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Parvovirus B19 uptake is a highly selective process controlled by VP1u: a novel determinant of viral tropism

Remo Leisi[1,2], Nico Ruprecht[1], Christoph Kempf[1,2] and Carlos Ros[1,2]*

[1]Department of Chemistry and Biochemistry, University of Bern, Freiestrasse 3, 3012 Bern, [2]CSL Behring AG, Wankdorfstrasse 10, 3000 Bern 22, Switzerland

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*Corresponding author: Department of Chemistry and Biochemistry, University of Bern, Freiestrasse 3, 3012 Bern. Phone: +41 31 6314349. Fax: +41 31 6314887.
E-mail: carlos.ros@ibc.unibe.ch

Abstract

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 internalizes UT-7/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 non-erythroid cell lines showed, that the N-terminal VP1u binding was restricted to 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 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.

Introduction

Human parvovirus B19V (B19 virus; 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, during pregnancy or in individuals with underlying immune or hematologic disorders, B19V may cause more severe syndromes such as hydrops fetalis, arthropathies and severe cytopenias (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; Gb4Cer) is the cellular receptor of B19V (5). The tissue distribution of Gb4Cer and additional cellular factors, highly restricted to the erythroid lineage, define the extraordinary 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, α5β1 integrin (12) and Ku80 autoantigen (13) have been proposed as potential co-receptors for B19V infection. While Ku80 might facilitate virus attachment to certain cell types (13); α5β1 integrin was proposed to function as co-receptor 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

66 molecules, it was observed that B19V cannot stably bind membrane-associated Gb4Cer
67 *in vitro* (14).

68 The small, nonenveloped, icosahedral B19V capsid consists of 60 structural
69 subunits, of which approximately 95% are VP2 (58 kDa) and 5% VP1 (83 kDa) (15). VP1
70 is identical to VP2 with the exception of an additional N-terminal region of 227 amino
71 acids, the so-called “VP1 unique region" (VP1u) (16). VP1u is the immunodominant part
72 of the capsid (17), (18). A poor immune response against VP1u has been linked to
73 persistent infections (19). The clustering of strong neutralizing epitopes in the VP1u
74 region, and in particular at the N-terminal region, denotes the existence of important
75 motifs required for the infection. The VP1u region of parvoviruses is not accessible on
76 the capsid surface but it becomes exposed during virus trafficking along the endocytic
77 pathway (20), (21), (22). The intracellular exposure of VP1u is thought to be important
78 for endosomal escape (23), (24), (25), (26), (27) and nuclear targeting (28), (22), (29). In
79 sharp contrast to other parvoviruses, VP1u of B19V becomes accessible to antibodies
80 upon binding to the Gb4Cer receptor (30), (31). The specific role of the early exposure of
81 VP1u at the cell surface has not been elucidated. However, considering that VP1u is the
82 immunodominant part of the capsid, its early conformational change should render
83 accessible motifs that are required for the infection. We hyphothesized that the receptor-
84 mediated rearrangement of VP1u prepares the capsid for a second interaction required
85 for virus internalization.

86 By using recombinant full-length and truncated versions of VP1u, we have
87 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.

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 fibroblasts MRC-5 and the human erythroleukemia cell lines K562 and KG1a, were purchased from ATCC. Erythrocytes were obtained from the blood transfusion service (BSD SRK, Bern). UT7/Epo cells were cultured in RPMI 1640 with 5% fetal calf serum (FCS) and 2 U/ml recombinant human erythropoietin (EPO); KU812Ep6 cells in RPMI 1640 with 10% FCS and 6 U/ml EPO; HepG2 and MRC-5 cells in MEM/EBSS with 5% FCS; K562 and KG1a cells in IMDM with 10% and 20% FCS, respectively. HeLa and HEK 293 cells were cultured in 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 (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; aa 30-42) were kindly provided by S. Modrow (Regensburg, Germany). Both antibodies were derived from healthy adults with high titers against B19V and reported as highly neutralizing (32). The polyclonal rabbit anti-PLA_2 antibody (1033/34) was obtained as previously described (30). A rat anti-FLAG mAb was purchased from Agilent Technologies (Santa Clara, CA). The CD32 (Fc gamma RII) mAb was obtained from BD Biosciences (San Jose, CA). Mouse mAb antibodies against early endosomes (EEA1, 70521), late endosomes (M6PR, 2733 2G11) and lysosomes (LAMP1, H4A3) were purchased from Abcam (Cambridge, MA).

