Characterization of Three Paralogous Members of the Mammalian Vaccinia Related Kinase Family*

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Members of the novel vaccinia related kinase (VRK) protein family are characterized by notable sequence homology to the vaccinia virus-encoded B1 kinase (vvB1). vvB1 plays an essential role in viral DNA replication, and Boyle and Traktman have demonstrated that VRK enzymes complement the replication defect of a temperature-sensitive viral mutant defective in vvB1 (Boyle, K., and Traktman, P. (2004) J. Virol. 78, 1992–2005). This mammalian kinase family comprises three members, VRK1, VRK2, and VRK3. We have annotated the gene structure for the members of this family and have characterized the enzyme activity and subcellular localization for the human and mouse proteins. VRK enzymes show robust autophosphorylation activity and will phosphorylate casein; VRK2 enzymes show modest autophosphorylation activity and will also phosphorylate casein. The VRK3 proteins have key amino acid substitutions that disrupt invariant motifs required for catalytic activity, rendering them enzymatically inert. The VRK1 and VRK2 proteins contain COOH-terminal extracatalytic sequences that mediate intracellular localization. VRK1 proteins possess a basic nuclear localization signal and are indeed nuclear; the extreme C termini of the VRK2 proteins are highly hydrophobic, and the proteins are membrane-associated and colocalize with markers of the endoplasmic reticulum. The NH2-terminal region of the VRK3s contains a bipartite nuclear localization signal, which directs these proteins to the nucleus. Our findings provide the basis for further studies of the structure and function of this newly discovered family of protein kinases.

Post-translational modification of proteins plays a major role in the coordination of cellular events. Reversible phosphorylation of serine, threonine, or tyrosine residues drives the transmission of signals from the cell surface to the nucleus, modulates faithful progression through the cell cycle, and regulates metabolic pathways. Phosphorylation in eukaryotes is largely conducted by a single superfamily of proteins, the protein kinases. To underscore the significant role that kinases serve in the eukaryotic cell, it has been determined that there are 518 cellular proteins and the viral B1 kinase stimulate our interest, and we initiated a detailed characterization of the structure and function of this new family. Ongoing data base searches have led our laboratory and others to identify additional vvB1-related proteins; we now know that the VRK family comprises three members, VRK1, -2, and -3 (22).

In this study, we present a characterization of the human and mouse VRK family in toto. We have annotated the genomic structure of the six genes and present a thorough analysis of primary sequence identities. We have used reverse transcription coupled with PCR (RT-PCR) to amplify and clone transcripts representing the human and mouse VRK family and have extended the existing analyses of mRNA tissue expression profile. We have also generated and purified recombinant protein preparations in order to characterize the enzymatic activity of these proteins, and we have generated expression constructs in order to analyze the intracellular localization of each of the proteins. As described herein, we show that the three VRK proteins show distinct profiles of intracellular localization.

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The abbreviations used are: vvB1, vaccinia virus-encoded B1 kinase; VRK, vaccinia related kinase; hVRK, mVRK, cVRK, and dVRK, human, mouse, C. elegans, and D. melanogaster VRK, respectively; RT, reverse transcriptase; ORF, open reading frame; GFP, green fluorescent protein; CK, casein kinase; DAPI, 4′,6-diamidino-2-phenylindole.
and enzymatic activity. These analyses open the way for future studies aimed at understanding the biological role of each of these proteins in regulating cellular events.

EXPERIMENTAL PROCEDURES
Cell Culture and Reagents
Human thymidine kinase-negative 143B osteosarcoma cells (TK−), mouse L 929 fibroblasts, and African green monkey BSC40 epithelial cells were maintained as monolayers in Dulbecco’s modified Eagle’s medium, supplemented with 5% fetal bovine serum (Invitrogen) in the presence of 5% CO2. Restriction endonucleases, the Klenow fragment of Escherichia coli DNA polymerase I, high fidelity Taq polymerase, calf intestinal alkaline phosphatase, DNase, and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA) or Roche Applied Science. Moloney murine leukemia virus reverse transcriptase was obtained from Invitrogen. RNAsin RNase inhibitor and oligodiT25-28 were purchased from Amersham Biosciences. The following plasmids were used in this study: p3×FLAG-7.1 (Sigma), pcDNA3 (Invitrogen), pEGFP-N1 (Clontech, Palo Alto, CA), pET16b (Novagen, Madison, WI), and pTMI-3×FLAG-Nde (a derivative of pTM1) (23, 24). 32P-labeled nuclease triphosphates (α-32P and γ-32P) were obtained from PerkinElmer Life Sciences.

Identification of VRK Family Members and Multiple Sequence Alignments
The cDNA sequences of the VRK family members were found in public and private data bases as submissions whose deduced amino acid sequences possessed similarity to the previously reported veb1 kinase (19) or VRKI (20, 21) primary sequences. To identify the vaccinia-related kinase family members, we used mouse and human VRK amino acid sequences as a query sequence in BLAST searches (25) of the NCBI RefSeq and GenBankTM nucleotide sequence records. We acquired and determined cDNA sequences that would comprise the VRK family. The following RefSeq and GenBankTM nucletide sequence records were acquired and used in this study: hVRK1, NM_003384; hVRK2, AB000450; hVRK3, NM_170292; Drosophila melanogaster VRK (dVRK), NM_062784. From these accession numbers, we obtained the deduced amino acid sequences of the relevant open reading frames and DNA sequence that served as a basis for primer design for RT-PCR amplification. Analyses of the global amino acid sequence similarity between members of the VRK family was performed with the neighbor-joining method. All sequences were obtained from the RefSeq and GenBankTM nucleotide sequence databases. The following plasmids served as templates for amplification: pET16b-mVRK1, pcDNA3-mVRK1(3′)-MYC, pET16b-mVRK2(3′)-D, and pET16b-mVRK3(3′)-D primers, digested with BglII, and then ligated into BamHI-digested calf intestinal alkaline phosphatase-treated pET16b, generating pET16b-hVRK3. These and subsequent PCR-derived clones were sequenced by the chain terminator method with BigDye Terminator 1.0 or 3.0 (Applied Biosystems; Foster City, CA) and then compared with the sequences from the accession numbers listed above. Plasmids were prepared for transfection with Qiagen EndoFree Plasmid Maxi Kits (Qiagen, Valencia, CA).

