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Mutually exclusive acetylation and ubiquitylation among enzymes involved in glucose metabolism

Sudharsana Rao Ande[1], G Pauline Padilla-Meier[1], and Suresh Mishra[1,2,*]

[1]Department of Internal Medicine; University of Manitoba; Winnipeg, MB Canada; [2]Department of Physiology; University of Manitoba; Winnipeg, MB Canada

The posttranslational modification (PTM) in protein occurs in a regiospecific manner. In addition, the most commonly occurring PTMs involve similar residues in proteins such as acetylation, ubiquitylation, methylation and sumoylation at the lysine residue and phosphorylation and *O*-GlcNAc modification at serine/threonine residues. Thus, the possibility of modification sites where two such PTMs may occur in a mutually exclusive manner (ME-PTM) is much higher than known. A recent surge in the identification and the mapping of the commonly occurring PTMs in proteins has revealed that this is indeed the case. However, in what way such ME-PTM sites are regulated and what could be their relevance in the coordinated network of protein function remains to be known. To gain such potential insights in a biological context, we analyzed two most prevalent PTMs on the lysine residue by acetylation and ubiquitylation along with the most abundant PTM in proteins by phosphorylation among enzymes involved in glucose metabolism, a fundamental process in biology. The analysis of the PTM data sets has revealed two important clues that may be intrinsically associated with their regulation and function. First, the most commonly occurring PTMs by phosphorylation, acetylation and ubiquitylation are widespread and clustered in most of the enzymes involved in glucose metabolism; and the prevalence of phosphorylation sites correlates with the number of acetylation and ubiquitylation sites including the ME-modification sites. Second, the prevalence of ME-acetylation/ubiquitylation sites is exceptionally high among enzymes involved in glucose metabolism and have distinct pattern among the subset of enzymes of glucose metabolism such as glycolysis, tricarboxylic acid (TCA) cycle, glycogen synthesis, and the irreversible steps of gluconeogenesis. We hypothesize that phosphorylation including tyrosine phosphorylation plays an important role in the regulation of ME-acetylation/ubiquitylation sites and their similar pattern among the subset of functionally related proteins allows their coordinated regulation in the normal physiology. Similarly their coordinated dysregulation may underlie the disease processes such as reprogrammed metabolism in cancer, obesity, type 2 diabetes, and cardiovascular diseases. Our hypothesis provides an opportunity to understand the regulation of ME-PTMs in proteins and their relevance at the network level and is open for experimental validation.

Keywords: posttranslational modification, phosphorylation, TCA cycle, glycolysis, glycogen synthesis, gluconeogenesis

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*Correspondence to: Suresh Mishra;
Email: mishra@cc.umanitoba.ca

The posttranslational modification (PTM) has a fundamental role in the regulation of protein function in diverse biological processes including cell signaling, transcription, and metabolism, and their dysregulation have been implicated in a number of prevalent diseases such as cancer, type 2 diabetes, and cardiovascular diseases.[1,2] The number of proteinogenic amino acids that are subjected to PTM are ~20 times fewer than various types of PTMs identified so far.[1] This would imply that the majority of the amino acids that undergo PTM are potential site for multiple modifications. Increasing evidence

