Human P54 and P56 proteins are tetratricopeptide proteins that are encoded by two closely related genes, ISG54 and ISG56. These genes are induced strongly but transiently when cells are treated with interferons or double-stranded RNA or infected with a variety of viruses. We observed that, although double-stranded RNA or Sendai virus infection induced the two genes with similar kinetics, their induction kinetics in response to interferon-β were quite different. The induction kinetics by virus infection were also different between two cell lines. Functionally the two proteins were similar. Like P56, P54 bound to the translation initiation factor eIF3 and inhibited translation. However, unlike P56, P54 bound to both the “e” and the “c” subunits of eIF3. Consequently, P54 inhibited two functions of eIF3. Like P56, it inhibited the ability of eIF3 to stabilize the eIF2, GTP, and Met-tRNAi (39, 40). The mouse proteins, on the other hand, bind to eIF3e and block the ability of eIF3 to promote formation of the 48 S pre-initiation complex containing the initiation factor eIF3, eIF3ε, and eIF3c, both of which contain the PCI (proteasome, eukaryotic initiation factor 3) motif.

A major component of the innate immune response to virus infection is the viral stress-inducible genes. These genes are not expressed or expressed at a low level in uninfected cells, but they are transcriptionally induced immediately after virus infection (1). In some cases, even subviral particles or inactivated virions can induce them (2, 3). In addition, viral gene products, such as, double-stranded RNA or Sendai virus infection (1). In some cases, even subviral particles or inactivated virions can induce them (2, 3). In addition, viral gene products, such as, double-stranded RNA (dsRNA) (4) or single-stranded RNA (5) or virally induced cytokines, such as type I interferons (IFNs), can induce many of these genes (6, 7). The different inducers use distinct signaling pathways that converge on the same cis-element in their promoters, the IFN-stimulated response elements (8–11). Members of the IFN regulatory response factors (IRFs) recognize IFN-stimulated response elements and drive transcription either by themselves or in combination with STAT proteins (12). In the cytoplasm, virus infection or dsRNA activates IRF-3 and IRF-7, which dimerize, translocate to the nucleus, and bind to IFN-stimulated response elements (13–15). IFN, on the other hand, uses the transcription factors ISGF-3, composed of STAT-1, STAT-2, and IRF-9, which is activated by the JAK-STAT signaling pathway (16, 17). Activation of IRF-3 or IRF-7 by dsRNA occurs by two partially overlapping but distinct pathways. Extracellular dsRNA is recognized by Toll-like receptor 3, which activates the protein kinase TANK-binding kinase-1 using the adaptor proteins, Toll/IL-1 receptor domain-containing adaptor-inducing IFN-β and TRAF-3 (18–20). TANK-binding kinase-1 phosphorylates IRF-3 on multiple residues (21, 22), but additional phosphorylation of IRF-3 by a phosphatidylinositol 3-kinase 3-kinase-dependent pathway is required for its complete activation (23). Cytoplasmic dsRNA, produced during virus replication, is recognized by the RNA helicases, Rig-I and Mda-5 (24, 25), which use the mitochondria-anchored adaptor protein INF-β promoter stimulator to activate TANK-binding kinase-1 (26). Viral single-stranded RNA is recognized by TLR-7 or TLR-8 leading to the activation of IRF-3 and IRF-7 (27, 28).

Among the most prominent members of the human viral stress-inducible gene family are ISG56 (IFIT1) and ISG54 (IFIT2) (29, 30), both of which are strongly induced in response to IFN, dsRNA, and infection by many viruses (31). Human ISG56 and ISG54 are related and linked genes located on chromosome 10 (32). The encoded proteins P56 and P54 are related, have 42% sequence conservation (1), and contain multiple tetratriocopeptide repeat motifs (33). We have previously reported that human P56, mouse P56, and mouse P54 inhibit translation by inhibiting the action of the initiation factor eIF3, a multisubunit protein complex (34, 35). The P56 proteins bind to one of the two specific subunits of eIF3, eIF3ε, and eIF3c, both of which contain the PCI (proteasome, COP9 signalosome, eukaryotic initiation factor 3) motif (36–38). eIF-3 has several functions in the multistep process of translation initiation, many of which are not affected by the P56 proteins (39, 41, 42). However, binding of human P56 to eIF3ε blocks the ability of eIF3 to stabilize the ternary complex of eIF2, GTP, and Met-tRNAi (39, 40). The mouse proteins, on the other hand, bind to eIF3c and block the ability of eIF3 to promote formation of the 48 S pre-initiation complex containing...
the 40 S ribosomal subunit, the ternary complex, elF4F, and mRNA (41).

