Interaction of p190A RhoGAP with eIF3A and Other Translation Preinitiation Factors Suggests a Role in Protein Biosynthesis

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The negative regulator of Rho family GTPases, p190A RhoGAP, is one of six mammalian proteins harboring so-called FF motifs. To explore the function of these and other p190A segments, we identified interacting proteins by tandem mass spectrometry. Here we report that endogenous human p190A, but not its 50% identical p190B paralog, associates with all 13 eIF3 subunits and several other translational preinitiation factors. The interaction involves the first FF motif of p190A and the winged helix/PI1 domain of eIF3A, is enhanced by serum stimulation and reduced by phosphatase treatment. The p190A/eIF3A interaction is unaffected by mutating phosphorylated p190A-Tyr308, but disrupted by a S296A mutation, targeting the eIF3A interaction is unaffected by mutating phosphorylated eIF3A and several other translational preinitiation factors. The interaction involves the first FF motif of p190A and the winged helix/PI1 domain of eIF3A, is enhanced by serum stimulation and reduced by phosphatase treatment. The p190A/eIF3A interaction is unaffected by mutating phosphorylated p190A-Tyr308, but disrupted by a S296A mutation, targeting the only other known phosphorylated residue in the first FF domain. The p190A/eIF3 complex is distinct from eIF3 complexes containing 56K1 or mammalian target of rapamycin (mTOR), and appears to represent an incomplete preinitiation complex lacking several subunits. Based on these findings we propose that p190A may affect protein translation by controlling the assembly of functional preinitiation complexes. Whether such a role helps to explain why, unique among the large family of RhoGAPs, p190A exhibits a significantly increased mutation rate in cancer remains to be determined.

Members of the Rho GTPase family act as binary switches to control many cell biological processes. They perform this function by cycling between GTP-bound “on” and GDP-bound “off” states. Proteins that regulate this cycling include guanine nucleotide exchange factors (GEFs),³ which activate Rho family members by eluting Rho-bound GDP and replacing it with the more abundant GTP, and by GTPase activating proteins (GAPs), which enhance the low intrinsic GTPase activity of Rho family members, causing their inactivation. In their GTP-bound active state Rho family members are capable of interacting with a diverse set of effector proteins, which mediate their various effects (1, 2).

Remarkably, the human genome includes 68 genes encoding RhoGAP-like proteins (2, 3). Among the first discovered, p190A RhoGAP (encoded by the ARHGAP35 gene and hereafter referred to as p190A) was identified as a tyrosine-phosphorylated 190-kDa protein that forms a complex with p120 RasGAP in Src-transformed or growth factor-stimulated cells (4–6). The 1499-residue human p190A protein includes an N-terminal GTPase-like segment, followed by four so-called FF domains (7), and a C-terminal RhoGAP catalytic region (8). No recognized motifs map to the ~700 amino acids between the FF domain array and the RhoGAP segment, but parts of this region have been implicated in p120 RasGAP binding, in the regulation of RhoGAP substrate specificity and activity (9, 10) and, together with two adjacent FF domains, in controlling the functionally important localization of p190A to F-actin protrusions (11). Interestingly, a survey of somatic mutations in 4,742 exome sequences from 21 tumor types identified ARHGAP35 as a novel, highly significantly mutated gene in 15% of endometrial cancers, as well as in 2% of the total tumor cohort (12). This finding was in line with earlier reports that had implicated p190A as a potential tumor suppressor (13–15).

Conserved from yeast to mammals, FF domains are ~60 amino acid protein-protein interaction motifs characterized by two conserved phenylalanine residues (7). Arrays of FF domains are found in just six human proteins, namely in the TCERG1 and TCERG1L transcription elongation regulators, in the PRPF40A and PRPF40B splicing factors, and in the ~50% identical p190A and p190B RhoGAP proteins. Others previously found that p190A interacts with the serum-inducible transcription factor TFII-I via its first FF domains, and that this interaction is disrupted upon PDGF-stimulated phosphorylation of Tyr-308 within this protein segment (16). However, whether growth factor regulated cytoplasmic sequestration of PIC, preinitiation complex; 56K1, ribosomal protein S6 kinase 1; FDR, false discovery rate; IP, immunoprecipitation.

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³ This article contains supplemental Tables S1 and S2.
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³ The abbreviations used are: GEF, GDP/GTP exchange factor; FF domain, protein segment characterized by two conserved phenylalanine residues; GAP, GTPase activating protein; mTOR, mammalian target of rapamycin; PBB, phosphate binding buffer; PCI, proteasome, COP9 signalosome, eIF3;
p190A RhoGAP Interacts with eIF3A

