Dynamics of NF-κB and IκBα studied with GFP-fusion proteins.
Investigation of GFP-p65 binding to DNA
by fluorescence resonance energy transfer (FRET$^1$)

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**Abbreviations:** CPRG, chlorophenol red-β-D-galactopyranoside; CFP, Cyan Fluorescent
Protein; CHO, Chinese Hamster Ovary cells; FRET, Fluorescence Resonance Energy
Transfer; GFP, Green Fluorescent Protein; HEX, hexachloro-carboxyfluorescein;
HUVEC, Human Umbilical Vein Endothelial Cells; RHD, Rel Homology Domain; TET,
tetrachloro-carboxyfluorescein; β-TrCP, beta-transducin repeat-containing protein; YFP,
Yellow Fluorescent Protein
SUMMARY
We investigated the dynamics of the transcription factor NF-κB by using fusion proteins of the p65-subunit with mutants of GFP. GFP-NF-κB chimeras were functional both in vitro and in vivo as demonstrated by electrophoretic mobility shift assays and reporter gene studies. GFP-p65 was regulated by IκBα similar to wild type p65 and associated with its inhibitor even if both proteins were linked to a GFP-protein. This was also verified by FRET microscopy and studies showing mutual regulation of the intracellular localization of both GFP-chimera. Incubation of GFP-p65 with fluorescently labeled NF-κB binding oligonucleotides also resulted in FRET. This effect was DNA-sequence specific and exhibited saturation characteristics. Application of stopped-flow fluorometry to measure the kinetics of FRET between GFP-p65 and oligonucleotides revealed a fast increase of acceptor fluorescence with a plateau after about 10 milliseconds. The observed initial binding rate showed a temperature-dependent linear correlation with the oligonucleotide concentration. The association constant calculated according to pre-steady state kinetics was 3 x 10^6 M\(^{-1}\), whereas equilibrium binding studies implied significantly higher values. This observation suggests that the binding process involves a rapid association with a rather high off-rate, followed by a conformational change resulting in an increase of the association constant.

INTRODUCTION
Nuclear factor κB (NF-κB) or Rel-proteins are members of a family of eukaryotic transcription factors, which share structural and functional similarities. They are characterized by the presence of a so called Rel-homology domain, RHD, with a length of about 300 amino acids and their active DNA-binding forms are homodimeric or heterodimeric complexes consisting of combinations of members of this protein family. Their common structural feature, the RHD, is important for dimerization, DNA-binding and regulatory binding to inhibitory molecules of the IκB family (for review see 1-3). The NF-κB/Rel-factors can be divided into different functional classes. The most important distinction is the presence of transactivating domains in p65 (RelA), RelB, c-Rel and v-Rel subunits, which are not contained in p50 and p52 subunits. The latter Rel-family members originate from p100 and p105 precursors, respectively, by proteolytic cleavage of an inhibitory, IκB-like sequence from the proform. In most cells, NF-κB complexes are normally localized to the cytosol as inactive complexes with inhibitory IκB proteins. However, at least in certain cell types, mainly of the hematopoetic cell lineage, particular NF-κB members are constitutive in the nucleus, where they have essential roles in the cellular proliferation and differentiation (2). The most abundant NF-κB dimers, p50/p65 and p65/p65, which are important for the inducible expression of genes involved in inflammation, are maintained in the cytosol as inactive complexes with IκB proteins (IκBα, IκBβ and IκBε), which mask their nuclear localization sequence by binding to the RHD (2,4,5). In these cases, the activation of NF-κB is tightly regulated by signal transduction pathways leading to the phosphorylation, ubiquitynylation and proteasomal degradation of IκB molecules (for review see 6). It is generally assumed that binding of different signaling molecules like TNFα to the corresponding receptors on the cell surface, leads to an oligomerization of receptors (7)
and cytosolic adapter molecules (like TNF-receptor associated factors, TRAF’s), followed by activation of downstream kinases like NIK (NF-κB inducing kinase), or MEKK1 (MAPK/ERK kinase kinase-1) (8-10). These kinases phosphorylate and activate so-called IκB kinases (IKK1/IKK-α and IKK2/IKK-β), which are part of a multi-protein complex termed the signalosome that is responsible for the signal-induced phosphorylation of IκB molecules on two Ser-residues close together in the N-terminal regulatory domain (11-15). This phosphorylation triggers the ubiquitylation of IκB by β-TRCP variants (16,17) and its degradation by proteasomes. The proteolytic elimination of IκB molecules leads to the release of NF-κB and the unmasking of its nuclear localization sequence, followed by translocation to the nucleus and binding to cognate DNA-sequences. The occurrence of different NF-κB dimer isoforms, the formation of complexes with distinct IκB molecules, which exhibit diverse phosphorylation and degradation characteristics (2,18), and modifications in the signaling cascades leading to release of NF-κB provide enormous variation for the activation of NF-κB. Moreover, it has been shown that different members of the NF-κB family bind slightly distinct DNA sequences with different affinities (19), which represents an additional level of transcriptional regulation of NF-κB dependent genes. The classical NF-κB consensus sequence 5’GGGRNNYYCC3’ (R indicates purine and Y is pyrimidine) is an imperfect palindrome, which in itself allows a rather high level of variation (2). Interestingly, the p65/p65 homodimer can bind to just one half site of the target sequence, thereby expanding the number of potential NF-κB regulated genes dramatically. Differences in the second half site lead to variations in the association constants (20).

