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ENDO-GLYCOSIDASE F AND PEPTIDE N-GLYCOSIDASE F RELEASE THE GREAT MAJORITY OF TOTAL CELLULLAR N-LINKED OLIGOSACCHARIDES: USE IN DEMONSTRATING THAT SULFATED N-LINKED OLIGOSACCHARIDES ARE FREQUENTLY FOUND IN CULTURED CELLS

Hudson H. Freeze and Ajit Varki

Cancer Biology Program, UCSD Cancer Center and Department of Medicine, University of California at San Diego, T-012 San Diego, CA 92093

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Subtle modifications of N-linked oligosaccharides are known to mediate important biological functions. However, many of these modifications are destroyed by chemical treatments used to release oligosaccharides from the proteins. Therefore, enzymatic release of the intact, modified oligosaccharides is preferred. We have used a mixture of endoglycosidaseF and Peptide: N-glycosidaseF to release most, if not all, of the [2-^{3}H]Man-labelled N-linked oligosaccharides from a wide variety of mammalian cell lines. This approach combined with biosynthetic radiolabelling could, therefore, be used to identify novel, rare or transient modifications of N-linked oligosaccharides. This treatment indeed released metabolically incorporated [$^{35}SO_4$] from each of 9 different cell lines demonstrating that sulfated N-linked oligosaccharides are much more common than previously believed.

The complete structural analysis of N-linked oligosaccharides is best done after they are released from the peptide backbone. This allows fractionation and analysis based solely on the properties of the sugars rather than on variable amounts of peptide found in glycopeptides. Most glycosidic linkages are stable to harsh chemical treatments such as hydrazinolysis or strong base, which are used to release N-linked oligosaccharides from the peptide backbone (1). However, these methods can destroy, O-acetyl, phosphoryl and sulfate esters found on the sugars (2-5). Since such substitutions can be physiologically significant, (2,4,6,7,13-16) it is important to develope non-degradative methods to release oligosaccharides.

Endoglycosidases H,-D and -CII cleave a limited range of N-linked oligosaccharides (1,8) and, at best, release only about half of the N-linked oligosaccharides of most mammalian cells (9). On the other hand N-acetylglucosaminidaseF (EndoF) and Peptide:N-glycosidase F (PNGaseF) have recently been shown to cleave both high-mannose and complex-type chains found on randomly selected

Abbreviations used: EndoH, β-endoglucosaminidase H; EndoF/PNGaseF, endoglycosidase preparation which contains both endoglycosidase F and peptide:N-glycosidase F activities; PNGaseA, peptide N-glycosidase from almond emulsion.

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individual glycoproteins (10). In this study we used the latter two enzymes to release the great majority of the [2-^{3}H]Man labelled N-linked oligosaccharides from a wide variety of mammalian cell lines. Furthermore, all of these cell lines were found to contain sulfated N-linked oligosaccharides, showing that this modification is very widespread.

