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SUBSTRATE RECOGNITION BY UDP-*N*-ACETYL-α-D-GALACTOSAMINE: POLYPEPTIDE *N*-ACETYL-α-D-GALACTOSAMINYLTRANSFERASE. EFFECTS OF CHAIN LENGTH AND DISULPHIDE BONDING OF SYNTHETIC PEPTIDE SUBSTRATES*

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ABSTRACT

A synthetic peptide AcTPPP, based on a threonine-containing sequence present in bovine myelin basic protein, is a potent acceptor of glycosyl transfer from UDP-*N*-acetylgalactosamine catalyzed by extracts of baby hamster kidney (BHK) cells or rabbit lymph node tissue. In contrast, the disulphide-linked peptide $(AcTCPPP)_2$, based on a glycosylated sequence present in the hinge region of rabbit immunoglobulin G, is not an acceptor and inhibits glycosylation of AcTPPP. Extension of the cystine-containing peptide at the *N*-terminus produced weak acceptors but strong acceptors resulted when the cystine residue was reduced to form monomeric peptides. The acceptor specificity of the *N*-acetylgalactosaminyltransferase activity of BHK cells is very similar to that of rabbit lymph node tissue. The results indicated that tissues actively secreting immunoglobulin do not contain a transferase activity adapted specifically for glycosylation of sequences containing cystine residues, and suggested that addition of an *N*-acetylgalactosamine to a threonine residue in the hinge region of rabbit immunoglobulin takes place during biosynthesis prior to the formation of the inter-chain disulphide bridge of fully assembled immunoglobulins.

INTRODUCTION

The first step in assembly of *O*-glycan chains in mammalian cells involves enzymic transfer of an *N*-acetylgalactosamine residue from UDP-*N*-acetylgalactosamine to the hydroxy group of a serine or threonine residue in nascent polypeptides[1]. The UDP-GalNAc:polypeptide GalNAc-transferase activity has been studied extensively in extracts of various tissues[2–4] and has been purified[5,6]. However, its exact substrate specificity remains obscure. No sequence homology could be detected[4] surrounding the glycosylated serine and threonine residues in ovine submaxillary gland mucin, a glycoprotein containing many *O*-glycan chains, and a

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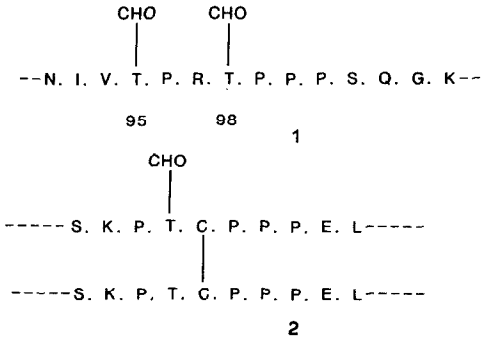
similar finding was obtained for the multiple glycosylation sites of human chorionic gonadotrophin[7] and human immunoglobulin D[8]. The purified GalNAc-transferase of hepatoma AH66 cells was shown[5] to utilise with similar efficiency a variety of different apoprotein acceptors, but the sequences of the regions containing the glycosylation sites were not determined. However, the present consensus is that a single enzyme may be capable of glycosylating different peptide sequences provided certain constraints are met. On the basis of computer prediction of protein secondary structure, Aubert *et al.*[9] suggested that the peptide segments at sites of *O*-glycosylation are capable of participating in β-turn structures. This would be consistent with the high content of proline surrounding sites for *O*-glycosylation (Scheme 1) in human myelin basic protein[10,11] (**1**) and rabbit immunoglobulin[12] G (**2**).



Scheme 1. Sites for glycosylation in bovine myelin basic protein[10,11] (MBP) (**1**) and rabbit immunoglobulin[12,13] (IgG) (**2**).

