ELSEVIER

Biochimica et Biophysica Acta 1372 (1998) 174–186

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Functional reconstitution of membrane glycoproteins into lipid vesicles using lectin precipitation. Application to the VIP receptor

Assou El Battari [a,*], Mourad Zerfaoui [a], José Luis [b], Zuo-Liang Xiao [b], Azzeddine Kadi [b], Catherine Bellan [b], Dominique Lombardo [a], Jacques Marvaldi [b]

[a] *INSERM U.260, Faculté de Médecine, 27 Bd Jean Moulin, F-13385 Marseille Cedex 5, France*
[b] *CNRS URA 1924, Faculté de Pharmacie, 27 Bd Jean Moulin, F-13385 Marseille Cedex 5, France*

Received 19 January 1998; revised 17 March 1998; accepted 26 March 1998

Abstract

We studied the interaction of *n*-octyl-β-D-glucopyranoside-solubilized VIP receptors (VIPR) with wheat germ agglutinin and found that the addition of the lectin to the detergent extract led to the formation of aggregates that could be pelleted by high speed centrifugation. Resuspension of the pellet in the presence of the competing trisaccharide, *N*,*N*′,*N*″-triacetylchitotriose (TAC), dissociated the lectin from the complex without altering the precipitability of VIPR. The final pellet (referred to as TAC pellet) contained an average of 12% of total protein and 96% of total VIP binding activity with a typical rank order of potency for VIP-related peptides. Lipid analysis and electron microscopic examination indicated that the precipitated material was composed of lipid vesicles. VIPR molecules were shown to be integrally inserted in the liposomes because they could not be dissociated from the vesicles at pH 11 or with high salt concentration. By comparing the liposome-associated VIP binding activity in the presence and absence of detergent and by showing accessibility of VIPR to PNGase F, it was concluded that VIP binding sites were not simply trapped within the reconstituted vesicles but likely exposed at the external surface of the liposomes.

Keywords: VIP receptor; Reconstitution; Wheat germ agglutinin; Lipid vesicle

1. Introduction

Reconstitution experiments have contributed to the understanding of how receptors function and how their activity can be influenced by various factors such as membrane lipid composition or the ori-

Abbreviations: CMC, critical micellar concentration; ConA, concanavalin A (*Canavalia ensiformis*); DOPC, dioleoylphosphatidylcholine; DSA, *Datura stramonium* agglutinin; DSS, disuccinimidyl suberate; hGRF, human growth releasing factor; HEMBG, 20 mM Hepes pH 7.5, 2.5 mM EGTA, 5 mM $MgCl_2$, 1 mg/ml bacitracin and 20% glycerol; HEMBG/MPI, HEMBG buffer containing the following protease inhibitors: 1 mM iodoacetamide, 1 mM PMSF, 5000 U/ml aprotinin, 1 μg/ml leupeptin, 1 mM pepstatin and 1 mM orthophenanthrolin; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; OG, *n*-octyl-β-D-glucopyranoside; PACAP, pituitary adenylate cyclase-activating peptide; PHM, peptide histidine methionine amide; PNGase F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase; PS, PC and PE, phosphatidylserine, -choline and -ethanolamine, respectively; TAC, *N*,*N*′,*N*″-triacetylchitotriose; VIP, vasoactive intestinal peptide; VIPR, vasoactive intestinal peptide receptor; ^{125}I-VIP, monoiodinated vasoactive intestinal peptide; UEA, *Ulex europaeus* agglutinin; WGA, wheat germ agglutinin

* Corresponding author. Fax: +33 (4) 91830187; E-mail: Assou.Elbattari@medecine.univ-mrs.fr

0005-2736/98/$19.00
PII: S0005-2736(98)00055-8

entation of the receptor within the lipid bilayer [1]. In common practice, detergent extracts are mixed with the required lipids and vesicles are generated through the removal of the detergent. Depending on the type, the removal of detergents can be performed by dialysis, reverse-phase evaporation or gel filtration as well as by adding an insoluble detergent scavenger like polystyrene beads or by diluting the suspension below the critical micellar concentration (CMC) of the detergent. In the present study, we describe a lectin-based precipitation method that allowed us to concentrate and functionally reconstitute the vasoactive intestinal peptide receptor (VIPR) from the human melanoma cells IGR 39.

