

# Analysis of Cholecystokinin-binding Proteins Using Endo- $\beta$ -N-Acetylglucosaminidase F

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**ABSTRACT** We have previously shown that the cholecystokinin (CCK)-binding proteins in rat pancreatic plasma membranes consist of a major  $M_r$  85,000 and minor  $M_r$  55,000 and  $M_r$  130,000 species as revealed by affinity labeling with  $^{125}\text{I}$ -CCK-33 using the cross-linker, disuccinimidyl suberate. The glycoprotein nature of these species was investigated using endoglycosidase F (endo F) and neuraminidase treatment and wheat germ agglutinin-agarose chromatography. Treatment of affinity-labeled membranes with endo F resulted in increased electrophoretic mobilities of all three binding proteins, indicating removal of N-linked oligosaccharide side chains. Endo F treatment of each protein in gel slices indicated the following cleavage relationships:  $M_r$  85,000  $\rightarrow$  65,000;  $M_r$  55,000  $\rightarrow$  45,000;  $M_r$  130,000  $\rightarrow$  110,000. Using limiting enzyme conditions to digest each protein contained in excised SDS gel slices, three and four products, respectively, were identified for the  $M_r$  85,000 and 55,000 proteins. Similar treatment of the  $M_r$  130,000 protein revealed only the  $M_r$  110,000 product. These results indicated that the  $M_r$  85,000 protein has at least three, the  $M_r$  55,000 protein has at least four, and the  $M_r$  130,000 protein has at least one, N-linked oligosaccharide side chain(s) on their polypeptide backbone. Neuraminidase treatment of affinity-labeled membranes caused slight increases in the electrophoretic mobilities of all three proteins, indicating the presence of sialic acid residues. Solubilization of affinity-labeled membranes in Nonidet P-40 followed by affinity chromatography on wheat germ agglutinin-agarose revealed that all three CCK-binding proteins specifically interact with this lectin and can be eluted with *N*-acetyl-D-glucosamine. Analysis of the proteins present in the eluted fractions by silver staining indicated a significant enrichment for proteins having molecular weights corresponding to the major CCK-binding proteins in comparison to the pattern of native membranes. Taken together, these studies provide definitive evidence that the CCK-binding proteins in rat pancreas are (sialo)glycoproteins.

Cholecystokinin (CCK)<sup>1</sup> has many effects on its target tissues, including stimulation of secretory protein discharge by pancreatic acinar cells, contraction of gall bladder smooth muscle cells, and presumably a change in the activity of neuronal cells in brain (1, 2). In the pancreatic acinar cell, CCK increases calcium levels (3), the phosphorylation of ribosomal

protein S6 (4), and enzyme secretion (5) after interaction with its plasmalemmal receptor. Specific binding sites for CCK have been demonstrated in isolated pancreatic acini (6-8), and pancreatic membranes (9, 10), and, using light and electron microscopic autoradiography, a distinct polarized distribution of CCK receptors on the basolateral plasmalemma of pancreatic acinar cells has been shown in keeping with CCKs presumed site of action in this tissue (11, 12).

Progress toward characterization of the structural properties of the CCK receptor in pancreas has recently been made using affinity cross-linking techniques (12-14). We previously described the identification of a major CCK-binding protein of  $M_r$  85,000 and minor CCK-binding components of  $M_r$  55,000 and  $M_r$  130,000 in rat pancreatic plasma membranes using

<sup>1</sup> *Abbreviations used in the paper:* CCK, cholecystokinin; PMSF, phenylmethanesulfonyl fluoride; STI, soybean trypsin inhibitor; DTT, dithiothreitol; DSS, disuccinimidyl suberate; HMS, 25 mM HEPES, 5 mM  $\text{MgCl}_2$ , and 104 mM NaCl; MBS, *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester; NP-40, Nonidet P-40; WGA, wheat germ agglutinin; GlcNAc, *N*-acetyl-D-glucosamine; endoglycosidase, endo- $\beta$ -*N*-acetylglucosaminidase; endo F, endoglycosidase F.

homobifunctional chemical cross-linkers. Analysis of cross-linked samples in the absence of reductant by SDS PAGE revealed all of these proteins plus an additional  $M_r$  ~140,000 component. On the basis of these findings, we suggested that the  $M_r$  85,000 protein was involved, to some extent, in disulfide-linked complexes in native pancreatic membranes. Svoboda et al. (15) described the specific labeling of a  $M_r$  76,000 CCK-binding protein in rat pancreatic membranes. Under nonreducing conditions the  $M_r$  76,000 protein was not detectable but a  $M_r$  126,000 and a  $M_r$  96,000 protein were, strongly suggesting that under these conditions the  $M_r$  76,000 component was disulfide-linked to other membrane proteins. They did not observe a  $M_r$  55,000 component. Similarly, Sakamoto et al. (13) reported the specific labeling of a  $M_r$  80,000 binding protein in membranes isolated from dispersions of mouse pancreatic acini. Subsequently, several of these authors (14) went on to find that the  $M_r$  80,000 protein was involved in a disulfide-linked complex ( $M_r$  120,000) in native membranes. They concluded that the CCK receptor has a subunit structure consisting of two proteins: one  $M_r$  76,000 CCK-binding protein disulfide bonded to a  $M_r$  40,000 protein that does not bind CCK. Taken together the results of all the aforementioned studies suggest that the major CCK-binding protein in native pancreatic membranes and thus the minimal structure of the CCK receptor has an apparent  $M_r$  of 76,000–85,000 (71,000–81,000 native-corrected for the mass of CCK) that exists both free and disulfide bonded to a  $M_r$  40,000–55,000 protein. The relationship of the additional components observed to this complex is unknown.

