Supporting Information

Dube et al. 10.1073/pnas.1008509107

SI Materials and Methods

Cells. Freestyle human 293F cells, a derivative of 293 cells adapted for growth in suspension, were cultured in Freestyle Expression Media (Invitrogen) on a rotary platform to keep them in suspension or where indicated, were allowed to adhere to tissue-culture plates and grown in static culture. 293T cells were cultured in DMEM (Gibco) supplemented with 10% FBS, 1× penicillin-streptomycin (Gibco), and 500 μg/mL active G418 (Gibco). Human JurkatT lymphocytes, Daudi B lymphocytes, THP1 monocytes, and JY B lymphocytes (Victor Engelhard, University of Virginia, Charlottesville, VA) were maintained in RPMI 1640 media with 10% FBS, 1× penicillin-streptomycin, and 1 mM sodium pyruvate. Baby hamster kidney-21 (BHK-21) cells, human SNB-19 cells (glioblastoma cell line; Wendy Maury, University of Iowa, Iowa City, IA), and B95a cells (an adherent B lymphocyte line derived from marmoset; Fumio Kobune, National Institute of Infectious Disease, Tokyo, Japan and Roberto Cattaneo, Mayo Clinic, Rochester, MN) were maintained in DMEM supplemented with 10% FBS and 1× penicillin-streptomycin. Peripheral blood mononuclear cells (PBMCs) were isolated from human blood from healthy donors seronegative for HIV-1 and HIV-2 as well as hepatitis B and C after leukapheresis and subsequent density-gradient centrifugation; monocytes and primary blood lymphocytes (PBLs) were purified by countercurrent centrifugal cell elutriation as described previously (1) and cryopreserved before use. Thawed monocytes were either used immediately or differentiated into macrophages by culture in DMEM with 10% human serum for 2 wk. PBLs were cultured in DMEM supplemented with 10% FBS.

Antibodies and Fluorescently Tagged Marker Proteins. Anti-early endosome antigen-1 (EEA-1; cytosolic face of early endosome) and anti-GM130 (cis-Golgi marker) were purchased from BD Transduction Laboratories. H4B4 (against Lamp2 in the lysosome lumen) was acquired from the Development Studies Hybridoma Bank. Anti-Calnexin (luminal face of the endoplasmic reticulum) was obtained from Santa Cruz Biotechnology. Anti–trans-Golgi network (TGN) 46 was from David Castle (University of Virginia, Charlottesville, VA), initially purchased from AbDSerotech. Anti-Golgin97 (TGN) and Alexa Fluor 546 phalloidin (binds F-actin) were from Invitrogen. Anti-γ-tubulin [located at the microtubule-organizing center (MTOC)] was from Sigma-Aldrich. Anti-lysobisphosphatidic acid (LBPA; late endosome) and Rab11-GFP (pseudocolored red in micrographs shown) were from Jean Gruenberg (University of Geneva, Geneva, Switzerland) and Jim Casanova (University of Virginia, Charlottesville, VA), respectively. Secondary reagents were Alexa Fluor 547 anti-mouse (Invitrogen), protein A, Alexa Fluor 488 (Invitrogen), anti-rabbit Fab 488 (Jackson ImmunoResearch), and Alexa Fluor 647 goat anti-rabbit (Invitrogen). The lipid membrane label DiD was obtained from Invitrogen.

Receptor Binding Region (RBR) Cell-Surface Binding Assay by Flow-Cytometry. Fc-conjugated RBR was produced and purified, and cells were examined for cell-surface RBR binding, as described previously (2). Briefly, cells (kept at 4 °C for the entirety of the assay, unless otherwise noted) were lifted by pipetting with PBS^{++} (PBS with Ca^{2+} and Mg^{2+}), blocked for 20 min with either 3% BSA-PBS^{++} or 10% human serum in 3% BSA-PBS^{++} (for B lymphocytes and macrophages, which have Fc receptors), incubated for 90 min with 200 nM RBR-Fc, washed, incubated with a 1:300 dilution of protein A, Alexa Fluor 488, or (as indicated in legends) anti-rabbit Fab 488 for 45 min, washed two times, and fixed in 2% paraformaldehyde (PFA); 5×10^5 cells were used per sample. Cell-surface binding was quantified by flow cytometry.

