The vanilloid receptor 1 (VR1) is a cation channel predominantly expressed by primary sensory neurons involved in nociception. It was initially identified based on its activation by the vanilloid capsaicin [1]. VR1 has also demonstrated to be activated synergistically by noxious heat and acidic pH [2]. However, the idea that the endogenous cannabinoid receptor ligand anandamide and several lipoxygenase products, such as 15-hydroxy-eicosatetraenoic acid, are capable of activating VR1 [3,4] suggests that the participation of VR1 in various signal transduction pathways is not necessarily related to nociception in response to tissue inflammation. This emerging complexity of VR1 biology demands a deeper insight into the molecular features and biochemical properties of this ion channel. VR1 is related to the transient receptor potential-family of nonselective ion channels. Based upon sequence similarities to transient receptor potential-channels, a topological model was proposed for VR1 comprising six membrane spanning sequences per monomer and one additional pore forming sequence. The N- and C-terminal domains are proposed to be localized intracellularly (reviewed in [5]). The investigation of the biochemical characteristics of VR1, like the experimental assessments of its topology and its quaternary structure, its subcellular localization, and its post-translational modifications are just beginning. Recent data obtained by Kedei et al. [6] suggests a tetrameric quaternary structure for VR1 overexpressed in CHO cells as well as in a dorsal root ganglia (DRG) crude membrane fraction. The glycosylation state of VR1 was found to depend on the expression system. Remarkably, endogenous VR1 in rat DRG was not found to be glycosylated [6] in contrast to what would be expected for an ion channel localized at the neuronal plasma membrane. Olah et al. [7] reported a predominant localization of VR1 at the ER membrane when overexpressed in COS-7 cells and suggested the same localization for endogenous VR1 in the DRG. However, this finding appears to be inconsistent with electrophysiological data, particularly with single-channel recordings in the attached-patch configuration, which have been obtained from plasma-membrane-localized VR1 [1].

We addressed this apparent conflict and assessed the biochemical characteristics of VR1 such as its glycosylation state, quaternary structure and subcellular localization, when expressed in HEK293 cells and in the DRG-derived cell line F-11 (mouse neuroblastoma × rat dorsal root ganglia hybridoma). VR1 expressed in F-11 cells mimics the in vivo situation better than expressed in fibroblast-derived cell lines. In this study, we show that VR1 is
glycosylated when expressed both in HEK293 and in F-11 cells. VR1 localizes to distinct regions of the plasma membrane, especially neurite-like structures, in F-11 cells, whereas its localization in HEK293 cells appears more heterogeneous. Under limited denaturing conditions, the tetrameric quaternary structure of VR1 could be observed in both F-11 and HEK293 cells. Under native conditions, the receptor is part of a high-molecular-mass complex.

EXPERIMENTAL PROCEDURES

Cloning of rat VR1

Rat vanilloid receptor 1 (rVR1) was cloned from total RNA prepared from L4-L6 DRG of adult rats. Total RNA (2.5 μg) from rat DRG was reverse transcribed using OligoT and Superscript reverse transcriptase (Life Technologies) at 37 °C. RT-PCR was carried out with specific rVR1 forward and reverse primers designed using the GenBank sequence AF029310. Primers corresponding to nucleotides 73–91 and 2578–2597 and which incorporated the restriction sites for EcoR1 and XbaI were rVR1F–EcoR1 (5’-GGCGCGAA TTCGGAAAAGGATTGGGAACACG-3’) and rVR1R–XbaI (5’-GGCGCTCTAGATTATTTCTCCCCTCTTCCGACC-3’). Reverse transcribed reaction (1 μL) was used in 20 μL PCR amplifications containing 5% dimethylsulfoxide using Pfu polymerase (Life Technologies) after heating the sample to 95 °C for 5 min. PCR conditions were 95 °C, 30 s; 57 °C, 30 s; 72 °C, 90 s for 35 cycles. PCR was analysed by electrophoresis in a 0.9% agarose gel for the presence of a specific 2.5-kb rVR1 band. The band was excised from the gel and purified with UltraClean DNA Purification Kit (MOLBIO Laboratories, Inc.) using the manufacturer’s instructions. The cDNA was cloned into the EcoR1–XbaI site of pcDNA3.1 (+) (Invitrogen). The accuracy of the entire open reading frame was checked by DNA sequencing of both strands. The resulting sequence was identical to GenBank sequence accession number AF040873 that represents the complete ORF of rat vanilloid receptor-1-like protein 1 (VR1L1).

