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A hairpin-like fluorescent probe for imaging of NF- κ B transcription factor activity

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Abstract

Three oligodeoxyribonucleotides (ODN) covalently labeled with near-infrared (NIR) fluorochromes were synthesized and characterized with a goal of comparing *in vitro* a hairpin-based and a duplex-based FRET probe designed for the detection of human recombinant NF- κ B p50/p65 heterodimer binding to DNA. Using deoxyguanosine phosphoramidite with a phosphorus-linked aminoethylene (diethylene glycol) hydrophilic linker, we synthesized ODNs with internucleoside reactive sites. The hairpin loop amino linker was modified with IRDye 800CW (FRET acceptor), and the 3'-end was modified with Cy5.5 (FRET donor) using a dithio-linker. To obtain a duplex probe we conjugated Cy5.5 and 800CW to complementary strands at the distance of ten base pairs in the resultant duplex. No quenching of dyes was observed in either probe. The FRET efficiency was higher in the duplex (71%) than in the hairpin (56%) due to a more favorable distance between the donor and acceptor. However, the hairpin design allowed more precise ratiometric measurement of fluorescence intensity changes as a result of NF- κ B p50/p65 binding to the probe. We determined that as a result of binding there was a statistically significant increase of fluorescence intensity of Cy5.5 (donor) due to a decrease of FRET if normalized by 800CW intensity measured independently of FRET. We conclude that the hairpin based probe design allows for the synthesis of a dual fluorescence imaging probe that renders signal changes that are simple to interpret and stoichiometrically correct for detecting transcription factor – DNA interactions.

INTRODUCTION

NF- κ B is a fast-acting transcription factor with an established role in carcinogenesis and cancer progression due to NF- κ B - mediated control of expression of the genes involved in the anti-apoptotic response to tumor surveillance, regulation of tumor angiogenesis and cell motility¹. Several studies utilizing fluorescence energy transfer demonstrated that the interaction of NF- κ B proteins with fluorescent labeled ODNs can be measured directly^{2,3}. We previously reported ODN duplex probes carrying two covalently linked fluorescent dyes for detecting interactions with transcription factors that specifically recognize the DNA sequence encoded by the duplex^{4,5}. These probes encoding NF- κ B binding sequence were

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Supporting Information Available: The structure of ATG-dG-P nucleoside phosphoramidite synthon. This material is available free of charge via the Internet at <http://pubs.acs.org>.

used for determining p50/p65 protein-mediated interference with the fluorescence resonance energy transfer from the donor near-infrared dye (Cy5.5) to a near-infrared acceptor⁵. Near-infrared fluorochromes potentially allow FRET measurements in live tissues due to a lower absorption and scattering of light in the near-infrared range of light^{6, 7}. The detection of such protein-mediated interference with FRET was accomplished by either using a migration shift assay or plate-capture based assay^{5, 8}. However, a disadvantage of the probes based on ODN duplexes as opposed to a hairpin, i.e. single self-complementary ODN, is in the effort required for purification of components that constitute the probe. Moreover, since such a hairpin is capable of carrying an equal number of donor and acceptor dyes, variations occurring in the formulations of duplexes during their preparation would also be eliminated.

However, linking of two different fluorescent dyes to a single hairpin would require introducing 3'- and 5'-terminal linkers with differential reactivity towards dye derivatives, i.e. for example, 3'-thio and 5'-amino linkers⁹. After the modified ODN folds with the formation of a hairpin, the dyes linked to the ends of the ODN are in a close proximity resulting in partial or complete quenching⁹. Alternatively, the aminohexyl linkers bridging the dye to dC, dA or dG bases can also be used for the same purpose. In the latter case the linked dyes can potentially interact with nucleoside bases and/or get partially buried in the DNA duplex. As an alternative, we used previously developed novel phosphoramidite synthons that by design allow the amino-linkers to be positioned between any adjacent nucleosides, i.e. between any bases in the sequence¹⁰. This synthon was incorporated into a loop region of a hairpin probe. The 3'-end of the hairpin carried a reducible dithio bond enabling modifications with maleimides of cyanine dyes. We chose Cy5.5 maleimide was used as the donor and IRDye 800CW as the acceptor components of FRET pair for the following reasons: 1) the excitation maximum of the donor lies in the near-infrared range of light that corresponds to the maximum depth of penetration in tissue; 2) there is a large spectral emission/excitation spectral overlap suggesting high theoretical energy transfer efficiency; 3) both dyes are hydrophilic and 800CW dye is very stable. Here we report fluorescent and transcription factor binding properties of this newly designed hairpin probe.

