Separate elements are required for ligand-dependent and -independent internalization of metastatic potentiator CXCR4

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The C-terminal cytoplasmic domain of the metastatic potentiator CXCR4 regulates its function and spatiotemporal expression. However, little is known about the mechanism underlying constitutive internalization of CXCR4 compared to internalization mediated by its ligand, stromal cell-derived factor-1 alpha (SDF-1α)/CXCL12. We established a system to analyze the role of the CXCR4 cytoplasmic tail in steady-state internalization using the NP2 cell line, which lacks endogenous CXCR4 and SDF-1α. Deleting more than six amino acids from the C-terminus dramatically reduced constitutive internalization of CXCR4. Alanine substitution mutations revealed that three of those amino acids Ser^{344} Glu^{345} Ser^{346} are essential for efficient steady-state internalization of CXCR4. Mutating Glu^{345} to Asp did not disrupt internalization, suggesting that the steady-state internalization motif is S(E/D)S. When responses to SDF-1α were tested, cells expressing CXCR4 mutants lacking the C-terminal 10, 14, 22, 31 or 44 amino acids did not show downregulation of cell surface CXCR4 or the cell migration induced by SDF-1α. Interestingly, however, we identified two mutants, one with E344A mutation and the other lacking the C-terminal 17 amino acids, that were defective in constitutive internalization but competent in ligand-promoted internalization and cell migration. These data demonstrate that ligand-dependent and -independent internalization is genetically separable and that, between amino acids 336 and 342, there is a negative regulatory element for ligand-promoted internalization. Potential involvement of this novel motif in cancer metastasis and other CXCR4-associated disorders such as warts, hypogammaglobulinemia, infections and myelokathexis (WHIM) syndrome is discussed. (*Cancer Sci* 2007; 98: 373–379)

The chemokine receptor CXCR4 is a class-A G protein-coupled receptor (GPCR; reviewed in [1,2]) and its natural ligand is stromal cell-derived factor-1 alpha (SDF-1α)/CXCL12. CXCR4 also serves as the receptor for HIV type 1 (HIV-1). Many cell types express CXCR4, including peripheral blood lymphocytes, monocytes-macrophages, thymocytes, dendritic cells, endothelial cells, epithelium-derived tumor cells, microglial cells, neurons and hematopoietic stem cells. CXCR4 plays multiple biological roles from promoting development of neuronal networks to regulating migration of leukocytes, cerebellar granule cells and hematopoietic stem cells.[3–8] Analysis of knockout mice indicates that the CXCR4/SDF-1α system is essential for maintenance of hematopoiesis and intestinal vascularization.[9,10]

The CXCR4/SDF-1α system also functions in pathological processes, including autoimmune diseases, cancer progression and metastasis, and AIDS caused by HIV-1. Recently, metastasis of breast cancer cells was found to be regulated by the CXCR4/SDF-1α axis.[5] Similarly, other studies have found that metastasis of other malignancies was controlled by the CXCR4/SDF-1α system, including colon carcinoma[11] non-small cell lung cancer[12] and prostate cancer.[13] These observations suggest that the CXCR4/SDF-1α axis is a potential target for metastatic cancer therapy.

Warts, hypogammaglobulinemia, infections and myelokathexis (WHIM) syndrome is a rare combined immunodeficiency characterized by an unusual form of neutropenia. It is reported that the CXCR4 cytoplasmic tail is mutated and often truncated in WHIM syndrome.[14] Thus, determining the biochemical activity of the CXCR4 cytoplasmic tail should facilitate understanding of the pathogenesis of WHIM syndrome as well as suggest ways to control cancer metastasis.

Following SDF-1α binding, CXCR4 is activated, triggering multiple signaling cascades via Gα or β-arrestin 2 (reviewed in[15]). To desensitize activated CXCR4, the G protein-coupled receptor kinase (GRK) is recruited and phosphorylates serine residues on the CXCR4 cytoplasmic tail, thereby inactivating Gα-mediated signal. Simultaneously, CXCR4 is internalized in a clathrin-dependent manner. β-arrestin 2 competes with Gα for CXCR4 binding and can initiate signal transduction independent from Gα. β-arrestin 2 can also induce clathrin-dependent CXCR4 endocytosis. Thus, cell surface levels of CXCR4 transiently decrease after agonist binding but, several hours later, surface levels of CXCR4 return to normal. Most internalized CXCR4 is transported to lysosomes and degraded, but some internalized CXCR4 is recycled. It is reported that amino acids within the cytoplasmic tail are required for agonist-dependent endocytosis of CXCR4.[16–18]

By contrast, it is unclear how steady-state cell surface levels of CXCR4 are maintained in the absence of SDF-1α. Although cell surface levels of CXCR4 could be regulated at the transcriptional level, it is likely that primary regulation occurs post-translationally. Given that the cell surface levels of CXCR4 are positively correlated with cancer cells' ability to metastasize,[5,19] understanding the post-translational behavior of CXCR4 is likely to shed light on metastatic processes. Historically, cells expressing endogenous CXCR4 have been used for analysis of CXCR4 trafficking. However, as is the case with many G protein-coupled receptors (GPCR), CXCR4 trafficking is influenced by spontaneous oligomerization in the absence of ligand.[20–22] Thus, previous observations might not correctly model phenotypes seen in CXCR4 mutants.

In the present study, we analyzed the contribution of the cytoplasmic tail to the post-translational trafficking of CXCR4 in a cell line lacking both endogenous CXCR4 and SDF-1α. Using genetic approaches, we identified two amino acid motifs within the CXCR4 cytoplasmic tail; one that positively regulates

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spontaneous ligand-independent internalization and the other that negatively regulates ligand-dependent CXCR4 internalization.

Materials and methods

Cells. The glioblastoma cell line NP2, human embryonic kidney (HEK) 293T, and HeLa, cells were maintained in RPMI-1640 (Sigma, Tokyo, Japan) supplemented with 10% FBS (Japan Bioserum, Tokyo, Japan), penicillin and streptomycin (Invitrogen, Tokyo, Japan). All cell lines were incubated at 37°C in the humidified 5% CO_2 atmosphere.

Plasmids. Full-length CXCR4 cDNA was amplified from a plasmid kindly provided by Dr Shioda[23] using the following primers: sense, 5′-ACCGGTGCCACCATGGAGGGGATCAGT-ATATACACTTCAG-3′, and antisense, 5′-AGATCTCGCTGGA-GTGAAAACTTGAAGACTCAGACTC-3′. CXCR4 lacking the cytoplasmic tail (d-44) was amplified using the same sense primer and the antisense primer, 5′-AGATCTTGGCTCCAAGGAAA-GCATAGAGGATGGG-3′. Polymerase chain reaction (PCR) fragments were cloned into the *Age* I-*Bgl* II sites of pEGFP-C2 (Clontech, Palo Alto, CA, USA) to create pCXCR4 FL and pCXCR4 d-44, respectively. To construct pCXCR4 FL- and d-44-GFP, the *Sna* BI-*Bgl* II fragments from pCXCR4 FL and d-44 were cloned into the *Sna* BI-*Bgl* II sites of pEGFP-N2, respectively (Clontech). To construct pCXCR4 FL- and d-44-GFP flag, the *Sna* BI-*Bgl* II fragments from pCXCR4 FL and d-44 were cloned into the *Sna* BI-*Bgl* II sites of pEGFP-flag in which the following annealed oligonucleotides had been inserted into the *Bsr* GI site of pEGFP-N2: forward, 5′-GTACGACTAC-AAAGACGATGACGACTATAAGTAAGC-3′, and reverse, 5′-GGCCGCTTACTTATAGTCGTCATCGTCTTTGTAGTC-3′. To construct pCMMP CXCR4 FL- and d-44-GFP, pCXCR4 FL- and d-44-GFP were digested with *Not* I, blunted using T4 DNA polymerase, and further digested with *Age* I. The Age *I*-blunted *Not* I fragments of both constructs were cloned into the pCMMP eGFP plasmid that had been digested with *Bam* HI, blunted with T4 DNA polymerase, and digested with *Age* I. pCMMP CXCR4 FL- and d-44-GFP-flag were constructed using the same strategy. CXCR4 deletion and point mutants were PCR-amplified using the sense primer 5′-ACCGGTGCCACCATGGAGGGGATCAGTG-TGAAAACTTGAAGACTCAGACTC-3′ and the following reverse primers: d-6, 5′-AAGCTTGAGCTCGAGATCTCAG-ACTCAGACTCAGTGGAAAC-3′; d-10, 5′-AAGCTTGAGC-TCGAGATCTCAGTGGAAACAGATGAATGTCC-3′; d-14, 5′-AAGCTTGAGCTCGAGATCTCTGAATGTCCACCTCGC-TTTCC-3′; d-17, 5′-AAGCTTGAGCTCGAGATCTCACCTC-GCTTTCCTTTGG-3′; d-22, 5′-AAGCTTGAGCTCGAGATCT-CGGAGAGGATCTTGAGGCTGGACC-3′; d-31, 5′-AAGCTT-GAGCTCGAGATCTCGCTCACAGAGGTGAGTGCGTGC-3′; E343A, 5′-CGAGATCTCGCTGGAGTGAAAACTTGAAGAC-TCAGACGCAGTGGAAACAGATGAATGTC-3′; S344A, 5′-CGAGATCTCGCTGGAGTGAAAACTTGAAGACTCAGCCT-CAGTGGAAACAGATGAATGTC-3′; E345A, 5′-CGAGATC-TCGCTGGAGTGAAAACTTGAAGACGCAGACTCAGTGGA-AACAGATGAATGTC-3′; S346A, 5′-CGAGATCTCGCTGGA-GTGAAAACTTGAAGCCTCAGACTCAGTGGAAACAGATG-AATGTC-3′; S347E, 5′-CGAGATCTCGCTGGAGTGAAAA-CTTTCAGACTCAGACTCAGTGGAAACAGATGAATGTC-3′; H350E, 5′-CGAGATCTCGCTGGACTCAAAACTTGAAGAC-TCAGACTCAGTGGAAACAGATGAATGTC-3′; S347E/H350E, 5′-CGAGATCTCGCTGGACTCAAAACTTTCAGACTCAGA-CTCAGTGGAAACAGATGAATGTC-3′; and E343/345D, 5′-CGAGATCTCGCTGGAGTGAAAACTTGAAGAGTCAGAGT CAGTGGAAACAGATGAATGTC-3′. The PCR fragments were cloned into the *Age* I-*Bgl* II sites of pCMMP CXCR4 FL-GFP-flag, replacing wild-type with mutant CXCR4. Protein expression of each mutant in 293T cells was verified by Western blot analysis.