In this paper, we report the induction characteristic of human ISG54 and the function of human P54. We observed that the kinetics of induction of ISG54 and ISG56, in response to dsRNA, IFN, and Sendai virus (SeV), were quite distinct. As expected, recombinant human P54 bound to elF3 and inhibited translation. But unexpectedly it bound to both the “e” and the “c” subunits of elF3 and inhibited its two functions: stabilization of the ternary complex and the formation of the pre-initiation complex.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—The commercially available anti-FLAG M2 monoclonal antibody (Sigma) was used at a 1:2000 dilution for detection of elF3c, elF3c deletion mutants, and elF3e. The transfected human P54 was detected by the c-Myc 9E10 monoclonal antibody at a 1:1000 dilution (Santa Cruz Biotechnology, Santa Cruz, CA). A polyclonal antibody raised in goat against purified rabbit elF3 was also used, at a 1:1000 dilution, in these studies. This antibody recognized primarily the p110 subunit of elF3 (elF3c) and other elF3 subunits to a lesser extent (39).

Anti-human P54 polyclonal antibody, was raised by Biosynthesis Inc., Lewisville, TX, against a peptide encoding amino acids 314–331 of human P54 protein. Serum from injected rabbits was collected and tested for their anti-human P54 activity by Western blot; the polyclonal antibody was used at a dilution of 1:2000 in these studies.

**Constructs**—The full-length elF3e and elF3c constructs were described previously (40, 42). Deletion mutants of elF3c were generated as follows: elF3 (712–847 amino acids) by PCR then subcloned into pFLAG-CMV-2 (Kodak Scientific Imaging System) containing FLAG on the N-terminal domain: elF3 (Δ656–860 amino acids) by digesting the full-length of elF3c with PstI and religation.

Human P54 was obtained by reverse transcription-PCR; the cDNA sequence was inserted into Myc-pcDNA3 (42). Human P56 was described previously (41). All constructs were confirmed by having the ligated junctions and the insert sequenced.

**Purification of Recombinant Protein from Escherichia coli**—Human P54 was purified following the same procedure as described previously (42). Briefly, the cDNA encoding the full-length human P54 was subcloned in pET-15b vector (Novagen); the protein was expressed in bacterial strain BL21-DE3 pLys (Novagen) by induction with 1 mM isopropyl β-D-thiogalactopyranoside for 12 h at 30 °C. Human P54 was purified via nickel-affinity chromatography. Protein was then dialyzed against a high glycerol buffer (20 mM Tris-HCl, pH 7.9, 150 mM KCl, 50% glycerol) and stored at −20 °C.

**Cells, Cell Treatments, and Infection**—HT1080 human fibrosarcoma cells (43) and HEK 293 cells were all maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin.

Human IFN-β 1000 units/ml (Calbiochem) and dsRNA [poly(I)-poly(C)] 100 µg/ml (Amersham Biosciences) were added to the culture medium, and the cells were treated for the desired time periods as indicated in the figures. Cells (1 × 10⁶) were infected with Sendai virus (Sendai/Cantell, ATCC VR-907) at 80 HA units/ml for 1 h in serum-free Dulbecco’s modified Eagle’s medium. Medium was removed; the cells were washed, then reseeded with Dulbecco’s modified Eagle’s medium containing 10% serum and incubated for the time periods indicated in the figures.

**Transfection**—4 µg of each expression plasmids were transfected in HT1080 cells using FuGENE 6 (Roche Applied Science), according to the manufacturer’s protocol. After 18 h, cells were collected, and 50 µg of total cell extracts (as determined by the Bradford assay for protein, Bio-Rad) was loaded onto SDS-PAGE for straight immunoblots, otherwise lysates were used for immunoprecipitation as described below.

