Effect of inhibiting *N*-glycosylation or oligosaccharide processing on vasoactive intestinal peptide receptor binding activity and structure

Assou EL BATTARI,* Pierre FORGET, Francis FOUCHIER and Pierre PIC

CNRS URA 202, Université d'Aix-Marseille 1, Institut de Chimie Biologique, Place V. Hugo F-13331, Marseille Cedex 3, France

We used inhibitors of four steps of the glycosylation pathway to examine the contribution of carbohydrate moieties to the ligand-binding activity, cell-surface expression and apparent molecular mass of the human vasoactive intestinal peptide (VIP) receptor. Human melanoma IGR 39 cells, incubated for 60 h with the inhibitors tunicamycin, castanospermine, swainsonine or deoxymannojirimycin, under conditions where cell viability and protein synthesis were not affected, expressed VIP receptor species with different VIP-binding properties. The most pronounced effects on VIP binding were obtained with tunicamycin and deoxymannojirimycin, which respectively caused 80 % and 67 % inhibition. Treatment with either swainsonine or castanospermine resulted in only a 25–32 % decrease in VIP specific binding. Based on Scatchard analyses of data from competition experiments, the decrease in VIP-binding activity in either swainsonine- or deoxymannojirimycin-treated cells was due to a decrease in ligand affinity; the cell-surface number of VIP-binding sites remained unchanged. In contrast, tunicamycin and castanospermine caused decreases in the cell-surface number of functional VIP receptors without affecting affinity. Besides, the drug-treated cells produced VIP-binding proteins with different molecular masses and endoglycosidase H (Endo H) sensitivities. When compared with their counterpart synthesized in control cells, VIP-binding proteins produced by deoxymannojirimycin- or swainsonine-treated cells were smaller in size and exhibited the expected sensitivity to Endo H. No modification in the apparent molecular mass was observed in the presence of either castanospermine or tunicamycin. In addition, after Endo F digestion, all of the deglycosylated proteins migrated with the same electrophoretic mobility. Finally, processing in the presence of castanospermine led to an Endo H-resistant receptor species which showed an unexpected neuraminidase-sensitivity, indicating that, as in control cells, these receptors carry *N*-linked oligosaccharides with terminal sialic acid residues.

INTRODUCTION

Vasoactive intestinal peptide (VIP) is a 28-amino-acid neuropeptide with a widespread distribution and action in the organism [1]. The VIP receptor is an integral membrane protein to which VIP binds, triggering the activation of the enzyme adenylate cyclase [2]. The use of affinity-labelling techniques has provided much information regarding the biochemical characterization of VIP receptors from different origins [3], and more recently a VIP-binding protein has been purified from pig liver [4]. We and others have demonstrated that the VIP receptor, like several other membrane receptors, is a glycoprotein carrying *N*-linked oligosaccharide chains [5,6]. However, the functional significance of receptor glycosylation is not clearly understood at present. Glycosylation appears to be important in the activation of the nascent insulin [7], epidermal growth factor [8] and acetylcholine receptors [9]. In all cases, failure of glycosylation of these receptors results in an absence of ligand binding. On the contrary, glycosylation appears not to be required for expression, ligand binding or coupling to GTP-binding proteins (G-proteins) in several G-protein-linked families of transmitters [10–12]. For example, inhibition of *N*-glycosylation by tunicamycin does not affect expression or function of β -adrenergic receptors in either A431 cells or S49 mouse lymphoma cells [12,13]. In accordance with this, enzymic deglycosylation of the purified β -adrenergic receptor does not affect its agonist-binding properties [14].

To our knowledge, little information is presently available on the role of *N*-linked glycans with regard to the synthesis,

expression and ligand-binding properties of VIP receptors [15,16]. As an initial approach to evaluate the role of the carbohydrate moieties in VIP receptor function, we have demonstrated that inhibiting either oligosaccharide formation by tunicamycin or terminal glycosylation by swainsonine or monensin resulted in decreased VIP binding on the human colon adenocarcinoma HT29-D4 cells [15].

In the present study, we have investigated the role of *N*-linked oligosaccharides in the binding capacity of IGR 39 cells cultured in the presence of inhibitors of four steps of the glycosylation pathway; namely tunicamycin, castanospermine, deoxymannojirimycin and swainsonine. Tunicamycin inhibits the enzyme *N*-acetylglucosamine-1-phosphatetransferase and thereby prevents the formation of the first lipid-linked oligosaccharide intermediate GlcNAc-*P*-dolichol [17]. Castanospermine is a plant alkaloid which inhibits the endoplasmic reticulum enzyme α -glucosidase I. The oligosaccharide chains of the glycoproteins produced in the presence of this inhibitor retain three glucose residues and a variable number of mannoses [18,19]. (+)-1-Deoxymannojirimycin is a mannose analogue that specifically inhibits Golgi α -mannosidases IA/IB [20]. Its effect is to block the trimming of the first mannose residues from *N*-linked high-mannose oligosaccharides in the Golgi apparatus, and it therefore blocks the addition of terminal sugars which are needed to form complex-type oligosaccharide structures. Swainsonine is an indolizidine alkaloid which prevents the processing of high-mannose forms of glycoproteins into complex forms through the inhibition of the Golgi α -mannosidase II [21]. These inhibitors have been

Abbreviations used: VIP, vasoactive intestinal peptide; G-protein, GTP-binding protein; DSS, disuccinimidyl suberate; PBS, phosphate-buffered saline (NaCl, 8 g/l; KCl, 0.2 g/l; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 1.4 g/l; KH_2PO_4 , 0.2 g/l, pH 7.3); GlcNAc, *N*-acetylglucosamine; Endo F and H, endo- β -*N*-acetylglucosaminidases F and H; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; PMSF, phenylmethanesulphonyl fluoride; p[NH]ppG, guanosine 5'-[β -imidotriphosphate].

