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Charge Distribution of Flanking Amino Acids Influences *O*-Glycan Acquisition *in Vivo**

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The elements that regulate *O*-glycosylation are poorly understood. We have developed a novel *in vivo* system to analyze the role of flanking sequence on the modification of a single well characterized *O*-glycosylation site derived from human von Willebrand factor (PHMAQVTVGPGL). A secreted chimeric reporter protein, containing the human von Willebrand factor sequence, an antibody recognition epitope, and a heart muscle kinase site, was engineered and expressed in COS7 and MCF-7 cells. Glycosylated and non-glycosylated forms of the immunoprecipitated reporter were resolved electrophoretically and their relative amounts quantitated. Using mutational analysis we find that the glycosylation apparatus of COS7 cells can accommodate a broad range of changes in the flanking sequence without compromising glycosylation, but that the distribution of charged amino acids flanking the *O*-glycosylation site can have a profound influence on glycosylation with position −1 relative to the glycosylation site being particularly sensitive. A combination of acidic residues at positions −1 and +3 almost completely eliminates glycosylation of the reporter in both COS7 and MCF-7 cells. The overall density of charged amino acids is less important since substitution of acidic residues at position −2, +1, and +2 had no effect in the level of glycosylation observed.

The acquisition of carbohydrate side chains in *O*-glycosidic linkage to either Thr or Ser has a profound structural impact on a polypeptide backbone and thus underlies many unique physicochemical properties of heavily *O*-glycosylated molecules such as mucin-glycoproteins (1). In addition, *O*-glycans function as ligands for receptors modulating such diverse actions as lymphocyte trafficking (2), sperm-egg binding (3), and tumor cell adhesion (4). The first committed step of *O*-glycosylation is catalyzed by UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase (ppGaNTase).[1]

Since all hydroxyamino acids are not *O*-glycosylated, signals must exist to specify which Ser and Thr residues acquire *O*-glycans. From surveys of known *O*-glycosylation sites, it is apparent that charged residues are rarely found at positions flanking glycosylated Thr or Ser, while Pro and clusters of Ser and Thr often flank glycosylated residues (5–7). A large number of synthetic substrates have been examined which confirm that the rate of GalNAc addition is sensitive to the sequence surrounding Ser or Thr *in vitro* (7–10). However, despite intensive efforts, no consensus sequence has emerged (11, 12). This is due, in part, to the broad range of residues that the binding site of ppGaNTase can accommodate (7, 8, 13) and to the existence of multiple isoforms of ppGaNTase, which may have overlapping specificities (8, 9, 14, 15).

Recent methodological advances in the quantitation of carbohydrate present on a single Ser or Thr during solid phase protein sequencing have provided an assessment of the occupancy level of specific *O*-glycosylation sites of native proteins (11, 16, 17). It is not known whether the observed site heterogeneity reflects inter-animal variation or whether some sites are indeed variably occupied. Another limitation of this form of analysis is that it does not lend itself to the study of mutated forms of the glycoprotein.

In the present study we have examined a small chimeric reporter protein to determine the role played by flanking amino acids on *in vivo O*-glycosylation.

EXPERIMENTAL PROCEDURES

Construction of Vector Encoding Chimeric Reporter—The reporter protein (rHVF) contains the amino acid sequence PHMAQVTVGPGL, which is a known glycosylation site of human von Willebrand factor and which is an *in vitro* substrate for ppGaNTase (8). This sequence was linked to the insulin secretory signal (which is cleaved prior to entry into the Golgi apparatus), a Ni^{2+} binding site and the FLAG® octapeptide epitope (IBI) (to facilitate purification), and a bovine heart muscle kinase site to aid detection of the reporter protein (Fig. 1).

