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The dominant-negative action of a fusion protein containing the cytoplasmic domain of human immunodeficiency virus type 1 transmembrane protein gp41 in virus replication

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Abstract

We previously described a novel mode of downregulation of human immunodeficiency virus type 1 (HIV-1) Gag expression by a cytoplasmic domain fusion protein of the envelope (Env) transmembrane protein, β-galactosidase (β-gal)/706–856, which contains the cytoplasmic tail of gp41 fused at the C terminus of *Escherichia coli* β-gal. In the present study, we showed that this mediator conferred a dose-dependent dominant interference with virus infectivity. In the context of an HIV-1 provirus, this inhibitor downregulated steady-state Env expression. Paradoxically, Env overexpression suppressed β-gal/706–856-mediatd Gag downregulation. Sucrose gradient ultracentrifugation and confocal microscopy revealed that Gag, Env, and β-gal/706–856 had stable interactions and formed aggregated complexes in perinuclear regions. Moreover, Env overexpression hindered colocalization of Gag with β-gal/706–856 in the perinuclear region. Further cytoplasmic domain mapping analyses showed a correlation between the ability of cytoplasmic subdomains to downregulate Gag expression and the ability of these subdomains to stably interact with Gag. These studies show that redirection of Gag from its cytoplasmic synthesis site to a perinuclear compartment is a prerequisite for β-gal/706–856-mediated Gag downregulation. The results also illustrate that the dynamic interplay among Gag, Env, and β-gal/706–856 can modulate Gag and Env expression, thus controlling HIV-1 infection.

Introduction

In the late phase of the human immunodeficiency virus type 1 (HIV-1) life cycle, viral capsid Pr55 Gag precursor is transported to the plasma membrane where the precursor self-assembles into viral particles that bud out from the cell membrane (for a review see Ref. [1]). During or shortly after virus budding, the Pr55 Gag precursor is cleaved by the virus-encoded protease into matrix (MA, p17), capsid (CA, p24), p2 (sp 1), nucleocapsid (NC, p7), p1 (sp 2), and the C-terminal p6 domain [2–5]. Although the viral protease-mediated Gag precursor processing is not crucial for particle assembly and budding, this processing is essential for both virus maturation and infectivity [6–9].

It has well been documented that the interaction between the cytoplasmic domain of transmembrane protein gp41 and the MA protein, which occurs during virus assembly/budding or even before expression of Env and Gag on the cell surface, facilitates recruitment of the Env into buds where mature virions are released into

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medium [10–24]. In addition, alternations to the Gag or Env often affect the intracellular targeting and localization properties of their counterparts [25, 26]. Other studies further substantiate the notion that the interaction between gp41 and Gag is specific for the presence of the cytoplasmic tail of gp41 [26, 27] and that a fusion protein containing the gp41 cytoplasmic domain can bind to MA *in vitro* [28].

The cytoplasmic domain of gp41, encompassing residues 706–856, is characterized by the presence of three amphipathic α-helical segments, located at residues 824~856, 770~795, and 786~815, respectively, at its C-terminus (Figure 1A). Due to their cytolytic effects on cell membranes, these three motifs are referred to as the lentivirus lytic peptide (LLP)-1, LLP-2, and LLP-3, respectively. The cytoplasmic tail also contains sequences in regulation of Env expression on the cell surface [29, 30], basolateral targeting of virus budding in polarized cells [16, 17], and in interactions with cellular components such as induction of apoptosis [31, 32]. The residues between 750~763 and 764~785 in the cytoplasmic tail inhibit Env cell surface expression [33]. Internalization of Env from the cell surface is mediated by interaction of the AP-2 clathrin-adaptor complexes with the YSPL membrane-proximal, tyrosine-based signal in the cytoplasmic tail [29, 34, 35]. The conserved C-terminal dileucine motif in cytoplasmic tail is crucial for recruiting the AP1 clathrin adaptor [36]. It was also shown that Env interacts with TIP47, a protein required for the transport of mannose-6-phosphate receptors from endosomes to the *trans*-Golgi network and that the diaromatic motif located at residues 802 and 803 in the cytoplasmic tail is responsible for the retrograde transport of the Env to the *trans*-Golgi network [37].

Examining fusion proteins containing subdomains of the gp41 cytoplasmic tail, we previously showed correlation between the membrane binding ability and oligomerization potential of these cytoplasmic tail subdomains [38, 39], suggesting that the oligomerization and membrane-binding abilities of the cytoplasmic domain play crucial roles in Env assembly and virus infection. We also examined the effects of an *Escherichia coli* β-galactosidase (β-gal) and a cytoplasmic tail fusion protein, β-gal/706–856, on Gag expression and found that this fusion protein abrogates steady-state Gag expression, which may occur by an intracellular protein degradation pathway [40].

To understand the mechanistic insight into the mode of this fusion protein in antiviral action, in the present study we further examined the action of this inhibitor in Gag and Env expression in the context of HIV-1 proviral constructs. We show that β-gal/706–856 interferes *in trans* with wild-type (WT) virus infectivity through downregulation of steady-state Env expression in a *gag* gene-dependent manner. Remarkably, Env overexpression counteracts the impairing effect of β-gal/706–856 on Gag expression and prevents colocalization of Gag with β-gal/706–856 in the perinuclear region. Our study thus has an implication in designing anti-HIV-1 strategies targeting the Gag–Env interaction.

Methods

Cells, hybridoma, and antibodies

HEK293T and COS-1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). The $CD4^+$ T cell lines, CEM-SS and PM1, were cultured in RPMI containing 10% FBS. Hybridoma 902, Chessie 8, and 183 (clone H12-5C) were described previously [41]. All of these hybridomas were maintained in RPMI-1640 containing 10% FBS. Sheep anti-gp120 (cat. # 288) and rabbit antiserum to HIV-1 p25/p24 (cat. # 384) were previously described [42].

