Institut für Pharmazie[1], Freie Universität Berlin, Germany, and National Cancer Institute[2], National Institute of Health, Bethesda, USA

Fusion of the binding domain of Raf-1 kinase with green fluorescent protein for activated Ras detection by fluorescence correlation spectroscopy

U. Trier[1], Z. Olah[2], B. Kleuser[1] and M. Schäfer-Korting[1]

Ras proto-oncogenes play a central role in cell proliferation by the regulation of signal transduction pathways from receptors of the outer cell membrane to the nucleus via the activation of transcription factors. Wild-type Ras cycles between the activated GTP-bound and the inactivated GDP-bound state, and the GTPase reaction is a timer for the interaction between Ras-GTP and effector molecules such as Raf-1 protein kinase. Mutations of *ras* resulting in the loss of the intrinsic GTPase activity result in autonomous proliferation. Mutated Ras is found in a variety of human tumors. Therefore, monitoring of GTP-loaded conformation of Ras related proteins could be utilised in cancer diagnosis. To develop a fluorescence based bioassay we have coupled the gene for the N-terminal Ras binding domain (RBD) of Raf-1 protein kinase with the gene for the green fluorescent protein (GFP). The chimeric fusion protein RBDGFP was identified by immunoblotting and subsequently investigated by fluorescence correlation spectroscopy (FCS), a new analytical technology allowing the measurement of characteristic diffusion times of fluorescently labeled molecules. Molecular interactions increase the molecular weight and influence the diffusion time of RBDGFP. FCS diffusion value of the recombinant protein was in coincidence with the molecular weight of the construct. Fluorimetric measurements of RBDGFP versus GFP showed clearly that the recombinant protein contains functional GFP. Increased FCS transition times indicated the interaction of RBDGFP with its corresponding antibody. Suboptimal binding of the fusion protein to activated Ras, however, resulted in a modest influence on the diffusion value. Taken together our rational design and construct shows the way for a ready characterisation of novel GFP-connected fusion proteins employing FCS.

1. Introduction

The *ras* proto-oncogenes are postulated to play a major role in mitogenic signalling and cellular transformation [1] by regulating signal transduction pathways from cell surface receptors to the nucleus [2]. These receptors include muscarinic m_1 and m_2, α_2-adrenergic, thrombine and lysophosphatidic acid receptors. The mitogenic response can be inhibited by pertussis toxin indicating that an inhibitory G-protein mediates this signal cascade. Signals from the cell surface receptors to the interior of the cell result in the activation of Ras. The activated form in turn stimulates the ubiquitous mitogene activated protein (MAP) kinase cascade which proceeds through a defined sequence of protein-protein interactions and phosphorylations [3, 4].
Three well characterised *ras* genes (H-*ras*, K-*ras* and N-*ras*) belong to a superfamily of genes coding for small monomeric GTP binding proteins, which are highly homologous MW 21 000 Da proteins [5]. The Ras protein cycles between the GDP bound and GTP bound state. In resting cells, $p21^{ras}$ is in the inactive form. It is activated by guanine nucleotide exchange factors (GEFs) such as Sos or Cd 25 [6] resulting in the active GTP bound form. It returns to the inactive state via GTP cleavage due to the intrinsic GTPase activity of Ras. Ester hydrolysis is greatly enhanced by a second protein called GTPase activating protein (GAP) [7].
Downstream of Ras-activation a direct interaction between the GTP-bound form and the Ser/Thr-specific protein kinase Raf-1 can be observed [8, 9]. Raf with the three isoforms C-Raf, B-Raf and A-Raf possesses a binding domain for Ras. This Ras binding domain (RBD) of c-Raf is a polypeptide of 81 amino acids comprising the residues 51-131 [10, 11]. Expression of this domain leads to a sufficient binding of activated Ras [12, 13].
The event that turns a wild-type *ras* gene into an oncogene is a single point mutation leading to a single amino acid substitution in the encoded protein [5]. It is of interest that these point mutations result in the loss of the intrinsic GTPase activity and therefore induce non-controlled cell growth. Activating mutations are invariably found in the GTP binding regions of $p21^{ras}$ [14]. Indeed mutations found in codons 12, 13 and 61 of H-K-N-*ras* appear to be responsible for the initiation and progression of most human tumors [15] and *ras* gene mutations can be found in a variety of tumor types although the incidence varies greatly [16]. The highest incidences are found in adenocarcinomas of pancreas (90%), colon (50%) and lung (30%) as well as in thyroid tumors (50%) and in myeloid leukemia (30%).
These factors underline the central role of *ras* related genes and proteins in different phases of cancer and give significant support to the view that early detection of *ras* gene mutations of Ras related oncoproteins can indeed serve as a valuable marker in cancer diagnosis [16].
By principle the interaction between the oncogenic form of Ras and the Ras effector enzyme Raf-1 proteins kinase provides an excellent specificity for a bioassay. In addition, the discovery of genes coding for novel protein fluorophores and new advances in laser technology, especially fluorescence correlation spectroscopy (FCS), have enabled the development of a method where the interaction between Ras and Raf can be monitored. For visualisation by FCS fluorescence labelling of at least one binding component is required. The discovery of green fluorescent protein (GFP) gene offers the perspective to generate fluorescence marked proteins.
In the bioluminescent jellyfish *Aequorea victoria* light is produced when energy is transferred from the photoprotein aequorin to GFP. The gene encoding for the 238 amino acid residues of GFP [17] was cloned recently and its subsequent expression in heterologous systems established GFP as a novel genetic reporter system [18, 19]. GFP emits bright green light ($\lambda = 508/540$ nm) when exposed to UV light or blue light [20, 21]. The quantum yield or probability of emitting a photon once the molecule has been excited is 0.72–0.85, and the excited state lifetime is