VIPR is an integral membrane glycoprotein of 63–66 kDa belonging to the superfamily of seven hydrophobic domain receptors coupled to adenylate cyclase via the heterotrimeric G proteins. Based on the primary sequence data, rat and human VIPR consist of a polypeptide core of 52–56 kDa (including a signal peptide of 30 amino acids) carrying four potential *N*-glycosylation sites, three of which are located in the N-terminal extracellular domain [2–4]. Early studies from our laboratory indicated that mature *N*-glycosylation is required for VIP binding activity [5] and recently, Couvineau et al. [6] reported that *N*-glycosylation of at least two sites is crucial for correct delivery of the receptor to the plasma membrane. Using the human melanoma IGR 39 cells, we previously showed that the 63–66 kDa VIP binding protein could be converted to a 43 kDa species (based on SDS-PAGE) upon complete de-*N*-glycosylation and that at least three of the four potential glycosylations carried *N*-linked glycans [7].

The present work is a continuation of our previous efforts to evaluate the contribution of lipid environment [8] and *N*-glycosylation to receptor function [5,7]. Deriving advantage from the high avidity of VIPR towards WGA, we used this lectin to precipitate the receptor from an octyl-β-glucoside cell extract and found that endogenous lipids were also sedimented along with VIPR and other uncharacterized proteins. Removal of the lectin from the sedimented material led to the reformation of membrane vesicles as assessed by electron microscopy. Various analytical approaches were applied in order to determine association of VIPR with the lipids and to study binding parameters of the reconstituted VIPR.

2. Materials and methods

2.1. Reagents

N,*N*′,*N*″-Triacetylchitotriose (TAC), WGA, *n*-octyl-β-D-glucoside and metrizamide were from Sigma (St. Louis, MO, USA). Synthetic VIP was purchased from Neosystem (Strasbourg, France). ^{125}I-VIP (200 Ci/mmole) was iodinated by iodogen method and purified by HPLC as previously described [9]. The bee venom phospholipase A_2 was from Boehringer (Mannheim, Germany) and the cross-linker disuccinimidyl suberate (DSS) was from Pierce Chemical (Rockford, IL). *Flavobacterium meningosepticum* PNGase F was from Oxford Glycosystem (Abingdon, UK). All other chemicals were of the highest reagent grade available.

2.2. Cell culture

The melanoma cells IGR 39 [10] were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. When needed, cell cultures were metabolically labeled overnight in complete media with 100 μCi/ml Tran^{35}S-label (ICN Biochemicals, Irvine, CA).

2.3. Solubilization and lectin precipitation

Solubilization of IGR 39 cells was performed essentially as described earlier [11]. Briefly, cells were harvested in PBS and rinsed twice in the same buffer before solubilization. Cell pellet was then resuspended in solubilization buffer HEMBG/MPI containing 1% *n*-octyl-β-D-glucoside (OG), incubated at 4°C for 30 min with mild stirring followed by centrifugation at $100\,000\times g$ for 1 h in a TL100 Beckmann ultracentrifuge. The lectin WGA (0.5 mg/ml, final concentration) was then added to the $100\,000\times g$ supernatant (OG extract). After 20 min incubation at 4°C the clear OG extract became turbid, and a yellowish pellet (WGA pellet) was obtained after another centrifugation at $100\,000\times g$ for 15 min. In order to wash out the detergent, the WGA pellet was homogenized 3 times in a detergent-free HEMBG/MPI buffer by passing the suspension through a 25-gauge needle, followed by centrifugation at $100\,000\times g$ for 15 min. The last

pellet was treated similarly except that the WGA competing trisaccharide TAC was included to dissociate WGA-glycoprotein aggregates and the mixture was incubated for 15 min at room temperature. A white translucent pellet (TAC pellet) was obtained after another 100 000 × *g* centrifugation, resuspended in HEMBG/MPI and stored at 4°C until use.

