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Stable Expression of Functional CBP70 Lectin During Heat Shock

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Abstract CBP70 is a glycoslylated lectin that interacts through either glycan-lectin or protein-protein interactions. In addition, depending on its cellular localization, this lectin has different partners, for example, galectin-3, an 82-kDa ligand in the nucleus, or Bcl-2 in the cytoplasm. In this study, we observed the persistence of plurilocalized lectin CBP70 after two heat-shock treatments conducted either under mild conditions, i.e., , incubating the cells for 1 h at 42°C then for 1, 3, 5, 7, or 9 h at 37°C, or harsh conditions, i.e., incubation at 42°C for 1, 2, 4, 6, 8, or 10 h. By combining the information collected from biochemical, fluorocytometric, confocal, and affinity-chromatography analyses, we concluded that CBP70 persisted in HL60 cells and its N-acetylglucosamine-binding sites remained active after all the heat-shock treatments tested. These data and the previously published findings reviewed in this report concur in supporting the hypothesis that CBP70 could function as an organizer of multimeric assembly, leading to the formation of various complexes in different cellular compartments, according to the needs of the cell. J. Cell. Biochem. 77:615–623, 2000.

Key words: CBP70 lectin; heat shock; Bcl-2 interaction

The most primitive goal of living cells is to survive [De Maio, 1999], hence the need for an adequate protein machinery to allow them to resist to aggression and death. Although the precise mechanism underlying stress tolerance remains unclear, it is well known that proteins with essential roles in cellular physiology continue to be expressed when the cells are stressed [De Maio, 1999]. For instance, to ensure the protection of the cells, high quantity of various proteins, named heat-shock proteins or stress proteins (hsp), are synthesized [Gething and Sambrook, 1992; Hendrick and Hartl, 1993]. These proteins are present in unstressed cells and play important roles in the folding and the translocation of polypeptides across membranes. During stress, they are probably involved in the resolubilization of proteins that had been denatured by the event. However, not all of stress proteins are classified as hsp [De Maio, 1999]; increasing numbers of proteins, with chaperone-like activities, have been identified after cells were expressed to various reversible cell injuries. For instance, the lectins calnexin and calreticulin are active partners in the cell survival [Krause and Michalak, 1997; Trombetta, 1998]. Lectins are proteins that are able to associate with the glycosidic part of a glycoconjugate. The major contributions of the lectins involve their capacities to form multimeric complexes because they can participate in glycan-lectin and protein-protein interactions and thus can react with a multitude of proteins [Barondes, 1988]. In addition, some lectins are plurilocalized and plurifunctional, two features that further enhance the interest accorded investigations on the roles of these proteins [Kasai, 1997; Leffler, 1997].

Abbreviations used: Ab, antibody; Ig, immunoglobulins; GlcNAc, N-acetylglucosamine; BSA, bovine serum albumin; CBP, carbohydrate-binding protein; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline with $^{(+)}$ or without $^{(-)}$ Ca^{2+} and Mg^{2+}; PrPc, cellular prion protein; hsp, heat shock protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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For all these reasons, we examined further the expression of the carbohydrate-binding protein CBP70, a lectin that we first isolated from the nucleus of HL60 cells and which is able to bind glucose and N-acetylglucosamine (GlcNAc), the latter with a higher affinity [Felin et al., 1994]. More recently, this lectin was found to have multiple localizations [Hadj-Sahraoui et al., 1996]. In the nucleus, the protein is present throughout the interchromatin spaces and in the nucleolus. In the cytoplasm, CBP70 was localized in the endoplasmic reticulum, the Golgi apparatus, mitochondria, and the cytosolic fraction [Hadj-Sahraoui et al., 1996; Felin et al., 1997] (Rousseau et al., submitted). It should be noted that CBP70 is highly glycosylated and that the glycosylation pattern differs according to the protein's cellular localization [Rousseau et al., 1997]. The nuclear form of CBP70 possesses GlcNAc and fucose residues, whereas the cytoplasmic form does not [Rousseau et al., 1997]. Furthermore, CBP70 seems to be plurifunctional, as it interacts with different partners in the cytoplasm and the nucleus, participating in either protein-protein or glycoprotein-lectin interactions. For instance, in the nucleus of HL60 cells, CBP70 interact with galectin-3, a lactose-binding protein via a protein-protein interaction that can be disrupted by the addition of lactose [Sève et al., 1993, 1994], and an 82-kDa ligand via a glycoprotein-lectin interaction [Felin et al., 1997]. In NB4 cell nuclei, CBP70 was reported to interact with the cellular prion protein (PrPc) (Botti et al., submitted). Our recently obtained data have shown that, in the cytoplasm of HL60 cells, CBP70 could interact with Bcl-2, a well-known anti-apoptotic molecule, suggesting that CBP70 could be involved in programmed cell death (Rousseau et al., submitted).

