herbimycin A (*33*) were reported to be more specific for IKK-β than IKK-α. Whereas, these IKK inhibitors are competitive with ATP, other agents including BMS-345541 (*34*) and aspirin (*35*) are known to inhibit IKK-β through allosteric effects.

As far as we examined, the inhibitory action of carbonic adenine nucleosides on NF-κB correlated with the trihydroxylation of the carbonic ring and hydrogenation or amination of adenine nucleoside at position 2 ('X' in Fig. 1). Increase in non-specific and cytotoxic effects were observed upon halogenation of the position 2, suggesting that a strong negative charge in this position may induce interaction with many other kinases.

NAM showed a preferred inhibitory action on IKK-α activity over IKK-β. Cumulative evidences suggest that IKK-α has distinct and independent roles from IKK-β (*30*, *31*). Whereas, IKK-β gene knock-out induced fetal cell death because of massive apoptosis in liver tissues (*36*), IKK-α gene is dispensable for normal embryonic development although IKK-α-null mice have impaired secondary lymphoid tissues such as lymph nodes and Payer's patch in the intestine (*37*, *38*). Recent studies have revealed the involvement of IKK-α in the 'non-canonical' NF-κB activation pathway that lead to p65 and p100 phosphorylation (*8*, *30*). Whereas, p100 phosphorylation leads to its proteolytic processing to p52 NF-κB subunit, p65 phosphorylation is considered crucial for the transcriptional competence of NF-κB-mediated transactivation (*8*, *17*). IKK-α is also known to be involved in the phosphorylation of histone H3 that is involved in the chromatin remodelling by converting the repressive histone to transcriptionally active status (*39*, *40*). In this pathway, IKK-α is solely involved in the extracellular signalling elicited by lymphotoxin β, Blys/BAFF, RANK and CD40, independently of IKK-β (*41–44*). Moreover, constitutive NF-κB activation in some tumour cells, such as adult T-cell leukemia and multiple myeloma, appears to depend on the non-canonical pathway (*18*, *45*).

Thus, specific inhibition of IKK-α would lead to selective suppression of chronic immuno-inflammatory stimulation and carcinogenesis, in addition to the inhibition of HIV replication. These findings should foster the synthesis of improved analogues ameable to pharmacological development of effective agents for the treatment of pathological conditions where IKK-α plays a major role.

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CONFLICT OF INTEREST

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2. APOBEC3G/Vif システムによるHIV-1の複製制御

泉 泰輔，高折晃史

近年，新規の抗HIV-1宿主因子としてAPOBEC3G（A3G）が同定された．本分子は，APOBECファミリーに属し，ウイルスDNAにグアノシン（G）からアデノシン（A）に変異を導入することでウイルスの複製を阻害する．一方で，HIV-1は，アクセサリー分子の1つであるVifが細胞性因子であるCullin5，ElonginB/CとE3リガーゼ複合体を形成し，A3Gをユビキチン依存的に分解することで，A3Gの抗HIV-1活性から逃避している．したがって，HIV-1感染においては，A3GとVifのバランスはきわめて重要であり，HIV-1自身の多様性や進化にも影響してきていることが近年報告されている．このバランスを制御することは，新たな抗HIV薬の開発へとつながる可能性があり，本稿ではわれわれの研究成果もふまえて最新の知見を紹介したい．

はじめに

宿主ゲノムにはウイルス感染を阻止するためのいくつかの戦略が備わっている．この宿主応答は，これまで主な防御機構だと考えられてきた病原体の感染によって誘起される自然免疫および獲得免疫に対し，“intrinsic immunity（内因性免疫※1）”と称される．近年，この内因性免疫として働く因子が次々と同定されており，その中でもHIV-1を阻害する代表的なものがtripartite motif protein 5 alpha（TRIM5α）とapolipoprotein B mRNA-editing enzyme, catalyticpolypeptide-like（APOBEC）ファミリーメンバーである．本稿では，複数あるAPOBECファミリーの中でもプロトタイプであり特によく研究されているヒトAPOBEC3G（A3G）に関して，またその抗ウイルス活性はHIV-1以外のウイルス〔レトロウイルス，

[キーワード&略語]
A3G, Vif, Cullin5, ElonginB/C, PKA, MDM2

A3G：APOBEC3G（アポベック3G）
APOBEC：apolipoprotein B mRNA-editing enzyme, catalyticpolypeptide-like
Cul5：Cullin5（カリン5）
EloB/C：ElonginB/C（エロンジンB/C）
MDM2：murine double minute 2
NC：nucleocapsid（ヌクレオカプシド）
PKA：protein kinase A（プロテインキナーゼA）
Vif：Viral infectivity factor

※1 内因性免疫
内因性免疫は病原体による感染から宿主を守るために細胞に先天的に備わっている防御機構である．タンパク質レベルで病原体を認識し反応するが，抗体産生などの獲得免疫と異なり，その効果は持続的ではない．すなわち感染に対して即座に反応する動植物間で広く見受けられる防御機構の1つである．近年，APOBECファミリーを代表的にTRIM5など多くの内因性免疫因子が発見されている．

Restriction of HIV-1 replication by APOBEC3G/Vif system
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B型肝炎ウイルス（HBV）など〕にも及ぶが，本稿では特にHIV-1に関して深く解説したい．

1 研究背景

HIV-1はレトロウイルス構成分子であるGag，Pol，Envの他に6つのアクセサリー分子（Tat，Rev，Vpr，Vpu，Vif，Nef）を保持しており，いずれのタンパク質もウイルス感染に重要な役割を担っている．その中で，Viral infectivity factor（Vif）はレンチウイルス※2間において，高度に保存されたアミノ酸配列を有し，これまでレンチウイルスの感染に重要な因子であると考えられていた．サル免疫不全ウイルス（simian immunodeficiency virus：SIV）の各種のアクセサリー遺伝子を欠損させた変異株をサルに接種した際，Vif欠損変異株の感染性は野生型の0.005％しか認められず，エイズも発症しないことから，Vifは*in vivo*におけるウイルス複製に非常に重要な因子の1つであることが推測された．同じ頃，HIV-1の標的細胞にはVifを欠損したウイルスでも感染できる細胞（許容細胞）と野生型ウイルスでないと感染できない細胞（非許容細胞）の2種類のタイプに分けられることが見出された．2002年，Malimらのグループは，許容・非許容細胞間でのサブトラクション法により，標的細胞中に存在するHIV-1阻害因子としてA3Gを同定した[1]．

AID，APOBEC1，APOBEC2およびAPOBEC3を含むシチジン脱アミノ化酵素の一群を総称してAPOBECファミリーと呼ぶ．APOBEC3遺伝子は，ヒトにおいては22番染色体にAからHまでタンデムに並んでいる．近年，新たなAPOBECファミリーとしてAPOBEC4が同定された．本分子は1番染色体に存在し，哺乳類間でその存在が確認されているが，発現量が非常に低く抗HIV-1活性をもたない．

また一方，感染患者から分離されたHIV-1ゲノム中には高頻度でGからAへの変異が見出され，あるウイルス株では全体の60％のGに変異が及んでいた．この変異は単に逆転写のエラーによるものだと考えられており，長年の間その機序は不明のままであった．APOBEC3，特に3Gは逆転写の際にマイナス鎖DNAのCをUに変換する．この変異は，プラス鎖DNAに多数のGからAへの変異を導入し，結果HIV-1ゲノム中で確認された高頻度のG-to-A変異はA3Gによる影響であることが明らかになった[2)3)]．

> **※2 レンチウイルス**
>
> 一本鎖RNAのプラス鎖をゲノムとしてもち，逆転写酵素により複製過程で自身のゲノムRNAを鋳型としてcDNAを合成，宿主のゲノムDNAに挿入することで感染を成立させるウイルスをレトロウイルスという．レトロウイルスにはウイルスの構成に最小限必要な因子のみをもつシンプルレトロウイルスとそれ以外にも特徴的な因子をいくつか保持している複雑レトロウイルスに分類される．レンチウイルスは後者であり，代表的なウイルスとしてHIV-1などがあげられる．

2 A3Gの抗ウイルス作用（図1）

A3Gはウイルス出芽の際，Gagのヌクレオカプシド（NC）部位とウイルスゲノムあるいは細胞性RNAを介して結合することで粒子中へと侵入する[4)]．新たに出芽したウイルスは次の標的細胞に感染し，細胞中へとウイルスが侵入した後，コアの脱殻が起こる．脱殻後，逆転写がはじまりマイナス鎖cDNAが合成されるが，この過程で合成されるマイナス鎖cDNAのCをUに変換することで，HIV-1ゲノムに変異を導入する（図2-①）．A3Gの変異導入は5′末端から3′末端に進むにつれてその頻度は高くなり，最も変異の頻度が少ない部位が5′-LTRであり，逆に頻度の高い部位がnefの5′末端である．A3Gは一本鎖DNAにのみその脱アミノ化活性を有し，DNA-DNAやDNA-RNAなどの二本鎖に対しては活性を示さない．レンチウイルスはpolypurinetracts（PPT）というRNaseH耐性の特異的な配列を有し，PPTを利用してプラス鎖DNAの合成を行う．PPTにはG-to-A変異はまったくみられないことから，A3Gによる変異導入は5′末端LTRからPPTまでということになる[5)]．A3Gによる高頻度な変異導入は種々の機序でウイルスの複製を阻害している．まず，DNA中にUの存在は一般的に許容されず，uracil *N*-glycosidase（UNG）によってそのUは除去される．Uの除去で生じたニックはapurinic/apyrimidinic endonuclease-1（APE-1）などの宿主のDNA修復酵素によって分解される．断片化したDNAはその後宿主のゲノムにインテグレーションされることはなく，結果ウイルスの増幅は起こらない．2つ目には，変異導入されたウイルスDNAがインテグレーションされても，新たに合成されたプラス鎖DNAにはG-to-A変異が入っているため，スプライス部位のズレや終止コドンの導入によりウイルスタンパク質は機能不全に陥る．実際に，Gagの60％

