Glycoconjugate Journal 16, 685–689, 1999

SHORT COMMUNICATION

Glycosylation of stress glycoprotein GP62 in cells exposed to heat-shock and subculturing

Jerka Dumić, Gordan Lauc and Mirna Flögel*

Department of Biochemistry and Molecular Biology, Faculty of Pharmacy and Biochemistry, University of Zagreb, Ante Kovačića 1, 10000 Zagreb, Croatia

GP62 is a member of the stress glycoprotein family that was proposed to have a chaperone-like function in the heat-shock response. Using lectin blotting we have studied glycosylation of GP62 and determined that in addition to heat-shock, even simple subculturing of cells is a sufficient stimulus to provoke induction of GP62. Interestingly, both kinetics of induction and glycosylation of GP62 induced by subculturing were different than when GP62 was induced by heat-shock. While GP62 induced by heat-shock was recognized by SNA, DSA and PHA-E lectins, and not by BSA I, Con A, RCA I, SJA, UEA I, VVA, and WGA lectins, GP62 induced by subculturing was also recognized by RCA I and WGA lectins.

Keywords: **GP62, stress glycoproteins, stress, heat-shock, glycosylation**

Abbreviations: **Gal, galactose; Glc, glucose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; Man, mannose; Sia, sialic acid (N-acetylneuraminic acid); SDS, sodium dodecylsulfate; TBS, tris buffered saline (150 mmol/L NaCl, 50 mmol/L Tris/HCl pH 7.5); TBST, TBS supplemented with 0.1% Tween 20; BSA I, lectin I from *Bandeiraea (Griffonia) simplicifolia*; BSA II, lectin II from *Bandeiraea (Griffonia) simplicifolia*; Con A, Concanavalin A, lectin from *Canavalia ensiformis*; GNA, lectin from *Galantus nivalis*; DSA, lectin from *Datura stramonium*; MAA, lectin *Maackia amurensis*; PHA-E, lectin E_4 from *Phaseolus vulgaris*; PNA lectin from peanut (*Arachis hipogaea)*; RCA I, lectin from *Riccinus communis*; SJA, lectin from *Sophora japonica*; SNA, lectin from *Sambucus nigra*; UEA I, lectin from *Ulex europaeaus*; VVA, lectin from *Vicia villosa*; WGA, lectin from wheat germ (*Triticum vulgare*).**

Introduction

Glycosylation is an ubiquitous posttranslational modification that is used to direct, protect, and regulate activity of nearly all membrane, and many intracellular proteins [1,2]. When exposed to adverse conditions, *e.g.* to heat-shock, cells adapt to hostile environment by altering numerous metabolic processes, including the process of protein glycosylation [3]. For more than a decade it is known that heat-shock alters the activity of glycosyltransferases [4,5]. Specific stress-induced changes in glycosylation have been identified [6], but the exact molecular functions of these changes are not known. However, they appear to be an important segment of the cellular adaptation process since various forms of stress are associated with distinct glycosylation changes [7–9]. Within minutes after a heat-shock, while "classical" members of the stress response are not yet activated, calreticulin, an important cellular chaperone that recognizes $Glc_1Man_9GlcNAc_2$ structures and retains unfolded nascent glycoproteins in the endoplasmic reticulum [10,11], undergoes prompt changes in its glycosylation [6].

GP62, a 62 kDa stress glycoprotein was originally identified as a major heat-shock inducible glycoprotein in M21 cells that constitutively overexpress Hsp70, but subsequently it was also found to be induced by heat-shock in other types of cells [12]. Contrary to majority of other glycoproteins, GP62 was shown to be located in the cytoplasm [13]. Partial amino acid sequencing revealed some regions with significant homology to Hsp70, and it was suggested that GP62 might also have a chaperon-like function [14]. On two-dimensional gels it can be resolved into two isoforms (with p*I* values of 5.84 and 5.9) that were suggested to reflect subtle differences in glycosylation [14]. However, the data on glycosylation of GP62 is very limited. Until now it was only shown that GP62 from rat fibroblasts (M21 cells) binds to Con-A and WGA affinity columns [14].