**Immunoprecipitation and Immunoblotting**—Cell lysates were prepared as described (44). For immunoprecipitation of FLAG-tagged protein 300 µg of whole cell extract was mixed with 500 µl of low salt buffer (20 mM Tris/HCl, pH 7.5, 50 mM KCl, 200 mM NaCl, 1 mM EDTA, 20% glycerol, 0.05% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, and protease inhibitor) and 20 µl of M2 anti-FLAG-Sepharose beads (Sigma, pre-soaked with 3 µg of bovine serum albumin for 15 min) at 4 °C overnight. The immunocomplexes were washed with low salt buffer and resolved by 10 SDS-PAGE followed by Western blotting with antibody against Myc. Alternatively, 300 µg of cell lysate was incubate with 6 µl of antibody against P54 or elF3 in 500 µl of radioimmune precipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris/HCl, pH 8.0, 0.4 mM phenylmethylsulfonyl fluoride, and protease inhibitor) at 4 °C overnight. 20 µl of protein A-agarose (Roche Applied Science) was then added, followed by 4-h incubation at 4 °C. Samples were washed with radioimmune precipitation assay buffer and subjected to denaturing gel electrophoresis followed by Western blotting with an antibody against FLAG, or Myc.

**RNase Protection Assay**—RNA was isolated using RNAzol B reagent according to the manufacturer’s protocol (Tetlast, Friendswood, TX). RNase protection assays (RPAs) were performed with the RPA III kit (Ambion, Austin, TX) following the manufacturer’s protocol. The antisense probe to human P56 and P54 was generated first by cutting the cDNA with MboII and PvuII, respectively, and then transcribing with T7 or SP6 RNA polymerase respectively. For each sample, 20 µg of total RNA was used for RPA; protected mRNA levels were visualized by autoradiography and quantified by a PhosphorImager using Molecular Dynamics ImageQuaNT Software (Amersham Biosciences). Levels of actin mRNA were used as internal control; the antisense probe used was generated by cutting the cDNA with Hinfl, and then transcribing with SP6 RNA polymerase.

**In Vitro Translation Inhibition Assay**—A 0.5–µg aliquot of luciferase mRNA (Promega) was added to a 25-µl reaction of a rabbit reticulocyte lysate in vitro translation reaction (Promega) in the presence of dialysis buffer (0 mM) or increasing amounts of recombinant purified protein (100, 400, and 800 nM). The in vitro translations were performed with nucleasate-treated rabbit reticulocyte lysate under conditions recommended by the manufacturer. The reaction mixture was incubated at 30 °C for 2 h; following translation the newly synthesized 35S-labeled proteins were analyzed by loading 5 µl
of reaction on 10% SDS-PAGE, and incorporated radioactivity was quantitated using ImageQuaNT.

Initiation Factors and 40 S Ribosomal Subunit—eIF2, eIF3, and eIF4F were purified from rabbit reticulocytes lysate as described previously (45, 46). Free 40 S ribosomal subunits were purified using high salt sucrose gradient as described previously (47).

Generation of \([^{14}C]\)Met-tRNA, and \([^{32}P]TRAP\) mRNA—Radiolabeled Met-tRNA, was prepared by using Brewer’s yeast tRNA (Ambion), E. coli aminoacyl-tRNA synthetase, and \([^{14}C]methionine (56 mCi/mmol, PerkinElmer Life Sciences) as described previously (46). A 53-nucleotide-long radiolabeled mRNA transcript for tryptophan RNA-binding attenuation protein (TRAP) (48) was kindly provided by Nick Kaye and Eckhard Jankowsky (Case Western Reserve University, Cleveland, OH).

