Responsiveness of the state of O-linked *N*-acetylglucosamine modification of nuclear pore protein p62 to the extracellular glucose concentration

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O-linked *N*-acetylglucosamine (O-GlcNAc) modification has been described in many proteins, including nuclear pore glycoproteins. In the present study we investigated the effect of extracellular glucose on the level of modification of nuclear pore protein p62 by O-GlcNAc. We found that exposure of cells to a high concentration of glucose caused an increased level of modification of p62 with O-GlcNAc, whereas the modification of other proteins did not change detectably. The increased O-GlcNAc modification of p62 in response to glucose required the metabolism of glucose to glucosamine. The exposure of the cells to glucosamine itself also caused increased O-GlcNAc modifi-

cation, whereas mannosamine and galactosamine did not. Furthermore, changes in glucose concentrations within the physiological range induced the O-GlcNAc modification in p62 in rat aortic smooth-muscle cells, indicating that this modification of p62 might occur in an intact animal. These results imply that the ambient glucose concentration has an impact on the level of O-GlcNAc modification of proteins such as p62, and that functional changes in some of these proteins might ensue.

Key words: *N*-acetylglucosamine, O-glycosylation, transcription.

INTRODUCTION

Although many cellular proteins are known to be modified by the addition of O-linked *N*-acetylglucosamine (O-GlcNAc) [1–3], the biological role of this modification is not well understood. This modification involves the attachment of a single GlcNAc residue via O-glycosyl linkages to serine and threonine residues in the polypeptide and can be found in viral proteins, many DNA-binding proteins and transcription factors, nuclear pore proteins, oncogene products and cytoskeletal proteins [1–6]. Of these, the nuclear pore glycoproteins were the first O-linked GlcNAc proteins to be studied in detail [1,7–10]. A family of peripheral glycoproteins ranging in size from 54 to 210 kDa have been identified as part of the nuclear pore complex. The most abundant member of the nuclear-pore-complex proteins is p62, a protein with a molecular mass of 62 kDa [10–12]. Cordes et al. [11] cloned the cDNA encoding the rat nucleoporin p62 and examined its glycosylation. It was found that this pore protein contained at least three domains: a highly repetitive N-terminus, a Thr/Ser-rich central region and a helical C-terminus containing heptad repeats typical of the helical regions of filament-forming proteins such as myosin, tropomyosin and keratin [12].

There is some evidence that the O-GlcNAc modification on pore complex proteins might be essential for the active transport of mRNA or proteins and for the assembly of the components needed for recognition of the nuclear localization signal [13], although the function of this modification remains controversial. Because marked changes in the O-GlcNAc modification of certain proteins accompany changes in cellular physiology or occur in response to extracellular signals, this form of glycosylation is speculated to have a regulatory function [1–3,14].

The finding that glucose can regulate growth-factor gene expression in vascular smooth-muscle cells [15] and that glucose

metabolism to glucosamine is required for this transcriptional response to glucose [16,17] prompted us to determine whether protein modification by O-GlcNAc could be altered by ambient glucose concentrations. In the present study we used the monoclonal antibody RL2, which recognizes polypeptides containing O-GlcNAc residues [10,16–19], as a tool for identifying and quantifying the relative level of O-GlcNAc modification. Here we report marked changes in the O-GlcNAc modification of the nuclear pore protein p62 in response to the exposure of cells to high concentrations of glucose. This response requires the metabolism of glucose to glucosamine and can be mimicked by exposure of the cells to glucosamine. This result indicates that the O-GlcNAc state of p62, and perhaps that of other intracellular proteins, can be modulated in response to changing extracellular glucose concentrations and that these changes in the O-GlcNAc state of intracellular proteins could modulate cellular function.

EXPERIMENTAL

Cell culture

Normal rat kidney (NRK) cells and MDA468 cells were grown in DMEM (Dulbecco's modified Eagle's medium) containing 10% (v/v) fetal calf serum (FCS) (Gibco/BRL, Grand Island, NY, U.S.A.) containing 100 µg/ml penicillin (Sigma Chemical Co., St Louis, MO, U.S.A.) and 50 µg/ml gentamicin (Sigma) at 37 °C under air/CO₂ (37:3) in a humidified incubator. For studies on exposures to various sugars, exponentially growing cells were seeded at 5 × 10⁵ cells per 60 mm dish. When the cells reached 80% confluence, the medium was changed to glucose-free DMEM containing 10% FCS and 5 mM pyruvate; incubation was continued for an additional 15 h. Cells were then exposed to one of the following sugars: 20 mM glucose, 5 mM

Abbreviations used: DON, 6-diazo-5-oxonorleucine; GFAT, glutamine:fructose-6-phosphate amidotransferase; NRK, normal rat kidney; O-GlcNAc, O-linked *N*-acetylglucosamine; RASM cells, rat aortic smooth-muscle cells.

