CrkRS: a novel conserved Cdc2-related protein kinase that colocalises with SC35 speckles

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SUMMARY

We have isolated and characterised a novel human protein kinase, Cdc2-related kinase with an arginine/serine-rich (RS) domain (CrkRS), that is most closely related to the cyclin-dependent kinase (CDK) family. CrkRS is a 1490 amino acid protein, the largest CDK-related kinase so far isolated. The protein kinase domain of CrkRS is 89% identical to the 46 kDa CHED protein kinase, but outside the kinase domains the two proteins are completely unrelated. CrkRS has extensive proline-rich regions that match the consensus for SH3 and WW domain binding sites, and an RS domain that is predominantly found in splicing factors. CrkRS is ubiquitously expressed in tissues, and maps to a single genetic locus. There are closely related protein kinases in both the *Drosophila* and *Caenorhabditis elegans* genomes. Consistent with the presence of an RS domain, anti-CrkRS antibodies stain nuclei in a speckled pattern, overlapping with spliceosome components and the hyperphosphorylated form of RNA polymerase II. Like RNA polymerase II, CrkRS is a constitutive MPM-2 antigen throughout the cell cycle. Anti-CrkRS immunoprecipitates phosphorylate the C-terminal domain of RNA polymerase II in vitro. Thus CrkRS may be a novel, conserved link between the transcription and splicing machinery.

Key words: Phosphorylation, RS domain, Nuclear speckles, MPM-2, RNA polymerase II

INTRODUCTION

Several protein kinases have been isolated that are most closely related in their kinase domain sequence to cdc2, the major cell cycle kinase in fission yeast. Some of these proteins have been shown to resemble cdc2 further in requiring a cyclin partner for activity, and these proteins are called cyclin-dependent kinases (Cdk). Only some Cdks have roles in regulating the cell cycle. Most of the other Cdks (e.g. CDK7, CDK8 and CDK9) have been implicated in the regulation of transcription as protein kinases that phosphorylate the C-terminal domain (CTD) of RNA polymerase II. However, there are a number of other cdc2-related kinases (Crk) that either do not need to bind a cyclin to be active, or for which no cyclin partner has been identified. (If an activating cyclin partner is subsequently identified, the protein is reclassified as a Cdk.)

The Crk family comprises a diverse set of proteins that vary from 42 to 55% identity to cdc2 in the kinase domain (Meyerson et al., 1992). Individual Crk family members are often referred to by the one letter code of the amino acid sequence in the region corresponding to the 'PSTAIRE' α-helix of cdc2 that interacts with the cyclin.

The PCTAIRE proteins are most closely related to cdc2 (51-55% identity) (Meyerson et al., 1992; Okuda et al., 1992). There are three human PCTAIRE genes, and the proteins are abundant in nerve cells (Gao et al., 1996; Sladeczek et al., 1997). There are at least two PCTAIRE genes in the mouse, of which PCTAIRE-1 is abundant in differentiated spermatids and in adult neurones (Besset et al., 1999). A closely related protein kinase, PFTAIRE, is found in postnatal and adult nerve cells (Lazzaro et al., 1997), and has a *Drosophila* homologue (Sauer et al., 1996). However, the functions of both PCTAIRE and PFTAIRE kinases are unknown.

The PITSLRE kinase subfamily have been implicated in apoptosis (Beyaert et al., 1997; Lahti et al., 1995). There are three tandemly linked human PITSLRE genes (A, B and C) (Lahti et al., 1994) that give rise to eight isoforms (40-130 kDa) through alternative splicing (Xiang et al., 1994), and internal initiation in G_2 phase (Cornelis et al., 2000). The 110 kDa isoform localises to nuclear speckles and binds to the RNPS1 RNA binding protein (Loyer et al., 1998). The PISSLRE kinase is a 39 kDa protein kinase that is most closely related to PITSLRE (50% identity). It is subject to a complicated pattern of splicing, but has not been correlated with apoptosis (Brambilla and Draetta, 1994; Crawford et al., 1999; Grana et al., 1994).

Lastly, CHED (cholinesterase-related cell division controller) is a 418 amino acid serine/threonine kinase with 42% identity to human cdc2 (Lapidot-Lifson et al., 1992). CHED has the sequence PITAIRE in the 'PSTAIRE' α-helix and has been suggested to be involved in haematopoesis. Mouse bone marrow cells treated with antisense oligonucleotides directed against the CHED mRNA showed a reduction in the number of mature, polynuclear megakaryocytes, and an increase in the number of early, mononuclear cells (Lapidot-Lifson et al., 1992). CHED is also the closest relative to a protein kinase identified in mosquitos that have been subjected to stress. This kinase is not present in normal *Aedes aegypti* mosquitos, but is induced after trauma or bacterial innoculation (Chiou et al., 1998).

In this paper we report the cloning and characterisation of a Cdc2-related kinase, CrkRS, which may represent a novel kinase involved in the regulation of transcription and splicing.

MATERIALS AND METHODS

cDNA cloning

A partial CrkRS cDNA was isolated from a HeLa cDNA library in a polymerase chain reaction (PCR) screen for Cdc2-related genes. Sequences were amplified between degenerate oligonucleotides corresponding to the ATP binding site (kinase subdomain I) and kinase subdomain VII. Products from the primary reaction were then reamplified with two internal primers corresponding to the PSTAIRE (kinase subdomain III) region and kinase subdomain VI. A 270 nt PCR fragment was used to screen a HeLa cDNA library (a kind gift of Steve Hanks, Vanderbilt University) and several clones containing partial overlapping fragments of CrkRS were found. Secondary probes were generated and used to screen a human testis λgt10 cDNA library (Clontech) for the full-length clone. Sequencing reactions were carried out by the dideoxynucleotide chain termination method using Sequenase® sequencing mixes (USB).

Northern blotting

Northern blots were performed on multiple tissue mRNA blots (Clontech). Blots were probed using two different CrkRS cDNA [α-^{32}P]dCTP random-prime labelled probes. One probe was from subdomains I-V of the kinase domain of CrkRS and the other from the unique 3′ untranslated region (UTR) of CrkRS.

Southern blotting and genomic mapping

Filters were hybridised in Church buffer with [α-^{32}P]dCTP labelled probes. For the human genomic DNA blot, DNA was extracted from MRC5 diploid lung fibroblasts and 10 μg of DNA was digested with 100 units of *Bam*HI, *Eco*RI, *Hind*III or *Pst*I for a total of 19 hours at 37°C. DNA fragments were transferred to nylon membrane under alkaline conditions. Probes were generated from either the kinase domain (subdomains I to V) or the 3′ UTR. Filters were probed at 42°C for 20 hours, washed and exposed to preflashed film with an intensifying screen for 6-10 days at −70°C. The 3′ UTR probe recognised one band in *Bam*HI-, *Hind*III- and *Pst*I-digested DNAs, and one strong band and one faint band in *Eco*RI-digested DNA. The kinase domain probe recognised one band in the *Bam*HI and *Pst*I digests, and two bands in the other digests. CrkRS was mapped onto the mouse genome by restriction fragment length polymorphism analysis between interspecific crosses of *Mus spretus* and C57BL/6J mice through the Jackson Laboratory DNA mapping resource (Bar Harbor, ME) (Rowe et al., 1994). Filters were hybridised to the 3′ coding region of CrkRS (nucleotides 3662-4260), which is unique to CrkRS. CrkRS co-segregated with the mouse HoxB cluster, and therefore mapped to the distal end of mouse chromosome 11 (56.0 cM) (Mouse Genome Database (MGD), Mouse Genome Informatics, The Jackson Laboratory, Bar Harbor, ME.)

Expression and purification of GST-CrkRS C-terminal fusion protein

A *Hind*III fragment of the CrkRS C-terminal coding region (amino acids 1218-1490) was subcloned into the *Hind*III and *Not*I sites of pGEX-5X-1 (Pharmacia, Sweden) and expressed in *Escherichia coli* C41(DE3). Overnight cultures (grown in LB, 100 μg/ml ampicillin) were diluted 1:10 in prewarmed media plus 100 μg/ml ampicillin and 2% glucose, and grown with shaking at 37°C until mid-log phase (OD_{600nm} ~0.3-0.4). IPTG (final concentration 0.1-0.5 mM) and ethanol (final concentration, 3%) were added and cells were induced for 4 hours at either 30°C or 37°C. Cells were lysed and the fusion protein was purified on glutathione sepharose 4B according to the manufacturer's instructions (Pharmacia, Sweden).

Affinity purification of anti-CrkRS polyclonal antibodies

Rabbit polyclonal antisera were raised against a GST-CrkRS C-terminal fusion protein (GST-CCT). GST and a GST-CCT protein columns were constructed by crosslinking proteins to glutathione agarose beads with dimethyl pimelimidate-HCL (DMP; Sigma). Raw serum from inoculated rabbits was passed through the GST column to remove anti-GST antibodies and passed down a GST-CCT protein column. Anti-CrkRS antibodies were eluted from the column with glycine pH 2.5 and immediately neutralised with Tris pH 7.4.

Immunoblotting

Cell lysates were resolved by SDS-PAGE and transferred to Immobilon-P membrane (Millipore) and incubated with primary antibodies in TBS with 0.2% Tween-20. The working dilutions for the MPM-2 monoclonal antibodies (Upstate Biotechnology), affinity-purified anti-CrkRS antibodies, goat horseradish peroxidase anti-rabbit and anti-mouse secondary antibodies (ICN/Cappel) were 1:1000, 1:3000, 1:5000 and 1:3000, respectively. Immunoreactive proteins were visualised using enhanced chemiluminescence (Amersham).

In vitro coupled transcription/translation

CrkRS cDNA was subcloned into the *Nhe*I and *Eco*RI sites of pCI-neo (Promega) and transcribed and translated in vitro with the TNT® T7 Coupled Transcription/Translation System (Promega).

Cell culture and synchronisation

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 5% newborn calf serum, 5% fetal calf serum plus fungizone, penicillin, streptomycin and glutamine at 37°C in 10% CO_2. Cells were synchronised by sequential thymidine (Sigma) and aphidicolin (Sigma) treatment. Mitotic cells were obtained by using aphidicolin/nocodazole (Aldrich) treatment. The extent of synchronisation was determined by DNA flow cytometry analysis on a FACSort (Becton Dickinson).

Nuclear preparation and immunoprecipitation

HeLa cells were grown to 70-80% confluency, harvested and washed in PBS, and lysed in hypotonic buffer (20 mM Hepes (pH 7.4), 20 mM NaCl, 5 mM β-mercaptoethanol, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, protease inhibitor cocktail (BCL)) with a loose fitting dounce homogeniser. The lysate was centrifuged at 1000 rpm for 7 minutes, and the pellet extracted with nuclear extraction buffer (NEB) (50 mM Tris-HCL (pH7.4), 150 mM NaCl, 50 mM NaF, 2 mM Na_3VO_4, 40 mM β-glycerophosphate, 1 μM Microcystin LR (Calbiochem), 1-10 mM ATP-γ-S (Calbiochem), 5 mM EDTA (or EGTA), 0.1-0.5% sodium deoxycholate, 0.25-1%Tween-20, 5 mM β-mercaptoethanol and protease inhibitor cocktail (BCL, UK)] for 30-60 minutes at 4°C. The nuclear extract was centrifuged at 32,000 ***g*** (20 minutes, 4°C) to separate the insoluble fraction, and the supernatant centrifuged at 285,000 ***g*** (1 hour, 4°C). The soluble nuclear supernatant was pre-cleared with protein G or protein A beads (Pharmacia) for 1 hour. 0.1-0.5% Triton X-100, 8-10 μl of affinity purified anti-CrkRS antibodies and 25 μl of protein G or protein A were used per immunoprecipitation. After immunoprecipitating for a total of 2 hours at 4°C, the lysate was removed and the beads were washed four times with HSNEB (0.3-1 M NaCl+NEB) for 15 minutes. Anti-CrkRS immunocomplexes were resolved on either 7.5% or 12.5% SDS-PAGE and immunoblotted as above. Control rabbit IgG (Jackson ImmunoResearch Labs) and pre-cleared affinity purified anti-CrkRS were used as controls.