Cloning and protein expression. The DNA fragment encoding for VP1u was amplified from the infectious clone pB19-M20, kindly provided by S. Wong (the National Institutes of Health, Bethesda, MD) with restriction site overhang primers (forward WT VP1u 5'-ACTCAAGCTTAGTAAAGAAAGTGGCAAATG-3'; reverse VP1u 5'-AGTGGTACCGCTTGGGTATTTTTCTGAG-3'). The fragment was cloned into the pT7-FLAG-MAT-Tag-2 expression vector (Sigma, St. Louis, Miss) and transformed into *E. coli* XL10-Gold cells. Truncated proteins were cloned by using deletion primers and QuickChange PCR. Protein expression was carried out in *E. coli* BL21(DE3) cells and was induced with 1mM IPTG at OD_{600} ~0.6 for 4h at 37 °C. Recombinant VP1u proteins were purified twice with Ni-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_2 Ab (1 µg) for 1 h in native wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM

imidazole, 0.05% Tween 20, pH 8) and 3% BSA. Beads were washed twice to remove unbound anti-PLA_2 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 prelabeled VP1u protein was washed twice and eluted by native elution buffer (250 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 0.05% Tween 20, pH 7.2).

VP1u binding and internalization assays. Cells (4 x 10^5) were harvested, washed and resuspended in 100 µl RMPI 1640 without FCS. To avoid unintended binding of the Ab-labeled molecules to Fc receptors, we pre-incubated 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_2 Ab prelabeled) or with B19V (10^{10} virions) for 1h at 4 °C. For detection of binding, the cells were subsequently washed 4 times at 4 °C with PBS and processed for immunofluorescence (IF) or Western Blot (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 were 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, Axiovert 200M, Carl Zeiss). Images were processed by LSM Image Browser and BioImageXD software (33). VP1u was detected by WB with the antibody against the PLA_2 region and a secondary goat anti-rabbit Ab HRP. Detection and quantification of viral DNA was carried out by DNA extraction (DNeasy Blood and Tissue Kit, Qiagen) and subsequent qPCR with B19V

171 specific primers (Forward 5'-GGGCAGCCATTTTAAGTGTTT-3'; reverse 5'-
172 GCACCACCAGTTATCGTTAGC-3').

173 **Inhibition experiments.** To examine the inhibitory effect of the N-VP1u mAb (aa
174 30-42) on the recombinant VP1u or B19V internalization, we incubated the recombinant
175 protein and native virus with N-VP1u antibody for 1 h at 4°C prior to cell binding. Cells
176 were blocked with anti-CD32 Ab to avoid Fc receptor mediated binding (described
177 above).

178 To investigate the competition of the virus with recombinant VP1u, we incubated
179 the cells first with recombinant VP1u (WT and ΔN29, 150 ng; ΔC128, 75 ng) for 1 h at
180 4°C and then with 10^{10} B19 virions for one additional hour at 4°C. The subsequent
181 experimental steps were carried out as described above.

182 **Infectivity assay.** Cells (4 x 10^5) were resuspended in 100 µl of fresh culture
183 media and inoculated with 4 x 10^9 B19 virions for 30 min at 37°C. Cells were further
184 incubated for 3 days in 10 ml culture media, washed and fixed for IF. Progeny capsids
185 were detected with the anti-capsid antibody 860-55D.

