microarray only about 1%
were phosphorylated effectively, and about one-third of
the peptides showed some radiolabeling. These peptide sub-
strates were based on a variety of sites of protein phospho-
ylation in the PhosphoBase database and had a mean length
of nine residues to facilitate recognition. Contrary to its clas-
cification, KPI-2 did not phosphorylate Tyr-containing pep-
tides but was specific for Ser/Thr peptides. This agrees with
our previous results of KPI-2 autophosphorylation on Ser/Thr
residues and a lack of reactivity with poly(Glu-Tyr) as a sub-
strate. Thus, the Lmr group had been placed on a branch of the
kinase family tree with the well known Tyr-specific kinases
EGFR-HER and JAK-TYK (10) and is related by BLAST anal-
ysis to Trk Tyr kinase; however, the Lmr kinases need to be
considered specific Ser/Thr kinases.

The specificity of KPI-2 for peptide substrates revealed
reactivity with Pro-Ser and Pro-Thr sites in peptides from
bovine MBP. Interestingly KPI-2 did not phosphorylate a num-
ber of other peptides for known phosphosites in MBP, includ-
ing sites for mitogen-activated protein kinase (Thr-97), protein
kinase C (Ser-45, Ser-114, and Ser-150), and PKA (Ser-10,
Thr-33, Ser-54, Ser-109, Ser-131, and Ser-160). Phosphoryla-
tion of residues adjacent to Pro ((Ser/Thr)-Pro-X) is a hall-
mark of the so-called Pro-directed kinases (31, 32) such as
CDK, glycogen synthase kinase-3, or mitogen-activated pro-
tein kinases. However, these kinases are different from KPI-2
in that they react with Ser/Thr followed by Pro, whereas KPI-2
phosphorylated Ser/Thr preceded by Pro. Yet KPI-2 also phos-
phorylated residues followed by Pro such as the PITPP site in
protein phosphatase-1 (Thr-320) and the PRPT site in MBP.
KPI-2 phosphorylation of Thr-320 in PP1 reduces the activity
of the phosphatase and is removed by intramolecular autode-
phosphorylation. KPI-2 unlike other Pro-directed kinases is
not exclusively specific for Pro sites. Many of the KPI-2 reac-
tive sites had neighboring basic residues. In this way KPI-2
resembles PKA, which prefers (R/K)XXS as a substrate, but
with both peptide microarrays and with proteins substrates
these kinase are different from one another. Overall KPI-2
exhibited a unique pattern of substrate specificity as a Ser/Thr
kinase.

Results from the peptide microarray led us to test two
proteins as substrates for KPI-2. First, by far the best sub-
strate in the entire array was a peptide corresponding to
Ser-737 in the regulatory domain of CFTR. Indeed the R
domain of CFTR is an excellent substrate for KPI-2. Both
KPI-2 and CFTR are transmembrane proteins found in the
plasma membrane of cells, and phosphorylation of CFTR
regulates its channel activity (33–35). But it remains to be
determined whether KPI-2 phosphorylates full-length CFTR in
membranes and whether this has any functional conse-
quence. The other protein identified from the PepChip array is
phos-b, and we found that purified phos-b is indeed a sub-
strate for KPI-2. Phos-b was the first kinase substrate ever
discovered, by Fischer and Krebs (24) 50 years ago, and since
that time no other kinase has been found capable of phos-
phorylating the single site at Ser-15 in the N terminus of the
protein. To date the one and only phos-b kinase is a Ca2+-
and calmodulin-dependent kinase composed of four subunits
arranged in a tetramer of tetramers (24–26). Here we utilized
a kinase assay including EGTA to exclude this phos-b kinase
activity and effectively measured the activity of precipitated
KPI-2. This assay was used to show that the kinase activity of
KPI-2 was reduced by >60% in response to adding NGF to
living cells. A previous report (9) showed the NGF stimulation
of PC12 cells caused reduced mobility of KPI-2 in SDS-PAGE,
consistent with NGF-induced phosphorylation. That response
was blocked by chemical inhibitors K252a and chelerythrine.
Together the results suggest that NGF stimulates phos-
phorylation of KPI-2, perhaps involving protein kinase C, which
reduces its kinase activity. Other experiments have shown
that CDK5 phosphorylation of KPI-2 reduced its autophos-
phorylation (8). Thus, KPI-2 seems to have multiple sites of
phosphorylation that can inhibit its kinase activity. The avail-
ability of phos-b as a convenient and highly specific substrate
for KPI-2 will facilitate future studies of the regulation of this
unusual transmembrane kinase.

Acknowledgment—We thank Dr. William Reenstra, University of
Pennsylvania, for the generous gift of pGEX-CFTR-R domain plasmid.

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