The Anti-HIV-1 Editing Enzyme APOBEC3G Binds HIV-1 RNA and Messenger RNAs That Shuttle between Polysomes and Stress Granules*§

Received for publication, February 28, 2006, and in revised form, August 3, 2006. Published, JBC Papers in Press, August 3, 2006, DOI 10.1074/jbc.M601901200

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Deoxycytidine deaminases APOBEC3G (A3G) and APOBEC3F (A3F) (members of the apolipoprotein B mRNA-editing catalytic polypeptide 3 family) have RNA-binding motifs, invade assembling human immunodeficiency virus (HIV-1), and hypermutate reverse transcripts. Antagonistically, HIV-1 viral infectivity factor (Vif)4 encoded by human immunodeficiency virus type-1 (HIV-1) neutralizes a potent anti-HIV-1 defense system that occurs specifically in T lymphocytes and to a lesser degree in macrophages, thus explaining the efficient replication in T cells of wild-type HIV-1 but not HIV-1(Δvif) that lacks a functional vif gene (1–4). This antiviral defense system involves the deoxycytidine deaminases APOBEC3G (A3G) and its paralog APOBEC3F (A3F) (members of the apolipoprotein B mRNA-editing catalytic polypeptide 3 family), each of which contains two RNA-binding motifs and incorporates into assembling HIV-1 capsids where they cause lethal dC-to-dU hypermutations in the single-stranded viral DNA that transiently forms during reverse transcription (1, 2, 4–10). This single-stranded DNA, which has a minus sense, is synthesized using viral RNA as a template and is released from the DNA-RNA hybrid when the RNA is degraded by a ribonuclease H inherent in reverse transcriptase. Vif binds specifically to A3G and A3F and recruits a multisubunit ubiquitin ligase complex containing cullin 5 and elongins B-C that causes their polyubiquitination and proteasomal degradation, thereby ridding infected cells of these antiviral enzymes and precluding their incorporation into progeny virions (1, 2, 11–16).

Several lines of evidence suggest that A3G is tightly regulated intracellularly. Several human T cell lines contain substantial amounts of A3G and/or A3F but incorporate only small amounts into virions and are highly permissive for replication of HIV-1(Δvif) (17). This suggests that cellular factors can modulate the anti-HIV-1 activities of A3G and A3F. A3G is also transcriptionally induced in T cells by mitogenic factors that activate extracellular signal-regulated kinase (18) and in macrophages by interferon α (19). It was recently reported that A3G in proliferating T cells and HEK293T cells occurs in an enzymatically inactive form in a large ribonucleoprotein complex (20, 21). Treatment of this complex with RNase A released A3G.

*This work was supported by National Institutes of Health Grant AI49729. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§The on-line version of this article (available at http://www.jbc.org) contains Table S1 and Figs. S1 and S2.

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4 The abbreviations used are: Vif, viral infectivity factor; HIV-1, human immunodeficiency virus type 1; A3G and A3F, APOBEC3G and APOBEC3F, members of the apolipoprotein B mRNA-editing catalytic polypeptide 3 family of cytidine deaminases; APOBEC1, apolipoprotein B mRNA-editing catalytic subunit; P-bodies, mRNA processing bodies; LacZ, β-galactosidase; Stauf1, Staufen1; PABP, poly(A)-binding protein; PTB, polypyrimidine tract-binding protein; Gag, gag-specific antigen, the retroviral core structural proteins; HA, hemagglutinin; TEV, tobacco etch virus; LC/MS/MS, liquid chromatography/tandem mass spectrometry; PBS, phosphate-buffered saline; RT, reverse transcription; TAP, tandem affinity purification; TOF, time of flight.
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and activated its enzymatic activity (20, 21). This high M₉ complex is absent in resting T cells, which contain relatively little RNA, and the uncomplexed active A3G in those cells can apparently invade fully assembled wild-type HIV-1 capsids and cause their inactivation (21). These results strongly suggested that A3G associates intracellularly with RNA in a ribonucleoprotein complex(es), that this inhibits its enzymatic and anti-HIV-1 activities, and that this inhibitory association is controlled by mitogenic cytokines that activate T cells. Similarly, the cytidine deaminases APOBEC1 and activation-induced cytidine deaminase, which edit apolipoprotein B mRNA and control recombination and hypermutation of immunoglobulin genes, respectively, associate with RNAs and proteins that restrict their activities and substrate specificities (22–25). The concentric incorporations of A3F and A3G into assembling retroviral capsids also require functional interactions with RNAs and proteins (2, 26–30).

In this study, we identified proteins and RNAs that associate with A3G. We expressed a biologically active tandem affinity-tagged derivative of A3G (TAP-A3G) or a negative control TAP protein in human HEK293T cells and employed tandem mass spectrometry (LC/MS/MS) to identify proteins that specifically copurify with A3G. A3G is strongly associated with ribonucleoproteins that remain attached throughout a multistep purification procedure in physiological conditions. The proteins thereby identified coimmunoprecipitated specifically from cell extracts with A3G and A3F, including extracts of T lymphocytic cells. Many but not all of the TAP-A3G-associated proteins were released from A3G by RNase A, suggesting that they were linked via RNA. The large rapidly sedimenting A3G complex in cell extracts consists of polysomes, as indicated by changes in its sedimentation behavior caused by protein synthesis inhibitors. A3G was associated with the mRNA component of polysomes, and these A3G-mRNA complexes moved reversibly from polysomes into storage granules in conditions of stress. Conversely, cycloheximide drove dormant A3G-mRNA complexes back into polysomes. In agreement with this evidence, HIV-1 viral RNA and many cellular mRNAs coimmunoprecipitated with A3G. Nearly all of the A3G-associated proteins identified by mass spectrometry were reported previously to bind exclusively or intermitently to mRNAs.

**EXPERIMENTAL PROCEDURES**

**Cells and Expression Vectors**—Human embryonic kidney (HEK) 293T cells (ATCC), HeLa-C14 (HI-J clone) (31), and human leukemic T cell lines H9 (32) (AIDS Research and Reference Reagents Program, Division of AIDS, NIAID, National Institutes of Health) and H9LVifSN (H9.Vif) (33) (Didier Trono, University Medical Centre, Geneva, Switzerland) were maintained according to their supplier’s specifications. Cells and expression vectors were transfected with equimolar ratios of plasmid DNAs using Lipofectamine 2000 (Invitrogen) at 80 μg/ml (Roche Applied Science) and extracts prepared using I.PP150 buffer (10 mM Tris-Cl, pH 8.0, 0.1% Nonidet P-40, and 150 mM NaCl) supplemented with complete protease inhibitors (Roche Applied Science). The extracts were centrifuged at 1,500 × g for 5 min to sediment the nuclei, adjusted to equivalent protein concentrations using Bradford reagent (Bio-Rad), mixed with IgG-Sepharose (0.3 ml/ml cell lysate) (Amersham Biosciences), and allowed to rotate at 4 °C overnight. Some extracts were treated in the presence of RNase A, DNase-free (20 μg/ml) (Roche Applied Science) at 25 °C for 40 min prior to mixing with the IgG-Sepharose. The IgG-Sepharose with the bound TAP-A3G was pelleted by centrifugation, washed extensively with IPP150 buffer, and subsequently washed and suspended in TEV cleavage buffer (IPP150 buffer supplemented with 0.5 mM EGTA and 1 mM dithiothreitol). The samples were incubated with AcTEV (100 units/ml) (Invitrogen) and rotated at 4 °C overnight to release the A3G complexes. The IgG-Sepharose was pelleted and the supernatant containing the cleaved complexes was diluted with 3 volumes of IPP150 calmodulin binding buffer (IPP150 buffer supplemented with 10 mM β-mercaptoethanol, 1 mM magnesium acetate, 1 mM imidazole, 2 mM CaCl₂), mixed with calmodulin-Sepharose 4B (0.075 ml/ml supernatant) (Amersham Biosciences), and rotated at 4 °C for 1 h. The pelleted calmodulin-Sepharose was washed extensively with IPP150 buffer followed by IPP150 buffer without Nonidet P-40, and the protein complexes were eluted with EGTA-containing buffer (IPP150 calmodulin binding buffer without Nonidet P-40 and CaCl₂ supplemented with 2 mM EGTA). The eluted samples were concentrated using Amicon ultracentrifugal filter devices (Millipore), and aliquots were electrophoresed for analysis of A3G by Western blotting or for SYPRO Orange (Invitrogen) staining of the copurified proteins prior to mass spectrometry.