RBR Binding Assay by Immunofluorescence. Adherent cells were cultured on fibronectin-coated glass coverslips. Suspension cells were centrifuged onto alcian blue-coated coverslips at 4 °C as described in ref. 3. Cells were washed, fixed with 2% PFA, labeled with 1 μM DiD for 10 min, washed at least three times, and either left intact (nonpermeabilized) or permeabilized with saponin (0.05% saponin, 10 mM glycine, 10 mM Hepes, 10% FBS, pH 7.4) for 20 min. Cells were blocked for 20 min with either 3% BSA-PBS or 10% human serum in 3% BSA-PBS. Cells were then incubated for 45 min with Fc-conjugated wild-type (WT) RBR or 4mer mutant (binding-deficient) RBR (500 nM), washed two times, and incubated with a 1:300 dilution of protein A, Alexa Fluor 488 (Invitrogen), or (as indicated in legends) anti-rabbit Fab 488 for an additional 45 min. Fluorescent images were captured on a Nikon Eclipse TE2000 laser-scanning confocal microscope using a 100× objective. The resultant .ids files were converted to .tiff files using the Nikon EZ-C1 (Build 3.5) controller software. Pseudocoloring or uniform changes to the γ level were made using the EZ-C1 software and are indicated in the appropriate figure legends; .tiff files were then imported into Adobe Illustrator for the sole purpose of figure assembly and labeling.

Determination of the Intracellular Location and Orientation of the RBR Binding Site by Immunofluorescence. Suspension cells were prepared for immunofluorescence as above. To determine the orientation of the RBR binding site (luminal or cytoplasmic) (Fig. 2*A* and Fig. S2*A*), cells were fixed in 2% PFA, plasma membranes were labeled with 1 μM DiD (pseudocolored blue in Fig. 2*A* and Fig. S2*A*), and their membranes were either left intact (nonpermeabilized), permeabilized with saponin (as above), or permeabilized with digitonin [where indicated; 2 min at room temperature (RT), 1 μM digitonin in KHM buffer (110 mM potassium acetate, 2 mM magnesium acetate, 20 mM Hepes, pH 7.2)]; the latter treatment selectively permeabilizes the plasma membrane (*Text*). Cells were then blocked with 3% BSA-PBS and incubated with 500 nM Fc-conjugated RBR (Fig. 2*A*), the indicated cytosolic or luminal marker antibody, or the cytosolic marker control Alexa Fluor 546 phalloidin (Fig. S2*A*). Cells were then washed and incubated with protein A, Alexa Fluor 488 to detect RBR-Fc (Fig. 2*A*), or Alexa Fluor 547 anti-mouse to detect the organelle marker. To assess colocalization with organelle markers (Fig. 2*B* and Figs. S2*B* and S4), cells were fixed and processed as above (permeabilized with saponin) but not labeled with DiD. They were then incubated with RBR-Fc and an antibody against the indicated organelle marker, washed, and incubated with anti-rabbit Fab 488 to detect RBR-Fc and Alexa Fluor 547 anti-mouse to detect the organelle marker. To assess possible colocalization with recycling endosomes, 293F cells were transfected with Rab11-GFP before RBR binding (4), which was then detected with Alexa Fluor 647 anti-rabbit. Fluorescent images were captured and processed as above.

Pseudovirion Infection and Virus-Like Particle Entry Assays. Cell susceptibility to ebolavirus (EBOV) glycoprotein (GP)-mediated infection was examined using vesicular stomatitis virus (VSV) pseudovirions encoding GFP (in place of the coding region for VSV G) and bearing either VSV G or EBOV GP lacking its mucin-like domain (GPΔ). VSV pseudovirions were produced in BHK-21 cells as described previously (2, 5, 6). The expression plasmid for

GPΔ was from Gary Nabel (National Institute of Allergy and Infectious Diseases). In specified experiments, GPΔ was precleaved to its 19-kDa fusion-primed form (GP 19 kDa) by treatment with thermolysin (2, 5). Infection was quantified by flow cytometry (GFP expression) as described.

