The RNP protein, RNPS1, associates with specific isoforms of the p34\(^{cdc2}\)-related PITSLRE protein kinase in vivo

Pascal Loyer*,‡, Janeen H. Trembley*, Jill M. Lahti and Vincent J. Kidd§

Department of Tumor Cell Biology, St Jude Children's Research Hospital, Memphis, TN 38105, USA
*These two authors contributed equally to this work
‡Present address: INSERM U 49, Hopital Pontchaillou, 35033 Rennes cedex, France
§Author for correspondence
Accepted 25 March; published on WWW 14 May 1998

SUMMARY

The PITSLRE protein kinases are members of the p34\(^{cdc2}\) superfamily, with >20 different isoforms expressed from two linked genes in humans. PITSLRE homologues have been identified in mouse, chicken, Drosophila, Xenopus, and possibly Plasmodium falciparum, suggesting that their function may be well conserved. A possible role for a caspase processed PITSLRE isoform has been suggested by studies of Fas- and TNF-induced cell death. However, the function of these kinases in proliferating cells is still unknown. Here we demonstrate that the 110 kDa PITSLRE isoforms (p110) are localized to both the nucleoplasm and nuclear speckles, and that these isoforms specifically interact in vitro and in vivo with the RNA-binding protein RNPS1. RNPS1 is also localized to nuclear speckles, and its over expression disrupts normal nuclear speckle organization by causing the aggregation of many nuclear speckles into ~6 ‘mega’ speckles. This type of nuclear speckle aggregation closely resembles what occurs when cells are treated with several transcriptional inhibitors. These data indicate that the PITSLRE p110 isoforms interact with RNPS1 in vivo, and that these proteins may in turn influence some aspect of transcriptional and/or splicing regulation.

Key words: PITSLRE, Cell cycle, Nuclear speckle, RNA-binding, RNPS1, Transcription

INTRODUCTION

A large sub-family of p34\(^{cdc2}\)-related protein kinases, the PITSLRE kinases, exists in human, mouse, chicken, and Drosophila melanogaster (Bunnell et al., 1990; Xiang et al., 1994; Li et al., 1995; Sauer et al., 1996). Ectopic expression of one PITSLRE isoform, PITSLRE p58, results in a late telophase delay and markedly reduced cell growth (Eipers et al., 1991). Further analyses of the CHO cell lines ectopically expressing the p58, or a truncated PITSLRE p50 construct that mimics caspase cleavage of PITSLRE isoforms during TNF- and Fas-mediated apoptosis, demonstrated that decreased cell growth in these cultures was primarily due to sustained apoptosis (Lahti et al., 1995). In addition, it was shown that PITSLRE kinase mRNAs, steady-state protein levels, and enzyme activity increase dramatically in T-cells activated to undergo apoptosis by treatment with a Fas antibody. The larger PITSLRE isoforms (several p110 isoforms and a p65 isoform) are cleaved by caspase protease(s) during tumor necrosis factor (TNF)-and Fas-mediated apoptosis to generate a smaller ~46-50 kDa PITSLRE protein species that phosphorylates histone H1 (Beyaert et al., 1997; Tang et al., 1998). At least two different caspase activities, caspase 3 and caspase 8, are involved in the processing of the PITSLRE p110 isoforms during apoptosis induced by Fas receptor oligomerization. Bcl-2, crmA, and the peptide inhibitor zVAD block the caspase protease processing of the PITSLRE kinases during TNF- and Fas-mediated cell death. Thus, one or more of the PITSLRE kinase isoforms may be involved in apoptotic signal transduction.

While we have established a potential role(s) for the smaller caspase-processed PITSLRE isoform in apoptotic signaling and oncogenesis (Lahti et al., 1994, 1995), the function of the larger 110 kDa PITSLRE isoform(s) is unknown. These larger PITSLRE isoforms are expressed ubiquitously in all growing cells from at least two distinct genes (Xiang et al., 1994; Lahti et al., 1994). In addition, they contain unique amino-terminal sequence motifs, including the so-called RD (single letter amino acid codons for Arg and Asp) and RE (single letter amino acid codons for Arg and Glu) motifs. These regions are crucial for the sub-nuclear localization of certain splicing components (Fu, 1995; Neugebauer et al., 1995; Staknis and Reed, 1995; Hedley et al., 1995). Substantial evidence supports the association of splicing components to these sub-nuclear ‘speckles,’ as well as their relationship with the nuclear matrix (Spector, 1993; Spector et al., 1991; Xing et al., 1993, 1995; Carter et al., 1993; Wang and Manley, 1997). However, the exact relationship between these nuclear speckles, transcription, and splicing is still unclear. It is possible that these nuclear speckles represent important sub-nuclear compartments, but it is more likely that they function as storage depots for a number of proteins.
involved in regulating transcription and splicing (Spector et al., 1993; Spector, 1993; Misteli and Spector, 1997).

Here we demonstrate that a portion of the PITSLRE p110 protein kinase co-localizes with the splicing component SC-35, which is tightly associated with the nuclear matrix. A second, more soluble pool of nuclear PITSLRE p110 was also detected in the same cells. Using one of the PITSLRE p110 isoforms as bait, three distinct RNA or DNA binding proteins, including the RNP protein hRNPS1 and the autoimmune antigen SS-A/Ro, were isolated from a human B-cell expression library in a yeast two-hybrid screen. Murine RNPS1 was originally isolated by differential screening of a S-phase enriched Ehrlich ascites tumor cDNA library, and it contains the RNPI/2 recognition motifs and several Arg-Ser (RS)-repeats commonly associated with other RNA-binding proteins (Schmidt and Werner, 1993; Staknis and Reed, 1995).