Because CBP70 is both a lectin and a glycoprotein it can participate in multiple interactions. For instance, various glycosylated ligands can interact with its carbohydrate-recognition domain via a glycan-lectin interaction, depending upon its cellular localization [Felin et al., 1997]. In addition, numerous lectins can react with the glycosidic moieties of the different glycosylated isoforms of CBP70 [Rousseau et al., 1997]. Finally, CBP70 can interact via protein-protein interactions with other proteins, as was shown for galectin-3 [Sève et al., 1993]. In this latter case, it is noteworthy that the protein-protein association could be disrupted in favor of a glycan-lectin one, probably owing to a three-dimensional modification of the lectin when a sugar moiety interacted with the carbohydrate-recognition domain [Sève et al., 1994]. All these observations suggest that CBP70 is an active player in the formation of multimeric complexes.

While awaiting for CBP70 cDNA cloning and sequencing to furnish the necessary information concerning the role of the protein, heat treatment appeared to be a way to assess the physiological importance of this lectin. Most protein synthesis is stopped under stress treatment, while hsp are synthesized [Gething and Sambrook, 1992; Hendrick and Hartl, 1993; De Maio, 1999]. Consequently, a protein that persists during heat stress should play a fundamental role in cellular physiology. To evaluate such a potential for CBP70, HL60 cells were subjected to mild or harsh heat treatment.

The results of these experiments demonstrated that after heating the localization of CBP70 was not altered and a constant amount of the lectin remained expressed. In addition, even under the more drastic heating conditions, the GlcNAc-binding site remain active, this providing evidence that the lectin remained a functional molecular assembler.

MATERIALS AND METHODS

Cell Culture, Cell Morphology, and Heat Treatments

HL60 cells were grown in suspension in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco, France), 2 mM L-glutamine, 50 IU/ml of penicillin, and 50 mg/ml of streptomycin. Cells were grown at 37°C in a humidified 5% CO_2 atmosphere and maintained at a density of 2×10^5 to 1×10^6 cells/ml by resuspending the cells in fresh culture medium every 3 days. Cell morphology was analyzed using classical May-Grünwald-Giemsa staining.

Heat treatments were as follows : 1 h at 42°C then 1, 3, 5, 7, or 9 h at 37°C (mild conditions); or 42°C for 1, 2, 4, 6, 8, or 10 h (harsh conditions). Cells were used between passages 15 and 40.

Antibodies

Fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit immunoglobulin (Ig) antibod-

ies (Ab) were purchased from Dako A/S (Glostrup, Denmark) and peroxidase-conjugated goat anti-rabbit Ig polyclonal Ab was purchased from Sigma Chemical Co. (St. Louis, MO). Anti-CBP70 polyclonal Ab were obtained as previously reported [Sève et al., 1993].

Immunohistochemical Labeling and Confocal Analyses

Cells were spread on glass slides in a cytocentrifuge at 400 rpm for 5 min, quickly air-dried, then cells were fixed and permeabilized in acetone at 4°C for 10 min, and air-dried. These preparations were incubated with anti-CBP70 Ab (1:200) [Sève et al., 1994] for 1 h at room temperature. After 5 washes in Ca^{2+}- and Mg^{2+}-containing phosphate-buffered saline (PBS^+) supplemented with 1% bovine serum albumin (BSA), the cells were incubated for 30 min at room temperature with FITC-conjugated swine anti-rabbit Ig Ab (1:500). Finally, they were washed 5 times in PBS^+-1% BSA, followed by 2 washes in PBS^+. All incubations were performed in a humidified chamber at room temperature. Samples were examined by confocal laser scanning microscopy using a Bio-Rad MRC-600 confocal imaging system (Bio-Rad Microscience Ltd., Hertfordshire, UK) and an inverted Diaphot 300 Nikon microscope. Images were collected using an oil immersion lens (60×, NAI.4 plan Apochromat). For FITC, a krypton/argon ion laser (Ion Laser Technology, Salt Lake City, UT) was used at the 488-nm wavelength. For DAPI (4′,6-diamino-2-phenylindole dihydrochloride) excitation, an enterprise ion laser (Coherent Laser Group, Santa Clara, CA) was used at the 353-nm wavelength. FITC and DAPI images were merged to obtain pseudo-colored green for FITC and blue for DAPI, which specifically labels cell nuclei. Each image represents a single section for which the confocal system was adjusted to obtain a field depth of about 0.8 μm.

