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Y_2 receptor proteins for peptide YY and neuropeptide Y

Characterization as *N*-linked complex glycoproteins

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Affinity labeling using [^{125}I-Tyr^{36}]PYY and homobifunctional affinity crosslinking reagents of the rabbit Y_2 receptor for peptide YY(PYY) results in specifically labeled proteins of both M_r=50,000 to 60,000 and M_r=96,000 to 115,000 [1,2]. In this work the glycoprotein nature of affinity labeled Y_2 receptor proteins were investigated by enzymatic deglycosylation using neuraminidase, endoglycosidase F (endo F), *N*-glycosidase F (PNGase F), and *O*-glycanase treatment. Only *N*-glycosidase F and neuraminidase increased the electrophoretic mobility of the radiolabeled receptor bands, whereas all other glycosidases did not. PNGase F treatment of both radiolabeled receptor bands electroeluted from gel slices reduced the apparent molecular mass of by 16–17 kDa units, that is M_r=96,000 to 79,000 and M_r=60,000 to 44,000, indicating removal of *N*-linked oligosaccharide chains of similar size from both species. Neuraminidase treatment caused slight increases in the electrophoretic mobilities suggesting the presence of terminal sialic residues. It is concluded that the Y_2 binding proteins are *N*-linked complex (sialo)glycoproteins with a minimal core protein size of M_r=44,000. Furthermore, based on this sensitivity pattern of the glycosidases, the Asn-linked carbohydrate may be of the tri- or tetra-antennary complex type containing terminal sialic acid residues.

G-Protein coupled receptor; Glycosylation; Affinity labelling

1. INTRODUCTION

Neuropeptide Y (NPY), peptide YY (PYY), and pancreatic polypeptide (PP) are 36-amino acids regulatory peptides with a C-terminal tyrosylamide [3]. The three peptides constitute a family of regulatory peptides, characterized by a common and highly conserved tertiary structure; the PP-fold [4]. NPY is an important and widely distributed neuropeptide in central and peripheral neurones, whereas PYY is a gut hormone present in endocrine cells in the lower small intestine and colon [3,5,6]. Specific receptors for NPY and PYY have been characterized in different tissues, especially brain, from numerous species and neuroendocrine cell lines [5–10]. These receptors, which bind NPY and PYY with equal high affinity, have been classified into Y1 and Y2 receptors [5–11]. While the Y1 receptor has recently been cloned from rat hippocampus, and found to be a member of the superfamily of G-protein coupled receptors [12], no such information is available for the Y2 receptor. The G-protein coupled receptors share several structural features including seven transmembrane domains connected by intra- and extracellular loops, and epitopes for palmityolation and glycosylation [12]. Studies with adsorption of solubilized crosslinked Y_2 receptors to various lectin agarose beads have indicated, that the Y_2 receptor is a glycoprotein [1]. However, neither the carbohydrate nor the core protein components of these receptors have been biochemically characterized. Glycosidases have proven useful tools in characterization of the glycoprotein nature of cell surface receptors [13– 15]. On the basis of alteration in apparent molecular weight after treatment with glycosidases we now report that affinity labeled Y_2 receptors from kidney proximal tubules consists of protein cores of 44 and 75 kDa, that are both linked through asparagine-residues to 16–17 kDa carbohydrate components. Furthermore, the sensitivity pattern of the receptor for the glycosidases provides an approximation of the receptor carbohydrate structure.

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2. EXPERIMENTAL

2.1. *Materials*

Neuraminidase, endo-β-*N*-acetylglucosaminidase H (endo H), endo-β-*N*-acetylglucosaminidase F (endo F), and peptide-*N*-(*N*-acetyl-β-glucosaminyl)asparagine amidase (*N*-glycosidase F or PNGase F) were from Boehringer Mannheim Biochemicals (Martinsread, Germany). Endo-α-*N*-acetylgalactoaminidase O (*O*-glycanase) were from Genzyme Corporation (Boston, MA). Synthetic PYY and NPY were purchased from Peninsula Laboratories (Merseyside, UK). Ethylene glycolbis(succinimidylsuccinate) (EGS) and IODO-GEN were from Pierce.

2.2. *Production of [^{125}I-Tyr^{36}]PYY*

Synthetic PYY was iodinated and isolated as previously described in detail [15]. Briefly, PYY was iodinated with carrier-free Na^{125}I (Amersham) using the oxidative reagent IODO-GEN, and the reaction products purified by reversed phase HPLC.