* To whom correspondence should be addressed.

shown to affect the cell-surface expression and function of numerous glycoproteins (for review see [22]). The experiments carried out here demonstrate that failure of *N*-glycosylation considerably decreases VIP binding to IGR 39 cells by inhibiting cell-surface expression of functional VIP receptors. Our findings indicate also that, although terminal glycosylation seems not to be required for cell-surface expression, it directly influences the affinity of the VIP receptor for its ligand.

MATERIALS AND METHODS

Materials

Synthetic VIP was purchased from Neosystem (Strasbourg, France). The monoiodinated VIP was purified as previously described [23] and the specific radioactivity of the ^{125}I -VIP was estimated at 60×10^6 c.p.m./ng. Castanospermine and deoxymannojirimycin were from Genzyme (Boston, MA, U.S.A.). Tunicamycin, swainsonine, endoglycosidase H (Endo H), endoglycosidase F (Endo F) and neuraminidase were obtained from Boehringer (Mannheim, Germany). The cross-linker agent disuccinimidyl suberate (DSS) was provided by Pierce Chemical Co. (Rockford, IL, U.S.A.). *In vivo* labelling grade [^{35}S]methionine (> 100 Ci/mmol) and [$2\text{-}^3\text{H}$]mannose ($10\text{--}20$ Ci/mmol) were from Amersham (Amersham, Bucks., U.K.). Dulbecco's modified Eagle's medium (DMEM) was from Gibco (Cergy-Pontoise, France) and foetal calf serum (FCS) was from Flow Laboratories (Beyneix, Marseille, France). The SDS/PAGE protein molecular mass standards phosphorylase *b* (97.4 kDa), BSA (66.2 kDa), ovalbumin (42.7 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and hen egg white lysozyme (14.4 kDa) were from Bio-Rad (Richmond, CA, U.S.A.). All other chemicals were of the highest purity commercially available.

Cell culture conditions

Human melanoma IGR 39 cells [24] were routinely cultured as described [25]. Cells were plated at a density of $(15\text{--}20) \times 10^3$ cells/cm². Only cells at the stationary phase of growth were used. For inhibitor treatment, the culture medium was replaced by a new medium containing $1\text{ }\mu\text{g}$ of swainsonine/ml, $100\text{ }\mu\text{g}$ of deoxymannojirimycin/ml, $100\text{ }\mu\text{g}$ of castanospermine/ml or $1\text{ }\mu\text{g}$ of tunicamycin/ml and the culture was continued for an additional period of 60 h. During this period, cell culture media were changed three times, after each 18–20 h of incubation. After treatment, cells were used for binding assays, affinity labelling or biosynthetic labelling.

Incorporation of [^{35}S]methionine and [$2\text{-}^3\text{H}$]mannose

The sensitivity of IGR 39 cells to the inhibitors was determined by measurement of incorporation of [^{35}S]methionine and [$2\text{-}^3\text{H}$]mannose into macromolecules. Cells in 24-well plates were incubated in the absence or the presence of the inhibitors for 60 h, rinsed and incubated for 1 h in fresh serum-free medium which was either methionine- or glucose-free, depending on the tracer used. [^{35}S]Methionine was added to separate dishes in the absence or the presence of the appropriate inhibitors, followed by incubation for 4 h at 37 °C with methionine-deficient medium (containing 10% of the methionine found in normal DMEM)/10% FCS dialysed against distilled water. Cells to be labelled with [$2\text{-}^3\text{H}$]mannose were incubated for 4 h at 37 °C in glucose-deficient DMEM (containing 20 mg of glucose/l)/10% dialysed FCS. After removal of medium and three iso-osmotic washes with phosphate-buffered saline (PBS), cells were solubilized in 0.1 M-NaOH, precipitated with 10% trichloroacetic acid, and the labelled proteins were recovered by centrifugation at 1000 *g* for 15 min. The pellets were washed twice with PBS, redissolved in 0.5 M-NaOH and radioactivity was counted.

Binding of ^{125}I -VIP to IGR 39 cells and Scatchard analysis

Control and treated cells in 24-well plates [$(125\text{--}150) \times 10^6$ cells/well] were incubated with ^{125}I -VIP (final concentration 0.01 M) in the absence or the presence of native VIP (final concentration 0.1 μM) and the binding assay was performed as previously described [25]. The surface number of VIP receptors together with their affinities were determined by Scatchard analysis [26] of the binding performed in the presence of increasing amounts of native VIP (up to 0.1 M) as previously described [27]. The cell-bound radioactivity was then collected after solubilization in 0.5 M-NaOH and counted in a γ -radiation spectrometer.

