Protease Peptide Mapping of Affinity-Labeled Rat Pancreatic Cholecystokinin-Binding Proteins†

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ABSTRACT: Affinity-labeling probes with sites of cross-linking distributed along the ligand have been used to biochemically characterize the pancreatic cholecystokinin (CCK) receptor. Probes with photolabile sites spanning the receptor-binding domain have labeled a M_r = 85 000–95 000 plasma membrane protein, while a probe cross-linked via the amino terminus of CCK-33, far removed from the carboxyl-terminal receptor-binding domain, has labeled a distinct M_r = 80 000 protein. In this work, protease peptide mapping of the pancreatic proteins labeled by each of these probes has been performed to gain insight into the identities of the bands and to define domains of the labeled proteins. Photolabile decapeptide probes with sites of cross-linking at the amino terminus, mid region, and carboxyl terminus of the receptor-binding domain each labeled a M_r = 85 000–95 000 glycoprotein with a M_r = 42 000 core protein and similar *Staphylococcus aureus* V8 protease peptide maps. This confirms that each probe labels the same binding protein and the same domain of that protein. Serial slices through the broad labeled band were separately deglycosylated and protease-treated, demonstrating a single protein core with differential glycosylation. The CCK-33-based probe, however, labeled predominantly two proteins, one having similar sizes in its native and deglycosylated forms to that labeled by the decapeptide probes and a distinct M_r = 80 000 protein. Of note, the peptide map of the protein believed to be the same as that labeled by the shorter probes was different, suggesting that this probe labeled the binding subunit at a site distinct from that which was labeled by the short probes.

Cholecystokinin (CCK) represents a family of linear peptide hormones, ranging in length from 4 to 58 amino acid residues, with physiologic effects on multiple target tissues (Mutt, 1980). Among these, the target studied most extensively is the pancreatic acinar cell, where CCK stimulates enzyme secretion (Mutt, 1980). The pancreatic CCK receptor has been studied functionally (Miller et al., 1981), morphologically (Rosenzweig et al., 1983), and biochemically (Rosenzweig et al., 1983; Pearson & Miller, 1987, Pearson et al., 1987b; Powers et al., 1988a).

The first biochemical characterization of a pancreatic CCK-binding protein was performed by affinity labeling using ^{125}I-Bolton-Hunter-CCK-33 (Rosenzweig et al., 1982, 1983). That and subsequent similar studies identified a major M_r = 80 000 pancreatic plasmalemmal glycoprotein (variably described to range between M_r = 76 000 and M_r = 95 000) (Rosenzweig et al., 1982, 1983; Svoboda et al., 1983). However, extensive primary structure–activity data have localized the carboxyl-terminal heptapeptide as the biologically active part of CCK, which contains the receptor-binding domain (Villanueva et al., 1982; Ondetti et al., 1970). Since the sites of cross-linking of CCK-33-based probes are located in the amino terminus of that molecule, far removed from the receptor-binding domain, we have developed a series of "shorter" decapeptide probes that possess sites for cross-linking close to or within the receptor-binding domain (Pearson & Miller, 1987; Pearson et al., 1987b; Powers et al., 1988a; Klueppelberg et al., 1989). All of these probes have identified a distinct M_r = 85 000–95 000 pancreatic plasma membrane protein in affinity-labeling studies (Pearson & Miller, 1987; Pearson et al., 1987a,b; Powers et al., 1988a; Klueppelberg et al., 1989).

Questions have been raised about the nature of the proteins labeled by each probe since they migrate as broad bands on polyacrylamide gels. In addition, it has recently been observed that under certain conditions ^{125}I-Bolton-Hunter-CCK-33 can label two pancreatic proteins, one of which migrates in the same region as the M_r = 85 000–95 000 band labeled by the shorter probes (Madison et al., 1987).

In this work, we have applied protease peptide mapping, a technique that generates characteristic cleavage fragments (Cleveland et al., 1977), to better understand the broad nature of the labeled proteins, to gain insight into the identities of the pancreatic proteins labeled by each of a battery of CCK receptor probes, and to define domains of the labeled proteins. We have used probes with photolabile sites of cross-linking that span the theoretical receptor-binding domain [2-diazo-3,3,3-trifluoropropionyl-^{125}I-D-Tyr-Gly-[(Nle^{28,31})CCK-26-33], amino-terminal region; ^{125}I-D-Tyr-Gly-[(Nle^{28,31},6-NO_2-Trp^{30})CCK-26-33], middle region; and ^{125}I-D-Tyr-Gly-[(Nle^{28,31},pNO_2-Phe^{33})CCK-26-33], carboxyl-terminal region] as well as ^{125}I-Bolton-Hunter-CCK-33. Peptide mapping of labeled bands was performed both before and after enzymatic deglycosylation.

Materials and Methods

Materials. Synthetic CCK-8 was from Peninsula Laboratories (Belmont, CA). Purified natural porcine CCK-33 was from Professor Viktor Mutt (Karolinska Institutet, Stockholm, Sweden), and synthetic CCK-33 was from Peptides International (Louisville, KY). These peptides behaved identically with each other, but inadequate quantities of the natural hormone were available to use that exclusively. Na^{125}I (>1800 Ci/mmol) was from Amersham (Arlington Heights, IL) and ^{125}I-Bolton-Hunter reagent was from New England Nuclear (Boston, MA). Protease inhibitors included soybean trypsin inhibitor from Worthington Biochemicals (Freehold, NJ) and phenylmethanesulfonyl fluoride from Sigma (St. Louis, MO).

