Dynamic Interplay between O-Glycosylation and O-Phosphorylation of Nucleocytoplasmic Proteins

ALTERNATIVE GLYCOSYLATION/PHOSPHORYLATION OF THR-58, A KNOWN MUTATIONAL HOT SPOT OF c-Myc IN LYMPHOMAS, IS REGULATED BY MITOGENS*

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Previously, we reported that c-Myc is glycosylated by O-linked N-acetylglucosamine at Thr-58, a known phosphorylation site and a mutational hot spot in lymphomas. In this paper, we describe the production and characterization of two Thr-58 site-specific antibodies and use them to examine the modification of Thr-58 in living cells. One antibody specifically reacts with the Thr-58-glycosylated form of c-Myc, and the other reacts only with unmodified Thr-58 in c-Myc. Using these antibodies together with a commercial anti-Thr-58-phosphorylated c-Myc antibody, we simultaneously detected three forms of c-Myc (Thr-58-unmodified, -phosphorylated, and -glycosylated). It has been reported that Thr-58 phosphorylation is dependent on a prior phosphorylation of Ser-62. Mutagenesis of Ser-62 to Ala showed a marked decrease of Thr-58 phosphorylation and a marked increase of Thr-58 glycosylation. Growth inhibition of HL60 cells by serum starvation increases Thr-58 glycosylation and correspondingly decreases its phosphorylation. Serum stimulation has the opposite effect upon the modification status of Thr-58. A candidate kinase responsible for Thr-58 phosphorylation is the glycosynthase kinase 3 (GSK3). Lithium, a competitive inhibitor of GSK3, decreased Thr-58 phosphorylation and increased its glycosylation. Finally, we show that the Thr-58-phosphorylated form of c-Myc predominantly accumulates in the cytoplasm rather than the nucleus upon inhibition of proteasome activity. These data suggest that hierarchical phosphorylation of Ser-62 and Thr-58 and alternative glycosylation/phosphorylation of Thr-58 together regulate the myriad functions of c-Myc in cells.

c-Myc, the product of the c-myc protooncogene, is a helix-loop-helix leucine zipper (HLHLZ) protein that regulates gene transcription in cell proliferation, apoptosis, and metabolism (1). Two regions of c-Myc required for its biological activities are the N-terminal transcriptional activation domain (TAD) and the C-terminal basic-HLHLZ-specific DNA-binding domain (2). The HLHLZ domain mediates heterodimerization of c-Myc with its partner, Max, permitting binding to specific DNA sequences (2). c-Myc activity is precisely controlled at various levels, including transcription, translation, and post-translation (3). c-Myc can be phosphorylated at more than a dozen Ser and Thr residues (3). Phosphorylation at Thr-58 and/or Ser-62 in the TAD has been shown to be particularly important for regulating transformation of cells by c-Myc (4, 5). It has also been shown recently that c-Myc turnover appears to be regulated by the ubiquitin-proteasome pathway (6–9). Mutation of Thr-58 increases c-Myc stability (10–12), and phosphorylation of Thr-58 is associated with rapid degradation of c-Myc (12).

Our previous studies showed that the TAD of c-Myc is also glycosylated by O-linked N-acetylglucosamine (O-GlcNAc) (13) and that Thr-58 is a major glycosylation site of c-Myc (14). O-GlcNAc is an abundant posttranslational modification of nuclear and cytoplasmic proteins in eukaryotes (15). Virtually all known O-GlcNAc-modified proteins are also phosphorylated proteins that form reversible multimeric protein complexes, suggesting that O-GlcNAc may regulate protein phosphorylation, protein-protein interaction, or both (16, 17). Thr-58 is also a known mutational hot spot in lymphomas, and this mutation is thought to be involved in tumor progression (1). This evidence led us to the hypothesis that alternative modification of Thr-58 by O-phosphophate or O-GlcNAc regulates the c-Myc function differentially.