2.4. Gel filtration of solubilized VIPR

The OG-extracted VIP binding activity was analyzed by size-exclusion chromatography. A TSK Spherogel SW 4000 HPLC column was equilibrated with HEMBG/MPI containing 0.1% OG. Flow rate was 0.5 ml/min. The column was calibrated with molecular mass standards (thyroglobulin, 669 kDa; apoferritin, 443 kDa; β-amylase, 200 kDa; alcohol dehydrogenase, 150 kDa and carbonic anhydrase 29 kDa). 500 μl of OG extract (5.5 mg/ml) were applied to the column and aliquots of 300 μl were collected and analyzed for VIP radioreceptor assay as described below.

2.5. VIP radioreceptor assay

^{125}I-VIP binding was determined using the polyethylenimine soaked GF/F glass fiber filters and vacuum filtration as described earlier [11]. Cross-linking of ^{125}I-VIP to cells or TAC pellet was performed using 2 mM DSS [11]. After cross-linking, solubilization and reconstitution were performed as described above.

2.6. Metrizamide density gradient centrifugation

After cross-linking of ^{125}I-VIP to IGR 39 cells and solubilization, the TAC pellet containing ^{125}I-VIP-labeled glycoproteins was prepared as described above except that WGA pellet was resuspended in the presence of [^{14}C]DOPC (50 μCi/ml) as a tracer before the addition of the trisaccharide TAC. This mixture was then subjected to a discontinuous metrizamide density gradient as described [12], with slight modifications. Briefly, the final TAC pellet was resuspended in 1 ml Hepes/NaOH buffer pH 7.5 containing MPI and adjusted to 40% (w/v) metrizamide by the addition of a 60% metrizamide solution prepared in the same buffer. This solution was transferred into a 13.5 ml polypropylene centrifuge tube. Then 3 ml of 20% metrizamide was overlaid followed by another overlay of 3 ml of 10% metrizamide which, in turn, was covered with buffer (see below). Centrifugation was carried out at 100 000 × *g* for 2 h at 25°C in an SW 41 rotor. After centrifugation, a turbid band could be seen distinctly at the 40–20% metrizamide interface. Fractions of 500 μl were collected from the top to the bottom of the tube and radioactivity was determined.

2.7. SDS-polyacrylamide-gel electrophoresis

SDS-PAGE was performed according to Laemmli [13] using 1.5 mm thick slab gels containing 10% acrylamide. When needed for autoradiography, gels were exposed to Fuji-XR films at −80°C.

2.8. Lipid analysis and phospholipase A_2 treatment

Lipids were extracted from samples according to Folch et al. [14] and chromatographed on 0.25 mm thick silica gel (Merck, Darmstadt, Germany) in chloroform/methanol/methylamine (68:26:6, v/v). Lipid spots were visualized using phosphomolybdic acid (0.05%) in solution in acetic acid/sulfuric acid (25:1.25, v/v). In the phospholipase A_2 experiments, TAC pellet aliquots were adjusted to 500 μg protein/ml in HMBG (20 mM Hepes, 5 mM $MgCl_2$, 1 mg/ml bacitracin and 20% glycerol) containing MPI. BSA and $CaCl_2$ were then added to a final concentration of 1 mg/ml and 10 mM, respectively. Phospholipase A_2 dissolved in water was then added and samples were incubated for different periods at 25°C. Aliquots were then assayed for ^{125}I-VIP binding assay as described above.

2.9. Electron microscopy

WGA and TAC pellets were fixed with 2% glutardialdehyde in 0.1 M phosphate buffer pH 7.4, post-fixed with 1% osmium tetroxide, dehydrated through cold graded ethanol and embedded in Epon. The sections were then examined with a Jeol 100C microscope. Freeze-fracture electron microscopy was performed on material resuspended from WGA and TAC pellets, according to Lindstrom et al. [15].

2.10. PNGase F treatment

^{125}I-VIP-labeled cells and ^{125}I-VIPR-labeled vesicles (reconstituted from those cells) were incubated with PNGase F (100 U/ml) in 100 mM Tris/HCl pH 8.6 containing MPI and 10 mM orthophenanthrolin. Control samples did not receive the enzyme. The reaction mixtures were incubated at 37°C for 3 h and the samples were analyzed by SDS-PAGE, after addition of 5-fold concentrated SDS sample buffer.

2.11. Other procedures

The protein content of samples was measured using the bicinchoninic acid assay (BCA assay, Pierce) and phosphorus determination was carried out according to Van Veldhoven and Mannaerts [16]. The computer-fitted program LIGAND [17] was used to analyze the binding data.

