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MOLECULAR AND BIOSYNTHETIC HETEROGENEITY OF FUCOSYL GLYCOPROTEINS ASSOCIATED WITH RAT BRAIN SYNAPTIC JUNCTIONS

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Summary

The composition and biosynthesis of fucosyl glycoproteins present in rat brain synaptic membranes and synaptic junctions were investigated. Reaction with ^{125}I-labelled fucose-binding protein (*Lotus tetragonolobus*) following sodium dodecyl sulphate gel electrophoresis identified 6—8 fucosyl glycoproteins in synaptic membranes but only three major high molecular classes (M_r = 180 000, 130 000 and 110 000) in synaptic junctions. Affinity chromatography on concanavalin A-Sepharose resolved each of the synaptic junctional fucosyl glycoproteins into concanavalin A-positive and negative components indicating the presence of at least six high molecular weight fucosyl glycoproteins in synaptic junctions. Following the administration of [^{3}H]fucose synaptic membranes, synaptic junctions and post-synaptic densities incorporated isotope, the order of relative specific activities being synaptic membranes > synaptic junctions > post-synaptic densities. Fractionation of [^{3}H]-fucose-labelled synaptic junctions on concanavalin A-Sepharose revealed a time-dependent increase in the percentage of isotope associated with the concanavalin A-positive glycoproteins. The results demonstrate both molecular and biosynthetic heterogeneity of fucosyl glycoproteins associated with synaptic junctions.

Introduction

Glycoproteins present at central nervous system synapses have been proposed as mediators of a variety of synaptic functions ranging from recognition and adhesion during development to modulation of the activity of mature

synapses (for recent reviews see Refs. 1 and 2). Consistent with these proposed functions a wide variety of structurally diverse glycoproteins have been identified in synaptic membranes [3,4]. However, with the possible exception of a few components (e.g. the receptors for acetylcholine [5] and glutamate [6]), little is known concerning the precise functional role or localization of the majority of these proteins. In contrast to synaptic membranes isolated synaptic junctions and post-synaptic densities are associated with relatively few high molecular weight classes of glycoproteins [7—9]. These glycoproteins react with a variety of lectins, including concanavalin A [7—9] and most probably correspond to the receptors for concanavalin A which have been identified in the synaptic cleft using histochemical procedures [10,11]. The localization of these glycoproteins within the synaptic apparatus per se suggests that they may directly participate in synaptic interactions, and we have undertaken their molecular characterization with the aim of defining more specifically their possible functional role. In the present study we have used [^{3}H]fucose as a biosynthetic precursor, and the fucose-binding protein from *Lotus tetragonolobus* to investigate fucosyl glycoproteins associated with rat brain synapses. A preliminary report of this work has appeared [12].

Materials and Methods

Administration of isotope and preparation of subcellular fractions. [^{3}H]-Fucose (1—2 mCi) was dissolved in 10 mM sodium phosphate buffer, pH 7.4, containing 0.9% NaCl, and administered to ten 28—32-day-old Sprague-Dawley rats by intraventricular injections. Rats were killed at the indicated intervals between 1 and 24 h and the cortex removed for the preparation of subcellular fractions. A crude synaptic membrane fraction was prepared as described [13] and used for the isolation of synaptic junctions and post-synaptic densities according to the procedures of Cotman et al. [13]. In order to minimize contamination by sarcosyl-solubilized components post-synaptic densities were washed once by resuspending in 1.4 M sucrose and pelleting at 104 000 $\times g_{av}$ for 30 min. Isolated synaptic junctions were resuspended in 0.32 M sucrose and stored at −20°C until use. Post-synaptic densities were immediately solubilized by heating at 100°C for 3—5 min in 1% sodium dodecyl sulphate/4 M urea/10 mM mercaptoethanol. Microsomes were prepared from the post-mitochondrial supernatant as described [14].