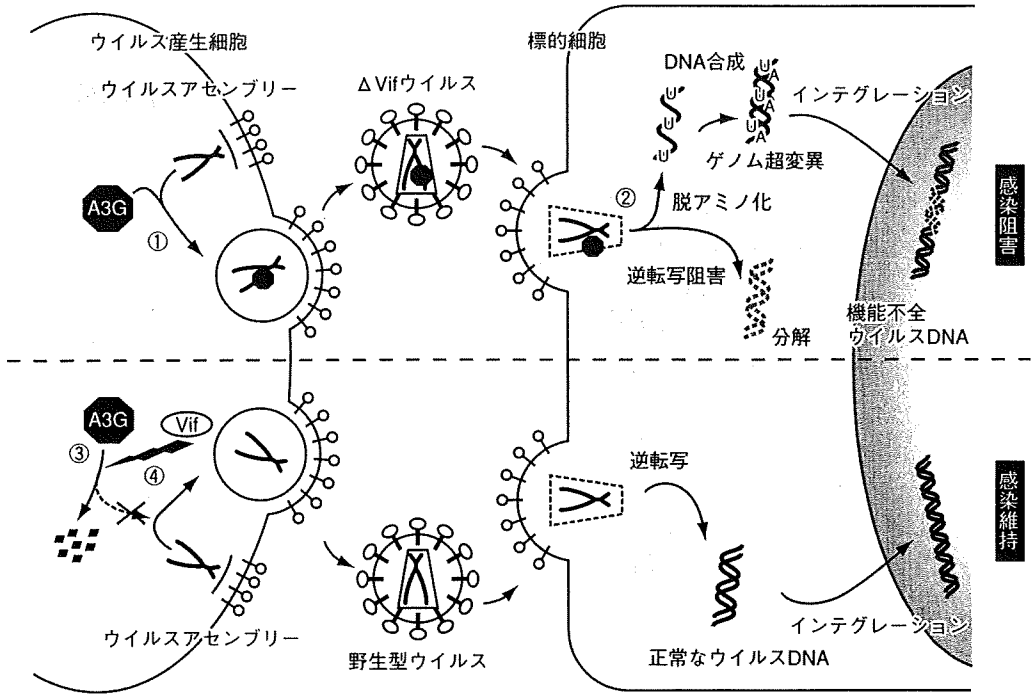

図1　APOBEC3GによるHIV-1の感染阻害機序
①Vif欠損ウイルスにおいて，A3Gは出芽ウイルス中に取り込まれ，②標的細胞中でゲノム超変異および逆転写阻害により感染を阻害する．一方，③HIV-1 Vifはウイルス産生細胞中のA3Gをユビキチン依存的に分解し，④ウイルス粒子中へのA3Gの侵入を阻害し，ウイルスの感染維持に働く

およびNefの90％の開始コドンはA3Gによって変異が導入されていることが報告されている（図1）．

さらに近年，脱アミノ化活性のないA3Gにも逆転写産物の生成を阻害する効果があることが報告された（図2）．本分子は，その活性部位をN末端とC末端に2カ所に所有しており，C末端の活性部位が酵素活性および抗ウイルス活性に必須であることが知られている．C末端の活性部位を欠損させたA3G変異体もウイルス粒子に侵入することは可能であり，ウイルスおよび宿主タンパク質と結合することで逆転写キネティクスを変化させ，結果逆転写の進行を止めていると考えられた．

最近，岩谷らがA3Gが逆転写過程のそれぞれの段階にどのように関与しているかを報告した[6]．彼らの報告では，A3Gは逆転写酵素依存的なDNA伸長反応は阻害するが，RNaseH活性およびNCを介したアニーリング過程は阻害していないことを見出した．具体的には，A3Gは，マイナス鎖strong-stop DNAおよびプラス鎖strong-stop DNAの合成，マイナスおよびプラス鎖cDNAの転移（First strand, Second strand転移），プラスおよびマイナス鎖DNAの伸長反応，の三段階を阻害している（図2-②～⑤）．また，GuoらはA3G発現細胞中で逆転写初期産物が減少する原因として，tRNAのプライミングが阻害されていることを報告している[7]．彼らの報告によると，AOBEC3G存在下では3′-LTR DNAが多量に形成されることから，A3GはtRNAの除去と分解を阻害していることが伺える（図2-⑥）．さらに，インテグレーションされない2-LTR DNAのシークエンス解析より，A3G存在下で，U5末端に本来なら存在しない$tRNA^{Lys3}$の3′末端由来と考えられる6塩基の付加がみられる．この現象は，A3GによるtRNA除去の阻害により引き起こされたと考えられ，結果ウイルスDNA末端に余分な配列が付加しているために，プラス鎖転移に支障をきたしていると推察される（図2-⑦）．しかし，本実験は野生型および変異型A3Gを過剰発現させた際得られた結果であり，*in vivo*発現レベルでの実験では変異型A3Gの抗ウイルス効果は確認され

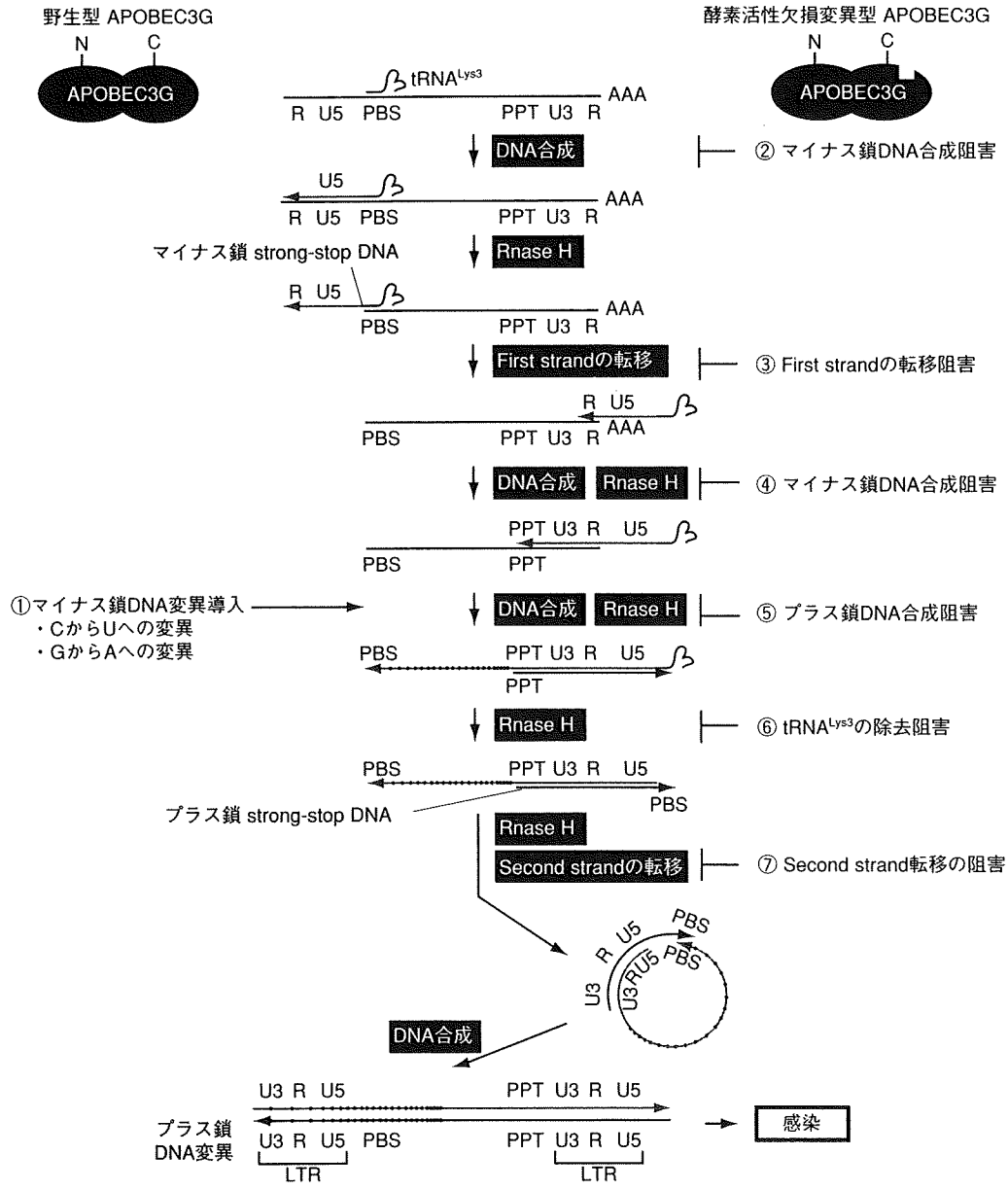

図2　HIV-1の逆転写過程とAPOBEC3Gによる阻害機序

A3Gの酵素活性によりPPTから5′末端への伸長中にCからUへの変異が導入される（①）．シチジン脱アミノ化活性を欠損した変異型A3Gは変異を導入することなく逆転写過程の各段階を阻害する．PBSに宿主のtRNA^{Lys3}が結合しマイナス鎖strong-stop DNAの合成（②）がはじまり，鋳型RNAはウイルスのRNaseH活性によって分解される．合成されたマイナス鎖strong-stop DNAは鋳型RNAの3′末端LTRに転移する（③）．転移されたマイナス鎖strong-stop DNAをプライマーとして，さらにマイナス鎖の伸長がはじまる（④）．同時にRNaseH活性により，鋳型RNAが分解されるが，PPTは分解されない．また，PPTをプライマーとしてプラス鎖の合成がPBSまではじまる（⑤）．マイナス鎖DNA3′末端に付加されていたtRNA^{Lys3}はRNaseH活性によって除去される（⑥）．プラス鎖strong-stopはマイナス鎖DNAのPBS部位に転移し，プラス鎖の全長が合成される（⑦）．マイナス鎖を鋳型に合成されたプラス鎖DNAはGからAへの変異が導入される．このように，A3Gは酵素活性依存的，非依存的に逆転写を阻害する

ず，この現象は未だ議論の余地がある．

3 Vifタンパク質によるA3Gの阻害機序

Vifタンパク質はHIV-1がA3Gから逃避するのに必須のタンパク質である．その詳細なメカニズムをここでは述べていきたい．VifはA3Gをユビキチン-プロテアソーム依存的な分解反応によって抑制している．VifがA3Gを分解するためには，A3Gおよび種々の宿主因子と結合する必要があり，Vifはそのアミノ酸配列中にいくつも重要な結合ドメインを有している（図3A）．その1つとして，VifはそのC末端に高度に保存されたSLQモチーフと呼ばれるドメインを有する．SLQモチーフはSLQ(Y/F)LA配列を有し，suppressors of cytokine signaling（SOCS）タンパク質のBC-boxに保存された配列と類似しており，VifがElonginC（EloC）と結合するのに必要なモチーフである．EloCは酵母のSkp1タンパク質と相同性の高いタンパク質であり，E3ユビキチンリガーゼ複合体の構成要素として知られている[8]．さらに，BC-box様SLQモチーフの上流に位置し，$HX_5CX_{17-18}CX_{3-5}H$配列を有するHCCHモチーフと呼ばれるドメインも重要である．本配列はCullin5（Cul5）と結合するのに必要な配列である．また，HCCHモチーフ中の2つのシトシンはzinc fingerドメインの中心アミノ酸であり，VifがZn^{2+}と結合することはVifの適切な構造形成に必須であり，Vifの機能維持に重要な役割を担っている．したがって，VifはHCCHモチーフとSLQドメインを用いて，EloB/C，Cul5，Rbx1とE3ユビキチンリガーゼ複合体を形成し，A3Gのユビキチン化を誘導している（図3B）[9)10)]．ユビキチン化されたA3Gはその後26Sプロテアソームで分解される．ユビキチン化は一般的にタンパク質のリシン残基に結合することが知られているが，最近の知見ではリシン残基をすべてつぶした変異体A3GもVifに感受性を示し，分解されることが報告された[11]．その報告によると，Vifが形成するE3ユビキチンリガーゼ複合体はA3Gだけでなく，Vif自身のユビキチン化も誘導しており，Vifの分解シグナルがA3Gも一緒に分解している可能性を示唆している．しかし，本現象は完全に証明されておらず，さらなる研究が必要であると考えられる．