Up to now, the binding of NF-κB to its cognate DNA sequence was mainly studied by electrophoretic mobility shift assays, which only allows equilibrium binding analysis. Using this technique, a rather slow binding of NF-κB to DNA was reported with a plateau after about 60 min in the presence of poly-dI/dC (21) or within approximately 5 min in the absence of an excess of unspecific competitor-DNA (22). However, the high DNA-binding affinity of NF-κB complexes, with association constants in the range of $10^{10}$ to $10^{13}$ M$^{-1}$ (19,23,24), would argue for much faster binding kinetics, which cannot be resolved by the electrophoretic mobility shift techniques used so far. Therefore, we generated a GFP-NF-κB fusion protein suited for the investigation of not only the dynamics of this transcription factor in living cells, but also the binding to DNA in vitro by applying the principle of fluorescence resonance energy transfer. This principle is based on the fact that energy transfer occurs between two fluorophores when they are in close proximity (< 10 nm) and the emission spectrum of the first fluorophore (the donor) overlaps with the excitation spectrum of the second (the acceptor). The energy transfer is dependent on the quantum yield of the donor, the degree of spectral overlap and the orientation of the fluorophores and declines with the 6th power of the distance. A close association of two suited fluorophores and excitation of the donor results in an increase of acceptor fluorescence and/or a decrease in donor fluorescence due to FRET (25-27). By applying this phenomenon we could measure the kinetics of the interaction between GFP-NF-κB and fluorescently labeled oligonucleotides using stopped-flow fluorometry. We found a rapid association reaching a plateau within about 10 milliseconds, and a subsequent slow decrease of the energy transfer. This phenomenon, as well as the
difference between association constants calculated from initial binding rates and those
derived from equilibrium binding studies, indicate that the rapid association is followed
by a conformational change of the NF-κB/DNA complex, which increases the affinity.
The technique that we describe should open a wide field of possible applications, for
instance the investigation of the interaction between various NF-κB family members and
different DNA sequences for drug screening of potential inhibitors. Moreover, it could be
very valuable for the analysis of the dynamics and biochemical characteristics of protein-
protein interactions in solution using CFP- and YFP-fusion proteins. In that respect, it
should have considerable advantages over conventional assays of macromolecular
interactions, which usually do not reflect the dynamics of the association, and also over
kinetics-based methods using proteins immobilized on chip-surfaces (like surface
plasmon resonance on BIAcore™ chips), which do not represent the behavior of
macromolecules in solution.

METHODS

Construction of GFP-NF-κB and CFP-IκBα variants.
The p65 subunit of NF-κB was amplified by PCR with Pfu DNA polymerase using the
sequences 5’AAA AAA GCT TCC ACC ATG GAC GAA CTG TTC C-3’ and 5’AAA
AGG ATC CAA GGA GCT GAT CTG ACT CAG-3’ for forward- and reverse primers,
respectively. The PCR-fragment was cloned in frame into the BamH I and Hind III sites
of pEGFP-C1 (Clontech Laboratories Inc., Palo Alto, USA) generating a fusion protein
of the mammalian codon-optimized enhanced GFP protein with p65 linked to its C-
terminus. A YFP-p65 construct was generated in the same way using pEYFP-C1
(Clontech). The CFP-IκBα construct (with CFP linked to the N-terminus of IκB) was
designed by transferring the coding region of IκB from the construct described (28) into
the pECFP-C1 plasmid (Clontech). An IκB-CFP construct, where the CFP-tag is linked
to the C-terminus of IκB was cloned by inserting a PCR-fragment without stop-codon
into the Nhe I site of the pECFP-C1 vector. All constructs were verified by restriction and
sequence analysis.

Cell Culture and Transfections
CHO cells were cultured in MEM-alpha medium (GIBCO-BRL Life Technologies,
Rockville, Maryland, USA), 293 and HeLa cells in DMEM medium (GIBCO-BRL), both
including 10% FCS and 2 mM glutamine. HUVEC were grown as described (29).
Transient transfections were performed with Lipofectamine-Plus™ reagent (GIBCO-
BRL) according to the manufacturer’s protocol. In brief, CHO cells (one well of a 6-well
plate, 10 cm²) were transfected with 1 µg DNA, 5 µl Plus reagent and 2 µl Lipofectamine
for 3.5 h; 293 cells with 1 µg DNA, 4 µl Plus reagent and 3 µl Lipofectamine for 4 h.
HeLa cells were transfected with 1.5 µg DNA, 5 µl Plus reagent and 4 µl Lipofectamine
for 5 h and HUVEC with 1.5 µg DNA, 8 µl Plus reagent and 4 µl Lipofectamine for 2.5
h. Stable transfection of CHO cells with GFP-p65 was performed with the construct after
linearization by Mlu I together with a puromycin resistance vector (pPUR, Clontech,
linearized by EcoR I) using the Lipofectamine protocol as described above. Selection was
performed with 50 µg/ml puromycin (Clontech) and single GFP-p65 expressing clones
were generated by limiting dilution and evaluation of the fluorescence.
Electrophoretic Mobility Shift Assays
Whole cell extracts from GFP-p65 expressing CHO cells were prepared by repeated freeze-thawing in buffer A (20 mM Tris/HCl, pH 7.9, 1 mM EDTA, 5 mM MgCl₂, 50 mM KCl, 1 mM DTT), and pelleting of cell debris at 14000 g for 30 min. Labeling of double-stranded NF-κB binding oligonucleotides and electrophoretic mobility shift assay were done as described (30). Imaging and quantification of protein-bound and free oligonucleotides was done with PhosphorImager equipment (Molecular Dynamics).

