Near-Infrared Fluorescent Oligodeoxyribonucleotide Reporters for Sensing NF- κ B DNA Interactions *In Vitro*

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Two types of reporters for optical sensing of NF- κ B p50 protein-oligodeoxyribonucleotide (ODN) duplex interactions were designed and compared *in vitro*. The reporters were based on the effect of fluorescence resonance energy transfer (FRET) between the pair donor Cy5.5 near-infrared (NIR) fluorochrome and either 800CW emitting fluorescence dye acceptor (800CW-Cy), or a nonemitting QSY 21 dye quencher (QSY-Cy). The donor and the acceptor dyes were covalently linked to the complementary oligonucleotides, respectively: Cy dye was conjugated to 3'-thiol, whereas 800CW or QSY21 were conjugated to a hydrophilic internucleoside phosphate amino linker. The reporters were tested initially using recombinant NF- κ B p50 protein binding assays. Both reporters were binding p50 protein, which protected oligonucleotide duplex from degradation in the presence of exonuclease. The incubation of 800CW-Cy reporter in the presence of control or IL-1 β treated human endothelial cells showed the uptake of the reporter in the cytoplasm and the nucleus. The measurement of NIR fluorescence ratio (i.e. Cy5.5/800CW) showed a partial loss of FRET and the increased Cy5.5 fluorescence in nontreated, control cells. Thus, the specific p50 binding to ODN duplex reporters affected the donor-acceptor fluorochrome pair. NF- κ B p50 exhibited the protective effect on FRET between NIR fluorochromes linked to the complementary strands of the reporter duplex.

Introduction

THE INITIAL DEMONSTRATION OF fluorescence resonance energy transfer (FRET) effect in complementary oligonucleotides lead to a conclusion that with further development, fluorescence techniques will be used in detecting and quantifying nucleic acid hybridization in living cells (Cardullo et al., 1988). So far, fluorescence gained acceptance as a method of choice for detecting high complementarity duplex formation predominantly in cell-free systems (Tyagi and Kramer, 1996; Bratu, 2006). For example, fluorescence detection is commonly used for multiplexing PCR products in real time in homogeneous assays [reviewed in (Shi, 2001), as well as in detecting single-nucleotide variations (polymorphisms) (Marras et al., 1999; Dubertret et al., 2001). The latter analyses are facilitated by using FRET from the high-quantum yield donor SYBR Green I fluorochrome to primer-linked acceptor dyes (Takatsu et al., 2004). Unlike ethidium and propidium cations, SYBR dyes do not intercalate and bind to the surface of ssDNA (Zipper et al., 2004).

The applications of fluorescent oligodeoxyribonucleotides (ODNs) can be expanded beyond detecting complementarity between the oligonucleotides. For example, the efficient detection of prokaryotic DNA-binding proteins has been reported upon *Escherichia coli* single-stranded DNA-binding protein interaction with a hairpin-like beacon carrying a pair of a quencher and a fluorochrome at the opposite termini (Li et al., 2000). In contrast to prokaryotic stabilizing proteins, the direct detection of transcription factors in homogenous assays is a complex task since transcription factors interact with double-helix sequence-specific elements and do not induce strand separation in the duplex. One of the potential strategies that enabled the detection of transcription factors is based on protein-mediated assembly of the binding sites using a pair of DNA duplexes carrying a fluorescent donor and an acceptor of fluorescence, respectively (Heyduk and Heyduk, 2002; Heyduk et al., 2003). This approach requires the use of 6-nucleotide long overhangs (encoding a half of the binding site each) that upon base pairing, assemble in a DNA duplex. This strategy was successfully applied for

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detecting bacterial CAP protein and p53 in cell-free extracts (Heyduk and Heyduk, 2002). However, the need to simultaneously deliver two halves of the reporter probe into the cells and the instability of 3'-overhang would limit potential applications in live cells. Alternatively, prestained oligonucleotide duplexes can be used for detecting specific protein-ODN duplex interactions using the effect of ODN duplex protection from the degradation by exonucleases (Chen et al., 2006). The method is based on high fluorescence of SYBR in DNA-bound state in the presence of the intact duplex. As demonstrated using NF- κ B detection in crude cell extracts, the reporter remains fluorescent if protected from the degradation by Exo III by dsDNA-bound protein; however, upon the degradation of dsDNA, the fluorescence of SYBR dye decreases (Chen et al., 2006). Though the above approach is applicable for homogeneous assays *in vitro*, it is doubtful that noncovalently bound fluorescent dyes can be useful for detecting transcription factor binding to the reporter ODN duplexes in live cells due to the potential fluorochrome exchange with cellular DNA.

