Vol. 118, No. 2, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 30, 1984 Pages 623-628

PANCREATIC CCK RECEPTORS: CHARACTERIZATION OF COVALENTLY LABELED SUBUNITS

Choitsu Sakamoto, Ira D. Goldfine, and John A. Williams

Cell Biology Laboratory, Mt. Zion Hospital and Medical Center, San Francisco, 94120

Departments of Physiology and Medicine, University of California, San Francisco, CA 94143

Received December 8, 1983

^{125}I-CCK was crosslinked with ultraviolet light to its receptor on pancreatic plasma membranes. The predominant labeled species following polyacrylamide gel electrophoresis had a molecular weight of 120,000 in the absence, and 80,000 in the presence of the reducing agent dithiothreitol. The M_r = 120,000 labeled band could be extracted, reduced and converted to M_r = 80,000. Moreover, peptide mapping with Staph aureus V8 protease showed a similar pattern for the 120,000 and 80,000 dalton bands. The crosslinked receptor could be solubilized with Triton X-100, absorbed to wheat germ agglutinin and eluted with N-acetylglucosamine. The results indicate, therefore, that the CCK receptor is a glycoprotein with subunits coupled by disulfide bonds.

The actions of CCK on pancreatic acinar cells are initiated by its binding to specific receptors located on the basolateral cell membrane (1,2). The binding characteristics of this receptor have been extensively studied both on intact cells and in isolated membrane fractions (2-6). Our laboratory and others have reported that it is possible to crosslink ^{125}I-CCK to its receptors and that, in the presence of reducing agent, major labeling of a 75-80,000 dalton protein is observed (7-9). We recently observed that when plasma membranes were prepared and crosslinking carried out in the absence of reducing agent, a major band with a M_r = 120,000 (9) was labeled. We suggested, therefore, that the CCK receptor consisted of a binding subunit coupled by disulfide bonds to a non-binding subunit. In the present study, using a much more efficient crosslinking protocol, we now demonstrate that the labeled M_r = 120,000 species contains the M_r = 80,000 species. Moreover, it is possible to solubilize the crosslinked receptor and demonstrate that it has carbohydrate moieties.

0006-291X/84 $1.50

MATERIALS AND METHODS

Methods:

Affinity crosslinking. Pancreatic plasma membranes were prepared from isolated pancreatic acini of Swiss Webster mice as reported previously (10). CCK_{33} was labeled with ^{125}I by conjugation with ^{125}I-Bolton Hunter reagent (11). Plasma membranes were incubated for 30 min at 24°C with 1 nM ^{125}I-CCK in 10 mM Hepes buffer (pH 7.4), and enriched with 5 mg/ml BSA, 1 mg/ml bacitracin and 0.2 mg/ml soybean trypsin inhibitor (2,9); CCK_8 was added at 100 nM to determine nonspecific binding. The incubation mixture was then centrifuged at 30,000 x g for 15 min at 4°C and the resulting pellet washed and resuspended at 0.3 mg/ml of protein in phosphate-buffered saline, pH 7.4. Crosslinking was performed by exposing the membrane suspension to UV light from a GE 275 watt sunlamp at a distance of 14 cm for 12 min at 4°C. In one case, crosslinking was carried out using disuccinimidyl suberate as previously reported (9).

When indicated, labeled membranes were solubilized with 50 mM Tris HCl, pH 7.4, containing 0.5% Triton X-100, 1 mM benzamidine, 0.5 mM PMSF, 1 mg/ml bacitracin at 24°C. Labeled extract was used for further analysis.

Electrophoresis. Crosslinked labeled membranes were solubilized in 62.5 mM Tris-HCl, pH 6.8, containing 2.3% sodium dodecyl sulfate. Electrophoresis was performed according to Laemmli (12), using 1.5 mm thick slab gels containing 7.5% acrylamide. Analysis of non-reducing and reducing conditions were performed by a two-dimensional technique. Crosslinked bands from this first electrophoresis were resolved by determining the radioactivity of slice sections. The appropriate gel slices were then pulverized, boiled for 5 min, and incubated at room temperature overnight with reducing agents. A second electrophoresis was then carried out similar to the first.

