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3pK, a New Mitogen-Activated Protein Kinase-Activated Protein Kinase Located in the Small Cell Lung Cancer Tumor Suppressor Gene Region

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***Not*I linking clones, localized to the human chromosome 3p21.3 region and homozygously deleted in small cell lung cancer cell lines NCI-H740 and NCI-H1450, were used to search for a putative tumor suppressor gene(s). One of these clones, NL1G210, detected a 2.5-kb mRNA in all examined human tissues, expression being especially high in the heart and skeletal muscle. Two overlapping cDNA clones containing the entire open reading frame were isolated from a human heart cDNA library and fully characterized. Computer analysis and a search of the GenBank database revealed high sequence identity of the product of this gene to serine-threonine kinases, especially to mitogen-activated protein kinase-activated protein kinase 2, a recently described substrate of mitogen-activated kinases. Sequence identity was 72% at the nucleotide level and 75% at the amino acid level, strongly suggesting that this protein is a serine-threonine kinase. Here we demonstrate that the new gene, referred to as 3pK (for chromosome 3p kinase), in fact encodes a mitogen-activated protein kinase-regulated protein serine-threonine kinase with a novel substrate specificity.**

The short arm of human chromosome 3 harbors a number of tumor suppressor genes. Two of these genes were cloned and characterized: the von Hippel-Lindau disease gene at 3p25 (33) and the hereditary nonpolyposis colorectal cancer gene at 3p21-p22 (6, 42). At least three other tumor suppressor loci are considered to be located on 3p: 3p25, which is frequently deleted in breast cancers (8); the 3p21 region, which is involved in the origin and/or development of small cell lung cancer (SCLC) and non-SCLC (4), testicular (37), ovarian (56), and renal cell (29, 63) carcinomas; and the 3p12-p13 region, which shows loss of heterozygosity or homozygous deletions of markers in several cases of breast cancer (8), SCLC (34, 44), and renal cell carcinoma (38). In the case of SCLC, heterozygous deletions of large segments of 3p are frequently observed, complicating the definition of the tumor suppressor locus. Recently homozygous deletions have been found in several SCLC-derived cell lines (13, 28), defining the putative SCLC gene locus to 3p21.3. At the same time, a 2-Mb DNA fragment from the 3p21-22 region, overlapping with the SCLC cell line NCI-H740 deletion, was shown to suppress tumorigenicity in nude mice when transfected into a mouse fibrosarcoma cell line (26). In the present study, *Not*I linking clones, which are markers of human genes (1), were used to identify transcribed sequences within the large NCI-H740 deletion. One of the genes identified within this region codes for a sequence which has extensive homology to the previously identified gene for mitogen-activated protein kinase-activated protein kinase 2 (MAPKAP kinase 2).

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MAPKAP kinase 2 was originally isolated from rabbit skeletal muscle (50). In vitro studies have shown that the mitogen-activated protein (MAP) kinase/extracellular signal-regulated kinase (ERK) isoforms are the only detectable MAPKAP kinase 2-reactivating enzymes in skeletal muscles and in PC12 cell extracts (50). However, recently it was shown that in several cell lines, MAPKAP kinase 2 was activated by chemical stress, heat shock, and osmotic shock but not in response to growth factors that activate the ERK1/ERK2 pathway (47). ERK is activated by threonine and tyrosine phosphorylation (3). This phosphorylation is catalyzed by a dual-specificity kinase capable of phosphorylating both threonine and tyrosine residues which is known as MEK (48). There are several MEK isozymes; two of them, MEK1 and MEK2, are functionally related (11, 59). Recently C-Raf was identified as an activator for MEK1 (30). All three Raf enzymes, C-Raf, A-Raf, and B-Raf, are capable of phosphorylating and activating MEK1 and MEK2 (30, 32, 57, 58).

In contrast to the three Raf enzymes and to MEK1 and MEK2, which show highly restricted substrate specificity, ERK1 and ERK2 have been reported to regulate a wide range of substrates by phosphorylation. These include transcription factors of the ets family (7, 22, 45, 55), phospholipase A2 (41), structural elements (24) and the protein Ser/Thr ribosomal S6 protein kinases (RSKs) (53), and MAPKAP kinase 2 (52). The consensus sequence for phosphorylation by ERK1 and ERK2 is Pro-Xaa-Ser/Thr-Pro (10, 23). The ERK1 and ERK2 substrates include those critical for transformation of NIH 3T3 cells by Ras or Raf, and coexpression of these oncogenes with catalytically inactive mutants of ERK2 abolished this induction (57). The protein Ser/Thr kinase RSK/MAPKAP kinase 1 is structurally and functionally distinct from MAPKAP kinase 2

(50, 51, 64). In this study, we show that the product of the newly isolated protein kinase gene from 3p, 3p kinase (3pK), can also be activated through the RAF-MEK-ERK pathway.

The small heat shock proteins Hsp25 and Hsp27 have been shown to be phosphorylated by MAPKAP kinase 2 at identical sites in vitro (27, 50, 52) and in vivo (21). Recent studies using a specific inhibitor of p38 kinase, an upstream activator of MAPKAP kinase 2, identified Hsp27 as a physiological substrate (12, 35). Both heat shock proteins are not phosphorylated by RSK/MAPKAP kinase 1, and conversely, two of the substrates of RSK/MAPKAP kinase 1, namely, the glycogen-binding subunit of protein phosphatase 1 and peptides related to the C terminus of S6 kinase II, are not phosphorylated by MAPKAP kinase 2 (52). However, both kinases phosphorylate glycogen synthase (at serine 7) and a glycogen synthase derived-peptide, GS peptide 1 (14, 52). Using GS peptide 1 analogs, it has been demonstrated that the substrate specificities for these two enzymes are different (51). When the same panel of peptides was tested with 3pK, a novel pattern of phosphorylation was observed, indicating that the substrate specificity of 3pK is different from that of RSK/MAPKAP kinase 1 and MAPKAP kinase 2.

MATERIALS AND METHODS

Sequence analysis. Searches of the GenBank database were performed by using BLAST on the National Center for Biotechnology Information file server (2). Amino acid alignments were generated with PILEUP (19). Sequences were read with an IBI Gel Reader and analyzed with the Genetics Computer Group package of programs (16) running on a VAX computer.

PCRs. Primers to 3pK sequences were designed by using the PRIMER program (36). Reactions were performed in 1× PCR buffer (Boehringer Mannheim). Samples were heated to 96°C for 5 min and amplified for 35 to 40 cycles of 96°C for 30 s, 5°C for 30 s, and 72°C for 1 min. PCR products were analyzed on 1.5 to 2% agarose gels.

