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Hypoxia and DNA-Damaging Agent Bleomycin Both Increase the Cellular Level of the Protein 4E-BP

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Abstract The 4E-binding proteins (4E-BPs) regulate the cap-dependent eukaryotic initiation factor 4E (eIF4E). The level of 4E-BP protein is regulated during early development of sea urchin embryos. Fertilization leads to the rapid disappearance of the protein that reappears later in development. We show that two important cellular stresses, hypoxia and bleomycin prolonged checkpoint mobilization provoked the overexpression of the protein 4E-BP in developing sea urchin embryos. Hypoxia resulted after 1 h in a reversible gradual increase in the protein 4E-BP level. At 20 h, the protein 4E-BP had reached the level existing in the unfertilized eggs. Bleomycin used as a DNA-damaging agent for checkpoint activation, provoked cell cycle inhibition and after prolonged exposure (20 h), induced the expression of the protein 4E-BP. The effect of bleomycin on 4E-BP protein overexpression was dose-dependent between 0.4 and 1.2 mM. The role of the overexpression of the protein 4E-BP is discussed in relation with cellular stress responses. J. Cell. Biochem. 99: 126–132, 2006.

Key words: 4E-BP; hypoxia; bleomycin radiomimetic; cell cycle checkpoint; sea urchin early development

Hypoxia is insufficient oxygen delivery for the demand of the tissue and cellular hypoxia can arise from physiological circumstances such as exercise or travel to high elevation, as well as from pathophysiologic conditions such as poorly formed tumor vasculature [review in Papandreou et al., 2005; Wouters et al., 2005]. The investigation of the hypoxia cellular response is of great importance since hypoxia impacts normal development, wound healing, and the malignant progression of a solid tumor. The cellular response to hypoxia depends on the severity of the hypoxic insult, and may result in complex gene expression changes designed to maintain cellular and tissue viability by involvement of transcriptional and post-transcriptional events. Severe hypoxia has cell cycle adverse effects and provokes apoptosis [review in Hammond and Giaccia, 2005]. Many results have been obtained in the past decade on the cellular mechanisms of hypoxia response [review in Papandreou et al., 2005]. Response to hypoxia is driven in a large part by a transcriptional program initiated by stabilization of hypoxia-inducible factors (HIF), which promote both angiogenesis and cell survival [reviews in Guillemin and Krasnow, 1997; Semenza, 2003; Cummins and Taylor, 2005; Wouters et al., 2005]. The hypoxia-inducible factor 1 (HIF-1) activates transcription of genes that are involved in crucial aspects of cancer biology and its discovery has opened an attractive field in anticancer therapeutics since inhibition of HIF-1 activity was shown to have marked effects on tumor growth [review in Semenza, 2003].

Beside HIF stabilization, hypoxia also results in all cell lines tested to date, in a rapid inhibition of protein synthesis, which occurs through the repression of the initiation step of mRNA translation [review in Wouters et al., 2005]. Inhibition of translation has been found to be mediated by two distinct mechanisms involving on the one hand the eukaryotic initiation factor eIF2α and on the other hand the eukaryotic initiation factor, eIF4E [Wouters

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et al., 2005]. The eukaryotic initiation factor, eIF4E, is implicated in cap-dependent translation and serves as a major control point for regulating the efficiency of mRNA translation by the cell [review in Richter and Sonenberg, 2005]. The eIF4E factor is the rate-limiting element of cap-dependent translation and is regulated by the small 4E-binding protein family, 4E-BPs [Pause et al., 1994; Haghighat et al., 1995]. The availability of eIF4E protein has been involved in the hypoxia responses in part by the phosphorylation of the 4E-BPs [review in Wouters et al., 2005]. Hypoxia negatively regulates the protein kinase mTOR resulting in the decrease of the phosphorylation of 4E-BP leading to its reassociation to eIF4E and the consequent inhibition of cap-dependent translation [Wouters et al., 2005]. No effect of hypoxia on the synthesis or level of 4E-BP has yet been described. Sea urchin early embryos contain a small 4E-BP protein of 16 kDa [Cormier et al., 2001] which is, as in mammalian cells under the control of the protein kinase mTOR [Salaun et al., 2003, 2004]. Early development of sea urchin is characterized by rapid cell divisions and a large increase in protein synthesis independent of transcription [Davidson, 1986; Epel, 1990; Gilbert, 2003]. The 4E-BP is fully degraded shortly after fertilization in correlation with the cap-dependent protein synthesis increase [Salaun et al., 2003]. Therefore, sea urchin early development offers the opportunity to analyze new insights into the regulation of 4E-BP under hypoxic stress.