Here we present evidence for the occurrence of both glycosylation and phosphorylation at Thr-58 in a cell line using both site- and modification state-specific antibodies. We also demonstrate the interplay between hierarchical phosphorylation of Ser-62/Thr-58 and alternative glycosylation/phosphorylation of Thr-58 in living cells.

EXPERIMENTAL PROCEDURES

Antibodies—A mouse anti-c-Myc antibody (C-33) is from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse anti-α-tubulin antibody (B-5–1–2) is from Sigma. A rabbit anti-phospho-c-Myc(Thr-58/Ser-62) antibody is from Cell Signaling Technology (Beverly, MA), and we designated this as a phosphorylated Thr-58-specific antibody (α-T58P).

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generate anti-glycosylated Thr-58 antibody (α-T58G) and anti-unmodified Thr-58 antibody (α-T58N), a synthetic glycosylated peptide (KKFELLPTPPLSLSRR) and a synthetic peptide (KKFELLPPTPLSLSRR) corresponding to amino acids 51–66 in the human c-Myc protein were used as antigens. After five immunizations in BALB/c mice, cells from the popliteal and inguinal lymph nodes were collected and fused with the P3X63Ag.653 myeloma line according to the standard procedures. After the HAT selection, supernatants were screened for the reactivity with respective antigen. Confirmation of specificity was obtained by dot-blot analysis.

Cell Culture and Transfection—Human embryonic kidney cell line 293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) (heat inactivated at 56°C for 30 min) at 37°C in humidified air with 5% CO2. Human promyelocytic leukemia cell line HL60 cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) FCS. Human T-cells from the popliteal and inguinal lymph nodes were used as antigens. After five immunizations (KKFELLPTPPLSLSRR) corresponding to amino acids 51–66 the cells were used in transfection.

Immunoprecipitation and Western Blotting—Cells were lysed in 50 mM Tris-HCl (pH 7.5), 0.5% (w/v) SDS, and 70 mM 2-mercaptoethanol, boiled for 5 min, and diluted with 4 volumes of 20 mM Tris-HCl (pH 7.4), 1% (w/v) Triton X-100, 0.25% (w/v) sodium deoxycholate, 250 mM NaCl, 1 mM LiCl, 0.5 mM MnCl2, 5 mM EDTA, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfon fluoride (PMSF), 1 µg/ml leupeptin, 2 µg/ml antipain, 10 µg/ml benzamidine, 10 units/ml aprotinin, 1 µg/ml chymostatin, and 1 µg/ml pepstatin. The extracts were centrifuged for 20 min at 12,000 g, and the clarified supernatants were then incubated with the indicated antibody for 2 h at 4°C. The immune complexes were precipitated with protein G-Sepharose 4 fast flow (Amersham Biosciences) and washed extensively with 20 mM Tris-HCl (pH 7.4), 1% (w/v) Triton X-100, 0.25% (w/v) sodium deoxycholate, 250 mM NaCl, and 5 mM EDTA.

For the detection of the proteins in Western blots, the immune complexes were suspended in SDS-PAGE sample buffer and boiled for 5 min. Proteins were separated in 7.5% SDS-PAGE, electrophoebtized onto polyvinylidene difluoride membrane (Millipore, Bedford, MA), and subjected to immunodetection using the appropriate primary antibody. Proteins were visualized by using horseradish peroxidase-linked anti-rabbit or anti-mouse immunoglobulin antibody. The membranes were blocked with 4% (w/v) bovine serum albumin in 10 mM Hepes (pH 7.9), 1% (w/v) soybean trypsin inhibitor, 0.1% (v/v) Triton X-100, and 0.25% (w/v) sodium deoxycholate, 250 mM NaCl, and 5 mM EDTA.

