Annexin 2: a Novel Human Immunodeficiency Virus Type 1 Gag Binding Protein Involved in Replication in Monocyte-Derived Macrophages

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Human immunodeficiency virus (HIV) replication in the major natural target cells, CD4⁺ T lymphocytes and macrophages, is parallel in many aspects of the virus life cycle. However, it differs as to viral assembly and budding, which take place on plasma membranes in T cells and on endosomal membranes in macrophages. It has been postulated that cell type-specific host factors may aid in directing viral assembly to distinct destinations. In this study we defined annexin 2 (Anx2) as a novel HIV Gag binding partner in macrophages. Anx2-Gag binding was confined to productively infected macrophages and was not detected in quiescently infected monocyte-derived macrophages (MDM) in which an HIV replication block was mapped to the late stages of the viral life cycle (A. V. Albright, R. M. Vos, and F. Gonzalez-Scarano, *Virology* 325:328–339, 2004). We demonstrate that the Anx2-Gag interaction likely occurs at the limiting membranes of late endosomes/multivesicular bodies and that Anx2 depletion is associated with a significant decline in the infectivity of released virions; this coincided with incomplete Gag processing and inefficient incorporation of CD63. Cumulatively, our data suggest that Anx2 is essential for the proper assembly of HIV in MDM.

Tissue macrophages, including microglia of the central nervous system, play a vital role in the establishment and maintenance of human immunodeficiency virus type 1 (HIV-1) infection. Furthermore, because macrophages appear to be less susceptible to HIV's cytopathic effects (14, 34) and can harbor virus for long periods of time, these cells are important potential reservoirs for infection, even after effective antiretroviral therapy has reduced the level of virus in peripheral blood lymphocytes (PBLs) and other susceptible CD4⁺ cells (21).

The HIV life cycle has been elucidated largely in primary CD4⁺ T cells and in T cell lines and less so in monocyte-derived macrophages (MDM). Recent insight into cell type-dependent differences in HIV assembly and budding have shown that in macrophages, virus assembly and budding take place at the limiting membrane of late endosomes, also referred to as multivesicular bodies (MVBs) (34, 35, 38); this process differs from that in CD4⁺ T lymphocytes (T cells), where HIV assembles and buds at the plasma membrane (20, for a review, see reference 27).

HIV assembly is known to be a highly ordered process (for a review, see reference 2) driven by the polyprotein precursor p55^{Gag} (16). Upon virus assembly and maturation, p55^{Gag} is cleaved by a viral protease, generating the structural proteins matrix (p17), capsid (p24), nucleocapsid (p7), and p6 (1). Virus assembly, whether intracellular or plasma membrane associated, localizes to specialized cholesterol-rich, detergent-resistant membrane microdomains known as lipid rafts. In cells supporting HIV budding from the plasma membrane, Gag

targeting to lipid rafts is thought to be modulated by a signal phospholipid, phosphoinositide (4,5)-biphosphate, and phosphoinositide (4,5)-biphosphate depletion results in Gag redistribution to CD63-positive late endosomes in HeLa cells (33). In turn, Gag transport to late endosomal compartments is likely driven by the AP-3 adaptor complex, which is also responsible for trafficking of CD63 itself (9). Interestingly, disruption of AP-3–Gag complexes not only prevents Gag transport to the late endosomes but also suppresses viral production in cell lines supporting plasma membrane HIV budding. These intriguing observations led to the hypothesis that Gag transport to the MVB is an intermediate destination on the way to the plasma membrane (9); however, in some cells types, such as macrophages, Gag docking at endosomal membranes results in virion assembly and subsequent intracellular budding. The basis for the differential localization of assembly remains largely undefined, but it has been proposed that differential binding of unknown cellular factors to HIV Gag products in macrophages in comparison with T cells may be responsible for differences in assembly sites (32).

Previous experiments in our laboratory demonstrated that, under nonactivating culture conditions, cells of the macrophage lineage can be infected with HIV but maintained in a state of low-level viral expression due to a partial block at the late stages of the viral life cycle (3, 4). These results implied a differential interaction between virus and cellular factors at late stages of the viral life cycle in the activated and nonactivated cells and provided a model for the identification of cellular proteins important in virus assembly, budding, or release in MDM. Using this model as a starting point, we have identified a novel interaction between the viral protein p55^{Gag} and annexin 2 (Anx2), an endosomal protein involved in vesicular trafficking and formation as well as exocytosis. We report direct binding between Anx2 and p55^{Gag} and collocation with CD63 in MDM during in vitro infection. Functionally, deple-

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tion of Anx2 during MDM infection resulted in not only a decrease in viral output but also in a defect in maturation and infectivity in those virions that were released from the cell.

MATERIALS AND METHODS

Virus production and detection. HIV-1_{YU-2} viral stock was prepared by transfection of 293 cells with YU-2 proviral genome (RF-1; a gift from M. Malim, King's College, London). Culture media were collected 48 h after transfection, and virus production in the precleared supernatant was measured using p24 enzyme-linked immunosorbent assay (ELISA) as per the manufacturer's instructions (Perkin-Elmer; NEK050); virus titer was determined in a multinuclear-activation galactosidase indicator (MAGI) assay as described previously (4). HIV-1_{JAGO} viral stock was obtained from the Virology Core at the Penn Center for AIDS Research (CFAR).

