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Probing into the role of conserved N-glycosylation sites in the Tyrosinase glycoprotein family

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Abstract N-linked glycosylation has a profound effect on the proper folding, oligomerization and stability of glycoproteins. These glycans impart many properties to proteins that may be important for their proper functioning, besides having a tendency to exert a chaperone-like effect on them. Certain glycosylation sites in a protein however, are more important than other sites for their function and stability. It has been observed that some N-glycosylation sites are conserved over families of glycoproteins over evolution, one such being the tyrosinase related protein family. The role of these conserved N-glycosylation sites in their trafficking, sorting, stability and activity has been examined here. By scrutinizing the different glycosylation sites on this family of glycoproteins it was inferred that different sites in the same family of polypeptides can perform distinct functions and conserved sites across the paralogues may perform diverse functions.

Keywords N-linked glycosylation · Tyrosinase · TRP-1 · TRP-2

Introduction

Post-translational modification of proteins is a common event in eukaryotic cells, of which, glycosylation is the most frequent [1]. The complex glycans may be attached to proteins by three distinct types of modifications; namely N-linked glycosylation of asparagines, O-linked glycosylation of serine or threonine and glycosylphosphatidylinositol derivatization of the carboxy-terminal end [2–4]. For a long time, the role of glycosylation of proteins was ambiguous. In the past two decades, significant development in the field of glycobiology has shed light on its importance. Glycosylation protects proteins from proteolytic degradation. Also, it is an efficient method to generate diversity as the glycans possess inherent structural variation. Of particular importance is modification by N-glycosylation. It is now known that N-linked carbohydrates play important roles in diverse biological processes such as protein folding and conformation, targeting of proteins to subcellular locations and extracellular sites (quality control), as well as cell–cell interactions [5–7]. Many studies show that they have a crucial role to play in cell-cycle progression and are essential for cell viability. Furthermore, many players of immune system, such as cytokines, antibodies and cellular receptors, are N-glycosylated [8–10].

Based on various biochemical, biophysical as well as genetic studies, it is currently acknowledged that N-glycosylation greatly influences the conformational dynamics of nascent polypeptide chains [11]. The increased efficiency of folding of glycosylated proteins may be a result of "chaperone-like" activity of glycans. Presence of glycans enhances the probability of achieving a correctly-folded conformation. Furthermore, covalently linked glycans also facilitate protein oligomerization by mediating the inter-subunit interactions and stabilizing the oligomer [12–18].

However, it is undetermined whether there exists any correlation among the sites of glycosylation and the subsequent effect in multiple-times glycosylated proteins; whether there is any pattern common to the conservation of glycans in a related family of glycoproteins.

Most families of proteins that undergo N-glycosylation have conserved set(s) of asparagine residues that can be

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putatively glycosylated. Are the roles of these glycans occurring at similar positions conserved over the family? We attempt to look into this question using the Tyrosinase-related protein (TRP) family, which includes three proteins, namely tyrosinase (the *albino* locus protein), TRP-1 (the *brown* locus product or gp75 or Tyrp1) and TRP-2 (the *slaty* locus product or DOPAchrome tautomerase or Tyrp2). They share a very high sequence identity (40–50%). All three proteins contain a signal peptide, a transmembrane domain and histidine- and cysteine- rich sequences. They have six to seven potential glycosylation sites containing a mix of both high-mannose type and complex glycans [19–21].

Role of proteins from the TRP family

The TRP family members are involved in the melanin producing pathways in melanosomes, present in epithelial cells. Tyrosinase is required for melanin synthesis through its action on tyrosine to form 3,4-dihydroxyphenylalanine and DOPAquinone. Post tyrosinase action, the melanin synthesis is divided into eumelanogenesis or pheomelanogenesis. The subsequent action of TRP-1 and TRP-2 determines the formation of eumelanin (Fig. 1). The

