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Arginine Methylation Inhibits the Binding of Proline-rich Ligands to Src Homology 3, but Not WW, Domains*

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Src homology 3 (SH3) and WW domains are known to associate with proline-rich motifs within their respective ligands. Here we demonstrate that the proposed adapter protein for Src kinases, Sam68, is a ligand whose proline-rich motifs interact with the SH3 domains of p59^{fyn} and phospholipase Cγ-1 as well as with the WW domains of FBP30 and FBP21. These proline-rich motifs, in turn, are flanked by RG repeats that represent targets for the type I protein arginine *N*-methyltransferase. The asymmetrical dimethylation of arginine residues within these RG repeats dramatically reduces the binding of the SH3 domains of p59^{fyn} and phospholipase Cγ-1, but has no effect on their binding to the WW domain of FBP30. These results suggest that protein arginine methylation can selectively modulate certain protein-protein interactions and that mechanisms exist for the irreversible regulation of SH3 domain-mediated interactions.

WW[1] and SH3 domains are structurally distinct modules that are functionally alike in that they bind proline-rich motifs within their corresponding ligands (1). The specificity of their interaction is mediated by unique sequences that correlate with binding pockets found on the surface of these domains (2–4). Expression, phage display, and peptide library screens have identified the components involved in determining ligand binding specificity. Subsequent structural analyses of SH3 and WW domains with respect to binding to their respective ligands have indicated how this precise interaction is achieved (5–11). Structural analysis of Src-like SH3 domains complexed with ligand reveals three points of contact, involving two hydrophobic interactions and a salt bridge. Salt bridge formation between a conserved acidic residue in the SH3 domain and the favored arginine residue, either N-terminal (class I ligand) or C-terminal (class II ligand) to the Pro-*X*-*X*-Pro motif, plays a key role in the specificity of this interaction. WW domains also display specificity in their binding to distinct proline-rich sequences. To date, four different WW domain binding motifs have been characterized, with each core motif binding to a subset of WW domains to the exclusion of the other motifs. They are: core I, PP*X*Y (where *X* signifies any amino acid; binds to the YAP (Ref. 12) and the Nedd4 WW domains (Refs. 13 and 14)); core II, PPLP (binds to the FBP11 (Refs. 9 and 15) and the FE65 WW domains (Ref. 16)); core III, a PGM motif, which, in addition to proline, glycine, and methionine residues, is also arginine-rich (binds to the FBP21 and FBP30 WW domains (Ref. 10)); and core IV, phosphoserine- or phosphothreonine-binding modules (binds to the Nedd4 and Pin1 WW domains (Ref. 17)).

WW domains and SH3 domains bind very similar or overlapping proline-rich motifs, suggesting that proteins harboring these domains might compete for the same ligands. A number of common Abl SH3- and FBP11 (formin-binding protein 11) WW-domain ligands have been identified (9), including the GSG domain-containing splicing factor SF1 (18). SF1 is an RNA-binding protein that belongs to the larger family of KH domain-containing proteins. These include heterogeneous nuclear ribonucleoprotein (hnRNP) K and FMRP (fragile X mental retardation protein) and the smaller GSG domain subfamily including Sam68, GRP33, GLD-1, and Qk1 (19–21). Sam68 is thought to serve as an adapter protein for Src kinases during mitosis (22–25) and has recently been identified as a functional homolog of the HIV-1 Rev protein, thereby implicating it in the post-transcriptional regulation of gene expression (26).

hnRNP K (the founding member of the KH domain-containing protein family), like many of the hnRNPs, is post-translationally methylated on arginine residues (27). This modification, in general, is likely to play an important role in RNA metabolism, signal transduction, and possibly the regulation of nucleic acid interactions (28–30). It has also been suggested

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[1] The abbreviations used are: WW, denotes two conserved tryptophan residues within this domain; SH3, Src homology 3 domain; YAP, Yes-associated protein; FBP, formin-binding protein; Pin1, peptidyl-prolyl *cis*/*trans*-isomerase; SF1, human splicing factor 1; Sam68, Src-associated substrate during mitosis 68 kDa; GRP33, glycine-rich protein 33 kDa; GLD-1, germline defective-1; GSG, GRP33 Sam68 GLD-1; hnRNP, heterogeneous ribonucleoprotein; Qk1, quaking protein 1; HIV-1, human immunodeficiency virus 1; KH, hnRNP K homology domain; AdoMet, *S*-adenosyl-L-methionine; RU, resonance unit(s); GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; MMA, N^G-monomethylarginine; DMA, N^G-,N^G-dimethylarginine; PLC, phospholipase C; TBST, Tris-buffered saline with Tween 20.

that arginine methylation might regulate protein-protein interactions (27, 31). This proposal stems from the fact that the SH3 domains of Src and Vav interact with hnRNP K (24, 32, 33) and the proline-rich domains responsible for these interactions are adjacent to RGG motifs that might be methylated.

Here we report that the Src kinase adapter protein Sam68, apart from binding SH3 domains, interacts with the WW domains of FBP30 and the putative spliceosome protein, FBP21. The proline-rich motifs that mediate these interactions are flanked by arginine-glycine (RG) repeats, which are reminiscent of recognition motifs for the type 1 protein *N*-methyltransferase. In what follows, we demonstrate that Sam68 peptides can be asymmetrically dimethylated on arginine residues. Furthermore, we show that when flanking arginine residues are methylated, Src-like SH3 domain binding to isolated Sam68 proline-rich motifs is dramatically reduced. By contrast, WW domain binding to these same Sam68 sequences remains largely unaffected by arginine methylation. These results identify a novel mechanism by which protein-protein interactions can be differentially regulated.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—The DNA constructs encoding myc epitope-tagged Sam68, glutathione *S*-transferase (GST)-PLCγSH3–1 GST-fynSH3, and GST-srcSH3 were described previously (15, 23, 34). GST-WW YAP, WW FBP11, WW FBP21, WW FBP30, B′ U1C, and Ld10 were described previously (9, 10). The DNA fragments encoding the Sam68 proline motifs P0, P1P2, P3, P4, and P5 were amplified by polymerase chain reaction using myc-Sam68f as a DNA template (34). The DNA were digested with *Bam*HI and *Eco*RI and subcloned in the respective sites of pGEX-KG (35). The sequence of the oligonucleotides are: P0, 5′-CGT GGA TCC AAG GAC CCG TCA GGT-3′ and 5′-GCG GAA TTC TCA AGC GCC TCC TCT GGG CCC AC-3′; P1P2, 5′-CGG GGA TCC CCC GCC ACC CAG CCG CCG-3′ and 5′-GCG GAA TTC TCA CGG CTG TGG CTG ACG GGG GC-3′; P3, 5′-AAC GGA TCC CCT GAA CCC TCT CGT GGT-3′ and 5′-GCG GAA TTC TCA AGC TCC TCT AGG TGG TCC AAC-3′; P4, 5′-CGT GGA TCC CCA GTG AGA GCT CCA TCA CC-3′ and 5′-GCG GAA TTC TCA CCC AGC TGT CCG AGC TCT TG-3′; P5, 5′-GCT GGA TCC CAG AGA ATA CCT TTG CCT-3′ and 5′-GCG GAA TTC TCA TAA TAG CCT TCA TAG CCT TC-3′. The sequence of the oligonucleotides used for the amplification of the PRMT2 SH3 domain are: 5′-CGT GGA TCC GCA ACA TCA GGT GAC TGT CCC-3′ and 5′-TAA GAA TTC TTA GAA GTA CTC TTC ATC CTG CC-3′; the DNA were digested with *Bam*HI and *Eco*RI and subcloned in the respective sites of pGEX-2TK (Amersham Pharmacia Biotech).

