Proteasome-mediated cleavage of the Y-box-binding protein 1 is linked to DNA-damage stress response

Alexey V Sorokin¹, Anastasia A Selyutina¹, Maxim A Skabkin¹, Sergey G Guryanov¹, Igor V Nazimov², Christina Richard³, John Th'ng³, Jonathan Yau³, Poul HB Sorensen^{4,5}, Lev P Ovchinnikov¹ and Valentina Evdokimova^{1,5,*}

¹Institute of Protein Research, Russian Academy of Sciences, Pushchino, Moscow Region, Russian Federation, ²Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russian Federation, ³Thunder Bay Regional Health Sciences Centre, Medical Sciences Division, Northern Ontario School of Medicine, Thunder Bay, Ontario, Canada, ⁴Department of Pathology, British Columbia Research Institute for Children's and Women's Health, and the University of British Columbia, Vancouver, British Columbia, Canada and ⁵Department of Pediatrics, British Columbia Research Institute for Children's and Women's Health, and the University of British Columbia, Vancouver, British Columbia, Canada

YB-1 is a DNA/RNA-binding nucleocytoplasmic shuttling protein whose regulatory effect on many DNA- and RNA-dependent events is determined by its localization in the cell. Distribution of YB-1 between the nucleus and the cytoplasm is known to be dependent on nuclear targeting and cytoplasmic retention signals located within the C-terminal portion of YB-1. Here, we report that YB-1 undergoes a specific proteolytic cleavage by the 20S proteasome, which splits off the C-terminal 105-amino-acid-long YB-1 fragment containing a cytoplasmic retention signal. Cleavage of YB-1 by the 20S proteasome *in vitro* appears to be ubiquitin- and ATP-independent, and is abolished by the association of YB-1 with messenger RNA. We also found that genotoxic stress triggers a proteasome-mediated cleavage of YB-1 *in vivo* and leads to accumulation of the truncated protein in nuclei of stressed cells. Endoproteolytic activity of the proteasome may therefore play an important role in regulating YB-1 functioning, especially under certain stress conditions.

The EMBO Journal (2005) **24**, 3602–3612. doi:10.1038/sj.emboj.7600830; Published online 29 September 2005

Subject Categories: proteins

Keywords: mRNA; nuclear localization; proteasome; specific cleavage; YB-1

Introduction

Y-box protein 1, also known as dbpB, has been initially cloned as a transcription factor that specifically recognizes the Y-box promoter element in a variety of different genes and designated YB-1, accordingly (Didier *et al*, 1988). Independently, we have cloned and characterized this 50 kDa protein (p50) as a major protein bound to messenger RNAs (mRNAs) in mammalian cells (Minich and Ovchinnikov, 1992; Evdokimova *et al*, 1995). At present, it is clear that YB-1/p50 is a nucleocytoplasmic shuttling protein involved in many DNA- and RNA-dependent events. When in the nucleus, this protein regulates transcription of many genes including those involved in cell proliferation and differentiation. It has also been implicated in repair, replication, recombination of DNA and alternative mRNA splicing (reviewed in Matsumoto and Wolffe, 1998; Kohno *et al*, 2003). When in the cytoplasm, it plays a key role in packaging of mRNAs into messenger ribonucleoprotein particles (mRNPs), and also regulates translational activity and stability of mRNA (Evdokimova *et al*, 2001; Skabkin *et al*, 2004). Distribution of YB-1 between nuclear and cytoplasmic compartments must be therefore stringently regulated. In accordance with this idea, it has been recently shown that although YB-1 is localized predominantly in the cytoplasm throughout the cell cycle, it moves to the nucleus at the G1 to S phase transition in a pattern similar to cyclin E (Jurchott *et al*, 2003). Nuclear accumulation of YB-1 is accompanied by transcriptional activation of cyclin A and B1 genes, which possess the YB-1 recognition element in their promoters. Translocation of YB-1 to the nucleus has also been shown to be activated by various insults including adenovirus infection, UV irradiation, hyperthermia or association with certain proteins such as tumor suppressor p53 or splicing factor SRp30c (reviewed in Kohno *et al*, 2003). Predominant nuclear localization of YB-1 is thought to be a characteristic of transformed cells, and was also associated with development of multiple drug resistance of cancer cells (Kuwano *et al*, 2004).

Experiments on a set of deletion mutants showed that the C-terminus of YB-1 contains one or two potential noncanonical nuclear localization signals (NLS) that are required for nuclear import of YB-1 (Stenina *et al*, 2001; Jurchott *et al*, 2003; Bader and Vogt, 2005). In addition, the C-terminal part of YB-1 possesses a cytoplasmic retention signal (CRS; see Figure 6F) that prevails over the NLS and ensures predominant cytoplasmic localization of YB-1 (Jurchott *et al*, 2003; Bader and Vogt, 2005). Recent data have suggested a novel mechanism for nuclear targeting of YB-1 that involves cleavage of its CRS-containing C-terminal part in response to thrombin stimulation of endothelial cells (Stenina *et al*, 2001). Truncated YB-1 exhibited nuclear localization and preserved its activity as a transcription factor facilitating

*Corresponding author. Department of Pediatrics, University of British Columbia, 3064-950 West 28th Avenue, Vancouver, British Columbia, Canada BC V5Z 4H4. Tel.: +1 604 822 2211; Fax: +1 604 875 3417; E-mail: evdokimo@interchange.ubc.ca

Received: 14 April 2005; accepted: 8 September 2005; published online: 29 September 2005

transcription of the platelet-derived growth factor (*PDGF*) gene (Stenina *et al.*, 2000), although the mechanism of proteolytic processing of YB-1 remained unclear.

Here, we report that cleavage of a short C-terminal YB-1 fragment containing the CRS is mediated by the 20S proteasome. Of physiological relevance, we found that association with mRNA prevents YB-1 cleavage and that only unbound, mRNA-free YB-1 may be targeted. Interestingly, ubiquitin and ATP were not required for proteolytic processing of YB-1 *in vitro*, albeit polyubiquitylated YB-1 species were observed in cells treated with proteasomal inhibitors. Limited proteolysis of YB-1 was triggered by DNA-damaging drugs and resulted in accumulation of the truncated YB-1 form in nuclear punctuate structures resembling nuclear speckles, zones of accumulation of transcriptional and splicing factors. We propose that specific cleavage of YB-1 mediated by the 20S

proteasome may generate a polypeptide with a potentially altered biological function.

