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Blood group-active carbohydrate chains on the receptor for epidermal growth factor of A431 cells

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The antigens expressed on the carbohydrate chains of the receptor for epidermal growth factor of A431 cells were studied by immunoblotting with monoclonal antibodies. Blood group A and the Type 1 based blood group ALe^b and Le^a antigens were detected as well as antigens associated with unsubstituted, monofucosylated and difucosylated Type 2 blood group chains. The Le^a and the difucosylated Type 2 antigen activities were abolished by treating the blotted receptor with endo-β-galactosidase, indicating that they are expressed on backbone structures of poly-lacto/neolacto type. (The term 'poly-lacto/neolacto' is used here to describe oligosaccharide backbone structures consisting of repeating Type 1, Galβ1-3GlcNAc (lacto) or Type 2, Galβ1-4GlcNAc (neolacto) sequences.) The glycosidic linkage of oligosaccharides to protein was investigated using Pronase digests of the receptor biosynthetically labelled with [^{3}H]glucosamine or [^{3}H]fucose. The oligosaccharides were alkali-resistant, consistent with N- rather than O-glycosidically linked chains. A proportion of [^{3}H]fucose-labelled glycopeptides was susceptible to endo-β-galactosidase, confirming the immunoblotting experiment using antibodies against the Le^a and the difucosylated Type 2 antigenic determinants. Oligosaccharides were released from the [^{3}H]fucose- and [^{3}H]-glucosamine-labelled glycopeptides by hydrazinolysis. Chromatography of the oligosaccharides on Bio-Gel P6 and Concanavalin A columns indicated a spectrum of oligosaccharides which include those of high mannose type labelled with [^{3}H]glucosamine, and a mixture of oligosaccharides labelled with [^{3}H]fucose and [^{3}H]glucosamine of bi- and multiantennary complex types of which a subpopulation is susceptible to digestion with endo-β-galactosidase.

Key words: EGF receptor/blood group antigens/monoclonal antibodies/carbohydrate antigens/epidermoid carcinoma cell line (A431)

Introduction

The receptor for epidermal growth factor (EGF) is a glycoprotein of mol. wt. ~170 K (Cohen, 1983). There is little information on the carbohydrate structures of this glycoprotein; however, evidence for the presence of N-glycosidically linked carbohydrate chains of high mannose and 'complex' types has been obtained from metabolic labelling experiments with [^{3}H]mannose and [^{3}H]fucose (Mayes and Waterfield, 1984) and polyacrylamide gel electrophoresis before and after treatment of the receptor with endo-β-N-acetylglucosaminidase F (Weber *et al.*, 1984). The presence of O-glycosidically linked oligosaccharides has not been investigated fully. Among several monoclonal antibodies to the EGF receptor of the epidermoid carcinoma cell line A431 (Schreiber *et al.*, 1981, 1983; Kawamoto *et al.*, 1983; Richert *et al.*, 1983; Gregoriou and Rees, 1983, 1984a; Schlessinger *et al.*, 1984), five have been shown to recognize blood group-related carbohydrate structures (Fredman *et al.*, 1983; Gooi *et al.*, 1983a and unpublished observations) as summarized in Table I. Since all five antibodies precipitate the EGF receptor from solubilized A431 cells, the inference has been that the receptor has blood group-active carbohydrate chains. However, their presence on the EGF receptor has not been demonstrated and it is essential to exclude the possibility that the blood group A and H antigens may be expressed on other components, for example, glycolipids that remain associated and co-precipitate with the solubilized receptor. We have investigated (i) the carbohydrate antigens expressed on the EGF receptor by immunostaining of the receptor glycoprotein, after purification by EGF-affinity chromatography and electrophoresis in SDS-polyacrylamide gels and (ii) the susceptibilities of the [^{3}H]glucosamine- and [^{3}H]fucose labelled carbohydrate chains to endo-β-galactosidase and to mild alkali. We present evidence that blood group-related antigens are expressed on the carbohydrate chains of EGF receptor of A431 cells and that a proportion of these are borne on backbone structures of poly-lacto/neolacto type that are susceptible to digestion with endo-β-galactosidase. In addition we have observed that the metabolically labelled carbohydrate chains of the receptor are alkali resistant as expected of N- rather than O-glycosidically linked oligosaccharides.

Results

The carbohydrate antigens expressed on the EGF receptor were investigated by immunostaining of the receptor glycoprotein blotted onto nitrocellulose. Of the monoclonal antibodies used, seven gave immunostaining of a major band of mol. wt. 175 K, corresponding to the receptor, and a diffuse band in the mol. wt. range ~126–138 K (Table II and Figure 1). The antigenic activities detected were: blood group A, ALe^b and Le^a and unsubstituted, monofucosylated and difucosylated Type 2 blood group sequences.

The major immunostained 175-K band co-migrated with the main silver-stained band in the purified receptor; the second diffuse band did not correspond to any silver-stained component in the purified receptor and has not been previously described. The Ii and the IgM^{WOO} antigenic determinants associated with unsubstituted, long backbone structures of poly-neolacto and lacto type, respectively (Feizi, 1981; Kabat *et al.*, 1982) were not detected by immunoblotting, even after neuraminidase treatment of the receptor glycoprotein. However, there was evidence for the presence of fucosylated poly-lacto and neolacto chains, as treatment of the receptor with endo-β-galactosidase almost completely

Table I. Blood group-related carbohydrate structures recognized by five monoclonal antibodies to the receptor for EGF of A431 cell lines

Monoclonal antibodies		Carbohydrate specificities assigned	
Designation	Reference	Structure	Reference
101	Richert *et al.*, 1983	Galβ1-3GlcNAc |1,2 Fucα	Fredman *et al.*, 1983
TL5	Schreiber *et al.*, 1983	GalNAcα1-3Galβ1-3/4GlcNAc |1,2 Fucα	Gooi *et al.*, 1983a
G49	Gregoriou and Rees, 1983, 1984a	GalNAcα1-3Galβ1-3GlcNAc |1,2 |1,4 Fucα Fucα	H.C. Gooi, M. Gregoriou, J. Picard, A. Rees and T. Feizi (unpublished observations)
29.1	Schlessinger *et al.*, 1984	GalNAcα1-3Galβ1-4GlcNAc |1,2 |1,3 Fucα ± Fucα	H.C. Gooi and T. Feizi (unpublished observations)
3C1B12	I. Lax, R. Kris and J. Schlessinger (unpublished)	Galβ1-4GlcNAcβ |1,3 3/6Gal Fucα	

abolished the immunostaining with anti-Le^a and with C14 antibodies (Figure 1B, Table II). The immunostaining with the anti-A, anti-ALe^b, M39 and 3C1B12 antibodies persisted after treatment with endo-β-galactosidase.