RESULTS

Specificity of Both Anti-Thr-58-glycosylated and Anti-Thr-58-unmodified c-Myc Antibodies—Thr-58 within the TAD of c-Myc has been independently identified as both a glycosylation (14) and a phosphorylation site (21). These findings suggest that there are three types of modifications at Thr-58 on c-Myc: unmodified, glycosylated, and phosphorylated. Although the phosphorylation status of Thr-58 has been studied extensively using an antibody specific for Thr-58-phosphorylated c-Myc (α-T58P) (12, 22–24), there has been little attention to the other forms.

To detect all three c-Myc forms simultaneously, we have generated two monoclonal antibodies; one is specific for Thr-58-glycosylated c-Myc (α-T58G), and the other is specific for Thr-58-unmodified c-Myc (α-T58N). The specificity of each antibody is shown in Fig. 1. For the characterization of the antibodies, we used four forms of synthetic TAD peptides, which have different modification status including phosphorylation at Ser-62. Because it has been reported that Ser-62 on c-Myc is also a phosphorylation site (21) and phosphorylation of Ser-62 may affect recognition by the antibodies, we also tested immunoreactivity against the Ser-62-phosphorylated peptide. α-T58N recognizes both the unmodified and the Ser-62-phosphorylated peptide but not the other two forms (Fig. 1A), indicating that α-T58N is specific for Thr-58-unmodified c-Myc and that the Ser-62 phosphorylation status does not affect its specificity. Although the sensitivity of α-T58G is lower than that of the other antibodies, α-T58G specifically reacts with the Thr-58-glycosylated peptide but not the other three forms (Fig. 1A). The epitope for α-T58G seems to be a peptide in the TAD including O-GlcNac-modified Thr-58 since it does not cross-react with all of several other synthetic O-GlcNac-modified peptides we have prepared (19) and the reaction between α-T58G and the Thr-58-glycosylated TAD peptide is not inhibited by 300 mCi GlcNac (data not shown). Moreover, α-T58G binds wild type c-Myc overexpressed in 293 cells but not with mutant c-MycT58A (Fig. 1B). It has been reported that c-Myc can be phosphorylated at more than a dozen Ser and Thr residues, including Ser-62 and Thr-58 (3). This evidence supports the occurrence of several bands in a broad band under PAGE. Glycosylation of c-Myc by O-GlcNac was confirmed by galactosyltransferase labeling (Fig. 1C), thus, we conclude that α-T58G is a Thr-58-glycosylated c-Myc-specific antibody, α-T58P reacts only with the Thr-58-phosphorylated peptide (Fig. 1A). Because it has already been reported that α-T58P recognizes c-Myc that is singly phosphorylated at Thr-58 and doubly phosphorylated at Thr-58 and Ser-62 but does not recognize c-Myc that is singly phosphorylated at Ser-62 or that is unmodified (12), we designate α-T58P as a Thr-58-phosphorylated c-Myc-specific antibody.

Reciprocal Glycosylation/Phosphorylation of Thr-58 on c-Myc upon Serum Stimulation—Using these antibodies, we detected Thr-58 phosphorylation status of endogenous c-Myc in HL60 cells, which is possible because of high levels of the c-myc in HL60 cells (25). c-Myc in lysates were concentrated by immunoprecipitation with a general anti-c-Myc monoclonal antibody
Fig. 1. Specificity of α-Thr-58 modification state-specific antibodies. A, the indicated amount of each synthetic c-Myc TAD peptide-bovine serum albumin conjugates were dot-blotted. TAD peptides used: TAD, KKFELPTPLPSRR; Thr-58-phosphorylated TAD, KKFELPT-P-O_4-SPLPSRR; Thr-58-glycosylated TAD, KKFELPTPLPSRR. Immunoblotting of three identical blots were done using three, 3, 7, or 10 μl of the immunoprecipitates (total 40 μl) were subjected to Western blotting with either C-33 or H9251. Ten μl of the immunoprecipitates were loaded). c-Myc is indicated along with the IgG heavy chain (IgH). B, human embryonic kidney cell line 293 cells (1 × 10^6) were transiently transfected with 2 μg of either pRSV-c-myc or pRSV-c-myc^{T58A} and cultured for an additional 36 h. Overexpressed c-Myc was immunoprecipitated from harvested cell lysates with an anti-c-Myc antibody (C-33). Three different amounts of the immunoprecipitates (total 40 μl) were subjected to Western blotting with either C-33 or α-T58G (in each panel, from left to right lane, 3, 7, or 10 μl of the immunoprecipitates were loaded). c-Myc is indicated along with the IgG heavy chain (IgH) from C-33. C, 293 cells (5 × 10^6) were transiently transfected with 1 μg of pRSV-c-myc and cultured for an additional 36 h. Overexpressed c-Myc was immunoprecipitated from harvested cell lysates with C-33. Ten μl each of the immunoprecipitates were subjected to either Western blotting with C-33 (lane 1) or on-blot galactosyltransferase labeling with UDP-[3H]galactose (lane 3). The same amounts of C-33 used for immunoprecipitation were loaded and subjected to the same assays as negative controls (lanes 2 and 4). c-Myc is indicated along with IgH from C-33. For the galactosyltransferase labeling, the membranes were cut at the position indicated by the arrows.