All molecular biological manipulations were carried out essentially as described (18). The expression vector pKN4, which encodes the chimeric reporter, was created as follows. Oligonucleotides KN4.1 (5′-CCCACCCGAGCCTTCGTTAACCCACATATGGCTCAAGTTACTGTGGGC-3′) and KN4.2 (5′-CCGGAATTCCCAATGATGCATACAGGAGGCCTGGGCCCACAGTAA CTTGAGCC-3′) were annealed and extended. The reaction products were cut with *Ava*I and *Eco*RI and cloned into the vector pGIR199, which contains the coding region for the insulin secretory signal and was a generous gift of K. Drickamer (19). The product vector (pKN1) was then cut with *Nsi*I and *Eco*RI and the extended products of oligonucleotides KN4.3 (5′-GTTATGCATCATTGGCATCACCACGCAAGAAGAGCATCTCACGACGTGCATGACTACAAAGACGATG-3′) and KN4.4 (5′-GGCGAATTCGTTGTAAAACGACTCATTTATCGTCATCGTCTTTGTAGTCATGC-3′) were cut and cloned into these sites, generating pKN3. pKN3 was then cut with *Nhe*I, filled with Klenow, ligated to *Eco*RI linkers, cut with *Eco*RI, and the linker-ligated fragment containing the reporter construct was cloned into the *Eco*RI site of the eukaryotic expression vector pcDL-SRα296 (Ref. 20; DNAX Research Institute of Molecular and Cellular Biology, Inc.) to create pKN4.

The *Pst*I-*Kpn*I fragment of pKN4 was cloned into PhagescriptSK m13 (Stratagene) to create m13SKN4. The dut⁻ung⁻strain RZ1032 was

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[1] The abbreviations used are: ppGaNTase, UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase; rHVF, recombinant human von Willebrand factor reporter protein; MOPS, 4-morpholinepropanesulfonic acid; Tricine, N-[2-hydroxy-1,1,bis(hydroxymethyl)ethyl]glycine.

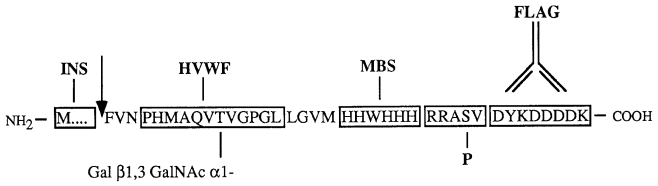

FIG. 1. **Schematic and amino acid sequence of the human von Willebrand factor chimeric reporter protein rHVF.** The pro-chimeric protein contains the insulin secretory signal (*INS*) fused to a 12-amino acid domain from the human von Willebrand factor (rHVF) containing a single, threonine glycosylation site (Galβ1,3GalNAcα1), followed in turn by a metal binding site (*MBS*), a bovine heart muscle kinase site (*P*), and the FLAG® antibody recognition octapeptide. The secreted form of the protein is cleaved immediately prior to the Phe residue shown to the *right* of the *arrow*.

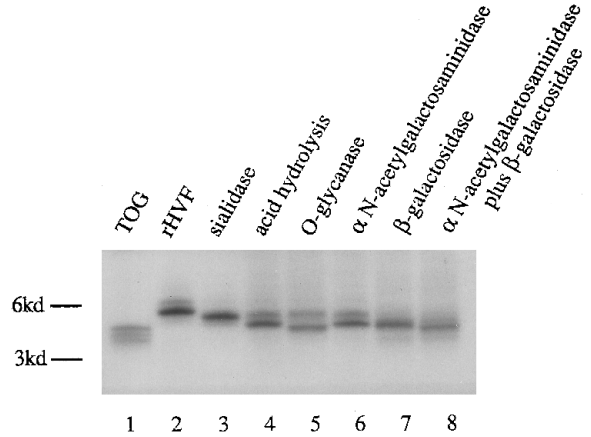

FIG. 2. **The chimeric reporter protein rHVF is *O*-glycosylated normally during secretion from COS7 cells.** Sialidase-digested material migrated more slowly than did acid-hydrolyzed rHVF (*lane 4*) due to incomplete enzymatic digestion, which may have been caused by the acetylation of a sialic acid residue or by a linkage to Gal or GalNAc that is a poor substrate. A labeled, purified mutant reporter protein containing a Thr → Gly substitution (*lane 1*) migrated similarly to the fully deglycosylated wild-type rHVF (*lanes 5* and *8*). In a mock transfection, no product the size of either the wild-type rHVF or the Thr → Gly mutant was observed (data not shown).