In vitro kinase assay

Anti-CrkRS IPs were washed once in kinase buffer (50 mM Tris-HCL (pH 7.4), 100 mM NaCl, 20mM β-glycerophosphate, 10 mM $MgCl_2$, 0.25% Tween-20, 0.1% Triton X-100, 50 μM cold ATP, 1 mM DTT and protease inhibitor cocktail (BCL)) and resuspended in 15 μl of

kinase buffer with 3.0 μCi of [γ-^{33}P]ATP for 30 minutes at 30°C. Samples were resolved on SDS-PAGE and quantified on a phosphoimager (Fuji) using MacBAS V2.5 and Image Reader V1.4E software. Exogenous substrates (0.4-1 μg per reaction) used were ASF (a kind gift of Angus Lamond, Dundee University), casein (Sigma), histone HI (BCL), myelin basic protein (MBP; Upstate Biotechnology) and yeast GST C-terminal domain of RNA polymerase II (GST-CTD; a kind gift of Jeff Corden, Johns Hopkins Medical School).

λ protein phosphatase reactions

200 U of λ protein phosphatase (Biolabs) was used per 50 μl reaction in (50 mM Tris-HCl (pH 7.5), 0.1 mM Na_2EDTA, 5 mM DTT, 0.01% Brij 35, 0.5% Tween-20, 0.1% Triton X-100 and 2 mM $MnCl_2$). To inhibit the phosphatase, 50 mM NaF, 10 mM Na_3VO_4, and 50 mm EDTA was used as recommended by the manufacturer. The reaction mix was incubated for 30 minutes at 30°C and stopped with SDS-sample buffer.

Immunofluorescence microscopy

Cells were fixed and permeabilised with 3% paraformaldehyde/0.5% Triton X-100. Affinity purified anti-CRKRS, MPM-2 and anti-SC35 antibodies were used respectively at 1:400, 1:400 and 1:2. FITC-conjugated goat anti-rabbit IgG, and Texas red-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Labs) were used at 1:200. Images were collected on a Nikon Optiphot microscope (Nikon) equipped with the MRC 1024 lasersharp confocal imaging system (Bio-Rad Laboratories). In the α-amanitin experiment, cells were incubated with 50 μg ml^{-1} of α-amanitin (Sigma) for 8 hours (Huang et al., 1994) prior to fixation.

Microinjection and YFP imaging

Cells were grown to 70-80% confluence on metasilicate-coated coverslips, transferred to a 0.15 mm ΔT dish (Bioptechs) and incubated in CO_2-independent medium without phenol red at 37°C. Cells were microinjected with yellow fluorescent protein (YFP)-tagged DNA constructs (0.1 mg/ml) using a semi-automatic microinjector (Eppendorf) on a Leica DMIRBE inverted microscope (Leica). Cells were fixed for 4-6 hours after microinjection. YFP was visualised by confocal laser scanning microscopy with a broad band FITC-filter set.

Expression and partial purification of CrkRS from insect cells

CrkRS tagged at the N-terminus with $(His)_6$ was subcloned into the transfer vector pVL1393 at the *Xba*I and *Not*I sites. Recombinant baculoviruses were generated using Baculogold™ (Pharmingen). 800 ml of exponential growing Sf9 (Spodoptera frugiperda) cells (cell density: 1-1.5×10^6) were infected with CRKRS recombinant baculovirus at a multiplicity of infection of five. Infected cells were harvested ~40 hours post infection. Unfortunately, most CRKRS was associated with the insoluble fraction.

RESULTS

CrkRS is a novel Cdc2-related protein kinase with an RS domain

We originally identified a 270 nucleotide cDNA product encoding part of the CrkRS kinase domain in a PCR screen undertaken to identify the cyclin A-associated kinase subsequently identified as Cdk2. We used the 270 nt PCR clone to screen a HeLa cell cDNA library and a human testis cDNA library, and isolated a series of overlapping clones that together generated a cDNA of 5550 bp with an open reading frame of 4473 nucleotides (Fig. 1A). The open reading frame encodes a protein of 1490 amino acids (164 kDa) that includes all 11 sub-domains conserved in protein serine/threonine kinases (Hanks et al., 1988). The open reading frame begins at the first ATG and encodes a protein of the correct size as judged by comparing the in vitro translation product with the endogenous protein (see below). The cDNA includes a translation stop codon followed by a 3′ untranslated region with a consensus polyadenylation signal (AATAAA) and a poly A tract. The catalytic domain is in the middle of the protein (residues 719-984) and is most closely related to the cdc2 family of protein kinases (42% identity to human cdc2), and has the sequence PITAIRE in the 'PSTAIRE' helix (Fig. 1A). The predicted ATP binding region of the kinase domain has adjacent threonine and tyrosine residues in analogous positions to threonine 14 and tyrosine 15 residues that are phosphorylated to inactivate cdc2 (Fig. 1A). CrkRS also has a threonine at an analogous position to the activatory threonine in the 'T-loop' of CDKs that is phosphorylated by Cdk-activating kinase (CAK) (reviewed by Lew and Kornbluth, 1996; Morgan, 1995) (Fig. 1A). A partial cDNA for CrkRS (KIAA0904) has been deposited in the database (Nagase et al., 1998).

A prominent feature of CrkRS is an arginine/serine rich (RS) domain (Fig. 1A,C), originally found in pre-mRNA splicing factors that are important in spliceosome assembly and in the regulation of alternative splicing (reviewed by Valcarcel and Green, 1996)). In the first 400 amino acids of CrkRS there are 21 RS motifs, and only one other RS motif in the remaining 1000 amino acids.

After the RS domain there is an acidic patch between residues 74 and 106 (38% glutamic acid or aspartic acid). Overall the protein has a pI of 7.4. Between the charged N-terminus and the catalytic domain are a number of proline/leucine rich repeats. The C-terminus is also rich in proline residues, including a run of 9 consecutive prolines. These N- and C-terminal regions contain the consensus binding sites for 15 class I and 3 class II SH3 binding sites (Alexandropoulos et al., 1995; Lim et al., 1994; Pawson and Schlessinger, 1993; Weng et al., 1995) (Fig. 1C). Of the class I sites there are 14 potential c-Abl SH3 binding sites, and one c-Src SH3 binding site. Furthermore, six of these have the sequence PPLP, which also acts as a ligand for the WW domain (Bedford et al., 1997).

The high proportion of proline and acidic residues in CrkRS indicated that the protein may contain 'PEST' regions that are correlated with protein degradation (Rogers et al., 1986). The PESTFIND program identified seven potential PEST sequences in CrkRS (triangular symbols in Fig. 1C).

CrkRS is conserved through evolution

A BLAST search of the NCBI database revealed that the closest relative to CrkRS is the CHED protein kinase (Lapidot-Lifson et al., 1992). The two proteins are 89% identical over 421 amino acids, including the kinase domain, but are completely unrelated at both the amino acid and nucleotide level outside this sequence (Fig. 1B). A BLAST search of the human genome revealed that the two proteins are encoded by separate genes; CHED is encoded by a gene on chromosome 7, whereas the CrkRS locus is on chromosome 17 (see below).

The next closest relative to CrkRS is the 1157 amino acid

A

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1 CTTTTTTCCCTTCTTCAGGTCAGGGGAAAGGGA

ATGCCCAATTCAGAGAGACATGGGGGCAAGAAGGACGGGAGTGGAGGAGCTTCTGGAACTTTGCAGCCGTCATCGGGAGGCGGCAGCTCTAACAGCAGAGAGCGTCACCGCTTGGTATCG  153
M  P  N  S  E  R  H  G  G  K  K  D  G  S  G  G  A  S  G  T  L  Q  P  S  S  G  G  G  S  S  N  S  R  E  R  H  R  L  V  S   40

AAGCACAAAGCGGCATAAGTCCAAACACTCCAAAGACATGGGGTTGGTGACCCCCGAAGCAGCATCCCTGGGCACAGTTATCAAACCTTTGGTGGAGTATGATGATATCAGCTCTGATTCC  273
K  H  K  R  H  K  S  K  H  S  K  D  M  G  L  V  T  P  E  A  A  S  L  G  T  V  I  K  P  L  V  E  Y  D  D  I  S  S  D  S   80

GACACCTTCTCCGATGACATGGCCTTCAAACTAGACCGAAGGGAGAACGACGAACGTCGTGGATCAGATCGGAGCGACCGCCTGCACAAACATCGTCACCACCAGCACAGGCGTTCCCGG  393
D  T  F  S  D  D  M  A  F  K  L  D  R  R  E  N  D  E  R  R  G  S  D  R S D R L H K H R H H Q H R R S R   120

GACTTACTAAAAGCTAAACAGACCGAAAAAGAAAAAAGCCAAGAAGTCTCCAGCAAGTCGGGATCGATGAAGGACCGGATATCGGGAAGTTCAAAGCGTTCGAATGAGGAGACTGATGAC  513
D  L  L  K  A  K  Q  T  E  K  E  K  S  Q  E  V  S  S  K  S  G  S  M  K  D  R  I  S  G  S  S  K  R  S  N  E  E  T  D  D   160

TATGGGAAGGCGCAGGTAGCCAAAAGCAGCAGCAAGGAATCCAGGTCATCCAAGCTCCACAAGGAGAAGACCAGGAAAGAACGGGAGCTGAAGTCTGGGCACAAAGACCGGAGTAAAAGT  633
Y  G  K  A  Q  V  A  K  S  S  S  K  E  S  R  S  S  K  L  H  K  E  K  T  R  K  E  R  E  L  K  S  G  H  K  D  R  S  K  S   200

CATCGAAAAAGGGAAACACCCAAAAGTTACAAAACAGTGGACAGCCCAAAACGGAGATCCAGGAGCCCCCACAGGAAGTGGTCTGACAGCTCCAAACAAGATGATAGCCCCTCGGGAGCT  753
H  R  K  R  E  T  P  K  S  Y  K  T  V  D  S  P  K  R  R  S  R  S  P  H  R  K  W  S  D  S  S  K  Q  D  D  S  P  S  G  A   240

TCTTATGGCCAAGATTATGACCTTAGTCCCTCACGATCTCATACCTCGAGCAATTATGACTCCTACAAGAAAAGTCCTGGAAGTACCTCGAGAAGGCAGTCGGTCAGTCCCCCTTACAAG  873
S  Y  G  Q  D  Y  D  L  S  P  S  R  S  H  T  S  S  N  Y  D  S  Y  K  K  S  P  G  S  T  S  R  R  Q  S  V  S  P  P  Y  K   280

GAGCCTTCGGCCTACCAGTCCAGCACCCGGTCACCGAGCCCCTACAGTAGGCGACAGAGATCTGTCAGTCCCTATAGCAGGAGACGGTCGTCCAGCTACGAAAGAAGTGGCTCTTACAGC  993
E  P  S  A  Y  Q  S  S  T  R  S  P  S  P  Y  S  R  R  Q  R  S  V  S  P  Y  S  R  R  R  S  S  S  Y  E  R  S  G  S  Y  S   320

GGGCGATCGCCCAGTCCCTATGGTCGAAGGCGGTCCAGCAGCCCTTTCCTGAGCAAGCGGTCTCTGAGTCGGAGTCCACTCCCCAGTAGGAAATCCATGAAGTCCAGAAGTAGAAGTCCT 1113
G  R  S  P  S  P  Y  G  R  R  R  S  S  S  P  F  L  S  K  R  S  L  S  R  S  P  L  P  S  R  K  S  M  K  S  R  S  R  S  P   360