Reporter Gene Assays
293 or HeLa cells were transiently transfected with a luciferase reporter construct under the control of the NF-κB dependent IkB promoter (28) and a β-galactosidase vector containing an ubiquitin-promoter (pUB6/V5-His/lacZ, Invitrogen, Groningen, Netherlands) as internal control. GFP-variants of p65 or IkBα, wt-p65 (31) or IkBα (28) were co-transfected with reporter and control vectors using Lipofectamine-Plus as described. Cell extracts were prepared by repeated freeze thawing in 0.1 M potassium phosphate buffer (pH 7.8). Luciferase activity was determined as described (32) and normalized to β-galactosidase activity determined with CPRG as substrate and colorimetric detection at 570 nm.

Morphological studies and FRET imaging
HUVEC or HeLa cells were transiently transfected with YFP-p65, CFP-IkB or both and investigated one day after transfection on a Nikon Diaphot TMD microscope using filter sets, which discriminate between CFP- and YFP-fluorescence (Omega Optical Inc., Brattleboro, Vermont, USA) and a cooled CCD-camera device (Kappa GmbH, Gleichen, Germany). For FRET microscopy studies, images were taken with the donor filter set (for CFP) and an acceptor filter set (XF88, Omega Optical Inc.) with excitation of the donor (440 nm), a 455 nm dichroic mirror and an emission filter for the acceptor (535 nm). Images were captured with both filter sets under identical conditions for control cells transfected with CFP and YFP, and cells transfected with IkB-CFP and YFP-p65. Ratio images were calculated by dividing the acceptor-filter image by the donor image (33) using NIH image software version 1.62. Nuclear import of GFP-p65 after addition of TNFα (200 IU/ml) to HUVEC was investigated by time series imaging using confocal laser scanning microscopy on a MRC600 equipment (BioRad, Munich, Germany).

Scanning and Stopped Flow Fluorometry
Extracts containing GFP-p65 were prepared from stable CHO-transfectants as for EMSA by repeated freeze thawing in buffer A, centrifugation at 100 000 g for 1 h at 4°C and filtration of the supernatant through a 0.45 μm filter. GFP-p65 extracts were incubated in buffer A with TET- or HEX-labeled double stranded oligonucleotides comprising the NF-κB binding site of the IkBα promoter (plus-strand: 5´-CTT GGA AAT TCC CCG-3´; minus-strand: 5´-TCG GGG AAT TTC CAA-3´ both labeled on the 5´-end). The competitor oligonucleotide had the same sequence but without label. The mutant oligonucleotide had the sequence 5´-TTA GAT TTC GTA GA-3´ with a 5´-TET-label and the corresponding complementary strand. Emission scans were performed
on a JASCO FP-920 spectrofluorometer with excitation at 482 nm (bandwidth 18 nm) and an emission bandwidth of 10 nm. Stopped flow fluorometry was performed with an Applied Photophysics SX-18MV equipment (Applied Photophysics, Leatherhead, Surrey, UK) at 15°C or 37°C with excitation at 490 nm and a 530 nm long pass emission filter. In total, 100 µl were shot into a flow cell with 1 cm light path. For monitoring the initial binding phase, fluorescence was recorded for 50 milliseconds with 2000 to 4000 data points and the recorded time course of fluorescence was fitted with an algorithm for a single exponential reaction. On- and off-rates were calculated from the concentration dependence of the initial binding rates observed (with \( k_{off} \) given by the slope of the linear curve fit, and \( k_{on} \) by the intercept on the y-axis, (34)). The association constant of the initial binding phase is defined as \( k_{on}/k_{off} \).

Investigation of longer time periods was done using 2000 data points for the initial 50 milliseconds and 2000 data sets for subsequent 1000 milliseconds.