We now report that the affinity labeled  $M_r$  85,000, 55,000 and 130,000 CCK-binding proteins are sialoglycoproteins using as criteria alteration in apparent molecular weight after endoglycosidase F (endo F) treatment (16) or neuraminidase treatment, and specific binding to wheat germ agglutinin (WGA). Digestion of proteins contained in bands excised from gels was carried out with limiting amounts of endo F to provide an approximation of the total number of N-linked oligosaccharides each contains. This work has appeared in abstract form (17).<sup>2</sup>

## MATERIALS AND METHODS

### Reagents

Synthetic CCK-8 was the generous gift of Dr. Miguel Ondetti (Squibb Institute for Medical Research, Princeton, NJ). Natural Porcine CCK-33 was obtained from Dr. Viktor Mutt (Gastrointestinal Hormone Research Laboratory, Karolinska Institutet, Stockholm, Sweden). Endo H was a gift from Dr. Paul Atkinson (Albert Einstein College of Medicine, Bronx, NY). Neuraminidase (EC 3.2.1.18) Type X, Bacitracin, phenylmethylsulfonyl fluoride (PMSF), and proteins used for molecular weight standards were obtained from Sigma Chemical Co. (St. Louis, MO). Soybean trypsin inhibitor was purchased from Worthington Diagnostics (Freehold, NJ). Monoiodinated Bolton-Hunter reagent was purchased from New England Nuclear (Boston, MA) (>2,000 Ci/mmol) or ICN Pharmaceuticals (Irvine, CA) (>1,500 Ci/mmol).

### Iodination of CCK-33

CCK-33 was acylated with <sup>125</sup>I-labeled Bolton-Hunter Reagent essentially as described by Sankaran et al. (6) and Rehfeld (19). Derivatized peptide was separated from underivatized peptide and unreacted, hydrolyzed reagent by chromatography on a column (1.6 × 100 cm) of Sephadex G-50 (superfine), equilibrated, and eluted with 0.5 M acetic acid, pH 2.40, containing 0.2% gelatin and 50 μM DTT as previously reported (12).

<sup>2</sup> This work was presented at a satellite meeting of the American Pancreatic Association entitled, "Pancreatic Physiology," held in Lake of the Ozarks, MO, November 1 and 2, 1983.

## Preparation of Pancreatic Plasma Membranes

Rat pancreatic plasma membranes were prepared from 125–150 g male rats (Charles River Breeding Laboratories, Charles River, MA) as previously reported (12). Membranes were stored under liquid N<sub>2</sub> as 300 μg suspensions (1 μg/μl) in HMS (25 mM HEPES, 5 mM MgCl<sub>2</sub>, and 104 mM NaCl) pH 7.4, containing 0.2% BSA, 0.01% soybean trypsin inhibitor (STI), 1 mM PMSF, and 1 mM bacitracin.

### Affinity-labeling Procedure

Membranes were affinity cross-linked with <sup>125</sup>I-CCK-33 in conjunction with disuccinimidyl substrate (DSS) or m-maleimidobenzoyl *N*-hydroxysuccinimide ester (MBS) essentially as described by Pilch and Czech (18). Membranes were thawed at room temperature and centrifuged for 2 min (12,000 g) in a Brinkmann Model 3200 Microfuge. Pellets were resuspended in 0.2 ml of HMS, pH 7.4, containing 0.2% BSA, 0.01% STI, 1 mM PMSF, 1 mM bacitracin, and <sup>125</sup>I-CCK-33 (1–2 nM) by gentle agitation. Incubations were carried out for 15 min at 23°C at the end of which time the tubes were placed on ice and 1 ml of ice-cold HMS, pH 7.4, containing 0.2% BSA and 0.01% STI was added to each tube. Membranes were pelleted by centrifugation at 12,000 g for 2 min and placed on ice. Pellets were resuspended in 98 μl of ice-cold HMS, pH 7.4, and 2 μl of a 2.5 mM solution of DSS or MBS freshly dissolved in dimethylsulfoxide was added yielding a final concentration of 50 μM of each reagent. Cross-linking was allowed to proceed for 5 min on ice followed by quenching with 20 mM Tris, pH 7.4 (supplemented with 50 or 20 mM 2-mercaptoethanol for MBS-containing tubes), and centrifugation at 12,000 g for 4 min to yield a final pellet for analysis by gel electrophoresis or endoglycosidase treatment.

