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HIV-1 Vif promotes the formation of high molecular mass APOBEC3G complexes

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Abstract

HIV-1 Vif inhibits the antiviral activity of APOBEC3G (APO3G) by inducing proteasomal degradation. Here, we studied the effects of Vif on APO3G *in vitro*. In this system, Vif did not cause APO3G degradation. Instead, Vif induced changes in APO3G that affected immunoprecipitation of the native protein. This effect required wt Vif and was reversed by heat-denaturation of APO3G. Sucrose gradient analysis demonstrated that wt Vif induced the gradual transition of APO3G translated *in vitro* or expressed in HeLa cells from a low molecular mass conformation to puromycin-sensitive high molecular mass (HMM) complexes. In the absence of Vif or the presence of biologically inactive Vif APO3G failed to form HMM complexes. Our results expose a novel function of Vif that promotes the assembly of APO3G into presumably packaging-incompetent HMM complexes and may explain how Vif can overcome the APO3G-imposed block to HIV replication under conditions of no or inefficient APO3G degradation.

Keywords

Vif; APOBEC3G; high molecular mass complexes; protein degradation

Introduction

HIV-1 and most other lentiviruses encode Vif (Virion infectivity factor), an accessory protein that is required for virus replication in primary $CD4^+$ T cells and monocytes. The host cell factor that inhibits virus replication in the absence of Vif was identified as APOBEC3G (APO3G) (Sheehy et al., 2002). APO3G belongs to a family of cytidine deaminases that in humans include APOBEC1, APOBEC2, seven genes designated APO3A through APO3H, as well as the activation-induced deaminase (AID) gene (for review see (Jarmuz et al., 2002; Wedekind et al., 2003)). In the absence of Vif, APO3G is active against a broad range of retroviruses but can also block hepatitis B virus whose replication cycle involves the reverse transcription of a pregenomic RNA intermediate (Seeger and Mason, 2000). Recently, another member of the APOBEC family, APOBEC3F, was shown to inhibit HIV-1 replication by a mechanism similar to that of APO3G (Wiegand et al., 2004; Zheng et al., 2004). Interestingly,

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Vif displays species specificity in its ability to interact with APO3G and the results from several independent reports suggest that the determinants of this species specificity map to amino acid 128 of APO3G (Bogerd et al., 2004; Mangeat et al., 2004; Schrofelbauer et al., 2004; Xu et al., 2004). It is now well established that Vif can reduce the cellular expression of APO3G and inhibit its incorporation into virions although the precise mechanism of the latter activity of Vif is still under investigation (Kao et al., 2003; Mariani et al., 2003; Marin et al., 2003; Sheehy et al., 2003; Stopak et al., 2003). The reduction in cellular expression has been attributed to the degradation of APO3G by cytoplasmic proteasomes (Conticello et al., 2003; Liu et al., 2004; Marin et al., 2003; Mehle et al., 2004b; Sheehy et al., 2003; Stopak et al., 2003; Yu et al., 2003). Based on these reports, degradation of APO3G involves binding of Vif to APO3G but also requires the interaction of Vif with components of an Skp1-cullin-F-box (SCF)-like complex that recognizes a conserved sequence motif located near the C-terminus of Vif (Mehle et al., 2004b; Yu et al., 2003). Other studies, however, found that Vif can prevent APO3G encapsidation even in the absence of noticeable degradation suggesting that intracellular degradation of APO3G may not be the sole mechanism by which Vif neutralizes its antiviral activity (Kao et al., 2007; Kao et al., 2003; Kao et al., 2004; Mariani et al., 2003; Mehle et al., 2004a; Opi et al., 2007).

APO3G is a cytoplasmic protein. Immunocytochemistry revealed diffuse cytoplasmic staining as well as accumulation of the protein in punctate cytoplasmic structures described as P bodies or stress bodies (Bogerd et al., 2006; Chen et al., 2006a; Gallois-Montbrun et al., 2007; Kozak et al., 2006; Niewiadomska et al., 2007; Opi et al., 2007; Wichroski et al., 2005; Wichroski et al., 2006). FPLC fractionation of APO3G containing cell lysates further revealed the assembly of APO3G into high molecular mass (HMM) complexes (Chen et al., 2006b; Chiu et al., 2005). Mass spectrometry of immunopurified HMM complexes identified a multi-subunit ribonucleoprotein complex (Chiu et al., 2006; Gallois-Montbrun et al., 2007; Kozak et al., 2006). The formation of high molecular mass complexes is not a unique property of APOBEC3G but was observed for APOBEC3F and APOBEC1 as well (Harris et al., 1993; Wang et al., 2007). Recent data suggest that APO3G antiviral activity is associated with its low molecular mass conformation (Kreisberg et al., 2006; Soros et al., 2007). Thus, the functional significance of HMM APO3G complexes for the control of retroviral replication remains unclear.

In this study, we have analyzed the effects of Vif on APO3G *in vitro*. We employed rabbit reticulocyte lysates to express Vif and APO3G either alone or in combination and assessed the impact of Vif on APO3G expression and stability. Rabbit reticulocyte lysates contain all the cellular components necessary for protein synthesis and post-translational modifications. In addition, we have previously employed reticulocyte lysates to investigate the ubiquitin-dependent proteasome degradation of CD4 (Chen et al., 1993). We found that Vif did not cause degradation of APO3G in our *in vitro* system. Instead, we found that binding of wild type (wt) Vif to APO3G interfered with the immunoprecipitation of native APO3G by epitope tag-specific antibodies. This effect was reversed by heat-denaturation of APO3G suggesting an effect of Vif on APO3G conformation. We hypothesized that wt Vif may induce aggregation of APO3G into high molecular mass complexes thereby rendering the protein inaccessible to antibodies for immunoprecipitation. Indeed, sucrose gradient fractionation of APO3G revealed the presence of puromycin-sensitive high molecular mass complexes in samples containing wt Vif. APO3G in the absence of Vif or in the presence of a biologically inactive Vif mutant failed to form similar high molecular mass complexes. Importantly, this effect of Vif on APO3G was not only observed in vitro but was also seen in transfected HeLa cells suggesting that it is a genuine property of Vif. The results from this study expose a novel effect of Vif on APO3G that does not require protein degradation and thus has important implications for our understanding of the role of Vif in the inhibition of APO3G antiviral activity.

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Results

In vitro interaction of Vif and APO3G

Co-immunoprecipitation studies performed in tissue culture previously demonstrated physical interactions between Vif and APO3G (Bogerd et al., 2004; Conticello et al., 2003; Mangeat et al., 2004; Mariani et al., 2003; Marin et al., 2003; Stopak et al., 2003; Xu et al., 2004; Yu et al., 2003). To further study functional interactions of Vif and APO3G, we employed an *in vitro* transcription/translation system where Vif and APO3G genes were separately transcribed using SP6 RNA polymerase and the resulting mRNA was used to direct protein synthesis in rabbit reticulocyte lysates. Our first goal was to verify that Vif and APO3G physically interacted in this *in vitro* system. To that end, we translated wild type (wt) Vif or a Vif mutant unable to inhibit the antiviral activity of APO3G (VifΔ23–43; (Khan et al., 2001)) as well as APO3G-MycHis in separate translation reactions. A mock reaction without mRNA was prepared in parallel. The two Vif proteins were metabolically labeled with [^{35}S] methionine/cysteine whereas APO3G and the mock sample remained unlabeled. The translation reactions were stopped after 60 min and lysates containing APO3G and either wt Vif or VifΔ23–43 were mixed at 1:1 ratios (20 μl each), supplemented with fresh lysate to a total volume of 150 μl and 50 μl each were incubated at 30°C for 0, 15, or 45 min. Similarly, mock lysates were mixed with lysates containing wt Vif or VifΔ23–43 and processed in the same manner. Samples were either analyzed directly by SDS-PAGE (Fig. 1, Total Protein) or subjected to immunoprecipitation with a polyclonal antibody to the Myc epitope tag present at the C-terminus of APO3G (Fig. 1, IP). As expected, Vif was not precipitated by the Myc-specific antibody in the absence of APO3G (Fig. 1, lanes 1–3 & 7–9). In contrast, wt Vif and VifΔ23–43 co-precipitated with APO3G (Fig. 1, lanes 4–6 & 10–12, respectively). These results confirm that Vif and APO3G are able to interact *in vitro* and that deletion of residues 23–43 in Vif did not affect its ability to interact with APO3G. Interestingly, pre-incubation of Vif with APO3G containing lysates failed to improve co-immunoprecipitation of wt Vif or VifΔ23–43 suggesting that Vif:APO3G complexes in both cases formed rapidly after mixing the samples.