GCATATTCAAGACATTCATCTTCTCATAGTAAAAAGAAGAGATCCAGTTCACGCAGTCGTCATTCCAGTATCTCACCTGTCAGGCTTCCACTTAATTCCAGTCTGGGAGCTGAACTCAGT 1233
A  Y  S  R  H  S  S  S  H  S  K  K  K  R  S  S  S  R  S  R  H  S  S  I  S  P  V  R  L  P  L  N  S  S  L  G  A  E  L  S   400

AGGAAAAAGAAGGAAAGAGCAGCTGCTGCTGCTGCAGCAAAGATGGATGGAAAGGAGTCCAAGGGTTCACCTGTATTTTTGCCTAGAAAAGAGAACAGTTCAGTAGAGGCTAAGGATTCA 1353
R  K  K  K  E  R  A  A  A  A  A  A  A  K  M  D  G  K  E  S  K  G  S  P  V  F  L  P  R  K  E  N  S  S  V  E  A  K  D  S   440

GGTTTGGAGTCTAAAAAGTTACCCAGAAGTGTAAAATTGGAAAAATCTGCCCCAGATCTGAACTGGTGAATGTAACACATCTAAACACAGAGGTAAAAAATTCTTCAGATACAGGGAAA 1473
G  L  E  S  K  K  L  P  R  S  V  K  L  E  K  S  A  P  D  T  E  L  V  N  V  T  H  L  N  T  E  V  K  N  S  S  D  T  G  K   480

GTAAAGTTGGATGAGAACTCCGAGAAGCATCTTGTTAAAGATTTGAAAGCACAGGGAACAAGAGACTCTAAACCCATAGCACTGAAAGAGGAGATTGTTACTCCAAAGGAGACAGAAACA 1593
V  K  L  D  E  N  S  E  K  H  L  V  K  D  L  K  A  Q  G  T  R  D  S  K  P  I  A  L  K  E  E  I  V  T  P  K  E  T  E  T   520

TCAGAAAAGGAGACCCCTCCACCTCTTCCCACAATTGCTTCTCCCCCACCCCCTCTACCAACTACTACCCCTCCACCTCAGACACCCCCTTTGCCTCCAACTACCAGCTCTT 1713
S  E  K  E  T  P  P  P  L  P  T  I  A  S  P  P  P  P  L  P  T  T  P  P  P  Q  T  P  P  L  P  P  L  P  P  I  P  A  L   560

CCACAGCAACCACCTCTGCCTCCTTCTCAGCCAGCATTTAGTCAGGTTCCTGCTTCCAGTACTTCAACTTTGCCCCCTTCTACTCACTCAAAGACATCTGCTGTGTCCTCTCAGGCAAAT 1833
P  Q  Q  P  P  L  P  P  S  Q  P  A  F  S  Q  V  P  A  S  S  T  S  T  L  P  P  S  T  H  S  K  T  S  A  V  S  S  Q  A  N   600

TCTCAGCCCCCTGTACAGGTTTCTGTGAAGACTCAAGTATCTGTAACAGCTGCTATTCCACACCTGAAAACTTCAACGTTGCCTCCTTTGCCCCTCCCACCCTTATTACCTGGAGGTGAT 1953
S  Q  P  P  V  Q  V  S  V  K  T  Q  V  S  V  T  A  A  I  P  H  L  K  T  S  T  L  P  P  L  P  L  P  P  L  L  P  G  G  D   640

GACATGGATAGTCCAAAAGAAACTCTTCCTTCAAAACCTGTGAAGAAAGAGAAGGAACAGAGGACACGTCACTTACTCACAGACCTTCCTCTCCCTCCAGAGCTCCCTGGTGGAGATCTG 2073
D  M  D  S  P  K  E  T  L  P  S  K  P  V  K  K  E  K  E  Q  R  T  R  H  L  L  T  D  L  P  L  P  P  E  L  P  G  G  D  L   680

TCTCCCCCAGACTCTCCAGAACCAAAGGCAATCACACCACCTCAGCAACCATATAAAAAGAGACCAAAAATTTGTTGTCCTCGTTATGGAGAAAGAAGACAAACAGAAAGCGACTGGGGG 2193
S  P  P  D  S  P  E  P  K  A  I  T  P  P  Q  Q  P  Y  K  K  R  P  K  I  C  C  P  R  Y  G  E  R  R  Q  T  E  S  D  W  G   720

AAACGCTGTGTGGACAAGTTTGACATTATTGGGATTATTGGAGAAGGAACCTATGGCCAAGTATATAAAGCCAGGGACAAAGACACAGGAGAACTAGTGGCTCTGAAGAAGGTGAGACTA 2313
K  R  C  V  D  K  F  D  I  I  G  I  I  G  E  G  T  Y  G  Q  V  Y  K  A  R  D  K  D  T  G  E  L  V  A  L  K  K  V  R  L   760

GACAATGAGAAAAGAGGGCTTCCCAATCACAGCCATTCGTGAAATCAAAATCCTTCGTCAGTTAATCCACCGAAGTGTTGTTAACATGAAGGAAATTGTCACAGATAAACAAGATGCACTG 2433
D  N  E  K  E  G  F  P  I  T  A  I  R  E  I  K  I  L  R  Q  L  I  H  R  S  V  V  N  M  K  E  I  V  T  D  K  Q  D  A  L   800

GATTTCAAGAAGGACAAAGGTGCCTTTTACCTTGTATTTGAGTATATGGACCATGACTTAATGGGACTGCTAGAATCTGGTTTGGTGCACTTTTCTGAGGACCATATCAAGTCGTTCATG 2553
D  F  K  K  D  K  G  A  F  Y  L  V  F  E  Y  M  D  H  D  L  M  G  L  L  E  S  G  L  V  H  F  S  E  D  H  I  K  S  F  M   840

AAACAGCTAATGGAAGGATTGGAATACTGTCACAAAAAGAATTTCCTGCATCGGGATATTAAGTGTTCTAACATTTTGCTGAATAACAGTGGGCAAATCAAACTAGCAGATTTTGGACTT 2673
K  Q  L  M  E  G  L  E  Y  C  H  K  K  N  F  L  H  R  D  I  K  C  S  N  I  L  L  N  N  S  G  Q  I  K  L  A  D  F  G  L   880

GCTCGGCTCTATAACTCTGAAGAGAGTCGCCCTTACACAAACAAAGTCATTACTTTGTGGTACCGACCTCCAGAACTACTGCTAGGAGAGGAACGTTACACACCAGCCATAGATGTTTGG 2793
A  R  L  Y  N  S  E  E  S  R  P  Y  T  N  K  V  I  T  L  W  Y  R  P  P  E  L  L  L  G  E  E  R  Y  T  P  A  I  D  V  W   920

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B

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CHED    1 MLPEDKEADS LRGNISVKAV KKEVEKK... ....LRCLLA DLPLPPELPG.....GD.DL SKSPEE...K K.TTTQLH..
CrkRS   635 LLPGGDDMDS PKETLPSKPV KKEKEQR... ....TRHLLT DLPLPPELPG.....GDLSP PDSPEP...K AITPPQQP..
CG7597  693 LPPGMNVLDL AGARSPSPGQ KKESDEKNVT SSGSANKSVL NLPMPPVIPGSEELSGDDDV IDSPEDFDAP AVGTVHGHGG
B0285   211 LPPNSQFMTP PPRPPPAPFS IPPPSVDIHFAATASFSLSSI PPPPPQTDGGASSSKRQDPL PMPPD..... ..........

  CHED    ....SKRRPK ICGPRYGETK EKDID.WGKLC VDKFDIIGII GEGTYGQVYK ARDKDTGEMV ALKKVRLDNE KEGFPITAIR
CrkRS     ....YKKRPK ICCPRYGERR QTESD.WGKRC VDKFDIIGII GEGTYGQVYK ARDKDTGELV ALKKVRLDNE KEGFPITAIR
CG7597  GPGTTRQRPV ILNRR..DSR NNVRD.WGERC VDVFEMIAQI GEGTYGQVYK ARDHHTNDMV ALKKVRLEHE KEGFPITAVR
B0285   SVKRIATRPL ITTRRGHATNRPSDSDSWYKTN LTHYTMLDQI GEGTYGQVYK AVNNLTGEQV ALKRVRLENE KEGFPITAIR

  CHED    EIKILRQLTH QSIINMKEIV TDKEDALDFK KDKGAFYLVF EYMDHDLMGL LESGLVH FYE NHIKSFMRQL MEGLDYCHKK
CrkRS     EIKILRQLIH RSVVNMKEIV TDKQDALDFK KDKGAFYLVF EYMDHDLMGL LESGLVH FSE DHIKSFMKQL MEGLEYCHKK
CG7597  EIKILRQLNH RNIVNLHEIV TDKQDAVEFR KDKGSFYLVF EYMDHDLMGL LESGMVD FNE ENNASIMKQL LDGLNYCHKK
B0285   EIKILRQLHH KNIVRLMDIV IDDISMDELK RTRANFYLVF EYVDHDLIGL LESKELVDFNK DQICSLFKQL LEGLAYIHNT

  CHED    NFLHRDIKCS NILLNNRGQI KLADFGLARL YSSEE.SRPY TNKVITLWYR PPELLLGEER YTPAIDVWSC GCILGELFTK
CrkRS     NFLHRDIKCS NILLNNSGQI KLADFGLARL YNSEE.SRPY TNKVITLWYR PPELLLGEER YTPAIDVWSC GCILGELFTK
CG7597  NFLHRDIKCS NILMNNRGKV KLADFGLARL YNADDRERPY TNKVITLWYR PPELLLGEER YGPSIDVWSC GCILGELFVK
B0285   GFLHRDIKCS NILVNNKGEL KIADLGLARL WEKE.SRLY TNRVITLWYR.PPELLLGDER YGPAIDVWST GCMLGELFTR

  CHED    KPIFQANQEL AQLELISRIC GSPCPAVWPD VIKLPYFNTM KPKKQYRRKL REEFVFIPAA ALDLFDYMLA LDPSKRCTAE
CrkRS     KPIFQANLEL AQLELISRLC GSPCPAVWPD VIKLPYFNTM KPKKQYRRRL REEFSFIPSA ALDLLDHMLT LDPSKRCTAE
CG7597  RPLFQANAEM AQLETISKIC GSPVPAVWPN VIKLPLFHTL KQKKTHRRRL REDFEFMPAP ALDLLDKMLD LDPDKRITAE
B0285   KPLFNGNNEF GQLELISKVC GSPNVDNWPE LTELVGWNTF RMKRTYQRRI REEFEHIMPREAVDLLDKMLT LNPEKRISAK

  CHED    QALQCEFLRD VEPSKCL.HQ ISLYGKIVMS YGVKSEEDRS RWA*                                    418
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CG7597  DALRSPWLRK INPDEMPTPQ LPTWQDCHEL WSKKRRRQMR EQQESLPPTV IASTKYQQHG ATMVGDA*          1157
B0285   EALNHPWIRS LEHTTVQPLK LPQHQDCHEM WSKKQKKSAR LGRQAEGSSG SGHSIRATSH PRGKAGDELE EIKISDFIFQ


