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Analysis of Interactions of Signaling Proteins with Phage-Displayed Ligands by Fluorescence Correlation Spectroscopy

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Fluorescent correlation spectroscopy (FCS) was used to measure binding affinities of ligands to ligates that are expressed by phage-display technology. Using this method we have quantified the binding of the 14-3-3 signaling protein to artificial peptide ligand. As a ligand we used the R18 artificial peptide expressed as a fusion in the cpIII coat protein that is present in 3 to 5 copies in an M13 phage. Comparisons of binding affinities were made with free R18 ligands using FCS. The result showed a relatively high binding affinity for the phage-displayed R18 peptide compared with binding to free fluorescently labeled R18. Quantification was supported by titration of the phage numbers using atomic force microscopy (AFM). AFM was shown to accurately determine phage numbers in solution as a good alternative for electron microscopy. It was shown to give reliable data that correlated perfectly with those of the viable phage numbers determined by classical bacterial infection studies. In conclusion, a very fast and sensitive method for the selection of new peptide ligands or ligates based on a quantitative assay in solution has been developed. (*Journal of Biomolecular Screening* 2008:766-776)

Key words: phage display, fluorescence correlation spectroscopy, atomic force microscopy, 14-3-3 protein, binding affinity

INTRODUCTION

TO ELUCIDATE THE INTERACTION BETWEEN SIGNALING PROTEINS and their target ligands is an increasingly important research theme. Investigating these interactions is indispensable to understand the underlying molecular mechanisms of signal transduction pathways. Even more importantly, this research is needed to reveal the factors that determine specificity in pathways involving a large number of proteins. Obviously, this may be significant for the development of new therapeutics that bind with high affinity to their signaling targets.

For most biological signal transduction pathways the lack of quantitative data for binding affinity between all interaction partners is a bottleneck for further understanding of the underlying molecular mechanisms. This is especially limiting to our understanding of factors that determine specificity in pathways that involve a large number of proteins. A good example is the 14-3-3 family of eukaryotic signaling proteins. The 14-3-3 proteins function in all eukaryotic organisms as dimeric proteins in a wide range of cellular processes, including signaling, apoptosis, cell division, and patterning during embryogenesis, by binding to many peptide ligands (for reviews, see van Hemert et al.[1] and Bunney et al.[2]). The molecular function and specificity of the enormous amount of target ligands is unknown. Several isoforms of the protein have been isolated in many species, for instance the human ζ-, τ-, σ-, and β-isoforms, but their specific biological functions are still hardly understood. There are indications that different isoforms have specialized developmental functions because they can display a tissue-specific distribution, for instance the human σ-14-3-3 isoform occurs only in epithelial cells.[3]

Phage display is a very promising technique for the identification of protein-protein interactions[4-8] offering the possibility to develop and to isolate new peptide ligands with desired binding affinities. Therefore it is the most ideal method for the isolation and characterization of ligands of 14-3-3 protein. During the 2 decades the technique has been in use, it has developed enormously because of many applications and technical improvements.[9] The phage-display technology is based on expression of foreign peptide or protein sequences as fusions to a phage capsid protein presented on the surface of a phage particle. Phage display is a well-established method to isolate new ligands from a

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complex pool of sequences that bind to a known binding partner (= ligate). The method offers the possibility of not only finding the best binder to a known ligate, but in addition, identifying alternative ligands. Because a physical link exists between the binding characteristics (i.e., surface characteristics) and the genetic information harbored by the phage, this is of great advantage, for instance, for the construction of tailor-made ligands.

A large spectrum of methods is available to study the binding affinity of an isolated ligand for its protein target, ranging from radioactive detection assays, enzyme-linked immunosorbent assay (ELISA), surface plasmon resonance (SPR) to microcalorimetry, fluorescent polarization, time-resolved fluorescence resonance energy transfer (TR-FRET), and fluorescence correlation spectroscopy (FCS). Nowadays, fluorescent techniques represent 1 of the most important detection methods as an alternative to radioactive assays because of their comparable sensitivity, safe handling, long lifetime compared with often used radiolabels, environmental friendliness, and because they are suitable for high-throughput screening.[10] A clear limitation, however is, that the fluorescent dye coupled to 1 of the binding partners may cause practical problems such as interference with binding affinity and/or ligand solubility as discussed recently by de Keijzer et al.[11] Methods as ELISA or SPR[12,13] do not struggle with this problem because they use no labels. However, in these assays, the target is immobilized, which limits these techniques strictly to in vitro studies. Moreover, the anchoring of the target molecule onto a solid surface may perturb the protein structure and its function.

Studies of binding in solution are enabled, for example, by measuring fluorescent polarization, TR-FRET (for a review, see Li et al.[10]) or FCS.[14-16] FCS studies fluorescence fluctuations caused by changes in number or orientation of single molecules diffusing in a tightly focused confocal detection volume (~1 fL). An interaction between 2 different molecules (e.g., binding of a small fluorescently labeled molecule with a fast diffusion time, due to its small size, to a larger ligand) results in a change, meaning an increase, of the diffusion time. In addition, FCS also gives information on the absolute number of fluorescent molecules in the detection volume; that is, it can be used to determine concentrations as well as the ratio of free and bound molecules.