Wt Vif affects immunorecogniton of APO3G

To analyze possible effects of Vif on APO3G stability *in vitro*, a time-course experiment was performed. To that end, Vif and APO3G were individually translated in reticulocyte lysates. In this case, APO3G protein was labeled with [^{35}S] methionine/cysteine whereas Vif proteins were synthesized in the absence of isotope. The translation reactions were stopped after 60 min, at which time Vif- and APO3G-containing lysates were mixed at 1:1 ratios (20 μl each) and fresh reticulocyte lysate was added to a total volume of 250 μl. The reaction mixture was instantly redistributed into 5 equal aliquots of 50 μl each, which were then incubated for up to 3 hr at 30°C. Individual aliquots were removed at different time points as indicated in figure 2 and stored on dry ice until all samples were collected. Samples (20% of total) from each time point were then either TCA precipitated (Fig. 2A, TCA) or immunoprecipitated (80% of each sample) using a polyclonal antibody against the Myc epitope tag of APO3G (Fig. 2A, IP). APO3G-specific bands were quantified and results from four independent experiments were plotted as percentage of the starting material (Fig. 2B). We found that incubation of wt Vif with APO3G did not have any discernible effects on the stability of APO3G over the three-hour observation period as evidenced by the fact that the amounts of total protein did not decrease with time (Fig. 2B, open circles). Surprisingly, however, there was a gradual decrease in the amount of APO3G immunoprecipitated by the antibody to the Myc epitope tag. Quantitation of the APO3G-specific bands (Fig. 2B, closed circles) showed that the amounts of APO3G recovered by immunoprecipitation rapidly decreased with a "half-life" of about 90 min. The reduction in immunoprecipitated APO3G cannot be explained by Vif-induced degradation of the protein since direct protein analysis (Fig. 2B, open circles) clearly verified

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the presence of constant amounts of APO3G at all times. This conclusion is further supported by our observation that the presence of 50 μM MG132 during the 3 hr incubation period did not prohibit the gradual loss of immunorecognition of APO3G (Fig. S1). The reduced immunoprecipitation of APO3G can also not be explained by proteolytic removal of the Myc epitope tag since the resulting shift in the mobility of the protein would be apparent in figure 2A. Thus, the most plausible explanation for the progressively reduced immunoprecipitation of APO3G by the Myc-specific antibody is direct or indirect interference by Vif. Of note, the observed effects were epitope tag independent since similar results were obtained with HA-tagged APO3G (data not shown).

A biologically inactive Vif mutant does not interfere with immunorecognition of APO3G

In figure 1 we demonstrated the ability of Vif to interact with APO3G *in vitro*. This raised the possibility that the reduced immunoprecipitation of APO3G in figure 2A was the result of steric interference by Vif. To address this issue, we performed a kinetic study of APO3G in the presence of the VifΔ23–43 variant, which is biologically inactive (Khan et al., 2001) but still capable of binding to APO3G *in vitro* (Fig. 1). The experimental procedure and quantitation of the results were the same as described for figure 2A/B. Consistent with the results from figure 2A, direct analysis of APO3G protein did not reveal any effects of VifΔ23–43 on APO3G stability (Fig. 2C, TCA). Interestingly, constant amounts of APO3G were recovered at all times by immunoprecipitation (Fig. 2C, IP) and quantitation of the data confirmed that VifΔ23–43 did not affect immunorecognition of APO3G (Fig. 2D). The different effects of wt Vif and VifΔ23–43 on the immunoprecipitation of APO3G are difficult to explain by steric interference of Vif with Myc-antibody binding to its target epitope since both wt Vif and VifΔ23–43 interacted with APO3G (Fig. 1). Our finding that VifΔ23–43 had no effect on APO3G immunoprecipitation also suggests that the increasing resistance of APO3G to immunoprecipitation in figure 2A was not the result of normal post-translational protein refolding but was caused by the addition of wt Vif to the sample.

As an additional control for the specificity of the effect of HIV-1 Vif on human APO3G, we analyzed the impact of HIV-1 Vif on African Green monkey APO3G (Agm-APO3G), which is resistant to HIV-1 Vif (Bogerd et al., 2004; Mangeat et al., 2004; Mariani et al., 2003; Schrofelbauer et al., 2004). The experiment was performed essentially as described for figure 2A/B employing wild type HIV-1 Vif and Agm-APO3G and the results are shown in figure S2. As expected, Vif had no effect on the stability of Agm-APO3G as judged by direct protein analysis (Fig. S2B, open circles). Importantly, wild type HIV-1 Vif also had no effect on the immunoprecipitation of Agm-APO3G (Fig. S2B, closed circles). Thus, the effect of wild type HIV-1 Vif on the conformation of native human APO3G observed in figure 2 is highly specific and dependent on the direct interaction of wild type HIV-1 Vif with human APO3G.

Heat-denaturation restores immunorecognition of APO3G

To test if the inaccessibility of APO3G to immunoprecipitation in figure 2A was due to a Vif-induced change in APO3G conformation, we subjected APO3G that had previously been exposed to wt Vif to heat-denaturation prior to immunoprecipitation. The experiment was essentially performed as described for figure 2A except that prior to immunoprecipitation by the Myc-specific polyclonal antibody, half of the APO3G sample was heated for 5 min at 95° C as detailed in the legend to figure 2. The results of the experiment are shown in figure 2E and the quantitative analysis of two independent experiments is shown in figure 2F. As expected, direct analysis of TCA precipitated protein confirmed the presence of constant amounts of APO3G throughout the 3 hr observation period (Fig. 2F, closed triangles). Also, immunoprecipitation of non-denatured protein revealed the same gradual loss of APO3G as observed in figure 2A (Fig. 2F, closed circles). Importantly, however, heat-denaturation of the protein samples prior to immunoprecipitation led to the recovery of similar amounts of APO3G

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by immunoprecipitation at all time points (Fig. 2F, open circles). These results support our model that interaction of wt Vif with APO3G induces a gradual yet reversible conformational change that affects APO3G recognition by epitope tag-specific antibodies.

Analysis of Vif-APO3G complexes

Tissue culture analyses have identified APO3G in two principal conformations: a low molecular mass (LMM) conformation of cytoplasmic APO3G and a high molecular mass (HMM) conformation, in which APO3G is associated with a variety of other host factors in a large RNA:protein complex in P-bodies or stress granules (Chiu et al., 2005; Gallois-Montbrun et al., 2007; Kozak et al., 2006; Wichroski et al., 2006). Newly synthesized APO3G is initially in the LMM conformation but assembles into HMM multi-protein complexes within approximately 30 minutes after synthesis (Soros et al., 2007). While this change of APO3G from LMM to HMM conformation is a natural process that does not require Vif, it is conceivable that the presence of Vif affects the kinetics of APO3G transition from LMM to HMM conformation.

We next studied the possible effects of Vif on APO3G conformation in our *in vitro* system. In this experiment, APO3G was metabolically labeled with [^{35}S] methionine/cysteine whereas wt Vif and VifΔ23–43 were translated in the absence of isotope as described for figure 2. *In vitro* synthesized [^{35}S]-labeled APO3G was incubated for 3 hr at 30°C with mock-translated lysate (Fig. 3 A, top panel), lysate containing wt Vif (Fig. 3 A, middle panel), or lysate containing VifΔ23–43 (Fig. 3A, lower panel). Samples were then layered onto a 15–40% sucrose gradient and centrifuged at 86,000 × g for 3 hr as reported (Kozak et al., 2006). Fractions were collected from the top of the gradient and subjected to 12.5% SDS PAGE. Fraction #1 was omitted from the analysis because it contained residual unincorporated isotope. APO3G proteins were visualized by fluorography. In the absence of Vif (Fig. 3A, top panel) or in the presence of VifΔ23–43 (Fig. 3A, bottom panel), APO3G was confined to the top of the gradient (fractions 2–6). In contrast, the presence of wt Vif resulted in a broader distribution of APO3G across the sucrose gradient (Fig. 3A, middle panel) and, in addition, small amounts of APO3G were recovered from the bottom of the gradient (fraction 19). Analysis of Vif in an experiment identical to the one shown in figure 3A except that Vif was metabolically labeled and APO3G remained unlabeled showed that Vif was largely confined to the upper fractions (Fig. 3B, fractions 2–8). We conclude that the presence of wt Vif promotes the assembly of APO3G into HMM complexes *in vitro*. The formation of *in vitro* APO3G HMM complexes was detergent-sensitive and complexes could be dissociated by addition of 0.5% SDS (data not shown).