In Vitro Methylation of GST Fusion Proteins—GST-methyltransferase fusion proteins GST-RMT1 (yeast) and GST-PRMT1 (rat) were previously described (36). GST-methyltransferase fusion proteins (2 μg) were incubated for 1 h at 30 °C (GST-RMT1) or 37 °C (GST-PRMT1 and blank) with 5 μg of the GST proline-rich fusion proteins P0, P1P2, P3, P4, or P5 in 0.5 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine ([^{3}H]AdoMet, 73 Ci/mmol) (NEN Life Science Products) and 25 mM Tris-HCl at pH 7.4 in a final volume of 30 μl. Reactions were stopped by the addition of an equal volume of 2× SDS-PAGE sample buffer, followed by heating for 5 min at 100 °C. Samples were then loaded onto a 10% SDS-polyacrylamide gel and stained with Coomassie, and the destained gel was soaked in EN^{3}HANCE (NEN Life Science) according to the manufacturer's instructions and visualized by fluorography (exposure time was 9 h).

Amino Acid Analysis of Methylated P3—GST-Sam68 P3 fusion protein was methylated as described above, with the exception that the total AdoMet concentration was raised to 200 μM by the addition of non-labeled AdoMet, and that the incubation was carried out for 6 h. The reaction mixture was precipitated in 25% trichloroacetic acid and then hydrolyzed with 6 N HCl *in vacuo* (110 °C for 20 h) in a vapor-phase Waters Pico-Tag apparatus. Hydrolyzed material was resuspended in 50 μl of water. Half of this material (25 μl) was mixed with 1 μmol each of N^G-monomethylarginine (MMA) and N^G,N^G-dimethylarginine (asymmetric) (DMA) as standards (both obtained from Sigma). The mixture was then loaded onto a Beckman AA-15 sulfonated polystyrene column (0.9 cm diameter × 11 cm height) that was pre-equilibrated with Na^+ citrate buffer (0.35 M in Na^+, pH 5.27) at 55 °C. Fractions were collected at approximately 1 ml/min. ^{3}H radioactivity was detected by adding 200 μl from each fraction to 400 μl of water, mixing with 5 ml of fluor, and counting in a scintillation counter. Additional 100-μl samples from each fraction were analyzed for nonradioactive methylated arginine standards by a ninhydrin method as described previously (37).

Synthesis of Arginine-methylated P3, P4, and Nef Peptides—Methylated and unmethylated peptides were synthesized by the W. M. Keck Biotechnology Resource Center (New Haven, CT). The 24-residue peptide sequence was biotin-GVSVR*GR*GAAPPPPPVPR*GR*GVGP (R* = N^G,N^G-dimethylarginine (asymmetric)) and was called P3*. The sequence of the P4* peptide was biotin-TR*GATVTR*GVPPPPTVR*GAPTPR. The sequence of the NEF* was biotin-WVGFPVTPQVPLR*PMTYKAA. The unmethylated forms of these peptides were called P3, P4, and NEF. The authenticity of the peptides was verified using mass spectroscopy.

Purification and Labeling of Fusion Proteins—GST fusion proteins were purified as described previously (38). Purified proteins were labeled with [γ-^{32}P]ATP using heart muscle kinase (Sigma catalog no. P-2645) (38). Unincorporated nucleotides were removed by purifying the sample over a Nick column (Amersham Pharmacia Biotech). For all purposes, 10–50 μg of protein were labeled; the typical specific activity was ~10^6 cpm/μg.

Blot Overlay and Pull-down Assay—Protein samples were separated by SDS-PAGE and transferred onto an Immobilon-P membranes (Millipore, Bedford, MA) by semidry electroblotting. The blots were blocked for 1 h at room temperature in TBST containing 1% nonfat dry milk. Blots were then incubated in binding buffer (TBST containing 1% milk and 0.5 × 10^6 cpm of probe/ml) overnight at 4 °C. Filters were washed in TBST four times, 15 min each, and exposed to film for 10 min. For blot overlay assays with the probes of biotinylated P3 and P3* peptides, a modified method, described by Sparks *et al.* (39), was used. 40 μg of peptide was precomplexed to 20 μl of streptavidin-horseradish peroxidase for 30 min at 4 °C. Blots were then incubated in binding buffer (10 ml of TBST containing 1% milk and all the precomplexed peptide) overnight at 4 °C. Filters were washed in TBST four times, 15 min each, and the horseradish peroxidase signal detected with the ECL kit (Amersham Pharmacia Biotech). Pull-down assays were performed as described previously (23).

Binding Assays—The binding assays were performed using a BIAcore Biosensor 2000® (BIAcore, Piscataway, NJ). The P3 and P3* peptides were immobilized in different flow cells of a streptavidin-coated BIAcore sensor chip (Sensor Chip SA, catalog no. BR-1000-32). 190 resonance units (RU) of the P3* peptide and 157 RU of the P3 peptide were bound to the chip. The P3 and P3* flow cells were linked so the same amount of analyte passed over each peptide. The concentration of analyte injected was 200 ng/μl (in phosphate-buffered saline) and 60 μl of analyte were injected in each case, at a flow rate of 10 μl/min. The *arrowhead* indicates the time at which injection of the analyte is stopped. A dissociation curve is observed as the flow of phosphate-buffered saline continues. Between assays, the chip was regenerated with 0.1 M NaOH. The integrity of the immobilized peptide, after multiple treatments with NaOH, was confirmed by starting and finishing each run series with the SH3 domain of Fyn. The RU values for the first and last Fyn SH3 domain runs were the same.

RESULTS

Sam68 Binds WW Domain—The WW domains of two formin-binding proteins, FBP11 and FBP21, associate with the pre-mRNA splicing factor, SF1 (9, 10). SF1 contains an extended KH domain (18) and, as such, is a member of the GSG protein family. To investigate whether this association with WW domain-containing proteins is a unique property of SF1 or is a general property of GSG proteins, we assessed the ability of other GSG family members (34), including Sam68, GLD-1, Qk1, and GRP33, to associate with the WW domains of FBP11, FBP21, YAP, and FBP30. WW domains derived from these proteins were expressed as GST fusion proteins and were used as affinity reagents in "pull-down" assays of cell lysates containing myc epitope-tagged GSG family members. The bound proteins were separated by SDS-PAGE and analyzed by immunoblotting with anti-myc antibodies. The WW domains of FBP21 and FBP30 bound Sam68 (Fig. 1*A*, *lanes 5* and *6*), and a weak interaction between GRP33 and the WW domain of FBP21 was also observed (Fig. 1*A*, *lane 23*). Sam68 is a known target for the SH3 domains of p59^{fyn} and Src kinases (22–24,