Construction of plasmids

pHXB2*myr*$^-$ was generated by substituting the *Sal*I-*Xho*I fragment from a *vpr*-, *vpu*-, and *nef*-encoding infectious pHXB2RU3 provirus [43] for the homologous segment in pHIVgpt*myr*$^-$ [44], which encodes a Gly-to-Ala substitution at residue 2 of MA in the *env*-defective pHIVgpt backbone [45]. To construct pHXB2*pro*$^-$, the *Sal*I-*Xba*I fragment in pHIVgpt*pro*$^-$ was replaced with the homologous sequence isolated from a *vpr*- and *nef*-encoding pHXB2R3 infectious clone. pHIVgpt*pro*$^-$ encodes a dysfunctional protease in which the catalytic site at residue Asp-25 of the *pro* gene in pHIVgpt is mutated to Asn [46]. pCDNA3-β-gal/752–775 was constructed using an

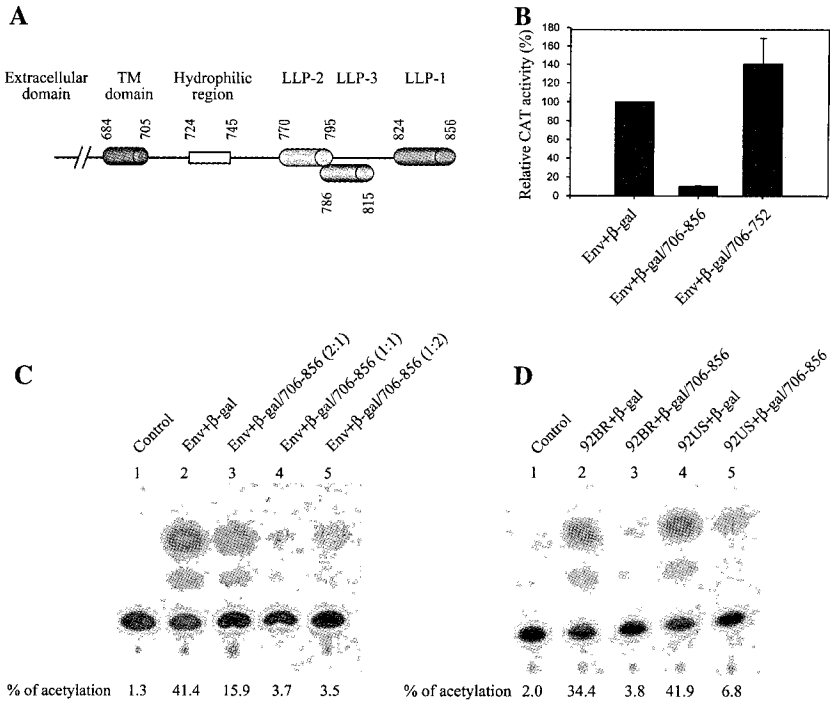

Figure 1. Dominant interference with HIV-1 infectivity by a cytoplasmic tail fusion protein. (A) Structural motifs in gp41 cytoplasmic domains including a highly hydrophilic region, and three positively charged amphipathic α-helices marked as LLP1, LLP2, and LLP3, are shown. The amino acid residues are numbered according to their positions in the Env of the HXB2 strain. (B) 293T cells were cotransfected with 7.5 μg each of pHXBΔBglCAT and pSVE7*puro* in the presence of 7.5 μg each of the pCDNA3-β-gal plasmids as indicated. (C) 293T cells were cotransfected with 5 μg each of pHXBΔBglCAT and pSVE7*puro* together with pCDNA3-β-gal/706–856 at different molar ratios of *env* to the β-gal/706–856 plasmids. (D) Cells were cotransfected with pHXBΔBglCAT and Env plasmids derived from the primary isolates, 92BR and 92US, in the presence of β-gal or β-gal/706–856 plasmids. To maintain the same total DNA amounts in all transfections, pSVE7(ΔKS)*puro*, which encodes a defective *env* gene, and pCDNA3 were added to the transfection mixtures that did not contain the *env*, β-gal, or 706–856 plasmids, respectively. Equal amounts of cell-free viruses, after RT activity normalization, from each transfection were used to challenge CEM-SS (B and C) or PM1 (D) cells, and CAT activity was measured. The background CAT level detected in the defective virus produced in the absence of Env was subtracted from the CAT activity of pseudotypes obtained from Env coexpressed with β-gal or β-gal/cytoplasmic tail fragments. The infectivity of the defective virus obtained from Env coexpressed with β-gal/706–856 or β-gal/706–752 was expressed as a percentage of that of the defective virus obtained from Env and β-gal coexpression. In (B), the diagrams represent the results from three independent experiments with the mean ± SD shown.

appropriate oligonucleotide containing an EcoRI linker and 856rXbaI as the primer and TM775 pSVE7*puro* as the template as previously described [38]. All the HIV constructs including pSVE7*puro*, pHXB2R3, pHXBΔBglCAT, pCX-Env, pHIVgpt and its related plasmids, were based on the HXB2 strain, which was derived from the IIIB isolate of subtype B HIV-1.

Plasmid DNA transfection

293T cells were transfected with plasmids using a standard calcium phosphate coprecipitation method [42]. COS-1 cells were transfected with plasmids by a DEAE-dextran transfection method as previously described [42].