suggests that this is indeed the case.[3,4] For example, more than ten different PTMs have been identified that occur on the lysine residue including abundantly occurring modification by ubiquitylation, acetylation, and methylation.[1,3] Similarly, serine, threonine, tyrosine, cysteine, arginine, and asparagine residues undergo multiple PTMs.[1,5] These PTMs are mutually exclusive and thus generate a great potential for cross-regulation. For example, serine-70 in CREB regulated transcription co-activator 2 (CRTC2) that undergoes phosphorylation and *O*-GlcNAc modification in a mutually exclusive manner.[6] The *O*-GlcNAc modification of CRTC2 at serine-70 has a role in the nuclear translocation and transcription of gluconeogeneic genes whereas phosphorylation at the same residue has been associated with cytoplasmic localization and proteasomal degradation of CRTC2.[6] As the PTM in protein occurs in a regiospecific manner this would indicate that the potential for modification sites where two **PTM**s may occur in a **m**utually **e**xclusive manner (ME-PTM) is greater than known. In addition, a number of PTMs are known to affect each other such as acetylation and phosphorylation, *O*-GlcNAc modification and phosphorylation, acetylation and ubiquitylation and so on.[3-5] Thus, the relationship between PTMs is far-reaching and involves modifications occurring at the same site in a mutually exclusive manner such as phosphorylation/*O*-GlcNAc modification, acetylation/ubiquitylation while in others these modifications may involve similar or dissimilar residues such as acetylation/ubiquitylation and acetylation/phosphorylation respectively (**Fig. 1**). For example, *O*-GlcNAc modification of a number of insulin signaling intermediates (e.g., insulin receptor, phosphoinositide-dependent kinase-1, insulin receptor substrate-1, glycogen synthase kinase-3β, etc.) has a role in the regulation of phosphorylation-dependent insulin signaling pathway.[7] However, their association in a biological context at the network level is not explored. Conventionally the PTM in proteins has been studied in a very limited manner in terms of throughput and various types of modifications mainly because of the lack of tools and technologies in the past. As a result, the scope to get insights at the network level was very limited in general. Recent advances in mass spectrometry and related technologies have created a surge in the large scale identification and mapping of a number of the commonly occurring PTMs in proteins in various biological and pathological contexts.[8-14] It is anticipated that systematic analyses of such unprecedented resources will reveal a number of indications concerning the regulation of protein function at the network level in various biological processes and how they are dysregulated in a disease process. This article is a small step in that direction. Here, we reveal one such novel insight that may have an important implication in the regulation of ME-acetylation/ubiquitylation sites in enzymes involved in glucose metabolism in the normal biology and in the altered metabolism in cancer, obesity, type 2 diabetes, and cardiovascular diseases.

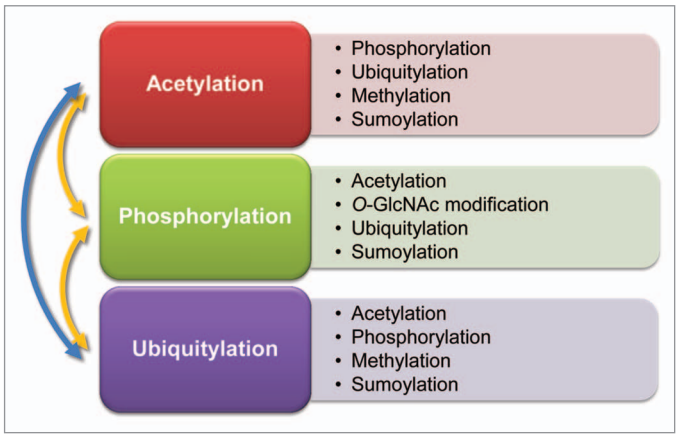

Figure 1. Schematic diagram showing the three most abundantly occurring PTMs in proteins (dark rectangles) provide a framework for the larger network of proteins as each one is known to influence multiple PTMs (light rectangles). Blue arrow, cross-regulation between PTMs occupying similar residues; golden arrow, cross-regulation between PTMs occupying dissimilar residues.

Phosphorylation, Ubiquitylation, and Acetylation Are Clustered Together Among Enzymes of Glucose Metabolism

To explore the possibility of ME-PTM sites in proteins at the network level we analyzed the known PTM sites in enzymes involved in glucose metabolism curated at the PhosphoSitePlus® (http://www.phosphosite.org).[15] The analysis of curated PTM data sets revealed that the prevalence of the three most commonly occurring PTMs in proteins are widespread among enzymes of glucose metabolism (see **Table S1**). Interestingly in majority of the enzymes these modifications are clustered together indicating an association among them (**Fig. 2**). Furthermore, in majority of the enzymes the number of phosphorylation sites present has an association with the acetylation and/or ubiquitylation sites (see **Table S1**). For example, in glucokinase with no acetylation and ubiquitylation site contains only three phosphorylation sites; whereas in hexokinase with 12 acetylation and ubiquitylation sites together, contains 13 phosphorylation sites (see **Table S1**). Similarly, in glucose-6-phosphatase with no acetylation or ubiquitylation site also lack phosphorylation site, whereas in glucose phosphate isomerase which contains 23 acetylation and ubiquitylation sites together also contain 14 phosphorylation sites (see **Table S1**). In addition, in a number of cases the known phosphorylation and acetylation/ubiquitylation sites are present within ±5 residues from each other (see **Table S1**). Most importantly, the acetylation and ubiquitylation site(s) are not identified independent of phosphorylation site(s) suggesting a close association between phosphorylation and acetylation/ubiquitylation in proteins. As phosphorylation, acetylation, and ubiquitylation are known to influence each other and regulate protein stability, function, and