Immunoblotting. Immunoblotting was performed as described.[24,25] The primary antibody was anti-green fluorescent protein (GFP) polyclonal antibody (Beckton Dickinson, San Jose, CA, USA). The secondary probe was EnVision+ (Dako, Glostrup, Denmark). Signals were visualized with an LAS3000 imager (Fuji Film, Tokyo, Japan) after treating the membranes with the Lumi-Light Western Blotting Substrate (Roche Diagnostics GmbH, Mannheim, Germany).

Flow cytometry. Cells were labeled with anti-CXCR4 antibodies recognizing the N-terminus conjugated with R-phycoerythrin (PE; 2B11, BD Pharmingen, San Diego, CA) or recognizing the second extracellular loop (12G5) conjugated with either PE or PE-Cy5 (Beckton Dickinson) for 30 min at 4°C. Cells were washed once with phosphate-buffered saline (PBS) supplemented with 1% FBS and analyzed by FACS Aria (Beckton Dickinson). To isolate GFP-expressing NP2 cells, cells were infected with murine leukemia virus (MLV)-based retroviral vectors as described.[25] Cells exhibiting similar green fluorescence intensities were gated and sorted by FACS Aria. Efficiency of internalization was measured by comparing mean fluorescence intensities for cell surface CXCR4 detected by a PE-labeled 2B11 monoclonal antibody before and after SDF-1α treatment (200 ng/mL, Peprotech EC, London, UK).

Microscopic analysis and imaging of cells. To judge a phenotype of a CXCR4 mutant, three independent scientists investigated the mutant cell phenotype under a fluorescent microscope (Olympus, Tokyo, Japan). Each scientist investigated more than 1000 cells for each mutant. More than 99% of cells of a mutant fell in the indicated phenotypic category. These phenotypes were unchanged for more than a year of continuous cultivation in tissue culture. For imaging, NP2 cells were grown on glass plates for more than 24 h, fixed in 4% formaldehyde in PBS for 5 min, stained with Hoechst 33258, mounted (Vectorshield, Vector Laboratories, Burlingame, CA, USA), and imaged using a confocal microscope META 510 (Carl Zeiss, Tokyo, Japan). A representative cell for each CXCR4 mutant carrying a wide cytoplasm was chosen such that the spatial resolution was high. The focal plane just above the glass surface was scanned with an optical thickness of approximately 1 μm. For the imaging of subcellular compartments, cells were incubated with either BODIPY TR ceramid, ER-Tracker Blue-White DPX, or Lysotracker Red DND-99 (Invitrogen) according to the manufacturer's protocol and imaged without fixation. Image brightness and contrast were processed by META510 software (Carl Zeiss). Unless noted, cells were imaged at ×630 magnification, the GFP signal was displayed in green, and Hoechst 33258-stained nuclei were blue. To visualize ligand-induced internalization, cells were treated with 200 ng/mL SDF-1α before fixation. The live cell imaging was performed using Leica DFC350FX system and the images were processed by FW4000 software (Leica Microsystems, Tokyo, Japan). Cells were plated on the glass-bottomed dish (Matsunami glass, Kishiwada, Japan) and incubated at 37°C in the humidified 5% CO_2 atmosphere during the monitoring.

Cell migration assay. Cell migration was measured using an HTS FluoroBlok Multiwell Insert System (8.0 μm pore size, BD Falcon) according to the manufacturer's protocol. For stimulation assays, cells were incubated without serum overnight before SDF-1α treatment (200 ng/mL). Cells were allowed to migrate overnight.

Statistical analysis. Significance of differences were determined by a Student's *t*-test. *P*-values less than 0.05 were considered significant.

RESULTS

Deleting 10 amino acids from the carboxyl end of CXCR4 alters the efficiency of constitutive internalization. Previous studies indicated that the cytoplasmic tail of CXCR4 amino acids 308–352 plays

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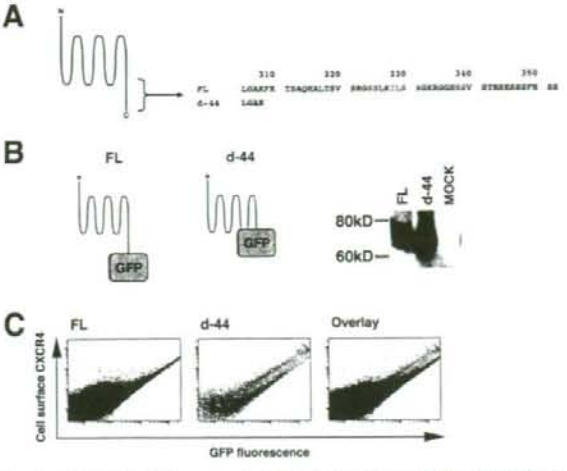

Fig. 1. The effect of stromal cell-derived factor-1 alpha (SDF-1α) treatment on NP2 cells expressing CXCR4 mutants. (a) Cells expressing d-17 were treated with SDF-1α, incubated at 37°C for the indicated times, fixed and imaged. The blue signal represents the Hoechst-stained nucleus. (Original magnification, ×630; bar, 10 μm). (b) FACS analysis to measure internalization efficiency of cell surface CXCR4 and mutant forms 2 h after SDF-1α exposure. The average and standard deviation from the indicated number of independent experiments are shown. Asterisks represent statistically significant difference from the FL levels ($P < 0.01$). (c) Cell migration assay to assess response of cells expressing CXCR4 and mutants to SDF-1α. The number of migrated cells in three to six randomly selected fields was counted and the average and standard deviation were calculated. (□) number of migrated cells in the absence of ligand; (■) migration in the presence of ligand. (*) statistically significant differences in the number of migrated cells between SDF-1α-untreated and -treated cells ($P < 0.01$).

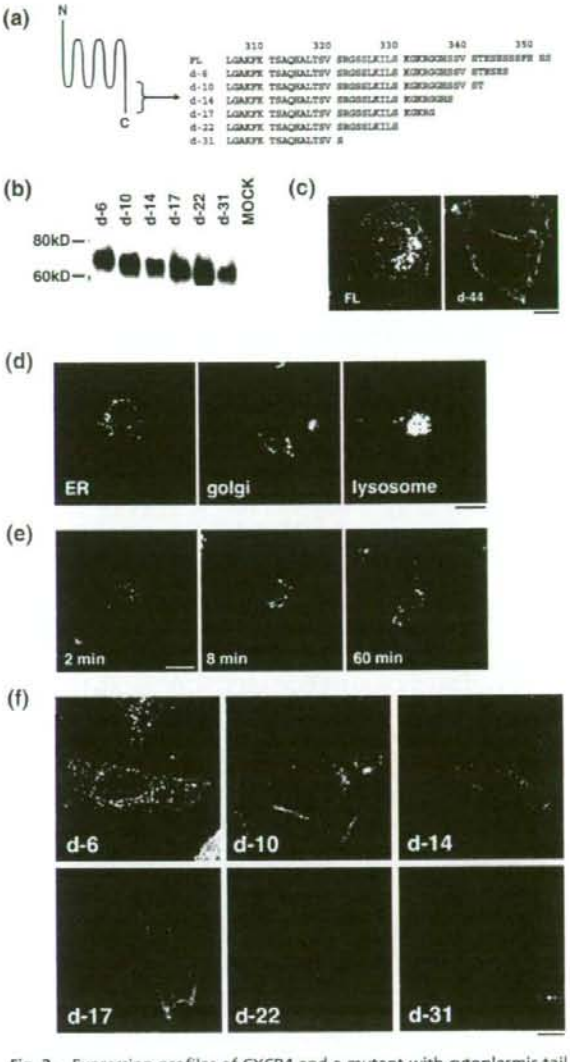

Fig. 2. Expression profiles of CXCR4 and a mutant with cytoplasmic tail deletion. (a) Schematic representation of CXCR4. The N-terminus CXCR4 is exposed in the extracellular space and the C-terminus is intracellular. Gray represents the lipid bilayer. The amino acid sequence of the cytoplasmic tail is shown. Residues in red are required for ligand-induced endocytosis. The CXCR4 d-44 mutant lacks amino acid 309–351. (b) Schematic representation and Western blot of FL and d-44 constructs. (c) Flow cytometry profiles of FL and d-44 expressed in 293T cells. The horizontal axis represents green fluorescence intensity indicative of green fluorescent protein (GFP)-tagged CXCR4 protein levels, and the vertical axis is PE-Cy5 fluorescence intensity, reflecting cell surface CXCR4 detected by the anti-CXCR4 antibody. GFP-positive cells expressing FL are colored in red (left) and those expressing d-44 in green (middle). The expressional differences between FL and d-44 is highlighted on the overlay plot (right).

a critical role in ligand-dependent internalization (Fig. 1a). Also, it has been shown in transfected cells that cell surface levels of CXCR4 lacking the cytoplasmic tail (equivalent to the d-44 mutant here) are higher than those of the full length, wild-type protein (hereafter designated FL), suggesting that the cytoplasmic tail of CXCR4 regulates steady-state internalization.[26,27] To confirm this, we constructed expression plasmids of CXCR4 FL and d-44 fused to GFP or GFP-FLAG at the C-terminus. Previous studies and data reported here indicated that CXCR4 function is not affected by this modification.[28] The expression of each construct was verified by Western blot analysis (Fig. 1b). Single cell-based quantitative analyzes revealed that the ratio of cell surface levels to the total amount of CXCR4 FL (Fig. 1c, left) was consistently lower than that of d-44 (Fig. 1c, middle) at any expression levels (Fig. 1c, right for the comparison). These data supported previous findings and demonstrate that constitutive internalization occurs at any level of CXCR4 expression.