The advantages and limitations of FCS over other methods have been discussed before (for instance, in Bahns et al.,[14] Lagerkvist et al.,[17] and Pope et al.[18]). In short, FCS offers a single-step method in a homogenous solution in contrast to SPR, for example, which is a multistep methods requiring washing steps; no separation is required of bound and unbound fluorescent ligand and it operates at a single molecule level offering advantages over ensemble measurements. Single molecule techniques can also be considered as complementary to ensemble measurements because they can discover properties that could not be predicted on the basis of binding or diffusion characteristics predicted from classical theory.[11] Compared with other single molecule techniques, FCS is less suited for detailed molecular analysis, but it is very fast, it offers extremely high sensitivity and therefore requires only small quantities, and, in principle, it gives the possibility to measure affinity constants. FCS is therefore 1 of the most prominent single molecule techniques for biological research.[15,19]

As proof of principle to establish whether phage display combined with FCS is an accurate approach and has significant advantages above other approaches to identify and characterize new peptide ligands of signaling proteins, we have studied the interaction between human and yeast 14-3-3 proteins and a previously identified peptide ligand called R18.[20]

The interaction between R18 and 14-3-3 protein was chosen as a model for the following reasons: (1) Binding of R18 displayed on the phage surface to 14-3-3 protein in solvent has been shown.[20] (2) R18 is bound as a nonphosphorylated ligand by τ-, β-, and ζ-isoforms of 14-3-3 protein.[20,21] (3) Isolated dimeric 14-3-3 proteins labeled with fluorescent tags and proven to be functional were available.[3] (4) The molecular binding mechanism is well known: the horseshoe-shaped, dimeric 14-3-3 protein has 2 equivalent binding sites 6.5 nm apart that act independently of each other. (5) The core R18 amino acid sequence WLDLE is bound by 14-3-3,[21] and 2 flanking cystidine residues that are 14 amino acids apart from each other form an S-S bridge. As a result, the bound sequence of the R18 peptide sequence "bulbs out" of its flanking sequences, and these therefore will not interfere with the fluorescent tag of the bound labeled 14-3-3 protein.

Previous work of Lagerkvist and others[17] has pioneered the use of FCS for analysis of phage-displaying monoclonal antibodies against hepatitis C virus and the soluble antigen. In a subsequent study, Bahns et al.[14] used FCS to further characterize interactions between M13 phages and antibodies. However, an application for the measurement of affinity constants for the interaction of phage-displayed peptide ligands and signaling proteins has not been reported yet.

In this work we show that the combination of phage display with FCS may be applied as a powerful method for the quantitative characterization of ligands binding to their partner ligate. By comparison of binding studies of 14-3-3 protein with free R18 peptide ligands, we show that the affinity of 14-3-3 protein for the R18 peptide is strongly influenced by the presence of additional surrounding peptide sequences.

MATERIALS AND METHODS

R18 peptide labeled with TAMRA

The R18 amino acid sequence NH_2-PHCVPRDLSWLDLEAN-MCLP-COOH labeled with tetramethylrhodamine (TAMRA) at the N-terminus was synthesized by Sigma (Sigma-Genosys Ltd, Cambridge, UK). MS spectra showed no degradation of the

peptide. It was stored at –20 °C as lyophilized powder. The peptide, designated as R18-TAMRA, was dissolved to 46 µM in 20 mM HEPES pH 7.01, aliquoted, and stored in the dark at –20 °C.

Construction of R18 sequence in the phage-display vector

E. coli strain DH5α (Invitrogen Life Technologies, Paisley, UK) was used for propagation and isolation of plasmids. All molecular handlings were performed according to standard methods.[22]

The clone GST-R18 in the vector pGEX-2T (Pharmacia, Buckinghamshire, UK) was a kind gift of Dr. Fu (Emory University, Atlanta, GA). The DNA sequence of the R18 insert was determined by BaseClear (Leiden, The Netherlands) using primer pGEX5′ (5′-GGGCTGGCAAGCCACGTTTGGTG-3′, synthesized by BaseClear). Based on this sequence, the oligonucleotides R18 sense (5′-*catgg*ccgacggggctcctcattgtgttccgagggatctgtcgtggcttg*atctaga*ggcgaatatgtgtcttccgggggccgctggggc-3′, overlapping *Nco*I site sequences and *Xba*I site in italics) and R18 antisense (5′-*ggcc*gccccagcggcccccggaagacacatattcgcc*tctaga*tcaagccacgacagatccctcggaacacaatgaggagccccgtcggc-3′, overlapping *Not*I site sequences and *Xba*I site in italics) were synthesized by Isogen (Amsterdam, The Netherlands). The oligonucleotides were phosphorylated, annealed, and ligated into dephosphorylated vector pHEN2[23] (a kind gift of Dr. Schots, Wageningen University) digested with *Nco*I and *Not*I, yielding plasmid pMP2043. Sequence determination of the independent clones pMP2043.1, pMP2043.2, and pMP2043.19 revealed a sequence as expected for R18 translationally fused to fd-geneIII.

Isolation of phages

E. coli strain TG1[24] was used for propagation of phages as it has been reported to be superior to strain XL1-Blue in phage production.[25] Vectors pHEN2, pMP2043.2, and pMP2043.19 were transformed into TG1 by standard methods.[22] For phage production, hyperphage M13K07ΔpIII (Progen Biotechnik, Heidelberg, Germany) diluted in water until multiplicity of infection (m.o.i.) of 10^{12} was used as helper phage. Essentially, the phage production protocol described before[24] was followed with modification as described by Progen (Heidelberg, Germany) and adapted to local conditions. In short, bacteria grown on Minimal Medium,[22] were transferred to liquid 2x TY medium[26] containing 1% glucose and cultured for 16 h at 37 °C. Subsequently, the precultures were 100× diluted in 2x TY medium, grown at 37°C until mid-log-growth phase, and subsequently infected by helper phage (20 phages/bacterium) for 60 min at 37 °C. Unattached helper phages were removed by centrifugation. Bacterial pellets were suspended in 2x TY medium supplemented with kanamycin (25 µg/mL) to select for helper phages and phage production was allowed to take place by growth at 30 °C for 16 h. All media were supplemented with carbenicillin (100 µg/mL) if appropriate. Phages were isolated from the growth medium by precipitation using PEG/NaCl (20% polyethylene glycol 6000, 2.5 M NaCl) and suspended in phosphate-buffered saline (PBS), pH 7.2 A 2nd precipitation of phages was carried out using again PEG/NaCl and suspending them in PBS, pH 7.2. Finally, the phage preparation was sterilized by gentle passing over a 0.45-µm filter (Minisart NML, Sartorius, Göttingen, Germany) and they were kept at +4 °C.

The titer of the phage preparations was determined as number of plaque-forming units (pfu)/mL by infecting TG1 cells with serial dilutions of phage preparations, and plating in triplicate on TYE media (8 g of NaCl, 10 g of Tryptone, 5 g of yeast extract) containing 1% glucose and supplemented with carbenicillin (100 µg/mL) or kanamycin (50 µg/mL).