興味深いことに，HIV-1 VifはヒトA3Gを中和できないことが当初より示されていた．HIV-1 Vifはアフリカミドリザル（african green monkey：AGM）のA3Gと結合することができず，これを抑制できない．逆に，SIV_{AGM} VifはヒトのA3Gを中和することができない．種間のA3Gはアミノ酸配列の相同性が高く，わずかな違いしか存在しない．したがって，これら種間のキメラを作製したところ，128番目のアミノ酸残基をヒトのアスパラギン酸（D）からAGMのリシン（K）に置換したA3G（D128K A3G変異体）はHIV-1 Vifの制御を受けないことが複数のグループにより報告され，128番目のアミノ酸がその種特異性を決定していることが判明した[12]．これによりA3G側の結合部位が同定されるとともに，Vif側の結合部位の検索もおおいに行われた．アスパラギン酸はマイナス電荷，リシンはプラス電荷を帯びている．つまり，AGMとヒトのA3GではVifに結合するアミノ酸の電荷が正負逆になっている．そこでSIV_{AGM}とHIV-1のVifにおいても，電荷が逆になっている部位をホモロジー解析により検索したところ，Vifの14～17番目のアミノ酸に違いがあることが明らかになった．HIV-1 Vifのアミノ酸配列はアスパラギン酸-アルギニン-メチオニン-アルギニン（DRMR）であり，プラス電荷を帯びるアミノ酸が多い一方で，SIV_{AGM}ではセリン-グルタミン酸-メチオニン-グルタミン（SEMQ）でマイナス電荷を帯びるアミノ酸が多い．HIV-1 Vifの14～17番目のアミノ酸（DRMR）をSIV_{AGM} Vif（SEMQ）に置換した変異型HIV-1 VifはAGM A3GおよびD128K変異型A3Gを抑制することがわかり，Vifの14～17番目のアミノ酸がA3Gとの結合に重要であることが示唆された．しかし，最近HIV-1の各種ウイルス株で特に保存されている部位に変異を導入し，変異型VifとA3Gの結合を調べたところ，40～44番目（YRHHY）と55～72番目（$VXIPLX_4LXIX_2YWXL$）がA3Gの結合に必要な領域であると他のグループより報告された[13]．また，当初結合部位であると考えられていた14～17番目（DRMR）はAPOBEC3Fとの結合部位であり，3Fはその他に74～79番目（TGERXW）とA3Gの結合部位と同じ55～72番目（$VXIPLX_4LXIX_2YWXL$）と3カ所のVif結合部位を有していることが明らかになった（図3A）．

また，われわれはリン酸化酵素であるprotein kinaseA（PKA）がA3Gの32番目のスレオニン（T32）をリ

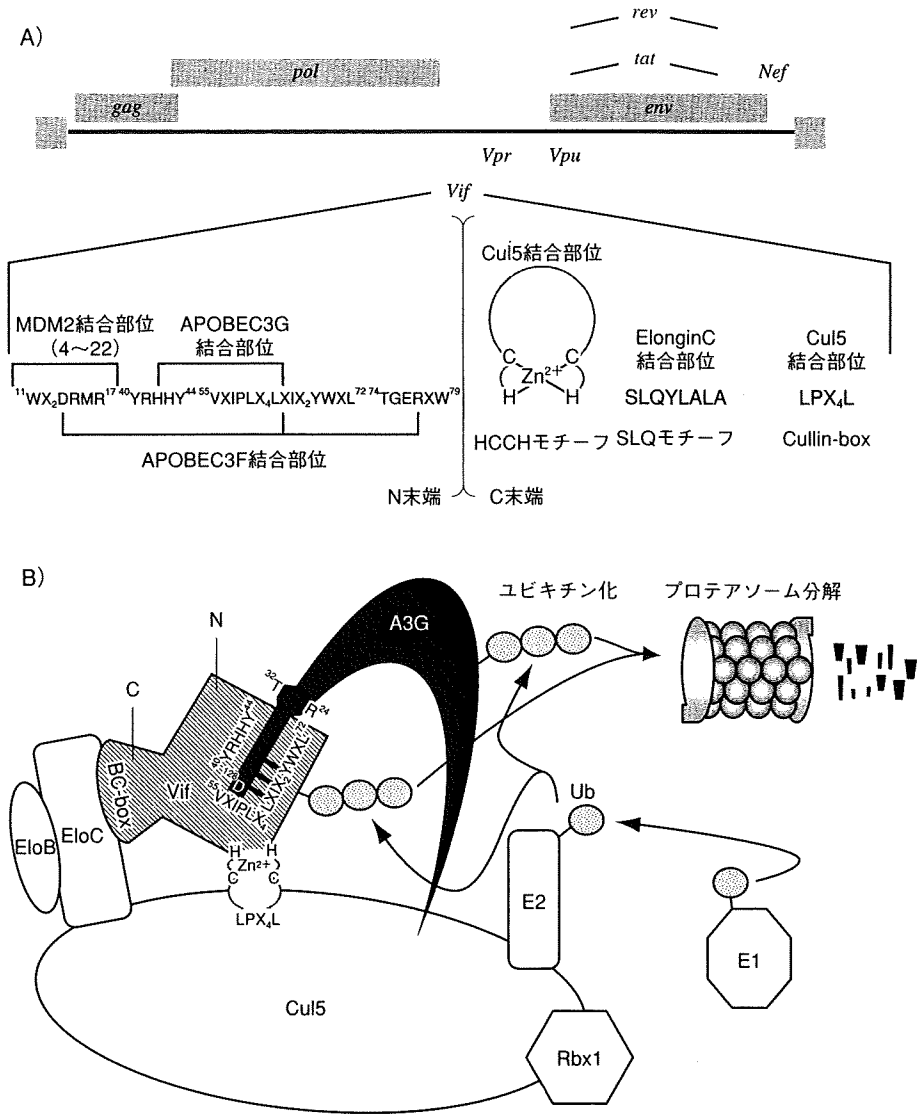

図3　HIV-1ゲノムとVif-Cullin5-ElonginB/C複合体（E3ユビキチンリガーゼ複合体）
VifはN末端からC末端にかけて数種のタンパク質と結合する．SLQモチーフはα-ヘリックス構造をとり，ElonginCと結合する．HCCHモチーフはCul5との結合に重要な部位であり，結合するにはZn^{2+}をそのモチーフ中に取り込む必要がある．Cul5との結合にはさらにCullin-boxも必要になる．N末端にはA3G，A3Fとの結合部位が存在する．疎水性残基を中心に構成される$^{55}VXIPLX_4LXIX_2YWXL^{72}$モチーフはA3GおよびA3F両方の結合に必要でありA3Gはさらにその近傍に1カ所，A3Fは2カ所にその結合部位が存在する．また，MDM2は最もN末端に結合し，A3G，A3Fと競合する

ン酸化することを明らかにした．リン酸化A3GはVif結合能を減少させ，安定化する．A3Gの立体構造をコンピュータ解析した結果，T32と24番目のアルギニン（R24）の間には1本の水素結合が形成されているが，リン酸化を受けるとその水素結合が2本になり，構造の柔軟性が損なわれVifの結合能が減少する可能性が示唆された[14]．これまでA3GのVif結合部位はD128のみが関与している「点」での結合だと考えられていたが，T32-R24間の水素結合が関与していることから，「面」での結合であることが示唆された．

4 A3G活性の調節機序

HIV-1がその標的細胞であるCD4陽性T細胞に感染するためには，細胞が活性化されている必要があり，休止期のCD4陽性T細胞はHIV-1感染に対して抵抗性を示すことが以前より知られていた．

1）GCboxとサイトカインによるA3G転写誘導

HIV-1は休止期の細胞中において，逆転写時およびウイルスゲノムの核移行前段階で感染が阻害されていることがわかっており，A3Gのような抗ウイルス因子による関与が疑われていた．T細胞中でのA3Gの転写は，複数の転写開始点を有するmultiple transcriptional start sites（TSS）を用いている．A3Gのプロモーター全長1,025bpを5′末端から少しずつ削って作製した欠損型プロモーターを使用した実験より，最小180bpがA3Gの転写に必要な領域であり，その中にはGC含量の高いGCboxがみうけられた．このGCboxには転写因子であるspecificity protein 1（Sp1）および3（Sp3）が結合していることがわかり，Sp1およびSp3依存的に転写されていることがわかった．また，A3Gの転写に関与するサイトカインの研究も多く行われており，これまでにIL-2，IL-7，IL-15が末梢血単核球中でのA3Gの発現を上昇させることがわかっている．さらに，細胞内シグナルタンパク質であるJAKおよびMAPKを刺激するとIL-2およびIL-15によるA3Gの転写活性が起きないことから，これらのサイトカインはJAK/MAPKを介してA3Gの転写誘導を起こしていると考えられる．A3Gの発現はHIV-1のもう1つの標的細胞である樹状細胞（DC）やマクロファージをインターフェロン-α，-β，-γ処理することによって成熟させた際にも上がる．しかし，これらサイトカインで処理した標的細胞はA3Gの発現が上がる一方でHIV-1の感受性も増す．A3GがHIV-1の感染阻害に働く抗ウイルス因子とするならば，この現象は一見矛盾している．したがって，A3Gは転写後何らかの細胞性因子によってその機能を制御されていることが示唆された．

2）A3Gがつくる RNA依存的なタンパク質複合体

近年，Chiuらは活性化CD4陽性T細胞や不死化した細胞株中でA3GはRNA依存的なタンパク質複合体（high molecular mass：HMM）を形成していることを明らかにした．293T細胞などにA3Gを導入し，その細胞溶解物をFPLCで解析すると700 kDa以上の複合体（HMM）が検出されるが，この細胞溶解物をRNaseAで処理すると60～100 kDaまでその大きさは減少する（low molecular mass：LMM）．したがって，この複合体はRNA依存的に形成されていることがわかった．興味深いことに，HMM型A3Gに酵素活性はなく，RNaseAで処理することでLMM型にするとその酵素活性が復活することがわかった．したがって，A3Gは細胞内のRNAやリボヌクレオタンパク質（RNP）と結合することで複合体を形成し，その酵素活性が不活化することで抗ウイルス機能を喪失していることがわかった．さらに，休止期CD4陽性T細胞中ではLMM型A3Gのみが発現しており，siRNAを使用してLMM型A3Gをノックダウンすると HIV-1感染に対して寛容になることから，LMM型A3Gは侵入してきたウイルスに対して（Vifの存在下であっても）その抗ウイルス活性を発揮できることがわかり，休止期CD4陽性T細胞にHIV-1が感染できない機構の1つと考えられた（図4 A）[15]．

HMM型には，その構成成分としてRNPであるStaufen RNA transporting granules, Ro, Prespliceosomesなどが含まれ，またレトロエレメントであるAluやhYをその複合体中に取り込んでいることが明らかになった．したがって，HMM型A3Gは侵入してきたHIV-1に対して抗ウイルス活性を示さないが，これらレトロエレメントの転移を阻害していることが示された（図4 B）[16]．

