Glycoconjugate Journal (1992) **9**:209–216

Comparison of the carbohydrate moieties of recombinant soluble Fc_ε receptor ($sFc_\varepsilon RII$/sCD23) expressed in *Saccharomyces cerevisiae* and Chinese hamster ovary cells. Different O-glycosylation sites are used by yeast and mammalian cells

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Received 31 December 1991 and revised 30 March 1992

Recombinant human soluble low affinity receptor for the Fc portion of IgE ($sFc_\varepsilon RII$/sCD23) was produced in *Saccharomyces cerevisiae* or Chinese hamster ovary cells and subjected to carbohydrate analysis. Applied methods included analytical SDS-PAGE, reversed phase HPLC, methylation analysis and sequential degradation with exoglycosidases. The results revealed that $sFc_\varepsilon RII$ derived from Chinese hamster ovary cells is glycosylated exclusively at Ser-147, containing mainly the trisaccharide Sia(α2-3)Gal(β1-3)GalNAc, whereas the yeast derived glycoprotein was glycosylated at Ser-167 and contained only α-mannosyl residues. It is shown here for the first time that different amino acids of a given protein can be O-glycosylated when expressed in yeast or Chinese hamster ovary cells.

Keywords: O-glycosylation in yeast, O-glycosylation in CHO cells; soluble $Fc_\varepsilon RII$; methylation analysis; sequential degradation with exoglycosidases

Two types of receptors for the Fc portion of IgE, that differ in their affinity and molecular structure, have been described on human leukocytes. A high-affinity receptor ($Fc_\varepsilon RI$), which exists as a tetrameric complex comprised of three different subunits of which one occurs twice, is found on basophils and mast cells [1]. A low-affinity receptor ($Fc_\varepsilon RII$), a single-chain molecule, previously known as CD23, is a B cell specific differentiation antigen which is expressed on mature IgM/IgD-positive B cells [2, 3]. Monocytes, platelets and eosinophils show a constitutive expression of $Fc_\varepsilon RII$ where it plays a role in the IgE-dependent cytotoxicity against parasites [4].

$Fc_\varepsilon RII$ is a molecule consisting of 321 amino acids with a hydrophobic transmembrane region of 25 amino acids (amino acids 22–47). It is expressed on the cell surface with its NH_2 terminus located in the cytoplasm and its COOH terminus outside the cell [2]. $Fc_\varepsilon RII$ positive cells release a proteolytic cleavage product of the extracellular part of the receptor, also known as soluble $Fc_\varepsilon RII$ ($sFc_\varepsilon RII$ or sCD23), which still binds IgE [5, 6].

Both expression of $Fc_\varepsilon RII$ on B cells and the level of $sFc_\varepsilon RII$ in serum are increased in patients with atopic diseases. In the case of soluble $Fc_\varepsilon RII$ most of the receptor is bound to IgE [6–9]. It was suggested that the membrane bound $Fc_\varepsilon RII$ or its soluble moiety plays an important role in the regulation of serum IgE level or in blocking the function of effector cells in allergy either by directly regulating IgE synthesis or by neutralizing free IgE.

The membrane bound $Fc_\varepsilon RII$ contains one potential N-glycosylation site at Asn-63 (-Asn-Val-Ser-). This tripeptide is located in the extracellular portion of the molecule in close proximity to the transmembrane domain of the protein. Since the soluble part of $Fc_\varepsilon RII$ is generated by cleavage of the polypeptide chain at Met-150 $sFc_\varepsilon RII$ does not contain this N-glycosylation site. The Pro-, Ser-, and Thr-rich region within the COOH terminal part of the protein, however, is a good candidate for O-glycosylation.

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In fact, evidence for O-glycosylation has been reported for natural as well as for recombinant $sFc_\varepsilon RII$ derived from Chinese hamster ovary (CHO) cells [6, 10]. Therefore, it has been assumed that the recombinant product secreted from yeast would also be glycosylated. As yeast synthesizes O-glycans containing mannose residues only [11], it can be expected that the structures of the oligosaccharides from $sFc_\varepsilon RII$ expressed in yeast (yeast-$sFc_\varepsilon RII$) would be different from that of $sFc_\varepsilon RII$ derived from CHO cells (CHO-$sFc_\varepsilon RII$).

In this study we report the characterization of the glycans of $sFc_\varepsilon RII$ derived from both yeast and CHO cells. As expected, the structures of the oligosaccharides from yeast- and CHO cell-derived $sFc_\varepsilon RII$ were different. Moreover, sugar chains were found to be attached to different amino acids, which to our knowledge has not as yet been reported for any other protein.

Materials and methods

Expression of $sFc_\varepsilon RII$ in yeast and CHO cells

Dehydrofolate reductase-deficient CHO cells transfected with the plasmid pSV2-dhfr-$sFc_\varepsilon R$ and *S. cerevisiae* strain GRF 18 (α-Leu-2-His-3) transfected with the plasmid p289b were cultured as described by Uchibayashi *et al.* [6]. The yeast supernatant was kindly provided by Dr. E. Falkner of this Institute. Assays for $sFc_\varepsilon RII$ were performed using an ELISA with monoclonal antibodies as described recently [6].