Northern (RNA) hybridization. DNA fragments used as probes were purified on a 1% low-melting-temperature agarose gel. DNA was labeled directly in agarose by using a Random Primed DNA Labeling Kit (Boehringer Mannheim) and hybridized to multiple-tissue Northern blots (Clontech) as instructed by the manufacturer. Each blot contains 2 μg of poly(A)$^+$ RNA from various human tissues.

Expression and purification of 3pK. An *Nco*I-*Sac*I fragment containing the entire coding region of the 3pK cDNA was cloned into pGEXKG. The recombinant plasmid was transformed into *Escherichia coli* TG1. Large-scale preparations of the transformed bacteria were induced to produce a fusion protein by the addition of 0.2 mM isopropylthiogalactopyranoside (IPTG). Cells were then harvested and lysed, and the fusion protein was purified by glutathione-agarose affinity chromatography (Pharmacia GST Gene Fusion System) essentially as described by the manufacturer.

Preparation of a 3pK-specific polyclonal antiserum. A glutathione *S*-transferase (GST)–3pK fusion protein was cleaved overnight with thrombin, and the 44-kDa 3pK protein was isolated from the gradient gel by electroelution. The electroeluted fragment was then used as an immunogen in rabbits to generate a 3pK-specific antiserum.

Expression of 3pK in Sf9 cells. A 1.3-kb *Eco*RI fragment containing the entire 3pK coding sequence was subcloned into the PVL 1392 transfer vector (InvitroGen). Then 0.5 μg of transfer vector containing the 3pK gene was transfected by lipofection into Sf9 cells along with *Bsa*36I-digested Bac Pak 6 viral DNA (Clontech). Recombinant baculovirus was isolated by plaque purification, using a standard protocol. For recombinant protein production, 2×10^6 Sf9 cells were infected with 3pK virus at a multiplicity of infection of 10 and lysed 48 h postinfection. Sf9 cells were assayed for expression of the recombinant protein by immunoblot analysis.

Metabolic labeling. HL60 cells were starved for 48 h in 0.01% serum. The cells were pelleted and washed twice with phosphate-buffered saline (PBS). The cells were resuspended in phosphate-free medium containing 0.01% serum and incubated for another 2 h. One millicurie of orthophosphoric acid was added to each sample, and the mixture was incubated for the indicated time period with serum stimulation.

Expression and purification of histidine-tagged C-Raf. The histidine-tagged C-Raf kinase was purified from Sf9 cells overexpressing C-Raf. Forty-eight hours after infection, a 50-ml culture of Sf9 cells was washed twice with PBS and lysed in 5 ml of buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 5 mM magnesium chloride, 0.1 mM EDTA, 10 mM sodium chloride, 1 mM dithiothreitol [DTT], 0.1 mM phenylmethylsulfonyl fluoride, 10 mM sodium fluoride, 0.4% Triton X-100). After 5 min of swelling, cells were sonicated three times for 30 s, mixed with 1 volume of buffer B (830 mM NaCl, 34% glycerol, 1.6 mM imidazole), and then incubated for 45 min at 4°C with continuous agitation. Cell debris were pelleted, and the supernatant was incubated for 1 h at 4°C with 2 ml of Ni^{2+}-resin (Pro BondTM Resin; InvitroGen) with continuous agitation. The resin was packed into a column and washed once with 5 ml of buffer C (10 mM HEPES [pH 7.9], 5 mM magnesium chloride, 0.1 mM EDTA, 50 mM sodium chloride, 1 mM DTT, 17% glycerol, 0.1 mM phenylmethylsulfonyl fluoride) supplemented with 0.8 mM imidazole, twice with 5 ml of buffer C–8 mM imidazole, and twice with 5 ml of buffer C–40 mM imidazole. The C-Raf protein was then eluted with 20 ml of buffer C–80 mM imidazole. The protein fractions were tested by immunoblotting and, after concentration and dialysis against kinase buffer, stored at −70°C.

Expression and purification of MEK1. Sf9 cells were infected with a baculovirus encoding MEK1. Forty-eight hours after infection, the Sf9 cells were washed twice with PBS and lysed in an equal volume of buffer D (50 mM β-glycerophosphate [pH 7.3], 1 mM EDTA, 1.5 mM EGTA, 0.1 mM vanadate, 1 mM DTT, 20 mM NaCl, 10 mM sodium fluoride, 1 mM benzamidine, 5% glycerol, 10 μg of leupeptin per ml, 10 μg of pepstatin per ml, 1% Triton X-100). After 5 min of swelling, cells were sonicated at 60% of the maximum speed three times for 15 s each time. The cell debris was pelleted (100,000 × *g* for 45 min), and the supernatant was incubated with DE52 resin equilibrated with buffer D (for 1 h at 4°C with continuous agitation) (0.2 g/ml). The DE52 resin was sedimented for 15 min at 1,000 × *g*, and the supernatant was concentrated and dialyzed against buffer E (40 mM HEPES [pH 7.2], 2 mM EGTA, 1 mM EDTA, 1 mM DTT, 10 mM sodium fluoride, 1 mM benzamidine). The supernatant was then loaded onto a Mono-S column (Pharmacia) equilibrated with buffer E. The column was washed with 5 ml of buffer E, and the protein was eluted with a 30-ml linear sodium chloride gradient (0 to 0.5 M) in buffer E. The MEK1 fractions were pooled on the basis of results of immunoblotting with antibody MEK C-18 (Santa Cruz Biotechnology, Inc.), concentrated, and dialyzed (MACROSEP; Filtron) against kinase buffer A (25 mM HEPES, 10 mM magnesium chloride, 1 mM DTT).

Expression and purification of histidine-tagged ERK2. The pCMV5 Erk2His6 construct was a generous gift from Melanie Cobb. The inserts were cut out by complete *Xba*I digestion followed by partial *Eco*RI digestion. The *Eco*RI-*Xba*I fragment was then ligated into the corresponding sites of pUC18. The bacterial pellet was resuspended in 50 ml of ice-cold buffer F (50 mM sodium phosphate [pH 8.0], 0.3 M sodium chloride, 10 mM 2-mercaptoethanol, 1% Nonidet P-40, 10 mM sodium fluoride, 0.5 mM Pefabloc) and sonicated on ice four times for 30 s each time. Following sedimentation of the lysate (100,000 × *g* for 45 min), the supernatant was applied to an Ni^{2+}-chelating Superose column (Pharmacia) equilibrated in buffer F with 1 mM imidazole. The column was washed with 20 column volumns of buffer F containing 10 mM imidazole followed by 30 column volumns of buffer F containing 20 mM imidazole. The protein was eluted with a 70-column-volume gradient of 20 to 300 mM imidazole in buffer F. The ERK2 fractions were pooled on the basis of results of immunoblotting with antibody ERK 1 (956/837 (Santa Cruz Biotechnology), concentrated, and dialyzed against kinase buffer A (25 mM HEPES, 10 mM magnesium chloride, 1 mM DTT).