Virus preparation and immunoblot analysis

Two days after transfection, cell-free virions were isolated through a 20% sucrose cushion, and equal volumes of cell and viral lysates were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blotting. The membrane blots were incubated with appropriate primary and secondary antibodies followed by enhanced chemiluminescent (ECL) detection. For quantitation, ECL photos of the blots within the linear range of the film were scanned using a Microtek ScanMarker 8700 (Zntennation Inc.; Hsinchu, Taiwan) and quantitated using MetaMorph software (Universal Imaging, Downing Town, PA). The percentages of Gag and gp41 levels in cells

and virions obtained from pCDNA3-β-gal/706–856 cotransfection relative to those obtained from pCDNA3 or pCDNA3-β-gal cotransfection were quantitated from three independent experiments and expressed as the mean ± standard deviation (SD). Alternatively, the relative percentages of Gag and gp41 levels in cells and virions are shown below each lane of the Western blots.

Infection of pseudotyped viruses, reverse transcriptase (RT), and chloramphenicol acetyltransferase (CAT) assays

Cell-free, Env pseudotyped viruses were assayed for virion-associated RT activity as previously described [47, 48]. The infectivity of the pseudotypes was assayed by challenging 1×10^6 CEM-SS cells with viruses containing 2.5×10^5–5×10^5 cpm RT activity by the spinoculation method as previously described [49], and CAT activity was determined as previously indicated [42].

Isolation of HIV-1 core structures

Immature cores were isolated from cell-free, concentrated viruses according to a previously described procedure [50]. After fractionation, the samples in each fraction were precipitated with 10% cold trichloroacetic acid. The viral proteins were then analyzed by Western blotting.

Confocal immunofluorescence microscopic studies

Transfected COS-1 cells were fixed by 4% paraformaldehyde, permeabilized by 0.25% Triton X-100, and then repeatedly incubated with each of the primary antibodies followed by incubation with appropriate, fluorescence-labeled secondary antibodies directed against the species from which the primary antibodies were generated. The immunostained cells were analyzed by confocal microscopy as previously described [40].

Results

Dominant interference with HIV-1 infectivity by a gp41 cytoplasmic tail fusion protein

We previously showed that an HIV-1 gp41 cytoplasmic domain fusion protein β-gal/706–856, which encodes the entire cytoplasmic tail of the HXB2 strain Env (Figure 1A) fused to the C terminus of the *Escherichia coli* reporter protein β-gal [40], can downregulate Gag expression in an *env*-independent manner. To characterize the antiviral action of β-gal/706–856, in the present study we first explored whether β-gal/706–856 can act as a dominant-negative inhibitor in interference with HIV-1 replication by a one-cycle virus infectivity assay [51]. The effect of pCDNA3-β-gal/706–856 was examined at a 1:1 molar ratio of an HIV-1 long-terminal-repeat (LTR)-driven pSVE7*puro env* plasmid to pCDNA3-β-gal/706–856. The infectivity of the defective virus pseudotyped with Env coexpressed with β-gal/706–856 was significantly reduced compared to that of the defective virus pseudotyped with Env coexpressed with β-gal (Figure 1B). Coexpression with β-gal did not affect the ability of Env to mediate virus infectivity (data not shown). The 706~752 segment (Figure 1A), which neither multimerizes nor binds to cellular membranes [38, 39], exhibited no dominant interference (Figure 1B). The dominant interference by β-gal/706–856 proceeded in an inhibitor dose-dependent fashion (Figure 1C). β-gal/706–856 also inhibited the infectivity of two macrophage-tropic 92BR and 92US isolates [48] in CXCR4- and CCR5-expressing PM1 cells (Figure 1D).

Effects of β-gal/706–856 on viral protein expression

To determine the mechanism responsible for dominant interference by this inhibitor, 293T cells were cotransfected with equal amounts of the pHXB2R3 provirus along with pCDNA3, pCDNA3-β-gal, or pCDNA3-β-gal/706–856 plasmids. Equal volumes of cell and virion lysates were resolved by SDS-PAGE followed by Western blotting using mouse Mabs 902, Chessie 8, and 183, which are specific for gp120, gp41, and CA p24, respectively. With β-gal/706–856 coexpression, the intracellular levels of Pr55, an uncleaved MA and CA intermediate (p41), and p24, which are all recognized by Mab 183, were reduced, but the virion-associated levels of Pr55 and its cleavage products were only slightly reduced compared to the control, which did not express β-gal, and to β-gal coexpression (Figure 2A, top panel). β-gal/706–856 also strikingly reduced the intracellular levels of the gp160 precursor, its cellular protease-catalyzed cleavage products, gp120 and gp41, and