3.25 ns [22]. The GFP chromophore consists of the cyclic tripeptide derived from ser-tyr-gly in the primary protein sequence [23] and is only fluorescent when embedded in the complete GFP protein. The chromophore is formed by a cyclisation reaction and oxidation step that requires molecular oxygen. These steps are either autocatalytic or use factors that are ubiquitous, since fluorescent GFP forms in a broad range of organisms. Unlike other bioluminescent enzyme reporters which require cofactors and substrates to emit light, GFP is an active fluorophore either alone or in chimera form with other polypeptides [24, 25]. Even in fusion with another protein domain it has the advantage to give greater sensitivity resolution and resistance to photobleaching than protein staining with fluorescently labelled antibodies [26]. Therefore fusion between the gene encoding for GFP with that of the RBD of Raf-1 protein kinase provides an excellent method for investigation of Ras-Raf interactions monitored by FCS.

The analytical strategy of FCS was introduced in the early 1970s to measure chemical kinetics of molecules and their associated modulations of molecular diffusability [27, 28]. The principle of FCS is based on the monitoring of fluorescence emitted from a small optically well-defined open volume element as a function of time. The recorded fluorescence emission signal is proportional to the number of fluorescent molecules in the probe volume. The temporal autocorrelation of the fluorescence signal fluctuation yields the average number of fluorophores in the volume element. The current demand for highly sensitive analytical methods in molecular biology stimulated advanced technological improvement obtained by Rigler and Eigen. Derived diffusion coefficients of the observed fluorescent molecule allow to distinguish between different states of these particles without physical separation [29, 30]. Therefore FCS is a useful technique for the examination of molecular interactions as well as their time dependence.

In the first FCS experiments the power of the technique was demonstrated by measuring the diffusion and binding kinetics of the small fluorescent drug ethidium to DNA. The original studies demonstrated a drastically slowed diffusion when ethidium intercalated to the stacked bases of DNA [31]. This early research formed the basis for the normalised mathematical autocorrelation function [32, 33] arising the possibility of FCS to measure the absolute fluorophore concentration and the determination of the number densities of macromolecules in solution [34, 35], in membranes [36] and on surfaces [37]. Moreover, molecular weights and molecular aggregation [38] were investigated applying this concept. As the limits of time resolution and sensitivity have been improved many innovative applications have been conceived. FCS has been used for studying binding activities between ligand and receptor [3], enzymatic activity [40], antibody antigen interactions and interactions of regulatory proteins with DNA/RNA [41]. As FCS opens up the possibility to analyse rapidly in small volumes this technique was used for screening [42, 43] as well as for single molecule detection studies [44]. The power of FCS for counting sparse molecules has recently been innovatively utilised by La Clair for the study of disfavoured reaction pathways [45]. Some of the most interesting new applications use FCS in combination with gene amplification techniques to multiply sparse genetic material into a multitude [46]. Eigen et al. have demonstrated the identification of particular RNA and DNA strands with the help of fluorescent primers in the context of viral pathogen analysis [47].

As FCS combines the high resolution of confocal optics with the precision of fluorescence spectroscopy for monitoring molecular interactions in a sample volume of only 1 fl and the spectroscopical properties of GFP seem ideally suited for FCS detection we combined both approaches. Here we report a genetic fusion between the gene encoding GFP with that of the RBD of Raf-1 resulting in the expression of the chimeric protein RBDGFP, the characterisation of the new recombinant protein and its binding properties.

A new class of fluoroprotein based reagents was designed, prototyped and tested to become a valuable tool for the early detection of onco-proteins. This is the first study applying the power of FCS in the search for new clinical methods in cancer diagnosis.

2. Investigations and results

The specific interaction between the oncogenic form of Ras and the Raf-1 protein kinase allows an excellent specificity for a bioassay in the diagnosis of cancer. Therefore, we have constructed a chimeric protein with the Ras binding domain of Raf-1 kinase and the green fluorescent protein. The fluorescent part of the molecule serves for detection of the interaction between activated Ras and the binding domain by fluorescence correlation spectroscopy. In the following the new recombinant protein is referred to as RBDGFP.