Activation of recombinant 3pK by ERK and MEK. ERK was activated as described for MAP kinase (40). Inactive ERK (2.5 μl, containing 20 μg of ERK per ml) was incubated with 2.5 μl of activated baculovirus-expressed MEK (containing 100 μg/ml) and 2.5 μl of protein kinase inhibitor (6 μM in water) for 3 min at 30°C. The activation of ERK was then initiated by the addition of 2.5 μl of 20 mM magnesium acetate–0.8 mM unlabeled ATP in buffer A (50 mM Tris-HCl [pH 7.4], 0.1 mM EGTA, 0.1% [vol/vol] 2-mercaptoethanol, 0.1 mM sodium orthovanadate). After 20 min at 30°C, activation of 3pK was initiated by adding 2.5 μl of 3pK (containing 600 μg/ml) and 12.5 μl of buffer A with 10 mM magnesium acetate, 0.2 mM unlabeled ATP, and 2.5 μM protein kinase inhibitor.

Activation of recombinant MEK by V-Raf. Activation of bacterially expressed MEK (3.5 μg) by baculovirus-expressed C-Raf (10 ng) was initiated by the addition of kinase buffer A containing 10 mM magnesium acetate and 0.2 mM unlabeled ATP. The reaction was carried out for 20 min at 30°C.

Peptide phosphorylation by 3pK. Recombinant 3pK was activated through the MEK/ERK kinase cascade as described above. Peptide phosphorylation was then carried out as described for MAPKAP kinase 2 (51). Phosphorylation of 30 μM synthetic peptide was initiated by adding kinase buffer B (50 mM sodium β-glycerophosphate [pH 7.0], 0.1 mM EDTA, 2.5 μM protein kinase inhibitor, 10 mM magnesium acetate, 0.1 mM [γ-^{32}P]ATP). After incubation for 10 min, a 40-μl sample was spotted onto a phosphocellulose filter (2 by 2 cm; Whatman P81). To remove unincorporated ATP, the filter was washed four times in 75 mM H_3PO_4 and once in acetone. The filter was air dried and then counted by liquid scintillation.

Nucleotide sequence accession number. The sequence shown in Fig. 2A has been deposited in GenBank under accession number U09578.

RESULTS

Detection and mapping of the 3pK gene. Human chromosome 3-specific and partially sequenced *Not*I linking clones (62) were checked for their presence in the SCLC cell line

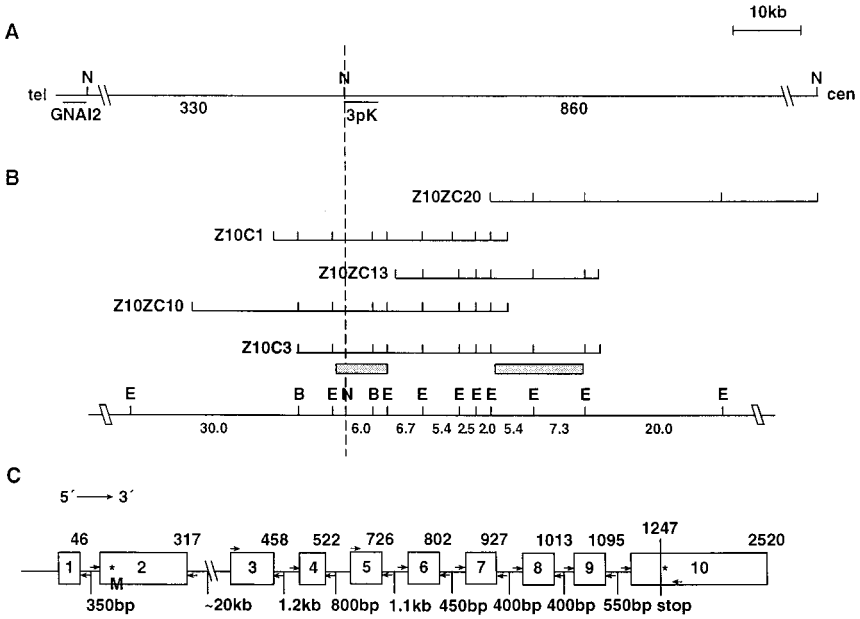


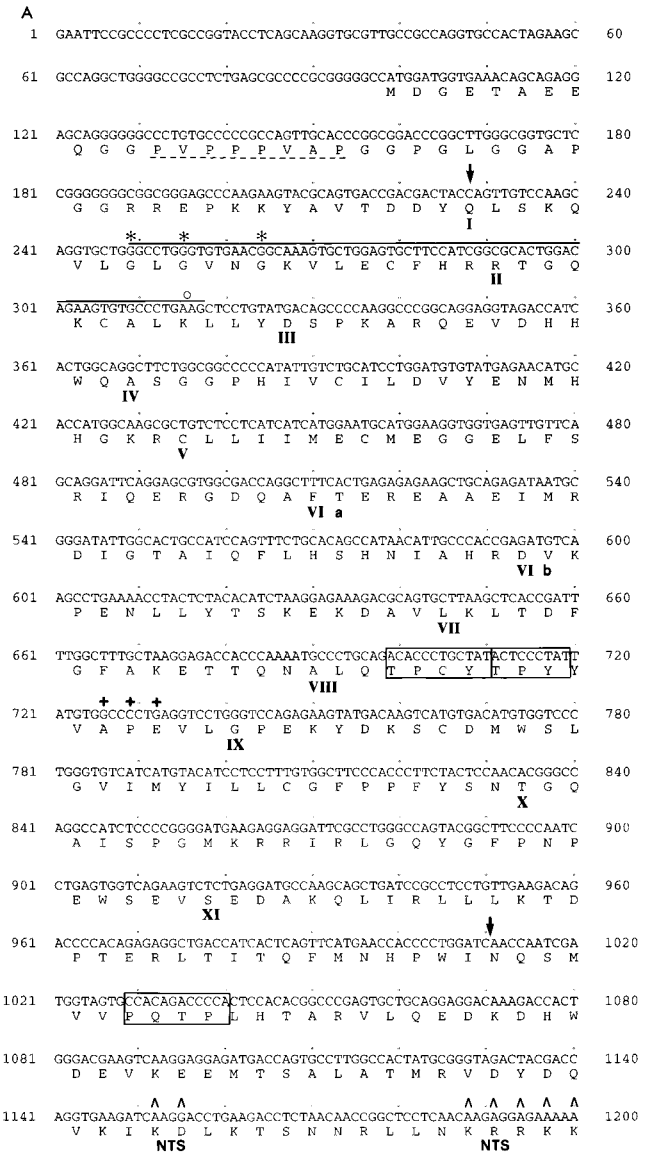
FIG. 1. Structure of the genomic locus and organization of the 3pK gene. (A) Large-scale *Not*I map of the 3pK gene region. GNAI2 is a previously mapped gene used as a reference marker on 3p. tel, telomere; cen, centromere. (B) Cosmid contig and *Eco*RI restriction map of the 3pK genomic locus. Fragment sizes are in kilobase pairs. Shadowed boxes indicate genomic fragments hybridizing to a 1,371-bp 3pK cDNA clone. (C) Exon-intron structure of the 3pK gene. Fragment sizes are in base pairs.