Virus-like particles (VLPs) carrying β-lactamase (Blam) were produced essentially as described in refs. 7 and 8. In brief, 293T cells in a 10-cm tissue-culture plate were cotransfected with 3 μg Blam-VP40 plasmid (Lijun Rong, University of Illinois at Chicago, Chicago, IL) and 1 μg GPΔ or 1 μg VSV G using Fugene 6, according to the manufacturer's instructions. Cells were incubated at 37 °C, and supernatants were harvested at both 24 and 48 h. Collected supernatants were then cleared of cell debris by centrifugation two times at 1,070 × *g* for 10 min at 4 °C. Cleared supernatant (buffered with 20 mM Hepes) was used directly in fusion experiments involving VLPs bearing VSV G. VLPs bearing GPΔ were concentrated by pelleting through 20% sucrose in an SW28 rotor at 82,705 × *g* for 2 h at 4 °C. The VLP pellet was then resuspended in virus resuspension buffer (10% sucrose in 130 mM NaCl, 20 mM Hepes, pH 7.4). VLPs were quick frozen in liquid nitrogen and stored at −80 °C. The presence of filamentous VLPs was confirmed by electron microscopy. To examine VLP entry, cells in 48-well dishes were spinfected with VLPs bearing EBOV GPΔ or VSV G in phenol red free Opti-MEM (Gibco) for 2 h at 380 × *g* at 4 °C in a tabletop centrifuge (7, 8). Spinoculation was performed in accordance with the β-lactamase entry assay (7, 8). Note that VLPs with no envelope protein give no entry signal when spinoculated onto cells in this manner (7). After 3 h at 37 °C, unbound virions were washed off with loading buffer (phenol red free DMEM supplemented with L-glutamine, 2.5 μM probenecid, 25 mM Hepes), and cells were loaded with the β-lactamase substrate coumarin cephalosporin fluorescein 2/acetoxymethyl ester (CCF2/AM; Invitrogen) diluted into loading buffer. Substrate-loaded cells were incubated in the dark for 1 h at RT, washed two times with loading buffer, and then incubated for 18 h in loading buffer containing 10% FBS in the dark at RT. Cells loaded only with CCF2/AM served as negative controls. The extent of CCF2/AM cleavage, detected by the change in dye emission from green to blue, was then evaluated by flow cytometry.

1. Gerrard TL, Jurgensen CH, Fauci AS (1983) Differential effect of monoclonal anti-DR antibody on monocytes in antigen- and mitogen-stimulated responses: Mechanism of inhibition and relationship to interleukin 1 secretion. *Cell Immunol* 82:394–402.
2. Dube D, et al. (2009) The primed ebolavirus glycoprotein (19-kilodalton GP1,2): Sequence and residues critical for host cell binding. *J Virol* 83:2883–2891.
3. Broderick L, Bankert RB (2006) Membrane-associated TGF-beta1 inhibits human memory T cell signaling in malignant and nonmalignant inflammatory microenvironments. *J Immunol* 177:3082–3088.
4. Balasubramanian N, Scott DW, Castle JD, Casanova JE, Schwartz MA (2007) Arf6 and microtubules in adhesion-dependent trafficking of lipid rafts. *Nat Cell Biol* 9:1381–1391.
5. Schornberg K, et al. (2006) Role of endosomal cathepsins in entry mediated by the Ebola virus glycoprotein. *J Virol* 80:4174–4178.
6. Takada A, et al. (1997) A system for functional analysis of Ebola virus glycoprotein. *Proc Natl Acad Sci USA* 94:14764–14769.
7. Tscherne DM, Manicassamy B, García-Sastre A (2010) An enzymatic virus-like particle assay for sensitive detection of virus entry. *J Virol Methods* 163:336–343.
8. Manicassamy B, Rong L (2009) Expression of Ebolavirus glycoprotein on the target cells enhances viral entry. *Virol J* 6:75–89.

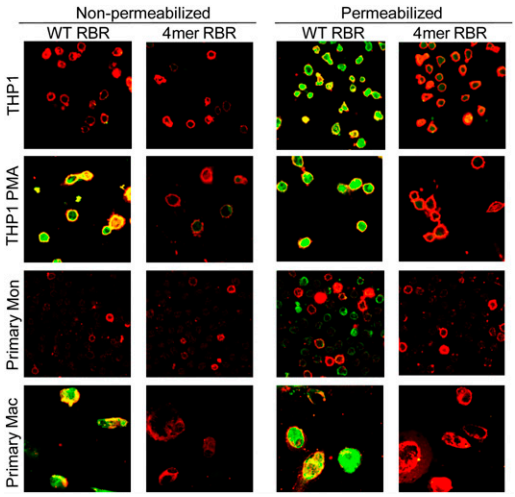

Fig. S1. There is a pool of RBR receptor inside (suspension) monocytes, but differentiated (adherent) macrophage cells display RBR receptor at the cell surface. THP1 cells were either grown in suspension as monocyte-like cells or treated with PMA for 24 h to differentiate them into adherent macrophage-like cells. Primary monocytes were collected by elutriation and frozen. On thawing, monocytes were either fixed immediately or cultured in the presence of 10% human serum for 2 wk and then treated with PMA for 24 h to allow differentiation into adherent macrophages. To analyze RBR binding, cells were fixed, their plasma membranes were labeled with DiD (red), they were either left intact (nonpermeabilized) or permeabilized with saponin, and then, they were incubated with Fc-conjugated RBR (500 nM) followed by anti-rabbit Fab 488 (green). Fluorescent images were captured and processed as in Fig. 1. Images represent findings from multiple experiments.

