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Characterisation of a Soluble Trypsin Fragment of GP130: A Neuronal Glycoprotein Associated With the Cytoskeleton

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A neuronal glycoprotein (GP130) that is associated with the cytoskeleton [Ranscht et al: J Cell Biol 99:1803–1813, 1984] remains insoluble in 0.1 M NaOH, a property typical of integral membrane proteins. At present it is possible to solubilise and hence isolate GP130 only under denaturing conditions. However, a large fragment of apparent molecular weight 120K is released into solution by trypsin. The fragment corresponds to the extracellular region of the glycoprotein as shown by the fact that it is released from live cultures of chicken sympathetic neurons and by its retention of concanavalin A-binding activity. The soluble extracellular fragment has been purified using mild biochemical techniques, which are expected to retain its biological activity. Measurement of the sedimentation coefficient, Stokes radius, and frictional ratio in addition to metal shadowing of the fragment show that it has a molecular weight of about 120K and is asymmetric, probably rod-shaped with a long axis of more than 20 nm.

Key words: proteolytic fragment, neurons, adhesion

Many plasma membrane glycoproteins are mobile in the plane of the membrane and are easily solubilised by nonionic detergents. However, a small but increasing number have been identified that appear to be insoluble under these conditions and in some cases have been shown to be immobile [2]. They are typically glycoproteins from cell junctions such as spot desmosomes [3], synaptic junctions [4], and zonulae adherens [5], or they are tightly associated with the cytoskeleton in nonjunctional membranes [6]. These glycoproteins may act as a link between the cytoskeletons of adjacent cells or the cytoskeleton and the extracellular matrix to give a tissue mechanical strength and individual cells the ability to produce and respond to mechanical forces. In addition, the function of the glycoproteins may require their immobilisation in discrete regions of the plasma membrane.

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A concanavalin A (Con A)-binding glycoprotein, GP130, has been identified associated with the "membrane skeleton" of neuronal cultures and chicken embryo brain by its insolubility in nonionic detergents [7]. It has been isolated by preparative sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and the monoclonal and polyclonal antibodies raised against it have been used to demonstrate its restriction to nervous tissue [1]. GP130 is present over the entire surface of cultured neurons from both the central and peripheral nervous system but is most abundant in adult chicken brain where it makes up about 0.2% of the total protein. The function of GP130 in the nervous system is a crucial but as yet unanswered question. Progress in investigating this question is hampered by the insolubility of GP130 except under denaturing conditions. In order to overcome this problem, I have isolated a large proteolytic fragment of GP130 that is freely soluble and that potentially retains some of the biological activity of the intact glycoprotein.

MATERIALS AND METHODS

Materials

Donkey anti-rabbit Ig (DAR-Ig) coupled to horseradish peroxidase (HRP) or biotin (B-DAR-Ig), biotinylated sheep anti-mouse Ig (B-SAM-Ig) and HRP-streptavidin were all obtained from Amersham. The Con A affinity column was prepared from cyanogen bromide activated Sepharose 4B (Pharmacia, Uppsalla, Sweden) and Con A (BDH, Poole Dorset) using 5 mg of Con A per ml of hydrated gel. Hydroxyapatite was a gift from M. Spencer.

Methods

Protein preparation. The neuronal membrane skeleton was prepared from adult chicken brain essentially as described previously [1]. Briefly, 30–40 g of adult chicken brains, stored at −20°C, were thawed and homogenised in 5% Nonidet P40, 10 mM Tris-Cl [tris(hydroxymethyl)aminomethane] pH 7.6, 1 mM dithiothreitol (DTT), and 1 mM phenylmethylsulphonylfluoride and centrifuged at 100,000g for 30 min. The insoluble pellet was separated on a 10–30% (w/v) discontinuous sucrose gradient in 10 mM Tris-Cl pH 7.6 and 0.2 mM DTT. The material at the 10–30% interface was collected, diluted five-fold in 10 mM Tris-Cl pH 7.6 and 0.2 mM DTT, and collected by centrifugation. This material was termed the "membrane skeleton." In some experiments embryonic chicken brains, frozen in liquid nitrogen, and stored at −70°C were used.

For large-scale preparation of the tryptic fragment, the "membrane skeleton" was resuspended in 50 mM Tris-Cl pH 7.6, 150 mM NaCl, 0.5 mM $CaCl_2$, and 0.5 mM $MnSO_4$ (buffer A) and treated with 0.1 mg/ml trypsin for 1–1.5 hr at 20°C, and the reaction was stopped by addition of an equal amount of soybean trypsin inhibitor. Insoluble material was removed by centrifugation for 2–3 hr at 200,000g. The supernatant was dialysed for several hours against buffer A to remove any remaining sucrose. In most cases, the insoluble pellet was re-extracted with buffer A, and in come cases the dialysed supernatant was centrifuged a second time.

The trypsin supernatant was applied to a 20 ml Con A affinity column, and the column was washed with 60 ml of buffer A, 60 ml of buffer A plus 0.5 M NaCl, and 20 ml of buffer A. The Con A-binding glycopeptides were eluted with buffer A plus 0.2 M methyl-α-D-mannosidase. The protein containing fractions were collected and

dialysed against 0.1 M sodium phosphate pH 6.8 containing 0.1 M Methyl-α-D-mannoside. The glycopeptides were applied to a hydroxyapatite column (1.6 × 8 cm) equilibrated in the same buffer, and the large tryptic fragment was eluted by an 80-ml gradient of 0.1–0.35 M sodium phosphate pH 6.8 containing 0.1 M methyl-α-D-mannoside.