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Protected amino acids and methylbenzhydrylamine resin were from Chemical Dynamics (South Plainfield, NJ), Peninsula Laboratories, and Applied Biosystems (Foster City, CA).

Receptor Probes. Peptides D-Tyr-Gly-[(Nle^{28,31})CCK-26-33], D-Tyr-Gly-[(Nle^{28,31},6-NO_2-Trp^{30})CCK-26-33], and D-Tyr-Gly-[(Nle^{28,31},pNO_2-Phe^{33})CCK-26-33] were synthesized as we described (Powers et al., 1988a,b; Klueppelberg et al., 1989). The identity of each peptide was verified by amino acid analysis and mass spectrometry. Each probe was iodinated by using the solid-phase oxidant *N*-chlorobenzenesulfonamide (Iodo-Beads, Pierce Chemical Co., Rockford, IL) and purified by reversed-phase HPLC to yield a specific radioactivity of 2000 Ci/mmol, as we have described (Pearson et al., 1986).

For the synthesis of the probe that contains a carbene precursor, the radioiodinated peptide ^{125}I-D-Tyr-Gly-[(Nle^{28,31})CCK-26-33] was acylated with 20 μmol of 2-diazo-3,3,3-trifluoropropionyl chloride (DTP) in 1 mL of 30% acetonitrile/70% triethylamine acetate, pH 5.0, for 35 min after the addition of 20 μL of *N*-methylmorpholine. This, too, was purified by reversed-phase HPLC to yield a product of 2000 Ci/mmol prior to usage.

CCK-33 (5–10 μg) was acylated with ^{125}I-Bolton-Hunter reagent according to the method of Rehfeld (1978) and purified by HPLC as we described (Shaw et al., 1987).

Pancreatic Membrane Preparation. Enriched pancreatic plasma membranes were prepared from 125–150-g male Harlan Sprague-Dawley rats as we described (Rosenzweig et al., 1983). In brief, excised pancreata were homogenized at 4 °C in 10 volumes of iced 0.3 M sucrose containing 0.01% soybean trypsin inhibitor, 1 mM phenylmethanesulfonyl fluoride, and 1 mM 2-mercaptoethanol. After filtration through gauze, the sucrose concentration of the homogenate was raised to 1.3 M by the addition of 2.0 M sucrose. This material was overlayered by 0.3 M sucrose and centrifuged at 149000*g* for 3 h. Membranes were collected at the 0.3–1.3 M interface, washed twice, and stored in Krebs–Ringers–Hepes (KRH) medium, pH 7.4, containing Hepes (25 mM), NaCl (104 mM), KCl (5 mM), $MgSO_4$ (1.2 mM), $CaCl_2$ (2 mM), KH_2PO_4 (1 mM), bovine serum albumin (0.2%), soybean trypsin inhibitor (0.01%), and phenylmethanesulfonyl fluoride (0.5 mM), at −70 °C. Under these conditions, membranes retained their binding activity for at least 2 months.

Affinity-Labeling Studies. Enriched pancreatic plasma membranes containing 100 μg of protein and 500 pM radioligand were incubated in 500 μL of KRH medium for 60 min at 25 °C, conditions shown to permit binding equilibrium. Bound and free radioligand were separated by centrifugation for 5 min at 10000*g*. The washed membrane pellet was resuspended to 98 μL of KRH medium without bovine serum albumin for chemical cross-linking or in 1.0 mL of similar medium for photolysis.

For chemical cross-linking of ^{125}I-Bolton-Hunter-CCK-33, *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS) or disuccinimidyl suberate (DSS) dissolved in dimethyl sulfoxide was added to final optimal concentrations of 5 and 50 μM, respectively. After 5-min incubations, the reactions were quenched by the addition of 100 μL of 0.2M Tris, pH 7.4, and the membranes were collected by centrifugation. For photolysis, the membranes were exposed to a 200-W medium-pressure Hanovia lamp through a Pyrex filter at 4 °C for 30 min (Powers et al., 1988a).

Following chemical cross-linking or photolysis, the membranes were pelleted by centrifugation and solubilized in sample buffer containing 4% sodium dodecyl sulfate (SDS), 10 mM EDTA, 15% sucrose, 0.0001% bromophenol blue, 0.125 M Tris (pH 6.8), and 0.1 M dithiothreitol. Samples were separated on 9% or 10% polyacrylamide slab gels (15.5 cm × 1.5 mm) with 5% stacking gels (2.0 cm × 1.5 mm) in the presence of SDS, according to the method of Laemmli (1970). Gels were frozen and exposed to X-ray film (Kodak XAR-5) at −70 °C for 12–24 h.

Electroelution. The autoradiographically visualized bands, or slices of those bands, were cut out of the gel and electroeluted for 12 h by using the electrophoretic concentrator (ISCO Model 1750, Lincoln, NE) with a cellulose dialysis membrane (molecular weight cutoff of 3500). Electroelution buffer was 0.01 M Tris–acetate, pH 8.6, and 0.1% Nonidet P-40, while the electrode chambers were filled with the same buffer at a higher concentration (0.04 M Tris).