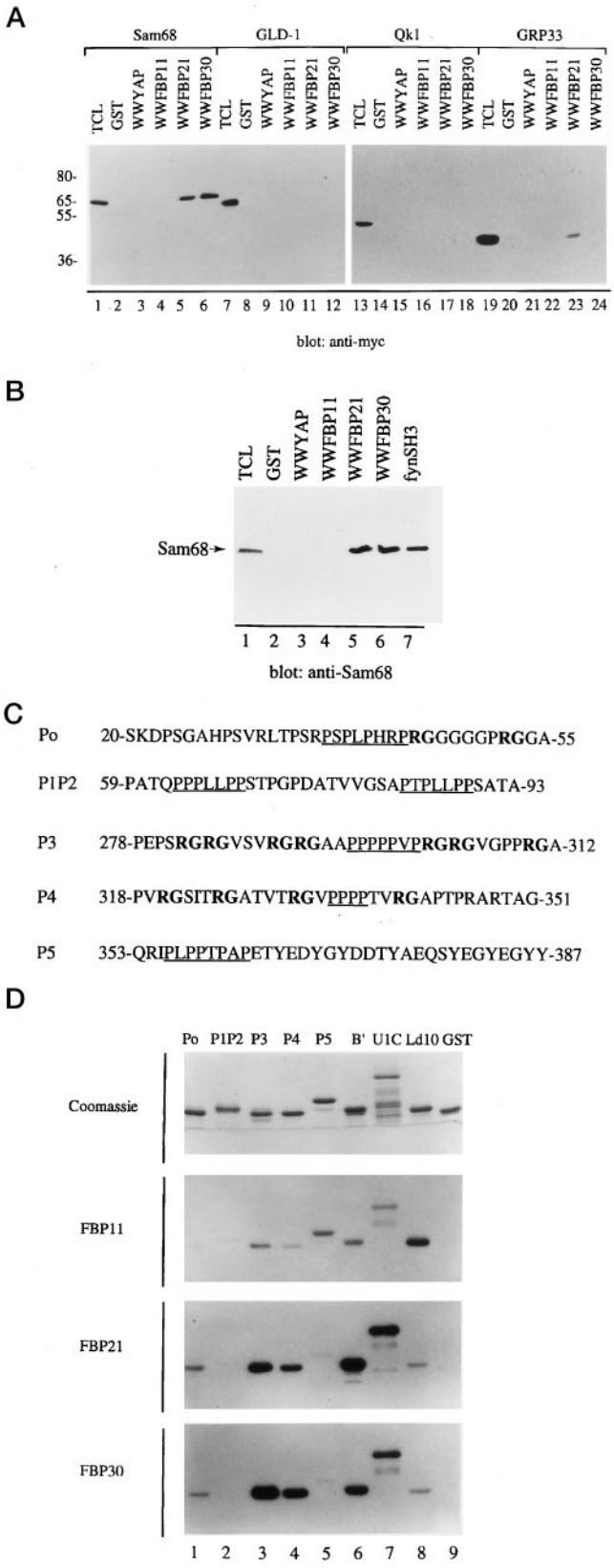

FIG. 1. **Sam68 associates with the WW domains of FBP21 and FBP30.** *A*, Sam68, but not other GSG proteins, including GLD-1, Qk1, and GRP33, associate with the WW domains of FBP21 and FBP30. HeLa cells transfected with myc-tagged Sam68, GLD-1, Qk1, and GRP33 were analyzed by GST pull-down assays using GST alone, and GST fusion proteins containing the WW domains of YAP, FBP11, FBP21, and FBP30 covalently coupled to beads. An aliquot of the total cell lysates (*TCL*) as well as the bound proteins were analyzed by SDS-PAGE and visualized by immunoblotting with anti-myc antibodies. The migration of the molecular mass markers is shown on the *left* in kDa. *B*, endogenous Sam68 associates with the WW domains of FBP21 and FBP30. Untransfected HeLa cells were lysed, and the cell lysates were incubated with beads containing GST alone or GST fusion proteins containing the WW domains of YAP, FBP11, FBP21, FBP30, or the SH3 domain of $p59^{fyn}$ as indicated. After multiple washes, the bound proteins were separated by SDS-PAGE and analyzed by immunoblotting with anti-Sam68 antibodies. The migration of Sam68 is indicated with an *arrow*. *C*, the amino acid sequences from Sam68 that were appended to GST resulting in GST-P0, -P1P2, -P3, -P4, and -P5 are shown. The proline-rich motifs are *underlined*, and the arginine-glycine (RG) repeats are shown in *bold letters*. *D*, determination of the relative affinities of the Sam68 proline motifs for the WW domains of FBP11, FBP21, and FBP30. GST alone or GST fusion proteins containing Sam68 P0, -P1P2, -P3, -P4, or -P5, as well as recognized ligands of FBP WW domain including GST-SmB′, -U1C, and -Ld10 were separated by SDS-PAGE, transferred to nitrocellulose, and either stained with Coomassie to visualize equal loading of the GST fusion proteins or incubated with ^{32}P labeled WW domain of FBP11, FBP21, or FBP30 in overlay assays. The binding of the WW domains to the GST fusion proteins was visualized by autoradiography.

33). We next investigated whether endogenous Sam68 bound to the WW domains of FBP21 and FBP30 with an affinity similar to that which it binds to the SH3 domain of $p59^{fyn}$. We found that this was indeed the case, as comparable levels of cellular Sam68 were observed in GST pull-down assays using the $p59^{fyn}$ SH3 domain or the FBP21 or FBP30 WW domains (Fig. 1*B*). These interactions are likely to occur through one or more of six distinctive proline-rich regions that occur within Sam68 (Fig. 1*C*). Inspection of the primary amino acid sequence of Sam68 revealed no PPLP or PPPY motifs, which is consistent with the fact that the WW domains of YAP and FBP11 that interact with these motifs do not associate with Sam68 (Fig. 1, *A* and *B*).

WW Domains Bind Proline-rich Sequences That Are Flanked by RG Repeats—Sam68 is reported to contain five proline-rich motifs (P1–P5) (23), and closer inspection reveals another motif in the N terminus of the molecule (P0). The amino acids surrounding these motifs are shown in Fig. 1*C*. To test the binding specificity of these Sam68 motifs, individual proline-rich sequences were linked as fusion proteins to GST and tested in a "blot overlay" assay for their ability to interact with the WW domains of FBP11, FBP21, and FBP30. Equal amounts of purified recombinant GST proteins containing Sam68 P0 to P5 and the known WW domain ligands SmB′, U1C, and Ld10 (10) were separated by SDS-PAGE and stained with Coomassie. Duplicate gels were transferred onto a polyvinylidene difluoride membrane and incubated with the indicated ^{32}P-labeled GST-WW domain (Fig. 1*D*). Blot overlay assays revealed that Sam68 proline-rich motifs P3 (RGRGAAPPPPPVPRGRG) and P4 (RGVPPPPTVRG) are the major binding sites for these WW domains of FBP21 and FBP30 (Fig. 1*D*). We noticed that both of these proline-rich motifs were flanked by multiple RG (arginine and glycine) repeats (Fig. 1*C*), dimers that are favored sites for dimethylation (40). In addition, human Sam68 expressed in Sf9 cells has been shown to contain asymmetric N^G,N^G-dimethylarginine residues (41). These findings prompted us to further investigate the nature of Sam68 methylation.