CrkRS     KVESGAGD      1100

B0285     FSENKVN*       684
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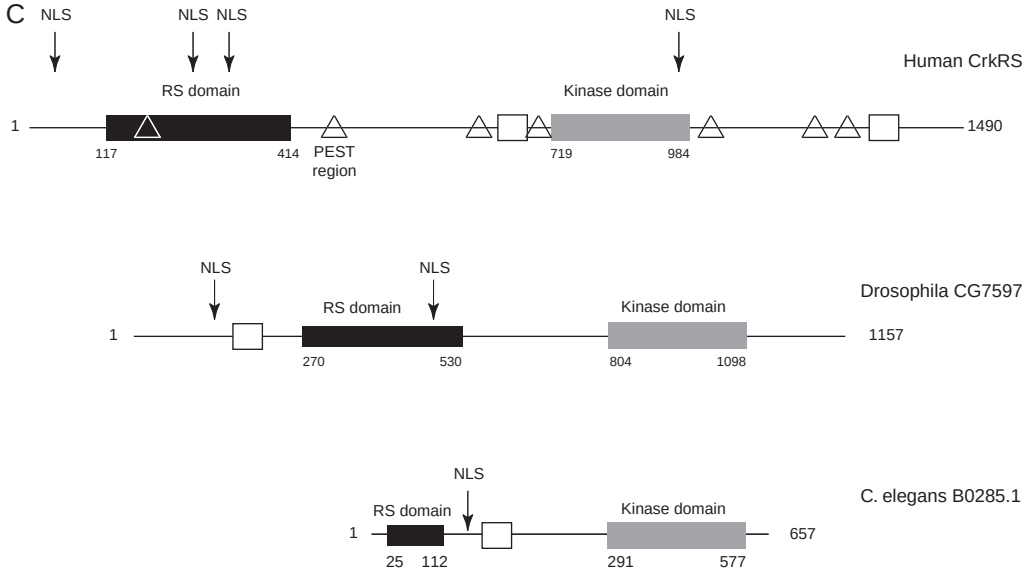


Fig. 1. CrkRS is a novel CDK-related protein kinase. **(**A) Nucleotide and predicted amino acid sequences of the CrkRS cDNA. CrkRS cDNA has an open reading frame of 4473 nucleotides encoding 1490 amino acids. The RS domain is in bold and the kinase domain is underlined. Residues corresponding to the conserved threonine and tyrosine phosphorylation sites of CDK1 are in bold and underlined. A run of nine proline residues is in italics. These sequence data are available from GenBank/EMBL/DDBJ, accession number AF227198. (B) Alignment of the amino acid residues encompassing the kinase domains of CHED, CrkRS, and the gene products of CG7597 (*Drosophila)* and B0285 (*C. elegans*). The CHED, CG7597, B0285.1 and B0285.2 sequences were obtained from GenBank (accession numbers A38197, AAF51738, CAA84302 and Z34533, respectively). Note, the *C. elegans* protein kinase is currently split into two genes in the database. The RS and proline-rich domain on clone B0285.2 was removed from the protein kinase domain on clone B0285.1 by the annotators because it did not resemble any CDKs in the database at that time (R. Durbin, personal communication). (C) Schematic diagrams of CrkRS and the gene products of CG7597 (*Drosophila)* and B0285 (*C. elegans)*. Bipartite nuclear localisation sequences (NLS) are indicated by arrows, RS domains by black rectangles, kinase domains by grey rectangles, PEST regions by triangles and SH3-binding domains by squares.

gene product of the CG7597 gene in *Drosophila*. This gene was sequenced as part of the *Drosophila* genome sequencing project, and maps to 78D7-E1 but there are no recorded alleles at this locus. This protein kinase is very likely to be the homologue of CrkRS because the protein kinase domains are 77% identical (Fig. 1B). In addition, the sequences C-terminal to the protein kinase domain are 48% identical (whereas CHED and CG7597 are only 27% identical in this region). The *Drosophila* protein also resembles CrkRS in that it has an RS domain at the N-terminus followed by a proline-rich region (Fig. 1C). Overall, CrkRS and the *Drosophila* protein are 41% identical.

There is also a 77 kDa protein kinase in the *C. elegans* database, hypothetical protein B0285, that is closely related to both CrkRS and CG7597. B0285 has been annotated as a putative CDK9 homolog, to which its protein kinase domain is 37% identical, but the sequence is much more similar to the CrkRS proteins. B0285 is 53% identical to human CrkRS and 54% identical to *Drosophila* CG7597; the 'PSTAIRE' helices are identical, and the region just C-terminal to the protein kinase domain is highly conserved (Fig. 1B). Moreover, in the 285 amino acids that are N-terminal to the B0285 protein kinase domain, there is an RS domain followed by a segment that is rich in proline residues, including two putative class I SH3 ligands. Thus, the overall structure of B0285 is very like that of human CrkRS and *Drosophila* CG7597 (Fig. 1C).

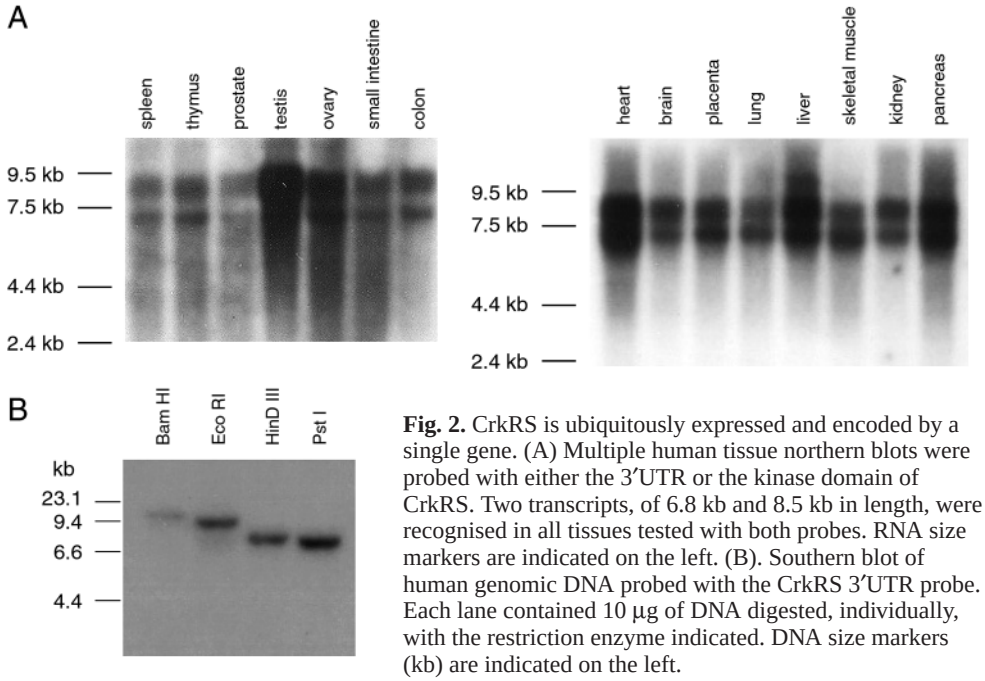


Fig. 2. CrkRS is ubiquitously expressed and encoded by a single gene. (A) Multiple human tissue northern blots were probed with either the 3′UTR or the kinase domain of CrkRS. Two transcripts, of 6.8 kb and 8.5 kb in length, were recognised in all tissues tested with both probes. RNA size markers are indicated on the left. (B). Southern blot of human genomic DNA probed with the CrkRS 3′UTR probe. Each lane contained 10 μg of DNA digested, individually, with the restriction enzyme indicated. DNA size markers (kb) are indicated on the left.

CrkRS is ubiquitously expressed in tissues and maps to a single genetic locuss

To determine the tissue distribution of CrkRS we screened a panel of RNAs from specific human tissues with two different probes. One was to the 3′ untranslated region (UTR) of CrkRS, which should be specific to the CrkRS mRNA. The second was to kinase domains I-V. Two primary transcripts were recognised, ~6.8 and 8.5 kb in length, and these were present in all tissues (Fig. 2A). An additional transcript of ~5.5 kb was detected specifically in the testis. The same pattern of transcripts was recognised by both probes under low or high stringency conditions. These findings show that CrkRS expression is ubiquitous, and suggest that the two primary transcripts arise by alternative splicing. Moreover, it is likely that the CrkRS mRNAs contain long 5′ UTRs that may indicate regulation at the translational level.

To determine whether CrkRS was encoded by a single gene we probed human genomic DNA with either the kinase domain (subdomains I-V) or the 3′ UTR probe. The 3′ UTR probe recognised one band in *Bam*HI-, *Hin*dIII- and *Pst*I-digested DNAs, and one strong band and one faint band in *Eco*RI digested DNA (Fig. 2B). The kinase domain probe recognised one band in the *Bam*HI and *Pst*I digests, and two bands in the other digests, showing that CrkRS is encoded by a single gene. Analysing the draft human genome sequence revealed that CrkRS is encoded by a gene containing seven exons on chromosome 17q21 (contig number NT010838). This agrees well with the position of the mouse CrkRS homologue that we mapped to the distal end of mouse chromosome 11 (56.0 cM, data not shown).

Affinity-purified anti-CrkRS antibodies recognise a 180 kDa nuclear protein that has associated protein kinase activity

To characterise the endogenous CrkRS product in human

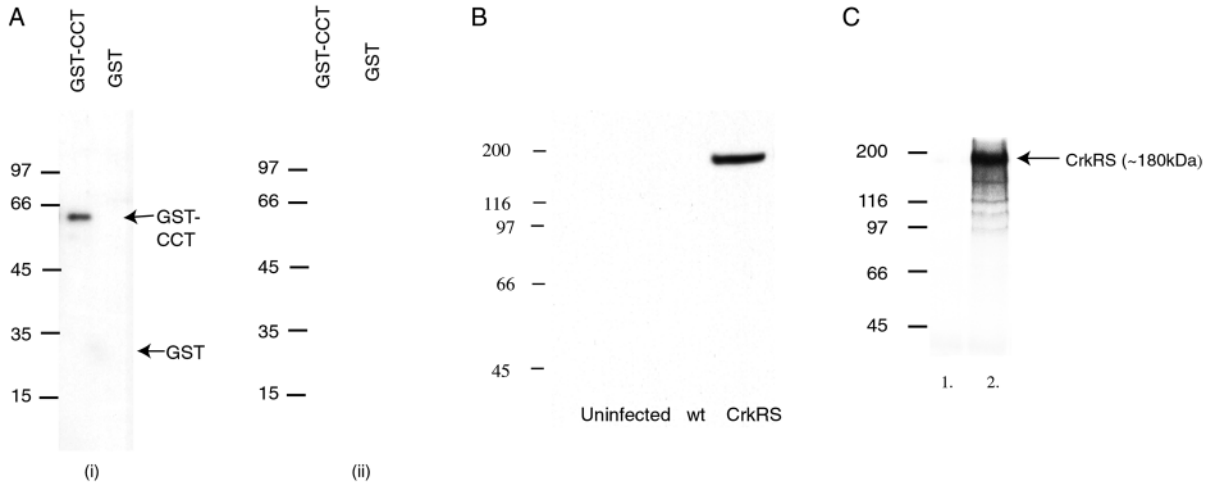


Fig. 3. CrkRS has an apparent M_r of 180 kDa on SDS-PAGE. **(**A) Affinity-purified anti-CrkRS antibodies are specific for CrkRS. Immunoblot of the GST-CrkRS C-terminal fragment (GST-CCT) fusion protein purified from *E. coli* and probed with affinity purified anti-CrkRS antibodies. The antibodies did not recognise the lane containing only GST (i), and pre-clearing the anti-CrkRS antiserum abolished the CrkRS signal (ii). (B) $(His)_6$-tagged human CRKRS expressed in Sf9 cells runs at 180 kDa. Anti-CrkRS immunoblot of lysates from Sf9 cells either uninfected, or infected with wild-type, or CrkRS-expressing baculovirus. (C) In vitro transcription/translation product of the CrkRS cDNA has an apparent M_r of 180 kDa. Full-length CrkRS was transcribed and translated in vitro from an expression vector containing the full length CrkRS cDNA (lane 2). An empty expression vector was used as a control (lane 1).

