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Fluorescence Labeling of Human Rhinovirus Capsid and Analysis by Capillary Electrophoresis

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The capsid of human rhinovirus serotype 2, consisting of four viral proteins, was fluorescence-labeled with fluorescein isothiocyanate and analyzed by capillary electrophoresis using UV and laser-induced fluorescence detection. Heat denaturation, proteolytic digestion, and receptor binding were applied for confirmation of the identity of the peak with the labeled virus. Incomplete derivatization with the fluorophore preserved the affinity of the virus for its receptor, indicating that its cell entry pathway is unperturbed by this chemical modification; indeed, an infectivity assay confirms that the labeled virus samples are infectious. The results show that fluorescence labeling of the viral capsid might lead to a valuable probe for studying infection processes in the living cell.

Fluorescence labeling is widely used for high-sensitivity detection and localization of biological molecules within the cell by fluorescence microscopy. Usually, the molecule of interest is visualized after fixation of the biological specimen, via reaction with a fluorescently labeled antibody or a nonlabeled antibody that is subsequently detected with a secondary antibody carrying the fluorophore. These methods require the specimen to be permeabilized to allow for access of the antibodies. However, both fixation and permeabilization might modify the antigenic properties of the molecule to be detected and it is often necessary to adjust the parameters in several experiments to obtain optimal results. Therefore, it is advantageous, if not indispensable, to use a molecule that itself carries the label and, consequently, does not require all of the aforementioned manipulations. This is particularly the case for studies aimed at following the internalization pathway of viruses in living cells. To avoid that attachment of fluorophores to the virus leads to a behavior different from the native virus several methods have been thought. Green fluorescent protein (GFP) was engineered into a viral protein, which was then incorporated into the virus during replication[1,2] or GFP was fused to a specific DNA-binding protein to be encapsidated in the virion when attached to the viral genomic DNA.[3] These methods can be used only where the virus allows for extensive modification of any of its proteins.

Human rhinoviruses (HRVs) are small icosahedral particles with a diameter of ∼30 nm. Four different capsid proteins comprise the viral shell, which is devoid of lipid and saccharides (for a review, see the work of Semler et al.[4]). They contain a single-stranded RNA genome with ∼7500 bases that results in a polyprotein upon translation in the host cell. This polyprotein is then autocatalytically processed to the mature structural and nonstructural (replicative) proteins. The small HRVs do not allow for any gross modification, and a capsid protein that carries GFP would not be incorporated into the virus. Therefore, GFP is not suitable for the fluorescence labeling of rhinoviruses. We thus explored the conventional method of modifying surface-exposed lysines chemically, labeled rhinovirus serotype 2 (HRV2) with fluorescein isothiocyanate (FITC), and characterized it by capillary electrophoresis (CE). Our present report demonstrates that HRV2 can be labeled with FITC in a way that preserves affinity for its receptor. This is a prerequisite for internalization studies to be performed with this probe in living cells.

EXPERIMENTAL SECTION

Instrumentation. CE with fluorescence (FL) detection was performed using two different instruments: a homemade apparatus that was equipped with a laser-induced fluorescence (LIF) detector (Ar-Laser, Laser-Physics, Reliant 50S-489, 50 mW, 488 nm) and an automated HP3D capillary electrophoresis system (Agilent, Waldbronn, Germany) that was equipped with a diode array UV−vis detector coupled with a broadband fluorescence detector (Argos 250B, Flux Instruments, Basel, Switzerland).

With the homemade instrument, an uncoated fused silica capillary (28.5/20.0 cm length, 50 μm inner diameter (ID); Composite Metal Services, Ltd.) was used. The capillary was positioned in still air without thermostating. The detector was placed at the cathodic side of the capillary. The emitted light was focused by a microscopic lens system and passed through a filter (cutoff of 500 nm). Light intensity was measured with a photomultiplier (Hamamatsu H5785, Shimokanzo, Japan). Injection was conducted hydrodynamically by lifting the capillary inlet by 3 cm for 10 s. Separation was performed at +7.5 kV at ambient temperature. Data collection was done with DataApex software (Prague, Czech Republic). The capillary was conditioned by flushing between all runs with ∼20 μL of background electrolyte (BGE).

In the automated HP3D instrument, a fused silica capillary (Composite Metal Services, Ltd.) with a total length of 59.0

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cm and effective lengths of 50.5 cm (UV detection) and 41.0 cm (FL detection), and an ID of 75 μm was used. The capillary was packed in a standard HP cassette and thermostated to 20.0 °C during all experiments. Injection was performed at 25 mbar for 4 s, and the voltage was +20 kV. UV absorption was measured at 205 and 260 nm. The excitation wavelength for FL detection was selected with a broadband filter (240−400 nm), and the emitted wavelength was measured at >520 nm using a cut-off filter. Data acquisition, storage, and analysis were performed using Agilent ChemStation Plus software.

Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) of the proteins was performed using a 12.5% gel after denaturation of the FITC-labeled virus sample by boiling in Laemmli sample buffer for 3 min. The gel was either stained with Coomassie Brilliant Blue or fluorescent bands were photographed on a UV transilluminator.

Reagents. HRV2 was produced and purified from infected cell pellets, as described previously.[5] The viral protein concentration was 6.0 mg/mL (0.70 μmol/L), as determined from an absorbance at 260 nm (A_{260}) of 7.7 for a 1 mg/mL solution,[6] in a 50 mmol/L HEPES buffer. The previously used TRIS buffer was replaced by the HEPES buffer in the present work to avoid reaction with FITC. Virus purity in this stock preparation was checked by CE from the virus peak and corrected.[7] A recombinant concatemer of five copies of ligand binding repeat number 3 of the very-low-density lipoprotein receptor (VLDLR), fused to maltose binding protein at its N-terminus and to a hexa-his tag at its C-terminus (MBP-V33333), was produced and purified as described.[8,9] It was used at a concentration of ~1.0 mg/mL. FITC isomer I was obtained from Sigma (Milwaukee, WI), and fluorescein sodium salt-CAPS solution was obtained from Fluka (Buchs, Switzerland). Proteinase K was obtained from Qiagen (Valencia, CA). All other chemicals were purchased from E. Merck (Darmstadt, Germany) and were used without further purification. Sephadex G-25 M material was taken from prepacked PD-10 columns (Amersham Bioscience, Uppsala, Sweden) to separate the labeled virus from excess FITC and byproducts. The SEC columns were equilibrated and the components were eluted in a 50 mmol/L borate buffer adjusted to pH 8.3 with NaOH. For CE analysis, a 50 mmol/L SDS solution was added to the fractions to a final concentration of 10 mmol/L SDS. If needed, the samples were diluted with separation buffer (100 mmol/L borate, pH 8.3, 10 mmol/L SDS). All solutions were prepared in double-distilled water, degassed by ultrasonication for 5 min, and centrifuged for 5 min in a tabletop centrifuge (Eppendorf, model 5415D, Hamburg, Germany) at 11 800 g prior to CE analysis.

Procedures. To label the capsid of native virus with FITC, a purified virus preparation was diluted (1:10 ratio) with labeling buffer (100 mmol/L sodium bicarbonate buffer adjusted to pH 9.0 with NaOH), and 9.5 μL of this solution was incubated with 0.5 μL of FITC stock solution (2 mmol/L in dimethyl sulfoxide (DMSO)) in darkness at 28 °C for 25 h. Final concentrations were 70 nmol/L virus and 0.1 mmol/L FITC, respectively (an ~1500-fold molar excess of FITC). Excess FITC was removed by size exclusion chromatography (SEC) on a self-packed Sephadex G25 column (bed volume of 450 μL, bed height of 1.7 cm) equilibrated and eluted with a 50 mmol/L borate buffer, at pH 8.3. Fractions (~40 μL each) were collected and 4 μL aliquots were mixed with 1 μL of SDS (50 mmol/L) before analysis by CE−LIF.

For heat denaturation, 8 μL of the FITC-labeled virus solution (fraction 5 from SEC) was mixed with 2 μL of 50 mmol/L SDS and incubated at 56 °C for 10 min by immersion in a water bath. Proteolytic digestion of the FITC-labeled virus was performed for 45 min at 45 °C after 1 μL of Proteinase K (concentration of 1.8 mg/mL in water) was added to 10 μL of virus solution that had been incubated at 56 °C.

Complexes between the soluble receptor fragment MBP-V33333[10] and HRV2 were formed by reaction in a 50 mmol/L borate buffer (pH 8.3) at room temperature for 5 min. A mixture of 7 μL of buffer, 1 μL of virus stock solution (concentration of 0.70 μmol/L), and 2 μL of receptor solution (concentration of 14.8 μmol/L) was formed, resulting in a virus molar concentration of 70 nmol/L and a receptor fragment molar concentration of 2.96 μmol/L (the receptor:virus molar ratio was 42:1). After 5 min, the mixture was diluted at a 1:1 ratio with separation buffer. Alternatively, 8 μL of FITC-labeled virus (from fraction 5 of the SEC) was mixed with 1 μL of receptor and incubated for 5 min; 2 μL of 50 mmol/L SDS then were added.