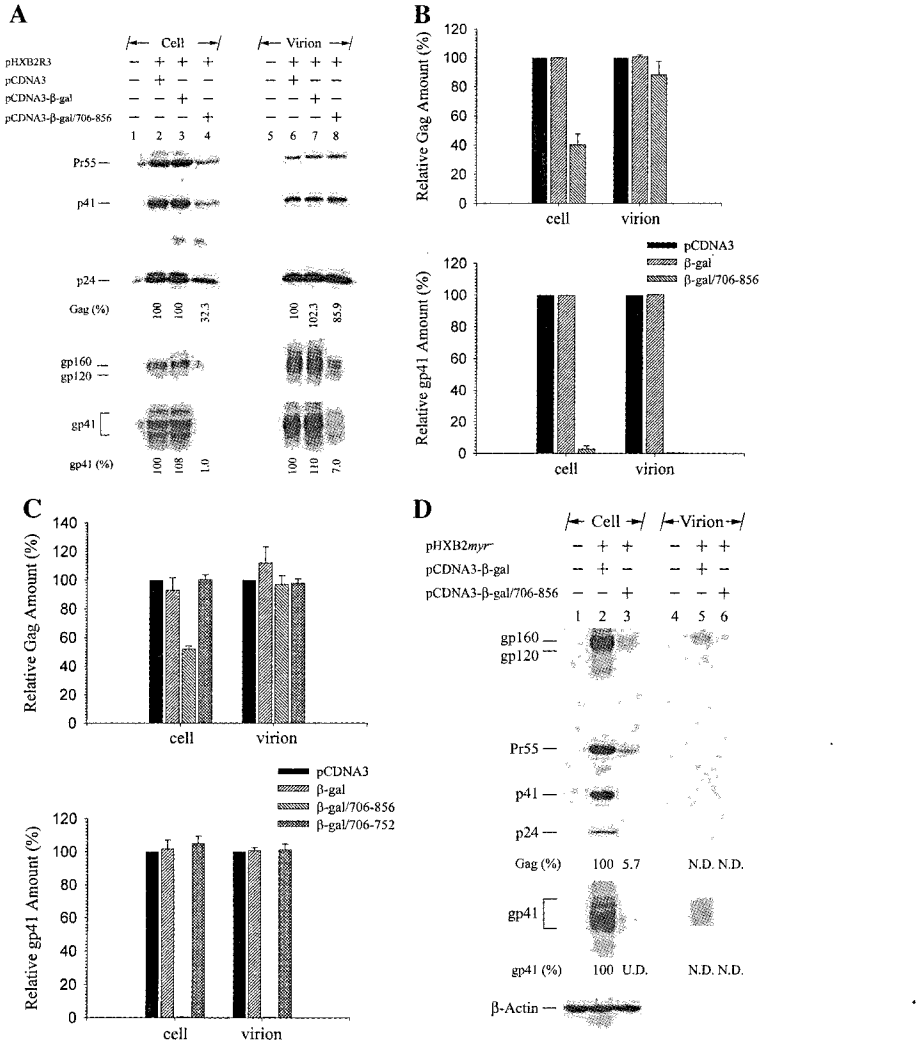

Figure 2. Effect of β-gal/706–856 on viral protein expression. (A) 293T cells were cotransfected with 5 μg of pHXB2R3 and 5 μg each of the pCDNA3 plasmids as indicated. Equal volumes of cell and virion lysates were analyzed by SDS-PAGE followed by Western blotting using Mabs 183, 902, and Chessie 8, respectively. (B) Gag and gp41 levels in cells and virions obtained from each transfection as shown in (A) are expressed as percentages relative to those of pCDNA3 cotransfection. Results from three separate experiments were quantified. (C) 293T cells were cotransfected with 5 μg of pHXB2*pro*$^-$ and 5 μg each of the pCDNA3 plasmids. Cell and virion lysates were then analyzed by Western blotting using sheep anti-gp120, and Mabs 183, Chessie 8, β-gal, or β-actin, respectively. The percentages of Gag and gp41 in cells and virions were quantitated. (D) Cells were cotransfected with 5 μg of pHXB2*myr*$^-$ and 5 μg each of the pCDNA3 plasmids, and cell and virion lysates were analyzed by Western blotting. The relative percentages of Gag and gp41 in cells obtained are indicated below each of the panels. U.D., undetectable; N.D., not determined.

the virion-associated gp120 and gp41 levels (Figure 2A, second and third panels, lanes 4 and 8). Quantitation from three independent experiments showed that β-gal/706–856 decreased the total intracellular Gag levels by 60%, but only decreased the total virion-associated Gag levels by 12%, compared to the control and β-gal coexpression (Figure 2B, top panel). The levels of cell- and virion-associated Env proteins, as measured by the gp41 amounts, were also strikingly reduced by this fusion protein (Figure 2B, bottom panel).

Effects of Gag structures on β-gal/706–856-modulated viral protein expression

To examine whether Gag processing affects β-gal/706–856-modulated Gag and Env expressions, 293T cells were cotransfected with pHXB2*pro*$^-$

and each of the pCDNA3 plasmids. Since pHXB2*pro*$^-$ encodes an Asp-to-Asn mutation at residue 25 of the protease gene, which inactivates the protease function, the Pr55 precursor synthesized was not cleaved to produce p41 or p24. Quantitation from three individual experiments showed that β-gal/706–856 reduced the intracellular Pr55 level to 52% of that obtained from pCDNA3 cotransfection, but did not affect the release of Pr55 premature virus-like particles into the medium (Figure 2C, top panel). The levels of intracellular and virion-associated Env proteins, as measured by the levels of gp41, were greatly reduced by β-gal/706–856 coexpression (Figure 2C, bottom panel). In contrast, β-gal/706–752 did not affect the intracellular or virion-associated levels of Gag or Env (Figure 2C).

To study whether Gag targeting to the plasma membrane is crucial for the modulating effect of β-gal/706–856 on Gag and Env expressions, pHXB2*myr*$^-$, which encodes a Gly-to-Ala substitution at residue 2 of the MA, was examined. Gag was barely detected in the culture medium (Figure 2D, top panel, lane 5) as removal of the Pr55 myristylation signal by this mutation severely impairs Gag targeting to the plasma membrane, as well as virus assembly and budding [6, 52–55]. Nevertheless, β-gal/706–856 still greatly depleted the intracellular levels of Gag and Env (Figure 2D, top and middle panels, lane 3).

Impairment of steady-state Gag expression by β-gal/706–856 in the absence of Env

We previously used pHIVgpt-derived constructs to study the effects of β-gal/706–856 on Gag expression. We then examined whether β-gal/706–856 can also downregulate expression of Gag encoded by another *env*-defective homologous proviral construct pHXBΔBglCAT. As previously observed [40], β-gal/706–856 still diminished the cell- and virion-associated levels of Gag proteins. Quantitation of three analyses showed that β-gal/706–856 reduced cell- and virion-associated Gag levels by 64% and 47%, respectively, whereas β-gal did not affect cell- and virion-associated Gag levels, compared to pCDNA3 cotransfection. The effect of this modulator on reduced steady-state Gag expression was specific since β-gal/706–752 did not affect Gag expression or budding (data not shown).