The interaction between hRNPS1 and PITSLRE p110 is specific, as demonstrated by the inability of smaller PITSLRE isoforms, such as p58, p65, and p90, to associate with hRNPS1 using in vitro binding assays and/or yeast two-hybrid assays. Domains required for hRNPS1/PITSLRE p110 interaction were mapped by in vitro binding assays using a series of deletion mutants. More importantly, we show that the human RNPS1 homologue is associated with the PITSLRE p110 protein kinase in vivo by co-immunoprecipitation (IP)/western blotting and indirect immunofluorescence (IF).

Intriguingly, interphase cells expressing the highest levels of a hRNPS1-Flag fusion protein did not contain the normal 30-40 punctate nuclear speckles usually observed (Fu and Maniatis, 1990); instead, they contained far fewer (~6), but much larger, ‘mega’ nuclear speckles containing the hRNPS1-Flag fusion protein, PITSLRE p110, and other SR proteins. When HeLa cells were treated with specific protein kinase and transcription inhibitors the nuclear distribution of PITSLRE p110 was frequently affected in a similar manner. Taken together, the data suggest that PITSLRE p110 and RNPS1 are important for some aspect of transcriptional and/or spliceosome regulation.

MATERIALS AND METHODS

Cell lines, labeling and isolation of proteins, cellular fractionation, western blotting and immunoprecipitations

All of the cell lines were either obtained from the American Type Culture Collection (ATCC) or kindly provided by Dr A. Thomas Look (St Jude Children’s Research Hospital). CEM-C7, HeLa and WI38 cells were cultured as previously described (Lahti et al., 1994, 1995; Spector, 1993; Misteli and Spector, 1997). All of the cell lines were either obtained from the American Type Culture Collection (ATCC) or kindly provided by Dr A. Thomas Look (St Jude Children’s Research Hospital). CEM-C7, HeLa and WI38 cells were cultured as previously described (Lahti et al., 1994, 1995; Spector, 1993; Misteli and Spector, 1997). Culture Collection (ATCC) or kindly provided by Dr A. Thomas Look (St Jude Children’s Research Hospital). CEM-C7, HeLa and WI38 cells were cultured as previously described (Lahti et al., 1994, 1995; Spector, 1993; Misteli and Spector, 1997). All of the cell lines were either obtained from the American Type Culture Collection (ATCC) or kindly provided by Dr A. Thomas Look (St Jude Children’s Research Hospital). CEM-C7, HeLa and WI38 cells were cultured as previously described (Lahti et al., 1994, 1995; Spector, 1993; Misteli and Spector, 1997).
cDNA inserts sequenced in their entirety as previously described (Xiang et al., 1994). DNA sequences were analyzed with the IntelliGenetics Suite and Genetics Computer Group (GCG) programs and the GenBank, EMBL, SWISS-PROT, and PIR databases (Molecular Resource Center, SJCRH).

**Production of recombinant proteins and in vitro binding assays**

The full-length human RNPS1 cDNA was subcloned into pBlueBacHis plasmid (Pharmingen), and the resulting construct was transfected into SF9 insect cells (Baculogold System, Pharmingen) for the production of baculovirus. Large-scale purification of the recombinant 6His-RNPS1 protein was performed using Ni²⁺-beads (Qiagen) under non-denaturing conditions according to the manufacturer’s instructions. The 6His-P2N100 protein was produced using Ni²⁺-beads (Qiagen) under non-denaturing conditions according to the manufacturer’s instructions. The 6His-P2N100 protein was produced in *E. coli* after subcloning of the 5′ end of the PITSLRE α2-2 cDNA, encoding the first 103 amino acids of this PITSLRE p110 isoform, in the PQE-10 vector (Qiagen). The full-length human RNPS1 cDNA was also cloned into the pGEX5X-2 vector (Pharmacia) to produce an RNPS1-GST fusion protein. Distinct fragments of RNPS1, encoding regions corresponding to amino acids 1-136 (-1), 1-206 (-2), 1-271 (-3), 1-288 (-4), 137-205 (ΔN), and 221-305 (RS), were cloned into pGEX expression vectors to generate the corresponding GST-RNPS1 fusion proteins. These fusion proteins were then used for in vitro binding assays to determine the requirements for RNPS1 interaction with the PITSLRE p110 isoforms. Two distinct RNPS1/PITSLRE p110 in vitro binding assays were performed. The various GST-RNPS1 fusion proteins (~10 μg) were bound to GSH beads and then incubated with 1 mg of total cell lysate from CEM-C7 cells at 4°C for 2 hours. After washing, the proteins bound to these beads were resuspended in 50 μl of sample buffer, boiled for 5 minutes and then separated by 10% SDS-PAGE. Proteins were transferred onto PVDF membrane and western blotting was performed using the affinity-purified P2N100 antisera as described above.

