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AUTHOR'S CORRECTION

HLA-Associated Viral Mutations Are Common in Human Immunodeficiency Virus Type 1 Elite Controllers

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Volume 83, no. 7, p. 3407–3412, 2009. GenBank accession numbers were erroneously omitted from the original publication. The GenBank accession numbers for the sequences derived from HIV elite controllers are EU517762 through EU517815 and EU517972 through EU518012 (as described in reference 25 of the original manuscript), EU873003 and EU873005 (as described in reference 26 of the original manuscript), and GU046566 through GU046604. The majority of the GenBank accession numbers for the sequences used to create the map of HLA-associated mutations appeared in references 7 and 9 of the original manuscript, and additional GenBank accession numbers are GQ303719 through GQ304249, GQ371216 through GQ372824, GQ398382 through GQ398387, and AY856956 through AY857186 (as described in a new publication [1]).

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解説［Ⅱ］

HIV Elite Controllers
—HIV感染症の自然制御—

三浦　聡之*

はじめに

後天性免疫不全症候群（Acuquired Immune Deficiency Syndrome：AIDS）はヒト免疫不全ウイルス（Human Immunodeficiency Virus type 1：HIV-1）の感染によって引き起こされ，1990年代前半までは，HIV感染＝AIDS＝死というイメージがあった．しかしながら，1996年ごろから利用できるようになった強力な抗ウイルス薬併用療法により，血液中のウイルス量を検出限界以下にまで抑制することが可能になり，AIDSによる死亡数は激減した．しかしながら，これらの抗ウイルス療法によっても，ウイルスを体内から完全に排除することは不可能であり，内服を中断すると再びウイルス量が増加してくる．したがって，感染者は生涯にわたり抗HIV薬を飲み続ける必要があり，長期の副作用が懸念されている．一方で，病気の進行速度は，患者間で大きく異なっており，なかには感染後20数年経過しても免疫力を保ち，抗HIV薬の投与を必要としない患者群も存在する．彼らは，HIV Controllersと呼ばれ，近年，これらにおけるウイルス複製コントロールの機序を解明することが，有効なワクチン開発の鍵となるという考えから，HIV Controllersを対象とした研究が盛んに行われている．ここでは，現在までの知見の一部を紹介する．

Ⅰ．HIV Elite Contollers

HIV-1は，CD4陽性細胞（主に，CD4陽性T細胞）に感染し，体内でCD4陽性T細胞が破壊されることにより，（一般的にCD4陽性細胞数が200/μL未満になると）感染者は各種の日和見感染症に罹患しやすくなる．かつて，血中のウイルス量の測定が商用ベースでは利用できなかった時代には，感染後7～10年以上を経ても，CD4数を500/μL以上（健常人におけるCD4陽性細胞数は500～1,000/μL）に保っている患者群は，長期未発症者（Long-term non-progressors：LTNP）と呼ばれ，その多くが研究対象とされてきた．しかしながら，彼らの多くは，最終的にはAIDSに進行することがわかってきた[1)]．

血中ウイルスの定量が可能になってからは，病気の進行速度が，その血中ウイルス量と強く相関することがわかってきたため[2)]，感染期間より，血中ウイルス量そのものが注目されるようになってきた．HIVに感染すると，3～4週の潜伏期の後，人によっては伝染性単核球症様の症状を呈する．この間，血中のウイルス量は，10^8 RNA copies/mLにも達し，一過性にCD4陽性T細胞数も減少する（**図1**）．急性期後，数年にわたる無症候期が続く．この間，血中ウイルス量はおおむね一定した量を保っており，この安定したウイルス量のことを「セットポイントウイルス量」と呼んでいる．平均的な未治療のHIV感染者では，この値はおよそ30,000 RNA copies/mL程度である．感染個体内でのウイルス複製の抑制には，HIV特異的細胞障害性T細胞（CTL）が非常に重要な役割をしていることが示唆されている[3,4)]．

中和抗体の誘導を目的としたワクチンの開発が，現在までの知見では非常に困難であり，血漿中ウイルス量が低ければ，AIDSになる確率が低く，他の人にウイルスを感染させる可能性も著しく低いことが報告されていることから[5)]，多くの研究者がHIV特異的CTLの誘導を目的としたワクチン開発を目指している．自らウイルス複製をこのレベル以下に抑制できるものが，最近，HIV Controllersと呼ばれるようになり，このうち，特に，商用の血中ウイルス定量で，検出限界以下（50－75 RNAcopies/mL）にまで，自らコントロールできるものを特に，HIV elite controllers（EC）

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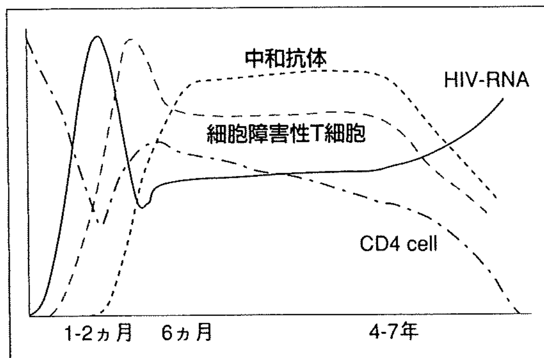

図1 HIV感染症の自然経過

と呼んでおり，その頻度は1%以下であると推察されている[1]．

次に要因ごとに，ウイルス因子，宿主遺伝的因子，免疫学的因子，と分けて解説したい．しかしながら，これらの要因は互いに重なりあう部分が多くある．

Ⅱ．ウイルス因子

ウイルス側の因子として最も知られたものは，アクセサリー遺伝子のひとつである*nef*の欠失(長さはさまざま)である．最初，一人のHIV陽性の輸血ドナーとその数名のレシピエントのすべてに，*nef*に大きな欠失が検出された[6]．これらの患者すべてが，いわゆるLTNPであったため，この*nef*遺伝子の欠失が一躍有名になった．以後も，小規模な研究で同様の報告がいくつか続き，LTNPにおいては，他のアクセサリー遺伝子の欠失などの報告も散見されている．

筆者らは，50人以上のECから，高用量(5～35mL)の血漿からウイルスを超遠心で濃縮し，すべての構造遺伝子の増幅を試みた．その結果，確かに数%のECでは*nef*遺伝子の欠失を認めたが，それ以外に遺伝子欠失は認められなかった[7]．さらに，ウイルスのアミノ酸配列をコドンレベルで，ECと未治療の高ウイルス血症患者群で比較したところ，elite controlに特異的と考えられるアミノ酸変化も認められなかった．想像に難くないが，ECからのウイルスそのものの分離は非常に難しい．筆者らは，血漿から増幅された*gag-protease*遺伝子をHIV-1の実験株NL4-3に組み込んだキメラウイルスを作製し，その増殖能を，50名以上のECと同等数の高ウイルス血症患者群の間で比較したところ，EC由来キメラウイルスは，有意に低下した増殖能を示した[8]．

一方で，Lassenらは，7人のEC由来の計36クローンのenvelopeタンパクの細胞侵入効率が，慢性未治療感染者群に比べ，有意に劣っていることを示した[9]．

未発表のデータであるが，筆者らも50名を超えるEC由来*envelope*で，同様な結果を得ている．しかしながら，重要なポイントとして，*gag-protease*および*envelope*に共通していたことは，進行感染者と比較しても遜色のない機能を持つウイルスに感染しているECが多くいたことである．

現時点で言えることは，弱毒株の感染によって説明できる例は多数あると考えられるが，その責任遺伝子は，EC間で共通とはいえない．また，弱毒株の伝播によるのか，または感染後に弱毒化されたのかは明らかにできていないが，少なくとも，*Gag-protease*に関しては後述するように，免疫からの逃避現象として出現している可能性が高いと考えられる．

Ⅲ．宿主遺伝的因子

宿主因子として，体内でのウイルス産生量に明らかに関連する因子は，HLAである．驚くべきことに，ECの30～40%は，HLA-B57を発現しており，20%はHLA-B27を発現している[10]．この非常に強いHLA分布の偏重は，強く細胞障害性T細胞の関与を支持するものである．これに関連する項目は，次章を参照されたい．一方で，HLA class Iの分布に偏りがあるからといって，必ずしも細胞性免疫だけが関与しているとは限らない．これらの遺伝子と強く連鎖不均衡を持つ他の遺伝子産物が病気の進行に関与していて，その結果としてHLAの分布に偏りが出てきている可能性は否定できない．

一般的な慢性HIV感染症患者における研究ではあるが，Fellay らは，whole genome association study により，ウイルスセットポイントに強く影響するヒトの遺伝子多型を見つけた[11]．最も強い関連が認められたのは，HCP5(HLA complex P5)というHLA-Bの近傍にある遺伝子で，ヒトの内因性レトロウイルスエレメント(human retroviral element)の中にあった．しかしながら，この多型は，HLA-B*5701と強い連鎖不均衡があるため，B*5701による効果を見ている可能性が高いが，HCP5遺伝子自体の産物が，レトロウイルスのpol遺伝子に類似することから，アンチセンス機序によるジーンサイレンシングなどが関与している可能性なども指摘されている．また，二番目に強い関連を示した多型は，HLA-C遺伝子の上流にあり，HLA-Cの発現レベルと強い関連があり，低い血中ウイルス

量と関連するアリルが，HLA-Cの高い発現と関連しているとされる．16名のECで，これら二つの遺伝子多型が見られるかを検討した研究があるが，それぞれ，0/16および4/16と低頻度であった[12]．