Results

Identification of the YB-1-specific protease as 20S proteasome

YB-1 purified from various cell extracts including rabbit reticulocyte lysate (RRL) often contains an additional 32 kDa band immunoreactive with anti-YB-1 antibodies (data not shown). This observation together with literature reports indicating that YB-1/dbpB can be specifically cleaved in endothelial cells (Stenina *et al.*, 2000) prompted us to search for proteins that may be involved in YB-1 cleavage. To identify these proteins, we performed a multistep purification procedure using RRL (Figure 1A). The resulting protease

Figure 1 Identification of the YB-1-specific protease as 20S proteasome. **(A)** Purification of protease that cleaves YB-1. **(B)** Selective cleavage of YB-1 by purified protease. YB-1, poly(A)-binding protein (PABP), glycogen phosphorylase (GP), glutathione *S*-transferase (GST), casein, tubulin or bovine serum albumin (BSA) were incubated with purified protease for 1 h and analyzed by SDS-15% PAGE and Coomassie staining. **(C)** Purified protease preparation was analyzed by Coomassie staining or Western using antibodies against $\alpha 5$ or $\alpha 6$ 20S proteasomal subunits. **(D)** List of polypeptides identified in the purified protease complex by mass spectrometry. BN, band numbers as in (C); Protein name, NCBI annotation; Score, generated by MASCOT tools; Proteasome subunit name is given in systemic nomenclature (Groll *et al.*, 1997). **(E)** Sedimentation distribution of the purified protease. Purified protease ($\sim 30 \mu\text{g}$) was fractionated by centrifugation at 45 000 r.p.m. (SW 60 rotor; Beckman Instruments) for 4 h through a linear 5–20% (w/v) sucrose gradient. Fractions (275 μl) were collected from the bottom and analyzed for the ability to cleave YB-1. Coomassie staining of YB-1 after incubation with the corresponding fractions is shown. 28S, 18S rRNAs and 9S α -globin RNA served as sedimentation markers. **(F)** Effect of proteasomal and protease inhibitors on YB-1 cleavage. YB-1 was incubated with purified protease preparation in the presence of the indicated inhibitors. After 60 min incubation, YB-1 and degradation products were resolved by SDS-15% PAGE and visualized by Coomassie staining.

preparation selectively cleaved YB-1 to give two fragments with relative molecular masses of about 32 and 22 kDa, while having no effect on other proteins tested (Figure 1B).

YB-1-specific protease was eluted from Superose 12 in the region corresponding to a molecular weight of about 700 kDa and remained intact even at 2 M NaCl, suggesting that it represents a highly stable multiprotein complex. Electrophoretic analysis of the purified protease preparation revealed that it consists of eight polypeptides within the size range of 22–32 kDa, plus one of ~90 kDa (Figure 1C, lane 1). As a similar subunit composition is characteristic of the 20S proteasome, we employed antibodies directed against human proteasomal subunits to test if these might be present in our protease preparation. Indeed, two of them were identified as $\alpha 5$ and $\alpha 6$ subunits of the 20S proteasome (Figure 1C, lanes 2 and 3). Furthermore, eight other major polypeptides were revealed by mass spectrometry as subunits of the 20S proteasome (Figure 1D). The 90 kDa polypeptide was identified as a heat-shock protein Hsp90 α (Hsp86), whose copurification with the 20S proteasome was previously reported (Tsubuki *et al*, 1994). The sedimentation coefficient of the purified protease complex was approximately 20S (Figure 1E), which is also characteristic of the 20S proteasome.

The cleavage of YB-1 was suppressed by specific proteasome inhibitors including MG132, YU102, epoxomicin, lactacystin and batenecin-5 but not by common protease inhibitor phenylmethanesulfonyl fluoride (PMSF) (Figure 1F), further indicating involvement of the 20S proteasome. Of further note, YU102, which is known to inhibit post-acidic activity of the 20S proteasome, markedly reduced YB-1 cleavage when added at low concentrations (0.2–5 μ M). Similarly, MG132, which inhibits both the post-acidic and chymotrypsin-like proteasomal activities, prevented YB-1 cleavage at a concentration of 10 μ M. In contrast, inhibitors preferably suppressing chymotrypsin-like activity of the proteasome (epoxomicin, lactacystin and batenecin-5) were significantly less efficient, suggesting that the post-acidic activity of the 20S proteasome is mainly required for YB-1 cleavage. Taken together, these results indicate that the 20S proteasome mediates selective cleavage of YB-1.

YB-1 cleavage is mediated by the 20S proteasome in a ubiquitin- and ATP-independent manner

To date, the majority of studies have been focused on the role of proteasome in the ubiquitin-dependent degradation pathway. This ATP-dependent pathway implies conjugation of multiple ubiquitin moieties to target proteins, which are then degraded by the 26S proteasomal complex (Hershko and Ciechanover, 1998). However, recent data indicate that the 26S proteasome and its catalytic core, the 20S proteasome, can also degrade some proteins in a ubiquitin- and ATP-independent manner (Orlowski and Wilk, 2003; Hoyt and Coffino, 2004). To address whether YB-1 might be cleaved by the 26S proteasome as well, we utilized a commercial human 26S proteasome or a preparation purified from RRL in parallel with preparations of the 20S proteasome. All proteasome preparations showed comparable activity in degrading a model fluorogenic substrate Suc-LLVY-AMC (Figure 2B); however, neither of the two preparations of the 26S proteasome stimulated YB-1 cleavage (Figure 2A). In contrast to casein, a known substrate of the 26S proteasome (Kisselev *et al*, 1999), YB-1 remained stable even after a prolonged 6 h

Figure 2 YB-1 cleavage is mediated by the 20S proteasome in a ubiquitin- and ATP-independent manner. (A) Comparison of proteolytic activities exhibited by various 20S and 26S proteasomal preparations toward YB-1. YB-1 (1.5 μ g) was incubated with purified 20S proteasome (0.5 μ g), commercial 20S proteasome (0.5 μ g), purified 26S proteasome (1.5 μ g) or commercial 26S proteasome (1 μ g). Incubations were performed for 1 h in a total volume of 20 μ l of buffer G. Coomassie staining of the gel is shown. (B) Proteolytic activities of proteasomes used in (A) were analyzed using fluorogenic peptide Suc-LLVY-AMC (100 μ M). Incubations were carried out for 1 h in a total volume of 200 μ l of buffer G. Fluorescence of released AMC was measured at 360 nm excitation and 430 nm emission. AMC extinction intensity upon cleavage of Suc-LLVY-AMC by purified 20S proteasome was taken as 100%. (C) Time course of protein cleavage by the 26S proteasome. For each time point, YB-1 (1 μ g) or casein (1 μ g) was incubated with commercial 26S proteasome (1 μ g) in a total volume of 20 μ l of buffer G. Degradation products were analyzed by SDS-15% PAGE and Coomassie staining. The bottom numbers indicate percentage of the remaining protein as measured from two independent experiments by densitometry, with values obtained for time 0 set as 100%. (D) Effect of ATP on YB-1 cleavage. YB-1 was incubated with 20S proteasome in buffer G in the presence or absence of ATP, an ATP-depleting system (10 mM glucose, 1 μ g/ml of hexokinase (HXK)) or an ATP-regenerating system (10 mM creatine phosphate (CP), 10 μ g/ml of creatine kinase (CK)), as indicated. Coomassie staining of the gel is shown. (E) Effect of the mRNA on YB-1 cleavage. YB-1 (1 μ g; 28 pmol) was incubated with α -globin mRNA (1 μ g; 5 pmol) at 30°C for 15 min in a total volume of 20 μ l of buffer G. YB-1-mRNA complexes were then either directly incubated with 20S proteasome for 60 min or pretreated with RNase A (0.15 μ g/ μ l) and micrococcal nuclease (0.05 U/ μ l) at 37°C for 30 min. Coomassie staining of the gel is shown. (F) Effect of the mRNA on proteolytic activity of 20S proteasome. 20S proteasome (0.5 μ g) was incubated with Suc-LLVY-AMC (100 μ M) in the presence or absence of α -globin mRNA (7.5 μ g). AMC extinction intensity upon cleavage of Suc-LLVY-AMC without mRNA was taken as 100%.

incubation (Figure 2C). These results suggest that although a role for the 26S proteasome *in vivo* cannot be ruled out, cleavage of YB-1 *in vitro* is mediated exclusively by the 20S proteasome.