[1]To whom all correspondence should be addressed: Phone: + 385 1 481 2158; Fax: + 385 1 4856 201; email: mfloegel@public.srce.hr

Using a set of 14 lectins that recognize different segments of carbohydrate structures we have analyzed glycosylation of GP62 in human glioblastoma cells exposed to heat-shock. We found that even a simple subculturing was sufficient stress to induce GP62 in these cells, while it was completely non-detectable in cells irradiated with UV-C light. Interestingly, carbohydrate structures on GP62 induced by subculturing were found to be different from those present on GP62 induced by heat-shock.

Materials and Methods

Materials

DSA, GNA, MAA, PNA, SNA, and WGA lectins labeled with digoxigenin, and anti-digoxigenin antibodies labeled with alkaline phosphatase were purchased from Boehringer Mannheim (now Roche Diagnostics). BSA I, BSA II, Con A, PHA-E, RCA I, SJA, UEA I, VVA, and WGA lectins labeled with biotin were purchased from Vector Laboratories (member of Boehringer Ingelheim Bioproducts Partnership, Heidelberg, Germany). Anti Hsp70, antibodies, and anti mouse IgG antibodies labeled with alkaline phosphatase were from SIGMA (St Lewis, MO), polyvinylidene difluoride (PVDF) membranes (Immobilon-P) from Millipore Corp (Bedford, MA, USA), and heat-inactivated fetal calf serum (FCS) from Life Technologies^{TM}.

Cell lines, culturing, and stress procedures

Human glioblastoma A1235 cells were asynchronously grown (37°C, 5% CO_2, relative humidity 95%) in the Dulbecco's modified Eagle's medium (SIGMA Cat No. D5648) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 µg/ml streptomycin and 100 U/ml penicillin. Cells were subcultured twice weekly to maintain cultures in exponential growth.

For heat treatment, cells were cultured in the T-75 plastic tissue-culture flasks. The flasks were horizontally submersed in well circulated, precision-controlled ($< \pm 0.1$°C) water bath at 42°C and incubated for 60 min. Control cells were submersed at 37°C for that time. Following heat-shock, cells were cultivated for 0, 4 or 24 h in standard conditions.

Cells were irradiated by UV-C light produced by Phillips UVT 15 W/G15T T8 lamp with emission maximum at 254 nm, and the intensity was determined using BioBlock VLX-3W dosimeter. Cells were grown in tissue culture dishes (60 × 15 mm), and the culture medium was briefly removed for the exposure. After the calculated exposure time (corresponding to the irradiation of 160, 320, 640 and 1280 Jm^{-2}), cells were supplemented with fresh medium and returned to the incubator and cultivated for 4 or 24 h.

To examine effects of subculturing, cells were washed twice with warm PBS, treated with trypsin (0.25% solution in PBS), and diluted with fresh medium to the final concentration of 1×10^6 cells in 5 ml. Cells were then transferred into tissue culture dishes (60 × 15 mm) and cultivated for 2, 4 or 24 h in standard conditions.

After the pre-determined incubation time, cells were washed twice with ice-cold PBS, scraped and centrifuged at 600 × *g* for 10 min. The cell pellet was resuspended in 0.04 ml 0.01 mol/L Tris/HCl, pH 7.4, 3 mmol/L $MgCl_2$, 10 mmol/L NaCl, 0.5% (v/v) Triton X-100, 1 mmol/L PMSF and additionally homogenized by sonication with ultrasound dezintegrator (MSE, 100 W, London, England) for 2 min (amplitude 5 µm, $\nu = 22$ kHz).

SDS-polyacrylamide gel electrophoresis

Denaturing SDS polyacrylamide gel electrophoresis was performed as described by Laemmli [15]. The samples were mixed with the SDS gel-loading buffer (1 : 1), containing 50 mmol/L Tris-HCl (pH 6.8), 0.3 mol/L 2-mercaptoethanol, 2% SDS, 0.1% bromphenol blue and 10% glycerol, and heated at 95°C for 5 min. Denatured proteins (10 g per lane) were loaded on the gel and separated using 8 V/cm for stacking (3.3% polyacrylamide), and 12 V/cm for the resolving (12% polyacrylamide) gel. Gels were run until the dye front reached the bottom of the resolving gel.