Preparation of Total Protein Extracts

In this study, 1×10^6 HL60 cells of each experimental incubation conditions were washed in PBS^+ and pelleted by centrifugation at 400*g* for 5 min. Pellets of cells were immediately lysed by adding 100 μl of boiling Laemmli solution [Laemmli, 1970] containing β-mercaptoethanol and disrupted with a pestle. Samples were then boiled for 5 min and insoluble material was removed by centrifugation at 13,000*g* for 5 min.

Affinity-Chromatography Procedures

N-Acetyl-β-D-glucosamine phenylisothiocyanate (Sigma, France) was immobilized on acetone-dehydrated Trisacryl GF 2000 M (IBF, Villeneuve-La-Garenne, France). The nuclear and cytoplasmic protein solutions prepared as previously reported [Rousseau et al., 1997] were adjusted to 0.7 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS^+) and were incubated with 1 ml of immobilized N-Acetyl-β-D-glucosamine overnight at 4°C under batch conditions [Sève et al., 1993]. After the columns were packed, the proteins specifically retained on the column were eluted with 0.2 M N-Acetyl-α-D-glucosamine (GlcNAc) and concentrated on Centricon-10 filters. Protein concentrations were determined using the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL).

Electrophoretic and Immunoblotting Analyses

Total protein extracts (10 μl) prepared from HL60 cells subjected to each experimental incubation condition were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% gel), according to Laemmli's procedure [Laemmli, 1970]. The resolved polypeptides were electrotransferred onto Immobilon-P (60 V at 4°C for 1 h) in 10 mM (cyclohexylamino)-1-propane sulfonic acid, (Caps) buffer at pH 10.7. The blots were incubated overnight in saturating Tris-buffered saline (TBS; 20 mM Tris-HCl (pH 7.4), 0.5 M NaCl) containing 5% BSA, then washed 3 times in TBS containing 0.5% Tween 20 (TBS-Tween) and incubated for 2 h with anti-CBP70 Ab (1:300). After incubation, the immunoblots were washed 3 times in TBS-Tween, incubated for 1 h at room temperature with peroxidase-conjugated polyclonal goat anti-rabbit Ig Ab (1:10 000), then washed 3 times in TBS-Tween and finally developed using the Enhanced Chemiluminescence (ECL) reagents (Amersham, Les Ulis, France).

Quantitative Flow Cytofluorometry

HL60 cells (1×10^8) grown in suspension were collected by low-speed centrifugation. After 2 washes in PBS^+-1% BSA, cells were permeabilized for 10 min in acetone at 4°C and incubated for 1.5 h at 4°C in the presence of anti-CBP70 Ab diluted 1:500 in PBS^+-1% BSA. After washing in PBS^+-1% BSA, HL60 cells were incubated for 1 h with FITC-conjugated