To investigate whether any of the carbohydrate chains of the receptor are O-glycosidically linked (alkali labile), Pronase glycopeptides of the [^{3}H]glucosamine- and [^{3}H]fucose-labelled rceptor were prepared and chromatographed on Bio-Gel P6 before and after mild alkali treatment. The profile of the [^{3}H]glucosamine-labelled glycopeptides showed three main areas of radioactivity (Figure 2A): in the excluded volume, peak I; a major included peak II and a smaller peak III. Redigestion of the glycopeptides with Pronase did not affect the elution of peak I on the Bio-Gel P6 column (results not shown). The [^{3}H]fucose-labelled glycopeptides gave one major peak corresponding approximately to peak II of the [^{3}H]glucosamine-labelled glycopeptides (Figure 2C). Neither the [^{3}H]glucosamine nor [^{3}H]fucose profiles were affected by the treatment of the glycopeptides with 0.1 M NaOH at 37°C for 48 h (results not shown) which releases and degrades O-glycosidically linked but not N-glycosidically linked carbohydrate chains.

Neuraminidase treatment of the [^{3}H]glucosamine-labelled glycopeptide peaks II plus III (Figure 2A inset) resulted in some reduction of the apparent mol. wt. of the glycopeptides concomitantly with the release of radioactivity corresponding to sialic acid (six hexose units). The effect of neuraminidase on the migration of the peak I glycopeptides was not investigated due to insufficient material.

The [^{3}H]glucosamine- and [^{3}H]fucose-labelled glycopeptides were treated with endo-β-galactosidase and rechromatographed on Bio-Gel P6 to detect the susceptible oligosaccharides and the released digestion products (Figure 2A, C). The results showed (i) a loss of radioactivity in the highest mol. wt. regions of the [^{3}H]glucosamine-labelled peak II and of the [^{3}H]fucose-labelled glycopeptides, (ii) a shift and an increased height of the apex of these peaks and (iii) the appearance of a new [^{3}H]fucose-labelled peak corresponding to 7–9 hexose units and amounting to 4% of the total radioactivity.

To ascertain whether any of the glycopeptide peaks represent clusters of short oligosaccharides attached to Pronase-resistant peptides, oligosaccharides were released from the labelled glycopeptides by hydrazinolysis and chromatographed on Bio-Gel P6 (Figure 2B, D). Peak I′ oligosaccharides released by hydrazinolysis from [^{3}H]glucosamine-labelled glycopeptides remained in the excluded volume of the Bio-Gel P6 column. Oligosaccharide peaks II′ and III′ and the major [^{3}H]fucose-labelled peak chromatographed in the apparent mol. wt. range 2600–6000, 1800–2600 and 2400–6000, respectively. The apparent mol. wt. ranges are calculated from the numbers of hexose units corresponding to elution fractions (Figure 3) and the quoted exclusion limit of the Bio-Gel P6. This calculation does not take account of the reduced K_{av} that results in the presence of sialic acid [(Yamashita *et al.*, 1977), sialic acid chromatographed as six hexose units under the conditions used in this study] and other possible substitutions with a negative charge, for example, SO_4^{2-} and PO_4^{3-}. Chromatography of the glycopeptides on Sephadex G50 produced a single broad included peak (results not shown) indicating that the mol. wt. of the peak I glycopeptides is not greater than 10 000, the exclusion limit of this gel.

Chromatography of pooled [^{3}H]glucosamine-labelled oligosaccharides released by hydrazinolysis, on a column of Con A-agarose, showed that 55% of the oligosaccharides of peak III′ bound to Con A and were specifically eluted with 500 mM α-methylmannoside consistent with a content of N-linked oligosaccharides of high mannose type (Cummings *et al.*, 1983). With peak II′ oligosaccharides 11% of radioactivity bound and was eluted from the Con A column with 10 mM α-methylglucoside consistent with a content of N-linked biantennary oligosaccharides (Cummings and Kornfeld, 1982): negligible additional radioactivity was eluted with 500 mM α-methylmannoside. Peak I′ oligosaccharides showed negligible binding to the Con A column. These and the non-binding peak II′ oligosaccharides may consist of multiantennary chains and complex type chains with fucosylation of their peripheral and core regions which do not bind to Con A (Yamashita *et al.*, 1980).

Table II. Summary of immunostaining results of the purified EGF receptor of A431 cells with monoclonal antibodies

Antibodies	Structure of determinants	Immunostaining	
		Before endo	After endo
TL5 (anti-A)	GalNAcα1-3Galβ1-3/4GlcNAc |1,2 Fucα	+	+
G49 and MH2 (anti-ALe^b)	GalNAcα1-3Galβ1-3GlcNAc |1,2 |1,4 Fucα Fucα	+	+
CF4 (anti-Le^a)	Galβ1-3GlcNAc |1,4 Fucα	+	–
19.9	Galβ1-3GlcNAc |2,3 |1,4 NeuAcα Fucα	–	–
C14	Galβ1-4GlcNAc |1,2 | 1,3 Fucα Fucα	+	–
H11 (anti-H)	Galβ1-4GlcNAc |1,2 Fucα	–	–
anti-SSEA-1*	Galβ1-4GlcNAc |1,3 Fucα	–	–
3C1B12	Galβ1-4GlcNAcβ1 |1,3 \ 3/6Gal Fucα	+	+
M39	Galβ1-4GlcNAcβ1	+	+
anti-I(Ma)*	Galβ1-4GlcNAcβ1 \ ^{6}Gal/GalNAc	–	–
anti-I Step*	Galβ1-4GlcNAcβ1 \ $^6_3$Galβ1-4GlcNAcβ1 Galβ1-4GlcNAcβ1/	–	–
anti-i McDon anti-i Den	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc	–	–
IgM^{WOO}	Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAc	–	–

The purified EGF receptor was transferred onto nitrocellulose paper after electrophoresis in SDS-polyacrylamide gels and immunostained with monoclonal antibodies before and after treament with endo-β-galactosidase as described in Materials and methods. Asterisk indicates those antibodies used in separate experiments to immunostain the EGF receptor after treatment with neuraminidase; +, positive immunostaining of 175-K and 126–138 K bands; –, no immunostaining; endo, endo-β-galactosidase.