To analyze whether cleavage of YB-1 by the 20S proteasome is ATP-dependent, cleavage reactions were carried out in the presence or absence of ATP. The ATP-containing reaction mixture was supplemented with an ATP-regenerating system consisting of creatine kinase and creatine phosphate. The reaction mixture lacking ATP contained hexokinase and glucose to eliminate any trace of ATP. As seen in Figure 2D, YB-1 was cleaved by the 20S proteasome independently of the presence or absence of ATP. It is also highly unlikely that YB-1 cleavage is ubiquitin-dependent, since both YB-1 and proteasome preparations used in this study showed electrophoretic homogeneity; no ubiquitin contamination was detected. Also, utilization of recombinant YB-1 purified from *Escherichia coli* excludes a possibility that YB-1 was originally ubiquitylated. These data indicate that YB-1 is cleaved by the 20S proteasome *in vitro* in an ATP- and ubiquitin-independent manner.

As the vast majority of cytosolic YB-1 is found exclusively in complexes with mRNAs (Minich and Ovchinnikov, 1992; Davydova *et al*, 1997), we next tested whether or not YB-1 binding to the mRNA affects its susceptibility to proteasomal cleavage. As seen in Figure 2E, preincubation of YB-1 with α -globin RNA abolished its cleavage by the 20S proteasome (lane 4), whereas treatment of YB-1-mRNA complexes with RNases fully restored sensitivity of YB-1 (lane 5). Inhibition of YB-1 cleavage in the presence of RNA was not due to the block of enzymatic activity of the 20S proteasome by RNA (Figure 2F). Therefore, binding to the mRNA protects YB-1, and only unbound, mRNA-free YB-1 appears to be a target for proteasomal cleavage.

20S proteasome cleaves YB-1 before Gly-220

Cleavage of YB-1 by the 20S proteasome was rapid, with a half-life of about 20 min (Figure 3A). Importantly, no intermediate products between full-length YB-1 and the 32 kDa YB-1 fragment were detected, suggesting that the 20S proteasome may endoproteolytically cleave YB-1 rather than processively degrade it from its terminal ends. However, prolonged incubation with proteasome caused additional cleavage of p32, as some minor degradation products became visible after 90 min incubation (Figure 3A, lane 9). To define the cleavage site on YB-1, we utilized various fragments that represent separate domains of YB-1 (Figure 3B). YB-1 is known to be composed of three domains including a short N-terminal Ala/Pro-rich domain (AP), an evolutionarily conserved cold-shock domain (CSD) in its central part and a C-terminal domain (CTD) with alternating negatively and positively charged amino-acid clusters (Wolffe, 1994). As seen in Figure 3C, the 20S proteasome specifically cleaved CTD to generate two subfragments of similar size and had no effect on the integrity of the N-terminal AP-CSD or YB-1 (1–204) fragments. Of further note, the larger fragment of the CTD (fragment II) showed the same mobility as the smaller fragment produced by cleavage of full-length YB-1 (Figure 3C, compare lanes 2 and 4). Because only two major fragments were generated by cleavage of both full-length YB-1 and the CTD, it is likely that the 20S proteasome cleaves

Figure 3 Identification of the cleavage site on YB-1. (A) Time course of YB-1 cleavage by the 20S proteasome. For each time point, YB-1 (1 μ g) was incubated with purified 20S proteasome (0.5 μ g) in a total volume of 20 μ l of buffer G. Coomassie staining of the gel is shown. (B) Schematic representation of YB-1 and its truncated fragments. The N-terminal AP, CSD and CTD are indicated. (C) Cleavage of YB-1 and its derivatives by the 20S proteasome. Full-length YB-1 and its fragments (1–1.5 μ g) were incubated with purified 20S proteasome (0.5 μ g) for 60 min. Peptides generated from full-length YB-1 are designated as peptides I and II. (D) Alignment of the full-length YB-1 sequence with those of peptides I and II, which were determined by N-terminal amino-acid sequencing.

YB-1 at a specific internal site that is located approximately in the middle of the YB-1 CTD.

To localize precisely the cleavage site, peptides I and II obtained by cleavage of full-length YB-1 were sequenced. Alignment of N-terminal amino-acid sequences of these peptides with the sequence of full-length YB-1 revealed that the larger YB-1 fragment is derived from its N-terminus and starts from Ser-2 (Figure 3D). Lack of the first Met can be explained by its co- or post-translational removal. The other peptide begins with Gly-220, suggesting that cleavage may occur immediately after glutamic acid (Glu-219). This is also consistent with the notion that a post-acidic activity of the 20S

proteasome was responsible for YB-1 cleavage (Figure 1F). These results demonstrate that the 20S proteasome cleaves YB-1 before Gly-220, removing a 105-amino-acid fragment from its C-terminus.

DNA-damaging drugs facilitate YB-1 cleavage *in vivo* in a proteasome-dependent manner

We next asked whether YB-1 cleavage may occur in the cell. Because YB-1 has been shown to regulate transcription of various stress-responsive genes, we considered the possibility that cleavage of YB-1 may be triggered by stress and required for nuclear translocation of YB-1. In the experiments described below, we utilized NIH3T3 cell lines, nontransformed or transformed with K-Ras. These cell lines were engineered to ectopically express N-terminal hemagglutinin (HA)-tagged YB-1, which allowed detection of both the full-length and truncated N-terminal YB-1 forms using highly sensitive and monospecific anti-HA antibodies. Screening of a broad spectrum of therapeutic agents revealed a substantial reduction of full-length YB-1 and an appearance of the 32 kDa fragment in

K-Ras-NIH3T3 cells treated with doxorubicin, a DNA-damaging drug that inhibits topoisomerase II activity (Figure 4A, lane 2). Noteworthy, the p32 polypeptide was detected by both anti-YB-1 and anti-HA antibodies, indicating that it was generated from the N-terminal part of YB-1. Neither 17-AAG, which interferes with the action of Hsp90, nor nocodazole, which disrupts microtubules, nor glucocorticoid dexamethasone, all displaying potent anticancer activity and causing various stress responses, affected YB-1 integrity (Figure 4A). We next tested whether cleavage of full-length YB-1 is specific to doxorubicin or other drugs of this class may exert a similar effect. Indeed, in cells treated with compounds causing DNA crosslinking (cisplatin) or inhibiting topoisomerase II (etoposide) or topoisomerase I (camptothecin) activities, full-length YB-1 was substantially cleaved to produce the 32 kDa truncated fragment (Figure 4B, lanes 3–5). In contrast, treatment with other drugs including those activating endoplasmic reticulum stress response (thapsigargin and brefeldin A) was not sufficient to stimulate YB-1 cleavage (Figure 4B, lanes 6–8). The cleavage of YB-1 was not caused