Radioactive labelling of CHO-$sFc_\varepsilon RII$

For metabolic labelling of $sFc_\varepsilon RII$ confluent CHO cells were harvested and suspended in 10 ml medium (1×10^8 cells ml^{-1}) with reduced glucose concentration (1.5 g Glc l^{-1} instead of 2.0 g Glc l^{-1}) containing [6-^{3}H]glucosamine hydrochloride (1 mCi, NEN). Labelled glucosamine was used as a precursor mainly for *N*-acetylgalactosamine. After 4 h incubation the supernatant containing about 7 μg of radioactively labelled $sFc_\varepsilon RII$ was harvested. About 350 μg of purified nonlabelled CHO-$sFc_\varepsilon RII$ were added as carrier.

Isolation and purification of recombinant $sFc_\varepsilon RII$

Cell-free supernatants from CHO cell or yeast culture were supplemented with PMSF and EDTA up to a final concentration of 10 mM and 5 mM, respectively. Yeast supernatants (≈10 l per batch) were concentrated to about 1 l in an Amicon concentrator equipped with a hollow fibre cartridge H1P10-8 and a LP1A pump.

Recombinant $sFc_\varepsilon RII$ preparations from both the CHO cell supernatant and the concentrated yeast supernatant were purified by immunoaffinity chromatography using a monoclonal antibody as described in [5]. $sFc_\varepsilon RII$-containing fractions were pooled and further purified by reversed phase HPLC (RP-HPLC; see Chromatographic procedures section).

Isolation of oligosaccharides by alkaline β-elimination

Trypsin-treated CHO-$sFc_\varepsilon RII$ was incubated at 37 °C in alkaline borohydride solution (0.8 M $NaBH_4$ in 0.05 M NaOH). After 48 h the excess of $NaBH_4$ was destroyed by dropwise addition of 2 M aqueous acetic acid, adjusting to a pH of about 5. The sample was dried by rotary evaporation. The residue was dissolved in methanol containing 1% by vol acetic acid and dried under a stream of nitrogen in a waterbath at 40 °C. This step was repeated four times. The residue was dissolved in bi-distilled water and applied to a Bio-Gel P-2 column in order to separate the released oligosaccharides from the peptides as well as from salt (see Chromatographic procedures section). The purified oligosaccharides were either directly submitted to methylation analysis or further separated by high pH anion-exchange chromatography (HPAEC, see Chromatographic procedures section).

SDS-PAGE and blotting

SDS-PAGE was performed as described by Laemmli [12] using 15% acrylamide separation gels. Following electrophoresis, the proteins were detected by staining with Coomassie blue or, after transfer to nitrocellulose, by reaction with enzyme-labelled lectins. The apparent molecular mass was determined using the Pharmacia LMW Calibration Kit.

Electrophoretic transfer to nitrocellulose (Schleicher & Schüll, 0.45 μm) was performed with a semi-dry apparatus (Sartorius SM 175 56) for 2 h at 1.1 mA cm^{-2} gel. The sheets were then incubated overnight at 4 °C in blocking solution (1% bovine serum albumin in Tris-buffered saline: 20 mM Tris-HCl, pH 4.5, 500 mM NaCl).

Glycoprotein detection after electrotransfer to nitrocellulose

Visualization of glycoproteins using concanavalin A and horseradish peroxidase was performed as described by Faye and Chrispeels [13]. The blocking and washing solutions, however, contained bovine serum albumin instead of gelatine. Detection of glycoproteins using soybean lectin or wheat germ agglutinin, both coupled to alkaline phosphatase was performed as follows: The lectins were coupled to alkaline phosphatase by incubating 1 mg of each lectin (Pharmacia) with 0.3 ml of enzyme solution (1 mg ml^{-1}, Sigma) for 2 h in 1 ml phosphate-buffered saline (PBS; pH 7.2) containing 0.2% glutardialdehyde. The solution was then dialysed against PBS and diluted with PBS to a final volume of 2 ml.

After blocking (see above), the nitrocellulose sheet was incubated in 10 ml Tris-buffered saline (TBS) containing 0.2 mg alkaline phosphatase-coupled lectin for 2 h and washed several times with TBS. For the alkaline phosphatase reaction the following stock solutions were

prepared: (i) solution of 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma; 50 mg ml^{-1} in dimethylformamide) and (ii) solution of Nitro blue tetrazolium (NBT, Sigma; 50 mg ml^{-1} in 70% dimethylformamide). The membrane was incubated in 10 ml of reaction buffer (100 ml Tris, pH 9.9, 100 mM NaCl, 5 mM $MgCl_2$) containing 33 µl BCIP and 66 µl NBT stock solution. After appearance of purple bands the reaction was stopped by rinsing the membrane with water.