HIVがCD4陽性T細胞に感染するときに，co-recepterとして，CCR5またはCXCR4を必要とする．白人では，CCR5遺伝子の32 base pairの欠失が知られている．このCCR5⊿32をホモ接合体として持つ者は，HIV感染に対して感受性が低いことがわかっており，ヘテロ接合体として持つ者も病気の進行が遅いという報告がある[13]．しかしながら，Pereyraらによる検討では，ホモ接合体は一人もおらず，ECでのヘテロ接合体の頻度も進行感染者と比べて違いはなかった[10]．Dolanらは，病気の進行に促進的に働くCCR5ハプロタイプを定義づけ，また，リガンドであるMIP1αをコードするCCL3L1遺伝子のコピー数が，病気の進行速度と負の相関があることも報告し，non-detrimentalなCCR5ハプロタイプと高いCCL3L1遺伝子コピー数の組み合わせが，ECで高い頻度で見られることを報告している[14]．

Ⅳ．免疫学的因子

1．適応免疫

1）HIV特異的CD4陽性T細胞

CD4陽性T細胞は，適応免疫のkey regulatorである．以前より，いわゆるLTNPでは，HIV特異的CD4陽性T細胞の増殖能が優れていると報告されていた[15]．Pereyraらは，IL-2とインターフェロンガンマをともに分泌するHIV特異的CD4陽性T細胞の頻度が，ECでは高いことを報告しており[10]，ごく最近では，Potterらが，HIV特異的CD4陽性T細胞の増殖能とIL-2分泌の程度が，抗ウイルス療法下の患者群に比べて，ECでは高かったことを報告している[16]．また，最近，一般的なHIV感染症では，HIV-1特異的CD4陽性T細胞上で，immunoregulatory molecules であるCTLA-4およびPD-1の発現が高まっているため，結果として，細胞が疲弊してしまっているとされる[17, 18]．ECにおいても，ECのHIV特異的CD4陽性T細胞では，CTLA-4のレベルが有意に低いことが示されており[18]，結果として，高い増殖能を保っている可能性がある．

2）CD8陽性T細胞（細胞障害性T細胞）

HIV-1特異的細胞障害性T細胞（Cytotoxic T Lymphocyte：CTL）がウイルス量のコントロールに主要な役割を果たしていることは，ほぼコンセンサスが得られている．ひとつには，図1に示すように，急性感染期において，ピークのウイルス量が低下し始めるとほぼ同時期に，HIV特異的CTLが検出されるようになること[3]，またサルのエイズウイルス感染モデルでは，抗CD8抗体の投与によりCTLを枯渇させると，血中のウイルス量が増加することが観察されている[4]．一般的な慢性HIV感染症において，その血中ウイルス量と相関する免疫学的パラメーターの探索は非常に多くあり，ここでそれらを紹介する紙面の余裕はない．大雑把にいうと，低い血中ウイルス量，あるいは病気進行の遅延と関連するものとして，①Gag特異的CTLとウイルスフィットネス，②CD8陽性T細胞のクオリティ，等が挙げられる．以下に，実際にECを対象にした研究を紹介する．

①Gag特異的CTLとウイルスフィットネス

HIVタンパクのうち，Gagが最も重要なCTLの標的であることは，多くの論文で明らかにされている[19]．ECにおける研究では，二つのグループが同様の結果を報告している[10, 20]．ECでは，HLA-B27あるいはB57を発現している者が多く，これらのアリルはGag（特にCapsid）タンパク内にCTLエピトープを持っている[21, 22]．しかしながら，この結果はECにおける偏ったHLA分布だけによるわけではないことがわかっている．

HIVの逆転写酵素はエラーが入りやすく，この高い変異率が，HIVの他に類をみない高い多様性の原因ともなっている．HIV特異的免疫によりウイルスに選択圧がかかると，HIVは容易に変異を獲得し，結果としてウイルスが免疫から逃避（エスケープ）する．これらエスケープ変異のなかには，ウイルスの複製能（フィットネス）を低下させるものが報告されている．ECの40%を占めるHLA-B57に拘束されるGagエピトープから，ほぼ普遍的に生じるエスケープ変異の一つは，ウイルスの複製能を低下させることが，試験管レベルでも，臨床上の観察でも認められている[23, 24]．

前述したように，EC由来*gag-protease*をもつキメラウイルスは有意に減弱した複製能を示したが，さらなる解析によりこれらは，特にHLA-B57を含めた，病気の進行に対して防御的に働くHLA class Ⅰタイプからの選択圧によってもたらされている可能性が高いことが示された[8]．さらに，HLA-B57患者由来のウイルスのシークエンスを解析すると，多くのECがそのエピトープ内に，高ウイルス血症患者には見られない稀な変異を持っており，ウイルスの複製能を著しく低下

させることが報告された[25]．それらのECは，エスケープバリアントを認識する特異的CTLを持っていることが報告されている．この二重の防御機構が，エリートコントロールに寄与している可能性が示された[25]．一方で，ECに多く認められるHLA-B51はGag内に標的エピトープが存在しないなど，上述した機序がすべてのECにあてはまるわけではないことも記しておく．

②CD8陽性T細胞のクオリティ

二つのグループからIFNガンマ/IL-2をともに産生するCTLの割合が，ECでは進行感染者群より有意に高かったことを報告されている[10, 20]．またBettsらは，ECにおいて，同じHLAによって拘束されるエピトープ特異的なCTLにおいても，ウイルス血症を認める患者群に比べて，多種類のサイトカインを産生するpolyfunctionalなCTLの割合が高かったことを報告している[26]．Miguelesらは，ECでは刺激に対して，HIV特異的CD8陽性T細胞の増殖能が認められたのに対して，抗ウイルス療法によって，同様に検出限界以下のウイルス量を達成している群では認められなかったことを報告している[27]．さらに，Saez-Cirionらは，EC由来の未刺激状態のCD8陽性T細胞が，同患者由来のCD4陽性T細胞にウイルスを試験管内で感染させたものに加えたところ，進行感染者群および，抗HIV療法群に比べて，ウイルス増殖をよりコントロールできたことを報告した[28]．また，非常に最近，Miguelesらは，ECのCD8陽性T細胞では，lytic granuleの量が増加しており，より効率的に感染細胞を殺すことができている可能性を示した[29]．

CD4陽性細胞の項でも述べたが，HIV特異的CTLでは，PD-1(programmed death-1)分子の発現が高まっており，これが細胞のdysfunctionに関連し，そのレベルは，血中のウイルス量と正の相関があることが示されている[30]．EC自体のサンプルを使用したデータは，筆者の知る限り一つであるが，進行感染者との差は明らかでなかった．しかしながら，上記すべてを通して，この良質なHIV特異的CD8陽性T細胞が，血中ウイルス量コントロールの原因なのか，その結果を見ているのかは定かでない．

2. 自然免疫

前述したように，感染個体内でのウイルス複製コントロールにCD8陽性T細胞が重要な役割を果たしていると考えられているが，急性感染期において，HIV特異的CTLが検出されるのは，ウイルス量がピークに到達するより若干遅れているとされている[31]．したがって，自然免疫系が果たしている役割も重要かもしれない．自然免疫を担う細胞のひとつであるNK(Natural Killer)細胞上のレセプターであるKIR(Killer Immunoglobulin-like Receptor)の特定のアリルと病気の進行速度には，関連があることがわかってきている．Activating NK cell receptorであるKIR3DS1アリルと，リガンドとして働く80番目のアミノ酸がイソロイシンであるHLA-Bw4(HLA-Bw4-80I)をともに持つ患者では，病気の進行が遅く，かつ試験管内では，この組み合わせが，強くHIVの複製を抑制することが証明されている[32, 33]．またinhibitory receptorであるKIR3DL1の特定のアリルとHLA-Bw4-80Iをともに持つ患者の病気の進行も遅いことが報告されている[33]．

重要なことに，HLA-Bw4-80Iは，最もprotectiveなHLA-B57を含んでいる．したがって，CTLによる効果に加え，一部のHLA-B57陽性者では，NK細胞による抗ウイルス効果も寄与している可能性がある．ECにおける研究では，20人の黒人ECにおいて，HLA-Bw4-80Iは17人に認められたが，KIR3DS1を持っていた者は2人しかおらず，両方持っていた者は，たったの1人であったことが報告されている[34]．もとより，KIR3DS1は黒人では稀なアリルであるため評価は難しいが，少なくとも，これらがエリートコントロールに必須でないことは明らかである．しかしながら，急性感染期に起きている現象が，その後のセットポイントウイルス量の決定に深く関与していると考えられているため，今後，自然免疫系の研究が重要となってくるかもしれない．

3. 液性免疫

前述したように，HIV感染症においては，感染個体内のウイルス産生コントロールへの中和抗体の寄与は高くないと考えられている．Pereyraらはheterologous virus由来envelopeを持つpseudovirusの中和抗体価は，ECでは進行感染者に比べて，有意に低かったことを報告している[10]．一方，EC由来の血漿が自己ウイルスを効率よく中和するために，ウイルス量がコントロールできているのではないかという疑問も生じる．これについては，数名程度の小規模のデータしか報告されておらず，進行感染者との間で，特に違いは報告されていない[35]．したがって，現時点での知見では，エリートコントロールに対する中和抗体の寄与は否定的である．

最近，Lambotteらは，recombinant gp120を標的と

したADCC(antibody-dependent cell cytotoxicity)を，10人のコントローラ(<400 RNA/copies/mL)と高ウイルス量群との間で比較したところ，コントローラでは，ADCCが有意に高かったことを報告している[36]．今後より規模の大きな研究での確認が必要であろう．

まとめ

ここ数年，ECに関して多くの論文が出されているが，解説したように特定の共通した機序は見つかっていない．HLAのバイアスを超えるような因子は見つからない可能性が高く，それぞれのelite controlが違った機序，あるいは複数の機序のコンビネーションの上に成り立っている可能性も高いと考えられる．有効なHIVワクチン開発の鍵が，本当にECに隠されているかは不明であるが，今後もこの領域の研究は継続されていくであろう．

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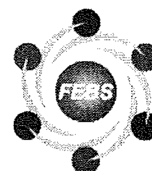

Requirement for microtubule integrity in the SOCS1-mediated intracellular dynamics of HIV-1 Gag

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ABSTRACT

Suppressor of cytokine signaling 1 (SOCS1) is a recently identified host factor that positively regulates the intracellular trafficking and stability of HIV-1 Gag. We here examine the molecular mechanism by which SOCS1 regulates intercellular Gag trafficking and virus particle production. We find that SOCS1 colocalizes with Gag along the microtubule network and promotes microtubule stability. SOCS1 also increases the amount of Gag associated with microtubules. Both nocodazole treatment and the expression of the microtubule-destabilizing protein, stathmin, inhibit the enhancement of HIV-1 particle production by SOCS1. SOCS1 facilitates Gag ubiquitination and the co-expression of a dominant-negative ubiquitin significantly inhibits the association of Gag with microtubules. We thus propose that the microtubule network plays a role in SOCS1-mediated HIV-1 Gag transport and virus particle formation.