To further examine the contribution of the cytoplasmic tail to post-translational trafficking of CXCR4, we devised a system utilizing the human NP2 glioma line: NP2 cells are flat and exhibit a large cytoplasmic space such that intracellular compartments can be well resolved under the microscope. NP2 cells also lack endogenous CXCR4[29] and SDF-1α (data not shown), both of which could potentially affect distribution of transduced CXCR4. However, NP2 cells are capable of appropriate signaling in response to CXCR4/SDF-1α interaction. We generated a series of CXCR4 deletion mutants lacking the cytoplasmic tail (Fig. 2a) and transduced them into NP2 cells using MLV vectors. Cells bearing similar green fluorescence intensities were collected by FACS sorter. The expression of each mutant was verified by Western blot analysis (Fig. 2b). Microscopic observations revealed that cells expressing FL were bordered by green fluorescence, and significant green fluorescence was detected in vesicular compartments of varying diameters lying close to the nucleus surrounding the nucleus (hereafter designated the FL phenotype, Fig. 2c, left). Vesicles around the nucleus were

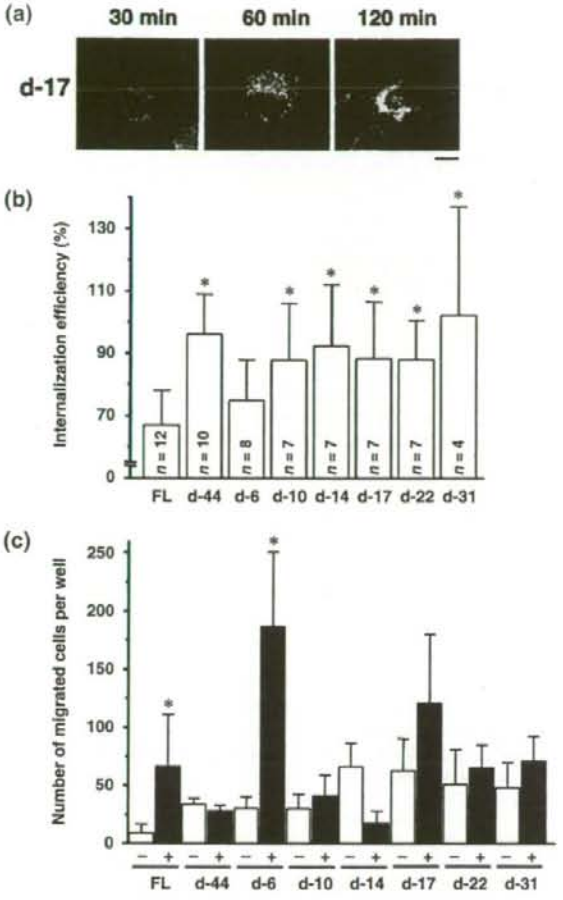

Fig. 3. Identification of the amino acids required for steady-state CXCR4 internalization. (a) Amino acid sequences of the cytoplasmic tail of FL and deletion mutants. Residues in red are required for ligand-induced endocytosis. (b) The protein expression of each mutant in 293T cells was verified by Western blot analysis. (c) Confocal micrographs of NP2 cells expressing FL and d-44 mutant proteins. The blue signal represents the Hoechst-stained nucleus. (Original magnification, ×630; bar, 10 μm.) (d) Confocal micrographs showing NP2 cells expressing CXCR4 FL stained with ER, Golgi, or lysosome organella markers. The organella marker signal is shown in red, the GFP signal is in green. The pixels that both red and green signals co-localized are shown in yellow. (Original magnification, ×630; bar, 10 μm.) (e) CXCR4 FL trafficking in the absence of SDF-1α in NP2 cells. Cell surface CXCR4 FL was labeled with an antibody conjugated with PE-Cy5 (red), incubated at 37°C for the indicated times, fixed and imaged. (Original magnification, ×630; bar, 10 μm.) (f) Confocal micrographs of NP2 cells expressing FL and mutant proteins. The intracellular vesicular green fluorescence reflecting steady-state internalization can be seen in the d-6 mutant. The blue signal represents the Hoechst-stained nucleus. (Original magnification, ×630; bar, 10 μm.)

mostly lysosomes, as demonstrated by fluorescent organella marker analyses in which cells expressing CXCR4 FL-GFP stained with the lysosomal marker yielded a substantial amount of co-localization signal. On the other hand, only a mall amount of co-localization signal was detected when the ER or Golgi markers were used (Fig. 2d), consistent with our biochemical fractionation (unpublished data) and previous publications.[16,27,28,30] The active constitutive internalization was visualized by labeling cell surface CXCR4 by PE-Cy5-conjugated monoclonal antibody followed by fluorescence imaging after cells were incubated at 37°C (Fig. 2e). The live cell imaging revealed that internalizing GFP-positive vesicles trafficked at an average velocity of 4.7 mm/h ($n = 15$), which is within the range of clathrin-dependent vesicular transport (2–20 mm/h), not that of caveolin-dependent vesicular transport (25–170 mm/h).[31–35] These data suggest that the FL is constitutively internalized from the cell surface to the cytoplasmic compartment. In sharp contrast, most green fluorescent signals from d-44 mutant-expressing cells were detected at the cell surface, and only a few small GFP-positive vesicles were seen in the cytoplasm near the nucleus (hereafter designated the d-44 phenotype, Fig. 2c, right). Similar observations were made in d-10, d-14, d-17, d-22 and d-31 mutant-expressing cells (Fig. 2f). The d-6 construct displayed a phenotype similar to FL, although the intracellular GFP signal was less prominent (Fig. 2c). Similar results were obtained in HeLa and 293 cells (data not shown). These data suggest that wild-type CXCR4 was trafficked to the plasma membrane but was internalized spontaneously. Thus, steady-state internalization appeared to be regulated by amino acids located between d-6 and d-10 (e.g. amino acids 343–346).

Steady-state and SDF-1α-induced CXCR4 internalization is genetically separable. Next, we investigated distribution of CXCR4 protein and cell migration after SDF-1α treatment. Confocal analysis showed that after SDF-1α exposure, cells expressing FL, d-6 and d-17 mutants showed GFP signals in intracellular compartments, which were enhanced 60 min after SDF-1α treatment, an effect most clearly shown in d-17-expressing cells (Fig. 3a). GFP signals from intracellular vesicles gradually disappeared 1–2 h after exposure to ligand. Such redistribution of GFP signals was not observed in cells expressing d-10, d-14, d-22, d-31 and d-44 (data not shown). Cell surface levels of CXCR4 before and after SDF-1α treatment were measured by FACS analysis undertaken with an antibody directed against the CXCR4 N-terminus, because that antibody did not interfere with ligand–receptor interaction (Fig. 3b). The downregulation of cell surface levels of FL 2 h after ligand exposure was 67.1 ± 11.1%, whereas that of d-44 was 96.3 ± 12.3% (average and standard deviation from 12 and 10 independent experiments, respectively), consistent with previous reports.[17,27,28] Ligand-induced downregulation of d-6 was 74.9 ± 12.9% ($n = 8$), similar to FL levels. Ligand-induced internalization was significantly less efficient in cells expressing d-10, d-14, d-17, d-22, d-31 and d-44 mutants when compared with FL ($P < 0.001$). Although the d-17 mutant supported ligand-facilitated internalization, as evidenced by microscopic observation, cell surface levels remained unchanged (Fig. 3a,b). This may be due in part to rapid recruitment of newly synthesized d-17 to the cell surface.

Next, we examined cells expressing CXCR4 mutants in response to SDF-1α. Migration results from intracellular signaling initiated by SDF-1α/CXCR4 interaction. Induction of cell migration by SDF-1α in cells expressing FL was 7.2-fold that of untreated cells ($P < 0.05$). In contrast, migration of cells expressing d-44 in response to SDF-1α was undetectable. These data are in agreement with a previous report.[26] The d-6 mutant, which is internalized upon SDF-1α treatment, supported ligand-promoted cell migration by 6.1-fold ($P < 0.01$) relative to untreated cells, similar to FL. Other deletion mutants tested did not display enhanced cell migration following ligand treatment, except for d-17, which showed modestly enhanced (1.9-fold) migration relative to untreated cells, which was not statistically significant. When basal migratory activities were compared, removal of six or more amino acids from the cytoplasmic tail appeared to potentiate migration in the absence of ligand (open bars, Fig. 3c). These data suggest that constitutive internalization is regulated independently of ligand-facilitated internalization.