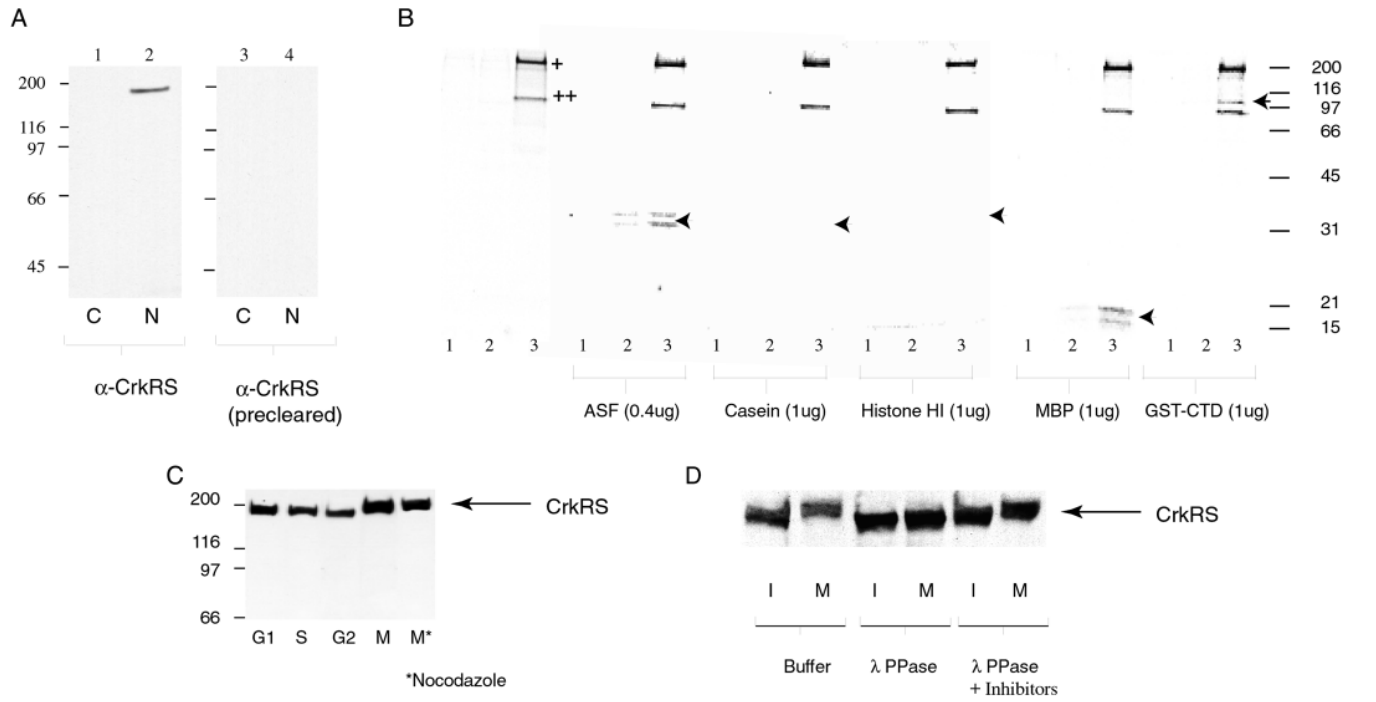


Fig. 4. CrkRS is a nuclear protein that is phosphorylated in a cell-cycle-dependent manner. **(**A) CrkRS is a nuclear protein. Immunoblots of cytosolic and nuclear fractions of HeLa cell lysate probed with anti-CrkRS antibodies (lanes 1,2) or precleared anti-CrkRS antibodies (lanes 3,4). (B) Anti-CrkRS immunoprecipitates have protein kinase activity. CrkRS was immunoprecipitated with anti-CRKRS antibodies and assayed for kinase activity in vitro. CrkRS (+) and an unidentified 85 kDa band (++) were phosphorylated in all anti-CrkRS immunoprecipitates (lanes 3). SR-type splicing factor ASF, casein, histone HI, myelin basic protein (MBP) and the GST-tagged CTD of yeast RNA polymerase II were added as exogenous substrates. Arrowheads indicate the position of the exogenous substrate. Kinase buffer alone (lanes 1)and anti-rabbit IgG immunoprecipitates (lanes 2) were assayed as controls. (C) CrkRS levels do not vary during the cell cycle. HeLa cells were synchronised by a thymidine/aphidicolin regime, released and collected at various times to enrich for different cell cycle phases; G_1 phase (G1), S phase (S), G_2 phase (G2), mitosis (M), and mitotic cells arrested with nocdazole (M*). Fractions were immunoblotted with anti-CrkRS antibodies. Note that CrkRS levels do not vary but CrkRS migrates more slowly on SDS-PAGE at mitosis. (D) CrkRS is a phosphoprotein. Anti-CrkRS immunoprecipitates were treated, or not, with λ protein phosphatase, in the absence or presence of phosphatase inhibitors and immunoblotted with anti-CrkRS antibodies. Note that both interphase (I) and mitotic (M) forms of CrkRS shift downwards after phosphatase treatment.

cells, we raised polyclonal antibodies against a GST-CrkRS fusion protein expressed in *E. coli*, and affinity-purified the antibodies as described in Materials and Methods. We used the unique C-terminal fragment of CrkRS (amino acids 1198-1490) as the antigen. These antibodies recognised GST-CrkRS expressed in bacteria, but not GST alone (Fig. 3A). Pre-incubating the antibodies with GST-CrkRS abolished the signal on the immunoblot (Fig. 3A). The anti-CrkRS antibodies specifically recognised a protein of apparent M_r 180,000 on immunoblots of cell lysates from Sf9 cells infected with a baculovirus expressing CrkRS, but not those infected with a control baculovirus (Fig. 3B). This was the same apparent M_r on SDS-PAGE as the in vitro translation product of the CrkRS cDNA (Fig. 3C). These results demonstrate that the antibodies are specific for CrkRS.

We used the anti-CrkRS antibodies to characterise CrkRS. On immunoblots of fractionated HeLa cell lysates, anti-CrkRS antibodies recognised a single protein band of 180 kDa in the nuclear fraction (Fig. 4A). No proteins were recognised by anti-CrkRS antibodies that had been pre-incubated with bacterially expressed GST-CrkRS (Fig. 4A). Both the CrkRS expressed using the baculovirus system, and the protein recognised by anti-CrkRS antibodies in HeLa cell lysates, had an apparent M_r on SDS-PAGE of 180,000, showing that we had isolated the full-length open reading frame of CrkRS (Fig. 3B; Fig. 4A). This was approximately 30 kDa larger than the M_r predicted by the CrkRS ORF. It was likely that the aberrant migration on SDS-PAGE was caused by the large number of proline rich regions and phosphorylation (see below).

We then assayed whether we were able to detect kinase activity in anti-CrkRS immunoprecipitates. We found that anti-CrkRS immunoprecipitates, but not immunoprecipitates using control rabbit IgG, were able to phosphorylate CrkRS itself and an, as yet unidentified, 85 kDa protein. When exogenous substrates were assayed, anti-CrkRS immunoprecipitates were able to phosphorylate the SR-type splicing factor ASF, myelin basic protein and a GST-fusion protein of the RNA polymerase II CTD (Fig. 4B). Neither casein nor histone HI were phosphorylated to a significant extent (Fig. 4B). Unfortunately, CrkRS expressed in baculovirus-infected cells proved to be highly insoluble and could not be purified to homogeneity, therefore we were unable to measure specifically its associated kinase activity. This also meant that we were unable to exclude the possibility that other kinases present in the anti-CrkRS immunoprecipitates are responsible for some of the phosphorylation activity.

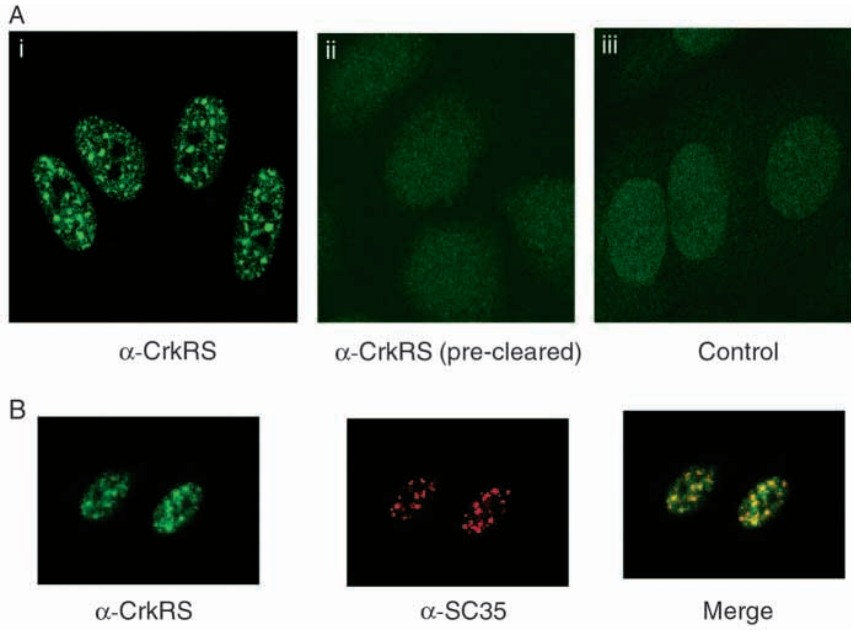


Fig. 5. CrkRS localises to SC35 nuclear speckles. (A) HeLa cells were fixed and immunostained with affinity-purified anti-CrkRS antibodies (i), precleared affinity-purified anti-CrkRS antibodies (ii) or control rabbit IgG (iii) and visualised by confocal laser scanning microscopy. (B) CrkRS mainly colocalises with SC35. To verify that CrkRS localises to nuclear speckles, cells were costained with anti-CrkRS antibodies and antibodies against SC35, a known nuclear speckle protein. The merged image shows the extent of overlap between CrkRS and SC35.

CrkRS is phosphorylated in a cell cycle-dependent manner

Given the number of potential PEST regions in CrkRS, we considered it possible that the levels of CrkRS vary through the cell cycle. Therefore, we immunoblotted whole cell lysates of HeLa cells synchronised at defined cell cycle stages. This analysis revealed that CrkRS was present at approximately constant levels throughout the cell cycle (Fig. 4C), but we noticed that CrkRS in the mitotic samples migrated more slowly on SDS-PAGE, which is often the hallmark of phosphorylation. To show whether this modification was due to phosphorylation, we treated anti-CrkRS immunoprecipitates with λ protein phosphatase in the presence or absence of phosphatase inhibitors. λ protein phosphatase was able to increase the mobility of mitotic CrkRS on SDS-PAGE, but only in the absence of phosphatase inhibitors, providing good evidence that CrkRS is phosphorylated in mitosis (Fig. 4D). Moreover, CrkRS from interphase cells migrated slightly faster on SDS-PAGE after phosphatase treatment, suggesting that Crk is also phosphorylated in interphase (Fig. 4D).

CrkRS is localised to SC35 speckles

To characterize CrkRS further, we performed an immunofluorescence analysis with anti-CrkRS antibodies. We found that anti-CrkRS antibodies exclusively stained the nucleus. Furthermore, CrkRS appeared to be localized in a discrete pattern of nuclear speckles and excluded from nucleoli (Fig. 5A). We observed a similar pattern whether the cells were prepared by fixing with paraformaldehyde or with methanol (data not shown). The staining was abolished when the antibodies were pre-incubated with purified GST-CrkRS, and was not observed with the control rabbit IgG (Fig. 5A).