Structured summary:
MINT-7014185: *Gag* (uniprotkb:P05888) and *SOCS1* (uniprotkb:O15524) *colocalize* (MI:0403) by *cosedimentation* (MI:0027)
MINT-7014239: *Cullin 2* (uniprotkb:Q13617) *physically interacts* (MI:0218) with *RelA* (uniprotkb:Q04206), *RBX1* (uniprotkb:P62877), *SOCS1* (uniprotkb:O15524), *elongin B* (uniprotkb:Q15369) and *elongin C* (uniprotkb:Q15370) by *pull-down* (MI:0096)
MINT-7014046: *gag* (uniprotkb:P05888), *SOCS1* (uniprotkb:O15524) and *tubulin alpha* (uniprotkb:Q13748) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)
MINT-7014269: *tubulin alpha* (uniprotkb:Q13748) *physically interacts* (MI:0218) with *Gag* (uniprotkb:P05888) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-7014036: *tubulin alpha* (uniprotkb:Q13748) and *SOCS1* (uniprotkb:O15524) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)
MINT-7014201: *Cullin 2* (uniprotkb:Q13617) *physically interacts* (MI:0218) with *RBX1* (uniprotkb:P62877), *SOCS1* (uniprotkb:O15524), *elongin B* (uniprotkb:Q15369) and *elongin C* (uniprotkb:Q15370) by *pull-down* (MI:0096)
MINT-7014257: *Gag* (uniprotkb:P05888) *physically interacts* (MI:0218) with *Ubiquitin* (uniprotkb:P62988) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-7014221: *Cullin 2* (uniprotkb:Q13617) *physically interacts* (MI:0218) with *Gag* (uniprotkb:P05888), *elongin C* (uniprotkb:Q15370), *elongin B* (uniprotkb:Q15369), *SOCS1* (uniprotkb:O15524) and *RBX1* (uniprotkb:P62877) by *pull-down* (MI:0096)

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1. Introduction

The human immunodeficiency virus 1 (HIV-1) employs multi-step and multi-factorial processes for producing progeny viruses during infection [1,2]. Virus must utilize the intrinsic transport machinery of the infected host cells to enable the active transport

Abbreviations: HIV, human immunodeficiency virus; SOCS1, Suppressor of cytokine signaling 1; KIR, kinase inhibitory region; MTOC, microtubule organizing center; Ub, ubiquitin; VLP, virus-like particle

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of viral proteins [3,4]. Several recent studies have identified cellular factors that modulate HIV-1 Gag trafficking and localization. These include AP-3δ, POSH, HP68, GGA and Trim22 [5–9]. Moreover, phosphatidylinositol-(4,5)-bisphosphate (PIP2) has been shown to control the targeting of Gag to the plasma membrane [10]. These findings point to a critical role of host cell factors in Gag assembly and release, but the precise molecular functions of these factors and the specific timing of their roles in this process remain largely unknown.

We recently reported that the suppressor of cytokine signaling 1 (SOCS1) is an inducible host factor during HIV-1 infection and plays an important role in the intracellular trafficking of Gag to the plasma membrane, resulting in the efficient production of HIV-1 particles [11]. Moreover, we have further shown that the function of SOCS1 in Gag trafficking and HIV-1 particle production is not principally due to the suppression of interferon/cytokine signaling, but is mediated via its interaction with the HIV-1 Gag polyprotein [11]. Importantly, the targeted depletion of SOCS1 results in the mistargeting and degradation of Gag in lysosomes, leading to a significant decrease in virus particle production [11].

In our current study, we have utilized SOCS1 as a molecular tool to further reveal the molecular mechanisms underlying the intracellular transport of HIV-1 Gag during viral infection. We reveal from our findings that SOCS1 regulates the Gag trafficking process via the microtubule-dependent cellular machinery. Furthermore, we find that Gag is also regulated by a ubiquitin signaling pathway which is accompanied by Gag ubiquitination. These findings shed new light on the mechanisms involved in the intracellular transport of HIV-1 Gag and provide important clues for the design of future novel therapeutic interventions against AIDS and related disorders.

2. Materials and methods

2.1. Antibodies

Antibodies (Abs) and fluorescent reagents were obtained from the following sources: rabbit polyclonal anti-myc (A-14) and rabbit polyclonal anti-SOCS1 (H-93) Abs (Santa Cruz Biotechnology); rabbit polyclonal anti-SOCS1 (Zymed Laboratories); mouse monoclonal anti-FLAG (M2), anti-α-tubulin, anti-acetylated-α-tubulin and anti-γ-tubulin Abs (Sigma, St. Louis, MO); rabbit polyclonal anti-stathmin antibody (Calbiochem); mouse monoclonal anti-myc antibody (9B11, Cell Signaling Technology); mouse monoclonal anti-cytokeratin 7, cytokeratin 18, vimentin and HIV-p24 Ab (Dako Cytomation). Immunoblotting, immunoprecipitation and immunofluorescent analyses were performed as described previously [11].

2.2. Plasmids and sequences

Expression constructs for SOCS1 have been described previously [12]. HIV-1 Gag constructs have also been described previously [13]. Stathmin cDNA was amplified by RT-PCR from a human kidney cDNA library using the primers 5′-AGCAAG CTTGCCACCATGGCTTCTTCTGATATCCAGG-3′ and 5′-GACGGATCC GTCAGCTTCAGTCTCGTCAG-3′ and then subcloned into the pcDNA3.1 vector. pcDNA3.1-myc-ubiquitin and its mutants were generated by PCR as described previously [14]. The siRNA sequences were as follows: SOCS1-siRNA, GGCCAGAACCTT CCTCCTCTT; control-siRNA, TCGTATGTTGTGTGGAATT. All expression constructs were validated by sequencing.

2.3. Microtubule-associated protein spin-down assays

Microtubule-associated proteins were collected using a microtubule-associated protein spin-down assay kit (Cytoskeleton, BK029) according to the manufacturer's instructions. Briefly, 293T cells were lysed in 0.5 ml of PEM buffer (80 mM PIPES, pH 6.9, 0.3% Triton X-100, 1 mM EGTA, 1 mM GTP, GTP, 1 mM $MgCl_2$) supplemented with 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM sodium orthovanadate, and 5 mM NaF. Cell lysates were incubated with taxol-stabilized microtubules, followed by ultracentrifuge at 100000×g for 40 min at 25℃.

2.4. Cell culture

The 293T, COS-1, COS-7, HeLa and HOS cell lines and mouse embryonic fibroblasts (MEFs) were cultured in DMEM supplemented with 10% FBS. SOCS1−/− MEF cells were cultured as described previously [12].

2.5. In vitro interaction analysis

The in vitro interaction between HIV-1 Gag and the SOCS1-E3 complex was analyzed as follows: ^{14}C-labeled recombinant proteins (SOCS1, elongin B/C, Rbx1, biotin labeled cullin 2, and HIV-1 Gag) were synthesized in a wheat germ cell-free system as described previously [15]. The synthesized proteins were subsequently incubated in 120 μl of reaction buffer (50 mM Tris–HCl, pH 7.6, 50 mM $MgCl_2$, 500 mM CH_3COOK, 0.1 mM DTT and 1 mg/ml BSA) and streptavidin magnetic beads (Promega, Madison, WI) at 23 °C for 1 h. The precipitated proteins were then washed three times with reaction buffer and subjected to autoradiography.

3. Results

3.1. SOCS1 aligns with microtubule forming fibrous structures

SOCS1 has been shown previously to localize at both the perinuclear region and the microtubule organizing center (MTOC) [16]. This finding indicated the possible involvement of the microtubule network in the regulation of Gag by SOCS1. We thus addressed whether the Gag transport system is in fact mediated by microtubule integrity and if SOCS1 enhances this process. Immunofluorescent analysis with α-tubulin antibodies revealed that endogenous SOCS1 forms punctate structures that align with the microtubule network (Fig. 1A). Importantly, these signals were completely abolished when the cells were stained with anti-SOCS1 antibodies that had been pre-absorbed with recombinant SOCS1 protein, confirming the specificity of this antibody (Fig. 1B). Parallel experiments revealed that SOCS1 does not colocalize with other cytoskeletal components such as actin or intermediate filaments (Fig. 1C).

3.2. SOCS1 promotes microtubule stability

Given our finding that SOCS1 can tightly associate with microtubules, we next addressed whether SOCS1 affects microtubule stability. Stabilized microtubules are frequently enriched in tubulin that has undergone post-translational modifications such as acetylation [17]. We found that the high expression of SOCS1 results in higher amounts of acetylated microtubules in three different cell lines when compared with control cells (Fig. 2A) and that this trend is dose-dependent in COS-7 cells (Fig. 2B). On the other hand, SOCS1−/− mouse embryonic fibroblasts (MEFs) exhibited lower levels of acetylated α-tubulin compared with wild-type MEFs (Fig. 2C). These results indicate that SOCS1 does indeed contribute to the stabilization of microtubules.