Glycosylation analysis

Electrophoretically separated proteins were semi-dry blotted onto Immobilon-P (PVDF) membranes according to Towbin [16]. Current of 0.8 mA/cm^2 of gel area was applied, and the blotting was stopped after 90 min. Blots were blocked overnight in TBS containing 3% bovine serum albumin.

Carbohydrate structures attached to proteins in the samples were analyzed using lectins labeled with digoxigenin or biotin [17]. Blocked PVDF membranes (blots) were washed 3 × 10 min in TBS containing 0.1 % Tween 20 (TBST). Blots were then incubated with lectins in TBS buffer containing 1 mmol/L $CaCl_2$ and 1 mmol/L $MgCl_2$ in the following dilutions: SJA - I : 3000, BSA I, BSA II, Con A, DSA, PHA-E, and VVA–1 : 2000; GNA, RCA I, SNA, WGA–1 : 1000, MAA–1 : 500; and PNA–1 : 100. After 60 min incubation with lectins, blots were washed 3 × 10 min in TBST. Lectins labeled with biotin were incubated with streptavidin-alkaline phosphatase (diluted 1 : 5000 in TBS), and lectins labeled with digoxigenin with anti-digoxigenin antibodies labeled with alkaline phosphatase (diluted 1 : 5000 in TBS) for 60 min at room temperature. Blots were washed 3 × 10 min in TBST, and developed with 0.02 mg/ml 5-bromo-4-chloro-3-indolyl phosphate and 0.04 mg/ml nitro-blue tetrazolium in 0.1 mol/L Tris/HCl, 0.1 mol/L NaCl, 50 mmol/L $MgCl_2$, pH = 9.5.

Other procedures

Protein concentrations in cell homogenates were determined by the method of Lowry [18].

Results and discussion

Using lectin Western blot we have analyzed glycosylation of stress glycoprotein GP62 in A1235 cells exposed to single episode of heat-shock (2 h at 42°C). As expected, this resulted in the appearance of significant amounts of GP62, which was undetectable in the control cells. SNA, DSA and PHA-E lectins recognized GP62, while other lectins listed in Table 1 did not (Fig. 1A). Based on this information, it is possible to identify some segments of carbohydrate structures attached to GP62. GP62 appears to contain a bianntenary complex-type oligosaccharide with at least one α(2,6)-linked sialic acid, N-acetyllactosamine and bisecting β(1,4)-linked N-acetylglucosamine.

While performing these experiments we noticed that heat-shock is not necessary for the induction of GP62, and that even simple subculturing of cells was a sufficient stimulus for the induction of GP62. However, the dynamics of the induction was somewhat different. When GP62 was induced by a heat-shock, it appeared within 4 h after the end of heat treatment and remained on a relatively high level for at least next 24 h (Fig. 1A). If the same protein was induced within a different pathway of cell adaptation, i.e. when cells were recovering from the treatment with trypsin, GP62 appeared after only 2 h, stayed on a high level during the following 2 h, and virtually disappeared after 24 h (Fig. 1B). Contrary to heat-shock and subculturing, when cells were exposed to UV-C radiation, GP62 did not appear (data not shown), suggesting that it is associated with some, but not all, cellular stress-response pathways [19].

Subculturing of cells is generally not considered to be a sufficient stimulus to cause activation of the stress response, and indeed we did not observe increase in Hsp70 in our samples. However, the process of subculturing includes trypsinization that is being used to break the connection between cells and the plastic surface. In addition to releasing cells from the surface, proteolytic enzymes inevitably cause partial digestion of numerous membrane proteins that subsequently get internalized. The exposure of their hydrophobic and/or denatured surfaces to the interior of the cell should be, and apparently is, a signal that causes activation of some kind of the cellular stress response. Although changes we observed were apparently not a part of the classical heat-shock response, some kind of cell response was definitively activated since the appearance of GP62 was very prominent and reproducible. Recently it was reported that the protein sequence of GP62 contains some areas of homology with Hsp70, and it was suggested that GP62 might also have a chaperone-like function [14]. If this hypothesis is correct, its activation following subculturing of cells might be involved in the restoration of homeostasis disturbed by the internalization of numerous truncated proteins.