Ternary Complex Assay—Ternary complex formation was performed as described previously (46). Purified eIF2 (8 pmol, 1.0 \(\mu\)g) was incubated with 10 pmol of \([^{14}C]\)Met-tRNA, and 100 \(\mu\)M GTP in a 100-\(\mu\)l reaction volume containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM MgCl\(_2\), 1 mM dithiothreitol, 0.3 IU of pyruvate kinase, and 4 mM phosphoenolpyruvate. To stimulate ternary complex formation, 30 pmol (20 \(\mu\)g) of purified eIF3 was also added to the reaction. Reactions were incubated for 10 min at 37 \(^\circ\)C and then immediately quenched with 3 ml of ice-cold 20 mM Tris-HCl, pH 7.5, 100 mM KCl, and 5 mM MgCl\(_2\) (quenching buffer). Ternary complex was bound to the nitrocellulose filter (25 mm, 0.45-mm pore size, Millipore) by adding the entire reaction mixture to the filter, followed by vacuum filtration. Filters were washed twice with 5 ml of quenching buffer, followed by vacuum filtration. Filters were dried, and radioactivity was then determined by liquid scintillation spectrometry. To test the effect of human P54 on ternary complex formation, 30 pmol (1.7 \(\mu\)g) of purified human P54 was preincubated with eIF3 for 10 min at 30 \(^\circ\)C prior to the addition to the ternary complex reaction mixture.

Preinitiation Complex Sucrose Gradient Analysis—The analysis was performed as described previously (41). Briefly, 30 pmol of eIF3 was preincubated either alone or with 30 pmol of P54 for 15 min at 30 \(^\circ\)C. 0.7 \(A_{260}\) unit (14 pmol) of purified 40 S subunits was then added, and the mixture was incubated for 10 min at 37 \(^\circ\)C. To this mixture, 30 pmol of Met-tRNA\(_S\), 30 pmol of eIF2 (3.8 \(\mu\)g), and 100 \(\mu\)M GTP were added, and the mixture was incubated further for 15 min at 30 \(^\circ\)C. 30 pmol of eIF4F was then added followed by a 30 min incubation on ice; then 2 pmol of \([^{32}P]TRAP\) mRNA was added to this reaction mixture, and the mixture was further incubated on ice for 30 min. The reaction was layered on 12 ml of 10–25% sucrose gradient, and centrifuged for 16 h at 20,000 rpm at 4 \(^\circ\)C (Beckman SW28Ti rotor). Sucrose gradients (10–25%) were prepared in buffer A (100 mM KCl, 20 mM Hepes-KOH, pH 7.5, 5 mM MgCl\(_2\), 2 mM dithiothreitol, and 100 \(\mu\)M GDPNP), as described previously (49). The gradients were unloaded via upward displacement using 60% sucrose, and UV absorbance was measured at 254 nm with an ISCO flow cell. Fractions of 1 ml were collected then directly measured for radioactivity by liquid scintillation spectrometry.

**FIGURE 1. Different kinetics of induction of ISG54 and ISG56 by IFN-\(\beta\).** Upper panel, HT1080 cells were treated with IFN-\(\beta\) (1000 units/ml) for 6, 12, and 24 h, then total RNA were extracted and used for RNase protection assay for ISG54 or ISG56 mRNAs; actin mRNA was used as the internal control. Lower panel, protected mRNA levels were quantified by phosphorimaging using Molecular Dynamics ImageQuaNT software. The levels of ISG56 and ISG54 mRNAs normalized by the levels of actin mRNA are shown. The highest levels of the two mRNAs were set at 100.