Recent experiments showed that near-infrared (NIR) fluorescence imaging can be applied for noninvasive detection and quantitation of receptor-ligand binding (Achilefu, 2004; Licha and Olbrich, 2005). Moreover, NIR self-quenched substrates enable detecting enzymatic activity *in vivo* (Weissleder et al., 1999; Bremer et al., 2002; Ntziachristos, 2006). Therefore, we set forth to investigate the utility of covalently linked NIR fluorochromes for detecting specific protein-DNA interactions *in vitro* and in the live cells. In this article we report the design and initial testing of two ODN duplex reporters based on NIR FRET.

Materials and Methods

Materials

Triethylammonium acetate (2M, TEAA), pH 7.0, was purchased from Glen Research (Sterling, VA, USA); Cy5.5 maleimide was from Amersham-GE Healthcare (Piscataway, NJ, USA); IRDye 800CW NHS ester was from Li-COR, (Lincoln, NE, USA). All other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

The following ODNs: 5'-CGGAAAGT*CCCTCATAGCT-3' (I) and complementary 5'-AGCTATGAGGGACTTCCG-Y-3' (II) were synthesized on 394 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA, USA) by using a standard protocol for phosphoramidite synthesis, where: Y stands for HOCH₂CH₂SSCH₂CH₂CH₂O-((O=(HO)P-O-linker; T* stands for T-O-P(OH)=O)-OCH₂CH₂OCH₂CH₂OCH₂CH₂NH₂. The latter internucleoside linker was introduced by using a novel thymidine phosphoramidite synthon (Tabatadze et al., 2008). This synthon contained a protected primary amino group linked via a hydrophilic triethylene glycol linker to phosphorus(V). ODNs were purified using reverse phase HPLC column (Microsorb MV100 C18, Varian, Lake Forest, CA, USA) eluted with a linear gradient of 2–50% acetonitrile in 0.1M TEAA, pH 7. ODNs were concentrated and precipitated using ethanol/sodium acetate. Electrospray mass-spectrometry showed the following m/z: Oligonucleotide I - 5904.2 (5904.0 calculated), Oligonucleotide II - 6087.6 (6087.9 calculated).

Chemical modification of oligodeoxyribonucleotides

Conjugation of 800CW mono N-hydroxysuccinimide ester (LI-COR, Lincoln, NE, USA) or QSY 21 N-hydroxysuccinimide ester was accomplished by adding dye solutions (16.7 mg/mL, DMSO in two 0.0075-mL portions) to amino linker-bearing ODN I (0.03 mL, 0.3 mM in 0.1 M NaHCO₃). The reaction mixture was incubated overnight at room temperature in the dark. The labeled oligonucleotide was purified by spin-chromatography using Bio Spin P6 microcolumns (Metellev et al., 2004), followed by C18 HPLC column (Microsorb MV100 C18, Varian, Lake Forest, CA, USA) eluted using a linear gradient of 2–50% acetonitrile in 0.1 M TEAA, pH 7; ODNs were precipitated using ethanol/sodium acetate. The covalent modification of ODN II via the reduction of 3'-hydroxypropyldithiopropyl linker (0.1 mL, 0.3 mM in 12 mM NaHCO₃) was performed by reducing with DTT (0.011 mL, 1 M solution) for 1 hour at room temperature, followed by ethanol/sodium acetate precipitation and dissolving in 0.1 M sodium phosphate buffer, pH 7.5. Cy5.5 maleimide (0.02 mL, 5 mg/mL DMSO) was then added in two increments within a period of 1 hour. The reaction mixture was kept in the dark overnight. The labeled oligonucleotides were purified as above. Electrospray mass-spectrometry showed the following: m/z: 800CW-I: 6888.9 (6888.0 calc); (Cy5.5-II: 7036.0 (7035.9 calc).