Sam68 Is Arginine-methylated by PRMT1 in Vitro—Using the yeast RMT1 and the mammalian PRMT1 protein arginine *N*-methyltransferases, we asked whether the arginine residues within the Sam68 proline-rich motifs were potential sites of methylation *in vitro*. The GST fusion proteins, containing the sequences depicted in Fig. 1*C*, were incubated with recombinant enzyme in the presence of the methyl donor *S*-adenosyl-L-[*methyl*-^{3}H]methionine. The analysis of the methylation reactions by SDS-gel electrophoresis revealed that P0, P3, and P4, all of which contain RG repeats, are methylated by both yeast and mammalian recombinant protein arginine *N*-meth-

yltransferases, whereas P1P2- and P5-containing fusion proteins are not methylated (Fig. 2*A*, *lower panel*). For the P0 and P3 methyl acceptors, we found that the RMT1 and PRMT1 enzymes were both very effective; however, PRMT1 was less effective in methylating the P4-containing fusion protein (Fig. 2*A*, *lanes 11* and *12*). In addition, we determined by amino acid analysis of these *in vitro* reaction products that the P0, P3, and P4 reactions are predominantly N^G,N^G-[*methyl*-^{3}H]dimethylarginine (asymmetric) with smaller amounts of N^G-[*methyl*-^{3}H]monomethylarginine present (Fig. 2*B* and data not shown).

Arginine Methylation Affects SH3 but Not WW Domain Binding—The proximity of RG repeats to proline-rich motifs suggests that protein-protein interactions between Sam68 and SH3 or WW domain-containing proteins may be regulated by arginine methylation. To test this hypothesis, we concentrated on the P3 proline-rich domain. The P3 motif binds the SH3 domains of p59^{fyn} and PLCγ (23) as well as the WW domains of FBP21 and FBP30 (Fig. 1), and is a good substrate for the type I protein arginine *N*-methyltransferase (Fig. 2). In addition, structure-function studies carried out by Weng *et al.* (7) have shown that Asp-99 of the Src SH3 domain (which forms a salt bridge with an arginine residue flanking the Pro-*X*-*X*-Pro motif of the P3 motif) is important for binding specificity. This study identified the P3 motif of Sam68 as an arginine-containing Asp-99-dependent binding site (7) and, therefore, a candidate for the hypothesized methylation-regulated interaction.

To test the possibility noted above, gel overlay assays were performed with probes of biotinylated P3 peptides, which were synthesized either with all the arginine residues asymmetrically dimethylated (P3*) or unmethylated (P3). Streptavidin-horseradish peroxidase was used as the secondary reagent to detect binding of the peptides to a panel of WW domains (FBP11, FBP21, and FBP30) and SH3 domains (PLCγ, p59^{fyn}, and the Vav C-terminal SH3), which were separated by SDS-PAGE (Fig. 3*A*). Each lane contained the same amount of GST fusion protein (data not shown). The unmethylated Sam68 P3 peptide bound the SH3 domains of PLCγ and p59^{fyn}, consistent with previous studies (23). Moreover, the P3 peptide bound the WW domains of FBP30 and FBP21 with a preference for the WW domain of FBP30. The Sam68 P3 peptide did not bind the C-terminal SH3 domain of Vav, the FBP11 WW domains, or GST alone, which served as negative controls (Fig. 3*A*). By comparison, the arginine-methylated peptide (P3*) did not bind the SH3 domain of PLCγ and bound the SH3 domain of p59^{fyn} with reduced affinity, whereas the binding of P3 to FBP30 WW domain remained unaffected by arginine methylation (the FBP21 WW domain may be slightly sensitive to the methylation status of the P3 ligand). These results indicate that the modification of arginine residues by methylation can interfere with the binding of a proline-rich ligand to the SH3 domains of PLCγ and p59^{fyn}. Analysis of the Sam68 P4 proline-rich motif yielded similar results, with arginine methylation not having an affect on FBP30 WW domain binding, but inhibiting Fyn SH3 domain binding (Fig. 3*A*).

To determine if these findings would hold true under native conditions, we made use of surface plasmon resonance as measured on a BIAcore 2000 Biosensor®. Biotinylated P3 and P3* peptides were immobilized to the surface of different flow cells of a streptavidin-coated sensor chip, thus allowing the same sample to flow over both peptides. Both peptides are thereby exposed to identical concentrations of each fusion protein containing either an SH3 or a WW domain. The binding profiles of these interactions (Fig. 3*B*) correspond well with the specificity results obtained using a blot overlay approach (Fig. 3*A*). The most dramatic effect is seen with the SH3 domain of PLCγ, which demonstrates little or no interaction with the methylated P3* peptide. The binding of P3 to the p59^{fyn} SH3 domain was also affected by arginine methylation, but to a lesser extent than that observed with the SH3 domain of PLCγ. The capacity of the FBP30 WW domain to bind the P3 peptide is not affected by arginine methylation, thus demonstrating the integrity of