The glycosylation of the hinge region of rabbit IgG is an intriguing example of selective glycosylation in a multimeric glycoprotein, since it has been shown[13–16] that only one subunit of the IgG dimer bears an *O*-glycan chain (Scheme 1). This asymmetry could be due to selectivity at the level of the glycosyltransferase or to the asymmetric assembly of nonglycosylated subunits with glycosylated subunits. The present paper examines the substrate specificity of GalNAc transfer to polypeptide. We have used homogenates of baby hamster kidney (BHK) cells since these cells contain high levels of GalNAc-transferase activity[17] and, for comparison, extracts of rabbit lymph nodes, a tissue actively engaged in biosynthesis of IgG.

EXPERIMENTAL

General procedures. — Reduction and alkylation of peptides was carried out by treatment in a volume of 0.25 mL with 10mM dithiothreitol for 1 h at room temperature, followed by treatment with 10mM iodoacetamide using the same conditions.

Synthetic peptides. — Peptides were synthesised by the solid phase

TABLE I

AMINO ACID COMPOSITION OF SYNTHETIC PEPTIDES EMPLOYED AS SUBSTRATES OF *N*-ACETYLGALACTOSAMINYLTRANSFERASE ACTIVITY

Peptide	*Constituent amino acids (molar ratios)*
AcTPPP (**3**)	Thr, 1.0; Pro, 2.9
AcSPPP (**4**)	Ser, 1.0; Pro, 3.3
$(AcTCPPPE)_2$ (**5**)	Thr, 0.9; Glu, 1.0; Pro, 3.2; Cys, 1.0
$(AcKPTCPPPE)_2$ (**6**)	Thr, 1.0; Glu, 1.0; Pro, 4.0; Cys, 0.4[a], Lys, 1.0
AcKPTCPPPE (**7**) | Bzl	Thr, 1.0; Glu, 1.1; Pro, 4.3; Benzyl Cys, 1.0; Lys, 1.0
$(AcSKPTCPPPE)_2$ (**8**)	Thr, 1.1; Ser, 0.9; Glu, 1.0; Pro, 4.1; Cys, 0.2[a], Lys, 1.0
AsSKPTCPPPE (**9**) | Bzl	Thr, 1.0; Ser, 0.9; Glu, 1.0; Pro, 3.9; Benzyl Cys, 1.0; Lys, 1.0
AcKPTPPPE (**10**)	Thr, 0.9; Glu, 1.0; Pro, 3.9; Lys, 1.0

[a]Cysteine content underestimated, see values obtained for S-benzylated derivatives.

method[18,19] using 1% crosslinked chloromethylated polystyrene resin. Attachment of the C-terminal aminoacid was by use of the caesium salt. Side-chain protecting groups were benzyl for serine, threonine, and cysteine; benzyloxycarbonyl for lysine; and benzyl ester for glutamic acid. Protection of α-amino groups was carried out with the *tert*-butyloxycarbonyl (BOC^t) group. Assembled peptides were removed from the resin with HBr in trifluoroacetic acid containing 1% anisole as scavenger. Removal of the *S*-benzyl group of cysteine was performed with sodium in NH_3. Conversion of SH-containing peptides to the disulphide form was carried out by air oxidation at pH 6.8 in the presence of a trace amount of Fe^{3+} ion.

Peptides were purified by gel filtration on a column (30 × 1 cm) of Sephadex G-10 with 50% (v/v) acetic acid as eluent, followed by ion-exchange chromatography on a column (30 × 1 cm) of SP-Sephadex C-25 with NaCl gradients. The peptides were monitored for purity by analytical l.c. (Waters Inc.) on a C18 Micro Bondapak column (40 × 0.4 cm) in 0.01M HCl and acetonitrile gradients (1%/min with a flow rate of 1.5 mL/min). The amino acid compositions (Table I) were determined by analysis with an LKB Model 4400 Autoanalyser after acid hydrolysis for 16 h at 110°.