186 **Statistical analysis.** Indicated error bars in qPCR histograms (Fig. 4C and D)
187 show the standard deviation (±SD) of the values obtained from four independent
188 experiments.

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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 anti-capsid antibodies and analyzed by immunofluorescence microscopy. While native virus and VLPs 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 internalizes UT7/Epo cells. To study the function of VP1u in the internalization process, we expressed the entire VP1u as recombinant protein in a prokaryotic system. Truncated VP1u proteins, lacking the N-terminal 29 amino acids (ΔN29), the C-terminal 128 amino acids (ΔC128), and its combination (ΔN29/ΔC128) were also expressed. The recombinant proteins were designed with a C-terminal MAT (His-Tag) and a FLAG Tag for purification and detection, respectively. Furthermore, we introduced a cysteine for protein dimerization or as possible modification site. The schematic representation of the expressed VP1u constructs is depicted in figure 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 prelabeled with a rabbit antibody against an epitope (aa 142-163) within the PLA_2 region, and a secondary Alexa Fluor 488 labeled anti-rabbit antibody. To test VP1u binding, we incubated UT7/Epo cells with prelabeled WT VP1u protein for 1h 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 prelabeled VP1u for 1h 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 endosome; and LAMP1, lysosome). As shown in figure 2B, an extensive co-localization of VP1u protein with markers of the endosomes/lysosomes was observed. These results indicate that VP1u binds and internalizes efficiently UT7/Epo cells, 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 denotes 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 (ΔN29) in this region and a truncation in the C-terminal part (ΔC128). The proteins were detected by WB and IF. In contrast to the results shown in figure 2, the following IF results were not achieved by anti-PLA_2 prelabeled 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 presence of anti-FLAG antibodies, which were subsequently detected by IF. Full-length VP1u (WT) and the N-terminal 99 aa fragment (ΔC128) showed both a strong internalization into UT7/Epo cells (Fig. 3B and C). In comparison, the N-terminal truncations (ΔN29; ΔN29/ΔC128) totally abolished the internalization. Similarly, ΔN29/ΔC128 showed no binding capacity compared with ΔC128. The truncated VP1u (ΔN29; ΔN29/ΔC128) did not bind to cells (Fig. 3D) and were not able to internalize (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 ΔC128 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_2 region in this process.

B19V and recombinant VP1u undertake the same internalization pathway. The full-length (WT) or the N-terminal (ΔC128) VP1u constructs were able to bind and to internalize into UT7/Epo cells independently of other viral capsid components. To elucidate whether the uptake mechanism of VP1u correspond 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. ΔC128 VP1u and virus internalization was assessed in the presence of a mAb against VP1u (aa 30-42; N-VP1u Ab 1418-1) (34) (Fig. 4). The cells were shortly trypsinized and washed to remove uninternalized 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 the VP1u 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 (ΔN29, ΔC128). While virus internalization was fully blocked in the presence of the full-length (WT) and ΔC128 VP1u, no significant effect was observed in the presence of the ΔN29 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 B19V internalization by an antibody targeting an epitope spanning residues 30 to 42 indicates, that this region is not directly involved in binding although 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 ΔC128 VP1u and whether a direct correlation exists to B19V internalization and infection. To this end, different erythroid and non-erythroid cells were employed. ΔC128 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 3 days post-infection 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 non-erythroid cell lines, HeLa, HEK 293, HepG2 and MRC-5 cells (Fig. 5). Expression