NCI-H740 deletion. The whole inserts, averaging 5 to 15 kb, were used as probes on Southern blots containing NCI-H740 and total human DNA as a control. Clones apparently deleted in NCI-H740 DNA were confirmed by PCR, using the same DNAs as templates (data not shown). The deleted clones were used as probes on Northern blots to identify expressed sequences and on pulsed-field gels to construct a long-range *Not*I map of the region (Fig. 1). NL1G210, one of the *Not*I linking clones located in the deletion, detected several mRNAs on a Northern blot containing poly(A)$^+$ RNA from different human tissues (Fig. 3A) and was shown to contain sequences of two previously unknown genes. The telomeric half of the clone, a 6-kb *Not*I-*Bam*HI fragment, detected a 1.8-kb transcript in the liver and kidney (see Fig. 3C), whereas the centromeric 4.5-kb *Not*I-*Bam*HI fragment contained sequences of a ubiquitously expressed gene encoding a 2.5-kb message (see Fig. 3B). When used as a probe on the pulsed-field gel blots containing *Not*I-digested human DNA, NL1G210 detected two *Not*I fragments, 330 and 860 kb (data not shown). The next telomeric *Not*I site from NL1G210 was shown to be close to the GNAI2 gene, also deleted in the NCI-H740 cell line (Fig. 1A). This was confirmed by, in addition to pulsed-field gel electrophoresis data, the chromosome jumping technique, using a chromosome 3-specific *Not*I jumping library (60) (data not shown). The next centromeric *Not*I site was located approximately 860 kb away from NL1G210 (Fig. 1A).

Cloning and sequencing of the 3pK gene. The 4.5-kb *Not*I-*Bam*HI fragment was used as a probe to isolate cDNA clones from a human heart cDNA library. A clone containing a 1,371-bp insert was isolated and completely sequenced (Fig. 2A). Sequence analysis revealed that the clone contained a 1,146-bp open reading frame, putatively encoding a 382-amino-acid protein with a predicted molecular mass of 42 kDa. Another clone, with a 1.8-kb insert, was shown to overlap with the previous clone from nucleotide position 643 (Fig. 2A) and most likely contains the entire 3′ untranslated region. A search of the GenBank database revealed a high sequence identity of the product of this gene to a number of serine-threonine kinases, especially to MAPKAP kinase 2 (Fig. 2B). Sequence identity ranged from 72% on the nucleotide level to 75% on the amino acid level. The new gene was named 3pK, and the clone, containing the entire open reading frame, was used for functional studies.

The deduced amino acid sequence of 3pK indicates the presence of a proline-rich motif at the N terminus which conforms to the SH3-binding consensus Xp0PpXP, where 0 represents a hydrophobic residue and p represents residues that tend to be proline (18, 60). The putative ATP-binding site including the reactive lysine residue located at the sequence motif AXK is well conserved in 3pK. Two putative MAP kinase phosphorylation site motifs are present in 3pK. One of them is located N terminal to the conserved APE sequence in kinase subdomain VIII, a location analogous to phosphorylation sites essential for the activity of cyclic AMP-dependent protein kinase (49), p34^{cdc2} (25), and RSK/MAPKAP kinase 1 (54). In MAPKAP kinase 1, this site has been shown to be phosphorylated by MAP kinase (54). TPY, a motif which is present in stress kinases (15, 31) and in STE 20 (43), is located in the same subdomain. The other MAP kinase recognition motif, PX(S/T)P, is located in 3pK, C terminal to the kinase domain. The same motif at an analogous position in MAPKAP kinase 2 is shown to be phosphorylated by MAP kinase (52). Intriguingly, the sequence KDLKTSNNRLLNKRRKK, close to the carboxy-terminal end of the protein, matches the consensus nuclear translocation signal KK10XKRRKK, which has been shown to be sufficient for nuclear localization (17). A related sequence is also present in MAPKAP kinase 2; however, this protein was not shown to migrate to the nucleus (18, 51).

3pK expression. The 1,371-bp cDNA clone was used as a probe on Northern blots containing poly(A)$^+$ mRNA from different sources. The 3pK gene was expressed in every human tissue examined (Fig. 3D). The levels of expression varied



