Synthesis and Application of Fluorescent Ras Proteins for Live-Cell Imaging

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Semisynthetic Ras proteins are efficient probes for cell-biology experiments. With a Bodipy FL fluorophore introduced at an appropriate site on the Ras peptide by solid-phase synthesis, the resulting Ras chimera is processed by the cellular machinery and the intracellular localization of the protein can then be visualized by means of confocal laser fluorescence microscopy at relatively low concentrations. The absence of a large N-terminal protein tag overcomes possible interferences in the interaction with cellular partner proteins. The fluorescence emission from Bodipy FL is continuous and disappears only after irreversible bleaching. These characteristics make Ras proteins with nonprotein fluorophores suitable for biophysical analysis. The easy accessibility of the lipopeptide moiety by chemical synthesis opens up numerous options for further biological investigations.

Introduction

The signal-transducing Ras proteins are critically involved in the orchestration of various important biological processes such as the control of growth and differentiation, and impaired Ras proteins are found in about 30% of all human cancers.[1] The biological activity of these small guanosine triphosphatases (GTPases) depends on their posttranslational modification with *S*-farnesyl (Far) and *S*-palmitoyl groups that are required for targeting the lipidated proteins to cellular membranes (Figure 1).

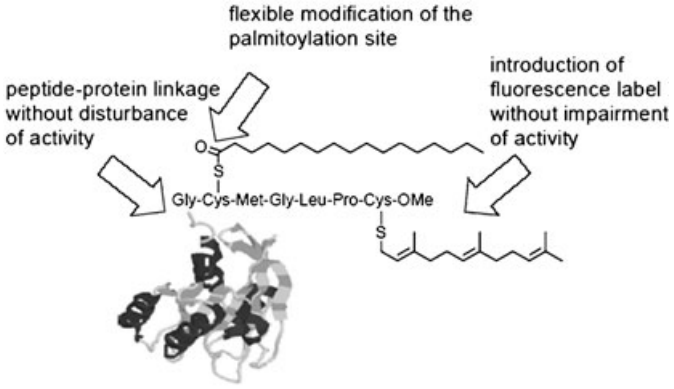

Figure 1. *Structural requirements for semisynthetic fluorescent N-Ras proteins.*

However, the precise biological roles of the different lipids found in the Ras isoforms (H-Ras is *S*-farnesylated and doubly *S*-palmitoylated, N-Ras is *S*-farnesylated and mono-*S*-palmitoylated, and K-Ras is only *S*-farnesylated) and the enzymatic processes by which these lipids are introduced are only partly understood. Thus, although Ras-farnesyltransferase is a well-characterized enzyme,[1] the putative Ras-palmitoylating enzyme and the intracellular site of *S*-palmitoylation have not been identified unambiguously.[2] Studies employing green fluorescent protein (GFP) and cyan fluorescent protein (CFP) labeled Ras proteins indicated that different Ras isoforms travel to the plasma membrane by different pathways and may be localized to different membrane subdomains according to their lipid modification and loading with guanosine diphosphate (GDP) or GTP.[3] While these very recent insights were gained by means of molecular genetic approaches, such techniques suffer from several drawbacks. With a mass of 26 kDa, as compared to 21 kDa for Ras, an influence of the biological fluorophore on the in vivo readout cannot generally be excluded, particularly if protein–protein interactions are involved. For instance, restrictions in the use of the GFP marker due to steric constraints and undesired interactions were reported in studies of tubule formation[4] and of the secretory pathway in yeast.[5]

An additional limitation comes from the overlapping emission and excitation spectra of the accessible fluorescent proteins (GFP, YFP, etc.); these overlaps restrict the utilization of fluorescence resonance energy transfer (FRET) assays or dual-wavelength confocal microscopy in this context.

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Clearly, the use of biologically fully active fluorescently labeled Ras proteins synthesized in vitro and carrying small fluorophores that do not interfere with the biological phenomena to be studied could serve to overcome these limitations. In addition, the application of such protein probes would give a control over the final protein concentration in the experiments that is usually not attainable with transfection methods. Furthermore, the availability of purified proteins in sufficient amounts would allow correlation of the results of cellular assays with in vitro observations, for example, gleaned from biophysical experiments. In particular, the control over the structure of the lipid modifications and the fluorophore gained by means of chemical synthesis provides the opportunity to introduce nonnatural lipids. Thereby, membrane anchors can be generated that are not accessible from the cellular machinery.[6]

In this paper we report in full detail[7] on the synthesis and preliminary biological characterization of Ras proteins that carry a small fluorophore at the C terminus, that are substrates for the cellular palmitoylation machinery, and whose intracellular localization can be successfully monitored by means of confocal laser fluorescence microscopy.

Results and Discussion

For the development of fluorescently labeled Ras proteins the following criteria had to be met:

1) The cysteine that is palmitoylatable in vivo (that is, Cys181 in N-Ras, see Figure 1) should readily be exchanged for other nonpalmitoylatable amino acids or amino acids that are modified with a stable thioether to allow for investigation of the palmitoylation.
2) A small fluorophore should be used that has a high quantum yield and does not bleach readily in laser light. It should not, or should only to a minor extent, interfere with the biological activity of the Ras protein and its interaction with the plasma membrane.
3) The synthesis strategy should allow for the flexible introduction of different fluorophores at different sites of the neo-Ras proteins.
4) The linker group between the modified C terminus and the core of the Ras protein should not interfere with the biological activity of the proteins.

For the synthesis of proteins that meet these criteria we have drawn on our earlier work.[6] Thus, an N-Ras mutant was generated that terminates with Cys181, which is the only surface-accessible cysteine. This truncated Ras was then coupled with synthetic peptides that carry a maleimide group at their N terminus (see Scheme 2). The maleimide group reacts with the SH group of the C-terminal cysteine to give a covalent addition product.

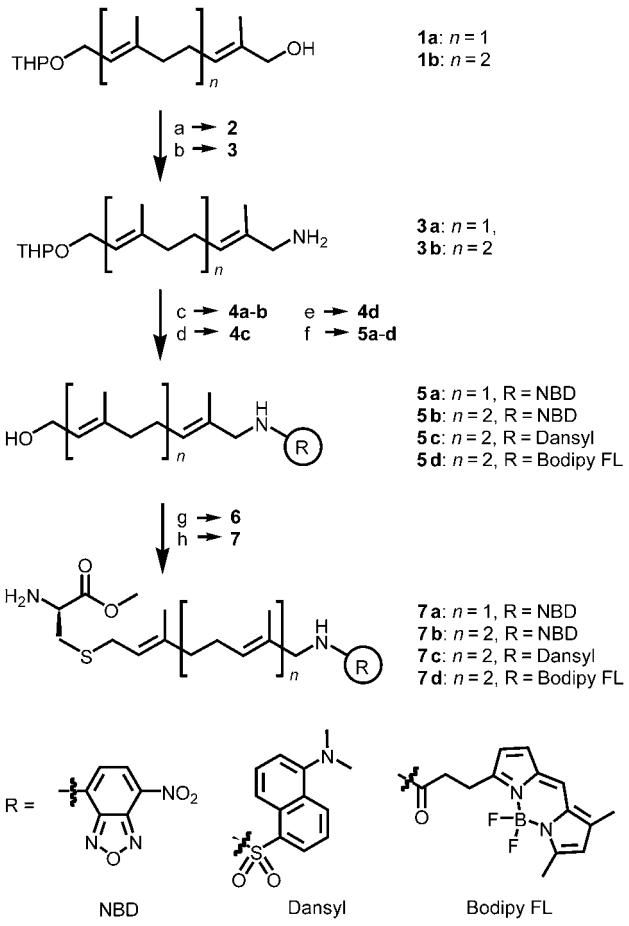
Synthesis of fluorescently labeled Ras peptides

In the first of two strategies investigated the fluorophore was introduced into the farnesyl residue. The use of peptides that contain a fluorescent or a photoactivatable group incorporated into a lipid group for biochemical investigations has been pursued before.[6,8] The positioning of the fluorophore was based on the findings that replacement of the farnesyl thioether by a linear alkyl chain does not reduce the PC12 cell-differentiating activity of the Ras chimera[6] and that Ras proteins in which the farnesyl group is replaced by almost completely saturated analogues are fully functional in an in vitro mitogen-activated protein (MAP) kinase activation system.[9] In addition, due to the enhancement of fluorescence in a hydrophobic environment, a higher quantum yield was expected upon insertion of a modified isoprenyl anchor into the plasma membrane.