In addition to regulation of translation and translation factors [review in Epel, 1990; Mathews et al., 2000], sea urchin early development allows the study of cell cycle regulation [Evans et al., 1983; Epel, 1990; Nurse, 2002]. It is noteworthy that sea urchin embryo contains the DNA-damage checkpoint of the cell cycle [Marc et al., 2002; 2004a,b]. The cell cycle checkpoint refers to mechanisms by which the cell actively halts progression through the cell cycle until it can ensure that an earlier process, such as DNA replication or mitosis, is complete [review in Hartwell and Kastan, 1994; Kastan and Bartek, 2004; Van Vugt et al., 2005]. Since hypoxia may induce DNA-damage checkpoint activation through reactive oxygen species (ROS) production [Barzilai and Yamamoto, 2004], it was of interest to compare the hypoxia response of the embryos with a DNA-damaging agent. We used bleomycin, a radiomimetic [Povirk, 1996] shown to be an efficient DNA-damage checkpoint inducer. Bleomycin provokes DNA double-strand breaks mobilizing the ATM pathway [Scott and Zampetti-Bosseler, 1985; Kaufmann and Kies, 1998] and producing ROS [Barzilai and Yamamoto, 2004]. The radiomimetic bleomycin provokes both G2 cell cycle delay and apoptosis in mammalian cells as a result of the checkpoint activation [Kaufmann and Kies, 1998; Vernole et al., 1998]. Interestingly, the 4E-BP protein was shown to be regulated by ATM phosphorylation under cell growth condition [Yang and Kastan, 2000].

We show here for the first time, that hypoxia and the radiomimetic drug bleomycin both induce expression of the protein 4E-BP. Therefore, the protein 4E-BP provides a molecular link between hypoxia cellular response and prolonged activation of the cell cycle DNA-damage checkpoint.

MATERIALS AND METHODS

Handling of Eggs and Embryos

Sphaerechinus granularis sea urchins were collected in the Brest area and maintained in running seawater. Spawning of gametes was induced by intracoelomic injection of 0.1 M acetylcholine. Eggs were collected in 0.22-μm Millipore-filtered seawater, rinsed twice and suspended in filtered seawater containing 0.1% glycine. Diluted sperm was added to the eggs and withdrawn after fertilization membrane elevation. Experiments were only performed on batches exhibiting greater than 90% fertilization and each experiment used eggs from a single female [Marc et al., 2002].

DNA Staining

At various times after fertilization, 0.2 ml aliquots of the embryos suspension were fixed overnight in 1 ml methanol/glycerol (3:1 v/v) in the presence of the DNA dye Hoechst (bisbenzimide: 0.1 μg/ml), were mounted in 50% glycerol and observed under fluorescence microscopy [Marc et al., 2002].

Embryo Treatments

For bleomycin treatment, eggs were dejellied by swirling 30 s in pH 5.0 filtered seawater, and rinsed twice in fresh seawater prior to fertilization. Twenty minutes post-fertilization, bleomycin (Aventis) was added to the incubation medium at the indicated concentration. Bleomycin was

removed from the incubation medium by rinsing the embryos twice in fresh seawater.

Hypoxia (oxygen level under 2 mg/l) was induced by incubation of the embryos 10 min post-fertilization, at the indicated dilution, in a closed chamber device preventing oxygen turnover. To relieve from hypoxia, the device was opened. Oxygen concentration in the embryos suspension was measured with oxygen electrodes DW3 Clark-type, Hansatech Instruments, King's Lynn, UK [Kupper et al., 2001].

Embryo Extracts and Protein Analysis

At different times following fertilization, whole embryo extracts were obtained by homogenization of 30 µl pelleted cells in 100 µl of buffer containing 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% β-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8 [Boulben et al., 2003].

Western Blot Analyses

Electrophoretic resolution of proteins was performed under SDS denaturing conditions on a 15% polyacrylamide gel according to Laemmli [1970]. Proteins were electro-transferred from the gel onto nitrocellulose membranes (Schleicher and Schuell). The membranes were saturated overnight in tris buffer saline (TBS) containing 0.1% Tween 20 and 10% skimmed milk. The protein 4E-BP was analyzed using rabbit polyclonal antibodies directed against human 4E-BP2 [Rousseau et al., 1996] used at 1:1,000. Rabbit polyclonal anti human actin (20–33, Sigma) was used at a 1:500 dilution. Secondary peroxidase conjugated antibodies (goat anti rabbit IgG antiserum, DAKO) were used at 1:5,000. Revelation was done by ECL method according to the manufacturer instruction (Amersham).

