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Research Paper

A sensitive fluorescence monitor for the detection of activated Ras: total chemical synthesis of site-specifically labeled Ras binding domain of c-Raf1 immobilized on a surface

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Abstract

Background: The Ras·GDP–Ras·GTP cycle plays a central role in eukaryotic signaling cascades. Mutations in Ras which stabilize activated Ras·GTP lead to a continuous stimulation of downstream effectors and ultimately to cell proliferation. Ras mutants which increase the steady-state concentration of Ras·GTP are involved in about 30% of all human cancers. It is therefore of great interest to develop a biosensor which is sensitive to Ras·GTP but not to Ras·GDP.

Results: The Ras binding domain (RBD) of c-Raf1 was synthesized from two unprotected peptide segments by native chemical ligation. Two fluorescent amino acids with structures based on the nitrobenz-2-oxa-1,3-diazole and coumaryl chromophores were incorporated at a site which is close to the RBD/Ras·GTP binding surface. Additionally, a C-terminal tag consisting of His_6 was introduced. The K_d values for binding of the site-specifically modified proteins to Ras·GTP are comparable to that of wild-type RBD. Immobilization of C-terminal His_6 tag-modified fluorescent RBD onto Ni-NTA-coated surfaces allowed the detection of Ras·GTP in the 100 nM range. Likewise, Ras·GTP/Q61L (an oncogenic mutant of Ras with very low intrinsic GTP hydrolysis activity) can also be detected in this assay system. Ras·GDP does not bind to the immobilized RBD, thus allowing discrimination between inactive and activated Ras.

Conclusions: The site-specific incorporation of a fluorescent group at a strategic position in a Ras effector protein allows the detection of activated Ras with high sensitivity. This example illustrates the fact that the chemical synthesis of proteins or protein domains makes it possible to incorporate any kind of natural or unnatural amino acid at the position of choice, thereby enabling the facile preparation of specific biosensors, enhanced detection systems for drug screening, or the synthesis of activated proteins, e.g. phosphorylated proteins involved in signaling pathways, as defined molecular species.

Keywords: Chemical protein synthesis; Fluorescent biosensor; Native chemical ligation; Ras binding domain of c-Raf1; Site-specific labeling

1. Introduction

In recent years, chemical synthesis by means of the native chemical ligation of unprotected peptides [1] has become a viable method for the preparation of a wide variety of biologically active proteins [2]. These include cytokines, enzymes (e.g. human secretory phospholipase A2 [3]), and effector molecules (e.g. the Ras binding domain (RBD) fragment of c-Raf1 [4]). Even integral membrane proteins, a class of proteins which will become of great significance in research in the near future, have been prepared by total chemical synthesis [5]. Furthermore, the expressed protein ligation technique [6] has enabled the production of polypeptide thioesters by recombinant means for use in native chemical ligation. The combination of chemically synthesized peptides with recombinant polypeptides greatly increases the versatility and appli-

Abbreviations: DAP, diaminopropionic acid; DMCA, 6,7-dimethoxycoumaryl-alanine; FRET, fluorescence resonance energy transfer; GppNHp, guanosine-5′-(*b,g*-imido)-triphosphate; HFIPA, hexafluoroisopropanol; His, 6×His-tag; mant, methylanthraniloyl; NBD, nitrobenz-2-oxa-1,3-diazole; RBD, Ras binding domain of c-Raf1

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cability of chemical protein synthesis. Since chemical synthesis is inherently not limited to the use of the 20 genetically encoded amino acids as building blocks, it is foreseeable that tailor-made proteins with customized properties suitable for research and for biotechnological applications can be prepared conveniently [7]. Examples of such custom-tailored proteins have already appeared in the literature. For instance, Sydor et al. [4] introduced a fluorescently labeled Trp analog into the RBD of c-Raf1 to study the kinetics of its binding to Ras, which belongs to the superfamily of small GTPases. In another example, Cotton and Muir [8] designed and assembled a dual-labeled fluorescence biosensor for Crk-II phosphorylation.

One of the challenging tasks in biochemistry will be concerned with the investigation and development of protein-based systems for use in the diagnosis of diseases and for use in drug design and discovery. An important aspect of these developments will be the specific immobilization of the monitoring protein on functionalized surfaces. The immobilization of enzymes and other proteins for chip or biosensor development has become increasingly important in the light of genome sequencing projects. First efforts to generate biosensors for in vivo measurements were developed in the early 1960s and led to glucose, lactate, urea, and glutamate/glutamine biosensors. All these biosensors employ enzymes (oxidoreductases) and provide their signal in the form of electroactive species which are detected by an electrode [9]. During the last decade, DNA chips have provided an efficient method for the detection of mutations in specific genes as diagnostic markers of particular diseases and for the detection of differences in gene expression levels [10]. In contrast, the design of protein-based biochips and their applications is still in its infancy [11,12].

An important system for the development of a biosensor is the Ras–Raf protein pair. The small GTPase Ras plays a major role in cell signaling events and cell proliferation. The interaction between Ras·GTP and its downstream effector c-Raf1 is of crucial importance for the regulation of cell division and differentiation [13]. Ras acts as a molecular switch which in its GTP-bound, but not in its GDP-bound state activates downstream signaling events. Certain mutations in Ras, such as G12V or Q61L, lead to an increase in the steady-state concentration of Ras·GTP due to lower hydrolysis activity, potentially leading to uncontrolled cell proliferation. Mutants with accelerated GDP dissociation rates can lead to similar effects. Ras mutants are involved in about 30% of all human cancers [14,15]. Therefore, it is of general interest, e.g. in cancer diagnostics, to establish a biosensor for the reliable determination of Ras·GTP in the presence of Ras·GDP.