In order to imitate the nature of the unmodified isoprenoid as much as possible, the fluorophore should be small, nonpolar, and compact. To this end, the NBD, dansyl, and Bodipy FL fluorophores were investigated (see Scheme 1). The diphenylhexatriene,[10,11] *N*-methylantranilate (Mant),[6,12] coumarine,[13] and fluorenyl[14] fluorophores were also considered but were not chosen because of insufficient fluorescence intensity and overlap with the optical properties of cellular chromophores. Thus, Mant-labeled Ras proteins[6b] cannot be detected in cellular experiments because the maxima in the absorption and emission spectra of the Mant chromophore (368 nm and 437 nm) nearly coincide with the values recorded for reduced nicotinamide adenine dinucleotide (NADH; 365 nm and 450 nm). For this reason the fluorescent isoprenoid analogue didehydrogeraniol[15] was also not suitable for our purposes.

For the synthesis of the lipid-modified peptides carrying the fluorescent label incorporated into the lipid group, the fluorescently labeled isoprenoids were first synthesized and attached to cysteine methyl ester (Scheme 1). The synthesis commenced with known alcohols **1a** and **1b** which can be obtained in two steps from commercially available geraniol and farnesol, respectively.[8d,16,17] The primary alcohols were then converted to form amines **3** in high yields by means of a Mitsunobu reaction with phthalimide[8d] and subsequent removal of the phthaloyl group with hydrazine. The attachment of the NBD group onto amines **3** proceeded best (65–67%) in an aqueous solvent in the presence of an inorganic base. The dansyl group was readily introduced by means of dansyl chloride, and attachment of Bodipy FL was achieved after activation of the carboxylic acid with a carbodiimide and *N*-hydroxybenzotriazole. Bodipy FL has its emission maximum at 515 mm and is considered to be less polar and to have a higher photostability than NBD.[18] Selective removal of the THP acetal under established conditions[19] yielded primary alcohols **5** in high yields after chromatography.

Subsequent Corey–Kim chlorination[20] gave the corresponding modified isoprenyl chlorides **6**. Compounds **6a**,**b**, and **d** were obtained in quantitative yield without any need for chromatographic purification. Finally, these chlorides were coupled to cysteine methyl ester by means of selective nucleophilic substitution by the SH group, which is more nucleophilic than



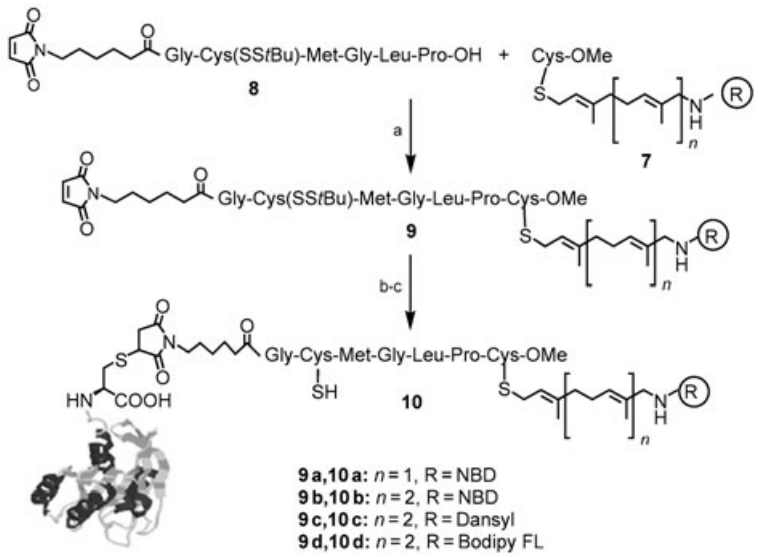
Scheme 1. *Synthesis of fluorescently labeled lipidated cysteines **7**: a) Phthalimide, PPh_3, DEAD, **2**: 79–81%; b) hydrazine, **3a**: 84%, **3b**: 95%; c) NBD-Cl, $NaHCO_3$ buffer (pH 8–9)/CH_3CNH, **4a,b**: 65%–67%; d) dansyl chloride, MeOH/THF, **4c**: 85%; e) Bodipy FL, EDC, HOBt, CH_2Cl_2, **4d**: 92%; f) PPTS, 60°C, EtOH, **5a**: 93%, **5b**: 94%, **5c**: 75%, **5d**: 92%; g) NCS, dimethylsulfide, −30→0°C, **6**: 75%–quant.; h) H-Cys-OMe, NH_3 in MeOH (1 M), 0°C, **7a,b**: quant., **7c**: 95%, **7d**: 55%. Bodipy FL = 4,4-difluoro-5-(2-pyrrolyl)-4-bora-3a,4a-diaza-s-indacene-3-propionyl, dansyl = 5-(dimethylamino)-1-naphthalenesulfonyl, DEAD = diethylazodicarboxylate, EDC = ethyl(dimethylamino)propylcarbodiimide, HOBt = 1-hydroxybenzotriazole, NBD = 7-nitro-4-benzofurazanyl, NCS = N-chlorosuccinimide, PPTS = pyridinium p-toluenesulfonate, THF = tetrahydrofuran, THP = tetrhydropyranyl.*

the unprotected amine. The reaction proceeds well in liquid ammonia according to a procedure described by Poulter and co-workers.[21] However, in our hands, the use of a 1 M solution of ammonia in methanol[22] proved to be more practical. *S*-alkylated cysteines **7** were isolated in high yields; *N*-alkylation was not observed.