**RESULTS**

**Functional integrity of GFP-p65-NF-κB**

We generated a fusion construct of the p65 NF-κB protein with a mammalian codon-optimized enhanced mutant of GFP (EGFP) linked to the N-terminus of p65. On the basis of the crystal structure of the p65 homodimer (20) or the p50/p65 heterodimer (35) bound to a cognate DNA sequence, we expected that the N-terminal GFP-tag would not interfere with binding of p65 to DNA. Moreover, the C-terminus comprising the two transactivation domains, which are essential for interaction with the transcriptional machinery, should remain unaffected by the N-terminal GFP. The C-terminal domain was also reported to be the target of a phosphorylation event that is essential for full transcriptional activity (36). To test whether the GFP-p65 fusion protein is still functional with respect to binding in vitro, we performed electrophoretic mobility shift assays with the fusion protein compared to wild type p65. CHO cells were transiently transfected with equal amounts of wild type p65 or GFP-p65 and extracts were prepared as described in the methods section. Incubation of these extracts with \(^{32}P\)-labeled NF-κB binding oligonucleotides and analysis of the electrophoretic retardation of protein-bound oligonucleotides revealed a comparable binding activity for both wild type and GFP-p65. The binding was specific for NF-κB in both cases as shown by competition with a 200-fold molar excess of unlabeled oligonucleotides and the inability of a mutant competitor to prevent binding (Fig. 1). These results indicate that GFP-p65 binds in vitro to the NF-κB cognate sequence as well as wild type p65. Our next aim was to verify whether GFP-NF-κB fusion proteins are also functional in vivo. For that purpose, we used reporter gene assays with a NF-κB dependent luciferase reporter. Transfection of HeLa cells with fusion proteins of p65 with GFP-variants (GFP or YFP) induced a strong up-regulation of luciferase reporter activity, which reached the same level as with wild type p65 (Fig. 2). The induction of luciferase activity could be blocked both for wild type p65 and for GFP- or YFP-p65 by co-transfection of a vector coding for IkBα, indicating that GFP-p65 variants are still binding in vivo to their physiologic inhibitor. Moreover, the same inhibition of NF-κB dependent reporter induction could be achieved by expressing a
fusion protein of IκBα with CFP linked either to the N- or the C-terminus, demonstrating that GFP-p65 or YFP-p65 still bind to a IκBα-GFP fusion protein. Similar results were obtained with 293 and CHO cells (data not shown). Taken together, these data indicate that GFP-p65 proteins are fully functional both *in vitro* and *in vivo*. Fluorescence microscopy of different cell types (CHO, 293, HUVEC, HeLa cells) that were transiently transfected with GFP-p65 revealed localization patterns dependent on the amount of protein expressed (Fig. 3A). Cells with low fluorescence intensity showed mainly cytosolic localization of the GFP-NF-κB fusion protein indicating complete binding to endogenous IκB molecules. However, cells with a high expression level of GFP-p65 exhibited a predominant nuclear fluorescence implying that the endogenous IκB was saturated leading to the nuclear translocation of the excess GFP-p65. Cotransfection of a YFP-p65 construct with a vector coding for CFP-IκB resulted in cytosolic localization of both proteins in nearly all of the transfected cells, as demonstrated by microscopy filter combinations that distinguish between the two GFP-variants. This observation supports the data obtained with reporter gene assays showing inhibition of NF-κB activity by CFP-IκBα fusion proteins. Interestingly, transfection of CFP-IκB without p65 revealed a predominant nuclear localization of the CFP-IκB fusion protein. This fusion protein has a significantly higher molecular weight than the postulated limit for passive diffusion through the nuclear pore complex, indicating an active nuclear import, as previously demonstrated for the wild type IκB (37,38). The interaction between YFP-p65 and CFP-tagged fusion proteins of IκBα in the cytosol of living cells was further supported by FRET microscopy using ratio imaging of donor and acceptor fluorescence. This imaging technique revealed a significantly higher ratio of acceptor to donor fluorescence at the excitation wavelength of the donor for cells transfected with CFP-IκB and YFP-p65 compared to control cells transfected with CFP and YFP investigated under the same conditions of transfection and imaging. Cotransfection of YFP-p65 with an IκB-CFP construct, coding for a fusion protein with the CFP-tag linked to the C-terminus, where it should be closer to the YFP-portion of p65 based on the known crystal structure (4,5) revealed an even higher ratio of acceptor to donor fluorescence. The ratio image indicated YFP-p65/IκB-CFP complexes in the cytosol, whereas that from the CFP/YFP control showed no remaining fluorescence signal (Fig. 3B). In addition to ratio imaging on a single cell level, we quantified the levels of fluorescence for a larger number of cells with donor and FRET-filter sets, in order to obtain average values, which should eliminate cell to cell variations of donor and acceptor expression. This quantitative evaluation showed a significantly increased ratio of acceptor to donor emission at the donor excitation wavelength and supported the FRET imaging results (data not shown).

We further verified the functional integrity of GFP-p65 by morphological analysis of transfected endothelial cells after administration of TNFα. Cells with a low expression level of GFP-p65 and a cytosolic localization, which reflect a physiological expression and regulation of the NF-κB fusion protein, showed a marked translocation of GFP-p65 into the nucleus after TNFα administration (Fig. 4), indicating degradation of the endogenous IκB and release of GFP-p65. Further investigation of GFP-p65 by fluorescence microscopy revealed subsequent nuclear export, which could be inhibited by leptomycin B, a drug that blocks nuclear export processes via exportin/crm1p (data not...
shown). This is in line with previous reports demonstrating nuclear export of p65 bound to its inhibitor IκB via a leptomycin B sensitive, crm1p-dependent transport (37,38).

**Fluorescence Resonance Energy Transfer between GFP-NF-κB and fluorescently labeled oligonucleotides**

After verifying the functional integrity of GFP-p65, we aimed to exploit the fluorescence properties of the fusion protein for studies on binding to its cognate DNA-sequence by applying the principle of fluorescent resonance energy transfer (FRET). The fluorescence characteristics of enhanced GFP, with an excitation peak at 488 nm and the maximum emission at 507 nm, suggested the use of acceptor fluorophores with excitation peaks in the range of 500 nm to 540 nm. Commercially available covalent tags for oligonucleotides in that range are TET (tetrachloro-carboxyfluorescein) and HEX (hexachloro-carboxyfluorescein), with excitation peaks at 522 nm and 535 nm, respectively. Incubation of GFP-p65 extracts with TET-labeled NF-κB binding oligonucleotides showed a significant increase of acceptor fluorescence (Fig. 5A), detected as a distinct shoulder on the emission scan (after subtraction of the TET-emission curve). Addition of increasing amounts of TET-labeled DNA to the GFP-p65 extract showed a saturation characteristics of the FRET effect calculated as ratio of acceptor to donor fluorescence at the excitation wavelength of the donor (Fig 5B). Using HEX-labeled DNA as acceptor, we observed only a slight increase of the acceptor fluorescence, but a significant decrease of the GFP-emission. This decrease was a DNA-sequence specific effect as shown by the restoration of the donor fluorescence after addition of unlabeled competitor DNA in excess (Fig. 5C). We assume that the rather small overlap of the GFP-emission with the excitation spectrum of HEX favors a donor quenching rather than an increase in acceptor fluorescence.