Brain membranes were prepared from 16–18-day-old embryo brains essentially as described by the method of Matus [8]. The membranes were extracted with 0.1 M NaOH as described by Luna et al [9] and were a gift from T. Allsopp.

Protein concentrations were determined by spotting samples onto filter paper and staining with Coomassie brilliant blue G250 [10] using bovine serum albumin (BSA) as a standard.

Cell cultures. Neuronal cultures essentially free of nonneuronal cells were obtained from sympathetic ganglia of 10-day-old chicken embryos as described previously [11].

SDS gel electrophoresis and immunoblotting. Proteins were separated by SDS-PAGE on minigels (0.4–0.5 mm thick) [12]. Con A-binding glycoproteins were detected directly on minigels using 1^{125} Con A as described previously [7]. For immunoblotting, proteins were transfered to nitrocellulose paper [13]. Nonspecific binding sites were blocked by overnight incubation with 0.05% Tween 20 in 10 mM Tris-Cl pH 8.4, 150 mM NaCl, 0.1% NaN_3 (buffer B) [14]. GP130 and its proteolytic fragments were detected by incubation with monoclonal or polyclonal rabbit antibodies (1:100) in buffer B with 3 mg/ml BSA followed by either B-SAM-Ig (1:100) in buffer B with 3 mg/ml BSA and streptavidin-HRP (1:1,000) in buffer B without NaN_3 for the monoclonal anti-GP130 or DAR-Ig-HRP (1:1,000) in buffer B without NaN_3 for the polyclonal anti-GP130. All incubations were carried out for 1 hr at room temperature and were followed by three 5-min washes in buffer B. Buffer B without NaN_3 was used before and after incubations with HRP-coupled components. Labeled protein bands were detected using 0.5 mg/ml diaminobenzidine, 0.3 mg/ml $CoCl_2$, 0.02% H_2O_2, in 1% DMSO, 10 mM Tris-Cl pH7.6, and 150 mM NaCl.

Protein bands stained with Coomassie were quantitated relative to actin and β-galactosidase by densitometry. Western blots were scanned using reflectance mode on a Joyce Loebl chromoscan 3. In both cases the area under the peaks was measured with a planometer.

Metal shadowing. Unidirectional shadowing was carried out by the method of Elliot and Offer [15] as modified by Shotton et al [16]. The large tryptic fragment was dialysed against 0.1 M ammonium acetate pH 7.6 and diluted with this buffer and glycerol to give a final concentration of 30% glycerol and 50 μg/ml protein. The solution was sprayed onto a freshly cleaved mica surface, dried under vacuum, shadowed at an angle of 10° with platinum at room temperature, and coated with carbon.

Determination of hydrodynamic parameters. The Stokes radius of the large tryptic fragment was determined by chromatography on a G200 Sephadex column (1 × 70 cm) using BSA 66,000 ADH 150,000, and β-amylase 200,000 as standards. The sedimentation coefficient was determined by comparison with known standards on sucrose gradients. Linear gradients of 7–28% sucrose in 10 mM Tris-Cl pH7.6 in H_2O and 3–12% sucrose in 10 mM Tris-Cl pH7.6 in D_2O (96.4% final D_2O concentration) were prepared in 5-ml ultracentrifuge tubes. Standard proteins (50 μg each) and 20 μg of the tryptic fragment were loaded onto each gradient, and centrifugation

was performed at 150,000g for 16 hr. Fractions (0.2 ml) were collected on an ISCO density gradient fractionator 640. The standard proteins were myoglobin ($S_{w,20}$ 2.0), ovalbumin ($S_{w,20}$ 3.5), BSA ($S_{w,20}$ 4.6), rabbit IgG ($S_{w,20}$ 7.0), and catalase ($S_{w,20}$ 11.3). The linearity of the gradients was checked using a refractometer, and the position of the proteins in the gradient was analysed by SDS-PAGE.

The molecular weight was calculated from the Stokes radius and the sedimentation coefficient using the following:

$$M = 6\pi N\eta as/(1 - \bar{v}\rho), \tag{1}$$

where the viscosity η for water at 20°C is 0.01002 g/cm, N is Avagadro's number, a is the Stokes radius in cm, s is the sedimentation coefficient at 20°C in water in seconds $\times 10^{-13}$, $\bar{v}$ is the partial specific volume, and the density ρ is 0.9982 g/cm^2. The partial specific volume ($\bar{v}$) for a glycoprotein will vary according to the amino acid composition, the polysaccharide composition, and the relative proportion the two components. Since these data are not available, $\bar{v}$ was determined using the sedimentation of the tryptic fragment compared to standards in sucrose gradients prepared in H_2O and D_2O [17,18]. The frictional ratio (f/f_0) of the tryptic fragment was calculated using the following:

$$f/f_0 = \frac{a}{\left(\frac{3M\bar{v}}{4\pi N}\right)^{1/3}}. \tag{2}$$

RESULTS

GP130 is an Intrinsic Membrane Protein

GP130 is exposed on the surface of neurons and is closely associated with the "membrane skeleton" under nondenaturing conditions [1]. However, there is no direct evidence that GP130 is an integral membrane protein. Most extrinsic membrane proteins, even those tightly bound to the membrane, under most other conditions are removed by 0.1 M NaOH. As shown in Figure 1A, GP130 was almost quantitatively retained in NaOH extracted membranes while the majority of extrinsic membrane proteins such as actin and tubulin were largely removed. It is possible that GP130 is extracellular and linked by disulphide bonds to an integral membrane protein. To test this, reduced and nonreduced samples of 19-day embryonic brain were analysed on Western blots, and GP130 appeared as a single band in both cases, although under nonreducing conditions the mobility is slightly higher, suggesting that GP130 may have internal disulphide bonds (Fig. 1B). The intensity of the band in the nonreduced sample was slightly less than the reduced sample, perhaps owing to a decrease in the antigenicity of the nonreduced protein and to a small amount of cross-linked material which does not enter the stacking gel. However there is no evidence for a specific disulphide bridged species consisting of GP130 and one or more membrane glycoproteins.