MATERIALS AND METHODS

Materials

The reagents for ODN synthesis and purification were obtained from Glen Research (Sterling VA). A nucleoside phosphoramidite synthon ATG-dG-P developed for introducing internucleoside linkers was synthesized using an approach described in¹⁰. The chemical structure of ATG-dG-P is shown in the Supplement. Cyanine fluorochrome Cy5.5 maleimide and Cy5.5 NHS ester were purchased from GE Healthcare (Piscataway NJ); IRDye 800CW NHS ester was obtained from Li-COR (Lincoln NE). All other chemicals were obtained from Sigma-Aldrich (St. Louis MO).

Oligodeoxyribonucleotide synthesis and chemical modification

The semi-automated ODN synthesis of three oligonucleotides (ODN1, ODN2 and H-ODN, the sequences are shown below and in Fig. 2), ODN purification and characterization were performed as in¹⁰. The capped 3'-dithiolinker was introduced by using a commercially available CPG derivative (Glen Research). The conjugation of Cy5.5 (donor) or IRDye 800CW (acceptor) fluorochromes to the internucleoside amino linkers was initially performed using ODN1, ODN2 and H-ODN, (Fig. 1). The conjugation of the donor fluorochrome (Cy5.5 mono maleimide) to H-ODN was performed after reducing hydroxypropyl dithiopropyl linker at the 3'-end of the hairpin with 1M DTT and precipitation of the obtained reduced intermediate from ethanol/sodium acetate. The

obtained ODNs were isolated on a C18-HPLC column using a gradient of acetonitrile in 0.1 M TEAA, pH 7.0, precipitated from ethanol/sodium acetate and characterized by using electrospray mass- spectrometry: ODN1-Cy5.5 5'-**AGCCTG***(Cy5.5) GAAAGTCCCACAT**CG**-3' m/z calc 7525.3, found 7527; ODN2-CW800 5'-**CGATG***(800CW)TGGGACTTTCCAG**GCT**-3' m/z calc 7633.2, found 7634; H-ODN: 5'-**CTGGAAAGTCCC**ACTG*AAGTGGGACTTTCCAGpO(CH₂)₃SS(CH₂)₃OH-3' m/z calc. 10257.4, found 10257, (*-denotes the amino linker, phosphorothioates shown in boldface).

Spectral measurements

All UV spectral measurements were performed at room temperature with a Cary 50 spectrophotometer (Varian). All fluorescence spectral measurements were carried out at room temperature in quartz microcuvettes using a Cary Eclipse fluorescence spectrophotometer (Varian).

Preparation of hairpin and ODN duplexes

Hairpin and ODN duplexes were prepared by adding a pre-specified amount of hairpin or mixing equimolar amounts of the corresponding ODNs dissolved in 25 mM Hepes, 1 mM MgCl₂, 50 mM NaCl, pH 7.4. The solutions were heated to 95°C for 5 min to dissociate any intrastrand secondary structures, and allowed to cool at room temperature to attain equilibrium. Hairpin and duplex formation were confirmed by measuring UV and fluorescence spectra.

Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed using a mixture containing hairpin or ODN duplexes 20 nM incubated 30 min at RT in a volume of 0.01 ml NF-κB p50/p65 solution in PPB. Samples were loaded and run on 15% TBE Ready Gels (Bio-Rad Laboratories, Hercules CA) using 0.5×TBE buffer. The gels were imaged and digitized using an Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln NE).

