Nuclear translocation of green fluorescent protein-nuclear factor κB with a distinct lag time in living cells

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Received 7 December 1998

Abstract A highly fluorescent mutant form of the green fluorescent protein (GFP) has been fused to the human nuclear factor κB (NF-κB) p50 and p105 (p50/IκBγ), a precursor protein of NF-κB p50. GFP-p50 and GFP-p105 were expressed in monkey COS-7 cells and human HeLa cells. Translocation of these chimeric proteins was observed by confocal laser scanning microscopy. GFP-p50 (without IκBγ) in the transfected cells resided in the nucleus. On the other hand, GFP-p105 (GFP-p50 with IκBγ) localized only in the cytoplasm before stimulation and translocated to the nucleus with stimulant specificity similar to that of native NF-κB/IκB. In addition, the translocation of NF-κB to the nucleus had a distinct lag time (a quiescent time) in the target cells. The lag time lasted 10–20 min after stimulation with hydrogen peroxide or tumor necrosis factor α. It was suggested that this might be due to the existence of a limiting step where NF-κB is released from NF-κB/IκB by the proteasome.

Key words: Nuclear factor κB; Green fluorescent protein; Nuclear translocation; Confocal laser scanning microscopy; Hydrogen peroxide; Tumor necrosis factor α

1. Introduction

Nuclear factor κB (NF-κB) and the other members of the Rel family of transcriptional activator proteins have been a focal point for understanding how extracellular signals induce the expression of specific sets of genes in higher eukaryotes. Unlike most transcriptional activators, this family of proteins resides in the cytoplasm and must therefore translocate into the nucleus to function [1]. NF-κB is the prototype of a family of dimeric transcription factors made from monomers that have approximately 300 amino acid Rel regions which bind to DNA, interact with each other, and bind the IκB inhibitors [2,3]. The inhibitors have 5–7 ankyrin (Ank) repeat domains, each of about 30 amino acids, which form a unit able to interact with Rel regions [4].

It is known that the inducible cytoplasmic form of NF-κB is assembled from the products of at least three different genes, encoding p105, IκB and p65 (RelA) [5]. p105 is a precursor protein of p50 and has a role as a Rel-specific inhibitor [6]. The N-terminal portion of p105 corresponds to the p50 subunit of the prototypical NF-κB complex. p50 either assembles with other Rel family members or alternatively self-assembles as homodimers [7–9]. The C-terminal portion of p105 corresponds to the IκBγ which is remarkable for the presence of Ank repeats resembling those present in IκBα [8]. In this regard, p105 binds to and sequesters other Rel-related proteins in the cytoplasm, thereby functioning as an IκB.

Activation of NF-κB is controlled by IκB which retains NF-κB in the cytoplasm because IκB masks the nuclear localization signals (NLS) of p50 and p65. Any one of several extracellular inducers activates one or more signal transduction pathways leading to the activation of protein kinases that phosphorylate p105 and IκB. Phosphorylated p105 and IκB are then recognized by ubiquitin-conjugating enzymes. Ubiquitinated p105 and IκB are then processed and degraded by the 26S proteasome to produce active NF-κB, which translocates to the nucleus. Finally nuclear NF-κB synergizes with other transcriptional activators to form a higher order enhancer complex [1].

Here, we studied by confocal laser scanning microscopy (CLSM) the translocation of NF-κB to the nucleus in living cells after stimulation. As the green fluorescent protein (GFP) provides a useful tool to trace chimeric proteins in living cells [10–14], we prepared a fusion protein between GFP and p50 or p105 (p50/IκBγ), a precursor protein of NF-κB p50. The former resided in the nucleus even without stimulation, but the latter was in the cytoplasm and translocated to the nucleus after stimulation with a distinct lag time. It seemed that this lag time was mainly due to the process releasing NF-κB from NF-κB/IκB by the proteasome [1,6].

2. Materials and methods

2.1. Construction of GFP-p50 and GFP-p105

The construct used for GFP-p105 (p50/IκBγ) in this paper is summarized in Fig. 1. GFP expression vector (pCMX-SAH/Y145F) was kindly given by Prof. K. Umesono and Dr. H. Ogawa (Nara Institute of Science and Technology). Human NF-κB gene was obtained from ATCC (Rockville, MD). NF-κB cDNA in Bluescript KS^- served as a template in the PCR amplification using appropriate oligonucleotide primers. For the generation of a GFP-p105 chimeric protein, NF-κB cDNA was amplified with oligonucleotides such that *Sal*I and *Nhe*I restriction sites were introduced at the 5′ and 3′ ends, respectively (Fig. 1). The fusion between GFP and N-terminal of p105 was achieved by *Sal*I and *Nhe*I restriction sites as described above. Construction of GFP-p50 was done by a similar procedure for GFP-p105.