Construction of bacterial expression plasmids
mVRK1, -2, and -3 inserts amplified by RT-PCR were digested with NdeI and BamHI and then ligated into pET16b DNA that had been digested with the same enzymes and treated with calf intestinal alkaline phosphatase; the resultant plasmids were designated pET16b-mVRK1, -2, and -3. Full-length mVRK1 and two splice variants, Δα (lacking exon 12) and Δβ (lacking exons 12 and 13), were isolated from the same RT-PCRs and identified after DNA sequencing of multiple plasmids recovered from E. coli transformants. pET16b-hVRK1 was generated in a similar fashion, except that the template for PCR amplification was the pcDNA3-hVRK1-MYC plasmid, generously provided by J. Nezu (Chugai Research Institute for Molecular Medicine, Ibaraki, Japan). Because the RT-PCR-amplified hVRK2 open reading frame (ORF) has an NdeI site at nucleotide position 898 (numbered from the adenine of the initiating ATG), the pET16b-hVRK2 was generated in two cloning steps, with the 3′ NdeI-BamHI fragment cloned first and then the 5′ NdeI-NdeI fragment inserted second, generating the final plasmid, pET16b-hVRK2. To prepare the hVRK3 insert, the hVRK3 RT-PCR product was amplified with bVRK3(3′)-UT and bVRK3(3′)-FLAG-D primers, digested with BglII, and then ligated into BamHI-digested and calf intestinal alkaline phosphatase-treated pET16b, generating pET16b-hVRK3. These and subsequent PCR-derived clones were sequenced by the chain terminator method with BigDye Terminator 1.0 or 3.0 (Applied Biosystems; Foster City, CA) and then compared with the sequences from the accession numbers listed above. Plasmids were prepared for transfection with Qiagen EndoFree Plasmid Maxi Kits (Qiagen, Valencia, CA).

Construction of mammalian expression plasmids
MYC-tagged versions of mVRK1, mVRK1Δ3, and mVRK1ΔΔ splice forms (originally generated by RT-PCR) were subcloned from the vaccinia virus expression vector pGS53 (28) into the BamHI site of pcDNA3, generating pcDNA3-mVRK1-MYC, pcDNA3-mVRK1Δ3-MYC, and pcDNA3-mVRK1ΔΔ-MYC. pET16b-mVRK2 and -mVRK3 plasmids served as templates for amplification with mVRK2(3′)-FLAG-U and mVRK2(3′)-D, and mVRK3(3′)-FLAG-U and mVRK3(3′)-D, respectively. These PCR products were digested with Asp718 and BamHI and then ligated into p3×FLAG-7.1 DNA that had been previously digested with the same enzymes and treated with calf intestinal alkaline phosphatase; the resultant p3×FLAG-mVRK2 and -mVRK3 plasmids served as templates for amplification with the primers hVRK2(3′)-FLAG-met-U and hVRK2(3′)-FLAG-D; the reaction products were digested with HindIII and BamHI and then ligated into appropriately digested p3×FLAG-7.1 DNA, generating p3×FLAG-hVRK2 and hVRK3

| TABLE I Primers used for amplifications |
|------------------------------------------|
| Primer                        | Sense/Reverse  |
|-----------------------------|---------------|
| hVRK1(3′)-UT                 | Sense/Reverse |
| mVRK1(3′)-U                  | Sense/Reverse |
| mVRK1(3′)-D                  | Sense/Reverse |
| mVRK2(3′)-UT                 | Sense/Reverse |
| mVRK2(3′)-D                  | Sense/Reverse |
| mVRK3(3′)-UT                 | Sense/Reverse |
| mVRK3(3′)-D                  | Sense/Reverse |
| mVRK2(3′)-FLAG-D             | Sense/Reverse |
| mVRK2(3′)-D                  | Sense/Reverse |
| mVRK3(3′)-FLAG-U             | Sense/Reverse |
| mVRK3(3′)-D                  | Sense/Reverse |

A. Punjabi and P. Traktman, unpublished results.
RT-PCR products were amplified with the hVRK3, hVRK2, \textit{FLAG}-U primer and hVRK3, hVRK2, \textit{FLAG}-D, and then were digested with Asp718 and NheI and ligated into appropriately digested p3\textsuperscript{FLAG}-hVRK3.

**Construction of GFP Fusion Plasmids**

The carboxyl terminus of hVRK2, corresponding to amino acids 314–508, was amplified from the p3\textsuperscript{FLAG}-hVRK2 plasmid construct with hVRK2\textit{GFP}-U and hVRK2\textit{GFP}-D (excludes stop codon). The amino terminus of hVRK3, corresponding to amino acids 1–149, was amplified from the p3\textsuperscript{FLAG}-hVRK3 plasmid construct with hVRK3\textit{GFP}-U and hVRK3\textit{GFP}-D. PCR products were digested with EcoRI and BamHI and then ligated into appropriately digested pEGFP-N1 plasmid DNA, generating pEGFP-hVRK2 C-Term and pEGFP-hVRK3 N-Term, respectively.

**Generation of mVRK1 Anti sera**

Generation of polyclonal antisera against hVRK1 is described elsewhere (28). Rabbit polyclonal antibodies against mVRK1 were generated commercially (Bethyl Laboratories Inc.), using a peptide from the amino terminus (R\textsuperscript{VKKAAQGRPGPAKRR}) that was conjugated to keyhole limpet hemocyanin.

**Northern Blot Analysis**

Multiple tissue Northern blots containing human or mouse poly(A) RNA (Clontech) were probed with a 509-bp XmnI fragment derived from the hVRK3 ORF. Membranes were probed with polyclonal sera prepared against mVRK2 and hVRK2, respectively. Probes were labeled with \( ^{32} \text{P}\text{-dATP} \) by priming with random oligohexanucleotides and filling in with the Klenow fragment of \( E. \text{coli} \) DNA polymerase I. Hybridization was performed in ExpressHyb solution (Clontech) at 68°C for 1 h; membranes were washed three times at room temperature with 2× SSC (150 mM NaCl, 15 mM sodium citrate (pH 7.0)) containing 0.1% SDS and then twice at room temperature with 0.2× SSC containing 0.1% SDS. Membranes were exposed at −80°C to Eastman Kodak Co. MS film with a BioMax MS intensifying screen for approximately 2 weeks.

**Immunoblot Analysis of VRK Expression**

4.5 µg of pcDNA3 or p3\textsuperscript{FLAG} constructs were introduced into 8 × 10\textsuperscript{5} BSC40 cells by transfection with LipofectAMINE Plus (Invitrogen). After a 24-h incubation period, cells were harvested, washed with phosphate-buffered saline (140 mM NaCl, 2 mM KCl, 10 mM Na\textsubscript{2}HPO\textsubscript{4}, 1 mM KH\textsubscript{2}PO\textsubscript{4}), and resuspended and boiled in 1× SDS-PAGE sample buffer (1% SDS, 1% β-mercaptoethanol, 50 mM Tris (pH 6.8), 10% glycerol). One-half of the sample of pcDNA3-transfected cells and one-fifth of p3\textsuperscript{FLAG}-transfected samples were fractionated on 12% SDS-polyacrylamide gels and transferred to nitrocellulose (Schleicher and Schuell). Membranes were probed with polyclonal sera prepared against mVRK1 or hVRK1 or with commercially available polyclonal or monoclonal antibodies directed against the MYC (Santa Cruz Biotechnology, CA, Santa Cruz, CA) or FLAG (Sigma) epitope tags. Horseradish peroxidase-conjugated secondary antibody and chemiluminescent developing reagents (Pierce ECL reagent) were used to visualize immunoreactive proteins.