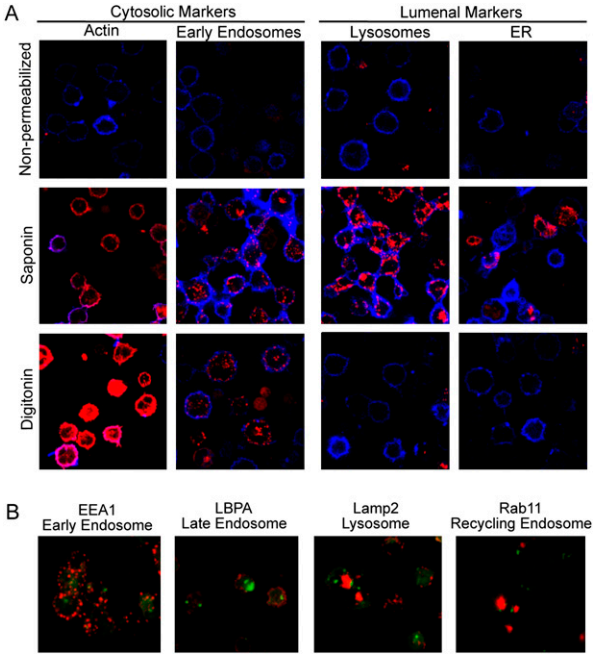

Fig. S2. Controls for RBR receptor localization. (*A*) In the same experiment as that shown in Fig. 2*A*, suspension 293F cells were fixed, and their plasma membranes were labeled with DiD (pseudocolored blue). Cells were then examined under three conditions: intact (nonpermeabilized), permeabilized with saponin (permeabilizes all membranes), or permeabilized with digitonin (permeabilizes only the plasma membrane; intracellular membranes remain intact). Controls for selective permeabilization (red) were: cytosolic markers: phalloidin (actin) and EEA1 (cytosolic face of early endosomes); luminal markers: H4B4/anti-Lamp2 (lumen of lysosomes) and Calnexin (lumen of endoplasmic reticulum [ER]). (*B*) In the same experiment as that shown in Fig. 2*B*, suspension 293F cells were fixed, permeabilized with saponin, and incubated with (rabbit) Fc-conjugated RBR (500 nM; green) and the indicated marker mouse antibody (red) followed by anti-rabbit Fab 488 and Alexa Fluor 547 anti-mouse secondary antibodies. In the case of Rab11, cells were first transfected with GFP-tagged Rab11 and then treated as above for RBR-Fc staining, except that the RBR-Fc was detected with Alexa Fluor 647 anti-rabbit. For this image (far right), GFP-Rab11 was pseudocolored red, and RBR-Fc was pseudocolored green for consistency with other panels in *B* (and Fig. 2*B* and Fig. S4). For images in *B*, RBR binding was analyzed at low laser intensity to focus on areas of brightest staining. Fluorescent images were captured and processed as in Fig. 1. Images represent findings from multiple experiments.

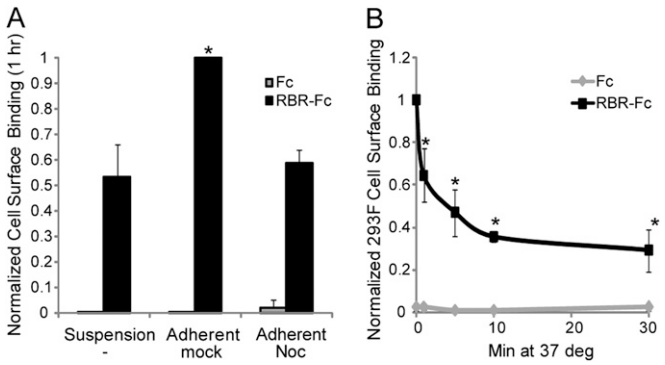

Fig. S3. Further evidence that the cell adhesion-induced differential of RBR binding to the cell surface depends on microtubules and actin filaments. (*A*) The effect of nocodazole (Noc) on RBR binding to the surface of 293F cells was analyzed as in Fig. 3*A* but after only 1 h of cell adhesion. Bars in *A* represent the average of three experiments done in duplicate, normalized to RBR binding to the cell surface of the adherent mock-treated sample. Error bars represent SD of the normalized data. Statistical significance compared with the suspension mock-treated sample was determined by Student *t* test (*$P < 0.004$). (*B*) 293F cells previously cultured in suspension were allowed to adhere for 18 h. The adherent cells were then lifted by pipetting with cold PBS^{++} and incubated at 37 °C for the indicated times (to allow membrane trafficking). Cells were chilled and then examined for RBR binding as described in Fig. 3. Error bars represent SD of the normalized data from three experiments, each performed in duplicate. Statistical significance compared with the 0 min/cold sample was determined by Student *t* test (*$P < 0.05$).