Deglycosylation. Endo-β-*N*-acetylglucosaminidase F (endo F) was purified from suspension cultures of *Flavobacterium meningosepticum* (American Type Tissue Culture Association, Rockville, MD) as described by Elder and Alexander (1982). Enzyme activity was determined by its ability to deglycosylate ovalbumin, with 1 unit defined as the amount of enzyme that deglycosylates 1 nmol of substrate in 1 h at 37 °C.

Following electroelution of labeled proteins, endo F digestion buffer (0.1 M sodium phosphate, pH 6.1, containing 50 mM EDTA, 0.1% Nonidet P-40, 0.1% SDS, and 1% 2-mercaptoethanol) was added to an equal volume of dialysis buffer to yield pH 6.2. For deglycosylation of N-linked simple and complex carbohydrates, 3 units of endo F was added per 50 μL of sample and the reaction mixture incubated for 12 h at 37 °C.

Protease Peptide Mapping. Limited proteolytic digestion was performed by using modifications to the method of Cleveland et al. (1977). In brief, sample buffer containing 0.125 M Tris, pH 6.8, 0.5% SDS, 10% glycerol, and 0.0001% bromophenol blue was added to an equal volume of the sample. *Staphylococcus aureus* V8 protease (537 units/mg, ICN, Lisle, IL) dissolved in 0.125 M Tris-HCl, pH 6.8, 0.1% SDS, and 1 mM EDTA was added to final concentrations of 0.75 μg/100 μL of sample. Proteolysis was carried out at 37 °C for 1 h. The digestion was terminated by the addition of 10 μL of 20% SDS/100 μL of sample, and the cleavage products were resolved on 15% polyacrylamide gels (15.5 cm × 1.5 mm) with 5% stacking gels (2.0 cm × 1.5 mm).

Alternatively, proteolytic digestion was performed within 5-cm stacking gels (Cleveland et al., 1977). Gel slices were loaded into sample wells and overlayered with sample buffer containing 0.125 M Tris-HCl, pH 6.8, 0.15% SDS, 1 mM EDTA, 20% glycerol, and 0.0001% bromophenol blue. Protease dissolved in the same buffer containing 10% glycerol was then added, and electrophoresis was initiated and continued until the dye front reached the bottom of the stacking gel, at which time the current was interrupted for 30 min to allow for proteolytic cleavage. The remainder of the electrophoresis was performed in the usual manner.

Gels were stained with Coomassie blue, dried, and exposed to X-ray film (Kodak XAR-5) at −70 °C for 1–21 days by using Du Pont Quanta III intensifying screens. The molecular weight values for affinity-labeled proteins were determined by interpolation on a plot of log M_r versus mobilities of standard proteins [myosin (M_r = 200000), β-galactosidase (M_r = 116150), phosphorylase *b* (M_r = 92500), bovine serum albumin (M_r = 66200), ovalbumin (M_r = 45000), carbonic anhydrase (M_r = 31000), α-chymotrypsinogen (M_r = 25000), β-lactoglobulin (M_r = 18000), and lysozyme (M_r = 15000)]. The full complement of studies, including the deglycosylation

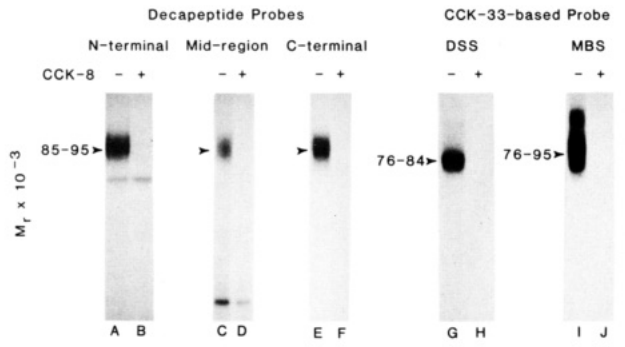

FIGURE 1: Rat pancreatic plasma membranes were affinity labeled with several radiolabeled CCK receptor probes, in the absence or presence of competing 0.1 μM CCK-8. Shown are representative autoradiographs of the SDS–polyacrylamide gels used to separate solubilized reaction products. Lanes a–f show the labeling of a M_r = 85 000–95 000 band by decapeptide probes cross-linked through photolabile groups positioned at the amino terminus, DTP-^{125}I-D-Tyr-Gly-[(Nle^{28,31})-CCK-26-33] (lanes A and B), in the mid region, ^{125}I-D-Tyr-Gly-[(Nle^{28,31},6-NO_2-Trp^{30})-CCK-26-33] (lanes C and D), and at the carboxyl terminus, ^{125}I-D-Tyr-Gly-[(Nle^{28,31},pNO_2-Phe^{33})CCK-26-33] (lanes E and F)of the receptor-binding domain. Right lanes (G–J) show the bands labeled by ^{125}I-BH-CCK-33. A major M_r = 76 000–84 000 band was labeled when DSS was used (lanes G and H), and a M_r = 76 000–95 000 band was labeled with MBS (lanes I and J).

and protease peptide mapping of electroeluted gel slices, was performed a minimum of three times (range three to five times) with each probe.