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glucosamine, 5 mM galactosamine or 5 mM mannosamine. In some experiments the cells were incubated with 40 μ M 6-diazo-5-oxonorleucine (DON) concurrently with the sugars. Rat aortic smooth-muscle (RASM) cells, prepared from the aortas of 250–300 g male Sprague–Dawley rats [15,16], were grown in DMEM with 10% FCS containing 2.5 mM glucose. For the stimulation, 30 mM glucose or 7 mM glucosamine was applied as described above.

Antibodies

A p62-specific polyclonal antiserum was raised as previously described [19]. The mouse monoclonal antibody directed against p62 (414) was purchased from Berkeley Antibody Co. (Richmond, CA, U.S.A.). Monoclonal antibody RL2, specific for O-GlcNAc-containing proteins, was a gift from Dr L. Gerace [10] (Scripps Clinic, La Jolla, CA, U.S.A.).

Immunoprecipitation and immunoblot analysis

Cells grown and treated as described above were scraped from the dishes with a rubber 'policeman', pelleted by centrifugation at 800 *g* and washed twice by centrifugation with cold PBS. The cell pellets were resuspended in an ice-cold buffer containing 20 mM Hepes, pH 7.9, 500 mM NaCl, 20% (v/v) glycerol, 1 mM dithiothreitol and 1 mM PMSF (Eastman Kodak Company, Rochester, NY, U.S.A.). The cells were disrupted by two cycles of freezing and thawing. The cell extract consisted of the supernatant obtained after centrifugation at 135000 *g* for 30 min at 4 °C in a TLA100.3 rotor (Beckman, Palo Alto, CA, U.S.A.). This high-salt extract was dialysed against binding buffer [20 mM HEPES/KOH (pH 7.9)/0.25 M KCl/2 mM MgCl₂/10 μ M ZnSO₄/1 mM dithiothreitol/10% (v/v) glycerol/1 mM PMSF] and the protein concentration was determined with the Bio-Rad D_c Protein Assay (Bio-Rad Laboratories, Hercules, CA, U.S.A.). For each immunoprecipitation reaction, extract containing approx. 600 μ g of protein was incubated with 4 μ g of affinity-purified RL2 antibody or anti-p62 antibodies in binding buffer at 4 °C for several hours. The immunoglobulin, bound to Protein G–Sepharose 4B (Pharmacia, Piscataway, NJ, U.S.A.), was pelleted and washed four times by centrifugation at 1000 *g*. The pellets were resuspended in SDS protein electrophoresis sample buffer, then boiled and analysed by SDS/PAGE [7.5% (w/v) gel]. The protein was transferred electrophoretically to a nitrocellulose membrane for immunoblot analysis. The immunoblots were developed with a 1:1000 dilution of affinity-purified antibodies and the bound antibodies were detected with the enhanced chemiluminescence system (Amersham, Arlington Heights, IL, U.S.A.).

RESULTS

Glucose stimulates glycosylation of several proteins in MDA468 cells

To investigate whether the extracellular glucose concentration could be reflected in the O-GlcNAc modification of intracellular proteins, we measured the O-GlcNAc level on proteins by using RL2 monoclonal antibody (Figure 1). The cell extracts from 20 mM glucose-treated MDA468 cells were used for immunological analysis with RL2 antibody. Exposure of MDA468 cells to 20 mM glucose resulted in a marked increase in O-GlcNAc signal on several proteins near 60 kDa and this detectability was competed away in the presence of 200 mM *N*-acetylglucosamine [17] (results not shown). This result demonstrates that extracellular glucose could affect the O-GlcNAc modification of certain cellular proteins.