2.3. *Preparation of basolateral membranes from rabbit proximal tubule*

The kidneys from female New Zealand White rabbits were excised and basolateral membranes prepared by differential centrifugation and Ca^{2+}/Mg^{2+} precipitation as described [16]. The membranes were suspended to a final protein concentration of 5 mg/ml in binding buffer, consisting of 25 mM HEPES, pH 7.4, containing in addition, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 5 g/liter bovine serum albumin (BSA), 0.1 g/liter bacitracin, 1 mM PMSF (phenylmethylsulfonyl fluoride), 0.5 g/liter leupeptin, 0.7 g/liter pepstatin, and stored at −80°C until use.

2.4. *Affinity labeling protocol*

Affinity labeling of PYY binding proteins was performed as previously described in detail [1]. Briefly, samples (usually 34–37 ml) of membranes (200 μg/ml) were incubated in binding buffer with 200 pM radiolabeled PYY for 60 min at 24°C. After centrifugation at 50,000 × *g* for 10 min at 4°C, the membranes were resuspended in 25 mM HEPES, 2.5 $CaCl_2$, and 1 mM $MgCl_2$, pH 7.4, and incubated with EGS at 1:100 dilution (final concentration 0.5 mM) for 20 min at 4°C. The membranes were then pelleted and solubilized in sample buffer (62.5 mM Tris-HCl, pH 6.8, containing 4.6% sodium dodesyl sulfate, 5% glycerol and 0.1% bromophenol blue with 0.1 M dithiothreitol) and subjected to polyacrylamide gel electrophoresis. This was performed with the buffer system of Laemmli [17] using 1.5 mm thick slab gels containing 10% acrylamide. Following 3–4 h of electrophoresis the gels were cut into 4 mm thick slices, which immediately were immersed in electroelution buffer.

2.5. *Deglycosylation of affinity-labeled proteins*

The optimal incubation conditions and times for the different glycosidases were determined in preliminary experiments. For enzyme treatment of individual radiolabeled proteins, these were located by counting gel slices in a gamma-counter. Each gel slice was then electroeluted for 5–7 h at 10 mA constant current in a model 422 electroeluter (Bio-Rad). The electroelution was performed in a buffer containing 50 mM Tris-HCl, pH 7.4, with 0.1% Triton X-100. The electroeluted radiolabeled proteins were then concentrated in Centricon 30 microconcentrators (Amicon, Danvers, MA), and treated with the different glycosidases. Next, the reaction products were separated by SDS-polyacrylamide gel electrophoresis using 1.0 mm thick mini-slab gels containing 10% acrylamide. The gels were fixed in 7% acetic acid and protein visualized by silver- or Coomassie blue-staining followed by autoradiography. *For treatment with neuraminidase*, electroeluted affinity labeled proteins were incubated in 50 μl of 50 mM HEPES, pH 5.0, containing 0.15 M NaCl and 5 mM $MgCl_2$ with 5 units of neuraminidase for 5 h at 37°C. *For endo H treatment*, the radiolabeled receptor proteins were incubated in 0.01 M sodium acetate buffer, pH 5.0, with 5 units of enzyme for 12 h at 37°C. *For endo F treatment*, the electroeluted affinity labeled proteins were incubated in 50 μl of 0.1 sodium phosphate buffer, pH 6.1, containing in addition 50 mM EDTA, 1% Nonidet P-40, 0.1% SDS, and 1% 2-mercaptoethanol with 10 units of enzyme for 4–24 h at 37°C. *For N-glycosidase F (PNGase F) treatment* we used the same buffer as for endo F with exception of the pH, which was changed to 8.0. The radiolabeled receptor proteins were incubated with 1 unit of PNGase F for 12–18 h at 37°C. *For O-glycanase treatment*, electroeluted affinity labeled proteins were first de-sialylated with neuraminidase. The proteins were then concentrated in Centricon 30 units and resuspended in 300 μl of 0.1 M sodium phosphate buffer, pH 6.1, containing 50 mM EDTA, 1% Nonidet P-40, 0.1% SDS and 1% 2-mercaptoethanol. Four miliunits of *O*-glycanase were added and the mixture was incubated for 12 h at 37°C.

2.6. *Wheat germ agglutinin (WGA)-agarose chromatography*

The electroeluted affinity labeled proteins and products of PNGase F digestion were resuspended in 50 mM of Tris-HCl, pH 7.4, containing 1% Zwittergent 3-12, and protease inhibitors as described [18], and incubated with prewashed WGA agarose beads (500 μl) by gentle agitation for 2 h at 4°C. Next, the incubation mixture was transferred to a small column and washed with 40 vol. of 50 mM Tris-HCl, pH 7.4, containing 0.25% Zwittergent 3-12. The column was then washed with 5 vols. of 0.3 M *N*-acetylglucosamide. Fractions of 1 ml were collected and counted in a gamma-counter.