**Immunofluorescence Microscopy**

Plasmid constructs encoding MYC-tagged hVRK1, mVRK1, mVRK1\textit{Δ3} and NH\textsubscript{2}-terminal 3×\textit{FLAG}-tagged mVRK2, mVRK3, hVRK2, and hVRK3 were introduced into 4 × 10\textsuperscript{5} BSC40 cells plated on LabTec chamber slides (Nunc, Naperville, IL), using LipofectAMINE 2000 (Invitrogen). After 24 h, medium was removed, and cells were washed three times with PBS and fixed at room temperature for 5 min by incubation with 50:50 (v/v) methanol/acetone. Fixed cells were washed twice and incubated with the following sera, as indicated in the figure legends: rabbit anti-FLAG (5 µg/ml), rabbit anti-casein (1:1000), mouse anti-phospho-paraformaldehyde and permeabilized with Triton X-100. DNA was stained with 4',6'-diamidino-2-phenylindole (DAPI). Images were captured with a Spot Camera on a Nikon DIAPHOT 200 microscope and processed using Spot Camera software (Diagnostic Instruments, Inc., Sterling, MI).

**Expression and Purification of Recombinant Proteins Produced in E. coli**

All pET16b VRK constructs were maintained in E. coli strain HMS174. Cultures were grown to an A\textsubscript{600} of 0.4, and protein expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM and infecting with λEC6 (encodes T7 RNA polymerase) (29) at a multiplicity of infection of 10. After a 20-min adsorption period at room temperature, induction was initiated by incubation at 37°C for 30 min followed by an additional 90 min at 30°C. Clarified lysates were prepared, and the NH\textsubscript{2}-terminal His-tagged VRK proteins were purified on Ni\textsuperscript{2+}-nitrilotriacetic acidagarose (Qiagen) resin. Protein concentrations were estimated using the Bradford method with bovine serum albumin as a standard.

**Expression and Purification of Recombinant VRK2 Produced in Mammalian Cells**

Generation of Recombinant Vaccinia Viruses Directing the Expression of VRK2—The mVRK2 ORF was subcloned from the pET16b construct into the NdeI-BamHI sites of the pTM1–3×\textit{FLAG}-NdeI vector. For the hVRK2 ORF, the internal NdeI site at position 898 was removed by overlap PCR without changing the predicted amino acid sequence. To produce this mutation, two sets of primer pairs were used to amplify the target region: (i) hVRK2\textit{pET}–D introduced the silent mutation and was used with the hVRK2\textit{pET}–U, and (ii) hVRK2\textit{pET}–D introduced the complement of the mutation and was used with hVRK2\textit{pET}–U. The p3\textsuperscript{FLAG}-hVRK2 plasmid was used as the template for these PCRs; a mixture of the products generated served as the template in a second round of amplification performed with hVRK2\textit{pET}–U and hVRK2\textit{pET}–D. The final product was digested with NdeI and BamHI then ligated into appropriately digested pTM1–3×\textit{FLAG}-NdeI plasmid DNA, generating pTM1–3×\textit{FLAG}-NdeI-hVRK2. Automated DNA sequencing was performed to ensure the presence of the desired nucleotide substitution and the absence of any spurious mutations. These constructs were used to generate recombinant vaccinia viruses in which the mVRK2 or hVRK2 gene is placed within the nonessential thymidine kinase locus under the control of a bacteriophage T7 promoter, as described (30). Viral stocks, designated t7T–3×\textit{FLAG}-mVRK2 and -hVRK2, were prepared from infected cytoplastic extracts by ultracentrifugation through a 36% sucrose cushion; titers were obtained by plaque assay on BSC40 cells. For VRK2 expression, 8 × 10\textsuperscript{5} BSC40 cells were co-infected with t7T7.5 (which expresses T7 RNA polymerase) (24) and one of the t7T–3×\textit{FLAG}-VRK2 viruses (multiplicity of infection of 2 each).

**Immunofluorescence Purification of VRK2—Infected cells were harvested at 8 h post-infection and were lysed in 900 µl of FLAG lysis buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml pepstatin) by incubation at 4°C on an end-over-end rotator for 15 min. Chromatin was removed by centrifugation at 800 × g. Cytosolic fractions were incubated at 4°C overnight with 60 µl of M2 anti-FLAG-agarose beads (Sigma); after extensive washing, the VRK2 was eluted with 30 µg of 3×\textit{FLAG} peptide (Sigma). Fractions were resolved by SDS-PAGE and monitored both by silver staining and immunoblot analysis with α-FLAG antibody. The concentration of purified VRK2 was estimated by comparison with known amounts of NH\textsubscript{terminal} 3×\textit{FLAG}-BAP protein (Sigma) in quantitative immunoblot analyses.

**In Vitro Kinase Assays**

Recombinant kinases were evaluated for autophosphorylation activity and for their ability to transfer phosphate to the exogenous substrate casein. 100 ng of bacterially expressed kinase (VRK1 and VRK3) or 25 ng of vaccinia virus-expressed kinase (VRK2) was assayed in 25-µl reactions containing 50 mM Tris (pH 7.5), 1 mM dithiothreitol, 5 µM rATP, and 10 µCi of \([γ-32\text{P}]\text{dATP} (3000 \text{Ci/mmol}), \) with or without the addition of 5 µg of casein (Sigma). Reactions were stopped by the addition of SDS-PAGE sample buffer. Reaction products were subjected to SDS-PAGE, and dried gels were visualized by autoradiography.

**Phosphoamino Acid Analysis of VRK2 Kinase Activity**

In vitro VRK2 kinase reactions were carried out as above, except for the inclusion of 20 µCi of \([γ-32\text{P}]\text{dATP}, \) and analyzed by phosphoamino acid analysis. Reactions were resolved in a 10% SDS-polyacrylamide gel, transferred to Immobilon P membrane (Millipore Corp., Bedford, MA), and visualized by autoradiography. Portions of the filter corre-sponding to phosphorylated casein and autophosphorylated VRK2 were excised and placed in 100 µl of constant boiling HCl at 110°C for 1 h. Phosphoamino acid analysis was performed by high voltage, thin layer
Comparative Analysis of the VRK Protein Kinase Family

The Vaccinia-related Kinases Share a High Degree of Identity to vvB1, Especially in Their Catalytic Domains—The striking similarity within the VRK family and between the VRK family and vvB1 can be seen through sequence alignments. The human VRK1 open reading frame encodes a 397-amino acid protein, and the open reading frames of mVRK1, mVRK1Δ, and mVRK1ΔΔ encode proteins of 441, 417, and 397 amino acids, respectively. The hVRK2 ORF encodes 508 amino acids, and the mVRK2 ORF encodes 503 amino acids. The hVRK3 ORF encodes 475 amino acids, and the mVRK3 ORF encodes 454 amino acids. When the amino acid sequences of the vvB1 and the mouse and human VRKs are subjected to alignment by the Clustal method, the high degree of overall identity among the VRKs and with vvB1 is apparent and is represented in Figs. 1A and 2. Fig. 1 summarizes the extent of amino acid identity among the VRK and vvB1 proteins. With respect to the human/mouse orthologs, the VRK1 enzymes show 87% identity, the VRK2 enzymes show 68% identity, and the VRK3 enzymes show 74% identity (A). When only the catalytic domains are considered, these numbers increase to 92, 83, and 78% identity, respectively (B). In terms of relatedness between paralogs, the hVRK1 and hVRK2 proteins show an overall identity of 44% (A) and an identity of 53% in their catalytic domains (B). The human VRK2 and VRK3 proteins show an overall identity of 23% (A), which rises to 34% when only the catalytic domains are considered (B). The human VRK1 and VRK3 proteins show an overall identity of 33% (A) and an identity of 32% in their catalytic domains (B). Comparisons of the murine paralogs gives similar results. The vvB1 amino acid sequence shares an overall identity of 39% with the murine VRK1 protein, 34% with the mVRK2 protein, and 27% with the mVRK3 protein.