Vif induces APO3G transition complexes

We next studied the kinetics of Vif-induced formation of HMM APO3G complexes *in vitro*. The experiment was performed essentially as described for figure 3A except that Vif and APO3G were incubated for various times (30 min, 90 min, 180 min) prior to sucrose gradient centrifugation (Fig. 4). At all times, significant amounts of APO3G were found in the upper fractions of the gradient (Fig. 4, all panels, fractions 2–4). Interestingly, an intermediate APO3G complex was detectable at the 30 minute and 90 minute time points (Fig. 4, fraction 14; top and middle panels) but was absent from the 180 minute sample. At the same time, APO3G in fraction 19 was undetectable in the 30 minute sample (Fig. 4, top panel, fraction 19) but was identified in increasing amounts in the later samples (Fig. 4, middle and lower panel, fraction 19). We conclude that conversion of APO3G from LMM to HMM conformation *in vitro* in the presence of Vif is a slow and gradual process that involves the formation of a distinct transition complex.

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Recently, Kozak et al. found that APO3G complexes from transfected 293T cells were sensitive to treatment with puromycin, a drug known to induce the release of nascent proteins from polysomes; their results strongly suggested that APO3G in untreated samples was bound to translationally active polysomes (Kozak et al., 2006). Interestingly, when puromycin was added to our *in vitro* reactions, the Vif-induced assembly of APO3G into intermediate (fraction 14) and heavy complexes (fraction 19) was completely inhibited as well (Fig. 4, puromycin). Thus, it appears that Vif promotes the association of APO3G with polysomes *in vitro*.

In Figure 3B we demonstrated that Vif was not part of the heavy APO3G complexes even though the formation of these complexes was Vif-dependent. To see if Vif was part of the APO3G transition complexes identified in figure 4 we metabolically labeled both Vif and APO3G and incubated the combined lysates for 30 or 90 minutes as described for figure 4. Samples were then subjected to sucrose gradient analysis as in figure 4. For comparison we analyzed APO3G in combination with either wt Vif (Fig. 5A) or VifΔ23–43 (Fig. 5B). In this experiment both APO3G and Vif are visible (Fig. 5). As expected, APO3G formed a distinct transition complex in the presence of wt Vif (Fig. 5A, APO3G) following 30 or 90 min incubation. Interestingly, wt Vif was found in fractions of the gradient up to and including fraction 14 (Fig. 5A, wt Vif). Consistent with our previous results, no transition complexes were observed in the presence of VifΔ23–43 and both VifΔ23–43 and APO3G were restricted to fractions 1–10 of the sucrose gradient (Fig. 5B). These results suggest that Vif may be part of APO3G transition complexes but subsequently dissociates as APO3G continues to form increasingly heavy complexes pelleting to the bottom of our gradients.

Effect of Vif on HMM association of APO3G in transfected HeLa cells

We recently identified an APO3G variant, APO3G-C97A, which was resistant to Vif-induced degradation but was still sensitive to Vif with respect to virus incorporation and antiviral activity (Opi et al., 2007). We also found that the ability of Vif to exclude APO3G from virions could be dissociated from its ability to cause proteasomal degradation of APO3G (Kao et al., 2007). From these observations we concluded that Vif had the ability to regulate APO3G encapsidation through degradation-independent mechanism(s). Furthermore, Soros et al. recently provided evidence that virus encapsidated APO3G occurs primarily from the LMM pool of *de novo* synthesized protein (Soros et al., 2007). Thus, the ability of Vif to promote the transition of APO3G from an LMM to a HMM conformation could be a way to prevent encapsidation of APO3G into HIV-1 virions in the absence of protein degradation.

To study the effect of Vif on HMM association of APO3G in tissue culture, HeLa cells were transfected with pcDNA-APO3G together with either pNL-A1 for the expression of Vif (Fig. 6A) or pNL-A1vif(−) as a *vif*-negative control (Fig. 6B). To prevent Vif-induced degradation of APO3G, a dominant negative Cul5 mutant, Cul5-Rbx (Yu et al., 2003), was cotransfected in this experiment. Cells were harvested 24 hr later and lysed as described in Materials and Methods. Post-nuclear extracts were overlaid onto 15–40% sucrose gradients and centrifuged for 3 hr at 86,000 × g. Twenty fractions (500 μl each) were collected from the top of the gradients and subjected to immunoblot analysis using an APO3G-specific peptide antibody (Fig. 6A/B, α-APO3G). The same blots were subsequently re-probed with a Vif-specific monoclonal antibody (α-Vif). In general, APO3G from transfected HeLa cells was more broadly distributed in the gradient than *in vitro* translated APO3G. This suggests that in tissue culture systems, APO3G has a greater propensity to assembly into HMM complexes irrespective of the presence or absence of Vif. Interestingly, the co-expression of Vif resulted in the appearance of a heavy APO3G complex sedimenting to the bottom of the sucrose gradient thus closely mimicking our *in vitro* results (Fig. 6A, fraction #20). This complex was not seen in the absence of Vif (Fig. 6B). Also, consistent with our *in vitro* analyses, Vif was absent from the heavy APO3G complexes (Fig. 6A, α-Vif). Our results suggest that the ability of Vif to

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promote the transition of APO3G from light to heavy complexes is not limited to the *in vitro* system but can be observed in tissue culture systems as well and is therefore likely to represent a functionally relevant property of Vif.

Discussion

HIV-1 Vif is essential for replication *in vivo* and in nonpermissive cell types in tissue culture. Several recent studies have reported that Vif can induce the degradation of APO3G when expressed in non-permissive target cells. This effect of Vif is manifested by a reduction of the half-life of APO3G in the presence of Vif when compared to *vif*-deficient controls (Conticello et al., 2003; Liu et al., 2004; Marin et al., 2003; Mehle et al., 2004b; Sheehy et al., 2003; Stopak et al., 2003; Yu et al., 2003). Furthermore, the decrease in APO3G protein levels was abrogated in the presence of proteasome inhibitors, indicating that Vif targeted APO3G for degradation by proteasomes (Conticello et al., 2003; Liu et al., 2004; Marin et al., 2003; Mehle et al., 2004b; Sheehy et al., 2003; Stopak et al., 2003; Yu et al., 2003). In support of this hypothesis, co-expression of Vif and APO3G was shown to result in poly-ubiquitination of APO3G, which is known to precede the proteasome-dependent degradation of proteins (for review see (Ciechanover et al., 2000)). Indeed, Yu et al demonstrated that Vif interacts with components of an ubiquitin ligase complex through a conserved motif near the C-terminus of Vif providing additional evidence that Vif regulates the degradation of APO3G (Yu et al., 2003). Thus, there is overwhelming evidence that Vif can induce the degradation of APO3G in tissue culture systems.

On the other hand, we and others have found that inhibition of APO3G encapsidation by Vif is not always accompanied by a proportional reduction in cellular APO3G (Kao et al., 2007; Kao et al., 2003; Kao et al., 2004; Mariani et al., 2003) and, more importantly, we have recently identified an APO3G variant that was resistant to Vif-induced degradation but was nonetheless excluded from HIV-1 virions in a Vif-dependent manner (Opi et al., 2007). Thus, there is increasing evidence that Vif can prevent encapsidation of APO3G not only through targeting the protein to a proteasomal degradation pathway but through additional as yet undefined mechanism(s).