MDM isolation and culture. Peripheral blood mononuclear cells were derived from concentrated whole blood obtained from the New York Blood Center. Cells were plated at a density of 4×10^6 /well in a six-well plate in Dulbecco's modified Eagle's medium (DMEM) supplemented with glucose, 5% fetal bovine serum, and 5% giant cell tumor supernatant (GCTS) containing granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, interleukin-1 (IL-1), IL-6, plasminogen activator, and collagenase (IGEN, Gaithersburg, MD). Peripheral blood lymphocytes were removed 24 h after plating and stimulated with phytohemagglutinin (1 μ g/ml) for 2 days and IL-2 (30 units/ml). For the small interfering RNA (siRNA) experiments, peripheral blood mononuclear cells or purified monocytes were obtained from the CFAR Immunology Core or from the New York Blood Center and plated at a density of 2.5×10^5 per well in a 24-well plate. Monocytes were differentiated in culture with DMEM with 5% fetal calf serum and 5% GCTS for 8 to 14 days before infection or siRNA treatment. Approximately 80 to 90% of the cells in the differentiated cultures were positive for the macrophages surface marker CD14, with some donor-dependent variability. For Gag binding experiments, MDM were cultured for 3 weeks and then transferred to either activating or nonactivating conditions (DMEM supplemented with 5% fetal calf serum and 5% GCTS or DMEM alone, respectively). After 5 days, MDM were infected with HIV-1_{YU-2} at an multiplicity of infection of 1.

HIV Gag immunoprecipitation. MDM were lysed in 0.5% Triton X-100, and the lysate precleared by mixing with rabbit serum and protein A-coupled agarose beads (Invitrogen) for 1 h. All incubations were performed at 4°C with the samples rotating at 8 rpm. Protein A beads were centrifuged, and rabbit polyclonal anti-Gag antibody (UP598 raised against HIV-1 p24 [a gift from M. Malim, King's College, London] or HIV-1 p17 antiserum [AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH]) was added to the precleared lysates and incubated overnight. Fresh protein A beads were then added to the antibody-lysate mix, left for 1 h, and washed twice with 0.5% Triton X-100. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the gel was fixed, stained with Coomassie brilliant blue R-250 in 47% methanol-7% acetic acid for 1 h, and destained in methanol-acetic acid for 24 h.

Mass spectrometry protein identification. Protein bands were excised and submitted to the Proteomics Core at the Genomics Institute at the University of Pennsylvania. Proteins were digested with trypsin and spotted onto a matrix-assisted laser desorption/ionization (MALDI) plate. A peptide mass fingerprint measurement for each sample was determined using a MALDI-time-of-flight (MALDI-TOF) mass spectrometer (Applied Biosystems Voyager Pro DE MALDI-TOF). For verification of the identified proteins, the trypsin-digested protein sample was analyzed with an LCQ Deca XP Plus mass spectrometer. Turbo-Sequest software converted the structural information from the peptide fragments to amino acid sequence data.

Annexin 2 protein production. Total RNA was isolated from differentiated macrophages by using Trizol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed with oligo(dT) as per the manufacturer's instructions (SuperScript First-Strand Synthesis System for RT-PCR; Invitrogen). The cDNA was amplified by PCR with Anx2-specific primers containing a hexahistidine tag sequence and appropriate restriction sites. The Anx2-encoding fragment was gel purified and cloned into vector pET-11 (Stratagene) under the control of the hybrid Lac operator/T7 promoter, followed by transformation of BL21-CodonPlus-RIL cells (Stratagene). Protein production was induced with IPTG (isopropyl- β -D-thiogalactopyranoside), and the cells were subsequently lysed in buffer containing 50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0, and 1 mg/ml lysozyme by sonication. The lysate was then centrifuged at 10,000 $\times$ g for 30 min, and the supernatant was saved for SDS-PAGE analysis and purification. To purify the protein, the lysate was incubated with a 50% Ni-nitrilotriacetic acid (NTA)

Superflow slurry (QIAGEN) (nickel matrix) and rotated at 4°C overnight at 8 rpm. The mixture was next loaded onto Poly-Prep chromatography columns (Bio-Rad). The matrix was washed twice with buffer containing 50 mM NaH₂PO₄, 300 mM NaCl, and 20 mM imidazole, pH 8.0. Purified His-tagged Anx2 was eluted five times with buffer containing 50 mM NaH₂PO₄, 300 mM NaCl, and 250 mM imidazole, pH 8.0, and the elution fractions were assayed for yield and purity with SDS-PAGE. Fractions enriched in Anx2 were concentrated 14-fold using a Millipore Ultrafree-15 30K filter device. The dilution and centrifugation steps were repeated four times to exchange the buffer and concentrate the purified protein to a final volume of 300 μ l.

Binding studies. For the cell-free binding assay, 2 μ g of purified Anx2-His protein (or 2 μ g of secreted amyloid precursor protein α -His [Sigma]) was mixed with 0.5 μ g of purified HIV-1_{SE2} p55^{Gag} (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) in 400 μ l buffer containing 50 mM NaH₂PO₄, 300 mM NaCl, and 20 mM imidazole, pH 8.0; the samples were incubated for 1 h at room temperature (RT) while rotating at 8 rpm. For binding in the presence of calcium, 100 μ M CaCl₂ was used. Ni-NTA matrix (QIAGEN) was added, and the samples were incubated for 1 h at 4°C while rotating. After centrifugation at 1,000 $\times$ g, the unbound fraction was collected. The matrix was washed four times, and bound proteins were eluted two times with 250 mM imidazole. Obtained fractions were analyzed by Western blotting with anti-Gag and anti-Anx2 antibodies.

For the cell-associated binding assay, 293T cells were transfected with 3 μ g of Anx-His or pCI-gag-PRE (a gift from C. B. Buck, John Hopkins University) or both. Twenty-four to 36 h later, the cells were lysed in 0.5% Triton X-100 and the protein concentration was determined (DC protein assay; Bio-Rad, California). Anx2-Gag complexes were pulled down using Gag immunoprecipitation as described above and analyzed in Western blotting with anti-Anx2 antibody. HIV p24 mouse monoclonal antibodies recognizing both p55^{Gag} and p24^{Gag} (24-4, 24-3, or 24-2; gifts from M. Malim, King's College, London) were used to detect Gag, and a rabbit polyclonal antibody specific for the N terminus of human Anx2 (Santa Cruz) or a mouse monoclonal antibody specific for the C terminus (BD Biosciences) was used to detect Anx2. The housekeeping GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene was used as a loading control and detected with a rabbit polyclonal antibody (AbCam).

