

# Parvovirus B19 Uptake Is a Highly Selective Process Controlled by VP1u, a Novel Determinant of Viral Tropism

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**The VP1 unique region (VP1u) of human parvovirus B19 (B19V) is the immunodominant part of the viral capsid. Originally inaccessible, the VP1u becomes exposed upon primary attachment to the globoside receptor. To study the function of the exposed VP1u in B19V uptake, we expressed this region as a recombinant protein. Here, we report that purified recombinant VP1u binds and is internalized in UT7/Epo cells. By means of truncations and specific antibodies, we identified the most N-terminal amino acid residues of VP1u as the essential region for binding and internalization. Furthermore, the recombinant VP1u was able to block B19V uptake, suggesting that the protein and the virus undertake the same internalization pathway. Assays with different erythroid and nonerythroid cell lines showed that the N-terminal VP1u binding was restricted to a few cell lines of the erythroid lineage, which were also the only cells that allowed B19V internalization and infection. These results together indicate that the N-terminal region of VP1u is responsible for the internalization of the virus and that the interacting receptor is restricted to B19V-susceptible cells. The highly selective uptake mechanism represents a novel determinant of the tropism and pathogenesis of B19V.**

Human parvovirus B19 (B19V) was discovered in 1975 (1) and is classified within the genus *Erythrovirus* of the family *Parvoviridae*. B19V is a prominent human pathogen, which is typically associated with a worldwide disease named erythema infectiosum or fifth disease, affecting mostly school-aged children between 5 and 14 years of age during winter and spring. The infection, which is transmitted primarily via the respiratory route, is generally resolved without further consequences. However, in pregnant women or in individuals with underlying immune or hematologic disorders, B19V may cause more severe syndromes, such as hydrops fetalis, arthropathy, and severe cytopenia (2, 3).

B19V has a remarkable tropism for erythroid precursor cells in the bone marrow (4), and their destruction accounts for many of the syndromes associated with B19V infection. The P antigen or glycosphingolipid globoside (globotetraosylceramide, or Gb4Cer) is the cellular receptor of B19V (5). The tissue distribution of Gb4Cer and of additional cellular factors highly restricted to the erythroid lineage defines the extraordinarily restricted tissue tropism of B19V (6, 7, 8, 9, 10). Although required for B19V attachment to cells, Gb4Cer is not sufficient to trigger the internalization step (11). Subsequently,  $\alpha 5\beta 1$  integrin (12) and Ku80 autoantigen (13) have been proposed as potential coreceptors for B19V infection. While Ku80 might facilitate virus attachment to certain cell types (13),  $\alpha 5\beta 1$  integrin was previously proposed to function as a coreceptor for virus uptake although the mechanism was not elucidated (12). In agreement with a complex binding/internalization process requiring the engagement of several cell membrane molecules, it was observed that B19V cannot stably bind membrane-associated Gb4Cer *in vitro* (14).

The small, nonenveloped, icosahedral B19V capsid consists of 60 structural subunits, of which approximately 95% are VP2 (58 kDa) and 5% are VP1 (83 kDa) (15). VP1 is identical to VP2 with the exception of an additional N-terminal region of 227 amino acids, the so-called VP1 unique region (VP1u) (16). VP1u is the immunodominant part of the capsid (17, 18). A poor immune response against VP1u has been linked to persistent infections (19). The clustering of strong neutralizing epitopes in the VP1u

region and, in particular, at the N-terminal region (N-VP1u) denotes the existence of important motifs required for the infection. The VP1u region of parvoviruses is not accessible on the capsid surface, but it becomes exposed during virus trafficking along the endocytic pathway (20, 21, 22). The intracellular exposure of VP1u is thought to be important for endosomal escape (23, 24, 25, 26, 27) and nuclear targeting (22, 28, 29). In sharp contrast to other parvoviruses, VP1u of B19V becomes accessible to antibodies (Abs) upon binding to the Gb4Cer receptor (30, 31). The specific role of the early exposure of VP1u at the cell surface has not been elucidated. However, considering that VP1u is the immunodominant part of the capsid, its early conformational change should render accessible motifs that are required for the infection. We hypothesized that the receptor-mediated rearrangement of VP1u prepares the capsid for a second interaction required for virus internalization.

By using recombinant full-length and truncated versions of VP1u, we studied the role of the receptor-induced structural rearrangement of VP1u in B19V internalization. We obtained solid evidence that the most N-terminal region of VP1u interacts with a host membrane component to trigger virus uptake. The expression of the cell membrane molecule recognized by VP1u was severely restricted and exclusively detected in cells that allowed virus internalization and infection. These results suggest that VP1u plays a pivotal role in the narrow tropism of B19V by controlling a highly selective uptake process.