**Kinetics of GFP-NF-κB binding to DNA**

The application of the FRET principle allows to obtain much more information on the binding process between proteins and DNA than the use of conventional techniques like electrophoretic mobility shift assays. One of the advantages is the possibility to determine the kinetics of the association by monitoring the time course of the FRET effect. We aimed to determine the kinetics of GFP-NF-κB binding to DNA by using stopped flow fluorometry, which is suited to measure changes in fluorescence in the millisecond range. Mixing of a cell extract from stable transfectants expressing GFP-p65 with a TET-labeled NF-κB binding oligonucleotide resulted in a distinct increase of acceptor fluorescence at the donor excitation wavelength reaching a plateau already after about 10 milliseconds (Fig. 6A). No temporal change of fluorescence was observed with a mutant TET-labeled DNA-sequence that does not bind to NF-κB.

For a further evaluation of the binding process, we investigated the concentration dependence of the observed initial binding rates at 15°C and 37°C deduced from single exponential fits between 1 and 10 milliseconds (Fig. 6B). Interestingly, we found rather high off-rates of $3.3 \times 10^2$ s$^{-1}$ and $2.4 \times 10^2$ s$^{-1}$ for 15°C and 37 °C, respectively. Given the on-rates deduced from Fig. 6B of $3.0 \times 10^8$ M$^{-1}$ s$^{-1}$ and $7.3 \times 10^8$ M$^{-1}$ s$^{-1}$, we calculate association constants $K_{\text{ass}}$ of $9.2 \times 10^5$ M$^{-1}$ and $3.0 \times 10^6$ M$^{-1}$ for 15°C and 37 °C,
respectively. These constants are significantly lower than postulated in previous reports, which claim association constants in the range of $10^{10}$ M$^{-1}$ for p65 homodimers as determined by electrophoretic mobility shift assays (Fujita et al., 1992). In order to clarify whether this difference in the calculated binding affinity from previously reported values is due to a reduced affinity of the GFP-fusion protein or a difference in pre-steady state versus equilibrium binding affinity, we applied the same technique of electrophoretic mobility shift titration assays that was previously used to determine the equilibrium binding constants. Constant amounts of GFP-NF-$\kappa$B extracts were incubated with increasing amounts of radioactively labeled NF-$\kappa$B binding oligonucleotides and subjected to gel electrophoresis. The relation of bound to free oligonucleotides was used to calculate the association constant by Scatchard analysis, and revealed a value of about $4 \times 10^8$ M$^{-1}$ (Fig. 7). This number is somewhat lower than reported for p65 homodimers, but still significantly higher than that calculated from off- and on-rates determined by stopped flow analysis. These data indicate that the GFP-portion of the fusion protein may cause some reduction in the equilibrium binding affinity. However, it is apparently not the reason for the distinct difference between the affinity determined by analysis of the pre-steady state kinetics and that observed under equilibrium conditions. One possibility would be that a second, slower reaction takes place, which increases the overall affinity. To test for this possibility, we investigated the FRET effect between GFP-p65 and TET-labeled oligonucleotides for longer time periods. These studies showed a rather slow decrease of FRET, which became noticeable after about 20 milliseconds (Fig. 8A). This decrease was apparently more prominent at 37°C with approximately 30% reduction of the FRET effect between 10 and 50 milliseconds, whereas it was only about 10% in the same time at 15°C (Fig. 6A). The decline was not due to a bleaching process, since GFP-p65 or TET-labeled oligonucleotide alone showed no reduction of fluorescence (data not shown). This second phase of the interaction between GFP-p65 and oligonucleotides could be fitted by a decay of fluorescence with the natural logarithm of time according to the formula:

$$\text{fluorescence} = k \times \ln(t)$$

Analysis of the second phase with different amounts of oligonucleotides could not detect any significant correlation between the constant $k$ and the oligonucleotide concentration (Fig. 8B). In principle, a dissociation of GFP-NF-$\kappa$B and the TET-labeled oligonucleotide could be a potential reason for the observed decrease of the FRET. However, in that case there should be a linear correlation between the natural logarithm of the fluorescence and time (39). Moreover, electrophoretic mobility shift assays indicated a strong, high-affinity binding of the oligonucleotide to GFP-p65 even after prolonged incubation. Therefore, we assume that the observed decrease of fluorescence is caused by a conformational change of the GFP-p65/DNA complex.