**Protein Identification by LC/MS/MS—SYPRO Orange-stained proteins resolved on one-dimensional SDS-polyacrylamide gels were prepared for digest by cutting the entire lane into 20–35 individual slices. Bands were excised manually or automatically using a 2DiDx sample preparation robot (Leap Technologies). Gel bands/plugs were placed in a ZipPlate for the entire digestion procedure. However, the ZipPlate was not used to perform any chromatographic cleanup of the samples.
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Rather, the plate was used as a filter to facilitate manual or automated tryptic digestion. Gel bands were destained in two changes of 100 mM ammonium bicarbonate (ambic) in 30% methanol. The plugs were dried with neat acetonitrile, reduced with 10 mM dithiothreitol, and alkylated with 50 mM iodoacetamide before being washed and dried again. Approximately 100 ng of sequencing grade trypsin (sequencing grade modified trypsin; Promega) in 20 mM ambic was added to each sample with sufficient buffer to immerse the gel plugs. Digestion proceeded overnight at 37 °C. Samples were acidified with the addition of 10 μl of 1% formic acid. Peptide extracts were collected by centrifugation, and the gel slices were subsequently extracted with two additions of 50 and 70% acetonitrile, respectively. Peptide extracts were brought to near dryness before being resuspended in 10 μl of 0.1% formic acid in preparation for mass spectrometric analysis. Tryptic peptides from digests were analyzed by nano-LC/MS/MS. Chromatography was achieved using an Eksigent nano-LC to generate a gradient using the following chromatographic conditions: for the mobile phase A, water, acetonitrile, formic acid, trifluoroacetic acid (95:4.89:0.1:0.01, respectively, v/v); for the mobile phase B, acetonitrile, isopropl alcohol, water, formic acid, trifluoroacetic acid (80:10:9.89:0.1:0.01, respectively, v/v). Mobile phase B was ramped from 2 to 45% over 40 min, increased to 80% in 5 min, and held for 5 min before being returned to starting conditions. Flow was regulated at 200 nl/min and directed through a 75-mm × 15-cm in-house packed column packed with Astrosil (5-mm particle size, 100 Å pore size, C18 reverse phase chemistry; Stellar Phases) coupled to a 5 μm tapered emitter (New Objectives). Prior to analytical chromatography, 5 μl of tryptic digest was injected onto a sample trap and washed with mobile phase A to remove salts and contaminants after which the trap was switched in-line with the analytical column. Tandem mass spectrometry data were collected using a Qstar XL hybrid time-of-flight mass spectrometer (Applied Biosystems) under the following conditions: spray voltage 1800–1900 V; TOF-MS scan m/z 400–1600, 0.5 s; TOF-MS/MS scan m/z 50–2000, 2.0 and 90 s exclusion; data-dependent product ion acquisition of the three most abundant +2 and +3 ions from the TOF-MS scan. Monoisotopic masses for data base searching were generated using Distiller (Matrix Science) and submitted to MASCOT version 1.9 (Matrix Science) for X!Tandem for protein identification. Masses were searched against the IPI data base (IPI Human version 3.13) (42) with the following parameters: digest agent, trypsin; fixed modification, cystine carboxamidomethylation; variable modifications, N/Q deamidation, methionine oxidation; 1 missed cleavage allowed, precursor and fragment ion mass tolerances of 0.1 Da. For X!Tandem analysis (43), the following parameters were used: fixed modification, cystine carboxamidomethylation, variable modification, methionine oxidation; refinement, methionine oxidation, N/Q deamidation; refinement of point mutations, one missed cleavage; digest agent, trypsin; no removal of redundant spectra; precursor and fragment ion mass tolerances of 100 ppm and 0.2 Da, respectively. Detailed analysis of protein identification data was carried out using Scaffold (Proteome Software), which is an implementation of the Peptide and Protein Prophet algorithms originally described by Keller and co-workers (44, 45) along with protein identification by X!Tandem. Positively identified proteins were selected using the following parameters: minimum peptide confidence 95%, minimum number of peptides 2, minimum protein confidence 95%. Any protein identified by three or fewer peptides was manually inspected to confirm identification.

Protein Analyses—Western immunoblotting and immunoprecipitations were described (13). Briefly, cells were lysed with RIPA (50 mm Tris-Cl, pH 7.4, 1% Nonidet P-40, 0.1% sodium deoxycholate, and 150 mm NaCl) with complete protease inhibitors followed by centrifugation at 1,500 × g for 5 min to sediment the nuclei. Postnuclear extracts were adjusted to equivalent protein concentrations using Bradford reagent, and equal aliquots (unless otherwise noted) were then used for Western immunoblotting (46) or immunoprecipitations (47). Some extracts were treated in the presence or absence of RNase A, DNase-free (20 μg/ml) at 25 °C for 20 min prior to analyses. Antibodies used for protein analyses were as follows: mouse anti-Myc 9E10, mouse anti-HA HA-7, rabbit anti-HA, mouse anti-luciferase LUC1, mouse anti-α-tubulin DM 1A, and mouse anti-β-actin peroxidase-conjugated AC-15 (Sigma); mouse anti-PABP1 and rabbit anti-YB1 (Abcam, Inc); mouse anti-PTB clone 1 (Invitrogen); rabbit anti-Ro (Santa Cruz Biotechnology); rabbit anti-A3G 9968, rabbit anti-Vif 2221, and HIV-IG 3957(AIDS Research and Reference Reagents Program, contributed by Dr. Warner C. Greene and Dr. Dana Gabuzda, NABI Biopharmaceuticals and NHLBI, National Institutes of Health, respectively); rabbit anti-human Stau1 (generously donated by Dr. Gabriella Boccaccio) and rabbit anti-L5 ribosomal protein (generously donated by Dr. Hua Lu).