Mammalian cells usually possess a population of microtubules that are resistant to the depolymerization effects of microtubule disorganizing reagents. We thus addressed whether SOCS1 impacts

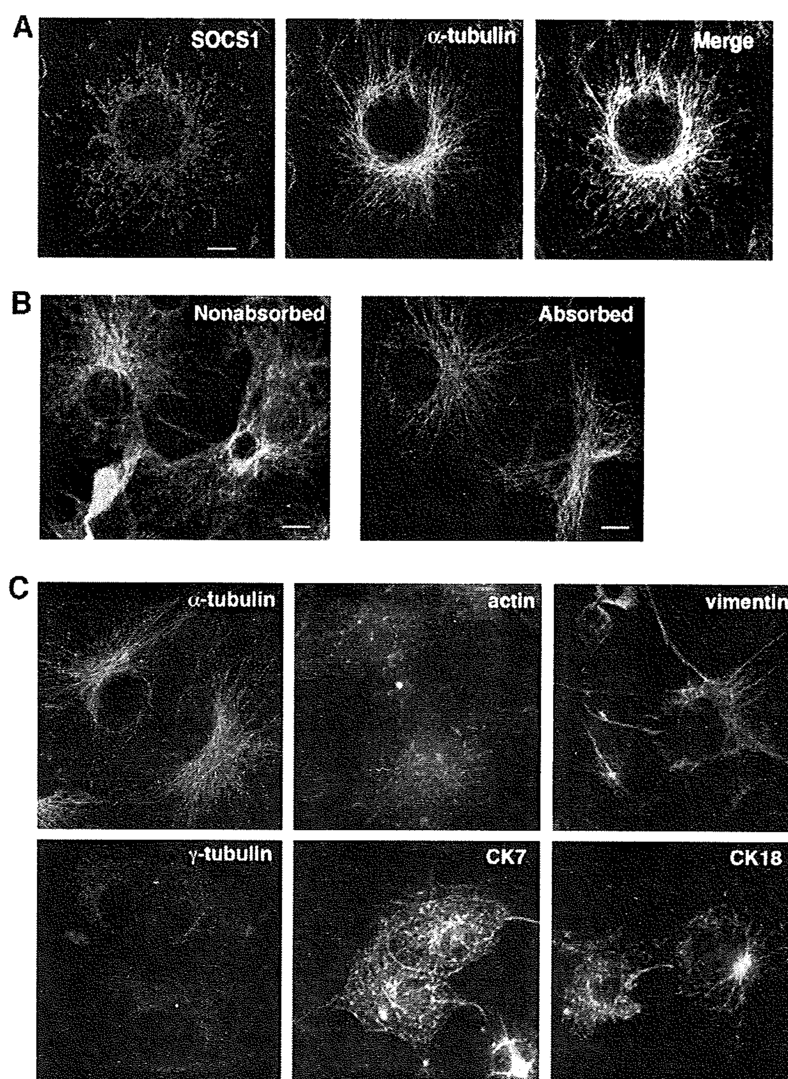

Fig. 1. SOCS1 colocalizes with microtubule forming fibrous structures. (A) COS-1 cells were fixed with 3% formaldehyde followed by 100% cold methanol, and then co-immunostained with polyclonal antibodies targeting SOCS1 (red) and monoclonal antibodies targeting α-tubulin (green). Cells were then analyzed by confocal microscopy. Scale bar, 10 μm. (B) COS-1 cells were immunostained with anti-α-tubulin monoclonal antibodies together with anti-SOCS1 polyclonal antibodies that had either been non-absorbed or pre-absorbed with GST-SOCS1 proteins. This was followed by confocal microscopy. Scale bar, 10 μm. (C) COS-1 cells were fixed with 3% formaldehyde followed by 100% cold methanol, then co-immunostained with polyclonal antibodies targeting SOCS1 (red) and monoclonal antibodies targeting various cytoskeletal components (green). Cells were then analyzed by confocal microscopy.

upon this property in this subpopulation of microtubules in COS-7 cells. The cells were transfected with either SOCS1 or control vector and then treated with 1 μM colchicine for 12 h to fully depolymerize the microtubules. Immunostaining with antibodies against acetylated α-tubulin showed that the SOCS1 expressing cells contained more polymerized microtubules compared with the control cells (Fig. 2D). These results indicate that SOCS1 might contribute to the microtubule stability required for Gag trafficking via this network.

3.3. SOCS1 enhances the association of HIV-1 Gag with microtubules

We next investigated the sub-cellular localization of HIV-1 Gag with SOCS1 and microtubules. COS-1 cells were transfected with Gag-GFP and after 24 h were fixed with 3% formaldehyde, followed by 100% cold methanol. The cells were then immunostained with anti-SOCS1 and anti-α-tubulin antibodies. Consistent with our earlier results, confocal microscopic analysis revealed that SOCS1 can form dotted filamentous structures in the cytoplasm along the microtubules, and that HIV-1 Gag colocalizes with these SOCS1 puncta (Fig. 3A).

To next determine whether cellular SOCS1 and Gag can together mechanically bind microtubules, and thus whether SOCS1 expression has any impact upon the interaction between Gag and microtubules, we performed microtubule pull-down analysis. 293T cells were transfected with either Gag-FLAG, myc-SOCS1, or a combination of these two plasmids, and the lysates from these transfected cells were subsequently incubated with taxol-stabilized microtubules and centrifuged to pellet the microtubule-associated proteins. The pellet fractions were then subjected to immunoblotting using either anti-myc or anti-FLAG antibodies. SOCS1 was found to be co-sedimented with microtubules irrespective of whether Gag had been co-transfected (Fig. 3B). The quantities of microtubule-bound Gag in the pellet fraction, however, were significantly increased when SOCS1 was co-transfected (Fig. 3B). These results together indicate that SOCS1 is itself a microtubule binding protein that may also mediate the interaction between HIV-1 Gag and microtubules.

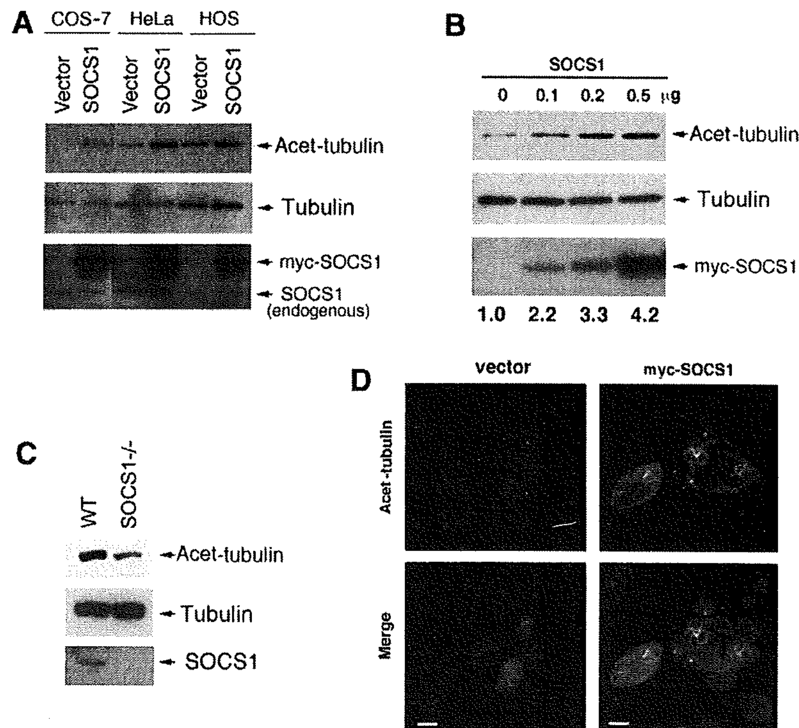

Fig. 2. SOCS1 enhances microtubule stability. (A) COS-7, HeLa or HOS cells were transfected with either empty vector or myc-SOCS1 for 48 h. Cell lysates were then subjected to immunoblotting analysis with antibodies against α-tubulin, acetylated α-tubulin or SOCS1. (B) COS-7 cells were transfected with various amounts of myc-SOCS1 as in (A). Cell lysates were then subjected to immunoblotting analysis with anti-α-tubulin, anti-acetylated-α-tubulin or anti-myc antibodies. Numerical values below the blots indicate acetylated α-tubulin signal intensities normalized by the unmodified α-tubulin intensity derived by densitometry. (C) Exponentially growing wild-type MEFs or SOCS1−/− MEFs were lysed and the cell lysates were immunoblotted with antibodies against α-tubulin, acetylated α -tubulin or SOCS1. (D) COS-7 cells were co-transfected with empty vector or myc-SOCS1, and then treated with colchicine (1 μM) for 12 h to depolymerize the microtubules. Cells were then fixed and immunostained with both anti-acetylated-α-tubulin (green) or anti-myc (red) antibodies and then stained with 4′,6-diamino-2-phenylindole (DAPI, blue), followed by confocal microscopy. Scale bar, 10 μm.