Infectivity Assay. Virus titer was determined as $TCID_{50}$ (tissue culture infectious dose), which is the virus concentration that lyses 50% of the cells.[11] Briefly, HeLa−H1 cells (Flow Laboratories) were grown in a minimal essential medium (MEM) that contained 10% fetal calf serum (Life Technologies) supplemented with penicillin and streptomycin in 96 well tissue culture plates until ~60% confluent. The cells were then challenged with 2-fold serial dilutions of the virus samples in MEM that contained 30 mmol/L $MgCl_2$ and 2% fetal calf serum. After incubation at 34 °C for five days, the plate was washed with water, stained with Crystal violet, and colorless wells were scored positive for virus infection. Wells appearing stained as a result of lack of infection were scored negative. Calculation of $TCID_{50}$ was performed as described.[11]

RESULTS AND DISCUSSION

Reaction of HRV2 with FITC. Virus was reacted with an ~1500-fold molar excess of FITC for 25 h at 28 °C and analyzed by CE with FL detection (Figure 1). Comparison of the electropherograms with the reagent blank (same components incubated in the absence of virus; see trace B in Figure 1) shows the appearance of several new fluorescing peaks (noted by asterisks in trace A in Figure 1). In the blank, there are two main peaks, most probably stemming from the excess FITC (FITC1) and its dimer (FITC2).[12] Five new peaks (asterisks) became apparent after reaction of the virus with FITC. All had a higher total mobility than the peaks in the FITC blank. Note that the virus preparation,

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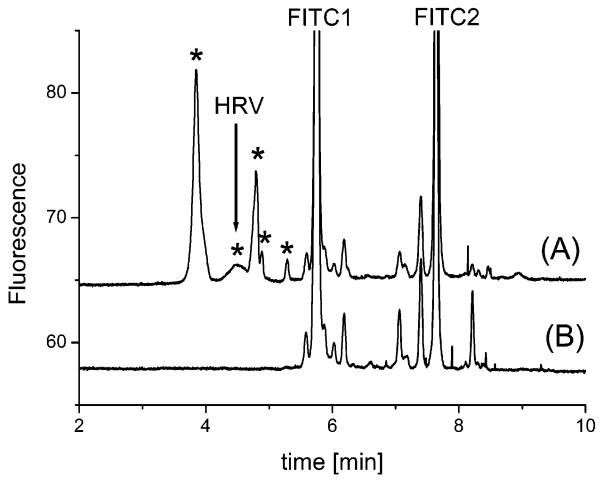

Figure 1. Electropherograms of the reaction products of human rhinovirus serotype 2 (HRV2) with fluorescein isothiocyanate (FITC). Trace A shows capillary electrophoresis (CE) after incubation of the virus with FITC for 25 h at 28 °C in the dark, whereas trace B shows that of a reagent blank (1 μL of 1:20 diluted virus was mixed with 8.5 μL of $NaHCO_3$ buffer (pH 9.0, 100 mmol/L) and 0.5 μL FITC (2 mmol/L in dimethyl sulfoxide (DMSO))). Before injection, the sample was diluted at a 1:10 ratio with a background electrolyte (BGE). Experimental conditions: broad band detector (Ex/Em: filter 240−400 nm/cut-off filter 520 nm); uncoated fused silica capillary (59.0/50.5 cm long, inner diameter (ID) of 75 μm); temperature, 20 °C; separation voltage, +20 kV; and separation buffer, 100 mmol/L sodium borate, pH 8.3, 10 mmol/L SDS. Injection at 100 mbar s. Asterisks indicate new peaks in the incubation solution that contains HRV2.

although purified by sucrose gradient centrifugation, might contain several contaminants (see also ref 7). The small, broad peak designated as "HRV2" in Figure 1 had an electrophoretic mobility of 23 × 10^{-9} m^2 V^{-1} s^{-1}, corresponding to that of HRV2, as previously determined.[13] The other peaks indicated by asterisks are contaminants that were strongly labeled (see below).

Separation of FITC-Labeled HRV2 by Size Exclusion Chromatography. Low-molecular-weight components were removed by SEC. For this purpose, 20 μL of the reaction mixture was loaded onto a Sephadex G25 column with a volume of ~450 μL and eluted with a borate buffer (pH 8.3, 50 mmol/L) by gravity. Fractions of 40 μL were collected manually and analyzed by CE with LIF detection. Electropherograms of fractions 4−7 (eluted at 160−280 μL) are shown in Figure 2. The reagent peaks (FITC1 and FITC2) are greatly reduced in the fractions that contain most of the virus (fraction 5; note that this fraction was diluted at a 1:10 ratio before electrophoresis), as compared to the reaction mixture prior to SEC (Figure 1), whereas their concentration increased in the subsequent fractions. The same is true for all other components in the sample, except from the peak tentatively identified as HRV2 and another component (labeled "C" in Figure 2). As these components elute in the void volume, they must be larger than 5 kDa, which is the exclusion limit of the column material. Similar results were obtained upon SEC on Sephadex G50 and G100 columns (data not shown). Therefore, HRV2 and C in Figure 2 have a relative molecular mass (Mw) exceeding 100 kDa (the exclusion limit of the Sephadex G100 column), which is in agreement with that of HRV2 (~8.5 MDa).