Confocal microscopy. HIV-1-infected MDM were grown in Lab-Tek glass chamber slides (Nalge Nunc International), fixed 4 to 7 days after infection with 4% paraformaldehyde, washed with phosphate-buffered saline (PBS), permeabilized either with 0.1% Triton X-100 for 5 min at RT or with methanol for 6 min at -20°C, and blocked in a solution of 10% goat serum. Blocked cells were incubated for 1 h with appropriate dilutions of primary antibodies, followed by subsequent incubation with Alexa-tagged chicken anti-mouse or chicken anti-rabbit secondary antibodies (Alexa Fluor 594 or Alexa Fluor 488; Molecular Probes) for 30 min at RT. The labeled cells were then stained with DAPI (4',6'-diamidino-2-phenylindole) (Molecular Probes) to visualize the nuclei, embedded in Vectashield mounting medium (Vector), and examined in a Zeiss LSM 510 confocal microscope (Carl Zeiss, Inc). Anx2 and CD63 were immunostained with the primary monoclonal antibodies obtained from BD Biosciences, clone 5 and clone H5C6, respectively. Gag was detected with p24 rabbit polyclonal serum no. 4250 (NIH AIDS Research and Reference Reagent Program), and p17 was detected with monoclonal antibody from ABI Advanced Biotechnologies. Statistics were performed using chi-square analysis for each donor.

siRNA treatment. siRNA was designed using the Dharmacon design website and synthesized by Dharmacon. The Anx2-specific target sequence (siAnx) is AAGGACAUUUAUUCGACACA, and nontargeting control siRNA (siScr) consists of a scrambled sequence of siAnx (ACACGAGAUAAUAUCGACUUG). siRNA was delivered intracellularly using Lipofectamine 2000 (Invitrogen, California) as per the manufacturer's instructions. MDM were treated with 200 nM siRNA in the growth medium described above without antibiotics for 4 to 6 h, washed, and refed with normal growth medium without antibiotics. After 3 days, the MDM were infected with HIV-1_{YU-2} in growth medium containing antibiotics at a multiplicity of infection of approximately 1. At subsequent time points, the cells were lysed in 0.5% Triton X-100 for Western blot analysis and the supernatants were assayed by MAGI assay or p24 ELISA. Statistical analysis was performed using a Student *t* test on the p24 and infectious unit values from three independent donors at day 4 after infection.

TEM. MDM were treated with siRNAs in duplicate wells of six-well plastic tissue culture plates (see above). After 3 days, 50 ng of p24 of HIV-1_{JAGO} (obtained from the Penn CFAR) was added to all MDM cultures and left for 16 h. Four days after infection, the cell culture supernatants were assayed for p24 output by ELISA and for infectious virus output by MAGI assay, and the cells were lysed for blotting or prepared for transmission electron microscopy (TEM). For microscopy, the cultures were fixed for 1 h in 2% glutaraldehyde and washed

TABLE 1. Identification of annexin 2 protein bands

Technique	Accession no. or GenInfo identification	Mass (kDa)	pI	Coverage (%) ^c	MOWSE ^d
MALDI-TOF ^a	18645167	38.576	7.6	50.0	1.66E+06
LCQ peptide sequencing ^b	AAH09564.1	38.6181		50.4	

^a Database searching using MS-FIT in ProteinProspector 4.0.4.
^b Database searching using SEQUEST Summary and Flicka programs.
^c Percentage of total protein sequence covered by matched peptides.
^d MOWSE, molecular weight search score.

in PBS, 500 µl of 1% gelatin (Sigma) was added to each well, and the scraped cells were pelleted at 5,000 rpm for 2 min on a tabletop microcentrifuge. The resulting cell pellet was further fixed in 2% osmium, washed in PBS and in water, and then dehydrated in increasing concentrations of ethanol (50% to 100%) followed by propylene oxide. The dehydrated cell pellets were embedded in plastic resin (Epon) for 3 days at 70°C, and 7-nm-thick sections were stained with uranyl acetate. A JEOL JEM 1010 (Tokyo, Japan) transmission electron microscope was used, and digital images were obtained using the computer program AMT 12-HR aided by a Hamamatsu ORCA charge-coupled-device camera (Danvers, Mass.).

Virus capture assay. Whole-virion precipitation was carried out as described previously (35). Briefly, aliquots of cell-free medium collected from infected macrophages at day 4 postinfection were incubated overnight at 4°C with 10 µg/ml anti-CD63 monoclonal antibody (FC-5.01; Zymed), followed by incubation with 30 µl of preblocked *Staphylococcus aureus* cells (10% stock) (Zysorbin; Zymed) for 1 h at RT. To control for nonspecific binding, a parallel precipitation was carried out without anti-CD63 antibody. Cells were pelleted, and the supernatants were assayed for p24 content and infectivity using p24 ELISA and MAGI assay, respectively. The captured p24 was calculated by subtraction of the p24 output remaining unprecipitated in the presence of anti-CD63 antibody from that in the supernatants precipitated without antibody. The efficiency of capture was expressed as the percentage of the value for the no-antibody control.

RESULTS

Identification of Anx2-Gag interaction. To identify host factors interacting with p55^{Gag} during the late stages of the HIV-1 life cycle, we compared the pattern of proteins coprecipitated with Gag from infected activated macrophages, which promote efficient virus release, with that obtained from quiescently infected macrophages in which HIV replication was suppressed posttranslationally (3, 4).