One of the most promising approaches to the development of biosensors involves the use of unnatural amino acids with tailored properties at specific sites in proteins of interest. It has been shown that a sequence of 81 amino acids from the N-terminal region of c-Raf1 (RBD) is sufficient for tight binding to Ras [16]. This interaction has been extensively investigated using kinetic and structural methods [17–19] as well as chemically synthesized RBD containing fluorescent Trp or the unnatural Trp analog N^1-methyl-7-azatryptophan at position 91. The latter example provided evidence that small fluorescent amino acids are suitable monitors for the binding event without perturbing the kinetics of this process [4]. However, the properties of those fluorescent residues were not appropriate for the development of a biosensor, because the fluorescent residues that were used displayed only low extinction coefficients and poor quantum yields. Furthermore, environmental effects had only a small influence on the emission intensity and/or the emission maximum of the fluorophores. Therefore, new small amino acids with favorable fluorescence properties had to be selected and incorporated into RBD.

In the present work, the synthesis of a sensor which can detect the potentially oncogenic Ras·GTP in the presence of Ras·GDP is described. To demonstrate the potential applicability of surface-immobilized proteins, we have chemically synthesized the RBD of c-Raf1 containing a suitable fluorescent amino acid in close proximity to the Ras binding site, and immobilized this detector protein on a derivatized surface. The properties of this protein biosensor for Ras and Ras mutants are described.

2. Results

2.1. Synthesis of fluorescent RBD

In earlier work it was shown that Trp or the Trp analog N^1-methyl-7-azatryptophan could be introduced by chemical protein synthesis into RBD in position 91 (see Fig. 1B) without loss of activity [4]. However, the results also showed that the poor sensitivity of the fluorescent chromophores would not allow the development of a biosensor. Therefore, two fluorescent amino acids based on the chromophores 7-nitrobenz-2-oxa-1,3-diazole (NBD) and 6,7-dimethoxy-coumaryl (DMCA) (Fig. 1A), which promised favorable properties (red-shifted emission maxima, higher extinction coefficients and quantum yields), were chosen for the incorporation into RBD. The red-shifted emission maxima of the fluorescent probes do not interfere with the Trp emission which permits the selective detection of binding events even in the presence of an excess of Trp residues. The NBD-containing amino acid has the additional advantage that its fluorescence is quite sensitive to the local environment.

Each amino acid was incorporated into RBD at position 91, replacing a leucine, using the strategy of native chemical ligation [1] and following the procedure described in [4] (see also Fig. 1B). The N-terminal segments, which contained the fluorescent amino acids, were synthe-

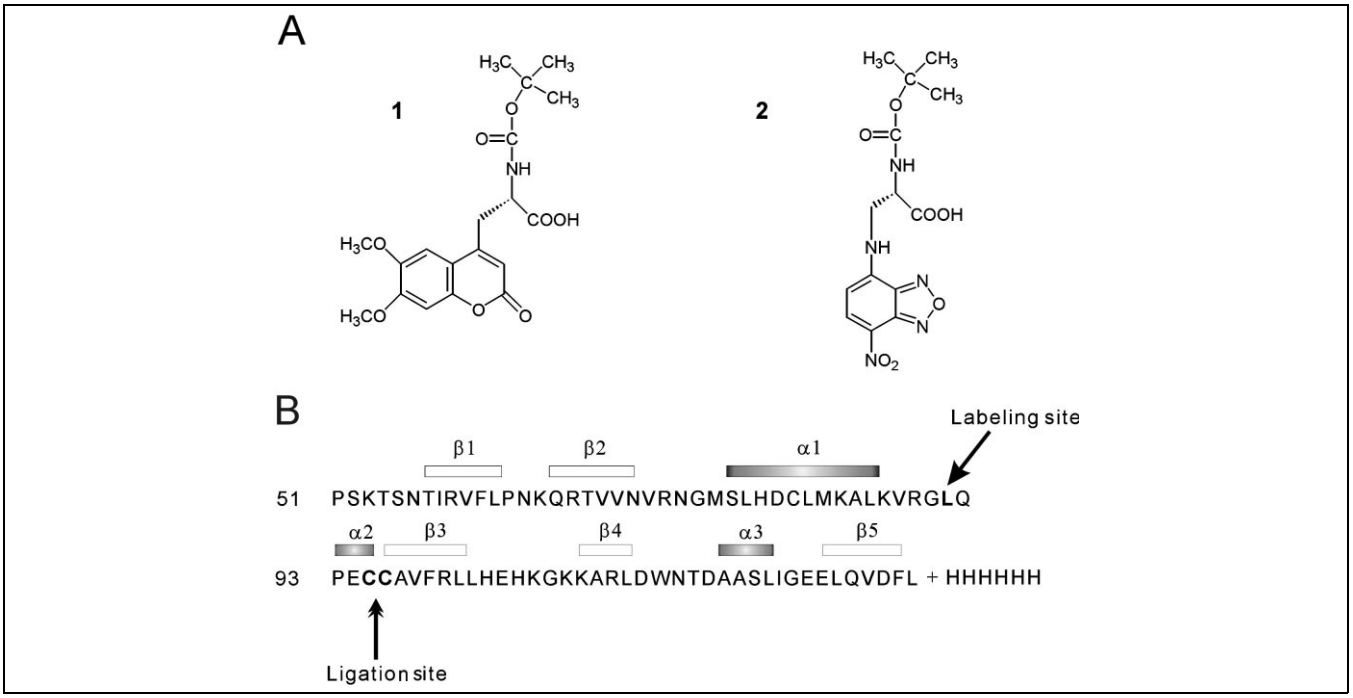

Fig. 1. (A) Structure of the incorporated amino acids: (**1**) *N*-Boc-L-(6,7-dimethoxy-4-coumaryl)-alanine and (**2**) N^2-Boc-N^3-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-L-2,3-diaminopropionic acid. (B) Sequence of the RBD. Secondary structure elements are indicated by white and gray bars above the sequence. The labeling site at position 91 and the ligation site between Cys 95 and Cys 96 are indicated by arrows.