**Fig. 1. Schematic depiction of the percentage identities between the complete ORFIs or catalytic domains of vvB1 and the VRKs.**

A. comparison of the sequence identities between the VRKs and vvB1. The hVRK1, -2, and -3 paralogs are connected pairwise by horizontal open arrows; the percentage of sequence identity between each pair is shown within an oval. The three pairs of hVRK and mVRK orthologs are connected by vertical, filled arrows; for each pair, the percentage of sequence identity is shown within a box. The percentage of sequence identity between each of the three mVRK paralogs and vvB1 is shown within a triangle placed on the appropriate connecting barbell. B, the overall architecture of vvB1 and the VRKs is shown. The catalytic domains are indicated by shaded boxes; for VRK1, -2, and -3, the number within these boxes represents the percentage of sequence identity between the catalytic domains of the murine and human orthologs. The numbers shown adjacent to the vertical arrows represent the percentage of sequence identity between the catalytic domains of the three human paralogs. For VRK1 and -3, the position of the NLSs is indicated by the checkered box.

**Computer Analysis**

Autoradiography films were scanned by a SAPHIR scanner (Lino-type-Heil Co., Hauppaug, NY). All sequence analyses were performed with Lasergene Software (DNASTAR, Madison, WI). Figures were labeled using Canvas software (Deneba Systems, Miami, FL).

**RESULTS**

Because of our continuing interest in the vaccinia virus-encoded B1 protein kinase (14, 18, 19), we were intrigued by the identification of the mammalian VRK1 kinases and by the appearance of other VRK-related expressed sequence tags and cDNAs in the public databases. We therefore set out to examine the structure and function of this novel family of vvB1-like protein kinases. Our first goal was to amplify and clone the human and mouse VRK ORFs. Using RT-PCR with preparations of total RNA from hTK cells, we were able to isolate the 1191-bp ORF of hVRK1, the 1527-bp ORF of hVRK2, and the 1425-bp ORF of hVRK3. Using RT-PCR with preparations of total RNA from mouse L 929 cells, we were able to isolate the 1323-, 1251-, and 1191-bp ORFs of mVRK1 and the splice variants mVRK1Δ and mVRK1ΔΔ; the 1512-bp ORF of mVRK2; and the 1362-bp ORF of mVRK3 (not shown). These products were used in preparation of the various VRK expression plasmids described under “Experimental Procedures.”

**Amplification of Human and Mouse VRK mRNAs by RT-PCR**—In order to investigate the VRK family, we utilized RT-PCR to obtain full-length cDNA clones of the human and mouse VRK1, VRK2, and VRK3.

**Membrane Association of VRK2**

In order to assess whether the VRK2s do associate with membranes and determine whether they are intrinsic or extrinsically associated, we treated transfected cell lysates with Na2CO3 (pH 11.5) (32). Twelve micrograms of p3FLAG-VRK2 and 4 µg of pEGFP-N1 were co-transfected into ~3.2 × 106 BSC40 cells, which were harvested 18 h later. Transfected cell monolayers were washed once in cold PBS and then scraped into 1.5 ml of PBS, and then cells were collected by low speed centrifugation. Cell pellets were washed with 100 mM NaCl and collected by centrifugation. The resulting cell pellets were resuspended in 300 µl of cold 100 mM Na2CO3 (pH 11.5) and then subjected to 10 strokes of a Dounce homogenizer. The cell lysates were incubated on ice for 30 min and then centrifuged at 150,000g for 30 min. The supernatant and pellet fractions were acetone-precipitated and then resuspended in equal volumes. Both fractions were subjected to analysis by SDS-PAGE and immunoblotting. Purity of fractionation was monitored by analysis of GFP partitioning with a mouse anti-GFP antibody (kind gift of Dr. J. Barbieri, Medical College of Wisconsin) at a dilution of 1:3000.

**Electrophoresis on a HITLE-7000 system (C.B.S. Scientific Co., Del Mar, CA) in phosphochromatography buffer (37.5% n-butyl alcohol, 25% pyridine, and 7.5% acetic acid) at 1800 V for 25 min, as described previously (18, 31). Phosphoserine, phosphothreonine, and phosphotyrosine standards were included and visualized by ninhydrin staining; radio-labeled amino acids were visualized by autoradiography.

**A**

Electrophoresis on a HTLE-7000 system (C.B.S. Scientific Co., Del Mar, CA) in phosphochromatography buffer (37.5% n-butyl alcohol, 25% pyridine, and 7.5% acetic acid) at 1800 V for 25 min, as described previously (18, 31). Phosphoserine, phosphothreonine, and phosphotyrosine standards were included and visualized by ninhydrin staining; radio-labeled amino acids were visualized by autoradiography.

**B**

Electrophoresis on a HTLE-7000 system (C.B.S. Scientific Co., Del Mar, CA) in phosphochromatography buffer (37.5% n-butyl alcohol, 25% pyridine, and 7.5% acetic acid) at 1800 V for 25 min, as described previously (18, 31). Phosphoserine, phosphothreonine, and phosphotyrosine standards were included and visualized by ninhydrin staining; radio-labeled amino acids were visualized by autoradiography.

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A comparison of vvB1 with the hVRK proteins gives similar results. The domain representation of vvB1 and the VRKs is illustrated in Fig. 1B; the gray boxes depict the catalytic domains, whereas the extracatalytic sequences are unshaded.

A detailed sequence alignment of the catalytic domains of the VRKs and vvB1, generated using the Clustal method, is shown in Fig. 2. Amino acids that are identical to vvB1 are boxed, and amino acids that are identical to the consensus (residues conserved in at least four family members) are shaded in yellow. All of the domains involved in the phosphotransfer reaction are present and identifiable in the VRKs. As described in more detail below, several key amino acid signatures that define the vvB1 kinase are contained in the VRKs and hence, we assert, define the family. The ATP binding motifs in subdomains I and II are indicated with a red overscore. The invariant lysine of subdomain II, usually required for ATP binding, is preceded by two aliphatic residues. Subdomains VI and VII, containing the invariant aspartic acid residues, are indicated with green overscores. The typical DFG motif that is present in subdomain VII of protein kinases is altered to a DYG motif in vvB1 and the VRKs. The APE motif found within subdomain VIII of the majority of protein kinases is more often a SIN motif in members of the casein kinase (CK) family (the defining branch of the CK group) (33). In the vvB1/VRK family (another branch of the CK group), a (P/S)xD motif is found in the corresponding position, as revealed by Clustal analysis (indicated in Fig. 2 with white lettering on a blue background).
a black overscore). It is of note that the PXD motif is present in vaccinia and variola viruses, but in all other poxviruses (e.g. goatpox virus, lumpy skin disease virus, sheeppox virus, Yaba monkey tumor virus, swinepox virus myxoma virus, ectromelia virus, and myxoma virus), the motif is SXD, as found in the VRKs.