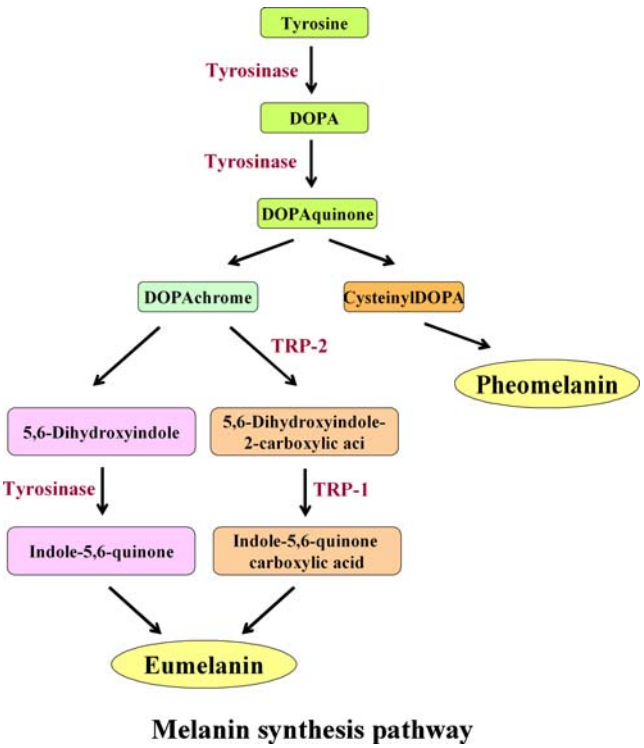

Fig. 1 The melanin synthesis pathway. The tyrosinase enzyme acts on tyrosine to initiate melanogenesis. Action of TRP-1 and -2 commit the pathway to Eumelanin formation

enzymes involved in formation of pheomelanin are undetermined so far [19, 22].

This family of genes has been conserved throughout vertebrate evolution. Many naturally occurring mutations in these genes lead to albinism in many species [23–25]. Similarly, the N-linked glycosylation sites in the tyrosinase family have been conserved throughout the vertebrate evolution. Taking the example of Asn350 site in mouse TRP-1, it is conserved in most members of TRP family, including the homologous proteins in birds and amphibians [20, 26]. Overall, members of this family share 40% amino acid sequence similarity and mammalian orthologues characteristically share more than 80% identity. However, they do harbor significant differences, such as distinct trafficking pathways in the cell and difference in glycosylation pattern (*i.e.* ratio of high-mannose to complex glycans) at individual glycosylation sites [27–29]. Study and characterization of potential N-glycosylation sites in members of this family nonetheless, gives insight into conservation and divergence of glycosylation with respect to function.

Analysis of glycans on TRP family

There have been studies carried out on mouse tyrosinase and TRP-1 and human tyrosinase, where putative glycosylation sites in these proteins were removed sequentially and the ensuing effect on activity and/or fate of the protein was determined [28, 30, 31]. However, direct investigation of glycosylation sites of TRP-2 has not been performed.

From a sequence alignment of human and mouse tyrosinase and TRP-1, it is observed that most of the Asn are conserved (Fig. 2). Those asparagines that formed a part of the sequence motif (Asn-X-Ser/Thr) are the putative glycosylation sites. Table 1 lists all the conserved Asn residues in TRP-1 and tyrosinase. The residues marked in bold are conserved and have glycans attached to them. The other Asn residues are conserved, but are not a part of the glycosylation motif. Asn 96 in TRP-1 and the corresponding Asn 86 in tyrosinase, are the first putative sites and are glycosylated both in mouse TRP-1 and tyrosinase. But their absence has no critical effect on the respective proteins. Asn 304 (TRP-1) is important for activity; its loss results in ER retention of the protein, but the corresponding Asn 290 in mouse tyrosinase is absent. This Asn is conserved in Human tyrosinase but its role has not yet been studied. Similarly Asn350 (TRP-1) and Asn337 (Tyrosinase), which are another set of conserved asparagines have different effects. In mouse TRP-1 and human tyrosinase, the protein trafficking and stability are affected when the glycan is removed, but there is no effect on the activity of mouse tyrosinase in the absence of the glycan. Other pairs of conserved Asn, show more similarity in function. In Asn104/94 pair, the Asn 104 in