3）HIV-1の細胞間伝播とA3G

HIV-1はまず単球系細胞に感染した後，T細胞に感染することが知られている．近年，HIV-1の細胞間伝播に関して興味深い現象が報告された．HIV-1感染CD16陽性単球はHIV-1感染に対して耐性を示す休止期CD4陽性T細胞を寛容化させ，HIV-1が単球からT細胞に伝播しやすくしているようだ．この現象は，ウイルスの拡散に重要であり，通常ウイルスフリーである脳細胞のような聖域にウイルスが感染するのに重要なステップとなっている．これについても，単球からの何らかの刺激が休止期T細胞中のLMM型A3GをHMM型に移行させることでその抗ウイルス活性が制御され，ウイルスの侵入を許している可能性も考えられる[17]．

では，ウイルス粒子に取り込まれるA3Gに関して

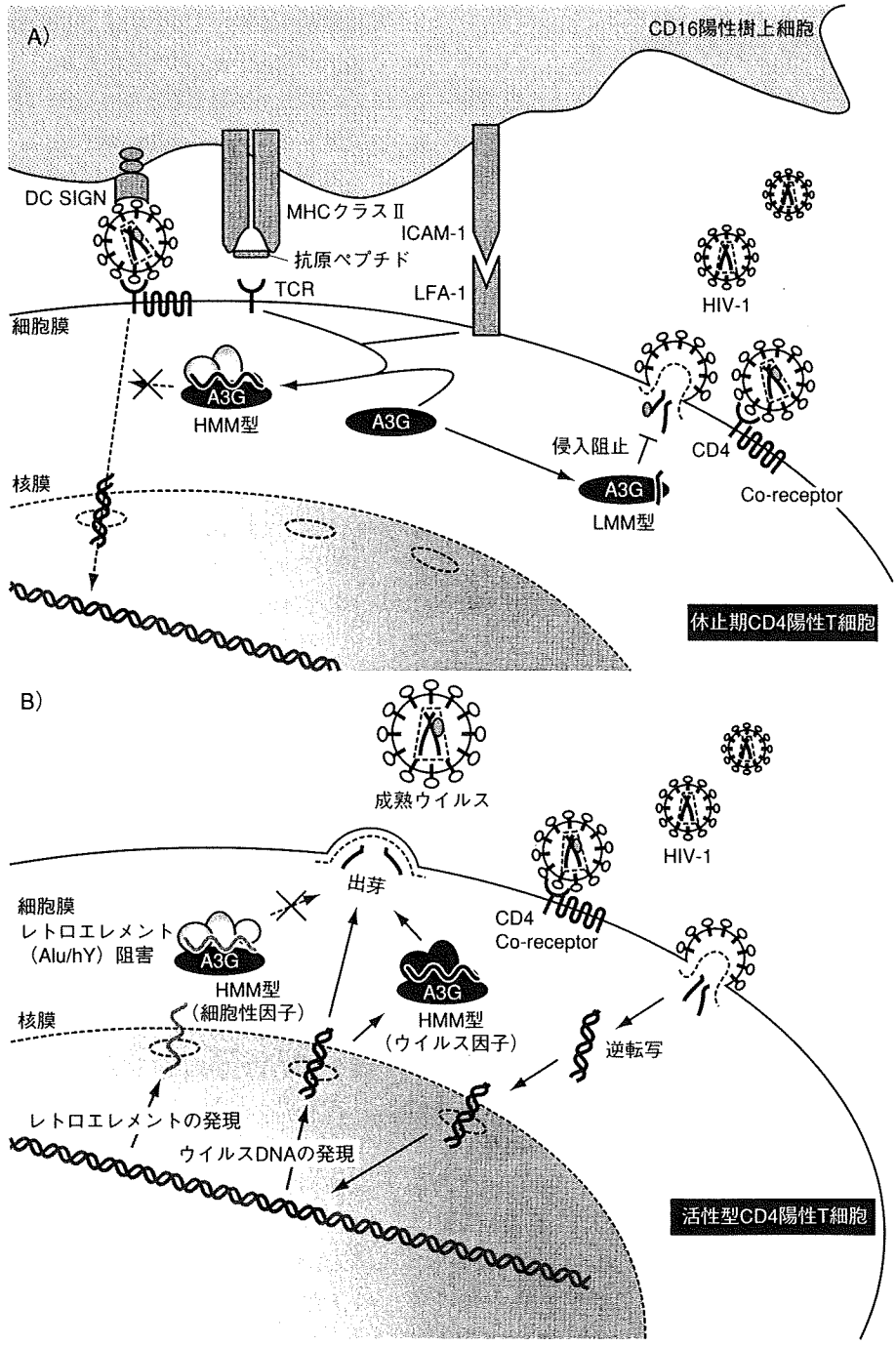

図4　活性化および休止期のCD4陽性T細胞中のAPOBEC3Gの動態と抗ウイルス作用

A）休止期CD4陽性T細胞では，A3Gは活性化状態であるlow molecular mass（LMM）型として存在している．LMM型A3Gは侵入してきたウイルスに対してその抗ウイルス活性を発揮し，感染を阻止する．単球からの刺激により，A3Gは不活性型high molecular mass（HMM）型となり，ウイルスの侵入を許す．B）活性型CD4陽性T細胞中では，A3Gはレトロエレメントなどの内在性RNAおよびリボタンパク質と複合体（HMM）を形成しており，不活性型になっている．プロウイルスが出芽する際，A3Gはウイルス粒子に取り込まれるが，細胞性因子と形成したHMM型は取り込まれず，ウイルスRNAおよびタンパク質と形成したHMM型が取り込まれる．HMM型A3Gは，内在性レトロエレメントの転移を抑制している

はどうか（図4 B）．A3Gは転写翻訳後，mRNA processing bodies（P-bodies）に局在する．P-bodiesは酵母や哺乳類の細胞質に存在し，翻訳されないmRNAなどを集積させ分解する細胞小器官である．A3GはこのP-bodiesで宿主RNPやRNAと複合体を形成し，HMM型になる．宿主由来因子と形成したHMM型はウイルスに侵入できない．しかし，ウイルスRNAが発現している感染細胞中では，新たに転写されたA3GはウイルスRNAやNCなどとHMM型を形成し，ウイルスに侵入する．ただし，ウイルス因子と形成したHMM型も同様に抗ウイルス活性はない．このウイルスが次の標的細胞に感染すると，逆転写中にウイルス由来のRNaseHが活性化する．このRNaseHはウイルスゲノムRNAを消化するだけでなく，HMM型A3Gに結合していたRNA自体も消化することで，A3Gは活性化し，その抗ウイルス活性を発揮するのである[18]．このようにして宿主とウイルスはお互いを利用しながら，生存競争を繰り広げている様がみてとれ非常に興味深い．

おわりに

以上のように，まったく新規の抗HIV-1宿主因子A3Gが同定されてから，わずかな年月しか経っていないが，膨大な量の研究が行われ，その機序がこれほどまでに早く解き明かされた分子は稀ではないだろうか．これらの知見は，ウイルスと宿主の関係，およびその進化を知るうえでも非常に重要な知見である．また，A3Gが抗HIV-1薬の標的に十分なりうることから臨床的にも重要な研究分野である．

これまで述べてきたようにHIV-1感染において，A3GとVifは絶妙なバランスの上に成り立ってその感染を成立させているがVifのA3G阻害効果は完全ではない．さらに，実験室レベルでの研究において，A3Gを過剰発現させると野生型HIV-1の感染をも完全に阻害する．われわれを含む複数のグループが現在A3Gを標的とした新薬の開発を行っており，最近興味深い低分子化合物がNathansらによって報告された[19]．彼らはVifによるA3Gの分解を阻害するような低分子化合物を30,000個のライブラリーの中からスクリーニングを行い，いくつかの分子を同定した．その中で，RN-18と名づけられた化合物は，濃度依存的にT細胞株へのHIV-1の感染を阻害するが，許容細胞を標的細胞に用いた場合その効果はみられなかった．RN-18を処理した細胞では未処理細胞に比べて，野生型HIV-1を感染させた際のA3G発現量は増加していたが，非感染細胞と比べると減少していた．つまり，RN-18はA3Gの発現を亢進させるわけではなく，Vifの中和機能を阻害していると考えられる．また，RN-18で処理した細胞中ではVifの発現量は未処理に比べて減少していた．したがって，RN-18はVifの代謝を促進することでA3Gを安定化させた結果，抗ウイルス作用を増強していると考えられる．

最近，われわれはVifをユビキチン化する新規のE3リガーゼとしてMDM2を同定した[20]．MDM2はがん抑制遺伝子であるp53をユビキチン化するE3リガーゼとしてよく知られているが，Vifも同様にユビキチン依存的に分解することを明らかにした．MDM2のVif結合部位はN末端4～22番目のアミノ酸に規定されており，VifのA3G結合能と競合する．MDM2はユビキタスに発現している酵素であるが，その発現量は厳格にコントロールされており，一般的に低くおさえられているのでMDM2によるA3G安定化能は低く，本機序も抗ウイルス作用に働くよりも耐性ウイルスの出現の一端を担っていると推測される．RN-18がVifの分解を促進する低分子化合物であるならば，もしかするとMDM2を含めたVifを標的としたタンパク質分解酵素に関与しているのかもしれない．仮にHIV-1がA3Gを完全に制圧してしまうと遺伝的に単一株のみしか出現せず，免疫細胞および薬剤の格好の餌食となってしまう．しかし，A3Gの過剰な寄与はウイルスゲノムに多大な損害を与え，ウイルス自体生存できなくなる．HIV-1はこのような矛盾した状況の中で絶妙なバランスを保ちながら感染を成立させており，それを打ち崩すような新薬の出現はそう遠くない未来に訪れるであろう．

本稿では多くは触れなかったが，APOBECファミリーには3Gをはじめ多くのサブタイプが存在し，3Gと同様に抗HIV-1活性を示すものもある．また，A3Gの抗ウイルス活性はHIV-1のみならず他のHTLV-1，HBV，アデノ随伴ウイルス，さらにはレトロトランスポゾンにまで及び，この分子が抗ウイルス自然免疫として重要な役割を担っていることは明らかである．しかし，APOBECの翻訳後修飾に関してはあまり解明されておらず，またウイルス制御以外の役割もよくわ

かっていない．今後，各サブタイプがどのような修飾を受け制御されているかを明らかにし，その機能の本質を見極めることはエイズ根治だけでなくその他の疾病に関しても重要な手がかりになると信じ本稿を締めくくりたい．

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Phosphorylation of APOBEC3G by protein kinase A regulates its interaction with HIV-1 Vif

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Apolipoprotein B mRNA-editing enzyme catalytic polypeptide–like 3G (APOBEC3G, referred to here as A3G) is a potent antiretroviral host factor against human immunodeficiency virus type 1 (HIV-1). HIV-1 viral infectivity factor (Vif) counteracts A3G by promoting its degradation via the ubiquitin-proteasome pathway. Recent studies demonstrated that protein kinase A (PKA) phosphorylates activation-induced deaminase (AID), another member of the APOBEC3 family. A3G has two putative PKA phosphorylation residues. Here we show that PKA binds and specifically phosphorylates A3G at Thr32 *in vitro* and *in vivo*. This phosphorylation event reduces the binding of A3G to Vif and its subsequent ubiquitination and degradation, and thus promotes A3G antiviral activity. Computer-assisted structural modeling and mutagenesis studies suggest that the interaction between A3G Thr32 and Arg24 is crucial for interaction with Vif. These data imply that PKA-mediated phosphorylation of A3G can regulate the interaction between A3G and Vif.