Discussion

Monoclonal antibodies have provided structural information on the backbone and peripheral regions of carbohydrate chains of glycoproteins and glycolipids (Feizi, 1981). They are of special value in the structural assignments of oligosaccharide sequences of the poly-lacto/neolacto series which often occur as mixtures of isomers that are difficult to separate (Hounsell *et al.*, 1981, 1984). We have used monoclonal antibodies to derive structural information on the carbohydrate chains of glycoproteins and glycolipids of erythrocytes (Childs *et al.*, 1978, 1979; Uemura *et al.*, 1983), teratocarcinoma cells (Childs *et al.*, 1983b) and the glycoproteins of lymphocytes (Feizi *et al.*, 1980; Childs and Feizi, 1981; Childs *et al.*, 1983a). In the present study, monoclonal antibodies directed against blood group-related structures were used to investigate the carbohydrate chains of the purified EGF receptor of the epidermoid carcinoma cell line A431.

Our studies establish, for the first time that blood group-related antigens are part of the 175-K EGF receptor glycoprotein of A431 cells, and, that only selected carbohydrate antigens on the surface of these cells are expressed on this receptor. The immunoblotting data before and after treatment of the receptor preparation with endo-β-galactosidase are consistent with the presence of the following backbone and

peripheral structures on this receptor:

(M39) Galβ1-4GlcNAcβ1-

(3C1B12) Galβ1-4GlcNAcβ1 (1,3 Fucα; 3/6Galβ1-)

(C14) Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1- (1,2 Fucα; 1,3 Fucα)

(TL5) GalNAcα1-3Galβ1-3/4GlcNAcβ1- (1,2 Fucα)

(G49) GalNAcα1-3Galβ1-3GlcNAcβ1- (1,2 Fucα; 1,4 Fucα)

(CF4) Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1- (1,4 Fucα)

The 126 – 138 K, diffusely migrating component in the EGF receptor preparation presumably has a high carbohydrate to peptide ratio. It is readily detected with the anti-carbohydrate antibodies that react with the 175-K receptor glycoprotein, but not with the silver stain. It does not correspond to any previously described form of the receptor glycoprotein (Cohen *et al.*, 1982; Mayes and Waterfield 1984; Weber *et al.*, 1984) and further investigations are required to establish its relationship to the main EGF receptor. The receptor preparation was obtained by affinity chromatography on an EGF-adsorbent. The diffusely migrating material may therefore be another form of the EGF receptor, although we cannot exclude the possibility that it is a degradation product or an unrelated glycoprotein which co-purifies with the receptor.

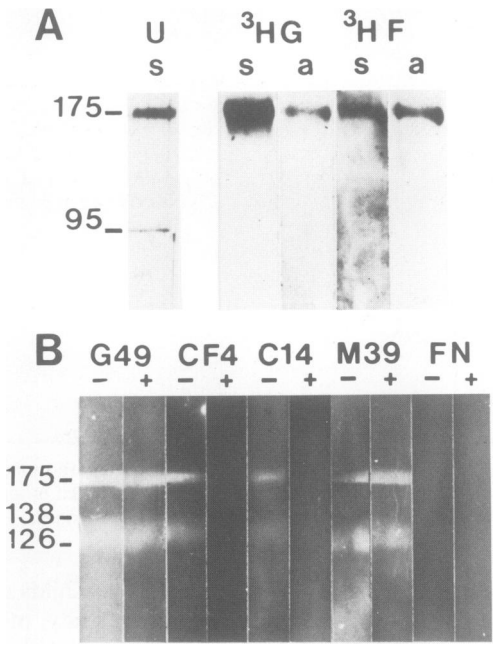

Fig. 1. SDS/polyacrylamide gel electrophoresis of EGF receptor preparations and immunostaining with monoclonal antibodies before and after treatment with endo-β-galactosidase. **Panel A**, preparations of unlabelled (U) and [^{3}H]glucosamine- and [^{3}H]fucose-labelled receptor (^{3}HG and ^{3}HF, respectively) were electrophoresed in SDS polyacrylamide gels and revealed by silver stain (s) or autoradiography (a) as described in Materials and methods. **Panel B**, ~1 μg samples of unlabelled receptor were electrophoresed in SDS/polyacrylamide gels and electrotransferred onto nitrocellulose; immunostaining was performed before (–) and after (+) treatment of the nitrocellulose strips with endo-β-galactosidase, as described in Materials and methods. The antibodies were EGR/G49, CF4, C14, M39 and, as a negative control, anti-fibronectin (FN).

Our objectives in the metabolic labelling of the EGF receptor were firstly to determine whether the EGF receptor contains O- as well as N-linked carbohydrate chains, and, secondly, to observe the effects of endo-β-galactosidase on these chains. Biosynthetic labelling with [^{3}H]glucosamine should lead to labelling of N-acetylgalactosamine and sialic acid as well as N-acetylglucosamine residues. Thus, N-acetylgalactosamine residues in the core regions of O-glycosidically linked oligosaccharides would be labelled in addition to N-linked chains. The [^{3}H]fucose would label O-or N-linked chains that are fucosylated in their core and/or peripheral regions. No release of radioactivity was detected after alkali treatment of the Pronase glycopeptides of the receptor labelled with either sugar. Thus, there was no evidence for the presence of labelled O-linked chains on the EGF receptor. Our findings are consistent with the observations of Mayes and Waterfield (1984) who found evidence for N- but not O-linked chains on the [^{35}S]methionine-labelled EGF receptor; they cited that endo-N-acetylgalactosaminidase did not alter the electrophoretic mobility of the EGF receptor immune precipitated from tunicamycin-treated A431 cells. Thus the EGF receptor differs from the receptor for low density lipoproteins which contains O- as well as N-linked chains (Cummings *et al.*, 1983).