Preparation of oligonucleotides duplexes

I/II duplexes (native, 800CW-I/Cy5.5-II (radiative FRET reporter) or QSY21-I/Cy5.5-II [nonradiative FRET (quenched) reporter] were prepared by combining the oligonucleotides at a 1:1 molar ratio unless otherwise noted in 25 mM HEPES, 1 mM MgCl₂ and 50 mM NaCl. The duplex mixtures were heated between 90°C and 95°C for 5 minutes to dissociate any intrastrand duplexes, and allowed to cool at room temperature.

Spectral measurements

Absorbance measurements were recorded using Cary 50 Bio UV/VIS spectrophotometer; fluorescence measurements were performed on Cary Eclipse fluorescence spectrophotometer (both from Varian, Walnut Creek, CA, USA). Fluorescence of Cy5.5 was excited at 675 nm. Fluorescence was recorded at 694 nm (Cy5.5) or 800 nm (800CW). Melting temperatures were determined using a Peltier temperature ramping bath in 2 degree mode in a temperature-controlled cuvette holder. To investigate p50 binding to ODN duplexes or p50-mediated protection against exonuclease degradation, human recombinant p50 protein (0.0043 mL p50 solution, 6 μ M, Alexis Biochemicals, Lausanne, Switzerland or Active Motif, Carlsbad, CA, USA) was added to FRET or quenched duplex reporters (0.08 mL final volume, 5.4 nM in 10 mM Tris, 100 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 0.1 mg/mL yeast tRNA, 10% v/v glycerol, 0.25 mM DTT, pH 7.5), and spectral measurements were taken at room temperature immediately and then every 1.5 minutes for 30 minutes. In control experiments, human serum albumin (HSA,

essentially fatty acid-free, Sigma St. Louis, MO, USA) or protein binding buffer were used as controls. Exonuclease III solution (Exo III, 1 U, Promega, Madison, WI, USA) was added and fluorescence was recorded immediately every 1.5 minutes for 1 hour.

Cell culture

Human umbilical vein endothelial cells (HUVEC, subculture 2, (Gimbrone and Cotran, 1975) were provided by Dr. Bill Lusinskas, (Vascular Research Division, Department of Pathology, Brigham and Women's Hospital). The cells were grown in 5% FBS, complete endothelial cell growth medium (EGM, Cambrex, Baltimore, MD, USA) until confluent. Cells were plated in the glass-coverslip chambers (Lab-Tek II, Electron Microscopy Sciences, Hatfield, PA, USA). Duplex reporters were added to cells in 5% FBS/EBM at the final concentration of 1 μ M (14 μ g 800CW-I/Cy5.5-II duplex probe/mL, 0.5 mL/well) and incubated at 37°C, 5% CO₂ for 4 hours. Cells were washed with 5% horse serum/PBS.

Fluorescence microscopy

Fluorescence images were acquired using Nikon TE2000-U inverted microscope equipped with a 100W Dia-illuminator and Nikon blue, Cy5, and 800 excitation fluorescence filter cubes. Images were acquired using CoolSnapHQ-M CCD (Photometrics, Tucson, AZ, USA). The results were processed using IP Lab Spectrum software (BD Bioimaging, Rockville, MD, USA). Region-of-interest analysis was performed by outlining the cell ROI using phase-contrast images and then pasting ROI for measuring fluorescence on 16-bit greyscale fluorescence images acquired using Cy5 and 800 filters. The ratios of 700/800 nm fluorescence were calculated and analyzed using Prism 4.0 (see statistical analysis). Cells were treated with IL-1 β (final concentration 2 pg/mL in EBM, Calbiochem, San Diego, CA, USA) at 37°C for 2 hours. The expression of E-selectin in response to IL-1 β treatment was detected by using anti-human E-selectin fragments (H18/7 F(ab')₂, a generous gift of Dr. Michael Gimbrone, Jr, Brigham and Women's Hospital) followed by FITC-labeled anti-mouse F(ab')₂ (10 μ g/mL) diluted in 2% serum in Hanks' solution. Cells were washed and postfixed in 2% formaldehyde in buffered PBS before acquiring the images.