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## MATERIALS AND METHODS

**Cells.** The human megakaryoblastoid cell line UT7/Epo was provided by E. Morita (Tohoku University School of Medicine, Japan), and the human erythroleukemia cell line KU812Ep6 was obtained from N. Ikeda (Fujirebio, Inc., Tokyo, Japan). The human epithelial carcinoma cell line HeLa, the human embryonic kidney cell line HEK 293, the human liver hepatocellular cell line HepG2, the normal human lung fibroblast cell line MRC-5, and the human erythroleukemia cell lines K562 and KG1a were purchased from ATCC. Erythrocytes were obtained from a blood transfusion service (BSD SRK, Bern, Switzerland). UT7/Epo cells were cultured in RPMI 1640 medium with 5% fetal calf serum (FCS) and 2 U/ml recombinant human erythropoietin (EPO); KU812Ep6 cells were cultured in RPMI 1640 medium with 10% FCS and 6 U/ml EPO; HepG2 and MRC-5 cells were cultured in minimal essential medium/Earle's balanced salt solution (MEM/EBSS) with 5% FCS; K562 and KG1a cells were cultured in Iscove's modified Dulbecco's medium (IMDM) with 10% and 20% FCS, respectively. HeLa and HEK 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 5% FCS. All culture media were supplemented with L-glutamine and penicillin-streptomycin.

**Viruses.** A B19V-infected plasma sample was obtained from our donation center (genotype 1) (CSL Behring AG, Charlotte, NC). Prior to experiments, the plasma was passed through protein G and protein L Sepharose affinity matrices (GE Healthcare) to remove any possible antibody, which might interfere with subsequent experiments. Baculovirus-expressed B19V-like empty capsids (virus-like particles [VLPs]) containing both VP1 and VP2 were kindly provided by R. Franssila (Helsinki, Finland). VP2-only empty particles were kindly provided by G. Elliott (Biotrin Technologies, Ltd., Dublin, Ireland).

**Antibodies.** The anti-B19V capsid monoclonal antibody (MAb) (860-55D) and the VP1u-specific MAb (1418-1; amino acids [aa] 30 to 42) were kindly provided by S. Modrow (Regensburg, Germany). Both antibodies were derived from healthy adults with high titers against B19V and were reported as highly neutralizing (32). The polyclonal rabbit anti-PLA<sub>2</sub> antibody (1033/34) was obtained as previously described (30). A rat anti-FLAG MAb was purchased from Agilent Technologies (Santa Clara, CA). The CD32 (FcγRII) MAb was obtained from BD Biosciences (San Jose, CA). Mouse MAb antibodies against early endosomes (70521; anti-EEA1), late endosomes (2733 2G11; anti-mannose 6 phosphate receptor [M6PR]), and lysosomes (H4A3; anti-LAMP1) were purchased from Abcam (Cambridge, MA).

**Cloning and protein expression.** The DNA fragment encoding VP1u was amplified from the infectious clone pB19-M20, kindly provided by S. Wong (National Institutes of Health, Bethesda, MD) with restriction site overhang primers (forward WT VP1u, 5'-ACTCAAGCTTAGTAAAGAAAGTGGCAAATG-3'; reverse VP1u, 5'-AGTGGTACCGCTTGGGTATT TTTCTGAG-3'). The fragment was cloned into the pT7-FLAG-MAT-Tag-2 expression vector (Sigma, St. Louis, MO) and transformed into *Escherichia coli* XL10 Gold cells. Truncated proteins were cloned by using deletion primers and QuikChange PCR. Protein expression was carried out in *E. coli* BL21(DE3) cells and was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at an optical density at 600 nm (OD<sub>600</sub>) of ~0.6 for 4 h at 37°C. Recombinant VP1u proteins were purified twice with Ni-nitrilotriacetic acid (NTA) magnetic agarose beads (Qiagen, Hilden, Germany) under native conditions to obtain a convenient purity.

**VP1u prelabeling for immunofluorescence.** Purified recombinant VP1u (50 ng) was bound to Ni-NTA magnetic agarose beads and incubated with primary rabbit anti-PLA<sub>2</sub> Ab (1 μg) for 1 h in native wash buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10 mM imidazole, 0.05% Tween 20, pH 8) and 3% bovine serum albumin (BSA). Beads were washed twice to remove unbound anti-PLA<sub>2</sub> Ab and subsequently incubated with secondary Alexa Fluor 488-labeled goat anti-rabbit Ab (4 μg) for 1 h in native wash buffer and 3% BSA. Bound pre-labeled VP1u protein was washed twice and eluted by native elution buffer (250 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10 mM imidazole, 0.05% Tween 20, pH 7.2).

**VP1u binding and internalization assays.** Cells (4 × 10<sup>5</sup>) were harvested, washed, and resuspended in 100 μl of RPMI 1640 medium without FCS. To avoid unintended binding of the Ab-labeled molecules to Fc receptors, we preincubated cells with a mouse anti-CD32 antibody (0.25 μg) (BD Biosciences) for 30 min at 4°C. Cells were incubated with VP1u constructs (50 ng; unlabeled or PLA<sub>2</sub> Ab pre-labeled) or with B19V (10<sup>10</sup> virions) for 1 h at 4°C. For detection of binding, the cells were subsequently washed four times at 4°C with phosphate-buffered saline (PBS) and processed for immunofluorescence (IF) or Western blotting (WB). For internalization, the cells were transferred without washing to 37°C for 30 min, trypsinized for 4 min at 37°C, and washed twice. Internalized virus or VP1u was detected by IF, WB, or quantitative PCR (qPCR) (only virions). A rat anti-FLAG MAb was used to detect the unlabeled VP1u constructs in IF experiments. B19V was detected by the human anti-capsid MAb (860-55D). Immunostained samples were visualized by fluorescence microscopy (Axiovert 35; Carl Zeiss, Feldbach, Switzerland) or confocal fluorescence microscopy (LSM 512 Meta or Axiovert 200 M; Carl Zeiss). Images were processed by LSM Image Browser and BioImageXD software (33). VP1u was detected by WB with the antibody against the PLA<sub>2</sub> region and a secondary goat anti-rabbit Ab conjugated to horseradish peroxidase (HRP). Detection and quantification of viral DNA were carried out by DNA extraction (DNeasy Blood and Tissue Kit; Qiagen) and subsequent qPCR with B19V-specific primers (forward, 5'-GGGCA GCCATTTAAGTGT-3'; reverse, 5'-GCACCACCAGTTATCGTTA GC-3').