Figure 4 DNA-damaging drugs induce YB-1 cleavage *in vivo* in a proteasome-dependent manner. (A) K-Ras-NIH3T3 cells stably expressing HA-YB-1 were treated for 14 h with doxorubicin (0.6 μ M), 17-AAG (1.7 μ M), nocodazole (0.2 μ M) or dexamethasone (10 μ M). Whole-cell extracts were analyzed by Western using anti-HA or anti-YB-1 antibodies. Unrelated RNA-binding protein TIAR served as a loading control. (B) K-Ras-NIH3T3 cells expressing HA-YB-1 were treated for 14 h with doxorubicin (0.6 μ M), cisplatin (10 μ M), etoposide (10 μ M), camptothecin (3 μ M), tunicamycin (5 μ M), thapsigargin (5 μ M) or brefeldin A (2 μ M) and analyzed as in (A). (C) K-Ras-NIH3T3 cells treated as above were analyzed by flow cytometry. Both detached and adherent cells were collected for the analysis. (D) K-Ras-NIH3T3 cells were treated for 24 h with 0.6 μ M of doxorubicin in the presence of ALLN (30 μ M), MG132 (20 μ M), Inhibitor I (50 μ M), PMSF (1 mM), leupeptin (2 μ M), pepstatin (2 μ M) or aprotinin (2 μ M). Whole-cell extracts were analyzed by Western using anti-YB-1 antibodies. (E) K-Ras-NIH3T3 cells expressing HA-YB-1 were treated with doxorubicin and MG132 (20 μ M) for 18 h, and corresponding cytosolic cell extracts were subjected to immunoprecipitation using rabbit anti-ubiquitin or preimmune antibodies. Immunoprecipitated proteins were then analyzed by Western using mouse anti-HA antibodies. Smear in the immunoprecipitates from cells treated with both doxorubicin and MG132 supposedly corresponds to polyubiquitylated HA-YB-1.

by apoptosis, as no significant elevation in a proportion of dead cells was observed by flow cytometry under these experimental conditions (Figure 4C). Also, despite a significant accumulation of mitotic cells in response to doxorubicin or cisplatin, YB-1 cleavage is unlikely to be a result of a cell cycle arrest, since nocodazole had no effect on YB-1 integrity, while causing accumulation of cells at G2/M (Figure 4C). It should be noted, however, that increased concentrations or prolonged incubation with the above drugs caused complete degradation of full-length YB-1 without generation of a truncated product (data not shown), suggesting that under conditions of a severe stress YB-1 may be degraded by proteasomes or due to activation of apoptotic programs.

To confirm that cleavage of full-length YB-1 in doxorubicin-treated cells was mediated by proteasomes, we employed proteasome and calpain inhibitors such as MG132, Inhibitor I and ALLN as well as common protease inhibitors (PMSF, leupeptin, pepstatin and aprotinin). As seen in Figure 4D, all proteasome but not protease inhibitors stabilized levels of full-length YB-1, indicating involvement of the proteasomal pathway. We also noticed that a minor portion of YB-1 in cells simultaneously treated with doxorubicin and MG132 is polyubiquitylated (Figure 4E), although it is unclear whether polyubiquitylation is required for specific cleavage or complete degradation of YB-1 via the traditional ubiquitin-proteasomal pathway.

Truncated YB-1 accumulates in the nucleus following DNA-damaging stress

To gain further insight and establish functional relevance of the N-terminal YB-1 fragment, we analyzed its subcellular localization. In agreement with the literature reports (Koike *et al*, 1997; Bader and Vogt, 2005), full-length YB-1 was localized predominantly in the cytoplasm, although a significant portion of YB-1 was also found in the nucleus (Figure 5A, compare lanes 1 and 2). Treatment with doxorubicin, cisplatin or etoposide resulted in reduction of full-length YB-1 in both cytoplasmic and nuclear compartments and accumulation of the 32 kDa YB-1 form in nuclear fractions (Figure 5A). These results were corroborated by immunofluorescence microscopy showing predominantly cytosolic HA-immunopositive staining in untreated cells and its nuclear accumulation in response to doxorubicin (Figure 5B). After 6 h of exposure to doxorubicin, more than 50% cells displayed mixed nuclear and cytosolic or purely nuclear HA-positive staining, as compared to less than 1% in untreated cells. A 16 h treatment resulted in significant accumulation of the HA-positive material in the nucleus. Notably, the HA immunoreactivity was concentrated in the punctuate structures resembling nuclear speckles that are known to be enriched with transcriptional and pre-mRNA splicing factors (Lamond and Spector, 2003). By contrast, another major mRNA-binding protein, PABP, exhibited diffuse cytosolic staining and its localization was not affected by doxorubicin (Figure 5B).

In order to determine whether cleavage of YB-1 is sufficient to trigger its nuclear localization, we first analyzed the effect of MG132, which was found to preserve YB-1 integrity (Figures 4D and 5D). As seen in Figure 5C, MG132 impeded nuclear accumulation of the HA-immunopositive material in doxorubicin-treated cells, suggesting that cleavage of YB-1 may be important for its accumulation in the nucleus.

To reinforce this point, we generated truncated YB-1(1–204) that lacked the CRS-containing C-terminal region, thereby mimicking the cleaved fragment. Consistent with previous reports (Stenina *et al*, 2001; Bader and Vogt, 2005), HA-tagged YB-1(1–204) exhibited predominantly nuclear localization (Figure 5E), confirming that loss of the C-terminal portion results in relocation of the truncated YB-1 molecule to the nucleus. We also noticed that YB-1(1–204) levels were not affected by doxorubicin treatment (Figure 5F, compare lanes 3 and 6), suggesting that the truncated YB-1 fragment is more stable than the full-length protein and that the C-terminal part is indeed important for YB-1 degradation via the proteasomal pathway. Together, these data indicate that proteasomal cleavage of YB-1 in response to DNA-damaging stress leads to nuclear accumulation of truncated YB-1.

Appearance of truncated YB-1 in the nucleus may predict multiple drug resistance

The above results are surprising in the light of previous reports showing positive correlation between nuclear accumulation of full-length YB-1 and resistance of various human cancer cells to certain DNA-damaging agents including cisplatin, mitomycin C and UV irradiation (Ohga *et al*, 1996; Koike *et al*, 1997). Moreover, nuclear localization of YB-1 has been proposed as a marker for patient outcome and multiple drug resistance in various cancers including breast, ovarian, colon, non-small-cell lung carcinomas and osteosarcomas (Bargou *et al*, 1997; reviewed in Kuwano *et al*, 2004). In the majority of these studies, however, nuclear accumulation of YB-1 was monitored by immunofluorescence microscopy or immunohistochemistry, the approaches that are unable to discriminate between the full-length and truncated YB-1 forms. We thus performed a pilot study using primary cancer cells from patients with tumors of a different origin. To analyze a possible correlation between drug sensitivities and accumulation of truncated YB-1, cancer cells were treated for 24 h with a panel of commonly used therapeutic agents, such as doxorubicin, carboplatin, cisplatin and taxol. As seen in Figure 6A, massive accumulation of a 32 kDa fragment immunoreactive with YB-1 antibodies in response to drug treatment was observed in breast, lung and ovarian cancer cells (lanes 1–15). For unclear reasons, levels of full-length YB-1 remained mostly unchanged despite a substantial increase of the p32 form. It is also unclear where this truncated YB-1 was accumulated, as quantities of the available material were not sufficient to separate nuclear and cytosolic fractions. Despite these limitations, generation of the truncated YB-1 protein in primary cancer cells of various origins in response to therapeutic agents raises the possibility that it is a truncated but not full-length YB-1 that is associated with multiple drug resistance of cancer cells.