The schematic diagram, presented in Fig. 1, demonstrates the construction of the pTrcHis vector including the chimeric genes coding for the RBDGFP fusion protein. *E. coli* cells were transformed with the engineered vectors and the chimeric fluoroproteins were expressed and subsequently purified by metal affinity chromatography. The pTrcHis vector contains a polyhistidine tag, genetically added to the N-terminus of the recombinant protein. This His_6-repeat binds to Ni^{2+} immobilised on the affinity resin allowing rapid and effective separation of the expressed protein from the crude homogenate. The protein bound to the resin can be recovered by elution with imidazole under non-denaturing conditions.

Crude homogenate and affinity purified proteins were subjected to gel electrophoresis and subsequently stained with

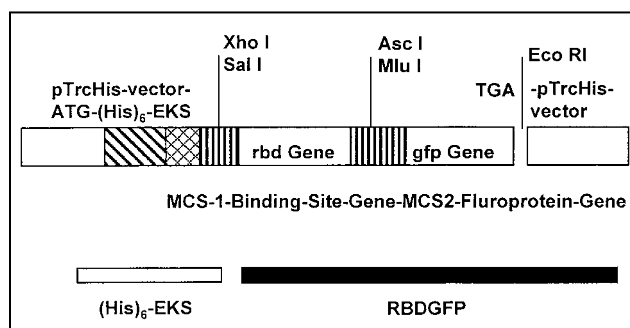

Fig. 1: Schematic diagram of the pTrcHisRBDGFP vector chimeric gene coding for the RBDGFP fusion protein. To subclone the *rbd* gene into the final plasmid vector, novel multi cloning sites (MCS) coding for the rare cutter restriction enzymes Sal I (MCS-1) and Asc I, Mlu I (MCS-2), respectively, were introduced in addition to the Xho I and Eco RI sites present in the pTrcHisA by LIC. The composite vector was constructed by inserting the GFP coding DNA at the Mlu I-Eco RI site of the LIC modified pTrcHisA containing the *rbd* gene. The ATG start codon followed by a $(His)_6$-repeat is provided by the parental vector along with a enterokinase cleavage site (EKS) that was used to facilitate affinity purification of the RBDGFP protein. The $(His)_6$-EKS was removed by protease digestion.

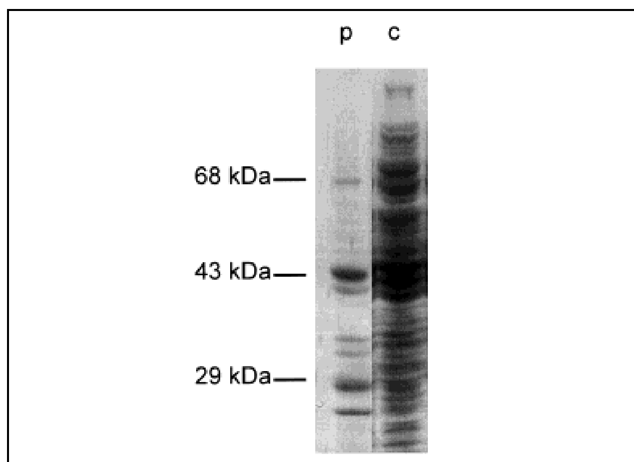

Fig. 2: Efficiency of the affinity purification. The crude *E. coli* homogenate (c) and purified protein (p) were electrophoresed on SDS-polyacrylamide gel and stained with Coomassie blue. The crude *E. coli* homogenate (right panel) reveals a variety of bands whereas in affinity purified sample (left panel) only a few bands are visible indicating the efficient purification by affinity chromatography. The dark band at 43 kDa corresponds to the expected location of RBDGFP.

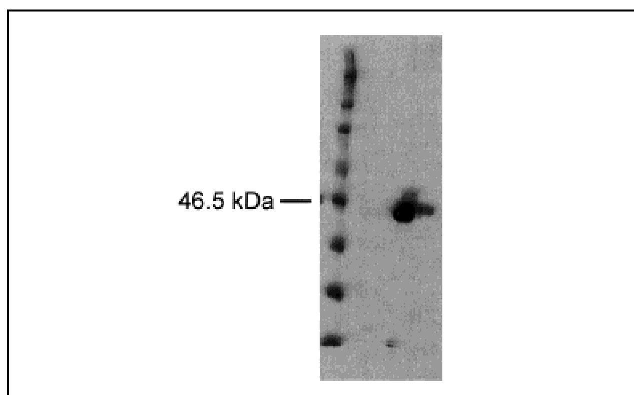

Fig. 3: Western blot of RBDGFP. Affinity purified samples were analysed by SDS gel electrophoresis and immunoblotting using Anti-Xpress antibody which is specific for the N-terminal leader peptide of RBDGFP. The major band below 46.5 kDa represents RBDGFP.

Coomassie blue. The gel depicted in Fig. 2 indicates the efficiency of the one-step purification. In contrast to the crude homogenate in the purified extract only a few bands are visible. The molecular weight of these bands varies from approximately 20 to 68 kDa. Molecular mass calculation of RBDGFP leads also to a value of 43 kDa. The following examinations should confirm if the expressed protein contains the Raf-1 binding domain as well as the fluorescent part.