We noted with interest that both the human and mouse VRK3 sequences have substitutions in several residues within these motifs that have been shown in other kinases to be required for catalysis; these amino acid alterations are indicated with blue boxes. The ATP binding motifs in subdomains I and II contain key changes; the glycines and neighboring amino acids in subdomain I have been replaced with amino acids containing larger, charged side chains (GXXPG → TRD-NQG, in hVRK3). These substitutions would presumably abrogate the ability of the domain to interact with the phosphates of ATP via backbone interactions, consequently blocking association with the phosphate donor. The VRK3s also have alterations in subdomain II; although the invariant Lys residue is retained, the typical aliphatic residues found in the NH2-terminal positions have been changed. Neither of the key Asp residues in subdomain VI and VII are maintained in VRK3. These residues are thought to interact with Mg2⁺ via salt bridges and in so doing to coordinate ATP. Finally, the Thr of the T/U/L/E motif within subdomain VIII, which we believe to correspond to the residue that is frequently phosphorylated in activated kinases (34), is altered to Asp, mimicking the negative charge introduced by phosphorylation. Taken together, the amino acid substitutions at these key residues elicited our hypothesis that the VRK3 enzymes would be catalytically inert, although they should retain substrate recognition and binding capabilities.

The alignment of the catalytic domains shown in Fig. 2A was subjected to analysis by the neighbor-joining method, and the resulting phenogram is shown in Fig. 2B. It is clear that there is a close phylogenetic relationship between orthologs and that the paralogs are more similar to each other than to vvB1. Overall, the high degree of identity among these proteins and the retention of characteristic sequence variants in key motifs supports their grouping into a novel family of vvB1-like protein kinases. Fig. 2C presents a phenogram derived from an alignment of hVRK1, hVRK2, hVRK3, cVRK (C. elegans), dVRK (D. melanogaster), and vvB1. D. melanogaster VRK is more closely related to the mammalian VRKs and vvB1 than is cVRK.

The VRKs Are Modular Enzymes with Significant Extracatalytic Domains—As suggested by their having molecular weights in the range of 50,000–60,000, and as illustrated in Fig. 1B, the VRKs have significant regions of extracatalytic sequence. The degree of conservation within these domains is high between the human and mouse orthologs, as is shown in detail in Fig. 3. This figure illustrates the positioning of the VRK extracatalytic sequences relative to the catalytic domain as well as presenting the sequence alignment among the family members. The extracatalytic sequences of VRK1 and VRK2 are located at the carboxyl terminus, whereas the extracatalytic sequence of VRK3 is located at the amino terminus (also noted in (22)). Alignment of the carboxyl termini of the VRK1 sequences demonstrates that mVRK1ΔΔ is most like hVRK1, with the mVRK1Δ and mVRK1 variants containing additional sequences; evidence for the generation of these variants by differential splicing will be discussed below. The carboxyl termini of the VRK1s are highly conserved, suggesting that these sequences will play important functional roles. This region contains a classical basic nuclear localization signal (NLS), which is marked by the wavy line.

The extracatalytic sequences found at the COOH termini of the VRK2 proteins are shown in Fig. 3B. The overall conservation within this domain is 50%, although it is apparent that the proximal and distal ends of these regions are highly conserved. Within the most carboxyl terminal region of VRK2, 17 of 22 amino acids are hydrophobic (indicated with an overscore); the PSORT program (available on the World Wide Web at psort.im.s.u-tokyo.ac.jp) predicts that this region may represent a transmembrane domain.

Fig. 3C presents an alignment of the NH2-terminal region of the VRK3 proteins; the overall identity within this region is 64.6%. A putative bipartite nuclear localization domain (KRGLNSSFETSPKKVK) is marked by the wavy line. In addition, hVRK3 contains additional sequences that are absent in our mVRK3 clones. We have been unable to find cognate sequences in any of the mVRK3 cDNAs or expressed sequence tags that have been submitted to public data bases. As described below, the additional sequence in hVRK3 represents an expansion of exon 5. Within this region, we have identified the repeated motif (KR)XSPQXT(KR) that could serve as a site of phosphorylation. Several computer algorithms predict that this sequence is a CDK5 recognition site (e.g. see, on the World Wide Web, www.cbs.dtu.dk/services/NetPhos/). There are five of these sequences in hVRK3, whereas only two are present in the mVRK3 sequence. In addition, when the total serine contents of hVRK3 and mVRK3 were examined, serines were found to comprise 11.5 and 9.2% of the total amino acid content, respectively, well above the average serine content of 6.9% (35).

Annotation of the Genomic Loci of the VRKs—We annotated the intron-exon structures of the mouse and human VRK genomic loci (Fig. 4). Genomic sequences were obtained from Celera and from NCBI. We annotated exon positions by aligning cDNAs with their corresponding genomic loci using the MegAlign software program (Lasergene, DNASTAR). This provided distances between exons and allowed for construction of diagrams that possess accurate distance representation. The genomic structures we derived were compared with annotations presented by Celera and NCBI. Our annotations agree with both sources, excepting Celera’s annotation of mVRK2. We ascribe this discrepancy to a difference in cDNA sequences used to annotate the exon structure; the mVRK2 cDNA used by Celera deviates and possesses rearrangements within the sequences encoding the catalytic domain.

According to Celera annotation, all of the VRK genomic loci are contained within mouse-human syntenic blocks. The hVRK1 locus spans ~80 kb on chromosome 14q32 and contains 12 exons. The mVRK1 locus spans ~38 kb on chromosome 12f12 and comprises 14 exons. The mVRK1 locus undergoes alternative splicing, with exon 12 being excluded in mVRK1Δ and exons 12 and 13 being excluded in mVRK1ΔΔ. As we have described above, no sequences corresponding to exon 12 or 13 are found in any of the known hVRK1 cDNA sequences. However, sequences that are conserved relative to exon 12 of mVRK1 are indeed found within the hVRK1 intron 11 (shown as a light gray box in Fig. 4). It is not clear why this region is not utilized as an exon during transcription of the hVRK1 gene, because consensus 5’ and 3’ splice sites are present (not shown). The catalytic domains of the VRK1s are encoded in exons 1–8, and the nuclear localization signal is located in exon 10.

The human VRK2 locus spans ~122 kb on chromosome 2p16, and the mVRK2 locus is of approximately the same size and is located on chromosome 11A3.3. The cDNAs we have used to annotate the VRK2 loci indicate that mature transcripts from these genomic loci represent 13 exons. In both of these genes,
the first exon of the cDNAs we have examined is untranslated and is indicated with a checkered box.