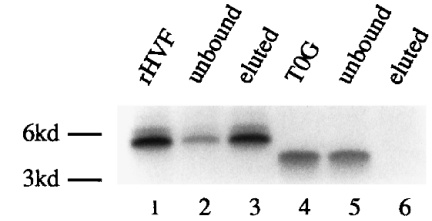

FIG. 3. **Wild-type rHVF (*lane 1*), but not a Thr → Gly mutant protein (*lane 4*), is recognized by the lectin jacalin.** Most rHVF is bound to a jacalin-affinity matrix and recovered with desorption with D-galactose (*lane 3*). A small quantity of rHVF failed to bind to jacalin matrix (*lane 2*). In contrast, all of the Thr → Gly mutant fails to bind to jacalin (*lane 5*).

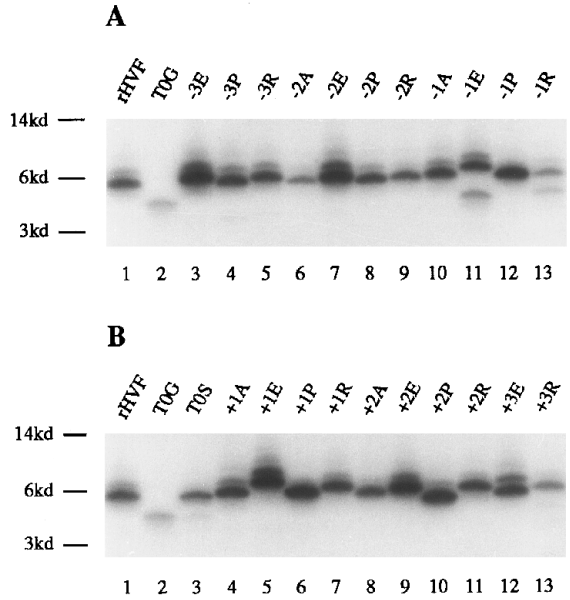

FIG. 4. ***O*-Glycosylation of the rHVF is insensitive to most changes in the sequence flanking the glycan linkage site.** *A* and *B*, site-directed mutagenesis was used to introduce single amino acid changes in the sequence from −3 to +3 flanking the Thr in the rHVF. Each amino acid was replaced by either Ala, Glu, Pro, or Arg (except the +3 position, where the Ala change was not obtained). In addition, Thr was mutated to both Gly and Ser. The occupancy of each mutated glycosylation site was then examined following secretion from COS7 cells (17). All of the reporter proteins shown, except the Thr → Gly mutant, consisted mainly of glycosylated protein and exhibited a distinct shift in mobility following mild acid hydrolysis (data not shown). The overall level of glycosylation was most sensitive to substitutions of a charged residue at the −1 position and, to a lesser extent, to substitution of Arg at the +3 position or Ser for Thr, as indicated by the lower, less intense bands in those samples. The labeling intensities in different lanes do not necessarily reflect the relative abundance of each mutant protein, due to the low concentration of ATP in the labeling reactions and to experimental deviation in the amount of material recovered from separate transfections (17). The values determined for the fraction of each mutant protein glycosylated are shown in Table I.

used to produce single-stranded uracil-containing m13SKN4 DNA for site-directed mutagenesis. Oligonucleotides for each of the single amino acid substitutions were degenerate and coded for either alanine and glutamic acid or proline and arginine. The oligonucleotide 5′-GGAA-GATACTGTTGACGGGAAACG-3′ is complementary to nucleotides 3465–3489 of pcDL-SRα296 and was used to screen by dideoxy sequencing for the desired mutants in m13SKN4, the *Pst*I-*Kpn*I fragments of which were then recloned into pcDL-SRα296. An infrared dye-labeled m13 −29 primer (LiCor) was used for confirming the sequence of double-stranded pKN4 mutant DNA on a LiCor automated sequencer.