sized on a resin designed to generate a thioester at the C-terminus after HF cleavage. The C-terminal segments were synthesized on a standard $-OCH_2$-phenylacetamidomethyl resin. An additional C-terminal six-residue histidine tag was added to allow immobilization on Ni-NTA surfaces [20,21]. All polypeptides were purified by reversed phase HPLC and identified by electrospray mass spectrometry. Chemical ligation of the unprotected peptide segments to yield the full-length RBD polypeptide was carried out as previously published [4]. The ligated polypeptides were obtained at a purity of $>98\%$ with recovered yields of 20–40% based on the N-terminal peptide segments.

After reconstitution of the lyophilized polypeptides in aqueous buffer, correct folding of the synthesized proteins was checked by circular dichroism (CD) spectroscopy (data not shown). All spectra are in good agreement with those of native RBD [4,22,23]. A comparison of CD data obtained from measurements of RBD/L91NBD-DAP/His with or without 5% hexafluoroisopropanol (HFIPA) showed only an increase of the absorption mainly at 225 nm in the presence of HFIPA whereas the negative ellipticity at 205 nm, indicative of α-helical structures, remained unaffected.

2.2. Fluorescence measurements

The fluorescence emission spectra of RBD labeled with DMCA and NBD-DAP are shown in Fig. 2. The maxima are at 425 nm and 538 nm, respectively, and are almost identical to those determined for the corresponding amino acids. The binding of Ras·GppNHp to these proteins results in a decrease of the fluorescence intensity which is of the order of 10% for the DMCA fluorophore and 20% for the NBD-DAP fluorophore. On the other hand, in the presence of Ras·GDP, the inactive state of Ras, the fluorescence intensities do not change, indicating that Ras·GDP, as expected, does not bind to RBD (data not shown).

The fluorescence properties of NBD- and DMCA-RBD can be modified by addition of small amounts of organic solvents. Addition of aprotic polar solvents such as dimethylsulfoxide or dimethylformamide leads to a general decrease of the fluorescence intensity (data not shown). However, HFIPA induces a considerable change in the fluorescence behavior which is most notable for RBD/L91NBD-DAP. 5% HFIPA induces not only a decrease of the fluorescence intensity, but also shifts the emission maximum from 538 nm to 528 nm. On binding of Ras·GppNHp, the fluorescence intensity then increases nearly 100% without altering the position of the band (Fig. 2C). Thus, use of this solvent potentially increases the sensitivity of the assay. Indeed, titrating RBD/L91NBD-DAP in the presence and absence of Ras·GppNHp with HFIPA shows clearly that the largest increase of fluorescence intensity after addition of Ras·GppNHp occurs in the range of 5–7.5% HFIPA (data not shown). At concentrations larger than 10% HFIPA, the change in fluorescence intensity is considerably reduced.