### Preparation of Endo F

Endo F was either obtained as a gift from Drs. John Elder and Stephen Alexander (Research Institute of Scripps Clinic) or prepared from suspension cultures of *Flavobacterium meningosepticum* (American Type Culture Collection, Rockville, MD) essentially as described by Elder and Alexander (16). Cells were separated from culture medium by centrifugation at 5,000 g for 10 min at room temperature. The supernatant was then brought to 80% saturated ammonium sulfate (560 g/l). After mixing for 1 h at 4°C and centrifugation at 10,000 g for 20 min, the precipitate that had collected along the walls of the centrifuge bottles was recovered by resuspension in 50% ammonium sulfate in 5 mM EDTA (100 ml). This was then centrifuged at 20,000 g for 15 min and the dark green precipitate obtained was "extracted" by resuspension in 0.01 M Na phosphate, pH 7.2, 150 mM NaCl, and 50 mM EDTA and vortexing. This suspension was centrifuged at 10,000 g for 2 min and the final supernatant applied to a column (1.5 × 55 cm) of Ultrogel Aca 54 (LKB, Bromma, Sweden) in 0.01 M Na phosphate, pH 7.2, 150 mM NaCl, 5 mM EDTA, and 0.05% NaN<sub>3</sub>. Aliquots (5 μl) of each column fraction were tested for endo F activity by assaying their ability to cleave oligosaccharides from ovalbumin or RNase B. The peak fractions were pooled, dialyzed against column buffer lacking NaN<sub>3</sub>, concentrated against Aquacide II-A (Calbiochem-Behring Corp., La Jolla, CA) and diluted 1:1 with glycerol. The enzyme was stored in 0.1 M Na phosphate pH 6.1, 50 mM EDTA and 50% glycerol at –20°C at a concentration of 1 U/μl. 1 U of activity is defined as that amount of enzyme that will deglycosylate 1 nmol of RNase B in 1 h at 37°C (as per New England Nuclear).

### Endo F Treatment of Affinity-labeled Samples

**MEMBRANES:** In preparation for digestion with endo F, affinity-labeled membrane pellets (300 μg) were resuspended in 0.2 ml of digestion buffer: 0.1 M Na phosphate, pH 6.1, containing 50 mM EDTA, 1% Nonidet P-40, 0.1% SDS, and 1% 2-mercaptoethanol. Samples were then heated at 100°C for 3 min and allowed to cool to room temperature. Endo F and/or buffer was then added to each membrane aliquot (50 μl) and digestion was allowed to proceed for 2 h at 37°C. An equal volume of SDS sample buffer was then added to each sample followed by heating at 100°C for 3 min and analysis by SDS PAGE.

**GEL SLICES:** For treatment of individual binding proteins, affinity-labeled membranes were resolved on 9% acrylamide gels under reducing conditions. The affinity-labeled binding proteins ( $M_r$  130,000,  $M_r$  85,000, and  $M_r$  55,000) were located by autoradiography of the dried, unfixed, unstained gels, and excised. Each gel slice was placed in a separate tube and rehydrated in 0.18 ml of endo F digestion buffer. The accessibility of enzyme to substrate was then increased by sequentially freeze-thawing the tubes through three cycles of alternate incubation in a dry ice-methanol bath/37°C water bath for 10 min each. Endo F and/or buffer was then added to each tube and the incubation allowed to proceed for 24 h at 37°C with gentle shaking. The incubation was

terminated by the addition of 0.05 ml of SDS sample buffer and heating at 100°C for 3 min. The samples (gel pieces and incubation buffer) were resolved on a second 9% acrylamide gel having a 4-cm stacking gel.

### Neuraminidase Treatment of Affinity-labeled Membranes

For neuraminidase treatment, affinity-labeled membrane pellets were resuspended in a final 0.2 ml volume of 50 mM HEPES, pH 5.0, containing 5 mM MgCl<sub>2</sub>, and 0.15 M NaCl with or without 1 U of neuraminidase (Sigma Type X, from *Clostridium perfringens*). After a 30-min incubation at 37°C, the membranes were pelleted and solubilized in preparation for electrophoresis.

### WGA-Agarose Chromatography

WGA was immobilized on agarose by reacting 10 mg WGA (Pharmacia Fine Chemicals, Uppsala, Sweden) with 5 ml of Reacti-gel (6X) (Pierce Chemical Co., Rockford, IL) in 0.1 M Na borate, pH 8.5 (5 ml), at 4°C for 48 h. After quenching with 0.1 M glycine in 0.1 M Na borate, pH 8.5, for 3 h at 23°C, the derivatized agarose was sequentially washed with 0.1 M NaHCO<sub>3</sub>, 0.5 M NaCl, 50 mM HEPES, pH 7.5, 0.5 M NaCl, 50 mM HEPES, pH 7.5, 0.1 M NaCl, and stored at 4°C in the latter buffer containing 0.05% Na azide. For affinity chromatography, 0.4 ml of WGA-agarose was equilibrated in 50 mM HEPES, pH 7.5, containing 0.1 M NaCl and 0.1% NP-40. After affinity labeling, membranes (300 µg) were solubilized in HMS containing 0.2% BSA, 0.01% STI, 1 mM PMSF, 1 mM bacitracin, and 2% NP-40 by gentle agitation and incubation on ice for 15 min. Insoluble material was removed by centrifugation at 100,000 *g*<sub>av</sub> for 60 min. The soluble fraction was diluted to 0.2% NP-40 by addition of 50 mM HEPES, pH 7.5, containing 0.1 M NaCl and added to the WGA-agarose beads. After incubation for 15 min at 23°C, the suspension was packed into a column (0.8 × 4 cm). The flow-through was recycled three times and the gel was sequentially washed with 2 ml each of 0.1 M NaCl, 0.5 M NaCl, and finally 0.3 M GlcNAc in 0.5 M NaCl all in 50 mM HEPES, pH 7.5, containing 0.1% NP-40. SDS sample buffer (0.1 ml) was added to each column fraction (0.2 ml) and the samples analyzed on SDS gels as described below.