Peptide mapping by limited proteolysis was performed as described by Bordier et al (13). Briefly, the whole lane cut from the first gel was applied with 1% agarose on the top of the second gel and overlaid with staphylococcus aureus V8 protease (30 µg/ml). The digestion proceeded in the stacking gel during electrophoresis. The resolving gel had an acrylamide concentration of 15%. Autoradiograms were obtained from the dried gels after exposure to Kodak Xomat AR film with a Dupont Cronex Lightning Plus enhancing screen at -70°C.

RESULTS

Pancreatic plasma membranes were incubated with ^{125}I-CCK_{33}, washed and crosslinked with either disuccinimidyl suberate or exposure to ultraviolet light. Following polyacrylamide gel electrophoresis, similar banding patterns were seen with the major band observed at M_r = 120,000 in the absence and M_r = 80,000 in the presence of reducing agent; a M_r = 55,000 band was also variably present (Fig. 1). Under the conditions used, however, the UV light procedure was considerably more efficient. Subsequent studies were, therefore, carried out with this procedure.

To determine the relationship between the M_r = 120,000 and 80,000 labeled proteins, these bands were extracted and subjected to a second electrophoresis in the presence and absence of dithiothreitol (Fig. 2).

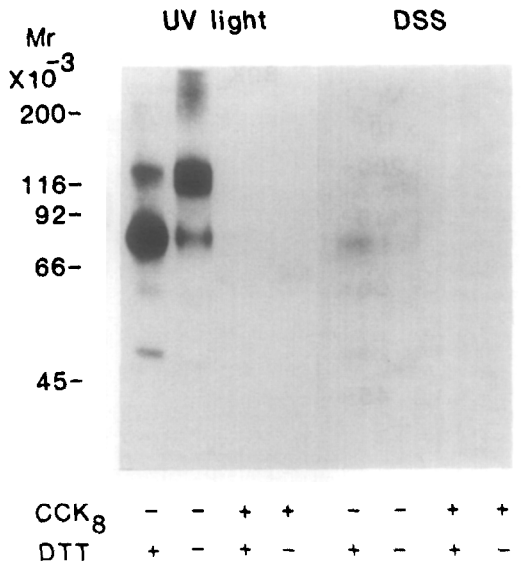

Fig. 1 Autoradiographs of CCK receptor crosslinked with UV light and disuccinimidyl suberate. Membranes were incubated with 1 nM ^{125}I-CCK in the presence or absence of 100 nM CCK_8. After binding, one half of samples were crosslinked with 0.1 mM disuccinimidyl suberate for 15 min at 4°C and the other half were crosslinked with UV light. Where indicated, membranes were then reduced with 50 mM dithiothreitol (DTT).

With or without further reduction, the M_r = 80,000 species again appeared as a discreet M_r = 80,000 band. Without reduction, the M_r = 120,000 band remained at M_r = 120,000, but after reduction it was significantly converted to M_r = 80,000.

In order to further compare the crosslinked bands labeled by ^{125}I-CCK, plasma membranes were labeled by UV light crosslinking, partially reduced with DTT to ensure the presence of both major bands, and the labeled bands subjected to peptide mapping in a second dimension following digestion with staphylococcus aureus V8 protease (Fig. 3). Similar labeled peptides were generated from the M_r = 120,000 and 80,000 bands, confirming that the same protein was labeled. The M_r = 55,000 band which is variably seen following gel electrophoresis generated some but not all of the labeled peptides. This behavior on gels is consistent with this labeled species being a proteolytic fragment of the labeled M_r = 80,000 band.

To assess the possible glycoprotein nature of the CCK receptor, the crosslinked CCK receptor was solubilized. The receptor was partially solubilized with 0.05% Triton X-100, and maximally solubilized with 0.5% of