MATERIALS and METHODS

Enzymes. EndoH was from Miles Laboratories, Peptide:N-glycosidase A (PNGaseA) was from Dr. Anthony Tarentino, New York State Department of Public Health (24,25), and Endo-β-N-acetylglucosaminidaseF/peptide:N-glycoside F (EndoF/PNGaseF) was a gift of Drs. John Elder and Stephen Alexander, Scripps Clinic and Research Foundation, La Jolla California. The notation EndoF/PNGaseF is used because the preparation contains a mixture of the two activities (11, 12).
Radiochemicals. [2-^{3}H]Man (10Ci/mmol) was from American Radiolabelled Chemicals, St. Louis, MO. and $^{35}SO_4$ from I.C.N. ^{35}S Methionine was from NEN.
Growth and labelling of cell lines was done in RPMI 1640 medium with 10% fetal calf serum in 5%CO_2 at 37^0 C. Subconfluent cell lines (10^7) were labelled with 590μCi of [2-^{3}H]Man and 500μCi of $^{35}SO_4$ for 2-3 days, harvested at 2000 x G for 10 minutes, and washed in ice-cold PBS.
Release and isolation of oligosaccharides. The labelled cells were dissolved in 500μl of 20mM phosphate buffer pH 7.0 containing 1% SDS/0.1M 2-mercaptoethanol (2-ME) and heated for 5 min at 100^{0}C. Following centrifugation at 10,000 x g for 5 min, the solubilized material was applied to a 0.7 x 59 cm column of Sephadex G-50 equilibrated in 20mM Tris HCl, pH 7.5, containing 0.2% SDS, and eluted with the same buffer. The radioactivity in the void volume (V_0) was pooled, and precipitated by adding acetone to a final concentration of 90%. After 3-5 hours at -20^{0}C the precipitate was collected by centrifugation at 2000 x g for 10 min. This precipitate, which contained 90-98% of both the ^{3}H and ^{35}S radioactivity, was redissolved in 50-100μL of 1% SDS/0.1M 2-ME by heating at 100^{0}C for 5 min. The sample was adjusted to 0.2% SDS, 0.02M 2-ME, 1%NP-40, and 0.1M sodium phosphate buffer, pH 6.3 (final volume 500μl). One unit of EndoF/PNGaseF was added (1u = 1nmol/hr) and incubated at 37^{0}C for 24 hours followed by an additional 0.5u of EndoF/PNGaseF for another 24 hours. The EndoH digestions of denatured proteins were carried out with 2mU (1mU - 1nmol/min) in 50mM citrate-phosphate buffer, pH 5.5 in the presence of 0.05% SDS and 0.5% NP-40. PNGaseA digestions of denatured proteins were carried out with 1mU of enzyme (1nmol/min) in 0.1M sodium acetate, pH 5.5 with 0.1% SDS and 0.4% NP-40. The reaction mixture was boiled for 5 min, centrifuged at 10,000 x g for 5 min, and the supernatant fluid re-applied to the Sephadex G-50 column.
Base/borohydride release of O-linked oligosaccharides. The V_0 pool of the "EndoF/PNGaseF resistant" material was precipitated with acetone, and treated with 0.2M NaOH with 0.2M $NaBH_4$ for 48 h at room temperature (13). After acidification and repeated evaporation in methanol the sample was reapplied to the Sephadex G-50 column.
Determination of [^{3}H]Man and [^{3}H]Fuc. Aliquots of each desalted sample were hydrolysed with 2N HCl at 100^{0}C for 4h. After drying, the released monosaccharides were desalted on small columns of Amberlite MB-3 and chromatographed on Whatman 3MM paper for 18-48 hours in n-butanol:acetic acid:water (4:1:5) (upper phase). Non-radioactive sugar standards were monitored with alkaline silver nitrate.

RESULTS

Principles of the Approach. Radiolabelled denatured macromolecules isolated from the void volume of a Sephadex G-50 column are treated with the enzymes and reapplied to the same column under identical conditions. The released material is clearly separated from the resistant species that remain in the Vo and is thus operationally defined as N-linked oligosaccharides (see Figures 1 & 2). Of course, very large or highly charged released oligosaccharides could still run in the V_0, and would not appear to be cleaved. The released material can then be freed of SDS and salt for further study (8).

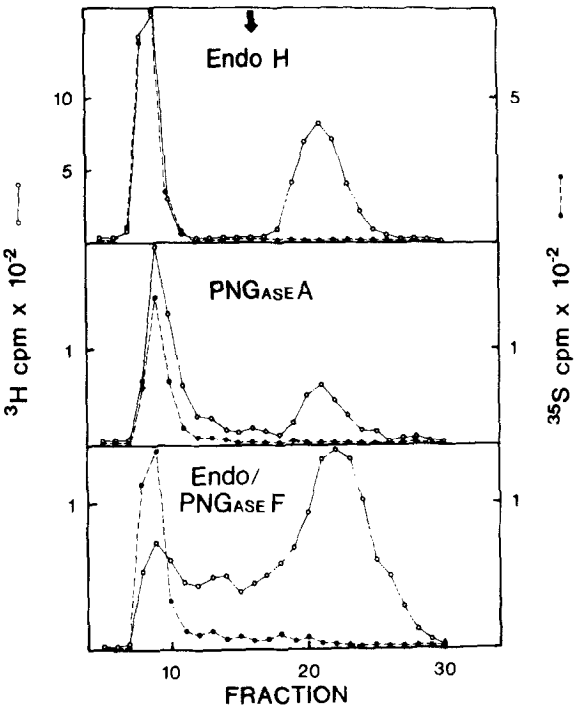

Figure 1. Sephadex G-50 Analysis of Endoglycosidase or Peptide:N-glycosidase Digestions of The Macromolecular Fraction from K-562 cells. The cells were labelled and the macromolecular fraction isolated from the V_0 of the Sephadex G-50 column as described. Separate aliquots were treated with EndoH(90h), PNGaseA (48h) or EndoF/PNGaseF (48h). The digests were analysed on Sephadex G-50. and fractions monitored for ^{3}H and ^{35}S radioactivity. The arrow represents the positon of elution of EndoF/PNGaseF released triantennary oligosaccharide from fetuin in a separate elution.