Chromatographic procedures

Final purification of the glycoprotein was performed using a Bakerbond C_{18} WP column (Baker, 4.6 mm × 250 mm, particle size 5 µm, pore diameter 30 nm) at 30 °C. Tryptic peptide maps were performed using either a Waters µBondapak C_{18} column (3.9 mm × 300 mm, particle size 10 µm, pore diameter 10 nm) or a Waters Deltapak C_{18} column (3.9 mm × 150 mm, particle size 5 µm, pore diameter 10 nm). In all cases the following solvents were used: solvent A, 0.1% trifluoroacetic acid (TFA) in water; solvent B, 0.1% TFA in acetonitrile. Gradient program 1 (0–2 min, 20% B; 2–26 min, 20–68% B [linear gradient]; 26–36 min, 68% B; flow rate 1 ml min^{-1}) was used for protein purification, program 2 (0–55 min, 0–55% B [linear gradient]; 55–70 min, 55% B; flow rate 1 ml min^{-1}) was used for the tryptic peptide maps. Before HPLC analysis, tryptic reaction mixtures were reduced by incubation with 3.5 M urea and 0.05 M DTT for 2 h.

Sialic acid-containing oligosaccharides were separated using a Dionex Bio LC system as described recently [14]. Briefly, purified oligosaccharides dissolved in water were separated on a CarboPak PA-1 column (Dionex) at room temperature using solvents A (0.1 M NaOH) and B (0.25 M sodium acetate in 0.1 M NaOH) for the following gradient program: 0–100% B in 50 min (linear gradient, flow rate 1 ml min^{-1}).

Desalting of samples and separation of oligosaccharides after sequential degradation with exoglycosidases were achieved by gel filtration using a Bio-Gel P-2 column (Bio Rad; -400 mesh, 0.5 cm × 50 cm) or a Bio-Gel P-4 column (Bio Rad; -400 mesh, 0.6 cm × 200 cm), respectively. Elution was performed at room temperature with 0.02% aqueous sodium azide.

For monitoring of radiolabelled compounds, aliquots of the fractions obtained were supplemented with scintillation cocktail (Rotiszint 2211; Roth, Karlsruhe, FRG) and tested for radioactivity in a liquid scintillation counter.

Enzymatic reactions

Trypsin treatment: RP-HPLC-purified $sFc_\varepsilon RII$ was dissolved in 1% w/v NH_4HCO_3 yielding a solution of 1 mg ml^{-1}. 1% w/w trypsin (sequencing grade, Boehringer Mannheim) was added. After 6 h incubation at 37 °C another 1% w/w trypsin was added and incubation was continued for 18 h.

Enzymatic deglycosylation either by neuraminidase (*Vibrio cholerae*) and endo-α-*N*-acetylgalactosaminidase (*Diplococcus pneumoniae*) or by α-mannosidase (jack beans) was performed according to the manufacturer's instructions. For sequential exoglycosidase degradation, neuraminidase and β-galactosidase (bovine testes) were used, applying the conditions suggested by the manufacturer. (All enzymes were purchased from Boehringer Mannheim.)

Peptide sequencing

Selected peak fractions from tryptic peptide maps were collected and dried in a SpeedVac concentrator. The peptides were redissolved in 75 µl 70% formic acid and directly applied to the cartridge of an Applied Biosystems model 477 A pulsed liquid phase sequencer. The sequencer cycles FIL-1, BEGIN-1 and NORMAL-1 were used as suggested by the manufacturer. Amino acid derivatives were detected by UV absorption at 269 nm. Retention times of the samples were compared to a set of standards.

Methylation analysis

Oligosaccharide alditols were permethylated [15] and then hydrolysed with 0.5 N H_2SO_4 in 90% acetic acid. Partially methylated alditol acetates obtained after sodium borohydride reduction and peracetylation were analysed by GLC/MS using the instrumentation and microtechniques described earlier [16].

^{252}Cf Plasma desorption mass spectrometry (PD-MS)

Mass spectra of tryptic peptides were obtained using a BIO-ION 20 time-of-flight mass spectrometer (BIO-ION Nordic AB, Uppsala, Sweden). Dried samples were dissolved in aqueous TFA (0.1%) and deposited on nitrocellulose-coated targets (BIO-ION). Spectral accumulation times ranged between 0.5 h and 12 h depending on the yield. The primary ion start rate was about 1000 fission fragments per second. Spectra were obtained at an acceleration voltage of 17 kV. Mass assignments were calculated from peak centroids using H^+ and Na^+ for calibration.

Results

Final purification of $sFc_\varepsilon RII$ produced in CHO cells and yeast

Final purification of CHO-$sFc_\varepsilon RII$ using RP-HPLC yielded two main peaks that contained $sFc_\varepsilon RII$. N-Terminal sequencing revealed that the earlier eluting peak was the correctly processed form, whereas the later eluting peak consisted of a polypeptide species still carrying part of the leader sequence. For isolation and further analyses of the oligosaccharides, both peaks were combined and used as a mixture. For all other experiments, only the correctly processed form of the protein was used.