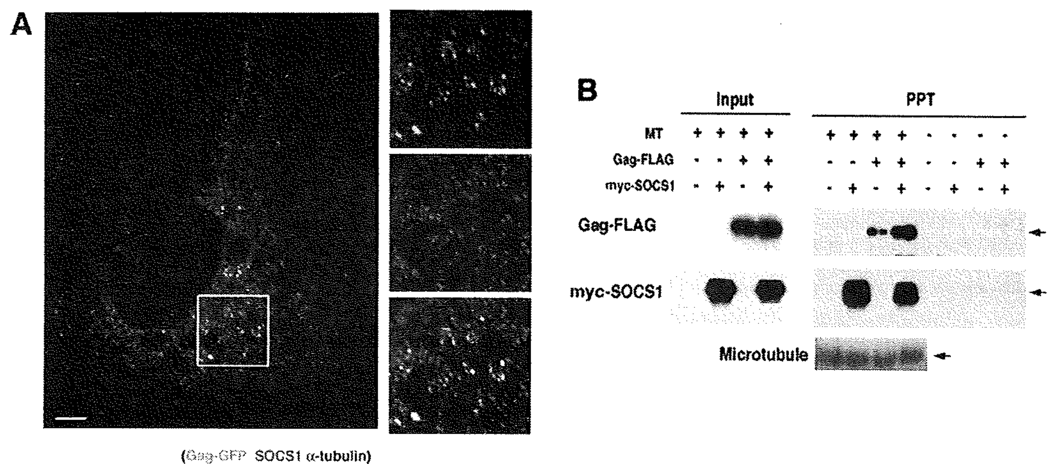

Fig. 3. SOCS1 enhances HIV-1 Gag associated with microtubules. (A) COS-1 cells transiently transfected with HIV-1 Gag-GFP were co-immunostained with antibodies targeting endogenous SOCS1 (red) and microtubules (α-tubulin, blue). The inset indicates the area shown at higher magnification in the right hand panels which reveal the colocalization of Gag-GFP with SOCS1 along the microtubules. Scale bar, 10 μm. (B) Cosedimentation of SOCS1 and HIV-1 Gag with polymerized microtubules. 293T cells were transfected with the indicated plasmids for 36 h. Cell lysates were then incubated with taxol-stabilized microtubules or control buffer and separated into precipitate (PPT) and supernatant fractions. Precipitate fractions were subjected to immunoblotting analysis with anti-myc or anti-FLAG antibodies.

3.4. Microtubule integrity is required for SOCS1 to function in HIV-1 particle formation

Our results shown above indicated that SOCS1 mediates the association of HIV-1 Gag with the microtubule networks. We next examined therefore whether SOCS1-mediated Gag trafficking, and the resultant HIV-1 particle production, are dependent upon an intact microtubule network. 293T cells were transfected with the HIV-1 molecular clone pNL4-3 and co-transfected with either empty vector alone or myc-SOCS1. After 24 h, the cells were washed with PBS and then cultured in the presence or absence of nocodazole for a further 6 h. Subsequent measurement of the p24 antigen levels in the cell supernatant by ELISA revealed nocodazole treatment significantly inhibited the enhancement of HIV-1 particle production in SOCS1-transfected cells more dramatically than in vector control transfected cells, and that this was

dose-dependent (Fig. 4A). Consistent with these results also, SOCS1 localization was observed to be significantly altered by nocodazole treatment, i.e. from a dotted filamentous structure along the microtubules to diffuse and larger aggregations in the cytoplasm (Fig. 4B). The use of trypan blue dye exclusion confirmed that cell viability was not affected by the nocodazole treatment (Fig. 4C).

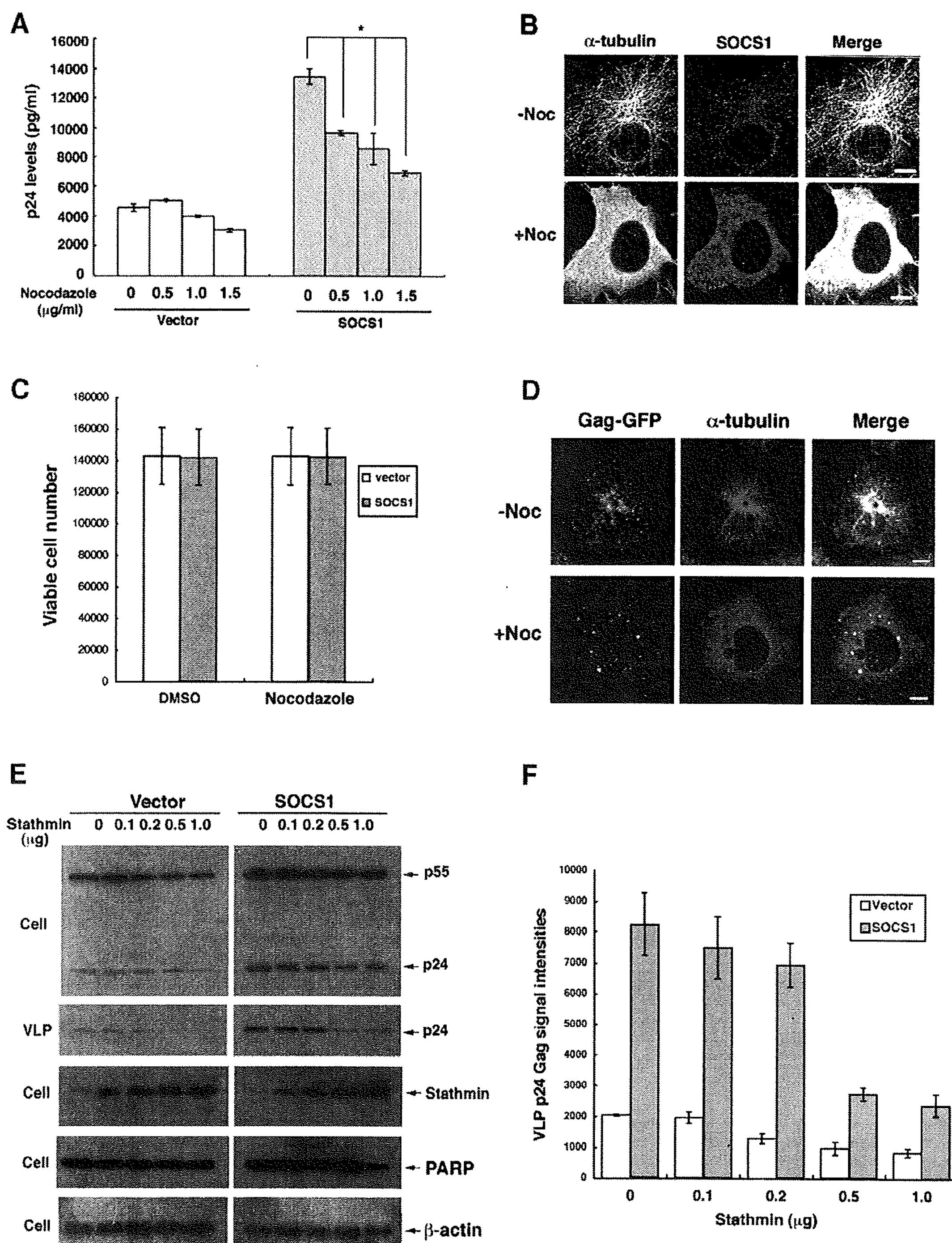

Fig. 4. Microtubule integrity is required for SOCS1 to function in HIV-1 particle formation. (A) 293T cells were transfected with pNL4-3, and co-transfected with either empty vector alone (Vector) or myc-SOCS1. After 24 h, cells were washed with PBS and then cultured with fresh media including the indicated concentrations of nocodazole for 6 h. Supernatant p24 antigen levels were measured by p24 ELISA . The data shown are the average ± S.D. of three independent experiments. $^*P \leq 0.05$, by the Student's *t*-test. (B and C) Mislocalization of SOCS1 in cells treated with nocodazole. COS-1 cells were treated with vehicle only or with nocodazole (2 μg/ml) for 6 h. Cells were then fixed and immunostained with both anti-SOCS1 (red) and anti-α-tubulin (green) antibodies, followed by confocal microscopy (B). Scale bar, 10 μm. The numbers of viable cells were calculated by trypan blue dye exclusion (C). (D) COS-1 cells transfected with Gag-GFP were treated with vehicle only or with nocodazole (2 μg/ml) for 6 h followed by immunostaining with anti-α-tubulin (red) antibody. Scale bar, 10 μm. (E and F) 293T cells were transfected with pNL4-3 and either vector or SOCS1, and co-transfected with various amounts of stathmin. At 36 h after transfection, cell lysate and supernatant virus-like particle (VLP) were processed for immunoblotting analysis with anti-p24, anti-PARP, anti-β-actin or anti-stathmin antibodies (E). VLP p24 Gag signal intensities, derived by densitometry, are shown in (F).