Site-directed mutagenesis

The QuikChange™ XL Site Directed Mutagenesis Kit (Stratagene) was used to introduce a point mutation into the cDNA changing the asparagine at position 604 into a threonine. Two primers were used, primer 1 (5’-GGAGATGGGAAGGACTAACTTCTTGGCTATGG-3’) and primer 2 (5’-CCATAGGGAGAGATGTCTTCCCACCTCAATC-3’) (TibMolBiol). The mutagenic codon is shown in bold; this generates a single nucleotide exchange (A1811C mutation) into the cDNA. The PCR reaction with the VR1-plasmid as template was performed under the conditions recommended by the manufacturer. The successful mutagenesis was confirmed by restriction digest with BbsI (NEB) and plasmid sequencing. The successful mutagenesis (A1811C) in the cDNA produced an additional restriction site for BbsI in the rVR1 plasmid. After restriction digestion, the rVR1 plasmid (7928 bp) generates three fragments with lengths of 5734, 1415, and 779 bp whereas the digest of the A1811C rVR1 plasmid results in four fragments 5734, 1263, 779 and 152 bp. The fragments after BbsI digestion were visualized on a 1.2% agarose gel with 0.5 μg·mL⁻¹ ethidium bromide (data not shown).

Transfection of cells and cell culture

HEK293 cells were cultured in modified Dulbecco’s Eagle’s medium (25 mM Hepes, sodium pyruvate, 1000 mg·L⁻¹ glucose with pyridoxine; Life Technologies, catalogue no. 22320-022), supplemented with 10% fetal bovine serum (GibcoBRL), 1% nonessential amino acids (Biochrom KG), 1% L-glutamine, 100 μg·mL⁻¹ streptomycin, and 100 μg·mL⁻¹ penicillin at 37 °C in a humidified atmosphere with 5% CO₂ on plastic tissue culture grade flask (Nunc).

F-11 cells (obtained from M. C. Fishman, Cardiovascular Research Center, Massachusetts General Hospital, Charlestown, MA, USA) were cultured in Ham’s F-12 media with Glutamax-I (Life Technologies), supplemented with 20% fetal bovine serum (GibcoBRL), 2% hypoxanthine/thymidine/aminopterin supplement (Biochrom KG), 100 μg·mL⁻¹ streptomycin, and 100 μg·mL⁻¹ penicillin at 37 °C in a humidified atmosphere with 5% CO₂ on plastic tissue culture grade flask (Nunc).

Both cell lines were transiently transfected with the rVR1 plasmid or A1811C rVR1 plasmid using Lipofectamine Plus (Life Technologies) according to the procedure recommended by the manufacturer. Plasmids were constructed as described above.

Indirect immunofluorescence and confocal microscopy

For indirect immunofluorescence coverslides were added to culture plates before seeding and transfection of cells. All following steps were carried out at room temperature; 48 h after transfection cells on the coverslides were washed once with NaCl/Pi, fixed with 3% paraformaldehyde for 1 h and washed three times with NaCl/Pi for 5 min. After a 10-min permeabilization with 0.4% Triton X-100 in NaCl/Pi, cells were incubated in glycine solution to quench unsaturated aldehydes for 20 min. Blocking was performed for 1 h with 5% normal goat sera (Dianova) in NaCl/Pi. For immunostaining, polyclonal anti-VR1 serum was diluted 1:500 in 5% normal goat sera/NaCl/Pi. The polyclonal anti-VR1 serum was raised in rabbits against the peptide VFKDMSVPGEK corresponding to the C-terminus of VR1. The antisemum was a kind gift of M. K.-H. Schäfer, Philipps-Universität Marburg, Germany.

Cy² conjugated goat anti-(rabbit IgG) Ig (Jackson Immuno- research Laboratories) was used in a 1:400 dilution in 5% normal goat sera/NaCl/Pi as the secondary antibody. Immunostaining was visualized by indirect immunofluorescence using confocal laser scanning microscopy (Radiance2000 Confocal Imaging Systems, Bio-Rad). The immunofluorescence was reduced to background levels in control samples with 200 μg·mL⁻¹ immunization peptide (Genosys) added to the primary antibody solution.