**RESULTS**

ISG56 and ISG54 Are Induced Differently—The kinetics of transcriptional induction of the human ISG56 and ISG54 genes in response to IFN-\(\beta\) treatment of HT1080 cells were measured using a quantitative RNase protection assay for the two mRNAs; actin mRNA served as the internal control (Fig. 1). There were no detectable levels of the ISG56 and the ISG54 mRNAs in untreated cells; both mRNAs were induced strongly by IFN-\(\beta\) treatment for 6 h, although ISG54 mRNA was induced to a higher level. The level of ISG54 mRNA remained constant over 24 h, but the level of ISG54 mRNA dropped sharply with time. These results demonstrated that, although the two genes are related, their kinetics of induction by IFN-\(\beta\) were quite different. These two genes can also be induced by added dsRNA, which signals through TLR-3, or by infection with SeV, which signals through RIG-I. ISG56 mRNA was induced much more strongly than ISG54 mRNA when the inducer was dsRNA, which signals through TLR-3, or by infection with SeV, which signals through RIG-I. ISG56 mRNA was induced much more strongly than ISG54 mRNA when the inducer was dsRNA (Fig. 2A). Unlike the dissimilar kinetics of their induction by IFN-\(\beta\), the two mRNAs behaved similarly in dsRNA-treated cells: the levels at 12 h were similar to those at 6 h and then they dropped sharply between 12 h and 24 h. When the inducer was SeV, a different pattern was observed. The two mRNAs were induced to similar levels at 6 h post infection, and
then the levels of both mRNAs dropped rapidly at 12 h; surprisingly, the levels were a little higher at 24 h and then slowly decreased (Fig. 2B). The second round of minor induction could have been mediated by IFN produced by the infected cells. In HEK 293 cells, the kinetics of induction by SeV were quite different (Fig. 3). Both mRNAs were induced quite strongly, and their levels stayed constant over time. Also, the levels for ISG54 and ISG56 mRNAs were very similar. The results shown in Figs. 1–3 demonstrate that the induction kinetics of the two closely related genes ISG56 and ISG54 were quite distinct. Moreover, the kinetics of their induction were inducer-specific, suggesting that the different signaling pathways, activated by the different inducers, may act upon the promoters of the two genes differently.

**P54 Interacts with eIF3**—To investigate the function of P54, the product of ISG54, we followed the lead of P56, the product of ISG56, which is known to interact with the translation initiation factor eIF3. When epitope-tagged P54 was expressed by transfection, it co-immunoprecipitated with eIF3 but not with pre-immune serum (Fig. 4A). As expected, transfected P56 also co-immunoprecipitated with eIF3 (Fig. 4C). Expression of P54 or P56 did not affect the level of expression of eIF3, as indicated by the level of its “c” subunit (Fig. 4B and D). Human P56 binds to the “e” subunit, but not the “c” subunit of eIF3. In the experiments shown in Fig. 5, we inquired whether the same was true for P54. Different epitope-tagged P54 and eIF3e were expressed in cells (Fig. 5B); when eIF3e was immunoprecipitated, P54 was associated with it (Fig. 5A). Unexpectedly, similar experiments with eIF3c showed that P54 associated with eIF3c as well (Fig. 5C and D); under the same conditions, we observed no interaction between eIF3c and P56 (41). The observed interaction between eIF3c and P54 was confirmed in another experiment. P54 was induced by IFN-β treatment of eIF3c-transfected cells, it was immunoprecipitated by an antibody to endogenous P54, and association of eIF3c was assayed by immunoblotting (Fig. 5E and F). These experiments clearly established that, like P56, P54 could bind eIF3e; but unlike P56, it could also bind eIF3c.

The interaction between eIF3e and P56 is mediated by the PCI domain of the first protein. In the next experiment, we investigated whether the same is true for P54/eIF3c interaction. For this purpose, we designed expression vectors for the PCI domain of eIF3c (PCI) and a mutant protein missing the PCI domain (open triangle) (Fig. 6A). Like the full-length protein, these proteins were expressed as epitope-tagged proteins (Fig. 6C). These proteins were immunoprecipitated, and the presence of P54 was examined. Both of these proteins and the full-length protein (fl) interacted with P54 (Fig. 6B, upper panel). The interaction with the PCI domain appears to be stronger, because, although much less of this protein was expressed (Fig. 6C) and immunoprecipitated (Fig. 6B, lower panel, lane 3), more P54 was co-immunoprecipitated (Fig. 6B, upper panel, lane 3). These results indicate that P54 interacts with at least
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P54 Inhibits Initiation of Translation at Two Steps—For testing the effects of P54 on translation, recombinant polyhistidine-tagged P54 was expressed in bacteria and purified by affinity chromatography. This protein inhibited in vitro translation of luciferase mRNA in a dose-dependent manner (Fig. 7); even at concentrations of <1 mM, translation was inhibited by 85%.