Cross-linking of ^{125}I -VIP to its receptor

Cross-linking was performed in the presence of 2 mM-DSS as previously described [25], except that the cross-linking reaction was quenched with 1.5 M-Tris/HCl, pH 9, instead of ammonium acetate, pH 8. After three washes of the cell monolayers with PBS, labelled receptors were extracted using SDS sample buffer containing 2% SDS [28]. Samples were then resolved by SDS/PAGE [28] and radioactive proteins were visualized by autoradiography.

Glycosidase treatments

After cross-linking, labelled receptors were recovered from the cell monolayers by extraction with SDS sample buffer [28]. Ice-cold acetone was then added to each SDS extract and proteins were allowed to precipitate by incubation overnight at $-45\text{ }^{\circ}\text{C}$. The precipitates recovered after 15 min of centrifugation at 1000 *g* were redissolved in Endo H/neuraminidase buffer (50 mM-sodium citrate, pH 5.5) or Endo F buffer (100 mM-sodium phosphate, pH 6.1), both containing 1% Nonidet P40, 0.1% SDS, 0.1% β -mercaptoethanol and 1 mM-phenylmethanesulphonyl fluoride (PMSF). Endo H, Endo F and neuraminidase were then added to final concentrations of 1 unit/ml, 10 units/ml and 1 unit/ml respectively, and the mixtures were incubated overnight at 37 °C. Control samples did not receive the enzyme. Each sample was analysed by SDS/PAGE after addition of an equal volume of 2-fold-concentrated SDS sample buffer.

SDS/polyacrylamide-gel electrophoresis

SDS/PAGE was performed as described [28] using homogeneous 1.5 mm thick slab gels containing 12.5% acrylamide. After electrophoresis, gels were stained, destained, dried and exposed on Fuji-XR films for 1–7 days at $-80\text{ }^{\circ}\text{C}$. The apparent molecular mass of the labelled bands was determined from a standard curve based on the mobility of molecular mass markers that were co-electrophoresed with the samples.

Miscellaneous

Protein concentration was determined using the bicinchoninic acid (BCA) protein assay reagent (Pierce).

The results described are representative of experiments carried out at least three times for Scatchard analyses and at least twice for the others.

RESULTS

The contribution of *N*-linked glycans to the size of the VIP receptor from IGR 39 cells

VIP-binding sites were first labelled with ^{125}I -VIP, cross-linked, extracted from cell monolayers using SDS sample buffer and treated with Endo F. The electrophoretic mobilities of glycosylated and deglycosylated VIP receptor are reported in Fig. 1.

Fig. 1. Endo F digestion of the VIP receptor

IGR 39 cells were affinity-labelled by ¹²⁵I-VIP using DSS and extracted using SDS sample buffer before an overnight precipitation with acetone. Precipitates were then redissolved in Endo F buffer and incubated in the absence (lane A) or presence (lane B) of Endo F (10 munits/ml) for 24 h at 37 °C. The samples were then resolved by SDS/PAGE. The positions of molecular mass standards are shown.

Table 1. Effect of the inhibitors on [³⁵S]methionine and [³H]mannose incorporation

IGR 39 cells in 24-well plates were exposed to tunicamycin or to oligosaccharide-processing inhibitors for 60 h at 37 °C. Cells were then rinsed and incubated in DMEM without serum (either methionine- or glucose-free) for 1 h, followed by a 4 h incubation with [³⁵S]methionine in methionine-free DMEM or with [³H]mannose in glucose-deficient DMEM (i.e. containing 20 mg of glucose/ml). All culture media also contained 10 % dialysed FCS. Cells to be counted did not receive the labelled compounds. After three isotonic washes, cells were solubilized in 0.1 M-NaOH and precipitated with 10 % trichloroacetic acid, and the incorporation of radioactive material into the trichloroacetic acid-insoluble fractions of the cell layers was determined. Each value represents the average of triplicate analysis of two separate experiments with S.E.M. values of less than 10 %. Values in parentheses are percentages of control values.

Treatment	10 ⁻³ × Cell no. (cells/well)	[³⁵ S]Methionine (c.p.m./μg of protein)	[³ H]Mannose (c.p.m./μg of protein)
Control	123 ± 18	73 889 (100)	8982 (100)
Tunicamycin	98 ± 4	63 361 (85.7)	2448 (27.2)
Castanospermine	126 ± 9	71 177 (96.3)	8534 (95.0)
Deoxymannojirimycin	113 ± 11	68 738 (93.0)	7768 (86.5)
Swainsonine	119 ± 6	71 444 (96.7)	8794 (98.0)

Lane A shows a major band of 63 kDa characteristic of the VIP receptor from IGR 39 cells [25]. Lane B shows a 43 kDa band generated by deglycosylation by Endo F of the 63 kDa glycoprotein. Thus Endo F treatment results in a loss of approx. 20 kDa. Longer exposures of the gel (e.g. up to 2 weeks) has revealed the presence of an additional faint 95–125 kDa protein which could be converted to 79.4 kDa by Endo F treatment (results not shown). This minor component, which could indicate a self-association of two receptor molecules, was variably represented from one experiment to another.