Fluorescence measurements using Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln NE)

The instrument utilizes simultaneous solid diode excitation of fluorescence at 685 and 785 nm and measures emitted light by using a dichroic mirror which transmits light above 810 nm ("800 channel" fluorescence band) and reflects light below 750 nm ("700 channel" fluorescence band). Fluorescence signals were processed to filter out scattered and stray light. The obtained images (16 bit TIFF) were analyzed, colorized, and fused using ImageJ 1.38 (NIH).

Calculation of experimental distance values (r)

The efficacy of energy transfer depends on the distance between the donor and acceptor dyes¹¹. The Förster distance R_0 ¹², where 50% of the donor excitation energy is transferred to the acceptor, is defined as: $R_0 = 9.78 \cdot 10^3 (\kappa^2 \eta^{-4} \phi_D J(\lambda))^{1/6}$ where ϕ_D is the fluorescent quantum yield of the donor, i.e. 0.28; κ^2 is a factor accounting for the relative orientation of transition dipole moments of donor and acceptor. Isotropic orientation of donor and acceptor in space was assumed so that $\kappa^2 = 2/3$ ¹³; η is the refraction index of the solvent (for water $\eta = 1.33$); $J(\lambda)$ is an overlap integral of the donor absorption spectrum and the acceptor emission spectrum and is given by

$$J(\lambda) = \frac{\int_0^{\infty} F_D(\lambda) \epsilon_A(\lambda) \lambda^4 d\lambda}{\int_0^{\infty} F_D(\lambda) d\lambda}$$

where $F_D(\lambda)$ is the fluorescence spectrum of the donor at wavelength λ ; and $\epsilon_A(\lambda)$ is the absorption spectrum of the acceptor at wavelength λ . The total electronic coupling was assumed as Coulombic coupling,

The donor and acceptor distance r was calculated as:

$$E = \frac{R_0^6}{R_0^6 + r^6}$$

$$E = 1 - \frac{F_{DA}}{F_D}$$

where E is the efficiency of energy transfer, R_0 the Förster distance, and F_D and F_{DA} the relative fluorescence intensity of the donor in absence and in presence of the acceptor respectively.

The efficiency of energy transfer (E) was calculated from donor quenching using the equation: $E = 1 - F/F_0$; where the steady-state relative fluorescence intensity is measured in the presence (F) and absence (F_0) of the acceptor^{14, 15}.

Molecular modeling was performed with Molecular Operating Environment v 2.0 software (MOE, Chemical Computing Group Inc., Montreal CA) using AMBER-94 force field parameter sets. The transition dipole moment directions of cyanine dyes were used as given in¹⁶.

Statistical analysis

All experiments were performed in triplicate. Values were expressed as the mean $\pm$ the standard deviation (SD) and compared using double-tailed unpaired t test with Welch's correction (Prism 4.0, GraphPad).

RESULTS

Using a two-stage synthesis that took advantage of differential conjugation chemistries, we synthesized a 32 bp ODN bearing thio- and amino- group terminated linkers that self-assembles into a hairpin structure (Fig 1, Fig. 2A). Consequently, an NF- κ B duplex binding site, (i.e. GGAAAGTCCC Ig-box derived p50/p65-binding sequence) is formed in the resultant oligonucleotide duplex as a result of self-complementarity of the ODNs (Fig. 2B).

Furthermore, the synthesized oligonucleotide was covalently modified with a pair of near-infrared fluorochromes capable of forming a FRET pair, i.e. one of the fluorochromes was conjugated to the internucleoside phosphate linker in the hairpin's loop (800CW, acceptor) and the other fluorochrome (Cy5.5, donor) was linked to the terminus. During the synthesis 5'-terminal phosphorothioates were introduced to achieve higher stability against endonucleases⁴, Zhang, 2009 #6739. The hairpin structure, and the sites of fluorochrome dye conjugation are shown in Fig. 2A. The linkers in the hairpin can theoretically exist in multiple conformations including limiting stretched or coiled states with the distance between nitrogen atoms of the linkers ranging from 23 to 70 Å. According to our