Primary monocytes were differentiated into MDM for 7 to 10 days and then either maintained in activating culture conditions or changed to nonactivating culture conditions as described in Materials and Methods. MDM were then infected 5 days later with an R5 HIV-1 isolate (HIV-1_{YU-2}), and p55^{Gag} polyprotein was immunoprecipitated from lysates of cells grown under either condition with a polyclonal antibody against p24^{Gag}. The specificity of the anti-Gag polyclonal antibody used in this study was confirmed by immunoprecipitation of p55^{Gag} from infected MDM lysates followed by Western blotting (data not shown).

After SDS-PAGE, the proteins that coprecipitated with Gag were visualized by Coomassie blue staining (data not shown). A band at approximately 35 to 40 kDa that was specific for the activating conditions was detected in two independent experiments; it was then identified as annexin 2 by mass spectrometry and LCQ peptide sequencing (Table 1). Additionally, non-muscle myosin II heavy chain 9 was identified as a potential Gag coprecipitant in one donor. However, nonmuscle myosin

II was seen under both activating and nonactivating conditions and was therefore not studied further.

Anx2 interacts with Gag in transfected cells and in a cell-free system. To confirm the mass spectrometry data, we analyzed Gag-coprecipitated products in Western blotting with anti-Anx2 antibody. Macrophages were productively infected either with HIV-1_{YU-2} or, to increase the efficiency of infection, with vesicular stomatitis virus G-pseudotyped pNL4-3. The cell lysates were used in Gag immunoprecipitation as described above, followed by immunoblotting for Anx2. The blot detected Anx2 coprecipitating with Gag from both HIV-

FIG. 1. Anx2 interacts with p55^{Gag} in MDM, transfected 293T cells, and cell-free systems. (a) Anti-Anx2 blot showing that Anx2 coprecipitates with Gag from MDM. Gag was immunoprecipitated from infected and uninfected MDM lysates with p24 rabbit polyclonal antiserum. Anx2 is present in the protein G-bound fraction from infected MDM only. (b) Anti-Anx2 blot showing Anx2-Gag coprecipitation from cotransfected 293T cells. Lysates from 293T cells transfected with a plasmid encoding the YU-2 proviral genome and Anx2-His or Anx2-His alone were precipitated with a rabbit polyclonal antiserum against Gag/p17. Anx2 coprecipitates with Gag in the doubly transfected cells. (c and d) Anti-Gag and anti-Anx2 Western blots. Purified Anx2-His protein precipitated on a nickel matrix after incubation with purified p55^{Gag} specifically coprecipitated p55^{Gag} (c, left panel). When His-tagged amyloid precursor protein was substituted for Anx2, no precipitation of Gag was detected (c, right panel). Incubation in the presence of 100 µM calcium enhanced Anx2-p55^{Gag} binding (d, left panel) without increasing nonspecific binding (d, right panel). Abbreviations: IgG, HC, immunoglobulin G heavy chain; IgG, HL, IgG light chain. B, bound fraction; w, wash. IP, immunoprecipitation; APP, amyloid precursor protein. Molecular masses: Anx2, 36 kDa; Gag/p55, 55 kDa; IgG, HC, 50 kDa; IgG, LH, 25 kDa.

FIG. 2. Reciprocal distribution of Anx2, Gag, and CD63 in macrophages. Infected macrophages were fixed at day 4 after infection and immunostained for Anx2 and Gag (A) or for CD63 and Gag (B); uninfected macrophages were immunostained for Anx2 and CD63 (C) as specified in Materials and Methods. Anx2 and CD63 are shown in red; Gag is shown in green. The insets show additional 2 $\times$ magnification of intracellular vesicles. Bar, 10 μ m.

1_{YU-2}-infected (data not show) and vesicular stomatitis virus G pseudotype-infected (Fig. 1a) MDM.

To further corroborate the interaction between Anx2 and p55^{Gag}, we performed several binding experiments. First, we cotransfected 293T cells with hexahistidine-tagged Anx2 (Anx2-His) cloned from MDM RNA and with HIV-1_{YU-2} proviral plasmid or with Anx2-His alone. Precipitation of the doubly transfected cell lysates with anti-Gag polyclonal serum showed efficient Anx2 coprecipitation (Fig. 1b). However, when the transfection with HIV-1_{YU-2} was omitted, no Anx2 was detected in the bound fraction (Fig. 1b). Anx2-Gag complexes were also detected in doubly transfected cells in an Anx2-His pull-down assay using a nickel-NTA matrix (data not shown).

Since it was possible that the Gag-Anx2 association was mediated by several proteins in a complex, we determined whether Anx2-His and p55^{Gag} could bind directly in a cell-free system. Purified Anx2-His protein was incubated with purified p55^{Gag}, and the bound complexes were precipitated with an Ni-NTA matrix. As demonstrated in Fig. 1c (left panel), elution of Anx2-His with imidazole also released p55^{Gag}, indicating the direct binding between these two proteins; this interaction was considerably enhanced by Ca²⁺ (Fig. 1d, left panel). No precipitated Gag was observed in the control experiments with an unrelated His-tagged protein (secreted amyloid precursor protein α), independent of the presence of Ca²⁺ (Fig. 1c and d, right panels). The specificity of the Gag-Anx2 binding was finally confirmed by immunoprecipitation of the prebound complex using anti-Gag polyclonal antibody (data not shown).

Anx2 colocalizes with Gag and CD63 in intracellular vesicles.