Effect of inhibitors on IGR 39 cells

Incubation of IGR 39 cells with the drugs that we selected for the present study was found to have no significant effect on cell

Fig. 2. Effect of glycosylation inhibitors on ¹²⁵I-VIP specific binding to IGR 39 cells

Cells in 24-well plates were incubated for the indicated periods in the absence (◆) or presence of tunicamycin (◇), castanospermine (□), deoxymannojirimycin (■) or swainsonine (▲), and specific binding was determined as described in the Materials and methods section.

growth, since after 60 h of incubation cell numbers were virtually the same in control and treated cells (Table 1). We therefore examined the effects of the inhibitors on protein biosynthesis and glycosylation. Protein biosynthesis and N-glycosylation were measured respectively as [³⁵S]methionine and [³H]mannose incorporated into trichloroacetic acid-precipitable material. Table 1 shows that there was no significant effect of the drugs on mannose or methionine incorporation into macromolecules, except for tunicamycin, by which mannose incorporation was inhibited by approx. 75 %, while methionine incorporation was only slightly affected (e.g. 14.3 %). It is noteworthy that, despite this considerable inhibition of N-glycosylation by tunicamycin, cells maintained their typical morphology throughout the duration of the study. In all cases, under the conditions used (e.g. inhibitor concentrations and duration of inhibitor treatment), we did not detect any effect on cell viability as determined by Trypan Blue exclusion (results not shown).

Effect of inhibitors on ¹²⁵I-VIP binding to IGR 39 cells

The earliest detectable effect of the drugs on VIP binding to IGR 39 cells occurred after an 18 h preincubation period. With the exception of tunicamycin, the effect of the inhibitors was maximal at 60 h and persisted for up to 72 h. For this reason, unless otherwise mentioned, all of the following studies were performed after 60 h incubations with the drugs. As shown in Fig. 2, no VIP binding could be detected after a 72 h exposure of IGR 39 cells to tunicamycin (1 μg/ml), while an approx. 80 % decrease was observed after 60 h of incubation. After this period, deoxymannojirimycin, at a concentration of 100 μg/ml, also decreased specific binding to 67 % of maximal. Tunicamycin and deoxymannojirimycin had the most pronounced effects compared with treatment with either swainsonine (1 μg/ml) or castanospermine (100 μg/ml), which respectively caused 25 % and 32 % decreases in VIP specific binding. On the basis of the above results, binding studies were conducted to determine whether receptors synthesized in the presence of these inhibitors had a modified affinity for VIP or a decreased number of VIP-binding sites. For this purpose, the 60 h incubation with the inhibitors was followed by measurement of ¹²⁵I-VIP binding in the absence or presence of increasing amounts of unlabelled VIP (Fig. 3).

Fig. 3. Scatchard plots of binding data from control and treated cells

After incubation for 60 h in the absence (control) or presence of glycosylation inhibitors, cells were incubated in the presence of ^{125}I -VIP and increasing amounts of native VIP. Binding was then determined and data were subjected to Scatchard analysis. (a) Scatchard analysis of binding data from control ($\blacklozenge$), tunicamycin ($\diamond$)- and castanospermine ($\blacksquare$)-treated cells. (b) Scatchard analysis of binding data from control ($\blacklozenge$), deoxymannojirimycin ($\blacksquare$)- and swainsonine ($\diamond$)-treated cells.

Competition binding with unlabelled VIP revealed that the affinity of the receptors in swainsonine-treated cells was slightly lowered, whereas the affinity of the receptors in deoxymannojirimycin-treated cells was to a much greater extent decreased (Fig. 3b). The K_d of normal receptors was 0.41 ± 0.12 nM and those of receptors from swainsonine- and deoxymannojirimycin-treated cells were 0.60 ± 0.18 nM and 1.33 ± 0.4 nM respectively. However, the number of surface VIP-binding sites on these cells remained unchanged (e.g. $170 \text{ fmol}/10^6$ cells). Similar Scatchard analyses were carried out after treatment with either castanospermine or tunicamycin. As shown in Fig. 3(a), there was a decrease in the number of high-affinity binding sites in both castanospermine- and tunicamycin-treated cells, from $170 \text{ fmol}/10^6$ cells (control) to $110 \text{ fmol}/10^6$ cells and $32 \text{ fmol}/10^6$ cells respectively. In these cases the parallel slopes of Scatchard plots indicated that the binding affinity had not been altered.

Characterization of VIP receptor structures obtained in the presence of the drugs

In order to determine whether the decrease in the VIP-binding capacity of IGR 39 cells induced by the drugs could be correlated with structural changes at the receptor level, control and inhibitor-treated cells were affinity-labelled and extracts were analysed by SDS/PAGE. As depicted in Fig. 4, the extract from control cells consisted, as expected, of two affinity-labelled proteins; a major 63 kDa and a minor 97–125 kDa protein (lane C). Because of the variation of these 97–125 kDa proteins, the following studies will focus exclusively on the 63 kDa component. Following swainsonine treatment, the estimated size of the VIP receptor was decreased by 7–8 kDa (Fig. 4, lane 2), whereas deoxymannojirimycin treatment provoked an increased electrophoretic mobility equivalent to the loss of approx. 15 kDa (lane 4). Visually, the intensity of the label was not affected by swainsonine treatment, whereas it was slightly decreased after incubation with deoxymannojirimycin. In extracts from castanospermine- and tunicamycin-treated cells, the same bands as in the control were detected (lanes 3 and 5). It is of interest to note that, whereas the label of the VIP-binding protein from tunicamycin-treated cells was barely detectable (lane 5), the major spot from castanospermine-treated (lane 3) cells was broader than the one from control cells. The result from tunicamycin-treated cells is unexpected, because these cells should have produced an aglyco-receptor with an apparent molecular