**Expression of Flag-tagged RNPS1 proteins, and immunolocalization of Flag-RNPS1, PITSLRE p110 isoforms, and SC-35 in human cells**

The full-length human RNPS1 and RNPS1-D4 cDNAs were subcloned into the PFlex expression vector (Holloway and Baum, 1996); kindly provided by Dr R. Bram, SJCRH). PFlex-RNPS1 or PFlex-RNPS1-D4 plasmids, encoding the Flag-tagged RNPS1 proteins, were transfected into HeLa cells and the protein localized by indirect immunofluorescence (IF) using a mouse monoclonal anti-Flag antibody (Kodak). Immunolocalization of the PITSLRE p110 isoforms and Flag-RNPS1 was accomplished by using the affinity-purified GN1 or P2N100 polyclonal antisera with the anti-Flag mAb M2 (5 μg/ml) to stain the MeOH-fixed transfected HeLa cells grown on coverslips. Appropriate secondary antibodies (FITC-goat anti-rabbit and rhodamine-goat anti-mouse, Southern Biotechnology) were included to allow fluorescent immunolocalization using an Olympus BX50 fluorescence microscope. Nuclei were visualized by staining with DAPI. Immunolocalization of PITSLRE p110 isoforms and SC-35 was performed in an identical manner, using the affinity-purified P2N100 polyclonal antisera, visualized with a FITC-goat anti-rabbit secondary antibody, and an SC-35 mouse monoclonal antisera (kindly provided by Dr T. Maniatis, Harvard), which was visualized with a rhodamine-goat anti-mouse secondary antibody.

Flag-tagged RNPS1 transfected HeLa cells were also harvested for co-immunoprecipitation studies. Proteins were isolated from the bulk population of transiently transfected HeLa cells using lysis buffer, and immunoprecipitations were performed as described above using the anti-Flag M2 mAb and Protein A agarose beads. These immunoprecipitates were then analyzed by separation on 10% SDS-PAGE, transfer to nitrocellulose membrane, and western blotting with the affinity-purified GN1 or P2N100 antisera (Lahti et al., 1995; Beyaert et al., 1997).

**RESULTS**

**Unidentified proteins co-immunoprecipitate with the p110 PITSLRE protein kinase isoforms**

Proteins from the human T-cell line CEM-C7 were labeled with [35S]methionine and the cell lysates immunoprecipitated (IP) with the GN1 polyclonal antibody, which recognizes many of the PITSLRE protein kinase isoforms (Xiang et al., 1994). This labeled PITSLRE-IP was compared with a pre-immune control from the same cell lysates, as well as a GN1 IP/competition (Fig. 1A). Several bands were observed in the GN1 IP corresponding to proteins of ~200, 130, 110, and 54 kDa. These proteins were not present in the pre-immune lane and they were greatly reduced when the GN1 antibody was competed with the GST-GN1 fusion protein (Fig. 1A). We have shown that many of the smaller PITSLRE protein kinase isoforms are only generated by caspase processing in response to apoptotic signals, whereas the 110 kDa PITSLRE isoforms are expressed ubiquitously in proliferating cells (Fig. 2A, and Beyaert et al., 1997). Therefore, the 110 kDa band observed in the GN1 IP of asynchronously growing CEM-C7 cells most likely corresponds to the larger PITSLRE α and β isoforms. To determine whether the co-immunoprecipitating 200, 130 and 54 kDa proteins were related PITSLRE isoforms or unrelated proteins, western blot analysis was performed in parallel. Both the GN1 and P2N100 (another PITSLRE-specific antibody described below) antibodies detected only the 110 kDa PITSLRE protein species in CEM-C7 cell lysates by western blot analysis (Fig. 1B, and data not shown). A pre-immune control IP of the same CEM-C7 cell lysate did not

Fig. 1. Identification of co-immunoprecipitating proteins in PITSLRE antibody immune complexes. (A) Analysis of [35S]methionine labeled proteins from CEM-C7 T cells by immunoprecipitation (IP) with the PITSLRE GN1 affinity-purified antisera (GN1 −). Also shown are a pre-immune control (pre −) and a GN1 IP done in the presence of the GST-GN1 fusion protein (GN1 +). (B) Analysis of CEM-C7 cell lysates (lysate), CEM-C7 lysate IP using GN1 (GN1), and a pre-immune control (pre) by SDS-PAGE followed by western blotting with the GN1 antibody. The location of the PITSLRE p110 is shown on the right of this panel.
contain any PITSLRE isoforms (Fig. 1B). These data demonstrate that the p110 PITSLRE isoforms are expressed in asynchronously growing CEM-C7 cells, that both the GN1 and P2N100 antibodies recognize these isoforms, and that PITSLRE immune-complexes from these cells contain several unidentified proteins. Many, if not all, p34cdc2-related protein kinases also have co-immunoprecipitating proteins that relate to their function and/or regulation (Draetta and Beach, 1988).

The PITSLRE p110 protein kinase isoforms co-localize with the spliceosome component, SC-35

To determine the subcellular localization of the various PITSLRE protein kinase isoforms, a panel of polyclonal antibodies was used. Antibodies were generated to: (1) the unique amino terminus of the PITSLRE α and β p110 isoforms (P2N100; Beyaert et al., 1997); (2) the amino terminus of the p58 isoform (GN1; Xiang et al., 1994; Lahti et al., 1995); (3) the conserved PSTAIRE-box region, denoted PITSLRE (PP8; Xiang et al., 1994; Lahti et al., 1995); and (4) the carboxyl terminus shared by all PITSLRE kinase family members (GC41; Xiang et al., 1994). All of these antisera were affinity purified (as described in Materials and Methods). Due to the high degree of DNA sequence conservation (>99%) between the human PITSLRE genes and their transcripts (Xiang et al., 1994; Lahti et al., 1994), it is not possible to distinguish the various p110 α and β isoforms, or some of the alternatively spliced variants, at the protein level. The availability of a reagent that specifically recognizes the larger 110 kDa PITSLRE isoforms, the P2N100 antibody, allowed us to determine the location of this subset of PITSLRE proteins in proliferating cells. Western blot analysis of several different human cell lines demonstrated that all express the p110 PITSLRE isoforms (Fig. 2A). Crude fractionation of the CEM-C7 cell lysates into nuclear and cytoplasmic components revealed that the 110 kDa PITSLRE isoforms are localized in the nuclear fractions (Fig. 2B). However, when detergent is added to the lysis buffer, a portion of p110 is associated with the cytoplasmic components, suggesting that two different nuclear pools of the protein (i.e. soluble and insoluble) exist. Reprobing this western blot with antibodies to GRP94 (a glucose responsive protein associated with the endoplasmic reticulum) and NuMA (a nuclear matrix protein) confirmed that the fractionation procedure worked as desired (Fig. 2B, middle and bottom panels; Little et al., 1994; Compton and Cleveland, 1993). GRP94 was present in the cytoplasmic fraction only when detergent was added to the lysis buffer, whereas NuMA was confined to the nuclear fraction.