**Inhibition experiments.** To examine the inhibitory effect of the N-VP1u MAb (aa 30 to 42) on recombinant VP1u or B19V internalization, we incubated the recombinant protein and native virus with N-VP1u antibody for 1 h at 4°C prior to cell binding. Cells were blocked with anti-CD32 Ab to avoid Fc receptor-mediated binding (described above).

To investigate the competition of the virus with recombinant VP1u, we incubated the cells first with recombinant VP1u (wild type [WT] and VP1u lacking the N-terminal 29 amino acids [ΔN29], 150 ng; VP1u lacking the C-terminal 128 amino acids [ΔC128], 75 ng) for 1 h at 4°C and then with 10<sup>10</sup> B19 virions for one additional hour at 4°C. The subsequent experimental steps were carried out as described above.

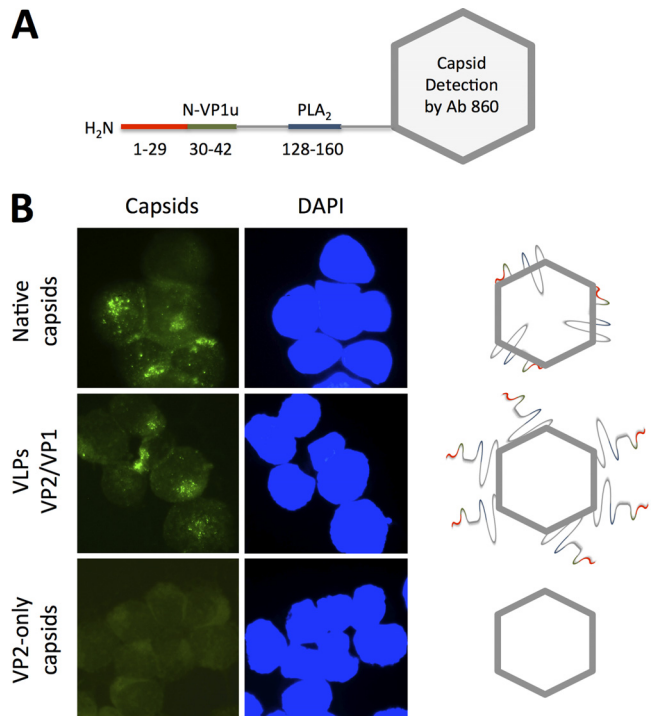
**Infectivity assay.** Cells (4 × 10<sup>5</sup>) were resuspended in 100 μl of fresh culture medium and inoculated with 4 × 10<sup>9</sup> B19 virions for 30 min at 37°C. Cells were further incubated for 3 days in 10 ml of culture medium, washed, and fixed for IF. Progeny capsids were detected with the anticapsid antibody 860-55D.

**Statistical analysis.** Error bars in qPCR histograms (see Fig. 4C and D) show the standard deviations (±SD) of the values obtained from four independent experiments.

## RESULTS

**The VP1u region is essential for virus internalization.** In order to examine the role of VP1 in B19V uptake, we compared the internalization of native virions and VLPs (empty capsids composed of VP1 and VP2) to VP2-only particles (empty capsids devoid of VP1). UT7/Epo cells were incubated with similar amounts of native virus, VLPs, and VP2-only capsids at 37°C for 30 min and subsequently trypsinized to remove uninternalized capsids. Cells were fixed, stained with anticapsid antibodies, and analyzed by immunofluorescence microscopy. While native virus and VLPs were internalized, no detectable signal was observed in cells incubated with VP2-only particles (Fig. 1). Since VP1u is the only protein domain lacking in VP2-only particles, this result suggests that the presence of VP1u as a component of the capsid is required for virus internalization.