A3G is a potent antiretroviral host factor against HIV-1. A3G is incorporated into budding virions and subsequently introduces cytidine-to-uridine mutations in the minus-strand viral DNA during reverse transcription in target cells, resulting in inhibition of HIV-1 replication[1–4]. On the other hand, HIV-1 Vif counteracts the antiviral activity of APOBEC3G to enable HIV-1 to replicate in nonpermissive target cells[5]. Vif forms an E3 ubiquitin-ligase complex with ElonginB/C and Cullin5 and degrades A3G through the ubiquitin-proteasome pathway[6,7]. Meanwhile, another APOBEC family protein, activation-induced deaminase (AID) is a B-cell specific cytidine deaminase that has a key role in somatic hypermutation (SHM) and class switch recombination (CSR), which enable diversification of immunoglobulin genes in germinal center B cells[8]. Recent studies have provided the evidence that PKA-mediated phosphorylation regulates the function of AID on CSR[9,10]. PKA phosphorylates Thr27 and Ser38 of AID embedded in a PKA consensus phosphorylation motif, R(R)X(S/T). Phosphorylation of these residues makes AID accessible to double-stranded DNA, leading to CSR. The first PKA consensus phosphorylation site (Thr27) of AID is conserved upstream of each cytidine-deaminase domain (CDD) of A3G (29-RRNT-32, 215-RHET-218) (**Fig. 1a**). These findings raise the possibility that A3G function is also regulated by PKA-mediated phosphorylation.

PKA is a key serine/threonine kinase downstream of the cyclic AMP signaling pathway. In an unstimulated state, PKA exists in the cytoplasm as an inactive heterotetramer holoenzyme consisting of two regulatory subunits and two catalytic subunits. Following stimulation, which elevates the intracellular cAMP concentration, cAMP binds to the regulatory subunits; subsequently, the catalytic subunits dissociate to become catalytically active, and they are able to phosphorylate serine or threonine in a specific motif R(R)X(S/T) on substrate proteins. The most common motif in PKA physiological substrates is RRXS, followed by RRXT, and then RXX(S/T)[11]. cAMP and PKA signaling have been shown to play an important role in the HIV-1 life cycle and AIDS pathogenesis. cAMP levels and PKA kinase activity of T cells among AIDS patients are known to be elevated[12] and affect host factors as well as viral factors. In host factors, PKA signals upregulate expression of a chemokine receptor, CXCR4 (ref. 13), and downregulate expression of another chemokine receptor, CCR5 (refs. 14,15). In viral factors, several groups have reported that PKA signals regulate the transcriptional activity of HIV-1 long terminal repeat[16,17]. PKA phosphorylates viral capsid[18] and Nef proteins[19], which results in the increasing spread of HIV-1. PKA is also involved in T-cell dysfunction of HIV-1–infected patients[20], and PKA antagonist was able to restore T-cell function in these patients by *in vitro* assays[12].

In this study, we examined whether human PKA can phosphorylate A3G and regulate its function. We show that PKA physically binds and specifically phosphorylates A3G at Thr32 and that PKA influences its antiretroviral activity.

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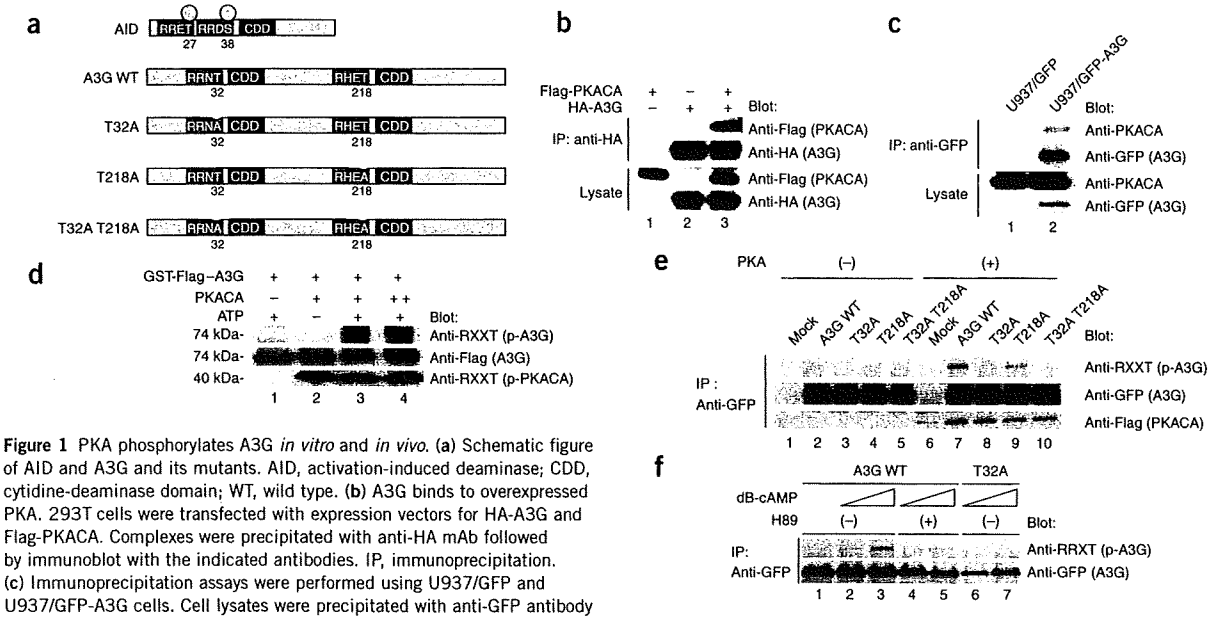

Figure 1 PKA phosphorylates A3G *in vitro* and *in vivo*. (**a**) Schematic figure of AID and A3G and its mutants. AID, activation-induced deaminase; CDD, cytidine-deaminase domain; WT, wild type. (**b**) A3G binds to overexpressed PKA. 293T cells were transfected with expression vectors for HA-A3G and Flag-PKACA. Complexes were precipitated with anti-HA mAb followed by immunoblot with the indicated antibodies. IP, immunoprecipitation. (**c**) Immunoprecipitation assays were performed using U937/GFP and U937/GFP-A3G cells. Cell lysates were precipitated with anti-GFP antibody followed by immunoblotting with the indicated antibodies. (**d**) PKA phosphorylates A3G *in vitro*. *In vitro* phosphorylation assays were performed as described in Methods. Phosphorylated GST-Flag–A3G was detected by Anti-RXXT PKA substrate antibody only when incubated with recombinant PKA and ATP. (**e**) PKA phosphorylates A3G *in vivo*. 293T cells were transfected with expression vectors for GFP-A3G, its mutants and Flag-PKACA as indicated. Cell lysates were immunoprecipitated with anti-GFP mAb and subjected to immunoblot with the indicated antibodies. (**f**) Treatment with the PKA-specific activator dB-cAMP induced phosphorylation of A3G, which was completely blocked by pretreatment with the PKA-specific inhibitor H89. 293T cells were transfected with expression vectors for GFP-A3G or T32A and treated with 50 μM H89 for 2 h followed by dB-cAMP for 4 h.

RESULTS

PKA specifically phosphorylates A3G *in vitro* and *in vivo*

First, we tested whether A3G physically interacts with human PKA catalytic subunit α type I (PKACA) by co-transfecting 293T cells with expression vectors for hemagglutinin (HA)-A3G and Flag-PKACA. Immunoblot analysis showed that Flag-PKACA was coprecipitated with HA-A3G (**Fig. 1b**, lane 3), but not in cells transfected with Flag-PKACA alone (lane 1) or HA-A3G alone (lane 2). We further examined whether A3G interacts with endogenous PKACA by immunoprecipitation of green fluorescent protein (GFP)-A3G from U937 cells stably expressing GFP or GFP-A3G. Endogenous PKACA was coprecipitated with GFP-A3G (**Fig. 1c**, lane 2), but not with GFP. To assess whether A3G can serve as a target for PKA-mediated phosphorylation, we performed *in vitro* phosphorylation assays using recombinant PKACA and GST-Flag–A3G proteins. The results showed that A3G was phosphorylated by PKACA in a dose-dependent manner (**Fig. 1d**, lanes 3 and 4), but not in the absence of PKACA (lane 1) or ATP (lane 2).

Whereas AID carries two PKA phosphorylation sites (Thr27 and Ser38) located upstream of the CDD, A3G possesses two putative target residues located upstream of each CDD: Thr32 and Thr218 (**Fig. 1a**). To examine whether A3G was phosphorylated by PKA *in vivo* and which putative site was phosphosylated, we performed *in vivo* phosphorylation assays with alanine-substitution mutants. Overexpression of PKACA induced phosphrylation of wild-type A3G and the mutant T218A (**Fig. 1e**, lanes 7 and 9), but not of T32A and T32A T218A (lanes 8 and 10), demonstrating that Thr32 is a crucial residue. Coimmunoprecipitaed Flag-PKACA was detected in each lane (below, lanes 7–10), suggesting that PKACA could bind not only to wild-type A3G but to all the mutants. Furthermore, treatment of transfected 293T cells with the PKA-specific activator, dibutyryl cAMP (dB-cAMP) induced the phosphorylation of A3G in a dose-dependent manner (**Fig. 1f**, lanes 2 and 3), an effect that was completely blocked by pretreatment with the PKA-specific inhibitor H89 (lanes 4 and 5), suggesting that this modification is specific. Treatment of monocytic U937 cells stably expressing A3G with dB-cAMP also induced the phosphorylation of A3G, which was completely blocked by H89 (data not shown). These indicate that activation of endogenous PKA signals can also induce A3G phosphorylation at Thr32. Taken together, these data indicate that PKA binds and specifically phosphorylates A3G at Thr32.