(C-33) and then visualized by Western blot analysis. As shown in Fig. 2A, several bands were immunoreactive with C-33. In addition to multiple phosphorylation events as mentioned above, it has been reported that the c-myc gene gives rise to two major species of human c-Myc proteins: Myc1, with an apparent molecular mass of 67 kDa; and a 64-kDa protein, Myc2 (3). This evidence predicts the occurrence of several bands under PAGE. The bands immunoreactive with α-T58G were in a smeared pattern and were similar to those recognized by α-T58N but not by α-T58P (Fig. 2A, long exposure). The immunoreactivities of each α-T58 antibody upon serum stimulation were quantified and normalized to the levels of C-33 (total c-Myc), as shown in Fig. 2B. The population of α-T58P-immunoreactive bands increased upon serum stimulation, which is similar to the pattern of the major C-33-immunoreactive bands (Fig. 2A, short exposure, and 2B). Conversely, the population of α-T58G-immunoreactive bands were high in growth-arrested cells and decreased upon serum stimulation (Fig. 2A, long exposure, and 2B). The population of α-T58N-immunoreactive bands also tended to decrease upon serum stimulation albeit to a smaller extent. Although the stoichiometry of each α-T58 antibody-immunoreactive band could not be estimated because of the different sensitivity of each antibody for the detection of each c-Myc forms (Fig. 1), these results indicate that alternative glycosylation/phosphorylation occur at Thr-58 on c-Myc in cells.

Inhibition of Ser-62 Phosphorylation Increases Thr-58 Glycosylation—It has been reported that phosphorylation of Thr-58 is dependent on a prior phosphorylation of Ser-62 (26). To examine the effect of Ser-62 phosphorylation on the modification status of Thr-58, we compared the modification status of wild type c-Myc and mutant c-Myc^{S62A}. Overexpressed c-Myc in the cell lysates was immunoprecipitated with C-33 and then visualized by Western blot analysis. As shown in Fig. 3, a similar protein level of both c-Myc were detected by C-33. Wild type c-Myc immunoreacted strongly with α-T58P and weakly with both α-T58N and α-T58G. Interestingly, both α-T58N- and α-T58G-immunoreactivities of mutant c-Myc^{S62A} were strikingly higher than that of wild type c-Myc, and mutant c-Myc^{S62A} did not immunoreact with α-T58P at all. These re-
Posttranslational Modification of Thr-58 and Ser-62 on c-Myc

Results indicate that both the glycosylation and phosphorylation of Thr-58 are affected by Ser-62 phosphorylation status.