Cell extracts. — BHK cells were grown at 37° in Glasgow modified Eagle's medium supplemented with 10% fetal calf serum. Confluent cultures (~10^8 cells) were harvested by scraping, washed with phosphate-buffered saline (Pi–NaCl) by centrifugation several times, and suspended in Pi–NaCl (0.5 mL) at 2°. An equal volume of 2.5% (w/v) Triton X-100–Pi–NaCl was added at 2°, the cells were lysed by freezing and thawing, and the supernatant obtained after centrifugation at 8000*g* for 15 min was used as source of enzyme. The supernatant containing 15–20 mg/mL of protein retained full activity for 1 week at 2° and was then discarded. Lymph node tissue was excised from the hind legs of a rabbit 9 days after priming by

injection of bovine serum albumin 10% (w/v) in complete Freund's adjuvant (0.5 mL). The tissue was homogenised at 2° in Pi–NaCl (5 mL) with an Ultraturrex homogeniser. The homogenate was filtered through a coarse-wire grid to remove tissue debris, and the filtrate was diluted with an equal volume of 2.5% Triton X-100–Pi–NaCl. The mixture was frozen and thawed several times and clarified by centrifugation, and the supernatant containing 3.8 mg/mL of protein was used as an enzyme source.

Enzyme assay. — Incubation mixtures[17] in duplicate contained, in Eppendorf tubes, UDP-*N*-acetyl[^{14}C]galactosamine (10 μL, 2 GBq/mmol, 4 nmol, Amersham International), 10mM $MnCl_2$ (10 μL), 0.25% Triton X-100–0.25M Tris·HCl buffer, pH 7.1 (40 μL), cell extract (20 μL, 0.07–0.36 mg of proteins), and acceptor (20 μL, 100 nmol) or water (20 μL). The mixtures (0.1 mL) were incubated at 37° for various times, and the reaction was stopped by addition of 0.02M $Na_2B_4O_7$–2mM EDTA, pH 9 (0.4 mL). The products were separated by passage through small columns (3 mL) of Bio-Rad AG 1-X8 (50–100 mesh, Cl^-) anion-exchange resin. The material eluted with 3 mL of water was pooled (3.5 mL) and aliquots (0.5 mL) were counted for ^{14}C radioactivity[17]. Control incubation mixtures lacking cell extract were processed similarly to measure the nonspecific breakdown of sugar nucleotide, which amounted to <1% of the radioactivity incorporated into exogenous or endogenous acceptors. Incorporation of radioactivity into endogenous acceptors was also measured by acid precipitation[17]. Incubation mixtures (0.1 mL) were cooled on ice, albumin (20 μL, 50 mg/mL) was added, followed by 1% (w/v) phosphotungstic acid in 2M HCl (1 mL). The precipitates were washed sequentially twice with 1% phosphotungstic acid, 10% (w/v) trichloro acetic acid (1 mL), 80% (v/v) ethanol (1 mL), and finally ether (0.5 mL), dissolved in M NaOH (0.5 mL), and assayed for radioactivity.

Characterization of products. — ^{14}C-Labelled products (0.5 mL) were applied to a column (1 × 77 cm) of Bio-Gel P-2 (100–200 mesh, Bio-Rad Laboratories), equilibrated at 2° with 0.1M pyridinium acetate, pH 5. The column was eluted with buffer at 5 mL/h, and aliquots (25–100 μL) of column fractions (1 mL) were assayed for radioactivity. Other ^{14}C-labelled samples were treated for 16 h at 37° with purified[17] *Trichomonas foetus* *N*-acetyl-α-D-galactosaminidase (0.082 U) in 0.05M sodium citrate buffer 0.2 mL at pH 5 (ref. 17). The digests were heated for 5 min at 100° and analysed by Bio-Gel P-2 chromatography as described above. The enzyme is specific for 2-acetamido-2-deoxy-α-D-galactopyranosyl linkages.