To determine the cellular localization of the 110 kDa PITSLRE isoform(s) more precisely, we analyzed human HeLa (transformed epithelial) and WI38 (normal fibroblast) cell lines by indirect-immunofluorescence (IF) (Fig. 3A, and data not shown). These cell lines were chosen for IF analysis since they represent both normal and transformed human cells, and since adherent cell lines are more amenable to IF analysis than cells that grow in suspension, such as the CEM-C7 cells. We observed both generalized nucleoplasmic and punctate nuclear staining in both cell lines using the P2N100 antibody (Fig. 3A). This staining pattern was effectively competed by preincubation of the antibody with the antigen, and the staining pattern is identical in all eukaryotic cell lines examined thus far (data not shown). In addition, the GN1 and GC41 antibodies detect the same punctate nuclear speckles as well as more diffuse nuclear and cytoplasmic staining ([Xiang et al., 1994; Lahti et al., 1994) and data not shown]. Since the P2N100 staining pattern was somewhat reminiscent of the spliceosome component SC-35 (Fu and Maniatis, 1990), simultaneous IF analyses of the p110 PITSLRE isoforms and SC-35 were performed using HeLa cells (Fig. 3A). During interphase, both the PITSLRE p110 isoforms and SC-35 are localized to the same sub-nuclear regions; SC-35 is closely associated with the nuclear matrix and is involved in RNA processing (Fu and Maniatis, 1990; Bennett et al., 1992). Conversely, in mitotic cells these punctate-staining regions disappear, and both the PITSLRE p110 isoforms and the SC-35 protein form a ‘halo’ which surrounds the dividing chromosomes (data not shown). Very similar IF results have been obtained previously using the
affinity-purified GN1 antibody to stain these same cells (L. Detiavud, J. M. Lahti and V. J. Kidd, unpublished results). These data suggest that a portion of the PITSLRE p110 isoforms co-localize with SC-35 in a sub-nuclear domain thought to be crucial for RNA processing events (Spector, 1993).

The PITSLRE p110 isoform(s) interact with an RNP family protein, RNPS1

To identify proteins that interact specifically with the p110 PITSLRE isoforms, a yeast two-hybrid screen was performed using one of the p110 PITSLRE protein kinase isoforms as bait. A full-length PITSLRE p110 cDNA was cloned into the PASCYH expression vector in-frame with the GAL4 DNA-binding domain. The yeast strain Y153 was co-transformed with the PASCYH-PITSLRE vector and a human B-cell library cloned into the PACT vector containing the GAL4 activation domain (Durfee et al., 1993). Approximately two million of the co-transformed colonies were screened by their ability to grow in the absence of tryptophan, leucine, and histidine, as well as their β-galactosidase activity. Thirty-five colonies were isolated from this initial screening for further study.

False positive clones were eliminated using two distinct approaches. First, PACT plasmids containing the potential p110 interactors were rescued from the Y153 yeast colonies and then used to retransform Y153 cells containing either p110 PITSLRE-PASCYH, p58 PITSLRE-PASCYH, cyclophilin-PASCYH or SNF1-PASCYH. The latter two plasmids are negative controls, encoding proteins that we had previously determined did not interact with any of the PITSLRE protein kinases (data not shown). In addition, the thirty-five Y153 yeast colonies containing the potential p110 interactors were segregated to lose the p110 PITSLRE-PASCYH plasmid they contained. The segregants were then mated to Y187 yeast strains containing either p110 PITSLRE-PASCYH, p58 PITSLRE-PASCYH, cyclophilin-PASCYH, or SNF1-PASCYH plasmids. Using these two distinct approaches, the same eight clones were shown to interact specifically with p110 PITSLRE, but not with the p58 PITSLRE or the unrelated cyclophilin or SNF1 proteins.

The DNA sequence from the first 200 bp of the 5’ and 3’ flanking regions of each of these eight clones was used to search the GenBank database. Of the eight clones, three were found to encode the human homologue of the murine RNPS1 protein (Badolato et al., 1995; Schmidt and Werner, 1993), a member of the RNP protein family. The other cDNAs correspond to the autoimmune antigen SS-A/Ro, an RNA/DNA-binding protein, and the DNA-binding protein CTFin-51 (Chan et al., 1990). Two of the three RNPS1 clones were partial cDNAs originating at amino acid 6 and 32 of the predicted ORF, while the last clone encoded a full-length cDNA identical, except for a single amino acid difference, to a previously reported human cDNA (Badolato et al., 1995). RNPS1 encodes a 305 amino-acid protein with a predicted size of ~35 kDa, containing an RRM RNA-binding domain midway through the predicted ORF as well as several RRS repeats in the carboxyl terminus (see Fig. 5B). The amino-terminal domain of the RNPS1 protein does not contain any apparent shared sequence motifs. It is of interest to note that the amino-terminal portion of PITSLRE p110 contains a large number of RE and RD repeats found in many proteins associated with the spliceosome (Xiang et al., 1994; Neugebauer et al., 1995). This RD family includes the U1 70K protein, which is involved in pre-mRNA splicing (Neugebauer et al., 1995). Clusters of these RS/RE protein sequence motifs have recently been shown by Hedley et al. (1995) to be necessary and sufficient for the speckled sub-nuclear localization associated with many of the splicing-related polypeptides. Thus, it is likely that the presence of these motifs in the p110 PITSLRE isoforms contributes to their localization to these regions of the nucleus (Fig. 3).