Our *in vitro* study was initiated by the desire to further study the mechanism of Vif-induced degradation of APO3G. We were encouraged in choosing an *in vitro* system by our previous success in demonstrating proteasome-mediated degradation of CD4 in the presence of Vpu in rabbit reticulocyte lysates (Chen et al., 1993). A significant advantage of the *in vitro* system was that proteins could be analyzed directly without prior immunoprecipitation since *de novo* synthesis of metabolically labeled proteins was strictly limited to exogenously supplied mRNA. Our initial immunoprecipitation analyses indeed revealed a gradual decrease of APO3G with time and produced kinetic profiles suggestive of protein degradation (Fig. 2B). However, direct analysis of TCA-precipitated, *in vitro*-translated proteins revealed that the reduced APO3G signals in the immunoprecipitates were not due to protein degradation but rather reflected an effect of Vif on APO3G that induced a gradual resistance to immunoprecipitation. In fact, APO3G protein was perfectly stable over the 3-hour observation period irrespective of the presence of wt or mutant Vif protein. It is not clear why Vif was unable to induce APO3G degradation *in vitro*. However, our previous *in vitro* studies on Vpu-induced degradation of CD4 employed lysates that had been supplemented with canine microsomal membranes (Chen et al., 1993). It is possible that these membrane preparations contributed factors crucial to proteasomal protein degradation. However, this was not further explored in the current study.

Instead, we used the fact that Vif did not cause degradation of APO3G *in vitro* as an opportunity to study additional degradation-independent effects of Vif on APO3G. Indeed, our *in vitro*

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study provided evidence that Vif may promote the sequestration of APO3G into packaging-incompetent HMM complexes. This conclusion was supported by our finding that the presence of Vif induced changes in APO3G that rendered the protein progressively less available to immunoprecipitation. This trend could be reversed by heat denaturation of APO3G prior to immunoprecipitation suggesting that wt Vif induced heat-labile changes in APO3G. Sucrose gradient analyses subsequently showed that the presence of wt Vif was associated with the transition of APO3G into high molecular mass complexes (Fig. 3–Fig. 5). These effects were specific for wt Vif and were not observed with a biologically inactive Vif mutant (Fig. 3A, bottom panel). From the data shown in figure 3A it appears that only a small fraction of the total APO3G assembled into heavy complexes while in figure 2 the majority of APO3G appears to become resistant to immunoprecipitation. This does not rule out a functional correlation between the two effects since it is possible that subjecting the samples to sucrose gradient centrifugation causes partial dissociation of the complexes. This is suggested by our finding that a significant amount of APO3G from transfected HeLa cells is identified in the upper (low molecular mass) fractions in the sucrose gradient shown in figure 6, while FPLC analyses identified the majority of APO3G from transfected HeLa cells in HMM complexes (Opi et al., 2007). Thus, we believe that the gradual transition from a low molecular mass to a high molecular mass conformation of APO3G most likely accounts for its increasing resistance to immunoprecipitation as observed in figure 2.

How Vif might promote the transition of APO3G from LMM to HMM conformations remains to be investigated. Both wt Vif and VifΔ23–43 interacted with APO3G in our *in vitro* analyses. In fact, VifΔ23–43 appeared to interact slightly more efficiently with APO3G than wt Vif (Fig. 1). These results are consistent with co-immunoprecipitation studies in HeLa cells that revealed solid binding of VifΔ23–43 to APO3G (data not shown). Interestingly, the interaction of Vif and APO3G was not improved by preincubating the proteins at 30°C. This suggested that binding of Vif to APO3G was a rapid process. The rapid binding of Vif to APO3G contrasts the slow and gradual increasing resistance of APO3G to immunoprecipitation as shown in figure 2. These kinetic differences argue against steric interference by Vif with antibody binding. Instead they suggest that the resistance of APO3G to immunoprecipitation is due to the conversion of APO3G from a low molecular mass to a high molecular mass conformation. Why only wt Vif can induce these changes is unclear. Both Vif and APO3G are nucleic acid binding proteins and APO3G HMM complexes were previously found to be sensitive to RNase-treatment (Chiu et al., 2005;Gallois-Montbrun et al., 2007;Kozak et al., 2006;Opi et al., 2007;Wang et al., 2007). Of note, rabbit reticulocyte lysates used in this study had been treated with micrococcal nuclease and our *in vitro* translation reactions were routinely stopped by addition of RNase A. Nevertheless, it is possible that APO3G or Vif mRNAs used for the *in vitro* translations were protected from complete RNase digestion and it is possible that the *in vitro* HMM APO3G complexes represent RNA-protein complexes similar to those observed in vivo. In support of this we found that the APO3G complexes dissociated in the presence of puromycin suggesting that they involved noncovalent interactions of APO3G and polysomes. We therefore propose a model, in which APO3G forms a complex with polysomes in reticulocyte lysates similar to those reported in transfected 293T cells (Kozak et al., 2006;Niewiadomska et al., 2007). We speculate that these Vif induced APO3G-polysome complexes are resistant to immunoprecipitation presumably because the C-terminal epitope tag on APO3G is inaccessible to antibodies in these complexes. Importantly, we were able to demonstrate Vif-induced heavy complexes of APO3G in HeLa cells (Fig. 6) and we observed gradual resistance of APO3G in transfected HeLa cells to immunoprecipitation by conformation-sensitive antibodies (data not shown). We therefore conclude that the effects of Vif on APO3G conformation described in this study reflect a process occurring *in vitro* as well as *in vivo*.

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Taken together our results provide evidence for a novel function of Vif that is likely to contribute to the exclusion of APO3G from viral particles by sequestering the protein in packaging-incompetent high molecular mass intracellular complexes. Such a function of Vif could be important early in infection when APO3G has not yet been eliminated from virus-producing cells by proteasome-mediated protein degradation.

Materials and Methods

Plasmids

The *in vitro* transcription vector pSP65 (Promega Biosciences, San Luis Obispo CA) was used for expression of *vif* and *APO3G* genes under the control of the SP6 promoter. For the expression of wt Vif, HIV-1 *vif* was PCR amplified from pNL4-3 and cloned as an *Eco*RI/*Bam*HI fragment into pSP65 (Chen et al., 1993) resulting in vector pSP-Vif. For the expression of VifΔ23–43, the mutant *vif* gene was PCR amplified from pNL-Al/VifΔ23–43 (Khan et al., 2001) and cloned between the *Eco*RI/*Bam*HI sites of pSP65 resulting in pSP-VifΔ23–43. For expression of human APO3G, pSP-hAPO3G was constructed by subcloning APO3G including its C-terminal Myc-His epitope tag from pcDNA-APO3G (Kao et al., 2003) into the pSP65 vector via its *Eco*RI and *Xba*I cloning sites. The following plasmids were used for expression in HeLa cells: pcDNA-APO3G for the expression of untagged human wt APO3G; pNL-A1 for the expression of Vif (Strebel et al., 1987), and pNL-A1vif(−) as a vif-negative control (Karczewski and Strebel, 1996). Plasmid pCul5-HA was a gift of Xiao-Fang Yu (Yu et al., 2003). An Rbx binding mutant, pCul5-Rbx-HA, was constructed by deleting residues 566–582 in pCul5-HA as described (Yu et al., 2003). The vector pSP-AgmAPO3G was used for expression of Agm-APO3G. This vector was constructed by subcloning Agm-APO3G including its C-terminal HA tag from pc-AGM-APO3G-HA (gift of Ned Landau) into pSP65.

Antisera

Immunoprecipitation of APO3G was carried out using a polyclonal antibody to the C-terminal Myc epitope tag (Sigma-Aldrich, St. Louis MO). For immunoprecipitation of Agm-APO3G a rat monoclonal antibody to its C-terminal HA tag was used (Roche Diagnostics, Indianapolis IN). A monoclonal antibody to Vif (MAb #319) was used for immunoblot analysis of Vif and was obtained from Michael Malim through the NIH AIDS Research and Reference Reagent Program (Simon et al., 1995). APO3G was identified using a rabbit polyclonal peptide antibody (ApoC17 (Opi et al., 2007)).