Polysome Analyses—Polysomes were prepared and analyzed as described previously (48) with some modifications. Transfected HEK293T cells were treated in the presence or absence of puromycin (0.5 μg/ml) (Sigma) at 37 °C for 30 min prior to lysis. H9 leukemic T cells were treated in the presence or absence of sodium m-arsenite (0.1 mM) (Sigma) at 37 °C for 1 h. Some samples were treated with cycloheximide (0.1 mg/ml) (Sigma) at 37 °C for 20 min prior to and subsequently during the 1 h arsenite treatment. Cells were lysed in RIPA supplemented with cycloheximide (0.1 mg/ml) and RNasin ribonuclease inhibitor (30 units/ml) (Promega). Postnuclear extracts adjusted for equivalent protein concentrations were layered onto 30-ml 15–40% sucrose gradients made in buffer B (0.01 mM Tris-Cl, pH 7.4, 0.25 mM KCl, 0.01 mM MgCl2) and centrifuged at 86,000 × g for 3 h. Fractions (1 ml), collected from the bottom, and the pellet resuspended in 1 ml of buffer B were used for Western immunoblotting and spectrophotometric A260 analyses. The total RNA isolated from 0.1-ml aliquots of each fraction using TRIzol reagent (Invitrogen), according to the manufacturer’s instructions, was electrophoresed in an agarose gel and visualized by ethidium bromide staining.

Immunofluorescence Microscopy—HEK293T cells were seeded at 10 5 cells/well in 4-well Permanox chamber slides (Nalge Nunc International) that had been pretreated with poly-L-lysine (0.1 mg/ml) at 25 °C for 20 min, and the cells were cultured overnight at 37 °C. The cultures were transfected with A3G-Myc expression vector using FuGENE 6 transfection reagent (Roche Applied Science). 24 h post-transfection the cul-
turedes were treated with arsenite (0.5 mM) in the presence or absence of cycloheximide as described above. Cells were fixed in 5% formaldehyde and 2% sucrose in PBS at 25°C for 20 min and permeabilized in 0.5% Nonidet P-40 and 10% sucrose in PBS at 25°C for 30 min. A3G-Myc was detected with rabbit anti-Myc (Sigma) or mouse anti-Myc 9E10 for colocalization studies with PABP1 or Stau1, respectively. PABP1 and Stau1 were detected with antibodies described above under “Protein Analyses.” Secondary antibodies were as follows: Alexa Fluor-488 goat anti-mouse IgG, Alexa Fluor-488 goat anti-rabbit IgG, Alexa Fluor-594 goat anti-mouse IgG, and Alexa Fluor-594 goat anti-rabbit IgG (Invitrogen). These antibodies reacted only with their species-specific primary antibodies. H9 leukemic T cells were seeded, treated with translational inhibitors, and processed as described above. Endogenous A3G was detected using a rabbit polyclonal antibody specific to the C terminus of A3G and produced as described previously (15). Slides were mounted in FluoroGuard (Bio-Rad) and examined under a Zeiss Axiovert 200 M deconvolution microscope.

**Viral Assays**—HIV-gpt virions were produced and titered using HeLa-CD4 cells (clone HI-1) and mycophenolic acid selection as described previously (36, 49, 50). For viral purifications, HIV-gpt(AΔvif) virions were produced in HEK293T cells cotransfected with pA3G-Myc or control vectors and purified by equilibrium sedimentation through a 20–60% sucrose gradient, as described previously (13). The peak viral fractions, identified by immunoblotting with HIV-IG, were pooled, and the proteins were precipitated with trichloroacetic acid (10% final) and with carrier yeast tRNA (100 μg/ml) (Sigma). The protein pellets were rinsed with cold acetone, air-dried, suspended in sample buffer, and analyzed by Western immunoblotting.

**Copurification of RNA**—To analyze the RNA in the A3G complexes, HEK293T cells cotransfected with pA3G-Myc and pHIV-gpt(AΔvif) or control vectors pLacZ-Myc and pcDNA3.1 were lysed in RIPA supplemented with RNasin ribonuclease inhibitor (30 units/ml), and immunoprecipitations were performed using mouse anti-Myc 9E10 as described above. The copurified RNAs in the immunoprecipitated complexes were recovered in TE buffer (10 mM Tris-Cl, pH 7.4, 1 mM EDTA) supplemented with 1% SDS and incubated at 100 °C for 10 min. The samples treated with RNase-free DNase I (40 units/ml) (Ambion) were subjected to agarose gel electrophoresis, and the recovered RNAs (20 ng/reaction) were used as templates in RT-PCR using the SMART PCR cDNA kit (Clontech). The PCR products were subsequently cloned into pCR2.1-TOPO vector (Invitrogen) and sequenced.

**RESULTS**

**Expression and Characterization of the TAP-A3G Protein**—Our goal was to use TAP to isolate TAP-A3G and negative control TAP complexes in physiological conditions from human cells. The N-terminal TAP tag that we chose for this purpose contains two IgG-binding domains derived from *Staphylococcus aureus* protein A, a cleavage site for TEV protease, and a calmodulin-binding peptide (51) (Fig. 1A). As a precondition for further work, the antiviral activity of TAP-A3G was tested by transiently cotransfecting HEK293T cells with pTAP-A3G and with vectors encoding either wild-type (HIV-gpt(wt)) or vif-deleted (HIV-gpt(AΔvif)) viruses produced in the presence of TAP-A3G or A3G-Myc expression vectors. The titers of the latter viruses were normalized relative to the titers of the same virus made in the presence of the control pcDNA3.1 empty vector (n = 3; error bars are ± S.E.). C, TAP-A3G fusion protein is down-modulated by Vif. HEK293T cells were cotransfected with expression vectors for TAP-A3G (left panel) or A3G-Myc (right panel) and with vectors for HIVHXB2-gpt(wt) or HIVHXB2-gpt(AΔvif) or a codon optimized HIV-1 HVifIIIB. Cell lysates were analyzed by Western blotting for A3G (upper panels), Vif (middle panels), or β-actin (lower panels). D, tandem affinity purification of TAP-A3G-Vif complex. Cell extracts from HEK293T cells transiently transfected with pTAP-A3G or pCMV-TAP were mixed on ice with extracts containing HIV-gpt(wt) or HIV8226-gpt(AΔvif) and tandem affinity purified. The purified samples were analyzed by Western immunoblotting for A3G (upper panel) and Vif (lower panel).
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**TABLE 1**

| Protein | EMBL-EBI accession |
|---------|--------------------|
| Ribonucleoproteins | |
| PTB | IP000183526 |
| 60-kDa S5-5A/Ro | IP00019450 |
| La | IP00009032 |
| hnRNP A2/B1 isoform B1 | IP00396378 |
| hnRNP C1/C2 isoform C1 | IP00216592 |
| hnRNP H1 | IP00479191 |
| hnRNP Q | IP00018140 |
| hnRNP R | IP00012074 |

| mRNA binding (active or dormant mRNAs)* | |
| PABP | IP00078522 |
| PABP4 | IP00012726 |
| Staufen1 | IP00000100 |
| PUR-α | IP00023591 |
| Utp1 (regulator of nonsense transcripts 1) | IP00034949 |
| FMRF (fragile mental retardation protein) | IP00016250 |
| YB-1 | IP00385699 |
| KOC | IP00165647 |
| CRDBP | IP00089557 |
| ELAV-1 | IP00039196 |
| 80-kDa cap-binding protein (CRP 80) | IP00019380 |
| Matrin-3 | IP00017297 |
| Interleukin enhancer binding factor 2 | IP00085198 |
| Interleukin enhancer binding factor 3 | IP00298788 |
| ZAP | IP00410069 |

| RNA helicases | |
| RNA helicase A | IP00215638 |
| U5–200 kDa | IP00420014 |
| MLEL1 | IP00027415 |
| MOV-10 | IP00444452 |
| Leukophysin | IP00155723 |
| DDX48 | IP00009328 |

| Ribosomal proteins | 31 ribosomal proteins identified |
|---------------------|-------------------------------|
| Other | |
| CAMK2δ | IP00065469 |
| Ubiquitin-protein ligase EDD1 | IP00026320 |
| Zinc finger CCHC domain protein 3 | IP00011550 |
| FUBP3 | IP00077261 |
| C6orf21 | IP00084746 |

* Other proteins in the table, including ribosomal proteins, helicases, and La also are known to intermittently associate with mRNAs.