PITSLRE p110 interacts with RNPS1 in vivo

The RNPS1 protein was produced by an in vitro transcription and translation (IVTT) reaction. The predicted size of the protein encoded by RNPS1 cDNA is ~35 kDa, but the [S35]methionine labeled translation products migrate at ~54 kDa, indicating that the hRNPS1 protein migrates anomalously on SDS-PAGE. This 54 kDa form of RNPS1 co-migrated with a protein species that is associated with the PITSLRE p110 immune complex in CEM-C7 cells (Fig. 4A). To determine whether the 54 kDa protein that co-immunoprecipitated with the p110 PITSLRE isoforms was, indeed, hRNPS1, we performed several complementary experiments. We isolated the [S35]methionine labeled 54 kDa protein found in the CEM-C7 cell GN1 IP, subjected it to partial V8 protease digestion and compared the resulting peptide fingerprint to an identical partial V8 protease digestion of the [S35]methionine labeled hRNPS1 IVTT (Fig. 4B). The patterns of the resulting partial V8 digests appear to be highly similar.

To further demonstrate an in vivo association between PITSLRE p110 and hRNPS1, the hRNPS1 cDNA was placed into the pFlex2 expression vector for transient transfection into human HeLa cells. Lysates from these transfected cells were then used for immunoprecipitation with the Flag mAb followed by western blotting with either the GN1 or P2N100 affinity-purified antisera. The PITSLRE p110 isoforms were detected by both the GN1 and P2N100 antibodies in these Flag mAb immune complexes (Fig. 4C). These proteins were not detected in the pre-immune control IPs (Fig. 4C). Further demonstration of hRNPS1 and PITSLRE p110 interaction in vivo is described below.