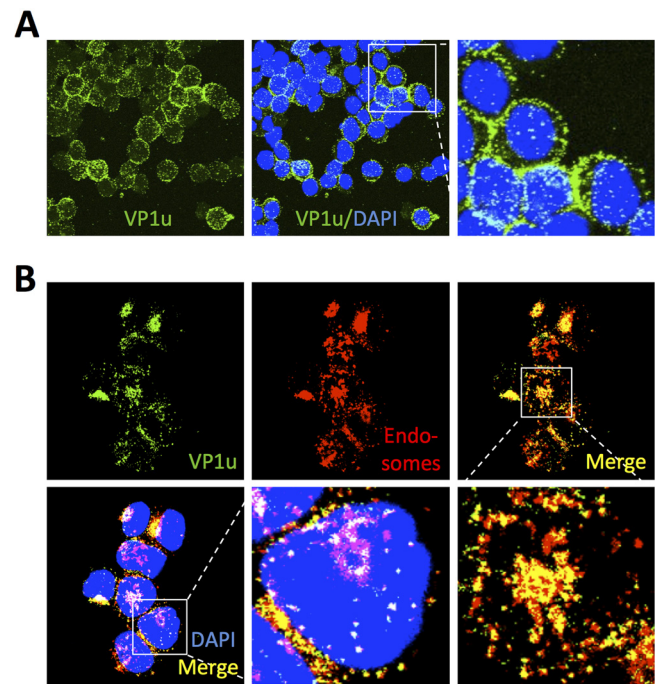
**Purified recombinant full-length VP1u binds and is internalized in UT7/Epo cells.** To study the function of VP1u in the internalization process, we expressed the entire VP1u as a recombinant protein in a prokaryotic system. Truncated VP1u proteins,



**FIG 1** Internalization assay with VP1u-containing and VP1u-lacking B19V capsids. (A) Schematic depiction of the B19V capsid and the VP1u region. In red is the truncated region ( $\Delta$ N29). In green is the epitope recognized by the N-VP1u MAb, and in blue is the epitope recognized by the PLA<sub>2</sub> Ab. (B) Internalization assay with different B19V capsids. Following binding at 4°C, UT7/Epo cells were incubated at 37°C for 30 min. Cells were trypsinized and washed to remove uninternalized particles and fixed for IF. Internalized capsids were detected with Ab 860-55D against capsids.

lacking the N-terminal 29 amino acids ( $\Delta$ N29), the C-terminal 128 amino acids ( $\Delta$ C128), and the combination ( $\Delta$ N29/ $\Delta$ C128), were also expressed. The recombinant proteins were designed with a C-terminal metal affinity tag ([MAT] His tag) and a FLAG tag for purification and detection, respectively. Furthermore, we introduced a cysteine for protein dimerization or as a possible modification site. The schematic representation of the expressed VP1u constructs is depicted in Fig. 3A. The proteins were expressed in BL21 *E. coli* and purified by means of the C-terminal MAT.

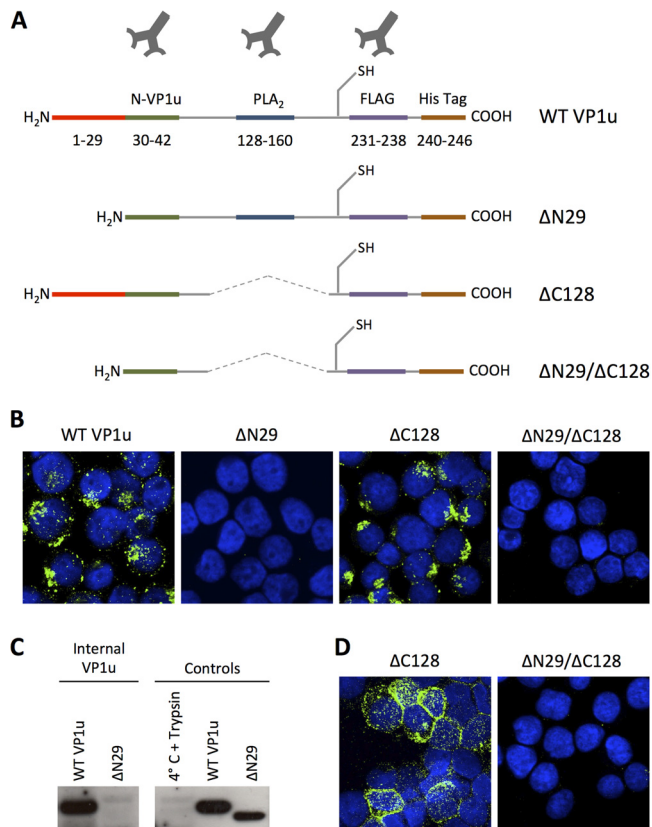
The recombinant full-length VP1u construct (WT) was pre-labeled with a rabbit antibody against an epitope (aa 142 to 163) within the PLA<sub>2</sub> region and a secondary Alexa Fluor 488-labeled anti-rabbit antibody. To test VP1u binding, we incubated UT7/Epo cells with pre-labeled WT VP1u protein for 1 h at 4°C. Subsequently, cells were washed and fixed for immunofluorescence. The results showed that the full-length recombinant VP1u protein binds UT7/Epo cells, showing an intense signal in most of the cells (Fig. 2A). To examine the internalization capacity of VP1u, we incubated the cells with pre-labeled VP1u for 1 h at 4°C and then allowed endocytosis at 37°C for 30 min. Cells were trypsinized to remove uninternalized VP1u and fixed for immunofluorescence. Endosomes/lysosomes were stained with specific markers (EEA1, early endosomes; M6PR, late endosomes; and LAMP1, lysosomes). As shown in Fig. 2B, extensive colocalization of VP1u protein with markers of the endosomes/lysosomes was observed.