The distribution of the various components present in the fractions of SEC (Figure 2), with respect to the peak area measured by LIF detection, is shown in Figure 3 in the form of a histogram (note that, for better visualization, the relative peak area of HRV2 is multiplied by a factor of 10). This makes it even clearer that an excess of FITC (and side products) is eluted after fraction

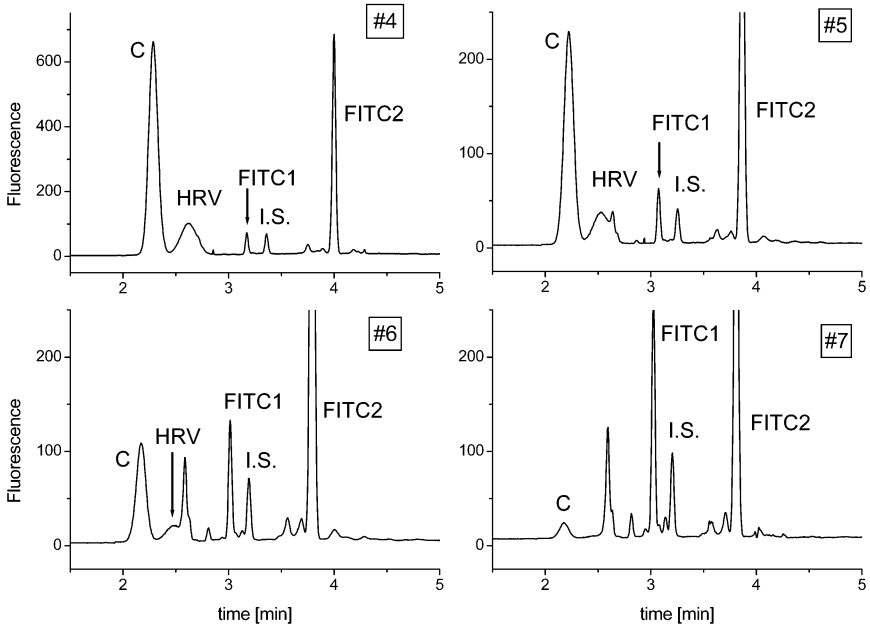

Figure 2. Electropherograms of fractions 4, 5, 6, and 7, collected after size exclusion chromatography (SEC) of HRV2 reacted with FITC. Peak legend is as follows: HRV, virus; C, high-molecular-mass contaminant; FITC1, reagent monomer; FITC2, reagent dimer; and I. S., internal standard (fluorescein). Twenty microliters of the reaction mixture were applied to a Sephadex G25 column with a bed volume of 450 μL and eluted with a borate buffer (pH 8.3, 50 mmol/L) and 40 μL fractions were collected. Prior to injection, 1 μL of sodium dodecyl sulfate (SDS) (50 mmol/L) was added to 4 μL of each fraction. Experimental conditions: laser-induced fluorescence (LIF) detection (Ex/Em: 488/520 nm). Uncoated fused silica capillary (28.5/20.0 cm, 50 μm inner diameter (ID)), positioned in still air without thermostating. Separation buffer was as in Figure 1. Hydrodynamic injection by lifting the injection inlet by 3 cm for 10 s. Separation voltage, +7.5 kV. Fractions 5, 6, and 7, but not fraction 4, are diluted at a 1:10 ratio with BGE. Analyses of fractions 4−7 are shown.

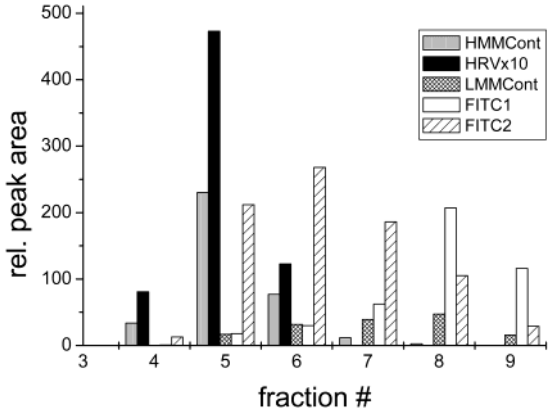

Figure 3. Distribution of virus, contaminant, and FITC byproducts in the individual SEC fractions. Conditions were as in Figure 2. Peaks are related to that of fluorescein at 1 nmol/L (internal standard). Peak areas for HRV2 were multiplied by a factor of 10. "HMM Cont" and "LMM Cont" denote high-molecular-mass and low-molecular-mass contaminants, respectively, whereas "FITC1" and "FITC2" are the peaks of the reagent monomer and dimer, respectively.

6, whereas the majority of the peak designated HRV2 is found in fraction 5, together with the high-molecular-mass component (C). However, because C was clearly differentiated by CE, it did not interfere with the determination and quantification of HRV2.