Recombinant 14-3-3 proteins

Recombinant human 14-3-3 σ-isoform proteins translationally fused to cyan fluorescent protein (CFP) or to yellow fluorescent protein (YFP; indicated as σ-14-3-3-CFP and σ-14-3-3-YFP, respectively)[3] were isolated from yeast as a dimeric protein using gel filtration and provided by Dr. van Hemert (Leiden University, The Netherlands) in 50% glycerol, 50 mM Tris-HCl pH 7.6, 300 mM NaCl, 1 mM DTT (37 and 5 µM solution, respectively), and stored at –20 °C. The yeast 14-3-3 protein BMH2 was provided by Dr. van Hemert as 16 µM solution in the same buffer and stored at –80 °C.

Theoretical concept of fluorescence correlation spectroscopy

FCS (for review, see Eigen and Rigler[15]) was used to analyze the fluorescence intensity fluctuations originating from single fluorescently labeled molecules diffusing in a confocal detection volume of less than 0.5 fL. The dependence of diffusion time on beam size and molecular weight of the diffusing components are addressed by Saxton.[27] Correlation of the intensity fluctuations over time yields the so-called autocorrelation function, $G(t)$. Using a 1-component model the experimental autocorrelation curves were fitted by:

$$G(t) = 1 + \frac{1}{N} \cdot \frac{1 - T + T \cdot e^{\left(\frac{-t}{\tau_t}\right)}}{\left(1 + \frac{t}{\tau_{diff}}\right) \cdot \sqrt{1 + \frac{t}{SP^2 \cdot \tau_{diff}}}},$$

where N denotes the number of fluorescent particles in the detection volume, T the fraction of fluorophores decaying through the triplet state, τ_t the triplet lifetime, τ_{diff} the diffusion time, and SP the structural parameter describing the confocal volume. The triplet state of the fluorophore indicates radiationless decay of the excited molecule to the ground state.

For 2 components, the 1-component model was extended to:

$$G(t) = 1 + \frac{1 - T + T \cdot e^{\left(-t/\tau_t\right)}}{N} \cdot \left(\frac{y}{1 + t/\tau_1 \cdot \sqrt{1 + t/SP^2 \cdot \tau_1}} + \frac{1 - y}{1 + t/\tau_2 \cdot \sqrt{1 + t/SP^2 \cdot \tau_2}} \right)$$

where y denotes the relative fraction of the 1st component, τ_1 the diffusion time of the 1st component, and τ_2 the diffusion time of the 2nd component. The ratio between the 2 components (e.g., free and bound dye) can be directly obtained from fitting the measured autocorrelation curves.

FCS measurements

FCS measurements were performed on a ConfoCor2 (Zeiss, Jena, Germany) using the 514-nm for YFP and 543-nm laser lines for TAMRA excitation, respectively. The fluorescence emission was passed through a 530- to 560-nm band pass (for detection of σ-14-3-3-YFP) or a 585-nm low-pass filter (for detection of R18-TAMRA) and detected using an avalanche photodiode. The signals were software correlated to obtain the autocorrelation functions. Calibration measurements with standard dyes (TAMRA and Rhodamine6G) were performed to determine the geometry and the size of the detection volumes before each series of measurements. Typically volumes of 0.170 and 0.155 fL were found for TAMRA and Rhodamine6G, respectively.

One volume of 10 to 50 nM R18-TAMRA diluted in binding buffer (20 mM HEPES pH 7.0, 150 mM KCl, 0.005% Tween-20) was mixed with an equal volume of 100 to 1000 nM σ-14-3-3-CFP or -BMH2 on ice for 15 min. Next, 10 µL of the mixture was transferred to an 8-well glass-bottom chamber (Nunc GmbH, Wiesbaden, Germany) and measurements were performed immediately. The presence of Tween-20 in the binding buffer was essential to prevent background binding of the TAMRA label to the glass surface.

Phages were diluted from stock solutions containing 5–10 · 10^{12} pfu/mL just before use in freshly prepared binding buffer. One volume of diluted phages was mixed with an equal volume of 10 nM σ-14-3-3-YFP. The 14-3-3 protein was allowed to bind to the phages for 15 to 20 min on ice, and subsequently 10 µL of the mixture was transferred to an 8-well glass-bottom chamber (Nunc GmbH). Measurements were performed immediately afterward.

For each autocorrelation curve, 5 individual measurements (30 sec each) were averaged. The ConfoCor2 software was used for fitting autocorrelation curves. When fitting curves with 2 or more components, the values of the diffusion times for the faster components were fixed, using the values that were determined in earlier experiments with only 1 component present in the solution. Likewise, for determination of binding kinetics, the values of the different components were kept fixed to fit the ratio of the different components.

Atomic force microscopy

Tapping mode atomic force microscopy (AFM) was done in air at room temperature using a Nanoscope IIIa (Veeco, Santa Barbara, CA). Phages were diluted in HPLC-grade H_2O using pipette tips of Sarstedt (product number 70.760); 2 µL of diluted phage solutions was deposited on freshly cleaved mica. The sample droplet spread over an area of 15 mm^2 and was dried under a gentle flow of dry nitrogen. Sixteen squares each of 36 μm^2 were imaged on various places of the sample.

RESULTS

Construction and production of R18-displaying phages

To display the R18 peptide on the outside of a phage, the phagemid vector pHEN2 was chosen because it is easy to handle during molecular manipulations because of its high copy number (e.g., cloning, nucleotide sequencing), the expression of the insert can be regulated because of the presence of the *lac* promoter upstream of the insert, and the fusion is made with the phage fd minor coat protein III (cpIII). This protein, located at only 1 proximal end of the phage, is absolutely required for infection of an *E. coli* host, but, surprisingly, it tolerates insertions (for a review, see Hoogenboom et al.[28]) probably because of the fact that it sticks out of the phage envelope. The translational fusion of the R18 sequence fused to the 5′ end of fd-geneIII resulted in vector pMP2043. Several independent clones with the correct nucleotide sequence were obtained designated pMP2043.1, pMP2043.2, and pMP2043.19.