Cell extract preparation

Nuclear extracts and cytoplasmic extracts were prepared using HUVEC cells grown to 80% confluency. Control and IL-1 β treated HUVEC cells were used. Cells were washed twice in 10 mL ice-cold PBS and resuspended in 500 μ L of buffer A (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 5 μ g/mL leupeptin, 100 μ g/mL aprotinin, 1 μ g/mL pepstatin) and incubated on ice for 15 minutes, followed by adding NP-40 to a final concentration of 0.5% and vortexing the cells for 10 seconds. The pellet was collected at 5500g, 20 seconds. The supernatant (cytoplasmic fraction) was transferred to a new tube. The nuclei pellet was resuspended in 150 μ L buffer C (20 mM HEPES, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 25%

v/v glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 5 μ g/mL leupeptin, 100 μ g/mL aprotinin, 1 μ g/mL pepstatin), incubated on the ice for 30 minutes with agitation. Samples were centrifuged at 12,000 rpm for 10 minutes at 4°C, and the supernatant was removed and stored at -80°C. The protein concentration was determined by Micro BCA Protein Assay (Pierce, Rockford, IL, USA).

Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed using a reaction mixture containing fluorescent and nonquenched I/Cy5.5-II duplex that was incubated for 30 minutes RT in a volume of 10 μ L in the presence of various concentrations of cell extracts in the protein binding buffer (10 mM Tris, 100 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 0.1 mg/mL tRNA, 10% v/v glycerol, 0.25 mM DTT, pH 7.5). Samples were loaded and run on 10% TBE Ready Gels (Bio-Rad Laboratories, Hercules, CA, USA) with 0.5 $\times$ TBE buffer. The gels were imaged and digitized using Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis

All experiments were performed in triplicate. Values are 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

Radiative and nonradiative (quenched) (FRET) in duplex reporters

Initially, we characterized the synthesized ODN reporters by comparing duplex melting temperatures of native, nonmodified ODN duplex to those formed by covalently modified ODNs.

The latter were synthesized by linking FRET acceptors (either 800CW NIR fluorochrome, or QSY21 quencher) to the first oligonucleotide (ODN I) via a novel hydrophilic internucleoside linker and by linking of Cy5.5 to the reduced S-S bond at the 3' end of the complementary ODN II (Fig. 1, Table 1). By using UV spectroscopy and Cy5.5 fluorescence intensity measurements (see Table 1), we determined that the native duplex melts at a slightly higher temperature than 800CW-I/Cy5.5-II duplex ($\Delta T^{\circ} = 5^{\circ}\text{C}$) and at approximately the same temperature as QSY21-I/Cy5.5-II duplex. Further, we measured absorbance spectra of 800CW-I/Cy5.5-II and QSY21-I/Cy5.5-II duplexes and calculated the arithmetic sum of absorption values of the corresponding ODNs comprising the duplex reporter probe (Fig. 2A and B). A simple comparison of the obtained arithmetic sum with the measured spectra showed that the latter was nearly identical to the former in the visible/NIR range of light. The near equivalence of summation values (red trace) and QSY21-I/Cy5.5-II duplex absorbance (green trace) in the ultraviolet range proved that the individual ODNs and the duplex were analyzed at the same total concentration of nucleotides. The quenching of Cy5.5 fluorescence by QSY21 resulted in a loss

of Cy5.5 fluorescence that was higher than the decrease of Cy5.5 fluorescence as a result of radiative energy transfer to 800CW dye (compare Fig. 2C and D), that is, FRET to 800CW dye resulted in 6-fold decrease of Cy5.5 fluorescence, whereas fluorescence quenching resulted in a 25-fold decrease of Cy5.5 fluorescence intensity.