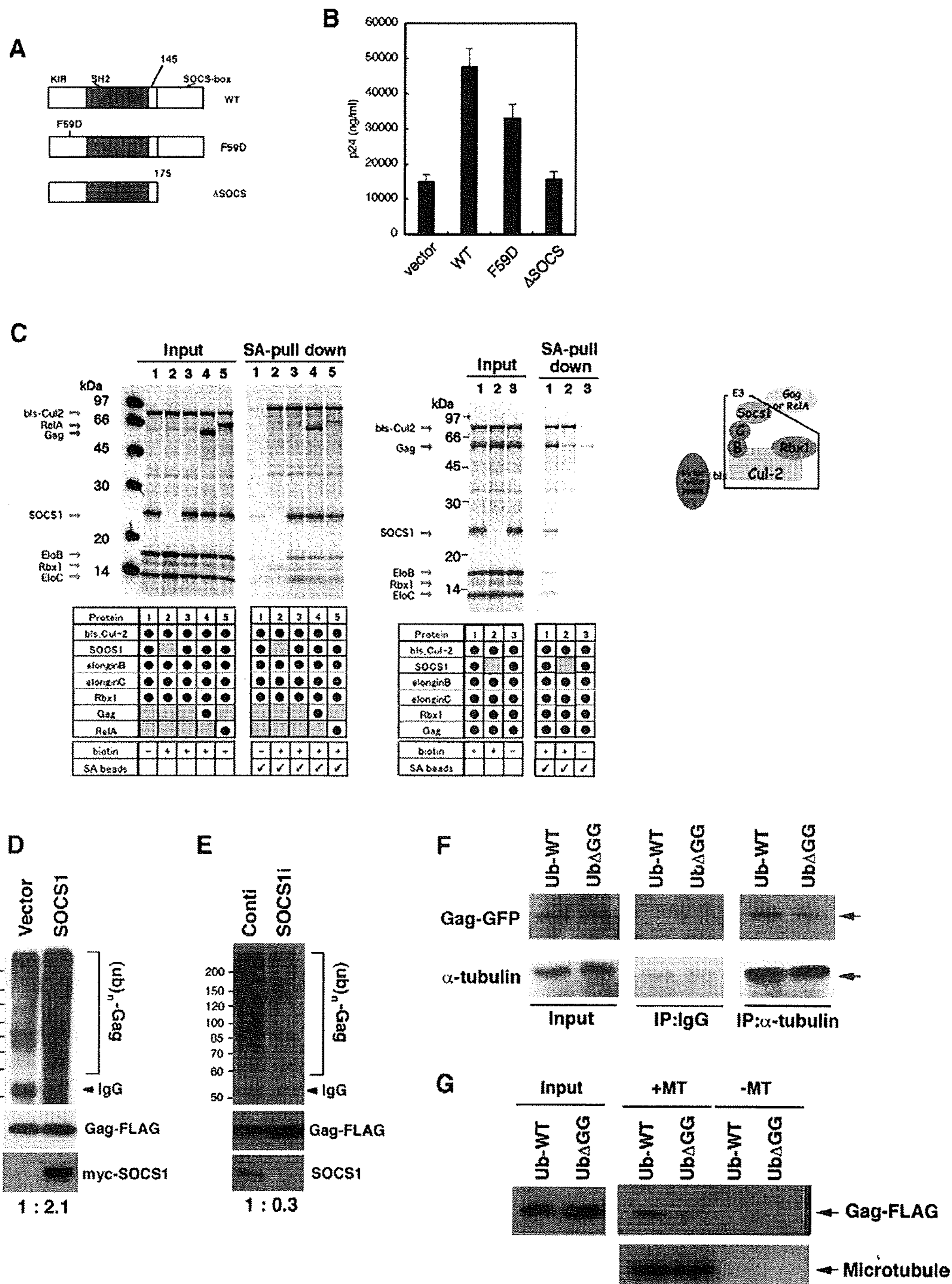

Fig. 5. SOCS1 enhances the ubiquitination of HIV-1 Gag and this affects the association of Gag with the microtubules. (A) Schematic represenation of SOCS1 mutants. (B) 293T cells were transfected with pNL4-3 and co-transfected with control vector, wild-type SOCS1 (WT), SOCS1-F59D mutant or ΔSOCS-box mutant. After 48 h, the p24 levels in the cell supernatants were measured by ELISA. (C) HIV-1 Gag associates with the ubiquitin ligase complex of SOCS1 in vitro. ^{14}C-labeled proteins (SOCS1, biotin labeled Cullin2, elongin B/C, Rbx1, HIV-1 Gag and RelA) were synthesized using a wheat germ cell-free system. Purified proteins were incubated in the indicated combinations for 1 h and subjected to co-purification with streptavidin magnetic beads. Captured proteins were then separated by SDS–PAGE followed by autoradiography. (D) 293T cells were co-transfected with Gag-FLAG, myc-tagged ubiquitin, and either empty vector (Vector) or SOCS1 expression construct. After 48 h, cells were lysed and denatured by boiling them in 1% SDS lysis buffer and diluted to RIPA buffer conditions, and Gag-FLAG proteins were immunoprecipitated (IP) with anti-FLAG antibody and processed for anti-myc immunoblotting to detect ubiquitinated Gag. Ubn, polyubiquitinated. Numerical values below blot indicates the relative levels of ubiquitinated Gag normalized by the amount of total Gag. (E) 293T cells were co-transfected with Gag-FLAG, myc-tagged ubiquitin, and either control-siRNA or SOCS1-siRNA. After 48 h, cell lysates were immunoprecipitated with anti-FLAG antibody and processed for anti-myc immunoblotting to detect ubiquitinated Gag. Ubn, polyubiquitinated. Numerical values below blot indicates the relative levels of ubiquitinated Gag normalized by the amount of total Gag. (F) 293T cells were transfected with Gag-GFP and SOCS1, and co-transfected with either myc-Ub-WT or myc-UbΔGG. After 24 h, cell lysates were harvested and subjected to immunoprecipitation analysis with anti-α-tubulin antibody or non-immunized mouse IgG (IgG) followed by immunoblotting analysis with the indicated antibodies. (G) 293T cells were transfected with Gag-FLAG and SOCS1, and co-transfected with either myc-Ub-WT or myc-UbΔGG. After 24 h, cell lysates were harvested and then incubated with taxol-stabilized microtubules (+MT) or control buffer (−MT), and separated into precipitate and supernatant fractions. Precipitate fractions were collected and then subjected to immunoblotting analysis with either anti-FLAG or anti-α-tubulin (microtubule) antibodies.

Furthermore, a parallel experiment revealed that the Gag-GFP puncta formed larger aggregations in the cytoplasm upon nocodazole treatment (Fig. 4D).

To further delineate the role of microtubule integrity in HIV-1 particle formation, we next performed experiments in which we co-transfected SOCS1 and pNL4-3 with or without the microtubule-destabilizing protein stathmin. Stathmin expression efficiently blocked the effects of SOCS1 upon HIV-1 particle formation in a dose-dependent manner (Fig. 4E and F). Cell viability was not strongly affected as revealed by immunoblotting analysis of either poly (ADP-ribose) polymerase (PARP) or β-actin (Fig. 4E). Our findings together indicate therefore that microtubule integrity may be required for SOCS1 to function in Gag assembly and release.

3.5. *SOCS1 facilitates the ubiquitination of HIV-1 Gag*

Our previous study has revealed that the SOCS-box of SOCS1 is required for both HIV-1 particle production and the enhancement of Gag association with microtubules [11]. The mechanism by which SOCS1 inhibits cytokine signaling is mediated by the inhibition of kinase activity through its N-terminal kinase inhibitory region (KIR) [18]. We next examined whether SOCS1 mutants lacking the function of either KIR (SOCS1-F59D) or SOCS-box (ΔSOCS) affected virus particle production. Our ELISA results indicate that the SOCS-box deletion mutant (ΔSOCS) of SOCS1 fails to promote virus production, whereas the KIR mutant, F59D, of SOCS1 partially enhances HIV-1 particle production when co-transfected with pNL4-3 in 293T cells (Fig. 5A and B). These data again suggest that the function of SOCS1 in HIV-1 particle production is not principally due to the suppression of interferon/cytokine signaling, but is mediated by its direct interaction with the HIV-1 Gag via the function of the SOCS-box.

The SOCS-box-mediated function of SOCS1 is chiefly exerted via its ubiquitin ligase activity [19]. Indeed, several reports have demonstrated that Gag ubiquitination is related to its membrane association and particle release, although the function of HIV-1 Gag ubiquitination remains unclear [20,21]. We thus explored the possibility that SOCS1 modulates the ubiquitination of HIV-1 Gag, leading to enhanced virus particle formation. We initially examined the specific interaction of HIV-1 Gag with the SOCS1-E3 ligase complex. Purified SOCS1 and its E3 component proteins (biotinylated-Cullin 2, elongin B/C and Rbx1) in addition to HIV-1 Gag were synthesized in a wheat germ cell-free system and then subjected to pull-down assays using streptavidin coated magnetic beads. We found that Gag was co-purified with a SOCS1-E3 complex comprising SOCS1-elongin B/C-Rbx1-Cullin2 in a similar manner to RelA, a previously reported SOCS1 binding protein (Fig. 5C, left). Significantly, in the absence of SOCS1, both elongin B and C were not co-purified with Cullin 2 probably due to the unsteady condition of the E3 complex without SOCS1, and the amount of bound Gag was also reduced (Fig. 5C, right).These result indicate that the SOCS1-E3 complex associates with HIV-1 Gag and may promote its ubiquitination.

We next addressed whether SOCS1 affects the ubiquitination of HIV-1 Gag. Immunoprecipitation analysis with cells co-transfected with Gag-FLAG and myc-tagged ubiquitin, with or without SOCS1 co-transfection, revealed that SOCS1 overexpression significantly enhances the ubiquitination of the Gag protein (Fig. 5D). In contrast, the targeted depletion of SOCS1 by siRNA significantly reduced the amount of ubiquitinated Gag (Fig. 5E). These results indicate that SOCS1 could indeed be a potent ubiquitin ligase for HIV-1 Gag.

To clarify the biological significance of Gag ubiquitination via SOCS1, we performed an experiment using a dominant-negative ubiquitin construct lacking two C-terminal glycines (residues 75–76). This mutant ubiquitin (UbΔGG) cannot become conjugated to target substrates, but can bind noncovalently to ubiquitin interacting domains [14]. By immunoprecipitation analysis we revealed that the levels of HIV-1 Gag associated with microtubules were significantly reduced in cells expressing UbΔGG, as compared with those expressing wild-type ubiquitin (Ub-WT) (Fig. 5F). This trend was further revealed by a microtubule sedimentation experiment showing that the expression of UbΔGG reduced the amount of Gag associated with microtubules when compared with the expression of Ub-WT (Fig. 5G). These results together indicate a link between ubiquitin signaling and the microtubule-mediated Gag dynamics involved with HIV-1 particle formation.