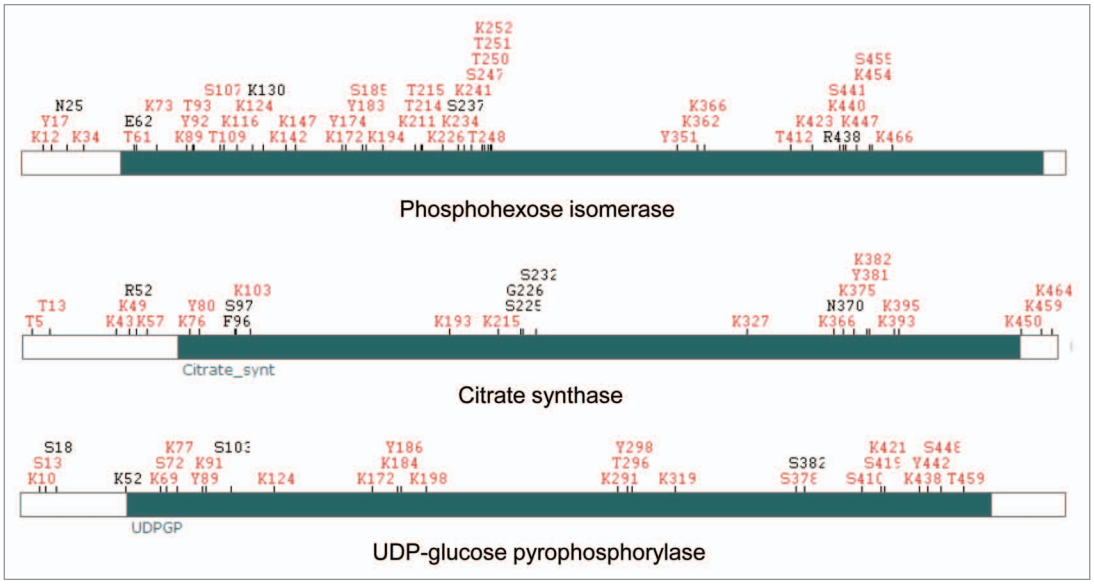

Figure 2. Representative diagrams showing the most commonly occurring PTMs are clustered among enzymes of glucose metabolism. The schematic diagram of phosphohexose isomerase, citrate synthase, and UDP-glucose pyrophosphorylase is shown as a representative of glycolysis, TCA cycle, and glycogen synthesis. Adapted from PhosphoSitePlus® (ref. 15) under Creative Common Licenses (http://creativecommons.org/licenses/by/2.5/).

translocation,[16] this would mean that these modifications have important roles in the regulation of enzymes involved in glucose metabolism. In contrast to acetylation and ubiquitylation, other commonly occurring PTMs on the lysine residue such as methylation and sumoylation appears to be relatively very rare or absent among enzymes involved in glucose metabolism as only one methylation site have been identified in triose-phosphate isomerase, pyruvate kinase, and citrate synthase, and 4 methylation sites have been mapped in lactate dehydrogenase (see **Table S1**). It is interesting to note that methylation in triose-phosphate isomerase and pyruvate kinase occurs at a ME-acetylation/ubiquitylation site confirming the notion of multiple modifications at the same residue in a mutually exclusive manner (see **Table S1**). However, the functional consequences of these modifications on a mutually exclusive site are not known. It is highly likely that the functional consequences of these modifications on a mutually exclusive site would be different and may regulate the enzyme activity either directly or indirectly through interacting partners. It would be interesting to know how multiple PTMs at the same site affect protein level, function, or distribution and how they are regulated.