NF- κ B p50 protein binding effects in duplex solutions

The addition of human recombinant p50 protein that specifically binds to the kappa-B box sequence (5'-GGAAAGTCCC-3') interfered with nonradiative FRET and resulted in a very rapid partial dequenching of Cy5.5 fluorescence in the case of QSY21-I/Cy5.5-II duplex (Fig. 3A). With the addition of an excess of the complementary ODN linked to a quencher (QSY21-II), the relative dequenching of Cy5.5 fluorescence was decreased. However, NF- κ B p50 protein had no effect on radiative FRET efficacy in 800CW-I/Cy5.5-II duplex reporter (not shown). Exonuclease Exo III, an ODN degrading enzyme, caused rapid loss of both nonradiative (Fig. 3B) and radiative FRET (Fig. 3C). If Exo III was added to the reaction mixture containing p50 and the duplex (either QSY21-I/Cy5.5-II, Fig. 3B, or 800CW-I/Cy5.5-II, Fig. 3C) we observed a significantly delayed loss of radiative FRET and a slow-down in dequenching. In the absence of p50, the increase

of Cy5.5 fluorescence due to the duplex degradation was almost instantaneous in the case of 800CW-I/Cy5.5-II (Fig. 3C, trace 1). However, the rate of Exo III-mediated QSY21-I/Cy5.5-II reporter dequenching was slower (Fig. 3B) and the addition of QSY21-II ODN contributed to the dequenching delay, suggesting that Cy5.5 fluorochrome was accessible for quenching by QSY21 in solution.

We further tested whether the observed time delay of FRET loss in the presence of recombinant p50 and exonuclease activity could be used in detecting cells that express activated NF- κ B transcription factor (Baeuerle and Henkel, 1994; Baldwin, 1996; Brand et al., 1997). We used either control HUVEC or HUVECs treated with IL-1 β to activate NF- κ B regulated transcription. The cells were then incubated with 800CW-I/Cy5.5-II or QSY21-I/Cy5.5-II duplexes. Low fluorescent QSY21-I/Cy5.5-II duplexes did not provide a signal high enough for analysis of cell fluorescence intensity (see below and Fig. 5). In contrast, 800CW-I/Cy5.5-II FRET reporter fluorescence changes enabled to localize Cy5.5 fluorescence distribution within the cells (Fig. 4A and B) and to register Cy5.5 channel fluorescence with green fluorescence resulting from cell surface labeling with the anti-E selectin H18/7 F(ab')₂ fragments. Anti-human E-selectin antibody fragments were binding to the surface of IL-1 β -activated cells and showed some internalization (compare Fig. 4E and D). There was no colocalization of green and red fluorescence, that is, anti-E-selectin antibody fragments and 800CW-I/Cy5.5-II FRET reporters were distributed in distinct intracellular compartments. Some accumulation of the duplex in cell nuclei was clearly present (see Fig. 4A, B, and E). Cells with the highest Cy5.5 NIR fluorescence (red, Fig. 4E) showed the lowest expression of E-selectin (green).

The expression of activated NF- κ B transcription factor proteins was verified by performing EMSA in the presence of I/Cy5.5-II reporter (i.e. non-FRET, always "on" duplex probe). The assay showed concentration-dependent protein binding and shifting of fluorescent band in the presence of either nuclear, or cytoplasmic protein fractions isolated from the IL-1 β treated cells (see Fig. 4F, lanes 3, 4, 6, and 7). We observed very low levels of EMSA signal in the presence of control, nonactivated cell extracts (see Fig. 4F, lanes 2 and 5). In the presence of either nuclear or cytoplasmic proteins, fluorescence intensity of the shifted band in IL-1 β treated cells was 8–10 times higher than in control cells (control: 2–3%, experiment: 22–24% of total fluorescence per lane).