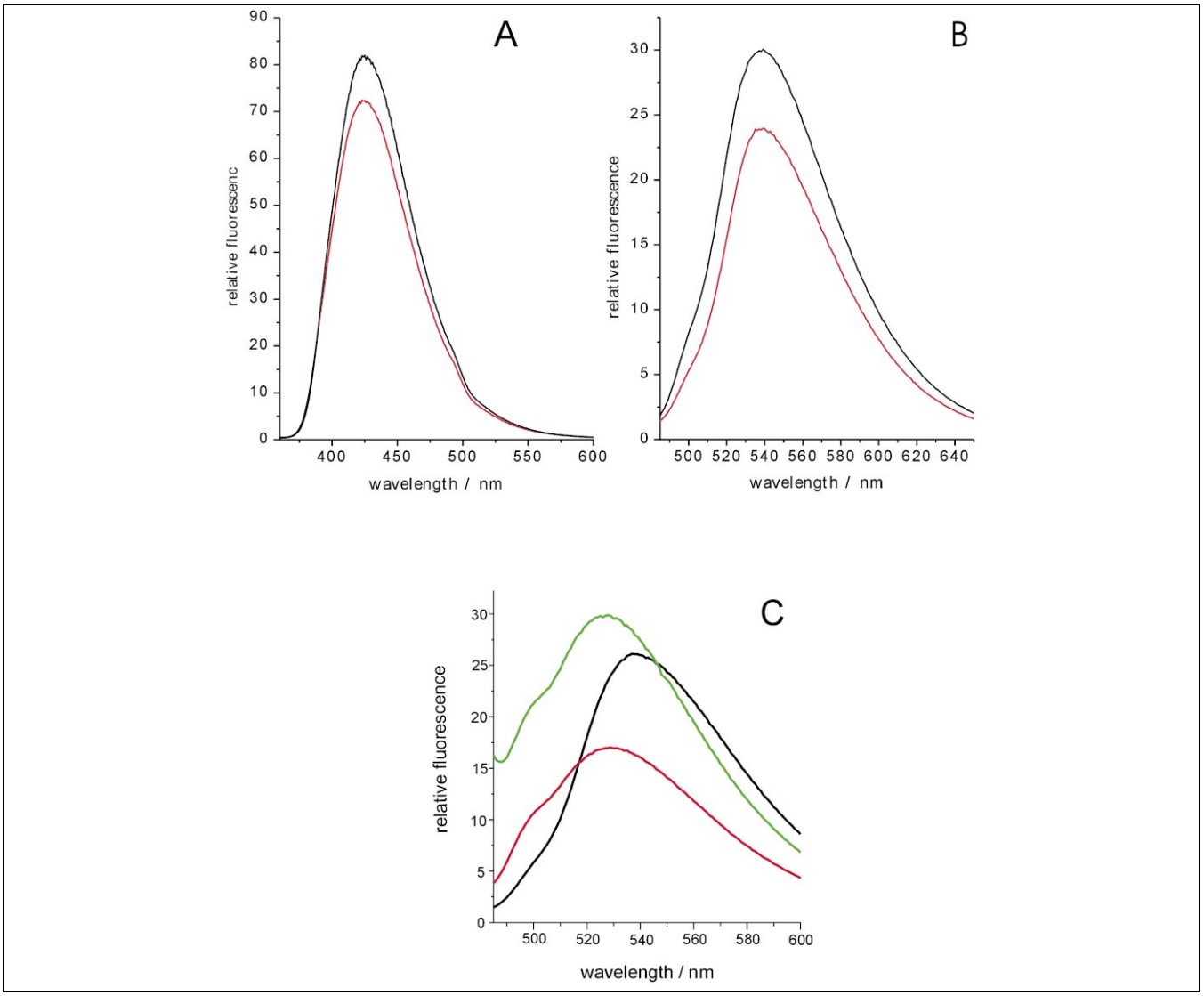

Fig. 2. (A) Fluorescence emission of RBD/L91DMCA in the presence of Ras·GppNHp (red) and without Ras·GppNHp (black). Excitation at 350 nm. (B) Fluorescence of RBD/L91NBD-DAP in the presence of Ras·GppNHp (red) and without Ras·GppNHp (black). Excitation at 465 nm. (C) Fluorescence emission of RBD/L91NBD-DAP after addition of 5% HFIPA to the buffer, in the presence of Ras·GppNHp (red) and without Ras·GppNHp (green). The black line shows the fluorescence emission of RBD/L91NBD-DAP without HFIPA and without Ras·GppNHp.

2.3. *Stopped-flow experiments*

The binding process of RBD to Ras·GppNHp, which occurs in the subsecond time range, can be followed by stopped-flow techniques. The kinetic parameters derived from these experiments are compiled in Table 1. For a detailed description of the kinetic analysis used here, see Sydor et al. [19]. Calculation of the K_d values from the association and dissociation rate constants shows that wild-type RBD displays a dissociation constant of 170

Table 1
Kinetic parameters for the interaction of chemically synthesized (ch) RBD proteins with Ras·GppNHp at 25°C

reaction	k_{diss} (s^{-1})	k_{ass} ($M^{-1}s^{-1}$)	K_d (nM)
1. wt RBD (ch)+Ras·mGppNHp	5.9	3.5×10^7	170
2. RBD/L91NBD (ch)+Ras·GppNHp	11	1.8×10^7	610
3. RBD/His (ch)+Ras·mGppNHp	5.9	1.2×10^7	490
4. RBD/L91NBD/His (ch)+Ras·GppNHp	14	2.7×10^7	520
5. RBD/L91DMCA (ch)+Ras/Y32W·GppNHp	10	2.1×10^7	480

Reactions 1 and 3: kinetics monitored using mant fluorescence of Ras·mGppNHp. Reactions 2, 4 and 5: kinetics monitored using fluorescence of labels or FRET.

nM towards activated Ras. The introduction of a His tag or the modification at position 91 by the fluorescent analogs increases this value to about 500 nM. A comparison of the k_{ass} and k_{diss} data indicates that the lowered binding affinity is due to a combination of a decrease in k_{ass} and an increase in k_{diss}.

It should be noted that energy transfer between mantGppNHp at the active site of Ras and NBD-DAP-containing RBD occurs if the sample is excited at 366 nm. The emission can then be monitored above 530 nm. This observation provides another method of detecting the specific interaction between Ras·mantGppNHp and RBD/L91NBD-DAP in situations in which both proteins carry a label, for example in screening for inhibitors of the interaction.

In the case of RBD/L91DMCA, the emission above 398 nm (excitation 334 nm) did not provide an adequate signal for stopped-flow measurements. Therefore, a Trp mutant of Ras (Ras/Y32W) was used for fluorescence resonance energy transfer (FRET) experiments in which Trp was the donor to DMCA as an acceptor. In this way it was possible to determine the K_d value for the interaction of Ras/Y32W·GppNHp and RBD/L91DMCA (see Table 1).

In preliminary experiments (data not shown), it was also shown that fluorescence lifetime measurements can also be used to monitor the RBD–Ras interaction. Although there was no change in the lifetime of the DMCA label on interaction with Ras (5.8 ns), the fluorescence lifetime of the NBD label decreases from 4.6 to 2.8 ns on complex formation.

2.4. Immobilization experiments

The RBDs containing the fluorescent probes DMCA and NBD-DAP and a His_6 tag at the C-terminus were immobilized on opalescent microtiter plates from Qiagen with Ni-NTA-coated wells (see schematic representation in Fig. 3). The binding capacity of the plates is approximately 10 pmol/well for globular 25 kDa proteins. To prevent unspecific binding of proteins to the polymer surface, the wells were pre-incubated with a buffer containing 5% fat-free milk powder. Subsequently, RBD/L91NBD-DAP/His was added at various concentrations (from 100 nM to 2 μM protein). After overnight incubation, the wells were extensively washed with buffer B and buffer B containing Tween 20 (0.05%), and the fluorescence intensity on the surface was measured. Since unspecific binding of non-His-tagged protein was still quite high, an additional washing step with 50 mM imidazole was included. RBD/L91NBD-DAP/His fluorescence was excited at 485 nm and the emission was monitored at 538 nm in a Fluoroskan device. Due to the lack of availability of suitable