In vitro transcription and translation

For the *in vitro* synthesis of run-off transcripts, all plasmids were initially linearized at the 3' end of the coding sequences using appropriate restriction enzymes. Plasmids pSP-Vif and pSP-VifΔ23–43 were linearized with *Bam*HI whereas pSP-APO3G was linearized using *Xba*I. Subsequent *in vitro* transcription of the linearized DNA was performed using an *in vitro* transcription kit (SP6 Riboprobe system; Promega Biosciences) according to the manufacturer's instructions. RNA resulting from the transcription of 1 μg of plasmid DNA was suspended in 50 μl of RNase-free water. *In vitro* transcribed RNA was used to direct the *in vitro* translation of proteins in rabbit reticulocyte lysates. Typically, 5 μl of RNA was mixed with 40 μl of rabbit reticulocyte lysate (Promega Biosciences) in the presence or absence of 10 μl of [^{35}S] methionine/cysteine (Redivue Pro Mix (10 mCi/ml); Amersham Biosciences Piscataway NJ) and incubated for 60 min at 30°C. Translation reactions were terminated by addition of RNase A (50 μg/ml) to the samples. Proteins were then either subjected to immunoprecipitation or directly analyzed by TCA precipitation and SDS-PAGE.

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TCA precipitation and immunoprecipitation

For direct analysis of *in vitro* translated proteins, proteins were precipitated with trichloroacetic acid (TCA) prior to gel electrophoresis to remove unincorporated isotope. For that purpose, 50 µl of cold 25% TCA was added to 10 µl of translation mix while vortexing. The sample was then incubated on ice for 10 min followed by a brief spin in an Eppendorf microcentrifuge (10 sec, 14,000 × g) to pellet protein aggregates. The supernatant containing unincorporated isotope was discarded and the pellet was washed twice with cold 2% TCA. The pellet was finally suspended in 100 µl of sample buffer (2% SDS, 1% β-mercaptoethanol, 1% glycerol, and 65 mM Tris-hydrochloride (pH 6.8)) and neutralized by addition of 1 N NaOH. The samples were boiled for 5 min and loaded onto 12.5% polyacrylamide-SDS gels.

Immunoprecipitation of *in vitro* translated proteins was carried out using appropriate antibodies as described in the text. Briefly, polyclonal antibodies to the Myc epitope or to Vif (Vif93) were adsorbed on protein A sepharose beads (Sigma-Aldrich) whereas rat anti-HA antibodies were adsorbed on Protein G sepharose beads (Amersham) for 1 hr at 4°C on a rotating wheel The beads were then washed once with cold Triton-Wash Buffer (50 mM Tris pH 7.4,300 mM NaCl, 0.1% Triton X-100). Antibody-adsorbed beads were then suspended in 1 ml of cold 0.1% BSA/PBS, 40 µl of translation reaction mix was added and beads were incubated for 1 additional hour at 4°C on a rotating wheel. After incubation, the beads were washed 3 times with 750 µl of Triton-Wash Buffer. Bound proteins were solubilized by boiling the beads in 100 µl of sample buffer and were then separated on 12.5% polyacrylamide-SDS gels. Gels were soaked in 1 M sodium salicylic acid for 30 min and dried. Radioactive bands were visualized by fluorography. Quantitation of the radioactivity of the respective bands was performed with a Fuji BAS 2000 Bio-Image Analyzer.

Immunoblotting

For immunoblot analysis of sucrose gradient fractions, 75 µl of each fraction were combined with 25 µl of 4x sample buffer (8% sodium dodecyl sulfate, 250 mM Tris-HCl, pH 6.8, 20% 2-mercaptoethanol, 20% glycerol). Proteins were heated for 10 to 15 min at 95°C with occasional vortexing of the samples prior to SDS-PAGE; proteins were transferred to PVDF membranes and reacted with appropriate antibodies as described in the text. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Piscataway NJ) and visualized by enhanced chemiluminescence (ECL, Amersham Biosciences).

Sucrose gradient fractionation

For the analysis of *in vitro* synthesized APO3G, lysates were incubated for different times with lysates containing either wt Vif or the Vif Δ23–43 mutant. After incubation, the samples were layered over 10 ml of 15–40% sucrose gradients made in buffer B (0.01 M Tris-Cl, pH 7.4, 0.25 M KCl,0.01 M $MgCl_2$) and centrifuged at 86,000 × g for 3 h (Kozak et al., 2006). For the analysis of APO3G complexes in HeLa cells, transfected cells were lysed on ice in 400 µl of NP40 lysis buffer (50 mM Tris-HCL pH 7.4, 1% NP40, 0.1% deoxycholate (DOC), 150 mM NaCl, and protease inhibitor cocktail). Nuclei were removed by centrifugation for 5 min at 1,500 × g. Post-nuclear extracts were overlaid onto a 15–40% sucrose gradient and centrifuged for 3 hr at 86,000 × g (Kozak et al., 2006). Nineteen to twenty fractions were collected from the top of the gradient and the samples were combined with 175 µl 4x sample buffer and heated for 5 min at 95°C. Aliquots of each fraction were loaded on 12.5% SDS-PAGE gels. For the analysis of [^{35}S]-labeled *in vitro* translated proteins, gels were soaked in 1 M sodium salicylic acid for 30 min and dried. Radioactive bands were visualized by fluorography. Quantitation of the radioactivity of the respective bands was performed with a Fuji BAS 2000 Bio-Image Analyzer. Proteins from HeLa extracts were analyzed by immunoblotting.

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Supplementary Material

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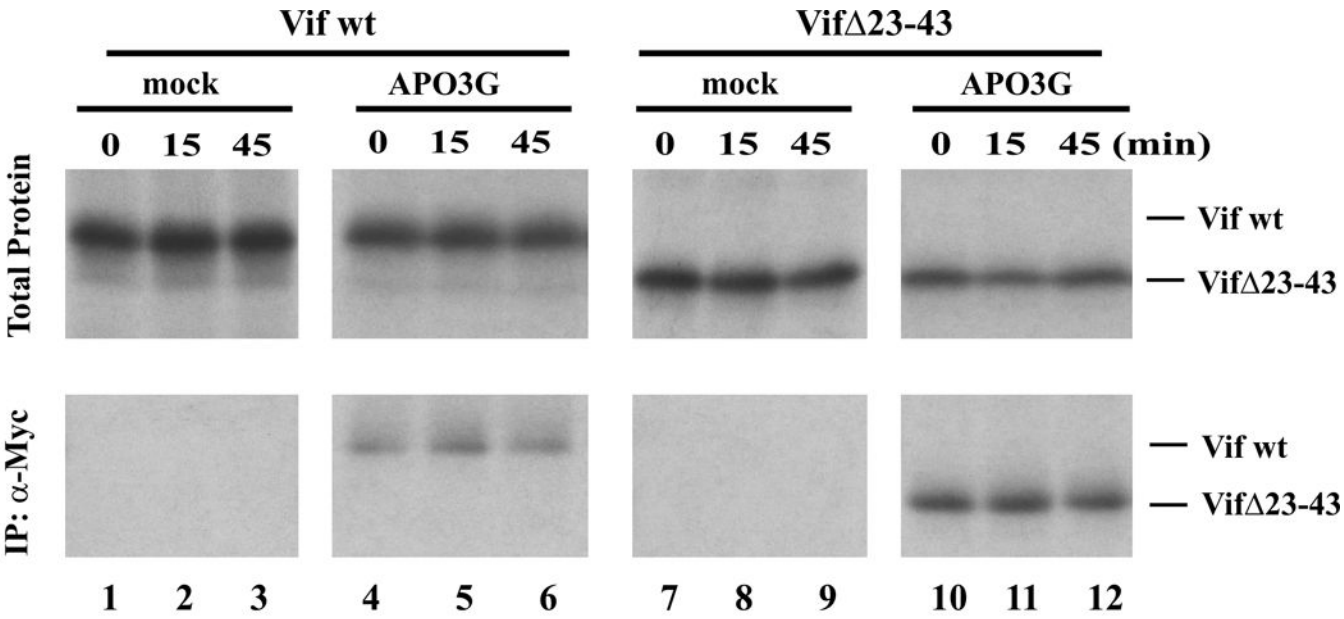

Fig. 1.