RESULTS AND DISCUSSION

As described earlier[17], BHK cell extracts catalyzed the incorporation of radioactivity from UDP-[^{14}C]GalNAc into the peptide acceptor **3** (Fig. 1a). When 100 nmol of peptide **3** was used, the incorporation increased up to 8 h of incubation at 37°. After 16 h at 37°, 2.4–3.4 nmol (0.32–0.43 × 10^6 c.p.m.) of GalNAc had been transferred. Addition to the incubation mixture after 8 h of fresh UDP-

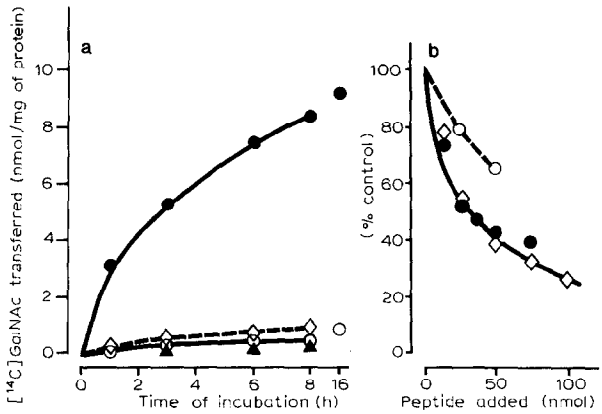


Fig. 1. *N*-acetylgalaactosaminyltransferase activity of BHK cells. (a) Transfer of *N*-acetylgalactosamine from UDP-GalNAc to various acceptors catalyzed by BHK cell extrats: peptide **3** (●), peptide **4** (▲), peptide **5** (○), and endogenous (◇). Duplicate incubation mixtures (0.1 mL) contained cell extract, Mn^{2+} ions, sugar nucleotide (4 nmol), peptide (100 nmol), or no peptide acceptor. Incorporation of radioactivity into exogenous or endogenous products was measured by the ion-exchange assay after incubation at 37° for the periods indicated. (b) Inhibition of GalNAc transfer into peptide **3** (○, ●) or endogenous acceptors (◇) by peptide **5**. The incubation mixtures contained cell extract, Mn^{2+} ions, sugar nucleotide, and peptide **5** (0–100 nmol) alone (◇) or in addition to 25 nmol (●) or 50 nmol (○) of peptide **3**. Incorporation of radioactivity into products was measured by ion-exchange (○,●), or acid precipitation (◇) assays after incubation for 3 h at 37°. The results are expressed as a percentage of the incorporation measured in the absence of inhibitory peptide. See Table I for peptide structures.

[^{14}C]GalNAc (4 nmol) and incubation for a further 8 h resulted in the total incorporation of 6.3–7.1 nmol of [^{14}C]GalNAc into the peptide. The structure of peptide **3** is based on a sequence adjacent to threonine 98 of bovine myelin basic protein (**1**). Although naturally occurring **1** is not a glycoprotein, it can serve as an acceptor for GalNAc transfer catalyzed by extracts of submaxillary glands and other tissues[20]. Under these conditions, threonine residues at both positions 95 and 98 (Scheme 1) may be glycosylated[10,11]. Extensive work by Young and assoc.[21,22] has shown that peptide **3** contains a minimum structure required for glycosylation by the GalNAc-transferase activity of porcine submaxillary glands. Interestingly, we observed that substitution of threonine by serine, as in peptide **4**, abolished the acceptor activity of BHK cell extracts (Fig. 1a).