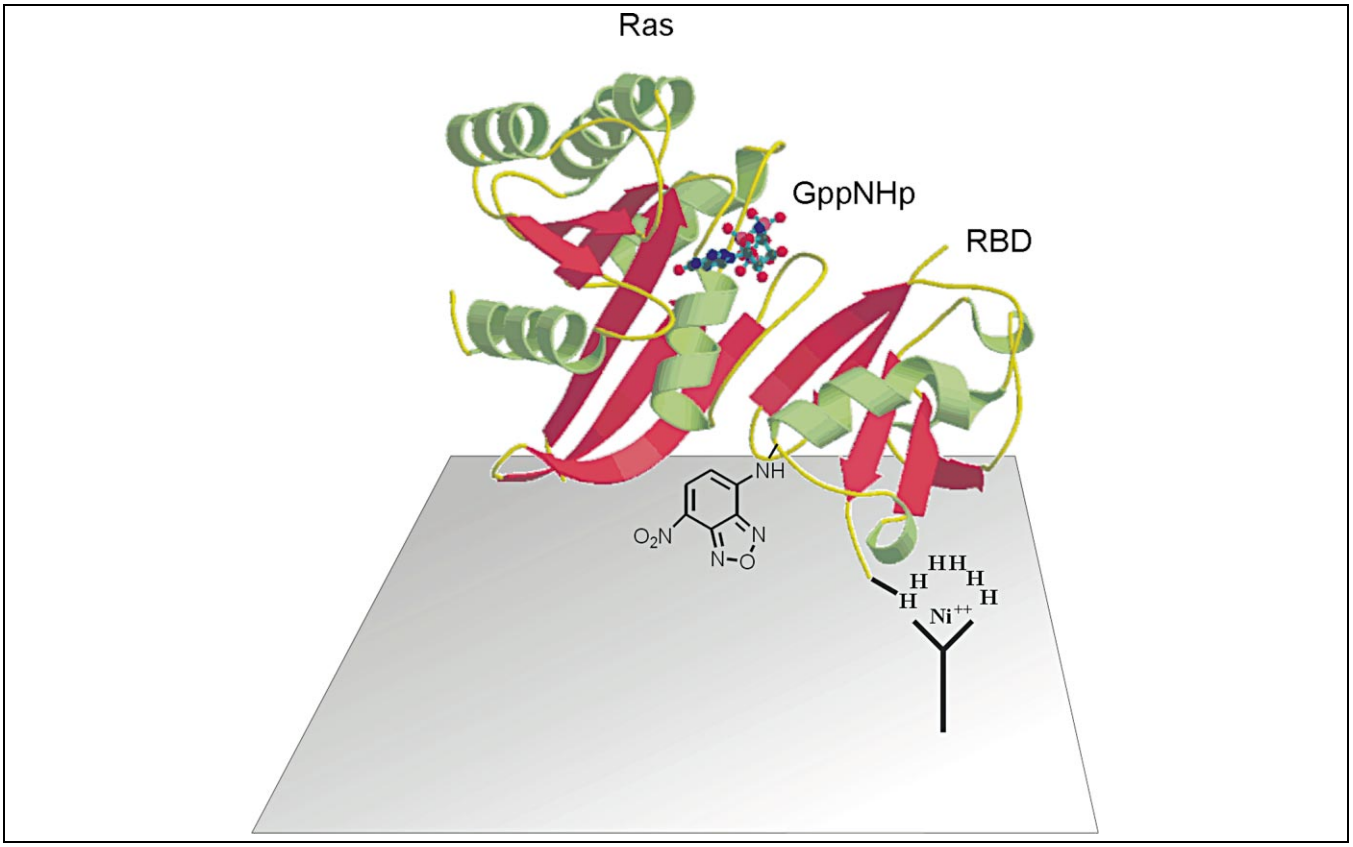

Fig. 3. Immobilization of RBD/L91NBD-DAP via the C-terminal His tag on a Ni-NTA-coated surface. Ras·GppNHp interacts with the immobilized RBD at its binding interface consisting of two β-sheets (modelled from the Rap1a:RBD structure [9a]). The fluorescence label is shown close to this binding interface between RBD and Ras.