3. RESULTS AND DISCUSSION

The present study was undertaken to characterize the carbohydrate and protein parts of the Y_2 receptor structure. Several groups have reported successful affinity labeling of receptors for the PP-fold family of peptides [1,2,9,19,20]. However, NPY receptor glycosylation have been indicated by indirect data only, such as adsorption of radiolabeled receptor protein to various lectins, where it is difficult to tell whether it is the receptor protein or an associated protein that contains the carbohydrate [1]. Furthermore, analysis of the Y_2 receptor core proteins might also provide information on why two proteins of M_r=50,000–60,000 and M_r=96,000– 115,000 are specifically labeled in affinity cross-linking studies in rabbit kidney tubules [1,2]. To our knowledge this is the first direct study of the glycosylation pattern of NPY and PYY receptors.

To determine whether the kidney PYY receptor is a glycoprotein we tested whether the receptor is sensitive to treatment with *N*-glycosidase F (PNGase F). PNGase F hydrolyses all common Asn-linked oligosaccharides (high mannose, as well as hybrid and complex types) from glycoproteins to give free oligosaccharides and free protein. The asparagine residue to which carbohydrate was linked is converted to an aspartic acid

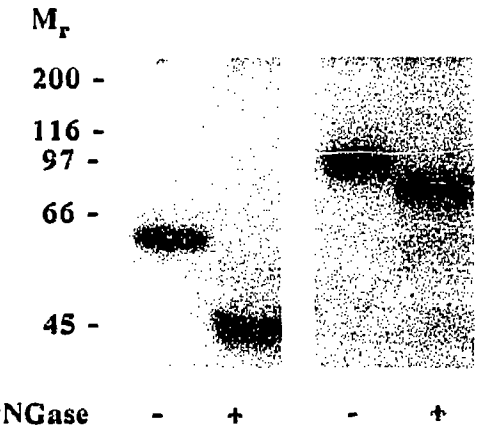

Fig.1. Autoradiograph of affinity labeled and electroeluted Y_2 receptors from rabbit kidney tubules treated with PNGase F. Lanes 1 and 3 represent the M_r=60,000 and M_r=96,000 PYY binding proteins. Lanes 2 and 4 represent these proteins after PNGase treatment. The autoradiograph is representative of 5 experiments. Positions of marker proteins are indicated.

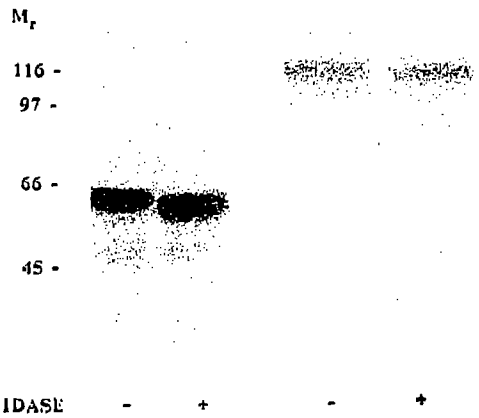

Fig. 2. Effect of neuraminidase treatment of PYY binding proteins from rabbit kidney. This autoradiograph of an SDS-polyacrylamide gel is representative of 5 experiments.

[21,22]. As shown in Fig. 1, a similar decrease of approximately 16–17 kDa in the apparent molecular weights of the radiolabeled bands was observed, when affinity labeled and electroeluted protein were treated with PNGase F. Thus, the M_r=96,000 radiolabeled band shifted to M_r=79,000, and the M_r=60,000 band decreased to M_r=44,000. The use of glycosidases on the Y_2 receptor was successful only after partial purification by preparative polyacrylamide electrophoresis and electroelution of the individual radiolabeled protein bands. To follow up these data. both the cleaved and original radiolabeled bands were subjected to WGA-agarose chromatography. The M_r=96,000 and M_r=60,000 bands were adsorbed by the lectin agarose, and could be recovered by elution using 0.3 M *N*-acetylglucosamine, whereas the cleaved products were repelled by the lectin agarose, as the radioactivity from those proteins were found in the wash fractions (data not shown). Taken together these results suggests, that the M_r=79,000 and M_r=44,000 proteins represent the *N*-linked carbohydrate-free polypeptide cores of the Y_2 receptor. The data does not show, whether the higher or the lower of the original affinity labeled bands is the best candidate for the true Y_2 receptor binding protein. However, since the carbohydrate content of both affinity labeled bands were similar, it could be speculated that the higher band may contain the lower band plus a distinct additional protein with a molecular weight of approximately 35,000. This associated protein could for example be a G-protein subunit cross-linked to the Y_2 receptor. In support of this notion, the association between the Y_2 receptor and its G-protein seems to be rather tight, since solubilization of kidney membranes results in functionally intact Y_2 receptor-G-protein complexes [2,18]. Alternatively, the lower band may be a degradation product of the higher band. We are currently persuing these explanations both by limited proteolysis of the two core proteins and by utilizing monoclonal antibodies to identify G-protein subunits.