Fig. 4. Molecular mass changes in the VIP receptor from IGR 39 cells on treatment with glycosylation inhibitors

IGR 39 cells were incubated in the absence (lane 1) or the presence of swainsonine (lane 2), castanospermine (lane 3), deoxymannojirimycin (lane 4) or tunicamycin (lane 5) for 60 h. VIP receptors were then radioactively labelled using DSS, extracted using SDS sample buffer and resolved by SDS/PAGE. The positions of molecular mass standards are indicated.

mass of 40–43 kDa, similar to that of the Endo F-deglycosylated receptor, as in the case with β -adrenoreceptors [13]. To ascertain that the receptor labelled in the presence of tunicamycin consisted of a residual pool of native receptors instead of a newly synthesized receptor population, incubation with tunicamycin was continued for an additional period of 24 h to allow the complete internalization of the pre-existing receptors. ^{125}I -VIP binding and cross-linking assays were then performed. For the same reasons, similar experiments were conducted with castanospermine-treated cells. As shown in Fig. 5, the affinity-labelled band from tunicamycin-treated cells had completely disappeared (lane 3), whereas both the intensity of the signal and the apparent molecular mass of the ^{125}I -VIP-binding protein from castanospermine-treated cells remained unchanged (lane 2). This result indicates that the labelled protein from tunicamycin-treated cells observed after a 60 h incubation period consists in a pre-existing population of native receptors, whereas the 63 kDa protein from castanospermine-treated cells could

Fig. 5. Influence of long-term treatment on VIP receptors from castanospermine- and tunicamycin-treated cells

IGR 39 cells were cultured in the absence (lane 1) or presence of either castanospermine (lane 2) or tunicamycin (lane 3) for 84 h. VIP receptors were then radioactively labelled using DSS, extracted using SDS sample buffer and resolved by SDS/PAGE.

Fig. 6. Digestion by Endo H of VIP receptors derived from control and treated cells

IGR 39 cells were incubated in the absence (lanes A and B) or the presence of swainsonine (lanes C and D), deoxymannojirimycin (lanes E and F) or castanospermine (lanes G and H) and the radiolabelled extracts were precipitated with acetone and redissolved in Endo H/neuraminidase buffer. Endo H (final concentration 1 unit/ml) was then added to the appropriate samples and the mixtures were incubated for 24 h. Shown are receptor preparations without Endo H digestion (lanes A, C, E and G) and after Endo H treatment (lanes B, D, F and H). The positions of molecular mass standards are shown.

correspond to a *de novo* synthesized receptor. Consequently we considered that the VIP receptors labelled in the presence of tunicamycin consisted of the same molecules as in control cells. For this reason, we did not perform any additional structural characterization of these receptors.

Endo H sensitivity of VIP receptors from control and inhibitor-treated cells

Inhibition of endoplasmic reticulum glucosidase I or Golgi α -mannosidases generally results in Endo H-sensitive forms of glycoproteins. To test this possibility, the affinity-labelled proteins were extracted from control and inhibitor-treated cells and their Endo H sensitivities were assessed. Fig. 6 shows that the VIP receptors from control cells were resistant to Endo H (lanes A and B). In contrast, the receptors from swainsonine- and deoxymannojirimycin-treated cells exhibited the expected sensitivity to Endo H (lanes C–F). The VIP receptor from swainsonine-treated cells was partially sensitive to Endo H treatment, with a decrease in the 56 kDa species by approx.

Fig. 7. Digestion by Endo F of VIP receptors from control and inhibitor-treated cells

IGR 39 cells were incubated in the absence (lanes A and B) or the presence of deoxymannojirimycin (lanes C and D), castanospermine (lanes E and F) or swainsonine (lanes G and H) 125 I-VIP-cross-linked receptors were extracted and treated with 10 units of Endo F/ml, exactly as described for Fig. 1. Shown are receptor preparations without Endo F treatment (lanes A, C, E and G) and after Endo F digestion (lanes B, D, F and H). The positions of molecular mass standards are shown.

10 kDa (lanes C and D). Digestion of the receptor from deoxymannojirimycin-treated cells resulted in the conversion of the 48 kDa species into a 43 kDa component, which should be the fully deglycosylated receptor, as it migrates in a similar way to the Endo F-deglycosylated receptor (lanes E and F). In contrast, the VIP-binding proteins derived from castanospermine-treated cells were completely resistant to Endo H (lanes G and H). In an effort to establish this Endo H sensitivity, we have performed different experiments in which the enzyme concentration and the duration of the treatment were enhanced. Despite all of these procedures, we did not succeed in demonstrating Endo H sensitivity of the receptors from castanospermine-treated cells (results not shown).