Endo-β-galactosidase treatment of both the [^{3}H]glucosamine- and [^{3}H]fucose-labelled glycopeptides followed by chromatography on Bio-Gel P6, showed that, in each case, the susceptible carbohydrate chains were those of the highest apparent mol. wt. eluting within the main, included radioactive peaks. Only with the [^{3}H]fucose-labelled glycopeptides was the release of low mol. wt. oligosaccharides detected with certainty. Their structures require investigation since they eluted as oligosaccharides with higher apparent mol. wt. (7 – 9 hexose units) than the theoretical minimum structures, tetra- and pentasaccharides (5.5 and 6.5 hexose units, respectively) that would be predicted to be released by this enzyme from oligosaccharides and Le^a and C14 antigen activities as shown below.

Galβ1-3GlcNAcβ1-3Gal (1,4 Fucα) (Le^a)

Galβ1-4GlcNAcβ1-3Gal (1,2 Fucα; 1,3 Fucα) (C14)

The finding of only small amounts of radioactivity in the low mol. wt. products after endo-β-galactosidase digestion of the [^{3}H]glucosamine-labelled glycopeptides may be due to the known interconversion and hence dilution of the [^{3}H]-glucosamine into other sugars, i.e., N-acetylgalactosamine and sialic acid (Yurchenco *et al.*, 1978). The interconversion of fucose is minimal by comparison. The loss of radioactivity in the ascending part of the main radioactive peak and the increased radioactivity with peaks at fractions 86 and 81 with the [^{3}H]fucose and the [^{3}H]glucosamine labels, respectively, following treatment with endo-β-galactosidase, may represent the release of branched oligosaccharides of higher mol. wt.

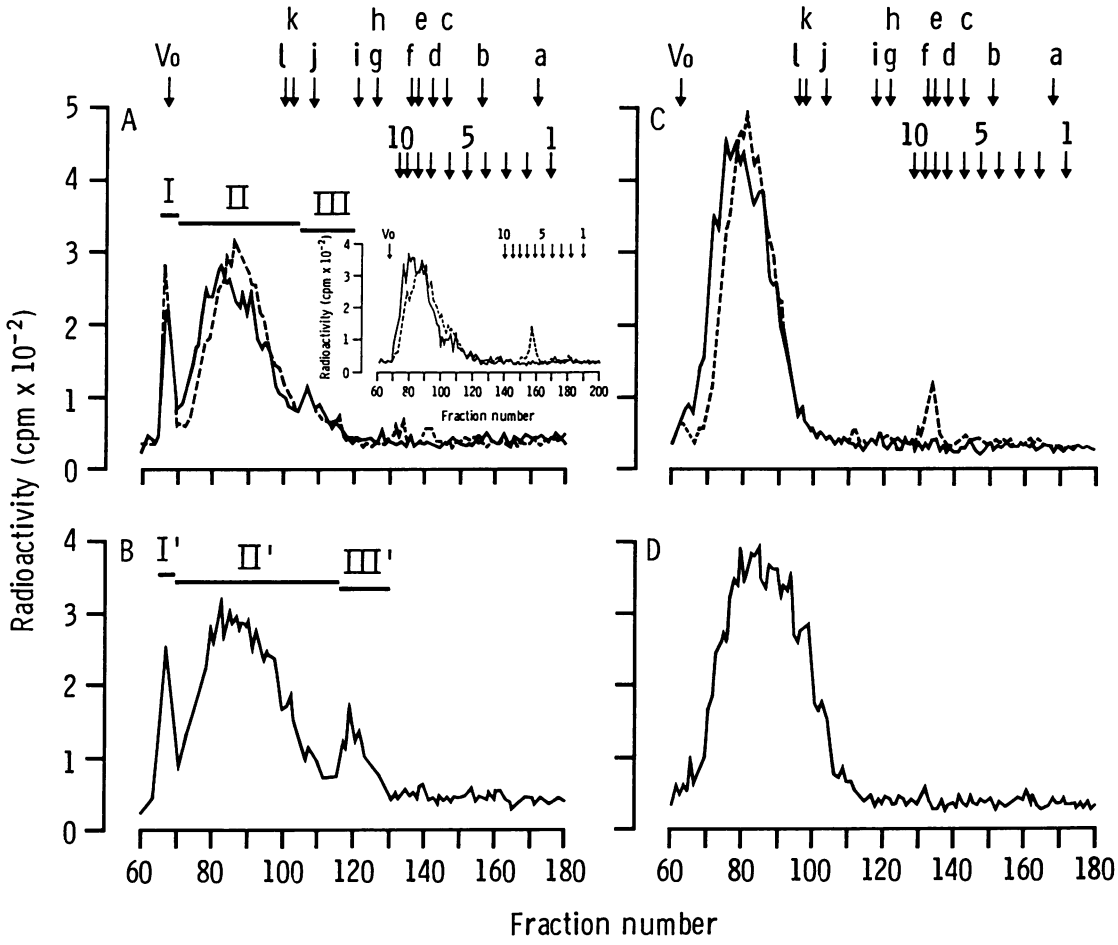

Fig. 2. Chromatography on Bio-Gel P6 of [^{3}H]glucosamine- and [^{3}H]fucose-labelled Pronase-glycopeptides and hydrazinolysis-released oligosaccharides of the EGF receptor. In **panels A** and **C**, [^{3}H]glucosamine and [^{3}H]fucose-labelled glycopeptides, respectively were chromatographed on the Bio-Gel P6 column before (———) and after (— — —) treatment with endo-β-galactosidase. In the inset to **panel A**, peak II plus III glycopeptides were chromatographed before (———) and after (— — —) treatment with neuraminidase, as described in Materials and methods. In **Panels B** and **D**, [^{3}H]glucosamine- and [^{3}H]fucose-labelled oligosaccharides, respectively, released from the Pronase glycopeptides by hydrazinolysis were chromatographed. Arrows indicate positions of glucose oligomers (1 – 10) obtained by partial acid hydrolysis of Dextran T2000 and ^{3}H-labelled oligosaccharide alditol standards ($a-l$) as described in Materials and methods.