Identification of HRV2 by Its Heat Lability. It is well-known that, at temperatures of >50 °C, HRVs undergo a structural change, which leads to the loss of the viral capsid protein VP4 (Mw = 7.4 kDa) and the release of the genomic RNA.[14−16] The remaining subviral particle is unstable in the presence of SDS and disintegrates into its components—the capsid proteins VP1, VP2, and VP3 (Mw = 32.9, 29.0, and 26.1 kDa, respectively). Therefore, incubation at elevated temperature should result in the appearance of five new UV-absorbing CE peaks and the disappearance of the original peak corresponding to the viral particle. By FL detection, only those components that have undergone labeling should deliver a signal, in particular, the most accessible capsid protein, VP1.

SEC fraction 5 (with the highest virus concentration) was incubated at 56 °C for 10 min, and SDS then was added to bring the concentration to 10 mmol/L, and the sample was analyzed by CE (Figure 4, trace B). When compared to the sample that was not heated (trace A), it becomes clear that the broad peak with a migration time of ∼4.5 min (LIF detection, upper panel at 5.5 min; UV detection, middle and lower panels) disappeared upon heating. Indeed, a new, fluorescent peak appeared (indicated by an arrow in the upper panel), whereas five new peaks were observed upon detection at 205 nm. Finally, detection at 260 nm (see the lower panel) identified one of these peaks as free RNA that must have been present within the viral particle before incubation at 56 °C. The four other peaks (P1−P4) most probably correspond to the proteins arising upon dissociation of the capsid in the presence of SDS. VP1 is fluorescent (see upper panel in Figure 4 and see Figure 5), indicating that it had incorporated FITC; this is in accordance with it being most strongly exposed in the intact virus and contributing the majority of the lysines that can be modified with FITC.

This was confirmed with denaturing SDS−PAGE analysis of the FITC-labeled virus. The gel electropherograms are shown in

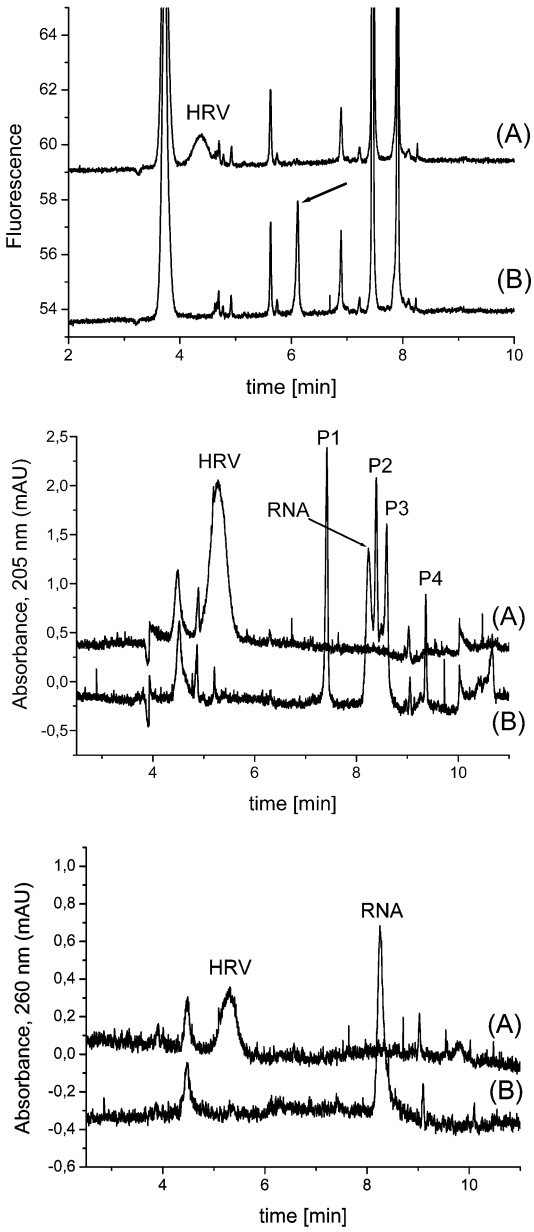

Figure 4. CE of virus before (trace A) and after (trace B) incubation at 56 °C for 10 min. Four microliters of fraction 5 from SEC was mixed with 1 μL SDS (50 mmol/L) before injection. Top panel shows fluorescence (FL) detection, middle panel shows UV absorbance detection at 205 nm, and lower panel shows UV absorbance detection at 260 nm. Experimental conditions were as in Figure 1.

Figure 5, with direct fluorescence detection of the bands (panel A), as compared to staining with Coomassie brilliant blue (panel B). In accordance with the results obtained by CE, VP1 is the most strongly labeled, whereas VP2 is less labeled and VP3 is not labeled at all.