2.2. Transfection of plasmid DNA

COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 100 U/ml penicillin G and 100 μg/ml streptomycin and 10% FBS from Biowhittaker (Walkersville, MD). HeLa cells were cultured in Eagle's minimal essential medium from Nissui (Tokyo) supplemented with 10% FBS. The cells were electroporated in cold K^+-PBS buffer with 20 μg of plasmid DNA at 300 V and 950 μF using Gene Pulser (Bio-Rad). COS-7 and HeLa cells transfected with the GFP-p105 or GFP-p50 plasmid DNAs were cultured in 35 mm dishes for a few days and were used for the experiments.

2.3. Western blot analysis

Western blot analysis was done following a previously described procedure [15]. To prepare whole cell lysate, collected cells were sus-

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PII: S0014-5793(99)00002-2

pended in NP-40 lysis buffer (20 mM HEPES, pH7.9, 50 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 0.1% (v/v) Nonidet P-40, 1 mM dithiothreitol, 10 mM sodium orthovanadate) supplemented with 10 μg/ml leupeptin and 0.5 mM phenylmethylsulfonyl fluoride and allowed to stand on ice for 30 min. The suspension was clarified by centrifugation (20 000×*g*, 20 min). After centrifugation, the resulting supernatants were solubilized by treatment with Laemmli buffer at 100°C for 3 min and separated by electrophoresis in 10% SDS-polyacrylamide gel. The electrophoresed proteins were transferred to PVDF membrane with an electroblotter. After blocking with 0.5% casein, the membranes were probed with 10 μg/ml of anti-GFP antibody and treated with FITC-labelled anti-rabbit IgG (10 μg/ml). The amount of FITC-labelled IgG bound to each protein band was determined by Fluoro Imager 595 (Molecular Dynamics).

2.4. Confocal laser scanning microscopy

The transfected cells were washed with fresh medium and were observed by a confocal laser scanning fluorescence microscope using a krypton/argon ion laser from Bio-Rad (MRC-1024) [16,17]. GFP fluorescence was excited at 488 nm and its emission was observed through a band filter (506–538 nm). The observation chambers were kept at 37°C.

3. Results

A highly fluorescent mutant form of GFP has been fused to the human NF-κB p50 and p105 (p50/IκBγ), a precursor protein of NF-κB p50. To develop a highly efficient, fluorescent version of NF-κB, we used GFP-p50 and GFP-p105 chimeras with a 28 kDa GFP variant fused in frame to the second amino acid of the human NF-κB gene (Fig. 1). The GFP variant (pCMX-SAH/Y145F), from jellyfish *Aequorea victoria*, contains a serine-to-alanine substitution at position 65 (S65A mutation), which makes the resulting chromophore more fluorescent than the wild-type GFP [11,18].

When the GFP-p105 chimera is introduced into cultured cells, a fusion polypeptide with a predicted molecular mass of 133 kDa is produced as shown by Western blot analysis (Fig. 2). This molecular mass was consistent with the sum of p105 and GFP (28 kDa).

Because the S65A variant of the GFP chromophore is resistant to photobleaching, it could used for CLSM to observe GFP-p105 during extended periods (0.5–1.0 h) of time. Then, using CLSM, we examined transfected cells, COS-7 and HeLa cells, for subcellular localization of the chimeric GFP-p50 and GFP-p105 proteins (see Fig. 3). GFP-p50 (without IκBγ) was found only in the nucleus without stimulation. However, GFP-p105 (GFP-p50 with IκBγ) resided in the cytoplasm and translocated to the nucleus with stimulant specificity similar to that of native NF-κB/IκB. Thus, the GFP was functional as a chromophore in essentially all of the expressing cells in this mammalian system.

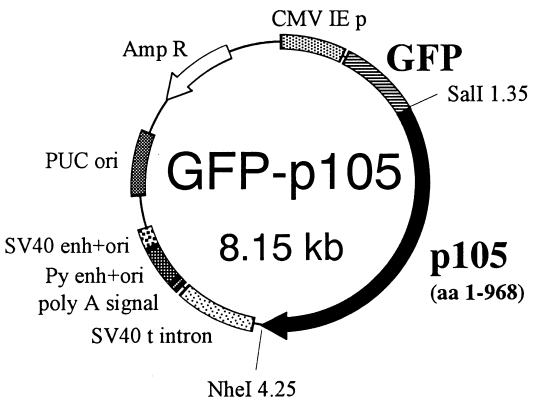

Fig. 1. The construction of GFP-p105 (p50/IκBγ). p105 was fused at its N-terminus to the GFP in pCMX-SAH/Y145F.