Identification of CXCR4 S(E/D)S as a ligand-independent internalization motif. The above data indicated that the carboxy-terminal four

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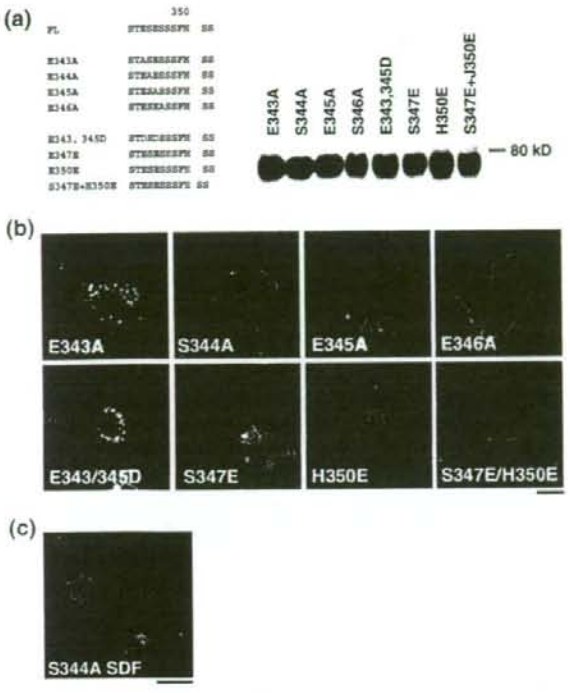

Fig. 4. Characterization of the SDF-1α-independent internalization motif of CXCR4. (a) Left, amino acid sequences of CXCR4 FL and substitution mutants. Letters in red indicate introduced mutations. Right, protein expression of each mutant in 293T cells was verified by Western blot analysis. (b) Confocal micrographs of NP2 cells expressing each mutant. The blue signal represents the Hoechst-stained nucleus. (Original magnification, ×630; bar, 10 μm.) (c) NP2 cells expressing the S344A mutant treated with SDF-1α for 2 h are shown. The blue signal represents the Hoechst-stained nucleus. (Original magnification, ×630; bar, 10 μm.)

amino acids (ESES; residues 343–346) likely function in ligand-independent CXCR4 internalization. To further characterize which amino acids are required for ligand-independent internalization, we generated alanine substitution mutants for each of the four amino acids in the context of FL and examined their phenotypes (Fig. 4a). Protein expression of mutants was verified in Western blot analysis (Fig. 4a). Among the four mutants, the E343A mutant showed the FL phenotype, while the others displayed the d-44 phenotype in the absence of ligand (Fig. 4b). These data demonstrate that Ser^{344}-Glu^{345}-Ser^{346} constitute the core motif for SDF-1α-independent CXCR4 internalization. Both E345A and S346A mutants exhibited the Thr^{342}-Glu^{343}-Ser^{344} sequence adjacent to the original SES sequence. However, this 'SES-like' motif did not support constitutive internalization, suggesting that Thr cannot substitute for Ser to maintain functionality as a constitutive internalization motif. We reasoned that if such a motif requires an acidic amino acid between two serine residues, changing Glu to Asp should maintain the motif's function. Thus, we constructed a mutant in which Glu was replaced with Asp (E343/345D; Fig. 4a). Also, to determine whether two adjacent SES sequences could augment the FL phenotype, we substituted Ser^{347} with Glu (S347E), creating an additional SES motif next to the original SES one (Fig. 4a). As controls, we created H350E and S347E/H350E mutants (Fig. 4a). Expression of these mutants was verified by Western blot analysis (Fig. 4a). Interestingly, the E343/345D mutant retained the FL phenotype (Fig. 4b), indicating that an acidic residue is required to maintain function of the constitutive internalization motif. S347E showed an intermediate phenotype in which numerous fine GFP-positive vesicles were seen close to the nucleus (Fig. 4b). These data indicate that the two adjacent SES sequences do not augment the FL phenotype but actually interfere with steady-state internalization. Both H350E and S347E/H350E mutants also showed an intermediate phenotype (Fig. 4b), suggesting that more than three acidic amino acids close to the SES motif may inhibit its function, potentially by generating a negative charge cluster. Overall, we conclude that the SDF-1α-independent internalization motif is located at amino acids 344–346 of the CXCR4 cytoplasmic tail.

Finally, we analyzed phenotypes of the S344A mutant in greater detail. Two hours after SDF-1α treatment, cells expressing this mutant showed accumulation of GFP signals at perinuclear regions, similar to the d-17 mutant (Figs. 1a and 4c). FACS analysis revealed that cell surface levels of S344A decreased to $70.8 \pm 11.7\%$ ($n = 7$) following SDF-1α treatment relative to untreated cells, almost as efficient as FL (Fig. 3b). Migratory activity of cells expressing the S344A mutant was stimulated 3.0-fold by SDF-1α, while that of cells expressing FL assayed in parallel showed a 5.8-fold increase relative to untreated cells. These data demonstrate that the S344A mutant, which is defective in constitutive internalization, can undergo ligand-dependent internalization and stimulate migration. Along with the d-17 data, our observations strongly suggest that genetic elements required for the ligand-dependent and -independent internalization are separable.

Discussion

We demonstrated here that CXCR4 is constitutively internalized in the absence of SDF-1α and that steady-state trafficking of CXCR4 is regulated by its cytoplasmic tail. We show that the three amino acid motif, Ser^{344}-Glu^{345}-Ser^{346}, within the cytoplasmic tail is essential for efficient steady-state internalization of CXCR4. Our work indicates that ligand-independent internalization of CXCR4 is genetically separable from ligand-dependent internalization: mutants defective in steady-state internalization (d-17 and S344A) were competent to respond to SDF-1α-promoted internalization signals. That residues required for ligand-dependent endocytosis (Ser324, 325, 330, 338, 339, Ile^{328} Leu^{329} and Lys^{331}; summarized in Fig. 1a)[16–18] do not overlap with those required for ligand-independent internalization, further supports the idea that these activities are separable.

Interestingly, the d-17 mutant displayed SDF-1α-promoted internalization, whereas the d-14 and d-22 mutants did not. These data suggest that an element between amino acids 336 and 342 negatively regulates ligand-initiated CXCR4 internalization. We are currently determining what amino acids are required for that motif. What is unique about the constitutive internalization motif is its position effect in terms of the distance of the motif from the "body" of the receptor. SES-like motifs can be found in the cytoplasmic tails of both CXC-chemokine receptors (for example, CXCR3) and CC-chemokine receptors including CCR2, CCR5 and CCR7. Indeed, these receptors share similar amino acid sequences in which two acidic amino acids (mostly Asp) positioned between the 36th and 45th amino acids of the cytoplasmic tail, where Ser and Thr residues are often in the close proximity to the acidic amino acids but positively charged amino acids, are infrequent. We hypothesize that for the ligand-independent internalization motif to function, the SES motif or its equivalent must be positioned at approximately the 40th residue of the cytoplasmic tail.

Many GPCR, including α1a-adrenoceptor, and the μ-opioid receptor, are spontaneously internalized.[36,37] Therefore, we conclude that various GPCR actively and continuously undergo endocytosis in the absence of ligand in a manner similar to

CXCR4 and hypothesize that the function of constitutive receptor internalization is to fine-tune the threshold at which cells sense ligand. Cells should be able to rapidly post up- and downregulate cell surface levels of CXCR4 using post-translational mechanisms. Such regulation should enable cells to migrate toward SDF-1α-rich tissues as needed and should also prevent inappropriate cells from migrating.

Our work is relevant to cancer cell metastasis and the pathogenesis of WHIM syndrome. Cell surface levels of CXCR4 positively correlate with cancer cells' ability to metastasize.[5,19] We hypothesize that enhanced metastatic capabilities of cancer cells could be due in part to mutations that disrupt the function of SES motif, which would result in upregulation of cell surface levels of signal-competent CXCR4 (as exemplified by the E344A mutant). As for WHIM syndrome, it was recently reported that it is due to mutations within CXCR4's cytoplasmic domain.[14,38] Interestingly, these mutations result in loss of SES motif. We predict that loss of the SES motif should increase cell surface CXCR4 levels. Although CXCR4 mutations generated here are not identical to reported WHIM mutations, the d-10 mutant resembles mutations seen in WHIM syndrome, and it exhibits enhanced basal cell migratory activity. Increased cell surface CXCR4 or increased migratory potential may contribute to WHIM pathogenesis. The response of d-10-expressing cells to SDF-1α, however, was not as robust as that of cells derived from WHIM.[39,40] This discordance may be partly due to the cell type differences, as we have employed a glioblastoma cell line for our studies.

Thus, CXCR4 is a potentially important therapeutic target not only for cancers but for other conditions such as HIV-1 infection, chronic autoimmune disease, and genetic disorders including WHIM syndrome. CXCR4 also plays critical roles in embryogenesis, homeostasis and inflammation. Although there are potential caveats for treating cancer with CXCR4 antagonists, our data furthers the understanding of mechanisms regulating CXCR4 and could be useful in devising therapeutic strategies.

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Structure-activity relationship study of CXCR4 antagonists bearing the cyclic pentapeptide scaffold: identification of the new pharmacophore†

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A highly potent CXCR4 antagonist **2** [cyclo (-D-Tyr[1]-Arg[2]-Arg[3]-Nal[4]-Gly[5]-)] has previously been identified by screening cyclic pentapeptide libraries that were designed based on pharmacophore residues of a 14-residue peptidic CXCR4 antagonist **1**. In the present study, D-Tyr and Arg in peptide **2** were replaced by a bicyclic aromatic amino acid and a cationic amino acid, respectively, and their binding activity for CXCR4 was evaluated for identification of the novel pharmacophore.