3. Results

3.1. Solubilization and lectin precipitation

The human melanoma IGR 39 cells were chosen for our solubilization and reconstitution experiments because these cells have been shown to express a very high number of VIP receptors [10]. Detergent solubilization was performed in the presence of 1% *n*-octyl-β-glucoside which, as described earlier [11], among several detergents was the most efficient in extracting active VIP receptors. About 70% of the initial VIP binding activity could be recovered in the $100\,000\times g$ supernatant. To further prove the soluble nature of VIPR, 0.5 ml of OG extract (5 mg/ml protein) was loaded on an SK Spherogel SW 4000 HPLC column preequilibrated in the solubilization buffer containing 0.1% detergent. As shown in Fig. 1A, specific VIP binding was found to partition between the void volume (V_0) and the included volume (V_t) with an elution volume corresponding to an apparent molecular mass of 200 kDa (Fig. 1B). This value is close to the mass of the ^{125}I-VIP-labeled VIPR we previously characterized on another cell line, HT 29 cells [11]. This value is significantly larger than the 63–65 kDa displayed in

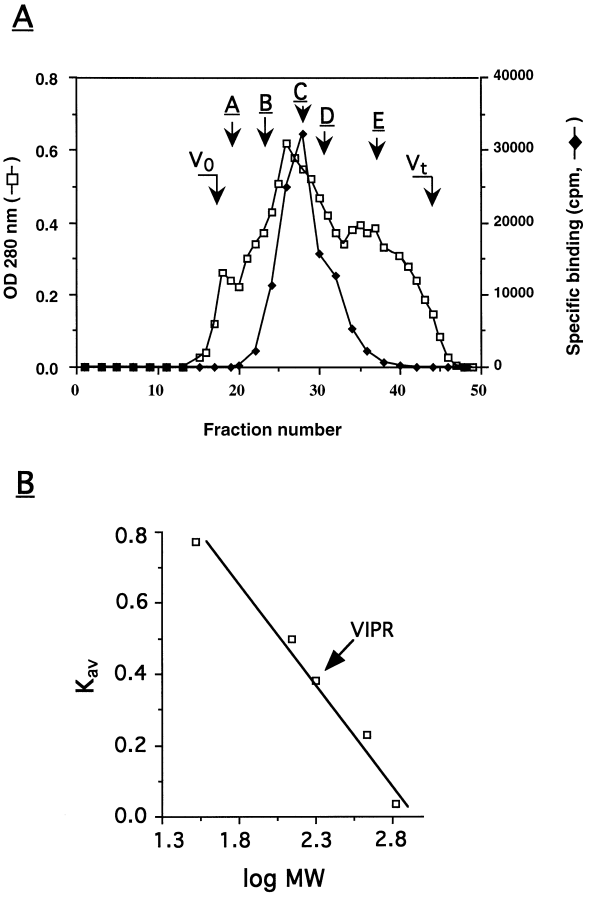

Fig. 1. Analysis of detergent-solubilized IGR 39 cells by HPLC size-exclusion chromatography. (A) A 0.5 ml aliquot of OG extract was run on a TSK Spherogel SW 4000 column, equilibrated with HEMBG/MPI containing 0.1% OG and calibrated with the following standards: thyroglobulin, 669 kDa (A); apoferritin, 443 kDa (B); β-amylase, 200 kDa (C); alcohol dehydrogenase, 150 kDa (D); carbonic anhydrase, 29 kDa (E). The void volume (V_0) is defined as the elution volume of blue dextran (2000 kDa) and the total volume (V_t) is defined as the elution volume of phenol red. (B) Estimation of soluble VIPR molecular weight. The regression curve was constructed by plotting the logarithmic values of molecular weights of various marker proteins (see above) against the distribution coefficient $K_{av}=(V_e-V_0)/(V_t-V_0)$ where V_e is the elution volume of standard proteins.