Lithium Decreases Thr-58 Phosphorylation and Increases Thr-58 Glycosylation

A candidate kinase responsible for Thr-58 phosphorylation is GSK3 (4, 5, 12, 26). It has been reported that lithium inhibits GSK3 activity (27, 28) by competition for magnesium (29). To examine the effects of lithium on the modification status of Thr-58, wild type c-myc transiently transfected 293 cells were treated with the indicated concentrations of LiCl. After treatment for 2 h, c-Myc was immunoprecipitated from each harvested cell lysates with C-33. Five μl each of the immunoprecipitates was subjected to Western blotting with either C-33, α-T58N, α-T58G, or α-T58P. c-Myc is indicated along with IgH from C-33. B, densitometry was performed on the representative film shown (all experiments were performed in triplicate). The quantified immunoreactivity of each α-T58 antibody at each time point was normalized to the corresponding C-33 immunoreactivity. The normalized immunoreactivities of each α-T58 antibody were plotted as relative immunoreactivity to that of 0 h point. Abbreviations used are shown in the legend of Fig. 1.

Fig. 2. Reciprocal glycosylation/phosphorylation of Thr-58 upon serum stimulation. A, human promyelocytic leukemia cell line HL60 cells (1 × 10⁶) were maintained in serum-free RPMI 1640 for 48 h and then stimulated with 10% (v/v) serum. Cells were harvested at three time points (lane 1, without serum stimulation; lane 2, 0.2 h after the stimulation; and lane 3, 1 h after the stimulation). Endogenous c-Myc was immunoprecipitated from each cell lysates with C-33. Five μl each of the immunoprecipitates was subjected to Western blotting with either C-33, α-T58N, α-T58G, or α-T58P. c-Myc is indicated along with IgH from C-33. B, densitometry was performed on the representative film shown (all experiments were performed in triplicate). The quantified immunoreactivity of each α-T58 antibody at each time point was normalized to the corresponding C-33 immunoreactivity. The normalized immunoreactivities of each α-T58 antibody were plotted as relative immunoreactivity to that of 0 h point. Abbreviations used are shown in the legend of Fig. 1.

Fig. 3. Mutation of Ser-62 to Ala causes a marked increase of Thr-58 glycosylation and a severe decrease of Thr-58 phosphorylation. 293 cells (1 × 10⁶) were transiently transfected with pRSV-c-myc or pRSV-c-mycS62A and cultured for an additional 36 h as described in the legend of Fig. 1. Overexpressed c-Myc was immunoprecipitated from each harvested cell lysates with C-33. Five μl each of the immunoprecipitates was subjected to Western blotting with either C-33, α-T58N, α-T58G, or α-T58P. c-Myc is indicated along with IgH from C-33. Abbreviations used are shown in the legend of Fig. 1.

Fig. 4. Lithium affects Thr-58 modification status. A, 293 cells (1 × 10⁶) were transiently transfected with pRSV-c-myc and cultured for an additional 36 h as described in the legend of Fig. 1. Two hours before harvesting, cells were treated with 0–40 mM of LiCl. Overexpressed c-Myc was immunoprecipitated from each harvested cell lysates with C-33. Five μl each of the immunoprecipitates was subjected to Western blotting with either C-33, α-T58N, α-T58G, or α-T58P. c-Myc is indicated along with IgH from C-33. B, densitometry was performed on the representative film shown (all experiments were performed in triplicate). The quantified immunoreactivity of each α-T58 antibody was normalized to the corresponding C-33 immunoreactivity and plotted as shown in the legend of Fig. 2. Abbreviations used are shown in the legend of Fig. 1.
was immunoprecipitated with C-33 or α-T58G from the cells cultured in the presence of the indicated ion for 16 h and then visualized by Western blot. As shown in Fig. 5, α-T58P immunoreactivity of c-Myc significantly decreased in the presence of lithium under all three culture conditions, and the lithium action was most significant when cells were stimulated with serum. Interestingly, α-T58G immunoreactivity of c-Myc increased in response to lithium when cells were stimulated with serum, indicating a reduction of Thr-58-phosphorylated c-Myc and a reciprocal increase of the Thr-58-glycosylated form. In the absence of lithium or potassium, serum stimulation causes a large increase in Thr-58 phosphorylation and a corresponding reproducible decrease in Thr-58 glycosylation (Figs. 2 and 5 and data not shown). As it has been reported that potassium affects GSK3 activity (30), potassium also had an effect on the Thr-58 modification status albeit to a smaller extent (Fig. 5).