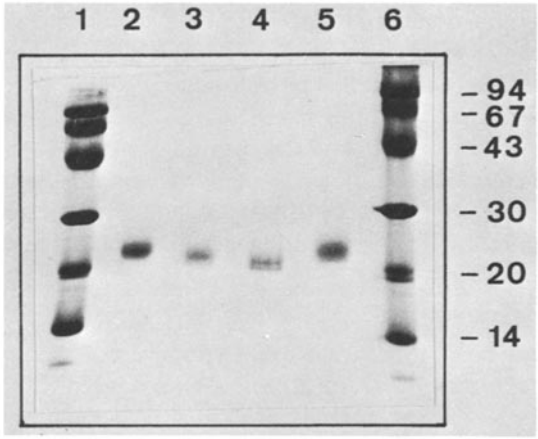

Figure 1. SDS-PAGE of CHO cell-derived $sFc_{\varepsilon}RII$. Proteins were reduced with DTT, separated on a 15% polyacrylamide gel and stained with Coomassie blue; molecular mass scale in kDa; lane 1, lane 6, Pharmacia LMW Calibration Kit; lane 2, lane 5, untreated CHO-$sFc_{\varepsilon}RII$; lane 3, neuraminidase-treated CHO-$sFc_{\varepsilon}RII$; lane 4, neuraminidase and O-glycosidase-treated CHO-$sFc_{\varepsilon}RII$.

Also, yeast-$sFc_{\varepsilon}RII$ was purified by RP-HPLC as a final step. In this case only the correctly processed form was found.

SDS-PAGE and affinoblotting of CHO-$sFc_{\varepsilon}RII$ before and after treatment with glycosidases

The apparent molecular mass of untreated $sFc_{\varepsilon}RII$ determined by SDS-PAGE was M_r 25,000. Treatment of the protein with neuraminidase and with endo-α-*N*-acetylgalactosaminidase (O-glycosidase) resulted in an increase in electrophoretic mobility, indicating that the protein was glycosylated (Fig. 1).

After electrotransfer to nitrocellulose, the untreated CHO-$sFc_{\varepsilon}RII$ showed positive reaction both with wheat germ agglutinin (WGA; specific for *N*-acetylneuraminic acid and *N*-acetylglucosamine) and soybean lectin (SBL; specific for *N*-acetylgalactosamine and to a lower degree for galactose). As expected, the desialylated glycoprotein no longer reacted with WGA, whereas the reaction with SBL still occurred. After complete deglycosylation of the protein neither of the two lectins reacted (data not shown).

Identification of the glycosylation site of CHO-$sFc_{\varepsilon}RII$

Purified radiolabelled CHO-$sFc_{\varepsilon}RII$ was digested with trypsin. The resulting (glyco)peptides were reduced and separated by RP-HPLC. Peak fractions detected by absorption (UV, 214 nm) were collected and an aliquot of each peak was tested for radioactivity in a scintillation counter (Fig. 2). Radioactive peaks (peaks 1, 5, 6 in Fig. 2) were submitted to sequence analysis. Apart from minor amounts of other peptides, each of these peaks contained the peptide with the following amino acid sequence

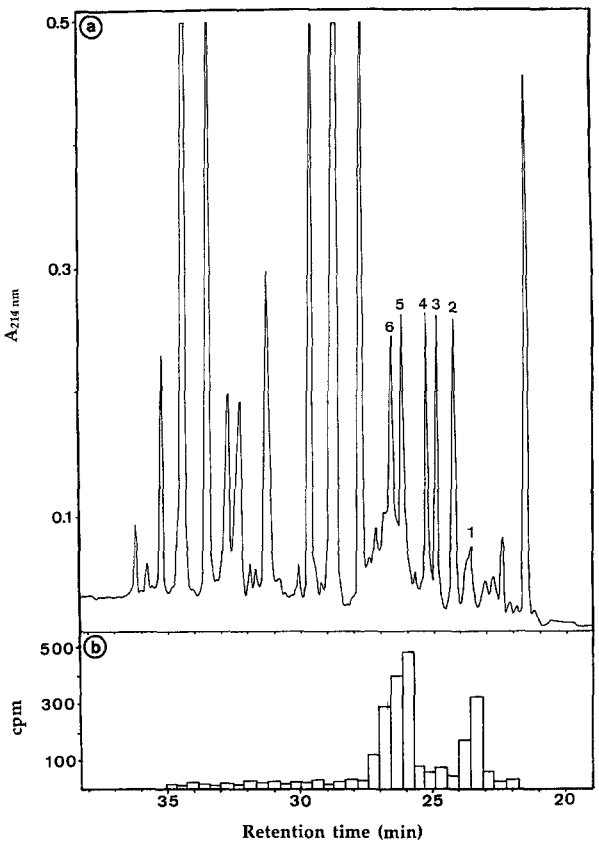

Figure 2. Tryptic peptide map of $sFc_{\varepsilon}RII$ derived from CHO cells. CHO-$sFc_{\varepsilon}RII$, metabolically labelled using [^{3}H]glucosamine, was treated with trypsin. Following reduction with DTT, resulting peptides were fractionated by RP-HPLC using a Waters Deltapak C_{18} column (3.9 mm × 150 mm) and a linear gradient of acetonitrile (0–55% in 55 min) in 0.1% TFA. (a) Absorbance was monitored at 214 nm; numbered peaks were further analysed by amino acid sequencing. (b) Aliquots of the peak fractions were monitored for radioactivity by liquid scintillation counting.