RNPS1 colocalizes with the PITSLRE p110 isoforms and spliceosome components

The distinct spliceosome-localization motifs in RNPS1 suggested that it might be found in the nuclear speckles associated with other splicing components. Attempts to raise polyclonal hRNPS1 antisera were unsuccessful. Therefore, we examined the subcellular localization of the Flag tagged-hRNPS1 to determine whether it co-localized with the PITSLRE p110 protein kinases and SC-35. As mentioned above, the PITSLRE p110 isoforms are prominently localized to a subnuclear compartment that is associated with the nuclear matrix. This ‘speckled’ nuclear localization pattern is characteristic of proteins associated with spliceosome complexes, such as SC-35 (Fu and Maniatis, 1990; Spector et al., 1991; Spector, 1993). The Flag tagged-hRNPS1 protein was found in similar nuclear speckled regions (~30-50 major nuclear sites in interphase cells) when the transfected HeLa cells were analyzed within 16-24 hours of transfection (Fig. 3B and C). Curiously, some of these cells appear to have re-
Fig. 3. Analysis of PITSLRE p110 and hRNPS1 cellular localization by indirect immunofluorescence (IF). (A) Comparison of p110 PITSLRE protein kinase and SC-35 splicing factor localization in HeLa cells using the P2N100 polyclonal antisera and an SC-35 monoclonal antibody. Both appear to be localized to similar nuclear speckles, which is confirmed by their overlapping co-localization (P2N100/SC-35). Bar, 20 μm. (B) HeLa cells were transiently transfected with a hRNPS1-FLAG expression construct, and the cells examined by IF with the P2N100 polyclonal and FLAG monoclonal antibodies. Note the altered number and appearance of large, 'mega,' nuclear speckles in the cells overexpressing hRNPS1-FLAG; much of the PITSLRE p110 protein has been recruited to these mega speckles. Examination of the same cells by phase microscopy reveals that these mega speckles coincide with the nuclear interchromosomal granules (dark spots). (C) Another example of nuclear speckle rearrangement in HeLa cells overexpressing the hRNPS1-FLAG protein and its effects on PITSLRE p110 distribution within the nucleus. Examples of the effects of lower levels of RNPS1 expression on nuclear speckle structure are shown in the panel on the far right. The larger arrows indicate cells expressing lower levels of RNPS1, while the smaller arrow indicates a cell expressing a higher level of protein. (D) HeLa cells transiently transfected with a PITSLRE p110-FLAG expression construct and examined by IF with either a FLAG polyclonal antisera (FLAG), SC-35 mAb (SC-35), or both (FLAG/SC-35). Note that while overexpression of PITSLRE p110 results in the loss of some of its nuclear speckling, SC-35 localization to nuclear speckles is unaffected. (E) HeLa cells transiently transfected with the hRNPS1-FLAG expression construct examined by IF with the FLAG polyclonal and SC-35 monoclonal antibodies. Even though the staining patterns of hRNPS1 and SC-35 clearly overlap in the mega speckles, not all of the SC-35 antigen has been recruited to these structures. The arrows indicate SC-35 localized on the outer periphery of these mega speckles, while the arrowheads indicate the regions of RNPS1/SC-35 overlap in the same structures. (F) HeLa cells treated with the transcription/protein kinase inhibitor DRB. The localization of PITSLRE p110 and SC-35 in these cells was examined by IF with the P2N100 polyclonal and SC-35 monoclonal antibodies. (G) HeLa cells treated with the transcription inhibitor actinomycin D. The localization of PITSLRE p110 and SC-35 was examined as described for F. Note that in contrast to the DRB-treated cells, actinomycin D eliminates much of the punctate nuclear staining pattern normally observed with P2N100, but it does not eliminate the SC-35 nuclear speckles.
organized their nuclear speckles, depending on the level of RNPS1-Flag protein expressed. Instead of the normal ~30-50 nuclear speckles, ~6 giant speckles (mega-speckles) were observed in cells expressing the highest levels of RNPS1 (Fig. 3B and C). An identical pattern of nuclear speckling was seen with either the affinity-purified PITSLRE P2N100 antibody (Fig. 3B and C) or the SC-35 mAb (Fig. 3E); in fact, the IF staining for these nuclear antigens clearly superimpose upon one another (Fig. 3B,C,E). It may be of interest to note that the hRNPS1 and SC-35 antigens do not appear to overlap completely when examined by IF (Fig. 3E). The SC-35 protein appears to be more heavily concentrated at the periphery of these ‘doughnut-shaped’ speckles (Fig. 3E, arrows). The possible significance of this is briefly discussed below. The IF data for hRNPS1 and the PITSLRE p110 isoforms is consistent with the in vitro and in vivo association between these proteins, and it suggests that they are localized to nuclear speckles that overlap with those containing SC-35. Thus, the hRNPS1 protein identified by two-hybrid interactive cloning with the PITSLRE cDNA appears to be associated with PITSLRE p110 isoforms in vivo by several different criteria. Interestingly, the number of cells expressing the RNPS1-Flag protein, in either the normal nuclear speckles or the mega-speckles, decreases dramatically. In fact, RNPS1-Flag positively staining cells are not present after ~48-60 hours. This suggests that the effects of overexpressing RNPS1, an RNA-binding protein of unknown function, are potentially lethal. Such lethality may result from the inhibition of either transcriptional or splicing activities. It should also be noted that overexpression of PITSLRE p110 in the same HeLa cells does not result in similar aggregation of nuclear speckles (Fig. 3D), consistent with results using murine PITSLRE cDNA expression constructs (Malek and Desiderio, 1994). In addition, PITSLRE p110 overexpression does not appear to significantly alter the localization of SC-35 (Fig. 3E), whereas overexpression of the related Clk/Sty protein kinase does (Colwill et al., 1996b). In an attempt to determine whether PITSLRE p110 function is more relevant to transcription or splicing, several inhibitors of transcriptional activity were used to treat HeLa cells prior to IF analysis with the P2N100 and SC-35 antibodies. Cells were treated with either 5,6-dichlorobenimidizole riboside (DRB), a nucleoside analog that inhibits CTD phosphorylation and mRNA production in vivo (Tasmm et al., 1976; Payne and Dahmus, 1989), H8 (an isoquinoline sulphonamide derivative that is a potent inhibitor of RNA polymerase II-associated CTD kinase activities (Serizawa et al., 1993) or α-amanitin (which blocks mRNA synthesis by binding preferentially to RNA polymerase II). The localization of p110 and SC-35 was affected similarly with all of these compounds, with both proteins aggregating into fewer, larger speckles (Fig. 3F; data not shown). Conversely, in HeLa cells treated with actinomycin D, which complexes with guanine residues in DNA and prevents mRNA synthesis by RNA polymerase II (Drapkin et al., 1994), PITSLRE p110 is no longer localized to nuclear speckles whereas SC-35 is (Fig. 3G).

**The PITSLRE p110 protein kinases bind to an amino-terminal domain of hRNPS1**

What hRNPS1 structural motifs are required for its
interaction with the PITSLRE p110 isoforms? A GST-
hRNPS1 fusion protein (containing amino acids 1-288 of
hRNPS1, lacking a portion of the RRS repeats, continues to
bind the PITSLRE p110 IVTT products (data not shown).
This result suggests that the ‘complete’ RRS domain of
hRNPS1 is not required for this interaction. To address this
point, we generated five additional GST-hRNPS1 fusion
proteins, GST-hRNPS1-1 (1-136), -2 (1-206), -3 (1-271), -4 (1-288; RNPS1-4), and -RS, 221-305) to determine the minimal
region of hRNPS1 that is required for binding to the p110
PITSLRE isoforms. GST-hRNPS1-3 lacks much of the RRS
domain; hRNPS1-2 ends at the boundary between the RNA
binding and RRS domains; hRNPS1-1 contains only the
amino-terminal domain; hRNPS1-ΔRN contains all of the RNA
binding and RRS domains; and hRNPS1-RS contains only
the RRS-repeat domain of the protein (Fig. 5B). These
hRNPS1-GST fusion proteins were incubated with CEM-C7
cell lysates to determine whether the same PITSLRE
isoforms from these cell lysates were bound. After incubation
and extensive washing, the protein(s) bound to the hRNPS1-
GST beads, or the GST beads alone, were analyzed by SDS-
PAGE and western blotting with the P2N100 antibody (Fig.
5A). The PITSLRE p110 isoforms were detected in
association with five of the GST-hRNPS1 fusion proteins, -1,
-2, -3, -4 and -AN, but not with the hRNPS1-RS protein, GST,
or hRNPS1-GST beads that were not incubated in the
presence of the CEM-C7 cell extract. These results demonstrate that the PITSLRE p110 isoforms from CEM-C7
cell extracts interact with the amino-terminal and RNA-
binding domains of hRNPS1 (inclusive of amino acid residues 1-220; Fig. 5B), and that this association does not involve the RRS sub-domain.