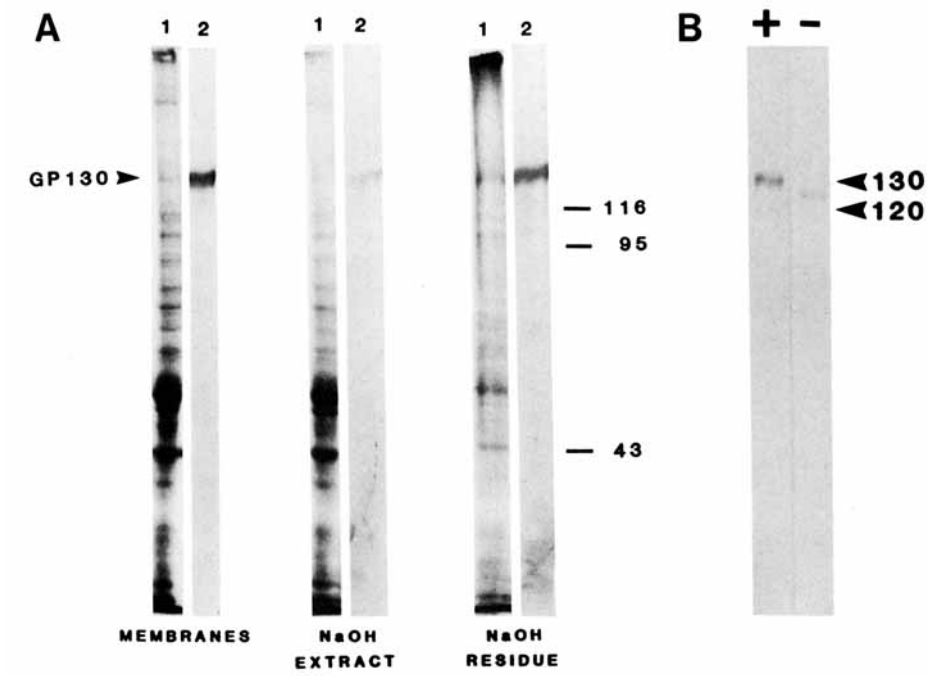


Fig. 1. A) GP130 is retained in NaOH-extracted membranes. Membranes from 18-day-old chicken embryo brains were extracted with 0.1 M NaOH for 30 min at 4°C, and the insoluble material was collected by centrifugation at 100,000g. Protein (2.5 μg) was separated on SDS-PAGE and either (1) stained with Coomassie brilliant blue or (2) analysed for GP130 content on Western blots. Molecular weight standards are β-galactosidase (116 kD), phosphorylase b (95 kD), and actin (43 kD). B) Nonreduced GP130 has a lower apparent molecular weight than reduced GP130. Whole brain homogenates from 19-day-old embryo brains were treated with sample buffer with (+) or without (−) 1% 2-mercaptoethanol, and equal amounts of protein were analysed on Western blots. The position of reduced GP130 and the 120-kD fragment are shown.

Proteolysis of GP130

A detergent-extracted "membrane skeleton" from adult chicken brain was treated with two different proteases for increasing time as shown in Figure 2. Whereas, V8 protease rapidly degraded GP130 to a number of bands about 60 kD, trypsin produced a much larger fragment of approximately 120 kD, which appeared to be relatively stable, persisting in large quantities after 1 hr at 37°C. About 50% of GP130 was degraded to the 120-kD fragment before significant quantities of the 60-kD fragments were produced. If GP130 was denatured by SDS, it was rapidly and completely degraded by trypsin, indicating that production of the 120-kD fragment was dependent on the native conformation of GP130 (data not shown).

Only a small fraction of intact GP130 remains in the 100,000g supernatant following extraction by 1% deoxycholate (data not shown). However, more than 50% of the 120-kD trypsin fragment was found in the supernatant with or without detergent. The insoluble material remaining after trypsin treatment is difficult to sediment, and trace amounts are often present in the supernatant. The pellet usually occupies a large volume after centrifugation, and although most of the 120-kD fragment can be