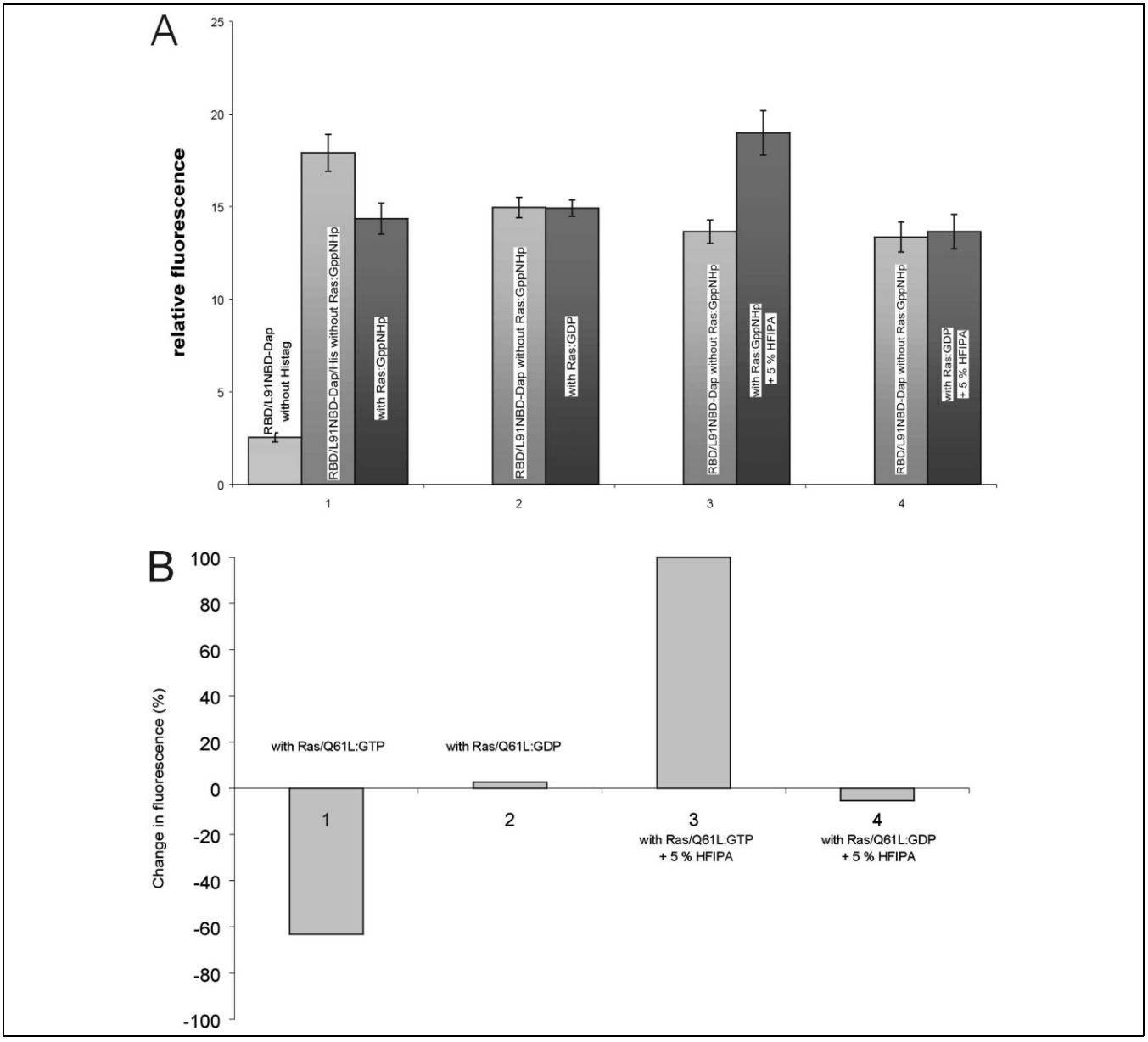

Fig. 4. (A) Data of fluorescence measurements on surface with Ras·GppNHp (lanes 1 and 3) or Ras·GDP (lanes 2 and 4; shown in black) and without these proteins (shown in gray) in the presence of HFIPA and without. All measurements were done with a filter pair of 485 nm (excitation) and 538 nm (emission) and were performed at least 10 times. (B) Differences of fluorescence intensities in the presence of Ras/Q61L·GTP or GDP without and with 5% HFIPA.

filter pairs for this apparatus, similar experiments were not performed with DMCA-labeled RBD.

The results are shown in Fig. 4. The binding of RBD/L91NBD-DAP/His_6 tag can be detected easily from the fluorescence signal (lane 1, second column). Unspecific binding is quite low, as can be seen from the data obtained from immobilized RBD lacking the His tag (lane 1, first column). Adding Ras·GppNHp to the well with bound His-tagged RBD decreases the fluorescence by about 20% (lane 1, third column), a value comparable to that measured in solution. The concentration of Ras·GppNHp was varied between 150 nM and 2 μM. The fluorescence intensity changes about 20% at concentrations above 500 nM Ras·GppNHp. Decreasing the amount of activated Ras to 300 nM reduced the fluorescence difference to about 8%. This is in harmony with the measured K_d value for the interaction.

As was expected from the solution experiments (Fig. 2C) the addition of 5% HFIPA decreased the fluorescence yield of the surface-bound RBD significantly. Addition of activated Ras then resulted in a ca. 30% increase in fluorescence (Fig. 4, lane 3). The interaction was detectable down to ca. 500 nM Ras·GppNHp. Under the present experimental conditions, reliable data are not obtained at lower concentrations.

Preliminary measurements performed in the presence of

complete HeLa cell extracts still demonstrated a significant change in fluorescence intensities in the absence as well as in the presence of 5% HFIPA. Although in these experiments the background fluorescence was increased, the detection level of Ras·GppNHp was not negatively influenced.

Addition of Ras·GDP at concentrations of 0.5–5 μM to the RBD-containing wells did not result in a change of fluorescence intensity, regardless of the addition of HFIPA (Fig. 4, lanes 2 and 4).

It was of interest that binding of oncogenic mutants of Ras to RBD/L91NBD-DAP/His also resulted in a detectable signal. This is the case, as demonstrated in Fig. 4B in which the fluorescence differences between bound- and unbound Ras/Q61L are plotted. Again, the GDP-bound form does not induce any fluorescence change. It should be noted that in these experiments, Ras/Q61L·GTP could be used directly, since the GTP hydrolysis rate of this mutant is 65 times slower than that of wild-type Ras [14].

3. Discussion

The results presented here show that chemical synthesis of fluorescently labeled RBD protein combined with its immobilization on a commercially available microtiter plate can lead to a bio-surface which can be used to detect the oncogene product Ras in its activated form. The system reported here is not yet fully optimized in terms of sensitivity, but it seems likely that significant improvements can be made. Some of these are discussed below.