DISCUSSION
We generated a fusion protein of p65 NF-$\kappa$B with enhanced GFP on the N-terminus and could demonstrate the functional integrity of this chimeric protein both in vitro and in vivo by the use of electrophoretic mobility shift and reporter gene assays, respectively. Under normal conditions of gel retardation assays, where an excess of oligonucleotides is used, we observed binding of GFP-p65 to its cognate DNA motif, which was equivalent
to that of wild type p65. However, this does not rule out the possibility that the affinity is lower under non-saturating conditions. In fact, electrophoretic mobility shift assays with various non-saturating concentrations of NF-κB binding oligonucleotides suggested a slightly lower affinity of GFP-p65 compared to values reported for wild type p65 (19), which might be the result of the GFP-moiety. Furthermore, the difference between reported affinities and those that we found in electrophoretic mobility shift assays might have been caused by subtle differences in the oligonucleotide sequence. Nonetheless, using reporter gene assays, we found an equivalent activity in vivo of GFP-p65 and wild type p65, with respect to the induction of NF-κB dependent promoters. Moreover, the GFP-fusion protein was inhibited by IκB, as is wildtype p65, and could also be blocked by a fusion protein of IκB and CFP, demonstrating that the transcription factor can bind in vivo to its physiologic inhibitor even when both proteins are linked to a GFP-variant. These findings were further supported by FRET microscopy using ratio imaging of donor and acceptor fluorescence and by morphological analysis, showing that CFP-IκB and YFP-p65 prevent each other from translocating into the nucleus. Transient overexpression of GFP-p65 without its inhibitory molecule IκB led to distinct fluorescence localization pattern depending on the expression level. While cells with rather low fluorescence exhibited a cytosolic localization of GFP-NF-κB, cells with a high expression level showed a predominant nuclear fluorescence. We assume that the cytosolic localization of GFP-p65 in cells with low expression is rather due to binding to endogenous IκB than due to a putative nuclear export sequence that was recently postulated to be active in COS cells (40). This notion was supported by the observation that TNFα induced a nuclear translocation of cytosolic GFP-p65, which can be explained by a degradation of bound endogenous IκB, while it is unlikely to affect a putative nuclear export mechanism of p65. Moreover, transfection experiments using low amounts of the GFP-p65 vector revealed a predominant cytosolic fluorescence, whereas co-expression of the same amount of GFP-p65 with an excess of unlabeled p65 lead to a significant shift from cytosolic to nuclear fluorescence, indicating that the endogenous IκB is saturated by the unlabeled p65 (data not shown). It might be dependent on the cell type, whether the localization of p65 is regulated by IκB molecules or by an intrinsic nuclear export sequence. This view is supported by the observation that overexpression of p65 leads to a predominant cytosolic localization in COS cells (40), whereas the majority of endothelial cells exhibit a nuclear localization. Our results on nuclear translocation of cytosolic GFP-p65 after addition of TNFα to transfected HUVEC is also in line with a very recent report, showing nuclear import of a GFP-p65 fusion protein in fibroblasts in response to IL-1β (41). The localization that we observed for CFP-IκB in the absence of co-transfected p65 is further support for the current assumption that IκB is actively transported into the nucleus instead of a passive diffusion as formerly suggested (42,43). The fusion protein is significantly larger than the threshold value for diffusion through the pore complex, whereas wild type IκB would be small enough for such a passive translocation. In a recent report, it has been demonstrated that there is an active nuclear import of IκB in vitro, which requires the ankyrin repeats (44). However, the quantitative relation of this active transport compared to a potential passive diffusion is not known in detail for the in vivo situation. Our results imply that the active nuclear import might constitute the major
portion of the transport, or is at least capable of transferring nearly all of the IκBα, since we find in vivo a predominant nuclear fluorescence after overexpression of CFP-IκB.

After verifying the functional integrity of GFP-p65 in various assays, we aimed to exploit its fluorescence properties for binding studies with fluorescently labeled DNA using FRET technology. We were able to monitor a significant change of the emission curve with different fluorophores that we used as labels for the NF-κB binding oligonucleotides. Incubation of GFP-p65 with TET-labeled oligonucleotides resulted in a significant increase in acceptor fluorescence, whereas incubation with HEX-labeled DNA caused rather a decrease of donor and only a slight augmentation of acceptor fluorescence. The observed difference between TET- and HEX-labeled oligonucleotides in energy transfer experiments with GFP-p65 might result from the smaller overlap between the emission spectrum of GFP and the excitation spectrum of HEX compared to that of TET, which would favor a consumption of the absorbed energy by other mechanisms, like molecular oscillations, instead of emission of acceptor fluorescence. However, for both fluorophores the detected spectral shifts were DNA-sequence specific, as shown by competition experiments or labeled oligonucleotides that do not bind to NF-κB. Our data on the kinetics of the binding process between NF-κB and DNA by using stopped flow fluorometry of the FRET effect between GFP-p65 and TET-labeled oligonucleotides provide important information for the dynamics of this transcription factor. In principle, stopped-flow measurements of binding reactions can be achieved, at least in certain cases, by monitoring changes of the intrinsic tryptophan fluorescence caused by conformational changes. However, the application of functional GFP fusion proteins and FRET analysis seems especially useful in stopped-flow measurements, since it circumvents the need for highly purified protein preparations, which are normally necessary for determination of the intrinsic fluorescence and which often retain only part of the original physiologic activity.