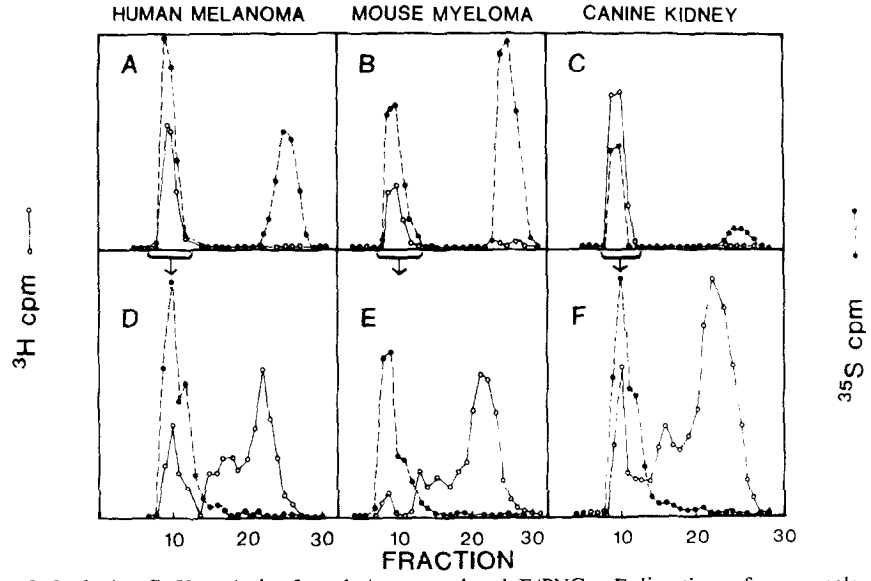

Figure 2. Sephadex G-50 analysis of crude lysates and endoF/PNGaseF digestions of macromolecular fractions. Cell lines were labelled with [^{3}H]Man/$^{35}SO_4$, solubilized and applied to the Sephadex G-50 column as described (Panels A,B and C). Aliquots (1%) of each fraction were monitored for ^{3}H and ^{35}S radioactivity. The macromolecular fractions were pooled, precipitated with acetone, treated with EndoF/PNGaseF, and reapplied to the column (Panels D,E and F). The three examples shown in this figure are FM9 (human melanoma), P3x63Ag8 (mouse myeloma), and MDCK (canine kidney). Because of the wide variations in the amount of radioactivity incorporated into each cell lines, the scales for ^{3}H and ^{35}S cpm are not shown on the figure. The radioactivity (^{3}H and ^{35}S cpm x 10^{-3}) loaded on the column in each case was as follows: Panel A (252/255), Panel B (110/872) and Panel C (162/593).

Release of N-linked Oligosaccharides from Total Cellular Proteins. We used this approach to release the [2-^{3}H]Man labelled macromolecular materials from a variety of cultured mammalian cell lines. As an example, the human erythroleukemia cell line K-562 was first labelled with [2-^{3}H]Man and $^{35}SO_4$ and aliquots of the V_0 from Sephadex G-50 were treated separately with EndoH, PNGaseA or EndoF/PNGaseF and reapplied to the same column. As shown in Figure 1, EndoH released only a restricted size class of [^{3}H]-labelled oligosaccharides (52%), and none (<0.2%) of the ^{35}S label. PNGaseA treatment released 45% of the ^{3}H label of 7% of the ^{35}S label and EndoF/PNGaseF treatment released 85% of the ^{3}H label and 20% of the ^{35}S label. Clearly the EndoF/PNGaseF digestion was the most effective. Note that in the latter two digestions, there was considerable heterogeniety in the size/charge of the released ^{3}H and ^{35}S labelled oligosaccharides. Since EndoH digestion gave no (<0.2%) ^{35}S release, this suggests that the ^{35}S radioactivity found outside the V_0 region in the case of both the EndoF/PNGaseF and the PNGaseA treatments is highly significant (see also Figure 2). No significant radioactivity ran in the position expected for free sulfate or monosaccharides, showing that sulfatases, mannosidases and/or fucosidases are not present in any of these enzyme preparations. The release of the label was not due to proteolysis since analysis of [^{35}S]Met-labelled cellular material digested with EndoF/PNGaseF for 60 hours gave no significant release of radioactivity from macromolecular fractions (<2%) (data not shown).

The same approach was then used to test a variety of cultured mammalian cell lines. The results shown in Table I indicate that EndoF/PNGaseF released the great majority of ^{3}H-Man label in each case, and a significant proportion of $^{35}SO_4$. This indicates that sulfated N-linked oligosaccharides are quite common in a diverse variety of mammalian cells.