To investigate the biochemical basis of the observed inhibition of translation, we examined the effects of P54 on the two functions of eIF3 that had been shown to be impaired by other members of this family (47, 50). eIF3 stabilizes the ternary complex of eIF2, GTP, and Met-tRNAi (compare bar 3 with bar 2, 4).

FIGURE 7. Inhibition of translation by recombinant P54. Luciferase mRNA was translated in the presence of increasing amounts of purified recombinant P54. Translated radiolabeled luciferase was analyzed by SDS-PAGE and quantified using phosphorimaging (Molecular Dynamics). Translation is shown as a percentage of a dialysis buffer control (100% translation). Data are presented as averages of three experiments.

two regions of eIF3c, the PCI domain and another region in the N-terminal portion of the protein.

P54 Inhibits Initiation of Translation at Two Steps—For testing the effects of P54 on translation, recombinant polyhistidine-tagged P54 was expressed in bacteria and purified by affin-
Fig. 8). As reported previously inclusion of P56 in the reaction completely blocked this stabilization (bar 4, Fig. 8). P54 also blocked it, but only partially (bar 5, Fig. 8). Thus, like P56, P54 could bind to eIF3e and block the same function of eIF3, albeit weakly.

Another function of eIF3 in translation initiation is to facilitate the formation of the 48 S pre-initiation complex (Fig. 9C), composed of the 40 S ribosomal subunit and the 20 S complex of eIF3, ternary complex, eIF4F, and mRNA (Fig. 9A). Formation of the pre-initiation complex was severely inhibited by P54 (Fig. 9D), although there was no effect on the stability of the 20 S complex (Fig. 9B). Thus, like mouse P56 and mouse P54, human P54 could bind to eIF3e and inhibit formation of the pre-initiation complex. The above results demonstrated that P54 inhibited two functions of eIF3, both of which are essential for initiation of protein synthesis.

**DISCUSSION**

ISG54 and ISG56 are widely used as marker genes for detecting signaling by the JAK/STAT pathway or the IRF-3/IRF-7 pathway. Because the genes are evolutionally related and induced by the same signaling pathways and the encoded proteins are similar, it is often assumed that the two genes are interchangeable with respect to their inducibility and functions. The results presented here clearly demonstrated that the above assumptions are only partially correct. Both genes are induced by viral stresses and both inhibit translation, but their induction kinetics and modes of action were quite different.

The induction kinetics of the two genes in response to IFN-β (Fig. 1) were quite different. Both mRNA were induced to high levels after 6 h of treatment, but the ISG56 mRNA level decreased with time, whereas ISG54 mRNA stayed at the same level over 24 h. The levels of the corresponding proteins paralleled the levels of the mRNA (data not shown); induction of P54 was transient, whereas cellular P56 level was high for 24 h. The observed difference in the induction kinetics of ISG56 mRNA and ISG54 mRNA could be caused by differences at the transcriptional level or at post-transcriptional level. For example, it is possible that both mRNAs are induced similarly, but transiently, and ISG54 mRNA turns over much more rapidly than ISG56 mRNA. However, this possibility is unlikely, because the induction patterns of the two mRNA were very similar when dsRNA or SeV was used as the inducer (Fig. 2). If differential stability of the two mRNAs is not the critical factor, one has to conclude that the two genes are induced differentially. Sequence analysis of the promoter regions of the two genes revealed that the ISG54 promoter contains two binding sites for IRF and none for NFκB, whereas the ISG56 promoter contains two IRF-binding sites and one NFκB-binding site.

Another difference was observed between the two cell lines, HT1080 and HEK 293, in induction of these genes by SeV (Fig. 3). Both genes were induced much more strongly in the HEK 293 cells, and the mRNA levels stayed high over 24 h in HEK
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293 cells but not in the HT1080 cells. One can speculate that the observed differences in the kinetics are due to a difference between the two cell lines at the level of down-regulation of gene induction. A potential candidate for such a role is the protein SIKE (Suppressor of IKK epsilon), which blocks IRF-3 activation (51). If the level of SIKE in HT1080 cells is much higher than that in 293 cells, it will account for the observed differences. Since SIKE does not block the NFκB branch of RIG-I signaling, one would predict that the kinetics of induction of a NFκB-driven gene, such as A20 (52), will be similar in the HT1080 and the HEK 293 cells.