Affinity chromatography and gel electrophoresis. Samples (200—300 μg protein) for gel electrophoresis and for affinity chromatography on concanavalin A-Sepharose were solubilized in 1% SDS/4 M urea/10 mM mercaptoethanol by heating at 100°C for 5 min. Prior to affinity chromatography samples were dialysed against 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1% sodium dodecyl sulphate (buffer A). Concanavalin A-Sepharose columns were washed with H_2O followed by buffer A immediately prior to the application of sample. Fresh columns were used for each sample. Following the elution of unretained components with buffer A, retained proteins were eluted with buffer A containing 5% α-methyl-D-mannopyranoside. Positive and negative fractions were precipitated overnight at 4°C following the addition of KCl to a final concentration of 0.14 M and precipitates were collected by centrifugation

(10 000 × g_{av}, 15 min). Recovery of radioactivity from the columns was generally in the range of 65—80% and 80—90% of the radioactivity was recovered in the precipitated fractions. KCl precipitates were used for the determination of radioactivity (see below) or for gel electrophoresis.

Electrophoresis on 7% polyacrylamide cylindrical gels was as described elsewhere [14] and on 10% polyacrylamide slab gels according to Laemmli [15]. Protein standards used for the estimation of apparent molecular weights included β-galactosidase (130 000), phosphorylase (94 000), bovine serum albumin (68 000), ovalbumin (45 000) and chymotrypsinogen (23 000). In some experiments resolubilized fractions from the affinity column were briefly dialysed against buffer A to reduce the KCl concentration immediately prior to electrophoresis. Similar results were obtained with or without inclusion of the dialysis step. Labelling of disc and slab gels with ^{125}I-labelled concanavalin A or ^{125}I-labelled fucose-binding protein (*L. tetragonolobus*) was as described previously [7,8]. The labelling and washing buffers contained 0.1% bovine serum albumin when fucose-binding protein was used. Control labelling experiments were done in the presence of 5% α-methyl-D-mannopyranoside (concanavalin A) of 5% α-L-fucose (fucose-binding protein). Gels were stained with Coomassie blue, and either sliced for counting [8] or dried for the preparation of radioautograms [9].

Determination of radioactivity. For the determination of radioactivity, membrane samples were precipitated with 10% trichloroacetic acid in the presence of 100 μg of bovine serum albumin. The precipitates were washed once with 10% trichloroacetic acid and then dissolved in 1 N NaOH for the determination of protein and radioactivity as described [3]. KCl precipitates obtained from the affinity columns were washed twice with acetone and once with 10% trichloroacetic acid prior to dissolving in 1 N NaOH for the determination of radioactivity.

Other methods. Concanavalin A was iodinated with ^{125}I using the lactoperoxidase procedure described previously [7]. Fucose-binding protein was iodinated using the chloramine T procedure exactly as described [16]. In each case the iodinated lectin was separated from free ^{125}I by chromatography on Sephadex G-25. The specific activity of the lectins generally was within the range of 0.2—0.3 μCi/mg. Protein was determined by the procedure of Lowry et al. [17].

Materials. ^{125}I and [6-^{3}H]fucose were purchased from New England Nuclear. Concanavalin A was obtained from Sigma and fucose-binding protein (*L. tetragonolubus*) from Miles Biochemicals. Concanavalin A-Sepharose was from Pharmacia.

Results

Identification of fucosyl glycoproteins

Synaptic junctions and synaptic membranes were isolated from rat cortices and fractionated by SDS gel electrophoresis as described in Materials and Methods. Reaction of the separated proteins with ^{125}I-labelled fucose-binding protein showed the presence of 7—8 molecular weight classes of fucosyl glycoproteins in synaptic membranes and three major classes, with apparent molecular

weights of 110 000, 130 000 and 180 000, in the synaptic junction fraction (Fig. 1). The latter glycoproteins correspond to the three major concanavalin A-binding proteins previously identified in this fraction (Refs. 8 and 9, and Fig. 2).