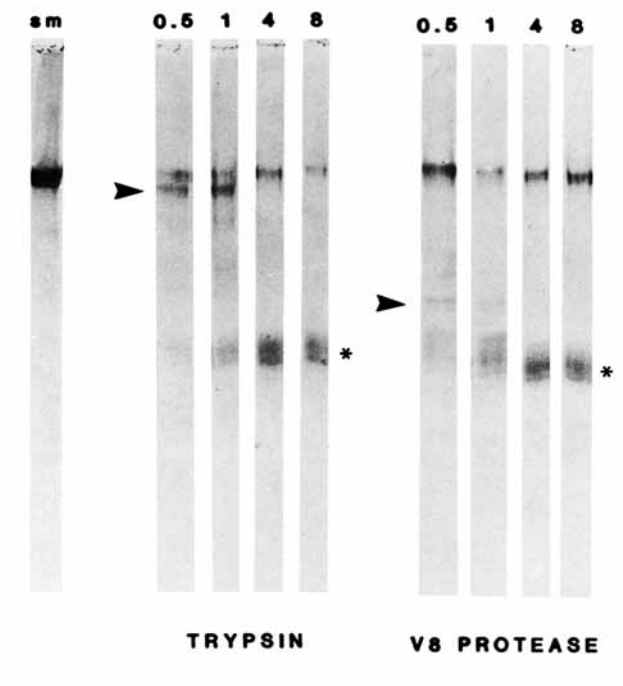


Fig. 2. Proteolysis of GP130 from the "membrane skeleton." (sm) is the "membrane skeleton" fraction. Numbers represent incubation times in hours with 0.1 mg/ml trypsin in 50 mM Tris-Cl pH7.6 and 100 mM NaCl at 37°C. The reaction was quenched by the addition of SDS sample buffer and heating to 100°C, and the samples were analysed on Western blots. Arrowheads indicate the largest fragment produced by each protease. (*) shows the series of similar molecular weight fragments produced by both proteases after extensive incubation.

extracted into the supernatant, a small fraction still remains insoluble. However, even in the absence of detergent, the 120 kD in the supernatant is completely soluble (Fig. 3), and the small amount of GP130 that failed to sediment is found in the void volume of the column as expected.

The 120-kD Fragment is the Extracellular Region of the Glycoprotein

The solubility of the 120-kD fragment in the absence of detergent suggests that trypsin has removed the transmembrane portion of the parent protein. If this is so, then the 120-kD fragment must represent either the extracellular region or the cytoplasmic region of the glycoprotein. To distinguish between these possibilities, live cultures of sympathetic neurons were treated with trypsin, and, as shown in Figure 4, a large fragment of about 120 kD was removed from the surface. Trypsin treatment does not permeabilise the cells or affect their viability [1]. This result is consistent with the 120-kD fragment being the extracellular region of the glycoprotein. Surface receptors involved in Ca^{2+}-dependent cell adhesion are generally protected from proteolysis by Ca^{2+} [19]; however, digestion of GP130 not only proceeded in the presence of Ca^{2+} but was more efficient (Fig. 3). Release of the 120-kD

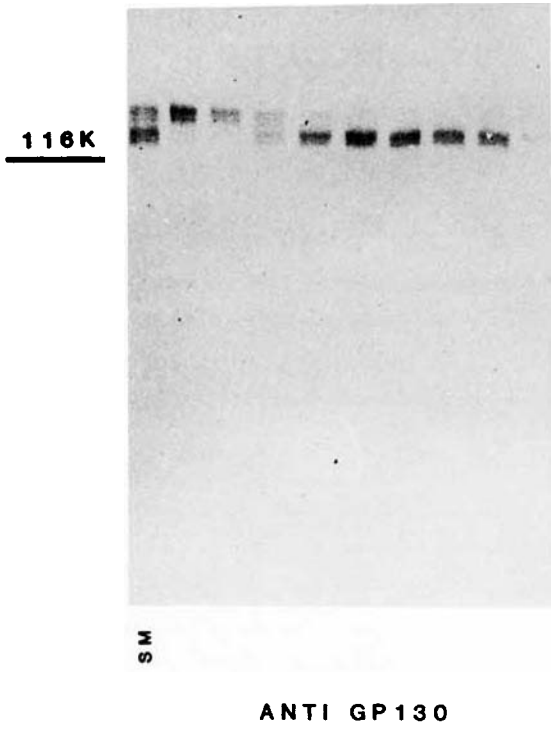


Fig. 3. Solubility of the 120-kD fragment. A "membrane skeleton" fraction was treated with 0.1 mg/ml trypsin for 1 hr at room temperature followed by addition of trypsin inhibitor. Most insoluble material was removed by centrifucation at 100,000g for 1 hr, and 0.5 ml of the supernatant was applied to a Sepharose 4B column (1.2 × 12 cm) in 10 mM Tris-Cl pH 7.6 and 100 mM NaCl, and 0.7-ml fractions were collected. The supernatant or starting material (SM) and column fractions were analysed on a Western blot. Fraction 1 represents the void volume. The position of β-galactosidase (116 kD) is shown.

fragment from neurons occurred only in the presence of trypsin, and the amount of fragment released depended on both trypsin concentration and the time of incubation.

The 120-kD fragment obtained from brain retains the Con A-binding activity of the glycoprotein and thus appears to be the extracellular region of the GP130. Some or all of the antigenic sites for both the polyclonal and the monoclonal antibodies raised against GP130 are present in the 120-kD fragment as shown by its detection on Western blots. The other portion of GP130 removed by trypsin is of interest since this portion of the molecule may be responsible for anchoring GP130 to the membrane and the cytoskeleton. If trypsin acts by cleaving GP130 at essentially one site near the external surface of the membrane, then the remaining fragment of approximately 10 kD may be present in the trypsin-treated insoluble pellet. Attempts to find this fragment with the polyclonal antibody by Western blots of this material electrophoresed on 15% polyacrylamide gels were unsuccessful (data not shown). This suggested that either the antibodies do not recognise this portion of GP130 or the 10-kD fragment was further degraded into small pieces.