Results

Affinity Labeling. Each monofunctional photoaffinity-labeling probe, DTP-^{125}I-D-Tyr-Gly-[(Nle^{28,31})CCK-26-33], ^{125}I-D-Tyr-Gly-[(Nle^{28,31},6-NO_2-Trp^{30})CCK-26-33], and ^{125}I-D-Tyr-Gly-[(Nle^{28,31},pNO_2-Phe^{33})CCK-26-33], labeled a single band in the range M_r = 85 000–95 000, and CCK-8 competed for this labeling in a concentration-dependent manner (Figure 1). The migration of this band was not altered when the electrophoresis was performed under reducing or nonreducing conditions or when the membranes had been prepared in the total absence of reductants.

^{125}I-Bolton-Hunter-CCK-33 labeled a major M_r = 76 000–84 000 band when DSS was used for cross-linking (Figure 1) and a broader band ranging between M_r = 76 000 and M_r = 95 000 when MBS was used (Figure 1). No differences were observed in this pattern after affinity labeling with purified natural CCK-33 or synthetic CCK-33. Labeling of these bands was also inhibited by CCK-8 in a concentration-dependent manner.

Decapeptide Photoaffinity Ligands. S. aureus V8 protease (SAP) was only minimally active in cleaving the native M_r = 85 000–95 000 species labeled by DTP-^{125}I-D-Tyr-Gly-[(Nle^{28,31})CCK-26-33], ^{125}I-D-Tyr-Gly-[(Nle^{28,31},6-NO_2-Trp^{30})CCK-26-33], and ^{125}I-D-Tyr-Gly-[(Nle^{28,31},pNO_2-Phe^{33})CCK-26-33]. The fragments that could be generated under these conditions, however, were similar after labeling with each probe (Figure 2). After deglycosylation of the M_r = 85 000–95 000 bands, however, SAP became quite active, generating characteristic fragments.

Endo-β-acetylglucosaminidase F (endo F) treatment shifted the M_r = 85 000–95 000 band labeled by the photoaffinity probes to a major M_r = 42 000 band, with minor bands of M_r = 55 000 and M_r = 27 000 (Figure 2). The intensity of the M_r = 55 000 band was related to the time of digestion, with less labeling of this band after prolonged incubation. The M_r = 27 000 band was observed only inconsistently.

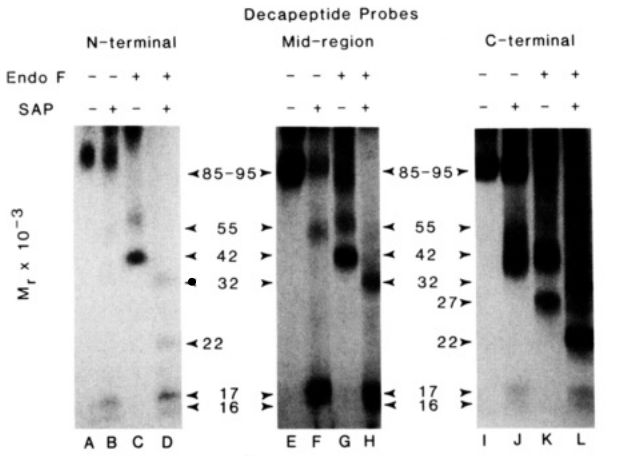

FIGURE 2: Deglycosylation and protease peptide mapping of pancreatic membrane proteins labeled by amino-terminal probe, DTP-^{125}I-D-Tyr-Gly-[(Nle^{28,31})CCK-26-33] (lanes A–D), mid-region probe, ^{125}I-D-Tyr-Gly-[(Nle^{28,31},6-NO_2-Trp^{30})CCK-26-33] (lanes E–H), and carboxyl-terminal probe, ^{125}I-D-Tyr-Gly-[(Nle^{28,31},pNO_2-Phe^{33})-CCK-26-33] (lanes I–L). After pancreatic membranes were affinity labeled and products were resolved on SDS–polyacrylamide gels, the labeled bands were visualized by autoradiography and electroeluted. In this figure are seen autoradiographs of subsequent gels in which these labeled proteins were rerun after no treatment (lanes A, E, and I) or treatment with endo F and/or SAP. Endo F treatment shifted the M_r = 85 000–95 000 species to a major band of M_r = 42 000 (lanes C, G, and K), while additional bands at M_r = 27 000 (lane K) and M_r = 55 000 (lanes C and G) were observed inconsistently. Proteolytic cleavage of the native glycoproteins with *S. aureus* V8 protease (SAP) (lanes B, F, and J) was relatively inefficient, with most radioactivity migrating with starting material, and with fragments of M_r = 52 000, 17 000, and 16 000 observed. After deglycosylation of the M_r = 85 000–95 000 species, SAP was more active, generating products of M_r = 32 000, 22 000, 17 000, and 16 000 (lanes D, H, and L). The relative amount of radioligand linked to individual fragments varied between probes, but was reproducible for each probe.