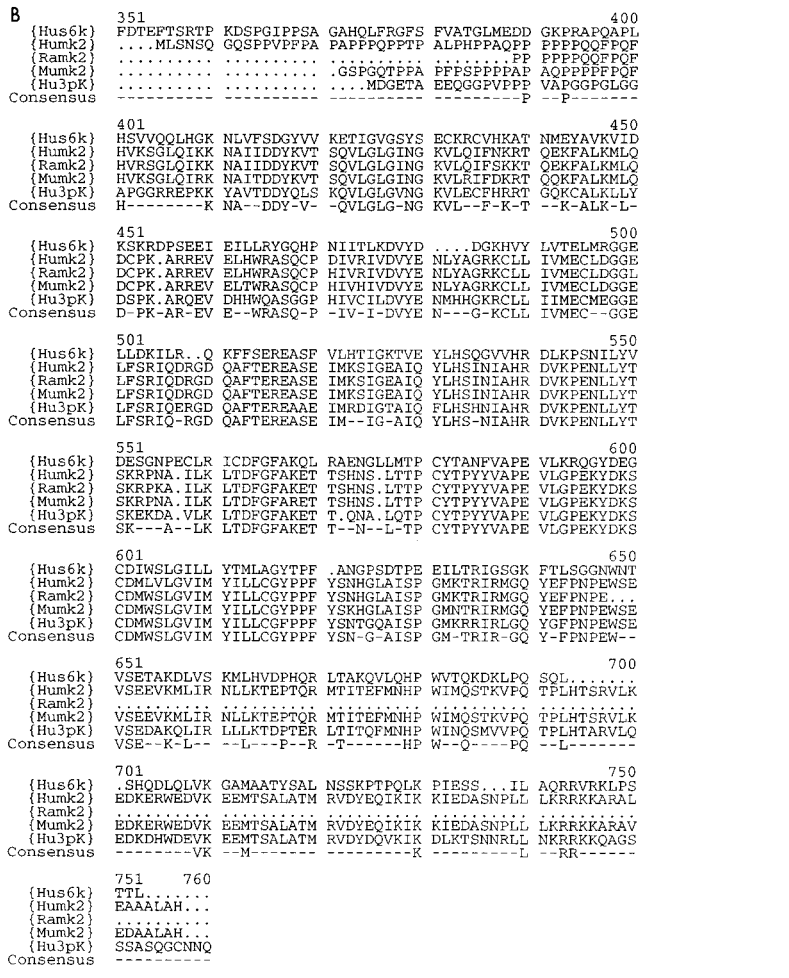


FIG. 2. 3pK cDNA and protein sequence and amino acid alignment of the protein to different MAP kinase-activated protein kinases. (A) Nucleotide and predicted amino acid sequence of the 3pK gene. Potential 3pK phosphorylation sites are marked by open boxes. The dashed line indicates the potential SH3-binding site. The GXGXXG sequence is indicated by asterisks, the conserved lysine is marked by a circle, and the APE sequence is indicated by +++. The region between the arrows marks the kinase domain, and roman numerals I to XI indicate the subdomains of the kinase domain. The consensus nuclear translocation signal (NTS) is indicated by carats. (B) Amino acid sequence alignment of 3pK with other known MAP-activated protein kinases from different organisms. Hus6k, human S6 kinase; Humk2, human MAPKAP kinase 2; Hu3pK, human 3pK; Ramk2, rabbit MAPKAP kinase 2; Mumk2, mouse MAPKAP kinase 2.

significantly, being especially high in the heart, skeletal muscle, and kidney; expression in the brain and placenta was barely detectable. Control hybridization of these blots with an actin probe showed roughly equal amounts of RNAs in all lanes (data not shown). A similar expression pattern was observed in mouse tissues (Fig. 3E). In eight different human tumors examined, high levels of expression were detected only in the HL60 promyelocytic leukemia cell line (Fig. 3F); the functional significance of this observation is unclear.

Organization of the 3pK genomic locus and exon-intron structure of the gene. The 4.5-kb *Not*I-*Bam*HI fragment of the NL1G210 linking clone was used as a probe for screening a human genomic cosmid library to isolate overlapping clones covering the entire 3pK genomic locus (Fig. 1B). Subsequently, the end probe from cosmid z10c3, a 3.4-kb *Eco*RI fragment, was used for chromosome walking to expand the contig. The end *Eco*RI restriction fragments of pWE15 cosmids were determined by hybridization of T3 and T7 primers to *Eco*RI digests of the cosmids. When using the 3pK 1,371-bp cDNA clone as a probe on isolated cosmids, we found that cosmid z10c3 contained all coding sequences of the gene (Fig. 1B). An *Eco*RI restriction map was constructed for the genomic region spanning about 85 kb (Fig. 1B). The genomic *Eco*RI restriction fragments were ordered by hybridization of *Bam*HI fragments of cosmid z10c3 to all cosmids digested with *Eco*RI. The map was verified by hybridizing cosmids to each other.

Exon-intron boundaries of the 3pK gene were determined, using PCR amplification of cosmid z10c3 DNA in comparison

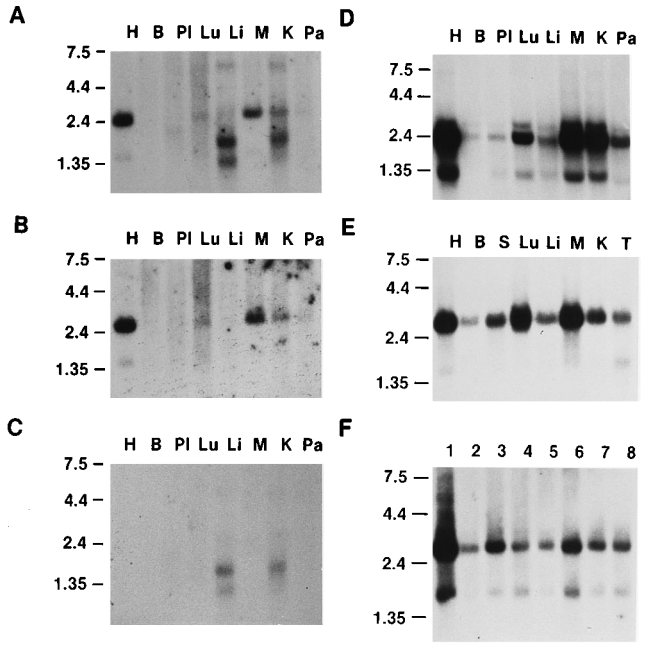


FIG. 3. Expression studies of the 3pK gene. Multiple-tissue Northern blots (Clontech) were used to detect expression of the 3pK gene. (A) The 10.5-kb whole insert of the NL1G210 linking clone was used as a probe on a Northern blot containing poly(A)$^+$ mRNA from different human tissues: H, heart; B, brain; Pl, placenta; Lu, lung; Li, liver; M, skeletal muscle; K, kidney; Pa, pancreas. (B) The 4.5-kb *Not*I-*Bam*HI half of the same linking clone was used as a probe on the same blot. (C) The 6-kb *Not*I-*Bam*HI half of the same linking clone was used as a probe on the same blot. (D) The whole 1,371-bp insert of the 3PK cDNA clone was used as a probe on the same blot. (E) The cDNA probe used on the mouse Northern blot, containing mRNA from various tissues: H, heart; B, brain, S, spleen; Lu, lung; Li, liver; M, skeletal muscle; K, kidney; T, testis. (F) The same probe hybridized to a Northern blot, containing mRNA from different human cancer cell lines: 1, promyelocytic leukemia HL60; 2, HeLa cell line S3; 3, chronic myelogenous leukemia K-562; 4, lymphoblastic leukemia MOLT-4; 5, Burkitt's lymphoma Raji; 6, colorectal adenocarcinoma SW480; 7, lung carcinoma A549; 8, melanoma G361. Sizes are indicated in kilobases.

with the entire open reading frame of the cDNA. The difference in size of the PCR products between the cosmid DNA and the cDNA indicated the presence of an intron(s). PCR fragments amplified from the cosmid DNA were directly sequenced or subcloned into a TA cloning vector (Invitrogen) and then sequenced. The total of 10 exons were detected (Fig. 1C), and the exon flanking sequences were used for mutation detection by PCR–single-strand conformation polymorphism analysis in 50 SCLC cell lines. No point mutations were detected.