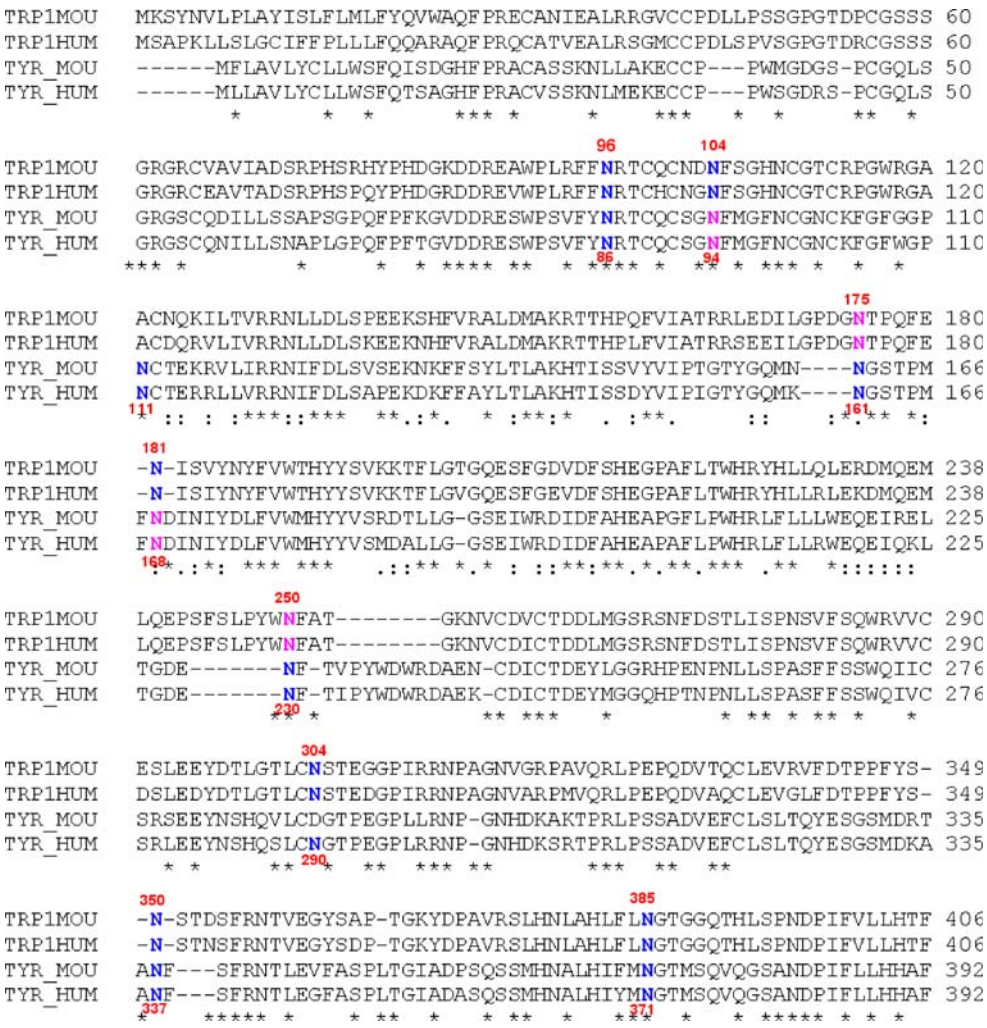

Fig. 2 Alignment of TRP-1 (mouse and human) with tyrosinase (Mouse and Human) sequences to highlight the conserved asparagines residues. The residues marked in blue are conserved and are a part of the glycosylation motif (Asn-X-Ser/Thr). The residues marked in *red* are conserved, but do not form a part of the glycosylation motif

TRP-1 of mouse is glycosylated in the wild-type protein, but there is no effect in the protein processing and maturation in its absence. The corresponding Asn 94 in tyrosinase does not have the glycosylation motif. Asn 111, a putative glycosylation site lacking a glycan in mouse tyrosinase does not have a counter part in the TRP-1 proteins. A glycan is absent in the putative site Asn 161 in mouse tyrosinase, and the corresponding Asn 175 in TRP-1 does not have the glycosylation motif. The absence of Asn 181 in Mouse TRP-1 does not affect the protein activity and its corresponding Asn 168 does not have a glycosylation motif. Other pairs of conserved Asn residues show similar trends. Asn with glycans missing or when glycans of Asn do not show any apparent activity in TRP-1/Tyrosinase, usually correspond to similar Asn residues with glycans missing or apparently not active or which do not have a corresponding conserved Asn in the other protein also. The last glycosylation site in TRP-1/Tyrosinase (Asn385/371) is conserved in both proteins and the corresponding glycans are important for their function.

From the above mentioned observations (also listed in Table 1) it can be deduced that there are two N-glycosylation sites that have not been well conserved in vertebrates, namely at Asn 181 and Asn 304 in TRP-1. Nonetheless these glycans play important roles with the N-glycan at Asn 181 affecting the glycoprotein transport through Golgi complex and the N-glycan at Asn 304 being essential for processing by Golgi complex [30, 31].