(numbers refer to the soluble part of $Fc_{\varepsilon}RII$ only):

136 140 145 147
Leu-Ala-Thr-[Cys]-Thr-Pro-Pro-Ala-Ser-Glu-Gly-(Ser)-Ala-Glu-

150 155 160
Ser-Met-Gly-Pro-Asp-Ser-Arg-Pro-Asp-Pro-Asp-Gly-Arg

Cys-139 could not be identified, but is inferred on the basis of the cDNA sequence. In all cases Ser-147 was detectable in significantly smaller amounts than expected from the yields of Ser-144, Ser-150, and Ser-155. Figure 3 shows the Ser-yield (pmol Ser from cycle 1 to 25, corresponding to amino acids 136–160) of the sequence analysis of peak 5 in Fig. 2a. This indicates that at least a major part of CHO-$sFc_{\varepsilon}RII$ contains the carbohydrate moiety attached to Ser-147. The two Thr residues which also could be substituted by glycans were detected in amounts which were

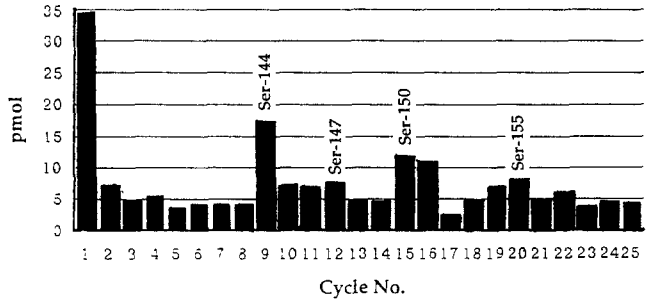

Figure 3. Histogram of Ser yields of the sequencing run of CHO-sFc$_\varepsilon$RII peak 5 (see Fig. 2a). Cycles with Ser identified as the amino acid present are indicated.

expected for nonglycosylated Thr residues, indicating that no carbohydrates are attached to Thr-138 and Thr-140.

Characterization of the oligosaccharides of CHO-sFc$_\varepsilon$RII

Oligosaccharides were released from the glycoprotein by β-elimination and separated according to charge by a Dionex high-pH anion-exchange chromatography (HPAEC) system (not shown). Two oligosaccharide fractions, A and B, containing one and two negative charges, respectively, were isolated and submitted to methylation analysis (Table 1). Fraction A contained 3-substituted galactose and 3-substituted GalNAcOH in about equimolar proportions, which indicates a monosialylated Gal(β1-3)GalNAcOH disaccharide. In fraction B, 3-substituted galactose and 3,6-disubstituted GalNAcOH were found, indicating an disialylated disaccharide. Due to the small amount of starting material, the peak eluting in the void volume (which may contain neutral oligosaccharides) was not further purified or characterized.

Table 1. Methylation analysis of oligosaccharides from CHO-sFc$_\varepsilon$RII. Oligosaccharide alditol fractions isolated from metabolically radiolabelled CHO-sFc$_\varepsilon$RII by β-elimination and HPAEC fractionation were permethylated and hydrolysed. The partially methylated alditol acetates obtained after reduction and peracetylation were analysed by capillary GLC/MS. Results are expressed in peak ratios of the alditol derivatives found. 2,4,6-GalOH, 2,4,6-tri-*O*-methylgalactitol; 1,4,5,6-GalN(Me)AcOH, 2-deoxy-2-(*N*-methyl)acetamido-1,4,5,6-tetra-*O*-methylgalactitol; 1,4,5-GalN(Me)AcOH, 2-deoxy-2-(*N*-methyl)acetamido-1,4,5-tri-*O*-methylgalactitol. Values are based on 1,4,5,6-GalN(Me)AcOH (fraction A) or 1,4,5-GalN(Me)AcOH (fraction B) = 1.0.

	Oligosaccharide alditol fraction	
Alditol derivative	*Fraction A*	*Fraction B*
2,4,6-GalOH	0.8	0.7
1,4,5,6-GalN(Me)AcOH	1.0	–
1,4,5-GalN(Me)AcOH	–	1.0

