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Expression and Activity of p67 Are Induced during Heat Shock[1]

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p67, a cellular glycoprotein, protects eIF2α from phosphorylation by inhibitory kinases such as PKR and HCR. p67 promoter contains heat shock element (HSE). To investigate whether this HSE of p67 has any role during heat-shock, rat tumor hepatoma cells were transiently transfected with CAT reporters linked to p67 promoter with HSE and without HSE. Heat shock induced CAT activity when p67 promoter contained HSE and this induction was not observed when HSE was deleted from the p67 promoter. In response to heat-shock, the endogenous p67 mRNA was also induced to more than 36-fold, and much of it translated into protein which was modified by GlcNAc moieties. The time of induced glycosyl modification at the later stages of the heat-shock correlates with the reduced level of eIF2α phosphorylation. During later stages of the heat shock of animal cells, there is a preferential translation of a small class of messages encoding heat shock proteins. Our results suggest that the expression and activity of p67 are induced at the later stages of the heat-shock, and may be involved in the preferential translation of the heat-shock messages.

In response to heat shock, several control mechanisms operate at various levels, including transcription, RNA processing, and translation (1). Mammalian cells when exposed to heat (5-10 °C above the normal growth temperature), rate of protein synthesis declines rapidly within 10-20 minutes and more than 70-90% of the cellular proteins cease their synthesis (2-5). The cessation of protein synthesis correlates with the increase level of phosphorylation of the smallest α-subunit of eukaryotic initiation factor 2 and several other initiation factors (5-6). eIF2 when phosphorylated at its α-subunit, sequesters the cellular limiting factor, eIF2B which has guanidine exchange activity. This leads to the inhibition of ternary complex formation by phosphorylated form of eIF2, and thus shuts-off of protein synthesis (7-8). At later stages of the heat stress, there is a preferential translation of a small class of mRNAs encoding heat shock proteins (1-2). At the same time the level of eIF2α decreases to the normal level (5). The heat shock genes contain heat shock elements (HSE) that are responsible for the induced expression of these genes (1-2). The heat shock mRNAs contain heat shock elements at their 5′UTR, and this promotes efficient translation (9). At the promoter region of p67, contains the heat shock element (10).

p67, a cellular glycoprotein, protects eIF2α subunit from phosphorylation by inhibitory kinases such as HCR and PKR (11-21). Under normal growth conditions, kinases are present in active form in mammalian cells, but they cannot phosphorylate eIF2α because of bound p67 (12-22). p67 contains multiple O-linked GlcNAc moieties and these glycosyl residues are necessary for p67 activity to protect eIF2α from inhibitory phosphorylation (13,15-17). Under stress conditions such as viral infections, the p67-deglycosylase activity is induced and this inactivates p67 upon deglycosylation (20-21). The molecular mechanism for the regulation of eIF2α phosphorylation during heat stress is poorly understood (1-2). In this study we have investigated the significance of the presence of the heat shock element of p67. Our study revealed that the heat shock element present at the p67 promoter is required for its expression during heat shock of rat hepatoma cells, and that the activity of p67 correlates with the decrease level of eIF2α phosphorylation at the later stages of the heat-shock.

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The abbreviations used are: eIF2, eukaryotic initiation factor 2; CAT, chloramphenicol acetyltransferase; DNA, Deoxyribonucleic acid; bp, base pair(s); kb, kilobase pair(s); p67, eIF2 associated 67 kDa glycoprotein; PKR, double-stranded RNA-dependent protein kinase (eIF2α kinase); hsp, heat shock protein; HSE, heat shock element; HCR, heme regulated protein synthesis inhibitor (eIF2α kinase); PCR, Polymerase chain reaction.

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MATERIALS AND METHODS

Cell culture and heat shock. The cloned cell line KRC-7, derived from Reuber H35 rat hepatoma cells, was kindly provided by John Koontz (University of Tennesse, Knoxville). KRC-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc.), containing 5% (v/v) fetal calf serum, 5% (v/v) calf serum, 100 units/ml penicillin, 50 μg/ml streptomycin, and 10 mM sodium pyruvate at 37°C incubator containing 5% CO_2 and 95% air. For heat shock treatment, 6×10^5 cells were seeded onto 100 mm tissue culture dishes containing 10 ml of medium. Cells at 37°C were then subjected to heat-shock at 42°C for various times as indicated in the figure legends.