Moving on to the conserved glycans, there are three most conserved glycans in the TRP family. They are the N-glycans at sites corresponding to Asn 96, Asn 350 and Asn 385 of Mouse TRP-1. Asn 385 of TRP-1 is critical for proper processing and maturation in endoplasmic reticulum (ER). Its loss affects early trafficking in the ER. However, its counterpart in tyrosinase *i.e.* Asn 371 appears to play a role in processing events outside the ER. Nevertheless, the glycans at Asn 337 in tyrosinase seem to participate in functions similar to that of Asn385. The corresponding N-glycan for this Asn 337 at Asn 350 of TRP-1 on the other hand is a major determinant of the glycoprotein

Table 1 Comparative roles of the conserved asparagine residues in TRP-1 and tyrosinase (mouse/human): effect of removal of glycosylation sites

Putative glycosylation sites on human/mouse TRP-1	Human (TRP-1)	Mouse (TRP-1)	Human (tyrosinase)	Mouse (tyrosinase)	Putative glycosylation sites on human/mouse tyrosinase
Asn96	No studies done	No effect	Not studied	Lowered activity of protein	Asn86
Asn104		No effect	Not studied	No glycosylation motif	Asn94
			Not studied	No glycan present	Asn111
Asn175		No glycosylation motif	Not studied	No glycan present	Asn161
Asn181		May effect transport through Golgi	Not studied	No glycosylation motif	Asn168
			Not studied	No effect	Asn230
Asn304		ER retention	Not studied	Absent	Asn290
Asn350		Lowered stability, increased susceptibility to proteolytic digestion; but glycan processing occurs normally	ER retention	No effect	Asn337
Asn385		Early trafficking affected; no maturation to complex glycosylated form	Lowered activity of protein	Lowered Activity of protein	Asn371

stability in the endocytic pathway. Asn 96, though well conserved, has not shown any specific effect on stability or function or trafficking till date [30, 31].

Comparison of sequence alignment shows that glycan binding sites most crucial to the TRP family activity, transport and stability are also conserved in TRP-2. However, the potential functions of these sites in TRP-2 remain to be determined. A conserved domain analysis between the three proteins shows that as in tyrosinase and TRP-1, TRP-2 also has only one major domain, the tyrosinase domain, which constitutes the majority of its sequence (Fig. 3).

Concluding remarks

Members of TRP family are glycoproteins with N-linked oligosaccharides. Their polypeptide chains have conserved potential N-glycosylation sites of which three are the most conserved. While the sites themselves may be conserved, the potential role of each site in maturation, trafficking, stability and function of the glycoprotein varies. We may conclude from the TRP family of glycoproteins that conserved N-glycosylation sites across species can participate in discrete roles during protein sorting, maturation, stability and function and that the role of each individual

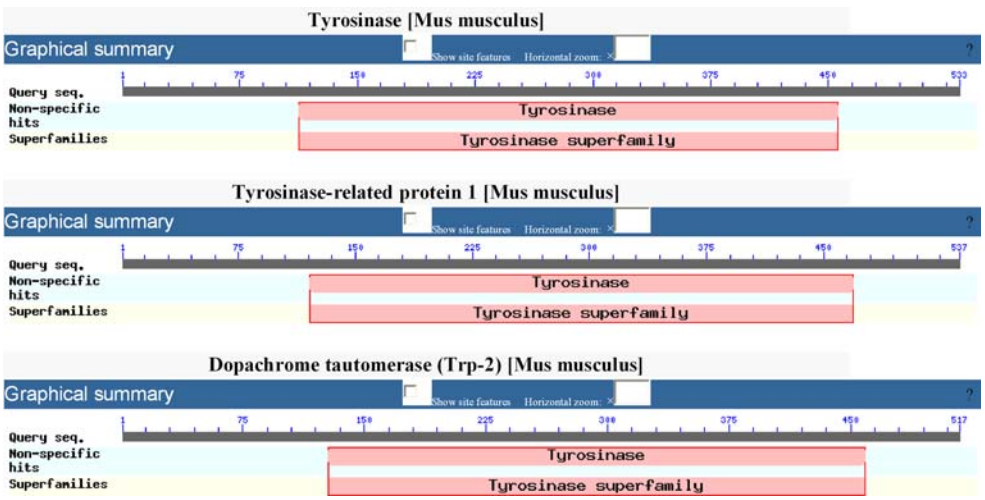

Fig. 3 Conserved domain search for tyrosinase, TRP-1 and TRP-2 from *Mus musculus* carried out by the NCBI server. The graphical summary shows that there exists one major domain in all the proteins, the tyrosinase domain, which constitutes majority of the protein sequence

site may not be retained during evolutionary process. The individual conserved sites on TRP family paralogues can perform different functions.

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