All Three Thr-58 Modifications of c-Myc Are Accumulated in the Cytoplasm upon Inhibition of Proteasome-mediated Degradation—It has recently been reported that phosphorylation of Thr-58 is associated with degradation of c-Myc by the ubiquitin-proteasome pathway (12). To examine whether the modification status at Thr-58 is related to proteolysis or not, we tested the effect of proteasome inhibition on the Thr-58 modification status in 293 cells. Moreover, to characterize the localization of the accumulated c-Myc forms after the proteasome inhibitor treatment, biochemical subcellular fractionation was performed. Endogenous c-Myc was immunoprecipitated with C-33 and then visualized by Western blot. As shown in Fig. 6, c-Myc localized mainly in the nuclear fraction and a trace amount of c-Myc was also detectable in the cytoplasmic fraction at shorter times (both 0.2 and 2 h) after the addition of the proteasome inhibitor, ALLN. Because α-tubulin was detected only in the cytoplasmic fraction, we concluded that proper subcellular fractionation was achieved (Fig. 6). Interestingly, c-Myc accumulated largely in the cytoplasmic fraction rather than the nuclear fraction upon longer times of treatment of the cells with ALLN (both 4 and 8 h). Similar results were obtained when cells were treated with another proteasome inhibitor, MG132 (20 μM, data not shown). Finally, we found that the accumulated c-Myc in the cytoplasmic fraction is predominantly immunoreactive with α-T58G although all modification-specific α-T58 antibodies immunoreact with both the cytoplasmic and nuclear forms of c-Myc (Fig. 6).

DISCUSSION

It is now well known that O-GlcNAc-modification sites resemble phosphorylation sites (15), and in the case of some proteins including c-Myc (14), estrogen receptor β (31), SV-40 large T antigen (32), and eNOS (33), O-GlcNAc and O-phosphate compete for the same site. In this study, using both site- and modification status-specific antibodies, we confirmed the occurrence of both O-GlcNAc and O-phosphate at Thr-58 on c-Myc in cell lines. We also characterized the Thr-58 modification status of c-Myc in living cells. Earlier study suggest that Thr-58 phosphorylation is dependent on the prior phosphorylation of Ser-62 (26). Our data show that mutation of Ser-62 increases Thr-58 glycosylation and decreases Thr-58 phosphorylation, indicating that Thr-58 glycosylation occurs prior to its phosphorylation (Fig. 7).

Earlier studies suggest that GSK3 is likely responsible for the phosphorylation of Thr-58 (4, 5, 12, 26) and that lithium acts as an inhibitor of GSK3 (27, 28). When cells were treated
with lithium, we observed a decrease of Thr-58 phosphorylation and a corresponding increase of Thr-58 glycosylation. The increase of Thr-58-glycosylated c-Myc by lithium treatment appears to be a reciprocal action with respect to inhibition of GSK3-mediated phosphorylation. This idea is supported by earlier studies that have shown that pharmacological manipulation of the phosphorylation state of cells in turn dramatically affects the levels of O-GlcNAc in a reciprocal manner (34, 35). Because these lithium effects were most significant when cells are concomitantly stimulated with serum, O-GlcNAc modification at Thr-58 on c-Myc may play an important role in the early stage of mitogenic stimulation analogous to Ser-62 phosphorylation (Fig. 7). As reported earlier, Ser-62 phosphorylation is likely mediated by ERK (12) and/or cyclin-dependent kinase (36). Likewise, Thr-58 glycosylation/deglycosylation is likely catalyzed by O-GlcNAc transferase/O-GlcNAcase (37–39). Thus, the interplay between alternative glycosylation/phosphorylation of Thr-58 and hierarchical phosphorylation of Ser-62/Thr-58 might be regulated by these enzymes (Fig. 7).