Helper phages are required for the isolation of phages from phagemids. However, a drawback of this system is that relatively large quantities of phages with wild-type cpIII are isolated in addition to phages with recombinant cpIII. Therefore, use was made of the helper phage M13K07ΔpIII, lacking the genetic information for cpIII,[29] because we did not have a selection method for phages displaying the R18 peptide readily available. In this way, theoretically, all phages produced will have a recombinant cpIII.

Phages were produced from bacterial strains containing phagemid pMP2043.2, pMP2043.19, or pHEN2. As control, the same isolation procedure was applied to a bacterial strain lacking a phagemid (indicated as "TG1"). The titer of the phage preparations was determined on carbenicillin-containing media. For unknown reasons, the titer of pMP2043-containing phages was found repeatedly to be 10 to 25 times higher (3.9–8 · 10^{12} pfu/mL) than that of pHEN2-containing phages (0.3 · 10^{12} pfu/mL). On kanamycin-containing media, phages could have propagated that contain the genetic information from the helper

Table 1. Binding of Fluorescently Labeled R18 Peptide to σ-14-3-3 Fused to Autofluorescent Protein CFP Determined by FCS with Detection at 530–560 nm

Ligand (Fluorescently Labeled)	*Ligate*	τ_1^a (μs)	τ_2^a (μs)	*Fraction$_2$ (%)*	τ_3^a (μs)	*Fraction$_3$ (%)*	*Binding Constant*
—	σ-14-3-3-CFP	N.D.	—	—	—		—
Dye TAMRA	—	24 ± 2	—	—	—		—
R18-TAMRA	—	N.D.	50 ± 2	100	—		—
R18-TAMRA	σ-14-3-3-CFP	—	50	48	215 ± 14	52	69 nM ± 22 pM
R18-TAMRA	BMH2	—	50	44	178 ± 7	56	—
R18-TAMRA	BSA	—	50	>99	260	<1	—

CFP, cyan fluorescent protein; FCS, fluorescence correlation spectroscopy; N.D., not detectable; TAMRA, tetramethylrhodamine.
a. τ_{1-3} are component diffusion times. Data are the mean of 5–10 independent experiments each performed in 5-fold.

phage. Titers found on these media were in the order of 10^8 pfu/mL and equal for all preparations. These results show that almost all phages that were isolated from pMP2043-containing bacteria contain the genetic content of the R18-geneIII fusion.

FCS measurements of binding of R18 peptide to 14-3-3 protein

The σ-isoform of human 14-3-3 protein was used as ligate in binding studies with phages displaying R18 on their surface. First, binding between σ-14-3-3 and free R18 peptide was analyzed to establish whether detection of binding of R18 by the 14-3-3 protein using FCS was feasible. Furthermore, the experimental conditions were optimized using this system. In the binding experiments, ligates were fused to autofluorescent proteins CFP and YFP because in forthcoming biological experiments fluorescently labeled protein had to be used (see below). YFP or CFP fluorescent tags do not interfere with ligand interaction,[3] but they cause a substantial increase in molecular weight of the protein (the expected molecular weight of monomeric 14-3-3 protein without and with the autofluorescent protein is 27 and 57 kDa, respectively) and therefore will influence the observed diffusion time.

In FCS, the ligand, usually the smallest molecule, is fluorescently labeled, and its fate is followed during the interaction with its binding partner or ligate, usually the largest molecule. To this end, TAMRA-labeled R18 peptide was allowed to bind to dimeric σ-14-3-3 protein labeled with CFP (indicated as σ-14-3-3-CFP)[3]. To detect the interaction, the dye TAMRA was chosen to label the ligand because it is known as a suitable dye for FCS, and its emission does not overlap with that of CFP ($\lambda_{emission}$ 480 nm). No background signal could be detected from the CFP tag when a 585-nm low-pass filter was used to cut off the CFP emission (**Table 1**). Test measurements with a series of KCl concentrations in the binding buffer (50, 100, 150, 250, and 500 mM) showed that the specificity of the binding was increased by the addition of KCl with an optimum at 150 mM (data not shown). Addition or omission of DTT to the binding buffer had no detectable effect and was therefore omitted. $MgCl_2$, an important molecule described for functioning of 14-3-3 protein, showed no effect on the binding and was therefore also omitted from the binding buffer. Reproducible results were obtained by using 5 nM R18-TAMRA and 100 nM σ-14-3-3-CFP in the binding experiments and when binding was allowed to take place for 15 to 20 min on ice or at ambient temperature, apparently, after this time equilibrium was established.

The TAMRA dye could be detected specifically in the experimental settings established with a diffusion time of 24 μs. Free TAMRA-labeled R18 peptide showed a diffusion time of 50 μs, without a significant amount of free dye. For binding of the TAMRA-labeled R18 peptide to the 14-3-3 protein BMH2, without a fluorescent tag, a diffusion time of 178 μs was obtained (**Table 1**). The binding of TAMRA-labeled R18 peptide to 14-3-3 proteins was specific as shown by the control experiment in which BSA was used. In this experiment no binding of R18 was observed, that is, no increase of the diffusion time (**Table 1**).

The diffusion time of TAMRA-labeled R18 peptide increased to 215 μs when σ-14-3-3-CFP protein was added (**Table 1**). **Figure 1** depicts 2 typical FCS measurements for free TAMRA-labeled R18 and binding to the σ-14-3-3-CFP protein.

FCS was also used to determine the binding constant of TAMRA-labeled R18 peptide to σ-14-3-3-CFP. For this purpose, the concentration of σ-14-3-3-CFP was varied between 5 and 1000 nM and the ratio of bound and free R18-TAMRA was determined from fitting the measured autocorrelation curves (**Fig. 2**, circles). For estimating the binding constant the data were fitted with the assumption of a 1-site binding component model (**Fig. 2**, line), yielding a value of 69 nM ± 22 pM.