Results

Proteomic Identification of p190A-associated Proteins—In HeLa cell extracts fractionated by differential centrifugation, p190A predominantly resides in the detergent-soluble membrane fraction, with some protein also found in the nuclear pellet and cytosol (Fig. 1A). A similar distribution was previously reported for Rat-1 fibroblast p190A (6). To identify proteins associated with endogenous p190A, we used a p190A antibody affinity matrix to purify potential complexes from the detergent-soluble membrane fraction of serum-starved and growth factor-stimulated HeLa cells, using a non-immune IgG affinity matrix as a control. After extensive washing, bound proteins were eluted, size fractionated by SDS-gel electrophoresis, and visualized by silver staining. In both growth factor-stimulated and serum-starved samples, a complex mixture of proteins was eluted from the p190A but not the control matrix (shown for the growth factor-stimulated sample in Fig. 1B). For identification the affinity-enriched and SDS-PAGE-fractionated proteins were subjected to analysis by microcapillary liquid chromatography tandem mass spectrometry (LC-MS/MS). Remarkably, the 20 identified proteins with the highest number of MS/MS spectra (spectral counts) include p190A/ARHGAP35 itself and 14 translation preinitiation complex subunits (17), including 11 of the 13 eIF3 subunits (18), two eIF4F subunits (eIF4A1 and eIF4G1), and poly(A)-binding protein PABPC1 (Table 1). No previously reported p190A/ARHGAP35 interacting proteins were among the top 20 identified proteins, whereas supplemental Table S1, listing 230 proteins with at least two spectral counts in either sample, includes β2-catenin/CTNNB1 (19), present in both growth factor-stimulated and serum-starved samples, and p120 RasGAP/RASA1, represented by three unique peptides in the growth factor-stimulated sample. Of note, whereas the spectral counts for p190A in the former sample were only 128 and 172, suggesting that serum stimulation enhances the interaction between p190A and its associated proteins. To determine whether a complex between p190A and translation initiation factors is also detectable in unfractionated HeLa extracts, we performed a second proteomic analysis. In this analysis, performed without SDS-PAGE size fractionation of affinity-enriched proteins, a largely overlapping set of translation preinitiation factors, including 12 eIF3 subunits, was detected, although in this case only eIF3A and eIF3B ranked among the first 20 identified proteins.

TFII-I is the only function of p190A FF domains is called into question by the fact that zebraria, Drosophila melanogaster, and Caenorhabditis elegans p190 RhoGAP orthologs include similar FF domain arrays, but lack obvious TFII-I orthologs. Thus, to shed further light on the functions of this and other p190A segments, we set out to identify proteins associated with endogenous human p190A. Here we report our unexpected finding that p190A, but not its 50% identical p190B paralog, interacts with eIF3A and exists in a complex with several other translational preinitiation complex subunits. We mapped the interacting segments to an N-terminal p190A region that includes the first FF domain, and to the winged helix-fold of the eIF3A PCI (Proteasome, COP9 signalosome, eIF3) domain. The interaction between p190A and eIF3A is stable and unaffected by mutating Tyr308. However, a non-phosphorylatable S296D mutation, targeting the only other known phosphorylated residue in the first FF domain, disrupts the interaction, whereas a non-phosphorylatable S296A, but not a phosphomimicking S296D mutation, disrupted the interaction, which together with other evidence argues that the interaction between p190A and eIF3A is regulated by phosphorylation. Because some functionally important preinitiation complex subunits are missing from the detected complex, we propose a role for p190A in translational regulation, by controlling the assembly of functional preinitiation complexes.

TABLE 1

Multiple translation preinitiation complex subunits are among the 20 most abundant proteins associated with membrane-associated p190A

| UniProt ID | Gene | Gene ID | SC starved | SC stimulated |
|------------|------|---------|------------|---------------|
| Q14152     | EIF3A| 8661    | 128        | 172           |
| P55884–2   | EIF3B| 8662    | 92         | 141           |
| Q04637–3   | EIF4G1| 1981   | 80         | 128           |
| Q99613     | EIF3C| 8663    | 84         | 117           |
| Q97262     | EIF3L| 51386   | 71         | 99            |
| P60228     | EIF3E| 3646    | 47         | 81            |
| O15371     | EIF3D| 8664    | 38         | 68            |
| O00303     | EIF3E| 8665    | 32         | 73            |
| Q7L2H7     | EIF3M| 10480   | 32         | 69            |
| O15372     | EIF3H| 8667    | 30         | 67            |
| Q9NRY4–2   | ARHGAP35| 2909  | 45         | 52            |
| Q5JSZ5     | PRRC2B| 84726  | 40         | 56            |
| O75821     | EIF3G| 8666    | 26         | 61            |
| P11940–2   | PABPC1| 26986  | 30         | 50            |
| Q13347     | EIF3I| 8668    | 30         | 50            |
| Q8IVT2     | MISP  | 126353  | 24         | 49            |
| P07837     | TUBB  | 203068  | 19         | 37            |
| P60842     | EIF4A1| 1973   | 12         | 40            |
| P60709     | ACTB  | 60      | 11         | 40            |
| P49792     | RANBP2| 5903   | 16         | 29            |

Multiple translation preinitiation complex subunits are among the 20 most abundant proteins associated with membrane-associated p190A. Shown are gene symbols, locus IDs, and spectral counts (SC) observed in serum-starved and growth factor-stimulated samples.
p190A RhoGAP Interacts with eIF3A

Among the top 35 p190A-associated proteins (supplemental Table S2).