than the triantennary neolacto-tetradecasaccharide standard (*k*). This oligosaccharide itself was obtained by digestion of a ceramide neolacto-pentadecasaccharide with endo-β-galactosidase (Scudder *et al.*, 1984). Alternatively, the released oligosaccharides may be relatively short, sialylated structures with disproportionately small K_{av} in the Bio-Gel P6 column. These questions are being further investigated. The elution profile of the ^{3}H-labelled peak I glycopeptides was not affected by this enzyme thus they may have highly branched backbones of poly-lacto/neolacto type or contain substitutions conferring resistance to this enzyme (Childs *et al.*, 1983a, 1983b). They may also contain multiple sialic acid residues or other substituents with negative charge resulting in a disproportionately low K_{av} value.

The external EGF-binding domain of the receptor contains ~30% of carbohydrate (Weber *et al.*, 1984). Our observations provide evidence for the occurrence of N-linked chains with backbone structures of poly-lacto and neolacto types and peripheral monosaccharides conferring blood group and other polymorphic antigen activities in addition to the usual 'complex' and high mannose oligosaccharides detected previously. The heterogeneity of carbohydrate structures in the backbone and peripheral regions of the carbohydrate chains

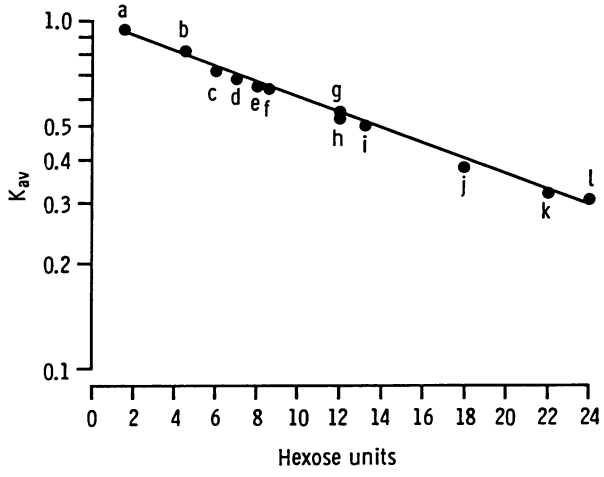

Fig. 3. Calibration of Bio-Gel P6 column with oligosaccharide standards. ^{3}H-Labelled oligosaccharide alditols of known structures designated $a-l$ (see Materials and methods) were chromatographed on a Bio-Gel P6 column (1.5 x 145 cm) equilibrated in 0.25 M NH_4HCO_3. Elution positons (K_{av}) are plotted against the mol. wt. expressed as equivalent hexose units as defined in Materials and methods.

of the receptor may reflect the cell to cell variation in the expression of carbohydrate antigens in the A431 cell line which we have noted by immunofluorescence (S. Thorpe and T. Feizi, unpublished observations). It will be interesting to investigate whether these heterogeneities correspond to different stages of the cell cycle and whether they arise from repeated modifications during recycling of the receptor. These backbone and peripheral glycosylations (which are also found on glycolipids) would be different on EGF receptors derived from different cell types. Thus it is unlikely that any particular structure among them is a prerequisite for EGF binding. On the other hand, certain lectins (Carpenter and Cohen, 1977) and antibodies (Schreiber *et al.*, 1983; Gregoriou and Rees, 1984a), known to bind to carbohydrate structures, inhibit EGF binding to a substantial extent or may trigger a biological response. These effects may represent indirect perturbation of the EGF-binding site on the receptor and raise the exciting possibility that the carbohydrate chains may be receptors for endogenous regulators, distinct from EGF, which modulate the cellular response to the growth factor.

Materials and methods

Isolation of EGF receptor

The human epidermoid carcinoma cell line A431 (received from Dr. E. Adamson, Department of Zoology, University of Oxford) was grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 5% foetal calf serum (DMEM/FCS), penicillin and streptomycin. For biosynthetic labelling, the cells were grown to 50% confluence and the medium replaced with fresh DMEM/FCS containing either D-[6-^{3}H]glucosamine hydrochloride (sp. act. 35 Ci/mmol) or L-[6-^{3}H]fucose (sp. act. 25 Ci/mmol), Amersham International, Amersham, UK, at 5 μCi/ml for 48 h. Unlabelled or biosynthetically-labelled cells were harvested using phosphate-buffered saline (PBS), pH 7.4, containing 5 mM EDTA and extracted with PBS containing 1% Triton X-100, 10% glycerol (v/v), 20 mM benzamidine and 1 mM EDTA. The extract was centrifuged at 100 000 *g* for 60 min and the EGF receptor isolated from the supernatant by affinity chromatography on an Affigel-EGF column as described previously (Cohen *et al.*, 1980; Gregoriou and Rees, 1984b) with the exception that benzamidine was omitted from the wash and eluate solutions of the biosynthetically-labelled receptor for Pronase digestion. After electrophoresis in SDS-polyacrylamide gel, as described below, and silver staining, the preparation of unlabelled receptor showed a major band of apparent mol. wt. 175 K and a minor band of 95 K (Figure 1A). Autoradiography of the [^{3}H]glucosamine- and [^{3}H]fucose-labelled receptor showed, in each case, a single radioactive band of apparent mol. wt. 175 K corresponding to the main silver-stained band (Figure 1A).