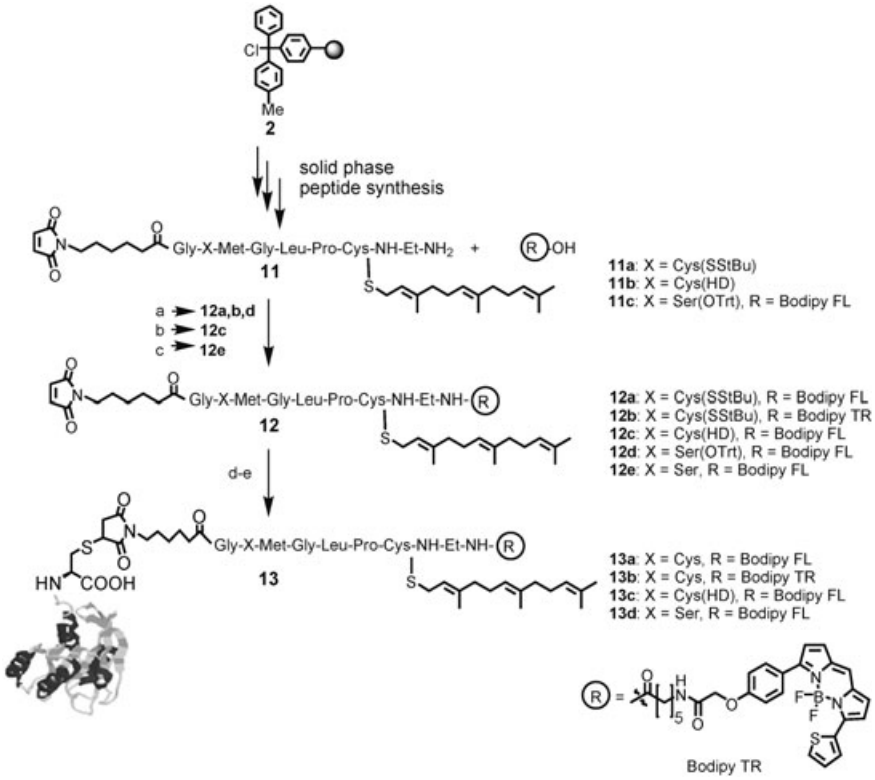
For the synthesis of maleimido-modified N-Ras peptides incorporating a fluorescent label in the isoprenoid group (Scheme 2), peptide **8** was assembled on the solid phase as described previously.[6c,d] Its coupling with fluorescent cysteine methyl esters **7** proceeded uneventfully to yield maleimido-modified lipidated peptides **9** in high yields.

In the second strategy investigated the fluorophore was attached to the peptide chain. Based on the notion that the C-terminal methyl ester present in all native Ras proteins most probably only serves to enhance the lipophilicity of the C-terminus of the proteins,[23,24] it was decided to replace the C-terminal methyl ester by a functional group that incorporates a fluorophore. Fluorophores Bodipy FL and Bodipy TR (see Scheme 3) were chosen due to their advantageous optical properties (see below). To minimize possible steric interactions between the fluorophores and the protein, an ethylenediamine spacer was introduced between the fluorophores and the Ras peptides or protein.

The synthesis of the fluorescently labeled Ras peptides is shown in Scheme 3. Peptides **11** were assembled on a polystyrene resin equipped with a 4-methyltrityl linker. After attachment of 9-fluorenylmethoxycarbonyl (Fmoc) protected ethylenediamine and removal of the Fmoc group, the peptide was synthesized by employing Fmoc-protected amino acid building blocks including *S*-farnesylated and *S*-hexadecylated Fmoc-cysteine.[6,25] After N-terminal deprotection of the final glycine residue, maleimidocaproic acid was attached to the N terminus. Peptides **11a** and **11b**, which incorporate the five C-terminal amino acids found in native N-Ras, were released from the solid support by treatment with dilute TFA. Under these conditions, the acid-labile farnesyl group and the *S*-*t*Bu disulfide protecting function of the second cysteine are unharmed. Peptide **11c** incorporating a fairly labile serine *O*-trityl blocking group was released from the solid support by employing a mixture of CH_2Cl_2, trifluoroethanol, and acetic acid in a 3:1:1 ratio. The Bodipy FL and Bodipy TR labels were then attached to peptides **11** by employing EDC/HOBt or HBTU/HOBt as condensing reagents to give fluorescently labeled peptides **12a–d** in high yields. The trityl protecting group was then released from *O*-protected compound **12d** under mildly acidic conditions to form **12e**.



Scheme 2. *Synthesis of fluorescently labeled Ras proteins **10**: a) EDC, HOBt, triethylamine, CH_2Cl_2, **9a**: 76%, **9b**: 64%, **9c**: 84%, **9d**: 77%; b) N-Ras, buffer (pH 7.4), Triton X-114, 4°C; c) DTE, buffer (pH 7.4), 37°C. DTE = 1,4-dithio-D,L-erythritol.*



Scheme 3. *Synthesis of fluorescently labeled Ras proteins* ***13****: a) EDC, HOBt, triethylamine, CH_2Cl_2,* ***12a****: 76%,* ***12b****: 64%,* ***12d:*** *84%; b) HBTU, HOBt, DIPEA, CH_2Cl_2,* ***12c****: 23%; c) TFA (1%), CH_2Cl_2,* ***12e****: 65% (**a** and **c**); d) N-Ras, buffer (pH 7.4), Triton X-114, 4°C; e) DTE, buffer (pH 7.4), 37°C. Bodipy TR = 4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)phenoxy, DIPEA = diisopropylethylamine, HBTU = 2-(1*H*)-benzotriazol-1-yl-1,1,3,3-tetramethyluronium hexafluorophosphate, TFA = trifluoroacetic acid.*

Synthesis of fluorescently labeled Ras proteins

Maleimido-modified and fluorescently labeled peptides **9** and **12a**, **b**, **c**, and **e** were then coupled to the N-Ras protein by employing a method described previously.[6] Briefly, an oncogenic Ras G12V mutant terminating in a cysteine residue at position 181 was generated by introduction of a stop codon in positions 182 and 183 of N-Ras complementary DNA (cDNA) and the resulting PCR product was cloned into the *Escherichia coli* ptac expression vector. Expression was performed in *E. coli* strain CK600 K. The C-terminal cysteine of the obtained Ras mutant is the only surface-accessible cysteine; when exposed to the maleimido-modified peptides in Triton X114 buffer it adds chemoselectively to the double bond. Protein isolation is facilitated by separation of the coupling buffer into a detergent-rich phase and an aqueous phase upon warming to 37°C. The lipidated proteins accumulate in the Triton-phase, while nonlipidated proteins remain in the aqueous phase. The proteins were purified by diethylaminoethyl (DEAE) ion-exchange chromatography and characterized by sodium dodecylsulfate (SDS) PAGE (Figure 2). In addition, MALDI mass spectrometric investigation of the coupling products proved that the lipidated peptides were attached only once to each Ras protein. Finally the *S-t*Bu disulfide protecting function still attached to the coupling products obtained was cleaved by treatment with dithiothreitol (DTE) at 37°C.

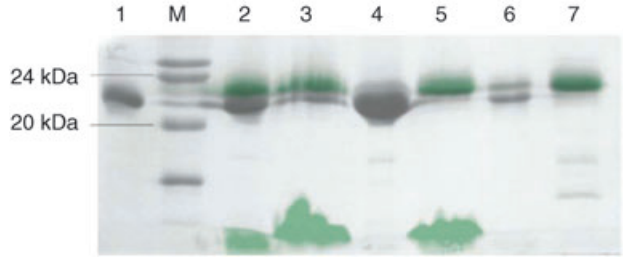


Figure 2. *Results of the synthesis and purification of semisynthetic Ras protein* ***13a****. Separation of coupled and uncoupled protein was performed by extraction with Triton X114. Excess of uncoupled protein in the Triton phase was removed by washing with aqueous buffer. Samples were analyzed by SDS-PAGE. Overlay of coomassie staining (black) and fluorescence image (green). Lanes: 1) Truncated N-Ras (1–181), M) molecular weight marker, 2) reaction mixture after 16 h, 3) Triton phase after extraction, 4) aqueous phase after extraction with Triton, 5) Triton phase after washing with aqueous buffer, 6) aqueous phase after washing of Triton phase, 7) coupling product after DEAE column.*

Ras protein and peptides were employed in equimolar amounts to give access to the desired fluorescently labeled Ras chimera in multimilligram amounts. Ras proteins **13a**–**d** were obtained in yields of 10–25%. For **13d** no deprotection was performed.