To further address whether generation of truncated YB-1 may be associated with increased drug resistance, we monitored YB-1 levels in K-Ras-transformed NIH3T3 cells growing in the presence of doxorubicin over a 9-day period. Levels of full-length YB-1 were substantially decreased in both cytosolic and nuclear fractions during the first 5 days of treatment (Figure 6C and D). During the same period, accumulation of the truncated 32 kDa YB-1 fragment in nuclear fractions was evident (Figure 6D, lanes 2–6). From day 5 on, levels of full-length YB-1 were gradually elevated and prevailed over those of the truncated protein (Figure 6D, lanes 7–10). This was

Figure 5 Truncated YB-1 accumulates in the nucleus following DNA-damaging stress. (A) NIH3T3 cells stably expressing HA-YB-1 were treated with doxorubicin (0.6 μ g/ml), etoposide (10 μ M) or cisplatin (10 μ M) for 14 h. Western of the corresponding cytosolic (C) and nuclear (N) fractions is shown. (B) NIH3T3-HA-YB-1 cells were treated with doxorubicin for the time indicated and analyzed by immunofluorescence microscopy using mouse anti-HA and rabbit anti-PABP antibodies, followed by secondary Alexa Fluor 488 anti-mouse and Alexa Fluor 594 anti-rabbit antibodies. Nuclei were visualized with DAPI. Micrographs were taken at $\times 100$ magnification. (C) NIH3T3-HA-YB-1 cells were treated for 16 h with doxorubicin and 20 μ M MG132, as indicated. HA-YB-1 was detected by immunofluorescence microscopy using anti-HA antibodies. Micrographs were taken at $\times 40$ magnification. (D) Western showing cytosolic (C) and nuclear (N) fractions of cells treated as in (C). (E, F) NIH3T3 cells were transiently transfected with vector alone or with plasmids coding for the HA-tagged full-length or truncated YB-1 forms. At 24 h following transfection, cells were processed for immunofluorescence microscopy (E), or cultured for additional 18 h in the absence or presence of doxorubicin. Whole-cell extracts from untreated or doxorubicin-treated cells were then analyzed by Western using anti-HA or anti-PABP antibodies (F).

also concurrent with restoration of cell growth (Figure 6B), which is probably attributed to development of doxorubicin resistance in surviving cells. Of further note, there was no difference between the levels of full-length YB-1 in nuclei of doxorubicin-sensitive and doxorubicin-resistant cells (Figure 6D, compare lanes 1 and 10). The levels of full-length YB-1 likely reflect a proliferative status of the cell, and therefore a predictive value of the nuclear localization of full-length YB-1 has to be reconsidered. Instead, the levels of truncated YB-1 were elevated immediately after doxorubicin

treatment and kept high in cells growing in the presence of doxorubicin, suggesting that appearance of truncated YB-1 in the nucleus may be indicative of increased resistance to doxorubicin and potentially to other cytotoxic agents.

Discussion

In this study, we show that YB-1 is targeted for specific endoproteolytic cleavage by the 20S proteasome that removes its C-terminal 105 amino-acid residues containing the CRS.

Figure 6 Truncated YB-1 accumulates in various cancer cells in response to stress. (A) Primary cancer cells derived from pleural fluids of the indicated human tumors (for details, see Richard *et al*, 2005) were exposed to doxorubicin (0.3 μ g/ml), carboplatin (180 μ M), cisplatin (8.8 μ M) or taxol (9.4 μ M) for 24 h. Whole-cell extracts were then prepared from surviving adherent cells and analyzed by Western using anti-YB-1 or anti-PABP antibodies. (B) Effect of doxorubicin on a long-term cell survival. K-Ras-NIH3T3 cells ectopically expressing HA-YB-1 were grown in the presence of doxorubicin (0.3 μ g/ml) for 9 days. Adherent cells were counted each day as described under Materials and methods. Error bars denote the standard error between replicates. Cytosolic (C) and nuclear (D) fractions obtained at each time point were analyzed by Western using anti-HA antibodies. Blots were re-probed with PABP and histone H3 antibodies to show equal loading and purity of the corresponding fractions. (E) A model for YB-1 cleavage/degradation. Translational activation or mild stress may trigger YB-1 dissociation from mRNA followed by endoproteolytic cleavage by 20S proteasomes, which supposedly recognize a hairpin-like loop of the YB-1 CTD created by positive and negative amino-acid clusters. Under severe stress conditions, YB-1 is likely to be polyubiquitinated and degraded via the classical ubiquitin-proteasomal pathway. (F) Domain organization of YB-1 with the indicated NLS and the CRS is given according to Stenina *et al* (2001), Jurchott *et al* (2003) and Bader and Vogt (2005). The arrow indicates cleavage site determined in this study. Below are listed known molecular partners of YB-1 (for review, see Kohno *et al*, 2003), whose interaction might be affected by YB-1 cleavage.

The resulting N-terminal 32 kDa polypeptide appears to be accumulated in nuclei of cells exposed to DNA-damaging drugs. Endoproteolytic cleavage of YB-1 by the 20S proteasome may therefore be considered as a highly specific mechanism regulating YB-1 localization and functioning in the cell.

Protein degradation through the proteasomal pathway is known to be a tightly regulated process in which attachment of multiple ubiquitin molecules to the target protein usually serves as a signal for protein degradation (Hershko and Ciechanover, 1998; Goldberg, 2003). The majority of polyubiquitinated proteins are targeted for degradation by the 26S proteasome, which consists of one or two 19S regulatory

complexes attached to the core 20S proteasome. The 19S complex is thought to be responsible for recognition and unfolding of target proteins in an ATP-dependent manner and for transferring them into the inner catalytic chamber of the 20S proteasome.