The pattern of anti-CrkRS staining resembled the ‘nuclear speckles’ that correspond to perichromatin fibrils (PFs) and interchromatin granule clusters (IGCs) observed by electron microscopy when cells are stained with antibodies against components of the splicing machinery (Mintz et al., 1999). Current evidence indicates that IGCs may be storage sites for spliceosome proteins and that sites of active transcription surround the IGCs, suggesting that spliceosomal proteins may be mobilised from the IGCs to active transcription sites (reviewed by Huang et al., 1997). To demonstrate whether CrkRS colocalised with nuclear speckles we co-stained cells with antibodies against CrkRS and with antibodies against a component of the spliceosome, the SC35 protein (Fu and Maniatis, 1992a; Fu and Maniatis, 1992b) (kind gift of A. Lamond, Dundee University). We found that the staining pattern of CrkRS significantly overlapped with that of SC35, suggesting that CrkRS might be associated with nuclear speckles (Fig. 5A). The speckled pattern of CrkRS staining was not altered by treatment with either RNase, or DNase, or both (Fig. 6A), in contrast to the disruption of snRNP staining after RNase treatment as visualised by the Y12 antibody (Fig. 6A). Thus, like SC35, CrkRS was not primarily localised to speckles through its association with either chromatin or RNA. To explore further the possibility that CrkRS was associated with nuclear speckles we treated cells with the RNA polymerase II inhibitor α-amanitin. This was shown to cause a decrease in the number and an increase in the size of nuclear speckles. We found that α-amanitin caused similar changes in the immunofluorescence pattern with both SC35 and anti-CrkRS antibodies (Fig. 6B). CrkRS colocalized with nuclear speckles throughout interphase. In mitosis, CrkRS dispersed throughout the cell and was excluded from condensed chromosomes (Fig. 6C).

CrkRS is a constitutive MPM-2 antigen

The change in the pattern of anti-CrkRS staining in mitotic cells could have been due to the change in its phosphorylation state. A number of proteins phosphorylated in mitosis have been recognised by the MPM-2 monoclonal antibody, a phospho-epitope specific antibody. Several MPM-2 antigens migrated at a similar size to CrkRS, therefore we immunoblotted anti-CrkRS immunoprecipitates with the MPM-2 antibody. This demonstrated that CrkRS was recognised by the MPM-2 antibody in both mitosis and

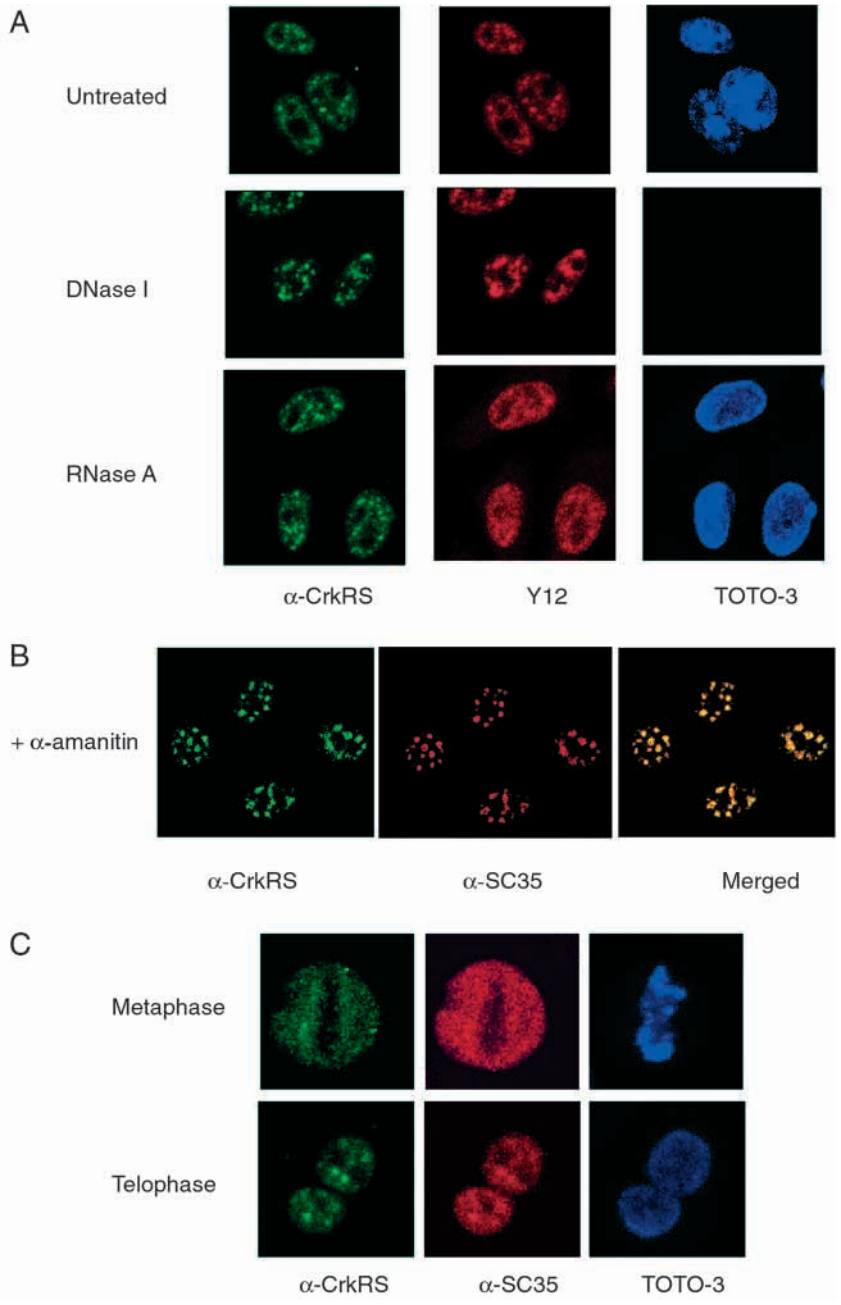


Fig. 6. CrkRS strongly associates with nuclear speckles. **(**A) CrkRS remains associated with nuclear speckles after DNase and RNase treatment. Hela cells were fixed and left untreated (top row) or treated with DNase 1 (middle row) or with RNase A (bottom row). Cells were costained with anti-CrkRS (green), anti-snRNPs (Y12, red) and a DNA marker (TOTO-3, blue). The localisations of CrkRS and snRNPs did not alter significantly in DNase I-treated cells. In RNase A-treated cells, the localisation of CrkRS did not alter significantly but the anti-snRNP staining was less punctate and more uniformly distributed throughout the nuclei. (B) α-amanitin alters the pattern of both CrkRS and SC35. In HeLa cells treated with α-amanitin both anti-CrkRS (left) and SC35 immunofluorescence patterns (middle) alter to one of fewer, larger and rounder speckles and these colocalise (merged image). (C) Localisation of CrkRS during metaphase and telophase. At metaphase (top), anti-CrkRS (left) and anti-SC35 (middle) immunofluorescence patterns become diffusely distributed throughout the cells, and do not stain the chromosomes (right). At telophase (bottom), anti-CrkRS antibodies and anti-SC35 both stain the reforming nuclear speckles.

interphase (Fig. 7A). The recognition of CrkRS by MPM-2 was dependent on phosphorylation because staining was abolished by treating samples with λ phosphatase (Fig. 7A). On immunofluorescence, MPM-2 was shown to stain interphase cells in a speckled pattern and we found that this pattern of staining overlapped with anti-CrkRS staining (Fig. 7B). Thus CrkRS is a novel MPM-2 antigen in both interphase and mitotic cells.

The RS domain of CrkRS plays the major role in targeting CrkRS to the nuclear speckles

To determine which region of CrkRS is required to target CrkRS to nuclear speckles we generated a series of different deletion mutants of CrkRS. These were tagged in-frame at the C-terminus with yellow fluorescent protein (YFP). The full-length CrkRS-YFP protein colocalised with nuclear speckles. N-terminal deletions showed that the first 414 amino acids of CrkRS, which included the RS domain, were required for the protein to localise to nuclear speckles (Fig. 8, compare i with ii-v). All but one of the other deletion mutants were localised uniformly within the nucleoplasm but were excluded from the nucleoli (Fig. 8ii-iv). A mutant that contained only the last 284 amino acids of CrkRS and lacked any consensus bipartite nuclear localisation signals, remained in the cytoplasm (Fig. 8v). To refine this analysis we generated a fusion protein between YFP and the first 414 amino acids of CrkRS. This fusion protein was predominantly localised to nuclear speckles (Fig. 8vi). However, all further deletions did not localise properly to nuclear speckles (Fig. 8B, compare vi with vii-ix). Furthermore, these deletion mutants accumulated in the nucleoli. Thus the first 414 amino acids, including the RS domain, were absolutely required for the proper targeting of CrkRS to nuclear speckles.

DISCUSSION

CrkRS is a novel protein kinase that has been conserved through evolution

In this paper we have reported the cloning and characterisation of CrkRS, a novel cdc2-related protein kinase of 164 kDa. This protein is encoded by a single gene that maps to human chromosome 17q21. The mRNAs encoding CrkRS are expressed in all tissues, as two primary transcripts of 8.5 kb and 6.8 kb. There is also a transcript of ~5 kb in the testis. Because the mRNA is present in all tissues, including non-proliferating tissue such as the heart and brain, we think it

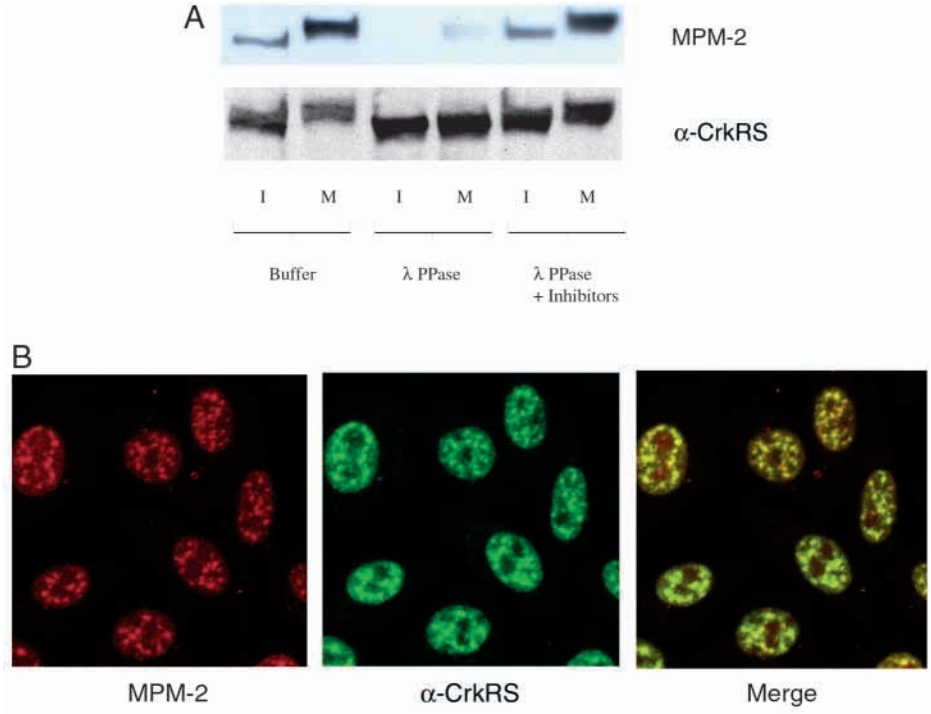


Fig. 7. CrkRS is an MPM-2 antigen. **(**A) Immunoblot. Anti-CrkRS immunoprecipitates from interphase and mitotic HeLa cell fractions were incubated in buffer alone, with λ protein phosphatase, or with λ protein phosphatase plus phosphatase inhibitors, and immunoblotted with the MPM-2 mAb (top) or with anti-CrkRS antibodies (bottom). CrkRS was recognised by MPM-2 mAbs in a phosphorylation-dependent manner. (B) CrkRS and MPM-2 colocalise during interphase. HeLa cells were fixed and stained with MPM-2 mAb (left) and anti-CrkRS antibodies (middle). The patterns overlap in the nuclear speckles (Merge).

unlikely that CrkRS plays a role in regulating progression through the cell cycle per se. It is more likely to be involved in regulating transcription by phosphorylating RNA polymerase II, given that this is the role of most of the other non-cell-cycle-related CDKs. If this is the case then the RS domain at the N-terminus of CrkRS would provide a unique link between this class of kinases and the splicing machinery.