Suppression of β-gal/706–856-mediated downregulation of Gag expression by Env overexpression

Therefore, these studies together showed that β-gal/706–856 reduced cell-associated Gag levels regardless of Env expression or not and that β-gal/706–856 decreased virion-associated levels of Gag only in the absence of Env expression. These observations suggest that Env may overcome the depleting effect of β-gal/706–856 on Gag expression. To test this hypothesis, 293T cells were cotransfected with pHIVgptGag, which encodes the *gag* gene, along with the β-gal or β-gal/706–856 plasmid in the presence of an increasing amount of pCX-Env, a pCAGGS-based Env expression plasmid (Chen, unpublished result). The pCAGGS vector contains the cytomegalovirus virus immediately early enhancer/chicken β-actin promoter. With β-gal coexpression, Env coexpression did not greatly affect the cell- and virion-associated levels of Gag (Figure 3, top panel). However, Env interfered with the downregulation effect of β-gal/706–856 on the intracellular and extracellular Gag levels in an *env* plasmid dose-dependent manner (Figure 3, bottom panel). Quantification of total intracellular and virion-associated levels of gp160, gp120, and gp41 in β-gal and β-gal/706–856 coexpression showed that the total levels of Env proteins in β-gal/706–856 coexpression were 23, 51.7, and 72.6% of those of Env proteins in β-gal coexpression when 1, 2, and 4 μg of pCX-Env were added into transfections, respectively. This observation indicated that a fraction of Env molecules was also downregulated by β-gal/706–856 with Gag under this experimental condition.

Molecular interactions among Env, Gag, and β-gal/706–856

We previously demonstrated that the detergent-containing, sucrose gradient ultracentrifugation method as previously described by Wyma et al. [27] can detect the authentic Env-Gag and Gag-gp41 cytoplasmic domain interactions [40, 50]. This approach provides a simple and convenient method to determine detergent-resistant, molecular interactions among Env, Gag, and β-gal/706–856. When viral particles obtained from cotransfection of pHXB2*pro*$^-$ and β-gal or β-gal/706–856 plasmid were analyzed, β-gal did

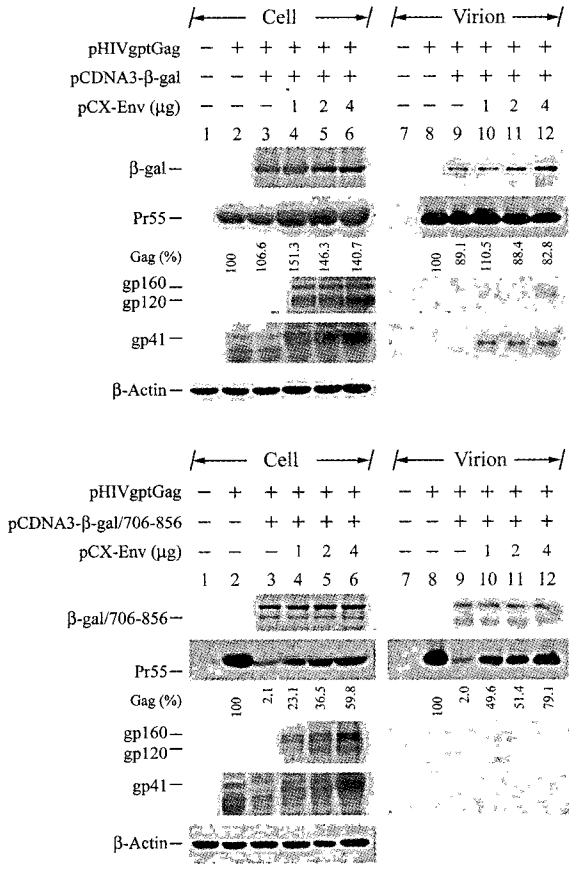

Figure 3. Effect of Env overexpression on β-gal/706–856-mediated Gag downregulation. 293T cells were cotransfected with 20 μg of pHIVgptGag and 2 μg each of the β-gal or β-gal/706–856 plasmids in the presence or absence of an indicated amount of the Env plasmid. Cell and virion lysates were analyzed by Western blotting, and the relative Gag amounts in cells and viruses were determined.

not co-appear with the Gag core, whereas a significant fraction of the Env proteins still positioned with the Gag core (Figure 4, top panel). In contrast, a significant fraction of β-gal/706–856 and a small fraction of Env were associated with the Gag core to bottom fractions of the gradient (Figure 4, bottom panel). These results indicate that the cytoplasmic tail, per se, forms a detergent-resistant, tertiary complex with Gag and Env. Interestingly, the fractions of gp160 and gp41 proteins associated with Gag were remarkably decreased when coexpressed with β-gal/706–856 compared to those of Env associated with Gag when β-gal was coexpressed (Figure 4).