### Gel Electrophoresis and Autoradiography

Cross-linked membrane pellets or gel slice digests were solubilized in sample buffer consisting of 0.125 M Tris, pH 6.8, containing 4% SDS, 10 mM EDTA, 15% sucrose, 0.1 M DTT, and 0.01% bromophenol blue, heated at 100°C for 3 min and run on 9% acrylamide slab gels (11 cm × 0.75 or 1.5 mm) containing 2 mM EDTA according to the method described by Laemmli (20). After electrophoresis, the gels were fixed and stained with Coomassie Brilliant Blue (21) or with the silver stain (22). The dried gels were exposed to Kodak XAR-5 x-ray film for 2–21 d using a Dupont Cronex light intensifying screen.

## RESULTS

To determine whether the CCK-binding proteins identified in rat pancreatic plasma membranes are glycoproteins, we initially chose to test their sensitivities to treatment with endo F. This enzyme has been shown to cleave both mannose-rich and complex N-linked oligosaccharide side chains en bloc from glycoproteins (16). As shown in Fig. 1, when affinity-labeled membranes were treated with endo F, a consistent decrease in the apparent molecular weights of the CCK-binding proteins was observed. These results suggested the following cleavage pattern: *M*<sub>r</sub> 130,000 to >110,000; *M*<sub>r</sub> 85,000 to >65,000; *M*<sub>r</sub> 55,000 to >45,000. These assignments were confirmed by treating each individual binding protein, obtained by excision from an unfixed gel, with endo F and analyzing the cleavage products (Fig. 2).

Assuming that the cleavage products shown in Figs. 1 and 2 represent the affinity-labeled binding proteins, devoid of N-linked oligosaccharide chains, it was next of interest to attempt to "capture" intermediate cleavage forms of each protein in order to estimate the total number of N-linked oligosaccharide side chains present in each of these proteins. To this end, affinity-labeled membrane proteins were resolved by

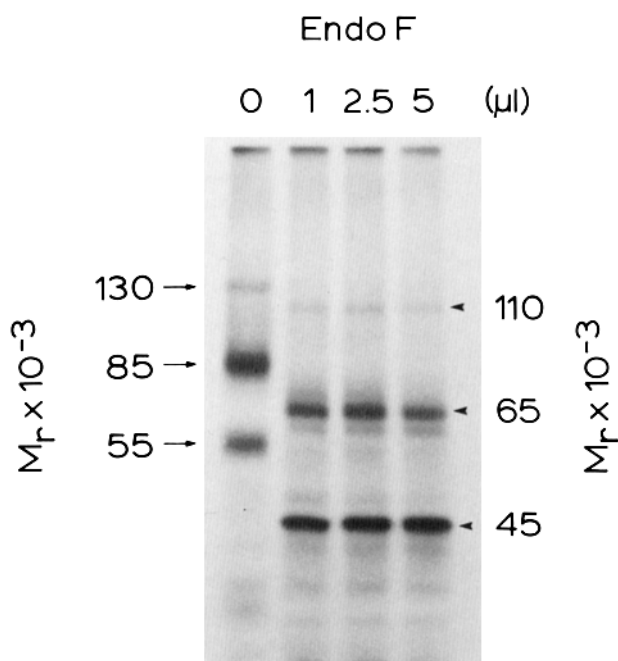


FIGURE 1 Endo F treatment of affinity cross-linked plasma membranes. Rat pancreatic plasma membranes (300 µg) were affinity cross-linked with <sup>125</sup>I-CCK-33 using DSS as described. Cross-linked membranes were resuspended in 200 µl of endo F sample buffer and boiled for 3 min. After cooling, endo F and/or buffer was added to each 50-µl aliquot and the samples incubated at 37°C for 2 h. Incubations were terminated by the addition of electrophoresis sample buffer. The samples were resolved on a 9% SDS gel and the dried gel was subjected to autoradiography. Arrows indicate the migration positions of untreated binding proteins. Arrowheads indicate final endo F cleavage products. In this experiment, 1 µl of enzyme is equal to 5 U of activity.

SDS PAGE and the CCK-binding proteins localized by autoradiography of the dried, unfixed, unstained gel, excised, and subjected to limited endo F digestion as described in Materials and Methods. The results obtained (Fig. 3) indicated that the *M*<sub>r</sub> 55,000 component contains at least four, the *M*<sub>r</sub> 85,000 component contains at least three, and the *M*<sub>r</sub> 130,000 component contains at least one N-linked oligosaccharide side chain(s) within their structures.