**FIG 2** Binding and internalization of recombinant VP1u. Recombinant full-length VP1u (WT) was labeled with the primary PLA<sub>2</sub> Ab and a secondary Alexa Fluor 488-labeled Ab. (A) The pre-labeled WT VP1u (50 ng) was incubated with UT7/Epo cells for 1 h at 4°C. Cells were washed with PBS, and bound VP1u was detected by confocal fluorescence microscopy. (B) Internalization of pre-labeled WT VP1u into UT7/Epo was allowed for 30 min at 37°C. Cells were trypsinized, washed, and fixed for IF. The endocytic pathway (red) was stained by anti-EEA1 (early endosomes), anti-mannose 6-phosphate receptor (late endosomes), and anti-LAMP1 (lysosomes) antibodies, and signal was detected by confocal microscopy.

These results indicate that VP1u binds UT7/Epo cells and is internalized efficiently, and, similar to native B19V, the protein enters the endocytic pathway.

**The most N-terminal region of VP1u is essential for binding and internalization.** The very N-terminal part of VP1u harbors a cluster of neutralizing epitopes, which suggests that it has important functions in B19V infection. To determine whether this part of VP1u mediates the attachment and internalization into UT7/Epo cells, we used a short truncation ( $\Delta$ N29) in this region and a truncation in the C-terminal part ( $\Delta$ C128). The proteins were detected by WB and IF. In contrast to the results shown in Fig. 2, the IF results described here were not achieved by anti-PLA<sub>2</sub>-pre-labeled VP1u protein. Instead, UT7/Epo cells were incubated with unlabeled VP1u constructs at 4°C, washed, and stained with anti-FLAG antibodies. Internalization of VP1u constructs at 37°C was carried out in the presence of anti-FLAG antibodies, which were subsequently detected by IF. Both full-length VP1u (WT) and the N-terminal 99-aa fragment ( $\Delta$ C128) showed strong internalization into UT7/Epo cells (Fig. 3B and C). In comparison, the N-terminal truncations ( $\Delta$ N29 and  $\Delta$ N29/ $\Delta$ C128) totally abolished the internalization. Similarly,  $\Delta$ N29/ $\Delta$ C128 showed no binding capacity compared with  $\Delta$ C128. The truncated VP1u proteins ( $\Delta$ N29 and  $\Delta$ N29/ $\Delta$ C128) did not bind to cells (Fig. 3D) and were not internalized (Fig. 3B and C). These findings indicate that the N-terminal 29 amino acids are essential for VP1u binding and internalization. Furthermore, the results obtained with  $\Delta$ C128



**FIG 3** Identification of the VP1u region responsible for binding and internalization. (A) A linear schematic representation of the recombinant full-length VP1u (WT) and the truncated proteins ( $\Delta$ N29,  $\Delta$ C128, and  $\Delta$ N29/ $\Delta$ C128) is shown. Important regions and epitopes are indicated with different colors: red, the truncated N-terminal region; green, epitope recognized by the neutralizing N-VP1u MAb; blue, epitope recognized by the PLA<sub>2</sub> Ab; violet, FLAG tag used for detection; brown, MAT (His tag) used for purification. The inserted cysteine for protein dimerization is shown as a sulfhydryl side chain. Antibodies used are schematically shown above their corresponding epitopes (N-VP1u Ab, PLA<sub>2</sub> Ab, and anti-FLAG Ab). (B) Internalization assay with full-length (WT) and truncated versions ( $\Delta$ N29,  $\Delta$ C128, and  $\Delta$ N29/ $\Delta$ C128) of VP1u. Recombinant VP1u proteins were incubated with UT7/Epo cells in the presence of anti-FLAG antibody for 30 min at 37°C. VP1u-mediated uptake of anti-FLAG antibody was detected postfixation by IF with a secondary Alexa Fluor 488-labeled Ab. (C) Unlabeled VP1u was internalized for 30 min at 37°C and detected by Western blotting. (D) Binding assay with  $\Delta$ C128 and  $\Delta$ N29/ $\Delta$ C128 VP1u. Cells were incubated with unlabeled VP1u for 1 h at 4°C and subsequently washed. Bound VP1u was detected by IF with an anti-FLAG antibody.

VP1u demonstrate that the N-terminal 99-amino-acid region is not only required but also sufficient to trigger the internalization and exclude an involvement of the PLA<sub>2</sub> region in this process.

**B19V and recombinant VP1u undertake the same internalization pathway.** The full-length (WT) or the N-terminal ( $\Delta$ C128) VP1u constructs were able to bind and to be internalized into UT7/Epo cells independently of other viral capsid components. To elucidate whether the uptake mechanism of VP1u corresponds to that of the native virus, we sought to show, on one hand, that their internalization is inhibited by the same antibody and, on the other hand, that both compete during the uptake process.  $\Delta$ C128 VP1u and virus internalization were assessed in the presence of an MAb against VP1u (aa 30 to 42; N-VP1u Ab 1418-1) (34) (Fig. 4). The cells were briefly trypsinized and washed to remove uninter-