Analysis of material resistant to EndoF/PNGaseF. In all cases, a small amount of the ^{3}H label remained in the V_0 region following digestion. One possible explanation for this material is that [2-^{3}H]Man was converted into [^{3}H]Fuc. To determine this, we subjected the EndoF/PNGaseF resistant fractions to strong acid hydrolysis and found that a variable fraction of the radioactivity was indeed in [^{3}H]Fuc (3-31%) (see Table 1). Another possible reason for the presence of resistant material is that the label is in O-linked oligosaccharides. To answer this question, aliquots of the EndoF/PNGaseF resistant fractions were treated with mild base/borohydride(13) under conditions that release O-linked oligosaccharides and then analyzed on Sephadex G-50. The results presented in Table 1 show that a major proportion of the resistant label was released in each case. The profiles of the released label were quite different from those obtained with EndoF/PNGaseF digestions (not shown). When the

TABLE 1. CELL LINES STUDIED FOR RELEASE OF N-LINKED OLIGOSACCHARIDES

NAME	CELL TYPE	% Radioactivity Released by Treatment with EndoF/PNGaseF[a]		% Radioactivity Released by Base/Borohydride after EndoF/PNGaseF[b]		Minimum % N-linked Oligos Released[c]	% Fuc in resistant fraction[d]
		^3H	^{35}S	^3H	^{35}S		
K562	Human Erythroleukemia	85	20	7.5	11	92	31
DS-19	Friend Erythro-leukemia	85	7	_[e]	_[e]	85	10
MDCK	Canine Kidney	86	14	3.0	22	89	30
BAEC11	Bovine Aortic Endo-thelial	81	11	2.0	1	83	30
FM9	Human Melanoma	96	10	1.4	3	97	3
$FM9_{met}$	Human Melanoma (metastatic variant)	88	4	3.6	2	91	23
T291	Human Lung Adeno-carcinoma	74	10	17	3	89	28
P3x63Ag8	Murine Myeloma	80	12	8.6	5	88	30
293	Human Embryonic Kidney	59	7	25	5	79	22

a) The released radioactivity (Y) is expressed as a percentage of the original amount (X) in Sephadex G-50 Vo i.e., Y/Xx100.

b) An aliquot of the endoF/PNGaseF resistant fraction (V_O of G-50 column after EndoF/PNGaseF treatment) was treated as described. The released radioactivity (Z) is expressed as a percent of the original starting radioactivity (X) i.e. Z/X x 100.

c) This estimate assumes that all of the ^3H label originally in the macromolecular fraction is either in N-linked or O-linked oligosaccharides, and that all ^3H not released by base is N-linked. The value is calculated as $\frac{\%\text{release by endoF/PNGaseF} \times 100}{(100 - \%\text{ released by base})}$

d) as described under Material and Methods

e) Sample lost during workup

composition of some of these released fractions was studied by acid hydrolysis and paper chromatography, they were found to contain predominantly of [^{3}H]Man, variable amounts of [^{3}H]Fuc, and only a small fraction that migrated with an internal [^{14}C] mannitol standard (data not shown). This implies that in many cells, Man exists in O-linked oligosaccharides, but most of these residues are not linked directly to the protein.

Since we can account for nearly all of the ^3H label, only a small portion of the remaining [^{3}H]-Man could be in EndoF/PNGaseF resistant N-linked oligosaccharides. Thus, implying that the enzymes can release most, if not all of the [2-^{3}H]Man-labelled N-linked oligosaccharides from so many different cells lines and suggests that essentially the entire range of such structures may be susceptible to the enzymes.

DISCUSSION

There are very few instances where *specific* N-linked oligosaccharide structures have been shown to encode *specific* biological functions that are of proven benefit to the organism. In two of the best established examples, the targetting of newly synthesized lysosomal enzymes (6) and the anticoagulant action of heparin (14), it is modifications such phosphorylation and sulfation that are the critical determinants of functional specificty. We believe that the discovery of other specific biological roles for sugar chains may depend upon the ability to identify and study other such minor and/or transient modifications of these molecules. In this study we have shown that a combination of EndoF and PNGaseF released most, if not all of the [2-^{3}H]Man-labelled N-linked oligosaccharides from a wide variety of cell lines. The released oligosaccharides could then be easily isolated and purified for further analysis.

The synthesis of sulfated N-linked oligosaccharides has previously been described only in certain cell types, such as those of pituitary (15-17) and endothelial (18) origin. In the case of the pituitary hormones, PNGaseF has recently been successfully used to extensively characterize the sulfated N-linked oligosaccharides (16,17). However,the existence of sulfate esters on certain pure glycoproteins (19-21) and on virus glycoproteins synthesized in several cell lines (22,23) suggests that such structures might very well be quite common. Our study shows that this is indeed the case. Metabolically-labelled $^{35}SO_4$ was specifically released by EndoF/PNGaseF from every one of a wide variety of cell lines. The ability to obtain pure labelled oligosaccharides under non-degradative conditions now allows further analysis of their structure. We are currently studying the detailed structure of these sulfated molecules in several of these cell lines. Our preliminary results suggest that there is considerable structural diversity and complexity in these molecules.

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