It was particularly interesting to find that glycosylation of GP62 induced by subculturing was different from glycosylation of GP62 induced by heat-shock (Table 1, Fig. 1). In addition to SNA, DSA and PHA-E lectins, which recognized

Table 1. Lectin analysis of GP62 glycosylation. Glycosylation of GP62 induced by heat-shock and subculturing of cells was analyzed by lectin Western blot analysis using 14 different lectins. Cells were collected after different times of incubation and analyzed as described under *Materials and methods*. The samples where GP62 was positively identified are marked with "+", and where it was not identified with "−". Samples which were not analyzed are marked with "N/A".

Lectin	*Approximate specificity*	*GP62 in cells exposed to heat shock*				*GP62 in cells after subculturing*			
		control	*0 h*	*4 h*	*24 h*	*control*	*0 h*	*4 h*	*24 h*
BSA I	α-Gal	−	−	−	−	−	−	−	−
BSA II	α/β-GlcNAc	N/A	N/A	N/A	N/A	−	−	−	−
Con A	α-Man	−	−	−	−	−	−	−	−
DSA	GlcNAc-β(1,4)-GlcNAc = Gal-β(1,4)-GlcNAc	−	−	+	+	−	+	+	−
GNA	Man-α(1,3) α(1,6) α(1,2)	N/A	N/A	N/A	N/A	−	−	−	−
MAA	Sia-α(2,3)	N/A	N/A	N/A	N/A	−	−	−	−
PHA-E	biantennary structures containing bisecting β(1,4)-GlcNAc	−	−	+	+	−	+	+	−
PNA	Gal-β(1,3)-GalNAc > α/β-Gal	N/A	N/A	N/A	N/A	−	−	−	−
RCA I	β-Gal > α-Gal	−	−	−	−	−	+	+	−
SJA	α/β-GalNAc > α/β-Gal	−	−	−	−	−	−	−	−
SNA	Sia-α(2,6)-GalNAc	−	−	+	+	−	+	+	−
UEA I	α-L-Fuc	−	−	−	−	−	−	−	−
VVA	GalNAc-α(1,3)-Gal = αGalNAc	−	−	−	−	−	−	−	−
WGA	GlcNAc-β(1,4)-GlcNAc	−	−	−	−	−	+	+	−