4. Discussion

In our current study, we report that microtubule integrity is required for SOCS1 to facilitate Gag trafficking and virus particle production. We demonstrate from our experiments that (1) SOCS1 colocalizes with HIV-1 Gag along microtubules; (2) both SOCS1 and HIV-1 Gag are co-purified with microtubules and SOCS1 can augment the association of Gag with microtubules; (3) an intact microtubule network is required for the function of SOCS1 during Gag trafficking; (4) SOCS1 facilitates Gag ubiquitination; and (5) Gag association with the microtubules is significantly reduced when a dominant-negative Ub mutant is overexpressed. These results together indicate that SOCS1 can regulate the trafficking and stability of HIV-1 Gag via the microtubule-related cellular machinery, which may be in turn enhanced by Gag ubiquitination.

SOCS1 was identified initially as a negative regulator of signaling downstream of cytokines [22–24] and has been shown to localize at both the perinuclear region and the microtubule organizing center (MTOC) in cells [16]. We show from our current data that SOCS1 also forms dotted filamentous structures in the cytoplasm emanating from the perinuclear region, including the MTOC, to the cell periphery. A recent report has also indicated that Gag colocalizes at the MTOC with HIV-1 RNA and is subsequently transported to the cell periphery [25]. These observations together indicate that SOCS1 might facilitate the trafficking of HIV-1 Gag from the MTOC toward the plasma membrane by utilizing the intrinsic transport machinery of infected host cells.

The plus-end directed transport system along the microtubules could provide a means for the targeting of virus capsid proteins to the site of virus assembly and budding in the vicinity of the plasma membrane [26]. This ante-grade transport system is utilized by several viruses, such as herpes simplex virus type 1 (HSV-1), vaccinia virus and African swine fever virus (ASFV) [26–29]. Significantly, we have demonstrated in our present study that HIV-1 can utilize the microtubule-dependent transport mechanism, which may in turn be enhanced by SOCS1. Consistent with this notion, Leblanc et al. have demonstrated previously using a monoclonal antibody raised against unprocessed Gag that intracellular Gag puncta can travel along microtubules [30]. Our current microtubule pull-down analyses also clearly indicate that SOCS1 associates with Gag on microtubules and can enhance this interaction. This in turn might accelerate the intracellular trafficking of Gag to the plasma membrane along these structures, although the topological details are still unknown. Consistent with this observation, a plus-end microtubule motor KIF4 has been shown previously to associate with HIV-1 Gag and to enhance Gag trafficking [31,32]. These results further demonstrate the relevance of microtubule network in the trafficking of the HIV-1 Gag.

The involvement of the microtubule cytoskeleton in Gag assembly and HIV-1 particle egress is somewhat controversial [3,26,33,34]. However, several reports have presented convincing data to indicate the importance of this network in HIV-1 assembly

and propagation [3,35,36]. Our current study further demonstrates that the microtubule depolymerizing reagent, nocodazole, or the expression of microtubule-destabilizing protein stathmin, significantly inhibits the enhancement of HIV-1 particle production by SOCS1, suggesting a possible role of the microtubule network in regular HIV-1 particle production.

Our previous report indicated that the targeted depletion of SOCS1 results in the prominent perinuclear accumulations of HIV-1 Gag in 293T cells [11]. Our current study shows that nocodazole treatment or stathmin expression only slightly affects Gag release in non-SOCS1 overexpressing cells. This difference might be attributable to the following two possibilities. First, SOCS1 may affect Gag at multiple points during trafficking and assembly, and a critical point could be prior to the microtubule-mediated events that can be affected by nocodazole or stathmin. Second, there are multiple pathways to the delivery of exogenously expressed Gag protein from the cytoplasm to the plasma membrane in addition to microtubule-directed transport. Furthermore, we are currently uncertain whether the Gag association with microtubules is mediated by other microtubule binding proteins in cooperation with SOCS1, or whether SOCS1 directly associates with HIV-1 Gag on the microtubules. Further careful analysis must be performed to elucidate these possibilities.

Acknowledgements

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Retrovirology

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Short report

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Reactivation from latency displays HIV particle budding at plasma membrane, accompanying CD44 upregulation and recruitment

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Abstract

Background: It has been accepted that HIV buds from the cell surface in T lymphocytes, whereas in macrophages it buds into intracellular endosomes. Recent studies, on the other hand, suggest that HIV preferentially buds from the cell surface even in monocytic cells. However, most studies are based on observations in acutely infected cells and little is known about HIV budding concomitant with reactivation from latency. Such studies would provide a better understanding of a reservoir for HIV.

Results: We observed HIV budding in latently infected T lymphocytic and monocytic cell lines following TNF-α stimulation and examined the upregulation of host factors that may be involved in particle production. Electron microscopy analysis revealed that reactivation of latently infected J1.1 cells (latently infected Jurkat cells with HIV-1) and U1 cells (latently infected U937 cells with HIV-1) displayed HIV particle budding predominantly at the plasma membrane, a morphology that is similar to particle budding in acutely infected Jurkat and U937 cells. When mRNA expression levels were quantified by qRT-PCR, we found that particle production from reactivated J1.1 and U1 cells was accompanied by CD44 upregulation. This upregulation was similarly observed when Jurkat and U937 cells were acutely infected with HIV-1 but not when just stimulated with TNF-α, suggesting that CD44 upregulation was linked with HIV production but not with cell stimulation. The molecules in endocytic pathways such as CD63 and HRS were also upregulated when U1 cells were reactivated and U937 cells were acutely infected with HIV-1. Confocal microscopy revealed that these upregulated host molecules were recruited to and accumulated at the sites where mature particles were formed at the plasma membrane.

Conclusion: Our study indicates that HIV particles are budded at the plasma membrane upon reactivation from latency, a morphology that is similar to particle budding in acute infection. Our data also suggest that HIV expression may lead to the upregulation of certain host cell molecules that are recruited to sites of particle assembly, possibly coordinating particle production.

Findings

It has been thought that HIV particles assemble and bud at the plasma membrane (PM) in T lymphocytes and HeLa cells, but at the endosomes in macrophages, suggesting that such endosomal targeting may be essential for HIV budding in macrophages [1-6]. However, recent studies using the inhibitors of the endocytic pathway and membrane-impermeant dyes have revealed that the PM is the primary site for HIV assembly and particle budding even in macrophages and that particles accumulate at the endosomes through endocytosis [7-9]. Nevertheless, these studies are based on observations in acutely infected cells and little is known about HIV budding concomitant with reactivation from latency. Latently infected resting T cells are known to serve as a stable reservoir for HIV during anti-retroviral therapy and to produce infectious particles upon cell reactivation. Studies on HIV production from latently infected cells upon reactivation are necessary for a better understanding of HIV pathogenesis, although some studies have indicated intracellular accumulation of particles in chronically or latently infected cells [10,11]. Here, we employed J1.1 cells that were Jurkat T lymphocytic cells latently infected with HIV-1, and U1 cells that were U937 monocytic cells latently infected with HIV-1, and observed HIV particle budding following reactivation.

We initially tested the dose of TNF-α, and temporally monitored cell growth and HIV particle production after stimulation (Fig. 1A). J1.1 cells proliferated equally regardless of the dose of TNF-α, and the particle production levels increased to 50 ng/ml TNF-α. In contrast, proliferation of U1 cells was inhibited in a dose-dependent manner, and the highest level of particle production was observed at 50 ng/ml. We thus used 50 ng/ml TNF-α for further experiments. To avoid nonspecific stimulation by changing the medium, we added TNF-α directly to the culture medium, and this led to the higher dose of TNF-α required in our study than in other reports [12,13].

Electron microscopy was carried out to examine where particle budding occurred in J1.1 and U1 cells upon reactivation (Fig. 1B). Little or no particles were produced in either cell line before TNF-α stimulation (Fig. 1B, most left panels), consistent with previous reports [11-14]. Upon stimulation, nascent budding particles were visible on the surface of nearly all J1.1 cells, similar to the case with U1 cells (Fig. 1B, arrowheads). Unexpectedly, particles in intracellular vesicles were rarely seen in both J1.1 and U1 cells (Fig. 1B, arrow). The findings were confirmed by immunoelectric microscopy using anti-HIV-1 p17MA antibody (Fig. 1B, most right panels). Next, their parental cell lines, Jurkat and U937 cells, were infected with HIV-1, and particle production in acute infection were examined by electron microscopy. Particle budding was observed predominantly at the PM of both Jurkat and U937 cells (Fig. 1C, arrowheads) but some U937 cells displayed budding into intracellular compartments (Fig. 1C, arrows). Immunoelectric microscopy indicated similar results (Fig. 1C, most right panels). For quantification, we counted the number of cells containing particles at the PM alone or that of cells containing particles at both intracellular vesicles and the PM (Fig. 1D). Budding at the PM was prominent, regardless of whether cells were acutely or latently infected, or T lymphocytic or monocytic, suggesting that unlike chronically infected cells [10], HIV particles are most likely budded from the PM in latently infected cells, although it cannot be ruled out in this experiment that the particles observed in extracellular spaces might be released by exocytosis.