We have previously reported the specific affinity labeling of a *M*<sub>r</sub> 85,000 protein in rat pancreatic plasma membranes using the heterobifunctional SH, NH<sub>2</sub> reactive reagent MBS (23; L. D. Madison, S. A. Rosenzweig, and J. D. Jamieson, manuscript submitted for publication). As shown in Fig. 4, the *M*<sub>r</sub> 85,000 CCK-binding protein identified by MBS cross-linking contains three N-linked oligosaccharide side chains with a major digestion product of *M*<sub>r</sub> 65,000. This lends evidence in support of this protein being identical to the *M*<sub>r</sub> 85,000 protein labeled using DSS. It should be noted that in the case of the *M*<sub>r</sub> 85,000 protein, even at the highest dose of endo F used (Fig. 3), complete cleavage was not obtained as minor labeled bands were detected above and below the major *M*<sub>r</sub> 65,000 cleavage product (see also Figs. 1 and 4). This was consistently observed for the *M*<sub>r</sub> 85,000 protein labeled with either DSS or MBS. It is not known whether this lack of complete cleavage was due to inaccessibility of the cleavage site(s), because the cross-linking made these sites inaccessible, or was due to lack of sufficient endo F for complete digestion.

When endo H (which cleaves mannose-rich N-linked oli-

gosaccharides exclusively [24]), was used in similarly designed experiments, no change in the electrophoretic mobilities of any of these proteins was detected (data not shown). These results suggested but do not prove that all the N-linked oligosaccharides identified by endo F treatment might be

complex. Because most complex oligosaccharides contain terminal sialic acid residues, we tested the affinity-labeled binding proteins for sensitivity to neuraminidase. Fig. 5 shows that the electrophoretic mobilities of all three binding proteins were increased by neuraminidase treatment, supporting the classification of these proteins as sialoglycoproteins. Whether all of the N-linked oligosaccharide side chains identified contain sialic acid (and are thus complex) is currently under investigation.

Additional support for the sialoglycoprotein nature of the affinity-labeled CCK-binding proteins was obtained by chromatography on WGA-agarose (WGA specifically binds to sialic acid- and GlcNAc-containing glycoproteins [25, 26]). As shown in Fig. 6A, all three CCK-binding proteins bound to the lectin and could be specifically eluted with GlcNAc. However, it is not known whether each protein directly interacted with the lectin or indirectly by association with other membrane glycoproteins in mixed micelles. Based on the pattern of silver stained proteins shown in Fig. 6B, it can be seen that the GlcNAc-eluted fractions were enriched in proteins having  $M_s$  corresponding to those of the major CCK-binding proteins. However, it is not known whether these silver-stained proteins represent the CCK-binding proteins exclusively or these proteins plus additional pancreatic plasma membrane glycoproteins having similar molecular weights. Nonetheless, it is clear that WGA-agarose affinity chromatography of pancreatic membranes yields a several-fold enrichment in these glycoproteins thus providing both an effective method for the partial purification of CCK receptors and additional evidence of their glycoprotein nature.

#### DISCUSSION

Many polypeptide hormone receptors have been considered to be glycoproteins on the basis of their interactions with various lectins (27-30). In the present study, we used sensitivity to cleavage by endo F to initially determine whether the CCK-binding proteins in pancreatic membranes were glyco-

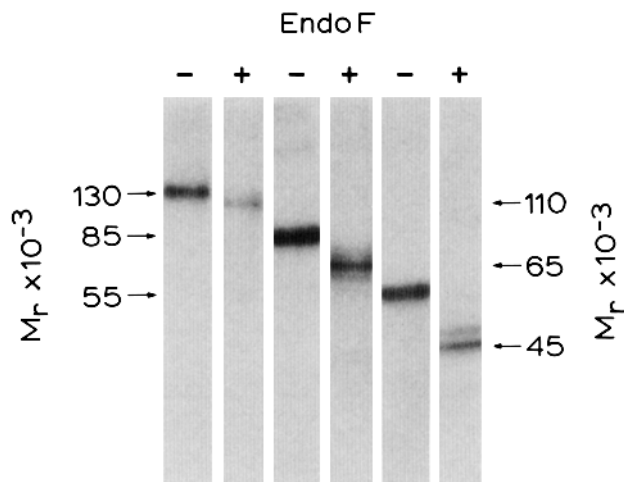


FIGURE 2 Endo F treatment of individual affinity-labeled CCK binding proteins. Affinity cross-linked plasma membranes were divided into several equal parts (50  $\mu$ g of membrane protein) and electrophoresed on a 9% gel. The unfixed, dried gel was autoradiographed and the radioactive bands were located. Gel bands were excised, rehydrated in sample buffer and their porosity increased by sequential freeze-thawing as described. Enzyme and/or buffer was then added and the samples incubated for 24 h at 37°C. Incubation was terminated by addition of 50  $\mu$ l of SDS sample buffer and the gel slices and buffer loaded onto a 9% gel. After electrophoresis, the gels were fixed, stained, and subjected to autoradiography. Plus signs indicate those samples that were treated with 20 U of endo F. Arrows on left indicate the positions of untreated binding proteins. Arrows on right indicate positions of the major endo F cleavage products.