In vitro interaction of Vif and APO3G. *In vitro* transcription plasmids encoding wt Vif, VifΔ23–43, and human APO3G were linearized at their 3' ends followed by *in vitro* transcription and translation in rabbit reticulocyte lysates as described in Materials and Methods. The translation reaction was allowed for 60 min at 30°C and then stopped by addition of RNase A (50 μg/ml). Vif proteins were synthesized in the presence of [^{35}S] methionine/cysteine. APO3G protein and mock samples were not metabolically labeled. Vif-containing lysates were mixed at equal ratios with either mock lysate (mock) or APO3G-containing lysate (APO3G). Samples were divided into equal fractions and incubated for 0, 15, or 45 min at 30°C. An aliquot of each sample (20%) was analyzed directly after TCA precipitation (Total Protein). The remaining samples (80%) were immunoprecipitated with a polyclonal Myc antibody (lower panel). Note that only Vif proteins are visible since APO3G was unlabeled. The positions of wt Vif and VifΔ23–43 in the gel are indicated on the right.

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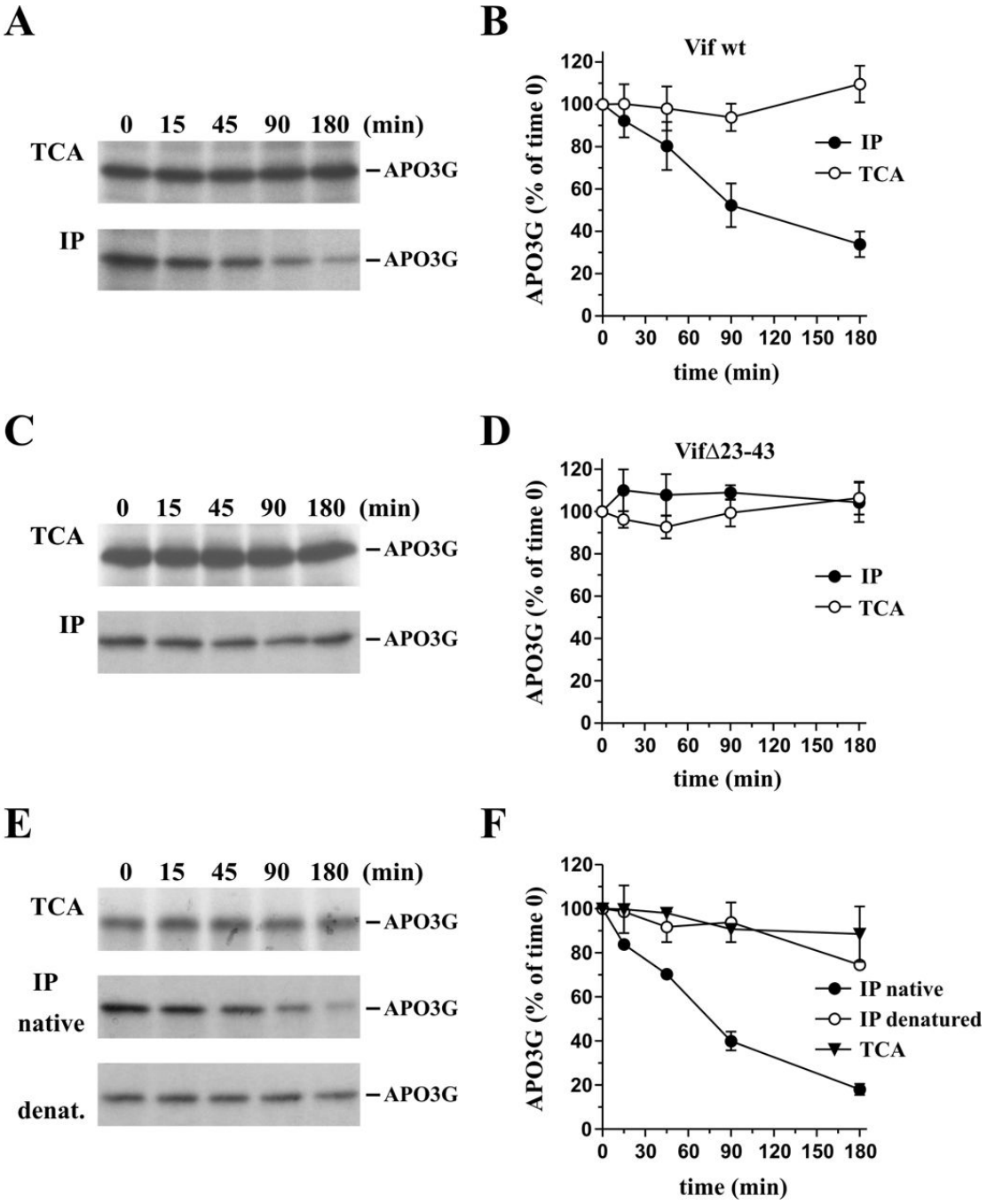

Fig. 2.

Post-translational effect of Vif on APO3G *in vitro*. Vif (unlabeled) and APO3G ([^{35}S]-methionine/cysteine-labeled) were translated separately in reticulocyte lysate for 60 min at 30° C. Equal amounts of the lysates were mixed and fresh reticulocyte lysate was added to a total volume of 250 µl. The mixture was immediately divided into 5 samples (50 µl ea.), which were then incubated for 0, 15, 45, 90, or 180 min at 30°C. **(A & B)** Analysis of wt Vif. **(A)** For total protein analysis, 20% of the samples from each time point was precipitated with TCA and subjected to SDS-PAGE (top panel). Remaining samples (80%) were immunoprecipitated using a polyclonal Myc antibody prior to SDS-PAGE (lower panel). **(B)** The APO3G-specific bands from panels A were quantitated using a Fuji BAS 2000 Bio-Image Analyzer and the

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results were plotted as a function of time. Error bars reflect standard error from four independent experiments. **(C & D)** Analysis of VifΔ23–43. The experiment is identical to panels A & B except that VifΔ23–43 was employed instead of wt Vif. Error bars reflect standard error from four independent experiments. **(E & F)** Heat denaturation rescues immunoprecipitation of APO3G exposed to wt Vif. **(E)** The experiment was done essentially as described for panels A & B. For total protein analysis, 20% of the samples from each time point was precipitated with TCA and subjected to SDS-PAGE (top panel). The remaining samples (80%) were divided into 2 equal parts. One half was immunoprecipitated using a polyclonal Myc antibody as in panel A. The second half was first denatured by adding an equal volume of 2x sample buffer and heating the sample at 95°C for 5 min. Prior to immunoprecipitation, the sample volume was increased to 1 ml using phosphate-buffered saline to reduce detergent and 2-mercaptoethanol levels in the sample. Immunoprecipitation was then carried out as before at 4°C using a polyclonal Myc antibody (lower panel). **(F)** The APO3G-specific bands from panel E were quantitated as in panel B. Error bars reflect standard error from two independent experiments