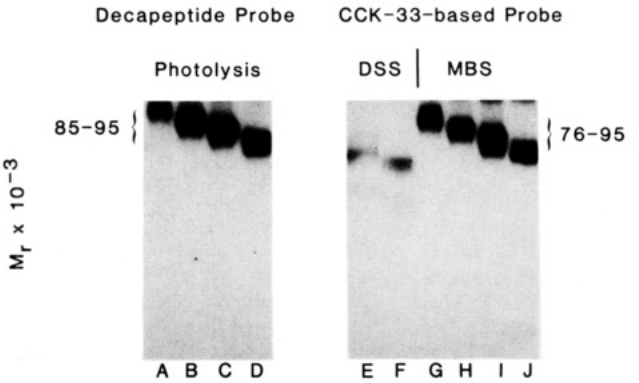

FIGURE 3: Repeat electrophoresis of slices of affinity-labeled pancreatic membrane proteins. Following affinity labeling with ^{125}I-D-Tyr-Gly-[(Nle^{28,31},pNO_2-Phe^{33})CCK-26-33] (lanes A–D) and ^{125}I-BH-CCK-33 with DSS (lanes E and F) and MBS (lanes G–J), the labeled species were resolved on 10% gels, and the visualized bands (see Figure 1) were cut into slices. The labeled proteins were then electroeluted and rerun on a second (15%) gel. The different portions of each band migrated in the same relative positions on a second gel.

The SAP-generated fragments of the deglycosylated M_r = 85 000–95 000 bands labeled by each of the probes were of identical sizes, but there were differences in the relative intensities of labeling that were dependent on the radioligand used (Figure 2).

When the M_r = 85 000–95 000 band was cut into four slices, the proteins from these individual slices migrated on a second gel in the same respective positions (Figure 3). When each of the four slices was separately subjected to deglycosylation and proteolytic mapping, differences provided insight into the nature of the broad band. SAP digestion of the native gel slices

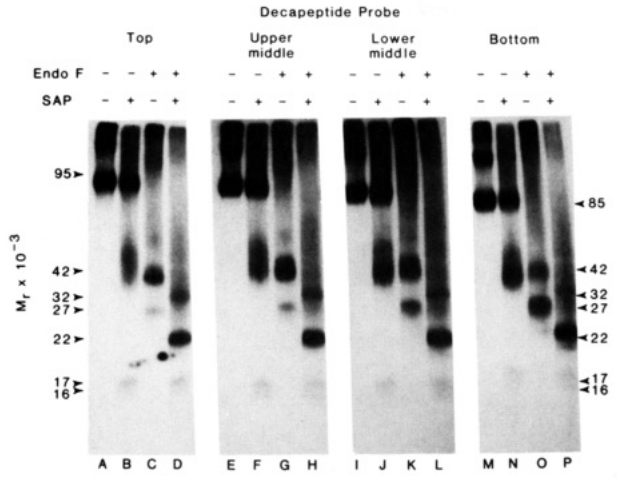

FIGURE 4: Deglycosylation and protease peptide mapping of pancreatic membrane proteins labeled by ^{125}I-D-Tyr-Gly-[(Nle^{28,31},pNO$_2$-Phe^{33})CCK-26-33]. The labeled M_r = 85000–95000 band was cut into four slices, with each electroeluted and rerun after no treatment (lanes A, E, I, and M) or treatment with endo F and/or SAP. SAP digestion of all native slices was relatively inefficient, with much radioactivity migrating with starting material (lanes B, F, J, and N). Fragments of M_r = 17000 and 16000 were generated from each slice, while the larger fragments reflected subtle size differences [M_r = 37000–50000 (lane B), M_r = 33000–43000 (lane N)]. Endo F shifted the radioactivity to a major M_r = 42000 band, with additional bands at M_r = 55000 and 27000. The M_r = 55000 band was more prominent after deglycosylation of the upper slices (lanes C and G), whereas the M_r = 27000 band was more prominent after deglycosylation of the lower slices (lanes K and O). Proteolytic cleavage after deglycosylation generated identical fragments (M_r = 32000, 22000, 17000, and 16000) from all four slices (lanes D, H, L, and P).