The fact that both proteomic experiments detected all but one or two eIF3 subunits suggests that p190A associates with the eIF3 complex, which serves as a scaffold for larger preinitiation complexes involved in mRNA 5′-cap binding and in the scanning mechanism responsible for identifying the correct translational start codon (18). The presence of PABPC1 and eIF4F subunits eIF4A1 and eIF4G1 also implicates such a p190A-containing complex in the initiation of mRNA translation. Further supporting this idea, supplemental Table S1 includes the two remaining eIF3 subunits (eIF3J and eIF3K), the remaining eIF4F subunit (eIF4E; the mRNA cap-binding protein), two paralogs of eIF4A1 (eIF4A2 and eIF4A3), the PABPC1 paralog PABPC4, and 40S small ribosome subunit proteins RPSA, RPS3, RPS3A, RPS8, RPS14, RPS18, RPS19, and RPS25. Finally, PRRC2C (proline-rich coiled-coil 2C), a member of the mRNA interactome (20) was found associated with eIF3A, eIF3E, eIF3F, eIF3H, and eIF3I in previous proteomic studies (21–24).

Confirmation of the p190A/eIF3 Association—To rule out an obvious artifact, we tested whether the rabbit polyclonal antibody used to affinity purify p190A complexes cross-reacts with one or more translation factors. To rule this out, we used a mouse monoclonal antibody to precipitate p190A from HeLa cells, and analyzed the presence of eIF3A, eIF3B, eIF3C, eIF3D, and eIF3H by immunoblotting. In this and additional co-immunoprecipitation (co-IP) experiments we also increased the stringency of washes by using ionic detergent containing RIPA rather than Triton buffers. Under these more stringent conditions all five tested eIF3 subunits again co-precipitated with p190A (Fig. 2A). Moreover, p190A, eIF3B, eIF3C, eIF3D, and eIF3H each co-precipitated with eIF3A (Fig. 2B). Translational preinitiation complexes include the 40S small ribosomal subunit and bind to the 5′ cap of mRNAs to initiate translation (17). However, RNase treatment did not affect the co-precipitation of p190A and the above five eIF3 subunits, arguing that complex formation does not require RNA (not shown). Arguing that the detected interaction is specific for p190A, no association between the five tested eIF3 subunits and endogenous p190B RhoGAP was detected in similar co-IP experiments (Fig. 2C).

Because a role for p190A in translational initiation was unanticipated, we performed a further specificity test by repeating the co-IP experiments with HeLa cells in which either p190A or eIF3A had been knocked down. Using ON-TARGET plus siRNA Smartpools, near complete knockdown of p190A and substantial knockdown of eIF3A was achieved (Fig. 3, A and C). In cells lacking p190A, the p190A antibody did not co-IP any of the five analyzed eIF3 subunits, and vice versa, p190A and the four other eIF3 subunits were not precipitated by the eIF3A antibody when eIF3A was knocked down (Fig. 3, B and D). The latter observation is compatible with the recent finding that eIF3A knockdown disintegrates the entire eIF3 complex (25). Taken together, these results provide strong evidence that the detected interaction occurs in vivo.

p190A Interacts with eIF3A: Mapping of the Responsible Domains—Because the 20 proteins with the highest number of spectral counts in Table 1 include 11 of 13 eIF3 subunits, we speculated that p190A might interact with one or more subunits of this complex. As a first test, we analyzed the stability of the interaction between p190A and eIF3A, eIF3B, eIF3C, eIF3D, and eIF3H by subjecting p190A immune complexes to washes of increasing ionic strength. Fig. 4 shows that among the five subunits analyzed, the interaction between p190A and the eIF3A scaffolding subunit of the eIF3 functional core (26) is most stable, with some interaction detectable even after 0.6 M NaCl RIPA buffer washes. Retention of eIF3A in p190A immune complexes even after removal of other eIF3 subunits suggests it may represent a key interface for p190A/eIF3 interaction. Consistent with this interpretation, RNAi knockdown of eIF3A abolished the interaction of p190A with four other eIF3 subunits (Fig. 3D). To obtain more direct evidence that p190A associates with eIF3A, and to map the interacting protein domains, we performed pulldown experiments with GST fusion proteins representing various p190A segments (Fig. 5A). In these assays, eIF3A was brought down by a GST fusion protein representing the p190A N-terminal GTPase domain, and more strongly by a protein representing the adjacent FF domains. However, eIF3A did not detectably interact with a

FIGURE 2. eIF3 subunits co-precipitate with p190A, and vice versa. A, five endogenous HeLa cell eIF3 subunits co-immunoprecipitate with endogenous p190A. B, p190A, eIF3B, eIF3C, eIF3F, and eIF3H co-precipitate with eIF3A. C, endogenous p190B precipitated from HeLa cells does not detectably associate with the five tested eIF3 subunits.
fusion protein representing the RhoGAP domain, or with GST itself (Fig. 5A). In similar experiments with His-tagged eIF3A fusion proteins, p190A interacted with an eIF3A fusion protein that included the winged helix-fold (27) of its PCI domain (residues 404–495), but not with fusion proteins representing other parts of the protein (Fig. 5B). Although these results do not rule out that other translation factors contribute to the detected interaction, they do suggest that an interaction between the N-terminal p190A GTPase/FF domain segment and the eIF3A PCI domain winged helix-fold plays an important role.