By measuring the ratios of fluorescence intensities at 700 (Cy5.5, Fig. 5A) and 800 nm (800) we attempted to monitor 800CW-I/Cy5.5-II FRET reporter protection from degradation in live, IL-1 β -stimulated endothelial cells (Fig. 5A). We determined that the differences between measured Cy5.5/800 ratios in treated and control (nontreated cells) were statistically significant ($p < 0.001$). The microscopy quantitation results above were corroborated by using high-sensitivity NIR fluorescence measurements of cell suspensions in 96-well plates (Fig. 5B and C). HUVECs incubated with the non-FRET duplex probe (I/Cy5.5-II) showed the uptake of fluorescence that was independent of cell treatment with IL-1 β (Fig. 5B). However, if a FRET reporter was used, the nontreated cells had a higher Cy5.5 fluorescence intensity than the IL-1 β treated ones (Fig. 5C) at various numbers of cells used in the assay.

FIG. 1. Nucleotide sequence of ODN duplex reporter. FRET effect was achieved by linking Cy5.5 (R) as a donor of fluorescence, and 800CW (X) as acceptor. Nonradiative FRET was achieved by using QSY21 (X) as an acceptor. The QSY21 and 800CW were conjugated to the internucleoside phosphate via tri(ethylene glycol)amino linker.

TABLE 1. OLIGONUCLEOTIDE DUPLEXES AND THEIR PROPERTIES

Duplex	Duplex ^a	Melting temperature, °C (method)	FRET/quenching efficiency
I/II	5'-CGG AAA GT* C CCT CAT AGC T-3' 3'-YGCC TTT CAG GGA GTA TCG A-5'	64 (UV)	N/A
800CW-I/ Cy5.5-II	5'-CGG AAA GT ^{800cw} C CCT CAT AGC T-3' 3'-Cy5.5-S-GCC TTT CA G GGA GTA TCG A-5'	59 (FRET)	82.9 ± 2.3
QSY 21-I/ Cy5.5-II	5'-CGG AAA GT ^{QSY21} C CCT CAT AGC T-3' 3'-Cy5.5-S-GCC TTT CA G GGA GTA TCG A-5'	65 (dequenching)	90.8 ± 4.0

^aFor non-modified ODN: Y is HOCH₂CH₂SSCH₂CH₂CH₂O-(O=(HO))P-O-; T* is T-O-P((OCH₂)=O)-OCH₂CH₂OCH₂CH₂OCH₂CH₂NH₂

FIG. 2. UV/VIS absorbance and fluorescence spectra of the (A) FRET duplex reporter probe and its components: 800CW-I (blue); Cy5.5-II (black); duplex probe (green); the arithmetic sum of 800CW-I and Cy5.5-II absorbances (red); (B) QSY21-I/Cy5.5-II duplex probe spectra: QSY21-I (blue); Cy5.5-II (black); duplex probe (green); the arithmetic sum of QSY21-I and Cy5.5-II absorbances (red); (C) fluorescence spectra demonstrating FRET effect between Cy5.5-II donor ODN component (trace 1) and 800CW-I acceptor ODN (trace 2); 3 – 800CW-I/Cy5.5-II FRET effect; (D) fluorescence spectra of I/Cy5.5-II (trace 1) and QSY21-I/Cy5.5-II probe (trace 2). Duplexes were obtained by using 1:1 ratio of complementary ODNs. Fluorescence was excited at 675 nm (Cy5.5 maximum).

FIG. 3. NF- κ B p50 binding-mediated FRET effects, dequenching and duplex protection. (A) p50 destabilization of QSY21 quenching in QSY21-I/Cy5.5-II with the resultant partial loss of quenching of Cy5.5 fluorescence in QSY21-I/Cy5.5-II probe (1:1 ratio, triangles), in QSY21-I/Cy5.5-II probe (2:1 ratio, squares), p50 protein was added at $t = 0$ min (arrow); (B) p50 protection effect against Exo III degradation.