Isolation of the 120-kD Fragment

For large-scale preparations of the native 120-kD fragment, the trypsinisation step was normally carried out at room temperature for 1–1.5 hr at which point 50–

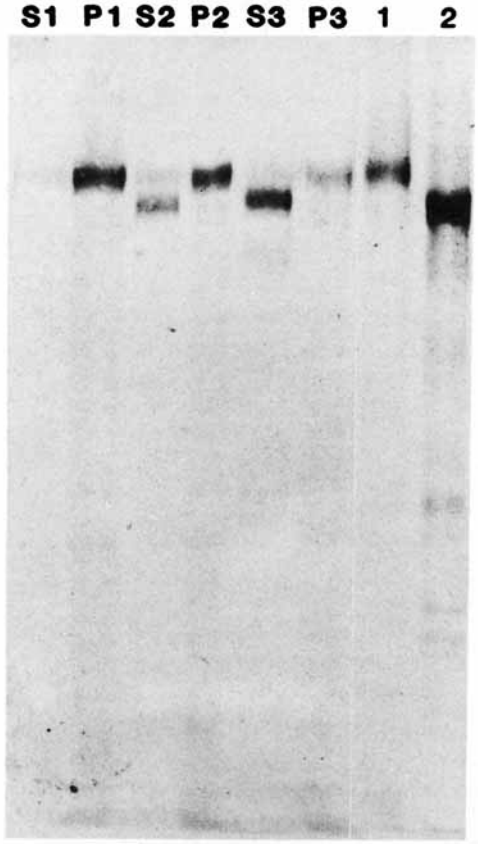


Fig. 4. Trypsinisation of live cultures of sympathetic neurons. The neurons were removed as a sheet and incubated for 30 min at 37°C in 0.02 ml of Hanks' balanced salt solution (BSS) with 10 mM EGTA (S1, P1), 0.1 mg/ml trypsin in BSS and 10 mM EGTA (S2, P2), 0.1 mg/ml trypsin in BSS with 10 mM $CaCl_2$ (S3, P3), followed by the addition of trypsin inhibitor to a final concentration of 0.1 mg/ml. The cells were pelleted at 500g for 5 min, and 1/8 of the pelleted cells and 3/8 of the supernatant were analysed on Western blots. Lane 1) semipurified fraction containing GP130 from adult chicken brain. Lane 2) semipurified fraction containing the 120kD fragment from adult chicken brain.

60% of the material recognised by the antibodies was the 120-kD fragment, while most of the remainder was intact GP130. It is probable that some GP130 was totally degraded in this period, since the sum of GP130 and the 120-kD fragment was less than the starting material. In some cases, the trypsinised pellet was treated a second time with trypsin to increase the yield.

The 120-kD fragment was isolated using Con A affinity chromatography and hydroxyapatite. Hydroxyapatite bound the 120-kD fragment tightly, whereas all the remaining Con A-binding glycopeptides were eluted with lower concentrations of phosphate buffer (Fig. 5). Phosphate buffer (0.4 M) was used to complete the elution of the 120-kD fragment in this experiment, although a gradient up to 0.35 M was used routinely. Because small amounts of Con A were often eluted from the Con A affinity column, the hydroxyapatite column was normally used as the last step in the purification. The sample was applied in 0.1 M phosphate buffer with 0.1 M methyl-

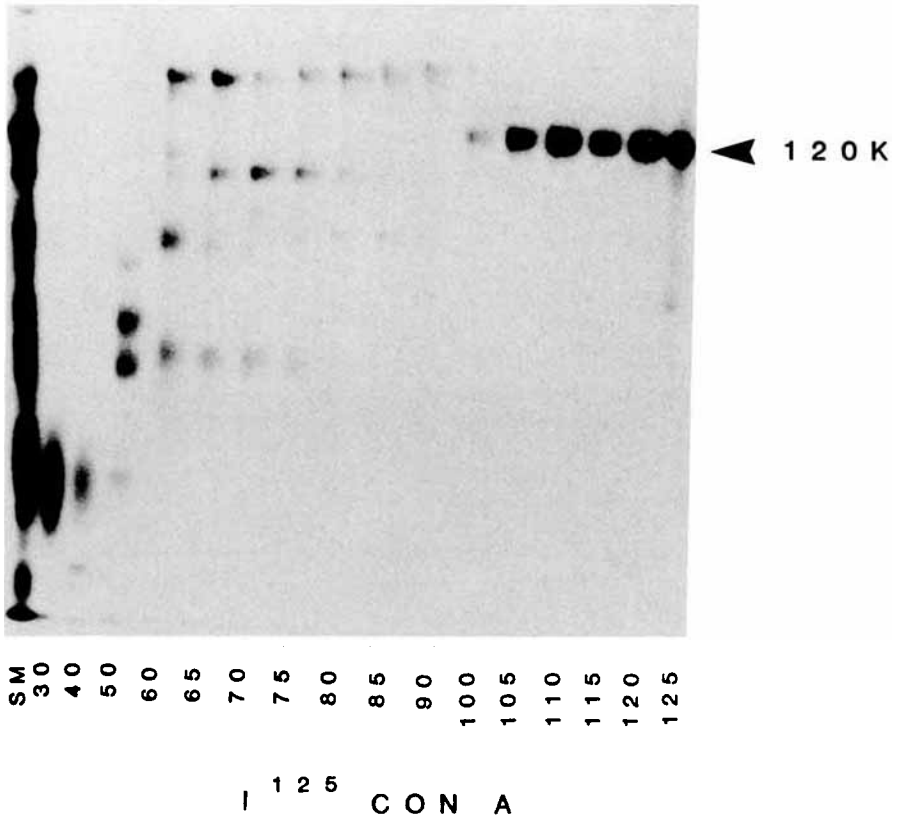


Fig. 5. Purification of the 120-kD fragment on hydroxyapatite. A 100,000g supernatant of a trypsin-treated "membrane skeleton" fraction (SM) was applied to the column, and proteins were eluted with a 200-ml gradient of 0.01–0.3 M sodium phosphate pH 6.8, and 2.5 ml fractions were collected. A 0.4 M step was applied at fraction 105. The glycoproteins in the fractions were detected with I^{125} Con A.