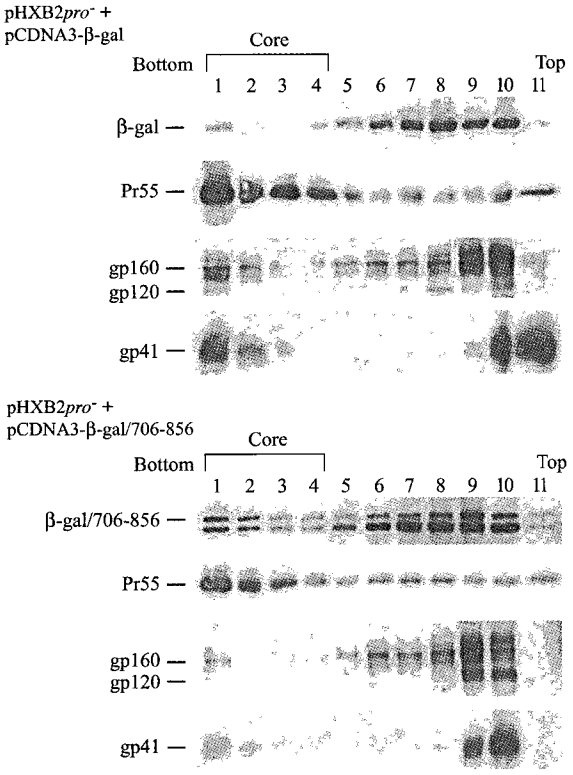

Figure 4. Analysis of interactions among Gag, Env, and β-gal/706–856. Concentrated virions obtained from cells cotransfected with 15 μg of pHXB2*pro*⁻ and 7.5 μg each of the β-gal and β-gal/706–856 plasmids were lysed with STE buffer containing detergent, and then analyzed by sucrose gradients without detergent. Samples in each fraction were precipitated with trichloroacetic acid and analyzed by Western blotting.

Immunofluorescence analysis of coexpressed viral proteins

We then assessed the intracellular localization of Env and Gag expressed from a *pro*-deficient HXB2 provirus with β-gal or β-gal/706–856 coexpression using Mabs β-gal, sheep anti-gp120, and a rabbit anti-p25/p24 antiserum (cat. # 384). When coexpressed with β-gal, Gag, Env, and β-gal coappeared in the peripheral cytoplasm, but they were not colocalized in the perinuclear region (Figure 5A, top panel). When coexpressed with β-gal/706–856, Gag, Env, and β-gal/706–856 were colocalized perinuclearly and in the cytoplasm as punctuate or aggregated structures (Figure 5A, bottom panel). We also used a functional *pro*-encoding HXB2R3 provirus to confirm the

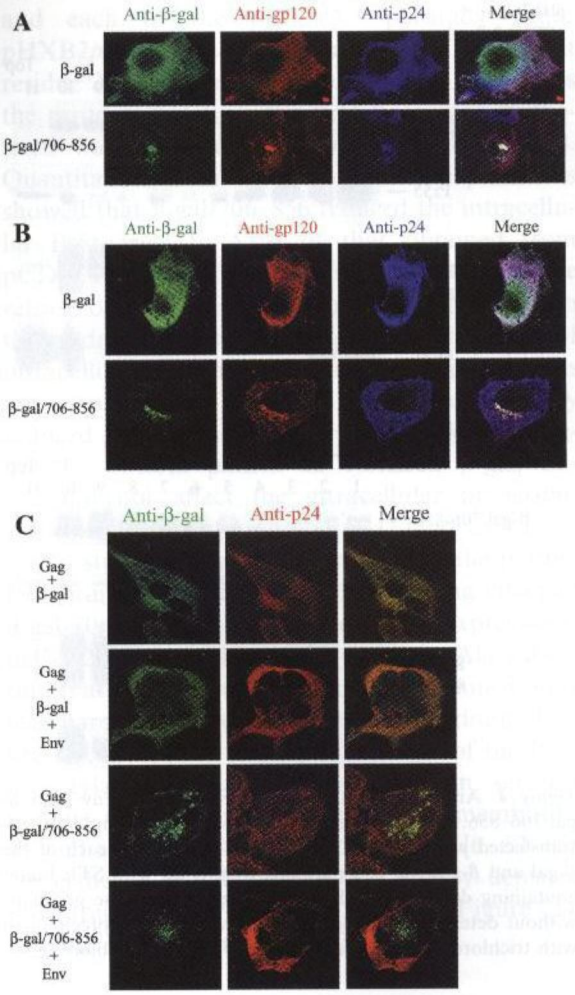

Figure 5. Immunofluorescence analysis of coexpressed viral proteins. Three millions COS-1 cells were cotransfected with 4 μg of pHXB2*pro*$^-$ and 1 μg each of the β-gal or β-gal/706–856 plasmids, and 2×10^4 transfected cells were grown on cover slips in 24-well plates. Transfected cells were immunostained with Mab anti-β-gal, rabbit anti-p25/p24 (cat. # 384), sheep anti-gp120, and appropriate fluorescence-labeled secondary antibodies. (B) Cells were cotransfected with 4 μg of pHXB2R3 along with 1 μg of pCDNA3-β-gal or pCDNA3-β-gal/706–856, and transfected cells were immunostained as indicated in (A) expect that another rabbit anti-GST/p24 (APR432) was used. (C) Cells were cotransfected with 3 μg of pHIVgptGag and 1.5 μg of the β-gal or β-gal/706–856 plasmid in the presence or absence of 1.5 μg of the Env plasmid. Cells were successively incubated with rabbit anti-GST/p24, rhodamine-labeled anti-rabbit IgG, β-gal Mab, and FITC-labeled anti-mouse IgG. Immunostained cells were analyzed by confocal microscopy.

intracellular colocalization of Gag, Env, and β-gal/706–856 by using another rabbit anti-p24 (APR432), which was generated by immunizing rabbits with a bacterially expressed glutathione-S-transferase (GST)-p24 recombinant protein, to stain Gag. Again, Gag, Env, and β-gal were distributed in the cytoplasm with some degree of co-appearance but they did not co-exist in perinuclear membranes as aggregated complexes (Figure 5B, top panels). However, Gag, Env, and β-gal/706–856 formed aggregated structures in the perinuclear cytoplasmic region (Figure 5B. bottom panels). These results collectively indicate that β-gal/706–856 specifically interacts with and directs Gag and Env together to a perinuclear cytoplasmic region to form aggregated structures.