ME-Acetylation/Ubiquitylation Sites Are Prevalent among Enzymes of Glucose Metabolism

Protein modification by acetylation and ubiquitylation that occur at the lysine residue are the two most abundant PTMs after phosphorylation. As the prevalence of other PTMs occurring at the similar residues (i.e., lysine, serine, threonine, and tyrosine) is relatively low, it may be presumed that the occurrence of ME-PTM sites involving acetylation and ubiquitylation would be the most abundant than any other two PTMs that can potentially occur in a mutually exclusive manner at a common residue. Furthermore, the PTM by phosphorylation being the most abundant PTM is best suited to have a role in the regulation of ME-PTM sites in proteins in general. Recently there have been an increase in the large scale identification and mapping of acetylation and ubiquitylation sites in proteins.[3,8,10,17] Furthermore, a number of common sites have been identified in a wide range of proteins that undergo both acetylation and ubiquitylation in a mutually exclusive manner.[3,17,18] The analysis of phosphorylation, ubiquitylation, and acetylation among enzymes of glucose metabolism revealed that the ME-acetylation/ubiquitylation sites are extensive among enzymes of glucose metabolism whereas other potential ME-PTMs such as on serine/threonine residues have not been identified so far among them (see **Table S1**). Most importantly, the ME-acetylation/ubiquitylation sites are distinctly distributed among the subset of enzymes of glucose metabolism indicating its importance in the regulation of enzyme function at the network level. For example, the prevalence of ubiquitylation sites in the first three enzymes of the glycolytic pathway that metabolize 6C-compounds is higher than acetylation sites (see **Table S1**). However, the percentage prevalence of ME-acetylation/ubiquitylation sites among acetylation sites is much higher (50–66%) than the percentage prevalence of ME-acetylation/ubiquitylation sites among ubiquitylation sites (12–16%). In contrast, the percentage prevalence of ME-acetylation/ubiquitylation sites in majority of the TCA cycle enzymes is higher (50–100%) among ubiquitylation sites than acetylation sites (15–42%). In addition, unlike glycolytic and TCA cycle enzymes which catabolize glucose, the ME-acetylation/ubiquitylation site is absent in enzymes involved in the anabolic pathway of glycogen synthesis (i.e., UDP-glucose pyrophosphorylase

and glycogen synthase) and in enzymes that catalyze irreversible reaction of gluconeogenesis (i.e., glucose-6-phosphatase and fructose biphosphatase). In fact, no ubiquitylation site has been reported so far in glucose-6-phosphatase and fructose biphosphatase whereas glucose-6-phosphatase also lack acetylation site (see **Table S1**).

As our knowledge of acetylation and ubiquitylation sites among various proteins will increase, the ME-acetylation/ubiquitylation sites are expected to increase accordingly. However, the underlying mechanisms that determine whether a lysine residue has to undergo acetylation, ubiquitylation, or other potential modification are not known. From a naïve point of view, the regulation of ME-PTMs at a common residue would require involvement of at least 5 proteins: a substrate and a pair of enzymes for the cycling of each dynamic modification involved. As PTMs have important roles in protein–protein interactions, one such possibility may involve modification status of the substrate protein itself or their interacting partners in a protein complex. As phosphorylation is the most abundant modification in proteins and the lack of ME-acetylation/ubiquitylation sites independent of phosphorylation sites (see **Table S1**) would suggest that phosphorylation may be a requisite for the ME-PTMs at a common site in proteins. Similar to acetylation and ubiquitylation, the crosstalk between serine/threonine phosphorylation and *O*-GlcNAc modification, and acetylation and methylation are other well-known examples of ME-PTM sites along with phosphorylation sites in the vicinity.[5,19,20] Furthermore, in our analysis of the most commonly occurring PTMs among enzymes involved in glucose metabolism we noticed that the occurrence of tyrosine phosphorylation in the cluster of the commonly occurring PTMs including ME-PTM sites is reasonably higher. Moreover, the percentage prevalence of tyrosine phosphorylation sites was found to be higher among enzymes of TCA cycle, thus more closely correlated with ME-acetylation/ubiquitylation sites among ubiquitylation sites (**Fig. 3**). A similar existence of tyrosine phosphorylation among proteins involved in phosphorylation/*O*-GlcNAc crosstalk have been reported.[5,19] Collectively, this would indicate a role for tyrosine phosphorylation in the regulation of interplay between PTMs including the ME-PTM sites in proteins.