Preparation of cell homogenate

At 48 h after transfection the cells from culture dishes were washed once with cold NaCl/Pi and harvested in NaCl/Pi. The following steps were carried out at 4 °C. The cells were gently centrifuged for 5 min at 500 g and then resuspended in 0.25 mM sucrose/Tris/mg (0.25 mM sucrose, 50 mM Tris/HCl, pH 7.4,
5 mM MgSO_4_ containing one protease inhibitor cocktail tablet for 50 mL buffer (Complete™, Boehringer Mannheim) and 1 mM phenylmethylsulfonyl fluoride. Cells were homogenized in a glass homogenizer. The homogenate was frozen in liquid nitrogen and stored at −70 °C until use.

**Western blot analysis**

Aliquots (20 µg) of proteins in loading buffer were subjected to SDS/PAGE (10%). Bands of protein were electroblotted onto nitrocellulose membranes (Schleicher & Schuell). Membranes were soaked for 2 h in NaCl/Tris/Tween (20 mM Tris buffer, pH 7.5, 150 mM NaCl, 0.1% Tween 20) with 5% nonfat dry milk and subsequently incubated for 1 h with polyclonal anti-VR1 serum (see above, dilution 1 : 500) in NaCl/Tris/Tween with 5% nonfat dry milk. After washing, blots were incubated for 1 h with a goat anti-(rabbit IgG) Ig coupled to either alkaline phosphatase or horseradish-peroxidase (dilution 1 : 1000).

Immunoreactive bands were visualized by either alkaline phosphatase or horseradish-peroxidase (dilution 1 : 1000). Immunoreactive bands were visualized by either alkaline phosphatase staining or horseradish-peroxidase (dilution 1 : 1000).

**Deglycosylation with N-glycanase and endoglycosidase H**

Deglycosylation with N-glycanase (Boehringer Mannheim) and endoglycosidase H (Boehringer Mannheim) was performed in a volume of 50 µL with 60 µg of cell homogenate. N-Glycanase reaction was carried out in 20 mM sodium phosphate buffer, pH 7.5, containing 50 mM EDTA and 0.02% sodium azide, with 0.1% SDS, 50 mM 2-mercaptoethanol and 0.05% bromphenol blue. After adding 2 U N-glycanase the reaction was incubated at 37 °C for 60 min.

The endoglycosidase H reaction was carried out in 100 mM sodium phosphate buffer pH 6.6 with a SDS concentration > 0.1% and 1 µL benzonase. After adding 5 mM of endoglycosidase H, the reaction was also incubated at 37 °C for 60 min.

Thirty micrograms of a membrane preparation from the electric organ of *Torpedo californica* containing mainly nicotinic acetylcholine receptor was used as a positive control. After incubation, the probes were separated by electrophoresis on a 10% Laemmli gel. Western blot analysis was performed as described above.

**Cross-linking with dimethyladipimidate**

Total cell homogenate was prepared as described above. Forty micrograms of HEK293 cell homogenate were centrifuged for 15 min at 13 000 g. Proteins from the resulting pellets were extracted in a total volume of 50 µL with 1% dodecyl maltoside in NaCl/P_i with 0.2 mM triethanolamine, pH 8.0, was cross-linked with 1 mg of dimethyladipimidate for 1 h at room temperature) was separated on the same gels with 1/4 volume of 2 × SDS sample buffer (125 mM Tris/HCl (pH 6.8), 20% glycerol, 4% SDS, 0.7 mM 2-mercaptoethanol and 0.05% bromphenol blue).

Western blot analysis was performed as described above. Immunoreactive bands were visualized using ECL reagent.

**RESULTS**

The cloned *VR1* from rat DRG RNA reveals identity to *VR1L1*, but differs from the published *VR1L1* sequence.

We cloned the rat *VR1* into vector pcDNA3.1(+) from total RNA, prepared from L4-L6 DRG of adult rats. We obtained a sequence identical to rat *VR1L1* (accession no. AB040873). The sequence of *VR1L1* represents the open reading frame of the rat *VR1* (accession no. AF029310) as cloned by Caterina *et al.* [1] except for three single nucleotide exchanges in codons at position of amino-acid 18 relative to the N-terminus [VR1, 132GAG; VR1L1, 52GAT; rat vanilloid receptor 1-like protein 2 (VR1L2, accession no. AB041029), 52GAT], amino-acid 96 (VR1, 366CCG; VR1L1, 286CAG; VR1L2, 286CAG) and amino acid 440 (VR1, 1398GTC; VR1L1, 1318GTT; VR1L2, 1138GTT). These differences lead to two changes in the amino-acid sequence, namely D18E and P96Q in VR1L1. The last difference leads to no change in the Val at position 440. These results have been confirmed in an independent cloning (data not shown, E. Wade, Grunenthal GmbH, Aachen, Germany, personal communication). We propose that the *VR1L1* sequence represents the true Sprague–Dawley rat *VR1* sequence (Fig. 1).