Cell lysate preparation. Cells were harvested, washed with phosphate-buffered saline (PBS, GIBCO), and lysed with lysis buffer [20 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM $Mg(OAc)_2$, 2 mM dithiothreitol] (15). Cell lysates were centrifuged at 15,000 rpm in a Beckman Microfuge B for 10 minutes. The protein concentration in the lysate was measured by Bio-Rad protein assay kit using bovine serum albumin as standard.

Metabolic labeling with [^{35}S]-methionine. KRC-7 cells were grown to 60%-70% confluency and incubated in serum-free DMEM lacking methionine for 15 minutes at 37°C (control) or at 42°C (heat shock). The labeling was initiated by adding 0.1 mCi/ml of [^{35}S]-methionine (specific activity 1175 Ci/mmole, American Radiochemicals) to the cells and continued incubation for additional 30 min. Cells were lysed in lysis buffer (15). 15μg of the [^{35}S]-methionine-labeled cell lysates were analyzed by SDS-PAGE followed by flourography.

In vitro phosphorylation of cell lysates and immunoprecipitation. 60 μg of proteins from different cell lysates were incubated in a reaction mixture (25μl) containing 20 mM Tris-HCl (pH 7.8), 100 mM KCl, 10μg BSA, 2 mM dithiothreiotol, 1 mM $MgCl_2$, 40 μM ATP, and 10μCi [γ^{32}P]-ATP at 37°C for 10 minutes. The radiolabeled eIF2α subunit was immunoprecipitated using eIF2α antibody and Protein A agarose. The immunoprecipitates were then analyzed by 15% SDS-PAGE followed by autoradiography (15).

Western blot analysis. 80 μg protein samples from cell lysates (control or heat-shock) were analyzed in 15% SDS-PAGE, transferred to Biotrace NT membranes, and immunoblotted with p67 mono- and polyclonal antibodies following the procedure as described (17).

Northern blotting. Cells were grown and subjected to heat shock for various intervals. After harvesting, total RNA was isolated from cells by the guanidium isothiocyanate method (24). RNA concentrations were determined. 10μg of total RNA was analyzed on 0.8% agarose gel, transferred to nylon membrane, and hybridized with a p67 probe (1 kb, concentration 5×10^5 cpm/ml). This probe was synthesized by *in vitro* transcription of pGEM-p67, using [α-^{32}P]-UTP and T7 RNA polymerase following the manufacturer's protocols (Ambion). The probe from the blot was stripped off, and rehybridized with a radiolabeled probe for β-actin [127 bp long fragment from pTRI-β-actin-125-Rat (Ambion)].

Preparation of pCAT-p67 promoter. For the preparation of pCAT-p67 promoter (−655 to +30), the forward primer (primer A: 5′GCAATGAAGCTTGGATGGGGAGG3′, −655 to −633) with a HindIII site and the reverse primer (primer B: 5′GCTCAGTCGACCTTCACCCACTTCC3′, +30 to +6) with a SalI, were synthesized. These two primers and the pGEM7-1.7 kb 5′UTR as a template were used to amplify the fragment using Taq DNA Polymerase. The PCR amplified product was purified by Wizard PCR Prep (Promega) and digested with HindIII and SalI. The digested fragment was subcloned at HindIII and SalI sites located upstream of the promoterless CAT gene in pCAT-basic vector (Promega). The authenticity of the sequence was confirmed by DNA sequencing using Sequencing II kit (USB). The primers used in these experiments were synthesized using the facilities of the DNA Synthesis Laboratory at the University of Nebraska-Lincoln and Life Technologies, Inc.