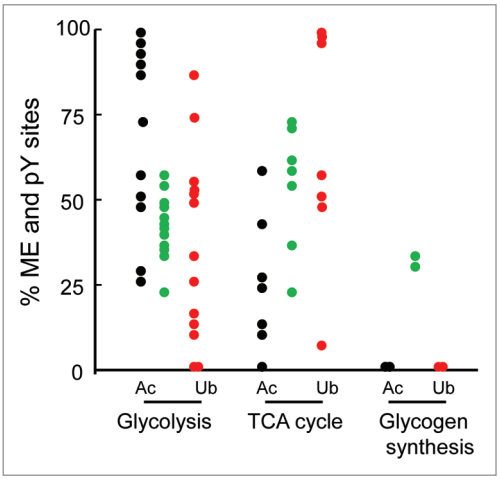

Figure 3. Scattered diagram showing the relationship between ME-acetylation/ubiquitylation sites and tyrosine phosphorylation (pY) sites among enzymes of glycolysis, TCA cycle, and glycogen synthesis. Enzymes that catalyze irreversible reaction of gluconeogenesis (i.e., glucose-6-phosphatase and fructose biphosphatase) are not included because no ubiquitylation site has been reported so far in glucose-6-phosphatase and fructose biphosphatase whereas glucose-6-phosphatase also lack acetylation site. Each dot represents one enzyme. Black dot, ME-acetylation/ubiquitylation sites among acetylation sites; red dot, ME-acetylation/ubiquitylation sites among ubiquitylation sites. The percentage of pY sites among total phosphorylation sites are shown with green dots. For further details see **Table S1**.

The Prevalence of Tyrosine Phosphorylation in Proteins with ME-Acetylation/Ubiquitylation Sites Is Exceptionally Higher

To further explore the possibility of the involvement of tyrosine phosphorylation in the regulation of ME-acetylation/ubiquitylation sites, we analyzed a recently reported large scale data set of ubiquitylated proteins.[3] In this study a total of 753 ubiquitylated peptides were identified belonging to 471 different proteins and out of 753 ubiquitylated peptides 152 were found to contain both ubiquitylation and acetylation sites.[3] A careful analysis of 152 ubiquitylated/acetylated peptides revealed that 130 (85%) of them have modification site(s) that undergoes ubiquitylation and acetylation in a mutually exclusive manner (**Fig. 4**). Interestingly, the prevalence of tyrosine phosphorylation among peptides having ME-acetylation/ubiquitylation sites (i.e., 130 out of 152) was found to be significantly higher (93%) than peptides with no ME site (i.e., 14 out of 22, 63%; **Fig. 4**) and even higher than ubiquitylated proteins with no acetylation reported (53% in a total of 601 ubiquitylated peptides).[3] Together, this would point toward an association of tyrosine phosphorylation in the interplay between acetylation and ubiquitylation in proteins. It is interesting to note that the prevalence of tyrosine phosphorylation among proteins with ME-acetylation/ubiquitylation sites was found to be even higher (93%) than its prevalence among serine/threonine phosphorylated/*O*-GlcNAc modified proteins (68%).[5] Collectively, this would suggest a more general role of tyrosine phosphorylation in the regulation of interplay between PTMs occupying similar residues including the ME-PTM sites.

Concluding Remarks

The prevalence of ME-acetylation/ubiquitylation sites is exceptionally high in a number of enzymes of glucose metabolism known to contribute to the altered metabolism in cancer cells. However, the functional status of ME-acetylation/ubiquitylation sites in the context of cancer metabolism is not known. Currently,