Figure 1 Western blot showing changes in RL2 signal from cellular proteins in response to treatments with glucose

MDA468 cells were pre-starved overnight with glucose-free DMEM containing 10% FCS, then incubated for an additional 24 h with (Glu) or without (—) 20 mM glucose. Equal amounts of protein from the high-salt cell extracts were loaded on each lane of a 7.5% (w/v) polyacrylamide gel for separation by SDS/PAGE. The resolved proteins were analysed by Western blotting probed with the monoclonal RL2 antibody, which detects O-GlcNAc. The positions of molecular mass markers are indicated (in kDa) at the left.

Exposure of MDA468 cells to glucosamine also increased the RL2-detectable signal on the Western blot of the proteins near 60 kDa, although an increase in signal was also seen on the numerous other proteins in the cell extract (Figure 2A, TCL). This effect of glucosamine could also be seen on proteins immunoprecipitated with the RL2 antibody (Figure 2A, IP) but, again, the most marked effect was on the proteins in the 60 kDa range of the gel. The immunoprecipitation of these proteins could be blocked by preincubation of the RL2 antibody with GlcNAc but not with GlcN or mannosamine (results not shown, and R. Konrad, personal communication).

Because the most abundant nuclear pore glycoprotein, p62, is known to be modified by O-GlcNAc, we tested whether p62 was one of the proteins in the 60 kDa range of the gel shown in Figures 1 and 2(A) that underwent changes in RL2 detectability. Using the monoclonal anti-p62 antibody 414, we immunoprecipitated p62 from cell extracts derived from glucose- or glucosamine-treated MDA468 or NRK cells and Western blotted with either anti-p62 antibody or RL2. The Western blot probed with the p62 antibody showed a relatively even loading of p62; however, when this blot was probed with RL2, a marked change in signal was seen. In the glucose-deprived cells, a very faint signal was detected on the immunoprecipitated p62, whereas the p62 from cells treated with either glucose or glucosamine displayed a markedly increased RL2 signal. Densitometric analysis showed that the RL2 signal in the p62 band was increased 7–8-fold with glucose treatment and up to 13-fold with glucosamine treatment. Thus extracellular glucosamine seems to be more effective than glucose in inducing changes in O-GlcNAc modification on proteins. Furthermore, this evidence indicates that one of the proteins in the 60 kDa range of the gel that changes RL2 signal in response to glucose is the nuclear pore protein p62.

Figure 2 Treatment of cells with either glucose or glucosamine resulted in an increased level of O-GlcNAc modification of p62

(A) MDA468 cells were pre-starved overnight with glucose-free DMEM containing 10% FCS, then treated for 24 h with 5 mM glucosamine in glucose-free DMEM containing 10% FCS. Both total cell lysate (left panel) and the immunocomplex with the anti-O-GlcNAc (α O-GlcNAc) RL2 (right panel) were analysed by RL2 Western blotting as described for Figure 1. (B) MDA468 cells (left panel) or NRK cells (right panel) were pre-starved with glucose-free medium containing 10% FCS, then exposed for a further 24 h to either 20 mM glucose (Glu) or 5 mM glucosamine (GlcN) in the same medium. Cell extracts were used for immunoprecipitation (IP) with anti-p62 (α -p62) antibody; the immunocomplexes were further analysed with either p62 or RL2 antibodies. The positions of molecular mass markers are indicated (in kDa) at the left.

Glucose induces p62 O-GlcNAc modification through its metabolism to glucosamine

The metabolism of glucose to hexosamines is controlled by the enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT) [20]. The glucosamine synthesized from glucose by GFAT can be further metabolized to UDP-GlcNAc, which is a direct substrate for protein glycosylation. As a further test of whether the changes in p62 O-GlcNAc content in response to glucose resulted from the metabolism of glucose to glucosamine by GFAT, we inhibited this enzyme with the glutamine analogue DON. Glucose-deprived MDA468 cells were treated for 24 h with either 20 mM glucose or 5 mM glucosamine in either the presence or the absence of 40 μ M DON. The p62 was immunoprecipitated with a rabbit polyclonal p62 antibody and the immunoprecipitated p62 was analysed by Western blotting with 414 (Figure 3A) or RL2 (Figure 3B) antibody. Although the quantity of p62 loaded on the gel remained relatively constant, 40 μ M DON completely blocked the effect of glucose on the detectability of p62 by RL2 (Figure 3, lanes 4). However, the effect of glucosamine treatment on p62 detectability with RL2 was not blocked by DON, presumably because glucosamine could enter the hexosamine pathway downstream of the DON-inhibited GFAT (Figure 3, lane 5). This result indicates that GFAT is necessary for the effect of glucose on the detectability of p62 with RL2. Furthermore, this result and the previous result, showing that free GlcNAc blocks the RL2 signal, further support the notion that it is the O-GlcNAc modification of p62 that is being detected by RL2.