Antiviral activity of phosphorylated A3G

We next examined the antiviral activity of phosphorylated A3G using VSV-G–pseudotyped NL4-3 luciferase reporter viruses. In the absence of PKA signals, A3G and its mutants showed similar antiviral activity against both wild-type and Δ*vif* viruses (**Fig. 2a**). Cellular expression and virion-incorporation levels of A3G and its mutants were also similar (**Fig. 2b**), suggesting that substitution of threonine to alanine at positions 32 or 218 did not influence virion incorporation or antiviral activity of A3G without PKA signals. In contrast, when PKACA was overexpressed, wild-type A3G and T218A showed more potent antiviral activity than nonphosphorylated mutants (T32A and T32A T218A) against wild-type viruses (**Fig. 2c**, gray bars), whereas they showed similar antiviral activity against Δ*vif* viruses (white bars), suggesting that phosphorylation of A3G did not affect antiviral activity itself. Cellular expression and virion-incorporation levels of wild-type A3G and the T218A mutant were less reduced by Vif than those of nonphosphorylated mutants (T32A and T32A T218A; **Fig. 2d**, lanes 4 and 8 as compared with lanes 6 and 10). These data suggest that, in the presence of PKA signals, wild-type A3G and the

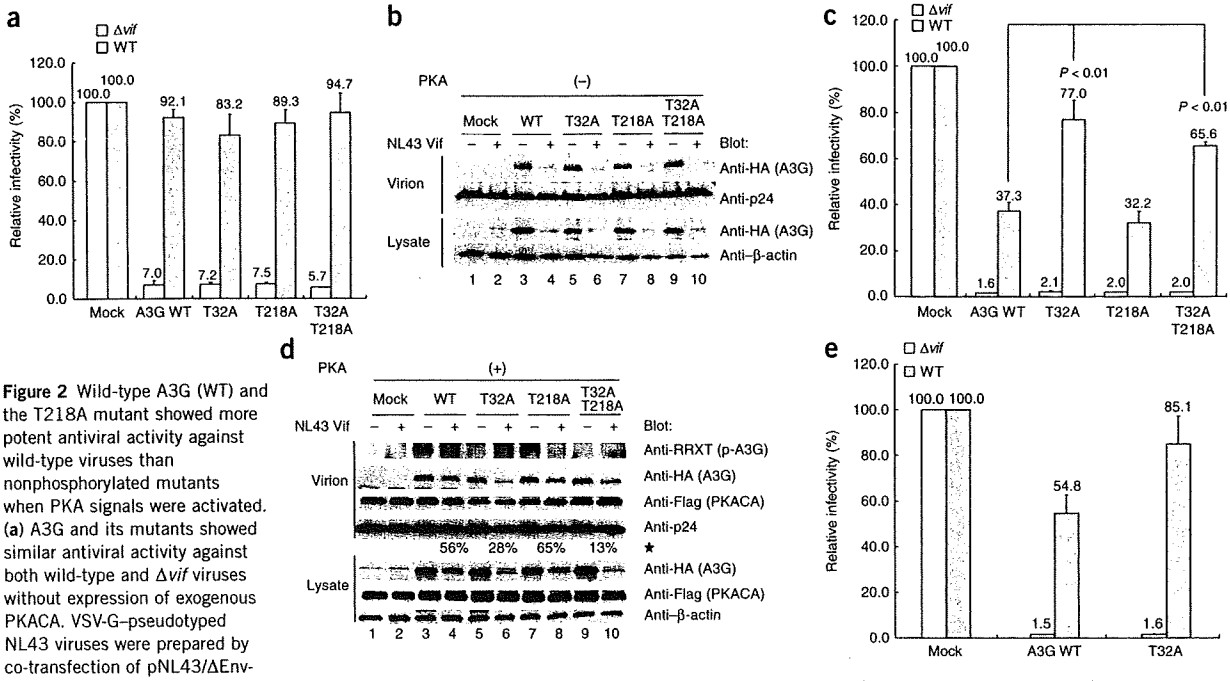

Figure 2 Wild-type A3G (WT) and the T218A mutant showed more potent antiviral activity against wild-type viruses than nonphosphorylated mutants when PKA signals were activated. (**a**) A3G and its mutants showed similar antiviral activity against both wild-type and Δ*vif* viruses without expression of exogenous PKACA. VSV-G–pseudotyped NL43 viruses were prepared by co-transfection of pNL43/ΔEnv-Luc or pNL43/ΔEnv/Δ*vif*-Luc and pVSV-G together with pcDNA3/HA-based vectors (mock, wild-type A3G and its mutants) in the absence of exogenous PKACA. Luciferase activities of target cell lysates were measured and presented as the percent infectivity relative to the value of each virus without expression of A3G. Values represent the average of three independent experiments and error bars represent s.d. from the average. IP, immunoprecipitation. (**b**) Cellular expression and virion incorporation of A3G and its mutants in the experiment shown in **a**. Virions were precipitated by ultracentrifugation and subjected to immunoblotting with the indicated antibodies. Cell lysates of producer cells were also analyzed by immunoblotting. (**c**) Wild-type A3G and the T218A mutant showed more potent antiviral activity against wild-type viruses than nonphosphorylated mutants when exogenous PKACA was overexpressed. Values are presented as described in **a**. Values represent the average of three independent experiments and error bars represent the s.d. from the average. (**d**) Cellular expression and virion incorporation of A3G and its mutants in the experiment shown in **c**. Star indicates the reduction rate of A3G in cell lysates as described in Methods. (**e**) Wild-type A3G showed more potent antiviral activity against wild-type viruses than T32A, when producer cells were treated with dB-cAMP.

T218A mutant showed more potent antiviral activity only against the wild-type viruses than did the nonphosphorylated mutants because they are more resistant to neutralization by Vif (**Fig. 2c,d**). Virion-incorporated A3G was also detected using an anti-RRXT antibody (lanes 3 and 7), suggesting that phosphorylated A3G was incorporated into virions. Additionally, PKACA was also incorporated into virions, as reported previously[18] (**Fig. 2d**, 'Anti-Flag (PKACA)'). When transfected 293T cells were treated with dB-cAMP, wild-type A3G also showed more potent antiviral activity than the T32A mutant only against the wild-type viruses, although to a lesser extent than observed in assays with PKACA overexpression (**Fig. 2e**). These findings suggest that PKA-mediated phosphorylation of A3G affects the neutralization of antiviral activity of A3G by Vif.

Antiviral activity of phosphomimetic A3G mutants

To further investigate the importance of PKA-mediated phosphorylation in the antiviral activity of A3G, we made phosphomimetic mutants (which mimicked phosphorylation by substituting threonine with aspartic acid; **Fig. 3a**) and examined their antiviral activity using VSV-G–pseudotyped luciferase reporter viruses. In contrast to non-phosphorylation mutants, phosphomimetic mutants of the first PKA phosphorylation site (T32D and T32D T218D) showed more potent antiviral activity against wild-type viruses than wild-type A3G and T218D even without PKA signals, whereas they showed similar antiviral activity against Δ*vif* viruses (**Fig. 3b**), again suggesting that these mutations did not affect the A3G's antiviral activity itself. Cellular expression levels of the T32D and T32D T218D mutants were less reduced by Vif than those of wild-type A3G and T218D (**Fig. 3c**, lower middle, lanes 6 and 10, as compared with lanes 4 and 8). Consequently, the T32D and T32D T218D mutants were more incorporated into wild-type virions than wild-type A3G and T218D (**Fig. 3c**, above, lanes 6 and 10 as compared with lanes 4 and 8).

To test the possibility that T32D is resistant to Vif-induced degradation, Vif sensitivity was analyzed by transfecting 293T cells with expression vectors for HA-A3G wild type and T32D together with increasing amounts of Vif expression vectors. Cell lysates were subjected to immunoblot with the indicated antibodies (**Fig. 3d**). Cellular expression levels of wild-type A3G were downregulated to 38% in response to Vif expression, but expression of T32D remained at 73%, even in the presence of Vif (**Fig. 3d**, top blot and graph). Coimmunoprecipitation assays showed that the T32D and T32D T218D mutants bound less efficiently to Vif than wild-type A3G and T218D (**Fig. 3e**, above, lanes 4 and 6, as compared with lanes 3 and 5). Finally, *in vivo* ubiquitination assays showed that ubiquitination of T32D and T32D T218D by Vif was clearly reduced as compared with that of wild-type A3G and T218D (**Fig. 3f**, above, lanes 5 and 7, as compared with lanes 4 and 6), indicating that phosphomimetic A3G mutants of Thr32 have more potent antiviral activity only against wild-type viruses because these mutants bound to Vif with less efficiency, were less ubiquitinated by Vif and were more

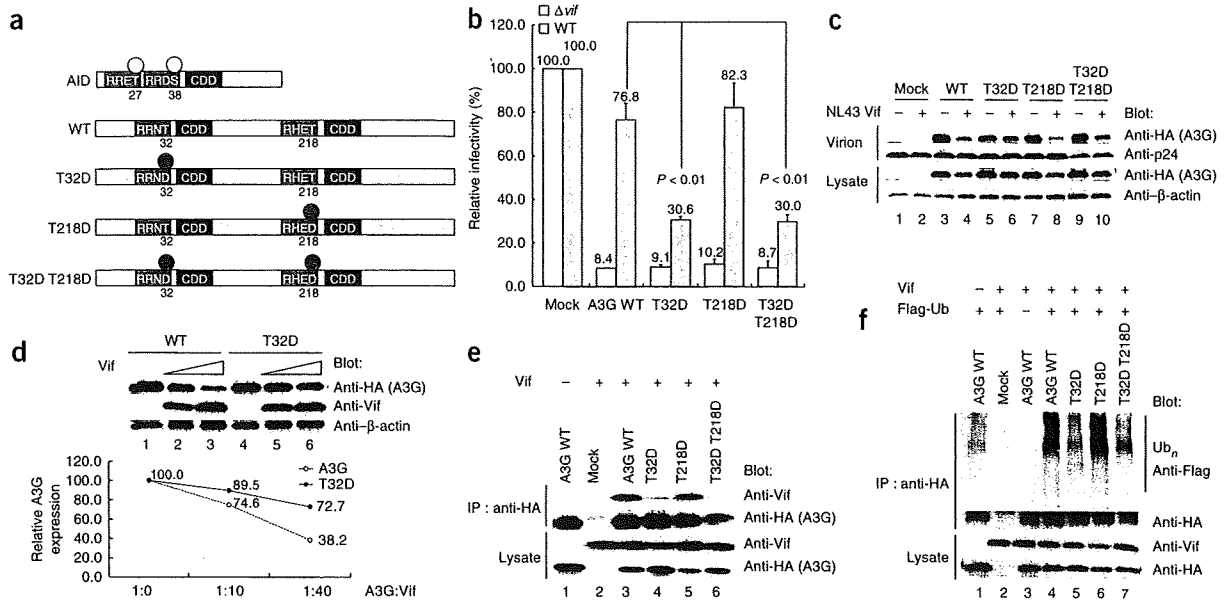

Figure 3 Phosphomimetic A3G mutants of the first PKA phosphorylation site showed more potent antiviral activity against wild-type viruses because they were more resistant to Vif-induced ubiquitination and degradation. (**a**) Schematic figure of phosphomimetic mutants of A3G. AID, activation-induced deaminase; CDD, cytidine-deaminase domain; WT, wild type. (**b**) Phosphomimetic mutants of the first PKA phosphorylation site (T32D and T32D T218D) showed more potent antiviral activity against wild-type viruses than did wild-type A3G and the T218D mutant. Values are presented as the percent infectivity relative to the value of each virus without expression of APOBEC3G. Values represent the average of three independent experiments and error bars represent the s.d. from the average. (**c**) Cellular expression and virion incorporation of A3G and its mutants in the experiment shown in **b**. (**d**) The T32D mutant was more resistant to Vif-induced degradation than wild-type A3G. 293T cells were transfected with expression vectors for HA-A3G, HA-T32D and Vif. Cell lysates were subjected to immunoblotting with the indicated antibodies. (**e**) The T32D and T32D T218D mutants had lower binding affinity to Vif than wild-type A3G and T218D. 293T cells were transfected with expression vectors for HA-A3G, phosphomimetic mutants and Vif. Cell lysates were immunoprecipitated with anti-HA mAb and analyzed by immunoblot with the indicated antibodies. (**f**) The T32D and T32D T218D mutants were less ubiquitinated by Vif than wild-type A3G and T218D. *In vivo* ubiquitination assays were performed as described in Methods. Flag-Ub–conjugated A3G proteins were detected as smears by immunoblotting with anti-Flag mAb.

resistant to Vif-induced degradation. Taken together with previous data, this suggests that PKA-mediated phosphorylation antagonizes Vif-induced degradation of A3G.