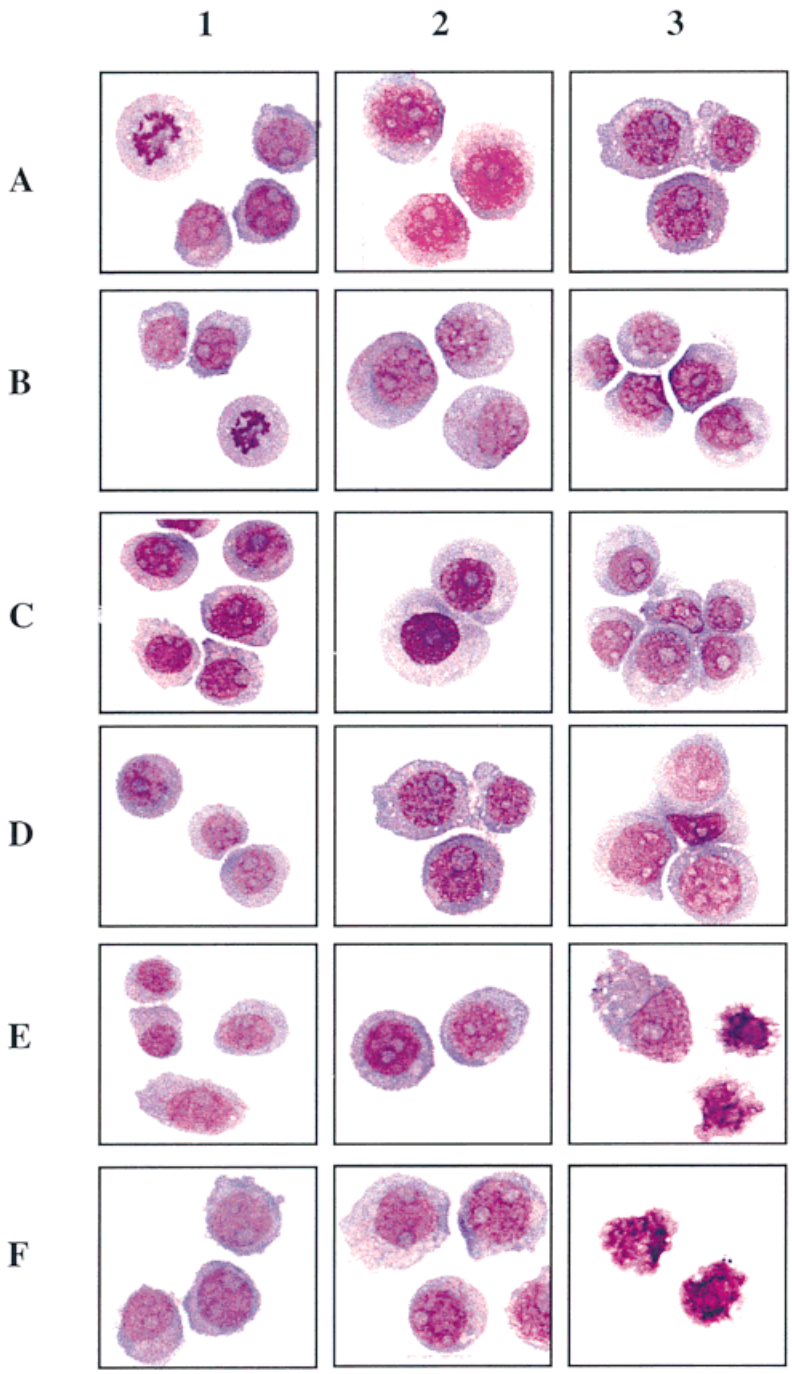

Fig. 1. Morphology of HL60 cells during heat stress. HL60 cells were incubated: at 37°C for 1 h (**A1**), 2 h (**B1**), 4 h (**C1**), 6 h (**D1**), 8 h (**E1**), or 10 h (**F1**); for 1 h at 42°C (**A2**), then at 37°C for 1 h (**B2**), 3 h (**C2**), 5 h (**D2**), 7 h (**E2**), or 9 h (**F2**), or at 42°C for 1 h (**A3**), 2 h (**B3**), 4 h (**C3**), 6 h (**D3**), 8 h (**E3**), or 10 h (**F3**) and stained with May-Grünwald-Giemsa.

swine Ig Ab (Dako A/S) diluted 1:500 in PBS^+-1% BSA. Cells were then washed twice in PBS^+ and the fluorescence intensity of 10,000 cells from each experimental incubation was recorded using a FACScan analyser (Becton-Dickinson, San Jose, CA). Control experiments: HL60 cells were incubated with either the buffer alone, or FITC-conjugated swine anti-rabbit Ig Ab.

RESULTS

Analysis of HL60 Cell Morphology After Heat-Shock Treatment

Changes in cell morphology in response to mild or severe heat shock were monitored using optical microscopy (Fig. 1). May-Grünwald-Giemsa staining showed that after 1 h of treatment at 42°C, followed by an incubation at