Biological evaluation of the fluorescently labeled Ras proteins

In order to investigate if the semisynthetic Ras proteins are biologically active we employed a method reported previously.[6]

If oncogenic versions of such proteins are microinjected into PC12 cells, the free cysteine in the protein is recognized by the cellular palmitoylation machinery and the doubly lipidated proteins are formed in vivo. They then induce a quantifiable outgrowth of neurites, thereby indicating localization at the plasma membrane.

The use of proteins **10**, shown in Scheme 2, met with frustration. When the NBD group was attached to the isoprenoid, NIH3T3 or MDCK cells microinjected with the corresponding N-Ras lipoproteins showed distinct fluorescence signals in a confocal fluorescence microscope after excitation at 488 nm. However, staining of the plasma membrane (as was expected for a functional Ras protein) was not observed and the NBD-modified protein did not display differentiation activity in the PC12 cell assay (Figure 3). A Ras chimera incorporating a dansyl fluorophore in the lipid group showed good PC12 differentiation potency (Figure 4) but could not be visualized by fluorescence microscopy. If a Bodipy FL group was attached instead, visualization by fluorescence microscopy was efficient but the protein did not localize to the plasma membrane or induce PC12 cell differentiation.

Although not successful, these experiments allowed us to narrow down the fluorophores that would be chosen for subsequent experiments. Thus, introduction of the dansyl label resulted in a biologically active lipoprotein that could not, however, be used due to practical limitations of cell imaging. It turned out that the weak absorptivity ($\approx 4000\ cm^{-1}\,M^{-1}$ at 372 nm) and only moderate fluorescence quantum yield of the fluorophore combined with a high autofluorescence background of the cell did not allow detection of the construct after microinjection. Consequently this fluorophore was not used further. On the other hand, introduction of NBD or Bodipy FL groups led to Ras chimeras that can be visualized by confocal microscopy but that lost differentiating efficiency in the PC12 cell assay.

A possible explanation for this lack of biological activity might be that the polar fluorophores "loop back" towards the aqueous phase, rather than remaining in the hydrophobic bilayer. This has been observed for both fluorophores when integrated into phospholipid fatty acyl chains.[26]

However, Ras constructs with a NBD or Bodipy FL group incorporated into the isoprenoid were found to localize to endomembranes like the Golgi apparatus (data not shown). Thus, in principle, they are able to bind to membranes and it is more likely that the NBD or Bodipy FL fluorophore in the isoprenoid might interfere with consecutive steps such as the palmitoylation reaction.

Taken together, these findings indicated that the fluorophore should not be incorporated into the lipid group but rather attached to the peptide chain.

Consequently, Ras protein **13a** was subjected to biological evaluation. When microinjected into PC12 cells, the Bodipy FL modified Ras protein **13a** was accepted by the cellular palmitoylation machinery and induced a differentiated phenotype (Figure 5). At concentrations of 150 μM in the injection needle,

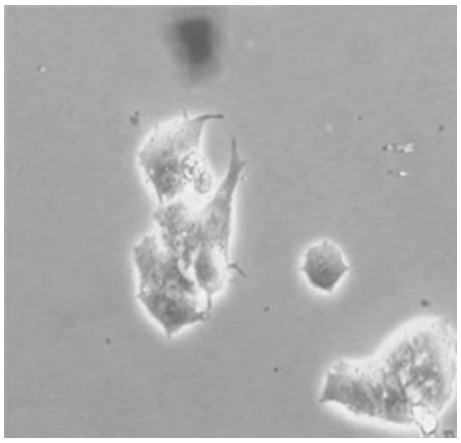


Figure 3. *PC12 cells after microinjection with oncogenic NBD-labeled Ras protein **10b**. Since no transformation could be observed, these conjugates seem not to be active in the living cell.*

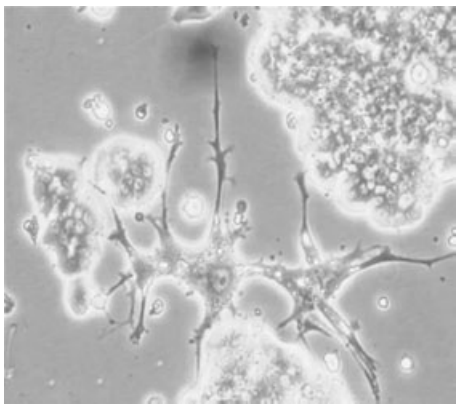


Figure 4. *Transformation of PC12 cells after microinjection with the oncogenic dansyl-labeled Ras protein **10c**. The picture indicates the biological activity of this protein conjugate.*

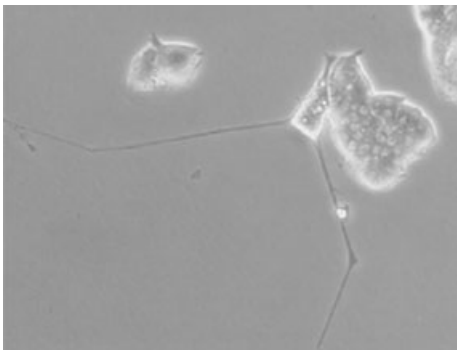


Figure 5. *Transformation of PC12 cells after microinjection with the Bodipy FL labeled Ras protein **13a**. The picture proves the biological activity of this protein conjugate.*

it reached a maximum differentiation efficiency of 76%, which is in the range of the efficiency observed for the analogous semisynthetic Ras protein bearing a methyl ester at the C-terminus (88%) and for the wild-type Ras protein itself (84%).

Encouraged by the finding that protein **13a** is indeed biologically active, we microinjected it into MDCK and COS7 cells and inspected them by confocal fluorescence microscopy (Figure 6a,b). Due to the excellent properties of the Bodipy FL fluorophore protein, concentrations of only 10 μM in the injection needle were sufficient to obtain satisfactory images.

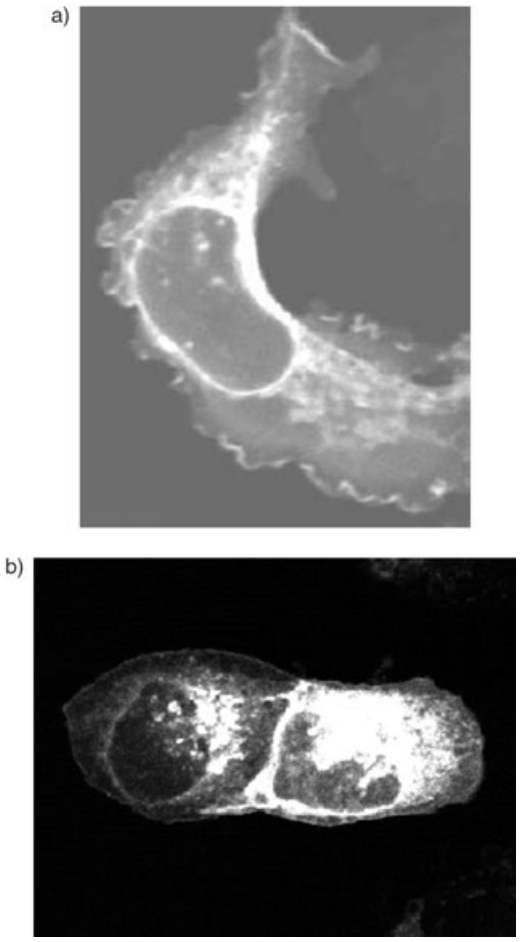


Figure 6. *Localization of the Bodipy FL labeled Ras protein **13a** after microinjection of 50 μM protein solution into a) COS-7 cells and b) MDCK cells. The cells were incubated for 4 h after injection and the Bodipy FL fluorophore was excited with the laser line 488 nm. In both cell lines, selective plasma-membrane and Golgi staining is observed.*

The Ras construct with a Bodipy FL group as a substitute for the carboxymethyl function inserted into endomembranes of cells within one hour of microinjection. It induced a distinct staining of the plasma membrane of MDCK or COS7 cells after further incubation at 37°C for three to four hours with a clear prevalence of the fluorescence signal in the endomembranes.