Cell Culture and Transfections—COS7 cells and MCF-7 (generously provided by R. Hilf) were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2. Cells were split every 3–4 days. Transfections were performed on cells at approximately 70% confluence using DNA prepared with Promega's "midi" prep kit and Lipofectamine reagent (Life Technologies, Inc.) according to the manufacturer's protocol. At 18 h post-transfection, fresh medium was added and then subsequently harvested 72 h post-transfection. The medium was centrifuged (13,000 × *g*; 10 min) prior to immunoprecipitation. In some experiments, following transfection, cells were cultured at 23 °C for periods of 18–72 h.

Immunoprecipitation, Labeling, and Protein Analysis—To precipitate the reporter protein, 40 μl of 25% anti-FLAG agarose affinity gel (IBI) was added directly to 0.5 ml of cell culture medium. The mixture was rocked at 4 °C overnight, pelleted, washed three times in Buffer A, (30 mM MOPS, pH 7.0, 100 mM NaCl, and 0.1% Tween 20), and then resuspended in 30 μl of 20 mM HEPES, pH 7.0, 75 mM NaCl, and 15 mM $MgCl_2$ containing 5 μCi of [γ-^{32}P]ATP (DuPont NEN) and 5 units of bovine heart muscle kinase (Sigma). Following incubation at 30 °C for 1 h, the beads were washed three times in buffer A and brought to a final volume of 15 μl in 50 mM Tris, pH 7.0, 12% glycerol, 4% SDS, 2% β-mercaptoethanol, and 0.01% Coomassie G-250. A sample (3 μl) was run on a 16.5% T, 6% C resolving phase Tricine SDS-polyacrylamide gel electrophoresis mini-gel containing both stacking and separating phases as described (21). The gels were dried, and reporter protein visualized by autoradiography or with a Molecular Dynamics PhosphorImager.

For the pulse-chase analysis, COS7 cells transfected with either wild-type or mutant expression vector, were pulsed 18 h post transfection for 10 min with 200 μCi/ml [^{35}S]Met in 0.5 ml of Met-free Dulbecco's modified Eagle's medium, washed twice with phosphate-buffered saline, then incubated in 1 ml of complete Dulbecco's modified Eagle's medium containing 5% fetal bovine serum at 37 °C for periods ranging from 0 to 2 h. The cells were then washed twice with ice-cold phosphate-buffered saline and lysed (1% Tween 20, 0.1% SDS, 50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) in

TABLE I

A comparison of the occupancy in vivo versus the rate of GalNAc transfer in vitro for 36 rHVF-derived reporter constructs

The values given in parentheses are standard deviations taken from at least three separate experiments.

	Reporter designation	Peptide sequence							Glycosylation *in vivo* (%)		GalNAc incorporation *in vitro* (relative to rHVF)[a]
									37 °C	23 °C	
1	**rHVT**	**Ala**	**Gln**	**Val**	**Thr**	**Val**	**Gly**	**Pro**	**>95**	**>95**	**100**
2	−3E	Glu			Thr				>95	ND	4
3	−3P	Pro			Thr				>95	ND	6
4	−3R	Arg			Thr				>95	ND	3
5	−2A		Ala		Thr				>95	ND	13
6	−2E		Glu		Thr				>95	ND	ND
7	−2P		Pro		Thr				>95	ND	11
8	−2R		Arg		Thr				>95	ND	8
9	−1A			Ala	Thr				>95	ND	61
10	−1E			Glu	Thr				76 (±3)	>95	15
11	−1P			Pro	Thr				>95	ND	173
12	−1R			Arg	Thr				63 (±6)	>95	6
13	TOG				Gly				0	0	0
14	TOS				Ser				83 (±6)	>95	ND
15	+1A				Thr	Ala			>95	ND	83
16	+1E				Thr	Glu			>95	ND	162
17	+1P				Thr	Pro			>95	ND	362
18	+1R				Thr	Arg			>95	ND	44
19	+2A				Thr		Ala		>95	ND	502
20	+2E				Thr		Glu		>95	ND	171
21	+2P				Thr		Pro		>95	ND	344
22	+2R				Thr		Arg		>95	ND	253
23	+3A				Thr			Ala	ND	ND	8
24	+3E				Thr			Glu	>95	ND	8
25	+3R				Thr			Arg	83 (±6)	ND	7
26	−3,−1E	Glu		Glu	Thr				>95	>95	ND
27	−2,−1E			Glu	Thr				74 (±6)	88 (±1)	ND
28	−1,+1E			Glu	Thr	Glu			51 (±3)	77 (±1)	ND
29	−1,+2E			Glu	Thr		Glu		63 (±3)	84 (±1)	ND
30	−1,+3E			Glu	Thr			Glu	11 (±1)	42 (±1)	ND
31	−3,−2E	Glu	Glu		Thr				>95	ND	ND
32	−2,+1E		Glu		Thr	Glu			>95	ND	ND
33	−2,+2E		Glu		Thr		Glu		>95	ND	ND
34	−2,+3E		Glu		Thr			Glu	>95	ND	ND
35	−2,+1,+2E		Glu		Thr	Glu	Glu		>95	ND	ND
36	−1,+3R			Arg	Thr			Arg	42 (±6)	ND	ND