The mouse and human VRK3 genes span 50 and 25 kb, respectively, and are located on chromosomes 19q13 and 7q2, respectively. Both of these genes contain 15 exons, of which the first two are untranslated. Exon 5 of hVRK3 contains the previously mentioned (K/R)xSPQXT(K/R) repeats that are reduced in number in mVRK3 exon 5. The bipartite NLS is located in exon 4 of both the human and mouse VRK3. We and others have also identified the existence of an hVRK3 pseudo-gene on chromosome 4p15.33 (1).

VRK mRNAs Are Expressed in a Broad Range of Tissues—Nezu et al. (20), in their initial identification of hVRK1 and hVRK2, presented Northern blot studies that pointed to a ubiquitous tissue distribution. To complement these studies, we examined the tissue distribution of hVRK3 and mVRK3 using the Clontech multitissue Northern blots. Filters containing poly(A)^+ RNA from a panel of human and mouse tissues were hybridized with a radiolabeled XmnI fragment representing the 5' portion of the hVRK3 open reading frame and an internal 3' SfcI fragment derived from mVRK3. In all human and mouse tissues examined, the probe detected a single species of ~1.9 kb (not shown).

Expression and Purification of Recombinant VRK Proteins—In order to evaluate the catalytic activity of the VRKs, we generated recombinant human and mouse VRK1, -2, and -3, either in bacterial or mammalian hosts. The VRK1s and VRK3s proved to be amenable to expression in prokaryotic cells, whereas the VRK2s were subject to degradation. Therefore, the VRK1s and VRK3s were expressed in E. coli, and the VRK2s were expressed in mammalian cells using recombinant vaccinia viruses.

E. coli HMS174 cells harboring pET16b constructs of hVRK1, mVRK1, mVRK1Delta, mVRK1Alpha, hVRK3, and mVRK3 were induced to express His-tagged proteins, which were subsequently purified by affinity chromatography. Examination of the VRK preparations by SDS-PAGE and silver staining indicated that they had been purified to near homogeneity (Fig. 5B). Expression from the pET16b vector is predicted to add 2,600 to the Mr,

5 R. J. Nichols and P. Traktman, unpublished data.
6 J. Nezu, personal communication.
of each protein, yielding a predicted $M_r$ of 47,600, 51,300, 50,100, and 47,600 for hVRK1, mVRK1, mVRK1Δ, and mVRK1ΔΔ, respectively. As shown in Fig. 5B, each protein migrates somewhat more slowly than expected (apparent $M_r$ 54,800, 59,300, 54,300, and 53,400, respectively). The predicted molecular weights for the hVRK3 and mVRK3 proteins are 53,100 and 51,100; the apparent molecular weights were 60,900 and 58,400, respectively. In each case, the observed $M_r$ was ~5,000–8,000 larger than predicted; how much of this discrepancy is due to intrinsic properties of the individual proteins or to post-translational modification has not been determined.

Overexpression of the VRK2 proteins was achieved by using the hybrid vaccinia virus-T7 expression system in BSC40 cells, and the 3×FLAG-tagged proteins were purified by immunoaffinity chromatography. The predicted molecular weight of both 3×FLAG-VRK2 proteins is 60,800; the observed molecular weights were 61,200 and 61,700, respectively. We typically observed a minor co-purifying species of ~70,000; based on our prior experience with this system, we believe this protein to be a member of the Hsp70 family.2

The VRK1 and VRK2 Genes Encode Active Protein Kinases, whereas the Protein Encoded by the VRK3 Gene Lacks Enzymatic Activity—The recombinant VRK protein preparations described above were tested in vitro for kinase activity. The VRKs are classified as members of the casein kinase group of protein kinases (1), and Zelko et al. (21) and Lopez-Borges et al. (36) have shown that casein can serve as a suitable substrate for the VRKs. Therefore, we chose casein as a test substrate for all the VRKs. The kinase activity of mVRK1, mVRK1Δ, and hVRK1 have been examined by Nezu et al. (20) and by the Lazo group (36, 37), respectively, as well as by our laboratory. We included them in our assays to provide a complete profile of VRK activity and to serve as reference points for mVRK1ΔΔ, the previously uncharacterized mVRK1 splice variant we have described in this report. In addition, our data represent the first biochemical characterization of the VRK2 and VRK3 proteins. Purified proteins were incubated with $\gamma^{-32}P$-ATP in an appropriate buffer in the presence or absence of casein. Reactions were resolved by SDS-PAGE and visualized by autoradiography. As can be seen in Fig. 5A, all of the VRK1 proteins exhibited robust autophosphorylation activity. The extent of autophosphorylation of the mVRK1 splice variants appears to be equal, suggesting that the major sites of autophosphorylation lie in the regions common to the three proteins. Casein also serves as an exogenous substrate for the VRK1 proteins (marked by the circle); we have consistently found that the mVRK1 proteins appear to phosphorylate casein more efficiently than does hVRK1.

The VRK2 proteins exhibit a modest level of autophosphorylation activity, with mVRK2 preparations consistently incorporating more phosphate than hVRK2. In addition, the VRK2 proteins phosphorylate the exogenous substrate casein to a greater extent than do any of the VRK1 proteins. In stark contrast, we did not detect any kinase activity in any preparations of the human or mouse VRK3 proteins, as measured either by autophosphorylation or substrate phosphorylation. As described above, the primary sequence of the VRK3s reveals several substitutions at key residues known to be essential for the catalytic activity of protein kinases in general. We predicted that these substitutions would render the VRK3 proteins catalytically inert, which was verified by our experimental results. Although we have only monitored autophosphorylation activity and the ability to phosphorylate a single exogenous substrate, we believe that the VRK3 proteins do in fact lack enzymatic activity while retaining a predicted substrate-binding domain.

The VRK2s Are Serine/Threonine Kinases—The VRK1 proteins were previously shown to have serine/threonine specificity (21, 36). The VRK2 proteins are also predicted to be kinases with serine/threonine specificity, due to the sequence between the two invariant aspartic acid residues (amino acids D160VKAANLLLGYKNPDQVYLAD in hVRK2) (3). To verify this prediction, we performed phosphoamino acid analysis on
the radiolabeled products generated in vitro by our VRK2 preparations: casein and autophosphorylated hVRK2 and mVRK2. The phosphoamino acid profile generated by hVRK2 and mVRK2 was identical. As shown in Fig. 5C, autophosphorylation of the VRK2 proteins occurs predominantly or solely on threonine residues, whereas casein was found to be phosphorylated predominantly or solely on serine residues.