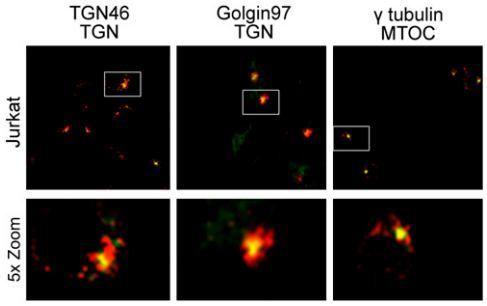

Fig. S4. The pool of RBR receptor is associated with the TGN and MTOC in suspension lymphocytes. Jurkat T lymphocytes were fixed, permeabilized with saponin, and incubated with (rabbit) Fc-conjugated RBR (500 nM; green) and the indicated marker mouse antibody (red) followed by anti-rabbit Fab 488 and Alexa Fluor 547 anti-mouse secondary antibodies. RBR binding was analyzed at low laser intensity to focus on the area of brightest staining. *Lower* shows a 5× enlarged view of a cell (boxed) from the panel directly above. Fluorescent images were captured and processed as in Fig. 1. Images represent findings from multiple experiments.

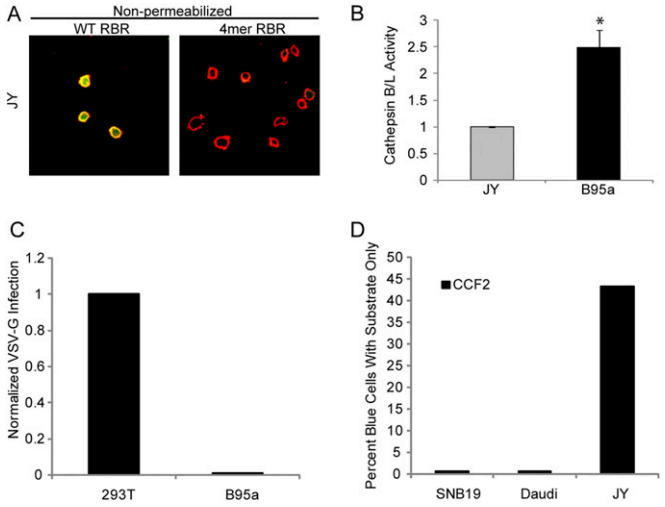

Fig. S5. Extended analyses of ebolavirus GP-mediated entry into adherent B cells. (*A*) Adherent JY B lymphocytes were fixed, their plasma membranes were labeled with DiD (red), and then, they were incubated with Fc-conjugated RBR (500 nM) followed by anti-rabbit Fab 488 (green). Fluorescent images were captured and processed as in Fig. 1. Images represent findings from multiple experiments examining at least five fields per sample. (*B*) JY and B95a adherent lymphocytes were lysed in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40), insoluble material was sedimented in a microcentrifuge at 4 °C, and cellular-protein concentration was determined for each sample by a Bicinchoninic acid (BCA) assay. Cathepsin B+L activity was then measured essentially as described in ref. 1; 14 μg of cleared cell lysate (in 20 μL of lysis buffer) were combined with 80 μL reaction buffer (100 mM sodium acetate, pH 5.0, 1 mM EDTA, 4 mM DTT) and 100 μL of substrate solution (100 μM Z-Phe-Arg-AMC; Invitrogen) and incubated for 1 h at 37 °C. Fluorescence was measured in black 96-well plates in a Spectramax Gemini plate reader with an excitation of 360 nm and emission of 460 nm. Averaged data from three experiments done in duplicate and normalized to Cathepsin B+L activity in JY cells are shown. Error bars represent SD; asterisk indicates significance ($P < 0.015$) based on Student *t* test. (*C*) 293T and B95a cells were challenged with control VSV bearing VSV G as in Fig. 5*C*. Infection was analyzed by flow cytometry and normalized to the infection of 293T cells at an equivalent multiplicity of infection (MOI). (*D*) SNB19, Daudi, and JY cells were incubated with the β-lactamase substrate CCF2/AM alone (with no VLP challenge) and analyzed by flow cytometry to determine background substrate cleavage.

1. Ebert DH, Deussing J, Peters C, Dermody TS (2002) Cathepsin L and cathepsin B mediate reovirus disassembly in murine fibroblast cells. *J Biol Chem* 277:24609–24617.