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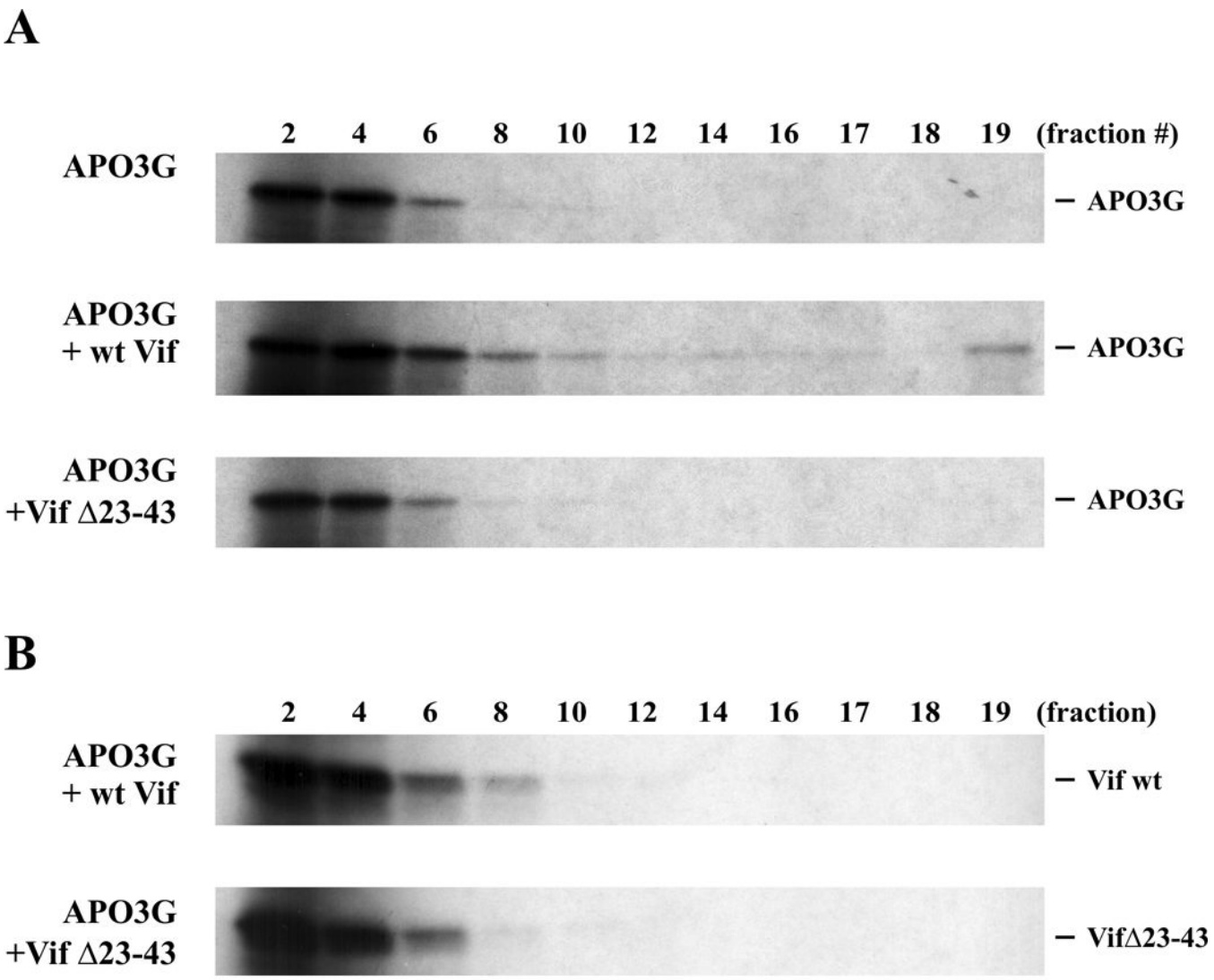

Fig. 3.

Sucrose gradient analysis of *in vitro* translated APO3G. **(A)** *In vitro* synthesized [^{35}S]-labeled APO3G (20 µl) was incubated for 3 h at 30°C with an equal volume of mock-translated lysate (top panel) or with an equal volume of lysate containing unlabeled wt Vif (middle panel) or VifΔ23–43 (lower panel). After incubation, the samples were layered onto 10 ml gradients of 15–40% sucrose in buffer B (0.01 M Tris-Cl, pH 7.4, 0.25 M KCl, 0.01 M $MgCl_2$ (Kozak et al., 2006)) and centrifuged for 3 h at 86,000 × g. Nineteen fractions (530 µl ea.) were collected from the top of the gradient and combined with 175 µl of 4x sample buffer. Aliquots were separated by SDS-12.5% PAGE and APO3G protein was visualized by fluorography. **(B)** The experiment was repeated as in panel A except that Vif was now metabolically labeled and APO3G remained unlabeled. The mock-translated control was omitted here since no labeled protein was present in the mock lysate. Sucrose gradient analysis and gel electrophoresis were performed as in panel A.

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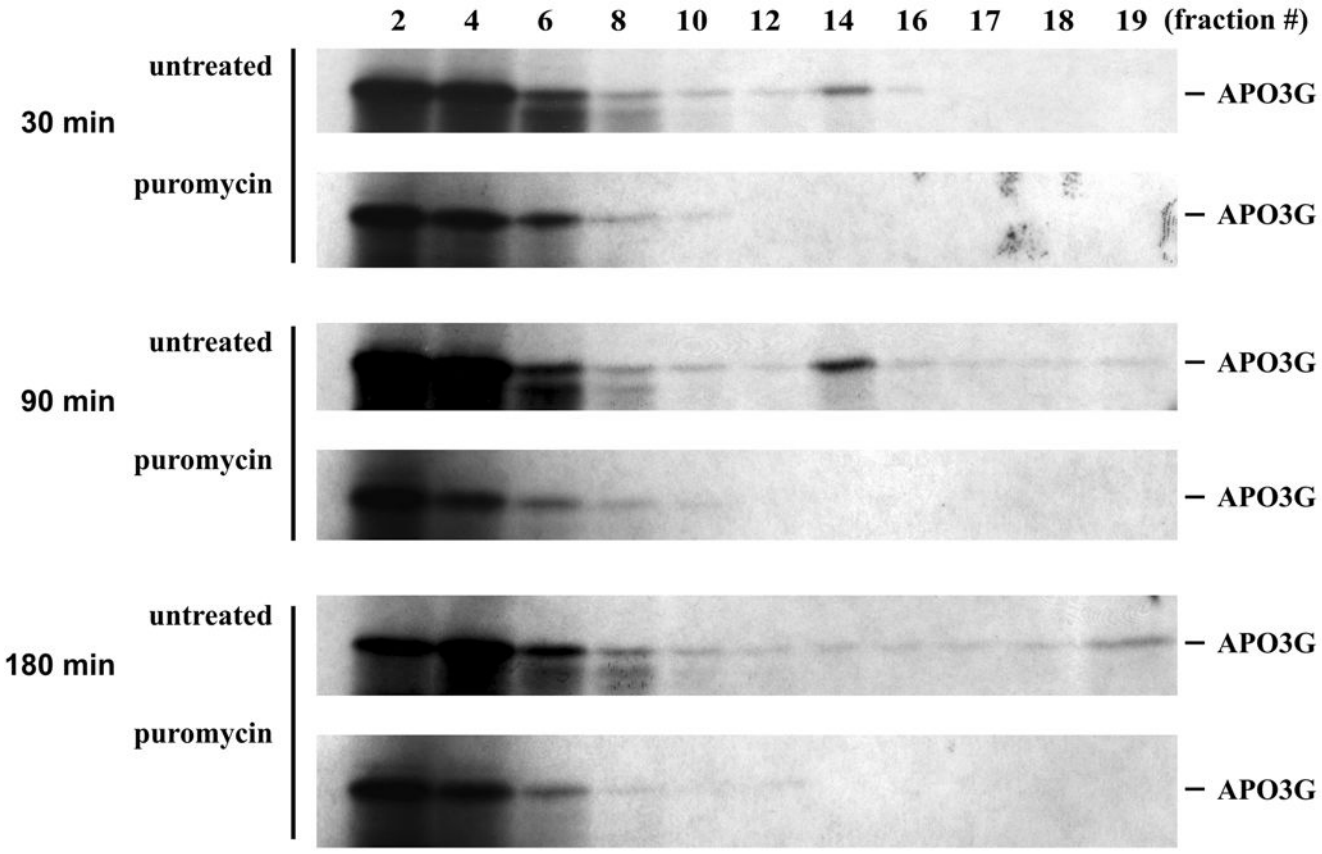

Fig. 4.

Kinetic analysis of APO3G high molecular mass complexes. *In vitro* synthesized [^{35}S]-labeled APO3G (20 µl) was incubated for 30, 90, or 180 minutes at 30°C with an equal volume of lysate containing unlabeled wt Vif. Samples were either incubated alone (untreated) or in the presence of puromycin (0.5 µg/ml; puromycin). After incubation, the samples were layered onto 10 ml gradients of 15–40% sucrose and processed as in figure 3A.