For the purposes of the present application (i.e. detection of unlabeled Ras in its active form), a suitable fluorescent probe should be small in size so that it can be incorporated as close to the protein backbone as possible without disturbing protein–protein interactions. The photochemical properties of the fluorescent amino acids should permit the sensitive detection of protein structural changes caused by the binding event, and in a system such as the present one in which large structural changes do not occur on binding, this means that the label must be placed close to, but not in, the binding interface. In other words, a compromise has to be made between a position which still detects the bound protein partner but which does not perturb its K_D. The results obtained show that this has, in principle, been achieved, but it is quite possible that positions in the sequence can be found which undergo more profound changes of environment without interfering with the interaction. In addition to these requirements, for some applications which could be envisaged, the photochemical stability of the chromophore should also be high enough to allow the high excitation intensities necessary for single molecule detection experiments.

The fluorescent unnatural amino acids (L-DMCA and L-NBD-DAP) which were chosen for the present work fulfil some of these requirements. L-NBD-DAP, which has already been used in in vivo translation experiments [26,27], and L-DMCA are both comparable in size to Trp, and were chosen for this reason. Their fluorescence maxima, at 538 nm and 425 nm, are well separated from that of Trp at ca. 340 nm. Quantum yields and extinction coefficients are also much more appropriate than those of tryptophan derivatives, and in the case of the NBD label, the excitation wavelength (ca. 480 nm) is in a range where standard lasers can be used. On the other hand, the photochemical stability is not satisfactory for methods which require high excitation intensities. Although the binding of Ras·GppNHp to RBD/L91NBD in single molecule experiments could be detected, the chromophore was bleached very rapidly (A. Koltermann, personal communication). An extension of the present work to include dyes known to be suitable for single molecule detection would certainly be feasible.

A further property of interest of the fluorescent labels used is their potential for FRET measurements. We have used them as acceptors in the present work (resonance energy acceptors in the following pairings: Trp/DMCA and mGppNHp/NBD-DAP). Both combinations led to adequate FRET signals in the stopped-flow experiments described. The labels used could also be used as donors if suitable acceptors were introduced into Ras. Since powerful methods have recently been introduced for site-specific labeling of GTPases of the Ras superfamily [28], an acceptor molecule could be introduced into the C-terminal region of Ras. This would be an attractive possibility for the NBD-labeled protein, since its excitation wavelength is suitable for currently available lasers and the emission wavelength is appropriate for transfer to several commonly used dyes.

The results reported on the interaction of the fluorescently labeled RBD molecules with Ras show that the affinity is unaffected (see Table 1) by the modification and is still in the 500 nM range. The fluorescence change (quenching) seen on interaction of Ras with labeled RBD is relatively modest, but this situation is improved by the inclusion of 5% HFIPA as cosolvent. This solvent decreases the fluorescence of free (or surface-bound) RBD/L91NBD-DAP, but there is a large increase in fluorescence on interaction with Ras, presumably due to 'sheltering' of that region of the RBD molecule from the cosolvent by the bound Ras. Using immobilized RBD/L91NBD-DAP/His, the lower limit for reliable detection of activated Ras was ca. 300 nM. This is partly the result of a combination of a not particularly high affinity and a less than optimal signal change on binding Ras. It is likely that this can be improved significantly. The filter combination, which was limited by the apparatus used, was not optimal, since the emission filter wavelength was too long to take most advantage of the changes induced on binding. The loss of affinity in the protein construct used appears to be mainly due to the C-terminal His tag, as can

be seen from the data in Table 1. Although the C-terminus is quite far away from the binding site (see Fig. 3) it still appears to interfere somewhat with the binding process. It might therefore be of interest to choose another site, e.g. in the loop region amino acids 113–118.

Compared to ELISA methods using antibodies to identify proteins, the approach described here has the general advantage that the specific detection of Ras·GTP in the presence of Ras·GDP becomes possible. This differentiation cannot be achieved using antibody-based techniques, due to the small conformational differences between the GDP and the GTP states of Ras, and to the metastable nature of the individual states.

Chemical synthesis of proteins provides the necessary tools to incorporate specifically designed amino acids at specific sites of the protein molecule [2], so that a suitable compatible chemistry for the immobilization can be selected. Increasing the capacity of the immobilizing surface is not likely to have a major influence, since the fraction of immobilized RBD molecules occupied by Ras will still be the same as with a lower capacity at a given Ras concentration. However, more fluorescent indicator on the surface means more overall emission intensity, which might improve the signal to noise ratio.

4. Significance

The work described here has provided evidence that the chemical synthesis of proteins and their immobilization on surfaces is a viable tool for the design of biosensors. As an example, the biologically and medically important Ras/RBD system was chosen and it could be shown that the binding of Ras·GTP to RBD could be observed with high sensitivity even in the presence of Ras·GDP. Oncogenic mutants with impaired hydrolysis activity for GTP could also be detected, demonstrating the potential of the system in cancer diagnostics and in screening of Ras·GTP inhibitors and/or substances which increase the GTP hydrolysis rate in oncogenic Ras mutants. Native chemical ligation, in combination with expressed protein ligation methods for making recombinant polypeptide thioesters [6,28], will enable the approach described here to be applied to even larger proteins including enzymes, receptors, and even membrane proteins [5]. The combination of site-specific chemical incorporation of fluorescent amino acids into the target protein with site-directed immobilization of the modified protein onto specialized surfaces such as Si, SiO_2, or Au constitutes a general approach for the development of biosensors and screening systems for drugs, inhibitors, and other protein ligands.

5. Materials and methods

tert-Butyloxycarbonyl (Boc)-protected amino acids were purchased from Peptides International. 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate was obtained from Spectrum (Gardena, CA, USA). The Boc-amino acyl-OCH_2-phenylacetamidomethyl resins and *N,N*-diisopropylethylamine were bought from Applied Biosystems. Trifluoroacetic acid was from Halocarbon (Hackensack, NJ, USA). Hydrogen fluoride was purchased from Matheson. N_α-Boc-L-DAP was bought from Bachem. All other chemicals were obtained from Fluka in the highest purity available.