Fractionation of glycoproteins on concanavalin A-Sepharose

Synaptic junctional proteins were fractionated on columns of concanavalin

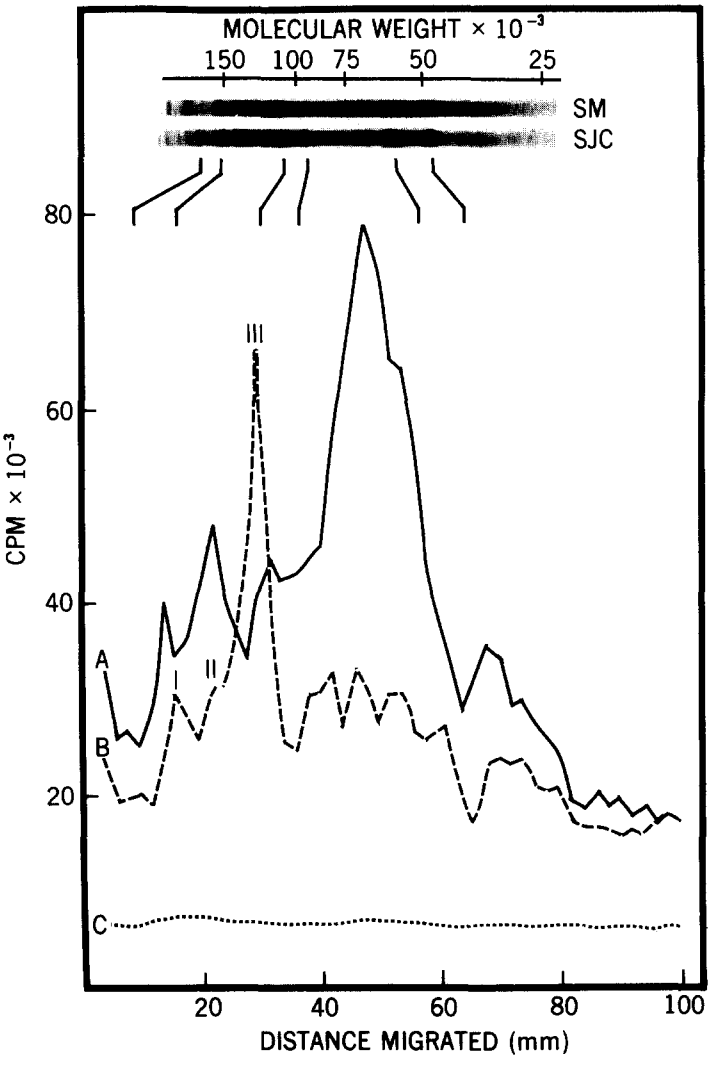


Fig. 1. Reaction of synaptic membrane and synaptic junctional glycoproteins with ^{125}I-labelled fucose-binding protein. Isolated fractions (200 μg protein) were separated by disc gel electrophoresis and reacted with ^{125}I-labelled fucose-binding protein as described in Materials and Methods. Gels were stained with Coomassie blue (photographs) and then sliced for the determination of radioactivity. (A) synaptic membranes; (B) synaptic junctions; (C) synaptic membranes in the presence of 5% α-L-fucose. I—III, glycoproteins with apparent molecular weights of 180 000, 130 000 and 110 000, respectively. SM, synaptic membranes; SJC, synaptic junctions.