Nucleotide Binding to the p190A GTPase Domain Is Not Required for eIF3A Binding—The N-terminal GTPase domain of p190A binds GTP, and although two guanine nucleotide-binding deficient S36N or 201KCD203 to 201DCV203 mutants both retained in vitro RhoGAP activity (8, 28), the former mutant lacked obvious in vivo activity when expressed in NIH 3T3 cells (28). Because eIF3A interacts with an N-terminal p190A segment that includes the GTPase domain, we tested whether the ability to bind nucleotide affects eIF3A binding. To this end we introduced the previously characterized 201DCV203 double mutation in our p190A1–512-GST fusion construct, and analyzed its ability to pulldown eIF3A. Suggesting that nucleotide binding is not required for eIF3A binding in this setting, no difference in the amount of eIF3A pulled down by the wild-type and mutant GST proteins was observed (Fig. 6A).

**The First FF Domain of p190A Binds eIF3A**—Next, we analyzed which of the four FF domains are required for eIF3A binding. As shown in Fig. 6B, of four GST proteins that each cover a single FF domain, only the one representing the first such domain brought down eIF3A. This first FF domain includes Tyr308, whose PDGF-stimulated phosphorylation was previously found to disrupt an interaction with transcription factor TFII-I (16). For this reason, and because growth factor stimulation appeared to cause an increase in the amount of several proteins associated with p190A (Table 1 and supplemental Table S1), we analyzed whether λ-phosphatase treatment of HeLa extracts prior to immunoprecipitation affected the p190A-eIF3A association. Arguing that phosphorylation of one or both proteins enhances their interaction, the amount of eIF3A co-precipitating with p190A was reduced by phosphatase treatment. The addition of a phosphatase inhibitor mixture partially, but reproducibly, prevented this reduction (Fig. 6, C and D).

A p190A S296A Mutation Abolishes the Interaction with eIF3A—The interaction between p190A and TFII-I is disrupted by growth factor-stimulated Tyr308 phosphorylation (16). However, non-phosphorylatable Y308A and phosphomimetic Y308D mutants of the p190A272–324-GST fusion protein representing the first FF domain, showed little difference in their ability to pulldown p190A (Fig. 7A). The only other p190A first FF domain residue identified as phosphorylated in the PhosphoSitePlus database is evolutionary conserved Ser296. Arguing that phosphorylation of one or both proteins enhances their interaction, the amount of eIF3A co-precipitating with p190A was reduced by phosphatase treatment. The addition of a phosphatase inhibitor mixture partially, but reproducibly, prevented this reduction (Fig. 6, C and D).

A p190A S296A Mutation Abolishes the Interaction with eIF3A—The interaction between p190A and TFII-I is disrupted by growth factor-stimulated Tyr308 phosphorylation (16). However, non-phosphorylatable Y308A and phosphomimetic Y308D mutants of the p190A272–324-GST fusion protein representing the first FF domain, showed little difference in their ability to pulldown p190A (Fig. 7A). The only other p190A first FF domain residue identified as phosphorylated in the PhosphoSitePlus database is evolutionary conserved Ser296. Arguing that phosphorylation of one or both proteins enhances their interaction, the amount of eIF3A co-precipitating with p190A was reduced by phosphatase treatment. The addition of a phosphatase inhibitor mixture partially, but reproducibly, prevented this reduction (Fig. 6, C and D).

**FIGURE 3.** Co-precipitation of p190A and eIF3 subunits is prevented by knockdown of eIF3A or p190A. HeLa cells were transfected with a siRNA control, or with p190A (panels A and B) or eIF3A (panels C and D) siRNA Smartpools. After transfection cells were cultured for 72 h prior to lysis and precipitation for immunoprecipitation. Panels A and C show the levels of p190A or eIF3A knockdown achieved. Panels B and D show that p190A or eIF3A knockdown prevents the co-precipitation of the proteins indicated to the right. The control lanes in panels B and D show p190A co-precipitation of the indicated eIF3 subunits from untreated HeLa cells.