Identification of A3G-associated Cellular Proteins—As seen by SDS-PAGE, numerous proteins reproducibly copurified with TEV-cleaved TAP-A3G but not with the TEV-cleaved TAP negative control (Fig. 2A). The most abundant protein in the gels was the TEV-cleaved TAP-A3G. Equal sections of each gel lane were digested in situ with trypsin, and the peptides were sequenced by LC/MS/MS mass spectrometry. The identification of specific proteins in the TAP-A3G preparation was based on the numbers of correspondent peptide sequences, their uniqueness in the tryptic peptide data base, and their absence from the CMV-TAP negative control preparation. Consistent with the large number of proteins and their reproducible patterns seen in the stained gels (e.g. Fig. 2), a total of 109 A3G-associated proteins were identified with substantial reproducibilities and probabilities ≥95% in data from independent experiments. A representative list of these proteins is shown in Table 1 (a comprehensive list is in supplemental Table S1). Most strikingly, nearly all of the A3G-associated proteins bind RNA and/or single-stranded DNA and are components of ribonucleoprotein complexes. More specifically, almost all of these proteins that had been studied previously were known to exclusively or intermittently associate with translationally active and/or dormant mRNAs. This major group includes

FIGURE 2. Tandem affinity purification of A3G and associated proteins. A, a comparison of proteins that copurify with TAP-A3G or with the negative control CMV-TAP protein. Cell extracts of HEK293T cultures transiently transfected either with TAP-A3G or CMV-TAP expression vectors were purified with an IgG-Sepharose affinity column, TEV-cleaved, adsorbed onto a calmodulin-Sepharose 4B column, and eluted with EGTA-containing buffer. The samples were electrophoresed in a 4 –12% acrylamide gradient SDS-polycrylamide gel, and the proteins were visualized by SYPRO Orange staining. The affinity-purified TEV protease-cleaved CMV-TAP negative control protein, which contains 38 amino acids and consists almost exclusively of the calmodulin-binding domain, was not seen in our stained gels. The gel lanes were digested with trypsin and further analyzed by LC/MS/MS mass spectrometry. Numerous proteins copurified with TAP-A3G but not with CMV-TAP. B, similar analysis showing the effect of RNase A digestion on the proteins that copurified with TAP-A3G. Note that the levels of the protein bands marked by → were unaffected by RNase A treatment. The * mark the bands which are no longer detectable whereas the + mark bands that were substantially reduced after RNase A treatment. The three samples were analyzed by electrophoresis, with intervening empty gel lanes used to eliminate cross-contaminations, which would have interfered with subsequent sequencing and mass spectrometric analyses. The empty gel lanes were digitally removed in order to limit the figure size and to facilitate visual comparison of the stained gel lanes.

substantially eliminated by VifHXB2, as seen by the greater infectivities of the corresponding wild-type virus (Fig. 1B). Western blot analysis of the HEK293T cell lysates showed that TAP-A3G and A3G-Myc were similarly down-modulated (decreased in quantity) relative to the Δvif negative controls by VifHXB2 and also by a codon-optimized VifHXB2 designated HVifIIIB (40) (Fig. 1C). These results support evidence that Vif neutralizes A3G by eliminating it from virus-producing cells (13, 14, 16).

We showed previously that Vif associates with A3G in cell extracts (13). To determine whether this is also true of TAP-A3G, HEK293T cell extracts containing TAP-A3G or the negative control, CMV-TAP protein were mixed with extracts containing VifHXB2 or HVifIIIB, incubated on ice for 20 min, and then tandem affinity-purified. This purification involves binding to IgG-Sepharose, elution by cleavage with TEV protease, adsorption onto calmodulin-Sepharose, and elution with EGTA. Both Vifs bound to TAP-A3G but not to CMV-TAP, and the Vif-A3G complexes were successfully isolated by tandem affinity purification (Fig. 1D). Considered together, these results indicate that the N-terminal TAP tag does not interfere with A3G antiviral activity or with Vif and that TAP-A3G is a suitable bait for purifying A3G-containing complexes from extracts of human cells.
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A

HEK293T cells

Anti-Myc IPs

Anti-PTB IPs

RNase A

LacZ-Myc

A3G-Myc

A3G-Myc

LacZ-Myc

LacZ-Myc

IgG

A3G-Myc

PABP1

Ro

Stau1

YB-1

hnRNP Q

hnRNP H1

C

HEK293T cells

Anti-HA IPs

Control

A3F-HA

LacZ-HA

A3F-HA

LacZ-HA

IgG

A3G

PABP1

hnRNP H1

hnRNP Q

hnRNP H1

FIGURE 3. Characterization of A3G and A3F complexes. A, cell extracts from HEK293T cultures expressing A3G-Myc or LacZ-Myc, treated or untreated with RNase A, were immunoprecipitated with anti-Myc antibody (left panel) or anti-PTB antibody (right panel). The immunoprecipitates were immunoblotted with anti-Myc antibody (upper panels) and with antibodies to PABP1, Ro, Stau1, YB-1, hnRNP Q, and hnRNP H1 (left panels). All seven proteins specifically coimmunoprecipitated with A3G, but these associations were eliminated by treatment with RNase A. PTB coelectrophoreses with the IgG heavy chain, and therefore could not be resolved following immunoprecipitations. B, specific interaction of endogenous A3G with PABP1, PTB, hnRNP Q, and hnRNP H1. H9 and H9.Vif cells that constitutively synthesize Vif were lysed and immunoprecipitated with PABP1, PTB, hnRNP Q, and hnRNP H1 antibodies. Left frame, the cell lysates were analyzed by Western blotting for A3G (upper panel) and Vif (middle panel). Right frame, anti-PABP1, anti-PTB, anti-hnRNP Q, and anti-hnRNP H1 immunoprecipitates were analyzed by Western blotting for A3G. C, specific association of A3F-HA with PABP1 and YB-1. HEK293T cell cultures transfected with A3F-HA or LacZ-HA expression vectors were lysed and immunoprecipitated with anti-HA antibody. The samples were analyzed by Western blots to determine the A3F-HA association with PABP1 (left panel) and YB-1. A3F-HA is a YB-1 related protein.