α-D-mannoside, and the column was washed with this buffer to remove Con A and many Con A-binding peptides. The 120-kD fragment was eluted with a phosphate gradient as described in "Methods." The purification is summarised in Table I and Figure 6. Approximately 0.36 mg of the 120-kD fragment was obtained from 80 g of adult chicken brain.

Molecular Characterisation of the 120-kD Fragment

The molecular weights for the intact gylcoprotein (130K) and for the large tryptic fragment (120K) are based on SDS-PAGE, which is generally inaccurate for glycosylated proteins. Alternative methods of determining the molecular weight of GP130 are not easily available because of its insolubility. However, the physical size and shape of the 120-kD fragment has been investigated using gel filtration, density gradient sedimentation, and unidirectional shadowing. The Stokes radius of the 120-kD fragment was determined by gel filtration against standards to be 5 nm (Table II), corresponding to a globular protein of about 220 kD. This result can be explained in

TABLE I. Summary of Purification of the 120-kD Fragment*

	Total protein (mg)	Percentage of GP130 or 120-kD fragment	Purification	Yield (%)
Fraction 1:				
Membrane skeleton	218	2.6	1	100
Fraction 2:				
Trypsin supernatant	19.3	5.6	2.2	19
Fraction 3:				
Elution off Con A column	0.88	60.0	23.0	9.0
Fraction 4:				
Elution off hydroxyapatite column	0.36	95.0	36.5	6.0

*The total protein was measured by spotting samples onto filter paper in triplicate as described in "Methods." The GP130 or 120-kD fragment was quantitated on Western blots. The 120-kD fragment in fraction 3 was measured by densitometry of the Coomassie-stained band and known amounts of actin and β-galactosidase. The GP130 or 120-kD fragment in fractions 1, 2, and 4 was measured by comparison with the 120-kD fragment in fraction 3 assuming no difference in the binding of the antibodies to GP130 and 120-kD fragment. No correction was made for the Coomassie binding to the glycoproteins; therefore, the absolute values are only approximate.

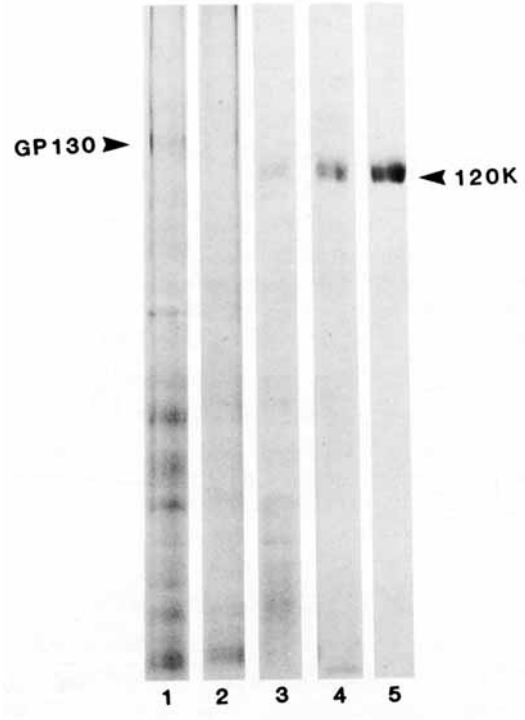


Fig. 6. Steps in the purification of the 120-kD fragment. Lane 1) "membrane skeleton" fraction isolated from the 10–30% interface of a discontinuous sucrose gradient as described in "Methods." Lane 2) insoluble material after trypsin treatment at 0.1 mg/ml for 1.5 hr at room temperature. Lane 3) 200,000g supernatant after trypsin treatment. Lane 4) glycopeptides eluted from the Con A affinity column. Lane 5) collected peak of 120-kD fragment from the hydroxyapatite column. The proteins were detected with Coomassie brilliant blue, and the position of GP130 and the 120-kD fragment are shown.

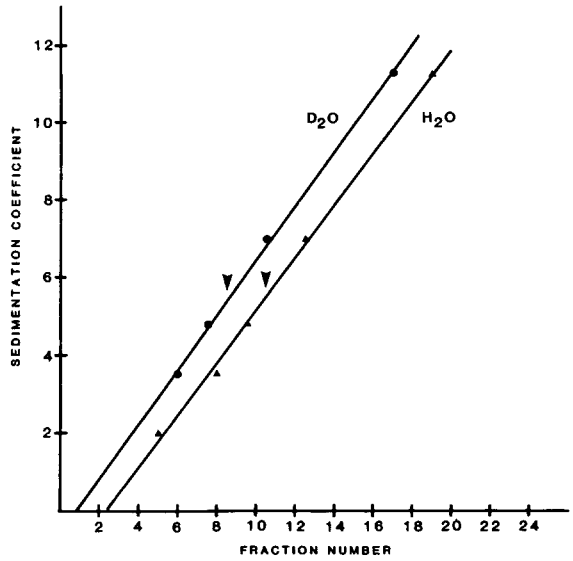


Fig. 7. Measurement of the sedimentation coefficient of the 120-kD fragment in H_2O and D_2O sucrose gradients. The standard proteins are catalase, rabbit IgG, BSA, ovalbumin, and myoglobin. The sucrose gradients were linear from fraction 6 to 22 for D_2O and from 4 to 22 for H_2O. The myoglobin peak was not in the linear portion of the D_2O sucrose gradient. The H_2O gradient was 7–28% sucrose, and the D_2O was 3–12% sucrose. The position of the 120-kD fragment in each gradient is shown by the arrowheads.