c-Myc is a highly unstable protein (40), and proteolysis of c-Myc is mediated by the ubiquitin-proteasome pathway (6–9). Recently, it has been shown that ubiquitinated c-Myc is phosphorylated at Thr-58 (12). Although we could not identify the Thr-58 modification status of the ubiquitinated c-Myc, our data show that all three forms of Thr-58-modified, but predominately Thr-58-phosphorylated form, are accumulated by proteasome inhibitor treatment. These results suggest that the major target of c-Myc for proteolysis is the Thr-58-phosphorylated form (Fig. 7) although Thr-58 phosphorylation is not always required for the proteolysis. This idea is not in conflict with the reports that degradation of c-Myc appears not to require Thr-58 phosphorylation in the absence of Ser-62 phosphorylation (12) and that there are no significant differences between c-Myc (T58A) and wild type c-Myc regarding the ubiquitination level (24). On the other hand, the turnover rate of c-Myc may be modulated by its modification status because it has been demonstrated that mutation of Thr-58 increases c-Myc stability (8, 10–12). Moreover, it has been proposed that O-GlcNAc/O-phosphate modulates proteasome-mediated proteolysis (41, 42). We also observed a large accumulation of c-Myc in the cytoplasm by the proteasome inhibitor treatment, although in higher eukaryotic cells the proteasome is mainly localized both in the cytoplasm and the nucleus (43), and c-Myc is a nuclear protein. Our data suggest that the modification status of Thr-58 affects the translocation efficiency of c-Myc for the degradation system, which in turn affects the turnover rate of c-Myc.

What are the role(s) of the Thr-58-modifications? Thr-58 is in the TAD and is the most frequently mutated amino acid in lymphomas (1). Mutation of Thr-58 can enhance focus formation in a cotransformation assay with Ras (4, 5), although conflicting reports surround the effect of Thr-58 mutation in c-Myc transactivation activity (21, 26). It has been proposed that the biological activities of c-Myc are modulated by its interaction with other factors in addition to heterodimerization with Max (1, 2, 44–46). There are increasing numbers of proteins identified to interact with the c-Myc N terminus including the TAD, such as TATA box-binding protein, (47), p107 (48), α-tubulin (49), BIN1 (50), protein associated with Myc (51), TRRAP (52), and NF-Y (53). The alternative glycosylation/phosphorylation of Thr-58 and its interplay with Ser-62-phosphorylation may participate in regulating the formation of different functional complexes with these c-Myc-binding proteins.

There are increasing numbers of putative c-Myc target genes related to various cellular functions including energy metabolism (1). Interestingly, glucose deprivation of c-Myc-overexpressing cells was found to induce extensive apoptosis, and that is thought to be linked to increased lactate dehydrogenase A expression (54). O-GlcNAc addition on proteins is catalyzed by O-GlcNAc transferase and uses the substrate UDP-N-acetylglucosamine, which is the end-product of the hexosamine biosynthetic pathway (55). This pathway is strongly affected by intracellular glucose concentration (55). Therefore, the apoptotic feature of c-Myc-overexpressing cells by glucose deprivation may possibly be a consequence of an alteration of the O-GlcNAc/O-phosphate status of c-Myc including Thr-58. This idea is in agreement with a recent report that reciprocal modification by O-GlcNAc/O-phosphate of transcription factors, such as Sp1, may function as a mechanism for regulating glucose-responsive gene transcription (56). Thus, alternative glycosylation/phosphorylation are thought to be essential and functional modifications of nuclear and cytoplasmic proteins in eukaryotes. It will be important to clarify the distinct roles of O-phosphate and O-GlcNAc at Thr-58 for the understanding of the myriad functions of c-Myc in cells.

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