The hinge region of rabbit IgG contains[12] a glycosylated threonine residue in a sequence similar to that surrounding threonine 98 of myelin basic protein **1**. The major difference is the insertion of half-cystine between the threonine residue and the triprolyl sequence. In the intact four-chain immunoglobulin molecule, the half-cystine residue participates in an inter H-chain disulphide bridge (**2**). We synthesised peptide **5** which contains this structural feature (Table I) and tested its ability to act as a substrate for glycosylation. The peptide was a very poor acceptor (Fig. 1a), incorporating after 3 h at 37° 0.26–0.53 nmol of [^{14}C]GalNAc/mg of homogenate protein, as compared with ~5 nmol/mg of homogenate protein for an

incubation mixture containing an identical concentration of peptide **3**. Hence, it appears that insertion of a half-cystine residue into the TPPP sequence interferes with substrate recognition by the GalNAc-transferase activity of BHK cell extracts. The results shown in Fig. 1 indicate, however, that peptide **5** can affect the activity of the enzyme. As shown previously[17], crude BHK cell extracts incorporate [^{14}C]GalNAc into endogenous acceptors to ~10% of the rate of incorporation given by suitable peptide acceptors, *e.g.*, peptide **3**. This low level of incorporation into endogenous acceptors (Fig. 1a) was significantly inhibited by peptide **5** (Fig. 1b). Peptide **5** also inhibited the glycosylation of peptide **3** (Fig. 1b). Half-maximal inhibition of incorporation was obtained by use of about equimolar amounts of peptides **3** and **5** in the incubation mixture, showing that the inhibition was due to competitive binding of peptide **5** to the enzyme and not to some impurity in the peptide **5** preparation. The data shown in Fig. 1b indicate an apparent K_i of 0.58mM for peptide **5**, a value close[17] to the K_m of 0.48mM for peptide **3**. Hence, peptides **3** and **5** possess similar binding affinities for the BHK cell activity.

N-Terminal extension of peptide **5** markedly increased the rate of glycosylation of the threonine residue. This conclusion is illustrated (Fig. 2) by the acceptor activities of peptides **6** and **8** which contain additional lysine and proline (peptide **6**), or serine, lysine, and proline (peptide **8**) residues. These residues were placed in an order identical to the sequence known[12] to be glycosylated in the hinge region of rabbit IgG. The incorporation of [^{14}C]GalNAc into these peptides, measured

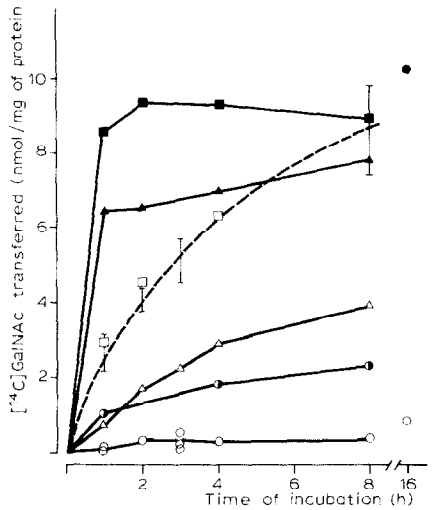


Fig. 2. Glycosylation of various peptides by BHK cell extracts. Duplicate assay mixtures (0.1 mL) contained BHK cell extract, Mn^{2+} ions, peptide (100 nmol), and UDP-[^{14}C]GalNAc (4 nmol); incubation at 37° was for the periods indicated. The compositions of peptide **3** (-----), **5** (○), **6** (△), **7** (▲), **8** (◑), **9** (□), and **10** (■) are shown in Table I. Three different BHK cell homogenates were used to collect these data. Each assay of a particular peptide included an assay using peptide **3** and the bars indicate the range of values obtained using the latter acceptor. The incorporations are corrected for nonspecific breakdown of sugar nucleotide and incorporation into endogenous acceptors.

after 1–8 h incubation at 37°, was 5–7 times greater than the incorporation into peptide **5**. Even so, the incorporation into these peptides was lower by 50–60%, as compared with peptide **3** (Fig. 2). Conversion of peptides **6** and **8** to single-chain peptides by benzyl substitution of the reduced peptides resulted in compounds that were similar (peptide **9**), or more efficient (peptide **7**) acceptors for glycosylation as compared with peptide **3**. Hence, an effective peptide acceptor for GalNAc transfer is one that is based on the glycosylated sequence of rabbit IgG, provided the threonine reaction site is surrounded by two or more aminoacid residues on both the *N*-terminal and *C*-terminal sides. The cysteine residue still appears to place some constraint on acceptor activity, since peptide **10**, which has a similar sequence to peptide **7** but lacks a benzylated cysteine residue, exhibited an enhanced acceptor activity (Fig. 2).