Introduction

The chemokine receptor CXCR4 is a membrane protein, which belongs to the G-protein coupled receptor family.[1,2] Interaction of CXCR4 with its endogenous ligand stromal-cell derived factor-1α (SDF-1α)/CXCL12 induces various physiological functions: chemotaxis,[3] angiogenesis,[4,5] neurogenesis,[6,7] *etc.* in embryonic stage. On the other hand, CXCR4 is also relevant to multiple diseases: AIDS,[8,9] cancer metastasis,[10] progress of leukemia,[11] rheumatoid arthritis,[12] *etc.* in adulthood. Actually, CXCR4 has been reported to be a potential drug target against these diseases. Thus, CXCR4 antagonists are useful for development of potent therapeutic agents against these diseases.[13-15] To date, various CXCR4 antagonists such as AMD3100[16,17] and KRH-1636[18] have been reported.

A β-sheet-like 14-residue peptide **1** was previously identified by structure optimization of an 18-residue cyclic peptide polyphemusin isolated from horseshoe crabs (Fig. 1).[19,20] In the downsizing of **1**, a cyclic pentapeptide **2** was developed by screening libraries based on four pharmacophore residues [Arg, Arg, 3-(2-naphthyl)alanine (Nal), D-Tyr] found by alanine scanning of **1**.[21]

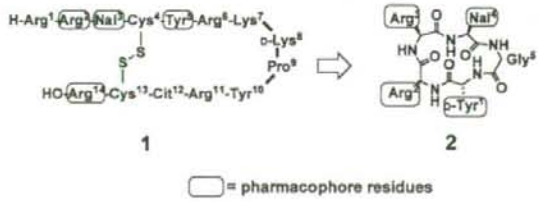

Fig. 1 Development of a cyclic pentapeptide **2** based the pharmacophore of a CXCR4 antagonistic peptide **1**. Cit = L-citrulline, Nal = L-3-(2-naphthyl)alanine.

We have studied structure-activity-relationships of **2** by various modifications.[22,23] In this paper, design of cyclic pentapeptide library based on the previous structure-activity relationship data led to development of novel analogues of **2** to explore new pharmacophore moieties.

Biological results and discussion

Substitution of a large aromatic amino acid for D-Tyr[1] of 2

Our previous data of alanine-scanning of **2** suggested that D-Tyr[1] or Arg[2] was not optimalized.[24] Thus, we attempted to replace these functional groups. According to other previous reports, potent CXCR4 antagonists absolutely contain aromatic and cationic groups.[25] It suggests that these functional groups are involved in binding to CXCR4 mediated by hydrophobic and electrostatic interaction. To evaluate significance of the hydrophobic interaction by aromatic rings, D-Tyr[1] of **2** was replaced by an L/D-bicyclic aromatic amino acid. In addition, four epimers were synthesized to evaluate effects of configuration of amino acids of the 1- and 2- positions (Fig. 2). Compounds **3c** and **3d** with replacement of D-Tyr[1] by D-3-(1-naphthyl)alanine (D-Nal(1)) showed high CXCR4 binding activity (IC_{50} = 0.043 and 0.078 μM, respectively, Table 1), although the potencies were approximately one-third or fifth of that of the parent compound **2** (IC_{50} = 0.015 μM, Table 1). Similarly, compounds **5c** and **5d**, replaced by D-Trp at the 1-position, showed 5–10 fold lower CXCR4 binding activity (IC_{50} = 0.15 and 0.070 μM, respectively, Table 1) than the parent compound **2**. On the other hand, compounds **4c** and **4d** did not show strong CXCR4 binding activity. These data indicates that the spatial position of aromatic ring is essential for the expression of CXCR4 binding activity. In addition, a series of **a** or **b** except for **5a** did not show strong CXCR4 binding activity (all IC_{50} values > 0.3 μM, Table 1). These data indicate that the chirality of L/D-Arg[2] was not important for the expression of CXCR4 binding activity, whereas the chirality of Nal(1)[1] and Trp[1] is influential. The

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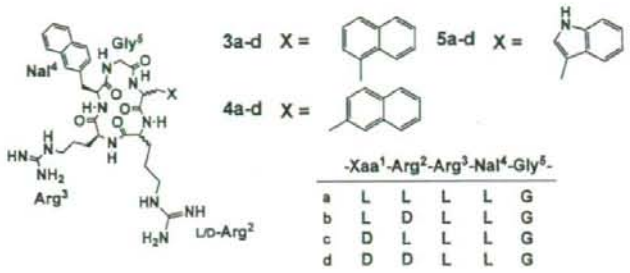

Fig. 2 Structures of compounds having substitution of an L/D- bicyclic aromatic amino acid for Tyr[1].

Table 1 Inhibitory activities of the synthetic compounds against binding of [^{125}I]-SDF-1α to CXCR4

Compound no.	IC_{50}/μM[a]	Compound no.	IC_{50}/μM[a]
2	0.015	**3c**	0.043
3a	0.3–2.0	**4c**	> 2.0
4a	0.3–2.0	**5c**	0.15
5a	0.22	**3d**	0.078
3b	0.3–2.0	**4d**	0.3–2.0
4b	0.3–2.0	**5d**	0.070
5b	> 2.0		

[a] IC_{50} values are the concentrations for 50% inhibition of the [^{125}I]-SDF-1 binding to Jurkat cells. All data are the mean values for at least three experiments.

Table 2 Inhibitory activities of the synthetic compounds against binding of [^{125}I]-SDF-1α to CXCR4

Compound no.	IC_{50}/μM[a]	Compound no.	IC_{50}/μM[a]
2	0.015	**6c**	0.3–2.0
6a	> 2.0	**7c**	0.3–2.0
7a	0.3–2.0	**8c**	> 2.0
8a	> 2.0	**6d**	> 2.0
6b	> 2.0	**7d**	0.3–2.0
7b	0.045	**8d**	0.3–2.0
8b	> 2.0		

[a] IC_{50} values are the concentrations for 50% inhibition of the [^{125}I]-SDF-1 binding to Jurkat cells. All data are the mean values for at least three experiments.

dependence of CXCR4 binding activity on the chirality at the 1-position might be caused by a conformational change of the peptide backbone.

Shuffling cationic and aromatic amino acids at the 1- and 2-positions of cyclic pentapeptides

An analogue of **2**, having substitution of Arg[1] and D-4F-phenylalanine[2] for D-Tyr[1] and Arg[2], respectively, was recently found as a strong CXCR4 antagonist.[22] To evaluate effects of the sequential difference of cationic and aromatic groups at the 1- and 2-positions on CXCR4 binding activity, Arg and a large aromatic amino acid (Nal(1), Nal or Trp) were shuffled in the pentapeptide, and four epimers were synthesized in a similar manner (Fig. 3). Synthetic compounds except for **7b** did not show CXCR4 binding activity up to 0.3 μM (Table 2). In particular, a series of **6** and **8** did not show CXCR4 binding activity despite of difference of the chilality of amino acids at the 1- and 2-positions (**6c**, **8d** > 0.3 μM, **6a**, **6b**, **6d**, **8a**, **8b**, **8c** > 2.0 μM). On the other hand, a series of **7**, which introduced L/D-Nal at the 2-position, did not show a serious reduction of CXCR4 binding activity. These data indicated that Nal(1) or Trp might not be appropriate as the amino acid introduced at the 2-position, possibly due to spatial configuration of aromatic rings. **7b** showed the highest CXCR4 binding activity among compounds in this library. Interestingly, **7b** has the opposite chirality and order of the aromatic residue at the 1- and 2-positions compared to the parent compound **2**.

Evaluation of anti-HIV activity and cytotoxicity

Anti-HIV activity and cytotoxicity of compounds **5c**, **5d** and **7b** that showed moderate CXCR4 binding activity and have a characteristic sequence and conformation were evaluated. Since CXCR4 is a coreceptor for an X4-HIV-1 entry, CXCR4 antagonists have anti-HIV activity.[8,9] Anti-HIV activities of compounds **5d** and **7b** (EC_{50} = 0.19 and 0.26 μM, respectively, Table 3) were nearly equal

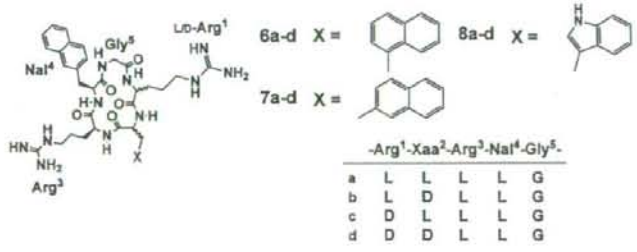

Fig. 3 Structures of compounds having L/D-Arg[1] and an L/D-bicyclic aromatic amino acid.[2]

Table 3 Anti-HIV activity and cytotoxicity of the synthetic compounds

Compound no.	$EC_{50}/\mu M^a$	$CC_{50}/\mu M^b$
AZT	0.077	> 10
1	0.044	> 10
2	0.15	> 10
5c	0.70	> 10
5d	0.19	> 10
7b	0.26	> 10

[a] EC_{50} values are based on the inhibition of HIV-induced cytopathogenicity in MT-4 cells. [b] CC_{50} values are based on the reduction of the viability of MT-4 cells. All data are the mean values for at least three experiments.

to that of **2** (EC_{50} = 0.15 μM, Table 3). Interestingly, CXCR4 binding activity of **5d** (IC_{50} = 0.070 μM, Table 1) was lower than that of **7b** (IC_{50} = 0.045 μM), whereas anti-HIV activity of **5d** (EC_{50} = 0.19 μM, Table 3) was slightly higher than that of **7b** (EC_{50} = 0.26 μM). In addition, all tested compounds did not show significant cytotoxicity (CC_{50} > 10 μM, Table 3).