[a] Taken from Ref. 8.

0.5 ml. The lysates were spun at 14,000 × *g* for 10 min, diluted 2-fold, and the reporter immunoprecipitated from the cleared lysate as described above.

Analysis of O-Glycans—To characterize acquired glycans, immunopurified and labeled rHVF was treated with sialidase while bound to FLAG affinity matrix (Fig. 2, *lane 3*; 2 units/ml) or, alternatively, COS7 culture medium containing secreted rHVF was hydrolyzed in mild acid to remove sialic acid residues prior to neutralization, immunoprecipitation, and labeling (*lane 4*; 0.1 N H_2SO_4 for 1 h at 80 °C, then equinormal NaOH). Acid-hydrolyzed material was treated with *O*-glycanase (*lane 5*), α-*N*-acetylgalactosaminidase (*lane 6*), β-galactosidase (*lane 7*), or a combination of α-*N*-acetylgalactosaminidase and β-galactosidase (*lane 8*).

As an additional test for the presence of *O*-glycans, COS7 culture media containing the wild-type rHVF or Thr → Gly mutant proteins were ultrafiltered through a Centricon-30 column, and 100 μl of jacalin-agarose, which recognizes oligosaccharides with the core structure Galβ1,3GalNAc in an α anomeric linkage to Ser or Thr (22). Material failing to bind to the jacalin-agarose was recovered by immunoprecipitation, while the bound species were desorbed by elution with 0.1 M D-galactose, then immunoprecipitated and analyzed as described above.

RESULTS AND DISCUSSION

Glycosylation of a Chimeric Reporter Protein in COS7 Cells—Wild-type rHVF was *O*-glycosylated when expressed from COS7 cells acquiring a common *O*-glycan structure consisting of sialic acid residues attached to a Galβ1,3GalNAc core. rHVF migrated as a single species with a relative molecular mass of approximately 6,000 Da (Fig. 2, *lane 2*). Ablation of the potential *O*-glycosylation site by mutation of the single Thr → Gly reduced the apparent mass of the reporter by approximately 1,500 Da (Fig. 2, *lane 1*). Patterns obtained by glycosidase digestion following mild acid hydrolysis were consistent with the presence of a sialidated Galβ1,3GalNAc core oligosaccharide structure; specifically, digestion with *O*-glycanase or a combination of α-*N*-acetylgalatosaminidase and β-galactosidase caused the deglycosylated species to co-migrate with the Thr → Gly mutant rHVF (Fig. 2, *lanes 5* and *8*). Fractionation on jacalin-agarose, a lectin that interacts with sialated Galβ1,3GalNAc structures (22), resulted in the binding of greater than 70% of rHVF, which could be nearly completely desorbed with D-galactose (Fig. 3).