To understand the mechanism underlying Env overexpression suppressing Gag downregulation, we examined the intracellular localization of Gag expressed with β-gal or β-gal/706–856 in the presence or absence of Env coexpression. In β-gal coexpression, Gag and β-gal were all distributed in the entire cytoplasm without any apparent colocalization in the perinuclear region regardless of Env overexpression or not (Figure 5C, first and second panels). In β-gal/706–856 coexpression, a fraction of Gag was found to be colocalized with β-gal/706–856 in the perinuclear region to form patches or speckles in the absence of Env overexpression (Figure 5C, third panel). However, Gag and β-gal/706–856 were not colocalized in the perinuclear region as aggregated structures in the presence of Env overexpression (Figure 5C, fourth panel). These observations indicate that overexpression of Env hinders Gag and β-gal/706–856 to form aggregated structures in the perinuclear region, which may explain why Gag is less downregulated by β-gal/706–856 when Env is overexpressed.

Determinants in the cytoplasmic tail crucial for downregulation of Gag expression

The above and our previous results [40] indicate that Gag, but not Env, is the primary target of β-gal/706–856. Thus, we further characterized the underlying molecular mechanism of cytoplasmic tail-mediated Gag downregulation. The effects of β-gal fusion proteins containing progressive truncations from the C terminus of the cytoplasmic domain [38] on Gag expression were examined. Sequences encompassing residues 706~844, 706~813, 706~795, and 706~775, just like the 706~856 sequence, all downregulated Gag

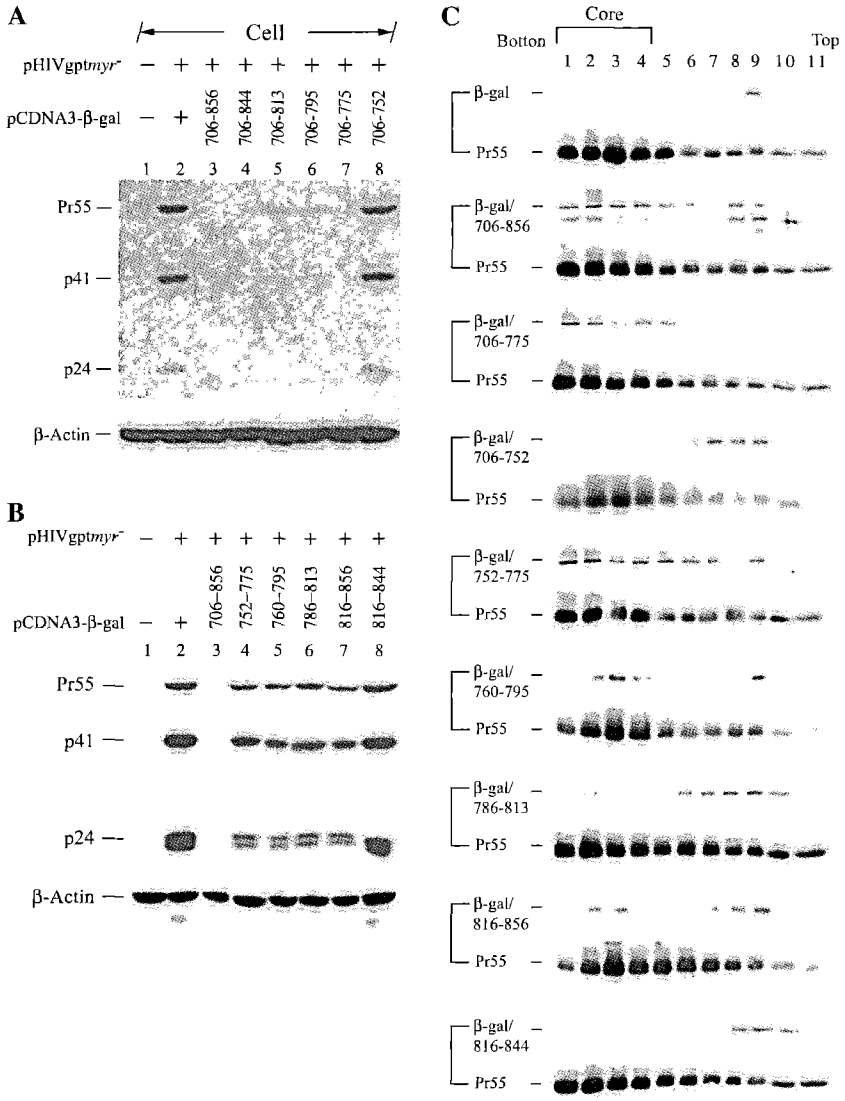

Figure 6. Characterization of cytoplasmic subdomain fusion proteins in Gag expression and interaction with Gag. (A) and (B) 293T cells were cotransfected with 10 μg each of pHIVgpt*myr*⁻ and β-gal fusion plasmids as indicated, and cell lysates were analyzed by Western blotting. (C) Cells were cotransfected with 15 μg of pHIVgpt*pro*⁻ and 3.75 μg each of the cytoplasmic subdomain fusion plasmids. Cell-free virions collected were analyzed by detergent-containing sucrose gradient centrifugation as described in Figure 4.

expression (Figure 6A, lanes 3–7) whereas the 706~752 sequence did not affect Gag expression (Figure 6A, lane 8). This result also suggests that the region between residues 752–775 is critical for Gag downregulation. Indeed, the 752~775 sequence caused Gag downregulation (Figure 6B, lane 4). Also, each of the LLP-1, LLP-2, and LLP-3 motifs spanning residues 816~856, 760~795, 786~813, respectively, was able to downregulate Gag expression (Figure 6B, lanes 5–7) whereas the 816~844 sequence did not cause Gag downregulation (Figure 6B, lane 8). These results indicate that the C-terminal two-thirds segment of the cytoplasmic tail contains multiple determinants capable of downregulating Gag expression.