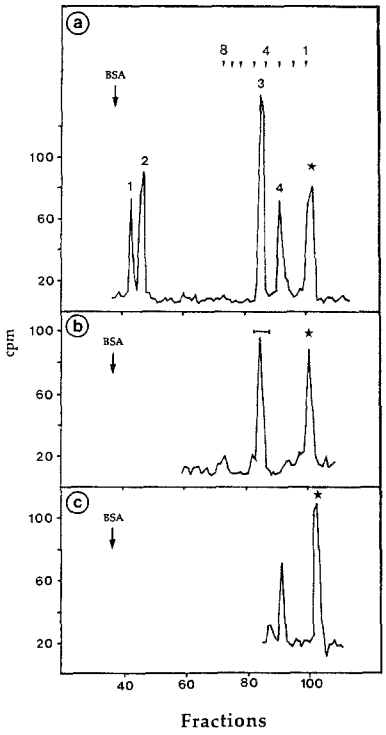

Figure 4. Sequential degradation of radiolabelled CHO-sFc$_\varepsilon$RII oligosaccharide alditols with exoglycosidases. Standards and reaction products were chromatographed on a calibrated Bio-Gel P-4 column (-400 mesh; 0.6 cm × 200 cm) at room temperature and hydrostatic pressure using 0.02% aqueous sodium azide as eluent. Fractions (0.4 ml) were collected and monitored for radioactivity. [^{3}H]mannose (★) and bovine serum albumin (BSA) were added to each sample as internal standards. (a) Standard mono- and oligosaccharides: (1) NeuAc(α2-3)Gal(β1-3)GalNAcOH; (2) NeuAc; (3) Gal(β1-3)GalNAcOH; (4) GalNAcOH; numbers (1–8) with small arrows indicate elution positions of isomaltosyl oligosaccharides (IM 1–8); (b) CHO-sFc$_\varepsilon$RII oligosaccharides after treatment with neuraminidase; (c) CHO-sFc$_\varepsilon$RII oligosaccharides (pooled as indicated by the bar in (b)) after treatment with β-galactosidase.

In a second experiment, the whole oligosaccharide mixture was submitted to methylation analysis, which demonstrated the presence of about 15% of unsubstituted (= terminal) galactose and 85% of 3-substituted galactose (data not shown). Thus, it may be concluded that total CHO-sFc$_\varepsilon$RII glycans also contain small amounts of neutral disaccharides.

The anomeric configurations of the glycosidic linkages of the monosaccharide constituents were determined by sequential degradation with exoglycosidases using neuraminidase (from *V. cholerae*) and β-galactosidase (from bovine testes). As shown in Fig. 4b, neuraminidase-treated oligosaccharides co-eluted with the authentic standard disaccharide Gal(β1-3)GalNAcOH (peak 3 in Fig. 4a). After treatment of this disaccharide with β-galactosidase the resulting product (Fig. 4c) co-eluted with GalNAcOH (peak

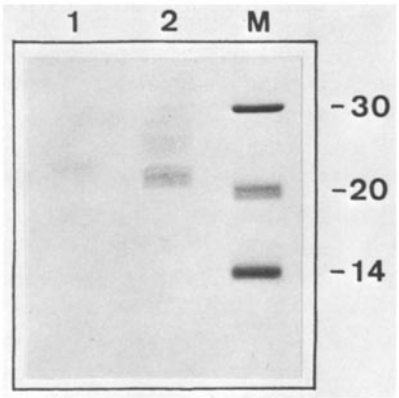

Figure 5. SDS-PAGE of sFc$_\varepsilon$RII derived from yeast. Proteins were reduced with DTT, separated on a 15% polyacrylamide gel and stained with Coomassie blue: M, Pharmacia LMW Calibration Kit (scale in kDa); lane 1, untreated yeast-sFc$_\varepsilon$RII; lane 2, yeast-sFc$_\varepsilon$RII after treatment with α-mannosidase. Higher molecular weight bands derive from impurities of the α-mannosidase preparation.

4 in Fig. 4a). From these results a (β1-3) linkage between galactose and *N*-acetylgalactosamine can be deduced.

SDS/PAGE and affinoblotting of yeast-derived sFc$_\varepsilon$RII before and after treatment with α-mannosidase

RP-HPLC-purified yeast-sFc$_\varepsilon$RII was treated with α-mannosidase (from jack beans) and analysed on an SDS gel together with the untreated glycoprotein. As shown in Fig. 5, the electrophoretic mobility of yeast-sFc$_\varepsilon$RII increased upon treatment with α-mannosidase. In another experiment, the affinity of yeast-sFc$_\varepsilon$RII to concanavalin A (ConA) was tested. Untreated sFc$_\varepsilon$RII showed positive reaction with ConA while α-mannosidase-treated sFc$_\varepsilon$RII was no longer recognized by ConA (data not shown). These results indicate the presence of sugar chains containing mannosyl residues.

Identification of the glycopeptide of yeast-sFc$_\varepsilon$RII by comparative tryptic peptide mapping

Both untreated and α-mannosidase-treated yeast-sFc$_\varepsilon$RII were digested with trypsin. Reaction products were reduced and applied to RP-HPLC. Comparing the two peptide maps (Fig. 6), it is obvious that peak 2 (retention time 27.7 min in panel a) of the untreated glycoprotein shifted to a longer retention time (28.4 min, peak 5 in panel b) when the protein was deglycosylated. All other peaks remained unchanged. This increase in hydrophobicity upon treatment with α-mannosidase strongly suggests that peak 2 of the untreated sample contains a glycopeptide.