FCS measurements of binding of R18 displayed on phage surface to 14-3-3 protein

A 1st set of experiments, in which the experimental settings for the measurement of binding of 14-3-3 protein by R18-displaying

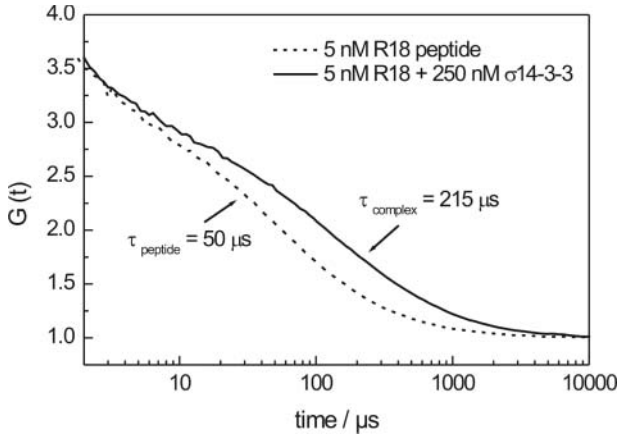

FIG. 1. Normalized fluorescence correlation spectroscopy (FCS) autocorrelation curves for free tetramethylrhodamine (TAMRA)-labeled R18 peptide (dashed line) and TAMRA-labeled R18 peptide after incubation with σ-14-3-3-CFP (solid line). The graph depicts the time dependence of the autocorrelation function, $G(t)$. Both experimental traces are the average of 5 individual measurements.

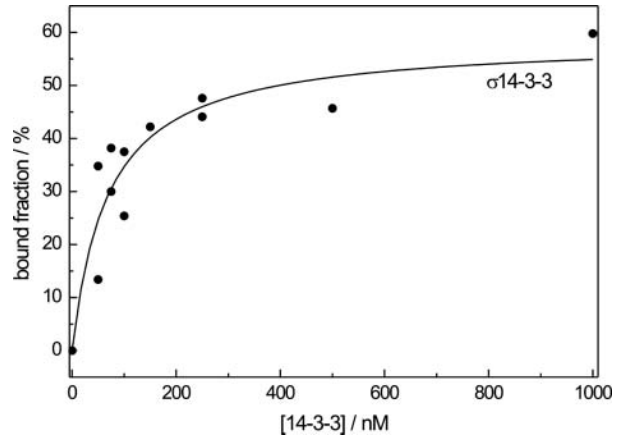

FIG. 2. Binding curve for binding of tetramethylrhodamine (TAMRA)-labeled R18 peptide to σ-14-3-3-CFP (black circles). Each data point was obtained as the average from fits of 3 autocorrelation curves. The line represents a fit to the data based on a 1-site binding model.

phages using FCS was tested, showed a high amount of background fluorescence probably due to growth media residues that have been precipitated together with the phages. This background fluorescence was also observed in a control sample that was obtained by using an identical isolation procedure for cultures of *E. coli* TG1 cells lacking a phagemid, as for the isolation of phages (data not shown). Therefore a 2nd precipitation of the phage preparation was performed. No decrease in the titers was observed (data not shown), and, just as important, after this additional purification step no background fluorescence was observed in the FCS measurements anymore.

Binding of σ-14-3-3 fused to YFP (indicated as σ-14-3-3-YFP) to R18-displaying phages was assessed using the binding conditions set before with σ-14-3-3-CFP and R18 peptide except that the fluorescent dye (YFP) was excited with a 514-nm laser line and a 530- to 560-nm band pass filter was used to follow the fate of the σ-14-3-3-YFP ligand. In these former experiments, 5 nM R18-TAMRA (equal to $3 \cdot 10^{12}$ molecules/mL) in reaction with 100 nM σ-14-3-3-CFP (equal to $6 \cdot 10^{13}$ molecules/mL) gave reproducible results, meaning a 20-fold excess of ligate.

When no ligate was added to the 14-3-3-YFP-labeled protein, a diffusion time of 141 µs was observed (**Table 2** and **Fig. 3**, dotted line). An additional component with a diffusion time of ~2000 µs appeared when phages isolated from cultures with pMP2043-containing bacteria (indicated as phage 2043) were added (**Table 2** and **Fig. 3**, solid line) indicating that binding occurred. Two independently-obtained phage isolates derived from 2 different clones (pMP2043.2 and pMP2043.19) showed indistinguishable results (**Table 2**). In contrast, phages isolated from control cultures with pHEN2-containing bacteria (indicated as phage-pHEN2) showed a diffusion time similar to samples when no ligate was added to the 14-3-3-YFP–labeled protein (152 µs) indicating that no binding occurred (**Table 2** and **Fig. 3**, dashed line). These results show clearly that specific binding of R18-displaying phages by its ligate 14-3-3 protein can be determined by FCS.

Subsequently FCS was used to determine the binding constant for binding of σ-14-3-3-YFP to the phages displaying the R18 peptide. **Figure 4** shows the ratio of free and bound σ-14-3-3-YFP for binding to phage 2043.2 and phage 2043.19. The minor coat protein cpIII contains 3 to 5 copies per phage at 1 proximal end of the phage. For calculations we assumed binding of 1 molecule dimeric 14-3-3 protein to 1 phage according to conventional 1st order kinetics. Binding of more 14-3-3 copies cannot be excluded but is unlikely because of steric hindrance. We have not found any indications of binding of multiple 14-3-3 proteins to 1 phage as could be indicated by better fitting with other settings based on multiple binding partners or by spikes in the correlation curves. Based upon this assumption, we found K_D: $1.6 \cdot 10^7$ phages/µL, which corresponds to about 30 pM.