Conclusion

Our first approach screening cyclic pentapeptides, which have substitution of a bicyclic aromatic amino acid at the 1-position, disclosed that D-3-(1-naphthyl)alanine and D-Trp at the 1-position might be alternative pharmacophore moieties, and that introduction of D-amino acid at the 1-position was required to form an optimal cyclic pentapeptide backbone. In addition, compound **5d** showed high anti-HIV activity, comparable to that of compound **2**.

A cyclic pentapeptide library based on shuffling cationic and aromatic amino acids at the 1- and 2-positions of compound **2** was designed. As a result, the order of a cationic amino acid and an aromatic amino acid is significant to maintain strong CXCR4 binding activity of analogues of **2**. Compound **7b**, however, showed the highest CXCR4 binding activity among the present synthetic cyclic pentapeptides. **7b** was proven to be a new type lead, because of the difference of the order of cationic and aromatic residues, and also showed high anti-HIV activity. Finding of compound **7b** indicated that Arg^1 and $D\text{-}Nal^2$ may be novel pharmacophore moieties in the combination with Nal^4 and Arg^5. To date, pharmacophore functional groups have been identified to be two guanidino, naphthyl and phenol groups derived from two Arg, Nal and D-Tyr in the cyclic pentapeptide scaffolds. In this study, only guanidino and naphthyl groups have been proven to be indispensable for CXCR4 binding activity. The present data will provide useful approaches for simple designs of new low molecular weight CXCR4 antagonists. These results might also give valuable insights for understanding the ligand-receptor interactions.

Experimental

Chemistry

Cyclic peptides were synthesized by Fmoc-based solid-phase synthesis on 2-Chlorotrityl resin followed by cleavage from the resin, cyclization with the diphenylphosphoryl azide and deprotection, as reported previously.[21]

Cell culture

Human T-cell lines, Jurkat cells and MT-4 cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum.

Virus

An X4 HIV-1 infectious molecular clone pNL4-3 was obtained from the AIDS Research and Reference Reagent Program.[26] The virus NL4-3 was obtained from the culture supernatant of 293T cells transfected with the pNL4-3. Aliquots of the viral stocks were stored at −80 °C until use. The titer of virus stocks was determined by endpoint titration of 5-fold limiting dilutions in MT-4 cells.

CXCR4 receptor binding assay

Jurkat cells were harvested and centrifugated at 1000 rpm for 5 min. Cells were then resuspended in RPMI buffer (20 mM HEPES, 0.5% bovine serum albumin) and placed in silicone-coated tubes (5.0×10^5 cells/120 μL). Cold SDF-1 (final concentration 1 μM, 15 μL/well) and various concentrations of test compounds (10% DMSO, 15 μL/well) were added to the above tubes followed by addition of [^{125}I]-SDF-1 (Perkin-Elmer Life Sciences, 0.05 nM, 15 μL/well). After 1 h's incubation on ice, oil (dibutyl phthalate:olive oil = 4:1 (v/v), 500 μL/well) was added followed by centrifugation at 14,000 rpm for 2 min. After removal of aqueous and organic layers and cutting the bottoms from the tubes, the bottoms were placed in RIA-tubes and the CPM was counted by γ-counter. Inhibition percentage of FC131 analogs against the binding of [^{125}I]-SDF-1 was calculated by the following equation.[27]

Inhibition (%) = (Et–Ea)/(Et–Ec) × 100

Et: the quantity of radioactivity in the absence of a test compound

Ec: the quantity of radioactivity in the presence of cold SDF-1α as a test compound

Ea: the quantity of radioactivity in the presence of a test compound

Anti-HIV assay

Anti-HIV-1 activity was determined based on the protection against HIV-1-induced cytopathogenicity in MT-4 cells. Various concentrations of test compounds were added to HIV-1 infected MT-4 cells at multiplicity of infection (MOI) of 0.001 and placed in wells of a flat-bottomed microtiter tray (2.0×10^4 cells/well). After 5 days' incubation at 37 °C in a CO_2 incubator, the number of viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.

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MINI-REVIEW

Roles of the interactions between Env and Gag proteins in the HIV-1 replication cycle

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List of Abbreviations: AP, adaptor protein; CA, p24 capsid; CT, cytoplasmic tail; DRM, detergent-resistant membrane; EE, early endosome; Env, Env glycoprotein; ER, endoplasmic reticulum; ESCRT, endosomal sorting complex required for transport; HIV-1, human immunodeficiency virus type 1; HTLV-1, human T-cell leukemia virus type 1; LE, late endosome; MA, p17 matrix; MDCK, Madin-Darby canine kidney; M-PMV, Maizon-Pfizer monkey virus; MPR, mannose 6-phosphate receptor; MuLV, murine leukemia virus; MVB, multivesicular bodies; NC, p7 nucleocapsid; $PI(4,5)P_2$, phosphatidylinositol-(4,5)-bisphosphate; PM, plasma membrane; RE, recycling endosome; SIV, simian immunodeficiency virus; TEM, tetraspanin-enriched microdomain; TGN, trans-Golgi network; TIP47, tail-interacting protein of 47 kDa; VLP, virus-like particle; VSV, vesicular stomatitis virus.

Key words
Env glycoprotein, Env incorporation, membrane microdomains, Gag, human immunodeficiency virus type 1.

ABSTRACT

The Env and Gag proteins of HIV-1 are the two major structural proteins of this retrovirus. The interactions between Env and Gag proteins and their regulation in HIV-1 are required for several steps of the replication cycle, involving not only virus assembly, specifically Env incorporation, but also entry steps after virus maturation. A large number of host factors and certain membrane microdomains appear to engage both in transport/trafficking of Env and/or Gag proteins, and in the interactions of these two proteins. The present review briefly summarizes our current knowledge regarding the roles of the interactions between Env and Gag proteins in the virus replication cycle.

The Env and Gag proteins of HIV-1, like those of other retroviruses, are two major structural proteins of the virus. The Env protein is synthesized as a precursor (gp160), glycosylated and oligomerized in the ER. Gp160 is then processed into gp120 and gp41 by a host protease and transported to the cell surface, eventually incorporating into virions to form infectious virus particles. The Gag proteins, however, are synthesized as a polyprotein named $Pr55^{Gag}$ on cytosolic polysomes and transported to PM. $Pr55^{Gag}$ is necessary and sufficient for the assembly of non-infectious VLP. During or shortly after virus release from the virus-infected cells, $Pr55^{Gag}$ is proteolytically cleaved by the HIV-1 protease, yielding the mature Gag proteins MA, CA, NC, and p6. This processing is an essential step for forming mature and infectious virus particles that are able to start the next round of infection. During the late steps of the HIV-1 replication cycle, Env and Gag ($Pr55^{Gag}$) proteins should interact with each other when Env is

incorporated into virions. In contrast, virion maturation and the Env-Gag interaction must be coordinated when the progeny viruses enter into the target cell and initiate a new round of infection. In the present review, we will focus on the roles of the interactions between Env and Gag proteins of HIV-1 during its replication cycle, especially Env incorporation and virus entry steps.

SYNTHESIS AND TRANSPORT/TRAFFICKING OF ENV

Human immunodeficiency virus type 1 Env is synthesized as a 160-kD precursor on ribosomes associated with the ER. This precursor protein (gp160) is cotranslationally glycosylated and oligomerized before going through the Golgi complex. Further trimming of oligosaccharide chains, resulting in the formation of mature N-linked oligosaccharide chains, is performed from the high-mannose core precursor during the Env transport in the Golgi complex. The majority of oligomeric Env is trimeric. Gp160 is processed or cleaved by a cellular enzyme, furin, or other enzymes within or near the TGN to generate the mature surface glycoprotein (SU) (gp120) and transmembrane protein (TM) (gp41), both of which form a complex through a non-covalent interaction. This Env processing is essential for transport of the Env complex to PM and membrane fusion induced by the Env (1).

The gp120-gp41 complex undergoes endocytosis after reaching the cell surface. Several lines of evidence indicate that a membrane-proximal, Tyr-based (YXXL) sequence in the gp41 CT and analogous motifs are responsible for rapid internalization of HIV-1, HIV-2, and SIV Env (2, 3). The internalization is mediated by the interaction of the $\mu 2$ chain of the AP-2 clathrin adaptor complexes with the Tyr-based motif (4, 5), although the Tyr-motif can also interact with the $\mu 1$ chain of the AP-1 clathrin adaptor complexes (6). Interestingly, this motif is also critical for basolateral targeting of virus budding in polarized MDCK cells (7). In addition, a C-terminal dileucine motif is considered to mediate endocytosis of HIV-1 Env through interaction with clathrin and AP-2 (8). It has also been reported that the C-terminal dileucine motif affects the cell surface expression and subcellular localization of Env through interaction with AP-1 (9). Rapid internalization of HIV-1 Env could restrict the susceptibility of virus-infected cells to the host immune response to help viral evasion. TIP47 (tail-interacting protein of 47 kDa), which is mainly located in the cytoplasm, has been shown to be necessary for retrograde transport (LE to the TGN) of MPR (10). Like MPR, HIV-1 Env was also reported to bind to TIP47 through its gp41 CT, specifically Y802W803 residues (11). This binding allows HIV-1 Env to be targeted from LE to the TGN. Mutation of this motif in the gp41 CT abolished both TIP47 binding and the targeting of the Env to the TGN. As we discuss in detail below (12), the interaction between HIV-1 Env and TIP47 is also thought to be required for Env incorporation into virions. Although targeting of HIV-1 Env to the PM through intracellular CTLA-4-containing secretory granules has been reported (13), little is known about how the HIV-1 Env is transported from the TGN to the PM. Current understanding of intracellular trafficking of Env and Gag proteins is illustrated in Figure 1.

