Supporting Information

Gianni et al. 10.1073/pnas.1212597109

SI Materials and Methods

β3-Integrin Silencing, Western Blot (IB), and Cell-ELISA of Silencing. Lentiviruses expressing two different sh-RNAs to β3-integrin (ITGB3), pseudotyped with vesicular stomatitis virus G glycoprotein, were produced by transfection of pFuGW-ITGB3-1 or pFuGW-ITGB3-2 and additional appropriate plasmids into 293T cells (all kindly provided by the authors of ref. 1), harvested 48 h later, and immediately used to transduce 293T cells. The transduced (sh-β3) cells were monitored for effective silencing 7–10 d later. Two to three independently generated cell pools were obtained for each cell line. They were assayed for extent of silencing, and one for each cell line was then used for following assays. The sh-β3 cells remained stably silenced for several months. The control mock-silenced cells were generated similarly by means of plasmid pFuGW-control. Cell surface expression of αvβ3-integrin was measured by cell-ELISA, as described (2). Briefly, wt-293T or sh-β3 cells were grown in 96 well plates, reacted with MAb L609 to the αvβ3-heterodimer or with MAb AP3 to β3-integrin subunit for 2 h at 4 °C, paraformaldehyde-fixed, and then reacted with anti-mouse peroxidase, followed by *o*-phenylenediamine (Sigma-Aldrich) at 0.5 mg/mL and reading the optical density at 490 nm. IB analysis was carried out by means of PAb AB1932 (Chemicon).

Transgene Expression and NF-κB Activity. Control or sh-β3 cells, or K562 cells negative or positive for αvβ3-integrin, were transfected by means of Arrest-in (Thermo Scientific) or Lipofectamine2000 Plus reagent (Invitrogen) with plasmids encoding firefly luciferase under a NF-κB regulated promoter (NF-κB-luc) (800 ng/2 × 10^5 cells) and Renilla luciferase (luciferase:renilla ratio 130:1), plus TLR2-FLAG or pcDNA 3.1 empty vector (50 ng/2 × 10^5 cells), as indicated. Alternatively, 293T cells were transfected with wt-Dyn or Dyn-K44A plasmids (3). After transfection with NF-κB-luc plasmid, cells were incubated with pre-exhausted medium for 2–3 d before use. To prepare the pre-exhausted medium, the respective (293T or K562) cells were grown, and the medium was replaced with fresh medium containing 1% or 5% (vol/vol) FCS, incubated with the cells for 2–3 d, and thereafter filtered twice through 0.22 μm filters. The transfected cells were then exposed to the indicated viruses (UV-irradiated or not) for indicated times or stimulated with LPS (Sigma-Aldrich, # L2630) (100 ng/mL), which induced a strong TLR2 response. Luciferase activity was quantified by means of Dual-Glo luciferase reporter assay system (Promega) and expressed as luciferase/renilla ratio (4). For detection and quantification of endogenous Iκ-Bα, wt- or sh-β3 293T cells, transfected or not with TLR2, were infected with UV-inactivated wt-HSV (wt-HSV-UV) or R9710 (20 PFU/cell). Lysates were harvested at 2, 4, and 6 h after infection in buffer containing 20 mM Hepes, 250 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% Nonidet P-40, plus protease and phosphatase inhibitors (Sigma-Aldrich), and separated by SDS PAGE. Iκ-Bα was detected by IB with anti–Iκ-Bα Ab (Cell Signaling). NF-κB activation leads to degradation of Iκ-Bα, visible as a decreased band. The blots were also reacted with anti-tubulin Ab (Sigma-Aldrich). Quantification of the bands, expressed as DUs, was performed by Image-J software, and expressed as ratio of the DU of band of interest relative to that of tubulin, in the same sample. Data were expressed as percentage of the Iκ-Bα/tubulin value in uninfected cells.

Infection. The β-gal–expressing R8102 is an otherwise wt-HSV-1 (F) carrying the reporter Lac-Z under the α27 immediate-early promoter (5). R7910 carries the deletion of α0 gene (ΔICP0) (6). Cells were infected at the indicated multiplicities with extracellular virions, previously pelleted by ultracentrifugation. Following virus absorption to cells for 90 min at 37 °C, virus inoculum was removed, and monolayers were rinsed twice with medium. Infection with β-gal–expressing R8102 was stopped at 6 h after infection and revealed by X-gal staining (5). For virus yield determinations, cells were frozen at 4 h (0 time) or 24 h after virus absorption. The virus was titrated by plaque assay in Vero [HSV-1(F)] or U2OS (R7910) cells.