Sequence specific deletion: generation of pCAT-p67 promoter ΔHSE (−655 to +30; Δ−326 to −310). Selective deletion of the heat shock responsive sequence was performed by PCR overlap-extension technique of Pease and coworkers (24). Initially, two different PCR products were synthesized. For the preparation of pCATΔHSE, the primers used were: reaction1, Primer A/Primer D (5′GTCATTAAGTAGATT/GGTTATATTGATATAAAAGGAAT3′ −295 to −309/−333 to −355 in reverse orientation); reaction 2, Primer C/Primer B (5′TTATATCAATATAACCAATCT/ACTTAATGACTCATCTAT3′ −348 to −328/−304 to −287 in the forward orientation). The two PCR products obtained were mixed and fused together. The temperature for the fusion reaction was the same as the annealing temperature used in the PCR reaction (50°C). The fused products were amplified with Primer A and Primer B. The amplified DNA was subcloned into pCAT basic vector.

Transient transfection. Approximately, 3×10^6 KRC-7 cells were transiently transfected with appropriate constructs by lipopolyamide mediated transfection according to manufacturer's protocols (LipofectAMINE™, Life Technologies, Inc.). 5μg of pSV-β-gal from Promega was used as an internal control. 48 hours after transfection, cells were harvested, and assayed for CAT and β-galactosidase activities.

CAT and β-galactosidase assays. The CAT activity was measured by the CAT assay kit (Promega) according to the manufacturer's instructions. In brief, cells were harvested, washed twice in phosphate-buffered saline (pH 7.4), and lysed in reporter lysis buffer (Promega). The cell debris was removed by centrifugation. Aliquots were used for CAT assays following the manufacturer's protocol. Cell extracts from untransfected cells and cells transfected with the pCAT basic vector alone were used as negative controls. The β-galactosidase activity was analyzed using a commercial enzyme assay system (Promega). The CAT activity was normalized with the β-galactosidase activity. The relative CAT values are the average of three independent experiments. The mean of the CAT activities relative to pCAT basic activity ± S.D. are presented in all the figures.

RESULTS AND DISCUSSION

Previously, we reported that at the p67 promoter, the heat shock element is present at −326 to −310 nucleotide upstream of the translational initiation codon (10). To test whether this element is involved in induced expression of p67 gene during heat shock, KRC-7 cells were transiently transfected with pCAT-p67 construct containing heat shock element (−326 to −310) upstream of a CAT reporter gene. The promotor activity was determined by the analysis of CAT expression in the transfected cell extracts before and after heat shock at 42°C for 45 minutes. Heat shock induced the CAT activity by 3 fold over the control cells transfected with pCAT-p67 promotor at 37°C. When the HSE (−326 to −310) was deleted, no significant increase in CAT activity was observed compare to pCAT-p67 promoter at 42°C (Fig. 1). To test for the effectiveness of the heat shock, the rate of protein synthesis was measured by metabolically labeling KRC-7 cells with [^{35}S]-methionine followed by the analysis of the newly synthesized proteins (Fig. 2). The results reveal that the rate of radiolabeling is more than 80% lower in heat shocked cells as compared to control cells grown at