Immunoblotting of EGF receptor

Purified, unlabelled receptor was treated at 90°C for 5 min with 5% (w/v) SDS, 2% (v/v) β-mercaptoethanol and electrophoresed in 10% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS according to Laemmli (1970). Electrotransfer onto nitrocellulose (0.1 μm pore size, Sartorius Instruments Ltd., Sutton, Surrey, UK) was as described by Towbin *et al.* (1979). Immunostaining with monoclonal anti-carbohydrate antibodies was performed as described previously (Childs *et al.*, 1983b) with the exception that Triton X-100 was omitted from wash solutions. Blotted receptor was immunostained before and after treatment with neuraminidase from *Vibrio cholerae* (Behringwerke AG, Marburg, FRG) or endo-β-galactosidase isolated from *Bacteroides fragilis* (Scudder *et al.*, 1983). For neuraminidase treatment, nitrocellulose strips were incubated at 37°C for 2 h with 200 mU of neuraminidase per ml of solution containing 150 mM NaCl, 20 mM NaOAc pH 5.8, 1 mM $CaCl_2$ and 0.02% NaN_3. Strips were washed in PBS before use. For endo-β-galactosidase treatment, strips were incubated at 37°C for 2 h with 250 mU of endo-β-galactosidase per ml of solution containing 150 mM NaCl, 50 mM NaOAc buffer pH 5.8, 3% bovine serum albumin (BSA) and 0.02% NaN_3.

Preparation and chromatography of radiolabelled glycopeptides and oligosaccharides of the EGF receptor

[^{3}H]Glucosamine- or [^{3}H]fucose-labelled receptor in a solution containing 0.2 M Tris buffer pH 8.0, 0.5% sodium deoxycholate, 5 mM ethanolamine and 10% glycerol was precipitated at 4°C for 16 h in 70% (v/v) aqueous ethanol in the presence of 3 mg/ml of BSA as a carrier. Following centrifugation at 2000 *g* for 15 min, the precipitate was dried under a nitrogen stream, reconstituted in 2.5 ml of 0.2 M Tris buffer pH 8.0 containing 2 mM $CaCl_2$ and digested with 2.5 mg of Pronase (Sigma, grade VI) at 37°C for 24 h under toluene. A further 2.5 mg of Pronase was added at 24 h intervals and after 72 h the digestion was terminated by heating at 100°C for 10 min. Any insoluble residues were removed by centrifugation and the radiolabelled glycopeptides were desalted on a Sephadex G15 column (1.5 x 8 cm) equilibrated in 7% (v/v) propan-1-ol in water. An aliquot (3.6 x 10^3 c.p.m.) of [^{3}H]glucosamine-labelled glycopeptides was further digested with 0.5 mg of Pronase (as described above) with a further addition of 0.5 mg after 24 h. Digestion was terminated after 48 h by heating at 100°C for 10 min.

For endo-β-galactosidase digestion, the desalted glycopeptides were treated at 37°C for 18 h with 400 mU of endo-β-galactosidase per ml of solution containing 50 mM NaOAc buffer pH 5.8 and 0.2 mg/ml of BSA. For neuraminidase treatment [^{3}H]glucosamine-labelled glycopeptides were treated at 37°C for 2 h with 50 mU of neuraminidase per ml of solution containing 150 mM NaCl, 50 mM NaOAc buffer pH 5.8, 1 mM $CaCl_2$ and 1 mM $MgCl_2$. For base treatment, glycopeptides were incubated at 37°C for 48 h with 0.1 M NaOH and then neutralized with HCl.

For hydrazinolysis, EGF-receptor glycopeptides either [^{3}H]fucose-labelled (2.2 x 10^4 c.p.m.) or [^{3}H]glucosamine-labelled (1.7 x 10^4 c.p.m.) were dried overnight in a vacuum oven at 105°C, dissolved in 0.3 ml anhydrous hydrazine and incubated at 105°C for 6 h. Samples were evaporated to dryness *in vacuo* at 60°C and traces of residual hydrazine removed by three further evaporations following the addition of 0.1 ml toluene. Hydrazine-released oligosaccharides were dissolved in 0.3 ml saturated $NaHCO_3$ and re-N-acetylated by the addition of 30 μl acetic anhydride in 10 μl aliquots at 15 min intervals. Prior to chromatography the pH was adjusted to 8.0 by the addition of 2 M NaOH.

Glycopeptides and oligosaccharides were chromatographed on a Bio-Gel P6 column (1.5 x 145 cm) equilbrated in 0.25 M NH_4HCO_3, containing 0.02% NaN_3.

[^{3}H]Glucosamine-labelled oligosaccharides released by hydrazinolysis were chromatographed on a 2 ml column of Concanavalin A (Con A)-agarose (8 mg Con A/ml agarose, Sigma) as described by Cummings and Kornfeld (1982). The column was equilibrated in a solution containing 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.02% NaN_3. Bound oligosaccharides were eluted stepwise with the above solution containing either 10 mM α-methylglucoside or 500 mM α-methylmannoside.

Oligosaccharide standards

The Bio-Gel P6 column was calibrated using Dextran T40 (mol. wt. 40 000), an acid hydrolysate of Dextran T2000 (Pharmacia Fine Chemicals, Milton Keynes, UK) prepared according to the method of Whelan (1961), and the following oligosaccharides which were used as the [^{3}H]alditols after reduction with sodium [^{3}H]borohydride as described previously (Scudder *et al.*, 1984);

(a) Glc
(b) Galβ1-4GlcNAc
(c) Galβ1-3GlcNAcβ1-3Galβ1-4Glc
(d) Galβ1-3[Fucα1-4]GlcNAcβ1-3GalB1-4Glc
(e) Fucα1-2Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc
(f) Galβ1-4GlcNAcβ1-3[Galβ1-4GlcNAcβ1-6]Gal
(g) NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc
(h) Galβ1-4GlcNAcβ1-3[Galβ1-4GlcNAcβ1-6]Galβ1-4GlcNAcβ1-3Gal
(i) Galβ1-4GlcNAcβ1-2Manα1-3[Galβ1-4GlcNAcβ1-2Manα1-6]Manβ1-4GlcNAc
(j) NeuAcα2-3Galβ1-3[NeuAcα2-6]GlcNAcβ1-3Galβ1-4Glc
(k) Galα1-3Galβ1-4GlcNAcβ1-3[Galα1-3Galβ1-4GlcNAcβ1-6]Galβ1-4GlcNAcβ1-3[Galα1-3Galβ1-4GlcNAcβ1-6]Galβ1-4GlcNAcβ1-3Gal
(l) Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-6[Galβ1-4GlcNAcBβ1-2]-Manα1-6([Galβ1-4GlcNAcβ1-4][Galβ1-4GlcNAcβ1-2]Manα 1-3)Manβ1-4GlcNAc

It was not established which one of the four branches in oligosaccharide *l* terminated with the Galβ1-4GlcNAcβ1-3 sequence (underlined).