To further confirm the identity of the four peaks with protein, the sample was incubated with Proteinase K and analyzed by CE (Figure 6). Comparison of the electropherograms prior to digestion (trace A in Figure 6) and after digestion (trace B in Figure 6) shows that the four peaks disappeared upon proteolytic digestion, with several new (peptide) peaks (denoted by asterisks) appearing. This is particularly apparent for the single peak that is observed by LIF detection (see the arrowed feature in the upper

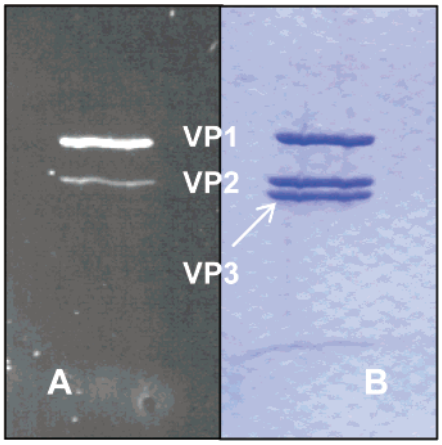

Figure 5. Denaturing SDS−PAGE of the FITC-labeled virus particle; the virus was heated to 95 °C for 3 min in a Laemmli sample buffer and proteins were separated on a 12.5% gel ((A) direct FL detection and (B) stained with Coomassie brilliant blue. Relative molecular masses of VP1, VP2, and VP3 were Mw = 32.9 kDa, Mw = 29.0 kDa, and Mw = 26.1 kDa, respectively.

panel of Figure 6). As seen at 260 nm, the RNA peak remained unchanged by the treatment with Proteinase K and was even higher, in comparison with that in the experiment shown in Figure 4. This is most probably due to digestion of contaminating RNases, which might easily degrade the RNA, by the Proteinase K.

The Labeled Material Specifically Interacts with a Viral Receptor. The viral surface becomes modified upon reaction with FITC as it reacts with ϵ-amino groups of surface-accessible lysines. Lys1224, which is located at the apex of an exposed loop of VP1, is directly involved in receptor recognition; therefore, we asked whether the labeling would prevent the virus from binding. The FITC-labeled virus was incubated with a receptor derivative that specifically interacts with the native virus.[5] A recombinant concatemer of the very-low-density lipoprotein receptor (VLDLR) engineered to contain five copies of module 3 fused to maltose binding protein (MBP) at the N-terminus and to a hexa-his tag at the C-terminus (MBP-V33333) was used.[8−10] This artificial receptor molecule binds strongly to all minor group HRVs in their native conformation.[16] Fraction 5 from SEC (see Figures 2 and 3) was incubated with MBP-V33333 and analyzed by CE. Monitoring the separation by FL detection (Figure 7, upper panel) and UV detection (Figure 7, middle and lower panels) demonstrates that the HRV2 peak disappeared after reaction with the receptor. Instead, a broad peak appeared that corresponds to the complex between HRV2 and the receptor molecules. This is substantiated in Figure 8, where the same experiment was conducted with unlabeled HRV2. In this case, the virus peak is not obscured by fluorescent contaminants, allowing one to clearly discern between the virus (upper panel), receptor (middle panel), and virus−receptor complex formed in the presence of excess receptor (lower panel).

An approximate comparison of the areas of the virus peak with the FITC standard (with time-normalized areas of the fluorescent