Reverse Transcription and q-RT-PCR. Total RNA was purified with Total RNA Isolation kit (Macherey-Nagel) according to the manufacturer's instructions; the procedure included digestion with DNase. Total RNA (1 μg/20 μL reaction mix) from mock- or HSV-infected cells, positive or negative for TLR2, wt, or sh-β3, was reverse transcribed with a high-capacity cDNA reverse transcription kit (Applied Biosystems). Real-time PCR primers were the inventoried TaqMan gene expression assays (Applied Biosystems) (see list in Table S1). Reactions were performed in triplicates in a 20 μL volume that included cDNA (150 ng), gene expression assay (1 μL), and TaqMan gene expression master mix (10 μL) (Applied Biosystems). Real-time PCR reactions were performed on an Applied Biosystems Prism 7300 sequence detection system. Samples were normalized relative to GADPH Ct. To compare mock and infected cell samples, relative changes in gene expression were determined using the $2^{-\Delta\Delta Ct}$ method. Data represent average of triplicates and were expressed as fold-variations relative to the uninfected cell value. Variations ≥ twofold relative to the corresponding value of mock-infected cells were considered significant.

Cytokine Quantification. To determine the amount of IFN-β and IL10 produced by wt- or sh-β3 cells, negative or positive for TLR2 as indicated, the cell culture media were harvested 24 or 48 h after infection with R7910. IFN-β was detected by means of VeriKine kit (Pestka Biomedical Laboratories, PBL IFN Source) and human IL10 by Elisa kit (Thermo Scientific, Pierce). Media were added to the precoated wells in a 1:1 ratio with the kit dilution buffer for 1–2 h, according to the manufacturer's instructions. The bound cytokines were revealed with appropriate antibodies conjugated to peroxidase plus substrate and reading the optical density at 450 nm. Standard quantities of the purified cytokines were run in parallel for relative quantification.

BIAcore. Kinetic measurements of the interaction between soluble gH/gL and soluble αvβ3-integrin purified from 293-B3 AVAP cells (a gift of Stephen Nishimura, University of California, San Francisco, CA) were made with a Biacore 2000 instrument (Biacore AB) as described (7). MAb LS-C44264 to AP (LifeSpan BioScience) was used to capture the integrins on the surface of the sensor chip. Measurements were made at 25 °C, and soluble gH/gL was injected immediately after integrin capturing at a flow rate of 50 μl/min.

Coimmunoprecipitation of β3-Integrin by TLR2-Flag. Cells were transfected with plasmids encoding αvβ3-integrin, TLR2-flag, or both. TLR2 was immunoprecipitated 24 h later by means of anti-flag MAb (Sigma-Aldrich). The proteins retained by protein A/G Sepharose were separated by SDS/PAGE and reacted with anti–β3-integrin Ab (4).

gH_t/gL. The purification and properties of the soluble gH_t/gL were described (2).

1. Gerold G, et al. (2008) A Toll-like receptor 2-integrin beta3 complex senses bacterial lipopeptides via vitronectin. *Nat Immunol* 9(7):761–768.
2. Gianni T, et al. (2010) Herpes simplex virus glycoproteins H/L bind to cells independently of alphaVbeta3 integrin and inhibit virus entry, and their constitutive expression restricts infection. *J Virol* 84(8):4013–4025.
3. Gianni T, Gatta V, Campadelli-Fiume G (2010) alphaVbeta3-integrin routes herpes simplex virus to an entry pathway dependent on cholesterol-rich lipid rafts and dynamin2. *Proc Natl Acad Sci USA* 107(51):22260–22265.
4. Leoni V, Gianni T, Salvioli S, Campadelli-Fiume G (2012) Herpes simplex virus glycoproteins gH/gL and gB bind Toll-like receptor 2, and soluble gH/gL is sufficient to activate NF-κB. *J Virol* 86(12):6555–6562.
5. Cocchi F, Menotti L, Mirandola P, Lopez M, Campadelli-Fiume G (1998) The ectodomain of a novel member of the immunoglobulin subfamily related to the poliovirus receptor has the attributes of a bona fide receptor for herpes simplex virus types 1 and 2 in human cells. *J Virol* 72(12):9992–10002.
6. Kawaguchi Y, Bruni R, Roizman B (1997) Interaction of herpes simplex virus 1 alpha regulatory protein ICP0 with elongation factor 1delta: ICP0 affects translational machinery. *J Virol* 71(2):1019–1024.
7. Chesnokova LS, Hutt-Fletcher LM (2011) Fusion of Epstein-Barr virus with epithelial cells can be triggered by αvβ5 in addition to αvβ6 and αvβ8, and integrin binding triggers a conformational change in glycoproteins gHgL. *J Virol* 85(24):13214–13223.