Discussion

In this report, we performed initial testing of two types of alternative ODN duplex “sensing” reporters designed for detecting specific protein–DNA interactions. Duplex reporters were encoding NF- κ B p50 human β 2-microglobulin kappa-B box sequence (5′-GGAAAGTCCC-3′) (Gobin et al., 2003) (Fig. 1). The first reporter (800CW-I/Cy5.5-II) was based on radiative fluorescence energy transfer between the donor of fluorescence (Cy5.5) covalently linked to the first ODN and a NIR acceptor (800CW dye) covalently linked to the complementary ODN using a novel internucleoside phosphate amino linker (Tabatadze et al., 2008), (Figs 1 and 2C). The second reporter (QSY21-I/Cy5.5-II) carried nonfluorescent QSY21 acceptor of Cy5.5 fluorescence linked to the complementary ODN instead of the fluorescent acceptor of Cy5.5 fluorescence, thereby causing efficient quenching (Fig. 2D). We expected that as a result of specific binding of p50 to the ODN reporter, the interaction between Cy5.5 donor with the acceptor of fluorescence would be stabilized against the degradation by exonucleases *in vitro* and in living cells. This would result in a lower fluorescence in cells expressing activated p50 that is proteasome-processed p105 (Palombella et al., 1994) than in control cells.

It is currently accepted that in addition to resonance energy transfer to the quencher (FRET quenching, that is, Forster-Coulomb and Dexter dynamic modes of quenching), non-FRET mechanism is also common in dual-labeled ODN reporters (Johansson and Cook, 2003; Metelev et al., 2004; Johansson, 2006) and that the formation of kinetically stable ground-state complexes between the fluorochrome and quencher is also highly probable. Therefore, we expected that the initial binding of p50 to the duplexes could potentially interfere with energy transfer and with close interactions between the dye pairs and result in the initial increase of Cy5.5 fluorescence due to either radiative or nonradiative energy transfer. The results of UV/VIS spectroscopy and melting behavior studies suggested the potential formation of QSY21-Cy5.5 pairs (that would be evident by blue-shifted absorbance of Cy5.5 (Mujumdar et al., 1993)). However, our absorbance spectra measurements suggested no apparent close interaction between the dye pairs (Fig. 2A and B). This was confirmed, in part, by melting temperature measurements. Though the methods used for determining the melting temperatures (i.e. the temperatures corresponding to 50% strand separation) in native and covalently modified duplexes were different, the measurements of radiative FRET loss in 800CW-I/Cy5.5-II duplex or dequenching

(1) kinetics of dequenching in QSY21-I/Cy5.5-II probe (1:1 ratio) in the absence of p50; (2) kinetics of dequenching in QSY21-I/Cy5.5-II probe (2:1 ratio) in the absence of p50; (3) a delay in dequenching in the presence of p50 and QSY21-I/Cy5.5-II probe (1:1 ratio); (4) a delay in dequenching in the presence of p50 and QSY21-I/Cy5.5-II probe (2:1 ratio); (C) (1) kinetics of radiative FRET loss in 800CW-I/Cy5.5-II probe (1:1 ratio) in the absence of p50; (2) p50 protection effect against Exo III degradation and resultant FRET loss in 800CW-I/Cy5.5-II probe (1:1 ratio).

FIG. 4. Fluorescence microscopy of HUVEC (red—Cy5.5 fluorescence, green—FITC fluorescence): (A) treated with IL-1 β (2 pg/mL) and incubated with 800CW-I/Cy5.5-II (1 μ M); (B) no IL-1 β treatment, incubated with 800CW-I/Cy5.5-II (1 μ M); (C) autofluorescence of control HUVEC; (D) control cells, no IL-1 β treatment, incubated with 800CW-I/Cy5.5-II, stained with anti-E-selectin F(ab')₂, and FITC-anti F(ab')₂; (E) cells treated with IL-1 β (2 pg/mL), incubated with 800CW-I/Cy5.5-II, stained with anti-E-selectin F(ab')₂/FITC-anti F(ab')₂; (F) EMSA of I/Cy5.5-II in the presence of HUVEC extracts: (1) I/Cy5.5-II alone, (2) I/Cy5.5-II and control nuclear extract (126 ng/ μ L); (3) I/Cy5.5-II and nuclear extract (126 ng/ μ L), cells treated with IL-1 β ; (4) same as above, the concentration of nuclear extract (196 ng/ μ L); (5) I/Cy5.5-II and control cytoplasmic extract, concentration (57 ng/ μ L); (6) I/Cy5.5-II and cytoplasmic extract (126 ng/ μ L), cells were treated with IL-1 β ; (7) same as above, the concentration of cytoplasmic extract (211 ng/ μ L). The arrowhead points to shifted ODN bands.

