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Nuclear translocation of the Y-box binding protein by ultraviolet irradiation

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Abstract The Y-box binding protein, YB-1, is a member of a DNA binding protein family with a structurally and functionally conserved cold shock domain. Using Western blotting and immunohistochemical methods, larger amounts of YB-1 were detected in the cytosol, particularly at the perinuclear region, than in the nucleus of human cancer cells. UV irradiation increased accumulation of YB-1 in the nucleus at 20 min and thereafter. This translocation of YB-1 into the nucleus by UV irradiation was blocked by the protein kinase inhibitor H-7, but not HA-1004. Both green fluorescent protein (GFP)-YB-1 and GFP-YB-1C with the C-terminus (248–317) of YB-1 were located mainly in the cytosol, but GFP-YB-1ΔC with a deletion at the C-terminus of YB-1 was located in the nucleus. YB-1 is translocated into the nucleus by UV irradiation, possibly through a protein kinase C-mediated signal transduction pathway, and the C-terminal region of YB-1 might be important for cytoplasmic retention of YB-1.

Key words: YB-1; UV irradiation; Nuclear translocation; Protein kinase C; Green fluorescent protein

1. Introduction

The human protein, YB-1, belongs to the Y-box binding protein family. Each of these proteins has been shown to posses three principal domains. The N-terminal domain, which is rich in proline and alanine, is thought to function as a transcriptional regulation domain [1,2]. The middle portion, termed the nucleic acid binding domain or cold shock domain (CSD), binds to the Y-box, an inverted CCAAT box, in the promoter region of many genes; this domain is highly conserved in evolution [1,3,4]. Finally, the C-terminal region, also termed the charged zipper domain, contains alternating positively and negatively charged regions and has been implicated in protein-protein interactions [1,2,5].

YB-1 was initially cloned by screening a human B-cell expression library for proteins that interact with the Y-box in the promoter region of major histocompatibility complex class II genes [6]. The YB-1 protein has been shown to bind to single-stranded DNA sequences, damaged DNA, and RNA [1,3,7]. Binding of YB-1 to its consensus sequence has been shown to be enhanced in response to UV irradiation and chemical agents [8–10]. The YB-1 gene product, which is ubiquitous in many human tissues, is overexpressed in human cancer cell lines resistant to cisplatin, a platinum-containing anticancer drug that has been shown to damage cell DNA [11]. Transfection of YB-1 antisense oligonucleotides into cells confers an increased sensitivity to cisplatin as well as to mitomycin C and UV irradiation [11].

The Y-box consensus sequence has been found to be present in the promoter region of many genes. Genes that include this sequence in their *cis*-regulatory element include those encoding thymidine kinase, proliferating cell nuclear antigen, DNA polymerase α, epidermal growth factor receptor [1,3,4], as well as multidrug resistance 1 (MDR1) [12].

We have previously shown that the inverted CCAAT box in the human MDR1 gene promoter is required for promoter activation by various environmental stimuli including carcinogens, anticancer agents, and UV irradiation [8–10,13–15]. We therefore hypothesized that MDR1 is a stress-induced gene, activated by the binding of the nuclear protein, MDR-NF1, to the MDR1 promoter region [16]. We previously cloned the MDR-NF1 cDNA from a human colon library and showed that it is identical to YB-1 cDNA [11,17]. This enhanced expression of the human MDR1 gene in response to DNA damaging agents may be due to the enhanced binding of YB-1 to the inverted CCAAT box (Y-box) consensus sequence on the MDR1 promoter [8–10]. Bargou and colleagues have recently reported that nuclear localization and an increased level of YB-1 are closely associated with intrinsic MDR1 gene expression in primary human breast cancers [18].

The further analysis of the intracellular state or localization of YB-1 should be critical to understand how MDR1 gene promoter and other Y-box-containing promoters are activated in response to UV irradiation or other genotoxic stimuli.

2. Materials and methods

2.1. Materials

Propidium iodide was purchased from Sigma (Tokyo, Japan). H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride) and HA-1004 (*N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide dihydrochloride) were purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA).

2.2. Antibodies

Polyclonal anti-YBC antibodies to YB-1 were prepared against a 15-amino acid peptide (residues 299–313) (Fig. 3A) [11]. Monoclonal antibodies to DNA topoisomerase IIα were prepared as described previously [19].

2.3. Cell culture

A line of human epidermoid cancer cells, KB, were grown in monolayer culture in Eagle's minimum essential medium (Nissui Seiyaku Co., Tokyo, Japan) containing 10% newborn calf serum (Sera-lab Ltd., Sussex, UK), 292 μg/ml glutamine, 100 μg/ml kanamycin, and

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100 units/ml penicillin at 37°C in a humidified atmosphere of 5% CO_2 [20].