RESULTS

Effect of Hypoxia on the level of 4E-BP Expression

We took advantage of the physiological disappearance of the protein 4E-BP shortly after fertilization in sea urchin [Salaun et al., 2005] to analyze the effect of hypoxia on the level of the protein. Figure 1 illustrates the cellular level of the 4E-BP protein as judged from immunoblotting experiments using specific anti-4E-BP antibodies at different times following fertilization. The 16 kDa protein 4E-BP rapidly disappeared and slowly reappeared

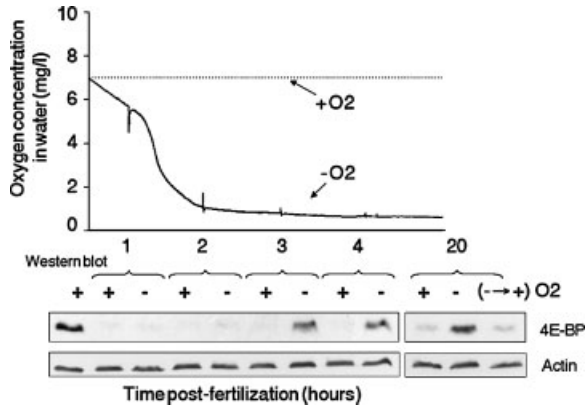

Fig. 1. Hypoxia regulates the protein 4E-BP expression in sea urchin embryo. Sea urchin eggs (15% suspension) were fertilized and after 10 min, placed in a closed chamber ($-O_2$), or in an open chamber ($+O_2$). At 4 h, part of the embryos in the closed chamber was transferred in an open chamber ($- \rightarrow +$) to restore oxygen level (~7 mg/l). Oxygen level was measured with a Clark electrode. At the indicated time after fertilization whole protein extracts were performed and analyzed after electrophoresis for the presence of 4E-BP by immunoblotting. Immunoblotting with anti-actin antibodies was used as a loading control. NF: unfertilized eggs.

in the control embryos in concordance with previous results [Salaun et al., 2005]. In the hypoxia condition, which occurred after 2 h as judged from the oxygen concentration in seawater (below 2 mg/l), the reappearance of 4E-BP occurred at 3 h post-fertilization, that is, as early as 1 hour after hypoxia condition. At 20 h post-fertilization, whereas the expression of 4E-BP in the control embryos was barely detectable, the level of the protein in the hypoxia embryos was comparable to the initial level in the unfertilized embryos (Fig. 1). Correlatively, we observed a delay in early development in the hypoxia conditions both in the kinetic of cell divisions and hatching event (data not shown). Nonetheless, no sign of cytolysis, or apoptosis was observed under phase contrast microscopic analysis. When the embryos were replaced after 4 h under normal conditions, almost all of the embryos fully recovered progression in early development and the level of 4E-BP returned after 20 h to the basal level observed in untreated embryos (Fig. 1; right side). When hypoxia was applied 30 h post-fertilization, hatching was inhibited, gastrulation did not occur and again, 4E-BP protein expression was induced and reached the initial unfertilized egg level 20 h later (data not shown). Therefore, hypoxia induced 4E-BP protein expression in sea urchin embryos.

Bleomycin Impedes Sea Urchin Early Development

The radiomimetic bleomycin, known to affect the DNA-damage checkpoint of mammalian cell cycle (see Introduction) was applied to sea urchin embryos. When bleomycin was added to embryos 20 min post-fertilization, a delay in the first cell cycle was observed as judged by the occurrence of the first cleavage under phase contrast microscope (Fig. 2). The delay in cell cycle was dependent on the concentration of bleomycin (Fig. 2). Therefore, sea urchin cell cycle is affected by the radiomimetic as reported for mammalian cells involving a mobilization of the DNA-damage checkpoint, which results in a delay in the cell cycles [Kaufmann and Kies, 1998]. Analysis of the morphology of chromatin using Hoechst dye showed that the evolution of chromatin correlated with the delay observed in the first cleavage (Fig. 3). The delay in cell cycle judged as the number of nuclei was again dependent on the concentration of bleomycin (Fig. 3; compare the time at which one DNA spot separated into two, and the time at which the two DNA spots separated into four). After 20 h in bleomycin, when the control embryos had reached blastula stage, the bleomycin-treated cells were strongly delayed in development. The number of cells was estimated as the number of individual nuclei labeled spots. Control embryos contained around 300 cells, and bleomycin-treated embryos 150, 67, and 26 cells for concentrations of bleomycin of respectively 0.4, 0.8, and 1.2 mM (Fig. 3). Bleomycin-treated embryos, therefore, perform one to three less rounds of cell division compared to control. When bleomycin was removed from the incubation medium at 3 h post-