was not either detected in the erythroleukemia K562 and KG1a cells or in mature erythrocytes (Fig. 6), revealing strong variations in the expression of the internalization co-receptor within the erythroid lineage. These results reveal a strict correlation between expression of the internalization co-receptor 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 extraordinary 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), 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 full-length or as truncated protein versions. VP1u was truncated either at the C-terminus by 128 amino acids (ΔC128), including the PLA_2 motif, or at the N-terminus by 29 amino acids (ΔN29), a region particularly rich in neutralizing epitopes (17). The purified full-length (WT) and ΔC128 VP1u, but not the ΔN29 truncated version, were able to bind and to internalize 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 antibody (mAb 1418-1) (32), against an epitope adjacent to this region (epitope 30-42) (34), was able to disturb VP1u internalization and to efficiently block the internalization of B19V. Moreover, blocking of the co-receptor 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 co-receptor, which is required for virus internalization. Despite the importance of the antibody response in the control of infections caused by B19V, their mechanisms of action remain 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 when engineering vaccine-based approaches to control B19V infections.

The expression of the internalization co-receptor appears largely more restricted than Gb4Cer. All the non-erythroid cells tested expressed variable levels of Gb4Cer (11), (13), (36), however, none of them expressed detectable levels of the internalization co-receptor. Among the cells from the erythroid lineage, UT7/Epo and

343 Ku812Ep6 cells expressed abundant amounts of the co-receptor, but it was
344 undetectable in the leukemia KG1a and K562 cell lines, pre-treated or not with phorbol
345 12-myristate 13-acetate. Similarly, mature erythrocytes did not express the
346 internalization co-receptor. Apart from poorly understood intracellular factors, the sharp
347 variations in co-receptor expression along the erythroid differentiation might also
348 contribute to the variations in susceptibility of erythropoietic cells to B19V (37). A full
349 correlation was found between co-receptor expression and virus internalization and
350 infection. α5β1 integrin was proposed as a potential co-receptor for B19V (12).
351 However, the highly restricted expression profile of the internalization co-receptor does
352 not resemble that of α5β1 integrin, which is expressed in many cell types (13).
353 Moreover, experiments to verify a connection between α5β1 integrin and B19V infection
354 were unsuccessful. Pre-treatment of UT7/Epo cells with activating (N29) or inhibitory
355 (P4C10) β1 integrin antibodies had no effect on B19V internalization and infection.
356 Similarly, co-localization of B19V with the ubiquitous CD29 (β1 integrin subunit) or
357 CD49e (α5 integrin subunit) was not observed (unpublished data). UT7/Epo cells, which
358 allow VP1u binding, virus internalization and infection, do not express detectable levels
359 of Ku80 (31). However, Ku80 may function as a primary receptor for B19V attachment
360 in certain cells that do not express Gb4Cer (13). Further studies are in progress to
361 identify the restricted molecule recognized by VP1u and the dynamic interactions
362 leading to B19V internalization.

363 Revealing the events that govern virus binding and internalization is essential to
364 understand the basis of viral tropism and pathogenesis and to develop efficient antiviral
365 strategies interfering with the early steps of the infection. Our study reveals a pivotal

366 role of VP1u in B19V internalization and provides insights into the molecular basis of the
367 extraordinary narrow tissue tropism of B19V.

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513 ## Figure Legends

514 **Figure 1. Internalization assay with VP1u-containing and VP1u-lacking B19V**
515 **capsids.** (A) Schematic depiction of the B19V capsid and the VP1u region. In red is the
516 truncated region (ΔN29). In green is the epitope recognized by the N-VP1u mAb and in
517 blue is the epitope recognized by the PLA_2 Ab. (B) Internalization assay with different
518 B19V capsids. Following binding at 4 °C, UT7/Epo cells were incubated at 37 °C for 30
519 min. Cells were trypsinized and washed to remove uninternalized particles and fixed for
520 IF. Internalized capsids were detected with Ab 860-55D against capsids.