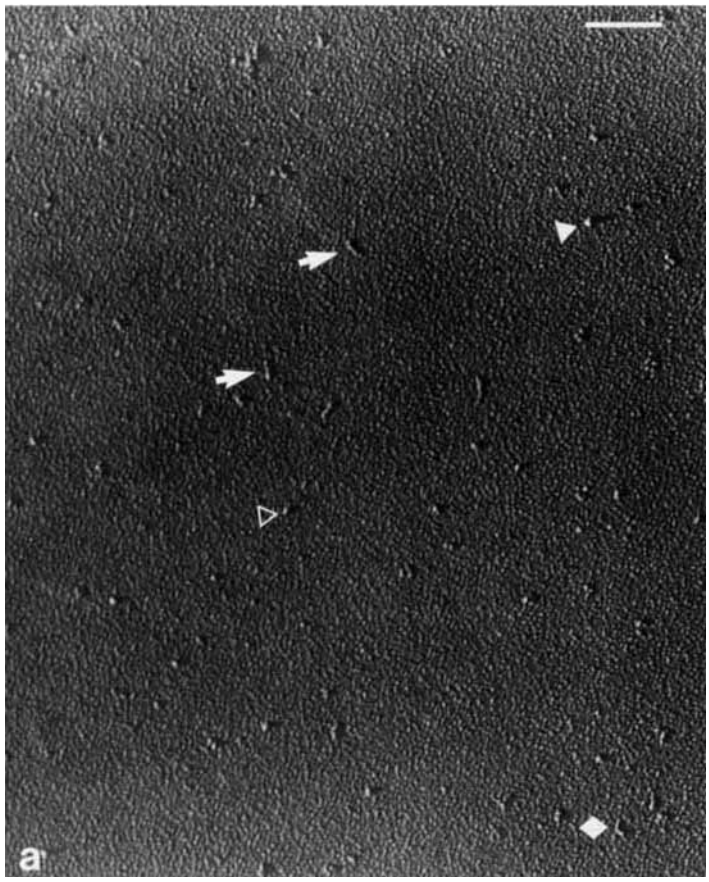
TABLE II. Summary of Molecular Properties of the 120-kD Fragment*

Stokes radius	5 nm
$S_{20,w}$	5.5
Partial specific volume	0.73
Molecular weight	115,000
Frictional ratio	1.55
Length	22–24 nm

*The Stokes radius was measured by gel filtration as described in "Methods." The sedimentation coefficient and partial specific volume were measured from sucrose gradients in H_2O and D_2O. The molecular weight and frictional ratio were calculated from equations 1 and 2, respectively, and the length was measured from unidirectional shadowing.

two ways. Either the 120-kD fragment is asymmetric in shape, or it is present in solution as a dimer.

To resolve this question, the sedimentation coefficient for the 120-kD fragment was determined since the molecular weight for a glycoprotein can be calculated from the Stokes radius, the sedimentation coefficient, and the partial specific volume ($\bar{v}$) as described in "Methods." Within experimental error, the sedimentation coefficient was 5.5 in both H_2O and D_2O gradients when compared to the standard proteins (Fig. 7). Thus, it is reasonable to use 0.73 (the average value for the standard proteins) for $\bar{v}$. The molecular weight of the large trypsin fragment is calculated to be 115K from



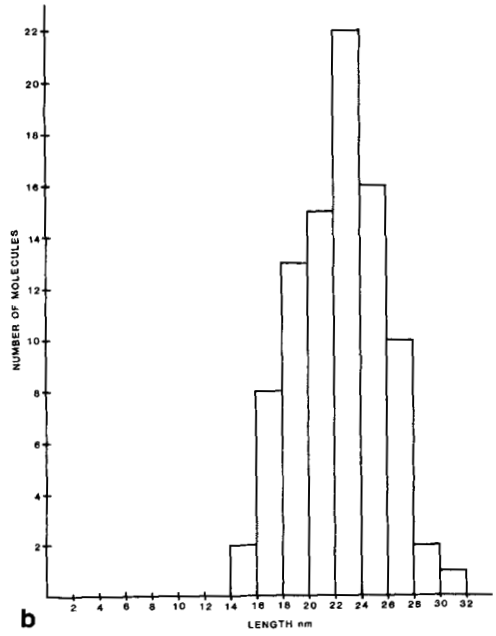


Fig. 8

equation 1, only slightly less than that observed by SDS-PAGE. This suggests that unlike many glycoproteins [20], the 120-kD fragment and GP130 have a comparatively normal mobility on SDS gels. This is confirmed by measuring the Rf of both the 120-kD fragment and GP130 in SDS-PAGE over a range of acrylamide concentrations. In both cases, the mobility of the glycoproteins change in a similar way to the standard proteins, showing that they do not bind an anomalous amount of SDS. Thus, 10 kD may be taken as a reasonable estimate for the portion of the glycoprotein removed by trypsin.

If the molecular weight of the fragment is 115–120K, then the most plausible explanation of the comparatively large Stokes radius is that the 120-kD fragment is asymmetric in shape. The asymmetry can be estimated from the frictional ratio using equation 2. For the 120-kD fragment, f/f_0 is 1.55, indicating a moderately asymmetric or highly hydrated molecule. For comparison, subfragment 1 of myosin has a similar molecular weight and sedimentation coefficient [21] and has dimensions of 16 × 6 nm maximum width [22].