To examine the relative intracellular locations of Anx2 and Gag, we visualized the expression of both proteins in HIV-1-infected MDM by using laser scanning confocal microscopy. Although the staining pattern for Gag was somewhat diffuse because of abundant virus expression, clear accumulation of Gag fluorescence was seen at the limiting membrane and interior of perinuclear vesicles ranging from 0.8 to 4 μ m in diameter; these were also positive for Anx2 (Fig. 2A). The size of these vesicles is consistent with previous descriptions of MVBs/late endosomes in HIV-laden MDM (35, 38). Therefore, to further characterize the sites of Gag and Anx2 collocation, we probed the distribution of both proteins with respect to the late endosomal marker CD63. In agreement with published observations (35, 38), Gag accumulation was consistently observed in the CD63-positive late endosomal compartment in infected macrophages (Fig. 2B). In turn, analysis of Anx2 collocation with markers for early endosomes (EEA1) (data not shown) or late endosomes (CD63) demonstrated preferential collocation of Anx2 and CD63 in the perinuclear region (Fig. 2C). Of note, the somewhat diffuse staining pattern for Anx2 reflects its cytosolic and membrane-bound activity (39) and often cannot be avoided. Together these data suggest that the Anx2-Gag interaction takes place on the late endosomal membranes, which are known to be sites of viral assembly and budding in macrophages (28, 35, 38).

Anx2 downregulation results in suppression of viral replication. To test the functional relevance of Anx2 in HIV rep-

FIG. 3. Depletion of Anx2 in MDM results in decreased viral replication. MDM were treated with Anx2-specific siRNA (siAnx520) or with nontargeting control siRNA (siAnxScr) and infected with HIV-1_{YU2} at day 3 after the treatment. Culture medium was collected and cells were lysed at 2, 4, and 7 days postinfection (p.i.). (A) The Anx2 level in cell lysates was analyzed by Western blotting at the indicated time points after infection. After Anx2 detection, membranes were stripped and reblotted for GAPDH as a control for total protein loads. (B and C) Release of p24^{Gag} (B) and infectious units (C) in culture medium were measured using p24^{Gag} ELISA and MAGI assay, respectively. (D) Intracellular Gag expression was monitored by Western blotting at days 2, 4, and 7 after infection. This experiment is representative of data from three independent donors.

lication, we examined viral production in MDM where Anx2 expression had been decreased by siRNA methodology (45). MDM were treated with an Anx2-specific siRNA (siAnx) or transfection control siRNAs consisting of a scrambled sequence of siAnx (siScr). The siAnx effectively decreased the steady-state levels of Anx2 beginning 3 days after transfection, with a peak of suppression at 7 to 10 days (corresponding to 4 to 7 days after infection) (Fig. 3A); the treatment did not affect control GAPDH expression. Additionally, the control siRNAs had no effect on the level of Anx2. On a per-cell basis, Anx2 downregulation by siRNA treatment occurred in 70 to 85% of the treated cells as determined by indirect fluorescent-antibody assay (data not shown) and lasted from 5 to 10 days with some donor-to-donor variation. Three days after siRNA transfection, the cultures were infected with HIV-1_{YU-2} and virus replication was monitored for p24^{Gag} antigen and infectious particle release.

The concentration of p24^{Gag} in the supernatants of the Anx2 siRNA-treated cultures was decreased in comparison with that for the control cells, concomitant with the reduction in Anx2. In the representative experiment shown in Fig. 3B, at days 4 and 7 after infection, siAnx-treated cells had 2.8- and 8.3-fold decreases in p24^{Gag} release, respectively, relative to the cells treated with a control siRNA. Strikingly, this was accompanied by 5.5- and 16.8-fold reductions in infectious particles quantified using MAGI assay for the same data points (Fig. 3C). The difference in p24^{Gag} and infectious unit release between siAnx- and siScr-treated cells was statistically significant ($P < 0.02$) at day 4 after infection based on data pooled from three independent donors.

Notably, there was a consistent delay in Gag protein synthesis and processing in the Anx2-depleted cells over the course of infection (Fig. 3D). Recently, Anx2 was implicated as a cofactor for HIV-1 entry (25), and thus the decrease in intracellular Gag may result from the inefficient virus attachment to Anx2-depleted cells. However, the sharp decline in the infectivity of released virions suggests that the restricted spread of infection can be at least in part due to the production of replication-incompetent viral particles.

Anx2 depletion is associated with decreased infectivity and aberrant Gag processing. The data in Fig. 3 implied that the major difference between the experimental and control cells may be not at the level of p24/Gag release in the supernatant but rather in the infectivity of the released particles. To extend these observations, we first evaluated the efficiency of Gag release, determined as a ratio of intracellular to extracellular p24^{Gag}. Of note, the p24 ELISA used in our study recognizes both unprocessed Gag and processed capsid protein p24^{Gag}, as was detected using 293 cells transfected with Gag YU-2-encoding vectors (data not shown). Despite a reduction in overall Gag expression in the siAnx-treated cell lysates (Fig. 3D) the efficiencies of Gag release were similar under either condition at day 4 after infection (Fig. 4A). However, when we calculated the number of infectious units per nanogram of extracellular p24^{Gag} (Fig. 4B), a notably smaller proportion of the released Gag was associated with functional HIV particles after Anx2 depletion, suggesting that there is a functional defect in the virions produced. The difference in normalized infectious units between siAnx- and siScr-treated cells was statistically signifi-

FIG. 4. Depletion of Anx2 results in aberrant virus production. MDM were treated with siRNA as indicated and infected with HIV-1_{YU2} at day 3 after the treatment. Supernatants and cell lysates were collected at day 4 after infection. (A) Gag was measured in the lysates and cell-free supernatants by using p24 ELISA, which recognizes both p24^{Gag} and unprocessed p55^{Gag}. The efficiency of Gag release was determined as a percentage of intracellular Gag with respect to the extracellular Gag. (B) Infectious units in the cell-free supernatants were measured using MAGI assay and normalized by extracellular Gag measured in panel A. A decrease in the IU/p24 ratio indicates the production of replication-defective virions in the cells treated with siRNA specific for Anx2. (C) Cell-free supernatants were lysed and used in immunoprecipitation with anti-Gag rabbit polyclonal serum followed by Western blotting with anti-p24 mouse monoclonal antibody. The right panel shows the ratio of p24 band intensity to that of p55 measured using NIH ImageJ software. Error bars indicate standard deviations.

cant ($P < 0.02$) at day 4 postinfection based on data obtained from three independent experiments.