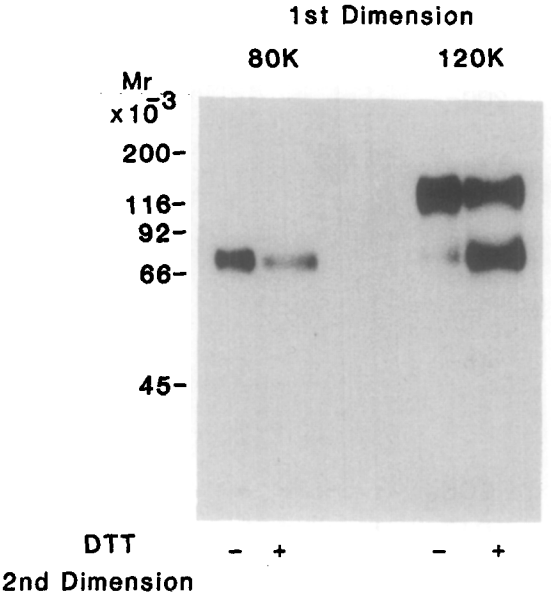

Fig. 2 Effect of DTT on ^{125}I-labeled CCK receptor of pancreatic plasma membranes. Gels from a first electrophoresis, performed without reduction, were sliced into 2 mm section. Receptor bands corresponding to 120 and 80 K were then extracted, incubated either with or without 100 mM dithiothreitol, and subjected to a second electrophoresis.

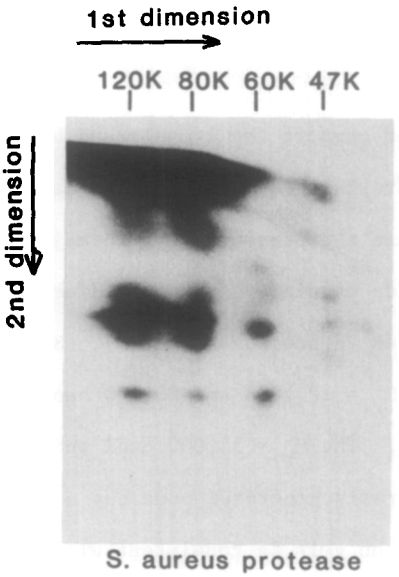

Fig. 3 Peptide mapping of ^{125}I-labeled CCK receptor with staphylococcus aureus V8 protease. After crosslinking, partial reduction with 5 mM dithiothreitol and electrophoresis in 7.5% acrylamide, a whole lane was cut out and rinsed with 125 mM Tris-HCl, 0.1% sodium dodecyl sulfate at room temperature for 40 min. The lane from the first gel was then fixed on the top of a 15% acrylamide gel, overlaid with staphylococcus aureus V8 protease, and subjected to electrophoresis.

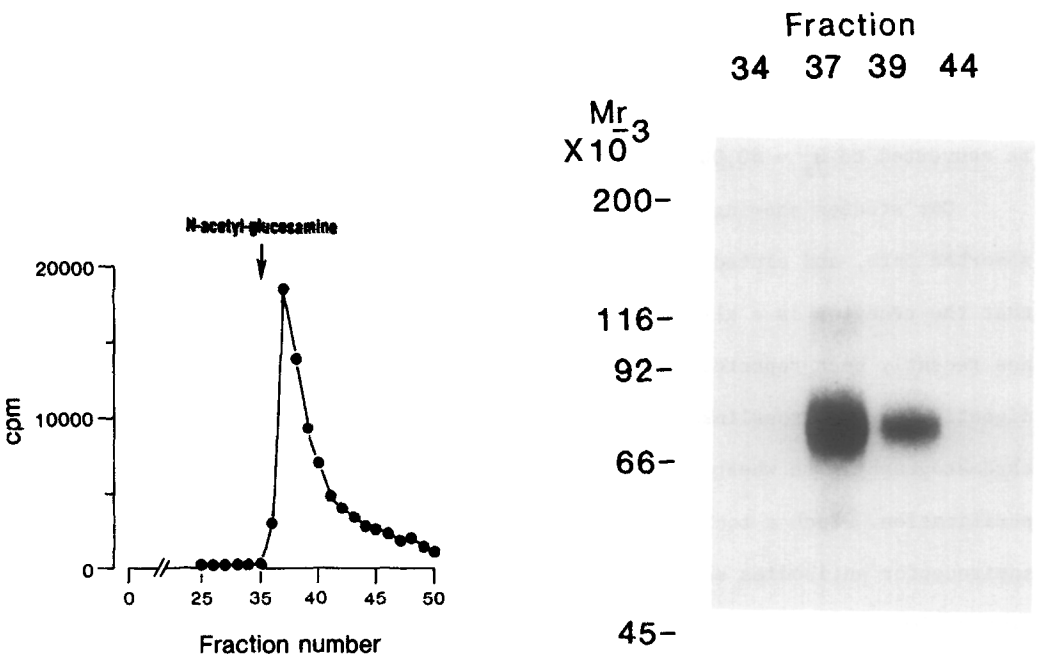

Fig. 4 Chromatography of ^{125}I-labeled CCK receptor on wheat germ agglutinin-agarose. Membranes crosslinked to ^{125}I-CCK were solubilized with 0.5% Triton X-100, and centrifuged. The supernatant was applied to a column of wheat germ agglutinin-agarose equilibrated with 50 mM Tris-HCl, pH 7.4, containing 0.2% Triton X-100 and washed with 35 ml of the same buffer. Absorbed protein was eluted by the addition of 0.3 M N-acetylglucosamine. Eluted samples were concentrated and analyzed by electrophoresis and autoradiography.