The Anti-Xpress antibody which is specific for the N-terminal leader peptide of the chimeric protein was used for immunoblotting experiments as it is demonstrated in Fig. 3. This Western blot analysis established that only the main band possessed the specific antibody tag in accordance with the calculated molecular weight suggesting the complete expression of the chimeric protein.

The C-terminus of the fusion protein represents the fluorescent part of the molecule. By the previous results indeed no information is obtained about the fluorescence properties of the recombinant construct. Therefore FCS and fluorimetric measurements served for fluorescence characterisation of RBDGFP in comparison to native GFP. Fig. 4 presents the excitation and emission spectra of the fluoroprotein and GFP. It is clearly shown that the excitation as well as the emission maxima are identical for both proteins. Blue light is maximally absorbed at 395 nm with a minor peak at 470 nm and green light is emitted at 509 nm. In contrast to native GFP the second and minor excitation peak of RBDGFP at 470 nm is less distinctive, whereas the excitation maximum at 395 nm is enhanced.

Additionally FCS was applied for the further identification of RBDGFP. Fluctuations in the fluorescence intensity of RBDGFP diffusing through the confocal volume element were measured. Correlation of the fluorescence signals leads to the characteristic diffusion time of 259 μs. As the molecular weight can be calculated from the transition value we studied various proteins with a defined molecular weight in order to obtain a standard curve. The diffusion times correlated well with the molecular weight of the investigated molecules as comparison data from Evotec (Hamburg, Germany; personal communication) were used. The diffusion times presented in the Table indicate that

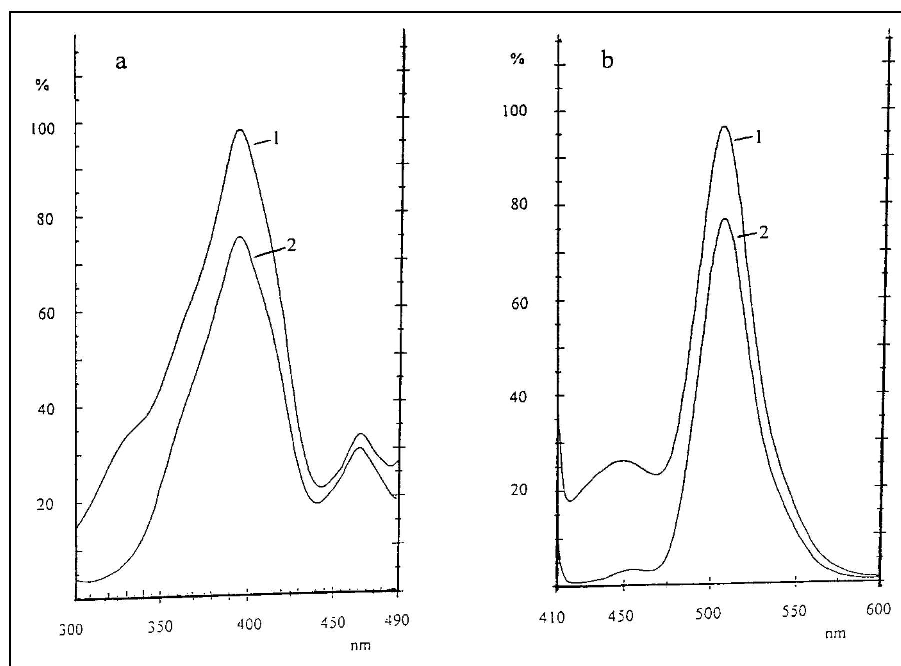

Fig. 4:
Excitation (a) and emission (b) spectra of RBDGFP (1) versus GFP (2). Excitation of the fluoroproteins reveals the characteristic maxima at 395 nm and 470 nm, whereas the ratio of both peaks varies. RBDGFP shows an increased excitation maximum and in comparison to the enhanced main peak a decreased shoulder at 470 nm. Similar observations can be made in the emission spectra of the proteins. Fusion of GFP to the Ras binding domain of Raf-1 does not affect the emission maximum at 509 nm but leads to variations in the curve amplitude.

the diffusion time of 259 μs is well in accordance to the expected molecular weight of 43 kDa.
RBDGFP even in nanomolar concentrations is easily detected by FCS. The chimeric protein gives a stable fluorescence signal when excited at 488 nm. RBDGFP shows better resistance to photobleaching in comparison to commonly used fluorescein dyes. Additionally the FCS triplet fraction (T) which represents the amount of excited molecules without emitting fluorescence light was about 15%. These fluorescence properties resulted in a standard deviation of the diffusion time less than 6% (Table).