**Expression of VR1 in different cell types**

As a prerequisite for further biochemical studies, we transfected different cell lines with *VR1* cDNA and probed cell
lysates at 48 h post-transfection with a polyclonal antiserum directed against the C-terminus of rat VR1. The transient transfection efficiency was about 70% for the HEK293 cells and about 50% for the F-11 cells, as assessed by indirect immunofluorescence (see below). Both in lysates from transiently transfected HEK293 and F-11 cells, the anti-VR1 serum specifically detected a diffuse protein band at \( \approx 115 \text{kDa} \) and a doublet band at \( \approx 100 \text{kDa} \) and \( \approx 97 \text{kDa} \) (Fig. 2). These bands were not detected in nontransfected cells nor when the immunization peptide was added to the primary antibody solution to block the VR1-antigen-specific antibodies. The anti-VR1 immunoreactive protein band at 97 kDa is in agreement with the calculated molecular mass of rat VR1. The presence of the two other anti-VR1 immunoreactive bands at slightly greater apparent molecular masses suggested post-translational modification of VR1. One possibility was that the post-translational modification was in the form of an Asn-linked glycosylation of VR1.

**VR1 is part of a large protein complex in transfected cells**

The determination of the quaternary structure of a ligand-gated ion channel is important for the interpretation of ligand binding data. Furthermore, many receptors are part of multiprotein complexes, and protein–protein interactions may determine receptor function. Therefore, we assessed the quaternary structure of VR1 in transfected HEK293 and F-11 cells. We used the blue native PAGE system [8] for the separation of cell lysate components under native or controlled denaturing conditions; 4–16% polyacrylamide gradient gels were used for the separation. After electroblotting, the blotting membranes were probed with the anti-VR1 serum.

When solubilized by \( n \)-dodecyl maltoside under non-denaturing conditions, anti-VR1 immunoreactivity appears on the blotting membrane at positions corresponding to the gel pockets (Fig. 3A). Thus, VR1 is part of a protein complex too large to enter the gel. When limited amounts of SDS were added, anti-VR1 immunoreactivity showed up predominantly at the positions of VR1 dimers, but also at the position of trimers and tetramers as deduced from the migration of glutamate dehydrogenase oligomers that were used as molecular mass standards (Fig. 3A). Thus, in the presence of SDS the complex dissociates and VR1 oligomers become visible. As a control, we disrupted the protein–protein interactions of VR1 by applying urea and SDS to the cell lysates to observe the VR1 monomer. The monomer appeared as a double band, consistent with a potential glycosylation of the receptor. When crosslinked by dimethyladipimidate, VR1 monomers and dimers were observed. This suggests that the dimer is particularly stable (Fig. 3B).

**VR1 is predominantly localized at the plasma membrane in F-11 cells**

As the literature contains conflicting data on the subcellular localization of VR1 [7,10], we assessed the subcellular localization of the receptor by indirect immunofluorescence using confocal laser scanning microscopy of transiently VR1-transfected HEK293 and F-11 cells. In HEK293 cells,
anti-VR1 immunoreactivity shows up at intracellular membranes, the major proportion in the ER, and at the plasma membrane (Fig. 4). When expressed in F-11 cells, anti-VR1 immunoreactivity shows up predominantly at the plasma membrane and localizes to neurite-like structures. In several cases, a punctuate immunoreactivity was observed (Fig. 4). This suggests that VR1 may be capable of clustering in distinct regions of the plasma membrane. The

Fig. 3. Blue native PAGE of cell lysates from HEK293 cells transiently transfected with the VR1-plasmid (A) and cross-linking with dimethyl adipimidate (B). (A) Cell lysates were separated under native or controlled denaturing conditions as indicated. Under native conditions the VR1 complex is too large to enter the gel. With increasing amounts of SDS anti-VR1 immunoreactivity appeared predominantly at the position of VR1 dimers, but also trimers and tetramers become visible. (B) Lysate of HEK293 cells transiently transfected with the VR1-plasmid VR1 was cross-linked with dimethyl adipimidate and applied to a 4–16% Blue Native gel. Following Western blot analysis, VR1 monomers and dimers were observed. DTT, dithiothreitol.