Using time-resolved FRET analysis, we could show that the association between GFP-p65 and corresponding oligonucleotides occurs within several milliseconds. The first reports using equilibrium binding studies based on electrophoretic mobility shift assays proposed a very slow binding process with a plateau after about 60 min (21), which seems rather unlikely given the high affinity of this transcription factor for its cognate DNA-sequence, with an association constant in the range of 10^{10} M^{-1} to 10^{12} M^{-1} (19). It has to be noted that the binding reaction in the study of Zabel and Baueerle (21) was done in the presence of 100 µg/ml poly-dI/dC, which is usually added in electrophoretic mobility shift assays to improve the specificity of the technique. However, later studies of the same group demonstrated that this compound has to be omitted in experiments measuring the affinity of binding, since it is a significant competitor of the binding process with an IC_{50}-concentration of about 38 µg/ml and also influences the kinetics of association. Gel retardation assays in the absence of poly-dI/dC revealed an equilibrium of the binding process within less than 5 minutes (22), which is apparently faster than the time limit that can be resolved by that technique. Our present study is to our knowledge the first report on the kinetics of binding between a NF-κB molecule and its cognate DNA sequence in solution, using a technique that is suited to resolve reactions in the millisecond range. Interestingly, the affinity that we calculated based on k_{on} and k_{off} rates derived from pre-steady state kinetics is significantly lower than the value derived from Scatchard analysis of data obtained by electrophoretic mobility shift assays using the
same GFP-p65 preparation. Although we found somewhat lower steady state affinities for the GFP-p65 fusion protein compared to values reported for wild type p65 homodimers (19), the GFP-moiety is apparently not the reason for the observed difference between the association constant calculated from pre-steady state kinetics and that derived from equilibrium binding studies. A possible explanation for this difference is that the binding process includes a fast association with lower affinity, followed by a slower reconfiguration of the complex leading to an increase of the association constant. Stopped-flow analysis of the FRET effect for 1 second actually indicated a conformational transition after the first binding process, resulting in a decrease of FRET. This decrease was not due to bleaching of fluorophores and it could not be explained by dissociation of GFP-p65 and TET-labeled DNA, since it did not follow the kinetics of a dissociation process. Moreover, electrophoretic mobility shift assays, which were done after 15 min of incubation, still showed a strong, high-affinity binding. The observed decrease of fluorescence might be caused by an increased distance between the GFP-tag and the TET-label, or alternatively by a decrease of the distance between the two GFP-molecules of a GFP-p65 homodimer, resulting in a mutual donor quenching and a subsequent decrease of acceptor fluorescence. The on-rate that we measured for the first stage of binding between GFP-p65 and DNA is comparable or even higher than values reported for other DNA-binding proteins. However, the off-rate of $2.4 \times 10^2$ s$^{-1}$ that we observed for this initial phase, seems considerably higher than the off-rates reported for these proteins (34,45,46). It seems possible that a subsequent decrease of the off-rate due to a conformational change results in a corresponding increase of the association constant. A decrease of $k_{off}$ to a number in the range of $10^9$ s$^{-1}$, as described for RNA polymerase (34), would lead to a corresponding increase of the association constant from $3 \times 10^6$ M$^{-1}$ to $10^8$ - $10^9$ M$^{-1}$, which would be in line with our data obtained with equilibrium binding assays.

It has to be noted, however, that these values that are derived from in vitro studies cannot reflect the complexity of the situation in vivo, where the conditions for diffusion and accessibility of NF-$\kappa$B binding sequences are probably significantly different. Nevertheless, our study demonstrates that GFP-fusion proteins are not only useful to investigate the dynamics of a great variety of proteins in living cells, but also to monitor the biochemical characteristics of macromolecular interactions in solution by the means of time-resolved measurements of FRET in the millisecond range. The application of this technique to the investigation of protein-protein interactions using appropriate GFP-variants might expand our understanding of these interactions considerably.

Acknowledgements
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References
Fig. 1: Binding of GFP-p65 to its cognate DNA sequence.
Extracts from CHO cells that were transiently transfected with wild type p65 (wt-p65) or GFP-p65 were subjected to electrophoretic mobility shift assays with $^{32}$P-labeled NF-κB binding oligonucleotides in the absence (-) or presence (+) of a 200 fold molar excess of unlabeled competitor oligonucleotides of the same DNA sequence (comp.) or a mutated sequence (mut. comp.). The localization of wild type p65 (*) and GFP-p65 (#) is indicated.

Fig. 2: Fusion proteins of p65 or IκBα with GFP-variants are functional in reporter gene assays.
HeLa cells were transiently transfected in triplicates with a luciferase reporter construct containing a NF-κB dependent promoter (0.5 µg per 6-well), a plasmid coding for β-galactosidase under the control of an ubiquitin-promoter (NF-κB independent) as internal control (0.5 µg) and p65, IκBα or GFP-variants thereof, as indicated (0.05 µg of the p65-construct and 0.45 µg of the IκBα vector or an empty control vector). Cell extracts were prepared and luciferase, as well as β-galactosidase activity determined as described in the methods section. Luciferase activity normalized to β-galactosidase is expressed as fold induction of control.

Fig. 3: Microscopy of YFP-p65 and CFP-IκBα in living cells.
A) Human endothelial cells (HUVEC) were transfected with YFP-p65 (left panel), CFP-IκBα (middle panel) or both (right panel). Images were taken with a cooled CCD camera one day after transfection using the appropriate filter sets for CFP and YFP, respectively. The image in the right panel showed the same fluorescence pattern with both filter sets.
B) FRET microscopy using ratio imaging: HeLa cells were transfected with equal amounts of CFP and YFP or IκB-CFP and YFP-p65 and images were taken with the donor filter set and the acceptor filter set as described in the methods section. The ratio images represent the division of the acceptor-filter images by the donor-filter images. In the case of the negative control, fluorescence is higher with the donor-filter set, whereas for the IκB-CFP/YFP-p65 pair it is higher in the acceptor filter set, resulting in a distinct ratio image.