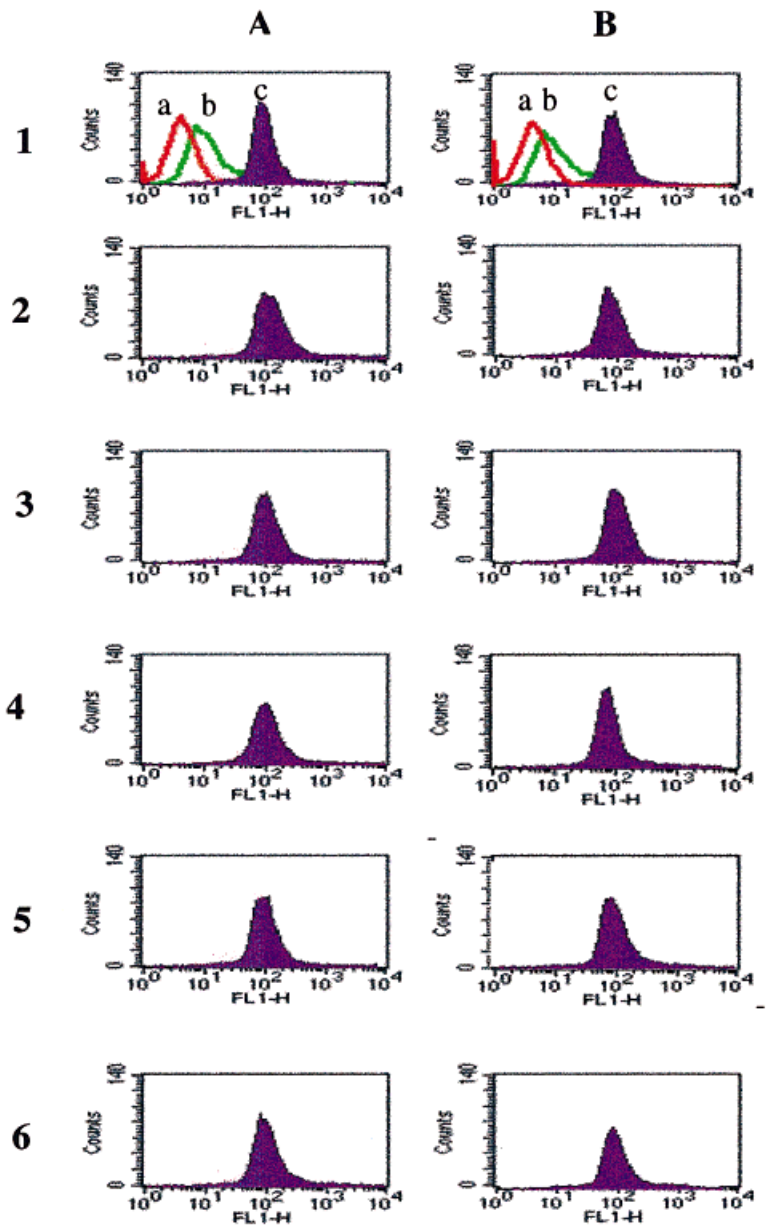

Fig. 2. Quantitative flow cytofluorometry analysis of CBP70 expression during heat-shock stress. Counts = cell number, FL1-H = fluorescence intensity expressed in arbitrary units (log^{10} scale). Peaks represent the mean fluorescence intensity of 10,000 cells. **A1:** HL60 cells incubated at 37°C with buffer alone (a), FITC-conjugated Ab alone (b), or anti- CBP70 Ab then FITC-conjugated Ab (c). **B1:** HL60 cells incubated for 1 h at 42°C with buffer alone (a), FITC-conjugated Ab alone (b), or anti-CBP70 then FITC-conjugated Ab (c). CBP70 expression was analyzed in HL60 cells incubated for 1 h at 42°C then at 37°C for 1 h (**A2**), 3 h (**A3**), 5 h (**A4**), 7 h (**A5**), or 9 h (**A6**), or in HL60 cells incubated at 42°C for 2 h (**B2**), 4 h (**B3**), 6 h (**B4**), 8 h (**B5**), or 10 h (**B6**).

37°C for 1–9 h, HL60 cell morphology was unaltered (Fig. 1, lanes 2A–F) compared with the HL60 cells subjected only to 37°C (Fig. 1, lanes 1A–F), whereas maintenance of the cells at 42 °C for 1-10 h progressively led to disruption of cell structure (Fig. 1, lanes 3A–F).

Expression of CBP70 Under Heat Stress

CBP70 expression under conditions of heat-shock stress was followed by fluorescence-activated cell sorting (FACS) (Fig. 2) and immunoblotting analyses (Fig. 3). Examination of FACS data indicated that regardless of the treatment, HL60 cells showed similar CBP70 labeling intensity (Fig. 2A2–A5 mild or; B2–B5 harsh conditions). Immunoblotting experiments confirmed that CBP70 remained unchanged (Fig. 3B,C) under the conditions tested. However, under mild heat-shock conditions a 70-kDa polypeptide was observed (Fig. 3B, lanes 1–6), whereas after the harsh heat stress, two polypeptides, 70 and 60 kDa, were recognized by the Ab raised against CBP70 (Fig. 3C, lanes 1–5).