Table: FCS diffusion time and molecular weight of various fluorescent molecules. Binding of RBDGFP to anti-Xpress antibody

	MW (Da)	Diffusion time (μs)	Standard deviation (μs)
Rhodamine 6 G	650	67	1.3
GFP	27000	217	2.3
RBDGFP	43000	259	6
RBDGFP + Anti-Xpress antibody	183000	336	19
antibody	140000	382	23
prim./sec. antibody	280000	486	19

Our next investigation based on the theory that the diffusion time of RBDGFP should be enhanced after the specific interaction with a binding protein. As we have demonstrated by immunoblotting, the Anti-Xpress antibody shows considerable binding affinity to the fluoroprotein construct. Antibody addition to the fluoroprotein induced a significant increase in diffusion time (Table). The diffusion value of 336 μs comprises the transition time of the fluoroprotein alone as well as that of the complex as there is still unbound protein in the investigated sample. The changed diffusion value can be visualised in the correlation curve leading to a curve shift to the right. The enhancement was specific as in control experiments the addition of a non-specific antibody or the combination of GFP with the Anti-Xpress antibody did not change transition parameters.
Of note FCS measures the fluorescence coded by the C-terminus of the fusion protein whereas the antibody binds to the N-terminal His_6-tag. This again indicates the complete expression of the Ras binding domain framed by the leader peptide and the GFP.
Our next consideration was the examination of the interaction between the Ras binding domain of our fluoroprotein with activated or mutated Ras. Wild-type Ras was activated by a GTP derivative which is resistant to enzymatic hydrolysis and subsequently incubated with RBDGFP. Interaction was monitored by FCS. No changes in diffusion time were observed. This can be expected because of the disadvantageous proportion of the molecular weight. Optimal FCS conditions require the smaller and faster diffusing component to be labelled in order to obtain measurable changes in diffusion time which is not the case here. To solve this problem an antibody specific for Ras was added. This should increase the diffusion time from 259 to about 450 μs when complete binding takes place. Experimental data, however, showed only a small influence on transition values suggesting suboptimal interaction of activated Ras to its binding domain.

3. Discussion

We have expressed a fusion protein consisting of the Ras binding domain of the protein kinase Raf-1 and the green fluorescent protein and tested it by FCS. As described by other groups we could show that GFP is well suited for fluorescence measurements [48, 49].
Rapid photobleaching is one of the major problems of a variety of fluorescent markers even in FCS with high intensity. In coincidence with other groups our results indicate that GFP is quite stable when illuminated at 488 nm [19, 50]. A further problem in FCS examinations is the transfer of fluorescent molecules to the triplet state resulting in a loss of emitted fluorescence light. In comparison to fluorescein with a triplet state of more than 50% only 15% of the excited GFP molecules are transferred to the triplet energy level. GFP needs to be in an oxidised state to fluoresce because chromophore formation depends on an oxidation of Tyr-66 [51]. Fluorescence is fully revealed when expressed in *E. coli* indicating that the cyclisation reaction is an autocatalytic process. It was shown that GFP in fusion with other proteins revealed an even higher sensitivity and resolution signal [51]. Therefore it was of interest to compare the fluorescence parameters of GFP alone with that of our recombinant chimera protein. FCS allows direct monitoring of the count rate which represents the fluorescence intensity. As expected the count rate of RBDGFP was enhanced. The excellent spectral properties decreased the detection limit allowing the measurement of the fusion protein in the nanomolar range. Our experiments clearly demonstrated that the fluorescence labelled molecule and its corresponding partner must be present in a relative pure solution for monitoring of specific interaction by FCS, especially in low concentrations. Otherwise fluorescent background noise in combination with non-specific binding conceals the fluorescence signal triggered by the specific interaction.
Moreover the spectral properties of the fluoroprotein in contrast to native GFP were studied. Fluorimetric measurements showed clearly that the recombinant protein contains functional GFP due to the identical excitation and emission maxima of RBDGFP in comparison to that of GFP. Additionally variations in the minor peaks and peak ratio were detected. Analogous effects can be observed in excitation spectra, where GFP dimerisation results in a reduction in the absorption at 470 nm and a concomitant increase in absorption at 395 nm, too. As FCS detection confirmed RBDGFP to exist as a single molecule the ratio variation is due to the fusion of GFP with the binding domain.
For engineering of the recombinant protein the pTrcHis vector was used. This vector offers the feature of N-terminal six tandem histidine residues for purification and the Xpress epitope for simple detection. Affinity chromatography with a Ni-charged resin and elution with imidazole allows the fusion protein to be separated from the crude *E. coli* homogenate and its detection by the Anti-Xpress antibody. This gentle purification under non-denaturing conditions was effective and sufficient for the measurement of the recombinant protein by FCS.
In FCS experiments the drug diffusion time of the fluorescence marked molecule is measured. Complex formation increases the molecular weight of the fluorescent agent and therefore slows down the diffusion value. Therefore it is of advantage if the molecular weight of the binding partners differs considerably, whereby the smaller molecule must be labelled. For FCS investigations the Anti-Xpress antibody confirming the N-terminal epitope of the recombinant protein was used. Binding of the antibody to RBDGFP increased the molecular weight and therefore slowed down diffusion time which indicated clearly the

complete expression of the fusion protein. Therefore by principle FCS analysis of RBDGFP allows the detection of Ras mutations.