Transient Expression of Epitope-tagged VRK Family Members in Mammalian Cells—To facilitate analysis of the intracellular localization of the VRK proteins, we generated plasmid constructs encoding COOH-terminally MYC-tagged VRK1 and NH2-terminally 3\times\text{FLAG}-tagged VRK2 and VRK3 under the transcriptional regulation of the cytomegalovirus immediate early promoter. BSC40 cells were transfected with pCDNA3-hVRK1\text{MYC}, mVRK1\text{MYC}, -mVRK1\Delta\text{MYC}, or -mVRK1\Delta\text{MYC} or p3\times\text{FLAG}-hVRK2, hVRK3, -mVRK2, or -mVRK3, and expression of the various proteins was monitored by immunoblot analysis of cellular lysates (Fig. 6). We have generated antibodies against mVRK1 and hVRK1 and show here their reactivity against transiently expressed proteins; the VRK1 proteins were also detected with an antibody directed against the MYC epitope tag (\alpha\text{-MYC}). An \alpha\text{-FLAG} antibody was used to detect the 3\times\text{FLAG}-tagged VRK2 and VRK3 proteins. In all cases, proteins of the predicted molecular weight were observed.

Subcellular Localization of the Vaccinia-related Kinase Proteins—Accurate and effective signal transduction depends upon the appropriate intracellular localization of protein kinases and their substrates. The subcellular localization of the VRK proteins was therefore determined by performing indirect immunofluorescence staining of transiently transfected cells. Zelko et al. (21) retrieved mVRK1 in the nuclear fraction of cellular lysates and postulated that the basic sequence R\text{\textsuperscript{556}}KKK would be the functional NLS of mVRK1; Lopez-Borges and Lazo (36) demonstrated that the NH2-terminal portion of VRK1 did contain the NLS by monitoring the localization of GFP fusion proteins containing either the intact hVRK1 ORF or a COOH-terminal fragment. The results we present in Fig. 7A confirm and extend these results; epitope-tagged hVRK1, mVRK1, mVRK1\Delta, and mVRK1\Delta\Delta all showed...
a nuclear localization upon transient expression in BSC40 cells.

A distinct pattern of subcellular localization was observed for transiently expressed human and mouse 3×FLAG-VRK2 (Fig. 7B). These proteins showed intense juxtanuclear staining as well as reticular staining that extended throughout the cytoplasm. There was no indication of nuclear localization and no staining of the plasma membrane. To explore the intracellular localization of the VRK2 proteins further, we performed double-labeling experiments using both the α-FLAG antibody and antibodies directed against proteins whose localization is diagnostic of specific intracellular organelles. The localization of VRK2 was clearly distinct from the staining pattern seen with antibodies specific for the following markers: ε-COP, part of the coatamer of COP-I transport vesicles (38); ERGIC-53, a mannose-specific lectin that acts as a cargo receptor for glycoprotein transport and marks the endoplasmic reticulum-to-Golgi intermediate compartment (39, 40); and Golgin, a member of the granin family of proteins and a peripheral membrane protein found on the cytoplasmic face of the Golgi apparatus (41) (data not shown). In contrast, as shown in Fig. 7B, the pattern of VRK2 localization seen with the α-FLAG antibody resembled the staining pattern observed for protein-disulfide isomerase and p63 (not shown), two resident protein components of the endoplasmic reticulum (42, 43). The merged images confirm and emphasize the extensive degree of overlap seen in the reticular pattern of cytoplasmic staining; in addition, a ring of VRK2 staining around the nuclear periphery was also seen.

As mentioned above, the predicted primary sequence of the VRK3 proteins contains a putative bipartite nuclear localization sequence within the amino-terminal region: KLEEPQRLNSSFETSPKKVKW. Consistent with this prediction, transiently expressed human and mouse 3×FLAG-VRK3 showed a clear nuclear localization that overlapped with the DAPI-stained image, as shown in Fig. 7C.

Analysis of Determinants within the Extracatalytic Domains of VRK2 and VRK3 That Direct the Intracellular Localization of GFP Fusion Proteins—Based on the organization of the VRK2 and VRK3 proteins (see Figs. 1B and 3) and the PSORT analysis described above, we hypothesized that the localization determinants responsible for the intracellular localization of VRK2 and VRK3 would lie in the carboxyl- and amino-terminal extensions of these proteins, respectively. To investigate this hypothesis, we generated GFP fusion constructs in which the carboxyl-terminal 194 amino acids of hVRK2 or the amino-terminal 149 amino acids of hVRK3 were fused upstream of GFP. The fusion proteins were expressed transiently, and the pattern of GFP fluorescence was monitored (Fig. 8). Indeed, the COOH terminus of hVRK2 led to a reticular, cytoplasmic pat-
treated with Na$_2$CO$_3$ and the membranes were isolated by ultracentrifugation, we observed that GFP partitioned with the membrane pellet (Fig. 6B). These data indicate that the VRK2s are integrally associated with membranes.

**DISCUSSION**

Given our longstanding interest in the regulation of the poxvirus life cycle by virally encoded protein kinases, the demonstration that diverse eukaryotic species encode proteins with significant homology to the vaccinia B1 kinase was of significant interest. The B1 protein kinase is required for replication of vaccinia DNA, and we had hypothesized that if there were sequence homology between the B1 and VRK kinases there might also be functional similarity. In a contemporaneous manuscript from our laboratory, we present data demonstrating that the hVRK1 and mVRK1 can complement a temperature-sensitive mutant of vaccinia virus encoding a defective B1 kinase (28). The cellular VRK1 proteins restore the ability of this virus to direct efficient genome replication and complete a productive infectious cycle at the nonpermissive temperature. These data suggest that vvB1 and VRK1 share functional as well as structural similarity, making the future identification of viral and cellular substrates of significant interest. In support of the possibility that the VRK1 proteins might participate in the replication, repair, or transmission of the cellular genome, it is of interest that RNAi-mediated repression of the VRK-like kinase encoded by *C. elegans* causes an embryonic lethal phenotype characterized by defects in cell division (see, on the World Wide Web, nematoda.bio.nyu.edu). Furthermore, Lazo et al. have demonstrated that hVRK1 can phosphorylate portions of the p53 protein when they are presented as glutathione S-transferase fusion proteins *in vitro* (36). Cumulatively, these data suggest that the VRK1 proteins, as well as vvB1, might participate in the processes of DNA replication and/or repair.

VRK1, VRK2, and VRK3 Comprise a Novel Family of Eukaryotic Kinases—Our goal for the studies described herein was to isolate and characterize the human and mouse proteins that comprise the family of vvB1-related VRKs. We were able to identify three sets of orthologous mammalian proteins with a significant degree of homology to vvB1 and VRK1. Analysis of multiple sequence alignments of the entire ORFs or their catalytic domains reinforced the conclusion that these kinases merit designation as a family of three paralogs that are conserved in the human and mouse genomes.