These latter observations led us to conclude that the amount of CBP70 detected by FACS

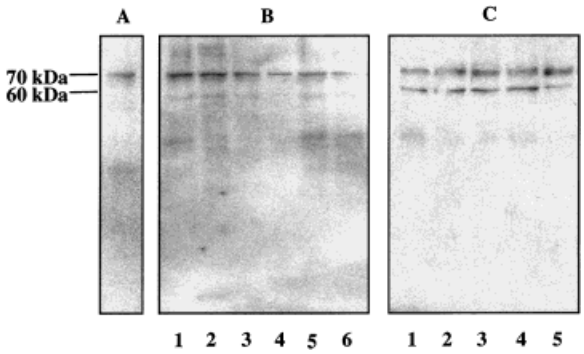

Fig. 3. Expression of CBP70 during heat-shock stress. A total protein extract of HL60 cells (10 μg) was incubated at 37°C (**A**); for 1 h at 42°C (**B**, **lane 1**) or 1 h at 42°C, then at 37°C for 1 h (**B**, **lane 2**), 3 h (**B**, **lane 3**), 5 h (**B**, **lane 4**), 7 h (**B**, **lane 5**) or 9 h (**B**, **lane 6**) or only at 42°C for 2 h (**C**, **lane 1**), 4 h (**C**, **lane 2**), 6 h (**C**, **lane 3**), 8 h (**C**, **lane 4**), or 10 h (**C**, **lane 5**). Samples were then subjected to SDS-PAGE (12% gel), electrotransferred and immunoblotted with the polyclonal Ab raised against CBP70.

cytofluorometry was lower under severe heat-shock stress, if we consider that, in the intact cells, the 60-kDa peptide is recognized by the Ab. However, CBP70 expression detected by immunoblotting appeared to be the same. These findings led us to conclude that most likely only CBP70 is recognized during FACS analysis.

Localization of CBP70 Under Stress Conditions

Under mild heat-shock conditions, we observed that CBP70 was always located in the nucleus and the cytoplasm (Fig. 4B–G, lanes 1–3) compared with the HL60 cells controls (Fig. 4A, lanes 1–6). Under harsh conditions, no difference in the CBP70 localization was noted (Fig. 4C–G, lanes 4–6) compared with the control (1 h at 42°C; Fig. 4B, lanes 1–6). Taking into consideration the immunoblot, showing the presence after severe heat-shock treatment of 60- and 70-kDa polypeptides, and the stable distribution of antigens recognized by the anti-CBP70 Ab after all heat treatments, we conclude that the two proteins have similar localizations.

Lectin Activity of CBP70 Is Conserved During Heat-Shock Treatment

The persistent expression of CBP70 provides no information concerning the activity of its carbohydrate-recognition domain. Therefore, we performed affinity chromatography on GlcNAc columns of nuclear and cytoplasmic extracts of HL60 cells exposed to severe heat stress for 10 h. When the nuclear (Fig. 5, lane 1) or cytoplasmic protein fraction (Fig. 5, lane 2) was passed through the column, CBP70 was recovered in the fraction specifically eluted with GlcNAc. Consequently, in addition to the constant presence of the CBP70 under the two heat-shock conditions tested, it appeared that the CBP70 carbohydrate-binding site remains active, even under the most severe conditions used in this study.

DISCUSSION

By comparing two heat-shock treatments we were able to demonstrate that both the localization and level of expression of the CBP70 in heat-stressed HL60 cells were similar, compared with control HL60 cells. In addition, we showed that the carbohydrate-recognition domains of CBP70 remain active regardless of heat-shock administered.

Under harsh conditions, in addition to CBP70, a 60-kDa polypeptides was also recognized by the anti-CBP70 Ab. This 60-kDa polypeptide was found during the HL60 cells differentiation and probably corresponds to a glycoform of CBP70, as previously postulated [Hadj-Sahraoui et al., 1996]. Because the 60-kDa polypeptide was not retained on the GlcNAc column, it appears not to have a functional GlcNAc-recognition domain. FACS analysis indicated stable Ab labeling in all heat-treated cells, thereby leading us to conclude that only CBP70 was recognized by the anti-CBP70 Ab as assessed with this technique. These data are in agreement with our biochemical analyses, which showed that the amount of CBP70 appeared similar under both conditions.