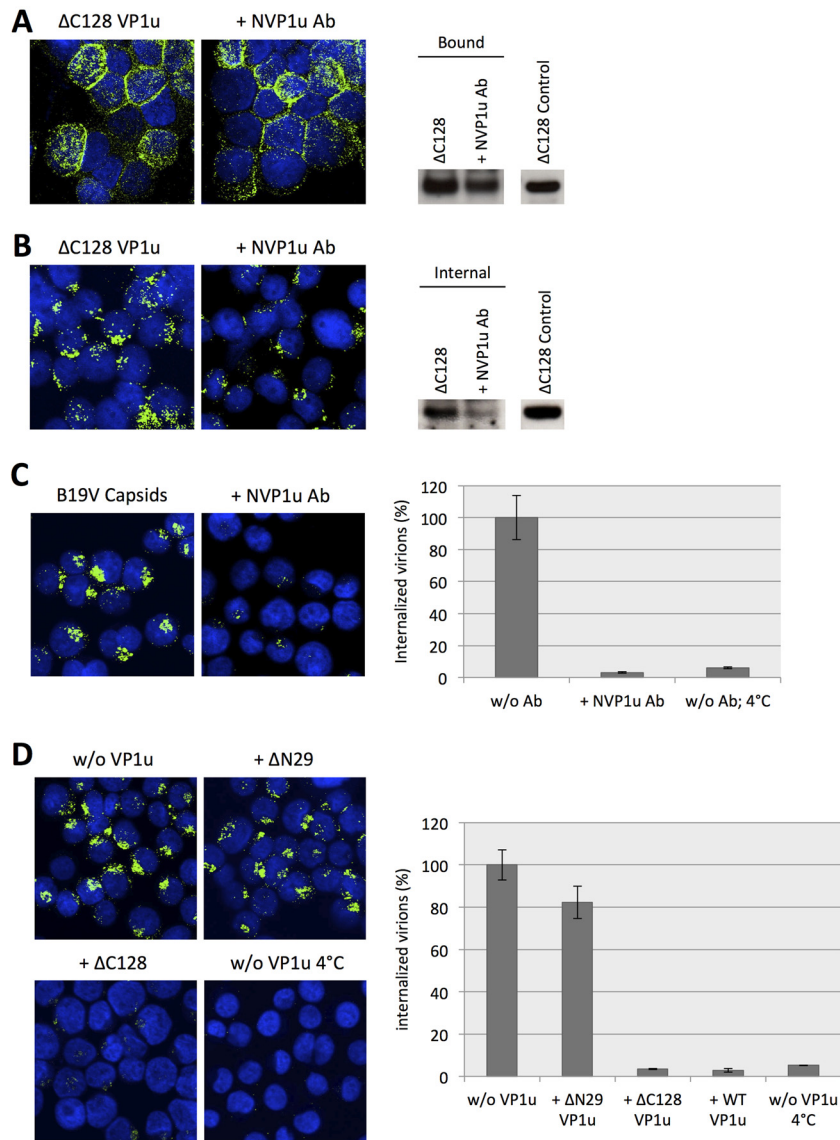
nalized VP1u or virus and prepared for immunofluorescence, WB, or quantitative PCR. The results showed that the presence of N-VP1u MAb had no significant effect on VP1u binding (Fig. 4A) but impaired VP1u internalization (Fig. 4B). Similarly, virus internalization was strongly inhibited in the presence of N-VP1u MAb (Fig. 4C). The stronger inhibition of the virus compared to that of the VP1u protein can be explained by the higher N-VP1u Ab-to-target ratio in the case of B19V. Furthermore, virus uptake was evaluated in the presence of a 50-fold excess of recombinant full-length (WT) or truncated VP1u versions ( $\Delta$ N29 and  $\Delta$ C128). While virus internalization was fully blocked in the presence of the full-length (WT) and  $\Delta$ C128 VP1u proteins, no significant effect was observed in the presence of the  $\Delta$ N29 VP1u (Fig. 4D). Therefore, native virus and VP1u uptake involves the same mechanism, which strongly suggests that the N-terminal part of VP1u is the region of the capsid responsible for B19V uptake. The inhibition of VP1u and of B19V internalization by an antibody targeting an epitope spanning residues 30 to 42 indicates that this region is not directly involved in binding although it is adjacent to critical regions required for uptake.

**VP1u binding is highly restricted and correlates with virus internalization and infection.** To further study the cell membrane component recognized by VP1u, we examined by immunofluorescence which cell types allow binding of  $\Delta$ C128 VP1u and whether a direct correlation exists to B19V internalization and infection. To this end, different erythroid and nonerythroid cells were employed.  $\Delta$ C128 VP1u was incubated with cells at 4°C to allow binding, stained, and detected by IF. In parallel experiments, B19V internalization was tested as described above, and virus infection was examined at 3 days postinfection by the detection of progeny viral capsids. The results showed that the cell membrane structure recognized by VP1u was exclusively expressed in UT7/Epo and KU812Ep6 cells, which were also the only cell types that allow virus internalization and infection (Fig. 5 and 6). The VP1u-interacting receptor was not detectable in the nonerythroid cell lines, HeLa, HEK 293, HepG2, and MRC-5 cells (Fig. 5). Expression was not detected in either the erythroleukemia K562 and KG1a cells or in mature erythrocytes (Fig. 6), revealing strong variations in the expression of the internalization coreceptor within the erythroid lineage. These results reveal a strict correlation between expression of the internalization coreceptor and B19V internalization/infection. The restrictive expression of the VP1u-interacting partner evokes the narrow tissue tropism of B19V.