The presence of *Drosophila* and *C. elegans* homologues with very similar protein kinase domains, and with N-terminal RS domains separated from the protein kinase domain by a proline rich region, indicates that this protein kinase has been conserved through animal evolution. (There does not appear to be a homologue in *Saccharomyces cerevisiae*.) CrkRS and the *Drosophila* CG7597 protein are 77% identical in the protein kinase domain and 41% identical overall. The *C. elegans* protein B0285 is 53% identical in the protein kinase domain and 45% identical overall to CrkRS. In the proline-rich region between the RS and the protein kinase domains, B0285 has two class I SH3 ligands (PxxxPxxP) that are also the most abundant type of SH3 ligand in human CrkRS.

The nearest neighbour to CrkRS in humans is the 46 kDa CHED protein kinase. The kinase domains of these two proteins are very similar (89% identical) but they are encoded by two separate genes. CHED was implicated as a protein kinase involved in haematopoesis by antisense experiments on mouse bone marrow cells. After antisense treatment there was a shift in the proportion of cells from mature, polynuclear megakaryocytes to early, mononuclear cells (Lapidot-Lifson et al., 1992). The antisense oligonucleotide used in these experiments, 5′-ATTGACTGGGGAAAA has 3 mismatches to the analogous sequence in Crk 7, 5′-AGCGACTGGGGGAAA. Therefore, it is unlikely that CrkRS would also have been eliminated in these experiments, although this remains a formal possibility.

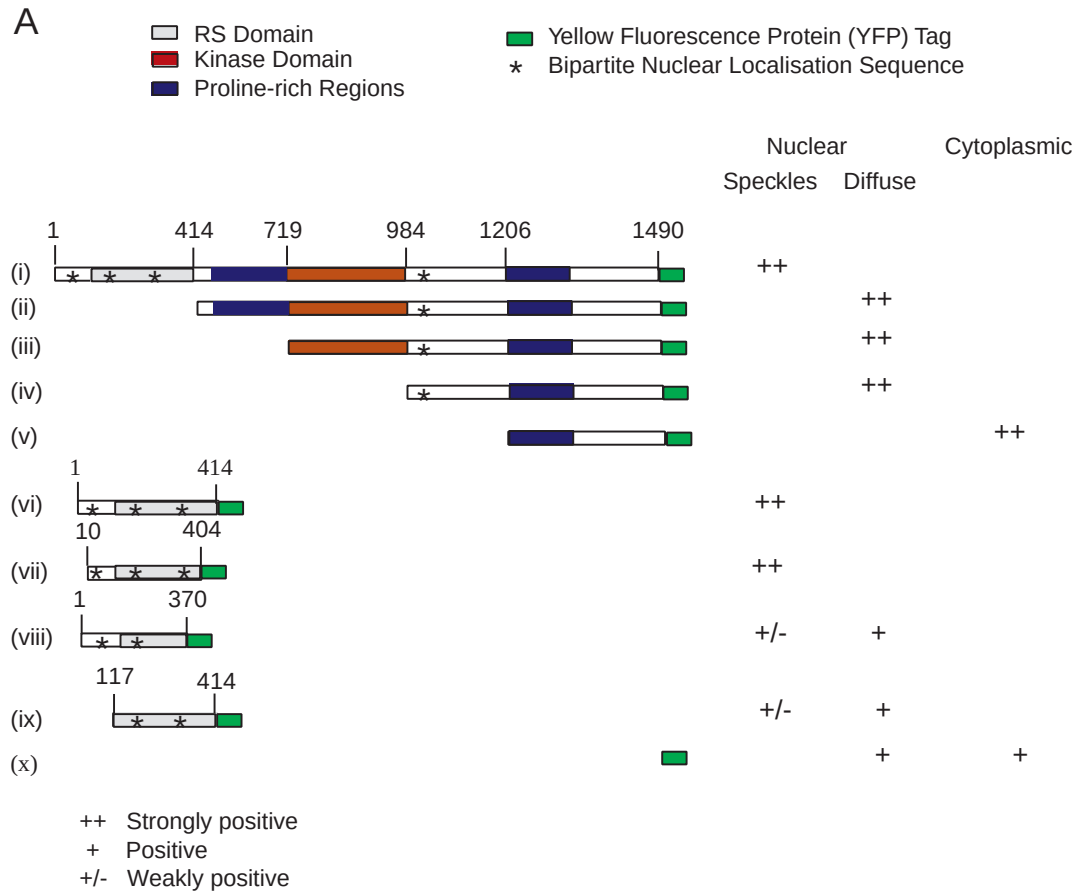
The ATP binding site of CrkRS contains an adjacent threonine and tyrosine in analagous positions to those used to regulate cdc2 and CDK2. Therefore, CrkRS could be regulated by post-translational modification by dual-specificity threonine/tyrosine kinases and phosphatases in an analagous fashion to cdc2. However, CrkRS-associated kinase activity does not appear to vary through the cell cycle when assayed using myelin basic protein and GST-CTD (data not shown).

At present we do not know whether CrkRS needs to bind to a cyclin partner to be activated (if it does, this would make it a cyclin-dependent kinase), or whether, like other members of the Crk family such as PITSLRE, it does not need an activatory cyclin. Thus far we have not found an association between CrkRS and a number of candidate cyclins including cyclins K, T and M (data not shown). However, CrkRS might interact with an as yet uncharacterised cyclin, such as cyclins L, O or P identified in the draft human genome sequence (Murray and Marks, 2001). The tightly associated ~85 kDa protein that coimmunoprecipitates with CrkRS is an attractive candidate for an activatory partner.

The CrkRS sequence provides an almost embarrassing number of candidate protein-protein interaction domains. There are three class II SH3 binding sites, corresponding to the consensus PxxPxR/K (Alexandropoulos et al., 1995), which are potential interaction sites for proteins such as Crk. There are 14 potential ligands for the c-Abl SH3 domain corresponding to the consensus PxxxxPxxP, and one to the c-Src SH3 domain, RxxPxxP (Alexandropoulos et al., 1995). Because CrkRS is a nuclear protein, an interaction with the nuclear c-Abl kinase (Van Etten et al., 1989) is more likely than one with c-Src, which is cytoplasmic. The conservation of proline-rich regions in the CrkRS proteins in all species suggests that this feature of the protein kinase is important for its function.

CrkRS is a novel nuclear speckle kinase

Subcellular fractionation and immunofluorescence studies show that CrkRS is a nuclear protein. The sequence of CrkRS has four regions that match the consensus for a bipartite nuclear localisation signal (NLS) (Dingwall and Laskey, 1991; Makkerh et al., 1996); three are in the N-terminus and the



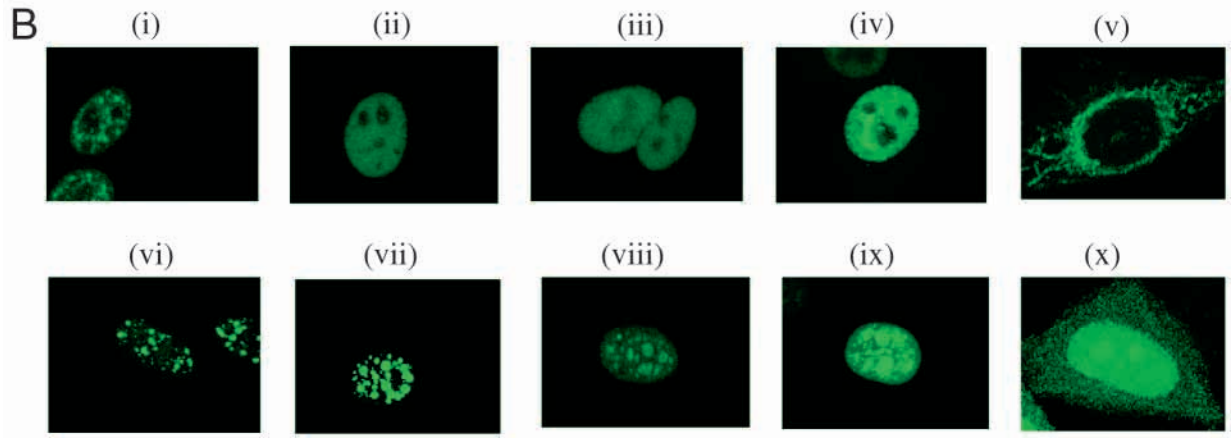


Fig. 8. The RS domain of CrkRS is required for its localisation to nuclear speckles. **(**A) Schematic diagram of the CrkRS deletion constructs (i-x). The YFP tag (green), kinase domain (red) proline-rich regions (blue), RS domain (grey) and the bipartite nuclear localisation sequences (stars) are shown. Constructs (i-x) were microinjected into HeLa cells and the subcellular localisation of each fusion protein assayed by confocal laser scanning microscopy. (B) Representative fluorescence images. Each image is representative of 60 microinjected cells 4-6 hours after microinjection. Constructs were assayed twice in two separate experiments.

fourth at the C-terminus. By deletion analysis we have found that the first three NLS motifs are sufficient to localise CrkRS to the nucleus. More specifically, a large proportion of CrkRS appears to be localised to discrete structures in the nucleus that appear as speckles, and this localization depends on the RS domain in CrkRS. A proportion of these structures stain with antibodies against components of the splicing machinery and against RNA polymerase II (reviewed by Spector, 1993). CrkRS is very strongly associated with the nuclear matrix. In subcellular fractionation studies the bulk of CrkRS remains in the insoluble fraction of the nucleus, even after nuclease and detergent treatment (data not shown). Thus CrkRS might act

as both an enzyme in modulating the activity of RNA polymerase II and/or the splicing machinery, and as a scaffold between these components and the nuclear matrix.

A phosphorylated form of the major subunit of RNA polymerase II is recognised in interphase by the MPM-2 monoclonal antibody (Albert et al., 1999). MPM-2 primarily recognises mitotic phosphoproteins (Davis et al., 1983) but in interphase it stains nuclei in a speckled pattern. This pattern overlaps with that of CrkRS, and CrkRS from both interphase and mitotic cells is recognised on immunoblots by MPM-2. These results, allied with our finding that anti-CrkRS immunoprecipitates can phosphorylate the CTD of RNA polymerase II in vitro, raises the possibility that CrkRS regulates RNA polymerase II activity. However, CrkRS does not co-purify with the RNA polymerase II holoenzyme (J. Parvin, personal communication), indicating that it is probably not part of the core transcriptional apparaus. In addition, we are unable to exclude the possibility that a co-immunoprecipitating kinase, and not CrkRS itself, is responsible for phosphorylating RNA polymerase II CTD, although the balance of probability remains that CrkRS does phosphorylate the CTD because this is the physiological substrate of a number of the other Cdc2-family members in the cell. Thus, CrkRS may be involved in, for example, transcription or alternative splicing in response to cues such as differentiation. CrkRS co-immunprecipitates with p150, an as yet unidentified protein recognised by the CC-3 monoclonal antibody raised against phosphoepitopes of the RNA polymerase CTD (Albert et al., 1999) (data not shown).