SDS-PAGE by the ^{125}I-VIP-labeled VIPR (this report) and the 52–56 kDa predicted from primary sequence data [2–4]. This discrepancy may be explained by the interaction of the receptor with lipids and detergent molecules. In fact, membrane proteins typically bind detergent and lipids up to 30–100% of their weight [18,19]. Alternatively, OG-extracted

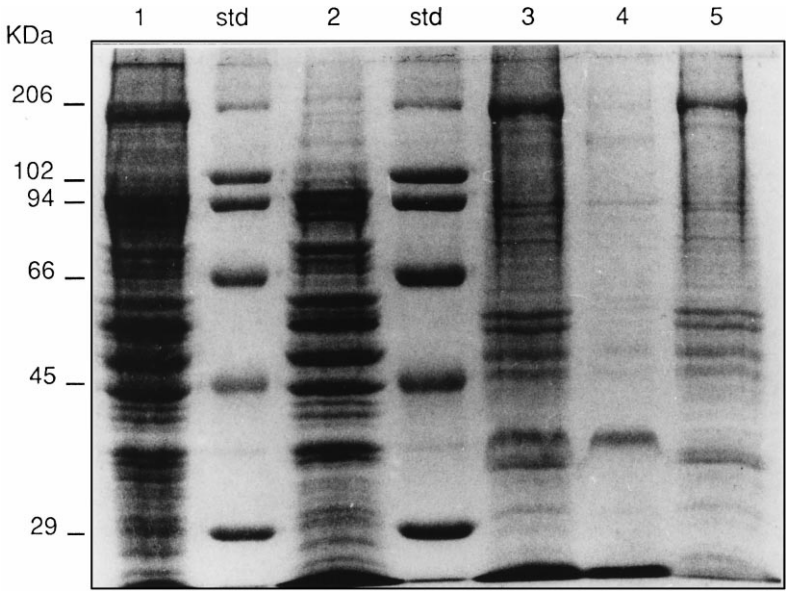

Fig. 2. SDS-PAGE analysis of proteins from various fractions. After solubilization and WGA/TAC precipitation, samples from various fractions were resolved on a 12% acrylamide gel under non-reducing conditions. Lanes: 1, OG extract; 2, WGA supernatant; 3, WGA pellet; 4, TAC supernatant; 5, TAC pellet. Reference protein size markers are indicated on the left of the figure.

VIPR may form oligomeric receptors which may be associated to other proteins such as G proteins [20].

Our previous investigations on the glycosylation of VIP receptor have demonstrated that VIPR bound avidly to the immobilized lectin WGA and could be efficiently recovered from the column in the presence 3 mM TAC [5,7]. We took advantage of this high avidity of VIPR towards WGA to precipitate the receptor from an OG extract of IGR 39 cells, using a soluble form of the lectin. After dissociating the WGA pellet by the trisaccharide TAC, another pellet was obtained (TAC pellet). As shown in Fig. 2, several major proteins present in the original OG extract (lane 1) were eliminated during the first WGA precipitation step (lane 2). However, there was no significant difference in protein pattern between WGA pellet (lane 3) and TAC pellet (lane 5). In fact, only few proteins including WGA dimer 36 kDa protein came into solution after the addition of the trisaccharide TAC (lane 4). This result shows the efficacy of TAC in dissociating WGA-glycoprotein complexes and indicates that the majority of proteins precipitated with WGA are hydrophobic glycoproteins since they remain precipitable after the removal of the lectin.

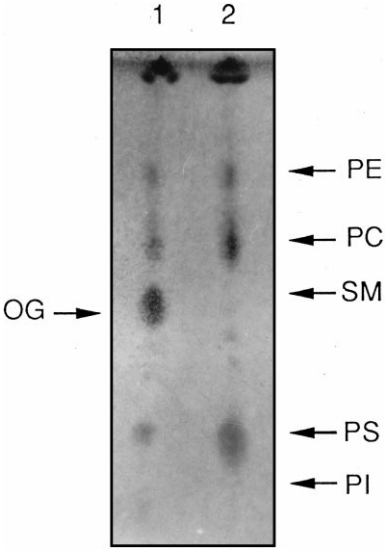

Fig. 3. Lipid analysis of OG extract and TAC pellet. Lipids (0.3 μmol) were isolated from the OG extract (lane 1) or the TAC pellet (lane 2) and chromatographed in chloroform/methanol/methylamine (68:26:6, v/v). Lipid spots were visualized using phosphomolybdic acid. Reference lipid markers are indicated on the right and the position of OG is indicated on the left. PE, PC, PS and PI, phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol, respectively; SM, sphingomyelin.