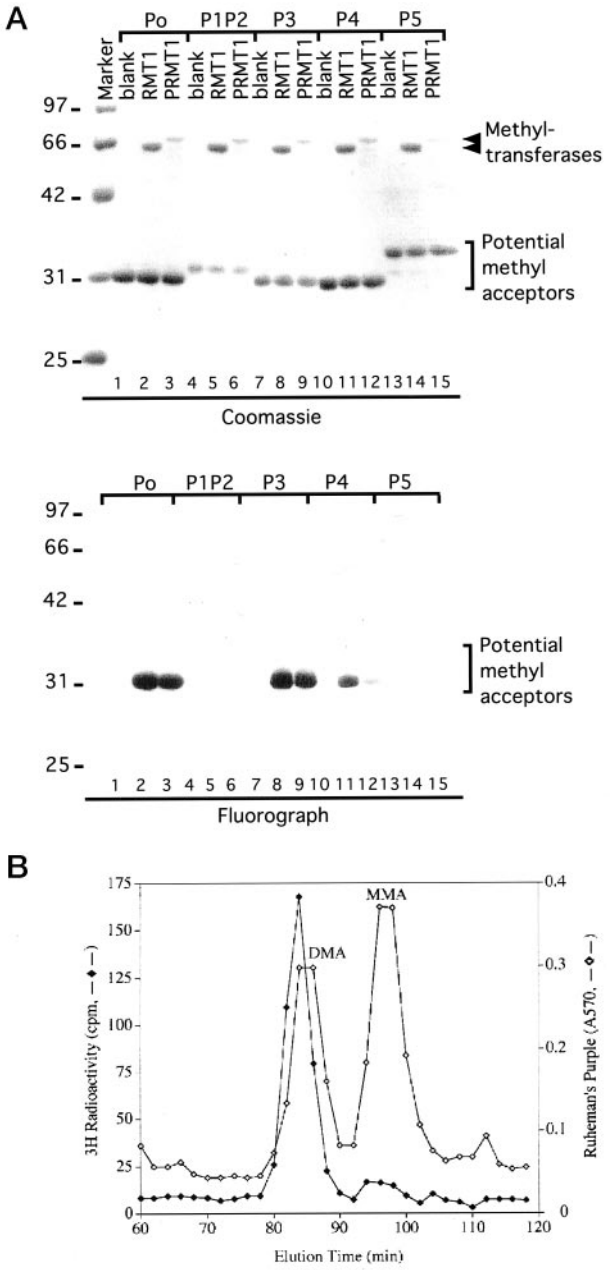

FIG. 2. **Sam68 is arginine-methylated *in vitro* by PRMT1 and RMT1.** *A*, methylation of Sam68 proline motifs *in vitro*. Recombinant yeast RMT1 and mammalian PRMT1 methyl-arginine transferases were incubated with Sam68 proline motifs *in vitro* in the presence of [^{3}H]AdoMet. The proteins were separated by SDS-PAGE, the gel stained with Coomassie (*upper panel*), and the methylated proteins visualized by fluorography (*lower panel*). The molecular mass markers are shown on the *left* in kDa. The positions of the methyltransferase and the potential methyl acceptors are shown. *B*, identification of the ^{3}H-methylated arginine residues in substrate P3, determined by amino acid analysis. The GST fusion protein containing proline motif P3 of Sam68 was methylated *in vitro* with PRMT1 and hydrolyzed with 6 N HCl. The presence of MMA and DMA (*closed diamonds*) was observed by fractionating the hydrolyzed amino acids on a sulfonated polystyrene column using unlabeled MMA and DMA as standards (*open diamonds*). Similar results were seen when motif P0 and P4 were used as methyl acceptors (data not shown).

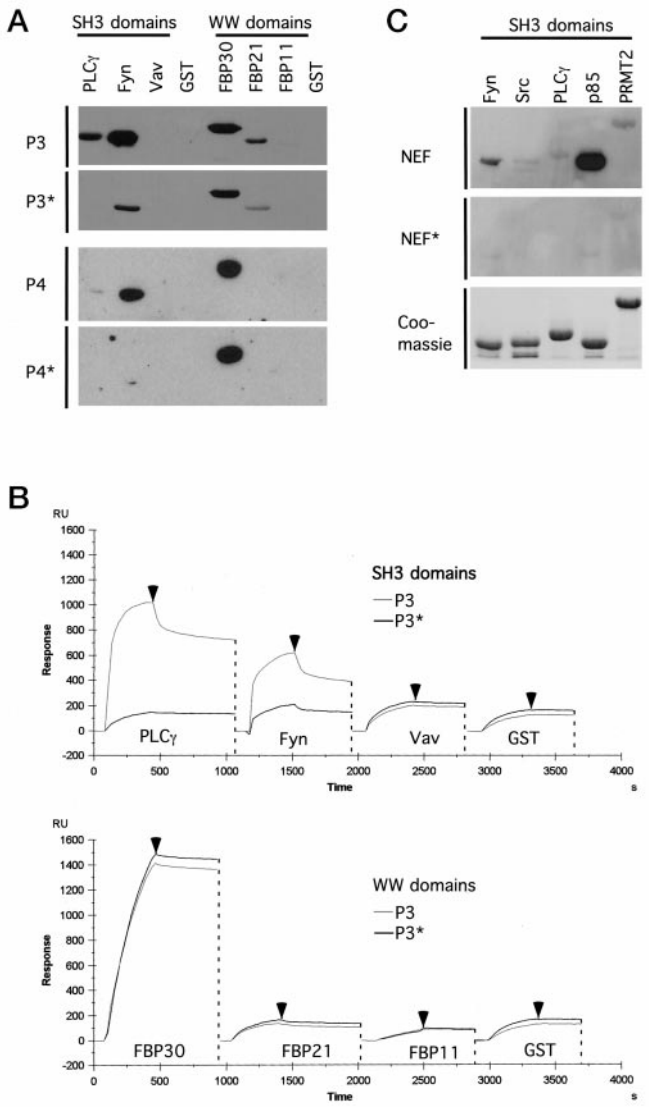

FIG. 3. **Arginine methylation of the Sam68 P3 and P4 peptides prevents SH3 domain binding but has no effect on WW domain binding.** *A*, Arginine methylation of peptides, corresponding to Sam68 proline-rich P3 and P4 motifs, reduces their affinity for SH3 domains. GST alone or GST fusion proteins containing the SH3 domain of PLCγ-1, $p59^{fyn}$, and the C-terminal SH3 domain of Vav were separated by SDS-PAGE. Duplicate filters were probed with either a biotinylated unmethylated P3 or P4 peptide or a peptide where the arginines have been replaced with asymmetrically dimethylated arginines (P3* and P4*). The presence of the bound peptides was visualized using streptavidin-horseradish peroxidase, followed by chemiluminescence. *B*, comparison of the binding profiles of WW and SH3 domains to the P3 peptide in its unmethylated (P3) or an arginine-methylated (P3*) state. BIAcore technology was used to assess the binding of GST-fused WW domains (FBP30, FBP21, and FBP11) and SH3 domains (PLCγ, Fyn, Vav) to P3 and P3* peptides. The peptides were immobilized in different flow cells of a streptavidin-coated BIAcore sensor chip. The *arrowhead* indicates the time at which injection of the analyte is stopped. Between assays, the chip was regenerated with 0.1 M NaOH (*dotted line*). Interaction between ligand and analyte is indicated by an increase in RU value. Time is measured in seconds. The amount of analyte injected in each case was 200 ng/μl. *C*, methylation of a single arginine residue within the Nef proline-rich motif prevents associate with the SH3 domains of p85 and $p59^{fyn}$. GST fusion proteins containing the SH3 domain of Src, PRMT2, p85, $p59^{fyn}$, and PLCγ-1 were separated by SDS-PAGE. Duplicate filters were probed with either a biotinylated unmethylated Nef peptide (NEF) or a peptide where the arginines have been replaced with asymmetrically dimethylated arginines (NEF*). A third gel was Coomassie-stained as a loading control. The presence of the bound peptides was visualized using streptavidin-horseradish peroxidase, followed by chemiluminescence.

the P3* peptide. These BIAcore results further demonstrate that arginine methylation inhibits a subset of SH3 domain interactions.

The $p59^{fyn}$ SH3 domain is sensitive to the arginine methylation of both the P3 and the P4 sequence (Fig. 3*A*). These peptides, however, contain multiple arginine residues. To determine if a single arginine methylation event is sufficient to disrupt an SH3 domain interaction and to better understand, at the molecular level, how arginine methylation might prevent the binding of proline-rich peptides to SH3 domains, we turned to the $p59^{fyn}$-Nef interaction. The crystal structure of the $p59^{fyn}$ with the HIV-1 Nef protein has been determined (42), and Nef contains one arginine residue within its SH3 domain interacting motif. To test whether the methylation of this single arginine residue had any affect on $p59^{fyn}$ SH3 domain binding, we performed gel overlay assays, as described above, with probes of biotinylated Nef peptides, which were synthesized either with the arginine residue asymmetrically dimethylated (NEF*) or unmethylated (NEF). A panel of SH3 domains (PLCγ, $p59^{fyn}$, Src, p85, and PRMT2) were separated by SDS-PAGE (Fig. 3*C*). Each lane contained the same amount of GST fusion protein. The unmethylated Nef peptide bound the SH3 domains of $p59^{fyn}$ and p85. This binding is abrogated by arginine methylation, thus allowing us to speculate (see under "Discussion" and see Fig. 4) as to the possible mechanisms by which this modification can regulate protein-protein interactions.