As a follow-up experiment, extracellular Gag was analyzed by immunoprecipitation from the lysed medium of infected cells followed by Western blotting. Figure 4C shows nearly complete processing of the Gag released from the control cells, whereas in the siAnx-treated cells there was a relative abundance of unprocessed p55^{Gag}. Since we did not observe significant differences in either the viability or total protein amounts in the experimental and control MDM, it is unlikely that the p55^{Gag} in the supernatant originated from dead cells, but rather it is likely that it reflects a deficiency in viral maturation.

Gag association with late endosomes and intracellular virion assembly/maturation is inhibited in Anx2-depleted macrophages.

To examine possible alterations in the intracellular maturation of virions, we analyzed particle formation in Anx2-depleted MDM with confocal and transmission electron microscopy. For the confocal experiments, we used a double-label immunofluorescence assay with a monoclonal antibody that recognizes only the mature form of the p17^{Gag} matrix protein together with a p24 polyclonal serum that recognizes both processed and unprocessed Gag. This system enabled us to differentiate between cells that were competent for virus maturation (p55^{Gag}⁺/p17^{Gag}⁺ phenotype) and those in which Gag processing was likely impaired (p55^{Gag}⁺/p17^{Gag}⁻). As it is shown in Fig. 5A, punctate Gag fluorescence was detected in most of the MDM treated with control siRNA. This collocated with a strong p17^{Gag} signal, identifying mature HIV particles. In agreement with published data (3, 4) the assembled virions were seen primarily in perinuclear vesicles, likely late endosomes as documented in Fig. 2B, and on the plasma membrane at probable sites of virus egress.

In contrast, in the majority of siAnx-treated macrophages the Gag staining pattern was diffuse, with no evident local accumulation of fluorescence. Furthermore, no signal for p17^{Gag} was detected in these cells, reflecting a defect in virus assembly and maturation. To quantify this observation, we collected data from two experiments with independent monocyte donors. Overall, mature particles were seen in approximately 70% of the infected cells in control cultures, whereas only 30% of infected cells exhibited a p55^{Gag}⁺/p17^{Gag}⁺ phenotype after siAnx treatment. (Fig. 5C).

Next, we studied the changes in virus assembly and maturation induced by Anx2 depletion at an ultrastructural level using transmission electron microscopy. MDM were treated with either control or Anx2-specific siRNA and then infected with HIV-1_{JAGO}. HIV-1_{JAGO} induces significant cytopathicity in MDM and was chosen to facilitate screening of TEM ultrathin cell sections prepared as described in the Materials and Methods. The Anx2 expression level was monitored by Western blotting in parallel cultures from the same donors (data not shown). To ensure unbiased selection of cells from the EM sections, 10 random fields from the outer edges and inner portions of the ultrathin sections from each condition were selected, and the cells were inspected for electron-dense virus particles and imaged at magnifications of $\times 7,500$ and $\times 30,000$. Twelve of 29 macrophages tested from a control siRNA-treated culture showed overwhelming evidence of particle accumulation in intracellular vesicles (Fig. 6A), in agreement with previous TEM reports on HIV maturation in MDM (35, 38). However, when the Anx2 protein levels were reduced, we could not detect any mature HIV particles either in intracellular vesicles or at the plasma membrane in any of 30 cells tested (Fig. 6B). Instead, we observed only possible immature viral particles with membrane bilayer structures (data not shown), consistent with the aberrant Gag processing noted in the virus immunoprecipitation and confocal microscopy experiments. However, immuno-EM analysis is required to identify Gag-positive immature particles, and this study is currently in progress. Notably, no significant morphological perturbation of the vesicular structures was noted in the Anx2-depleted macrophages at a magnification of either $\times 30,000$ magnification

FIG. 5. Anx2 depletion impairs virus maturation. (A) Infected macrophages stained with a monoclonal antibody against the mature form of the p17 matrix protein (red) or with anti-Gag polyclonal serum (green) at day 4 after infection. The merged panel shows that the p17 signal for mature HIV particles is collocated with regions of Gag accumulation, indicating sites of virus assembly and maturation (yellow). (B) HIV-1_{YU-2}-infected, Anx2 siRNA-treated macrophages. Despite efficient p55^{Gag} production visualized by the diffuse green staining, neither discrete sites of Gag accumulation nor mature virions were observed. (C) Efficiency of virion maturation was quantified as a percentage of infected cells positive for maturation marker (p55^{Gag+}/p17^{Gag+}) with respect to the total number of infected cells (p55^{Gag+}). Data from two independent donors are shown; the range of infected cells was 53 to 100 per condition.

(Fig. 6B) or $\times 7,500$ (data not shown). The TEM experiment was repeated using macrophages from an independent donor, with a similar pattern. The observed difference between siAnx- and control siRNA-treated cells with respect to HIV particle formation was statistically significant (chi-square test, $P < 0.01$).

Because virus assembly is initiated by anchoring of Gag on the limiting membranes, these results implied that the association between Gag and endosomal membranes may be decreased in the Anx2-knocked-down MDM. Therefore, we analyzed the rate of CD63 incorporation into viral particles by using a virus capture assay with anti-CD63 monoclonal antibody, as described in Materials and Methods. Only about 10% of Gag released from siAnx-treated cells was precipitated by the CD63 antibody, in comparison with 30 to 40% for control cells (Fig. 6C), concomitant with the reduction in infectivity (data not shown). Collectively, our data demonstrated that Anx2 depletion interferes with proper HIV assembly and maturation and is associated with a reduction in CD63 incorporation into virions.