The splicing machinery has been shown to be regulated by phosphorylation. For example, the phosphorylation of RS proteins alters their sequence specific binding to RNA, and, therefore, can regulate alternative splicing (Fetzer et al., 1997; Tacke et al., 1997). The SRPK and Clk/Sty protein kinase families regulate the localisation of RS proteins. The SRPK1 protein kinase is specific for RS proteins (Gui et al., 1994b) and can cause the disassembly of nuclear speckles (Gui et al., 1994a). Furthermore, high levels of SRPK1 inhibit an in vitro splicing reaction (Gui et al., 1994a). The Clk/Sty protein kinase has a less extensive RS domain than the one in CrkRS, but this domain is important for interactions between Clk/Sty and splicing factors (Colwill et al., 1996). When Clk/Sty is overexpressed, it causes a redistribution of splicing factors from nuclear speckles to the nucleoplasm (Colwill et al., 1996). Overexpressing CrkRS to a high level also causes RS proteins and RNA polymerase II to become more evenly distributed through the nucleoplasm (data not shown), indicating an increase in transcriptional activity. However, it is an exciting possibility that CrkRS differs from Clk/Sty and SPRK in directly phosphorylating the RNA polymerase II CTD. Thus, CrkRS could represent a novel, evolutionarily conserved RNA polymerase II CTD kinase that might directly link transcription with the splicing machinery.

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REFERENCES

Albert, A., Lavoie, S. and Vincent, M. (1999). A hyperphosphorylated form of RNA polymerase II is the major interphase antigen of the phosphoprotein antibody MPM-2 and interacts with the peptidyl-prolyl isomerase Pin1. *J. Cell Sci.* **112**, 2493-2500.

Alexandropoulos, K., Cheng, G. and Baltimore, D. (1995). Proline-rich sequences that bind to Src homology 3 domains with individual specificities. *Proc. Natl. Acad. Sci. USA* **92**, 3110-3114.

Bedford, M. T., Chan, D. C. and Leder, P. (1997). FBP WW domains and the Abl SH3 domain bind to a specific class of proline-rich ligands. *EMBO J.* **16**, 2376-2383.

Besset, V., Rhee, K. and Wolgemuth, D. J. (1999). The cellular distribution and kinase activity of the Cdk family member Pctaire1 in the adult mouse brain and testis suggest functions in differentiation. *Cell Growth Differ.* **10**, 173-181.

Beyaert, R., Kidd, V. J., Cornelis, S., Van de Craen, M., Denecker, G., Lahti, J. M., Gururajan, R., Vandenabeele, P. and Fiers, W. (1997). Cleavage of PITSLRE kinases by ICE/CASP-1 and CPP32/CASP-3 during apoptosis induced by tumor necrosis factor. *J. Biol. Chem.* **272**, 11694-11697.

Brambilla, R. and Draetta, G. (1994). Molecular cloning of PISSLRE, a novel putative member of the cdk family of protein serine/threonine kinases. *Oncogene* **9**, 3037-3041.

Chiou, J. Y., Huang, S. J., Huang, S. T. and Cho, W. L. (1998). Identification of immune-related protein kinases from mosquitoes (Aedes aegypti). *J. Biomed. Sci.* **5**, 120-126.

Colwill, K., Pawson, T., Andrews, B., Prasad, J., Manley, J. L., Bell, J. C. and Duncan, P. I. (1996). The Clk/Sty protein kinase phosphorylates SR splicing factors and regulates their intranuclear distribution. *EMBO J.* **15**, 265-275.

Cornelis, S., Bruynooghe, Y., Denecker, G., Van Huffel, S., Tinton, S. and Beyaert, R. (2000). Identification and characterization of a novel cell cycle-regulated internal ribosome entry site. *Mol. Cell* **5**, 597-605.

Crawford, J., Ianzano, L., Savino, M., Whitmore, S., Cleton-Jansen, A. M., Settasatian, C., d'apolito, M., Seshadri, R., Pronk, J. C., Auerbach, A. D. et al. (1999). The PISSLRE gene: structure, exon skipping, and exclusion as tumor suppressor in breast cancer. *Genomics* **56**, 90-97.

Davis, F., Tsao, T. Y., Fowler, S. K. and Rao, P. N. (1983). Monoclonal antibodies to mitotic cells. *Proc. Natl. Acad. Sci. USA* **80**, 2926-2930.

Dingwall, C. and Laskey, R. A. (1991). Nuclear targeting sequences – a consensus? *Trends Biochem. Sci.* **16**, 478-481.

Fetzer, S., Lauber, J., Will, C. L. and Luhrmann, R. (1997). The [U4/U6.U5] tri-snRNP-specific 27K protein is a novel SR protein that can be phosphorylated by the snRNP-associated protein kinase. *RNA* **3**, 344-355.

Fu, X. D. and Maniatis, T. (1992a). The 35-kDa mammalian splicing factor SC35 mediates specific interactions between U1 and U2 small nuclear ribonucleoprotein particles at the 3′ splice site. *Proc. Natl. Acad. Sci. USA* **89**, 1725-1729.

Fu, X. D. and Maniatis, T. (1992b). Isolation of a complementary DNA that encodes the mammalian splicing factor SC35. *Science* **256**, 535-538.

Gao, C. Y., Chauthaiwale, V. M., Rampalli, A. M. and Zelenka, P. S. (1996). Expression of alternatively spliced PCTAIRE-1 mRNA in PC12 cells and neonatal rat brain. *Gene* **176**, 243-247.

Grana, X., Claudio, P. P., De, L. A., Sang, N. and Giordano, A. (1994). PISSLRE, a human novel CDC2-related protein kinase. *Oncogene* **9**, 2097-2103.

Gui, J. F., Lane, W. S. and Fu, X. D. (1994a). A serine kinase regulates intracellular localization of splicing factors in the cell cycle. *Nature* **369**, 678-682.

Gui, J. F., Tronchere, H., Chandler, S. D. and Fu, X. D. (1994b). Purification and characterization of a kinase specific for the serine- and arginine-rich pre-mRNA splicing factors. *Proc. Natl. Acad. Sci. USA* **91**, 10824-10828.

Hanks, S., Quinn, A. and Hunter, T. (1988). The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* **241**, 42-52.

Huang, S., Deerinck, T. J., Ellisman, M. H. and Spector, D. L. (1997). The dynamic organization of the perinucleolar compartment in the cell nucleus. *J. Cell. Biol.* **137**, 965-974.

Lahti, J. M., Valentine, M., Xiang, J., Jones, B., Amann, J., Grenet, J., Richmond, G., Look, A. T. and Kidd, V. J. (1994). Alterations in the PITSLRE protein kinase gene complex on chromosome 1p36 in childhood neuroblastoma. *Nat. Genet.* **7**, 370-375.

Lahti, J. M., Xiang, J., Heath, L. S., Campana, D. and Kidd, V. J. (1995). PITSLRE protein kinase activity is associated with apoptosis. *Mol. Cell. Biol.* **15**, 1-11.

Lapidot-Lifson, Y., Patinkin, D., Prody, C. A., Ehrlich, G., Seidman, S., Ben, A. R., Benseler, F., Eckstein, F., Zakut, H. and Soreq, H. (1992). Cloning and antisense oligodeoxynucleotide inhibition of a human homolog of cdc2 required in hematopoiesis. *Proc. Natl. Acad. Sci. USA* **89**, 579-583.

Lazzaro, M. A., Albert, P. R. and Julien, J. P. (1997). A novel cdc2-related protein kinase expressed in the nervous system. *J. Neurochem.* **69**, 348-364.

Lew, D. J. and Kornbluth, S. (1996). Regulatory roles of cyclin dependent kinase phosphorylation in cell cycle control. *Curr. Opin. Cell Biol.* **8**, 795-804.

Lim, W. A., Richards, F. M. and Fox, R. O. (1994). Structural determinants of peptide-binding orientation and of sequence specificity in SH3 domains. *Nature* **372**, 375-379.

Loyer, P., Trembley, J. H., Lahti, J. M. and Kidd, V. J. (1998). The RNP protein, RNPS1, associates with specific isoforms of the p34cdc2-related PITSLRE protein kinase in vivo. *J. Cell Sci.* **111**, 1495-1506.

Makkerh, J. P. S., Dingwall, C. and Laskey, R. A. (1996). Comparative mutagenesis of nuclear localization signals reveals the importance of neutral and acidic amino acids. *Curr. Biol.* **6**, 1025-1027.

Meyerson, M., Enders, G. H., Wu, C. L., Su, L. K., Gorka, C., Nelson, C., Harlow, E. and Tsai, L. H. (1992). A family of human cdc2-related protein kinases. *EMBO J.* **11**, 2909-2917.

Mintz, P. J., Patterson, S. D., Neuwald, A. F., Spahr, C. S. and Spector, D. L. (1999). Purification and biochemical characterization of interchromatin granule clusters. *EMBO J.* **18**, 4308-4320.

Morgan, D. O. (1995). Principles of CDK regulation. *Nature* **374**, 131-134.

Murray, A. W. and Marks, D. (2001). Can sequencing shed light on cell cycling? *Nature* **409**, 844-846.

Nagase, T., Ishikawa, K., Suyama, M., Kikuno, R., Hirosawa, M., Miyajima, N., Tanaka, A., Kotani, H., Nomura, N. and Ohara, O. (1998). Prediction of the coding sequences of unidentified human genes. XII. The complete sequences of 100 new cDNA clones from brain which code for large proteins in vitro. *DNA Res* **5**, 355-364.

Okuda, T., Cleveland, J. L. and Downing, J. R. (1992). PCTAIRE-1 and PCTAIRE-3, two members of a novel cdc2/CDC28-related protein kinase gene family. *Oncogene* **7**, 2249-2258.

Pawson, T. and Schlessinger, J. (1993). SH2 and SH3 domains. *Curr. Biol.* **3**, 434-442.

Rogers, S., Wells, R. and Rechsteiner, M. (1986). Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* **234**, 364-368.

Rowe, L. B., Nadeau, J. H., Turner, R., Frankel, W. N., Letts, V. A., Eppig, J. T., Ko, M. S., Thurston, S. J. and Birkenmeier, E. H. (1994). Maps from two interspecific backcross DNA panels available as a community genetic mapping resource. *Mamm. Genome* **5**, 253-274.

Sauer, K., Weigmann, K., Sigrist, S. and Lehner, C. F. (1996). Novel members of the cdc2-related kinase family in Drosophila: cdk4/6, cdk5, PFTAIRE, and PITSLRE kinase. *Mol. Biol. Cell* **7**, 1759-1769.

Sladeczek, F., Camonis, J. H., Burnol, A. F. and Le Bouffant, F. (1997). The Cdk-like protein PCTAIRE-1 from mouse brain associates with p11 and 14-3-3 proteins. *Mol. Gen. Genet.* **254**, 571-577.

Spector, D. L. (1993). Nuclear organization of pre-mRNA processing. *Curr. Opin. Cell Biol.* **5**, 442-447.

Tacke, R., Chen, Y. and Manley, J. L. (1997). Sequence-specific RNA binding by an SR protein requires RS domain phosphorylation: creation of an SRp40-specific splicing enhancer. *Proc. Natl. Acad. Sci. USA* **94**, 1148-1153.

Valcarcel, J. and Green, M. R. (1996). The SR protein family: pleiotropic functions in pre-mRNA splicing. *Trends Biochem. Sci.* **21**, 296-301.

Van Etten, R. A., Jackson, P. and Baltimore, D. (1989). The mouse type IV c-abl gene product is a nuclear protein, and activation of transforming ability is associated with cytoplasmic localization. *Cell* **58**, 669-678.

Weng, Z., Rickles, R. J., Feng, S., Richard, S., Shaw, A. S., Schreiber, S. L. and Brugge, J. S. (1995). Structure-function analysis of SH3 domains: SH3 binding specificity altered by single amino acid substitutions. *Mol. Cell. Biol.* **15**, 5627-5634.

Xiang, J., Lahti, J. M., Grenet, J., Easton, J. and Kidd, V. J. (1994). Molecular cloning and expression of alternatively spliced PITSLRE protein kinase isoforms. *J. Biol. Chem.* **269**, 15786-15794.