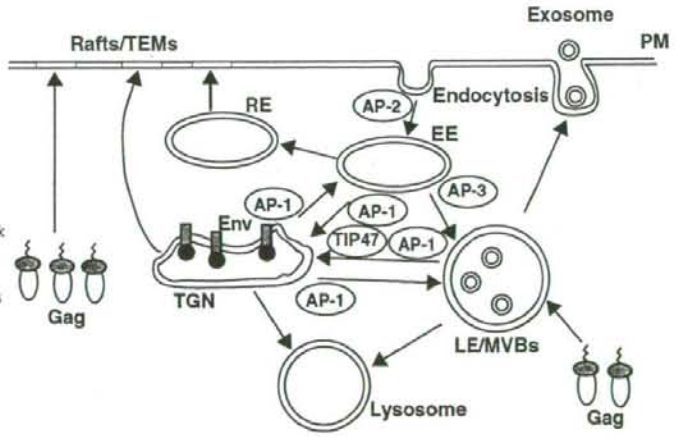

Fig. 1. Sorting pathways for assembly and release of HIV-1. Env proteins are transported from the TGN to the PM, undergoing endocytosis to the EE. The Env could traffic back to the PM through the RE. Gag proteins are targeted to the PM directly or through the LE/MVB. A large number of host factors such as AP complexes, and TIP47 are thought to be involved in trafficking of Env and/or Gag proteins. Membrane microdomains at the PM such as lipid rafts and TEM appear to be a scaffold for virus assembly, including Env-Gag and Gag-Gag interactions.

SYNTHESIS AND TRAFFICKING OF GAG

Like other retroviruses, HIV-1 Gag proteins play an essential role in virus assembly and release. The Gag proteins themselves are necessary and sufficient for the formation of VLP. The $Pr55^{Gag}$ consists of p17 MA, p24 CA, p7 NC, p6, and two spacer peptides, SP1 (originally called p2) and SP2 (originally called p1). Gag proteins are involved in the following retrovirus steps: (i) membrane binding and PM targeting; (ii) Gag-Gag multimerization; (iii) incorporation of the viral genome and Env proteins into virions; and (iv) budding and release of virions (14, 15). The N-terminal myristate and a cluster of highly basic residues in MA are required for membrane binding of Gag. As will be discussed later, these basic residues are also involved in PM targeting. The region stretching from the C-terminal domain of the CA to part of the NC is considered to be important for Gag-Gag multimerization. HIV-1 budding and release are dependent on the interaction between p6 and host proteins, named ESCRT (endosomal sorting complex required for transport)-I, -II, -III complexes (16–18), which mediate delivery of cargo proteins into the lumen of the lysosome for degradation.

Gag proteins are synthesized as a precursor named $Pr55^{Gag}$ on cytosolic polysomes, and eventually targeted to the PM. Two models have been proposed for intracellular trafficking of HIV-1 Gag to the PM (Fig. 1). In one model, HIV-1 Gag or virus particle is targeted to the PM through MVB/LE. Although HIV-1 Gag is mainly localized in the PM in most of the cell types, many studies have shown that Gag and virus particles are observed in MVB/LE (19–23). One of the above studies demonstrated that newly synthesized Gag proteins appear in MVB/LE before trafficking to the PM (22). In this model, Gag assembly and HIV-1 release occur at the PM if fusion of MVB with the PM is rapid, whereas the virus assembles and buds into MVB if targeting of Gag to the PM is slow (24). It is noteworthy that other retroviruses such as HTLV-1, MuLV, and M-PMV use MVB and other endosomal compartments for trafficking of Gag proteins to the PM (25–28). In contrast, another model proposes that HIV-1 Gag or virus particles are directly targeted to the PM, probably via interaction between the MA of $Pr55^{Gag}$ and the lipid $PI(4,5)P_2$ (29, 30) without being routed through the MVB/LE. The following two independent results demonstrate that $PI(4,5)P_2$ is a key lipid that regulates HIV-1 Gag targeting to the PM (29). (i) Reduction of cellular $PI(4,5)P_2$ levels inhibits Gag localization to the PM and HIV-1 production. (ii) Induction of $PI(4,5)P_2$-rich intracellular vesicles alters Gag localization from the PM to the endosomal compartment, resulting in reduced HIV-1 release. By using fluorescent imaging of HIV-1 Gag in living cells, nascent Gag is shown mostly to accumulate rapidly at the PM in HeLa and Jurkat T cells (31). A MA mutant that is defective in PM targeting shows a marked defect in virus production while accumulating its Gag proteins in MVB in HeLa cells (21). Furthermore, recent reports using several complementary approaches demonstrate that HIV-1 assembly/release occurs primarily at the PM, but not at MVB/LE in both 293T cells and primary macrophages (32, 33). A novel ultrastructural approach also showed that in primary macrophages, HIV-1 assembles on an intracellular PM domain, which contains the tetraspanin marker proteins and is connected to the cell surface (34).

Several AP complexes such as AP-1 (35), AP-2 (36), and AP-3 (37), which are involved in endocytic pathways, are reported to be important for subcellular localization of Gag and virus release. AP complexes are cytosolic protein complexes that play a major role in the 'post-Golgi network' including both the secretory and endocytic pathways (38, 39). MA, MA-CA junction, and H1 helix of MA are responsible for interactions with AP-1, AP-2, and AP-3, respectively, emphasizing the importance of MA regions in Gag targeting. Rab9, a small GTP-binding protein, has been reported to affect Gag localization and HIV-1 release, whereas another small GTP-binding protein, Rab11a, is also required for HIV-1 replication, although how this protein affects virus replication remains unknown (40). In addition, a ubiquitin ligase POSH is considered to be involved in HIV-1 release and Gag localization to the TGN (41). ABCE1/HP68, which was reported to be required for capsid assembly in murine cells (42), has been recently found to associate with HIV-1 Gag at the PM in simian cells until the onset of virus maturation (43). The double-stranded RNA-binding protein, Staufen 1, is also considered to play a role not only in genomic RNA trafficking but also in virus assembly by altering Gag-Gag multimerization (44, 45). Thus, a large number of host factors are involved in localization and/or trafficking of Gag, and release of HIV-1 particles.

ROLE OF ENV-GAG INTERACTIONS IN ENV INCORPORATION INTO VIRIONS AND VIRUS RELEASE

Lentiviruses, including HIV-1 and SIV, have TM protein (gp41 for HIV-1) with long CT compared with oncoretroviruses whose CT are 20–30 amino acid residues in length. The mechanism by which HIV-1 Env is incorporated into virions that are released from virus-infected cells is still not well understood. Several biochemical and genetic studies suggest that interaction between the gp41 CT of HIV-1 Env and MA is required for active incorporation. (i) Mutations in the MA region can block the Env incorporation into virions (46–48), whereas this incorporation defect can be restored by pseudotyping with heterologous Env

glycoproteins such as MuLV Env and vesicular stomatitis virus (VSV)-G or by removing the gp41 CT (47, 49, 50). (ii) Small deletions or truncations of the gp41 CT cause a defect in Env incorporation, and a single amino acid change in MA restores the defect (51). (iii) HIV-1 Env directs Gag budding to the basolateral surface of polarized epithelial MDCK cells (7, 52, 53). It has also been shown that the Tyr-based motif is involved in polarized budding of HIV-1 in T cells (54). (iv) The gp41 CT is essential for efficient Env incorporation into virions in most cell lines and in primary cell types such as human peripheral blood mononuclear cells (PBMC) and monocyte-derived macrophages (MDM), although some cell lines, including HeLa or MT-4 cells, are tolerant of gp41 CT truncation (55, 56). (v) Coexpression of $Pr55^{Gag}$ suppressed endocytosis of HIV-1 Env mediated by the gp41 CT (57). (vi) Association of HIV-1 Env with detergent-resistant membranes was mediated by interaction with $Pr55^{Gag}$ (58). (vii) Direct interaction between HIV-1 MA and gp41 CT has been demonstrated in an *in vitro* system (59). Thus, it is evident that the gp41 CT is essential for the incorporation of full-length HIV-1 Env into virions. The cell-type dependent requirement of the gp41 CT suggests that some host factors are involved in the interaction of the gp41 CT and MA and play a role in Env incorporation. Indeed, it has been argued that a host cytosolic protein, TIP47, plays roles in trafficking of Env (11) as well as bridging the gp41 CT of Env and Gag (12). Therefore, TIP47 is considered to be one of the host factors that are required for HIV-1 Env incorporation into virus particles. Although TIP47 also appears to be involved in Env retrograde transport from LE to TGN, suggesting that Gag and Env could meet on LE or TGN, where and how the trimolecular complexes are formed among Env, Gag, and TIP47 remains to be determined.