Conclusion

Taken together the resulting Bodipy FL containing Ras chimera gratifyingly fulfils all the desired criteria for a functional N-Ras protein. The lipoprotein is biologically active as demonstrated in the PC12 differentiation assay and can be visualized in conventional fluorescence microscopes at relatively low concentrations. The absence of a large N-terminal protein tag overcomes possible interferences in the interaction with cellular partner proteins. A further advantage of the Bodipy FL probe compared to GFP is that the latter shows "flickering" where phases of fluorescence emission alternate with time periods of inactive GFP.[27] The readout with Bodipy FL is continuous and disappears only after irreversible bleaching. These characteristics make Ras proteins with nonprotein fluorophores more suitable for biophysical analysis, for example, for reconstitution and interaction studies in artificial membranes.

The easy accessibility of the lipopeptide moiety by chemical synthesis opens up numerous options for further biological investigations. For instance, combination of a fluorophore or a biotin moiety at the C terminus with a photoactivatable benzophenone group incorporated into an analogue of the farnesyl group that does not impair plasma-membrane localization could yield Ras proteins that allow the identification of Ras-binding or -modifying cellular partners and their cellular localization.

Experimental Section

General procedures: ^{1}H and ^{13}C spectra were recorded on Bruker AC-250, Bruker AM-400, Varian Mercury 400, and Bruker DRX-500 spectrometers. The signal of the residual protonated solvent ($CDCl_3$ or CD_3OD) was taken as a reference (^{1}H: $\delta = 7.24$ ($CHCl_3$) or 3.31 ppm (CH_3OH); ^{13}C: $\delta = 77.0$ ($CHCl_3$) or 49.0 ppm (CH_3OH)). EI and FAB mass spectra were measured on a Finnigan MAT MS 70 workstation (FAB: 3-nitrobenzyl alcohol (NBA) was used as the matrix). ESI and HPLC/ESI mass spectra were measured on an HPLC/ESI-MS system with a Finnigan Thermoquest LCG spectrometer and a Hewlett Packard (Agilent, 1100 series) HPLC apparatus. CC 250/4 Nucleosil 120–5 C4 reversed-phase analytical columns were purchased from Macherey–Nagel. The following elution and detection conditions were used: flow: 1 mL min^{-1}; eluent gradient ($CH_3CN/H_2O/HCO_2H$): 19.95:79.95:0.1→89.85:9.95:0.1 over 40 min; detection wavelengths: 210, 310, 468, 515 nm. Specific rotations were measured with a Perkin–Elmer polarimeter 241.

Materials: Analytical chromatography was performed on E. Merck silica gel 60F_{254} coated plates. Flash chromatography was performed on Baker silica gel (40–65 μm). Size-exclusion chromatography was performed on Pharmacia Sephadex LH20. All solvents were distilled by using standard procedures. Commercial reagents were used without further purification. All peptide synthesis reactions were performed under argon.

[2,6-Dimethyl-8-(tetrahydropyran-2-yloxy)octa-2,6-dienyl]-(7-nitrobenzo[1,2,5]oxadiazol-4-yl)amine (NBD-NH-Ger-OTHP where Ger = geranyl; 4a): A solution of NBD-Cl (299 mg, 1.50 mmol) in acetonitrile (5 mL) was slowly added to a solution of H_2N-Ger-OTHP **3a** (253 mg, 1.00 mmol) in acetonitrile/25 mM $NaHCO_3$ buffer (1:1; 15 mL). After stirring for 1 h, more NBD-Cl (299 mg, 1.50 mmol) was added and the solution was stirred for 1 h. The re-

sulting black solution was poured into a separation funnel containing dichloromethane and brine. The organic layers were separated, dried over Na_2SO_4, and filtered, then the solvent was removed in vacuo. Purification of the resulting oil by flash chromatography with *n*-hexane/ethyl acetate (3:1) as the eluent yielded the desired product **4a** (279 mg, 0.67 mmol, 67%) as a reddish brown oil: R_f = 0.25 (*n*-hexane/ethyl acetate (3:1)); ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.68 (s, 3H, C(CH_3)-CH-CH_2-OR); 1.74 (s, 3H, NH-CH_2-C(CH_3)); 1.51–1.86 (m, 6H, CH_2 THP); 2.09 (t, J = 7.5 Hz, 2H, CH_2-C(CH_3)-CH-CH_2-O); 2.23 (td, J = 7.5, 7.2 Hz, 2H, NH-CH_2-C(CH_3)-CH-CH_2); 3.51–3.55 (m, 1H, O-CH(R)-O-CH_{2a}-CH_2); 3.88–3.92 (m, 1H, O-CH(R)-O-CH_{2b}-CH_2); 4.01 (dd, J = 12.0, 6.9 Hz, 1H, CH_{2a}-O-CH(R)-O-CH_2); 4.04 (s, 2H, NH-CH_2); 4.26 (dd, J = 12.0, 6.9 Hz, 1H, CH_{2b}-O-CH(R)-O-CH_2); 4.63 (dd, J = 4.4, 2.8 Hz, 1H, O-CH(R)-O); 5.35 (tq, J = 6.9, 1.2 Hz, 1H, CH-CH_2-O); 5.50 (tq, J = 7.2, 1.2 Hz, 1H, NH-CH_2-C(CH_3)-CH); 6.21 (d, J = 8.7 Hz, 1H, CH arom.); 6.51 (br s, 1H, NH); 8.49 (d, J = 8.7 Hz, 1H, CH arom.) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 14.7 (NH-CH_2-C(CH_3)); 16.4 (C(CH_3)-CH-CH_2-O); 19.7 (CH_2 THP); 25.5–25.9 (CH_2 THP, NH-CH_2-C(CH_3)-CH-CH_2); 30.7 (CH_2 THP); 38.9 (CH_2-C(CH_3)-CH-CH_2-O); 51.5 (HN-CH_2); 62.5–63.7 (CH-CH_2-O, CH_2 THP); 98.2 (CH THP); 99.3 (CH arom.); 121.4 (CH-CH_2-O); 128.9 (NH-CH_2-C(CH_3)-CH); 136.5 (CH arom.); 124.1, 128.9, 139.0, 143.9, 144.0, 144.3 (2×C_q Ger, 4×C_q arom) ppm; MS (EI): *m/z*: calcd for $[M+H]^+$: 416.2060; found: 416.2075; $C_{21}H_{28}N_4O_5$ (416.3).