Identification of the glycosylation site of yeast-sFc$_\varepsilon$RII

Peaks 2 and 5 in Fig. 6, panel a and b, respectively, were submitted to sequence analysis. Both showed the amino acid

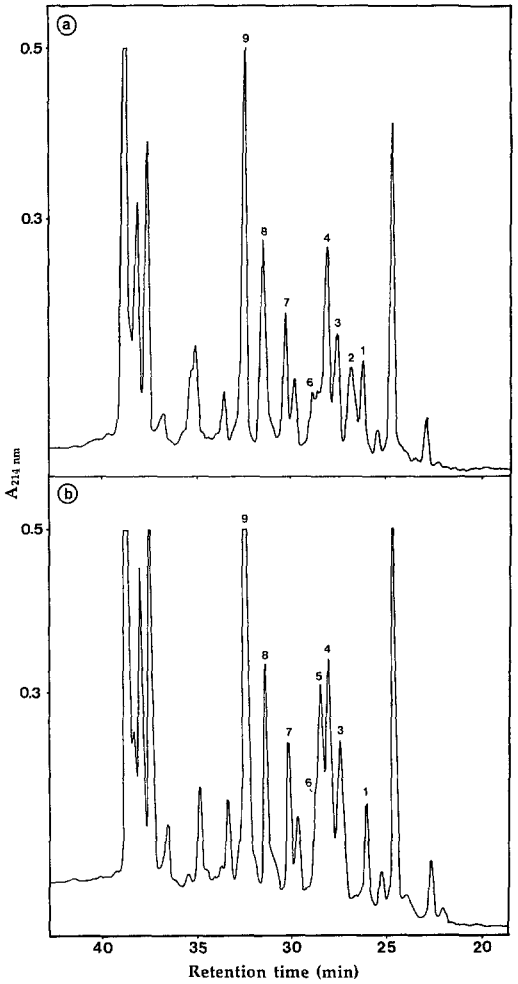

Figure 6. Tryptic peptide maps of sFc$_\varepsilon$RII derived from yeast. Yeast-sFc$_\varepsilon$RII was cleaved with trypsin before and after treatment of the glycoprotein with α-mannosidase. Following reduction with DTT, resulting peptides were fractionated by RP-HPLC using a Waters μBondapak C_{18} column (3.9 mm × 300 mm). Elution conditions were as described in Fig. 2. (a) Separation of fragments obtained from untreated glycoprotein. (b) Peptide map obtained from deglycosylated sFc$_\varepsilon$RII.

sequence of the C-terminal tryptic peptide:

163 167 172
Leu-Pro-Thr-Pro-Ser-Ala-Pro-Leu-His-Ser

Ser-167 was not detactable in peak 2 of the untreated sample, whereas the expected amount of Ser-167 was found in the corresponding peptide after treatment with α-mannosidase. This suggests that Ser-167 was the glycosylated amino acid. In addition, sequencing of the peptide which corresponded to the glycopeptide in CHO-sFc$_\varepsilon$RII (peaks 3, Fig. 6a,b) proved that Ser-147 in yeast sFc$_\varepsilon$RII was not glycosylated, as this serine was easily detectable (not shown).

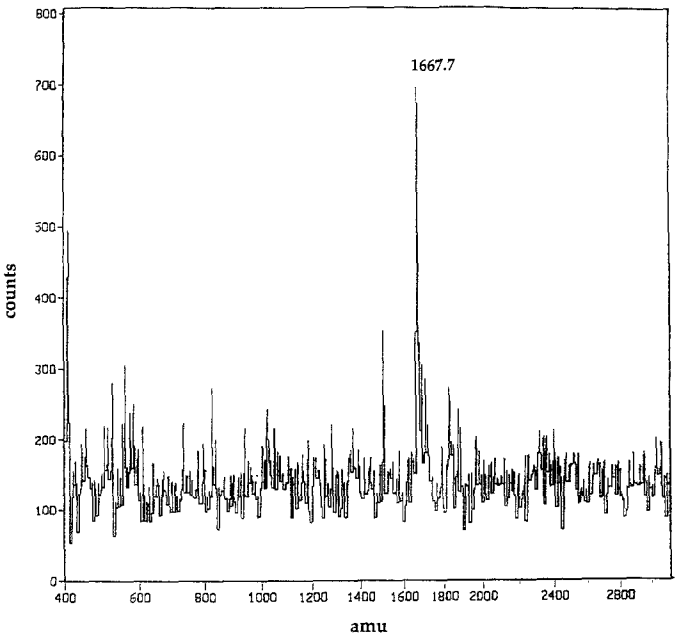

Figure 7. Plasma desorption mass spectrometry of yeast-sFc$_\varepsilon$RII. Tryptic peptide fraction 2 (peak 2 in Fig. 6a) was subjected to ^{252}Cf plasma desorption mass spectrometry. Molecular masses are given for the molecular ions ($[M + H]^+$). The peak at 1667.7 reflects the peptide carrying four hexose units.

^{252}Cf Plasma desorption mass spectrometry of the yeast-sFc$_\varepsilon$RII glycopeptide

The yeast-sFc$_\varepsilon$RII glycopeptide identified as described above was isolated and submitted to ^{252}Cf plasma desorption mass spectrometry. The resulting mass spectrum contained a major peak equivalent to a molecular mass of 1667.7 (Fig. 7). This value corresponds to a glycopeptide comprising amino acid residues 163–172, including four hexose units.

The peptide containing amino acids 136–162 was also submitted to PD-MS. In this case, only one peak equivalent to the expected molecular mass of the peptide without glycosylation (2703.9) was detected (not shown).