There are several questions as to the mechanism by which HIV-1 Env is incorporated into virions. (i) Are there any host factors that are required for Env incorporation in concert with or without TIP47? (ii) Where and how do Env and Gag proteins meet and assemble into HIV-1 particles? Env proteins are synthesized in the ER, processed in the Golgi, and transported to the PM, whereas Gag proteins are synthesized on free ribosomes in the cytosol, and transported to the inner phase of the PM. It has been suggested that complexes containing Env, Gag, and viral genomic RNA are preformed on endosomes before virus budding in the case of MuLV (25). Although it appears that HIV-1 Env directs virus release in polarized epithelial cells (52) and in rat neurons (60), accumulating evidence suggests that HIV-1 Env and Gag can interact intracellularly but assemble into virions only at the surface of cells. Several studies demonstrated that insertion of an ER retention signal at the C-terminus of HIV-1 Env or treatment with brefeldin A did not affect virus release without Env incorporation (61–63), suggesting lack of a functional interaction between Env and Gag proteins at an early step in Env transport. A study using SIV Env and Gag also shows that both proteins can interact intracellularly but assemble into virions only at the PM (64). In addition, strong colocalization of Gag and Env is observed at the PM using high-resolution confocal microscopy, suggesting that direct recruitment of Env by $Pr55^{Gag}$ to the assembly sites occurs at the PM (65). These results suggest that Env and Gag proteins could interact at the PM or on endosomes (or transport vesicles).

ROLE OF MEMBRANE MICRODOMAINS IN ENV-GAG INTERACTIONS AND HIV-1 ASSEMBLY

Cellular membranes of the intracellular organelles or PM are not homogeneous lipid bilayers but rather contain various lipid microdomains with distinct lipid and protein compositions. One type of microdomain that is highly rich in sphingolipids and cholesterol is called 'lipid raft(s)'. It is considered that eukaryotic cells use this microdomain as a scaffold for assembly of various molecules by which the cells perform several biological processes such as intracellular transport and signal transduction (66–68). Furthermore, enveloped viruses such as HIV-1 also use lipid rafts for their replication cycle, especially their entry and assembly (for review of lipid rafts and virus replication, see (69)).

It has been shown that HIV-1 envelopes contain relatively high amounts of sphingoglycolipids and cholesterol compared with the host plasma membrane (70, 71), suggesting that HIV-1 assembles in certain membrane domains, probably lipid rafts. Several lines of evidence support this hypothesis. HIV-1 Env proteins have been detected in detergent-resistant membrane (DRM) fractions (72, 73). It has been reported that palmitoylation of the two Cys residues in the gp41 CT is responsible for DRM association and Env incorporation (74). However, more detailed biochemical and virological analyses showed that Cys residues were not essential for Env incorporation and virus infectivity, suggesting that palmitoylation of the gp41 CT is not critical for virus replication (75, 76). The majority of Gag proteins have also been recovered in the DRM fractions (72, 73, 77–80). In addition, PM rafts are critical for HIV-1 assembly and release (80). Substitution of myristic acid for unsaturated fatty acids inhibits VLP production without altering the membrane-binding capacity of Gag (81). It has recently been demonstrated that depletion of cholesterol impairs membrane binding and high-order multimerization Gag (82). In terms of a role of lipid rafts in Env-Gag interactions, observation by

confocal microscopy demonstrated that HIV-1 Env and Gag on the cell surface colocalized with raft markers such as CD59 and GM1 in the virus-infected or transfected cells, but did not colocalize with non-raft markers like CD45 (72, 78). More recently, association of HIV-1 Env with DRM was found to be mediated by interaction with $Pr55^{Gag}$ (58). Importantly, a mutation in the matrix region that causes a defect in Env incorporation in $Pr55^{Gag}$ eliminated Env association with DRM. In addition, mutations in the gp41 CT that abrogate Env incorporation also eliminated Env association with DRM. These results suggest that Gag recruits Env into lipid raft domains where Env and Gag interact for Env incorporation. Thus, lipid rafts appear to be a membrane microdomain that is essential for assembly (interactions not only between Gag and Gag but also between Env and Gag) and release of HIV-1.

Tetraspanins are a superfamily of cell-surface proteins that are expressed in a variety of tissues and cell types. Members of this family form large integrated complexes for signal transduction and immune response called 'tetraspanin-enriched microdomains (TEM)' in association with various molecules (83). Several lines of evidence suggest that tetraspanins are involved in the infection of several viruses such as hepatitis C virus (HCV), HTLV-1, and HIV-1 (84). Furthermore, it has been suggested that TEM are involved in virus assembly/release of HIV-1 (85, 86). Colocalization was observed between a surface TEM marker such as CD63, and not only HIV-1 Env/Gag, but also ESCRT-I components, TSG101 and Vps28. Further studies will determine how HIV-1 proteins are recruited to the TEM and the relationship between lipid rafts and TEM.

ROLE OF ENV-GAG INTERACTIONS IN HIV-1 ENTRY

During or shortly after virus budding from the infected cells, HIV-1 undergoes virus maturation by proteolytic cleavage with viral protease. The Gag and Gag-pol proteins are processed into mature virion proteins MA, CA, NC, p6 (from Gag), protease (PR), reverse transcriptase (RT), and integrase (IN) (from pol). Virus maturation converts non-infectious virus particles with electron-lucent cores to infectious particles with electron-dense, conical cores. It has been assumed that immature virions are non-infectious (87–89) and inhibitors of viral protease are widely used clinically to treat HIV-1-infected patients. However, the nature of the infectivity block has not been well addressed, although previous findings suggested that it appears to be an uncoating step (90). Functional interaction between the gp41 CT and the MA region of $Pr55^{Gag}$ has been suggested by a large number of studies, many of which are discussed above. In addition, it has also been suggested that 'detergent-treated virions (viral cores)' contain substantial amounts of $Pr55^{Gag}$ and gp41, and that the gp41 CT mediates this interaction (91). The growing evidence indicates that the CT of retroviral Env glycoproteins affects virus-cell or cell-to-cell fusion. Cleavage of the Env CT by viral protease after virus release activates Env fusogenecity in certain retroviruses such as MuLV and M-PMV, suggesting cooperative regulation of membrane fusion and virus maturation (92–94). Furthermore, truncation of the Env CT causes enhanced fusion activity not only in HTLV-1 (95) but also in SIV (96, 97) and HIV-1 (49, 98, 99). These results raise the possibility that HIV-1 Env-mediated membrane fusion is modulated by Gag processing instead of cleaving the Env CT, although HIV-1 could also activate fusogenecity by cleaving the CT under certain conditions where a cholesterol-binding compound inhibits HIV-1 entry (100). Indeed, it has been shown that immature HIV-1 virions are repressed for membrane fusion with target cells through the interaction with the gp41 CT, indicating that interaction between the gp41 CT and $Pr55^{Gag}$ couples HIV-1 fusion to virus maturation (Fig. 2) (101–103). The fusion defect by protease-negative virions was found to be due to defects in neither CD4 binding nor six-helix bundle formation (102). A recent report using a panel of gp41 CT mutants further dissected the regions responsible for the fusion repression from those involved in the interaction of the gp41 CT and $Pr55^{Gag}$, demonstrating that removal of 28 or more amino acids relieves the dependence of HIV-1 fusion on maturation (104). Thus, stable association of the gp41 CT and $Pr55^{Gag}$ in immature virions is required but not sufficient for inhibition of fusion by immature virions. Interestingly, immature particles are reported to be more rigid than mature ones and truncation of the gp41 CT reduced the stiffness of immature virions to that of mature virions (105). Further studies are necessary to elucidate the mechanism of virus fusion modulated by the gp41 CT and Gag processing.

CONCLUSIONS

In the present review, we discussed studies indicating that interactions between Env and Gag proteins are essential for Env incorporation into infectious HIV-1 virions, although Gag proteins are necessary and sufficient for formation of non-infectious VLP. Studies so far demonstrate that at least one host protein, TIP47, is required for the Env-Gag interaction during Env incorporation. Furthermore, virus maturation and fusogenecity of the HIV-1 Env are temporally and spatially regulated through the Env-Gag interaction. However, several questions still remain unanswered. In HIV-1, where and how do Env and Gag proteins assemble into infectious virions with genomic RNAs? What types of host factors are required for the Env-Gag

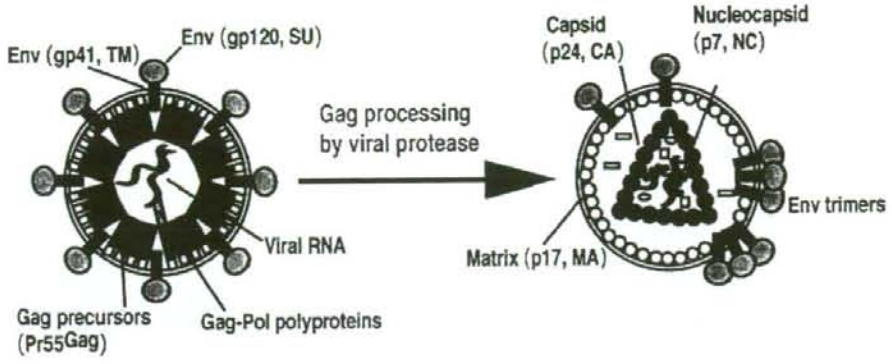

Fig. 2. Possible model for coupling of virus-cell fusion to virus maturation. Gag processing allows Env trimers to diffuse and cluster in the plane of the viral membrane to form fusion-active, higher-order complexes.

interactions and Env incorporation with or without TIP47? Which membrane microdomains, lipid rafts or TEM or both, are highly involved in virus assembly, including Gag-Gag and Env-Gag interactions? Which stage of virus-cell fusion is regulated via Gag processing and the gp41 CT? Further studies to answer these and related questions will provide not only important virological findings but also useful information to develop anti-HIV-1 drugs with new modes of action.

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