Expression and purification of 3pK protein. The full-length 3pK cDNA was cloned into the bacterial expression vector pGEXKG to produce a fusion protein with GST at the N terminus, permitting purification of 3pK protein by affinity chromatography. Upon induction with IPTG, a GST-3pK fusion protein of the predicted size of approximately 70 kDa was produced. Initially the induced protein was detected by silver staining and also in immunoblots with a GST antibody. The GST-3pK fusion protein was purified by glutathione-agarose chromatography. The N-terminal GST domain was released from the fusion protein by thrombin cleavage. The thrombin-cleaved products were separated on a sodium dodecyl sulfate (SDS)–5 to 20% acrylamide gradient gel, and the predicted 44-kDa 3pK protein was detected. To study the expression of 3pK in mammalian cells, we prepared polyclonal antibodies against 3pK. To determine the antibody specificity, recombinant bacterial GST-3pK and baculovirus-expressed 3pK proteins were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotted with this antiserum (Fig. 4A). The antiserum detects a doublet at approximately 43 kDa and another, faster-migrating form at about 39 kDa in both Sf9 and bacterial cell lysates. The relative amounts of the 39-kDa and the 43-kDa material varied between preparations, suggesting that the 39-kDa form is a proteolytic fragment.

Serum stimulates phosphorylation of 3pK in HL60 cells. To characterize the endogenous 3pK protein, HL60 cells were used since they were observed to express high levels of 3pK mRNA. To test whether endogenous 3pK is a phosphoprotein and whether phosphorylation is mitogen regulated, HL60 cells

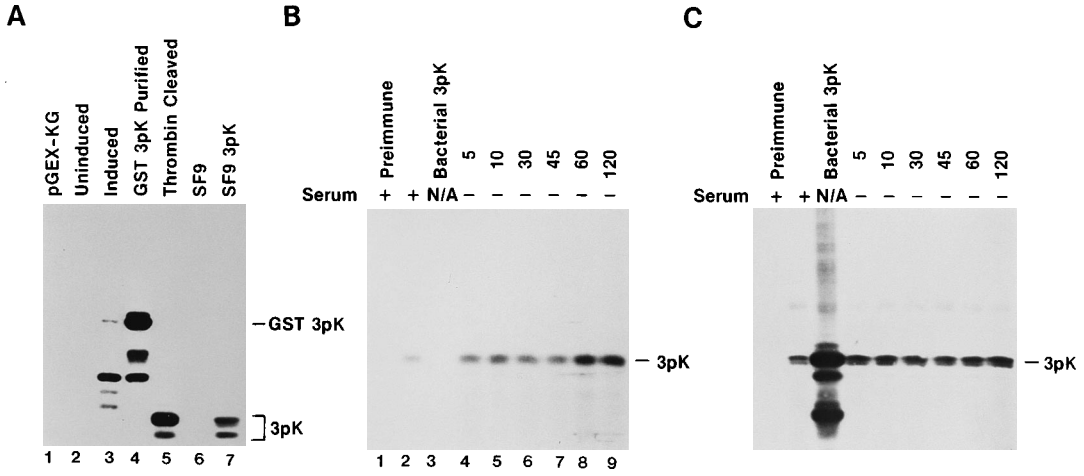


FIG. 4. (A) Expression of 3pK in *E. coli* and in Sf9 cells. *E. coli* and Sf9 cell extracts were prepared from cells harboring a GST vector control (lane 1), uninduced GST-3pK (lane 2), and induced GST-3pK (lane 3); aliquots of purified GST-3pK were loaded before (lane 4) and after (lane 5) thrombin cleavage. Lysates from uninfected Sf9 cells or cells infected with 3pK-expressing baculovirus were loaded in lane 6 and 7, respectively. Proteins were separated on an SDS–10% gel and immunoblotted with an antibody raised against bacterially expressed 3pK. (B) In vivo analysis of 3pK proteins in HL60 cells. Serum-starved HL60 cells were metabolically labeled with 1 mCi of orthophosphoric acid per ml for 30 min. After 5, 10, 30, 45, 60, and 120 min of serum stimulation, 3pK protein was immunoprecipitated from lysates adjusted to be equal in protein content. Lanes: 1 and 2, lysates from unstarved HL60 cells preincubated with and without preimmune serum; 3, bacterial thrombin-cleaved 3pKinase protein; 4 to 9, various time points for serum stimulation. Samples were resolved by SDS-PAGE, and phosphoproteins were visualized by autoradiography. (C) Immunoblotting with 3pK antiserum of the SDS gel in panel B.

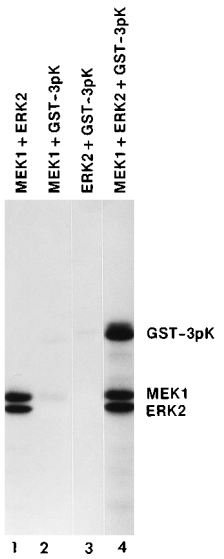


FIG. 5. Phosphorylation of 3pK by MEK1-activated ERK2. Bacterially expressed GST-3pK protein was incubated for 20 min at 30°C with baculovirus-expressed activated MEK1 (lane 2), inactive bacterially expressed ERK2 (lane 3), and baculovirus-expressed activated MEK1 and inactive bacterially expressed ERK2 (lane 4) in kinase buffer containing [^{32}P]ATP and $MgCl_2$. Kinase reactions were performed as described in Materials and Methods. The reactions were terminated with 2× Laemmli sample buffer, and the mixtures were heated for 5 min at 100°C. The proteins were separated by SDS-PAGE and visualized by autoradiography.

were labeled with ^{32}P and serum stimulated for different times. Cell lysates from stimulated and unstimulated cells were immunoprecipitated with a 3pK antibody. A ~43-kDa doublet was detected in both lysates (Fig. 4B). Upon serum stimulation, ^{32}P incorporation into 3pK became detectable after 5 min and further increased to plateau levels after 60 min. The absolute levels of 3pK protein did not change during the time course of serum stimulation, as shown by Western blotting (immunoblotting) (Fig. 4C). Whereas only the 43-kDa doublet was seen in the ^{32}P-labeling experiment, the 39-kDa fragment was additionally detected by Western blotting.