Oligosaccharide *a* was purchased from Sigma Chemical company, *b* and *f*, synthesized chemically, were gifts of Dr. A. Veyrières; *c*, *d* and *e*, isolated from human milk, were gifts of Dr. Winifred M. Watkins; *g*, *j*, *i* and *l* isolated from human urine were gifts of Dr. G. Strecker; *h* and *k*, were obtained by endo-β-galactosidase treatment of ceramide neolacto-octasaccharide and ceramide neolacto-pentadecasaccharide (Scudder *et al.*, 1984) isolated from rabbit erythrocytes by P. Hanfland and M. Kordowics.

Under the conditions used for Bio-Gel P6 chromatography, galactose and fucose chromatographed as one glucose hexose unit, glucitol as 1.5, N-acetylglucosamine as 2.5, N-acetylglucosaminitol as 3.5 and sialic acid as six hexose

units (Figure 3). K_{av} was calculated as $\frac{V_e - V_o}{V_t - V_o}$ where V_e = sample elution volume: V_o = void volume measured with Dextran T40 and V_t = total volume of the gel bed measured with glucose.

Antibodies

The following mouse hybridoma antibodies were used: three with blood group A specificity: EGR/G49 antibody, raised against the EGF receptor of A431 cells (Gregoriou and Rees, 1983, 1984a) and found to react preferentially with di-fucosylated Type 1 blood group A chains, i.e., the blood group ALe^b structure, Table I (H.C. Gooi, M. Gregoriou, J. Picard, A. Rees and T. Feizi, in preparation); TL5 antibody (a gift of Dr. J. Schlessinger) also raised against the EGF receptor of A431 cells (Schreiber *et al.*, 1983) and found to recognize the blood group A trisaccharide on Type 1 or 2 backbone structures, Table I (Gooi *et al.*, 1983a); MH2 (gift of Dr. E. Lennox) raised against human colonic adenocarcinoma and found to have anti-blood group A specificity (Voak *et al.* 1980) and to resemble EGR/G49 in its preference for the ALe^b structure (Table II) (H.C. Gooi, A.D. Lowe, E. Lennox and T. Feizi, unpublished observations); anti-blood group H antibody, H11 (gift of Dr. W.M. Watkins) raised against a human lymphoid line and shown to recognize Type 2 based blood group H structure (Knowles *et al.*, 1982); an anti-Le^a antibody CF4 (gift of Dr. W.W. Young, Jr.) raised against neutral glycolipids of human meconium (Young *et al.*, 1983); M39 antibody (gift of Dr. P.A.W. Edwards) raised against human milk fat globules (Foster *et al.*, 1982) with specificity for the Type 2 backbone structure (Gooi *et al.*, 1983b; Uemura *et al.*, 1983); antibody C14 (gift of Professor R.W. Baldwin) raised against human colon adenocarcinoma and shown to have specificity for difucosylated Type 2 blood group chains (2,3-difucosyl-N-acetyllactosamine) (Brown *et al.*, 1983); anti-SSEA-1 (gift of Dr. D. Solter) raised against mouse teratocarcinoma cells (Solter *et al.*, 1978) with a specificity for α1-3 fucosylated Type 2 blood group chains (3-fucosyl-N-acetyllactosamine) (Gooi *et al.*, 1981; Hounsell *et al.*, 1981); antibody 3C1B12 raised against the EGF receptor of A431 cells (I. Lax, R. Kris and J. Schlessinger, unpublished work), and shown to recognize 3-fucosyl-N-acetyllactosamine (Table II) and to differ from anti-SSEA-1 in being more strongly inhibited by an oligosaccharide carrying this determinant on a branched backbone structure (H.C. Gooi and T. Feizi, unpublished observations) and antibody 19.9 (gift of Dr. H. Koprowski) raised against a human colon cancer line and shown to recognize a sialylated form of the Le^a antigen (Magnani *et al.*, 1982). As a negative control, anti-fibronectin, purchased from Sera-Lab, Ltd (Crawley Down, Sussex, UK), was used. In addition the following human monoclonal antibodies were used: anti-I Ma, anti-I Step and anti-i McDon, anti-i Den (Feizi, 1981) and IgM^{WOO} (Kabat *et al.*, 1982); these have specificities for unsubstituted long chain backbone structures of neolacto or lacto type as shown in Table II. As a negative control, serum from a healthy donor was used to which a Waldenstrom macroglobulin was added as described previously (Feizi *et al.*, 1980). The human antibodies were used as diluted plasma (1:300 Ma, Step, Woo and McDon; 1:1000 Den) and the hybridoma antibodies were used as undiluted culture supernatant (C14), or 1:100 diluted immune ascites (M39, MH2, H11, CF4, 3C1B12 and anti-SSEA-1 and anti-fibronectin), or purified IgG 18 and 10 μg/ml (EGR/G49 and TL5, respectively).

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References

Brown,A., Feizi,T., Gooi,H.C., Embleton,M.J., Picard,J.K. and Baldwin, R.W. (1983) *Biosci. Rep.*, **3**, 163-170.

Carpenter,G. and Cohen,S. (1977) *Biochem. Biophys. Res. Commun.*, **79**, 545-552.

Childs,R.A., Feizi,T., Fukuda,M. and Hakomori,S. (1978) *Biochem. J.*, **173**, 333-336.

Childs,R.A., Feizi,T. and Tonegawa,Y. (1979) *Biochem. J.*, **181**, 533-538.

Childs,R.A. and Feizi,T. (1981) *Biochem. Biophys. Res. Commun.*, **102**, 1158-1164.

Childs,R.A., Dalchau,R., Scudder,P., Hounsell,E.F., Fabre,J.W. and Feizi, T. (1983a) *Biochem. Biophys. Res. Commun.*, **110**, 424-431.