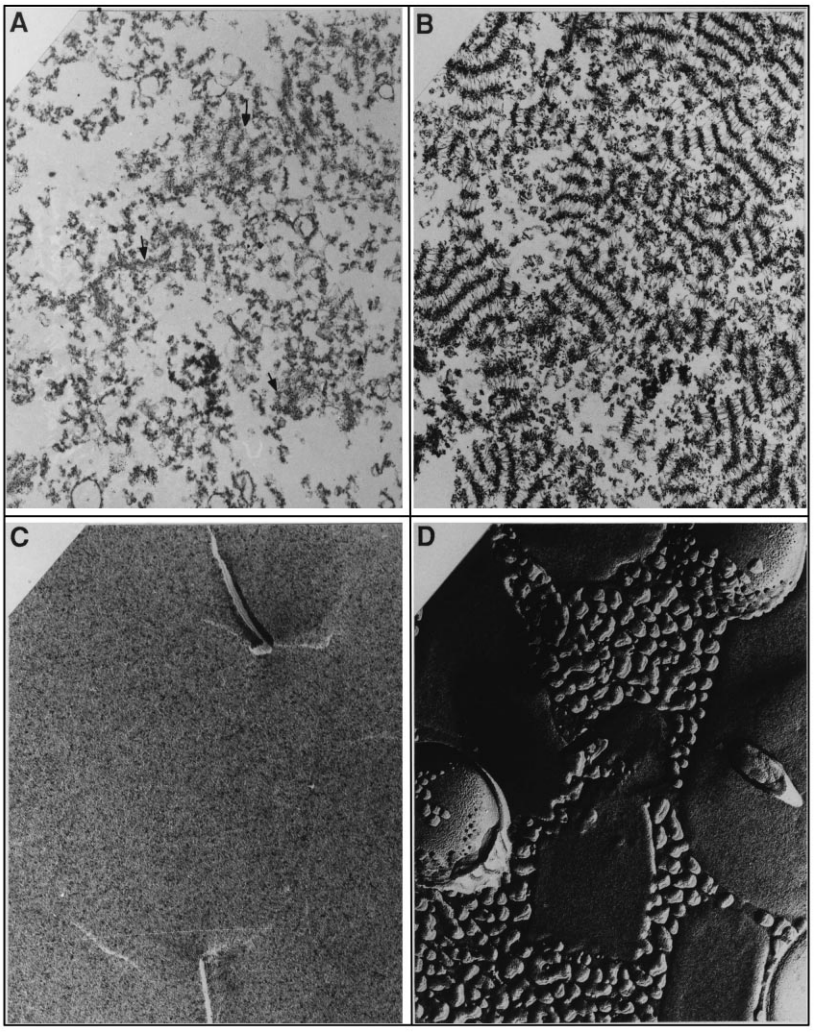

Fig. 4. Electron microscopy of the reconstituted lipid vesicles. Electron microscopic examination of sections from WGA pellet (A) and TAC pellet (B) (×100 000). (C,D) Electron microscopy of freeze-fracture replicas of material derived from WGA pellet (C) and TAC pellet (D), (×33 000).

3.2. Lipid composition of TAC pellet

In order to check whether precipitated glycoproteins were associated with lipids, we analyzed the lipid content of the TAC pellet vs. OG extract. As shown in Fig. 3, phospholipids PS, PC and PE, originally present in the OG extract, were recovered in the TAC pellet. Since lipids remained associated to glycoproteins throughout the various centrifugation steps, this result suggests that solubilization with OG only partially delipidates glycoproteins which may explain, in part, the apparent high molecular weight of VIPR observed by HPLC gel-exclusion chromatography (see above). Attempts to substitute endogenous lipids by exogenous phospholipids (PS, PC, PE, DOPC) with or without cholesterol were met with limited success and the recovery of active VIP binding sites was very poor and variable. For instance, the following combination (PE/PS/cholesterol, 56:19:25 in mol%) which was successfully used to reconstitute the OG-solubilized nicotinic acetylcholine receptor nAChR [21] was completely inefficient in our hands, i.e., less than 10% VIP binding activity reconstituted (data not shown).