3pK is phosphorylated by ERK. To determine potential upstream activators for 3pK, the bacterially expressed GST-3pK fusion protein was used as an in vitro substrate in protein kinase assays. Baculovirus-expressed and enzymatically active MEK1 was incubated with or without bacterially expressed, inactive GST-3pK and/or ERK2 in buffer containing [γ-^{32}P]ATP and Mg^{2+}. In the reaction mixture containing MEK1, ERK2, and GST-3pK, phosphorylation of the 70-kDa recombinant 3pK was detected (Fig. 5, lane 4). This protein band appears as a doublet with most of the label on the lower band. The level of phosphorylation was much lower in samples containing GST-3pK alone with MEK1 (lane 2) or ERK2 (lane 3), and only the smaller band was detectable, probably as a result of autophosphorylation. The phosphorylation of ERK2 by MEK1 is shown in lane 1. Similar results were obtained in kinase assays when ERK2 was replaced with bacterially expressed ERK1 (data not shown).

Comparison of the substrate specificities of 3pK with RSK/MAPKAP kinase 1, MAPKAP kinase 2, and calcium calmodulin kinase II. To study the substrate specificity of 3pK, we assayed a panel of 14 peptides used to evaluate the substrate specificity of MAPKAP kinase 2 relative to RSK/MAPKAP kinase 1 and calcium calmodulin kinase II (51). Members of this family of peptides are analogs of GS peptide 1, which was modeled after the N terminus of glycogen synthase sequence. Rates of phosphorylation are shown relative to that of peptide 1. 3pK phosphorylates itself upon incubation with MgATP at a very low level (data not shown). To minimize autophosphorylation, the peptide phosphorylation experiments were carried out with an incubation time of 10 min as described for MAPKAP kinase 2 (52) (Fig. 6A and B). The pattern of phosphorylation of the panel of peptides was unique for 3pK, with peptide 3 (KKVNRTLSVA) being the preferred substrate (Fig. 6A). The next-best substrate was peptide 4 (KKANRTLSVA), indicating a preference for a hydrophobic amino acid five residues N terminal to the phosphorylation site. On the basis of this comparison, peptide 3 was chosen as a substrate for subsequent assays of 3pK in vitro.

3pK is activated by ERK2 in vitro. To examine potential activation of 3pK by ERK2, the C-RAF–MEK–ERK protein kinase cascade was reconstituted in vitro. 3pK was incubated with or without baculovirus-expressed active C-Raf and inactive bacterially expressed MEK1 and ERK2 in kinase buffer with Mg^{2+} and ATP. Peptide 3 was added, and kinase activity was determined by the addition of [γ-^{32}P]ATP and Mg^{2+} for 10 min at 30°C. 3pK was activated when all three upstream kinases were present (Fig. 6B, column 1). In the absence of either C-Raf or ERK2, only low-level activation was observed (Fig. 6B, columns 2 and 3).

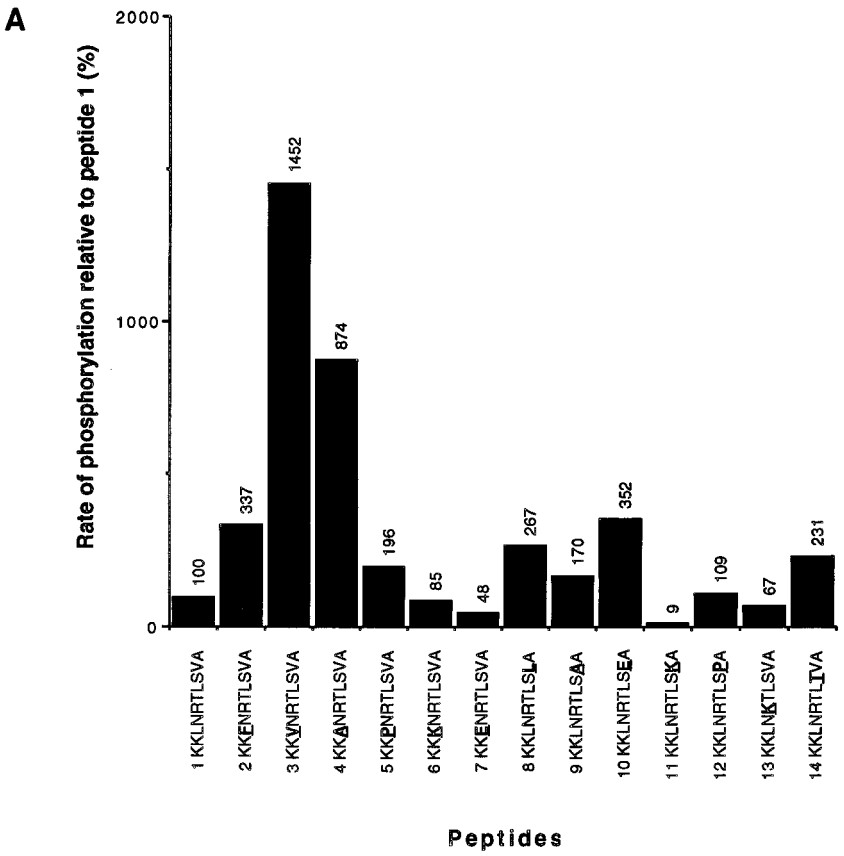
DISCUSSION

We report the cloning and characterization of a new MAP kinase-activated protein kinase, 3pK. *Not*I linking clones have been used as the starting material for detailed characterization of large genomic regions. *Not*I clones were chosen because the creation of a long-range *Not*I restriction map creates a framework into which more detailed mapping data can be integrated. Moreover, *Not*I linking clones have been shown to be good markers for human genes. About 90% of all *Not*I sites are located in CpG islands, closely connected with human genes, and therefore approximately 10 to 20% of all human genes contain *Not*I sites in their sequences (1). In the present study, the usefulness of *Not*I clones for the characterization of human genes is clearly demonstrated. The *Not*I linking clone NL1G210, deleted in SCLC cell line NCI-H740, detected two previously uncharacterized genes. One of the genes was investigated in detail in this study from two aspects: (i) the possible role of this gene in the development of lung cancers and (ii) its functional properties.

Cloning and sequencing of the full-length cDNA enabled the search for possible rearrangements or mutations of 3pK in SCLC cell lines. No structural alterations were detected, most likely eliminating 3pK as a candidate for an SCLC tumor suppressor gene. Consistent with this observation, recently published data (28) place 3pK outside of another SCLC homozygous deletion.

The high homology of 3pK to different serine-threonine kinases, especially to the recently described MAPKAP kinase 2 enzyme, suggested that 3pK is a MAP kinase-regulated serine threonine kinase. The sequences were more closely related in the kinase domain and diverged considerably at the amino and carboxy termini. A single potential SH3-binding site is present N terminal to the kinase domain where MAPKAP kinase 2 has two such sequences (18). The presence of the SH3-binding domain suggests that 3pK may interact with SH3-containing proteins.