3,7-Dimethyl-8-(7-nitrobenzo[1,2,5]oxadiazol-4-ylamino)octa-2,6-dien-1-ol (NBD-NH-Ger-OH; 5a): PPTS (445 mg, 1.77 mmol) was added to a solution of NBD-NH-Ger-OTHP (**4a**; 358 mg, 0.86 mmol) in ethanol. The reaction mixture was heated at 60 °C for 3 h, then poured into a separation funnel containing diethyl ether and brine. The organic layers were separated, dried over Na_2SO_4, and filtered, then the solvent was removed in vacuo. Purification of the resulting oil by flash chromatography with *c*-hexane/ethyl acetate (1.5:1) as the eluent yielded the desired product **5a** (268 mg, 0.81 mmol, 93%) as a reddish brown oil: R_f = 0.22 (*c*-hexane/ethyl acetate (1.5:1)); ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.61 (s, 3H, C(CH_3)-CH-CH_2-OH); 1.66 (s, 3H, NH-CH_2-C(CH_3)); 2.01 (t, J = 7.3 Hz, 2H, CH_2-C(CH_3)-CH-CH_2-O); 2.15 (td, J = 7.3 Hz, J = 7.0 Hz, 2H, NH-CH_2-C(CH_3)-CH-CH_2); 2.26 (br s, 1H, OH); 4.00 (s, 2H, NH-CH_2); 4.12 (d, J = 6.9 Hz, 2H, CH_2-OH); 5.31 (tq, J = 6.9, 0.8 Hz, 1H, CH-CH_2-OH); 5.43 (t, J = 7.0 Hz, 1H, NH-CH_2-C(CH_3)-CH); 6.15 (d, J = 8.7 Hz, 1H, CH arom.); 7.33 (br s, 1H, NH); 8.49 (d, J = 8.7 Hz, 1H, CH arom.) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 14.6 (NH-CH_2-C(CH_3)); 16.0 (C(CH_3)-CH-CH_2-O); 25.7 (NH-CH_2-C(CH_3)-CH-CH_2); 38.6 (CH_2-C(CH_3)-CH-CH_2-O); 51.6 (HN-CH_2); 59.1 (CH-CH_2-O); 99.3 (CH arom.); 128.6 (NH-CH_2-C(CH_3)-CH); 136.5 (CH arom.); 123.0, 129.2, 138.2, 143.9, 144.1, 144.5 (2×C_q Ger, 4×C_q arom.) ppm; MS (EI): *m/z*: calcd for $[M+H]^+$: 332.1485; found: 332.1498; $C_{16}H_{20}N_4O_4$ (332.4).

(8-Chloro-2,6-dimethyl-octa-2,6-dienyl)-(7-nitrobenzo[1,2,5]oxadiazol-4-yl)amine (NBD-NH-Ger-Cl; 6a): Dimethyl sulfide (21 mg, 0.34 mmol) was added dropwise to a solution of NCS (25 mg, 0.19 mmol) in dichloromethane (2 mL) with stirring at −40 °C under an argon atmosphere. The reaction mixture was then warmed to 0 °C with an ice bath and kept at this temperature for 5 min. The mixture was cooled back to −40 °C and a solution of NBD-NH-Ger-OH (**5a**; 57 mg, 0.17 mmol) in dichloromethane (2 mL) was added dropwise. The resulting muddy residue was then left stirring and allowed to warm to 0 °C over 1 h. The reaction mixture was maintained at 0 °C for another hour, after which time it was allowed to warmed to room temperature for a further 15 minutes. The resulting clear black solution was poured into a separation funnel containing diethyl ether (100 mL) and ice-cold brine. The layers were separated and the organic layer was washed once more with ice-cold brine. The combined aqueous layers were extracted with diethyl ether (2×20 mL). The combined organic layers were dried over Na_2SO_4 and filtered, then the solvent was removed in vacuo to yield product **6a** (60 mg, 0.17 mmol, quant.) as a reddish brown oil: R_f = 0.33 (*n*-hexane/ethyl acetate (3:1)); ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.73 (d, J = 1.2, 1H, C(CH_3)-CH-CH_2-Cl); 1.74 (br s, 1H, NH-CH_2-C(CH_3)); 2.11 (t, J = 7.3 Hz, 2H, CH_2-C(CH_3)-CH-CH_2-Cl); 2.23 (td, J = 7.3, 7.3 Hz, 2H, NH-CH_2-C(CH_3)-CH-CH_2); 4.05 (d, J = 5.5 Hz, 2H, NH-CH_2); 4.08 (d, J = 8.0 Hz, 2H, CH_2-Cl); 5.42 (tq, J = 8.0, 1.2 Hz, 1H, CH-CH_2-Cl); 5.48 (tq, J = 7.1, 1.1 Hz, 1H, NH-CH_2-C(CH_3)-CH); 6.21 (d, J = 8.7 Hz, 1H, CH arom.); 6.53 (t, J = 5.5, 1H, NH); 8.48 (d, J = 8.7 Hz, 1H, CH arom.) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 14.7 (NH-CH_2-C(CH_3)); 16.0 (C(CH_3)-CH-CH_2-Cl); 25.7 (NH-CH_2-C(CH_3)-CH-CH_2); 38.7 (CH_2-C(CH_3)-CH-CH_2-Cl); 41.0 (CH-CH_2-Cl); 51.4 (HN-CH_2); 99.3 (CH arom.); 121.0 (CH-CH_2-Cl); 128.3 (NH-CH_2-C(CH_3)-CH); 136.5 (CH arom.); 124.0, 129.3, 141.8, 143.9, 144.1, 144.3 (2×C_q Ger, 4×C_q arom.) ppm; MS (FAB, NBA): *m/z*: calcd for $[M+H]^+$: 350.1146; found: 350.1165; $C_{16}H_{19}N_4O_3Cl$ (350.8).

{*S*-[3,7-dimethyl-8-(7-nitrobenzo[1,2,5]oxadiazo-4-ylamino)octa-2,6-dienyl]}-L-cysteine methyl ester (Cys-(*S*-Ger-NH-NBD)OMe; 7a): A solution of NBD-NH-Ger-Cl (**6a**; 143 mg, 0.41 mmol) dissolved in dry THF (2 mL) was added to a solution of L-cysteine methyl ester hydrochloride (65 mg, 0.41 mmol) in a 2 M solution of ammonia in methanol (5 mL) with vigorous stirring at 0 °C under an argon atmosphere. The resulting clear solution was then left to slowly warm up for 1 h, before being poured into a separation funnel containing ethyl acetate (100 mL) and brine. The layers were separated and the organic layer was washed once more with brine. The combined aqueous phases were extracted with ethyl acetate (2×20 mL), dried over Na_2SO_4, and filtered, then the solvent was removed in vacuo to yield product **7a** (184 mg, 0.41 mmol, quant.) as a reddish brown oil: R_f = 0.34 (*n*-hexane/ethyl acetate (1:1)); $[\alpha]_D^{20} = -20.4°$ (c = 1, $CHCl_3$); ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.64 (br s, 3H, C(CH_3)-CH-CH_2-S); 1.73 (br s, 1H, NH-CH_2-C(CH_3)); 2.01–2.13 (m, 2H, S-CH_2-CH-C(CH_3)-CH_2); 2.17–2.35 (m, 2H, NH-CH_2-C(CH_3)-CH-CH_2); 2.85 (dd, J = 13.7, 5.7 Hz, 1H, β-CH_{2a} Cys); 2.97 (dd, J = 13.7, 4.9 Hz, 1H, β-CH_{2b} Cys); 3.14 (d, J = 7.6 Hz, 1H, Far CH_{2a}-S); 3.15 (d, J = 7.7 Hz, 1H, Far CH_{2b}-S); 3.75 (tdd, J = 7.6, 7.5, 7.5 Hz, 1H, α-CH Cys); 3.77 (s, 3H, CH_3 Cys); 4.02 (d, J = 5.0 Hz, 2H, NH-CH_2); 5.15 (ddq, J = 7.7, 7.6, 1.0 Hz, 1H, CH-CH_2-S); 5.41 (tq, J = 7.0, 1.2 Hz, 1H, NH-CH_2-C(CH_3)-CH); 6.16 (d, J = 8.7 Hz, 1H, CH arom.); 6.33 (d, J = 7.5 Hz, 2H, NH_2); 6.89 (br s, 1H, NH); 8.48 (d, J = 8.7 Hz, 1H, CH arom.) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 14.8 (NH-CH_2-C(CH_3)); 16.0 (C(CH_3)-CH-CH_2-S); 25.7 (NH-CH_2-C(CH_3)-CH-CH_2); 30.1 (C(CH_3)-CH-CH_2-S); 33.5 (β-CH_2 Cys); 38.8 (CH_2-C(CH_3)-CH-CH_2-S); 51.3 (HN-CH_2); 52.1 (CH_3 Cys); 52.8 (α-CH Cys); 99.2 (CH arom.); 120.4 (C(CH_3)-CH-CH_2-S); 128.0 (NH-CH_2-C(CH_3)-CH); 136.6 (CH arom.); 123.9, 129.1, 138.9, 144.0, 144.2, 144.4 (2×C_q Ger, 4×C_q arom.) ppm; MS (FAB, NBA): *m/z*: calcd for $[M+H]^+$: 450.1733; found: 450.1870; $C_{20}H_{27}N_5O_5S$ (449.5).