2.4. Subcellular fractionation

Cells were harvested, suspended in four packed cell volumes of buffer A (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, 0.5 mM DTT) for 20 min, and homogenized by 20 strokes with a loose-fitting Dounce homogenizer. The nuclear pellets were collected by centrifugation for 6 min at 4300×*g*, resuspended in five volumes of buffer A, and washed once by centrifugation. Proteins were extracted from the washed nuclei with high salt, followed by centrifugation of the nuclear extracts and dialysis against buffer D (20 mM HEPES (pH 7.9), 20% (v/v) glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT). 1% NP-40 (v/v) was added to the dialyzed nuclear extracts. The postnuclear fractions referred to the cytosolic fraction was adjusted to buffer D containing 1% (v/v) NP-40 conditions by the addition of stock solutions. Insoluble material was removed by centrifugation for 15 min in a Microfuge. Samples were stored at −80°C [21].

2.5. Western blot analysis

Western blots were performed as described previously [11]. Membranes were developed by chemiluminescence following the ECL protocol (Amersham).

2.6. Indirect immunofluorescence

Cells grown on coverslips were washed twice with cold phosphate-buffered saline (PBS), fixed with methanol for 15 min at −20°C, and rinsed with PBS. Cells were permeabilized with PBS-T (PBS containing 0.1% Triton X-100) for 30 min and then incubated for 1 h with rabbit anti-YBC antibodies diluted 1:500 in PBS-T. After washing with PBS-T, cells were incubated for 45 min with FITC-conjugated goat anti-rabbit IgG (Organon Teknika N.V., Turnhout, Belgium) diluted 1:100 in PBS-T. The cells were subsequently washed with PBS-T followed by PBS. Coverslips were mounted with 50% glycerol in PBS. The cell nuclei were stained with propidium iodide (200 ng/ml). The samples were examined by Nikon fluorescence microscopy with a Bio-Rad MRC1000 laser scanning confocal imaging system [22].

2.7. Construction of the green fluorescent protein (GFP)-YB-1

To obtain the GFP fusion construct for the expression of full-length YB-1 protein (GFP-YB-1), the plasmid pRc/CMV, into which was inserted a full-length YB-1 cDNA, was digested with *Hin*dIII to remove the insert. The YB-1 cDNA was subcloned into the *Hin*dIII site of the pEGFP-C1 expression vctor. (Clontech, Palo Alto, CA, USA). To obtain a construct expressing YB-1 from which the C-terminal domain had been deleted (GFP-YB-1ΔC), GFP-YB-1 expression plasmids were digested with *Sal*I to remove the C-terminal region of YB-1. To obtain a construct expressing YB-1 which had the C-terminal domain (GFP-YB-1C), GFP-YB-1 expression plasmids were digested with *Apa*I to obtain the C-terminal region of YB-1, and this cDNA was subcloned into the *Apa*I site of the pEGFP-C1 expression vector.

2.8. Transfection and confocal analysis

COS-7 cells and the human bladder cancer T24 cells were maintained in DMEM with 10% FBS at 37°C in a 5% CO_2 atmosphere. Cells were plated on glass coverslips at a density of 2×10^4 cells/cm^2 the night before transfection. Transfection was performed using Lipofectin (Gibco BRL, Bethesda, MD, USA) according to the manufacturer's protocol. Cells were incubated for 48 h following application of DNA. For confocal analysis, cells were washed with PBS twice, fixed in PBS containing 4% freshly prepared paraformaldehyde, and washed with PBS twice. Alternatively, coverslips were mounted directly on slides for observation. The samples were examined by Nikon fluorescence microscopy with a Bio-Rad MRC1000 laser scanning confocal imaging system [22].