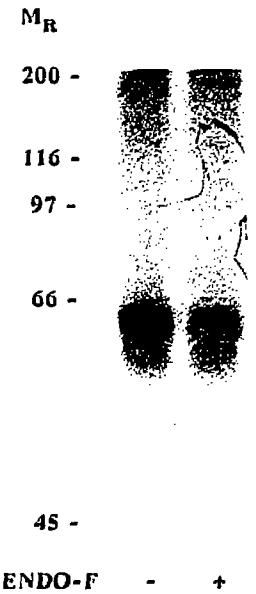

Fig. 3. Autoradiograph of endo F treatment of the radiolabeled M_r=60,000 species. The autoradiograph is representative of 6 experiments.

To investigate whether the complex *N*-linked carbohydrate contains terminal sialic acid residues, the Y_2 binding proteins were treated with neuraminidase. This enzyme cleaves sialic acid residues only. Fig. 2 shows that neuraminidase treatment increased the electrophoretic migration of both binding proteins. Thus, these PYY binding proteins can be classified as sialoglycoproteins. This observation is in line with the adsorption of the Y_2 receptor to WGA agarose beads, since this lectin binds sialic acid residues.

The Y_2 receptor sensitivity to endo F was tested in order to determine which type of *N*-linked oligosaccharides the Y_2 receptors carries. Endo F is known to cleave glycosidic bonds of the chitobiose core structure of many high-mannose and biantennary complex Asn-linked oligosaccharides. However, when electroeluted radiolabeled proteins were treated with endo F, no shift in the electrophoretic mobility of either the M_r=60,000 protein (Fig. 3) or the M_r=96,000 band (not shown) could be observed. The activity of endo F from different commercial sources was tested with ovalbumin as a substrate. The ovalbumin was treated exactly like the radiolabeled receptor proteins with respect to polyacrylamide gel electrophoresis, electroelution and endo F treatment. Endo F efficiently cleaved the ovalbumin associated oligosaccharides (data not shown). Thus, although PNGase F cleaves the carbohydrate of the PYY binding proteins, endo F has no effect. The Y_2 receptor resistance to endo F reduces the possibilities of carbohydrate structures to hybrid structures containing bisecting (peripheral) GlcNAc-linked β-(1,4) to the mannose core, and tri- and tetra-antennary complex chains. In addition, we tested endo H, that hydrolyses mannose-rich *N*-linked oligosaccharides exclusively [23]. This gly-

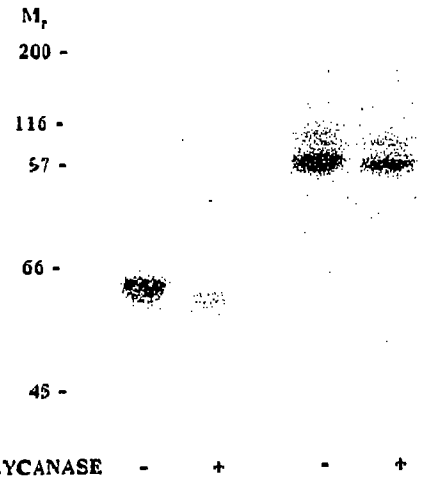

Fig. 4. Affinity labeled PYY binding proteins treated with *O*-glycanase. The proteins were desialyated using neuraminidase prior to *O*-glycanase treatment. The autoradiograph is representative of 5 experiments.

cosidase had no effect on the electrophoretic mobilities of the radiolabeled proteins, suggesting that all of the Asn-linked oligosaccharides identified by PNGase F treatment are of the complex type (data not shown).

Finally, the presence of *O*-linked carbohydrate structures on the Y_2 receptor proteins was probed by treatment with *O*-glycanase. This enzyme hydrolyses Gal-β(1,3)-GalNAc core disaccharides linked to serine or threonine residues of glycoproteins to give free oligosaccharides and an unsubstituted serine ot threonine group [24]. The radiolabeled Y_2 receptors were treated with neuraminidase prior to the *O*-glycanase treatment, since substituents, such as sialic acid residues present on either the galactosyl or *N*-acetylgalactoaminyl residues blocks the activity of *O*-glycanase. *O*-glycanase treatment of the de-sialylated PYY binding proteins did not further change the electrophoretic migration of the labeled proteins, as shown in Fig. 4. Consequently, no serine or threonine linked carbohydrate may be present on the receptor protein.

The results in the present paper identifies the PYY binding proteins as *N*-linked complex (sialo)glycoproteins, and characterizes the structures of the carbohydrate and protein parts of these receptors. These observations should facilitate further work on the functional importance of the carbohydrate component of the NPY and PYY receptor proteins.

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