Fig. 4. Indirect immunofluorescence with polyclonal anti-VR1 serum. VR1 transfected F-11 cells (A and B) show predominantly plasma membrane localization whereas in HEK293 cells (C and D) VR1 seems to localize mainly to intracellular membranes (ER).
anti-VR1 immunoreactivity at the plasma membrane is partly colocalized with F-actin as assessed by anti-VR1/-phalloidin double labeling experiments (data not shown). Taken together, our data suggests that VR1 is mainly localized at the plasma membrane and displays a distinct distribution pattern compared to transfected HEK293 or COS-7 cells [7]. In addition, our data suggests that this distinct subcellular distribution is clearer if the receptor is expressed in a neuronal DRG-derived cell line than if expressed in a fibroblast-derived cell line.

VR1 is a glycoprotein

Anti-VR1 immunoreactivity appeared as multiple bands in lysates of transfected HEK293 and F-11 cells (see above), which is characteristic of glycoproteins. To assess this potential post-translational modification of VR1, we incubated cell lysates from transfected cells with glycosidases. In order to distinguish between high mannose type glycosylation from complex glycosylation, we used endoglycosidase H (which only cleaves high mannose type sugars) or N-glycanase (which is capable of removing all types of sugars) for the deglycosylation of the VR1 containing cell lysates. The potential deglycosylation of VR1 was probed by anti-VR1 Western blotting subsequent to protein separation by SDS/PAGE according to Laemmli [9].

Incubation with endoglycosidase H abolished the upper band of the anti-VR1 immunoreactivity doublet band at \( \approx 100 \) kDa. The lower band of the doublet at 97 kDa increased proportionally in intensity, but the diffuse anti-VR1 immunoreactivity at 115 kDa was unaffected (Fig. 5). Neither the 115- nor the 100-kDa anti-VR1 immunoreactivity band were observed subsequent to incubation of the VR1-containing cell lysates with N-glycanase, and only the 97-kDa band showed up. These effects were observed both with lysates from transfected HEK293 and F-11 cells. Our data indicate that both high mannose-type glycosylation and complex glycosylation of VR1 occurs. As complex glycosylation is established in post-ER compartments such as the Golgi apparatus or the trans-golgi network, the presence of VR1 in a state of complex glycosylation demonstrates the presence of a post-ER VR1 population, in contrast to the predominant ER localization of VR1 suggested by others [7].

VR1 is exclusively glycosylated at Asn604

In order to determine the VR1 glycosylation sites, we used the PROSITE prediction tool (http://www.expasy.ch/tools/scnpsit1.html) for the detection of the Asn-X-Ser/Thr Asn-glycosylation consensus motif within the VR1 primary structures. Five potential Asn-glycosylation sites were detected, namely Asn170, Asn191, Asn213, Asn604, and Asn748. We then predicted the extracellular or intracellular localization of these Asn residues with respect to the receptor topology as predicted by the program TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0), and also as suggested by [1]. Asn604 is the only Asn residue within an Asn-glycosylation consensus motif predicted to be localized extracellularly (Fig. 6). Thus, if the topology prediction for VR1 is correct, Asn604 should be the only Asn-glycosylation site of VR1.

Using site-directed mutagenesis to generate a single nucleotide change, we obtained a A1811C mutation in the VR1 cDNA. This mutation results in a N604T exchange in the VR1 protein at the predicted NNSL consensus Asn-glycosylation site.

We then transfected HEK293 and F-11 cells with VR1 wild-type or N604T mutant VR1 cDNA and assessed the glycosylation state of the expressed VR1 using the deglycosylation assay, SDS/PAGE and anti-VR1 Western Blotting. Wild-type VR1 gave rise to the three-band anti-VR1 immunoreactivity pattern described above. Deglycosylation by endoglycosidase H affected the 100 kDa band, and deglycosylation by N-glycanase affected both the 115-kDa band and the 100-kDa band. In the case of the VR1 mutant, only a single anti-VR1 immunoreactivity band was seen at 97 kDa even in the absence of glycosidases (Fig. 7). Thus, Asn-glycosylation was completely abolished in the mutant VR1. These data demonstrate that Asn604 is the only Asn-glycosylation site of VR1. In addition, our data are consistent with an intracellular location of the other four potential Asn-linked glycosylation sites, as predicted by the VR1 topology model (Fig. 6).