3. Results and discussion

Treatment of cells with UV irradiation or drugs has been shown to induce binding of YB-1 to its Y-box [8–10]. We examined whether the cellular level of YB-1 was increased in response to UV irradiation in KB cells. Western blot analysis with anti-YBC antibodies showed no change of the cellular level of YB-1 until 6 h after UV irradiation (Fig. 1A). DNA topoisomerase IIα (topoIIα), a nuclear enzyme, also showed no change in its levels after UV irradiation (Fig. 1A). We next examined if the level of YB-1 was altered in the nucleus after UV irradiation. Western blot analysis showed that there was about 10 times more YB-1 in the cytosol than in the nuclear fraction (Fig. 1B). Increased amounts of YB-1 in the nucleus were observed 20 min after UV irradiation (Fig. 1B). A higher level of YB-1 was maintained up to 3 h in the nucleus after UV irradiation. By contrast, topoIIα showed no change in its levels in the nuclear fraction after UV irradiation. TopoIIα could not be detected in the cytosol fraction, indicating that the cytosol fraction was not contaminated with nuclear protein (Fig. 1B). The subcellular distribution of YB-1 in KB cells was also examined by indirect immunofluorescence microscopy. For comparison, the cells were also stained with propidium iodide which specifically stained the nuclei. The image of cells stained with anti-YBC antibody and propidium iodide showed that YB-1 was localized mainly in the perinuclear region (Fig. 1C). Consistent with this result, dbpA, a member of the YB-1 family, was

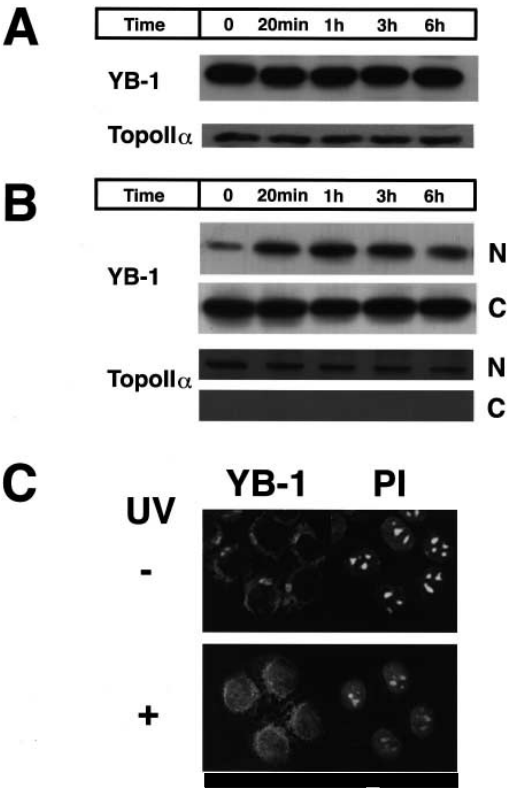

Fig. 1. Subcellular distribution of YB-1 and the effect of UV irradiation. A: Whole cell lysates of control untreated KB cells (indicated as 0 min) and cells exposed to UV irradiation (40 J/m^2) for the indicated time were assayed by immunoblotting using anti-YB-1 antibodies. Each lane contains 20 μg of total protein. The control topoIIα was also immunoblotted. B: Both nuclear extracts (N) and cytosolic fractions (C) of control untreated KB cells (indicated as 0 min) and cells exposed to UV irradiation (40 J/m^2) for the indicated time were assayed by immunoblotting using anti-YBC. Each lane contains 20 μg of total protein. The control topoIIα was also immunoblotted. C: Untreated KB cells and cells 1 h after UV (40 J/m^2) irradiation were stained with anti-YB-1 antibodies (YB-1) and viewed with a confocal laser scanning microscope. The nuclei were stained with propidium iodide (PI).