Because we treated portions of the extracts with RNase A in order to learn whether specific complexes were dependent on RNA. As shown by Western immunoblotting in Fig. 3A, all the tested proteins specifically coimmunoprecipitated in an RNase A-sensitive manner with A3G-Myc but not with LacZ-Myc. Control studies showed that these proteins were present in the initial cell extracts in amounts that were unaffected by the RNase A treatments (see supplemental Fig. S1A). Additional controls demonstrated that the RNase A treatments efficiently eliminated RNA from the extracts (see supplemental Fig. S1B). The fact that all seven of the antibodies that we tested confirmed the A3G associations indicated in Table 1 strongly supports the reliability of our mass spectrometric evidence. In addition, these results demonstrate that PABP1, YB-1, Ro, PTB, hnRNP H1, hnRNP Q, and Stau1 all bind A3G in an RNA-dependent manner.

We then determined whether the endogenous A3G in H9 T cells occurs in similar complexes. As a control for these studies, we used the H9.Vif cell line that stably expresses Vif and consequently contains negligible A3G (Fig. 3B, left panel) (17, 33). However, these experiments were hampered because available antibodies specific for A3G do not efficiently cause its immunoprecipitation (results not shown) and because only four of the proteins used in the previous studies, PABP1, PTB, hnRNP H1, and hnRNP Q, could be immunoprecipitated by their antisera. Based on these considerations, cell lysates derived from H9 or H9.Vif cultures were immunoprecipitated with these antisera, and the samples were analyzed by Western immunoblotting for the presence of A3G (Fig. 3B, right panel). In the samples derived from H9 cells, A3G coimmunoprecipitated with PABP1, PTB, hnRNP H1, and hnRNP Q. In agreement with the strong down-modulation of A3G in H9.Vif cells (Fig. 3B, left panel), A3G was absent in the immunoprecipitated samples derived from this cell line. In contrast, expression of Vif had no effect on the total cellular amounts of these proteins (see supplemental Fig. S1C).

A3F Associates with Some Proteins That Bind A3G—Because A3F is 48% identical in sequence to A3G and inhibits HIV-1 in patients by a closely related mechanism (5, 11, 39, 53), we deter-
mined whether it forms similar complexes with cellular factors. For this purpose, extracts of HEK293T cultures that had been transiently transfected with A3G-Myc expression vector and subsequently untreated or treated with puromycin were sedimented through sucrose gradients, and fractions were collected from the bottom. A, total cell lysates were analyzed by Western immunoblotting with specific antibodies for A3G and PABP1. α-tub, α-tubulin blot shows equivalent protein loading. B, the polysome and ribosomal components were detected by A260 absorbance of the fractions, and their presence was confirmed by Western blotting with specific antibody for the large ribosomal protein L5 as well as by agarose gel electrophoresis and ethidium bromide staining of the total RNA (28 S, 18 S, and tRNA) extracted from each fraction (C, lower panels). C, the indicated sucrose gradient fractions were analyzed by Western blotting with specific antibodies for A3G, PABP1, Stau1, and L5. 80S, single ribosomes. Control, untreated cells.

A3G Binds to Active Polysome-associated and Dormant mRNAs in HEK293T Cells and T Lymphocytes—The association of A3G with factors involved in protein synthesis, including ribosomal proteins and mRNA-binding proteins such as PABP1, implied that A3G might associate with polysomes. To test this hypothesis, cultures of HEK293T cells that expressed A3G-Myc were either untreated or treated for 30 min with puromycin before lysis and sedimentation in sucrose gradients. This analysis was based on previous evidence that puromycin causes rapid release of nascent polypeptide chains from polysomes and that the ribosomes then more slowly dissociate from the mRNA to form progressively smaller polysomes plus 80 S single ribosomes that lack mRNA (48, 54, 55). This secondary dissociation of polysomes occurs in many but not all cells. In
some cells this process is inefficient because the rate of protein synthesis initiation is adequate to offset the increased rate of ribosome detachment from the mRNA. As shown in Fig. 4A, the two cell lysates contained the same amounts of A3G-Myc and PABP1 proteins. The sedimentation profile of the poly- somes and 80 S single ribosomes was approximated by the \( A_{260} \) of the sucrose gradient fractions (Fig. 4B) and was further confirmed by analyzing the total RNA of each fraction (Fig. 4C, \( rRNA \) frame) and by immunoblot detection of the large ribosomal subunit protein L5 (Fig. 4C). The 28 S and 18 S ribosomal RNAs were present in the polysome and 80 S ribosome regions in the equimolar ratio (absorbance ratio \( \sim 2:1 \)) expected for associated 60 S and 40 S ribosomal subunits, and their sedimentation profiles in the gradients were consistent with our expectation that puromycin causes polysome disaggregation to form 80 S single ribosomes in these cells. As shown in Fig. 4C, most of the A3G and PABP1 from the untreated control cells cosedimented with the polysomes (i.e. in the pellet and in fractions 1–19), although substantial amounts also occurred in the lighter fractions that sedimented more slowly than 80 S single ribosomes (fractions 23–30). In contrast, treatment with puromycin caused a shift in sedimentation of the A3G and PABP1, with their complete movement out of the pellet and larger polysomes and with their accumulation in small residual polysomes and slowly sedimenting fractions. This puromycin-induced shift of A3G strongly suggests that the most rapidly sedimenting component of A3G in extracts from untreated cells is bound to translationally active polysomes. Moreover, the similarities of the PABP1 and A3G sedimentation profiles and the absence of A3G or PABP1 sedimentation peaks coincident with 80 S single ribosomes (fraction 21) imply that A3G binds to the messenger ribonucleoprotein component of polysomes rather than to the 80 S ribosomes. According to this interpretation, which is supported by evidence described below, the puromycin-induced movement of A3G into the slowly sedimenting upper fractions of the gradient is caused by release of A3G-mRNA complexes from the ribosomes. Stau1, which is known to bind to a subgroup of mRNAs in polysomes and in stress granules (56–58), has a sedimentation profile distinct from the majority of the A3G and PABP1 (Fig. 4C).

We expanded this analysis using H9 leukemic T cells. In these cells, the A3G and PABP1 sedimentation profiles were similar to those in HEK293T cells. However, puromycin caused only a slight disaggregation of polysomes in H9 cells, which made it more difficult to establish that the rapidly sedimenting A3G and PABP1 proteins were associated with polysomes (results not shown). Therefore, we used alternative and broader methods to investigate this issue. In particular, it is known that arsenite and cyanide block protein synthesis initiation of 5' capped mRNAs, resulting in release of these mRNAs from polysomes and their accumulation in a repressed form in cytosolic stress granules and processing bodies (P-bodies) (58–62). Moreover, these stress-induced shifts in mRNA localization are prevented and reversed when the movements of ribosomes on mRNA are slowed by cycloheximide, which inhibits the elongation steps of protein synthesis (58–63). Thus, stressors, including arsenite and cyanide, cause accumulation of dormant mRNAs, whereas moderate concentrations of cycloheximide inhibit ribosome release from mRNAs and causes dormant mRNAs to move into polysomes. Accordingly, the proportions of dormant and polysomal mRNAs can be reversibly shifted by stressors and cycloheximide, respectively.