A preparation of the 120-kD fragment was shadowed and, as shown in Figure 8a was observed to contain a number of short rod-like molecules of approximately 22–24 nm (Fig. 8b). This corresponds to a more asymmetric molecule than might be expected from the frictional ratio; however, the Stokes radius, as measured by gel filtration, may be erroneously small for rod-shaped molecules [23], leading to an underestimation of the frictional ratio. A number of bent rods could be seen, suggesting they may be fairly flexible, and occasionally rods of about twice the normal length were also observed. However, the preparation was quite heterogeneous as shorter rods, and more globular structures could also be identified. The heterogeneity may be due to variability in the protein preparation or to the fact that molecules with different orientations to the direction of shadowing will appear different, unlike rotary shadowing. For example, a molecule with its long axis parallel to the direction of shadowing will appear more globular, while there are examples of molecules that appear to have their long axis vertical as judged by the length of the shadow.

DISCUSSION

GP130 is neuronal glycoprotein that is associated with cytoskeletal components in a detergent-extracted membrane fraction from chicken brain. Little is known about the molecular nature of this association, the function of the glycoprotein, or its molecular structure. These questions have been difficult to tackle because of the insolubility of GP130. The isolation of a soluble proteolytic fragment of GP130 has provided information about the structure of the glycoprotein and may be useful in probing the function of GP130.

Trypsin treatment of the detergent-insoluble membrane skeleton or of live cultures of sympathetic neurons released a fragment of apparent molecular weight

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Fig. 8. a) Unidirectional shadowing of the 120-kD fragment. Arrows show typical rod-shaped molecules; the diamond shows a bent rod; the solid triangle shows an apparently globular molecule with an exceptionally long shadow; the open triangle shows smaller globular structures. The scale bar = 100 nm. b) Histogram of the length distribution of the rod-shaped molecules. The lengths of about 90 these molecules were measured using a microcompariter.

120K into solution. This corresponds to most, if not all, of the extracellular portion of the glycoprotein. The comparative stability of this fragment suggests that trypsin may have one major cleavage site in GP130, although extensive incubation with trypsin or V8 protease produce a second, very stable series of fragments of about 60 kD. Proteolytic cleavage sites often occur between functional domains and sometimes in flexible regions of a polypeptide chain (eg, fibronectin and myosin [24,21]) suggesting that the trypsin-sensitve site is in a flexible or more accesible region of the glycoprotein near the surface of the plasma membrane.

In some experiments (eg, Fig. 2), both the insoluble GP130 and the soluble 120-kD fragment appear as doublets, indicating that a short region, probably at the extracellular terminal, may be susceptible to proteolysis. No other major digestion products are observed between GP130 and the 120-kD fragment; therefore, it is reasonable to suppose that 10-kD fragment is removed as a single piece. However, this fragment could not be detected in the insoluble fraction with the antibodies available. Two pieces of evidence suggest that the 10-kD fragment is indeed integral to the plasma membrane: (1) 0.1 M NaOH failed to extract GP130 from membranes, and (2) there is no evidence for an alternative mechanism for anchoring G130 in the membrane (such as disulphide bonds) to a membrane protein, although GP130 may have intramolecular disulphide bonds.

A potential method of isolating the 120-kD fragment from the trypsin supernatant is affinity chromatography using either the monoclonal or polyclonal antibodies. However, this was unsuccessful because in both cases the antibodies only recognised the 120-kD fragment with high affinity after it was denatured in SDS. This was shown for the polyclonal antibody by an Ouchterlony double diffusion experiment (data not shown). All the antibodies had been raised against GP130 eluted from preparative SDS gels [1]; therefore, it was important to isolate the 120-kD fragment without denaturation both to raise antibodies which would recognise the native glycoprotein and for studies to determine the function of GP130.

GP130 has a large extracellular region of approximately 115–120 kD, which, as judged from gel filtration and shadowing experimemts, is asymmetric, probably rod-shaped. The remainder of GP130, about 10–15 kD of the polypeptide chain, is available to form the transmembrane and cytoplasmic portion of the molecule. A polypeptide of 2,500 daltons is required to form an α-helix that will traverse the membrane [25]; therefore, the 10-kD polypeptide could cross the membrane once and form a significant cytoplasmic region or traverse the membrane several times. Removal of this transmembrane polypeptide produces a dramatic change in the solubility of GP130, which is surprising in view of the small cytoplasmic region. However, many membrane proteins are oligomeric, and GP130 may have high-affinity binding sites within the membrane for itself or other neuronal membrane proteins, such as one of 90K, which is also found associated with the neuronal "membrane skeleton" [7]. An oligomeric complex of this nature may contribute to the insolubility of GP130 by providing a larger combined cytoplasmic region to interact with the cytoskeleton. The extracellular region does not appear to interact with itself, existing as a monomer in dilute solution. Thus high affinity homophilic interactions involving the external part of the molecule are unlikely.

The best characterised membrane skeleton is that of the erythrocyte, where the major glycoprotein responsible for linking the actin-spectrin network to the membrane is band 3. This glycoprotein has a large cytoplasmic domain (43 kD), makes several

loops through the plasma membrane, and does not have a large extracellular domain [26]. It is clear from the proteolysis experiments described here that GP130 has a different structure perhaps more analogous to that of glycophorin [27] or neuronal glycoproteins involved in cell adhesion. For example, N-CAM has a large extracellular region that is shed into the culture medium [28], while L1/N1LE/Ng-CAM may also be similar [29].

The function of GP130 is not known. However, owing to its interaction with the cytoskeleton and its abundance, it probably plays an adhesive or structural role in the nervous system. This is consistent with the molecular structure of the extracellular region, which may extend more than 20 nm away from the surface of the neuron to interact with molecules of the extracellular environment or surface receptors of adjacent cells. This distance is comparable with the intercellular distance between neurons and glial cells although rather smaller than the distance between pre- and postsynaptic membranes.

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