Maleimidocaproyl-glycyl-(*S*-*tert*-butyl)-L-cysteyl-L-methionyl-glycyl-L-leucyl-L-prolinyl-{*S*-[3,7-dimethyl-8-(7-nitrobenzo[1,2,5]-oxadiazo-4-ylamino)octa-2,6-dienyl]}-L-cysteine methyl ester (MIC-Gly-Cys(*S*tBu)Met-Gly-Leu-Pro-Cys(Ger-NH-NBD)OMe where MIC = maleimidocaproyl; 9a): HOBt (21 mg, 140 μmol) was added to a solution of MIC-Gly-Cys(*S*tBu)Met-Gly-Leu-Pro-OH[6c,d] (**8**; 80 mg, 93.2 μmol) and Cys-(*S*-Ger-NH-NBD)OMe (**7a**; 41.9 mg, 93.2 μmol) dissolved in dry CH_2Cl_2 (10 mL) at 0 °C under an argon atmosphere. EDC (21 mg, 112 μmol) was subsequently added. The reaction mixture was left stirring at room temperature for 12 h, diluted with ethyl acetate (50 mL), and extracted with 0.5 M HCl (2× 10 mL), 1 M $NaHCO_3$ (2×10 mL), and finally with brine (2×10 mL).

The combined organic layers were dried over $MgSO_4$, filtered, and concentrated in vacuo. Purification of the resulting oil by flash chromatography with an eluent gradient from *c*-hexane/ethyl acetate (1:1) to ethyl acetate/methanol (1:1) yielded the desired product **9a** (91 mg, 70.8 μmol, 76%) as a reddish brown oil: t_R = 26.03 min; $[\alpha]_D^{20} = -36.6°$ ($c = 1$, $CHCl_3$); ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 0.95$ (m, 6H, 2×ω-CH_3 Leu); 1.31 (m, 11H, CH_2 MIC, S*t*Bu); 1.50–1.70 (m, 13H, 2×CH_2 MIC, β-CH_2 Leu, γ-CH Leu, 2×CH_3 Ger); 2.00–2.20 (m, 9H, β-CH_{2b} Met, β-CH_{2b} Pro, γ-CH_2 Pro, SMe, CH_2 Ger); 2.20–2.40 (m, 6H, α-CH_2 MIC, β-CH_{2a} Met, β-CH_{2a} Pro, CH_2 Ger); 2.50–2.70 (m, 2H, γ-CH_2 Met); 3.00–3.15 (m, 6H, 2×β-CH_2 Cys, Ger CH_2-S); 3.46 (m, 2H, NCH_2 MIC); 3.70 (m, 1H, δ-CH_{2b} Pro); 3.70 (s, 3H, OCH_3 Cys); 3.80–3.95 (m, 5H, α-CH_2 Gly, δ-CH_{2a} Pro, α-CH_2 Gly′); 4.00–4.08 (m, 3H, NH-CH_2 Ger, α-CH Pro); 4.40–4.75 (m, 4H, α-CH Leu, α-CH Met, 2×α-CH Cys); 5.15 (m, 1H, CH Ger); 5.41 (m, 1H, CH Ger); 5.46 (m, 2H, Ger NH); 6.29 (d, $J = 8.7$ Hz, 1H, CH NBD), 6.79 (s, 2H, CH=CH MIC); 7.40–7.90 (m, 6H, NH); 8.49 (d, $J = 8.7$ Hz, 1H, CH NBD) ppm; MS (FAB, NBA): *m/z*: calcd for $[M+H]^+$: 1289.5; found: 1289.1; MS (HPLC/ESI, water/acetonitrile/formic acid): *m/z*: calcd for $[M+H]^+$: 1289.5; found: 1289.2; $C_{57}H_{84}N_{12}O_{14}S_4$ (1289.6).

Maleimidocaproyl-glycyl-(*S*-*tert*-butyl)-L-cysteyl-L-methionyl-glycyl-L-leucyl-L-prolyl-(*S*-farnesyl)-L-cysteyl-ethylenediamine (MIC-Gly-Cys(S*t*Bu)Met-Gly-Leu-Pro-Cys(Far)NH-Et-NH_2; 11a): The synthesis was carried out by using Fmoc chemistry under an argon atmosphere and starting with 4-methyltrityl chloride resin (loading of 2 mmol g^{-1}). A solution of dry FmocNH-Et-NH_2 (4.36 g, 11.0 mmol) in *N*-methylpyrrolidine (NMP) and DIPEA (2.84 g, 22 mmol) was added to the polymeric support (1 g, 2 mmol) swollen in dichloromethane. After the reaction mixture was shaken for 2 h, the resin was filtered and washed with 6×5 mL NMP. The deprotection of the Fmoc-NH-Et-NH-4-methyltrityl resin (186 mg, 0.16 mmol, loading of 0.87 mmol g^{-1}) was accomplished through repeated washing with a solution of 20% piperidine in *N*,*N*-dimethylformamide (DMF) for 10 min. The Fmoc-protected amino acids and MIC-OH (4 equiv), respectively, were coupled to the resin with HBTU (218 mg, 0.58 mmol), HOBt (117 mg, 0.77 mmol), and DIPEA (165 mg, 1.28 mmol) in DMF for 90 min after 5 min of preactivation. For capping, the resin was treated twice with 10% acetic anhydride in pyridine for 5 min; the deprotection was accomplished as described above. Between all the steps, the resin was washed thoroughly with DMF. The products were released from the resin by addition of 1% TFA in dichloromethane and consecutive shaking for 0.5 h. The solvent was removed by evaporation in vacuo under repeated addition of toluene for azeotropic removal of the acid. The purity (>90%) was sufficient for the subsequent labeling without further purification. Cleavage from the resin yielded the desired product **11a** (154 mg, 0.13 mol, 79%) as a yellowish oil: $t_R = 30.00$ min; $[\alpha]_D^{20} = 3.2°$ ($c = 1$, $CHCl_3$); ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 0.80$–1.00 (m, 6H, ω-CH_3 Leu); 1.10–1.35 (m, 11H, CH_2 MIC, S*t*Bu); 1.40–1.70 (m, 19H, 2×CH_2 MIC, β-CH_2 Leu, γ-CH Leu, 4×CH_3 Far); 1.85–2.10 (m, 7H, β-CH_{2b} Met, β-CH_{2b} Pro, γ-CH_2 Pro, SMe, 4×CH_2 Far); 2.24 (m, 4H, α-CH_2 MIC, β-CH_{2a} Met, β-CH_{2a} Pro); 2.53–2.61 (m, 2H, γ-CH_2 Met); 2.90 (m, 2H, NH-CH_2-CH_2-NH); 3.12 (m, 6H, β-CH_2 Cys, Far CH_2-S); 3.47 (m, 4H, NCH_2 MIC, NH-CH_2-CH_2-NH); 3.60 (m, 1H, δ-CH_{2b} Pro); 3.80 (m, 5H, α-CH_2 Gly, δ-CH_{2a} Pro, α-CH_2 Gly′); 4.00 (m, 1H, α-CH Pro); 4.38 (m, 3H, α-CH Met, 2×α-CH Cys); 4.50 (m, 1H, α-CH Leu); 5.07 (m, 2H, CH Far); 5.19 (m, 1H, CH Far); 6.69 (s, 2H, CH=CH MIC); 7.25–8.15 (m, 8H, NH) ppm; MS (FAB, NBA): *m/z*: calcd for $[M+H]^+$: 1207.6037; found: 1207.6161; MS (HPLC/ESI, water/acetonitrile/formic acid): *m/z*: calcd for $[M-H]^-$: 1206.6; found: 1206.6; $C_{57}H_{94}N_{10}O_{10}S_4$ (1207.7).