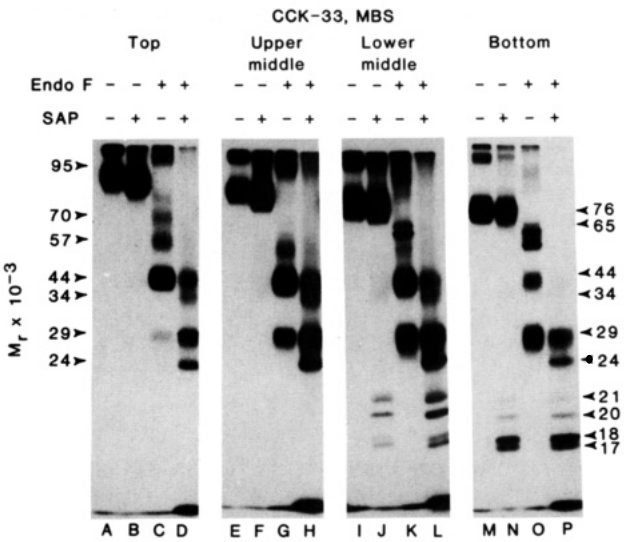

FIGURE 5: Deglycosylation and protease peptide mapping of pancreatic membrane proteins labeled by ^{125}I-BH-CCK-33 with MBS. The M_r = 76000–95000 band was cut into four slices, electroeluted, and rerun without treatment (lanes A, E, I, and M) and after treatment with endo F and/or SAP. The lower slices were much more sensitive to SAP than the upper slices, with enzyme treatment generating fragments of M_r = 21000, 20000, 18000, and 17000 (lanes J and N). Endo F deglycosylation of the slices demonstrated different core proteins in the upper slices and the lower slices, with the upper slices yielding a major band of M_r = 44000 and minor bands at M_r = 70000, 57000, and 29000, while the lower slices yielded a series of sharper bands around M_r = 65000. The deglycosylated upper slices were cleaved by SAP to products of M_r = 45000, 34000, 29000, and 24000 (lanes D and H). These fragments were also detectable after similar treatment of the lower slices (lanes L and P). In addition, SAP treatment of the deglycosylated lower slices produced the same M_r = 21000, 20000, 18000, and 17000 cleavage products (lanes L and P) as were seen without endo F treatment of these slices.

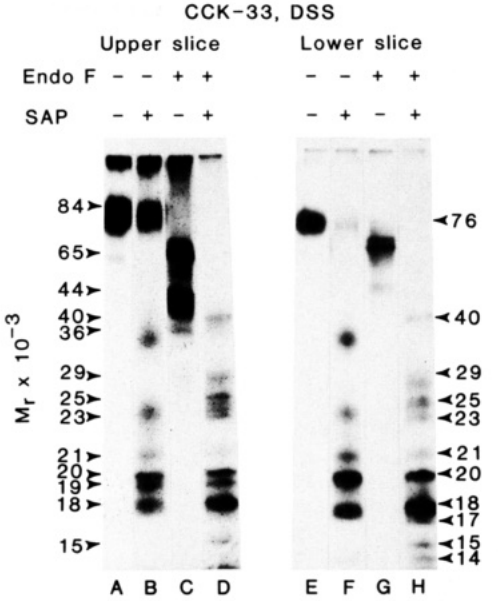

FIGURE 6: Deglycosylation and protease peptide mapping of pancreatic membrane proteins labeled by ^{125}I-BH-CCK-33 with DSS. The M_r = 76000–84000 band was cut into two slices, electroeluted, and rerun without treatment (lanes A and E) or after treatment with endo F and/or SAP. Treatment of the lower slice with SAP yielded cleavage to smaller fragments (lane F), while similar treatment of the upper slice was less effective, with a large amount of radioactivity migrating with starting material (lane B). Of note, an M_r = 19000 fragment was only seen after SAP digestion of the upper slice, and fragments of M_r = 17000 and 14000 were only detected after SAP digestion of the lower slice. Deglycosylation of both slices with endo F revealed a series of bands around M_r = 65000 (lanes C and G). However, deglycosylation of the upper slice yielded an additional cleavage product of M_r = 44000 (lane C). When SAP proteolysis was performed after deglycosylation, numerous cleavage products were observed (lanes D and H). Bands of M_r = 44000, 36000, 24000, and 19000 were seen only after digestion of the upper slice (lane D), while bands of M_r = 17000 and 14000 were seen only after cleavage of the lower slice (lane H).

generated larger fragments that differed in size (ranging from M_r = 37000–50000 in the upper slice to M_r = 33000–43000 in the lower slice) and smaller fragments of identical sizes (M_r = 17000 and 16000) (Figure 4). Deglycosylation of each slice revealed an identical major product of M_r = 42000; however, the pattern generated from each was slightly different. Identical endo F treatment produced relatively more labeling of the M_r = 55000 band from the upper slice and more labeling of the M_r = 27000 band from the lower slice (Figure 4). SAP digestion following deglycosylation, however, generated identical fragments from each slice (Figure 4).

^{125}I-Bolton-Hunter-CCK-33. Like the broad band labeled by the decapeptide ligands, when the band labeled by ^{125}I-Bolton-Hunter-CCK-33 was cut into slices, the proteins from the individual slices migrated on a second gel in the same respective positions (Figure 3).

Each of the slices of the band labeled by ^{125}I-Bolton-Hunter-CCK-33 after cross-linking with MBS (Figure 5) or with DSS (Figure 6) was treated similarly to those from the band labeled by the decapeptide ligands. Similar to the results with the decapeptide probes, the larger fragments generated by SAP treatment of the ^{125}I-Bolton-Hunter-CCK-33-labeled bands still reflected slight size differences, which were eliminated after deglycosylation (observed on overexposed autoradiograph not shown). The heterogeneity of the bands was best demonstrated by the differences in generating fragments from each slice of the native bands. Fragments of M_r = 21000, 20000, 18000, and 17000 were generated primarily