Our findings that YB-1 undergoes a limited proteolysis by the 20S proteasome that occurs in ubiquitin- and ATP-independent manner indicate a specific mechanism of YB-1 targeting. Several other proteins including α -synuclein, p21^{Cip1}, p53 and p73 were reported to be degraded by the 20S proteasome without ubiquitylation and, in some cases, without ATP (Tofaris *et al*, 2001; Touitou *et al*, 2001; Amici *et al*, 2004; Asher *et al*, 2005). In contrast to YB-1, however,

the above proteins were completely degraded by the 20S proteasome. The only known case of limited proteolysis catalyzed by the 20S proteasome has recently been reported for the translation initiation factors eIF4G and eIF3a (Baugh and Pilipenko, 2004). Both proteins were shown to be endoproteolytically cleaved by the 20S proteasome to produce large products with distinct functional properties. It remains unclear how the 20S proteasome could mediate a specific cleavage of YB-1, eIF4G and eIF3a. Supposedly, the targeted region should have certain properties that allow its specific recognition and endoproteolytic cleavage by the proteasome. The C-terminal region of YB-1 targeted for cleavage has two remarkable features. First, it contains a glycine–arginine–proline region that displays some similarity to the glycine–arginine-rich region (GRR) in NF- κ B p105 precursor, which is essential for processing of p105 by the 26S proteasome (Lin and Ghosh, 1996). A similar region has also been shown to be important for endoproteolytic cleavage and subsequent proteasomal degradation of the transcription factor SP1 (Su *et al*, 1999). Second, the C-terminal portion of YB-1 is unstructured owing to the presence of multiple proline residues, and is also thought to be able to form a hairpin loop structure via interaction between amino-acid clusters of opposite charge (Wolffe, 1994). The structural organization of YB-1 ideally meets the requirements of a so-called ‘loop model’ implying that a flexible segment of the polypeptide chain folded into a hairpin loop may be endoproteolytically cleaved by active sites of the 20S proteasome, followed by processive degradation of a disordered domain (Liu *et al*, 2003). The CTD of YB-1 is fused with a highly stable globular CSD that supposedly cannot enter the 20S annulus and thereby ceases further degradation (Figure 6E). We therefore propose that it is overall structure of the CTD but not a particular specific sequence that is responsible for recognition and cleavage of YB-1 by the 20S proteasome.

Since truncated YB-1 was found predominantly in the nucleus (Figures 5 and 6D), proteasomal cleavage of YB-1 is likely sufficient to trigger its nuclear accumulation. However, the presence of full-length YB-1 in the nucleus observed in this study and by other researchers (Koike *et al*, 1997; Jurchott *et al*, 2003) suggests that truncation of YB-1 does not serve simply to ensure its nuclear localization. Rather, coexistence of the truncated and full-length YB-1 forms in the nucleus may indicate an altered biological function of the former one. This notion is supported by reports showing that the truncated N-terminal YB-1 fragment is capable of recognizing a thrombin-responsive element but not the Y-box consensus sequence, and of inducing PDGF and tissue factor promoter activities much stronger than full-length YB-1 (Stenina *et al*, 2000, 2001). In contrast, an ability of truncated YB-1 to promote splicing was reported to be reduced (Stickeler *et al*, 2001). Truncated YB-1 retains the majority of binding sites for its known ligands (Figure 6F). However, some sites including those for RNA/DNA binding, YB-1 oligomerization and its association with certain proteins, such as p53, hnRNP K, EWS, Smad3 and YBAP1, are either entirely located on or extended to the split off fragment and may therefore be affected by the cleavage.

Of interest, the involvement of truncated YB-1 in stress response seems to have an ancient origin since a family of small closely related proteins that share over 45% sequence identity with the CSD of YB-1 also exists in bacteria. These

proteins are defined as cold shock proteins since some of them were first identified as being strongly induced at low temperatures. Later on, members of this widespread protein family were implicated in various cellular processes, including adaptation to low temperatures, cellular growth, nutrient and cytotoxic stress (Graumann and Marahiel, 1998). Interestingly, their expression is strongly restricted under optimal growth conditions.

Considering that truncated YB-1 was generated in response to genotoxic stress, it may play a role in transcriptional activation of stress-responsive genes or DNA repair. It is unclear, however, whether it is involved in primary stress response or triggers development of multidrug resistance. We favor the first possibility, as the mechanism leading to generation of truncated YB-1 was induced by DNA damage. Under optimal growth conditions, this mechanism is supposedly inactivated, since we did not observe truncated YB-1 in exponentially growing cells. It might therefore be predicted that abnormal activation of this mechanism may lead to a permanent generation of truncated YB-1, thereby increasing resistance of affected cells to DNA-targeting drugs and, perhaps, to other classes of therapeutic agents. Such a possibility is plausible for cancer cells, where the ubiquitin–proteasomal pathway is believed to be deregulated (Voorhees *et al*, 2003). A linkage between generation of truncated YB-1 and pleiotropic resistance of cancer cells to therapeutics needs further investigation to establish causal relationships.

Materials and methods

Reagents

YU102, ALLN, battenecin-5, epoxomicin, lactacystin, MG132, succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC) and 20S proteasome (cat.#PW8720) were purchased from BioMol Inc. Brefeldin A, camptothecin, cisplatin, DMSO, doxorubicin, DTT, etoposide, dexamethasone, nocodazole, PMSF, thapsigargin, tunicamycin, 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) and casein were purchased from Sigma and 26S proteasome (cat.# 539159) and proteasome Inhibitor I were from Calbiochem.

Expression constructs, proteins and antibodies

Expression plasmids and purification of recombinant YB-1 proteins were described previously (Evdokimova *et al*, 1995, 2001). pcDNA3-HA-YB-1(1–204) was generated by subcloning the *Nde*I–*Xho*I fragment encoding N-terminal *Sal*I-truncated YB-1 from pET-15b-YB-1 into the corresponding sites of pcDNA3-HA. pMSCV_{hygro}-HA-YB-1 was obtained by subcloning the *Hind*III fragment of pcDNA3-HA-YB1 into the *Hpa*I site of pMSCV_{hygro} (Clontech) after blunting the ends with T4 DNA polymerase. The following antibodies were used in this study: anti-YB-1 (Davydova *et al*, 1997), anti-PABP (gift from N Sonenberg), anti-TIAR (Transduction Laboratories), anti-HA (Babco), anti-histone H3 (Upstate), anti-ubiquitin (Santa Cruz) and anti-20S proteasomal subunits α 5 (p27K; clone IB5) and α 6 (p30K; clone 62A32) (gift from K Scherrer).

Purification of YB-1-specific protease activity and 26S proteasome

RRL (25 ml) obtained as described earlier (Evdokimova *et al*, 1995) was applied onto DEAE-cellulose (DE-52; Whatman) in an XK26/20 column (Amersham Pharmacia Biotech) equilibrated with buffer A (10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 5% sucrose, 10% glycerol (v/v)). After extensive wash with buffer A, the adsorbed material was eluted with buffer B (10 mM Tris-HCl (pH 7.6), 300 mM NaCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 5% sucrose, 10% glycerol (v/v)). Eluted samples were precipitated with ammonium sulfate (AS; 40% saturation), resuspended in 6 ml of buffer A and subjected to FPLC size-exclusion chromatography (350 ml of HiPrep Sephacryl S-300 in an XK26/70

column equilibrated with buffer A). Fractions displaying proteolytic activity toward YB-1 were collected, concentrated using Centricon-10 (Millipore) and loaded onto Mono Q HR 5/5 (Amersham Pharmacia Biotech) equilibrated with buffer C (10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 10% glycerol (v/v)). Bound proteins were eluted by a gradient ranging from buffer C to buffer D (10 mM Tris-HCl (pH 7.6), 1 M NaCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 10% glycerol (v/v)) and analyzed for proteolytic activity toward YB-1. The fraction displaying maximal proteolytic activity was eluted at 400 mM NaCl and then additionally purified using Superose 12 HR 10/30 (Amersham Pharmacia Biotech) equilibrated with buffer E (10 mM Tris-HCl (pH 7.6), 2 M NaCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 10% glycerol (v/v)). All steps were carried out at 4°C. Purified protease was concentrated with Centricon-10, dialyzed against buffer F (10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 2 mM MgCl₂, 50% (v/v) glycerol) and stored at -20°C. 26S proteasomes were purified as described above, except that all buffers contained 2 mM ATP, and fractions possessing proteasomal activity were identified by their ability to hydrolyze Suc-LLVY-AMC. In contrast to 20S, 26S peptidase activity of the resulting preparation was inhibited by 0.02% SDS or by removal of ATP, suggesting no cross contamination between 20S and 26S preparations.