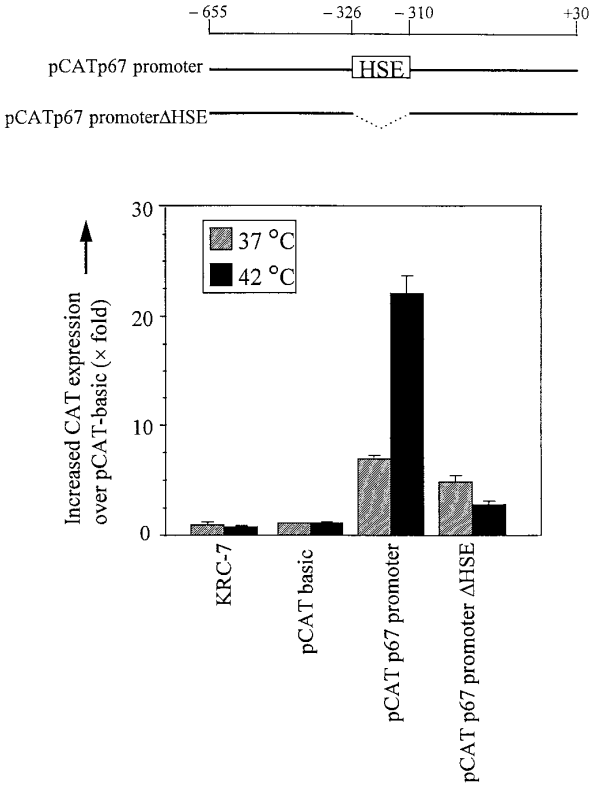

FIG. 1. Effect of heat shock on p67 promoter. KRC-7 cells were transiently transfected with different constructs as indicated with β-gal construct as an internal control. Forty-eight hours after transfection, β-galactosidase and CAT activities were measured from various samples. CAT activities were normalized with the β-galactosidase activities. Increased CAT expressions due to the p67 promoter elements were plotted. The first set from the left represents the CAT expression in KRC-7 cells. In the second set, similar transfection was performed with pCAT basic vector. The third and fourth sets represent the CAT expression in KRC-7 cells transfected with "pCATp67promoter" containing heat-shock element (HSE) and with "pCATp67promoterΔHSE", respectively. The light bars in each set indicate CAT expression in transfected cells at 37°C, and the dark bars indicate that at 42 °C. For details, see Materials and Methods.

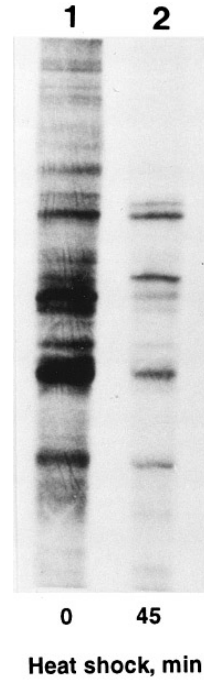

FIG. 2. Rate of protein synthesis during heat-shock of KRC-7 cells. KRC-7 cells were metabolically labeled with [^{35}S]-methionine while growing at 37°C (lane 1), or were subjected to heat-shock at 42°C for 45 min (lane 2). The radiolabeled proteins were analyzed by SDS-PAGE and fluorographed.

37 °C. This is consistent with the lower rate of protein synthesis during heat treatment of mammalian cells (2-6). We then examined the message level of p67 during heat shock. KRC-7 cells were grown to 70% confluency at 37 °C and were then subjected to heat shock at 42 °C for different time intervals. p67 message levels were measured by northernblotting (Fig. 3). Using Image Quant Software, the relative levels of p67 mRNA and β-actin mRNA at different times, were determined. The levels of p67 message were normalized against the levels of β-actin mRNA. The results suggest that the level of p67 message gradually increased with time of heat shock and peaked (>36-fold) at 45 minutes of heat-shock at 42 °C as compared to control cells grown at 37 °C. To examine whether this induced expression of the p67 message is indeed translated into protein, p67 protein levels at various times of heat shock were measured on westernblotting with polyclonal antibodies against p67 (Fig. 4). The data revealed that the protein levels of p67 also increased gradually with time of heat shock, and peaked at 45 minutes of incubation of the cells at 42 °C as compared to control cells grown at 37 °C. Interestingly however, the fold induction of the protein is significantly less as compared to the message levels indicating a major portion of the p67 mes-

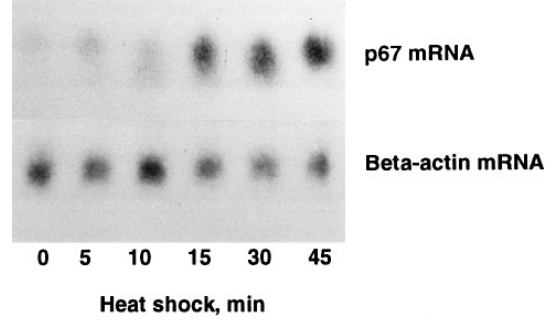

FIG. 3. Northern blot analysis of p67 mRNA in the control and heat-shocked tumor hepatoma cells (KRC-7). KRC-7 cells were grown at 37°C (lane 0 min), and were subjected to heat-shock at 42°C for 5, 10, 15, 30, and 45 min. RNA (10 μg) was used for Northern blotting with p67 cDNA probe (upper panel). The probe from the blot was stripped off and rehybridized with β-Actin cDNA probe (lower panel). In both panels, lane marked as "0" serves as controls.