Fig. 2. Western blot analysis of GFP-p105 (p50/IκBγ) using anti-GFP antibody. One major band, 133 kDa, was observed, shown in this figure.

Upon exposure to hydrogen peroxide or tumor necrosis factor α (TNF-α), translocation of GFP-NF-κB into the nucleus occurred mostly in the fluorescing target cells. A typical example of the time course of GFP-NF-κB fluorescence in the nucleus of target cells (COS-7 and HeLa cells) is shown in Fig. 4. With a lag time of 10–20 min after stimulation the fluorescence of GFP-NF-κB increased in the nucleus of the transfected cell, as shown in Fig. 4 (open circles). In contrast, the fluorescence intensity in the cytoplasm decreased with lag times, as shown in Fig. 4 (closed circles). The translocation of GFP-NF-κB was dependent on the concentration of the stimulants and on the temperature. As shown in Fig. 4A, 250 μM hydrogen peroxide was enough to induce the translocation of GFP-NF-κB from the cytoplasm to the nucleus in our present experimental conditions. Similar kinds of the nuclear translocation were also observed after stimulation with TNF-α (see Fig. 4B).

4. Discussion

In this paper we show that GFP-p50 in transfected cells (COS-7 and HeLa cells) occurred in the nucleus. On the other hand, GFP-p105 (GFP-p50/IκBγ) localized only in the cytoplasm before stimulation. Hydrogen peroxide or TNF-α at 37°C caused translocation of GFP-NF-κB from the cytoplasm to the nucleus with a distinct lag time (a quiescent time) as shown in Fig. 4. For the origin of this quiescent time, we speculated that it might be due to the process of degradation of IκB by the proteasome followed by the release of GFP-p50 (active form) from GFP-p105, because the processing of p105 (p50/IκBγ) comprised three successive reactions, phosphorylation, ubiquitination and degradation by the proteasome [1,18].

In our preliminary experiments at 25°C we found that

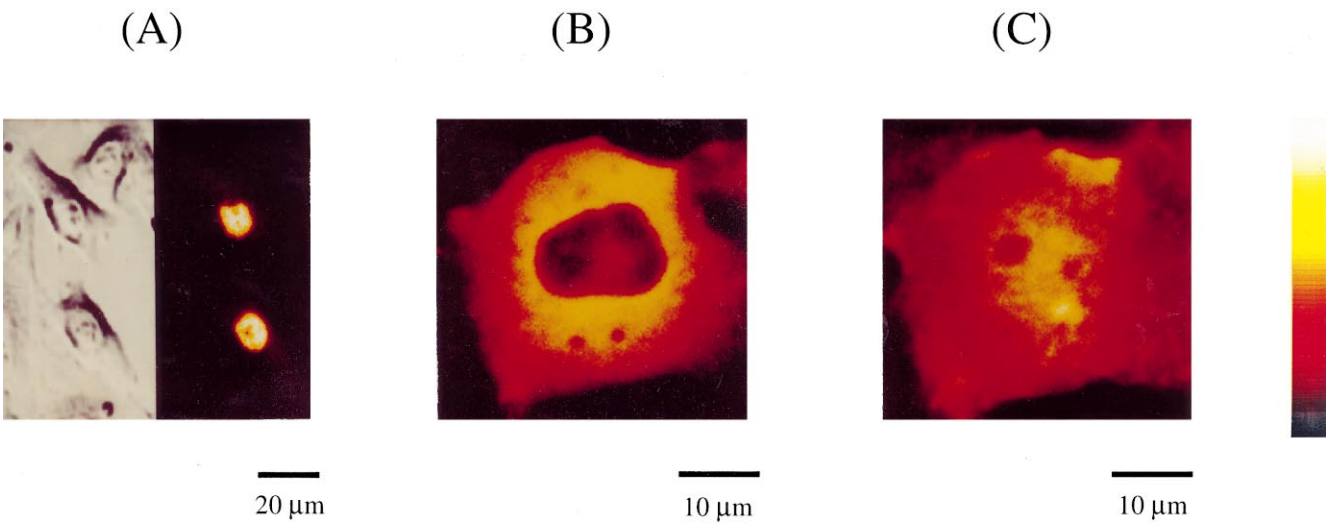

Fig. 3. CLSM images of GFP-p50 and GFP-p105 chimeric proteins at 37°C. A: GFP-p50 was expressed in COS-7 cells. A differential interference image (left) and a fluorescence pseudo-color image of GFP-p50 in the nucleus (right) are shown. B and C: Fluorescence pseudo-color images of the GFP-p105 transfected in a COS-7 cell before and 20 min after stimulation with hydrogen peroxide (250 μM), respectively.