## DISCUSSION

The molecular mechanism underlying B19V uptake is unknown. The sphingolipid globoside (Gb4Cer) is the receptor of B19V (5). However, binding of B19V to Gb4Cer is not sufficient for virus internalization (11). The virus might require further interactions with unknown cell membrane structures (14). The extraordinarily restricted tropism of B19V cannot be explained by the tissue distribution of Gb4Cer, which is not restricted to erythroid cells. Accordingly, other cell membrane components and poorly understood intracellular factors define the narrow B19V tropism.

The N terminus of VP1, the so-called VP1-unique region (VP1u), is the immunodominant part of the capsid (17, 18). The clustering of several neutralizing epitopes in the VP1u region denotes the existence of motifs with essential functions in the virus life cycle. Although originally not accessible to antibodies (35),

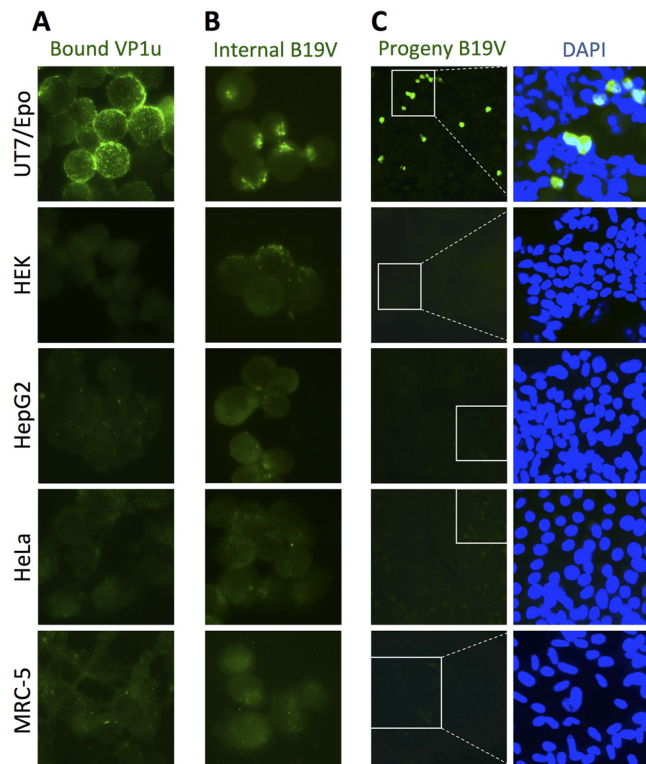


**FIG 4** B19V and the VP1u region share the same internalization pathway. (A) Unlabeled recombinant  $\Delta$ C128 VP1u (50 ng) alone or in the presence (+) of a 12-fold excess of N-VP1u Ab was incubated with UT7/Epo cells for 1 h at 4°C. Cells were washed, and cell-bound VP1u was detected by IF (anti-FLAG Ab) and WB (anti-PLA<sub>2</sub> Ab). (B)  $\Delta$ C128 VP1u was internalized alone or in the presence of N-VP1u Ab for 30 min at 37°C. Cells were briefly trypsinized, and internal VP1u was detected by IF and WB. (C) B19V internalization into UT7/Epo cells for 30 min was carried out in the presence (+N-VP1u Ab) or absence (w/o Ab) of 0.4  $\mu$ g of N-VP1u Ab. Internal capsids were stained with anticapsid Ab and detected by IF. In parallel, DNA of internalized virions was extracted and quantified by qPCR. (D) Prior to B19V internalization, UT7/Epo cells were incubated for 1 h at 4°C with recombinant VP1u proteins (150 ng of WT VP1u or  $\Delta$ N29 VP1u; 75 ng of  $\Delta$ C128 VP1u) corresponding to a 50-fold excess to the applied B19V. After 30 min at 37°C, internalized B19V was detected by IF and qPCR. Values of internalized virions were normalized to the value of internalization without Ab or recombinant VP1u. The sample at 4°C (no internalization) demonstrates the efficient removal of particles by trypsinization when internalization does not occur.

this protein domain becomes exposed following B19V binding to Gb4Cer receptor at the cell surface (30, 31). The extracellular exposure of VP1u, which contrasts with the intracellular exposure observed in other parvoviruses (20, 21, 22), suggests a possible function in B19V internalization.

In order to directly address the role of VP1u in B19V uptake, VP1u was cloned and expressed as a full-length or truncated protein. VP1u was truncated either at the C terminus by 128 amino acids ( $\Delta$ C128), including the PLA<sub>2</sub> motif, or at the N terminus by 29 amino acids ( $\Delta$ N29), a region particularly rich in neutralizing epitopes (17). The purified full-length (WT) and  $\Delta$ C128 VP1u