Substitution of Ser for Thr at the Glycan Acquisition Site—Ser acquires oligosaccharide to similar levels as Thr within the rHVF flanking sequence context *in vivo* (Fig. 4*B*, *lanes 1* and *3*). This is in contrast to *in vitro* glycosylation reactions in which Ser-containing substrates glycosylated 35–60-fold slower than Thr-containing analogues (9, 23, 24). Since there are an equal number of Ser and Thr *O*-glycosylation sites found in nature (5–7, 12), it has been argued that these *in vitro* differences are biologically insignificant (6). Our observation that both Ser and Thr are glycosylated to high levels *in vivo* strengthens this argument, but does not preclude a role for unique sequences surrounding Ser in facilitating enzyme recognition, as has been suggested by recent *in vitro* studies (10). However, we have recently demonstrated that a peptide in which Thr was replaced with Gly could inhibit polypeptide GalNAc transferase with a K_i that approximates the K_m for the Thr-containing

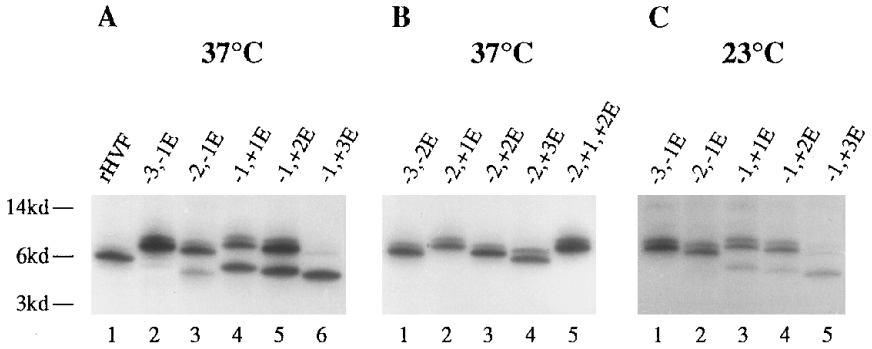

FIG. 5. **The influence of charge distribution, charge density, and the rate of secretion on *O*-glycosylation *in vivo*.** *A*, changes in the −1 position to Glu were combined with a scanning Glu mutation at all other positions from −3 to +3 flanking Thr, and the proteins were expressed in COS7 cells and analyzed (17). The +3 position was the most sensitive to change in the single-site mutant background, while, in contrast, changing the −3 position to Glu restored a wild-type level of glycosylation. *B*, changes in the −2 position to Glu were combined with a scanning Glu mutation, then expressed and analyzed (17). All of these double mutants were glycosylated normally, as was a triple charge mutant comprising three of the four residues immediately surrounding Thr. *C*, COS7 cells grown at 23 °C produced more fully glycosylated mutant rHVF relative to cells grown at 37 °C.

substrate (25). Thus, it appears that a hydroxyamino acid, *per se*, is not important for peptide recognition by the glycosylation apparatus.

Mutagenesis of the Flanking Amino Acids around the Glycosylation Site—The flanking sequence requirements for *O*-glycosylation *in vivo* are much less restricted than suggested by *in vitro* studies. Previously, we examined the *in vitro* glycosylation of a set of 52 peptides derived from the human von Willebrand factor sequence present in rHVF (8). Substitution of Glu, Arg, Ala, or Ile at positions +3, −3, and −2 markedly decreased the incorporation rate of GalNAc into the substrate. In addition, the presence of a charged residue at position −1 relative to the glycosylation site inhibited glycosylation *in vitro* (Table I). To examine the flanking sequence requirements for *O*-glycosylation *in vivo*, a series of single amino acid changes to Ala, Glu, Pro, or Arg were made from positions −3 to +3 relative to the Thr at the *O*-glycosylation site of the rHVF. Minor variations in mobility resulted from the amino acid changes, but in contrast to the results obtained *in vitro*, most of these mutations had little or no effect on the level of glycosylation *in vivo* (Fig. 4, *A* and *B*; Table I). However, when the −1 position was mutated to either Glu or Arg, there was a 24–37% decrease in site occupancy, which is in qualitative agreement with the *in vitro* data (Table I). A combination of glycosidase digestions and lectin fractionation confirmed that the lower molecular weight species in these samples represented non-glycosylated material, while pulse-chase experiments demonstrated that the glycosylated and non-glycosylated forms of rHVF disappeared from the intracellular pool of proteins at the same rate and were stable in cell culture media for at least 3 days (data not shown).