Phosphorylation affects interaction between Thr32 and Arg24

A3G consists of two tandem domains, the N-terminal and C-terminal domains. Thr32 is located in the N-terminal domain, which has been suggested to be crucial for interaction with Vif and for virion incorporation of A3G[21–25], although a complete picture of the interacting sites and molecules remains unclear. To obtain structural insights into the mechanisms by which Thr32 controls sensitivity to Vif-mediated neutralization, we conducted computer-assisted molecular modeling of the A3G N-terminal domain. The models constructed in this study included wild-type A3G, phosphorylated A3G (p-A3G), T32A and T32D. Thermodynamically optimized three-dimensional structures showed that the A3G N-terminal domain consists of a packed core structure with five β-strands and six α-helices, arranged from the N terminus to the C terminus in an α1-β1-β2/β2′-α2-β3-α3-β4-α4-β5-α5-α6 configuration (**Fig. 4a**). The configuration was the same among the four models and similar to that of the recently reported A3G catalytic C-terminal domain structure[26]. Asp128, a critical determinant of species-specific sensitivity of A3G to Vif[21–24], is located on the exposed β4-α4 loop (**Fig. 4a–d**, blue), as predicted by modeling based on the *Escherichia coli* cytidine deaminase[24]. Similarly, residues Tyr124 to Trp127, which contribute to virion incorporation, and Asp128 to Asp130, which contribute to interaction with Vif[25], are also exposed on the same protein surface (**Fig. 4a–d**, yellow and blue, respectively).

In the models, the amino acid at position 32 is located at the top of the β1 strand (**Fig. 4a–d**, red), on the same protein surface with residues 124–130 but separate from them. Consistently, no obvious differences in the conformations of these amino acids were detected among the models. Notably, the amino acid at position 32 is near Arg24 on the α1-β1 loop (**Fig. 4a–d**, orange). Arg24 is highly exposed to solvent and available to bind to negatively charged molecules, and its side chain is part of the same surface as that formed by residues 124–130 (**Fig. 4a–d**). The side chain of the amino acid at position 32 protrudes toward that of Arg24 in such a way that it could affect the movement of the Arg24 side chain. The hydrogen bonds between Arg24 and the side chains of the amino acids at position 32 are different among the models: Thr32 is predicted to form a single hydrogen bond with Arg24 (**Fig. 4e**), whereas phosphorylated Thr32 forms two hydrogen bonds (with oxygen atoms supplied by the phosphate group) (**Fig. 4f**). T32A resulted in a loss of hydrogen bonds (**Fig. 4g**). Finally, T32D, which has similar effects on Vif interaction as phosphorylation (**Fig. 3**), also formed two hydrogen bonds, with oxygen atoms supplied by the negatively charged side chain (**Fig. 4h**).

We also constructed models of the A3G N-terminal domain using the recently reported NMR structure of the A3G C-terminal domain (PDB 2JYW[26]) as a template. This procedure generated structural models similar to those obtained using the APOBEC2 crystal structure

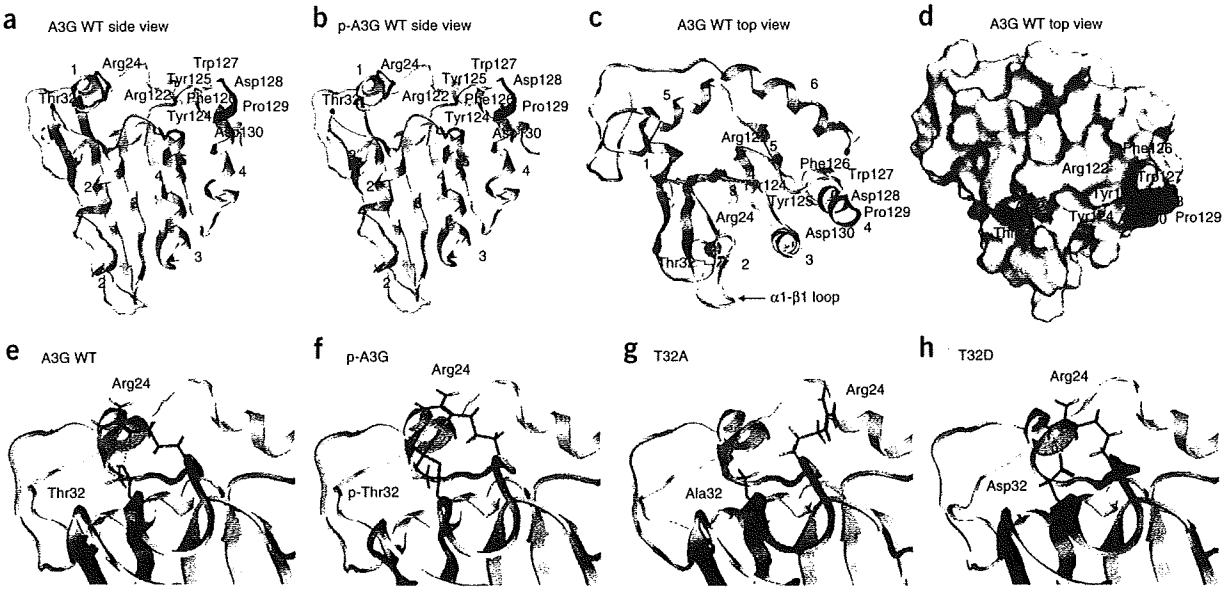

Figure 4 Structural models of the A3G N-terminal domain. The models were constructed by homology modeling using the X-ray crystal structure of APOBEC2. Configurations of five-β strands (blue numbers) and six α-helices (black numbers) forming a packed structure are indicated. (**a**,**b**) A3G wild type (WT) side view (**a**) and p-A3G wild type side view (**b**). Important residues are indicated: red, Thr32; orange, Arg24; yellow, residues contributing to virion incorporation; blue, residues contributing to Vif interaction, including Asp128. (**c**) A3G wild type top view. (**d**) Surface structure model of the A3G wild type N-terminal domain from the top view as shown in **c**. Thr32, Arg24 and Asp128 are shown in the same colors as in **a** and **c**. The yellow area indicates residues contributing to virion incorporation, and the blue area indicates those contributing to Vif interaction. (**e**–**h**) Close-up view around the position 32 residue of A3G N-terminal domain models: wild-type A3G (**e**), p-A3G (**f**), T32A (**g**) and T32D (**h**). The sticks represent the side chains of amino acids at positions 24 and 32. Orange dotted lines represent hydrogen bonds.

(**Supplementary Fig. 1** online). Phosphorylation or substitution of the amino acid at position 32 similarly influenced the formation of hydrogen bonds with Arg24 (**Supplementary Figs. 2a–d** online). However, because of the intrinsic 9-residue N-terminal deletion of the A3G C-terminal domain compared with the A3G N-terminal domain, an α-helix structure at the N terminus was missing when the NMR structure of the A3G C-terminal domain was used as a template for modeling (**Supplementary Fig. 1**).

Importance of Arg24 for Vif binding and virion incorporation

Because our structural models suggest that the interaction between Thr32 and Arg24 in A3G has an important role in virion incorporation and interaction with Vif, we examined the antiviral activity of the A3G mutants R24A and R24A T32D. Cellular expression levels of these mutants were adjusted by transfection with more plasmid DNA. R24A T32D was less resistant to Vif than T32D (**Fig. 5**, below middle, lane 10 compared with lane 8; a reduction rate of 42% compared with

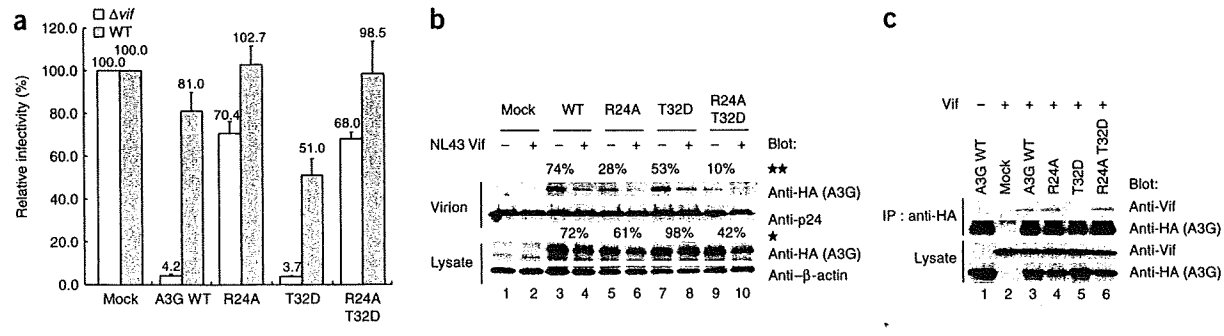

Figure 5 R24A mutation abrogates not only Vif resistance of the T32D mutant, but also the ability for virion incorporation. (**a**) Luciferase activities of target cell lysates were measured and presented as the percent infectivity relative to the value of each virus without A3G expression. Values represent the average of three independent experiments and error bars represent the s.d. from the average. (**b**) Cellular expression and virion incorporation of A3G and its mutants in the experiment shown in **a**. Virions were precipitated by ultracentrifugation and subjected to immunoblotting with the indicated antibodies. Cell lysates of producer cells were also analyzed by immunoblot. A single star indicates the reduction rate of A3G in cell lysates, whereas the double stars indicate the virion-incorporation rate of A3G, as described in Methods. (**c**) 293T cells were transfected with expression vectors for HA-A3G, its mutants and Vif. Cell lysates were immunoprecipitated with anti-HA mAb and analyzed by immunoblot with the indicated antibodies.

98%, respectively). Moreover, R24A substitution restored the binding of T32D to Vif (**Fig. 5c**, above, lane 6 compared with lane 5). Accordingly, infectivity assays showed that R24A T32D lost the potent antiviral activity of T32D against wild-type viruses (**Fig. 5a**), supporting the hypothesis that the interaction between Thr32 and Arg24 has a crucial role in interaction with Vif. In addition, the R24A and R24A T32D mutants showed impaired antiviral activity even against Δ*vif* viruses, because they were less efficiently incorporated into virions (**Fig. 5b**, above, lanes 5 and 9; incorporation rates were 28% and 10%, respectively). Therefore, these data suggest that Arg24 might also have an important role in virion incorporation.

DISCUSSION

In this study, we show that PKA specifically phosphorylates Thr32 on A3G and that PKA-mediated phosphorylation of A3G confers resistance to Vif-induced ubiquitination and degradation. We also show that the interaction between Thr32 and Arg24 has an important role in regulating A3G's interaction with Vif.