Each ^{14}C-labelled peptide was analysed by chromatography on Bio-Gel P-2 (Fig. 3). As shown, the radioactivity in each case was eluted in a position expected for the respective size of the glycopeptide synthesized. Minor peaks of radioactivity, eluted between fractions 82 to 87, represented free 2-acetamido-2-deoxy-D-galactose present in the incubation mixtures owing to nonspecific breakdown of the sugar nucleotide during the period of assay. Reduction and alkylation of the product obtained by glycosylation of the disulphide-bonded peptide **8** (Fig. 3e) produced a component of lower molecular size (Fig. 3f). The α-D linkage of the GalNAc residue transferred to the synthetic peptides was identified unequivocally by the sensitivity of these products to *T. foetus N*-acetyl-α-D-galactosaminidase (see Figs. 3a,e).

The lymph nodes are an important site of IgG synthesis and glycosylation. We considered it of interest, therefore, to examine the ability of extracts of rabbit lymph node tissue to utilize *in vitro* synthetic peptides containing half-cystine residues. The results obtained are shown in Table II. Unfortunately, rabbit lymph node tissue showed a rather high incorporation of radioactivity from UDP-[^{14}C]GalNAc into endogenous acceptors, presumably nascent or fully assembled IgG molecules. However, addition of peptides **3** or **10** to incubation mixtures increased the incorporation of radioactivity into products. After 3 h, no further incorporation appeared to take place, presumably owing to the instability of transferase activity in lymph node extracts or the presence of degrading enzymes, *e.g.*, glycosidases or nucleotidases. Peptide **5** was found to be a very poor substrate for glycosylation, yet it inhibited the incorporation into endogenous acceptors. The longer chain, disulphide-bonded peptide **6**, was a poor acceptor whereas the benzylated derivative **7** was a reasonably good acceptor, producing an incorporation significantly greater than that found in incubation mixtures containing only endogenous acceptors. The essential findings of this analysis are: (a) Rabbit lymph node extracts utilized efficiently peptides **3** and **10** which lack a cystine residue and differ in sequence from the glycosylated site in IgG; (b) peptide **5**, which contains a sequence identical to the glycosylated site in IgG, was not an acceptor. In these respects, therefore, the GalNAc-transferase activity of rabbit lymph nodes dis-