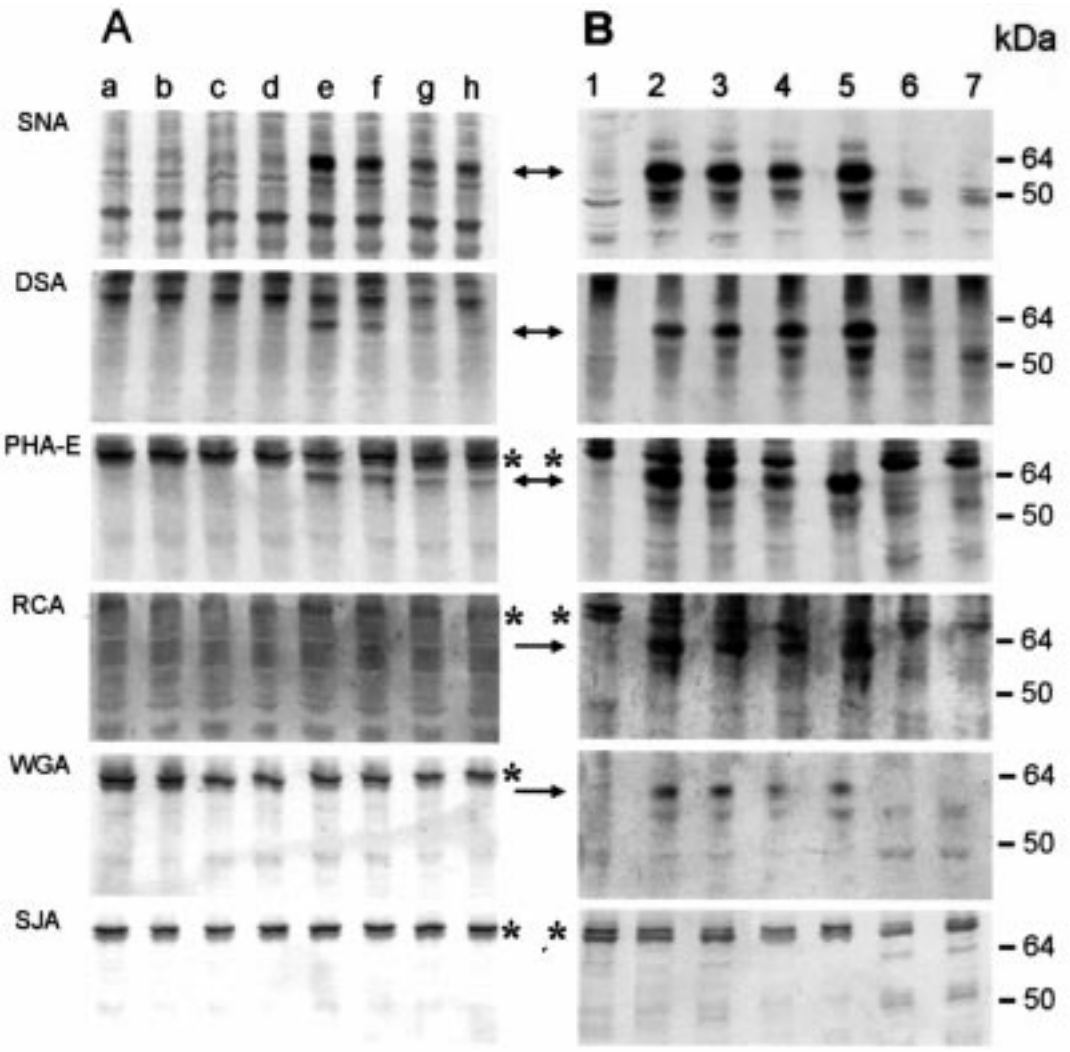

Figure 1. Lectin analysis of GP62 glycosylation. A1235 cells were homogenized, proteins (10 μg per lane) separated by denaturing SDS polyacrylamide electrophoresis and transferred onto PVDF membranes. Glycosylation patterns were analyzed using a set of 14 lectins listed in the *Materials and methods* section (only 6 patterns are shown, other 8 lectins did not recognize GP62). In one series of experiments cells were stressed by heat-shock at 42°C for 2 h and collected after 0 h (c, d), 4 h (e, f), or 24 h (g, h) of subsequent incubation. Control cells (a, b) were submersed at 37°C during that time. In the second series of experiments cells were either collected by scraping (1), or treated with 0.25% solution of trypsin in PBS, diluted with fresh medium and cultivated for 2 h (2, 3), 4 h (4,5), or 24 h (6, 7). The appearance, and glycosylation of GP62 (marked by arrows) was analyzed using lectins SNA, DSA, PHA-E, RCA I, WGA, and SJA lectins. Protein bands marked with asterix (*) are a consequence of secondary staining due to direct binding of streptavidin to proteins in the sample. They are absent from the blot stained with WGA lectin on the panel B because in this experiment WGA lectin labeled with digoxigenin was used.

GP62 induced by heat-shock, GP62 induced by subculturing was also recognized by RCA I and WGA lectins. Thus, in addition to carbohydrates present on GP62 induced by heat-shock, GP62 induced by subculturing contained terminal galactose and N-acetylglucosamine residues. It would be very interesting to learn whether these modifications of carbohydrate structures attached to GP62 in different forms of stress response are involved in the regulation or turnover of GP62. Although glycosylation is one of the major means of determining distribution and clearance of serum glycoproteins, to our knowledge until now it was not implicated in the regulation or turnover of intracellular proteins [2,20,21].

Acknowledgements

The authors thank professor Willem van Dijk from the Vrije Universiteit Amsterdam for helpful suggestions and comments. This work was supported by grants #006320 and #006320-2 from the Croatian Ministry of Science and Technology.

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Received 11 November 1999; revised 5 January 2000;
accepted 13 January 2000