MALDI-TOF mass spectrometry

For mass spectrometric identification, protease components were separated by SDS-12% PAGE, visualized by Coomassie blue staining, excised from the gel and subjected to tryptic digestion according to standard procedures. Mass spectrometry was performed using a MALDI-TOF mass spectrometer (Reflex III model; Bruker Analytic GmbH, Bremen, Germany) with 337 nm UV laser. Ion acceleration voltage was 20 kV. Spectra were collected in a positive ion reflector mode with delay extraction and identified via MASCOT database (www.matrixscience.com) using Peptide Mass Fingerprint Program.

Proteolytic assays and N-terminal sequencing

Incubations of 1–1.5 µg proteins with 0.1 µg proteasome were performed at 30°C for 1 h in a total volume of 20 µl of buffer G (30 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM CaCl₂, 2 mM MgCl₂, 50 µM ATP, 1 mM DTT, 5% (v/v) glycerol). Reactions were stopped by addition of 5 × Laemmli sample buffer, resolved by SDS-15% PAGE and stained with Coomassie. For mapping of the cleavage site, YB-1 cleavage products were separated by SDS-15% PAGE and transferred onto an Immobilon-P membrane in transfer buffer O (10 mM H₃BO₄ (pH 11), 0.02% (v/v) 2-mercaptoethanol) using a wet-blotter at 400 mA for 2 h. N-terminal sequencing of the peptides was performed in an automatic gas-phase sequencer (Applied Biosystems model 477A) equipped with an HPLC system 120A from the same manufacturer.

Cell cultures, subcellular fractionation and Western blotting

NIH3T3 cells were obtained from ATCC and grown in DMEM (Invitrogen) containing 10% calf serum (Invitrogen). Nontrans-

formed and K-Ras-transformed NIH3T3 cells were generated by retroviral transduction of NIH3T3 with empty vector pMSCV_{puro} (Clontech) or pMSCV_{puro}-GFP-K-Ras plasmid (gift from R Kay), respectively. Cell lines stably expressing HA-YB-1 were generated from nontransformed or K-Ras-transformed cells using pMSCV_{hygro}-HA-YB-1. Cells were lysed with buffer containing 20 mM Hepes-KOH (pH 7.8), 50 mM KCl, 2 mM MgCl₂, 2 mM DTT, 0.25% NP-40 and 20 µM MG132. Nuclear, mitochondrial and post-mitochondrial fractions were separated by sequential centrifugation at 1000g for 5 min, followed by centrifugation at 10000g for 15 min. The resulting post-mitochondrial supernatant was utilized as a cytosolic fraction. Nuclei were additionally purified from the re-suspended 1000g pellet fraction by spinning down through a 50% glycerol cushion and disrupted by sonication. Cell fractions were normalized for protein concentration using the Protein Assay kit (Bio-Rad). Immunoprecipitation and Western blotting were performed as described earlier (Evdokimova *et al*, 2001).

Immunofluorescence microscopy

NIH3T3 cells were grown on coverslips up to 50% confluency, rinsed with PBS, fixed in 3.7% paraformaldehyde-PBS for 10 min and permeabilized in PBS-0.5% Nonidet P-40 for 5 min. Coverslips were blocked with 5% milk in PBS for 30 min and incubated overnight in 0.5% milk-PBS with primary antibodies, followed by fluorophore-conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA). Slides were analyzed using a Zeiss Axioplan epifluorescent microscope equipped with a COHU-CCD camera.

Cell counting and flow cytometry

Cells (~1 × 10⁶/well; ~80% confluent) were seeded in six-well plates in triplicate and treated with various drugs, as indicated in the figure legends. Cells were counted every day over a 10-day period using a hemocytometer. For quantification of DNA content, cells were washed with PBS and re-suspended in 1 ml of propidium iodide (PI) solution containing 50 µg/ml PI, 0.1% sodium citrate, 0.1% Triton X-100 and 0.2 mg/ml RNase A. After incubation for 2 h at 4°C, cells were analyzed using a FACSCalibur flow cytometer (Beckton Dickinson) with the CellQuest program.

Acknowledgements

We thank Alexei Kisselev for helpful discussions, Klaus Scherrer for antibodies against proteasomal subunits, Nahum Sonenberg for anti-PABP antibodies, Rob Kay for pMSCV_{puro}-GFP-K-Ras construct and Evgeniya Serebrova for help in manuscript preparation. This work was supported in part by the 'Molecular and Cellular Biology' and 'Basic Sciences to Medicine' programs from Russian Academy of Sciences and by a grant from the President of the Russian Federation (#1959.2003.4) to LP, Russian Foundation for Basic Research (#98-04-48015) to VE and by the Johal Program in Pediatric Oncology Basic and Translational Research at the Child and Family Research Institute to PHBS and VE.

References

- Amici M, Sagratini D, Pettinari A, Pucciarelli S, Angeletti M, Eleuteri AM (2004) 20S proteasome mediated degradation of DHFR: implications in neurodegenerative disorders. *Arch Biochem Biophys* **422**: 168–174
- Asher G, Tsvetkov P, Kahana C, Shaul Y (2005) A mechanism of ubiquitin-independent proteasomal degradation of the tumor suppressors p53 and p73. *Genes Dev* **19**: 316–321
- Bader AG, Vogt PK (2005) Inhibition of protein synthesis by Y box-binding protein 1 blocks oncogenic cell transformation. *Mol Cell Biol* **25**: 2095–2106
- Bargou RC, Jurchott K, Wagener C, Bergmann S, Metzner S, Bommert K, Mapara MY, Winzer KJ, Dietel M, Dorken B, Royer HD (1997) Nuclear localization and increased levels of transcription factor YB-1 in primary human breast cancers are associated with intrinsic MDR1 gene expression. *Nat Med* **3**: 447–450
- Baugh JM, Pilipenko EV (2004) 20S proteasome differentially alters translation of different mRNAs via the cleavage of eIF4F and eIF3. *Mol Cell* **16**: 575–586
- Davydova EK, Evdokimova VM, Ovchinnikov LP, Hershey JW (1997) Overexpression in COS cells of p50, the major core protein associated with mRNA, results in translation inhibition. *Nucleic Acids Res* **25**: 2911–2916
- Didier DK, Schiffenbauer J, Woulfe SL, Zacheis M, Schwartz BD (1988) Characterization of the cDNA encoding a protein binding to the major histocompatibility complex class II Y box. *Proc Natl Acad Sci USA* **85**: 7322–7326
- Evdokimova V, Ruzanov P, Imataka H, Raught B, Svitkin Y, Ovchinnikov LP, Sonenberg N (2001) The major mRNA-associated protein YB-1 is a potent 5' cap-dependent mRNA stabilizer. *EMBO J* **20**: 5491–5502
- Evdokimova VM, Wei CL, Sitikov AS, Simonenko PN, Lazarev OA, Vasilenko KS, Ustinov VA, Hershey JW, Ovchinnikov LP (1995) The major protein of messenger ribonucleoprotein particles in somatic cells is a member of the Y-box binding transcription factor family. *J Biol Chem* **270**: 3186–3192