It is well known that the subcellular localization and the redistribution of the classical HSPs after heat stress are characteristic for each member and reflect, in part, its physiological function [Lindquist and Craig, 1988; Hendrick and Hartl, 1993; Jethmalani et al., 1997]. Then, for example, HSP70 is mostly cytoplasmic in unstressed cells, but it is translocated into the nucleus after heat-stress [Jethmalani et al., 1997]. In order to determine the potential function of CBP70 in normal unstressed and heat-stressed cells, the determination of its cellular localization was studied. Under mild and harsh conditions, no difference in CBP70 localization was noted. These data strongly suggested that CBP70 has potential important functions in both the cytoplasm and

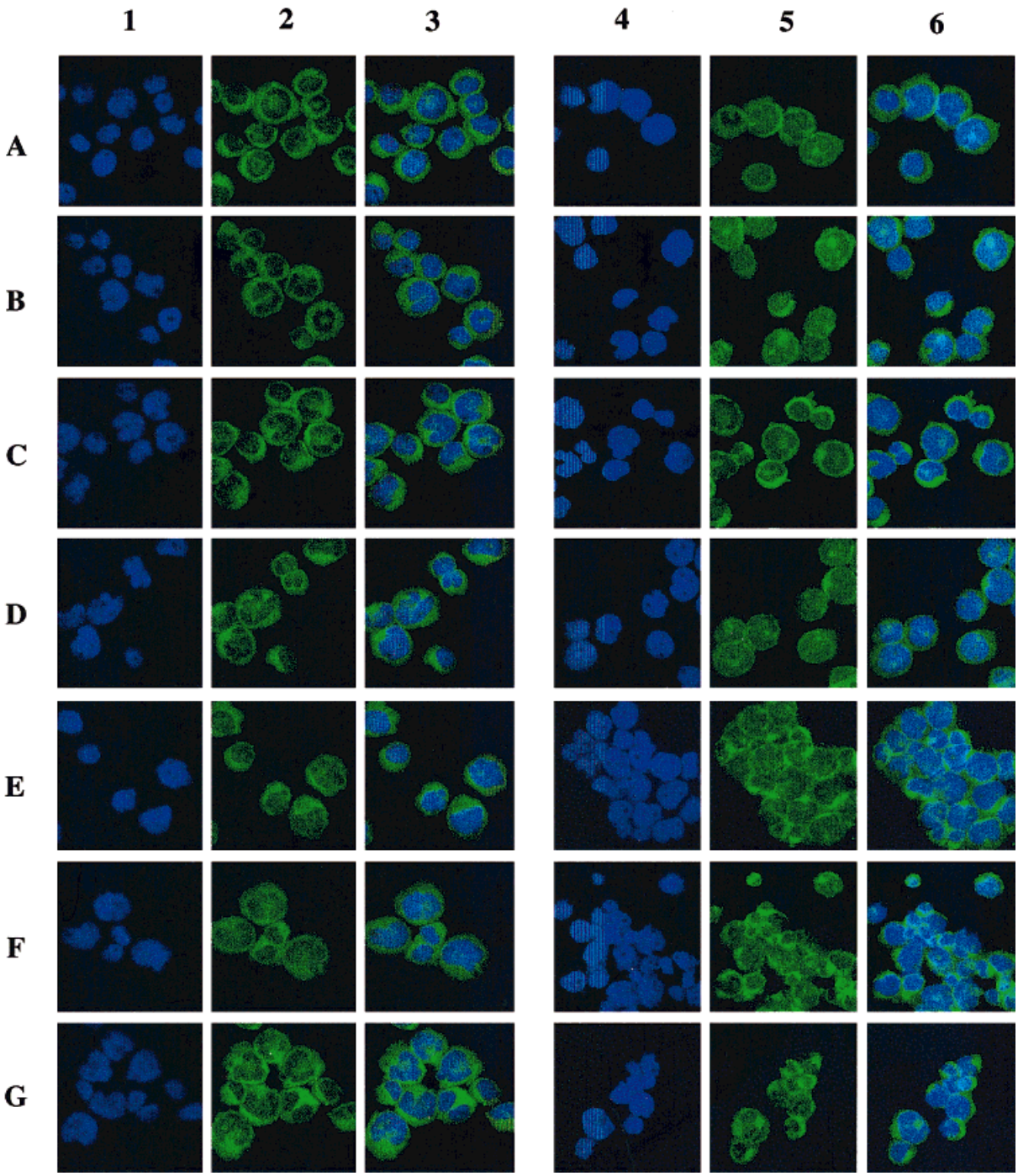

Fig. 4. Localization of CBP70 in HL60 cells during heat-shock stress. HL60 cells, incubated at various condition temperatures, were permeabilized with acetone, labeled with Ab raised against CBP70, and revealed with FITC-conjugated swine anti-rabbit Ig Ab, yielding a green fluorescence pattern. Cell nuclei are blue. HL60 cells were incubated either at 37°C (**A1–A6**), for 1 h at 42°C (**B1–B6**), for 1 h at 42°C, then at 37°C for: 1 h (**C1–C3**), 3 h (**D1–D3**), 5 h (**E1–E3**), 7 h (**F1–F3**), or 9 h (**G1–G3**) or at 42°C for 2 h (**C4–C6**), 4 h (**D4–D6**), 6 h (**E4–E6**), 8 h (**F4–F6**), or 10 h (**G4–G6**).