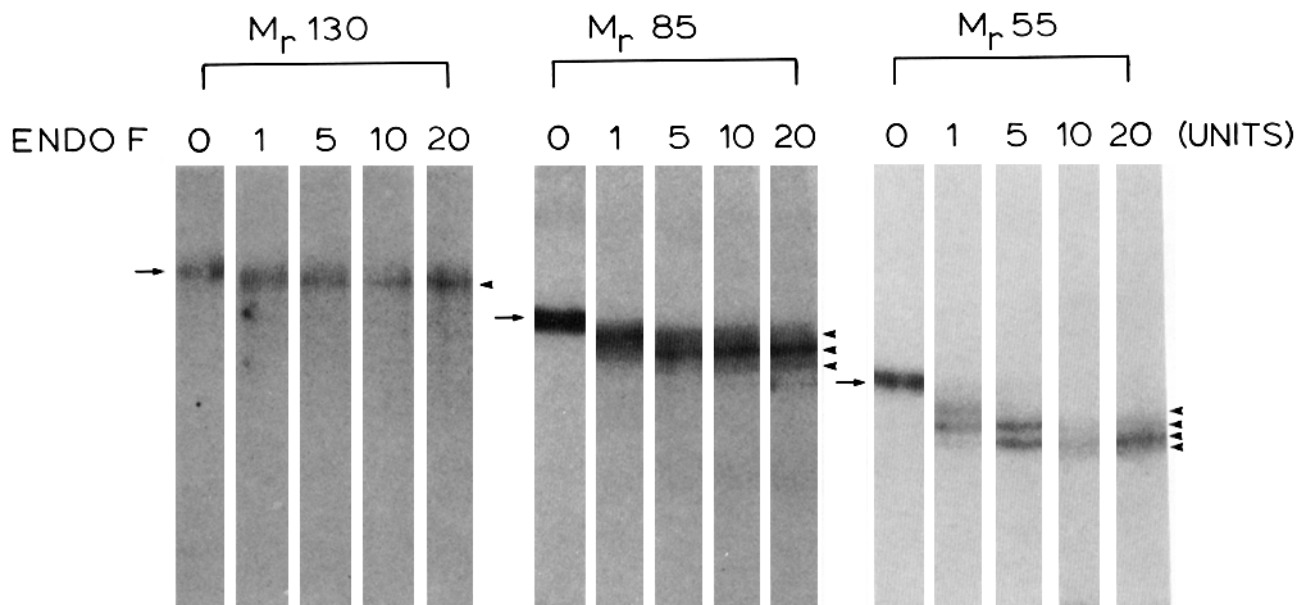


FIGURE 3 Dose-dependent endo F treatment of individual CCK-binding proteins. Individual affinity-labeled binding proteins were obtained and treated with various doses of endo F as described in the legend to Fig. 2. Arrows indicate the migration positions of untreated binding proteins. Arrowheads indicate the different species generated and thus indicate an approximation of the total number of N-linked oligosaccharide side chains present in each binding protein.  $M_r \times 10^{-3}$ .

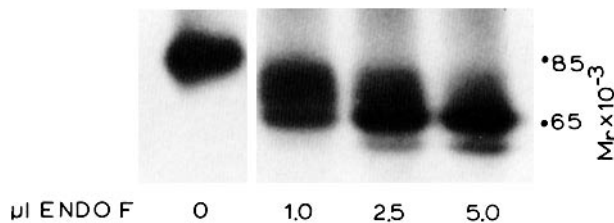


FIGURE 4 Endo F treatment of plasma membranes affinity cross-linked with MBS. Plasma membranes cross-linked to  $^{125}\text{I}$ -CCK-33 with MBS were resuspended in endo F buffer, boiled for 3 min and processed for endo F treatment as described in the legend to Fig. 1. For the purpose of clarity, only the  $M_r$  50,000–100,000 region of the gel is shown.

proteins. An added benefit to this approach was that it also enabled us to determine the minimal number of N-linked oligosaccharide side chains present on these proteins. Given that endo H treatment was without effect but that neuraminidase digestion affected all three components and that all could specifically bind to WGA, we suggest that some if not all of the endo F-sensitive N-linked oligosaccharide side chains are of the complex variety (31). Whether any of these proteins contain O-linked oligosaccharide side chains is currently under investigation.

We have previously shown (12) that the  $M_r$  85,000 component is the major CCK-binding protein in pancreas and that it exists both free and in disulfide linkage with another membrane protein to yield a  $M_r$  ~140,000 protein complex. This complex is only detectable in gels run in the absence of reductant. Sakamoto et al. (14) and Svoboda et al. (15) have made similar observations. Because the conditions we used for optimal endo F cleavage required the use of 1% 2-mercaptoethanol (16), it was not possible to study the disulfide-linked form of the CCK receptor. We are currently testing the efficiency of endo F cleavage of the CCK-binding proteins in the absence of reductant in order to analyze this complex. It should contain at least three N-linked oligosaccharide side chains contributed by the  $M_r$  85,000 protein. If the disulfide-linked subunit of this protein is also a glycoprotein, we should detect additional N-linked oligosaccharides by endo F cleavage.