Analysis of phage preparations by AFM

The most striking observation that was made in the FCS studies was a 2000-fold difference in binding constant of free R18 peptide with the ligate σ-14-3-3 protein compared with R18 fused to fd-cpIII. In this latter case, the R18 peptide is imbedded in a protein structure on the surface of the phage suggesting that the neighborhood of the peptide influences its binding characteristics. Alternatively, the huge difference in binding constant could be explained by supposing that more binding sites for 14-3-3 are present in the phage preparations than expected. Our studies thus far were based on the assumption

Table 2. Binding of Phages Displaying R18 Peptide on cpIII to Fluorescently Labeled σ-14-3-3-YFP Determined by FCS with Detection at >585 nm

Ligand (Fluorescently Labeled)	*Ligate*	*τ_1^a (μs)*	*τ_2^a (μs)*	*Fraction$_2$ (%)*	*Binding Constant^b*
σ-14-3-3-YFP	—	141 ± 13	—	—	
σ-14-3-3-YFP	Phage 2043.2	141	1949 ± 162	58 ± 3	33 pM +/– 12 pM
σ-14-3-3-YFP	Phage 2043.19	141	2619 ± 587	62 ± 4	
σ-14-3-3-YFP	Phage pHEN2	152 μs ± 24	N.D.	N.D.	

cpIII, coat protein III; FCS, fluorescence correlation spectroscopy; N.D. not detectable.
a. $\tau_{1\text{-}2}$ are component diffusion times. Data are the mean of 5-10 independent experiments each performed in 5-fold.
b. Binding constants for phages are based on pooled data from 4 independent measurement series. For each phage concentration, the amount of binding was fitted from 3 independent experiments, each performed in 5-fold.

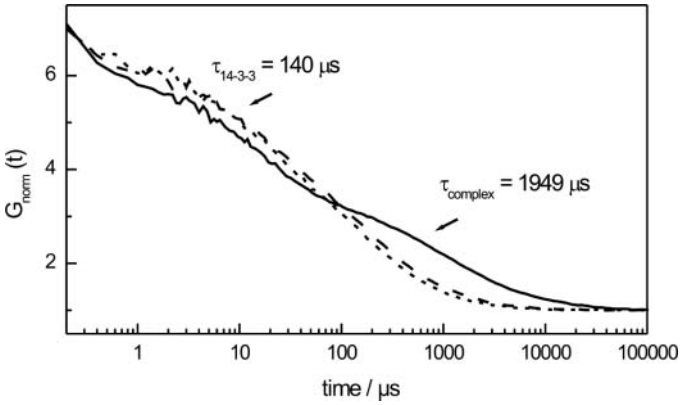

FIG. 3. Normalized FCS autocorrelation curves for free σ-14-3-3-YFP (dotted line), after incubation with phage pHEN2 (negative control, dashed line) and after incubation with phage 2043.2 (solid line). The graph depicts the time dependence of the autocorrelation function, $G(t)$. Each experimental trace is the average of 5 individual measurements.

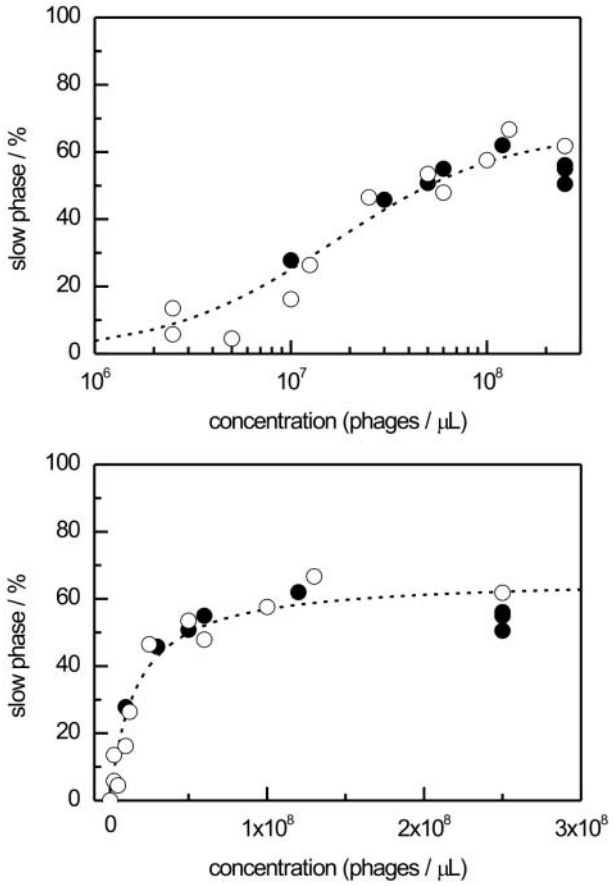

FIG. 4. Binding curve for binding of σ-14-3-3-YFP to phage 2043.2 (black circles) and phage 2043.19 (open circles). Each data point was obtained as the average from fits of 3 autocorrelation curves. The dashed line represents a fit to the data based on a 1-site binding model.

that 3 to 5 R18 copies per phage particle are present and that all R18 binding sites are present on viable phages.

To obtain further support for the estimated number and the integrity of phages present in the preparations we performed visualizations studies. In this way it was possible to determine whether smaller fragments (representing broken phage particles) were present in the phage preparation. Such fragments may contribute to binding to 14-3-3 protein as observed with FCS, but they are not viable and will therefore not show up as pfu. In a 1st approach using transmission electron microscopy (TEM) studies we were unable to characterize the average size of the phage particles in a reproducible way (data not shown). As an alternative, AFM was used to measure a large representative number of phage particles of 1 of the preparations used in FCS. Samples of phage 2043.2 were prepared on mica plates as a very thin outspread layer and imaged according to the scheme in **Figure 5A**. In this approach 4 squares of 36 μm^2 were imaged on various places of the sample (**Fig. 5A**). The experiment was repeated 3 times, each time with freshly prepared dilutions of the phage preparation. The images revealed that the phage particles were evenly distributed over the sample and no indication for the presence of aggregates was observed (**Fig. 5B**). In a typical experiment, the average number of phages per 6 × 6 μm^2 was 26 ± 6 for a 1000-fold dilution. Given the size of 15 mm^2 for a 2 μL droplet, a concentration of $5 \pm 2 \cdot 10^6$ phages/μL could be derived in this sample.

For 1 of the images, the lengths of the phages were determined. The length varied between 480 nm and 1320 nm with a median of 660 nm. In conclusion, no significant number of small phage particles was observed that might represent a large nonviable number of phages.