FIG. 5. Fluorescence measurements in HUVEC culture. (A) fluorescence intensity ratios (Cy5.5/800CW fluorescence) measured using HUVEC microscopy. Cells were either: (1) nontreated, (2) treated with IL-1 β in the absence of 800CW-I/Cy5.5-II probe or (3) incubated with 800CW-I/Cy5.5-II in the absence or the presence of IL-1 β . The results are presented as mean $\pm$ SD. Statistical significance was determined in groups of 20–30 cells/sample (two independent experiments); (B) comparative uptake of non-FRET (I/Cy5.5-II) duplex probe.

in QSY21-I/Cy5.5-II duplex (i.e. the increase of Cy5.5 donor fluorescence with the temperature increase) suggested that linking of 800CW (or QSY21) and Cy5.5 to complementary strands does not lead to duplex destabilization. A small increase of melting temperature in QSY21-I/Cy5.5-II duplex suggested a probability of dye pair interaction. This appears to be in line with the observation that Cy5.5 fluorescence intensity increased after adding recombinant p50 to the reporter (Fig. 3A), suggesting a partial destabilization of QSY21-Cy5.5 pair interaction. As predicted, both FRET and quenched reporters resulted in p50 protein-mediated protection effect against exonuclease-mediated degradation *in vitro* (Fig. 3B and C). This effect was especially apparent in the case of 800CW-I/Cy5.5-II FRET reporter since in the presence of Exo III, FRET disappeared within seconds.

One of the critical limitations of ODNs is their relatively inefficient uptake by cells. However, the experiments in HUVEC culture showed that duplex reporters were clearly taken up by cells (Fig. 4A and B) and that both IL-1 β activated and control cells showed approximately equal level of fluorescence, suggesting that IL-1 β treatment did not activate the uptake of ODN duplexes (Fig. 5B). The uptake by cells can be tentatively attributed to the previously reported effect of the increased cellular tropism of fluorescent ODN duplexes (Astrib-Fisher et al., 2004). When the duplexes were added to endothelial cells expressing active NF- κ B p50 protein (Fig. 4) or the untreated control HUVEC, the radiative FRET reporter showed a statistically significant difference in relative Cy5.5 fluorescence increase when compared to control cells (Fig. 5A). In this FRET reporter Cy5.5 donor fluorescence increased only as a result of degradation, and the presence of 800CW fluorochrome as acceptor of Cy5.5 fluorescence permitted accurate ratiometric measurements of NIR fluorescence in two separate channels in live cells, which could only augment the observed quantitative differences. Importantly, the experiments with endothelial cell extracts also suggested that it is not necessary for a duplex reporter to reside in the nucleus for reporting on mature (activated) p50 binding. The EMSA clearly demonstrated that the cytoplasm of IL-1 β treated cells contained high amounts of active p50 protein that binds to the duplex reporters (Fig. 4F).

Therefore, we performed synthesis and characterization of ODNs enabling formation of either radiative FRET pairs between the ODN-linked dyes, or, alternatively, the formation of nonradiative FRET dye pairs. The *in vitro* characterization of quenched reporter suggested that it could be potentially useful in detecting transcription factors in homogenous assays. Alternatively, NIR FRET reporters can be used as the ratiometric reporters of intracellular degradation and could sense the effect of specific protein–duplex interactions due to the fact

Note that there was no difference in the uptake in IL-1 β treated (squares) or in control cells (triangles); (C) Cy5.5 fluorescence in cells incubated in the presence of 800CW-I/Cy5.5-II FRET probe. Note that the control cells (triangles) have a higher Cy5.5 fluorescence signal because of the partial FRET loss.

that the duplexes were partially degradable by exonucleases. Since the sources of emitted NIR fluorescence can be detected in live tissues at the depth of several millimeters (Bogdanov et al., 2002; Ntziachristos et al., 2002), we expect that optimized duplex ODN reporter design could be potentially useful in detecting transcriptional activation on protein level in signal transduction imaging in live cells or animals.

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