Glucosamine, but not other hexosamines, stimulates the O-glycosylation of p62

Although a small portion of hexosamines such as galactosamine or mannosamine can be isomerized and metabolized to UDP-GlcNAc, these sugars are not considered major precursor substrates for O-GlcNAc modification. We therefore investigated

Figure 3 Effect of DON on glucose- and glucosamine-stimulated O-GlcNAc modification of p62

MDA468 cells were glucose-starved for 16 h. The cells were then exposed to either 20 mM glucose or 5 mM glucosamine for 24 h in the presence or absence of 40 mM DON as indicated. Total cell extracts were subjected to immunoprecipitation with rabbit polyclonal anti-p62 antibody (688) and further analysed by Western blot with either anti-p62 (414) (A) or RL2 (B) monoclonal antibodies. The positions of molecular mass markers are indicated (in kDa) at the left.

the effects of these other hexosamines on the O-GlcNAc modification of p62 (Figure 4). Glucose-deprived NRK cells were exposed to 5 mM glucosamine, galactosamine or mannosamine for 24 h and the immunoprecipitated p62 from the cell lysates was analysed by immunoblotting with either p62 (Figure 4A) or RL2 (Figure 4B) antibodies. Glucosamine treatment, but not the other hexosamines, resulted in an increase in the O-GlcNAc modification of p62 (compare Figures 4A and 4B). The relative O-GlcNAc levels on p62 were compared by densitometric analysis

Figure 4 Effects of hexosamine sugars on O-GlcNAc modification of p62

NRK cells were pretreated with glucose-free DMEM and then for 24 hrs with or without 5 mM galactosamine, glucosamine or mannosamine. Immunoprecipitations were performed with monoclonal antibody 414 from the high-salt cell extracts adjusted to contain equal amounts of protein. Immunoprecipitates were analysed by Western blot probed with either anti-p62 (414) (**A**) or RL2 (**B**) antibodies. The lower panels are charts of the relative band densities determined with a photometric scanner.

Figure 5 Time course of the effect of glucosamine on the O-GlcNAc modification of p62

MDA468 cells were pretreated with glucose-free DMEM. Cells were then treated with 5 mM glucosamine for the indicated durations. The p62 was immunoprecipitated with antibody (414) from the high-salt cell extracts containing equal amounts of protein. Immunoprecipitates were analysed by Western blots probed with either anti-p62 (414) (**A**) or RL2 (**B**) antibodies. The lower panels are charts of the relative band densities determined with a photometric scanner.

(Figure 4, lower panels) and only glucosamine resulted in the observed 7-fold increase in RL2-detectable O-GlcNAc.

Time course for the glucosamine effect on the O-GlcNAc modification of p62

A time-course study was performed to determine the kinetics of the glucosamine-induced O-GlcNAc modification of p62 in MDA468 cells (Figure 5). The cells were deprived of glucose overnight, then exposed for the indicated durations to 5 mM glucosamine. The maximal level of O-GlcNAc modification was reached approx. 18 h after the addition of glucosamine and stayed constant for an additional 20 h.

Level of O-GlcNAc modification of p62 responds to more physiological glucose concentrations in RASM cells

To observe the alterations in the level of glycosylation of p62 in NRK and MDA468 cells, these cells were deprived of glucose in the culture medium before exposure to high concentrations of sugars. Thus the changes in p62 glycosylation in these cells might not represent a phenomenon *in vivo* because an extracellular concentration of glucose approaching zero is not compatible with life. Indeed, the glucose-induced p62 glycosylation was maximal in these cells at a glucose concentration of 4 mM (results not shown). It has been shown previously that the effect of glucose on growth-factor gene regulation occurs at glucose concentrations that are an order of magnitude lower in MDA468

Figure 6 Effects of glucose and glucosamine on O-GlcNAc level on p62 in RASM cells