**FIGURE 4.** Among five tested eIF3 subunits, the p190A-eIF3A co-precipitation is most resistant to increased ionic strength. HeLa cell extracts were prepared by lysis cells in 50 mM NaCl containing RIPA buffer. p190A immunoprecipitates collected on Protein A-Sepharose beads were washed with 0.2 ml of RIPA buffer of the indicated ionic strengths, after which 50 µl of the washes were subjected to SDS-PAGE and immunoblotting using the indicated antibodies.
eIF3A, whereas no detectable eIF3A co-IPed with the S296A mutant (Fig. 7C). Finally, suggesting that p190A directly interacts with eIF3A, probing a blot of size fractionated HeLa proteins with a wild-type or S296A mutant p190A272–324-GST fusion protein, followed by detection of bound proteins with an anti-GST antibody, detected a 170-kDa protein (the size of eIF3A) with the wild-type but not with the mutant probe (Fig. 7D). The wild-type probe was also detected a smaller protein the identity of which remains unknown, but which may rep-resent an eIF3A degradation product or one of the other five PCI domain-containing eIF3 subunits. Thus, we conclude that a single in vivo phosphorylated serine residue in the first FF domain plays an important role in promoting the interaction of p190A and eIF3A.

**FIGURE 5. Mapping of the p190A and eIF3A interacting segments.** The indicated segments of p190A (diagram in panel A) and eIF3A (panel B) were produced as GST fusion proteins. The central region of p190A could not be made as a soluble fusion protein and has not been analyzed. The anti-GST or anti-His tag blots in panels A and B show the sizes and relative amounts of GST proteins used in the pull downs. The eIF3A Western blot (WB) in panel A show that eIF3A interacts weakly with the p190A GTPase domain (residues 1–268), and more strongly with a fusion protein representing all four FF domains (residues 268–512). Interaction is also observed with a fusion protein representing p190A residues 1–512. The p190A Western blot in panel B shows that only the winged helix (WH) segment of the eIF3A PCI domain interacts with p190A in this assay.
complexes that include eIF3, eIF4F, and the eIF2-GTP
the 40S small ribosomal subunit and eukaryotic initiation factor
involves binding of a 43S preinitiation complex, consisting of
occurs mainly at the rate-limiting initiation step, which

mutually exclusive.
taining S6K1, mTOR
and p190A was evident (Fig. 8
was part of either the eIF3
mTOR—
mTOR complex. Not
/H11032
-2-end m7GpppN cap

p190A May Control the Assembly of Functional Preinitiation
Complexes—The regulation of eukaryotic mRNA translation
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the 40S small ribosomal subunit and eukaryotic initiation factor
complexes that include eIF3, eIF4F, and the eIF2-GTP-Met-
tRNA ternary complex, to the mRNA 5'-end m7GpppN cap
structure. Subsequently, with the help of additional factors, the
43S complex scans along the 5'- untranslated region (5'-UTR)
to locate the initiation codon. Upon interacting with this codon,
eIF5-stimulated GTP hydrolysis of the eIF2-GTP-Met-tRNA
complex results in preinitiation complex dissociation, allowing
the joining of the 60S ribosomal subunit to form an 80S ribo-
some poised for translational initiation (30). Suggesting that
p190A may prevent the assembly of functional 43S preinitiation
complexes, although the p190A-associated proteins identified
include all subunits of the eIF3 and eIF4F complexes, no pep-
tides representing several other functionally important sub-
units were detected. Thus, peptides representing eIF1, eIF1A,
all three subunits of the eIF2 complex (eIF2S1, eIF2S2, and
eIF2S3), eIF4B, eIF5, and eIF5B are absent from supplemental
Table S1, although some of these proteins are included as low
spectral count, low confidence interactors in the more exten-
sive list (supplemental Table 2) of potential p190A-associated
proteins detected in our second proteomic analysis. For three of
the above proteins (eIF1A, eIF5, and eIF5B) we confirmed co-IP
with eIF3A, but not with p190A (Fig. 9). We hypothesize,
because p190A interacts with the winged helix/PCI domain
of eIF3A, and because cryoelectron microscopy suggests that a
horseshoe-like arrangement of the PCI domains of the six PCI
domain containing eIF3 subunits represents a major protein-
protein interaction interface of the eIF3 core complex (31, 32),
that p190A may control the rate of mRNA translation by con-
trolling the assembly of functional 43S preinitiation complexes.
Additional work is required to test the merits of this idea.

Discussion
By functioning as molecular on/off switches, Rho family
GTases play important roles in multiple cell biological pro-
cesses, including cell migration, endocytosis, and cytokinesis
(2). Given the importance of RhoA–C, Rac1–3, Cdc42, and
other Rho family members in cell biology, it may not be surpris-
ing that they are subject to intricate regulation. This is perhaps
best illustrated by the fact that the human genome includes 68
genes encoding RhoGAP-like proteins, and an even larger
number or genes predicting RhoGEFs (2, 33, 34). Many of these
proteins contain a variety of protein and/or lipid interaction
motifs, the significance of which remains largely unexplored.
This is what motivated the proteomic analysis of p190A
RhoGAP reported here.
Our finding that endogenous HeLa cell p190A associates
with multiple translational preinitiation complex subunits was
unexpected, given that no previous evidence had suggested a
role for p190A or its GTase substrates in the rate-limiting
initiation step of mRNA translation. However, our finding that
the first FF domain of p190A interacts with the winged helix/
PCI domain of eIF3A, and our identification of p190A Ser296 as
an in vivo phosphorylated residue required for this interaction,
clearly implicates p190A in this process. Mechanistically,
p190A might serve as an eIF3 anchoring protein that directs the
localized translation of mRNAs. Alternatively, by preventing
the assembly of complete preinitiation complexes, p190A
might affect the overall rate of mRNA translation. Our results
suggest the latter, given that eIF1, eIF1A, and other important
translational preinitiation complex subunits appear to be miss-
ing from the p190A complex. Suggestive evidence that the
p190A/eIF3A interaction is enhanced by phosphorylation
argues that the translation-related role of p190A may be regu-
lated by upstream signals. However, the sequence around Ser\textsuperscript{296} does not resemble any consensus phosphorylation site and no candidate protein kinase that might target this residue was suggested upon analysis of the human p190A sequence with the Scansite-3 algorithm.