DISCUSSION

At this point, the subcellular localization of VR1 is still controversial. Whereas Olah et al. [7] report a predominant localization of the receptor at the ER, patch clamp studies
with single channel recordings of VR1 in transfected cells require a plasma membrane localization of the receptor. Furthermore, Kedei et al. [6] recently reported differences in the presence or absence of glycosylation on VR1 depending on the cell line in which the receptor is expressed.

We here present the first biochemical characterization of the VR1 expressed in the DRG-derived F-11 cell line and thereby the first characterization when expressed in a neuronal cell line. We chose this cell line in order to have an expression system as close to the in vivo conditions as possible, but still yielding an amount of receptor large enough for biochemical studies. We also transfected HEK293 cells with VR1 to compare the results obtained in the two cell lines. There were no gross differences with respect to the quaternary structure analysis and the glycosylation state between VR1 in F-11 and in HEK293 cells. However, the subcellular localization of VR1 in F-11 cells appeared more distinct (predominantly at the plasma membrane) in F-11 cells than in HEK293 cells.

VR1 showed up as a glycoprotein in F-11 and HEK293 cells. We observed both high mannose-type and complex, i.e. endoglycosidase H-resistant glycosylation. By site-directed mutagenesis, we identified Asn604 as the only occupied glycosylation site out of five potential Asn-X-Ser/Thr

Fig. 6. Predicted membrane topology of VR1. Ankyrin repeat domains (ANK) and five potential Asn-X-Ser/Thr Asn-glycosylation consensus motifs are shown. The N- and C-terminus are located intracellularly. We show that Asn604 is the only Asn-glycosylation site of VR1. The other potential Asn-glycosylation consensus sites are located intracellularly, as predicted by the VR1 topology model.

Fig. 7. Deglycosylation with N-glycanase and endoglycosidase H of VR1 and VR1A1811C transfected F-11 cells (A) and HEK293 cells (B). Wild-type VR1 produced the three-band anti-VR1 immunoreactivity pattern also shown in Fig. 5. Deglycosylation by endoglycosidase H affected the 100-kDa band, and deglycosylation by N-glycanase affected both the 115-kDa band and the 100-kDa band. The N604T mutant VR1 only showed a single anti-VR1 immunoreactivity band showed up at 97 kDa even in the absence of glycosidases. Endo H, endoglycosidase H; N-Gly, N-glycanase.
consensus glycosylation sites present in the VR1 primary structure. The observation that Asn604, which represents the only predicted extracellular consensus site, was indeed the only site of glycosylation, supports the current model for the VR1 topology with the other consensus sites being localized intracellularly. The observation that this single glycosylation site occurs in different glycosylation states (high mannose and complex) may be explained in two ways: either there is a mixed population of mature receptors, or the high mannose-glycosylated subpopulation represents immature receptor molecules that reside still in the ER, but are about to leave for post-ER compartments to have the glycosyl structure trimmed and to be localized at the plasma membrane as mature receptor.

The subcellular localization of VR1 in F-11 cells as assessed by indirect immunofluorescence using confocal laser scanning microscopy appears to be predominantly at the plasma membrane. Within the plasma membrane, our data suggest a clustering at specialized structures and a localization to neurite-like structures. Consistent with this, anti-VR1 immunoreactivity was found to partially colocalize with the phalloidin stain for filamentous actin, suggesting contact with the cytoskeleton; however, more detailed studies are required to assess this.

Our data on the quaternary structure of VR1 are consistent with reports by others [6] who demonstrated a tetrameric structure of VR1. Cross-linking with dimethyladipimidate suggests that the tetramer may consist of a dimer of dimers. In our experiments, VR1 was seen as part of a high molecular mass complex under native conditions, and SDS was required to reveal VR1 dimers, trimers and tetramers. The question as to whether VR1 occurs as a homooligomer or heterooligomer or which other proteins may interact with VR1 is a major topic that needs to be addressed.

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