Discussion

In this study, glycans and glycosylation sites of recombinant sFc$_\varepsilon$RII produced both in yeast and in CHO cells were compared. As there is no consensus sequence (Asn-X-Ser/Thr) in this part of the protein N-glycosylation can be excluded. Natural and CHO cell-derived sFc$_\varepsilon$RII, however, were reported to be O-glycosylated [6, 10] although a detailed characterization of the carbohydrate part has not yet been published.

The oligosaccharide of CHO cell-derived sFc$_\varepsilon$RII consisted of the trisaccharide Sia(α2-3)Gal(β1-3)GalNAc as determined by sequential degradation with exoglycosidases and GC/MS following peracetylation/permethylation of the oligosaccharides. Small amounts of the asialo and disialo forms were also found. This is in good agreement with previous studies reporting that CHO cell-derived sFc$_\varepsilon$RII is susceptible to treatment with neuraminidase [10]. It has been reported recently that traces of *N*-glycolylneuraminic acid were detected in CHO-cell derived recombinant human glycoproteins [17]. Due to the small amounts of glycoprotein available we were not able to analyse the sialic acid residue in detail.

Metabolic labelling of the glycoprotein with [^{3}H]glucosamine resulted in incorporation of radioactivity in a single tryptic peptide, which eluted at different retention times from RP-HPLC due to different glycosylation. Sequencing of the radioactivity containing peaks (peaks 1, 5 and 6 in Fig. 2a) showed that each contained the glycopeptide [amino acids (AA) 136–162] among minor amounts of other peptides. In this peptide, the amount of the detectable Ser-147 was significantly lower than in the following Ser-containing cycles. A nonglycosylated peptide would not co-elute with a glycosylated one because of its higher hydrophobicity. Therefore, the residual amount of Ser-147 may be caused by partial hydrolysis of the oligosaccharide due to the long exposure of the peptide to the reaction conditions of the sequencing (12 cycles).

The glycopeptide of yeast derived sFc$_\varepsilon$RII was determined by comparing the tryptic peptide maps derived from both untreated and α-mannosidase treated sFc$_\varepsilon$RII. The glycopeptide eluted earlier from RP-HPLC than the deglycosylated one (peaks 2 and 5 in Fig. 6, panel a and b, respectively). Sequencing of the peptides showed that Ser-167 was not detectable in the peptide derived from the glycosylated protein (peak 2, Fig. 6a) whereas it was detected in the deglycosylated peptide (peak 5, Fig. 6b). Determination of the molecular mass of the glycopeptide derived from yeast-sFc$_\varepsilon$RII by PD-MS revealed that the majority of the glycans consisted of four hexyl residues. When analysed by SDS-PAGE, the molecular mass of the glycoprotein decreased upon α-mannosidase treatment to a value expected for completely unglycosylated sFc$_\varepsilon$RII. The rather large difference in molecular mass between the glycosylated and the deglycosylated protein is most likely to be due to the fact that carbohydrate parts often simulate a higher molecular mass than would be expected by summing up the masses of the components. On the basis of these results and previous reports on yeast glycoproteins [11, 18], we propose that the glycan consists of mannose exclusively. O-Glycosylation involving a Ser(Thr)-O-GalNAc linkage, which is most common in mammalian cells, has never been reported to occur in yeast. Therefore, it is very unlikely that this type of linkage is present in the yeast derived sFc$_\varepsilon$RII.

In contrast to the results obtained for CHO cell derived sFc$_\varepsilon$RII, Ser-147 in the corresponding peptide was easily detectable, indicating that Ser-147 was not glycosylated in yeast sFc$_\varepsilon$RII. Meanwhile, by sequencing the peptide AA

163–172 of the CHO-cell derived glycoprotein, Ser-167 was found not to be glycosylated. In addition, analysis of this peptide by PD-MS showed that the molecular mass was similar to that expected for the nonglycosylated peptide.

Both glycosylation sites are located in a Pro-, Ser- and Thr-rich region within the C-terminal part of the molecule. O-Glycosylation in such regions has been described previously for several glycoproteins (see, for example, [19, 20]).

The present finding that different glycosylation sites are used in yeast- and CHO cell-derived $sFc_{\varepsilon}RII$ may be due to the presence of Pro at the N-terminal side of Ser-167, which seems to stimulate the transfer of mannosyl residues from dolichol monophosphate mannose during yeast O-glycan biosynthesis [11]. On the other hand, it has been reported that Gly-residues located at the N-terminal side of Ser or Thr might inhibit O-glycosylation in yeast glycoproteins [21]. Therefore, glycosylation of Ser-147 in the yeast glycoprotein cannot be expected. To our knowledge, this is the first time that different O-glycosylation sites in a recombinant glycoprotein have been observed, depending on whether the protein is expressed in yeast or in mammalian cells.

Acknowledgement

We thank Dr E. Falkner from this institute for performing yeast fermentation and T. Gramanitsch, R. Spies, M. Scherer, S. Kühnhardt, and W. Mink for excellent technical assistance. Fruitful discussions with Professor G. Bodo and Dr G. R. Adolf are gratefully acknowledged.

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