Endo F treatment of 125 I-VIP-cross-linked receptors from control and inhibitor-treated cells

In order to establish whether the alterations in the VIP receptor properties could reflect modifications at the protein or the oligosaccharide level, 125 I-VIP-cross-linked receptors were extracted and exposed to Endo F for 18 h at 37 °C. The data in Fig. 7 illustrate the effects of Endo F treatment on the electrophoretic mobility of VIP receptors from control and inhibitor-treated cells. Clearly, after deglycosylation of the receptors, all of the proteins migrated with the same mobility and had the same apparent molecular mass of 43 kDa (Fig. 7). This fact, together with the Endo H sensitivity, strongly suggests that, at least in the case of deoxymannojirimycin- and swainsonine-treated cells, differential glycosylation may be primarily responsible for the observed alterations in binding activity and structure of the VIP receptor.

Neuraminidase characterization of the VIP receptor from castanospermine-treated cells

Since the VIP receptor from castanospermine-treated cells behaved electrophoretically like the control receptor, we used neuraminidase digestion to verify whether the former contain sialic acid residues. Neuraminidase treatment of extracts from control cells decreased the apparent molecular mass of the 125 I-VIP-labelled receptor by approx. 9 kDa (Fig. 8, lanes A and B). Digestion of extracts from castanospermine-treated cells also decreased the VIP receptor molecular mass, but to a lesser extent, with a loss of about 5 kDa (lanes C and D). This result

Fig. 8. Effect of neuraminidase on VIP receptors derived from control and castanospermine-treated cells

IGR 39 cells were incubated either in the absence (lanes A and B) or in the presence (lanes C and D) of castanospermine. After 60 h of incubation at 37 °C, cells were affinity-labelled, solubilized with SDS sample buffer and precipitated with acetone. The precipitates were redissolved in Endo H/neuraminidase buffer and neuraminidase was added to the appropriate samples (final concentration 1 unit/ml). The mixtures were then incubated overnight at 37 °C. Shown are receptor preparations without neuraminidase digestion (lanes A and C) or after neuraminidase treatment (lanes B and D). The positions of molecular mass standards are shown.

demonstrates the presence of sialic acid on the VIP receptor from castanospermine-treated cells and does not conform with the expected high-mannose structures usually found in such cells.

DISCUSSION

The VIP receptor of human melanoma IGR 39 cells is a glycoprotein of 63 kDa containing *N*-linked carbohydrate chains. Treatment of the 63 kDa species with Endo F generated a 43 kDa protein (Fig. 1) consistent with what we had already obtained with VIP receptors from another human cell line, HT29-D4 (45 kDa) [15]. Assuming that one molecule of ¹²⁵I-VIP (3 kDa) was cross-linked per receptor molecule, the result from Endo F deglycosylation suggests that the VIP-binding protein from these two cells of human origin resides on a polypeptide of 40–43 kDa.

Although the increase in electrophoretic mobility of the endo-F-deglycosylated receptor might not quantitatively correspond to a loss of about 30 % from the native molecule, *N*-glycans seem to be largely involved in the VIP receptor structure. Based on this fact, we have investigated the biosynthesis of the *N*-linked glycan chains of the human VIP receptor and their influence on receptor structure and function. For this, we used the glycosylation inhibitor tunicamycin and the oligosaccharide-processing inhibitors castanospermine, deoxymannojirimycin and swainsonine.

IGR 39 cells were incubated with tunicamycin in order to produce VIP receptors lacking *N*-glycosylation. After 60 h of exposure to the drug, these cells expressed only 20 % of the control number of VIP-binding sites (Fig. 3a). This result is in accordance with our previous report [15] and with the results obtained with the VIP receptor by Svoboda *et al.* [16], who found that tunicamycin treatment of the rat pancreatic acinar tumour cell line AR4-2J considerably altered VIP binding to these cells. From a structural point of view, instead of the expected 43 kDa VIP-binding protein (aglycoreceptor), tunicamycin-treated cells produced a weakly labelled 63 kDa protein similar to that found

in control cells (Fig. 4, lane 5). When tunicamycin treatment was continued for an additional period of 24 h, no 63 kDa labelled component was detected (Fig. 5, lane 3). This indicates that the 63 kDa protein detected in tunicamycin-treated cells represents a pre-existing population of VIP receptors. One likely explanation for the absence of cell-surface expression of VIP-binding activity induced by tunicamycin is the prevention of the formation and transfer of oligosaccharides on the nascent protein, causing it not to be further processed, as is the case with the insulin receptor [7]. Similar studies with the VIP receptor using 'heavy' amino acids (¹⁵N-, ¹³C- and ³H-labelled amino acids) could address this question. Another explanation would be that the aglycoreceptor was prematurely degraded in tunicamycin-treated cells. The present study cannot rule out such a possibility, since there is increasing evidence that tunicamycin decreases the intracellular stability of various glycoproteins [29,30]. Although sensitivity to tunicamycin is not a general feature of membrane glycoproteins, the *N*-glycan moieties are considered to be important for establishing the functional conformation of several receptors. Receptors for insulin, epidermal growth factor and acetylcholine synthesized in the presence of tunicamycin fail to bind ligands [7,9]. It has been assumed that carbohydrates, co-translationally added to the core peptide, induce the correct folding of the cysteine-containing ligand-binding domains of these receptors. This in turn promotes the formation of disulphide bridges, which were found to be essential for the acquisition of the ligand-binding activity (for review see [31]). This situation is in contrast with the one generally found with the β -adrenergic receptor which, like the VIP receptor, belongs to the G-protein-linked family of transmitters. The reason for this difference is an open question.