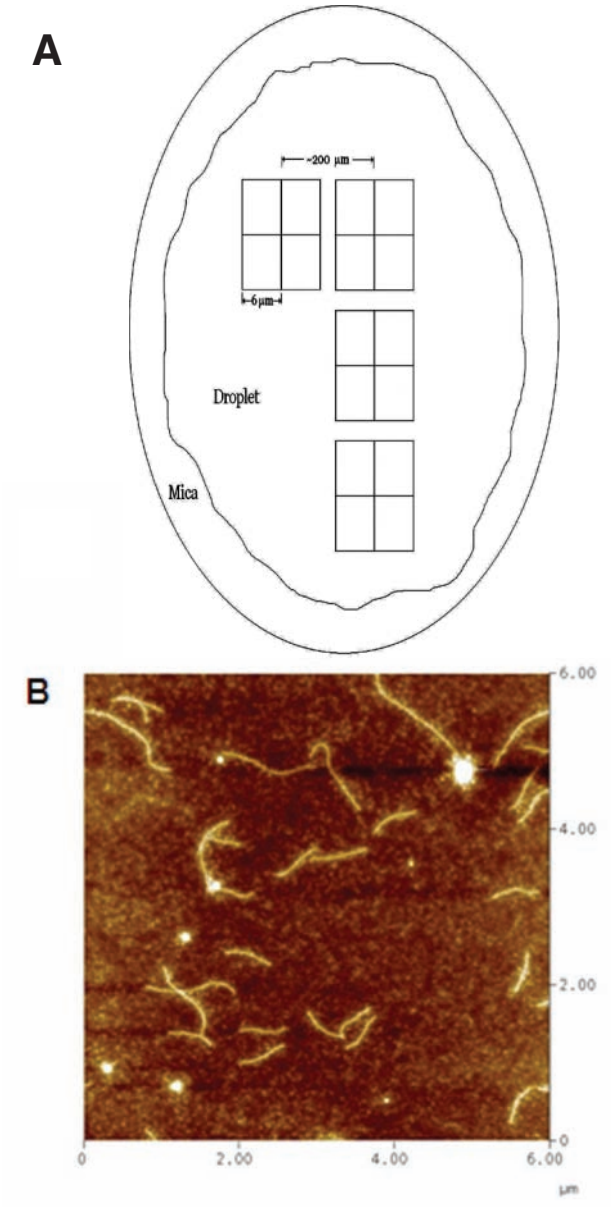

FIG. 5. **(A)** Scheme of the sample areas scanned by atomic force microscopy (AFM). **(B)** One of the 16 AFM images used to determine the density of viruses on the mica surface.

DISCUSSION

FCS offers the possibility to study the interaction of proteins with other molecules qualitatively and quantitatively in solution. The aim of this study was to show that phage display in combination with FCS is a very promising method for identifying new peptide ligands and characterizing the binding. For this purpose we investigated peptide ligands of the eukaryotic signaling protein 14-3-3 displayed by phage M13. To our knowledge, this is one of the very few reports studying a recombinant filamentous phage with FCS. Former work showed that it is possible to detect protein binding to phages with FCS. These studies employed M13 phages and labeled antibody against M13, antibody-displaying M13, or recombinant T7, the latter being an icosahedral-shaped phage.[17]

As proof of principle, the R18 peptide, a known ligand of 14-3-3 proteins,[20] was displayed on the surface of the recombinant M13 phage 2043 as a fusion with the minor coat protein cpIII. This approach was successful, as shown by the binding experiments analyzed using FCS. The recombinant phages bound specifically to 14-3-3 protein and the binding constant could be determined (**Table 2** and **Fig. 4**). Binding between the phages and dimeric 14-3-3 protein followed conventional 1st order kinetics up to a concentration of about $2 \cdot 10^8$ phages/µL (corresponding to ~0.3 nM). In contrast to a previous report,[17] no phage aggregates interfered with our measurements because of the use of low phage concentrations, avoiding shear forces when mixing the phage samples (no vortexing), short incubation times with the ligand, and the presence of a detergent, Tween-20, in the binding buffer.

The diffusion time of the fluorescently labeled 14-3-3 protein (σ-14-3-3-YFP) increased more than 10-fold when the R18-displaying phage was bound. From the diffusion time of ~2 ms (**Table 2**) fitted from our data we calculate a diffusion constant of $\sim 3.2 \cdot 10^{-12}$ m^2/sec. This value is considerably smaller than the diffusion constants of $\sim 1\text{–}2 \cdot 10^{-11}$ m^2/sec calculated from the FCS data of Lagerkvist et al.[17] and Bahns et al.[14] For this reason we also calculated the theoretical diffusion constant for a rod.[30] Assuming a length of 660 nm (as determined from the AFM images) and a diameter of 8 nm, we arrived at a diffusion constant of $3.51 \cdot 10^{-12}$ m^2/sec, which matches surprisingly well with our measured value. In this context it is worth noting that our diffusion times were fitted from experimental traces where about 60% of the labeled ligand was bound by phages, whereas in the former studies less than 30% of the fluorophores were bound to phage particles.

The recombinant phages 2043, harboring the R18 peptide sequence fused to the 5′ end of the fd-geneIII, were produced using M13 helper phages lacking the wild-type coat protein geneIII. Because phage display is based on the physical link between surface characteristics and genetic content of the phage, and because viable phages were produced, it was assumed that all phages would display the R18 peptide. A positive binding assay was not readily available nor was an alternative method to establish the R18-cpIII fusion at the protein level. Western blotting using cpIII antibodies to determine whether the fusion of R18 to cpIII was successful was not considered because cpIII migrates with an apparent molecular size of 66 kDa in protein gels,[31,32] and this size will not be altered by the R18 peptide having a predicted size of ~2 kDa. Moreover, antibodies to R18 were not available.