calculations the Förster distance between the NIR donor and acceptor pair (Cy5.5/800CW) corresponded to 68.8 Å. Actual FRET efficiency measurements obtained using the hairpin and duplex probes showed that FRET in the duplex probe was more efficient, i.e. 71% vs. 56% FRET in the hairpin. Based on these results we estimated that the distance between Cy5.5 and 800CW in the hairpin was close to the Förster distance, i.e. 66.3 Å, whereas in the duplex the distance was 59.3 Å. The latter difference in distances between the donor and acceptor fluorochromes was confirmed by applying AMBER-94 force field parameter sets in Molecular Operating Environment (MOE, Fig. 2A,B). The close quenching of the dyes was not anticipated due to the relative rigidity of the DNA duplex combined with the distance separating the donor and the acceptor that allows simplified assumption of Coulombic interaction as a dominant dipole coupling mechanism¹⁶. Spectral measurements demonstrated that the Cy5.5 absorbance spectrum in a hairpin linked to the 3'-Cy5.5 fluorochrome (Fig. 3A, broken line) was similar to that of the hairpin with two linked dyes, i.e. Cy5 and 800CW (Fig. 3A, solid line). This result suggests that both fluorochromes do not engage in close interactions i.e. there is no intramolecular pi-bond overlap resulting in static quenching of fluorescence.

The same was true for a duplex probe consisting of two separate complementary ODNs that were used as a control in our studies. The spectrum of the duplex also resembled the superimposed spectra of individual dyes linked to individual ODNs (Fig. 3B). The FRET effect in both hairpin and duplex was confirmed by measuring fluorescence spectra (Fig. 3C). After exciting fluorescence at 675 nm (i.e. at the Cy5.5 excitation maximum in water) we observed a decrease of Cy5.5 fluorescence in a two-fluorochrome conjugate (hairpin-Cy5.5-800CW) when compared to a single dye hairpin-Cy5.5 (Fig. 3C). We also measured a concomitant 120% increase of fluorescence intensity in the range corresponding to the 800CW fluorescence peak (i.e. 790–810 nm). Therefore, our observations confirmed the role of Cy5.5 as a donor in FRET. Even higher loss of Cy5.5 fluorescence due to FRET was observed in the case of a duplex probe after hybridizing with 800CW-linked oligonucleotide. These spectra were used to calculate the Förster distances (see above).

Since the dual-fluorochrome labeled hairpin duplex has a fixed donor and acceptor stoichiometry (1:1 mol/mol) we decided to investigate whether the fluorescence intensity ratio of the donor/acceptor changes after the binding of a heterodimer of p50 and p65 NF-κB proteins, i.e. whether the hairpin probe responds to the binding. We used an electrophoretic separation technique to separate hairpin ODN complexed with transcription factor from free hairpin (Fig. 4A lanes 5–8). We also compared the obtained results with the data acquired using the control NF-κB binding duplex composed of two complementary ODNs (Fig. 4A, lanes 1–4). To determine the efficacy of probe binding we initially used a 6-fold molar excess of NF-κB proteins over ODN probes during the incubation and tested ODNs labeled with a single Cy5.5 dye only (Fig. 4A lanes 1,2; 5,6). By measuring reference fluorescence intensity at 800 nm (excitation- at 780 nm), which is not appreciably affected by p50/p65 binding in dual-labeled probes (Fig. 4A, lanes 3,4 and 7,8) or by using a single dye labeled probe we determined that overall the hairpin was binding NF-κB proteins more efficiently, whether single-dye labeled (69±20% vs. 38±11%) or dual-labeled (76±2% vs. 69±16%), where the first number corresponds to the bound fraction of the hairpin, and the second corresponds to the bound duplex fraction. Moreover, the binding of dual-labeled probes on average appeared to be more efficient than binding of single-labeled ones although the differences were statistically insignificant. In the absence of p50/p65, only about 0.5% of total fluorescence was detectable in the upper part of the plate in both cases.