Unlike p190A, its closely related p190B paralog does not interact with eIF3 subunits. The specificity of the p190A/eIF3A interaction is further underscored by the fact that the interaction between full-length p190A and eIF3A is abolished by a p190A-S296A missense mutation. Ser\textsuperscript{296} is conserved in human p190B and Drosophila p190, but whether this residue is phosphorylated in these proteins is not known. Although p190A-Ser\textsuperscript{296} phosphorylation was detected in a global phosphoproteomic study (35), it remains unknown whether Ser\textsuperscript{296} phosphorylation changes upon serum stimulation. We note that FF domains are phosphoserine binding motifs (36, 37). The human eIF3A PCI domain GST fusion protein that binds p190A spans residues 404–495, and although none of the 29 phosphorylation sites identified in a proteomic analysis of the human eIF3 complex fall within this region (38), two global phosphoproteome studies identified eIF3A PCI domain residue Ser\textsuperscript{492} as being phosphorylated (39, 40). Whether phosphorylation of this residue affects the interaction between eIF3A and p190A is worth exploring.

Two proteins with canonical roles in translational control, S6K1 and mTOR, associate with the eIF3 complex in a mutually exclusive manner. Thus, in serum-starved cells, inactive S6K1 associates with the eIF3 complex, whereas stimulation of cells with a variety of factors leads to the formation of an eIF3 complex containing mTORC1 subunits mTOR and Raptor. Subsequent mTORC1-mediated activation of S6K1 causes the latter protein to dissociate and phosphorylate eIF4B (29), among other targets. More recent analyses implicated eIF3F as the eIF3 subunit responsible for both mTOR and S6K1 binding (41, 42).
**p190A RhoGAP Interacts with eIF3A**

Because p190A-eIF3 complex formation involves the eIF3A subunit, it is conceivable that eIF3 simultaneously interacts with p190A and S6K1 or mTOR. However, our proteomic analysis did not detect S6K1 or mTORC1 subunit peptides, and S6K1 or mTOR did not co-precipitate with p190A, suggesting that eIF3 forms distinct complexes with p190A, S6K1, or mTOR.

Others previously reported that ~20% of eIF3A associates with endoplasmic reticulum and plasma membranes, with the remainder of the protein localized in the cytosol (43). If p190A preferentially interacts with membrane-bound eIF3A, this would explain why eIF3 subunits and other preinitiation complex proteins had especially high spectral counts in our proteomic analysis of membrane-associated p190A, compared with our similar analysis of unfractionated HeLa cell extracts (compare supplemental Tables S1 and S2).

One final issue raised by our work is whether an additional translational function explains why a recent exome sequencing survey of 4742 human cancers of 21 distinct types identified the p190A encoding ARHGAP35 gene as the only human RhoGAP gene with a significantly above background mutation rate (12). Work to test whether cancer-associated missense mutations scattered throughout the entire p190A protein differentially affect its eIF3A binding and RhoGAP activity is in progress.

**Experimental Procedures**

**Cell Fractionation**—Approximately 1 × 10^8 80% confluent HeLa cells were kept in serum-free DMEM for 16 h and either used directly, or stimulated with DMEM containing 20 ng/ml of EGF, IGF1, and PDGF (Sigma) for 30 min. Cells were harvested by mechanical scraping, washed 3 times with phosphate-buffered saline (PBS), and allowed to swell in ice-cold hypotonic buffer (20 mM HEPES (pH 7.4), 5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 2 mM Na3VO4, 10 μg/ml each of aprotinin, pepstatin, leupeptin) for 30 min. Cells were disrupted by 20–40 strokes in a Dounce homogenizer and nuclear, cytosolic, and detergent-soluble and -insoluble membrane fractions were prepared by differential centrifugation. Briefly, nuclei were collected by a 10-min spin at 1,000 × g, resuspended in hypotonic buffer, and repelleted. The supernatant was subjected to a 1-h centrifugation at 285,000 × g at 4 °C to generate cytosolic and membrane fractions. The latter was resuspended in 0.5 ml of ice-cold Triton buffer (1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM Na3VO4, 10 μg/ml of aprotinin, pepstatin, and leupeptin) by vigorous pipetting, vortexing, and brief sonication. After a 30-min incubation of ice, another 1-h high-speed spin generated the detergent-soluble supernatant and insoluble pellets. The latter was resuspended in 0.5 ml of Triton buffer plus 0.5% sodium deoxycholate. Cell transfection with Lipofectamine 2000 (Invitrogen) followed the manufacturers protocol.