5.1. Synthesis of N-Boc-L-DMCA (*1*)

The fluorophore was synthesized according to Bennett et al. [24,25]. The α-amino group was protected with the Boc group with 1.1 equivalent Boc-ON in water/dioxane (1:1) and 1.5 equivalents of triethylamine. The enantiomeric purity was checked by HPLC using Teicoplanin as the chiral stationary phase [29,30]. The enantiomeric purity was better than 98%.

5.2. Synthesis of N^2-Boc-N^3-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-L-2,3-DAP (*2*)

4-Chloro-7-nitrobenz-2-oxa-1,3-diazole was coupled with N^2-Boc-L-2,3-DAP in ethanol/water 1:1 in the presence of 1.2 equivalents of triethylamine for 6 h at room temperature. The resulting mixture was purified by flash silica gel chromatography using dichloromethane/methanol 9:1.

5.3. Peptide synthesis

Solid phase peptide synthesis was performed manually or on a custom-modified 430A peptide synthesizer from Applied Biosystems, using Boc chemistry, in situ neutralization, and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate activation protocols [31,32]. Unnatural fluorescent amino acids were coupled manually using the above mentioned neutralization and activation protocol. All N-terminal peptide segments were synthesized on a resin that generates a C-terminal thioester after HF cleavage [33]. The peptides were deprotected and cleaved from the resin using anhydrous HF, precipitated with ether, dissolved in aqueous acetonitrile, and lyophilized. Peptides segments were purified by reversed phase HPLC after HF cleavage with a C4 column from Vydac (Hesperia, CA, USA) using linear gradients of buffer B (acetonitrile with 0.08% trifluoroacetic acid) in buffer A (water with 0.1% trifluoroacetic acid) and UV detection at 214 nm. Samples were analyzed by electrospray mass spectrometry with a Perkin-Elmer Sciex API-I quadrupole mass spectrometer.

5.4. Native chemical ligation

Ligation of the peptide segments in 6 M guanidinium hydrochloride, 300 mM phosphate at pH 7, and 1% thiophenol overnight produced the full-length polypeptide chain [1,34]. Purification and analysis were carried out as described above for the peptide segments.

5.5. Protein folding

The purified polypeptide chain was folded by dissolving 1 mg protein in 1 ml of buffer (100 mM NaCl, 50 mM Tris–HCl, 5 mM $MgCl_2$, 5 mM dithiothreitol (DTT), pH 7.4). The solution was gently stirred overnight at 4°C and after this the solution was shock frozen in liquid nitrogen and stored at −80°C. For CD spectroscopic measurements, the samples were dialyzed against 20 mM phosphate buffer at pH 7.4. CD spectra were recorded on a Jasco J-710 spectropolarimeter at 25°C in a quartz cell with 0.2 cm pathlength and a final protein concentration of 60 μM.

5.6. Fluorescence spectroscopy

All fluorescence spectra were measured on an Aminco-Bowman Series 2 luminescence spectrophotometer at 25°C. The proteins were dissolved in 100 mM NaCl, 50 mM Tris–HCl, 5 mM $MgCl_2$, 5 mM DTT at pH 7.4 at a concentration of 2 μM.

5.7. Fluorescence lifetime measurements

Frequency-domain fluorescence lifetimes of the proteins were measured with an ISS K2 Multifrequence Phase fluorometer using a saturated solution of POPOP in ethanol as a reference. RBD/L91NBD-DAP was excited at 460 nm and RBD/L91DMCA at 350 nm. The emitted light was monitored through filters with a cut-off wavelength of 400 nm for RBD/L91DMCA and without any filter for RBD/L91NBD-DAP.

5.8. Stopped-flow measurements

Stopped-flow measurements of the Ras–RBD interaction were carried out as described by Sydor et al. [19]. Excitation wavelengths were 334 nm for RBD/L91DMCA excitation, 436 nm for RBD/L91NBD-DAP excitation and 289 nm for Trp excitation in FRET experiments. Emitted light was monitored through filters with cut-off wavelengths of 398, 530, and 398 nm, respectively.

5.9. Preparation of H-Ras proteins

Wild-type and mutant H-Ras proteins were prepared from *Escherichia coli* using the ptac expression system as described [35]. The conversion into nucleotide-free H-Ras and nucleotide exchange against GppNHp or GTP was performed according to [36].

5.10. Preparation of HeLa cell extract

HeLa cells were grown in Dulbecco's Eagle's medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin solution. The cells were washed with a buffer containing 100 mM NaCl, 50 mM Tris–HCl, 5 mM $MgCl_2$ at pH 7.4 and lysed by ultrasonication. After lysis a mixture of EDTA-free protease inhibitors was added.

5.11. Immobilization of RBDs

Ni-NTA HisSorb Plates from Qiagen were incubated overnight with a buffer containing 150 mM NaCl, 50 mM KPi pH 7.4, and 5% fat-free milk powder (buffer A). The plates were washed with 150 mM NaCl, 50 mM KPi, 5 mM $MgCl_2$, 5 mM DTT, pH 7.4 (buffer B) and incubated with different concentrations of fluorescent and non-fluorescent RBDs for 12 h. The plates were subsequently washed with buffer B containing 0.05% Tween 20 and with pure buffer B.

5.12. Fluorescence measurements on HisSorb plates

The fluorescence of immobilized RBDs was measured with a Fluorskan Ascent FL from Labsystems using a filter pair with 485 nm as excitation wavelength and 538 nm as emission wavelength.

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