The use of endo F to probe CCK receptor structure has lent further insight into the lack of relation between the different CCK-binding proteins. Specifically, it was found that the  $M_r$  85,000 protein had three (~20,000 shift in  $M_r$ ) and the  $M_r$  55,000 protein had four (~10,000 shift in  $M_r$ ) N-linked oligosaccharide side chains. Assuming that under the conditions used we detected all the endo F products of each binding protein, then it is unlikely that the  $M_r$  55,000 protein is generated from the  $M_r$  85,000 protein by proteolysis as previously suggested (12–14), since it contains one more carbohydrate side chain than its putative precursor. Limited peptide mapping analysis of these proteins is consistent with this notion (17). In addition, the  $M_r$  130,000 component (~20,000 shift in  $M_r$ ) was found to possess only one N-linked oligosaccharide side chain suggesting that it is a distinct polypeptide and thus structurally unrelated to the  $M_r$  85,000 and  $M_r$  55,000 components. However, this must be interpreted with caution since it is possible that the  $M_r$  130,000 component contains more than one N-linked oligosaccharide and we are not resolving its endo F digestion intermediates on the 9% gels used in this study. Whether the  $M_r$  55,000 and  $M_r$  130,000

proteins are nearest neighbors to the  $M_r$  85,000 protein and as such become specifically labeled during cross-linking or are noncovalently associated subunits of a multicomponent receptor is not clarified by the present study.

The use of endo F to compare the polypeptide backbones of the  $M_r$  85,000 binding protein obtained by DSS or MBS cross-linking lends additional support to the relatedness of these two proteins and therefore to the hypothesis that the  $M_r$  85,000 protein is the major (CCK) recognition subunit of the CCK receptor. Furthermore, this kind of observation exemplifies the utility of endo F as a general probe of the core protein structure of glycoproteins. In combination with endo H cleavage data, endo F digestion can provide information on the number of N-linked oligosaccharide side chains present on a given glycoprotein as well as whether they are simple or complex. Additionally, removal of all N-linked glycans by endo F before peptide mapping studies should allow complete proteolysis to take place (32).

In summary, we used sensitivity to endo F digestion as an indication of the presence of N-linked oligosaccharide side chains on the CCK-binding proteins on rat pancreatic mem-

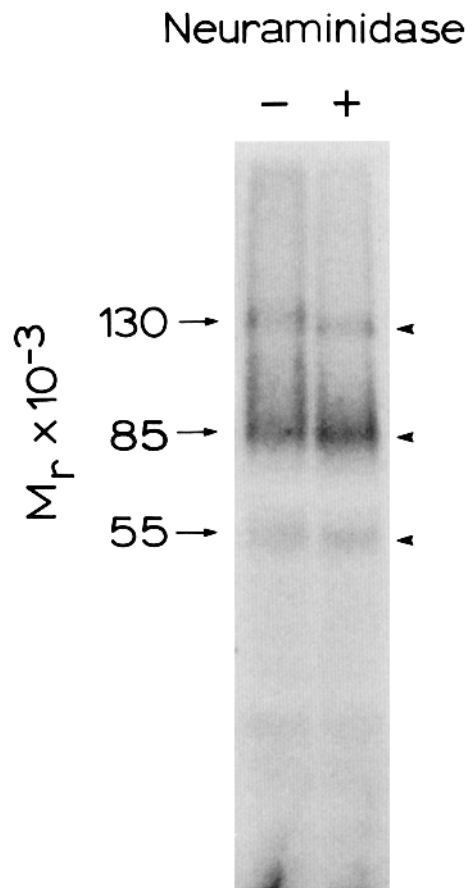


FIGURE 5 Neuraminidase treatment of affinity cross-linked plasma membranes. Plasma membranes were affinity cross-linked to  $^{125}\text{I}$ -CCK-33 with DSS as described and resuspended in a final 0.2 ml volume of 50 mM HEPES, pH 5.0 containing 5 mM  $\text{MgCl}_2$  and 0.15 M NaCl with or without 1 U of neuraminidase. After a 30-min incubation at 37°C, the membranes were pelleted and solubilized in preparation for electrophoresis. Samples were resolved on a 9% gel and following fixing and staining the gel was autoradiographed. Arrows indicate the migration positions of untreated binding proteins. Arrowheads indicate the migration positions of neuraminidase-treated binding proteins.