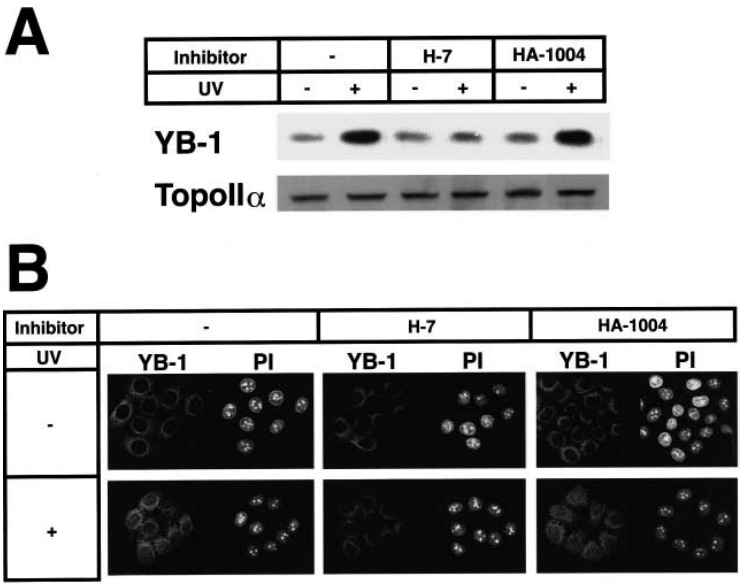

Fig. 2. Effect of protein kinase inhibitors on the nuclear accumulation of YB-1 induced by UV irradiation. A: H-7 and HA-1004 were added to cell cultures at 40 μM 90 min before UV irradiation, nuclear extracts of control untreated KB cells and cells 1 h after UV irradiation (40 J/m^2) were assayed by immunoblotting with anti-YB-1 antibodies. Each lane contains 20 μg of total protein. The control topoIIα was also immunoblotted. B: H-7 and HA-1004 were added to cell cultures at 40 μM 90 min before UV irradiation, control untreated KB cells and cells 1 h after UV irradiation (40 J/m^2) were immunostained by anti-YB-1 antibodies (YB-1) and viewed with a confocal laser scanning microscope. The nuclei were stained with propidium iodide (PI).

mostly located in the cytoplasm, particularly at the perinuclear region in human cancer cells [23]. Immunofluorescence data also showed an apparent increase of YB-1 in the nucleus of cells 1 h after UV irradiation (Fig. 1C). These results indicate that the nuclear translocation of the YB-1 was induced by UV irradiation.

The import of transcription factors into the nucleus is not a constitutive process, and appears to be modulated in response to external stimuli, cell cycle and development. Regulation of the subcellular localization of proteins involves the direct phosphorylation of the transported protein, masking of the nuclear localization signal(s), cytoplasmic retention by binding to an anchoring protein, and the interplay among these different mechanisms [24–26]. We previously reported that transcriptional activation of the MDR1 gene by UV irradiation is mediated through the Y-box motif [8,9]. This YB-1-mediated activation of the MDR1 promoter, however, is almost completely abrogated by a high concentration of the protein kinase C inhibitor H-7 [8,27], which suggests the possible involvement of the protein kinase in the nuclear translocation of YB-1. We examined the effect of protein kinase inhibitors, H-7 and HA-1004, on the nuclear translocation of YB-1 after UV irradiation. H-7 and HA-1004 inhibit a variety of protein kinases with distinct K_i values, but H-7 inhibits protein kinase C in comparison with HA-1004 [28,29]. Immunoblot analysis of nuclear protein showed that H-7 at 40 μM inhibited the nuclear translocation of YB-1 after UV irradiation, but not HA-1004 (Fig. 2A). Indirect immunofluorescence also showed that H-7, but not HA-1004, inhibited the nuclear translocation of YB-1 after UV irradiation (Fig. 2B). These results suggest that protein kinase C might specifically modulate the nuclear translocation of YB-1 induced by UV irradiation.

YB-1 does not have consensus sequence for the translocation of proteins to the nucleus, but it does have putative nuclear localization signals that consist of a cluster of three to six basic residues in a short peptide of four to nine amino acids [30]. YB-1 contains at least six such sequences of amino acids: 150–152, 185–192, 199–205, 242–247, 279–283, and 288–291, in a region of alternating blocks of predominantly acidic and basic amino acids (Fig. 3A). Moreover, YB-1 was mainly located in the cytoplasm. To assess which domain is important for cytoplasmic retention of YB-1, we generated plasmid constructs containing full-length YB-1 and its truncated form YB-1ΔC and YB-1C linked to GFP (Fig. 3B). Human bladder cancer T24 cells were transfected with GFP-

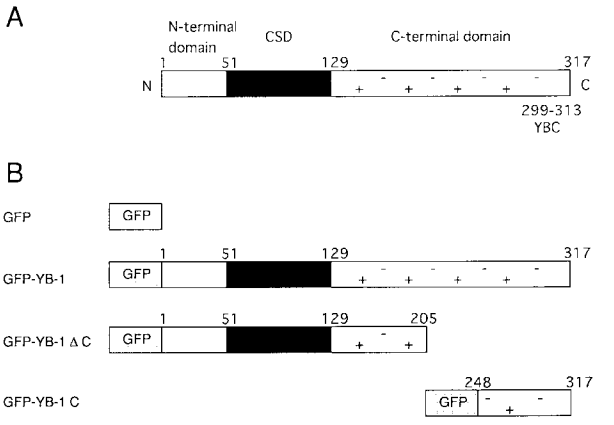

Fig. 3. Schematic representation of the YB-1 and GFP-YB-1 fusion proteins. A: Schematic drawing of the YB-1 and the position of the synthetic peptide used to prepare polyclonal antibodies to anti-YBC. The highly conserved CSD is the nucleic acid recognition domain. The N-terminal domain is involved in transcriptional regulation. The C-terminal domain, including the alternating basic/acidic amino acid cluster, is involved in protein-protein interactions. +, basic amino acids; −, acidic amino acids. B: Diagrams of the products of the GFP-YB-1 and its deletion constructs used in this study.