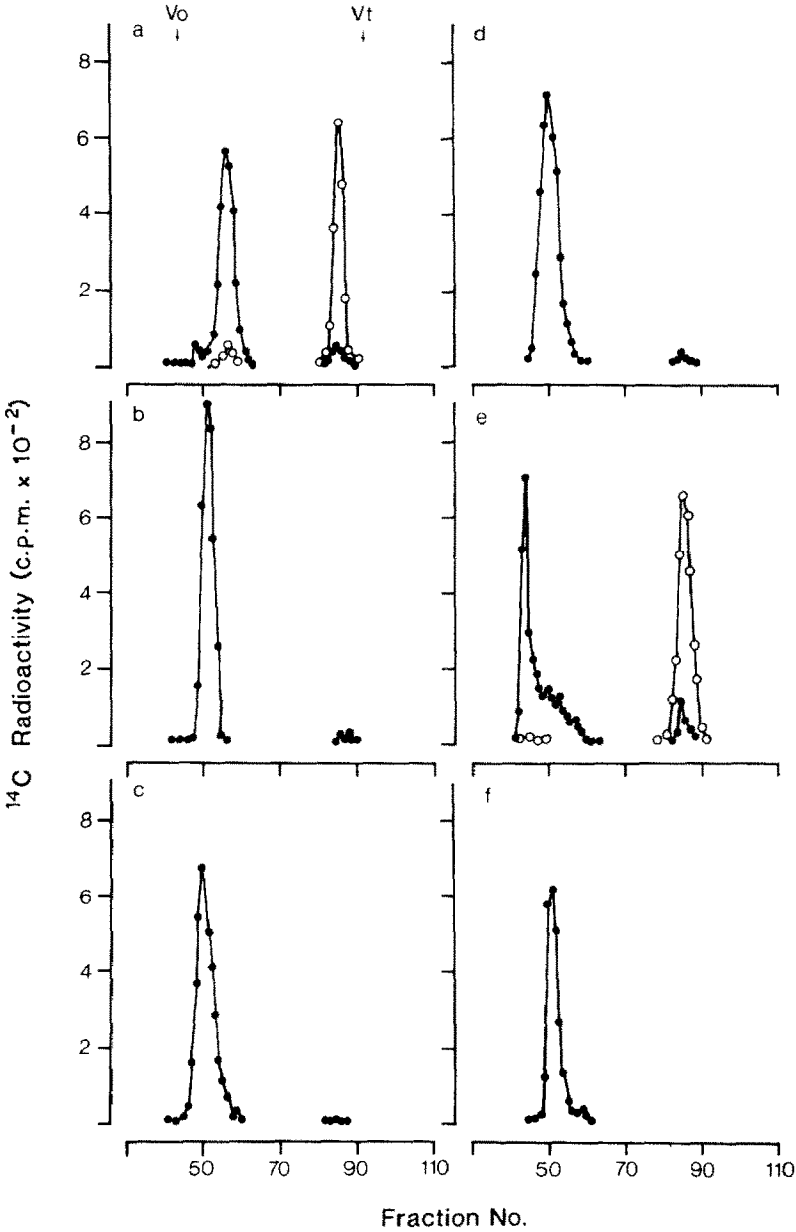


Fig. 3. Bio-Gel P-2 chromatography of [^{14}C]GalNAc peptides. Radioactive products obtained from incubation mixtures (see Fig. 2) containing synthetic peptides **3** (a), **10** (b), **7** (c), **9** (d), and **8** (e) containing ~80 000 c.p.m. were applied to a Bio-Gel P-2 column and eluted with 0.1M pyridinium acetate buffer. The profile obtained after reduction and alkylation of the major peak (Fractions 42–47) of (e) is shown in (f). Aliquots of column fractions (1 mL) were assayed for radioactivity. In (a) and (e) profiles for the untreated (●) or *T. foetus* *N*-acetyl-α-D-galactosaminidase-treated (○) products are shown. The void (Vo) and total (Vt) columns, as determined by use of bovine serum albumin and D-[^{14}C]glucose, are shown by the arrows.

TABLE II

INCORPORATION OF RADIOACTIVITY FROM UDP [^{14}C]GalNAc INTO SYNTHETIC PEPTIDES AND ENDOGENOUS ACCEPTORS CATALYZED BY EXTRACTS OF RABBIT LYMPH NODES[a]

Substrate	*Incubation time (h)*	*Incorporation*	
		C.p.m.	*nmoles*[b]
None	1	3 141	0.32
	3	8 540	0.87
3	1	13 743	1.4
	3	31 416	3.2
5	1	1 079	0.11
	3	2 945	0.30
6	1	5 006	0.51
	3	10 602	1.08
7	1	10 111	1.03
	3	20 713	2.11
10	1	20 615	2.1
	3	36 322	3.7

[a]Extrats of lymph nodes excised from an antigen-primed rabbit were incubated (76 μg of protein) with UDP-[^{14}C]GalNAc (4 nmol) in the presence or absence of various synthetic peptides (100 nmol). Incubation was for 1 h or 3 h at 37°, and incorporation of radioactivity into products was measured by the ion-exchange method. The average of duplicate experiments are shown. [b]Relative to 10 000 c.p.m.

played properties that are very similar to the enzyme activity of BHK cells. Thus, the GalNAc-transferase activity of rabbit lymph nodes did not appear to be adapted specifically to glycosylate peptide sequences containing a half-cystine or cystine residue.