One of the unexpected findings was the observation that human P54 interacts with both eIF3e and eIF3c proteins (Fig. 5). Among other members of the P56 family of proteins, we observed high specificity in their interaction with one or the other of the two subunits of eIF3. Human P56 interacts with the “e,” but not the “c” subunit, whereas mouse P56 and mouse P54 proteins interact with the “c,” but not the “e” subunit (40–42). Two other members of this family, human P58 and P60, do not interact with either the “e” or the “c” subunit of eIF3. Thus human P54 is the only family member to date that has been shown to interact with both subunits of eIF3. The interaction with eIF3c was mediated by at least two regions of the protein. As anticipated, the PCI domain of the eIF3c interacts with human P54 strongly; but the rest of the protein interacted as well (Fig. 6B). The interaction with the PCI domain was stronger, because much less of this protein precipitated more P54 (compare lanes 2 and 3, in Fig. 6B). Examples of P56 family of proteins interacting with either the C-terminal region of eIF3c, which contains the PCI domain, or the N-terminal region of eIF3c are known. Mouse P54 interacts only with the PCI domain of eIF3c, an interaction mediated by the N-terminal region of mouse P54 (42). In contrast, mouse P56 interacts only with the N-terminal region of eIF3c. Here, we observed that human P54 has retained both interacting properties of mouse P56 and mouse P54. It remains to be seen whether different domains of human P54 participate in the two interactions; our attempts to examine this possibility have so far been unsuccessful, because deletion mutants of P54 could not be expressed in human cells. The interaction between human P56 and eIF3e is mediated by the C-terminal regions of both proteins; the interacting domain of human P56 contains the last two tetratriopeptide repeat motifs and that of eIF3e contain the PCI motif, as revealed by yeast two-hybrid analyses (40). We anticipate that human P54 also interacts with the PCI domain of eIF3e.

Human P54 inhibited translation as efficiently as human P56, mouse P56, and mouse P54 (41, 42). Although human P54, compared with other proteins of this family, could inhibit two functions of eIF3 (Fig. 10), its efficiency of inhibiting translation in vitro was no higher than those of others. One of the reasons behind this observation could be that it was not as effective as human P56, in blocking the function of eIF3 to stabilize the ternary complex (Fig. 8). This functional difference may reflect a difference in the strength of binding between eIF3e and human P56 or human P54; it is also possible that the two proteins bind to different regions of eIF3e and hence affect its function differently. In contrast to its partial effect on ternary complex formation, human P54 was very efficient in blocking formation of the pre-initiation complex (Fig. 9D). Thus, it is likely that a mutant of human P54 that cannot interact with eIF3e and hence cannot affect ternary complex stabilization will still inhibit translation initiation as strongly as wild-type human P54. Note that the 20 S complex, composed of eIF3, ternary complex, eIF4F, and mRNA, was as stable in the presence of human P54 as in its absence (compare panels B and A, Fig. 9). This indicates that the partial effect of P54 on the stability of the ternary complex (Fig. 8) does not affect the stability of the 20 S complex. In contrast, in the presence of P56, no 20 S or 48 S complexes were formed (data not shown). Thus, human P54 affects eIF3 functions only selectively, a conclusion that we have extensively documented for mouse P56 (41). The current study extends our knowledge of how the P56 family of proteins blocks eIF3 functions by binding to its specific subunits (Fig. 10). Human P56 binds via the “e” subunit, and mouse P56 and mouse P54 bind via the “c” subunit; whereas human P54 bind to both subunits, and human P58 and P60 bind to neither. The functional characteristics of these proteins perfectly reflect their abilities to bind to the “c” or the “e” subunits of eIF3. They affect only two, among many, functions of eIF3 in translation initiation (41, data not shown). Between the two affected functions, human P56 inhibits only ternary complex stabilization, whereas mouse P56 and mouse P54 inhibit only 48 S pre-initi-

\[ F.\ Terezi and G.\ C.\ Sen,\ unpublished\ observation. \]
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