Furthermore, we determined the ratio of normalized fluorescence intensities of Cy5.5 donor at 700 nm and 800CW acceptor dye at 800 nm in ODN bands after separating the complex from the free ODN probes using electrophoresis. We compared the relative changes of

Cy5.5 dye fluorescence intensity after the probes were incubated in the presence of p50/p65 NF- κ B proteins. A lack of change in Cy5.5 intensity would indicate that after the protein heterodimer binds to either probe (i.e. duplex or hairpin) there is no change in FRET efficacy. An increase of Cy5.5 fluorescence intensity would indicate that the FRET efficacy decreased, i.e. that the protein binding interferes with FRET. First, we determined the ratios of fluorescence intensities (FI_{800}/FI_{700}) in free ODN probes.

The FI_{800}/FI_{700} ratio was higher in the case of the duplex than the hairpin (Fig. 4B). The reason for this is that during duplex preparation for EMSA analysis, the addition of a small excess of ODN carrying 800CW acceptor fluorochrome is required to ensure that the thermodynamic equilibrium is shifted towards the formation of a duplex from the individual single ODNs. In contrast, thermodynamic stability of hairpins is high at intermediate temperatures, relatively low ionic strength (<0.2 M) and the low ODN concentrations used in our study. Therefore, only a minor fraction of hairpins went through a hairpin-to-duplex transition as observed during the electrophoresis (Fig. 4A lanes 5, 7). In both single Cy5.5-dye labeled free duplex or free hairpin the intensity of fluorescence in the 700 nm channel was 2-times higher than in the case of double-labeled ODNs indicating the presence of FRET effect in double-labeled probes. The majority of the double-labeled hairpin ODN appeared as a yellow color band as an overlay of green (800 nm) and red (700 nm) pseudo-color fluorescence signals. The above was reflected in the ratios of measured band intensities (Fig. 4B) where in the case of a double-labeled duplex probe the ratio of fluorescence intensities (FI_{800}/FI_{700}) is closer to 1:1 than in the case of duplex probe. The intensity ratio itself reflects the relative concentrations of the two fluorochromes and is affected by quantum yields of fluorescence for each fluorochrome and the sensitivity of the light detector. The binding of NF- κ B proteins not only caused a shift in migration of the fluorescence band but also a change in the measured FI_{800}/FI_{700} ratio. There was a statistically significant decrease of this ratio measured in the band corresponding to the complex of a double-labeled hairpin probe with p50/p65 ($P<0.05$), Fig. 4A, asterisk. There was a much smaller decrease of the FI_{800}/FI_{700} ratio in the case of the duplex probe (Fig. 4B) and the measured differences were insignificant. This result can be interpreted as a consequence of a high signal in 800 nm fluorescence channel created by trailing of the excess of 800CW labeled ODN.

DISCUSSION

The detection of functional protein and nucleic acid complexes capable of regulating gene expression in live cells and whole organisms is one of the major goals of molecular imaging. The imaging of transcriptional activation in cells has been previously accomplished by using genetic constructs, e.g. knocked-in reporter expression cassettes^{17, 18}. However, the genetic approach is limited in that the imaging signal by design is produced by the protein product of gene expression, which introduces a number of uncertainty factors, which include mRNA and protein stability. These issues potentially complicate the interpretation of results.

We have investigated an alternative method that may allow the direct comparative analysis of transcription factor activity or transcription factor concentration in cells or animals. Several rapid acting primary transcription factors, including the important ubiquitous NF- κ B, undergo maturation in the cytoplasm as a result of the processing of precursor proteins in response to extracellular signaling. We previously investigated the potential of NF- κ B detecting probes, which were designed to take advantage of the NF- κ B protein-mediated interference with FRET within double-labeled ODN duplexes which encode the transcription binding sequences^{4, 5, 8}. In the current report we compared a single hairpin-forming ODN and a duplex forming ODN probe both of which carried two NIR dyes separated by a similar number of nucleotide base pairs. By using an electrophoretic assay for