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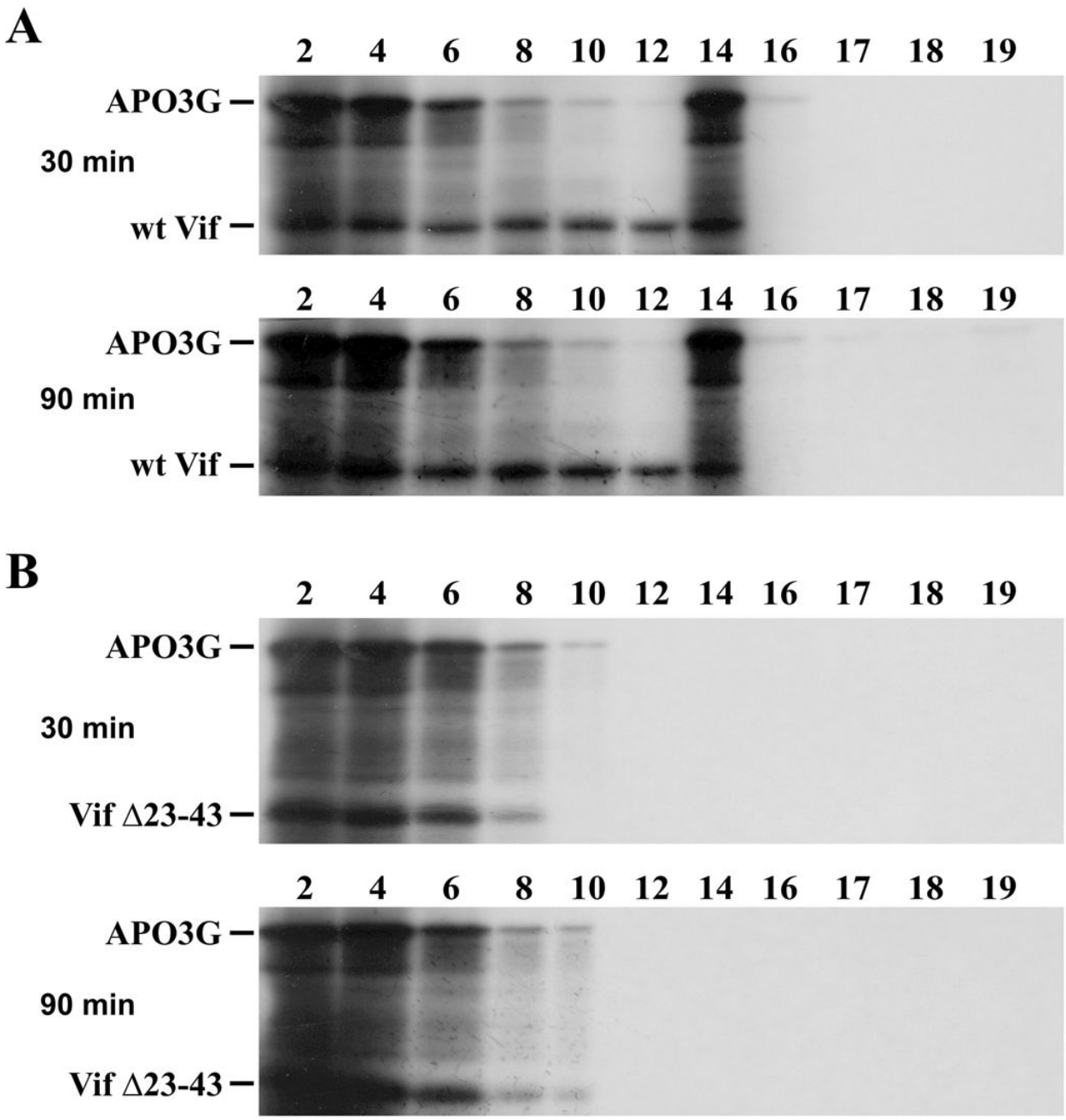

Fig. 5.

Association of Vif with APO3G complexes. *In vitro* synthesized [^{35}S]-labeled APO3G (20 μl) was incubated for 30, 90, or 180 minutes at 30°C with an equal volume of lysate containing [^{35}S]-labeled wt Vif **(A)** or VifΔ23–43 **(B)**. After incubation, the samples were layered onto 10 ml gradients of 15–40% sucrose and processed as in figure 3A.

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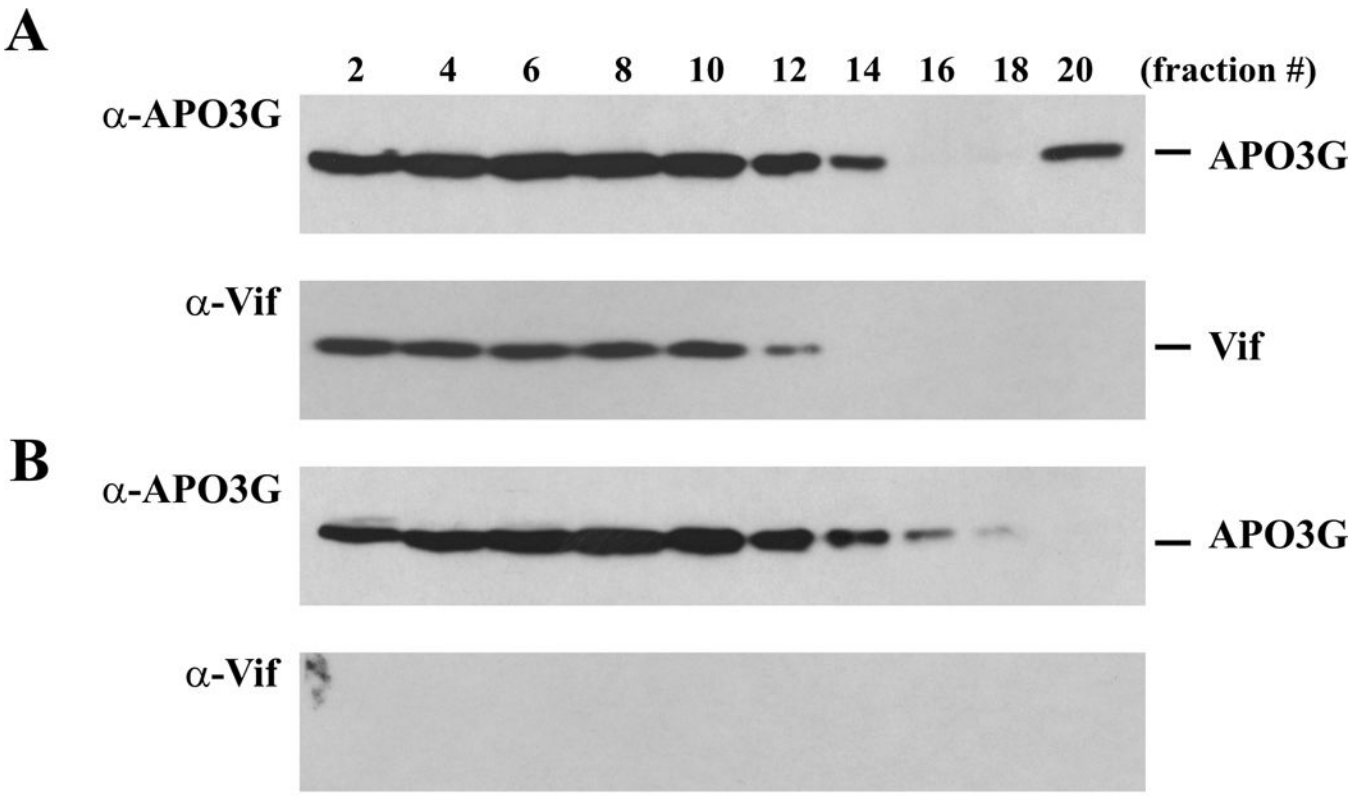

Fig. 6.

Vif promotes HMM complex formation of APO3G in transfected HeLa cells. HeLa cells were transfected with pcDNA-APO3G (3 μg), together 1 μg of pCul5-Rbx DNA and 2 μg of either pNL-A1 DNA **(A)** or pNL-A1vif(−) DNA **(B)**. Cells were harvested 24 h after transfection and lysed in 400 μl of NP40 lysis buffer (5 min, 4°C). Nuclei were pelleted (1,500 × g, 5 min), the post-nuclear supernatants were overlaid onto a 15–40% sucrose gradient as described for figure 3, and centrifuged for 3 h at 86,000 × g. Twenty fractions (500 μl each) were collected from the top of the gradient and indicated fractions were analyzed by immunoblotting using an APO3G-specific antibody (α-APO3G). The same blots were then re-probed with a Vif-specific monoclonal antibody (α-Vif).