Childs,R.A., Pennington,J., Uemura,K., Scudder,P., Goodfellow,P.N., Evans,M.J. and Feizi,T. (1983b) *Biochem. J.*, **215**, 491-503.

Cohen,S. (1983) *Cancer*, **51**, 1787-1791.

Cohen,S., Carpenter,G. and King,L.,Jr. (1980) *J. Biol. Chem.*, **255**, 4834-4842.

Cohen,S., Ushiro,H., Stoscheck,C. and Chinkers,M. (1982) *J. Biol. Chem.*, **257**, 1523-1531.

Cummings,R.D. and Kornfeld,S. (1982) *J. Biol. Chem., 257*, 11235-11240.

Cummings,R.D., Kornfeld,S., Schneider,W.J., Hobgood,K.K., Tolleshaug, H., Brown,M.S. and Goldstein,J.L. (1983) *J. Biol. Chem.*, **258**, 15261-15273.

Feizi,T. (1981) *Immunol. Commun.*, **10**, 127-156.

Feizi,T. Kapadia,A. and Yount,W.J. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 376-380.

Foster,C.S., Dinsdale,E.A., Edwards,P.A. and Norville,A.M. (1982) *Virchows. Arch. (Pathol. Anat.)*, **394**, 295-305.

Fredman,P., Richert,N.D., Magnani,J.L., Willingham,M.C., Pastan,I. and Ginsburg,V. (1983) *J. Biol. Chem.*, **258**, 11206-11210.

Gooi,H.C., Feizi,T., Kapadia,A., Knowles,B.B., Solter,D. and Evans,M.J. (1981) *Nature*, **292**, 156-158.

Gooi,H.C., Schlessinger,J., Lax,I., Yarden,Y., Libermann,T.A. and Feizi,T. (1983a) *Biosci. Rep.*, **3**, 1045-1053.

Gooi,H.C., Uemura,K., Edwards,P.A.W., Foster,C.S., Pickering,N. and Feizi,T. (1983b) *Carbohydr. Res.*, **120**, 293-302.

Gregoriou,M. and Rees,A.R. (1983) *Cell Biol. Internat. Rep.*, **7**, 539-540.

Gregoriou,M. and Rees,A.R. (1984a) *EMBO J.*, **3**, 929-937.

Gregoriou,M. and Rees,A.R. (1984b) *Biochem. Soc. Trans.*, **12**, 160-165.

Hounsell,E.F., Gooi,H.C. and Feizi,T. (1981) *FEBS Lett.*, **131**, 279-282.

Hounsell,E.F., Rideout,J.M., Pickering,N.J. and Lim,C.K. (1984) *J. Liquid Chromatogr.*, **7**, 661-674.

Kabat,E.A., Liao,J., Shyong,J. and Osserman,E.F. (1982) *J. Immunol.*, **128**, 540-544.

Kawamoto,T., Sato,J.D., Le,A., Polikoff,J., Sato,G.H. and Mendelsohn,J. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 1337-1341.

Knowles,R.W., Bai,Y., Daniels,G.L. and Watkins,W. (1982) *J. Immunogenet.*, **9**, 69-76.

Laemmli,U.K. (1970) *Nature*, **227**, 680-685.

Magnani,J.L., Nilsson,B., Brockhaus,M., Zopf,D., Steplewski,Z., Koprowski,H. and Ginsburg,V. (1982) *J. Biol. Chem.*, **257**, 14365-14369.

Mayes,E.L.V. and Waterfield,M.D. (1984) *EMBO J.*, **3**, 531-537.

Richert,N.D., Willingham,M.C. and Pastan,I. (1983) *J. Biol. Chem.*, **258**, 8902-8907.

Schlessinger,J., Lax,I., Yarden,Y., Kanety,H. and Libermann,T.A. (1984) in Greaves,M.F. (ed.), *Receptors and Recognition, Vol.* **17**: Monoclonal Antibodies to Receptors, Chapman and Hall, London, pp. 279-303.

Schreiber,A.B., Lax,I., Yarden,Y., Eshhar,Z. and Schlessinger,J. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 7535-7539.

Schreiber,A.B., Libermann,T.A., Lax,I., Yarden,Y. and Schlessinger,J. (1983) *J. Biol. Chem.*, **258**, 846-853.

Scudder,P., Uemura,K., Dolby,J., Fukuda,M.N. and Feizi,T. (1983) *Biochem. J.*, **213**, 485-494.

Scudder,P., Hanfland,P., Uemura,K. and Feizi,T. (1984) *J. Biol. Chem.*, **259**, 6586-6592.

Solter,D. and Knowles,B.B. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 5565-5569.

Towbin,H., Staehelin,T. and Gordon,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350-4354.

Uemura,K., Childs,R.A. Hanfland,P. and Feizi,T. (1983) *Biosci. Rep.*, **3**, 577-588.

Voak,D., Sacks,S., Alderson,T., Takei,F., Lennox,E., Jarvis,J., Milstein,C. and Darnborough,J. (1980) *Vox Sang*, **39**, 134-140.

Weber,W., Gill,G.N. and Spiess,J. (1984) *Science (Wash.)*, **224**, 294-297.

Whelan,W.J. (1961) in Whisler,R.L. and Wolfrom,M.L. (eds.), *Methods in Carbohydrate Chemistry*, Vol. **1**, Academic Press, NY, pp. 321-324.

Yamashita,K., Tachibana,Y. and Kobata,A. (1977) *J. Biol. Chem.*, **252**, 5408-5411.

Yamashita,K., Tachibana,Y., Nakayama,T., Kitamura,M., Endo,Y. and Kobata,A. (1980) *J. Biol. Chem.*, **255**, 5635-5642.

Young,W.W., Johnson,H.S., Tamura,Y., Karisson,K.-A., Larson,G., Parker,J.M.R., Khare,D.P., Spohr,U., Baker,D.A., Hindsgaul,O. and Lemieux,R.U. (1983) *J. Biol. Chem.*, **258**, 4890-4894.

Yurchenco,P.D., Ceccarini,C. and Atkinson,P.H. (1978) *Methods Enzymol.*, **50**, 175-204.

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