Consistent with these expectations, arsenite and cycloheximide caused shifts in the sedimentation profiles of polysomes as seen by \( A_{260} \) measurements and by assays for the L5 ribosomal protein (Fig. 5). Moreover, these shifts in the polysomes were accompanied by corresponding changes in sedimentation of A3G and PABP1. Specifically, in control untreated H9 cells the A3G and PABP1 sedimentation profiles were similar to those in HEK293T cells, with substantial amounts in the polysomes and in the subribosomal region (i.e. in fractions 23–31). Cycloheximide caused nearly all the A3G and PABP1 to shift out of the subribosomal fractions into the polysomes (Fig. 5B). Conversely, arsenite caused an opposite shift, with movements of A3G and PABP1 out of polysomes and into the subribosomal region. Cycloheximide blocked the arsenite effects and also induced a substantial movement of A3G and PABP1 out of the subribosomal region into polysomes. These results, which were also seen using cyanide as a stressor (results not shown), strongly suggest that large proportions of A3G and PABP1 are associated with mRNAs that move reversibly between polysomes and dormant storage complexes. In contrast, Stau1 is only partially associated with translationally active mRNAs and is consequently less affected by protein synthesis inhibitors (see Fig. 5B).

In agreement with the above evidence, immunofluorescence microscopy indicated that arsenite and other stressors caused almost all of the A3G-Myc and PABP1 to co-localize in cytosolic granules in HEK293T cells (see Fig. 6A). These A3G-Myc- PABP1 accumulations were dispersed and prevented by cycloheximide. Consistent with our sedimentation analyses (Figs. 4 and 5), arsenite had only a slight effect on the distribution of Stau1 granules (Fig. 6B), and these only partially overlapped with the A3G-Myc-PABP1 granules. Similarly, cytosolic A3G- PABP1 granules accumulated in arsenite-treated H9 T cells (Fig. 6C), and these accumulations were also prevented and reversed by cycloheximide (results not shown). We conclude that A3G and PABP1 localize on mRNAs that shuttle between polysomes and dormant granules. The fact that nearly all A3G and PABP1 localize in stress granules in arsenite-treated cells suggests that A3G is almost entirely bound to A3G-PABP1-mRNA complexes intracellularly as well as in cell extracts.

To further analyze this issue we performed RT-PCR analyses of the RNAs that coimmunoprecipitated with A3G-Myc from extracts of HEK293T cells that had been transiently transfected with pA3G-Myc or pLacZ-Myc in the presence or absence of pHIV-gpt(D2vif). Cell extracts containing 1.2 mg of total protein were immunoprecipitated with the 9E10 anti-Myc antibody, and DNA-free RNA was isolated from the washed immunoprecipitates. 50–60 ng of copurified RNA was recovered from the immunoprecipitates that contained A3G-Myc, whereas only trace amounts (~5 ng or less) were detected in the LacZ-Myc samples. These copurified RNAs were used as templates for RT-PCR using the SMART oligo(DT) and SMART PCR random primers. The analyses of amplified cDNA products
revealed a heterogeneous population of transcripts only for the A3G-Myc-derived samples and not for the LacZ-Myc controls (Fig. 6D). The heterogeneous profile of the transcripts was evident in both the presence and absence of HIV-1 RNA. The PCR products were cloned into the PCR2.1-TOPO vector, and several clones from each amplification were sequenced. The results indicated that the A3G-Myc immunoprecipitated from the cells lacking HIV-gpt (Δvif)/H9004vif contained mRNAs encoding for A3G, spastic ataxia of Charlevoix-Saguenay (sacsin), ubiquitin C, and protein phosphatase 2A. The A3G immunoprecipitated from the cells that contained HIV-gpt (Δvif) contained transcripts encoding HIV-1 Gag and glycyl-tRNA synthetase. Although we have not comprehensively identified all coimmunoprecipitated RNAs, these results strongly suggest that A3G associates with HIV-1 RNA and with mRNAs, in agreement with the results in Figs. 4–6. We used additional methods that confirmed the associations of HIV-1 RNA and A3G mRNA with A3G (see supplemental Fig. S2), and this evidence also implied that β-actin mRNA is substantially underrepresented in A3G-Myc immunoprecipitates compared with its abundance in the total RNA from cell extracts. This suggests that A3G may selectively bind to HIV-1 RNA and certain mRNAs but minimally to β-actin mRNA, in agreement with a previous in vitro binding analysis (5).

Diverse RNase A Sensitivities of A3G-Protein Linkages—The above studies and previous evidence (21) implied that many or all of the proteins that copurified with TAP-A3G are attached via RNAs. To investigate this, we repeated the TAP-A3G purification using both untreated and RNase A-treated cell extracts. A parallel purification was done using an RNase A-treated extract that contained the negative control protein CMV-TAP. As shown by electrophoresis, similar amounts of A3G were present in the untreated and RNase A-digested samples (Fig. 2B). However, the profile of A3G-associated proteins was different, with some components no longer detectable (Fig. 2B, labeled *) or substantially reduced (labeled +) after RNase A treatment. More importantly, several protein bands appeared to be substantially resistant to RNase A, implying that they might interact with A3G in an RNA-independent manner (Fig. 2B, labeled →). Control studies confirmed the efficient elimination of RNA by this treatment (results not shown).

All three lanes in the latter gel were processed and analyzed by LC/MS/MS mass spectrometry. The proteins in the negative control sample were nearly all keratin contaminants or heat
FIGURE 6. Immunofluorescence microscopy and RT-PCR analyses confirm the association of A3G with mRNA-PABP1 complexes. A–C, arsenite stress induces recruitment of A3G and PABP1 into stress granules. A and B, HEK293T cell cultures that had been transiently transfected with the A3G-Myc expression vector were untreated or treated with arsenite in the presence or absence of cycloheximide prior to immunostaining for A3G-Myc and PABP1 (A) or A3G-Myc and Stau1 (B). The arsenite treatment caused nearly all of the A3G-Myc and PABP1 to colocalize in cytosolic stress granules (A), whereas only partial colocalization of A3G-Myc with Stau1 in stress granules was observed (B). Cycloheximide reversed the effect of the arsenite treatment causing a cytosolic redistribution of the A3G-Myc and PABP1 (A and B). Note that the A3G-Myc vector was expressed in only some of the transfected HEK293T cells, whereas the PABP1 and Stau1 were in all cells. C, arsenite treatment of H9 cells caused nearly complete colocalization of endogenous A3G and PABP1 into stress granules. D, mRNAs specifically copurify with A3G-Myc. The lysates of HEK293T cells transiently cotransfected with A3G-Myc or LacZ-Myc control expression vectors, and HIV-gpt(Δvif) or pcDNA3.1 plasmids were immunoprecipitated with anti-Myc antibody. The copurified RNAs were used as templates for RT-PCR using a SMART PCR cDNA kit, and the PCR products were electrophoresed on a 1.2% agarose/ethidium bromide gel. The analyses of cDNA products revealed a heterogeneous population of transcripts only for the A3G-Myc-derived samples and not for the LacZ-Myc controls. Control, untreated cells. CHX, cycloheximide.
shock proteins, and these were subtracted from the protein data pool. Because our mass spectrometric data identifies tryptic peptides compatible with specific proteins rather than absolute peptide or protein quantities, additional criteria were needed for interpreting the data, and for this purpose we used in part the evidence in Figs. 2B and 3. Consistent with the fact that A3G was present in the RNase A-digested and -undigested lanes in equal quantities (Fig. 2B), the numbers of detected peptides diagnostic of A3G were the same in these samples. In contrast, the proteins for which the interaction with A3G was demonstrated to be RNase A-sensitive in our coimmunoprecipitation studies (see Fig. 3) were less abundant in the RNase A-treated preparation and showed fewer diagnostically concordant tryptic peptides and/or greatly reduced quantities of specific peptides relative to the amounts of the detected A3G peptides in the same preparation. Similar mass spectrometric results were obtained for ribosomal proteins, consistent with our sucrose gradient sedimentation evidence that A3G does not bind directly to 80 S ribosomes (Figs. 4 and 5). Based on this evidence, we classified proteins as RNase A-sensitive if the digestion substantially reduced the numbers of diagnostically concordant tryptic peptides that were detected and/or greatly reduced the quantities of specific peptides relative to the amounts of A3G peptides in the same preparation. In contrast, proteins were considered to be resistant only if the numbers and relative amounts of their corresponding peptides were unaffected by RNase A. By these criteria, nearly all of the A3G-associated proteins were attached in a manner that was at least partially sensitive to RNase A, consistent with Fig. 2B. The only apparent exceptions to this conclusion were three proteins, the antiretroviral protein ZAP, the ubiquitin-protein ligase EDD1, and the unknown protein C6orf21. Additional tests will be required to determine whether these or other proteins bind directly to A3G.