3.3. Electron microscopic examination

Due to the visible turbidity of the TAC suspension

and given that lipids were present in this fraction, we carried out electron microscopy on WGA and TAC pellets to assess whether these fractions were composed of lipid vesicles. As shown in Fig. 4A,B, a clear difference could be seen between WGA and TAC pellets. The TAC-sedimented material was composed of networks having a vineyard-like aspect suggestive of multilamellar structures, whereas WGA pellet examination showed a weak supramolecular arrangement, although some rough shapes could be seen from place-to-place (Fig. 4A, arrows). Evidence for this difference was also provided by freeze-fracture electron micrographs (Fig. 4C,D). Freeze-fracture electron microscopy showed the presence of numerous vesicles in the material resuspended from TAC pellet (Fig. 4D), while no vesicles could be detected in the WGA-precipitated material (Fig. 4C). Taken together, lipid analysis and electron microscopy suggest that lipid vesicles are formed during or shortly after the dissociation of lectin from the precipitated material.

3.4. Functionality of the reconstituted VIP receptor

In a first set of experiments, ^{125}I-VIP was covalently cross-linked to intact cells in the presence of 2 mM disuccinimidyl suberate and solubilization with OG was performed as described above. By following the same protocol of WGA/TAC precipitation, the final TAC pellet and supernatant were analyzed by SDS-PAGE and autoradiography. As shown in Fig. 5A (lane 2) a ^{125}I-VIP-receptor complex representing the typical 63 kDa VIP binding protein was predominantly recovered in the TAC pellet. We then assayed ^{125}I-VIP affinity labeling of the material recovered in the TAC pellet. Fig. 5B shows the incorporation of ^{125}I-VIP to a 63 kDa protein. This incorporation was blocked in the presence of 0.3 μM unlabeled VIP (Fig. 5B, +).

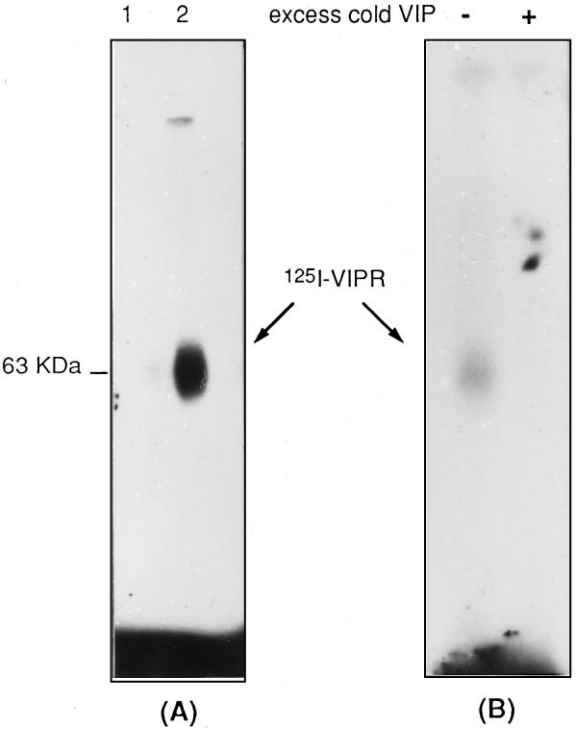

Fig. 5. SDS-PAGE of ^{125}I-VIPR. (A) After covalent cross-linking of ^{125}I-VIP to IGR 39 cells, solubilization and reconstitution of the ^{125}I-VIP-labeled material into lipid vesicles were performed as described in Fig. 1. Samples from the TAC supernatant (lane 1) and the TAC pellet (lane 2) were then resolved on a 12% polyacrylamide gel under reducing conditions. (B) ^{125}I-VIP was covalently cross-linked to the TAC pellet in the absence (−) or the presence (+) of 0.3 μM unlabeled VIP. Gels were then exposed to Fuji-XR films at −80°C for autoradiography.