Gene expression analysis based on cDNA microarrays has extensively been employed and has provided evidence for the modulation of host cellular gene expression upon HIV infection (replication and latency) [15-20]. Although numerous host genes are modulated upon HIV infection, it is conceivable that expression levels of host membrane components may change by feedback regulation upon HIV reactivation, as HIV requires host cell membrane for particle budding. A membrane contains a number of microdomains, enriched in cholesterol (i.e., rafts) and in tetraspanins (e.g., CD63 and CD81), which accumulate at sites of HIV budding [7,21-26]. It has been shown that TSG101, a component of endosomal sorting complex required for transport (ESCRT) is recruited to the sites of particle assembly and is responsible for HIV particle budding [27,28]. Thus we chose endosomal (EEA1, CD63, HRS, TSG101, and Syntaxin12) and PM (CD44 and SNAP23) markers and quantified their mRNA levels by qRT-PCR (Fig. 2A and 2B) using the primer sets shown in Additional File 1. Their properties and functions are as follows: EEA1 is a marker molecule for early endosome; HRS is an initial molecule for the ESCRT pathway; Syntaxin12 is a SNARE molecule for endosomal membrane fusion; CD44 is an adhesion molecule implicated in cell migration; SNAP23 is a SNARE molecule for PM fusion in the exocytic pathway. When the mRNA levels in J1.1 cells stimulated with TNF-α were compared with those in unstimulated J1.1 cells, CD44 gene expression was increased, but the other genes tested were largely unaltered. No significant upregulation of CD44 was observed when cells of its uninfected parental line, Jurkat, were similarly stimulated with TNF-α, indicating that the CD44 upregulation was not simply due to cell stimulation (Fig. 2A, upper). CD44 has been reported hardly expressed even at mRNA level in unstimulated Jurkat cells [29]. A similar analysis was carried out for U1 cells. Downregulation of CD44 has been reported for chronically infected monocytic cells [30]. Upon reactivation, CD44 upregulation was apparent but the endocytic molecules (CD63

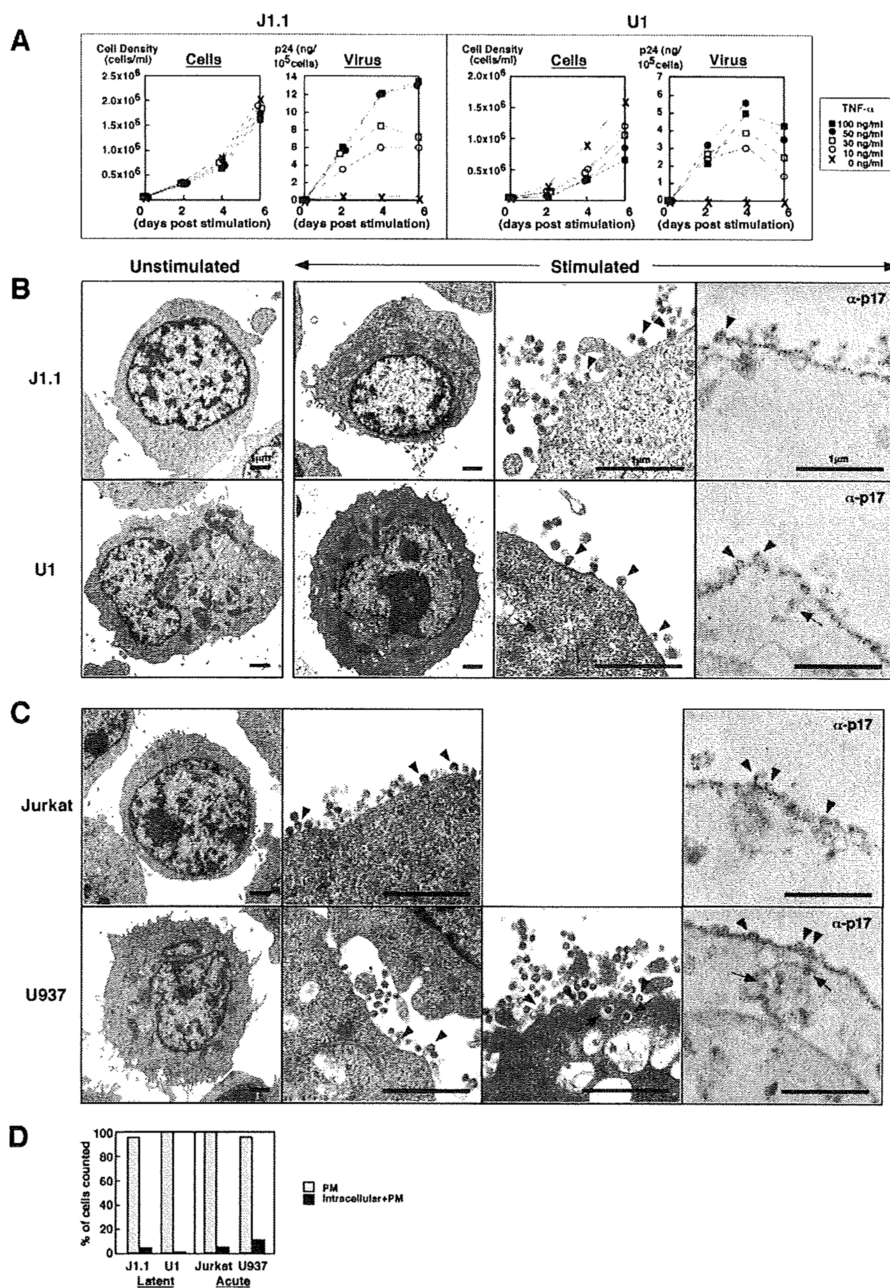

Figure 1

Reactivation of latently infected J1.1 and U1 cells displays HIV particle budding at the PM. (A) HIV production from J1.1 and U1 cells upon TNF-α stimulation. J1.1 and U1 cells were stimulated with TNF-α (~100 ng/ml). Levels of particle production were measured by p24 antigen ELISA. (B) HIV particle budding from J1.1 and U1 cells upon TNF-α stimulation. J1.1 and U1 cells stimulated with 50 ng/ml TNF-α were subjected to conventional electron microscopy and immunoelectric microscopy using anti-HIV-1 p17MA antibody. (C) HIV particle budding from acutely infected Jurkat and U937 cells. Jurkat and U937 cells were infected with HIV-1 (LAV strain) corresponding to 100–200 ng of p24CA antigen and were analyzed by electron microscopy. Arrowheads indicate budding particles and arrows indicate particles into intracellular vesicles in (B) and (C). (D) Semi-quantification of HIV-1 particle localization. Approximately 300 of particle-positive cells observed by conventional electron microscopy were sorted into the categories indicated.

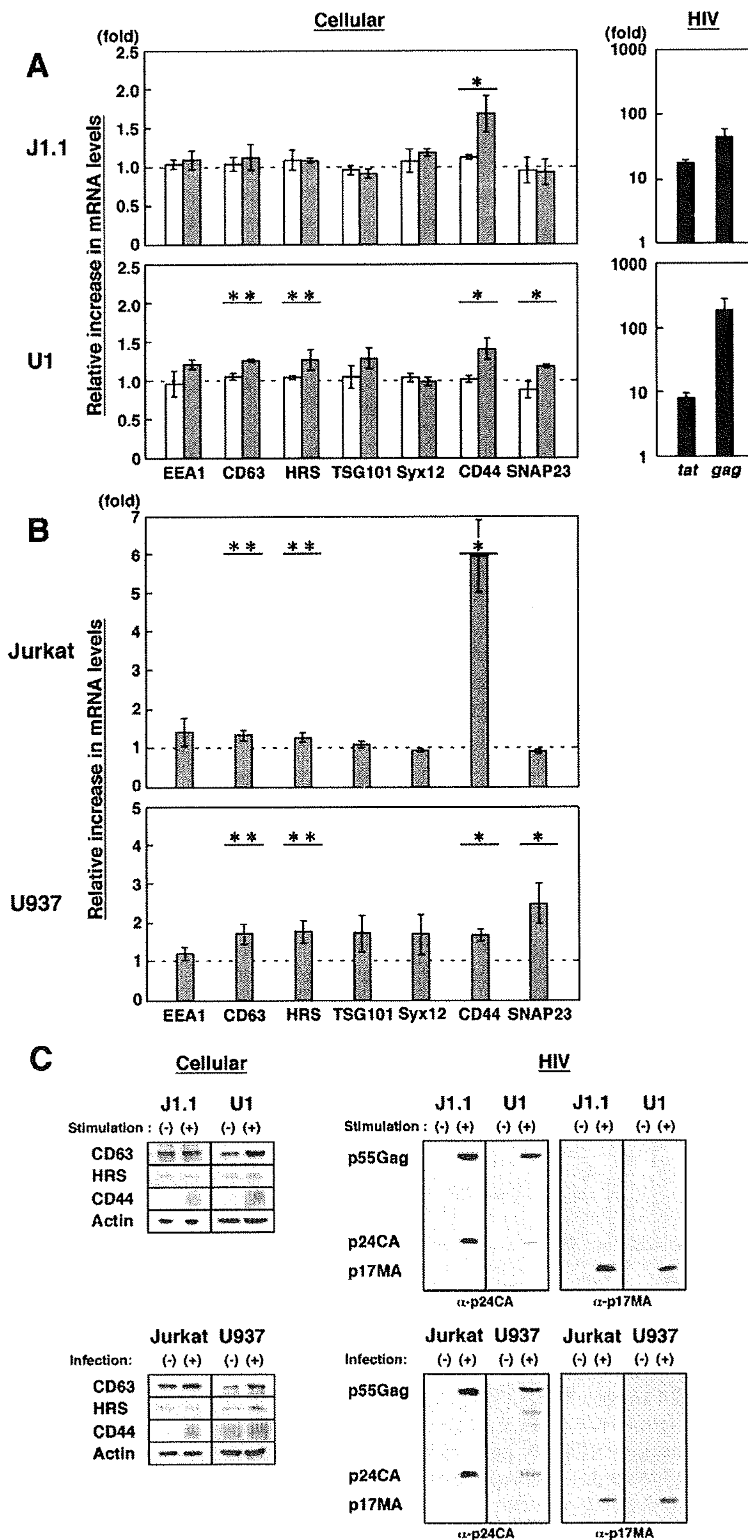

Figure 2 *(see legend on next page)*