In the presence of the α -glucosidase I inhibitor castanospermine, cells synthesized a 63 kDa VIP-binding protein (Fig. 4, lane 3) which was completely resistant to Endo H digestion (Fig. 6, lane H). It is unlikely that this VIP-binding protein consists of a residual pool of native receptors, because when castanospermine treatment was continued for an additional period of 24 h, no further modification was observed in either molecular mass or the intensity of the signal (compare Fig. 4, lane 3 and Fig. 5, lane 2). Furthermore, when digested by neuraminidase, the VIP receptor from castanospermine-treated cells lost about 5 kDa, indicating the presence of sialic acid residues. Although glycoproteins synthesized in the presence of castanospermine generally carry glucose-capped high-mannose oligosaccharides, the presence of sialylated *N*-glycans in castanospermine-treated cells has been observed in numerous studies [32–34]. Synthesis of complex *N*-glycans, even in the presence of a high concentration of this inhibitor, may be due to a processing pathway alternative to the main processing, which involves the sequential removal of glucose and mannose residues. Indeed, an endomannosidase activity, which can act on glucosylated high-mannose glycans to release Glc₁₋₃-Man saccharide, has been detected in Golgi apparatus from rat liver [35,36] and more recently in cultured HepG2 cells and thyroid slices [37]. This endomannosidase activity, which is not inhibited by castanospermine, provides an alternative processing pathway for the glucosylated high-mannose oligosaccharides to be converted to normal complex structures [36]. Involvement of such an endomannosidase is a possible explanation for the slight decrease in the cell-surface number of VIP-binding sites, which could be attributed to a retardation in receptor movement from the endoplasmic reticulum to the cell surface. Structural analyses of *N*-glycans produced in castanospermine-treated IGR 39 cells would be required to prove this point.

To determine whether altering the carbohydrate chains at the Golgi level results in changes in structure, cell-surface expression

or ligand-binding activity of the VIP receptor, cells were incubated with two inhibitors of Golgi mannosidases: deoxymannojirimycin and swainsonine. Based on the mechanisms of action of these substances, one would expect the VIP receptor to appear as a lower-molecular-mass species after incubation with them. Fig. 4 demonstrates that VIP-labelled proteins migrate with an apparent molecular mass of 48 kDa following deoxymannojirimycin treatment (Fig. 4, lane 4) and 56 kDa after exposure to swainsonine (Fig. 4, lane 2). These represent the native receptor in control IGR 39 cells. Furthermore, following treatment with either of these inhibitors, the VIP-binding proteins were found to be sensitive to Endo H digestion (Fig. 6, lanes D and F). This result is in accordance with the predicted structures, since deoxymannojirimycin-treated cells synthesize increased amounts of *N*-glycans of the high-mannose type [20] and swainsonine-treated cells synthesize hybrid chains [38]. In addition to the structural changes, the affinity of the receptor towards VIP was affected in both deoxymannojirimycin- and swainsonine-treated cells, the most pronounced effect being obtained after exposure of cells to the former. There was no change, however, in the number of VIP-binding sites after treatment with either inhibitor (Fig. 3b). These data are consistent with studies on other membrane glycoproteins [12,39,40]. It is of interest to note that among the four drugs used in this study, swainsonine had the smallest effect, which may suggest that partial complex chains are sufficient to maintain the correct affinity of the VIP receptor for its ligand.

The ability of VIP receptors from inhibitor-treated cells to interact productively with adenylate cyclase is a matter for further investigation. Cyclic AMP accumulation in response to VIP was diminished in deoxymannojirimycin- and swainsonine-treated cells, whereas the response to forskolin was essentially normal (A. El Battari & P. Pic, unpublished work). The effect of the GTP analogue guanosine 5'-[$\beta\gamma$ -imidotriphosphate p[NH]ppG on VIP binding was also explored with membranes derived from control, swainsonine- and deoxymannojirimycin-treated cells. Preliminary results showed that VIP receptors from deoxymannojirimycin-treated cells exhibited a normal capability to interact with the stimulatory G-protein G_s , as shown by the inhibition by p[NH]ppG of 125 I-VIP binding. These data suggest that the deoxymannojirimycin-induced alteration in the affinity might occur at the receptor level rather than on receptor- G_s coupling (A. El Battari, P. Forget, F. Fouchier & P. Pic, unpublished work).

This work was supported by the Université d'Aix-Marseille I and the Centre National de la Recherche Scientifique (CNRS URA 202). We are grateful to Professor J. Marvaldi for his helpful suggestions and to Dr. J. Pichon for his interest and support. We thank Mr. Roger Soirat, M. Jean-Jacques Roccabianca and Mr. Fernand Giannellini for their expert technical assistance, and Mrs. C. Sallam for improving the English of the paper.