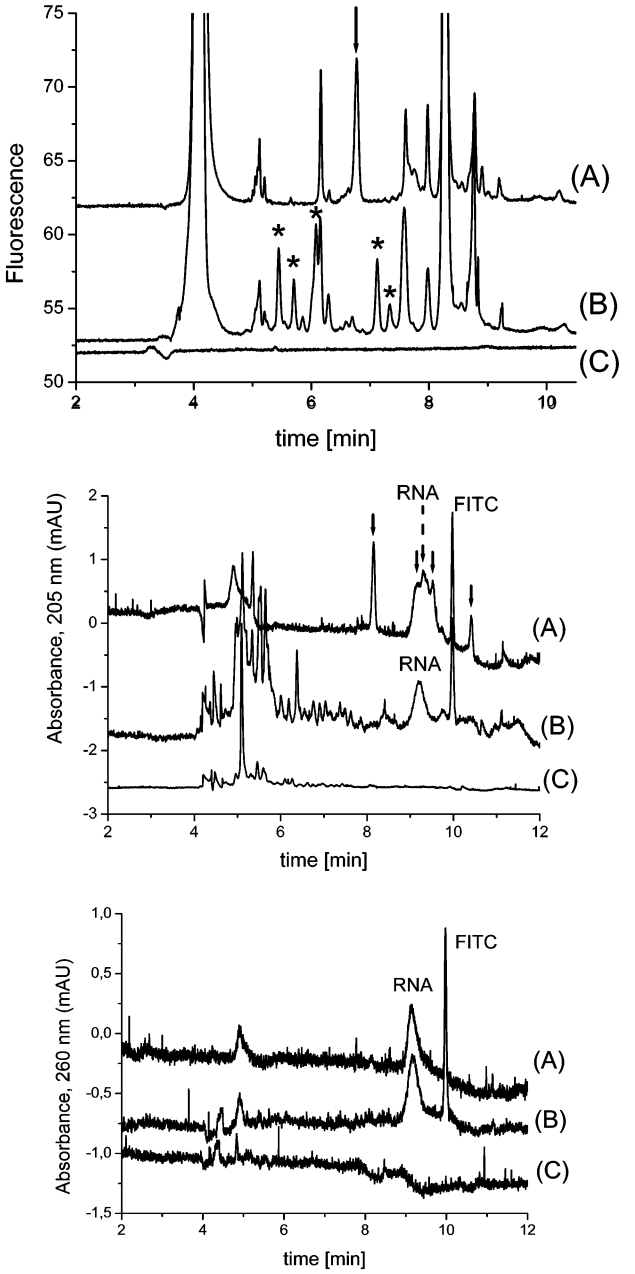

Figure 6. CE of the virus after incubation at 56 °C, as in Figure 4 (trace A), after digestion with Proteinase K (trace B), and that of the Proteinase K blank (trace C). Separation conditions were as in Figure 1. Asterisks denote new peaks appearing after proteolytic degradation; these correspond to protein fragments.

peaks) indicates that ~90 FITC molecules were attached per virus particle (when reacted with an ~1500-fold excess of FITC) that possesses 240 accessible lysines at the capsid surface. Therefore, this corresponds to a reaction yield of ~38%, assuming that no fluorescence quenching occurs. Indeed, it was found that identical peak areas were measured for the labeled virus and the sum of the labeled free proteins (as recorded after denaturation; see Figure 4). In case of quenching, the yield would be even higher.

It was found that labeling with an even 15 000-fold molar excess of FITC preserved bioaffinity toward the receptor fragment MBP-V33333. From these results, we conclude that the labeling did not modify the virus capsid completely. At least in this respect, the fluorescence-labeled virus remains biologically active and

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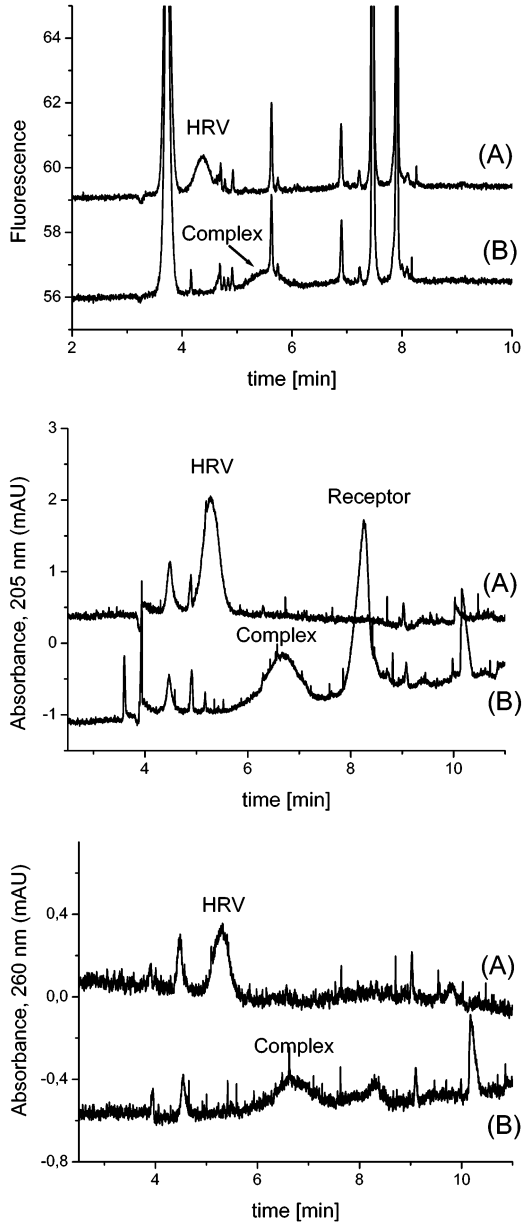

Figure 7. FITC-labeled HRV2 binds MBP-V33333: trace A is that of 8 μL of SEC fraction 5 plus 2 μL SDS (50 mmol/L) (control), and trace B is that of 8 μL of fraction 5 incubated with 1 μL of MBP-V33333 for 5 min at 28 °C, then 2 μL of SDS (50 mmol/L) was added and an aliquot was injected. Conditions were as in Figure 1.

should enter cells via its natural pathway. This was tested by an infection assay, as described below.

FITC-Labeled HRV2 Is Infectious. The virus was labeled with a 1500-fold and a 15 000-fold excess of FITC and separated from low-molecular-mass compounds by SEC. Infectivity was measured as described in the Experimental Section and compared to that of a virus incubated under identical conditions in the absence of FITC; the results are given in Table 1. It can be seen that infectivity is preserved upon labeling, although the $TCID_{50}$/mL values for the labeled samples are reduced by a factor of 5−20. This indicates that the labeled virus is indeed able to bind its cellular receptors and can be internalized into the host cells and initiate replication. Such labeled viruses might allow for monitoring of these processes by life-cell imaging using confocal fluorescence microscopy.