The in vitro activation experiments with p42 and p44 isoforms of ERK demonstrated that they can phosphorylate and catalytically active 3pK. Serum stimulation of HL60 cells also


A
Rate of phosphorylation relative to peptide 1 (%)
2000
1000
0
100
337
1452
874
196
85
48
267
170
352
9
109
67
231
1 KKLNRTLSVA
2 KKENRTLSVA
3 KKVNRTLSVA
4 KKANRTLSVA
5 KKPNRTLSVA
6 KKKNRTLSVA
7 KKENRTLSVA
8 KKLNRTLSLA
9 KKLNRTLSAA
10 KKLNRTLSEA
11 KKLNRTLSKA
12 KKLNRTLSPA
13 KKLNKTLSVA
14 KKLNRTLTVA
Peptides

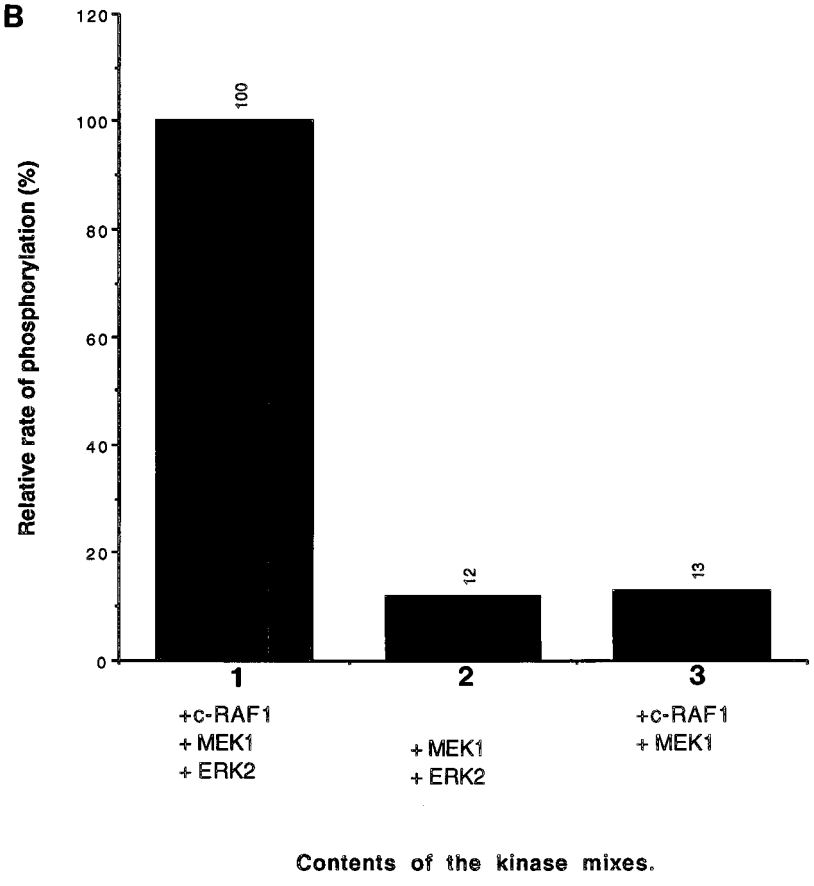

B
Relative rate of phosphorylation (%)
120
100
80
60
40
20
0
100
12
13
1
2
3
+c-RAF1
+ MEK1
+ ERK2
+ MEK1
+ ERK2
+c-RAF1
+ MEK1
Contents of the kinase mixes.

FIG. 6. Substrate specificity of 3pK. (A) Bacterially purified 3pK was incubated for 20 min at 30°C with or without baculovirus-expressed activated MEK1 and inactive bacterially expressed ERK2 in kinase buffer containing Mg^{2+} and ATP. Synthetic peptides were then added as indicated together with [γ-^{32}P]ATP and Mg^{2+}, and the mixtures were incubated for 10 min at 30°C. Experiments were performed as described in Materials and Methods. Rates of phosphorylation relative to that of peptide 1 are plotted. Similar results were obtained in several experiments. (B) Activation of 3pK. 3pK was incubated for 20 min at 30°C with the reconstituted protein kinase cascade consisting of baculovirus-expressed active C-Raf1 and inactive bacterially expressed MEK1 and ERK2 (column 1). The control include the mixture without active C-Raf1 (column 2) or without ERK2 (column 3). The synthetic peptide KKVNRTLSVA was incubated with [γ-^{32}P]ATP for 10 min at 30°C. Relative rates of phosphorylation of the synthetic peptide are plotted. Similar results were obtained in several experiments.

stimulated the phosphorylation of endogenous 3pK. As ERK1 and ERK2 are the major ERK kinase isoforms that become activated upon treatment of cells with serum, it appears likely that these enzymes may contribute to the in vivo phosphorylation.

Although we have shown here for 3pK activation in vitro that ERK1 and ERK2 can function as upstream activators, we also have to consider integration of 3pK into other signaling pathways. In fact, the potential phosphorylation site within the kinase domain overlaps with a potential stress kinase phosphorylation site. It was recently shown that MAPKAP kinase 2 becomes in vivo activated through a stress-activated kinase cascade (47). The MAP kinase isoform responsible for phosphorylating MAPKAP kinase 2 appears to be a homolog of yeast HOG, a component of the osmoregulatory pathway (5, 20, 47). Thus, there is more than one subfamily of mammalian MAP kinases with distinct but overlapping substrate specificities. Experiments are under way to examine whether 3pK is also activated by components of stress-related pathways.

In regard to the spatial distribution, two aspects of the sequence deserve consideration. The SH3-binding domain might allow interaction with an SH3-containing protein, some of which are known to mediate membrane translocation upon stimulation of growth factor receptors, as in the case of the epidermal growth factor receptor, GRB2, and SOS (39, 46). The other sequence element is a potential nuclear translocation sequence downstream of the kinase domain. Genetic analysis will be required to establish the roles of these sequences as determinants of the subcellular distribution of the enzyme. With respect to the related MAPKAP kinase 2, for which two SH3-binding domains and a potential nuclear translocation signal have been described, no data have been reported for their association with subcellular compartments (18, 51). In contrast, another MAP kinase-regulated kinase, MAPKAP kinase 1 or RSK-1, was shown to enter the nucleus upon treatment of HeLa cells with serum (9).

Comparison of a panel of 14 analogous peptides, which differ by one amino acid, as substrates for 3pK showed a pattern different from that previously reported for MAPKAP kinase 2, RSK/MAPKAP kinase 1, and calcium calmodulin kinase II (51). The peptide preferred by 3pK has the sequence K-K-V-X-R-X-L-Ser-X-X and differs at position 5 from those preferred by the other enzymes. Experiments are under way to identify a physiological substrate.

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