DISCUSSION

Methylation of Sam68—We have shown that Sam68 is arginine-methylated *in vitro* in the vicinity of its proline-rich motifs using peptides and GST fusion proteins (P0, P3, and P4) (Fig. 2*A*). It appears that the P0 and P3 motifs are much better substrates for methylation *in vitro* than are those of P4. Three out of the seven RG repeats that flank the Sam68 P3 proline-rich motif contain glycine residues at the *n*-1 position from arginine; P4 has 4 RG repeats preceded by either valine or threonine (Fig. 1*C*). A glycine residue at the *n*-1 position from arginine has been shown to be highly conserved among substrates tested for arginine methylation (40, 43–45). This may account for the difference in methylation between P3 and P4 (Fig. 2*A*). Surprisingly, bands reflecting methylated proteins corresponding to P0 appear similar in relative intensity to those of P3, even though both RG repeats in P0 are preceded by proline rather than glycine residues at *n*-1 positions. This finding suggests that, although many known arginine methyltransferase substrates fit the consensus (F/G)GGRGG(G/F) (30, 45), such a consensus may not accurately depict the enzyme substrate preference and may erroneously constrain the search for other potential substrates. Indeed, a search of protein data bases using overlapping motifs for Src-like SH3 domains and recognition motifs for type I protein arginine *N*-methyltransferase (like those found in Sam68) revealed a number of proteins that might be methylated and, therefore, regulate the binding of SH3 domains as discussed below. These proteins include hnRNP K and the Wiskott-Aldrich syndrome protein, both of which are SH3 domain ligands (32, 33, 46).

Selective Regulation of Protein-Protein Interactions by Arginine Methylation—Phosphorylation events within the domain itself (47) or adjacent to a P*XX*P motif (48) have been shown to play a role in the regulation of SH3 domain binding. Arginine methylation is apparently an irreversible modification, unlike protein phosphorylation (30). If this is the case, then this permanent modification provides a fixed switch, which could relieve competitive pressure or prevent protein-protein interactions altogether by increasing the complexity of motifs that harbor critical arginine residues. Our data indicate that the interplay between a subset of proline-rich motifs and the Src-

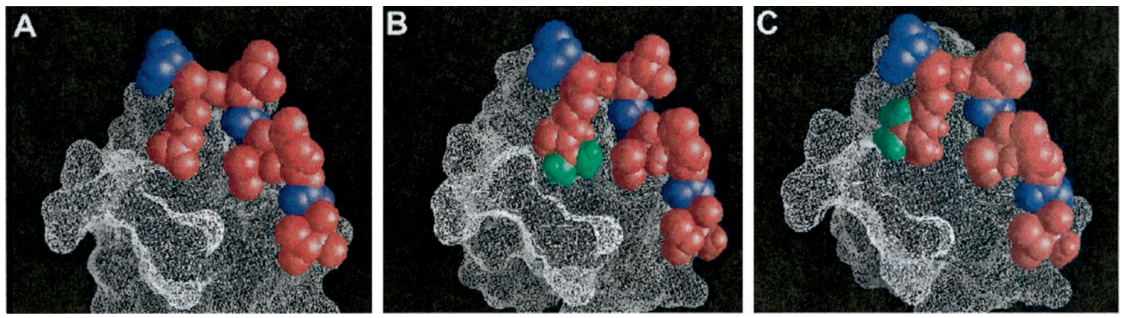

FIG. 4. **A model for arginine methylation inhibition of SH3 domain binding.** *A*, structure of the Fyn SH3 domain bound to a portion of the HIV-1 Nef ligand. Coordinates are from the Fyn-Nef complex (Protein Data Bank accession code 1AVZ; Ref. 42). The molecular surface of the Fyn SH3 domain is shown in mesh representation, and the interacting portion of Nef (sequence ^{71}TPQVPLRP^{78}) is shown with prolines colored *purple* and other atoms colored *red*. *B*, asymmetric methylation of one of the two acceptor nitrogens on the Arg guanidino group is shown with the methyl groups colored *green*. In this position, there is collision between the methyl groups and the SH3 surface, which can be accommodated only by partially obliterating the adjacent proline binding pocket. *C*, asymmetric methylation of the alternative nitrogen on the Arg guanidino group. This would sterically impair the key salt bridge formed between this nitrogen and a conserved acidic residue within the subset of SH3 domains that bind Arg-containing proline sequences. (Figure was constructed using Grasp; see Ref. 75).

like SH3 domains might be influenced by arginine methylation.

The SH3 domain module has apparently not extensively evolved as a component of proteins with nuclear functions, but rather is mainly found in cytoplasmic signaling and cytoskeletal proteins. A noteworthy exception includes c-Abl, where its SH3 domain does not require the presence of arginine residues in its ligands (8, 50). WW domains, on the other hand, are commonly found in nuclear proteins associated with splicing and transcriptional co-activation. These spliceosome-associated proteins include PRP40, FBP11, FBP21, and CA150 (10, 51–53), and the transcription activators are YAP and Npw38 (54, 55). Sam68 is predominantly nuclear but is thought to shuttle between the cytoplasm and the nucleus (56, 57). The fact that two Sam68 proline-rich sites (P3 and P4) bind both WW domains and SH3 domains supports the hypothesis that some SH3 and WW domains might compete for similar binding sites (9). Although the function of Sam68 is only suspected, it does become tyrosine-phosphorylated during mitosis and associates with several SH3 and SH2 domain-containing proteins (22–24, 33). Sam68 also binds RNA as a multimer, and this binding is regulated by tyrosine phosphorylation (34, 58). These observations suggest that Sam68 might function as an adapter protein for Src kinase in the cytoplasm or during mitosis (23, 25). The RNA binding function and the association with WW domains are probably nuclear functions of Sam68. Thus, the methylation of Sam68 may be a mechanism by which its cytoplasmic functions are switched to predominantly nuclear functions by preventing the interaction with cytoplasmic SH3 domain-containing proteins.

A Mechanism for Inhibition of SH3 Binding by Arginine Methylation—The exact mechanism by which asymmetric arginine dimethylation inhibits the binding of Pro-Arg-rich polypeptides to SH3 domains (but not to WW domains) is unknown. One possible explanation, however, emerges from analysis of the x-ray and NMR structures of several SH3 domains and their ligands (Fig. 4). A large subset of SH3 domains show specificity for an Arg residue located either 2–3 residues N-terminal (so-called class I ligands) or 1–2 residues C-terminal (so-called class II ligands) to the consensus P*XX*P binding motif (6, 8, 59). In either binding orientation, the ligand's arginine guanidino group makes a key salt bridge with a conserved Asp or Glu residue in the SH3 domain, equivalent to Asp-99 in the Src-SH3 domain (7, 42). The aliphatic portion of the Arg side chain packs along the back side of a conserved Trp residue (Trp-118 in Src), which forms a critical component of one proline binding site.