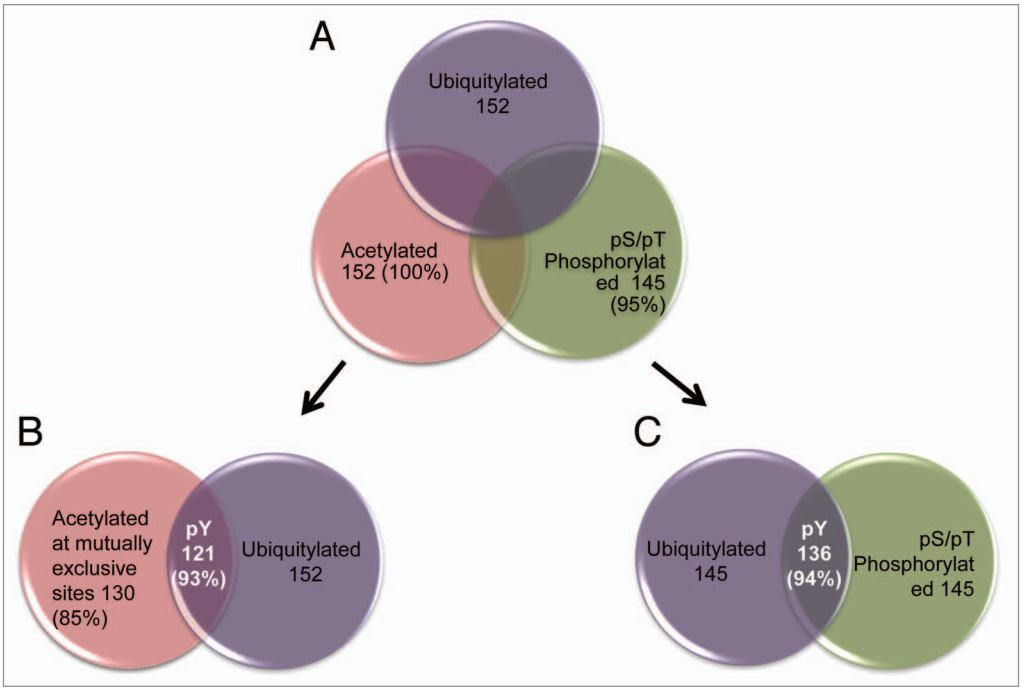

Figure 4. Venn diagrams showing acetylation and phosphorylation status of ubiquitylated proteins identified by Danielsen et al.[3] (**A**) The commonly occurring PTMs in proteins are clustered together, (**B**) high prevalence of tyrosine phosphorylation in a random set of proteins that undergo both acetylation and ubiquitylation (i.e., PTMs involving similar residues) in a mutually exclusive manner at the same site, and (**C**) high prevalence of tyrosine phosphorylation in a random set of proteins that undergo both acetylation and serine/threonine phosphorylation (i.e., PTMs involving dissimilar residues). pS, phosphorylated serine; pT, phosphorylated threonine; pY, phosphorylated tyrosine.

a number of small molecule inhibitors of acetylation cycling enzymes are at the various stages in the drug development as anti-cancer agents.[21,22] However, the underlying mechanisms involved in their action in cancer cells is not well understood. It is possible that their effect in part is mediated through altering acetylation/ubiquitylation or other associated PTMs among enzymes involved in glucose metabolism. Like ME-acetylation/ubiquitylation, other potential ME-PTMs may involve acetylation/methylation and methylation/ubiquitylation at the lysine residue, phosphorylation/*O*-GlcNAc modification at serine and threonine residues, phosphorylation/sulfation/nitration at the tyrosine residue, S-oxidation/acylation/prenylation/disulfide bond formation at cysteine residue, N-glycosylation/ADP-ribosylation at the asparagine residue etc. in various proteins. It would be interesting to know whether the incidence of phosphorylation including tyrosine phosphorylation among various combinations of ME-PTMs in proteins is similar to its association with acetylation/ubiquitylation and phosphorylation/*O*-GlcNAc modification. Protein modification by phosphorylation, ubiquitylation and acetylation—the three most abundantly occurring dynamic PTMs encompassing similar and dissimilar residues are intimately associated with each other and provide a framework for the larger network of proteins by integrating a number of other PTMs such as *O*-GlcNAc modification, methylation, acylation, and sumoylation (**Fig. 1**). It is anticipated that our hypothesis will stimulate studies of ME-PTM sites in the context of biological processes at the network level.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

SM wrote the manuscript and contributed in data interpretation, SRA and GPPM contributed in writing and data acquisition. All authors have read and approved the manuscript.

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Supplemental Materials

Supplemental materials may be found at: www.landesbioscience.com/journals/adipocyte/article/26070

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