Detection of cancer in the early, clinically asymptomatic phases is important for better therapeutic efficiency. As described mutated Ras is present in a variety of human tumours. The interaction between Ras and Raf-1 taking place only with mutated respectively activated Ras is an exciting tool for the early detection of the onco-protein. For this purpose FCS provides excellent conditions because this technology enables the visualisation of fluorescent molecules in nanomolar concentrations. Hence the engineered fusion protein was investigated for measurement of activated Ras. The protein itself possesses a molecular weight of 20 kDa. This means that the molecular mass of 43 kDa of the fusion protein is enhanced to 63 kDa after the interaction with Ras. This modest increase is not sufficient for monitoring by FCS. To avoid this problem Ras-specific antibodies were added in order to enlarge the complex and to improve the significantly higher FCS diffusion value. Unfortunately experimental data showed only a weak enhancement of the transition time. This indicates that the complex was built merely to a minor extent. From this investigation it can be concluded that the binding between mutated respectively activated Ras and the fusion protein is not sufficient. A variety of circumstances may be responsible for the low binding activity. As we have shown the fusion protein is fully expressed incorrect threedimensional folding in *E. coli* might occur.

In analogy to our experiments Nassar et al. [52] have performed Xray crystal structure analysis of the binding between the Ras related protein Rap-1 and the RBD. They also expressed these proteins in *E. coli* and showed an interaction of these components. As RBD alone sufficiently binds Ras it seems most likely that the addition of N-/C-terminal sequences disturbs binding activity. Possibly the fusion of RBD with GFP or the N-terminal His-epitope leads to a sterical blockade of the binding domain.

Changes in the recombinant fusion protein may solve the above mentioned problems of the non-sufficient binding between Ras and RBDGFP. The introduction of a spacer between RBD and the N- or C-terminal epitope might be useful to obtain more effective complex formation and to avoid sterical hindrance. Moreover using an eukaryontic expression system might improve binding.

Taken together our rational design and construction allows ready characterisation of novel GFP-connected fusion proteins employing FCS. The demonstrated technology will vastly accelerate preparation of polypeptide based diagnostics against uncharacterised gene products and other molecular targets of interest.

4. Experimental

4.1. Materials

Cell culture products were obtained from Life Technologies (Eggenstein). Phenylmethylsulfonylfluoride, protease inhibitors leupeptin, pepstatin A, aprotinin and general chemicals were purchased from Sigma (Deisenhofen) and Merck (Darmstadt). GFP protein was from Clontech (Heidelberg).

4.2. Preparation of the crude E. coli homogenate and affinity purification

RBDGFP was expressed in *E. coli* and purified according to the following procedure. To an overnight culture of the cells in LB-medium containing 50 μg/ml ampicillin 0.4 mM IPTG (Calbiochem, Bad Soden) was added. Following 4 h of permanent shaking at 37 °C cells were harvested by centrifugation at 10000 × g for 10 min. The following steps were performed at 4 °C. The supernatant was decanted and the bacterial pellet was subsequently resuspended in ice-cold Novagen (Boehringer Ingelheim Bioproducts Partnership, Heidelberg) binding buffer containing 1 mM PMSF, leupeptin, aprotinin and pepstatin A (each 20 μg/ml). To shear DNA cells were sonicated on ice. To remove debris cells were collected by centrifugation at 10000 × g for 10 min and extraction was repeated. Combined supernatants were centrifuged at 39000 × g for 20 min and used for affinity purification according to the manufacturers protocol (Novagen). The His-tag sequence of the recombinant protein binds to Ni^{2+} which are immobilised on a resin. After unbound proteins were washed away RBDGFP was recovered by elution with 1 M imidazole. The eluted fractions were controlled by FCS.

4.3. Immunoblotting

The expression of the fusion protein was studied using enhanced chemoluminiscence (ECL) Western blot analysis. Affinity purified RBDGFP was electrophoresed on 10% SDS-polyacrylamide gel according to Laemmli [53, 54]. Prestained proteins (lysozyme 14.3 kDa, β-lactoglobulin 18.4 kDa, carbonic anhydrase 29 kDa, ovalbumin 43 kDa, bovine serum albumin 68 kDa, phosphorylase B 97 kDa, myosin (H-chain) 200 kDa) from Calbiochem (Bad Soden) served as molecular mass markers for electrophoresis combined with biotinylated molecular weight standards (6.5; 14.5; 28; 37.5; 46.5; 57; 76; 105; 165 kDa) from New England Biolabs (Schwalbach) for the transfer to PVDF membrane (Millipore, Eschborn). Following electrophoresis the gels were immersed in transfer buffer (Tris-glycine, pH 8.3) for 15 min. Proteins were transferred from gels to PVDF membrane using a Biometra (Göttingen) tankblot (12 h, 100 mA). The membranes were blocked with 5% non-fat dried milk in TBST for 1 h under permanent shaking. Before the addition of primary antibody membranes were washed three times for 5 min with TBST. Then the membranes were incubated with the anti-Xpress antibody from Invitrogen (NV Leek, The Netherlands) as primary antibody diluted 1:5000 in TBST and 1% BSA. After washing three times a solution of the secondary antibody, horseradish peroxidase (HRP) linked antiserum from New England Biolabs (Schwalbach) diluted 1:1000 was added for 1 h under permanent shaking. HRP linked anti-biotin antibody from New England Biolabs served for detection of biotinylated molecular weight standards. Non-bound antibody was removed by three washing steps and membranes were incubated with LumiGlo detection solution (New England Biolabs). The light emitted by destabilised LumiGlo reagent was subsequently captured on X-ray film. All incubation and washing steps were performed at room temperature.