Interaction of Gag with cytoplasmic subdomains

To determine whether a stable interaction between Gag and these gp41 cytoplasmic subdomains is

required for Gag downregulation, virions collected from cells coexpressing Gag and various cytoplasmic subdomain fusion proteins were subjected to sucrose gradient equilibrium ultracentrifugation. In addition to the full-length cytoplasmic domain, the 706~775, 752~775, 760~795, 786~813, and 816~856 sequences all had stable interactions with Gag (Figure 6C). Just like β-gal, the 706~752 and 816~844 sequences did not show stable interaction with Gag (Figure 6C).

Discussion

We demonstrate herein that an HIV-1 Env cytoplasmic tail fusion protein, β-gal/706–856, interferes *in trans* with HIV-1 infectivity (Figure 1) by reducing steady-state Env expression (Figure 2). This inhibitor reduced steady-state Env expression in a *gag* gene-dependent manner since β-gal/706–856 does not inhibit Env expression in the absence of Gag expression [40]. In the context of the HXB2 provirus, β-gal/706–856 does not greatly affect virion-associated Gag levels but reduces intracellular and virion-associated Env levels and intracellular Gag levels (Figure 2). The effects of this inhibitor on virus infectivity and viral protein expression are specific since the N-terminal third fusion protein, β-gal/706–752, neither exhibited dominant interference in HIV-1 infectivity (Figure 1B) nor affected Gag or Env expression or virus budding (Figure 2C). These results together indicate that Gag, not Env, is the primary target of this inhibitor. Paradoxically, Env appears to counteract the downregulation effect of β-gal/706–856 on Gag expression (please refer to the text and Figure 2A–C). In support of this notion, overexpression of Env was observed to relieve the depleting effect of β-gal/706–856 on both the intracellular and virion-associated Gag levels (Figure 3).

In the context of proviral constructs, results of confocal microscopy showed that β-gal/706–856, but not β-gal, specifically interacted with and directed Gag and Env together to perinuclear cytoplasmic regions to form aggregated complexes (Figure 5A and B). Sucrose gradient ultracentrifugation analyses also demonstrated detergent-resistant, stable interactions among Gag, Env, and β-gal/706–856 (Figure 4). These results imply that interactions of Gag and Env with the cytoplasmic tail fusion protein and the targeting of Gag and Env to an intracellular site are required for β-gal/706–856-mediated Gag and Env downregulation. Interestingly, the association of Env with Gag was decreased when coexpressed with β-gal/706–856 compared to β-gal (Figure 4), supporting the idea that Env and β-gal/706–856 compete for binding to Gag. This feature of Env may thus counteract the downregulation effect of the inhibitor on Gag expression.

Results from cytoplasmic tail mapping analyses showed a correlation between the ability of cytoplasmic subdomains to downregulate Gag expression and the ability of these subdomains to stably interact with Gag (Figure 6). These observations further substantiate the notion that Gag downregulation by β-gal/706–856 requires the stable interaction between the cytoplasmic tail in the fusion protein and Gag. Interestingly, Env overexpression suppresses β-gal/706–856-mediated Gag downregulation (Figure 3) and prevents Gag localization with β-gal/706–856 in the perinuclear region (Figure 5C). These results together indicate that redirection of Gag from its cytoplasmic synthesis site to a perinuclear region is a prerequisite for cytoplasmic tail-mediated Gag degradation.

Based on the results presented above, we propose the following model to illustrate the dynamic interplay among Gag, Env, and β-gal/706–856 that can modulate Gag and Env expression. β-gal/706–856 does not downregulate Env expression unless Env is coexpressed with Gag. In the context of a 1:1 molar ratio of the HXB2 proviral DNA and β-gal/706–856 plasmids, Gag may be expressed in a larger quantity relative to those of Env and β-gal/706–856. Since β-gal/706–856 causes only a fraction of Gag molecules to detour from its normal maturation process to an intracellular site (Figure 5), it is conceivable that only Gag complexes present in the cytoplasm, but not those present on the plasma membrane or in budding processes, are targeted by β-gal/706–856-induced downregulation, resulting in decreased intracellular Gag levels but with only a slight effect on virion-associated Gag levels. Under this premise, the majority of Env synthesized may be trapped intracellularly by binding to Gag; the Env molecules, behaving as "bystanders", are therefore downregulated intracellularly as a unit of the Gag–Env complex, resulting in decreases in both the

intracellular and virion-associated Env levels. Paradoxically, in addition to being depleted as a complex with Gag, Env, in essence, possesses a characteristic of competing with β-gal/706–856 for binding to Gag, in particular, when Env is overexpressed, thus, counteracting β-gal/706–856-mediated Gag downregulation. Also, Env may interact with β-gal/706–856 to decrease the down-regulation effect of this inhibitor on Gag expression.

In the present study, we demonstrate the molecular action of β-gal/706–856 in dominant inhibition of HIV-1 infectivity and virus replication. Since Gag and Env are important structural components of HIV-1 and crucial for virus infectivity, this novel mode of dominant inhibition in HIV-1 replication by a cytoplasmic tail fusion protein and its underlying mechanisms should have implications in designing useful anti-HIV strategies targeting the gp41 cytoplasmic tail-MA interaction.

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