A3G Homo-oligomerization and Binding to A3F Are RNA-mediated, whereas Binding to Vif Is Not—A3G has been reported to form homo-oligomers as well as hetero-oligomers with A3F (5, 17, 39). To determine whether these associations are mediated by RNA, we coexpressed different epitope-tagged derivatives of A3G and A3F in HEK293T cells and performed coimmunoprecipitation assays from untreated or RNase A-treated cell extracts. For homo-oligomerization studies, extracts that contained A3G-Myc and A3G-Luc or Luc negative control proteins were analyzed (Fig. 7A). In the hetero-oligomerization assays, samples that contained A3F-HA and A3G-Myc or LacZ-Myc were used (Fig. 7B). In undigested samples, A3G-Myc formed a complex with A3G-Luc but not with Luc (Fig. 7A, right frame), and A3F-HA formed a complex with A3G-Myc but not with LacZ-Myc (Fig. 7B, right frame). However, both complexes were substantially reduced by RNase A treatments, although these treatments had no effect on the quantities of these proteins in the total cell extracts (Fig. 7, A and B, left frames). Similarly, we analyzed the RNase A sensitivity of A3G-Myc association with Vif, in that case employing extracts of HEK293T cells that contained HIVf<sub>1</sub> and A3G-Myc or LacZ-Myc. As shown in Fig. 7C, right frame, the A3G-Vif complex was RNase A-resistant. Indeed, the quantity of Vif that coimmunoprecipitated with A3G was reproducibly higher in the RNase A-treated sample than in the untreated sample. This strongly suggests that the binding of RNA to A3G and/or to Vif interferes with their association, presumably because the large RNA occludes the surface(s) needed for their interaction.

Most A3G-containing Complexes Are Excluded from HIV-1 Virions—In cells lacking Vif, A3G is relatively abundant and is efficiently incorporated into HIV-1 virions (1, 2, 4, 13, 14, 16,
To determine whether the A3G-containing complexes are similarly encapsidated, we purified HIV-gpt (H9004) virions from the medium of HEK293T cells that either expressed or lacked A3G-Myc. Equal amounts of purified virions as indicated by Gag p24 protein (Fig. 8, left frame, lower panel) were analyzed for the presence of A3G, PABP1, Ro, YB-1, PTB, and Stau1. As expected, the A3G was concentrated into the virions purified from A3G-expressing cells (Fig. 8, left frame, top panel). In contrast, PABP1, Ro, YB-1, and PTB were undetectable in both virus preparations, even upon longer exposure of these blots (Fig. 8, left frame) and despite their facile detection in the cell lysates (Fig. 8, right frame). These four proteins remained undetectable in additional blots in which twice the amounts of purified virions were analyzed (results not shown). Interestingly, a small amount of the YB-1-related protein that was seen in Fig. 3 was detected in virions independent of A3G presence. Consistent with previous evidence, we detected a small amount of Stau1 in HIV-1 virions (65, 66) independently of the presence of A3G. We did not detect A3G-dependent incorporation of any protein into HIV-1 virions.

DISCUSSION

Predominant A3G-mRNA Complexes—To identify cellular ribonucleoproteins that bind to A3G and thereby attenuate its enzymatic and anti-HIV-1 activities (20, 21), we used a stepwise approach involving proteomic, biochemical, and immunocytochemical methods. These methods worked reliably, and our results all suggest that A3G predominantly associates with mRNAs that are translated in a 5’-cap-dependent manner in both HEK293T and T lymphocytic cells. This category of mRNAs is translationally inhibited at an initiation step by arsenite, which causes these mRNAs to move from polysomes into a dormant form in stress granules (58–61, 67). Accordingly, sucrose gradient sedimentation analyses of cell extracts clearly suggested that A3G and PABP1 shifted reversibly between the polysome and dormant mRNA pools depending on treatments of the cells with the translational inhibitors puromycin and cycloheximide and with the stressors arsenite and cyanide (Figs. 4 and 5). For example, arsenite, which inhibits initiation factor eIF4E (67), caused polysome dissaggregation with formation of 80 S single ribosomes and release of A3G-PABP1-mRNA complexes that sediment slower than 80 S single ribosomes (Fig. 5). These arsenite-induced changes were coordinately blocked by cycloheximide, which inhibits elongation of nascent polypeptide chains, establishing that the arsenite effects were mechanistically coupled and not simply caused by cellular damage. Furthermore, moderate concentrations of cycloheximide in the absence of other inhibitors caused a shift of A3G-PABP1-mRNA in the opposite direction, out of the dormant pool and into the polysomes (Fig. 5). In accordance with these interpretations, immunofluorescence microscopy showed that arsenite induced nearly all of the intracellular A3G to colocalize with PABP1 in stress granules in HEK293T cells and H9 leukemic T cells and that these were dispersed by cycloheximide (Fig. 6, A and C). Some but not all of these A3G-containing stress granules also contained visible clusters of Stau1 (Fig. 6B).

In agreement with these results, the protein composition of the tandem affinity-tagged A3G complex that we purified from HEK293T cell extracts (Tables 1 and S1) was fully consistent with the expected compositions of polysomes plus stress granules (58, 68, 69), and we confirmed the associations of these proteins with A3G in all tested cases by coimmunoprecipitation from extracts of HEK293T cells and H9 T cells (Fig. 3). Based on these results, we assayed A3G-Myc and negative control LacZ-Myc immunoprecipitates for the presence of RNAs (e.g., see Fig. 6D and supplemental Fig. S2). Interestingly, HIV-1 RNA and A3G mRNAs were readily detected in the A3G-Myc
immunoprecipitates (supplemental Fig. S2). In contrast, mRNAs were absent in the negative control LacZ-Myc immunoprecipitates (Fig. 6D). Additional cDNA cloning studies unambiguously identified HIV-1 RNA and several cellular mRNAs, including A3G mRNA in the A3G-Myc immunoprecipitates. In agreement with a previous study (5), β-actin mRNA was underrepresented in the A3G-Myc immunoprecipitates compared with its relative abundance in total cellular RNA (supplemental Fig. S2).