Fig. S1. (*A*) Replication of R8102 in wt- and control mock-silenced 293T cells. Wt-293T cells (*Left*) and control mock-silenced 293T cells (*Right*) were exposed to 5–50 PFU/cell of R8102, a wt-recombinant HSV expressing β-gal driven by the α27 immediate-early promoter. β-gal expression was measured at 6 h after infection and expressed as arbitrary units (U). R8102 replication in wt-293T cells could not be differentiated from that in control mock-silenced cells. (*B*) Synthesis of selected immediate-early, early, and late HSV-1 proteins is not altered in sh-β3 293T cells. wt- or sh-β3 293T cells were infected with HSV-1(F) (10 PFU/cell). Lysates were prepared at 4, 8, and 16 h after infections and subjected to SDS/PAGE. The viral proteins ICP27 (immediate-early), thymidine kinase (TK) (early), and gC (late) were detected by WB with the following antibodies: MAb to ICP27, rabbit polyclonal antibody R161 to TK, and MAb 1483 to gC. The pattern of protein synthesis is very similar in wt- and sh-β3 293T cells, indicating that there was no defect in sh-β3 293T cells to be infected and to support infection.

Fig. S2. Expression of IL2Rα (*A*) but not of PTAFR (*B*) is greatly affected in β3-integrin–silenced 293T cells. Control mock-silenced (ctrl) or sh-β3 293T cells, transfected with irrelevant plasmid (−TLR2) or TLR2 (+TLR2), were cultured in pre-exhausted medium from 12 to 48–72 h after transfection and then infected with UV-inactivated wt-HSV-1(F) (50 PFU/cell) and harvested at 2 h p.i. or infected with R7910 (20 PFU/cell) and harvested at 6 h p.i. RNA was extracted and retrotranscribed by standard techniques for q-RT-PCR. To compare infected versus uninfected cell (uninfected) samples, relative changes in gene expression were determined using the $2^{-\Delta\Delta Ct}$ method. Data were expressed as relative mRNA levels relative to the uninfected cell value, taken as 1 (column, uninfected). Each value represents the average of triplicate samples ± SD.

Fig. S3. NF-κB activation in 293T cells following αvβ3-integrin cross-linking with MAb L230. 293T cells, transfected with TLR2 (TLR2+), or an empty plasmid (TLR2–) plus NF-κB-luc and Renilla luciferase plasmids were exposed 48–72 h after transfection to the anti–αvβ3-integrin function-blocking MAb L230 (40 μg/mL) or mouse control immunoglobulins (Ig) for 90 min. R7910 (R7910 +) or no virus (R7910 –) was then added in the same medium. Following virus adsorption, viral inoculum was removed, and cells were overlaid with medium containing MAb L230 or Ig. NF-κB-luc was quantified as in panel *A* of Fig. 2. This experiment provides evidence that integrin cross-linking with anti-integrin antibodies leads to NF-κB in both uninfected and infected cells; hence αvβ3-integrin exhibits an intrinsic ability to activate NF-κB.

Table S1. List of inventoried TaqMan Assay probes and their target genes

Gene	Inventoried TaqMan assay
ITGB3	Hs01001469_m1
ITGB6	Hs00168458_m1
IFNB1	Hs01077958_s1
IFNA1	Hs00855471_g1
IL2	Hs00174114_m1
IL10	Hs00174086_m1
SRC	Hs01082246_m1
IRF3	Hs01547283_m1
IRF7	Hs00185375_m1
TRIF	Hs0190712_m1
SYK	Hs00895377_m1
CARD9	Hs00364485_m1
IL1A	Hs00174092_m1
TNF	Hs00174128_m1
IL6	Hs000985639_m1
BCL3	Hs00180403_m1
IL2RA	Hs0090779_m1
LTBR	Hs00158922_m1
FASLG	Hs00181225_m1
GADPH	Hs02758991_g1