YB-1 plasmid, and treated with UV (40 J/m^2). GFP-YB-1 protein was mainly located in the perinuclear region of untreated cells (Fig. 4). By contrast, translocation of GFP-YB-1 was apparently observed 1 h after UV irradiation (Fig. 4). Endogenous YB-1 as well as exogenous YB-1 (GFP-YB-1) thus appeared to be accumulated in the nucleus under UV irradiation.

We further transfected GFP-YB-1 and its deletion constructs (GFP-YB-1ΔC and GFP-YB-1C) into COS-7 cells, and examined which domain in the YB-1 was critical for the cytoplasmic retention. The GFP-YB-1 protein as well as GFP-YB-1C were mainly located in the perinuclear region (Fig. 5B,D). By contrast, GFP-YB-1ΔC was mainly localized in the nucleus (Fig. 5C). GFP alone was localized in both cytoplasm and nucleus (Fig. 5A). The C-terminal domain might be important for the cytoplasmic retention or nuclear export of YB-1. However, the C-terminal domain does not have a nuclear export signal such as mitogen-activated protein kinase kinase [31]. Since this domain has been shown to be involved in the protein-protein interaction [1,2,5], we hypothesized that YB-1 could be retained in the cytoplasm by binding to an anchoring protein as observed in the representative transcription factor NF-κB interacting with IκB in the cytoplasm [32]. Using glutathione *S*-transferase (GST)-YB-1, affinity purification of such proteins associated with the C-terminal domain of YB-1 is now in progress.

We have previously reported that transfection of the YB-1 antisense oligonucleotides into cells resulted in their increased sensitivity to the cytotoxic effects of cisplatin, mitomycin C and UV irradiation, suggesting that YB-1 itself protects the cells against the effects of this genotoxic damage [11]. The Y-box proteins are integral components of a eukaryotic redox signaling pathway, serving as a scaffold for the assembly of other factors [33], suggesting that these Y-box proteins may be part of a mammalian stress signal transduction mechanism [34]. These studies indicate that YB-1 may itself have a key role in self-defense signaling mechanisms, being initiated in response to such environmental poisons as oxidative stress or DNA damaging reagents. Bargou et al. have recently reported that nuclear localization of YB-1 is closely associated with intrinsic MDR1 gene expression in primary human breast cancers [18]. Further study is required to determine whether MDR1 gene overexpression is closely associated with YB-1 activities in human breast cancers and other human tumors in the absence or presence of environmental stress.

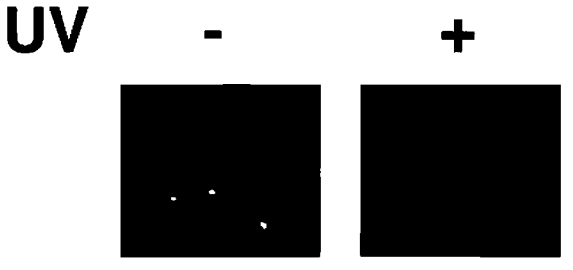

Fig. 4. Subcellular distribution of GFP-YB-1 and the effect of UV irradiation. Human bladder cancer T24 cells were transiently transfected with GFP-YB-1. Untreated cells and cells 1 h after UV (40 J/m^2) irradiation were analyzed by confocal microscopy.

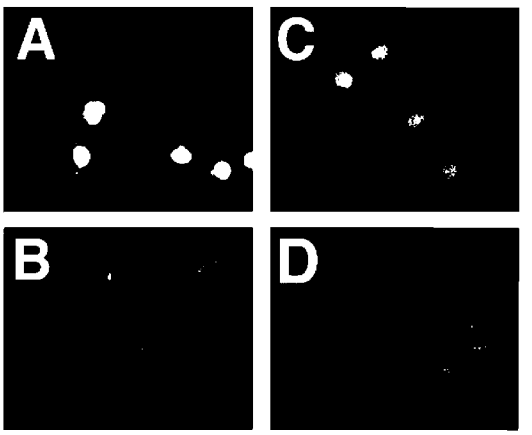

Fig. 5. Subcellular distribution of GFP, GFP-YB-1, GFP-YB-1ΔC and GFP-YB-1C. COS-7 cells was transiently transfected with GFP (A), GFP-YB-1 (B), GFP-YB-1ΔC (C), or GFP-YB-1C (D), and analyzed by confocal microscopy.

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