The TAMRA-labeled R18 peptide was successfully used to test the feasibility that the interaction of the 14-3-3 protein with its ligand could be measured in solution using FCS. Our data show (**Table 1**) that the R18 peptide binds specifically to the yeast 14-3-3 protein BMH2 and to the human σ-isoform of dimeric 14-3-3 protein, each with different diffusion times as

expected. Binding of R18 peptide to these 2 isoforms of 14-3-3 has not been shown before. However, our result is not so surprising, because binding of R18 peptide to several isoforms of 14-3-3 protein has been shown before.[20,21] It has been suggested that R18 peptide probably binds to a common motif in the binding site of all 14-3-3 protein isoforms. The binding of R18 to the dimeric protein σ-14-3-3-CFP followed a conventional 1st order kinetic (**Fig. 2**), indicating that the protein acts as a monovalent molecule, yielding a binding constant of 69 nM ± 22 pM. This number is very similar to the value of 70 to 90 nM as published for R18 peptide using a radio affinity chromatography binding assay and ζ-14-3-3 protein.[20] Theoretically, the very short distance of the 2 TAMRA-labeled R18 peptides bound by 14-3-3, estimated as 6.5 nm,[33] may contribute to quenching of the fluorescent dye. However, no quenching, instability, degradation, or release of the dye from the peptide was observed.

A rather unexpected result was the observation that 14-3-3 protein binds the R18 peptide ligand with a higher binding coefficient when it is imbedded in a protein context, as it is in the recombinant phage, than when it occurs as free peptide. The binding constant of free R18 peptide and R18 displayed by the phage was 69 nM ± 22 pM and 33 ± 12 pM, respectively (**Tables 1** and **2**). Visual inspection of our phage preparations using AFM indicated that this difference was not because there was a large number of binding sites present in our phage preparations in addition to viable phages. Rather, this result suggests that 2 R18 sequences displayed on the same phage particle may bind to one 14-3-3 protein resulting in a lower K_D. This might be of relevance to our understanding of how ligand binding to 14-3-3 protein may contribute to different effects in vivo. This is of even greater importance, because 14-3-3 is involved in a very wide range of cellular processes by binding to very many different ligands (for reviews, see Bunney et al.[2] and van Hemert et al.[34]).

Visual analysis using AFM provided essential data on the quality of our phage preparations. AFM imaging showed an even distribution of phages with no aggregates and most, if not all, phage particles were intact with an average length of 660 nm (**Fig. 5B**). This size is smaller than the published length of M13 phages (~800 nm) but might be explained by the fact that the length of the phage particle is determined by its genome size and our phage particles contain the recombinant phagemid pMP2043 whose size is smaller than that of M13 (~4.7 and ~6.4 kb, respectively). The lack of aggregates, which is essential for binding studies, is likely due to the relative low phage concentration in our preparations as has been reported before.[17] The concentration of phages determined by counting in AFM images was in agreement with the expected number of phages as established by pfu determination indicating that titer determination is a reliable method to predict the number of binding sites present in a phage preparation.

A major concern when using AFM for particle counting was that particles in the solution tend to accumulate at the edges of the drying droplet and that they are not evenly distributed over the sample. We could overcome this drawback by using very small volumes of phage preparations and spreading it out over a large area, using the purest water available and a quick drying method. Imaging of various dilutions of phage preparations turned out to be crucial to find the most optimal experimental setup. TEM or scanning electron microscopy is usually used to analyze phage preparations (e.g., Bahns et al.[14]), but we found AFM superior to TEM because of its higher sensitivity, low background, and good reproducibility.

In our combined study of phage display and FCS, use was made of fluorescently labeled 14-3-3 protein. The label was due to a C-terminal translational fusion with the autofluorescent protein YFP. In the past decade, many reports have shown the enormous potential of translational fusions with autofluorescent proteins in cellular biological research.[35] Recently, their use was extended to FCS studies including those in living cells.[36-38] The main advantage of autofluorescently labeled proteins over the use of proteins, to which the fluorescent dye is coupled chemically as usually done for FCS, is 2-fold. (1) The link of the fluorescent dye to the protein is made at the DNA level; therefore the fluorophore is stably coupled to the protein and there is no free fluorophore. (2) The function of the recombinant protein carrying the autofluorescent protein can be established both in vivo and in vitro prior to the FCS experiments. In this way, potential problems as reported for fluorescently labeled biomolecules with regard to binding affinity can be excluded. Indeed, no indication of instability of the 14-3-3-YFP protein was obtained. Additionally, bleaching, a well-known disadvantage of autofluorescent proteins, was not detected because the exposure time to the laser beam in FCS measurements is very short. Furthermore, we conclude that 1 molecule of dimeric 14-3-3 protein binds to 1 phage displaying the R18 peptide in 3 to 5 copies. This conclusion is based on the fact that no abundant spiking was observed during the FCS measurements and a binding curve for 1st order kinetic was satisfactory to fit our experimental binding data.

In summary, we have developed a very fast and sensitive method for quantitative measurement of ligate binding to phage-displayed ligands. In conventional methods employed for phage display, new ligands are isolated in a repeated cyclic process of large-scale phage production and recovery of potential ligands via affinity columns of binding partners, a method known as biopanning (for a review, see, e.g., Hoogenboom[39]). Characterization of the binding partners often makes use of ELISA or an alternative biochemical assay both of which usually exclude the determination on binding constants. The whole procedure of biopanning is laborious, very time consuming, and characterized by many pitfalls. Our data show that FCS offers an excellent alternative and less elaborate method for the isolation and characterization of new ligands. It should be noted, however, that FCS requires complex instrumentation and data processing,[18] which will limit general application. In contrast, for

instance, SPR measurements in biomolecular interactions are more commonly used and they offer a wide variety of essays.[13] Recently, interesting new applications of SPR measurements for phage-displayed peptides have been shown by the development of a bifunctional ligand display system.[40] It would be of great interest for future research to make comparisons of binding affinities of phage-displayed ligands determined with FCS and SPR methods using the same targets.

The results described in this article are promising with regard to future possibilities of FCS that include its use for high-throughput screening of large libraries of molecules for the presence of individual molecules that possess improved or more specific binding characteristics for signaling proteins such as 14-3-3. Such libraries may arise from ligand evolution. The combination of phage display, which enables the evolution of peptide sequences, and FCS is a powerful method for quantitative characterization. However, to be able to select individual molecules from very large phage libraries based on quantitative criteria, new technological developments are still needed to increase the throughput and selection criteria, for instance, using microfluidic sorting technology.

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