We first demonstrated the physical interaction between A3G and PKACA by immunoprecipitation asssays, although we cannot exclude the possibility that this interaction is indirect, through binding to PKA regulatory subunits. We also showed that PKA can phosphorylate A3G *in vitro* and *in vivo*. PKACA is sufficient to phosphorylate A3G *in vitro*. *In vivo*, PKACA overexpression or activation of endogenous PKA induced by dB-cAMP can promote the phosphorylation of A3G, suggesting that PKA specifically phosphorylates A3G *in vivo*. We also showed that virion-incorporated A3G is phosphorylated by PKA (**Fig. 2d**). PKA is anchored by AKAP and mainly exists at the plasma membrane or subcellular organelles. On the other hand, A3G resides in RNA-processing bodies in the cytoplasm. Where does PKA phosphorylate A3G? In HIV-1 infected cells, A3G is recruited into the plasma membrane through the HIV-1 Gag interaction. PKA might therefore target A3G during virion budding. Another possibility is that virion-incorporated PKA might target A3G in virions, because PKA has been shown to be incorporated into virions[18] (**Fig. 2d**). Further study is necessary to clarify this issue.

We next demonstrated that PKA signals increase the antiviral activity of A3G against wild-type viruses, because cellular expression and virion-incorporation levels of A3G are less reduced by Vif in the presence of PKA. Our data clearly indicate that PKA regulates the functional interaction between A3G and Vif, but not A3G antiviral activity *per se*, although we cannot completely exclude the possibility that PKA might regulate other physiological functions of A3G. In the case of AID, the T27A and S38A mutants lost deaminase activity only on double-stranded DNA, whereas they showed similar deaminase activity on single-stranded DNA, suggesting that phosphorylation by PKA does not affect deaminase activity itself, but instead induces conformational changes in AID, enabling it to access double-stranded DNA[9].

We investigated the mechanisms by which A3G phosphorylation confers Vif resistance. Phosphorylation of A3G at Thr32 reduces binding to Vif and A3G's subsequent ubiquitination, resulting in a Vif-resistant phenotype. Comparison of the structural models for the A3G N-terminal domain revealed that Thr32 interacts with Arg24, and its phosphorylation or substitution with a negatively charged amino acid would stabilize this interaction (**Fig. 4e–h**). The models suggest that the side chain of Arg24 in phosphorylated A3G or T32D mutant has a more rigid conformation than it does in dephosphorylated A3G or T32A mutant, because of increased Coulomb interactions with the negatively charged side chains. APOBEC2 has a corresponding loop to the α1-β1 loop where Arg24 is located, termed the L1 loop, whose basic amino acids are directly involved in joining inner domains through head-to-head interactions during tetramer formation[27]. Notably, the A3G α1-β1 loop has additional basic amino acids, Arg29 and Arg30, forming a positively charged protein surface. These observations suggest that the A3G α1-β1 loop is available to have a direct role in binding to the negatively charged surface of other molecules. If this were the case, the degrees of conformational freedom of Arg24 would crucially influence interaction efficiency and specificity.

As expected, the substitution of Arg24 to alanine abrogates Vif resistance of the T32D mutant and also impairs the ability for virion incorporation to some extent. Structural modeling of the R24A mutant protein showed that the hydrogen bond between residues 32 and 24 was missing, whereas no changes in the main chain structure were induced in other regions (**Supplementary Fig. 2e**). The data suggest that the R24A substitution mainly influences the degrees of conformational freedom and positively charged status of the protein surface around residue 24, although we cannot exclude the possibility that the R24A mutation induces allosteric changes in A3G conformation. Taken together, our data suggest that the amino acid at position 32 controls conformational changes of Arg24, which in turn influence A3G's interaction with Vif and molecules for virion incorporation.

Although PKA-mediated phosphorylation renders A3G resistant to Vif-induced neutralization, in fact, the pathology of most HIV-1–infected patients progresses to AIDS. A recent study reported that naturally occurring Vif point mutants with suboptimal anti-A3G activity induce drug resistance–associated mutations in the HIV-1 provirus[28]. In other words, the imbalance between the antiviral activity of A3G and the anti-A3G activity of Vif could determine HIV-1 genome diversity. Our data suggest that the phosphorylated A3G or T32D mutants are not fully resistant to Vif (**Figs. 2c** and **3b**), but they might cause this balance to be disturbed. PKA-mediated phosphorylation of A3G might contribute to genetic diversity of HIV-1. Further study will be required to clarify this issue. Finally, PKA-mediated phosphorylation regulates the functional interaction between A3G and Vif, which might be a target for new strategies to control HIV-1 replication.

METHODS

Plasmids and cell lines. We constructed an expression vector for HA-tagged human A3G, pcDNA3/HA-A3G, as previously described[29]. We generated expression vectors for A3G mutants (T32A, T218A, T32D, T218D, R24A) by QuickChange XL Site Directed Mutagenesis Kit (Stratagene) using pcDNA3/HA and pDON/EGFP vectors[4]. We amplified human PKACA by PCR and cloned it into pcDNA3/Flag. pNLA1-43Vif (a gift from H. Akari) was constructed by inserting a Vif fragment from NL4-3 into the subgenomic expression vector pNL-A1 (ref. 30), and this expressed all HIV-1 proteins except for *gag*, *pol*, and *vpr* products. We constructed pNL43/ΔEnv-Luc and pNL43/ΔEnvΔ*vif*-Luc as previously described[4]. HEK293T and M8166 cells were maintained as previously described[4]. We established U937/GFP and U937/GFP-A3G cells, expressing GFP and GFP-tagged A3G, respectively, by retroviral transduction of GFP and GFP-A3G, respectively, and maintained them with RPMI1640 containing 10% (v/v) FCS.

***In vitro* phosphorylation assays.** We incubated purified GST-Flag–tagged A3G prepared with a baculovirus expression system and recombinant PKACA (Upstate Cell Signaling Solutions) in an appropriate buffer (20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 25 mM glycerophosphate, 0.1 mM $NaVO_4$, 1 mM ATP, 1 mM cAMP) at 30 °C for 30 min, and analyzed them by immunoblot with phospho-(Ser/Thr) PKA substrate antibody (anti-RXXT) (Cell Signaling Technology).

***In vivo* phosphorylation assays.** We transfected HEK293T cells with expression vectors for GFP-A3G, its mutants, and/or Flag-PKACA by the calcium

phosphate method. At 36 h after transfection, we lysed cells in lysis buffer (25 mM HEPES, pH7.4, 150 mM NaCl, 1 mM $MgCl_2$, 0.5% (v/v) TritonX-100, 10% (v/v) glycerol, 25 mM β-glycerophosphate and 100 μM $NaVO_4$) and immunoprecipitated GFP-A3G with anti-GFP mAb and Protein A Sepharose beads (Amersham Biosciences) at 4 °C. We washed the beads with lysis buffer and analyzed them by immunoblot with Phospho-PKA Substrate Rabbit mAb (100G7) (anti-RRXT; Cell Signaling Technology) or anti-Flag mAb (M2) (Sigma-Aldrich). We also treated transfected HEK293T cells with 50 μM H89 (Sigma-Aldrich) for 2 h, followed by 100 μM or 500 μM dibutyryl cAMP (Sigma-Aldrich) for 4 h.

Infectivity assays with luciferase reporter viruses. We prepared Luciferase reporter viruses with or without Vif by co-transfection of pNL43/ΔEnv-Luc (wild type) or pNL43/ΔEnvΔ*vif*-Luc(*vif*) plus pVSV-G, together with expression vectors for A3G, its mutants, and/or Flag-PKACA by the calcium phosphate method[4]. We collected viruses in the supernatants 48 h after transfection and measured virus titers with an enzyme-linked immunosorbent assay kit for the p24 antigen (RETRO-TEK, ZeptoMetrix). We challenged an adjusted amount of viruses to target M8166 cells. At 24 h after infection, we lysed the cells in passive lysis buffer (Promega) and measured their luciferase activity with a Luminometer (EG & G Berthold). We presented values as percent infectivity relative to the value of wild-type or Δ*vif* virus without the expression of A3G. In **Figures 2d** and **5b**, band intensities were quantified using Image J software (http://rsb.info.nih.gov/ij/). Reduction rates of A3G by Vif were calculated as the ratio of A3G levels with Vif to those without Vif in cell lysates, normalized by the amounts of β-actin intensity. Additionally, in **Figure 5b**, virion-incorporation rates were calculated as the ratio of A3G levels in virions to those in cell lysates, normalized by the amounts of p24 and β-actin intensity, respectively.

Co-immunoprecipitation assays. To see the A3G-Vif interaction *in vivo*, we performed immunoprecipitation assays as described previously[4]. We transfected HEK293T cells with expression vectors for HA-A3G, its mutants, and Vif as indicated. At 36 h after transfection, we lysed cells in lysis buffer and immunoprecipitated complexes with anti-HA mAb (12CA5; F. Hoffmann-La Roche) and Protein A Sepharose beads at 4 °C. We washed the beads with lysis buffer and analyzed them by immunoblot with anti-HA or anti-Vif mAbs (#319; a gift from M. Malim through the AIDS Research and Reference Reagent Program)[31].

***In vivo* ubiquitination assays.** We transfected HEK293T cells with expression vectors for A3G, its mutants, Vif and Flag-tagged ubiquitin (Flag-Ub) as indicated. We treated cells with 10 μM MG132 for 6 h before harvest. At 48 h after transfection, we lysed cells in lysis buffer and immunoprecipitated them with anti-HA mAb and Protein A Sepharose beads at 4 °C. We washed the beads with lysis buffer and analyzed them by immunoblot with anti-HA, anti-Flag and anti-Vif mAbs.

Molecular modeling of the APOBEC3G N-terminal domain. We took the crystal structure of APOBEC2 at a resolution of 2.50 Å (PDB 2NYT[27]). APOBEC2 belongs to the APOBEC family and has cytidine-deaminase activity, as does A3G[32,33]. The sequence identity of A3G (Genbank NM_021822) N-terminal domain and APOBEC2 (PDB 2NYT; chain B) is ~29.5%. These functional and sequence similarities are sufficient to construct models with an r.m.s. deviation of ~1.5 Å for the main chain between the predicted and actual structures[34]. To minimize misalignments of the target sequence, the A3G N-terminal domain and the template sequence, APOBEC2, we used the multiple sequence alignment method with the sequences of APOBEC3A (Genbank NM_145699), APOBEC3C (Genbank NM_014508) and APOBEC3F (Genbank NM_145298). We generated multiple sequence alignments using 'MOE-Align' in the Molecular Operating Environment (MOE; Chemical Computing Group). We constructed three-dimensional models of the A3G N-terminal domain by the homology modeling technique using 'MOE-Homology' in MOE as previously described[35,36]. We optimized the three-dimensional structure thermodynamically by energy minimization using MOE and an AMBER99 force field[37]. We further refined the physically unacceptable local structure of the optimized three-dimensional model on the basis of evaluation by the Ramachandran plot using MOE. We constructed the three-dimensional model of A3G with phosphorylated Thr32 by adding the phosphate group to the side chain of Thr32 in the three-dimensional model of A3G using 'Molecular Builder' in MOE and by energy minimization. We also constructed the three-dimensional models of A3G T32A or T32D by the same method as the A3G model.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

K.S. designed research, performed research, contributed vital new reagents, analyzed data and wrote the paper; A.T.-K. designed research, analyzed data, wrote the paper and organized research; M.Y. and H.S. performed the structure modeling and wrote the paper; T.I., M.M., K.I. and T.S. prepared the materials and performed a part of research; T.U. analyzed data, drafted the paper and organized research.

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