Our sequence analysis reveals that the VRK family members share several distinct sequence variations within their catalytic domains that set them apart from the majority of protein kinases in general. These diagnostic motifs are most easily seen in subdomains II-IVK, VIB-HGDIK, VII-DYG, and VIIIP/S-I/X-D. It is likely that the three VRK paralogs arose by gene duplication followed by divergence, a process represented clearly by the acquisition of paralog-specific extensions to the N or COOH termini that mediate, at the very least, intracellular localization. The VRK proteins represent one of 90 kinase families with no closely related orthologs in yeast (48); the orthologs encoded in the genomes of *C. elegans* and *D. melanogaster* are recognizably, albeit more distantly, related to the VRK proteins. The dVRK (*D. melanogaster* homolog) and cVRK (*C. elegans* homolog) lack the distinguishing Tyr in the subdomain-VII DYG $\rightarrow$ DFG but retain the variation from APE $\rightarrow$ SXD in subdomain-VIII (not shown). As is shown in Fig. 2C, dVRK is more closely related to the mammalian VRKs than is cVRK, consistent with other evidence indicating that the fly kinome more closely parallels the human kinome (48). In this regard, it is worth noting that expression of dVRK during infections performed with the tsB1 virus at 39.7 °C restores the ability of the virus to complete a productive infectious cycle.$^5$

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$^5$ K. Boyle and P. Traktman, unpublished results.
hVRK1 and mVRK1 Encode Nuclear Serine/Threonine Kinases: Multiple Splice Variants of mVRK1 Are Expressed—In the initial report of a murine hVRK1-like protein, the protein was shown to possess an extended carboxyl terminus that had no cognate in the human ortholog. We have, however, detected three splice variants of the murine protein; the mVRK1Δ transcript encodes the smallest protein, which is most similar to the hVRK1 protein. Splice variants that retain one (exon 12; mVRK1Δ) or two (exons 12 and 13; mVRK1) additional exons are also expressed in the L929 cells from which we isolated our mRNA. Interestingly, sequences with a high degree of similarity to the murine exon 12 are found in the human VRK1 gene in a comparable intronic region. However, inclusion of these sequences in mature transcripts has not been detected, either by our laboratory or in the various cDNA sequences that have been submitted to the available data bases. This sequence appears to be flanked by perfect 5’ and 3’ splice sites. Moreover, since the primers we used for all of our RT-PCR analyses hybridized to regions overlapping the observed or predicted initiation and termination codons, additional splice variants of the VRKs representing variable 5’- or 3’-untranslated regions or exons would not have been amplified. Indeed, such splice variants do appear in various public databases (e.g., the recent submission of VRK2 splice variants and ongoing annotation of genomic sequences may lead to the identification of previously unrecognized, noncoding exons).

The human and mouse VRK1 enzymes have similar enzymatic properties, possessing robust autophosphorylation activity on serine and threonine residues.5 Although we have not determined the site of autophosphorylation, these enzymes do possess a T/V/L/E motif within subdomain VIII, whose Thr may correspond to the residue typically phosphorylated in other kinases upon their activation. The VRK1 kinases will also phosphorylate casein as an exogenous substrate; we have not observed any modulation of mVRK1 kinase activity by the sequences encoded in exons 12 and 13. This situation contrasts with other kinases, such as twitchin kinase, which possess regulator regions in their carboxyl termini that modulate kinase activity (49). The putative basic NLS is retained in all forms of VRK1 that we have isolated, and indeed all of the VRK1 enzymes we have characterized localize to the nucleus, as shown in Fig. 7. Such a localization pattern is consistent with our hypothesis that the VRK1 proteins may participate in cellular DNA metabolism.

The VRK2s Are Serine/Threonine Kinases Associated with the Endoplasmic Reticulum—The second member of the VRK family (hVRK2) was first identified by Nezu et al. (20) in a search for VRK1-related genes. They found that the VRK2 gene was transcriptionally active in all tissues tested; to date, this was the extent of direct investigations into this protein. Agoulnik et al. (50) have inadvertently investigated the effects of a VRK2 gene deletion in mice and showed that VRK2/vVRK2– mice were viable; these data were the by-product of investigations into POG, an adjacent gene that is transcribed in the opposite direction from mVRK2.

The identity between VRK2 and vvB1 is almost as high as that between VRK1 and vvB1. Using preparations of 3×FLAG-hVRK2 and 3×FLAG-mVRK2, we showed that these enzymes possessed the ability to undergo autophosphorylation on Thr residues and to phosphorylate the exogenous substrate casein on Ser residues. As described above for the VRK1 proteins, the Thr residue contained within the T/L/E motif in subdomain VIII represents a likely site on which the VRK2 proteins may undergo phosphorylation. Examination of the VRK2 primary sequence with PSORT or SCANSITE did not reveal any classical determinants of intracellular localization, although the extreme COOH terminus of these proteins is almost exclusively hydrophobic. The VRK2s were shown to co-localize with markers of the endoplasmic reticulum as well as concentrating in a juxtanuclear ring. Analysis of VRK2-GFP fusion proteins indicated that this localization pattern could be conferred by the COOH-terminal 194 amino acids of VRK2. The implied membrane association of VRK2 was verified by our demonstration that the mouse and human proteins partition with integral membrane components upon Na2CO3 fractionation of cell extracts. Refinement of how the hydrophobic tail of VRK2 might associate with intracellular membranes is an area for future study.

The VRK3s Are Catalytically Inactive, Nuclear Proteins—The VRK3s exhibit 27% identity to vvB1 and 33% identity to VRK1. Outside of the shared catalytic domain, the VRK3 proteins contain an NH2-terminal bipartite NLS that is functional, as assayed by indirect immunofluorescence analysis of transiently expressed 3×FLAG-VRK3 or VRK3 1–149-GFP. There is an additional repeated motif encoded within exon 5 of hVRK3 that is minimized in the corresponding exon of mVRK3. Within this region, there are five repeats of (K/R)XSPQXTX/K–R), which resembles a consensus site for CDK5-mediated phosphorylation and might serve as a regulatory site within the protein. Upon examination of the VRK3 catalytic domain, we observed the existence of several amino acid substitutions within key catalytic motifs that we predicted would ablate the ability of these proteins to catalyze the phosphotransfer reaction. Indeed, multiple preparations of recombinant VRK3 proved to be catalytically inert. hVRK3 is one of 50 human kinases that lack key catalytic residues (1) and falls into a category of proteins marked by retention of domains resembling canonical eukaryotic kinases. This category includes the kinase suppressor of Ras (51), kinase suppressor of Ras 2, titin, ILK, PSKH2, CASK, members of the STIK and Trbl families (1), and STRAD (Ste20-related adaptor (52). Inclusion of hVRK3 in this group presents the enticing hypothesis that this protein might function as a decoy or a scaffold within the cell. It is worth noting that the sequence motif within subdomain VIII (T/L/E) that we have suggested as a site for regulatory phosphorylation of the VRK1 and VRK2 proteins is altered to DLE in the VRK3 proteins. The acidic residue in this altered motif might mimic phosphorylation and engender a protein structure in which VRK3, although catalytically inactive, is constitutively primed for substrate binding.

In summary, this report lays the foundation for further functional and structural analysis of a largely unstudied family of protein kinases (20–22, 28, 36, 37). VRK1 is an active protein kinase found within the nucleus, VRK2 is an active protein kinase that associates with intracellular membranes, and VRK3 is a catalytically inert nuclear protein that retains the overall domain structure of a protein kinase and has an intact substrate recognition motif. Given the dominant role of protein kinases in intracellular signaling, it is likely that understanding the role of the VRK proteins will elucidate important facets of cell regulation.

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