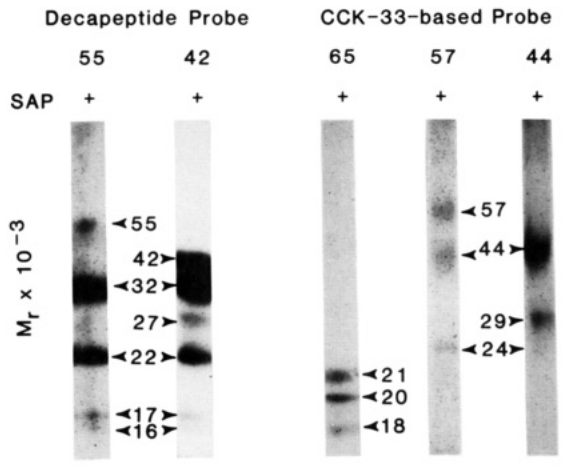

FIGURE 7: Protease peptide mapping of the pancreatic membrane proteins labeled by ^{125}I-D-Tyr-Gly-[(Nle^{28,31},pNO$_2$-Phe^{33})CCK-26-33] and ^{125}I-BH-CCK-33 with MBS, which have been deglycosylated with endo F. Individual bands were electroeluted and treated with SAP within the stacking gel. The M_r = 55 000 band and the M_r = 42 000 band seen after deglycosylation of the species labeled by short probes were cleaved to similar fragments of M_r = 32 000, 22 000, 17 000, and 16 000. An additional product of the M_r = 42 000 band migrated at M_r = 27 000. The ^{125}I-BH-CCK-33 labeled M_r = 57 000 and M_r = 44 000 bands were digested to similar fragments of M_r = 24 000. Additionally, the M_r = 44 000 band yielded a fragment of M_r = 29 000. The cleavage products of the bands of approximate M_r = 65 000 migrated differently at M_r = 21 000, 20 000, and 18 000.

from lower portions of the bands. SAP digestion of the upper portion of the bands following endo F treatment generated fragments of M_r = 34 000, 29 000, and 24 000.

When each slice was deglycosylated, it was clear that the series of bands in the region of M_r = 65 000 was derived from glycoproteins migrating in the lower molecular weight region of the M_r = 76 000–95 000 band labeled by using MBS (Figure 5) and of the M_r = 76 000–84 000 band labeled by using DSS (Figure 6). Deglycosylation of the upper half of these bands produced the M_r = 44 000 product (Figure 5 and 6). On the basis of patterns of deglycosylation, ^{125}I-Bolton-Hunter-CCK-33 was more likely to label the glycoprotein with the M_r = 44 000 core using MBS than DSS. Densitometric analysis of these patterns as well as data generated by cutting and counting bands confirmed these relationships.

Characterization of Individual Products of Endo F Treatment. To further characterize the endo F products, separate SAP digestions of the individual bands were performed. The M_r = 85 000–95 000 species labeled by the decapeptide photoaffinity ligand, ^{125}I-D-Tyr-Gly-[(Nle^{28,31},pNO$_2$-Phe^{33})CCK-26-33], was electroeluted from the first gel, treated with endo F, and resolved on a second gel (Figure 7). The major (M_r = 42 000) and the minor (M_r = 55 000) bands were cut from the gel and loaded on a third (15%) gel, where SAP digestion was performed within the stacking gel. Both produced identical fragments of M_r = 32 000, M_r = 22 000, M_r = 17 000, and M_r = 16 000.

The band labeled by ^{125}I-Bolton-Hunter-CCK-33 was treated similarly, with proteolytic mapping performed on individual deglycosylated bands. The M_r = 57 000 and M_r = 44 000 bands both yielded a fragment of M_r = 24 000. The bands around M_r = 65 000 yielded a different group of fragments, M_r = 22 000, 20 000, and 18 000.

Discussion

Pancreatic plasmalemmal proteins labeled by a battery of cholecystokinin (CCK) receptor probes have been studied by protease peptide mapping. Previously, it has been observed that at least two glycoproteins migrate in the band labeled by a CCK-33-based probe (Madison et al., 1987). In addition, a series of photoaffinity-labeling probes that are based on the carboxyl-terminal decapeptide of CCK have been developed and used to label a single broad M_r = 85 000–95 000 band (Pearson et al., 1987b; Powers et al., 1988a; Klueppelberg et al., 1989). These studies were designed to gain insight into identities of the proteins labeled by each probe, to define domains of the labeled proteins, and to better understand the basis of the broad nature of the labeled proteins.

We employed three CCK receptor probes with photolabile moieties located at the carboxyl terminus (at the position of Phe^{33}), the mid region (at the position of Trp^{30}), and the amino terminus of the theoretical receptor-binding domain. Recently, we have characterized and validated these probes chemically and biologically (Pearson et al., 1987b; Powers et al., 1988a; Klueppelberg et al., 1988). All are fully efficacious pancreatic secretagogues, with the carboxyl-terminal and amino-terminal probes of similar potency to native CCK-8 and the mid-region probe 70-fold less potent. All three probes have been reported to label a M_r = 85 000–95 000 pancreatic plasma membrane protein that is distinct from the predominant M_r = 80 000 protein (variably described to range from M_r = 76 000 to M_r = 95 000) labeled by ^{125}I-Bolton-Hunter-CCK-33 (Rosenzweig et al., 1982, 1983; Svoboda et al., 1982; Sakamoto, 1983). The unique nature of these M_r = 85 000–95 000 proteins has been confirmed by demonstration that they have different products of endo F deglycosylation than that of the M_r = 80 000 protein labeled by the CCK-33-based probe (Pearson et al., 1987a; Rosenzweig et al., 1984). Interestingly, it has been pointed out that minor bands labeled by CCK-33-based probes are dependent on the cross-linking reagent used (Madison et al., 1984); however, the major bands labeled by using DSS and MBS appear to represent the same protein (Madison et al., 1984). In this work, ^{125}I-Bolton-Hunter-CCK-33 labeled a broader band using MBS (M_r = 76 000–95 000) than using DSS (M_r = 76 000–84 000).