Anx2 is not expressed in peripheral blood lymphocytes. Having established that Anx2 is essential for HIV-1 particle maturation in macrophages, we next asked whether Anx2 had a role in virus replication in another HIV target cell type, PBLs. Therefore, we analyzed Anx2 expression in uninfected

PBLs by using Western blotting. As expected, Anx2 was detected in both the Triton X-100-soluble and Triton X-100-insoluble fractions of MDM lysates, which is in agreement with the presence of both a membrane/cytoskeleton-bound and a cytoplasmic form of this protein (42, 49). Anx2 was minimally, or not at all, detected in the soluble or insoluble fractions from donor-matched PBL lysates (Fig. 7A).

HIV infection is known to upregulate some of the host proteins that the virus requires for replication, such as cyclin T (23). Therefore, we assessed levels of Anx2 expression in both MDM and CD4⁺ T cells from uninfected and infected cultures by Western blotting. MDM infected with R5-tropic virus HIV-1_{YU-2}, HIV-1_{BAL}, or HIV-1_{ADA} were positive for Anx2 expression, as expected, with no evidence of increased expression in comparison with uninfected cells (Fig. 7B). PBLs were negative for Anx2 expression regardless of the infection status, thus indicating that the involvement of Anx2 in HIV-1 replication is restricted to macrophages.

DISCUSSION

In these experiments, we have identified Anx2 as a novel HIV-1 Gag binding partner. This interaction was first defined

FIG. 6. Anx2 depletion inhibits virus entry into MVBs. (A) Electron microscopy analysis of MDM transfected with scrambled siRNA and infected with HIV-1_{JAGO} for 5 days. The inset shows a detailed view of particles with lentiviral morphology in intracellular vesicles resembling MVBs. (B) Cryosection of MDM treated with siAnx520 and infected as for panel A. No mature HIV particles either in intracellular vesicles or at the plasma membrane were detected upon Anx2 downregulation. (C) Virus capture with anti-CD63 antibody. Cell-free supernatants of MDM treated with siRNA and infected with HIV-1_{YU-2} were precipitated by Zysorbin cells with or without anti-CD63 antibody. Captured p24 was calculated by subtraction of p24 output remaining unprecipitated in the presence of anti-CD63 antibody from that in the supernatants precipitated without antibody. Efficiency of capture was expressed as the percentage of the value for the no-antibody control. Error bars indicate standard deviations.

in MDM and, more importantly, was not detected in MDM that were “quiescently” infected; such cells have a block to HIV replication that we had previously mapped to the late stages of the viral life cycle (4). Analysis with confocal microscopy demonstrated that the Anx2-Gag interaction likely occurs at the limiting membranes of late endosomes/MVBs, which are known to be the site of HIV assembly and budding in MDM (35, 38). Significantly, virus replication was markedly decreased in MDM in which Anx2 had been transiently knocked down by siRNA methodology, indicating a potential functional relevance of the results obtained with the binding assays. Anx2 depletion was associated with a significant decline in the infectivity of the released virions. The decrease in Anx2 expression mediated by siRNA also coincided with incomplete Gag processing and with inefficient incorporation of CD63 into viral particles. The former is an important step in virion maturation, and the latter is a hallmark of assembly of viral particles in MVBs. Confocal and electron microscopy studies confirmed abnormal virus assembly and failure to complete maturation. Cumulatively, our data indicate that Anx2 is essential for the proper assembly of HIV in MDM, although precise delineation of its role in this process will require additional studies.

Nevertheless, we can suggest potential models based on our results and on the known Anx2 functions.

Anx2 is a member of a family of Ca^{2+} -dependent, cytosolic proteins that can bind cellular membranes, perhaps in response to intracellular Ca^{2+} mobilization. Anx2's diverse functions include membrane trafficking, endosomal formation, and aggregation of vesicles (11, 26, 48). Anx2 also associates with cholesterol in membranes (47), and it has been implicated in the process of exocytosis (10, 13, 24). Anx2 is expressed in a variety of cells, including monocytes, MDM, and microglia (5, 8, 44); remarkable exceptions are human PBLs (Fig. 7), the Jurkat T-cell line, and Raji B cells (references 17 and 25 and data not shown). In terms of its intracellular distribution, Anx2 was located to the plasma membrane and to early/recycling endosomes in HeLa and BHK cells and in the murine macrophage-like line JM774 (26); this differs from our observation that Anx2 associates with late endosomes in primary MDM (Fig. 2A). However, the distribution of Anx2 depends on cholesterol, and translocation of cholesterol to late endosomes in HeLa cells induced by the hydrophobic amine U18666 is accompanied by Anx2 redistribution to the same compartment (26). In our preliminary experiments we detected a substantial

FIG. 7. Annexin 2 is expressed in MDM but not in PBLs independently of infection status. (A) Donor-matched (donor A or donor B) uninfected PBLs and MDM in both the Triton-X-soluble and -insoluble fractions were probed for Anx2 protein expression. Anx2 was detected only in MDM in both fractions. (B) MDM were infected with various HIV-1 R5 strains, (YU-2, BaL, or ADA); lysates were taken 4 to 8 days postinfection and probed for Anx2 expression. Fifteen micrograms of total protein was loaded per well. (C) HIV-1_{YU-2}-infected and uninfected stimulated PBLs were probed for Anx2 expression by Western blotting. Twenty micrograms of total protein was loaded per well.

cholesterol accumulation in CD63-positive compartments in MDM, whereas in HeLa cells cholesterol was predominantly associated with the plasma membrane and with small peripheral vesicles that are most likely early endosomes (unpublished data). Therefore, it is possible that cell type-dependent cholesterol partitioning can account for the differential Anx2 intracellular distribution in different cell types.