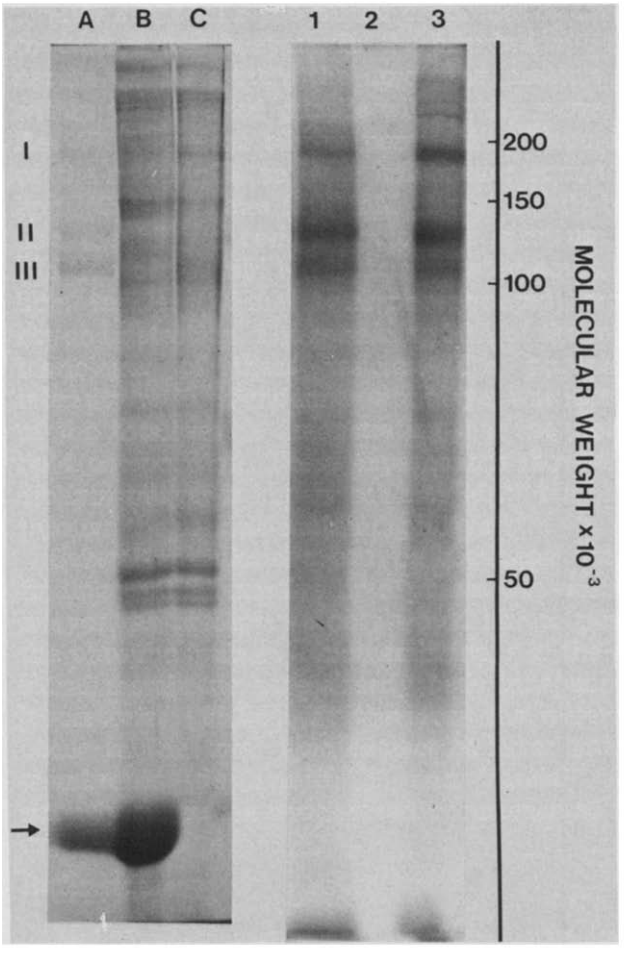


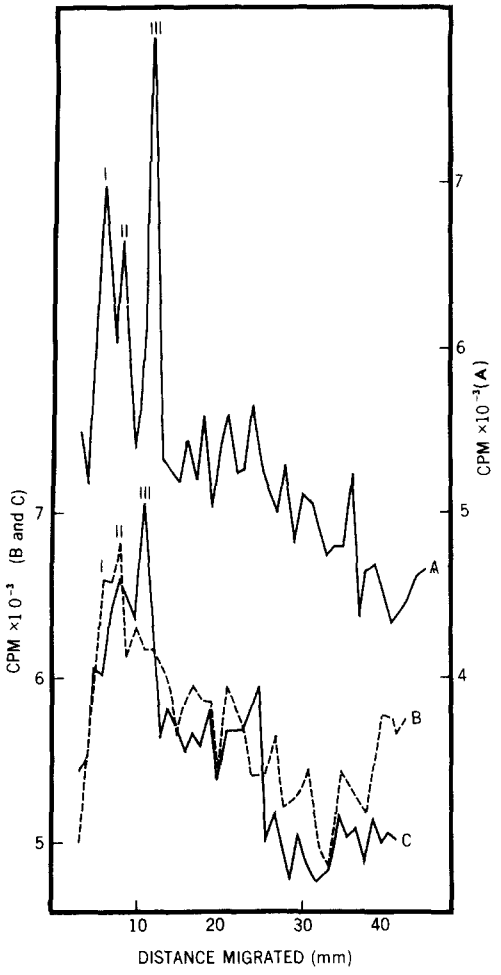
Fig. 2. Fractionation of synaptic junctional proteins on concanavalin A-Sepharose. Synaptic junctions were solubilized in sodium dodecyl sulphate and fractionated on concanavalin A-Sepharose as described in Materials and Methods. Total junctions and the retained and unretained fractions were analysed by electrophoresis on 10% polyacrylamide slab gels, reacted with ^{125}I-labelled concanavalin A, stained with Coomassie blue and radioautograms prepared. Columns represent protein stains (A—C) or radioautograms (1—3) of concanavalin-positive (A, 1) or negative (B, 2) fractions and total junctions (C, 3). I—III, glycoproteins with apparent molecular weights of 180 000, 130 000 and 110 000, respectively, and ↑, concanavalin A which is shed by the column.

A-Sepharose in the presence of 0.1% sodium dodecyl sulphate. Because previous authors have used 0.08% SDS in conjunction with lectin affinity columns [4] the efficiency of the present procedure was initially assessed by reacting slab gels of the retained and unretained fractions with ^{125}I-labelled concanavalin A. The results presented in Fig. 2 demonstrate that all concanavalin A-binding glycoproteins were retained by the column. Reaction of the column fractions with ^{125}I-labelled fucose-binding protein demonstrated that each molecular weight class was resolved into concanavalin A-positive and negative components (Fig. 3) although differential retention of individual glycoprotein classes

occurred. Thus glycoprotein fraction III (apparent M_r = 110 000) was preferentially retained whereas glycoprotein fraction I (M_r = 180 000) was more concentrated in the unretained fraction.