Maleimidocaproyl-glycyl-(*S*-*tert*-butyl)-L-cysteyl-L-methionyl-glycyl-L-leucyl-L-prolyl-(*S*-farnesyl)-L-cysteyl-ethylenediamino-Bodipy FL (MIC-Gly-Cys(S*t*Bu)Met-Gly-Leu-Pro-Cys(Far)NH-Et-NH-Bodipy FL; 12a): Compound **12a** was prepared from MIC-Gly-Cys(S*t*Bu)Met-Gly-Leu-Pro-Cys(Far)NH-Et-NH_2 (**11a**; 13.5 mg, 11.2 μmol) and Bodipy FL (3.2 mg, 11.2 μmol) by means of the procedure described for the synthesis of **9a**. Purification of the resulting oil by flash chromatography with an eluent gradient from *c*-hexane/ethyl acetate (1:1) to ethyl acetate/methanol (1:1) yielded the desired product **12a** (13.5 mg, 9.1 μmol, 81%) as a red oil: t_R = 29.48 min; ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 0.89$ (m, 6H, ω-CH_3 Leu); 1.30–1.45 (m, 11H, CH_2 MIC, S*t*Bu); 1.50–1.70 (m, 19H, 2×CH_2 MIC, β-CH_2 Leu, γ-CH Leu, 4×CH_3 Far); 1.80–2.40 (m, 27H, β-CH_2 Met, β-CH_2 Pro, γ-CH_2 Pro, SMe, 4×CH_2 Far, α-CH_2 MIC, NH-CH_2-CH_2NH, 2×CH_3 Bodipy FL); 2.40–2.60 (m, 2H, γ-CH_2 Met); 3.05–3.15 (m, 12H, 2×β-CH_2 Cys, Far CH_2-S, 2×CH_2 Bodipy FL, NH-CH_2-CH_2NH); 3.15–3.62 (m, 10H, NCH_2 MIC, NH-CH_2-CH_2NH, α-CH_2 Gly, δ-CH_2 Pro, α-CH_2 Gly′); 3.80–4.60 (m, 5H, α-CH Met, 2×α-CH Cys, α-CH Leu, α-CH Pro); 5.06 (m, 2H, CH Far); 5.17 (m, 1H, CH Far); 6.10 (s, 1H, CH Bodipy FL); 6.27 (m, 1H, CH Bodipy FL); 6.66 (s, 2H, CH=CH MIC); 6.88 (m, 1H, CH Bodipy FL); 7.00–7.80 (m, 8H, NH); 7.08 (s, 1H, CH Bodipy FL) ppm; MS (FAB, NBA): *m/z*: calcd for $[M+Na]^+$: 1503.70; found: 1503.22; MS (HPLC/ESI, water/acetonitrile/formic acid): *m/z*: calcd for $[M+H]^+$: 1481.7; found: 1481.5; $C_{71}H_{107}BF_2N_{12}O_{11}S_4$ (1481.8).

Protein synthesis:[6c,d] The N-Ras cDNA was subcloned into the ptac vector.[28] C-terminal truncation was performed by PCR with two stop codons introduced at positions 182 and 183. Expression and purification were carried out as previously described.[28]

Generation of semisynthetic neo-Ras proteins:[6] Prior to coupling, the *N-Ras*Δ181 protein was passed through a HiTrap gel filtration column (Amersham Pharmacia Biotech) in order to remove any excess of salts and the DTE required for storage of the protein. At all stages of the coupling reaction, all samples containing protein, unless otherwise stated, were kept at or below 4°C. 11% Triton X-114 buffer (Fluka) containing 30 mM tris(hydroxymethyl)-aminomethane (Tris)/HCl and 100 mM NaCl (1 mL) was added to a solution of the peptide dissolved in methanol (50 μL) in a 1.5 mL Eppendorf tube. The detergent solution was cooled to 0°C and an aqueous solution (1 mL) of the Ras protein (10 mg) in 20 mM Tris/HCl buffer containing 5 mM $MgCl_2$ (pH 7.4) was added. The coupling reaction was performed with stoichiometric amounts of peptide and protein under argon and incubated at 4°C for 16 h. The soluble supernatant was diluted with buffer (3 mL, containing 2 mM DTE). The mixture was heated to 37°C, which resulted in a separation of the detergent phase from the aqueous phase after centrifugation at room temperature. The aqueous phase was removed and extracted twice more with 11% Triton X-114 detergent solution (2×1 mL). The detergent phases were combined and washed three times with fresh buffer (3×7 mL). The protein extract was diluted to 2% Triton with fresh buffer and applied to a DEAE–sepharose column. For lipoproteins 13a–b, DTE was added up to a final concentration of 50 mM, and the solution was incubated for 1 h at 37°C. Bound protein was eluted with a sodium chloride gradient (0–1 M NaCl) and concentrated. The product was analyzed by MALDI-TOF MS (Perseptive Biosystems) and SDS-PAGE.

Transformation assay: The transformation assay was carried out as previously described.[6]

Cell culture and microinjection: MDCK and COS-7 cells were kept in 7.5% CO_2 conditions at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (Invitrogen, Karls-

ruhe). For fluorescence observation the cells were seeded at 2×10^5 per plate onto 35 mm dishes equipped with 12 mm glass coverslips (CELLocate 175 μm Eppendorf, Hamburg) and microinjected the next day. Microinjection was performed as previously described before.[29] Ras proteins were diluted to 50 μM in phosphate-buffered saline (PBS).

Confocal microscopy: Living cells were examined with a Nikon Eclipse TE 300 confocal microscope with a 60× water immersion objective and the BioRad v.3.1. software. The cells were examined in Hank's buffered salt solution four to five hours after microinjection.

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Keywords: cell imaging · fluorescence spectroscopy · lipoproteins · protein engineering · Ras proteins

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