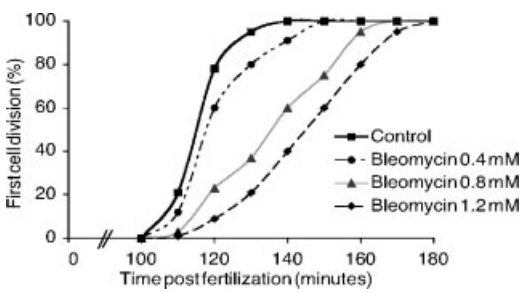

Fig. 2. Bleomycin affects the kinetic of the first cell division. Sea urchin eggs were fertilized. The embryos (5% suspension) were left in fresh seawater (control: continuous line) or treated (dotted line) after 20 min with various concentrations of bleomycin. At the indicated times, embryos (batches of one hundred) were scored by phase contrast microscopy for the first cell division.

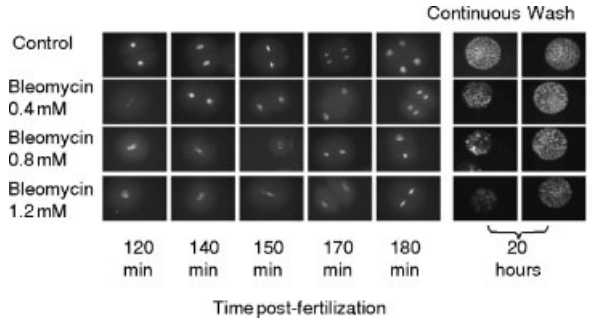

Fig. 3. Bleomycin delays early development. Sea urchin eggs were fertilized (5% suspension) and treated after 20 min with various concentrations of bleomycin. At the indicated time after fertilization, the chromatin state was observed by fluorescence microscopy of Hoechst stained embryos. Continuous: embryos treated by bleomycin during 20 h. Wash: embryos treated by bleomycin during 3 h, washed twice and transferred in fresh seawater.

fertilization, all the embryos recovered cell cycles upon further incubation in fresh seawater as shown at 20 h post-fertilization (Fig. 3; wash column compared to continuous column). The embryo recovery is indicative of a release in cell cycle inhibition due to release of the DNA-damage checkpoint effect [Lukas et al., 2004; Van Vugt et al., 2005]. As seen in Figure 3, recovered embryos conserved the initial delay induced by the radiomimetic (Fig. 3; compare 180 min evolution and 20 h evolution). Altogether, the radiomimetic bleomycin reversibly affects cell cycles of the sea urchin embryo most probably by the mobilization of the DNA-damage checkpoint of the cell cycle.

Bleomycin Induces Expression of the Protein 4E-BP

We investigated the effect of the bleomycin-induced stress on the level of 4E-BP protein. The level of 4E-BP protein was analyzed in the bleomycin-treated embryos and compared to the control embryos. As shown in Figure 4A, the disappearance of 4E-BP post-fertilization was observed (Fig. 4A; lanes b and c). Whereas the level of 4E-BP protein at 20 h was barely detectable in the control embryos, strikingly, 4E-BP was overexpressed in the bleomycin-treated (0.4 mM) embryos to a level comparable to the level in the unfertilized egg. In the embryos washed from bleomycin at 3 h post-fertilization, the level of 4E-BP protein was only slightly higher than the level in the control embryos (Fig. 4; lanes c.w and b.w). Since the embryos are released from the cell cycle

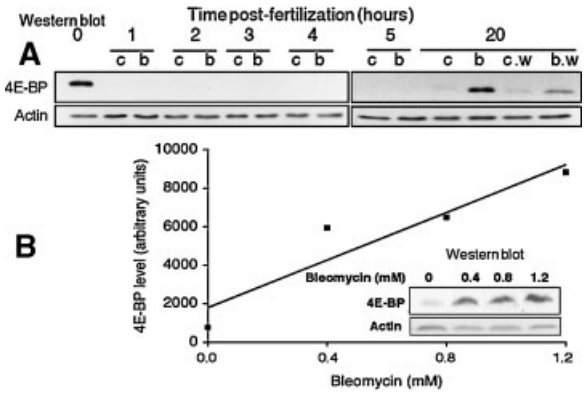

Fig. 4. Bleomycin affects expression of the protein 4E-BP. Sea urchin eggs (5% suspension) were fertilized and treated or not after 20 min with bleomycin. **A**: The embryos were treated with 0.4 mM bleomycin. Whole protein extracts from control embryos (c) or treated embryos (b) were prepared at different times and analyzed by immunoblotting with anti-4E-BP and anti-actin antibodies as described in materials and methods. Half of the embryos (w) were washed twice at 3 h post-fertilization and further incubated prior to immunoblotting analysis. **B**: The embryos were treated with 0.4, 0.8, or 1.2 mM bleomycin. Whole protein extracts were performed at 20 h and analyzed by immunoblotting with anti-4E-BP and anti-actin antibodies as described in materials and methods. The quantification of 4E-BP level from the immunoblots is indicated in arbitrary units as a function of bleomycin concentration. Insert: immunoblots.