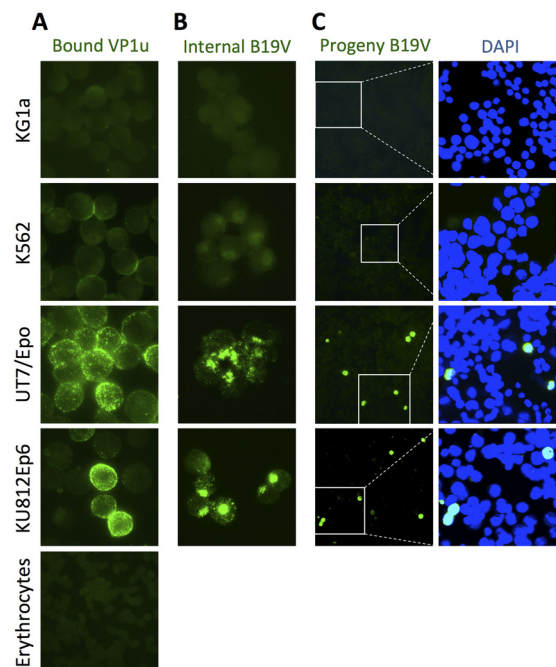
proteins, but not the  $\Delta$ N29 truncated version, were able to bind and to be internalized into UT7/Epo cells. These results indicate that the N-terminal part of VP1u, without the intervention of other capsid components, triggers the process of internalization. Sequence alignment with other erythroviruses reveals highly conserved clusters within the first N-terminal 35 amino acids, suggesting their possible function in receptor recognition and internalization. The role of the most N-terminal region of VP1u in the internalization process can also explain the strong neutralizing activity of the antibodies that target this region. In line with this interpretation, a naturally derived neutralizing monoclonal anti-



**FIG 5** Correlation of VP1u binding with B19V internalization and infectivity in different cell types. (A) Binding of  $\Delta$ C128 VP1u to cell lines derived from different tissues: UT7/Epo (bone marrow), HEK 293 (kidney), HepG2 (liver), HeLa (epithelial), and MRC-5 (lung). (B) Internalization of B19V into different cell lines. (C) Detection of capsid progeny in different cell lines. Cells were inoculated with  $10^4$  B19V virions per cell and harvested at 3 days postinfection. Progeny capsids were detected by the antibody 860-55D.

body (MAb 1418-1) (32) against an epitope adjacent to this region (epitope spanning residues 30 to 42) (34) was able to disturb VP1u internalization and to efficiently block the internalization of B19V. Moreover, blocking of the coreceptor by preincubation of cells with the full-length VP1u completely inhibited B19V uptake. Taken together, these results indicate that the early receptor-mediated exposure of VP1u at the cell surface mediates the interaction with a coreceptor, which is required for virus internalization. Despite the importance of the antibody response in the control of infections caused by B19V, the mechanism(s) of action remains unknown. The fact that VP1u is the immunodominant region of B19V (17, 18) and responsible for B19V internalization (this study) suggests that inhibition of virus uptake is a common and efficient mechanism of antibody-mediated B19V neutralization. Accordingly, a strong antibody response against the N-terminal region of VP1u should be considered in engineering vaccine-based approaches to control B19V infections.

The expression of the internalization coreceptor appears largely more restricted than that of Gb4Cer. All of the non-erythroid cells tested expressed variable levels of Gb4Cer (11, 13, 36); however, none of them expressed detectable levels of the internalization coreceptor. Among the cells from the erythroid lineage, UT7/Epo and KU812Ep6 cells expressed abundant amounts of the coreceptor, but it was undetectable in the leukemia KG1a and K562 cell lines, pretreated or not with phorbol 12-myristate



**FIG 6** Correlation of VP1u binding with B19V internalization and infectivity in different erythroid cells. (A) Binding of  $\Delta$ C128 VP1u to cell lines from different erythroid differentiation states (KG1a, K562, UT7/Epo, KU812Ep6, and erythrocytes). (B) Internalization of B19V into different cells and detection of internal capsids by IF. (C) Detection of capsid progeny in different cell lines. Cells were inoculated with  $10^4$  B19V virions per cell and harvested at 3 days postinfection. Progeny capsids were detected by the antibody 860-55D.

13-acetate. Similarly, mature erythrocytes did not express the internalization coreceptor. Apart from poorly understood intracellular factors, the sharp variations in coreceptor expression along the erythroid differentiation pathway might also contribute to the variations in susceptibilities of erythropoietic cells to B19V (37). A full correlation was found between coreceptor expression and virus internalization and infection.  $\alpha 5\beta 1$  integrin was proposed as a potential coreceptor for B19V (12). However, the highly restricted expression profile of the internalization coreceptor does not resemble that of  $\alpha 5\beta 1$  integrin, which is expressed in many cell types (13). Moreover, experiments to verify a connection between  $\alpha 5\beta 1$  integrin and B19V infection were unsuccessful. Pretreatment of UT7/Epo cells with activating (N29) or inhibitory (P4C10)  $\beta 1$  integrin antibodies had no effect on B19V internalization and infection. Similarly, colocalization of B19V with the ubiquitous CD29 ( $\beta 1$  integrin subunit) or CD49e ( $\alpha 5$  integrin subunit) was not observed (unpublished data). UT7/Epo cells, which allow VP1u binding, virus internalization, and infection, do not express detectable levels of Ku80 (31). However, Ku80 may function as a primary receptor for B19V attachment in certain cells that do not express Gb4Cer (13). Further studies are in progress to identify the restricted molecule recognized by VP1u and the dynamic interactions leading to B19V internalization.

Revealing the events that govern virus binding and internalization is essential to understanding the basis of viral tropism and pathogenesis and to developing efficient antiviral strategies that interfere with the early steps of the infection. Our study reveals a pivotal role of VP1u in B19V internalization and provides insights

into the molecular basis of the extraordinarily narrow tissue tropism of B19V.

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