The above interpretations are compatible with previous evidence concerning the compositions and dynamic movements of mRNAs between polysomes and stress granules (58–62, 68, 69). In this context, it should be understood that stress granules are believed to function as triage sites for holding dormant mRNAs prior to their return to polysomes or their transfer to P-bodies for degradation (59–61, 67). The distinctions between P-bodies and stress granules are not fully defined, and their components and locales often partially overlap, although it is generally believed that P-bodies comprise a less reversible end stage in mRNA degradation and silencing (59, 61). Because the A3G-PABP1-mRNA complexes in our analyses shuttled reversibly between polysomes and dormant pools, and because the dormant clusters accumulated when the cells were treated with the classical stressor arsenite, we infer that the A3G-containing dormant clusters are closely related to stress granules. Stress granules are also believed to contain Stau1, Purα, PABP1, CBP80, FRMP, RNA helicase A, MOV-10, hnRNP U, CAMK2, and Upf1, which all copurified with TAP-A3G (see Table 1 and supplemental Table S1) (58, 68, 69). Notably, however, the latter proteins have additional locations in cells. For example, Stau1 helps to localize some actively translated mRNAs at specific subcellular sites (56–58, 69), and it occurs together with Upf1 on specific polysomal mRNAs (57). It also binds to the HIV-1 TAR sequence and stimulates translation of HIV-1 RNA and other RNAs that have double-stranded hairpin regions (56). Our analyses are consistent with this expected broad functional and subcellular distribution of Stau1, which only partially overlaps with the subcellular distribution of A3G (Figs. 4–6). After completion of this work, it was reported that an unspecified proportion of A3G occurs in P-bodies (63). Although we did not detect any proteins unique to P-bodies in our purified TAP-A3G complexes, we cannot exclude the possibility that some A3G-mRNA complexes might eventually move from stress granules into P-bodies in steady-state conditions in cells. Indeed, this would be expected because mRNAs are ultimately degraded. In any case, our results strongly suggest that A3G is primarily and principally attached to mRNAs that contain PABPs, and that these primary complexes move reversibly between polysomes and stress granules depending on metabolic conditions of the cells. The reversible movement of A3G-PABP1-mRNA complexes between polysomes and dormant granules raises the possibility that A3G is a passenger on the mRNA and may not directly control its translational status, subcellular localization, or metabolic fate. Further studies are in progress to evaluate these issues.

Reliability of Our Proteomics/Mass Spectrometric Results—In evaluating this evidence, it is important that the TAP-A3G chimeric protein that we used as a bait has strong intracellular anti-HIV-1 activity, was efficiently neutralized by Vif (Fig. 1), and was affinity-purified from HEK293T cell extracts in physiologically appropriate conditions of pH and ionic strength. Although our gel electrophoresis and mass spectrometric data both revealed a surprisingly large number of proteins that bind to TAP-A3G but not to the TAP negative control (see Fig. 2, Table 1, and supplemental Table S1), our additional studies strongly supported the reliability of these identifications. Specifically, the TAP-A3G-associated proteins identified by mass spectrometry were confirmed in all tested cases by coimmunoprecipitations from HEK293T extracts with A3G-Myc using commercially available or donated antibodies in stringent assay conditions (Fig. 3A), and these proteins also coimmunoprecipitated specifically in all tests with A3F-HA (Fig. 3C) and with the endogenous untagged A3G from H9 T cells (Fig. 3B). In further agreement with our mass spectrometric evidence, we found that A3G was substantially associated with the mRNA component of polysomes and dormant stress granules in both HEK293T cells and H9 lymphocytic cells (Figs. 4–6). Thus, the large diversity of proteins that copurified with TAP-A3G is compatible with the metabolic complexity of mRNA. Our data strongly imply that the complexes we identified by mass spectrometry occur in T lymphocytes rather than only in transfected HEK293T cells, and that they are stable throughout multiple steps of affinity purification and, in the case of A3G-polysome complexes, during sucrose gradient sedimentation in a buffer containing 0.25 M KCl (Figs. 4 and 5). These considerations suggest that our LC/MS/MS identifications of A3G-associated proteins were accurate and biologically relevant.

Potential Functional Roles of A3G-associated Proteins—Although we have not analyzed effects of specific A3G-associated proteins on its anti-HIV-1 function, several of our observations are relevant to this issue. We did not detect any effects of Vif on the quantities of these proteins in cellular extracts, implying that they are not degraded by a piggyback mechanism. Nearly all of these proteins were also linked to A3G by RNase A-sensitive bonds, suggesting that they are only secondarily associated rather than directly attached to A3G. In addition, many of the A3G-associated proteins were not incorporated with A3G into HIV-1 virions (Fig. 8) or were incorporated independently of A3G (see below). Nevertheless, several of the identified proteins might influence HIV-1 infections. For example, murine ZAP inhibits replication of murine leukemia viruses (52), and our evidence suggests that the human ZAP homolog binds to A3G in a manner that appears to be substantially resistant to RNase A. Additional evidence will be required to determine whether human ZAP influences HIV-1 replication in an A3G-and/or Vif-dependent manner. From a functional perspective it is also notable that several of the A3G-associated proteins were shown previously to occur in HIV-1 virions (e.g. Stau1 (66)) or to bind HIV-1 proteins (e.g. Stau1 to the Gag precursor (65)) or HIV-1 RNA (e.g. PABP1 binds to a region in the Gag coding sequence and controls Gag expression (70), whereas Purα, YB-1, and Stau1 bind to the TAR sequence (56, 71–73)). Other proteins of potential interest include hnRNP Q that associates with APOBEC1 (74) and interleukin-binding proteins 2 and 3 (NF45 and NF90, respectively), which are essential for encapsidation and protein priming of hepatitis B viral polymerase (75).
Identification of APOBEC3G-Ribonucleoprotein Complexes

A C-terminal variant of NF90 confers resistance to HIV-1 by activating a cellular antiviral response (76).

A3G Incorporation into HIV-1 Virions—Our results confirm that A3G is incorporated efficiently into HIV-1 virions that assemble in HEK293T cells (Fig. 8) (2, 13–16, 64). In contrast, despite their intracellular abundances, we were unable to detect the A3G-associated proteins PABP1, YB-1, PTB, or Ro in purified HIV-1, even when large quantities of the virions were used and abundant A3G had been incorporated (see Fig. 8). Relative to their amounts in the cell extracts, A3G was highly concentrated in the virions, and the above proteins were excluded. We confirmed that a small amount of Stau1 was excluded. We confirmed that a small amount of Stau1 was

Acknowledgments—We thank Debra McMillen for expert assistance and advice concerning the mass spectrometric analyses; Gabriella Boccaccio for antiserum to Stau1; Hua Lu for antiserum to ribosomal protein LS; and our colleague Sheetal Golem for encouragement and for generously sharing resources and helpful advice.

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