Incorporation of [^{3}H]fucose into synaptic glycoproteins

The biosynthesis of synaptic junctional fucosyl glycoproteins was measured



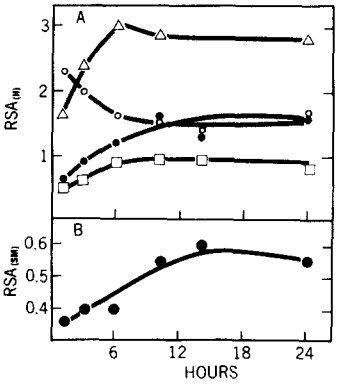


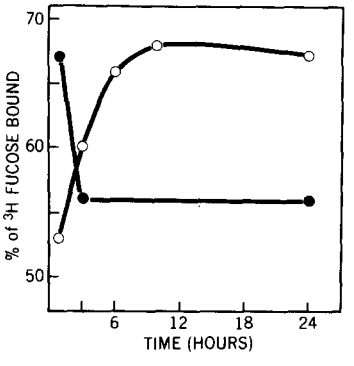
Fig. 3. Reaction of concanavalin A-positive and negative synaptic junctional fractions with ^{125}I-labelled fucose-binding protein. Synaptic junctions were solubilized in sodium dodecyl sulphate and fractionated on concanavalin A-Sepharose. Total (A), unretained (B) and retained (C) fractions were separated by disc gel electrophoresis and reacted with ^{125}I-labelled fucose-binding protein. Additional details are as in Fig. 1.

Fig. 4. Incorporation of [^{3}H]fucose into synaptic fractions. Rats received [^{3}H]fucose by intraventricular injection and subcellular fractions were prepared at the indicated times as described in Materials and Methods. △———△, synaptic membranes; ●———●, synaptic junctions; ○———○, microsomes; □———□, post-synaptic densities. The specific activity of the homogenate increased from 10 000 to 30 000 cpm/mg protein between 1 and 24 h. 1, 10 and 24-h points represent the averages of two separate preparations with variations of less than 10%. 3, 6 and 14-h points are the results of single experiments. (A) RSA$_{(H)}$, specific activity of fraction (cpm/mg protein)/specific activity of homogenate. (B) Specific activity of synaptic junctions relative to specific activity of synaptic membranes.

by following the incorporation of [^{3}H]fucose, a precursor which is uniquely incorporated into glycoproteins [18], into microsomes and synaptic organelles. Following the intraventricular administration of [^{3}H]fucose maximum incorporation of isotope into the microsomal fraction occurred within 1 h, the relative specific activity decreasing to a constant value by 10 h (Fig. 4A). In contrast to the microsomes, and consistent with a biosynthetic pathway involving glycosylation in the cell body followed by rapid transport to the nerve terminal, the relative specific activity of each of the synaptic subfractions increased two-fold before achieving constant values between 6 and 14 h after the administration of precursor. The slower incorporation of [^{3}H]fucose into synaptic junctions relative to synaptic membranes (Fig. 4B) suggests, in conjunction with the similar concentrations of fucose previously found in these fractions [19], that glycoproteins associated with the synaptic apparatus per se may be more stable than those present in the surrounding membranes.

Fractionation and characterization of [^{3}H]fucosyl glycoproteins

The incorporation of [^{3}H]fucose into synaptic junctional and synaptic membrane concanavalin A-positive glycoproteins was determined by resolving fractions which had been labelled for various time periods on concanavalin



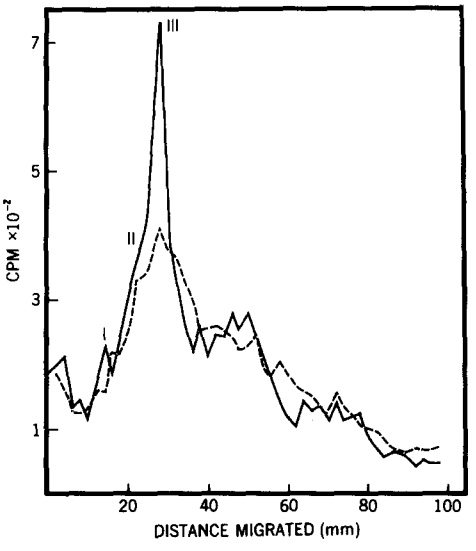


Fig. 5. The effect of time of the incorporation of [^{3}H]fucose into concanavalin A-positive and negative glycoproteins. Proteins of synaptic membranes and synaptic junctions labelled for the indicated times were fractionated on concanavalin A-Sepharose and radioactivity in the bound and unbound fractions determined as described in Materials and Methods. Recovery of radioactivity from the columns was between 65 and 80% and results are expressed as per cent of recovered radioactivity in the bound fraction. ○, synaptic junctions; ●, synaptic membranes.