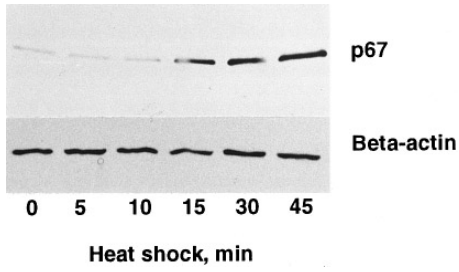

FIG. 4. Immunoblot analysis of p67 in control and heat-shocked KRC-7 cells. KRC-7 cells were grown at 37°C, and were subjected to heat-shock at 42°C for different intervals of time. Cells were then harvested and lysed. Protein (80 μg) was used for the analysis. p67 level was measured by standard immunoblotting using polyclonal antibody for p67 (upper panel). For protein loading control, similar immunoblot analysis was performed with β-actin monoclonal antibody (lower panel).

sages is degraded during heat shock treatment. Indeed, the mRNA degradation during heat shock is common in mammalian cells (1-2). To check whether the induced expression of p67 is properly modified by O-GlcNAc moieties, the levels of this modification were examined on a westernblot with p67 monoclonal antibody (Fig. 5). This monoclonal antibody against p67 recognizes the O-linked GlcNAc moieties of p67 (13,15-17,20-21). The results from this experiments clearly suggest that the p67 protein which is synthesized during heat shock, is also properly modified by O-GlcNAc moieties only after 30 minutes of heat shock of KRC-7 cells (Fig. 5, lane 30 min). The O-GlcNAc modifications of p67 are necessary for its activity to protect eIF2α from phosphorylation by inhibitory kinases (13,15-17,20-21). To examine whether the induced p67 activity did correlate with the decreased level of eIF2α phosphorylation, we therefore measured the level of eIF2α phosphorylation in KRC-7 cells that were heat-shocked for various times at 42 °C. The results are presented in Figure 6. Using Image Quant Software, the relative levels of eIF2α

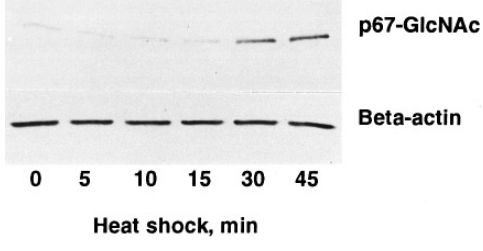

FIG. 5. Immunoblot analysis for GlcNAc modifications of p67 in control and heat-shocked KRC-7 cells. KRC-7 cells were grown at 37°C, and were subjected to heat-shock at 42°C for different intervals of time. Cells were then harvested and lysed. 80 μg protein was used for the analysis. The GlcNAc modifications of p67 were measured by standard immunoblotting using monoclonal antibody for p67 (upper panel). For protein loading control, similar immunoblot analysis was performed with β-actin monoclonal antibody (lower panel).