GFP-p105 chimeric proteins were homogeneously distributed in the cytoplasm as observed at 37°C, but they never moved to the nucleus after stimulation with hydrogen peroxide or TNF-α and formed bright inhomogeneous aggregates or vesicle-like structures which were less than 1 μm or so in diameter. As the optical resolution of fluorescence microscopy is about 0.5 μm, we were not able to determine the true diameters of the bright fluorescent aggregates or vesicles in the cytoplasm. As mentioned above, the processing of p105 (p50/IκBγ) requires the ubiquitin-proteasome pathway [1,18]. Therefore, we suppose that the above inhomogeneous aggregates might reflect the irregular complexes of GFP-p105 and 26S proteasome which digested GFP-p105 improperly and failed to release GFP-NF-κB.

Recently, it was reported that p50 was generated cotranslationally by a proteasome-mediated process in CHO-CD14 cells [6]. In the present paper, however, no significant expression of GFP-p50 was detected by Western blot analysis without stimulation (see Fig. 2). Our data do not exclude the possibility of cotranslational production of p50 and its independent function from that of p105, although it remains unclear why GFP-p50 was not produced in our experiments. This might be explained by the difference in cell types used in the experiments. On the other hand, several lines of evidence suggest that cytosolic p105 is degraded to p50 to be translocated into the nucleus [19,20] and our observation are supposed to have visualized these processes. This kind of processing of pre-existing protein seemed to be advantageous for a more efficient cellular response to the particular stimuli because no de novo protein synthesis is required.

Three major combinations of subunits are known for dimeric NF-κB complexes, p50/p50 and p65/p65 homodimers and a p50/p65 heterodimer. In the present experiment, there are three possibilities for the combination of dimer which was translocated from the cytoplasm to the nucleus: homodimer (GFP-p50/GFP-p50), homodimer (GFP-p50/p50) and heterodimer (GFP-p50/p65). Although we could not specify the subunit which associated with GFP-p50, it is most plausible that NF-κB was translocated from the cytoplasm to the nucleus as a homodimer (GFP-p50/GFP-p50), considering that GFP-p105 was overexpressed in the target cells.

The real-time observation of the spatio-temporal dynamics of NF-κB such as shown in this paper might illuminate the new aspect of the mechanism of signal transduction.

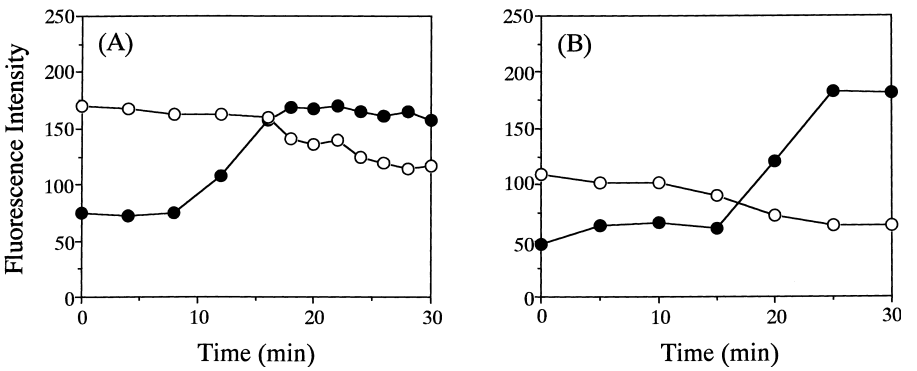

Fig. 4. Time courses of the fluorescence intensity changes of GFP-p105 in COS-7 cells after stimulation with 250 μM hydrogen peroxide (A) or 20 ng/ml TNF-α (B) at 37°C. Closed and open circles are the fluorescence intensities in the nucleus and in the cytoplasm, respectively.

Acknowledgements: We thank Prof. K. Umesono and Dr. H. Ogawa (Nara Institute of Science and Technology) for giving us the GFP expression vector (pCMX-SAH/Y145F).

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