4.4. Coomassie staining

Following electrophoresis the gels were simultaneously fixed with methanol/glacial acetic acid and stained with Coomassie brilliant blue R 250 (Serva, Heidelberg). Gels were immersed in a 0.25% methanol/acetic acid solution of the dye and placed on a slowly rotating platform for 2 h at room temperature. The staining solution was removed and excess dye was allowed to diffuse from the gel by destaining in a methanol/acetic acid solution without the dye under permanent shaking for 12 h. After destaining gels were stored in water containing 20% glycerol.

4.5. FCS

FCS was performed with a ConfoCor (Zeiss Jena/Evotec Hamburg) using a C-Apochromat 40×/1.2 W korr objective. The confocal volume element was illuminated by an $argon^{+}$-laser at the 488 nm line. An optical density filter OD 1.0 results in optimal illumination output. Measurements of RBDGFP in a concentration range between 1.9 and 9.9 nM were done at room temperature in eight chamber coverglasses (Nunc GmbH, Wiesbaden); recording time for all experiments was 30 s. For studying RBDGFP Anti-Xpress antibody interactions RBDGFP was used in a concentration of 1.3 nM and incubated with the antibody in a dilution of 1:25 for 5 min. For examination of interactions between RBDGFP and activated Ras, RBDGFP was incubated with wild-type Ras (Calbiochem, Bad Soden) in a ten-fold higher concentration and access guanosine 5′-O-(3-thiotriphosphate) tetralithiumsalt (GTP-γ-S · 4 Li, Alexis, Grünberg) for 5 min. To enhance molecular weight anti-pan-Ras antibody from Dianova (Hamburg) was added to the reaction mixture. Data evaluation was done with the FCS ACCESS software package (Zeiss/Evotec) using the one-component fit model.

References

1 Cowley, S.; Paterson, H.; Kemp, P.; Marshall, C. J.: Cell **77**, 841 (1994)
2 Calabretta, B.: Cancer Res. **51**, 4505 (1991)
3 Seger, R.; Krebs, E. G.: FASEB J. **9**, 726 (1995)
4 Block, C.; Tillmanns, H. W.; Meffert, R. M.: Nat. Struct. Biol. **3**, 244 (1996)
5 Barbacid, M.: Annu. Rev. Biochem. **56**, 779 (1987)