The structure of the Fyn SH3 domain with the interacting portion of the HIV-1 Nef protein is shown in Fig. 4*A* (42). Dimethylation on one of the two potential ω-nitrogen acceptor sites on the Nef ligand's Arg residue (*panel B*) results in collision with the SH3 interacting surface. Accommodating these bulky methyl groups would require movement of the conserved Trp residue with partial obliteration of the proline-binding pocket. In contrast, dimethylation of the other ω-nitrogen acceptor site on the Arg residue (*panel C*) sterically interferes with the Arg-Asp salt bridge. Consequently, either modification would impair SH3-ligand binding for these subsets of SH3 domains. It is also conceivable that dimethylation increases the energetic or entropic barrier for the ligand to adopt a left-handed polyproline II helix upon SH3 binding, since many ligands are structurally disordered prior to binding (42, 60). A similar model explaining why identical methylation events do not inhibit WW-domain binding awaits the structures of WW domain complexes with Pro/Arg-rich ligands.

The Role of Arginine Methylation—Five protein arginine methyltransferases have been identified in mammalian cells: PRMT1, PRMT2 (HRMT1L1), PRMT3, CARM1, and JBP1 (61–65). The arginine methyltransferase CARM1 associates with the p160 family of nuclear hormone receptor coactivators; this association enhances transcriptional activation by nuclear receptors (61). Methylation has been implicated in cellular signaling by Pollack *et al.* (63), who have shown that the Jak-binding protein 1 can act as a protein methyltransferase.

Immunolocalization studies have demonstrated that endogenous PRMT1 is predominantly nuclear and PRMT3 is cytoplasmic (65). In the nucleus the major population of arginine-methylated proteins are the hnRNPs. hnRNPs are a collection of about 20 nuclear proteins that bind pre-mRNA while it is in the nucleus and account for about 65% of the total N^G,N^G-dimethylarginine found in the nucleus (27, 66). The prominence of this modification in the hnRNP population points to its functional importance (12% of hnRNP arginine residues are methylated; Ref. 66). Indeed, Shen *et al.* (29) recently provided evidence that arginine methylation may directly or indirectly facilitate the export of some hnRNP proteins in a nuclear export assay, possibly through the prevention of cleavage by trypsin and other proteolytic enzymes (67–71). Therefore, it is conceivable that unmethylated proteins exported from the nucleus are subject to proteolysis within the cytoplasm. Another role for arginine methylation might involve mediating protein-RNA interactions (72–74). It has been shown that recombinant hnRNP A1 proteins missing their RGG boxes are incapable of binding to single-stranded RNA or promoting alternative splicing (74). However, methylation of the RGG box of Hrp1p, a yeast hnRNP, does not alter either its affinity or specificity for RNA ligands (49).

We have demonstrated a place for arginine methylation in the control of a particular class of protein-protein interaction. It is possible that proteins that coat pre-mRNA and accompany it out of the nucleus require the "pleiotropic" protection that arginine methylation provides in combating both proteolytic cleavage and the retention by cytoplasmic SH3 domain-containing proteins, thus allowing shuttling proteins a safe trip back into the nucleus.