Our results are consistent with, and extend, previous work concerning the specificity of GalNAc-transferase activity. Consideration of the peptide sequences surrounding sites that undergo glycosylation with GalNAc-transferase in various glycoproteins and prediction of secondary structures in these polypeptide segments have indicated a requirement for a regular spacing of amino acid residues that are likely to break or destabilise helices[4]. Thus, the preferred sites of *O*-glycosylation may occur in regions of little secondary structure which provide adequate access to the amino acid hydroxyl side-chains at the transferase active site. Hill *et al.*[4] have suggested that accessibility, rather than recognition, of amino acid sequences may be the predominant factor in sugar transfer catalyzed by GalNAc-transferase. This proposal is consistent with our present results which suggest that a cystine residue in a position adjacent to a threonine residue severely limits the potential of this site for glycosylation. One possible explanation could be related to the constraint placed on the local conformation of the glycosylation site by disulphide bond formation. A survey of the known glycosylated threonine and serine sites in glycoproteins showed that a half-cystine residue is invariably absent, except in the case of rabbit IgG. A half-cystine residue is present in a glycosylated sequence obtained from human chorionic gonadotrophin[6], but this residue is placed remotely from the substituted serine residue and it is unlikely to affect exposure of the glycosylation site.

However, our results indicated that a cystine residue, even when adjacent to a threonine residue, does not completely prevent glycosylation, provided an additional peptide sequence is present to impose a secondary structure appropriate for recognition or binding by the GalNAc-transferase. It remains an open question, therefore, whether the glycosylation of a threonine residue in the hinge region of rabbit IgG occurs before or after assembly of the IgG subunits into the four-chain molecule although, at present, the former model appears more likely. Other evidence suggested that the heavy and light chains of IgG are synthesised on separate polyribosome populations, *N*-glycosylated co-translationally, and assembled in the endoplasmic reticulum[23,24]. In non-IgG secreting cells, the GalNAc-transferase activity has been found to be enriched in smooth membranes and not in the rough endoplasmic reticulum[25]. Similarly, monensin, a drug that prevents migration in the mid region of the Golgi apparatus, blocked the addition of a GalNAc residue but not the *N*-glycosylation of nascent glycoproteins[26]. These results would indicate, if extrapolated to lymphoid tissue, that addition of a GalNAc residue occurs to the fully assembled, disulphide-bonded IgG molecule. However, kinetic data with plasma cells indicated that *O*-glycosylation may be an early event in glycoprotein maturation, occurring in the endoplasmic reticulum[27], and somewhat similar findings have been reported for gastric mucosal glycoproteins[28]. Whatever the temporal sequence of events in IgG maturation proves to be, the modulation imposed by a cystine residue in a position adjacent to a potential glycosylation site raises interesting questions. Rabbit IgG is unique in carrying a cystine residue in this region of the molecule. The cystine-free hinge region of IgD, for example, carries 3–4 *O*-glycans, probably substituted on both subunits, and human IgA is *O*-glycosylated at multiple sites in the hinge region. The highly glycosylalted hinge region of IgD (ref. 8) is believed[29] to play a role in blast transformation of target cells after antigen binding, as well as other well-defined effector functions in complement binding and initiation of subunit interactions[29,20]. Modification of this region in rabbit IgG by insertion of a cystine residue in the peptide sequence with a consequent modulatory effect on glycosylation by *N*-acetylgalactosaminyltransferase activity may play a role in the control of these biological properties of the different classes of the immunoglobulin molecule.

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