Fig. 6. Effect of time on the incorporation of [^{3}H]fucose into synaptic junctional glycoproteins. Synaptic junctions were isolated from brains 1 or 24 h after the administration of [^{3}H]fucose, proteins separated by disc gel electrophoresis as described in Materials and Methods, and gels sliced for the determination of radioactivity. 1 and 24-h samples contained 300 μg and 110 μg of protein, respectively. I—III, glycoprotein classes with apparent molecular weights of 180 000, 130 000 and 110 000, respectively. - - - - - -, 1 h labelling; ———, 24 h labelling.

A-Sepharose. The percentage of [^{3}H]fucose associated with the concanavalin A-positive fraction from synaptic junctions increased from 53% to 68% between 1 and 10 h after the administration of precursor (Fig. 5). During this period the counts recovered from the column in the retained and unretained fractions increased from 2200 to 9800 and from 2100 to 5200 cpm/mg applied protein, respectively, indicating that the increase in percent retained [^{3}H]-fucose was not due to a decrease in label associated with unretained glycoproteins. In contrast to synaptic junctions the percentage of isotope present in the synaptic membrane concanavalin A-positive fraction decreased between 1 and 3 h and then remained constant.

In order to relate the differential incorporation of [^{3}H]fucose into the concanavalin A fractions to individual molecular species synaptic junctions which had been labelled for 1 and 24 h were fractionated by gel electrophoresis (Fig. 6). Although the distribution of radioactivity was qualitatively similar following 1 and 24 h a relative increase in the labelling of glycoprotein fraction III occurred after the longer time. Glycoprotein fraction III was preferentially retained by concanavalin A (Fig. 3) and this may account in part for the time-dependent increase in label associated with the concanavalin A-positive fraction (Fig. 5).

Discussion

In the present paper we have shown that fucosyl glycoproteins associated with isolated rat brain synaptic junctions are both structurally and biosynthetically heterogeneous. Chromatography on concanavalin A-Sepharose resolved each class of glycoproteins into concanavalin A-positive and negative fractions, identifying a minimum of six high molecular weight fucosyl glycoproteins in synaptic junctions. Using similar methodologies isolated synaptic membranes have previously been shown to contain 25—30 fucosyl glycoproteins [3,4]. The heterogeneity of synaptic glycoproteins demonstrated in the present and earlier [3,4] studies may reflect (1) the association of structurally related glycoproteins with functionally distinct synaptic types; (2) the presence of a large variety of glycoproteins at individual synapses, or (3) microheterogeneity of the oligosaccharide chains associated with single glycoproteins, possibilities which cannot be differentiated on the basis of the present results. Zanetta et al. [4] recently suggested, however, that the diversity of synaptic membrane glycoproteins may at least in part be due to the functional variety of neurons present in rat cortex and a similar explanation may appy to the present findings. It should also be noted that some glycoproteins may be lost during the detergent extraction used for the preparation of synaptic junctions and a greater variety of species may be associated with the synapse in situ.

Glycoproteins associated with synaptic membranes and synaptic junctions displayed different kinetics of [^{3}H]fucose incorporation. Although the possible contribution of small amounts of microsomal contamination to the labelling pattern of the former fraction cannot be excluded (particularly at the shorter times) the results suggest that the synthesis and turnover of proteins present in different parts of the synaptic apparatus may be independently regulated. The time-dependent changes in the distribution of [^{3}H]fucose between synaptic

junctional glycoproteins (Figs. 5 and 6) is also consistent with a biosynthetic mechanism involving differential rates of synthesis and/or transport of individual glycoproteins. Recent studies have demonstrated independent turnover and transport of synaptic membrane fucosyl glycoproteins [20,21] in general agreement with the present findings. Further clarification of the metabolic relationships between synaptic regions and between synaptic proteins must, however, await the purification and characterization of individual components, experiments which are presently in progress.

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