6 Geyer, M.; Schweins, T.; Herrmann, C.; Prisner, T.; Wittinghofer, A.; Kalbitzer, H. R.: Biochemistry **35**, 10308 (1996)
7 Trahey, M.; McCormick, F.: Science **238**, 542 (1987)
8 Koide, H.; Satoh, T.; Makafuku, M.; Kaziro, Y.: Proc. Natl. Acad. Sci. USA **90**, 8683 (1993)
9 Moodie, S. A.; Willumsen, B. M.; Weber, M. J.; Wolfman, A.: Science **260**, 1658 (1993)
10 Wittinghofer, A.; Herrmann, C.: FEBS Lett. **369**, 52 (1995)
11 Emerson, S. D.; Madison, V. S.; Palermo, R. E.: Biochemistry **34**, 6911 (1995)
12 Emerson, S. D.; Waugh, D. S.; Scheffler, J. E.; Tsao, K. L.; Prinzo, K. M.; Fry, D. C.: Biochemistry **33**, 7745 (1994)
13 Herrmann, C.; Martin, G. A.; Wittinghofer, A.: J. Biol. Chem. **270**, 2901 (1995)
14 Barbacid, M.: Eur. J. Clin. Invest. **20**, 225 (1990)
15 Sidransky, D.; Tokins, T.; Hamilton, S. R.; Kinzler, K. W.; Levin, B.; Frost, P.; Vogelstein, B.: Science **256**, 102 (1992)
16 Bos, J. L.: Cancer Res. **49**, 4682 (1989)
17 Prasher, D. C.; Eckenrode, V. K.; Ward, W. W.; Prendergast, F. G.; Cormier, M. J.: Gene **111**, 229 (1992)
18 Chalfie, M.; Tu, Y.; Euskirchen, G.; Ward, W. W.; Prasher, D. C.: Science **263**, 802 (1994)
19 Carey, K. L.; Richards, S. A.; Lounsbury, K. M.; Macara, I. G.: J. Cell. Biol. **133**, 985 (1996)
20 Morise, M.; Slumomura, O.; Johnson, F. H.; Winant, J.: Biochemistry **13**, 2656 (1974)
21 Ward, W. W.; Cody, C. W.; Hart, R. C.; Cormier, M. J.: Photochem. Photobiol. **31**, 611 (1980)
22 Cubitt, A. B.; Heim, R.; Adams, S. R.; Boyd, A. E.: Trends. Biochem. Sci. **11**, 448 (1995)
23 Cody, C. W.; Prasher, D. C.; Westler, w. M.; Prendergast, F. G.; Ward, W. W.: Biochemistry **32**, 1212 (1993)
24 Ogawa, H.; Inouye, S.; Tsuji, F. J.: Proc. Natl. Acad. Sci. USA **92**, 11899 (1995)
25 Hampton, R. Y.; Koning, A.; Wright, R.; Rine, R.: Proc. Natl. Acad. Sci. USA **93**, 828 (1996)
26 Wang, S.; Hazelrigg, T.: Nature **369**, 400 (1994)
27 Elson, E. L.; Magde, D.: Biopolymers **13**, 1 (1974)
28 Ehrenberg, M.; Rigler, R.: Chem. Phys. **4**, 390 (1974)
29 Ehrenberg, M.; Rigler, R.: Rev. Biophys. **9**, 69 (1976)
30 Kask, P.; Piksarv, P.; Mets, U.; Pooga, M.; Lippman, E.: Eur. Biophys. J. **14**, 257 (1987)
31 Madge, D.; Elson, E. L.; Webb, W. W.: Phys. Rev. Lett. **29**, 705 (1982)
32 Elson, E. L.; Webb, W. W.: Annu. Rev. Biophys. Bioeng. **4**, 311 (1975)
33 Koppel, D. E.: Phys. Rev. **10**, 1938 (1974)
34 Weissman, M. B.; Schindler, H.; Feher, G.: Proc. Natl. Acad. Sci. USA **73**, 2776 (1976)
35 Sorscher, S. M.; Klein, M. P.: Rev. Sci. Instruments **51**, 98 (1980)
36 Fahey, P. F.; Koppel, D. E.; Barak, L. S.; Wolf, D. E.; Elson, E. L.; Webb, W. W.: Science **195**, 305 (1977)
37 Thompson, N. L.; Axelrod, D.: Biophys. J. **43**, 103 (1983)
38 Palmer, A. G.; Thompson, N. L.: Biophys. J. **52**, 257 (1987)
39 Bauer, B.; Neumann, E.; Widengren, J.; Rigler, R.: Biophys. Chem. **58**, 3 (1996)
40 Klingler, J.; Friedrich, T.: Biophys. J. **73**, 2195 (1997)
41 Schwille, P.; Oehlenschläger, F.; Walter, N. G.: Biochemistry **35**, 10182 (1966)
42 Rigler, R.: J. Biotechnol. **41**, 177 (1995)
43 Sterrer, S.; Henco, K.: J. Receptor and Signal Transduction Res. **17**, 511 (1997)
44 Wennmalm, S.; Edman, L.; Rigler, R.: Proc. Natl. Acad. Sci. USA **94**, 10641 (1997)
45 La Clair, J. J.: Proc. Natl. Acad. Sci. USA **94**, 1623 (1997)
46 Walter, N. G.; Schwille, P.; Eigen, M.: Proc. Natl. Acad. Sci. USA **93**, 12805 (1996)
47 Oehlenschläger, F.; Schwille, P.; Eigen, M.: Biochemistry **93**, 12811 (1996)
48 Crameri, A.; Whitehorn, E. A.; Tate, E.; Stemmer, W. P.: Nature Biotechnol. **14**, 315 (1996)
49 Terry, B. R.; Matthews, E. K.; Haseloff, J.: Biochem. and Biophys. Res. Com. **217**, 21 (1995)
50 Niswender, K. D.; Blackman, S. M.; Rohde, L.; Magnuson, M. A.; Piston, D. W.: J. Microbiol. **180** (2), 109 (1995)
51 Heim, R.; Prasher, D. C.; Tsien, R. Y.: Proc. Natl. Acad. Sci. **91**, 12501 (1994)
52 Nassar, N.; Horn, G.; Herrmann, C.; Scherer, A.; McCormick, F.; Wittinghofer, A.: Nature **375**, 554 (1995)
53 Blackshear, P. J.: Methods Enzymol. **104**, 237 (1984)
54 Laemmli, U. K.: Nature **227**, 680 (1970)

Received August 3, 1998
Accepted August 26, 1998

Prof. Dr. Monika Schäfer-Korting
Freie Universität Berlin
Institut für Pharmazie II
Königin-Luise-Str. 2–4
D-14195 Berlin