**RNPS1 interacts with the PITSLRE αβ2-1 and αβ2-2 isoforms, but not with the PITSLRE β1, PITSLRE α1 or PITSLRE α2-4 isoforms**

Using the yeast two-hybrid system we demonstrated that the hRNPS1 protein directly interacts with the p110 PITSLRE αβ2-2 protein kinase, but it did not interact with the p58 PITSLRE β1 isoform. This suggested that selective binding between the hRNPS1 protein and specific PITSLRE protein kinase isoforms was occurring. In an attempt to further map and define these interactions, we determined which PITSLRE isoforms interact with the hRNPS1 protein using a similar in vitro binding assay. The GST-RNPS1-4 fusion protein encoding a 288 amino acid peptide, which lacks the extreme carboxyl-terminal portion of the predicted ORF, was purified from bacteria. The resulting fusion protein migrates at the apparent size of 75 kDa when analyzed by SDS-PAGE (data not shown). Various PITSLRE isoforms were labeled with [35S]methionine by IVTT of the corresponding cDNA templates. These labeled PITSLRE proteins were then incubated with the GST-RNPS1 beads, and after stringent
washing these beads were analyzed by SDS-PAGE and autoradiography to determine which PITSLRE isoforms
bind to the GST-RNPS1 fusion protein. As a control, GST
beads were used for the incubations with the IVTT PITSLRE proteins. The PITSLRE β1, α1 and α2-4 isoforms did not bind to the GST-RNPS1 fusion protein, whereas the
PITSLRE α2-1 and α2-2 isoforms did (Fig. 6). None of the
[35S]methionine labeled PITSLRE proteins bound to GST
alone (Fig. 6). In addition, only the two largest IVTT products
of the p110 α2-1 and α2-2 PITSLRE isoforms, which were
generated by initiation from internal methionine residues
located within the first 91 amino acids of these isoforms,
bound to the GST-RNPS1 fusion protein (Fig. 6). IVTT
PITSLRE products generated by initiation from the third
internal methionine residue (found at position 156 and 154 of
the predicted PITSLRE α2-1 and β2-1 ORFs, respectively; Xiang et al., 1994) did not interact strongly with this GST-
RNPS1 fusion protein. The PITSLRE α2-4 isoform encodes a polypeptide initiating from a methionine residue corresponding to amino acid 225/223 of the PITSLRE αβ2-1 isoforms (Xiang et al., 1994); this α2-4 isoform did not interact with the GST-RNPS1 fusion protein either (Fig. 6). These results demonstrate that only the p110 PITSLRE αβ2-1 and 2-2 isoforms interact with hRNPS1, and strongly
suggest that the first 100 amino acids of the PITSLRE p110 isoforms defines the domain required for interaction with hRNPS1.
DISCUSSION

We have demonstrated that the RNA-binding protein hRNPS1 is associated with PITSLRE p110 isoforms in vivo. Furthermore, we have shown that hRNPS1 is an RNP/SR protein that localizes to 'nuclear speckles' commonly associated with splicing components such as SC-35 (Fu and Maniatis, 1990), and that overexpression of hRNPS1 appears to disrupt the normal structure and distribution of these nuclear speckles. The p34\(^{\text{cdc2}}\)-related protein kinase PITSLRE p110 is an RD-containing protein that co-localizes with SC-35 and RNPS1, suggesting that its function may be related to events that occur in nuclear speckles via its association with hRNPS1. The RD/RE sequences are located entirely in the amino terminus of the p110 isoforms (Xiang et al., 1994). The PITSLRE p110 isoforms and hRNPS1 interact with one another through physical association of their amino-terminal domains, as demonstrated by studies presented here. While high levels of hRNPS1 overexpression result in the aggregation of PITSLRE p110 and a substantial portion of SC-35 into 'mega' speckles, overexpression of p110 does not dramatically alter the distribution of SC-35. Furthermore, the effects of several transcriptional/protein kinase inhibitors on the nuclear distribution of PITSLRE p110 and SC-35 are similar to the effects of hRNPS1 overexpression.

The Clk/Sty protein kinases (distantly related members of the p34\(^{\text{cdc2}}\)-related and PITSLRE families of kinases) and SR protein kinase 1 (SRPK1) regulate the activity of splicing factors by phosphorylation (Colwill et al., 1996a,b; Gui et al., 1994a,b; Misteli and Spector, 1997). In this regard, overexpression of Clk/Sty results in the dissolution of nuclear speckles containing SC-35. However, in the current study we have shown that overexpression of the PITSLRE p110 kinase does not significantly disrupt SC-35 localization, suggesting that its function may not necessarily be linked to the regulation of RNA splicing events. Conversely, the isolation of hRNPS1, a RNP protein of unknown function, by two-hybrid screening with PITSLRE p110 argues that p110 may participate in regulating some aspect of nuclear speckle structure/function or that RNPS1 participates in some aspect of transcriptional regulation. The former possibility is suggested by the radical aggregation of nuclear speckles that occurs when hRNPS1 is overexpressed. The latter possibility is based upon the demonstration that the effect of hRNPS1 overexpression on the localization of PITSLRE p110 in HeLa cells is nearly identical to the effects of multiple transcriptional inhibitors (e.g. DRB, H8, \(\alpha\)-amanitin) on p110 distribution in the same cells. The interaction between hRNPS1 and PITSLRE p110 occurs with both the \(\alpha2\) and \(\beta2\) p110 isoforms in vitro and in vivo. Furthermore, hRNPS1 does not bind to any of the shorter PITSLRE isoforms that resemble caspase-processed isoforms. RNPS1 has also been identified in a two-hybrid screen using the murine Clk/Sty protein kinase as bait, but the interaction was not characterized (Colwill et al., 1996b).