- Goldberg AL (2003) Protein degradation and protection against misfolded or damaged proteins. *Nature* **426**: 895–899
- Graumann PL, Marahiel MA (1998) A superfamily of proteins that contain the cold-shock domain. *Trends Biochem Sci* **23**: 286–290
- Groll M, Ditzel L, Lowe J, Stock D, Bochtler M, Bartunik HD, Huber R (1997) Structure of 20S proteasome from yeast at 2.4 Å resolution. *Nature* **386**: 463–471
- Hershko A, Ciechanover A (1998) The ubiquitin system. *Annu Rev Biochem* **67**: 425–479
- Hoyt MA, Coffino P (2004) Ubiquitin-free routes into the proteasome. *Cell Mol Life Sci* **61**: 1596–1600
- Jurchott K, Bergmann S, Stein U, Walther W, Janz M, Manni I, Piaggio G, Fietze E, Dietel M, Royer HD (2003) YB-1 as a cell cycle-regulated transcription factor facilitating cyclin A and cyclin B1 gene expression. *J Biol Chem* **278**: 27988–27996
- Kisselev AF, Akopian TN, Woo KM, Goldberg AL (1999) The sizes of peptides generated from protein by mammalian 26 and 20 S proteasomes. Implications for understanding the degradative mechanism and antigen presentation. *J Biol Chem* **274**: 3363–3371
- Kohn K, Izumi H, Uchiumi T, Ashizuka M, Kuwano M (2003) The pleiotropic functions of the Y-box-binding protein, YB-1. *BioEssays* **25**: 691–698
- Koike K, Uchiumi T, Ohga T, Toh S, Wada M, Kohn K, Kuwano M (1997) Nuclear translocation of the Y-box binding protein by ultraviolet irradiation. *FEBS Lett* **417**: 390–394
- Kuwano M, Oda Y, Izumi H, Yang SJ, Uchiumi T, Iwamoto Y, Toi M, Fujii T, Yamana H, Kinoshita H, Kamura T, Tsuneyoshi M, Yasumoto K, Kohn K (2004) The role of nuclear Y-box binding protein 1 as a global marker in drug resistance. *Mol Cancer Ther* **3**: 1485–1492
- Lamond AI, Spector DL (2003) Nuclear speckles: a model for nuclear organelles. *Nat Rev Mol Cell Biol* **4**: 605–612
- Lin L, Ghosh S (1996) A glycine-rich region in NF-kappaB p105 functions as a processing signal for the generation of the p50 subunit. *Mol Cell Biol* **16**: 2248–2254
- Liu CW, Corboy MJ, DeMartino GN, Thomas PJ (2003) Endoproteolytic activity of the proteasome. *Science* **299**: 408–411
- Matsumoto K, Wolffe AP (1998) Gene regulation by Y-box proteins: coupling control of transcription and translation. *Trends Cell Biol* **8**: 318–323
- Minich WB, Ovchinnikov LP (1992) Role of cytoplasmic mRNP proteins in translation. *Biochimie* **74**: 477–483
- Ohga T, Koike K, Ono M, Makino Y, Itagaki Y, Tanimoto M, Kuwano M, Kohn K (1996) Role of the human Y box-binding protein YB-1 in cellular sensitivity to the DNA-damaging agents cisplatin, mitomycin C, and ultraviolet light. *Cancer Res* **56**: 4224–4228
- Orlowski M, Wilk S (2003) Ubiquitin-independent proteolytic functions of the proteasome. *Arch Biochem Biophys* **415**: 1–5
- Richard C, Matthews D, Duivenvoorden W, Yau J, Wright PS, Th'ng JP (2005) Flavopiridol sensitivity of cancer cells isolated from ascites and pleural fluids. *Clin Cancer Res* **11**: 3523–3529
- Skabkin MA, Kiselyova OI, Chernov KG, Sorokin AV, Dubrovin EV, Yaminsky IV, Vasiliev VD, Ovchinnikov LP (2004) Structural organization of mRNA complexes with major core mRNP protein YB-1. *Nucleic Acids Res* **32**: 5621–5635
- Stenina OI, Poptik EJ, DiCorleto PE (2000) Thrombin activates a Y box-binding protein (DNA-binding protein B) in endothelial cells. *J Clin Invest* **106**: 579–587
- Stenina OI, Shaneyfelt KM, DiCorleto PE (2001) Thrombin induces the release of the Y-box protein dbpB from mRNA: a mechanism of transcriptional activation. *Proc Natl Acad Sci USA* **98**: 7277–7282
- Stickeler E, Fraser SD, Honig A, Chen AL, Berget SM, Cooper TA (2001) The RNA binding protein YB-1 binds A/C-rich exon enhancers and stimulates splicing of the CD44 alternative exon v4. *EMBO J* **20**: 3821–3830
- Su K, Roos MD, Yang X, Han I, Paterson AJ, Kudlow JE (1999) An N-terminal region of Sp1 targets its proteasome-dependent degradation *in vitro*. *J Biol Chem* **274**: 15194–15202
- Tofaris GK, Layfield R, Spillantini MG (2001) Alpha-synuclein metabolism and aggregation is linked to ubiquitin-independent degradation by the proteasome. *FEBS Lett* **509**: 22–26
- Touitou R, Richardson J, Bose S, Nakanishi M, Rivett J, Allday MJ (2001) A degradation signal located in the C-terminus of p21WAF1/CIP1 is a binding site for the C8 alpha-subunit of the 20S proteasome. *EMBO J* **20**: 2367–2375
- Tsubuki S, Saito Y, Kawashima S (1994) Purification and characterization of an endogenous inhibitor specific to the Z-Leu-Leu-Leu-MCA degrading activity in proteasome and its identification as heat-shock protein 90. *FEBS Lett* **344**: 229–233
- Voorhees PM, Dees EC, O'Neil B, Orlowski RZ (2003) The proteasome as a target for cancer therapy. *Clin Cancer Res* **9**: 6316–6325
- Wolffe AP (1994) Structural and functional properties of the evolutionarily ancient Y-box family of nucleic acid binding proteins. *BioEssays* **16**: 245–251