Confirming previous work (Pearson & Miller, 1987; Pearson et al., 1987b; Powers et al., 1988a; Klueppelberg, 1989), we have demonstrated that each of the decapeptide probes labeled M_r = 85 000–95 000 proteins and that endo F treatment of these yielded major M_r = 42 000 bands. In addition, two minor products of M_r = 55 000 and M_r = 27 000 were seen. In previous work (Pearson & Miller, 1987), the M_r = 85 000–95 000 band was demonstrated to represent an N-linked complex sialoglycoprotein on the basis of its ability to be cleaved by neuraminidase and endo F but not to be cleaved by endo H or *O*-glycanase.

Taking the different molecular weights of the radioligand probes into account, we found the same pattern of labeling with ^{125}I-Bolton-Hunter-CCK-33 as with the decapeptide probes, with corresponding products of endo F treatment of M_r = 57 000, M_r = 44 000, and M_r = 29 000. In contrast to the short probes, ^{125}I-Bolton-Hunter-CCK-33 was also incorporated into endo F deglycosylation products of approximate M_r = 65 000 with the labeling of these components relatively more efficient in experiments performed with DSS than with MBS.

Slices cut from the broad predominant bands labeled by each probe continued to migrate in their respective positions on a second gel, supporting the presence of heterogeneity through the portions of the bands labeled by each probe. After digestion with *S. aureus* V8 protease (SAP), slices cut from the native M_r = 85 000–95 000 band labeled by the short probes generated large fragments that reflected slight size

differences. Interestingly, the analogous fragment generated from the bottom slice migrated on an SDS gel at a higher velocity and as a sharper band than that generated from the top slice of the native band. However, after deglycosylation, all SAP fragments generated from each slice were identical. These observations are consistent with the interpretation that the apparent heterogeneity through these bands reflects differences in glycosylation.

The minor M_r = 55000 band observed after endo F treatment was more prominent after shorter incubation times with enzyme and after digestion of upper portions of the labeled band. SAP mapping of this band and the M_r = 42000 core protein (Pearson et al., 1987a) resulted in fragments of identical sizes, suggesting that the M_r = 55000 band was still partially glycosylated. The M_r = 27000 band occasionally seen appears to be a proteolytic fragment of the M_r = 42000 protein core.

Due to the heterogeneity of labeling with ^{125}I-Bolton-Hunter-CCK-33, we were careful to separately treat each deglycosylation product with SAP. Various fragments could be generated from the M_r = 42000 protein labeled by the decapeptide probes (M_r = 34000, 27000, 22000, 17000, and 16000); however, only two fragments were generated from the M_r = 44000 protein labeled by the CCK-33-based probe (M_r = 29000 and 24000). We postulate that the M_r = 42000 and M_r = 44000 bands are identical proteins labeled by probes of different molecular weight and labeled in different regions. Since the smaller fragments should have been generated, but were not radiolabeled, the longer probe seems to be cross-linked to the receptor outside the ligand-binding domain.

Thus, three CCK receptor probes with photolabile moieties spanning the theoretical receptor-binding domain label the same M_r = 85000–95000 pancreatic plasma membrane glycoprotein, which has a protein core of M_r = 42000. The glycosylation of this glycoprotein is quite heterogeneous, accounting for its broad migration on SDS gels. A CCK-33-based probe labels at least two major plasmalemmal glycoproteins. It labels the same M_r = 85000–95000 species as that labeled by the shorter probes, but in a different domain, and it labels an additional protein of approximate M_r = 80000. The reproducibility of labeling of this protein with the "long" probe raises the likelihood of the biological importance of its association with the M_r = 85000–95000 protein that contains the hormone-binding domain. It may represent either a noncovalently associated subunit of the pancreatic CCK receptor or a near-neighbor molecule in the plasmalemma.

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Registry No. CCK, 9002-03-3; 2-diazo-3,3,3-trifluoropropionyl-^{125}I-D-Tyr-Gly-[(Nle^{28,31})CCK-26-33], 121524-95-6; ^{125}I-D-Tyr-Gly-[(Nle^{28,31},6-NO_2-Trp^{30})CCK-26-33], 121525-00-6; ^{125}I-D-Tyr-Gly-[(Nle^{28,31},pNO_2-Phe^{33})CCK-26-33], 121525-01-7; D-Tyr-Gly-[(Nle^{28,31})CCK-26-33], 121524-97-8; D-Tyr-Gly-[(Nle^{28,31},6-NO_2-Trp^{30})CCK-26-33], 121524-98-9; D-Tyr-Gly-[(Nle^{28,31},pNO_2-Phe^{33})CCK-26-33], 121524-99-0; ^{125}I-D-Tyr-Gly-[(Nle^{28,31})CCK-26-33], 121524-96-7; L-Gln, 56-85-9.

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