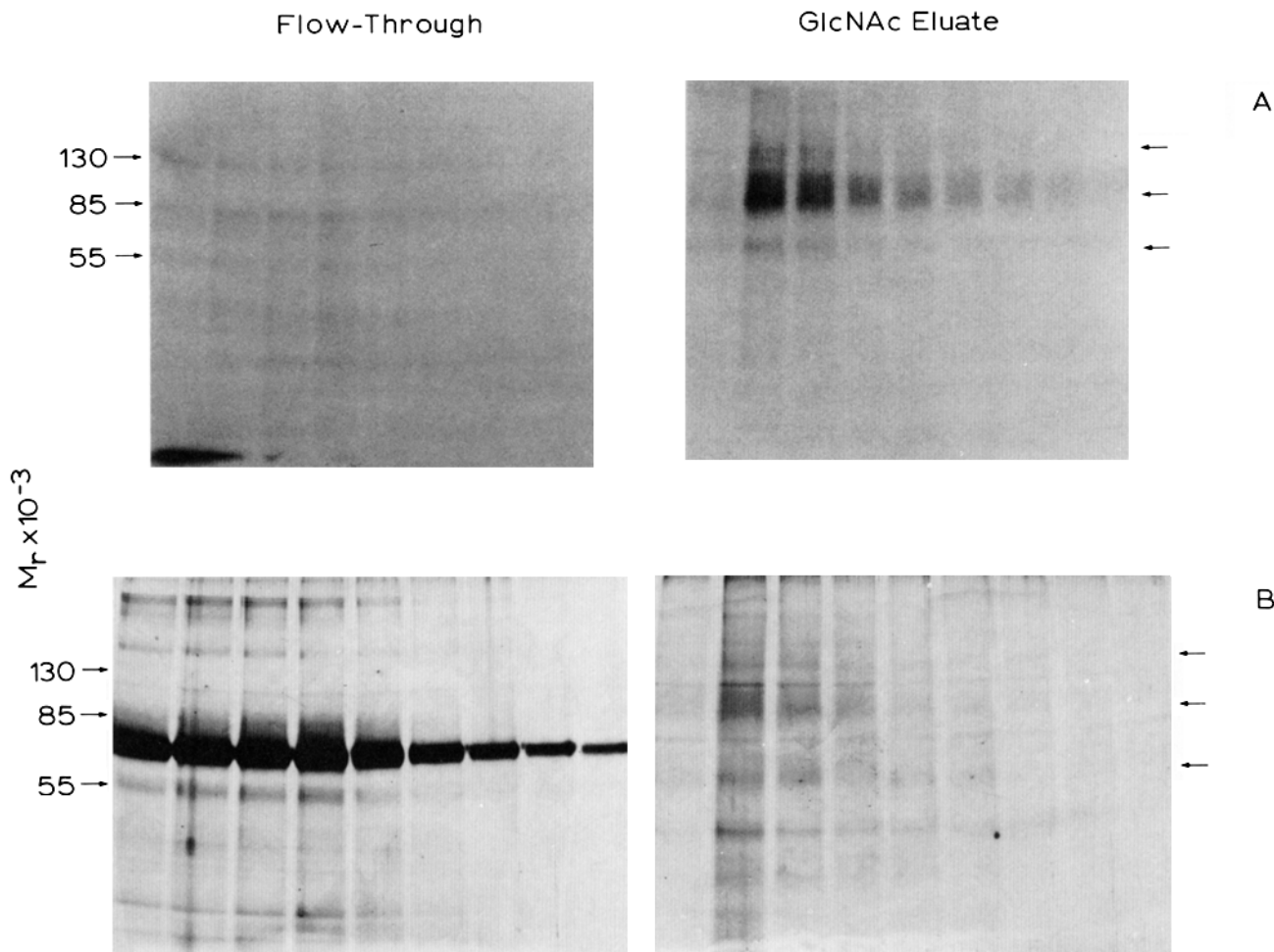


FIGURE 6 Chromatography of affinity-labeled CCK-binding proteins on WGA-agarose. Rat pancreatic plasma membranes (300  $\mu$ g) were affinity cross-linked to  $^{125}$ I-CCK-33 with DSS, solubilized with NP-40 and incubated with WGA-agarose as described. (A) Autoradiographic pattern of fractions 1-9 of the final flow-through and of the first nine fractions of the GlcNAc eluate. (B) Protein pattern of the corresponding gels in A stained with the silver stain.

branes. The advantage of this approach was demonstrated by the ability to additionally determine the minimal number of oligosaccharide side chains on the major  $M_r$  85,000 binding protein and therefore the  $M_r$  of its N-linked carbohydrate-free polypeptide core. This approach was also instrumental in allowing structural comparisons to be made between binding proteins labeled by different cross-linking reagents and thus provided convincing evidence of their structural relatedness.

Further studies are required for a more detailed analysis of the structure of the CCK receptor, including generation of specific immunologic probes. In this regard, it should be noted that in generating monoclonal antibodies to the epidermal growth factor receptor of A-431 cells, Richert et al. (33) obtained an antibody specific for human blood group H type I oligosaccharides. Although these antigenic determinants are present in O-linkage to serine or threonine residues (34), removal of N-linked oligosaccharide side chains using endo F may be a useful step in the preparation of antigens. The polypeptide backbones so obtained can then be used as immunogen in generating polyclonal and monoclonal antibodies, thus reducing the chance of obtaining carbohydrate directed antibodies, particularly in proteins having little or no O-linked oligosaccharides.

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