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REFERENCES

1. Pawson, T., and Scott, J. D. (1997) *Science* **278,** 2075–2080
2. Musacchio, A., Saraste, M., and Wilmanns, M. (1994) *Nat. Struct. Biol.* **1,** 546–551
3. Yu, H., Chen, J. K., Feng, S., Dalgarno, D. C., Brauer, A. W., and Schreiber, S. L. (1994) *Cell* **76,** 933–945
4. Macias, M. J., Hyvonen, M., Baraldi, E., Schultz, J., Sudol, M., Saraste, M., and Oschkinat, H. (1996) *Nature* **382,** 646–649
5. Sparks, A. B., Rider, J. E., Hoffman, N. G., Fowlkes, D. M., Quillam, L. A., and Kay, B. K. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 1540–1544
6. Feng, S., Chen, J. K., Yu, H., Simon, J. A., and Schreiber, S. L. (1994) *Science* **266,** 1241–1247
7. Weng, Z., Rickles, R. J., Feng, S., Richard, S., Shaw, A. S., Schreiber, S. L., and Brugge, J. S. (1995) *Mol. Cell. Biol.* **15,** 5627–5634
8. Alexandropoulos, K., Cheng, G., and Baltimore, D. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 3110–3114
9. Bedford, M. T., Chan, D. C., and Leder, P. (1997) *EMBO J.* **16,** 2376–2383
10. Bedford, M. T., Reed, R., and Leder, P. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 10602–10607
11. Chen, H. I., and Sudol, M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 7819–7823
12. Sudol, M., Bork, P., Einbond, A., Kastury, K., Druck, T., Negrini, M., Huebner, K., and Lehman, D. (1995) *J. Biol. Chem.* **270,** 14733–14741
13. Staub, O., Dho, S., Henry, P., Correa, J., Ishikawa, T., McGlade, J., and Rotin, D. (1996) *EMBO J.* **15,** 2371–2380
14. Gavva, N. R., Gavva, R., Ermekova, K., Sudol, M., and Shen, C. J. (1997) *J. Biol. Chem.* **272,** 24105–24108
15. Chan, D. C., Bedford, M. T., and Leder, P. (1996) *EMBO J.* **15,** 1045–1054
16. Ermekova, K. S., Zambrano, N., Linn, H., Minopoli, G., Gertler, F., Russo, T., and Sudol, M. (1997) *J. Biol. Chem.* **272,** 32869–32877
17. Lu, P. J., Zhou, X. Z., Shen, M., and Lu, K. P. (1999) *Science* **283,** 1325–1328
18. Arning, S., Gruter, P., Bilbe, G., and Kramer, A. (1996) *RNA* **2,** 794–810
19. Di Fruscio, M., Chen, T., Bonyadi, S., Lasko, P., and Richard, S. (1998) *J. Biol. Chem.* **273,** 30122–30130
20. Ebersole, T. A., Chen, Q., Justice, M. J., and Artzt, K. (1996) *Nat. Genet.* **12,** 260–265
21. Jones, A. R., and Schedl, T. (1995) *Genes Dev.* **9,** 1491–1504
22. Fumagalli, S., Totty, N. F., Hsuan, J. J., and Courtneidge, S. A. (1994) *Nature* **368,** 871–874
23. Richard, S., Yu, D., Blumer, K. J., Hausladen, D., Olszowy, M. W., Connelly, P. A., and Shaw, A. S. (1995) *Mol. Cell. Biol.* **15,** 186–197
24. Taylor, S. J., and Shalloway, D. (1994) *Nature* **368,** 867–871
25. Taylor, S. J., Anafi, M., Pawson, T., and Shalloway, D. (1995) *J. Biol. Chem.* **270,** 10120–10124
26. Reddy, T. R., Xu, W., Mau, J. K. L., Goodwin, C. D., Suhasini, M., Tang, H., Frimpong, K., Rose, D. W., and Wong-Staal, F. (1999) *Nat. Med.* **5,** 635–642
27. Liu, Q., and Dreyfuss, G. (1995) *Mol. Cell. Biol.* **15,** 2800–2808
28. Abramovich, C., Yakobson, B., Chebath, J., and Revel, M. (1997) *EMBO J.* **16,** 260–266
29. Shen, E. C., Henry, M. F., Weiss, V. H., Valentini, S. R., Silver, P. A., and Lee, M. S. (1998) *Genes Dev.* **12,** 679–691
30. Gary, J. D., and Clarke, S. (1998) *Prog Nucleic Acids Res. Mol. Biol.* **61,** 65–131
31. Aletta, J. M., Cimato, T. R., and Ettinger, M. J. (1998) *Trends Biochem. Sci.* **23,** 89–91
32. Van Seuningen, I., Ostrowski, J., Bustelo, X. R., Sleath, P. R., and Bomsztyk, K. (1995) *J. Biol. Chem.* **270,** 26976–26985
33. Weng, Z., Thomas, S. M., Rickles, R. J., Taylor, J. A., Brauer, A. W., Seidel-Dugan, C., Michael, W. M., Dreyfuss, G., and Brugge, J. S. (1994) *Mol. Cell. Biol.* **14,** 4509–4521
34. Chen, T., Damaj, B. B., Herrera, C., Lasko, P., and Richard, S. (1997) *Mol. Cell. Biol.* **17,** 5707–5718
35. Guan, K. L., and Dixon, J. E. (1991) *Anal. Biochem.* **192,** 262–267
36. Gary, J. D., Lin, W. J., Yang, M. C., Herschman, H. R., and Clarke, S. (1996) *J. Biol. Chem.* **271,** 12585–12594
37. Gary, J. D., and Clarke, S. (1995) *J. Biol. Chem.* **270,** 4076–4087
38. Kaelin, W. G., Jr., Krek, W., Sellers, W. R., DeCaprio, J. A., Ajchenbaum, F., Fuchs, C. S., Chittenden, T., Li, Y., Farnham, P. J., Blanar, M. A. *et al.* (1992) *Cell* **70,** 351–364
39. Sparks, A. B., Hoffman, N. G., McConnell, S. J., Fowlkes, D. M., and Kay, B. K. (1996) *Nat. Biotechnol.* **14,** 741–744
40. Najbauer, J., Johnson, B. A., Young, A. L., and Aswad, D. W. (1993) *J. Biol. Chem.* **268,** 10501–10509
41. Wong, G., Muller, O., Clark, R., Conroy, L., Moran, M. F., Polakis, P., and McCormick, F. (1992) *Cell* **69,** 551–558
42. Arold, S., Franken, P., Strub, M. P., Hoh, F., Benichou, S., Benarous, R., and Dumas, C. (1997) *Structure* **5,** 1361–1372
43. Ghosh, S. K., Syed, S. K., Jung, S., Paik, W. K., and Kim, S. (1990) *Biochim. Biophys. Acta* **1039,** 142–148
44. Rawal, N., Rajpurohit, R., Lischwe, M. A., Williams, K. R., Paik, W. K., and Kim, S. (1995) *Biochim. Biophys. Acta* **1248,** 11–18
45. Kim, S., Merrill, B. M., Rajpurohit, R., Kumar, A., Stone, K. L., Papov, V. V., Schneiders, J. M., Szer, W., Wilson, S. H., Paik, W. K., and Williams, K. R. (1997) *Biochemistry* **36,** 5185–5192
46. Banin, S., Truong, O., Katz, D. R., Waterfield, M. D., Brickell, P. M., and Gout, I. (1996) *Curr. Biol.* **6,** 981–988
47. Wu, Y., Spencer, S. D., and Lasky, L. A. (1998) *J. Biol. Chem.* **273,** 5765–5770
48. Comer, A. R., Ahern-Djamali, S. M., Juang, J. L., Jackson, P. D., and Hoffmann, F. M. (1998) *Mol. Cell. Biol.* **18,** 152–160
49. Valentini, S. R., Weiss, V. H., and Silver, P. A. (1999) *RNA* **5,** 272–280
50. Sawyers, C. L., McLaughlin, J., Goga, A., Havlik, M., and Witte, O. (1994) *Cell* **77,** 121–131
51. Neubauer, G., King, A., Rappsilber, J., Calvio, C., Watson, M., Ajuh, P., Sleeman, J., Lamond, A., and Mann, M. (1998) *Nat. Genet.* **20,** 46–50
52. Kao, H. Y., and Siliciano, P. G. (1996) *Mol. Cell. Biol.* **16,** 960–967
53. Abovich, N., and Rosbash, M. (1997) *Cell* **89,** 403–412
54. Komuro, A., Saeki, M., and Kato, S. (1999) *Nucleic Acids Res.* **27,** 1957–1965
55. Yagi, R., Chen, L. F., Shigesada, K., Murakami, Y., and Ito, Y. (1999) *EMBO J.* **18,** 2551–2562
56. McBride, A. E., Schlegel, A., and Kirkegaard, K. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 2296–2301
57. Chen, T., Boisvert, F. M., Bazett-Jones, D. P., and Richard, S. (1999) *Mol. Biol. Cell* **10,** 3015–3033
58. Wang, L. L., Richard, S., and Shaw, A. S. (1995) *J. Biol. Chem.* **270,** 2010–2013
59. Feng, S., Kasahara, C., Rickles, R. J., and Schreiber, S. L. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 12408–12415
60. Renzoni, D. A., Pugh, D. J., Siligardi, G., Das, P., Morton, C. J., Rossi, C., Waterfield, M. D., Campbell, I. D., and Ladbury, J. E. (1996) *Biochemistry* **35,** 15646–15653
61. Chen, D., Ma, H., Hong, H., Koh, S. S., Huang, S. M., Schurter, B. T., Aswad, D. W., and Stallcup, M. R. (1999) *Science* **284,** 2174–2177
62. Lin, W. J., Gary, J. D., Yang, M. C., Clarke, S., and Herschman, H. R. (1996) *J. Biol. Chem.* **271,** 15034–15044
63. Pollack, B. P., Kotenko, S. V., He, W., Izotova, L. S., Barnoski, B. L., and Pestka, S. (1999) *J. Biol. Chem.* **274,** 31531–31542
64. Scott, H. S., Antonarakis, S. E., Lalioti, M. D., Rossier, C., Silver, P. A., and Henry, M. F. (1998) *Genomics* **48,** 330–340
65. Tang, J., Gary, J. D., Clarke, S., and Herschman, H. R. (1998) *J. Biol. Chem.* **273,** 16935–16945
66. Boffa, L. C., Karn, J., Vidali, G., and Allfrey, V. G. (1977) *Biochem. Biophys. Res. Commun.* **74,** 969–976
67. Rajpurohit, R., Lee, S. O., Park, J. O., Paik, W. K., and Kim, S. (1994) *J. Biol. Chem.* **269,** 1075–1082
68. Baldwin, G. S., and Carnegie, P. R. (1971) *Science* **171,** 579–581
69. Brostoff, S., and Eylar, E. H. (1971) *Proc. Natl. Acad. Sci. U. S. A.* **68,** 765–769
70. Lischwe, M. A., Cook, R. G., Ahn, Y. S., Yeoman, L. C., and Busch, H. (1985) *Biochemistry* **24,** 6025–6028
71. Merrill, B. M., Lopresti, M. B., Stone, K. L., and Williams, K. R. (1987) *Int. J. Pept. Protein Res.* **29,** 21–39
72. Rajpurohit, R., Paik, W. K., and Kim, S. (1994) *Biochem. J.* **304,** 903–909
73. Ghisolfi, L., Joseph, G., Amalric, F., and Erard, M. (1992) *J. Biol. Chem.* **267,** 2955–2959
74. Mayeda, A., Munroe, S. H., Caceres, J. F., and Krainer, A. R. (1994) *EMBO J.* **13,** 5483–5495
75. Nicholls, A., Sharp, K. A., and Honig, B. (1991) *Proteins* **11,** 281–296