The recruitment of Anx2 to cholesterol-rich microdomains on the plasma membrane or internal vesicles probably underlies its general ability to bind lipid rafts and to coalesce them into stable membrane patches (reviewed in reference 39). In particular, Anx2 coordinates the formation of the assembly platforms required for membrane-bound F-actin polymerization. Mechanistically, Anx2 interacts with lipid rafts via a signal phospholipid, phosphoinositide (4,5)-bisphosphate; this binding initiates raft clustering, possibly through an annexin-annexin interaction, resulting in the formation of a protein scaffold with subsequent F-actin recruitment to the stabilized domains (18, 40). Because core lipid rafts are characterized by small size (less than 10 nm in diameter), high dynamics, and a short lifetime (1ms or less) (37), raft clustering and stabilization regulate the activation of a variety of raft-borne signaling or

trafficking processes, possibly including HIV assembly (reviewed in reference 12).

Structurally, all annexins are composed of two domains: an NH₂-terminal tail and a C-terminal protein core. The core harbors phospholipid binding sites and is highly conserved among family members, whereas the NH₂-terminal tail is unique and is involved in binding with a partner specific for each annexin. The annexin 2 binding partner is p11, or S100A10, a member of S100 Ca²⁺ binding protein family (15). The crystal structure of annexin 1 showed that the NH₂ tail is partially buried into the core under Ca²⁺-free conditions and is then exposed in the Ca²⁺-bound conformation (41). Interestingly, we detected an enhancement of the Anx2-Gag interaction in the context of increased Ca²⁺ concentration in the cell-free binding assay described in the legend to Fig. 1. This result points to the Anx2 amino terminus as a possible Gag binding site; the mapping of Anx2-Gag interaction sites is currently in progress.

Taking into account the known properties and the scaffolding characteristics of Anx2, our data fit a model in which Anx2 is involved in the organization of specialized Gag assembly platforms on endosomal membranes in infected macrophages. Although the initial Gag oligomerization may occur in the cytoplasm and may even be required for efficient membrane binding (36), Gag multimerization is greatly enhanced in the presence of phospholipids (6) and is thought to be driven by the larger coalesced raft domains (31), which differ from the raft cores by their higher floating density and thick electron-dense coat (22, 35). The mechanism for recruiting rafts into the segregated assembly platforms is unknown. However, identification of Anx2 as a Gag binding partner (Fig. 1) and its localization on the sites of Gag accumulation on endosomal membranes (Fig. 2A and B) suggest that these platforms may be regulated by Anx2, in agreement with its authentic cellular functions.

In the scenario proposed, depletion of Anx2 leads to raft destabilization and abortive Gag assembly, which is consistent with the diffuse Gag staining pattern that we observed in the majority of the knocked-down cells (Fig. 5B). It was demonstrated previously that some degree of Gag oligomerization occurs prior to membrane association and that a strong interaction with lipid rafts is not required for the primary membrane-bound Gag multimerization (30, 31, 36, 43). Therefore, it is possible that disruption of the coalesced rafts does not preclude the formation and budding of low-order multimers followed by release of noninfectious pseudoparticles; this would explain the Gag release from Anx2-depleted cells that was seen in these experiments (Fig. 4A). However, abortive assembly is likely to prevent proper incorporation of the Gag-Pol polyprotein precursor, resulting in the incomplete Gag processing seen in microscopy analysis of infected cells (Fig. 5B and 6B) and in the immunoprecipitation of extracellular Gag (Fig. 4C). Destabilization of lipid rafts induced by Anx2 downregulation could also account for inefficient incorporation of CD63 detected in a virion precipitation assay (Fig. 6C). CD63, being typically localized to a distinct tetraspan microdomain (46), is translocated to raft aggregates upon raft activation (19); this can explain the preferential integration of CD63 in HIV virions relative to the other late endosomal markers (35). Inhibition of raft clustering may therefore de-

crease the degree of spatial coincidence of the marker and budding particles, resulting in decreased CD63 abundance in virions.

Importantly, Gag collocation with CD63 was observed in a variety of cells independently of whether virus buds into intracellular vesicles or at the plasma membrane (29, 32). These observations provided the basis for the hypothesis that Gag trafficking to the late endosomes/MVBs is an intrinsic step of virus assembly that allows Gag to bind to the components of ESCRT machinery (9), followed by either budding into the MVB lumen or further transport to the plasma membrane, depending on the cell type. Gag anchoring to the endosomal membranes through an interaction with Anx2 may trigger viral assembly and thus define the intracellular mode of budding characteristic for macrophages.

While the aforementioned hypothesis seems most probable, other roles for Anx2 in HIV assembly and budding are possible. Gag binding of Anx2 may play a part in entrance into the endosomal pathway and aid in directing viral assembly into endosomal vesicles. Recently Dong et al. (9) showed that AP-3 is responsible for Gag trafficking to late endosomes in several cell lines; however, whether the same pathway holds true for macrophages remains to be elucidated. Additionally, recruitment of Anx2 to virus-containing vesicles may be necessary for exocytosis, although accumulation of virions in late endosomes would be expected if Anx2 acted only at this late stage. It is theoretically possible that Anx2 downregulation results in a global disruption of endosomal biogenesis and therefore affects HIV assembly and maturation nonspecifically. However, electron microscopy examination of Anx2-depleted macrophages did not reveal any significant morphological perturbation of endosomal compartments at a magnification of either $\times 30,000$ (Fig. 6B) or $\times 7,500$, rendering this possibility unlikely.

A recent report suggested that surface Anx2 promotes HIV entry into MDM through an interaction with phosphatidylserine present in infectious viral particles (25). Involvement of Anx2 in viral entry could explain the decrease in overall Gag expression detected in the siAnx-treated cultures at early time points after infection (Fig. 3D). However, our data argue for a more complex interaction between HIV and Anx2 that extends beyond attachment to the cell surface. Notably, a recent report demonstrated Anx2 incorporation into viral particles produced by macrophages (7), supporting Anx2 localization to the site of virus assembly observed in our study. Further studies to differentiate the roles of extracellular and intracellular Anx2 in HIV replication are under way.

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