Fig. 4: Cytosolic GFP-p65 is translocated to the nucleus in response to TNFα.
HUVEC cells were transiently transfected with GFP-p65. Cells with a low expression level, which showed cytosolic fluorescence, were investigated by confocal laser scanning microscopy followed by addition of TNFα (200 u/ml) and subsequent morphological analysis of the same cells.
Fig. 5: Binding between GFP-p65 and fluorescently labeled oligonucleotides can be monitored by FRET analysis.
A) Extracts from stable CHO-transfectants expressing GFP-p65 were incubated with TET-labeled NF-κB binding oligonucleotides (in the presence of 0.1 mg/ml Poly-dI/dC) and analyzed by scanning fluorometry with excitation of the donor fluorophore (GFP). The emission curve of the TET-fluorescence in the absence of GFP-p65 was subtracted from the emission scan of the sample containing both fluorophores. The resulting emission curve (GFP-p65/Tet-DNA, open squares, □) was compared with the GFP-p65 emission in the absence of labeled oligonucleotides (GFP-p65, filled circles, ●). B) Equal amounts of GFP-p65 extracts were incubated with increasing amounts of TET-labeled oligonucleotides and measured by fluorometry. FRET was determined by calculating the ratio of acceptor fluorescence to donor fluorescence. This ratio is plotted against the concentration of oligonucleotide. C) FRET between GFP-p65 and HEX-labeled oligonucleotides (determined as in A) shows a significant decrease of donor fluorescence (GFP-p65/Hex-DNA, open circles, ○) compared to GFP-p65 alone (thick line). Addition of an unlabeled competitor oligonucleotide in excess restores the donor emission (open triangles, ?)

Fig. 6: Kinetics of binding between GFP-p65 and fluorescently labeled DNA as measured by stopped flow fluorometry of the FRET.
A) GFP-p65 extracts were mixed with TET-labeled NF-κB binding oligonucleotides (Tet-DNA) or labeled non-binding oligonucleotides (mutant Tet-DNA) at 15°C in the mixing chamber of a stopped flow system and the acceptor fluorescence at the excitation of the donor was recorded for 50 milliseconds. B) Equal amounts of GFP-p65 extracts were mixed with increasing concentrations of TET-labeled NF-κB binding oligonucleotides and the kinetics of binding measured by stopped flow fluorometry for 50 milliseconds. The resulting time course of the acceptor fluorescence was fitted by a single exponential algorithm to obtain the initial binding rates. The observed rates for 37°C (filled squares, ■) and 15°C (open squares, □) are plotted against the concentration of the oligonucleotide and the corresponding correlation is calculated by linear regression. The y-axis intercept indicates the off-rate and the slope of the line is a measure for the on-rate of binding.

Fig. 7: Affinity of GFP-p65 for NF-κB binding oligonucleotides as determined by an equilibrium binding assay (electrophoretic mobility shift assay).
Constant amounts GFP-p65 extracts were incubated for 15 min with different concentrations of ³²P-labeled NF-κB binding oligonucleotides and subjected to native PAGE. NF-κB-bound and free radioactivity was quantified using a PhosphorImager and analyzed by Scatchard plotting. The equation and the correlation coefficient of the linear regression fit are given, as well as the deduced association and dissociation constant.
Fig. 8: Stopped-flow analysis of the FRET effect indicates a conformational transition of the GFP-p65/TET-DNA complex after the binding.

A) Kinetics of the FRET effect on a logarithmic time scale. The interaction between GFP-p65 and TET-labeled oligonucleotides was measured at 37°C by stopped flow fluorometry with 2000 data sets for the initial 50 milliseconds and 2000 data points for the subsequent 1000 milliseconds. Representative points are shown and fitted by a single exponential equation (dashed line) between 1 and 10 milliseconds, and by a logarithmic algorithm \( \text{fluorescence} = k \times \ln(\text{time}) \), solid line) between 10 and 100 milliseconds. The absolute fluorescence of the GFP-p65 extract was lower than in Fig 6A, resulting in a lower absolute amplitude of the fluorescence change. B) Equal amounts of GFP-p65 were analyzed with increasing concentrations of TET-labeled oligonucleotides and the second phase of the interaction (10 - 100 milliseconds) was fitted with the logarithmic algorithm. The calculated constant k is plotted against the oligonucleotide concentration indicating no concentration dependence of the second phase.
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**A**

![Graph of rel. fluorescence vs. seconds for Tet-DNA and mutant Tet-DNA](image)

**B**

![Graph of rate (1/sec) vs. DNA (µM)](image)

- **Tet-DNA**
  - Equation: \( y = 303.88x + 329 \)
  - \( R^2 = 0.9941 \)

- **Mutant Tet-DNA**
  - Equation: \( y = 734.21x + 244.09 \)
  - \( R^2 = 0.9508 \)
$y = -0.353x + 1.3695$

$R^2 = 0.9775$

$K_{ass} = 0.353 \times 10^{9}$

$K_{diss} = 2.83 \times 10^{-9}$
Dynamics of NF-kappa B and I-kappa-B-alpha studied with GFP-fusion proteins. Investigation of GFP-p65 binding to DNA by fluorescence resonance energy transfer (FRET)

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