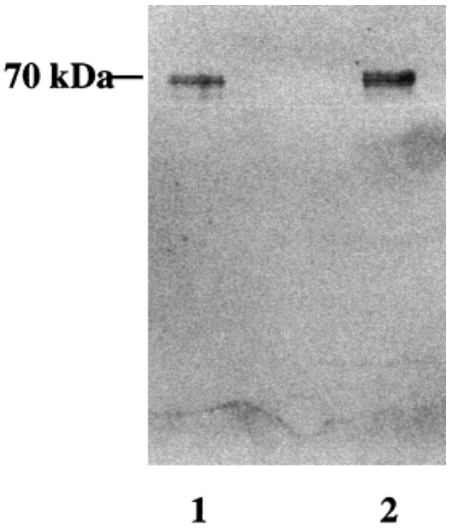

Fig. 5. Binding-site activity of nuclear and cytoplasmic CBP70 during heat-shock stress. Nuclear and cytoplasmic extracts of HL60 cells incubated for 10 h at 42°C were subjected to GlcNAc affinity chromatography. Specific eluted nuclear (**lane 1**) and cytoplasmic (**lane 2**) polypeptides (10 μg each) were subjected to SDS-PAGE (12% gel), electrotransferred, and immunoblotted with Ab raised against CBP70.

the nucleus compartment. It should be kept in mind that CBP70 has an active lectin domain and is also a glycoprotein [Rousseau et al., 1997]. These two properties increase the ability of CBP70 to interact with either glycoconjugates or lectins. In addition, a glycoprotein-lectin interaction could modulate a protein-protein interaction, thereby enhancing the possibility of protein assembly or disassembly [Sève et al., 1993, 1994; Yang et al., 1996].

In addition, the heat stress can influence protein activity. In the case of CBP70, a possible loss of activity of its lectin domain may result in a lost interaction with some of its characterized glycosylated ligands [Felin et al., 1997] (Botti et al., submitted; Rousseau et al., submitted) and then modify some of its function. For that reason, it was important to study the evolution of the activity of the CBP70 lectin domain under heat stress. The results obtained clearly demonstrated that the activity of the CBP70 lectin domain is still functional under mild and harsh heat-stress conditions. Thus, it appears that whatever the heat-shock treatment, the CBP70 retained its interaction with its various ligands. In this way, it is interesting to note that we have also determined, under the same heat-shock conditions, that the amount of Bcl-2 did not vary (data not shown).

The persistence of subcellular localization and lectin activity of CBP70 during heat stress argues for a fundamental role for this lectin as an active partner in cellular physiology. These data are in agreement with various results obtained by our group concerning the different ligands of CBP70 depending on its cellular localization. Since its characterization [Sève et al., 1993], CBP70 has been shown to be a protein able to interact with various partners, such as galectin-3 [Sève et al., 1993], PrPc (Botti et al., submitted) and an 82-kDa specific ligand [Felin et al., 1997] in the nucleus of HL60 and NB4 cells. Recently, we have reported that CBP70 could also bind to Bcl-2 in the cytoplasm (Rousseau et al., submitted). It should also be noted that PrPc was able to bind to the anti-apoptotically acting protooncogene protein Bcl-2, as shown with the yeast two-hybrid system [Kurschner and Morgan, 1995]. Moreover, it has been reported that galectin-3 could also interact with the prion protein in the nucleus [Schröder et al., 1994]. Taken together, these data strongly suggest that galectin-3, CBP70, Bcl-2 and the prion protein are able to interact either by glycan-lectin and/or by protein-protein interactions. The finding that CBP70 and Bcl-2 could interact leads us to hypothesize that this complex could contribute to protecting the cells from the death when exposed to such drastic conditions.

The above data support the notion that CBP70 might function as an organizer of multimeric assembly involved in the formation of various complexes acting in the different cellular compartments, according to the needs of the cells (Rousseau et al., submitted). Futher investigations are needed to elucidate the contribution of CBP70 to protection of the cell against death due to heat shock.

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