separating transcription-factor bound and free probes we could monitor changes in FRET efficacy by directly measuring a change in fluorescence intensity of the donor fluorochrome (Cy5.5) normalized by fluorescence of the acceptor (800CW) dye. The latter measurement did not depend on FRET efficacy since the fluorescence of the acceptor fluorochrome 800CW was excited at its characteristic maximum. The obtained results showed that the hairpin design had both advantages and disadvantages. The main advantage is in that the dye ratio per probe was much easier to control in the case of the hairpin, which resulted in more reliable measurements of the effects of NF- κ B proteins on donor fluorescence. The disadvantage was in the need to implement two different chemistries of dye conjugation in order to link one of the dyes to the ODN terminus in order to achieve a donor/acceptor 1:1 molar ratio.

In conclusion, we showed that dual-labeled fluorescent hairpin probes can be designed with two NIR dyes that form a FRET pair. With this design, an equimolar ratio of donor and acceptor dyes enables the detection of transcription factor protein binding to the probe. The relatively modest effect observed during the in vitro tests suggests that the probe design would benefit from further optimization in order to be useful for reliable intracellular imaging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

FRET	fluorescence resonance energy transfer
NIR	near infrared
ODN	oligodeoxyribonucleotide

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Figure 1. Synthesis of the probes

A- synthetic steps leading to dual near-infrared fluorescent-labeled hairpin H-ODN probe;
B- synthesis of fluorescent ODN1 and ODN2. Phosphorothioates are shown in bold. X- Cy5.5 carboxylate; Y- IRDye 800CW carboxylate, Z- Cy5.5 thiopyrrolidine dione residues.

Figure 2. Inter-fluorochrome distances based on molecular modeling

Ball-and-stick model of H-ODN (A) and the duplex probe ODN1/ODN2 (B) with the sequences shown below. Phosphorothioates are shown in bold. The calculated effective distances between the donor and acceptor NIR dyes is shown next to the models. The models were built in MOE, energy minimization was accomplished by applying AMBER-94 force field parameter sets.

Figure 3. Spectral analysis of ODN probes

A- absorbance spectra of Cy5.5- and dual Cy5.5/800CW labeled H-ODN probe; **B-** absorbance spectra of Cy5.5-ODN1, 800CW-ODN2 and the resultant dual fluorescent duplex probe; **C-** fluorescence spectra of Cy5.5-labeled hairpin and duplex probe showing Cy5.5 fluorescence spectra in the absence of FRET and the spectra of dual labeled probes with resultant FRET (shown by an arrowhead). Cy5.5 fluorescence was excited at 675 nm.

Figure 4. Electrophoretic mobility shift assay of NF-κB complex with ODN probes

A- dual wavelength imaging of PAGE gels in pseudo colors. Fluorescence intensities are: red -Cy5.5, green - 800CW; lanes: 1- Cy5.5ODN1/ODN2 duplex, 2- NF-κB p50/p65 complex with Cy5.5ODN1/ODN2 duplex; 3- dual labeled Cy5.5ODN1/800CW-ODN2 duplex probe, 4- p50/p65 NF- κB complex with Cy5.5ODN1/800CW-ODN2 ; 5- Cy5.5-H-ODN (a minor band represents a duplex form of H-ODN); 6- p50/p65 NF-κB complex with Cy5.5-H-ODN; 7- dual labeled hairpin Cy5.5/80CW-H-ODN; 8- p50/p65 NF-κB complex with dual labeled hairpin Cy5.5/800CW-H-ODN. The p50/p65 NF-κB complex migration is marked by an asterisk. The free duplex probe shown with an arrow (left); migration of the free hairpin probe is shown with an arrowhead (right). **B** - the ratio of fluorescence intensities of 800CW to Cy5.5 (F1800/F1700) measured using the EMSA gels (shown in A) showing a lack of difference between free (bar 1) and NF-κB p50/p65 bound (bar 2) Cy5.5-ODN1/800CW-ODN2 duplex probe (p=0.8) and significant differences between free Cy5.5/800CW-H-ODN hairpin probe (bar 3) and NF-κB p50/p65 bound hairpin probe (p=0.01), n=4.