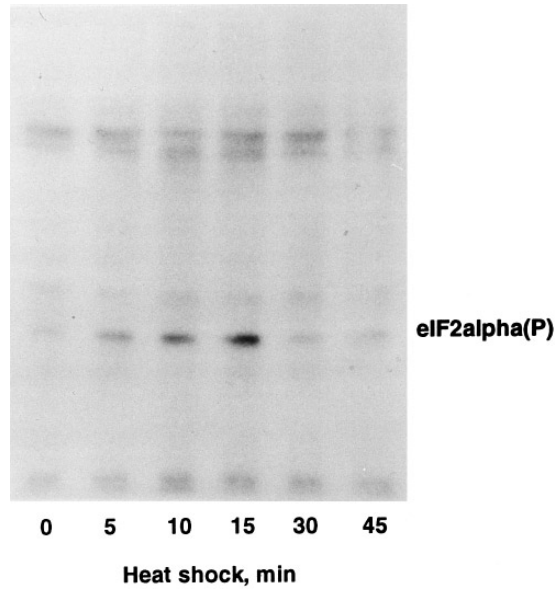

FIG. 6. Determination of the level of eIF2α phosphorylation in control and heat shocked KRC-7 cells. KRC-7 cells were grown at 37°C, and were subjected to heat-shock at 42°C for different intervals of time. Cells were then harvested and lysed. Protein (60 μg) was used for *in vitro* phosphorylation. Reaction conditions for eIF2α subunit phosphorylation were the same as described (12). The eIF2α subunit in the reaction mixtures was immunoprecipitated using eIF2α polyclonal antibody and protein A agarose. The immunoprecipitates were subsequently analyzed by SDS-PAGE followed by autoradiography. Phosphorylated eIF2α subunit in control cells (at 37 °C, lane "0") and in cells subjected to heat shock at 42°C for 5, 10, 15, 30, and 45 min was marked as eIF2alpha(P).

phosphorylation were measured and the results revealed that the level of eIF2α phosphorylation increased gradually until 15 minutes of heat shock (Fig. 6, lanes 0-15 min). Further heat treatment of KRC-7 cells for 30 and 45 minutes resulted in decreased levels of eIF2α phosphorylation (Fig. 6, lanes 30 & 40 min). These results indeed correlate with the increase activity of p67 (Fig. 5, lanes 30 & 40 minutes of heat shock).

The results presented in this study indicate that in rat tumor hepatoma cells (KRC-7), the synthesis of p67 is stimulated several fold at the message level and protein level when the cells are subjected to heat-shock (Figs. 3 & 4). At the promoter region are several regulatory elements, including HSE which perfectly matches to the heat shock core sequence 5′ GAANNTTCNNGAA 3′ (9). The DNA fragment containing this sequence when fused to a CAT reporter, its expression induced during heat shock whereas the similar DNA fragment containing no HSE element did not show any reporter activity (Fig. 1) suggesting that the induced expression of p67 during heat shock is due to the HSE element present at the p67 promoter.

At the early stages (15 minutes after heat-shock), eIF2α was heavily phosphorylated although the level of p67 was quite significant (Fig. 3, lane 15 min) but it

was not modified by GlcNAc moieties (Fig. 5, lane 15 min). In contrast, the level of eIF2α phosphorylation decreased sharply at 30 minutes of heat-shock when GlcNAc modification of p67 was very prominent suggesting that the activity of p67 depends upon its glycosyl modification and that the decrease level of eIF2α phosphorylation correlates with the increase activity of p67.

Heat-shock induces the post-translational modification of several initiation factors including eIF2α in animal cells (1-6). In HeLa cells, eIF2α is phosphorylated within 20 minutes of heat-shock treatment at 42.5 °C, and that this phosphorylation decreased sharply after 30 minutes of the heat-shock (5). In this report, we demonstrate that at this time p67 is significantly modified by GlcNAc moieties, and this correlates with the decreased level of eIF2α phosphorylation when KRC-7 cells were subjected to heat-shock treatment at 42°C. These results are consistent with our previous observation that the glycosyl residues on p67 possibly mask the phosphorylation sites on eIF2α and inhibits from phosphorylation by its inhibitory kinases (13,15-17,20-21).

In this report we have characterized p67 as a heat-shock protein. During heat shock, hsp mRNAs are preferentially translated than non heat-shocked cellular mRNAs. The synthesis of p67 increases by several fold after heat-shock treatment and thereby protects eIF2α from phosphorylation by the active kinases. This in turn helps hsp mRNAs to be translated and thus animal cells become thermotolerant. We previously hypothesized that p67 may be involved in preferential translation of some messages (12). This study and the fact that p67 is required for the translation of the messages, make it conceivable that the translation of hps mRNAs during the later stages of the heat-shock of animal cells may be such as an example for the preferential translation mediated by p67.

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