**Affinity Purification and Mass Spectrometry**—20 μg of affinity-purified rabbit polyclonal p190A antibody (Bethyl Laboratories, number A301-736A) was coupled to 0.2 ml of protein A-Sepharose by overnight incubation in 1% (w/w) formaldehyde at 4 °C. An aliquot representing 3.5 mg of total protein of the detergent-soluble HeLa membrane fraction was added to 0.1 ml of this affinity matrix or to 0.1 ml of a similarly prepared

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**FIGURE 8. p190A, mTOR, and S6 kinase form distinct complexes with eIF3A.** The input lanes in both panels show blots of cell lysates probed with the antisera indicated on the right. The lanes labeled “control IgG” show that eIF3A, p190A, mTOR, or S6 kinase are not precipitated by a non-immune antibody. Panel A shows that p190A co-IPs with eIF3A in both serum-starved and stimulated cells, whereas mTOR and S6 kinase show reciprocal interactions with eIF3A, as previously reported. Panel B shows that unlike eIF3A, mTOR and S6 kinase do not co-precipitate with p190A in either serum-starved or stimulated cells.

**FIGURE 9. Not all preinitiation complex subunits co-precipitate with p190A.** A, eIF3A co-precipitates with p190A, but eIF5, eIF5B, and eIF1A do not. B, all indicated proteins co-precipitate with eIF3A.
non-immune IgG control matrix, and incubated with constant gentle agitation at 4 °C for 4 h. After six washes with Triton buffer containing 10 mM NaF and 1 mM dithiothreitol (DTT), bound proteins were eluted with 0.1 M (pH 2.0) glycine buffer, the eluate neutralized with Tris-HCl (pH 8.0), and size fractionated by 10% SDS-PAGE. Coomassie-stained gel slices covering the entire molecular weight range were processed for analysis by microcapillary liquid chromatography tandem-mass spectrometry (LC-MS2) on an LTQ Velos mass spectrometer following a standard protocol at the Harvard Medical School Taplin Mass Spectrometry Facility. The acquired MS2 spectra were searched against the human UniProt protein sequence database (downloaded 02/04/2014) supplemented with sequences of known contaminants such as porcine trypsin. The target/decoy database approach was applied to control the false discovery rate (FDR) of peptide and protein annotations. To this end the target component of the protein database was complemented with a decoy component consisting of the same protein sequences in reversed C terminus to N terminus order (44). Searching was done using the SEQUEST algorithm (45) and spectra assignments filtered to an FDR of less than 1% using a linear discriminant analysis and combined discriminant score based on the following search score and peptide sequence criteria: Xcorr, dCn, peptide length, and the number of sites missed by trypsin cleavage (46). The probability of a peptide assignment to be correct was calculated using a posterior error histogram and the probabilities for all peptides assigned to a protein were combined to filter the data set for a FDR of less than 1%. Peptides with sequences matching more than one protein in the UniProt database were assigned to the protein with most matching peptides (46). In a subsequent experiment, candidate p190A-associated proteins were similarly analyzed without prior cell fractionation. Rather than subjecting affinity enriched proteins to SDS-PAGE fractionation, proteins were reduced and alkylated in solution basically as described previously (47). Proteins were then precipitated with chloroform/methanol, resuspended in 1 M urea, 50 mM HEPES (pH 8.5), and digested by sequencing grade trypsin (Promega). The generated peptides were analyzed by LC-MS2 in a 70-min gradient on an Orbitrap Fusion mass spectrometer equipped with an EASY-nLC 1000 autosampler/HPLC pump. The mass spectrometer was operated in a data-dependent mode with a full MS spectrum acquired in the Orbitrap followed by MS2 spectra acquired in the linear ion trap on the most abundant ions detected in the full MS spectrum. The data were essentially analyzed as described above except that the mass deviation of peptides were included into the linear discriminant analysis.

siRNA Knockdown—ON-Target plus siRNA SMART pools targeting human p190A or elf3A (Dharmacon-Thermo Scientific) were introduced into HeLa cells using DharmaFECT transfection reagent as specified by the manufacturer. Cells were processed for analysis 72 h after transfection.