inhibition under these conditions (see Fig. 3), high level of 4E-BP protein in the bleomycin-treated embryos is correlated with the maintenance of delayed early development. The level of 4E-BP protein was analyzed (Fig. 4B) in the embryos treated with varying concentration of bleomycin (0.4, 0.8, and 1.2 mM) by densitometric measurement of the 4E-BP signal on the immunoblots (Fig. 4B; insert). The expression of 4E-BP protein was proportional to the concentration of bleomycin (Fig. 4B), and therefore to the extent of the delay in early development (see Fig. 3). We, therefore, establish that bleomycin-induced stress of sea urchin embryos leads to expression of the protein 4E-BP.

DISCUSSION

The protein 4E-BP of sea urchin embryo was shown to be regulated at the level of its expression. First, 4E-BP decreased shortly after fertilization liberating the factor eIF4E for protein translation [Salaun et al., 2003, 2005]. Later in development, 4E-BP gradually reappeared from 10 h post-fertilization, and associated to eIF4E [Salaun et al., 2005]. We demonstrate that hypoxia induces a strong reversible overexpression of the 4E-BP protein. Using the radiomimetic bleomycin, we show that the first cell cycles of sea urchin early development are under the control of the DNA-damage checkpoint. The action of bleomycin was reversible indicating that the checkpoint could release the cell cycle blockage, a property of the checkpoint function [review in Van Vugt et al., 2005]. In the case of prolonged bleomycin stress, the expression of the protein 4E-BP was found to be strongly induced. Expression of 4E-BP was dose-dependent on bleomycin concentration and was mostly prevented when the embryos were removed from the drug. Therefore, 4E-BP expression appears to be a common feature of the hypoxic response on the one hand and of prolonged DNA-damage checkpoint mobilization on the other hand (Fig. 5). How the protein 4E-BP is upregulated under the stress conditions of hypoxia and DNA-damage checkpoint prolonged mobilization remains to be elucidated. Hypoxia and the radiomimetic bleomycin may both induce oxidative stress via ROS production [Barzilai and Yamamoto, 2004]. This effect may be related to the dFOXO regulation of 4E-BP in Drosophila [Tettweiler et al., 2005]. The upregulation of 4E-BP, shown to be critical for survival under dietetary restriction and oxidative stress resistance [Tettweiler et al., 2005] would function as a metabolic brake under stress conditions [Teleman et al., 2005]. Thus, the role of 4E-BP expression could be to buffer the amount of eIF4E and by that way impede cap-dependent protein translation, limiting energy spending, and assuring the survival of the cells (Fig. 5). Interestingly, in that context, 4E-BP would be a stress-induced protein necessary for cell survival. Another consequence of 4E-BP expression could be to participate in the apoptotic issue of the stressed cells (Fig. 5). It should be noted that hypoxia may lead to p53-mediated apoptosis [Hammond and Giaccia, 2005] and that DNA-damage checkpoint activation may also lead to apoptosis when DNA repair is not efficient [Hartwell and Kastan, 1994; Kastan and Bartek, 2004; Van

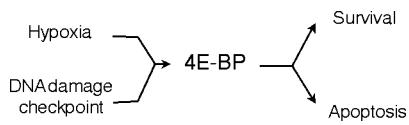

Fig. 5. Schematic model for 4E-BP implication in hypoxia and prolonged DNA-damage checkpoint mobilization (see text for details).

Vugt et al., 2005]. The apoptotic function of the protein 4E-BP through its association with eIF4E was suggested in mammalian cells [Clemens et al., 2000; Holcik and Sonenberg, 2005; Morley et al., 2005] and makes this attractive hypothesis of general concern. Whatever the role in cell survival or apoptosis, for the protein 4E-BP overexpression, its upregulation could result in preferential translation of mRNAs, which translate in a cap-independent manner, via an internal ribosome entry site (IRES) as documented under various stress conditions including irradiation and hypoxia [Holcik et al., 2000].

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