this detergent. When the solubilized receptor was applied to a wheat germ agglutinin-agarose column and washed, the noncovalently linked ^{125}I-CCK and about 90% of the applied protein passed through the column. The crosslinked receptor could then be eluted with N-acetylglucosamine as monitored by subsequent gel electrophoresis (Fig. 4).

DISCUSSION

Prior studies with photo and chemical crosslinking have elucidated the nature of several peptide hormone receptors. When ^{125}I-CCK is crosslinked to its receptor with either bifunctional chemical crosslinkers or by use of ultraviolet light, a similar banding pattern is observed. All previous studies have shown a major labeled species of M_r = 75 - 80,000 under reducing conditions (7-9). However, under non reducing conditions we also found a major band at M_r = 120,000 (9). As a result of the more efficient cross-linking with UV light, we now have been able to carry out studies using a

second gel electrophoresis which demonstrates that the M_r = 120,000 and M_r = 80,000 bands contain the same proteins, and that the M_r = 120,000 band can be converted to M_r = 80,000 by disulfide reduction.

Our studies showing that the crosslinked receptor can be solubilized, absorbed onto, and eluted from a wheat germ lectin column provides evidence that the receptor is a glycoprotein. A similar conclusion about this protein has recently been reported from studies of the effects of endoglycosidase digestion of the crosslinked receptor (14). More importantly, affinity chromatography with wheat germ agglutinin may be an important step in receptor purification. Such a technique coupled with CCK affinity chromatography or antireceptor antibodies should lead to both complete purification and elucidation of the molecular structure of the CCK receptor.

ACKNOWLEDGMENTS

This work was supported by NIH grant AM 26422 and the Elise Stern Haas Research Fund of Mount Zion Hospital and Medical Center. We thank Drs. Y. Takai and R. Roth for helpful discussion during the course of this work.

REFERENCES

1. Williams, J.A., Sankaran, H., Roach, E. and Goldfine, I.D. (1982) Am. J. Physiol. 243:G291-G296.
2. Sakamoto, C., Williams, J.A. and Goldfine, I.D. (1983) FEBS Lett. 151: 63-66.
3. Sankaran, H., Goldfine, I.D., Deveney, C.W., Wong, K.Y. and Williams, J.A. (1980) J. Biol. Chem. 255:1849-1853.
4. Jensen, R.T., Lemp, G.F., and Gardner, J.D. (1980) Proc. Natl. Acad. Sci. USA 77:2079-2083.
5. Steigerwalt, R.W. and Williams, J.A. (1981) Endocrinology 109:1746-1753.
6. Miller, L.J., Rosenzweig, S.A. and Jamieson, J.D. (1981) J. Biol. Chem. 256:12417-12423.
7. Svoboda, M., Lambert, M., Furnelle, J. and Christophe, J. (1982) Regul. Peptides 4:163-172.
8. Rosenzweig, S.A., Miller, L.J. and Jamieson, J.D. (1983) J. Cell Biol. 96:1288-1297.
9. Sakamoto, C., Goldfine, I.D. and Williams, J.A. (1983) J. Biol. Chem. 258:12707-12711.
10. Ponnappa, B.C., Dormer, R.L. and Williams, J.A. (1981) Am. J. Physiol. 240:G122-G129.
11. Sankaran, H., Deveney, C.W., Goldfine, I.D. and Williams, J.A. (1979) J. Biol. Chem. 254:9349-9351.
12. Laemmli, V.K. (1970) Nature 227:680-685.
13. Bordier, C. and Crettol-Järvinen, A. (1979) J. Biol. Chem. 254:2505-2567.
14. Rosenzweig, S.A., Madison, L.D. and Jamieson, J.D. (1983) J. Cell Biol. 97:401a.