It is evident that nuclear speckle organization is dynamic. In fact, recent work demonstrates that splicing factors leave large speckle domains and migrate to sites of gene activation and transcription (Misteli et al., 1997). There are many examples of speckle reorganization into fewer, but larger and rounder, domains following inhibition of either transcription or splicing (O’Keefe et al., 1994; Romac and Keene, 1995; Zeng et al., 1997). Disruption of pre-mRNA splicing by microinjection of either oligonucleotides or antibodies directed against snRNAs into HeLa cells resulted in reduced levels of nuclear transcription as well as the reorganization of splicing factors (O’Keefe et al., 1994). Similarly, when transcription was decreased using RNA polymerase II-specific inhibitors, SC-35 and RNA polymerase II large subunit accumulated in multiple,
enlarged nuclear speckle domains (Zeng et al., 1997). Overexpression of the RD/RE/RS domains of U1-70K also induced the formation of large nuclear foci containing SC-35 (Romac and Keene, 1995). The results described in this study are similar, but not identical, to those previously published experiments. Normally, PITSLRE p110 isoforms and SC-35 co-localize to nuclear speckles in proliferating cells (Fig. 3). Homogeneous nucleoplasmic staining is also observed with many of the PITSLRE-specific antibodies, and both PITSLRE p110 and SC-35 proteins are excluded from the chromosomes and mitotic apparatus during mitosis. Overexpression of hRNPS1 in HeLa cells resulted in a reorganized nuclear staining pattern that is similar to those associated with the inhibition of transcription (Fig. 3B and C). Instead of the normal 30-50 nuclear speckles, 8 to 20 large nuclear dots were observed. These ‘mega’ speckles increased in size and roundness as the number of normal nuclear speckles decreased. The PITSLRE p110 isoforms, detected by a polyclonal antibody specific for these isoforms, was also located in these mega-speckles, and diffuse nucleoplasmic p110 staining was lost. Overexpression of p110 does not cause a similar nuclear reorganization of SC-35 (Fig. 3D).

Since both hRNPS1 and a portion of the PITSLRE p110 protein kinases are localized to nuclear speckles, do they contribute to spliceosome function (e.g., assembly or disassembly), transcriptional regulation, or some other nuclear function? SR and SR-related proteins, such as RNPS1, are localized specifically to the nuclear speckle structures (Zahler et al., 1992; Hedley et al., 1995; Fu, 1995; Staknis and Reed, 1995; Manley and Tacke, 1996). However, speckle domains also contain the RNA polymerase II large subunit, RNAs, and various snRNPs (Blencowe et al., 1994; Mortillaro et al., 1996; Yeryv et al., 1996; Neugebauer and Roth, 1997; Gama-Carvalho et al., 1997). There are currently two theories regarding the function(s) of nuclear speckles. One theory suggests that nuclear speckles (also termed interchromatin granules) are storage sites for splicing components, and that gene transcription and RNA processing occur in the perichromatin fibrils that interconnect these speckles (Spector et al., 1991; Jimenez-Garcia and Spector, 1993; Spector et al., 1993; Spector, 1993; Misteli and Spector, 1997; Misteli et al., 1997). Another theory suggests that transcription and pre-mRNA processing occur within these nuclear speckles as well as the perichromatin fibrils (Xing and Lawrence, 1993; Xing et al., 1993, 1995; Carter et al., 1993; Moen et al., 1995). In fact, recent evidence supports the idea that RNA transcription and processing occur coincidentally, and that these activities are spread throughout the nucleus (Spector et al., 1993; Spector, 1993; Misteli and Spector, 1997). It has also been shown that actively transcribing cells have a more dispersed distribution of RNA polymerase II and splicing factors than less active cells (Neugebauer and Roth, 1997; Du and Warren, 1997; Kim et al., 1997). Thus, the nuclear speckles are greater in number, but smaller in size, in transcriptionally active cells. Treatment of cells with various transcriptional and protein kinase inhibitors (Fig. 3F and G) clearly indicates that the nuclear distribution of PITSLRE p110 isoforms is dramatically affected by both. The ability of actinomycin D to completely disperse the p110 nuclear speckles without affecting the apparent organization of the SC-35 nuclear speckles suggests that PITSLRE p110 function may, indeed, be linked to some aspect of transcriptional regulation. Further study of the PITSLRE p110 kinases, including, perhaps, an analysis of RNA polymerase II complexes, will help to determine whether their function in nuclear speckles is related to RNA splicing, transcription, or both.

We thank J. Grenet and G. Richmond for their excellent technical assistance, Dr S. Elledge for providing the yeast two-hybrid vectors and the human B-cell cDNA library, Dr R. Bram for helping with the yeast two-hybrid screens, Dr T. Maniatis for providing the SC-35 mAb, Dr L. Henesshot for providing the GRP94 antibody, Dr J. Engler for first pointing out the association of PITSLRE kinases with the nuclear matrix, and two anonymous reviewers for their helpful comments. We also acknowledge the assistance of Dr C. Naeve and the Molecular Resource Center of SJCRH in the production of oligonucleotides and DNA sequencing analysis. J.T. was supported by NIH Postdoctoral Training grant 5 T32 CA09346. This research was supported by a grant from the NIH to V.J.K. (GM44088), by a Cancer Center Core Grant to SJCRH (CA21765), and by the American Lebanese Syrian Associated Charities (ALSAC).

REFERENCES