Antibodies and Immunological Procedures—For immunoprecipitations 2.5 μg of antibody was coupled to Protein A-Sepharose by overnight incubation at 4 °C. Cells were lysed in 150 mM NaCl RIPA buffer, and cell extracts containing 0.8–1.0 mg of protein were incubated with the immobilized antibody for 4 h at 4 °C, after which the beads were washed six times with RIPA buffer of the same ionic strength supplemented with 10 mM NaF and 1 mM DTT, and processed for SDS-PAGE. Immunoblotting used the following antibodies at 1:1000 dilutions: mouse p190A monoclonal (BD Transduction Laboratories number 610150), rabbit p190A polyclonal (Bethyl Laboratories, number A301-736A), elf3A rabbit polyclonal (Cell Signaling, number 2538), affinity purified elf3B, elf3C, elf3D, and elf3H rabbit polyclonal antibodies (Bethyl Laboratories, numbers A301-760A, A300-376A, A301-758A, and A301-754A), and rabbit polyclonal anti-GST (Cell Signaling, number 2622) or anti-His-tag antibodies (Cell Signaling, number 2366). To analyze the role of phosphatase, protein samples were incubated with A-phosphatase (New England Biolabs) in the presence or absence of the recommended amount of the Halt Phosphatase Inhibitor Mixture (Thermo Scientific) at 30 °C for 30 min. Samples were washed six times with wash buffer prior to further processing. For quantification, films representing three experiments were scanned using an Epson Perfection V750 PRO and analyzed using ImageJ image analysis software. The elf3A band intensities were normalized relative to HA-p190A band intensities.

Recombinant Protein Preparation and Pulldown Experiments—E. coli BL21 cells harboring GST-p190A fusion constructs were induced with 0.2 mM isopropyl 1-thio-β-d-galactopyranoside for 3 h at 37 °C and harvested by centrifugation. Bacterial pellets were sonicated in 50 mM Tris (pH 7.6), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and a mixture of protein inhibitors (Roche Diagnostics). Proteins were purified on glutathione-Sepharose (Sigma) as suggested by the manufacturer. His-tagged elf3A protein segments were expressed in Escherichia coli BL21(DE3) and affinity purified using nickel-nitritolatriacetic acid-agarose (Qiagen) as recommended by the manufacturer. Pulldown experiments were performed by incubating 20 μg of immobilized p190A or elf3A proteins with 1 mg of total protein at 4 °C for 4 h. After six 1-ml 50 mM Tris-HCl (pH 7.6), 200 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 1 mM DTT washes, bound proteins were eluted with 50 mM Tris-HCl (pH 7.6), 500 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 mM EDTA, and 1 mM DTT and processed for SDS-PAGE.

Far Western Analysis—HeLa cell lysate (20 μg of protein) was subjected to SDS-PAGE and transferred to Immobilon PVDF membrane (Millipore). The membrane was washed with Protein Binding Buffer (PBB; 20 mM HEPES/KOH (pH 7.4), 50 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 10% glycerol), incubated for 3 h with PBB containing 5% bovine serum albumin (BSA) at 4 °C, and probed overnight with 10 μg of affinity purified GST-p190a-FF domain fusion protein at 4 °C in 10 ml of PBB containing 5% BSA. The blot was subjected to eight 10-min 0.1% Nonidet P-40 in PBB washes, incubated with anti-GST antibody (1:1000 Cell Signaling), ECL etc.

DNA Constructs and Transfection—The N terminally HA-tagged rat p190A expression vector RChAp190A has been described (48). RChAp190A-S296A and RChAp190A-S296D mutants were created by PCR mutagenesis. GST-p190A constructs were made by subcloning PCR-amplified cDNA fragments into the pGEX-KG vector, using EcoRI and Xhol. The

FEBRUARY 17, 2017 • VOLUME 292 • NUMBER 7 ASBMB JOURNAL OF BIOLOGICAL CHEMISTRY 2687
p190A RhoGAP Interacts with eIF3A

following GST-p190A fusion constructs were generated (numbers in parentheses indicate included amino acids): GST-GTPase(1–268), GST-N terminus (1–541), GST-FF1 (266–541), GST-FF2 (371–419), GST-FF3 (431–480), GST-FF4 (487–533), and GST-GRD (1227–1499). S296A/S296D, Y308A/Y308D, and Y310A/Y310D mutants in both the GST-FF1 and GST-FF1–4 vectors were created by PCR mutagenesis. The nucleotide-binding defective p190A GTPase double mutant has been described previously (8). His₆-tagged human eIF3A constructs were made by inserting PCR-amplified cDNA fragments into the pET28a (Addgene) vector using SacI and NotI. Numbers in parentheses for the following constructs again indicate included amino acids: His-eIF3A N terminus (1–400), His-eIF3A-PCI domain (404–495), His-eIF3A-spectrin repeat (496–924), His-eIF3A-10 AA repeat (926–1175), and His-eIF3A C terminus (926–1383). The integrity of all constructs was verified by sequence analysis.

**Author Contributions**—A. B. conceived and coordinated the study and wrote the paper. P. P. designed, performed, and analyzed most experiments. P. M. designed the cell fractionation and initial mass spectrometry analysis. J. A. W. contributed to experiments analyzing the role of p190A in translation. B. L. and M. B. provided technical assistance. W. H. designed and coordinated the mass spectrometry analysis and wrote the paper. P. P. designed, performed, and analyzed most of the results and approved the final version of the manuscript.

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