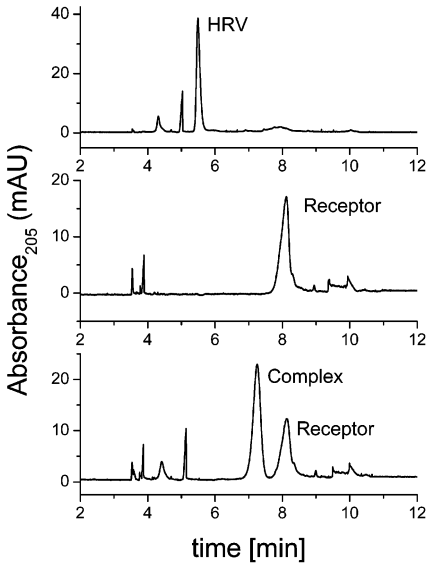

Figure 8. Affinity reaction of native, unlabeled virus with MBP-V33333 specifically binding HRV2. Top panel shows HRV2 in a HEPES buffer diluted at a 1:20 ratio with BGE. Middle panel shows MBP-V33333 diluted with BGE (1:5 ratio). Bottom panel shows that of a sample similar to that in the top panel but incubated with MBP-V33333 (1:5 ratio) diluted with BGE for 5 min at 28 °C; after the incubation, 2 μL SDS (50 mmol/L) was added and an aliquot was injected. Conditions for UV detection were as in Figure 1.

Table 1. Results of the Infectivity Assay of Capsid-Labeled HRV2[a]

virus concentration (μg/mL)	FITC concentration (mmol/L)	FITC: virus ratio in the reaction	$TCID_{50}$ (mL)
	FITC Labeled HRV2, Experiment 1		
0.075	0.1	1430:1	1.4×10^6
0.10	0.1	1430:1	6.5×10^6
	FITC Labeled HRV2, Experiment 2		
0.10	1.0	14300:1	1.0×10^7
0.09	1.0	14300:1	4.6×10^6
	Native HRV2		
0.10			5.7×10^7

[a] Labeling was performed with two different FITC concentrations. Excess FITC was removed using size exclusion chromatography (SEC) prior to the assay. Two experiments are shown.

CONCLUSIONS

Purified human rhinovirus serotype 2 (HRV2) was labeled with fluorescein isothiocyanate (FITC). Analysis of the product by capillary electrophoresis (CE) revealed several peaks, preventing unequivocal assignment of the peak corresponding to HRV2. Following size exclusion chromatography (SEC), an excess of FITC and its side products were removed, but two high-molecular-mass peaks eluted together with the virus in the void volume. One of these exhibited strong fluorescence, as seen by laser-induced fluorescence (LIF) detection. This material had a very low extinction in UV; its nature and origin remained unidentified (see also below). Upon incubation at 56 °C, this peak was unaffected while the smaller, broader peak (exhibiting a slightly higher apparent electrophoretic mobility than the native virus) disappeared and resulted in five new peaks. One of these was identified as RNA, released from the viral capsid, and the remaining four peaks were tentatively identified as the viral capsid

proteins VP1, VP2, VP3, and VP4; their protein nature was confirmed by proteolytic digestion. Further identification, as it was done, for example, in the case of bacteriophage T7 stained with Alexa dyes[17]) is beyond the scope of this paper.

We would have liked to identify the nature of the large peak corresponding to the contaminant. It is not stained with Coomassie blue, it is not affected by proteolysis, and it has very low UV absorbance; therefore, it is neither a polypeptide nor a nucleic acid. Its elution in the void volume in SEC suggests that it is a large molecule or aggregate. From its reaction with FITC, it must be deduced that it possesses reactive primary amino groups, which makes it likely that it is an oligosaccharide carrying several nonacetylated amino sugars. It should be mentioned that very similar results for capsid labeling were obtained by all 12 assays, which were conducted over several months. This high reproducibility also indicates the invariable presence of this compound in all highly purified virus preparations.

The effective mobility of the small broad peak decreased slightly from a value of 23×10^{-9} m^2 V^{-1} s^{-1} (the mobility of native HRV2) to $\sim 17 \times 10^{-9}$ m^2 V^{-1} s^{-1}, indicating a loss of negative charge upon FITC derivatization. This effect cannot be related to a substitution of the positively charged lysines by negatively charged FITC molecules. It is rather an effect introduced by interactions of the labeled virus with sodium dodecyl sulfate (SDS) present in the separation buffer. This peak was shifted upon incubation with a recombinant derivative of the cellular virus receptor. Since this reaction is known to be specific for the native virus, we conclude that the peak corresponds to a FITC-labeled virus that has conserved its native conformation. The native state was further confirmed by infectivity assays, revealing viral titers that are only 5−20 times lower than those of a native virus sample. This finding is important because it signifies that the labeled virus can be used in studies aimed at monitoring the very first event in viral infection, i.e., attachment to and internalization into the host cell.

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