Charge Distribution Is More Important Than Charge Density in Influencing Glycosylation—Our results indicate that multiple charged residues at specific positions adversely affect *O*-glycosylation. To explore the importance of flanking charged groups, the −1 position-to-Glu mutant was combined with a Glu scanning mutation of all other flanking positions, in turn, from −3 to +3. The glycosylation of most of these double mutants was further compromised beyond that of the single mutant, and ranged from 74 to 11% occupancy (Fig. 5*A*, *lanes 3–6*; Table I). A combination of Glu at both the −1 and −3 positions, however, restored glycosylation to wild-type levels, indicating that charged residues may both positively and negatively modulate *O*-glycosylation in a position-dependent fashion (Fig. 5*A*, *lane 2*). To distinguish between a generalized charge effect (charge density) and a position-specific charge effect (charge distribution) on *O*-glycosylation, a similar set of

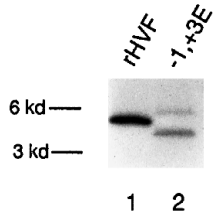

FIG. 6. **The human mammary carcinoma cell line from MCF-7 (29) was transfected with plasmid DNA encoding the wild-type rHVF (*lane 1*) or the −1/+3E mutant (*lane 2*).** The percent occupancy was >95% for the wild-type and 22% for the mutant protein.

double charge mutations was introduced in the flanking positions of the single mutant containing Glu at the −2 position. These double mutant forms of the reporter protein were all glycosylated to wild-type levels, except for the previously described double mutant containing Glu at the −1 and −2 flanking positions (Fig. 5*B*, *lanes 1–4*). Thus, charge distribution appears to be more important than charge density. To confirm this, a triple mutant was made where the −2, +1, and +2 positions were simultaneously changed to Glu; it was also glycosylated to the same level as the wild-type reporter (Fig. 5*B*, *lane 5*). Both positively and negatively charged residues are likely to display this positional effect on glycosylation, as the substitution of Arg at positions −1 or +3 (but at no other positions) resulted in slightly decreased levels of glycosylation; furthermore, the mutant with a double substitution of Arg at both positions −1 and +3 (corresponding to the most deleterious Glu double mutant) was glycosylated to 42% occupancy, lower than that of either single mutant (Table I).

To demonstrate that the effect of charged residues at positions −1 and +3 on *O*-glycosylation is not unique to the COS7 cell background, we compared the glycosylation of rHVF with the −1/+3E mutant in a human mammary carcinoma cell line, MCF-7. The occupancy of the wild-type glycosylation site was >95%, whereas the level for the −1/+3 mutant was 22% (Fig. 6).

The Occupancy Level of an O-Glycosylation Site Is Modulated by Temperature—When transfected cells were maintained at 23 °C, single-site mutants at the −1 position were all restored to wild-type levels (Table I). Moreover, the double mutants were glycosylated with higher efficiency, though still reduced compared to wild-type levels (Fig. 5*C*). One consequence of culturing cells at 23 °C is to increase the transit time of secreted proteins through the cell (26). Increased exposure to the Golgi apparatus could lead to enhanced levels of glycosylation;

abnormally long polylactosamine units are added to lamp glycoproteins secreted by Chinese hamster ovary cells maintained at 23 °C (26). Since glycoproteins traffic through cells at a different rate (27), this may represent a potential control point of glycosylation regulation. Alternatively, culture at 23 °C may induce expression of a new ppGaNTase activity since enhanced ppGaNTase activity is observed during thermotolerance development in Chinese hamster ovary cells (28).

Our findings indicate that although a wide range of flanking sequences are accommodated by the ppGaNTase, charged residues at specific positions severely impair *O*-glycosylation of single acceptor sites *in vivo* in at least two different cell backgrounds. Moreover, current studies with a diverse spectrum of naturally occurring single-site acceptors suggest that the importance of the −1 and +3 positions is widespread.[2]

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[2] K. Nehrke and L. A. Tabak, unpublished observation.