REFERENCES

- Said S. I. (1984) *Peptides* **5**, 143–150

- Laburthe, M. (1985) *Biochimie* **67**, 11–17
- Luis, J., Martin, J. M., El Battari, A., Marvaldi, J. & Pichon, J. (1988) *Biochimie* **70**, 1311–1322
- Couvaineau, A., Voisin, T., Guijarro, L. & Laburthe, M. (1990) *J. Biol. Chem.* **265**, 13386–13390
- El Battari, A., Luis, J., Martin, J. M., Fantini, J., Muller, J. M., Marvaldi, J. & Pichon, J. (1987) *Biochem. J.* **242**, 185–191
- Nguyen, T. D., Williams, J. A. & Gray, G. M. (1986) *Biochemistry* **25**, 361–368
- Olson, T. S., Bamberger, M. J. & Lane, M. D. (1988) *J. Biol. Chem.* **263**, 7342–7351
- Slieker, L. J., Martensen, T. M. & Lane, M. D. (1986) *J. Biol. Chem.* **261**, 15233–15241
- Merlie, J. P. & Smith, M. M. (1986) *J. Membr. Biol.* **91**, 1–10
- Klotz, K. N. & Lohse, M. J. (1986) *Biochem. Biophys. Res. Commun.* **140**, 406–413
- Jarvie, K. R., Niznik, H. B. & Seeman, P. (1988) *Mol. Pharmacol.* **34**, 91–97
- George, S. T., Ruoho, A. E. & Malbon, C. C. (1986) *J. Biol. Chem.* **261**, 16559–16564
- Boege, F., Ward, M., Jürss, R., Hekman, M. & Helmreich, E. J. M. (1988) *J. Biol. Chem.* **263**, 9040–9049
- Stiles, G. L. (1985) *Arch. Biochem. Biophys.* **237**, 65–71
- El Battari, A., Luis, J., Martin, J. M., Fantini, J., Muller, J.-M., Marvaldi, J. & Pichon, J. (1988) *Ann. N.Y. Acad. Sci.* **527**, 667–671
- Svoboda, M., De Neef, P., Tastenoy, M. & Christophe, J. (1988) *Eur. J. Biochem.* **176**, 707–713
- Tkacz, J. S. & Lampen, J. O. (1975) *Biochem. Biophys. Res. Commun.* **65**, 248–257
- Palamarezyk, G. & Elbein, A. D. (1985) *Biochem. J.* **227**, 795–804
- Saunier, B., Kilker, R. D., Tcaciz, J. S., Quaroni, A. & Herscovics, A. C. (1982) *J. Biol. Chem.* **257**, 14155–14161
- Fuhrmann, U., Bause, E., Legler, G. & Ploegh, H. (1984) *Nature (London)* **307**, 755–758
- Elbein, A. D., Dorling, P. R., Vosbeck, K. & Horisberger, M. (1982) *J. Biol. Chem.* **260**, 7665–7674
- Elbein, A. D. (1988) *Plant Physiol.* **87**, 191–195
- Martin, J. M., Darbon, H., Luis, J., El Battari, A., Marvaldi, J. & Pichon, J. (1988) *Biochem. J.* **250**, 679–685
- Aubert, C., Rougé, F. & Galindo, J. R. (1980) *J. Natl. Cancer Inst.* **64**, 1029–1037
- Luis, J., Martin, J. M., El Battari, A., Reynier, M., Marvaldi, J. & Pichon, J. (1989) *Eur. J. Biochem.* **180**, 429–433
- Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672
- Martin, J. M., Luis, J., Marvaldi, J., Pichon, J. & Pic, P. (1989) *Eur. J. Biochem.* **180**, 435–439
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Prives, J. & Bar-Sagi, D. (1983) *J. Biol. Chem.* **258**, 1775–1780
- Yanagishita, M. (1986) *Arch. Biochem. Biophys.* **251**, 287–298
- Olson, T. S. & Lane, M. D. (1989) *FASEB J.* **3**, 1618–1624
- Pan, Y. T., Hori, H., Saul, R., Saufort, B. A., Molyneux, R. J. & Elbein, A. D. (1983) *Biochemistry* **22**, 3975–3984
- Gross, V., Tran-Thi, T. A., Schwarz, R. T., Elbein, A. D., Decker, K. & Heinrich, P. C. (1986) *Biochem. J.* **236**, 853–860
- Foddy, L. & Hugler, R. C. (1988) *Eur. J. Biochem.* **175**, 291–299
- Lubas, W. A. & Spiro, R. G. (1987) *J. Biol. Chem.* **262**, 3775–3781
- Lubas, W. A. & Spiro, R. G. (1988) *J. Biol. Chem.* **263**, 3990–3998
- Moore, S. E. H. & Spiro, R. G. (1990) *J. Biol. Chem.* **265**, 13104–13112
- Tulsiani, D. R. P. & Touster, O. (1983) *J. Biol. Chem.* **258**, 7578–7585
- Akiyama, S. K., Yamada, S. S. & Yamada, K. M. (1989) *J. Biol. Chem.* **264**, 18011–18018
- Berke, B., Matlin, K., Bause, E., Legler, G., Peyrieras, N. & Ploegh, H. (1984) *EMBO J.* **3**, 551–556

Received 17 September 1990/8 April 1991; accepted 17 April 1991