Cells were grown in DMEM (containing 2.5 mM glucose) plus 10% FCS, then exposed to high glucose (30 mM) or glucosamine (7 mM) for 24 h. Total cell lysates were prepared and the p62 was immunoprecipitated with our polyclonal anti-p62 antibody (688). The precipitated proteins were analysed by Western blot probed with either polyclonal anti-p62 (688) (**A**) or monoclonal RL2 (**B**) antibodies. The lower panels are charts of the relative band densities determined with a photometric scanner.

cells than in vascular smooth-muscle cells [15,16]. Whereas most cells behave like MDA468 cells, which exhibit a saturated glucose response at physiological glucose concentrations, vascular smooth-muscle cells and perhaps other cell types might exhibit alterations in the O-GlcNAc level on proteins as the glucose concentrations vary within the physiological range or become even higher, as in uncontrolled diabetes mellitus.

RASM cells grown in medium containing 2.5 mM glucose were stimulated with either 30 mM glucose or 7 mM glucosamine for 24 h. O-GlcNAc levels on p62 were measured after immunoprecipitation with polyclonal p62 antibody (Figure 6). Densitometric analysis showed that O-GlcNAc RL2 signal on p62 increased approx. 7-fold at high glucose concentration, with an increase of up to 13-fold in the presence of glucosamine (Figure 6B, lower panel).

DISCUSSION

Nuclear pore proteins are often modified by O-GlcNAc; these sugar residues have been thought to have a role in nuclear transport because lectins that bind O-GlcNAc residues block the translocation of proteins across the nuclear envelope [21], and the depletion of O-GlcNAc glycoproteins inhibits transport *in vitro* [13,22]. However, Miller and Hanover [23] showed that nuclear pore glycoproteins can tolerate the alteration of their O-GlcNAc groups by the addition of a galactose residue without altering nuclear pore structure or nuclear function, including nuclear protein import and DNA synthesis. Therefore the function of O-GlcNAc modification in nuclear pore proteins is still unresolved.

The major finding of this study is that the degree of modification of the nuclear pore protein p62 by O-GlcNAc is markedly sensitive to changes in extracellular glucose concentration or to the addition of glucosamine to the culture medium. This change in modification did not seem to occur in all proteins detected by RL2, suggesting that only certain intracellular proteins undergo changes in O-GlcNAc modification in response to the availability of these sugars. This apparent specificity might relate to a more

rapid rate of turnover of O-GlcNAc on p62 than on other proteins.

Although the change in p62 O-GlcNAc modification was observed in three different cell types, the dose response to glucose differed in the vascular smooth-muscle cells from that in the MDA468 breast cancer cells and the NRK fibroblasts. In the latter cell types, the maximal response was observed at a glucose concentration of 4 mM only when glucose was provided to cells that had been previously deprived of glucose for several hours. Thus, at a physiological glucose concentration, the glycosylation of p62 is maximized, permitting no further increase in modification at higher glucose concentrations. In the vascular smooth-muscle cells the response could be observed at more physiologically relevant glucose concentrations, suggesting that this modification could be responsive *in vivo* to the fluctuations in the concentrations of glucose in response to feeding or as a result of the development of diabetes. Recently, pancreatic β -cells were also shown to alter the O-GlcNAc content in response to changes in the extracellular glucose concentrations *in vivo* [24], although the proteins undergoing this change have not been identified. The observation that high ambient concentrations of glucose or glucosamine increase the degree of O-GlcNAc modification on p62 in the vascular cells suggests that either these sugars stimulate the activity of O-GlcNAc transferase or else they inhibit the O-GlcNAc-selective *N*-acetyl- β -D-glucosaminidase [25]. Hart [3] has suggested that substrate availability might be one of means of modulating the activity of O-GlcNAc transferase; our results support this mechanism. That is, a blockade of GFAT activity blocked the glucose-induced increase in p62 glycosylation, whereas the provision of glucosamine increased modification. Other hexosamine sugars such as galactosamine and mannosamine did not effect the O-GlcNAc modification of p62, suggesting that there is an insufficient level of isomerization of these hexosamines to glucosamine to support the increased glycosylation of p62.

The functional relevance of these changes in p62 O-GlcNAc in response to changes in ambient glucose concentration is not clear, mainly because the role of O-GlcNAc in p62 function is

not understood. It remains to be seen whether these changes in p62 contribute to the known alterations in gene expression in response to glucose or glucosamine [15–17,26].

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