521 **Figure 2. Binding and internalization of recombinant VP1u.** Recombinant full-
522 length VP1u (WT) was labeled with the primary PLA_2 Ab and a secondary Alexa Fluor
523 488 labeled Ab. (A) The prelabeled WT VP1u (50 ng) was incubated with UT7/Epo
524 cells for 1 h at 4 °C. Cells were washed with PBS and bound VP1u was detected by
525 confocal fluorescence microscopy. (B) Internalization of prelabeled WT VP1u into
526 UT7/Epo was allowed for 30 min at 37 °C. Cells were trypsinized, washed and fixed for
527 IF. The endocytic pathway (red) was stained by anti-EEA1 (early endosomes), anti-
528 mannose 6-phosphate receptor (late endosomes), and anti-LAMP1 (lysosomes)
529 antibodies and signal was detected by confocal microscopy.

530 **Figure 3. Identification of the VP1u region responsible for binding and**
531 **internalization.** (A) A linear schematic representation of the recombinant full-length
532 VP1u (WT) and the truncated proteins (ΔN29, ΔC128, ΔN29/ΔC128) is shown.
533 Important regions and epitopes are indicated with different colors: (red) the truncated
534 N-terminal region; (green) epitope recognized by the neutralizing N-VP1u mAb; (blue)

epitope recognized by the PLA_2 Ab; (violet) FLAG-Tag used for detection; (brown) MAT (His tag) used for purification. The inserted cysteine for protein dimerization is shown as sulfhydryl side chain. Antibodies used are schematically shown above their corresponding epitopes (N-VP1u Ab, PLA_2 Ab, anti-FLAG Ab). (B) Internalization assay with full-length (WT) and truncated versions (ΔN29, ΔC128, ΔN29/ΔC128) of VP1u. Recombinant VP1u proteins were incubated with UT7/Epo cells in presence of anti-FLAG antibody for 30 min at 37 °C. VP1u-mediated uptake of anti-FLAG antibody was detected post-fixation 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 blot. (D) Binding assay with ΔC128 and ΔN29/ΔC128 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.

Figure 4. B19V and the VP1u region share the same internalization pathway. (A) Unlabeled recombinant ΔC128 VP1u (50 ng) alone or in the presence of a 12-fold excess of N-VP1u Ab were 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_2 Ab). (B) ΔC128 VP1u was internalized alone or in presence of N-VP1u Ab for 30 min at 37 °C. Cells were shortly trypsinized and internal VP1u was detected by IF and WB. (C) B19V internalization into UT7/Epo cells for 30 min was carried out in presence or absence of 0.4 µg N-VP1u Ab. Internal capsids were stained with anti-capsid 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 ΔN29 VP1u, 75 ng of ΔC128

558 VP1u) corresponding to a 50-fold excess to the applied B19V. After 30 min at 37 °C,
559 internalized B19V was detected by IF and qPCR. Values of internalized virions were
560 normalized to the value of internalization without Ab or recombinant VP1u. The sample
561 at 4 °C (no internalization) demonstrates the efficient removal of particles by
562 trypsinization when internalization does not occur.

563 **Figure 5. Correlation of VP1u binding with B19V internalization and**
564 **infectivity in different cell types.** (A) Binding of ΔC128 VP1u to cell lines derived from
565 different tissue. UT7/Epo (bone marrow), Hek 293 (kidney), HepG2 (liver), HeLa
566 (epithelial) and MRC-5 (lung). (B) Internalization of B19V into different cell lines. (C)
567 Detection of capsid progeny in different cell lines. Cells were inoculated with 10^4 B19
568 virions per cell and harvested 3 days post-infection. Progeny capsids were detected by
569 the antibody 860-55D.

570 **Figure 6. Correlation of VP1u binding with B19V internalization and**
571 **infectivity in different erythroid cells.** (A) Binding of ΔC128 VP1u to cell lines from
572 different erythroid differentiation states (KG1a, K562, UT7/Epo, KU812Ep6 and
573 erythrocytes). (B) Internalization of B19V into different cells and detection of internal
574 capsids by IF. (C) Detection of capsid progeny in different cell lines. Cells were
575 inoculated with 10^4 B19 virions per cell and harvested 3 days post-infection. Progeny
576 capsids were detected by the antibody 860-55D.