The pharmacology of the reconstituted VIPR was therefore investigated by competition between ^{125}I-VIP and VIP-related peptides. As shown in Fig. 6, the reconstituted receptor from IGR 39 cells exhib-

Table 1
^{125}I-VIP binding parameters of OG-solubilized and reconstituted VIPR

Sample	High affinity binding sites		Low affinity binding sites	
	K_d (nM)	B_{max}	K_d (nM)	B_{max}
OG extract	0.67 ± 0.12	710 ± 250	136 ± 64	7 850 ± 4 560
TAC pellet	0.36 ± 0.04	5 110 ± 320	110 ± 42	136 200 ± 53 000

OG-solubilized or reconstituted VIPR was assayed for ^{125}I-VIP binding in the presence or absence of 0.3 μM unlabeled VIP. The computer-fitted program LIGAND [17] was used to analyze binding data. Values represent the mean ± S.E. of three independent experiments performed in duplicate.
B_{max} = fmol/mg protein.

ited pharmacological characteristics consistent with those observed for the original membranes, VIP- > PACAP > helodermin > hGRF = PHM > secretin [10]. Glucagon did not interfere with VIP binding at all. Binding parameters of the reconstituted VIPR are reported in Table 1. Scatchard analysis of binding data indicates the presence of two classes of binding sites: high affinity binding sites with a K_d of 0.36 ± 0.04 nM and low affinity binding sites with a K_d of 110 ± 42 nM (Table 1). With regard to high affinity sites, these K_d values were reproducibly lower than the corresponding values of 0.67 nM in the OG extract (this paper) and 0.66 nM on intact cells [10]. These data indicate a slight increase in receptor affinity upon reconstitution. Moreover, compared to the OG extract, the binding capacity B_{max} (fmol/mg protein) was increased by 7- and 17-fold in high and low affinity binding sites, respectively. Re-solubilization of the TAC pellet by 1% OG did not lead to an increase in VIP binding activity indicating that all VIP binding sites of the reconstituted VIPR were accessible to the ligand (data not shown).

In another study, we compared the precipitability of VIP binding activity and [^{35}S]methionine/cysteine-labeled proteins, with WGA and lectins having different sugar specificities such as concanavalin A (ConA, specific for mannose), *Ulex europeus* agglutinin (UEA, specific for fucose) and *Datura stramonium* agglutinin (DSA, specific for GlcNAc oligomers and *N*-acetyllactosamine). As shown in Table 2, WGA and DSA, which respectively precipitated 12% and 10% of total ^{35}S-labeled proteins, were the most efficient in precipitating VIP binding activity (96% and 95%, respectively). However, while ConA could precipitate up to 32% of total ^{35}S-labeled proteins, it precipitated only 10% of VIP binding activity. These data suggest that lectin precipitation of VIPR is sugar-specific and is likely due to a direct interaction between the lectin and VIPR.

Table 2
Differential lectin precipitation of ^{35}S-labeled proteins and VIP binding activity

Lectins	Precipitated ^{35}S-labeled proteins (%)[a]	Precipitated VIP binding activity (%)[b]
WGA	12	96 ± 2.5
ConA	32	10 ± 4.0
UEA	8.5	22 ± 5.2
DSA	10	95 ± 3.5

Cells were metabolically labeled with [^{35}S]methionine/cysteine mixture overnight, solubilized with 1% OG and precipitated with the indicated lectins. Data are the mean of one representative experiment performed in triplicate. VIP binding data are the mean ± S.D. of three independent experiments performed in duplicate.
[a]% of TCA-precipitable radioactivity in OG extract.
[b]% of VIP binding activity in OG extract.

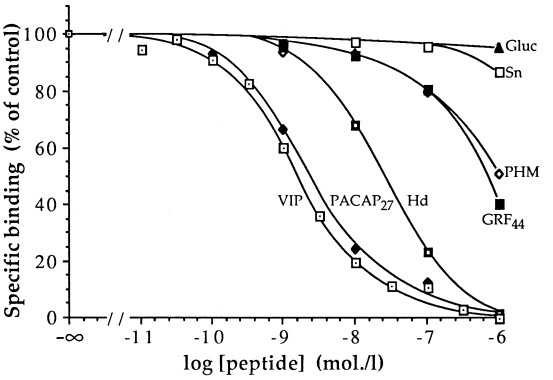

Fig. 6. Competition for ^{125}I-VIP binding by VIP and VIP-related peptides. Lipid vesicles reconstituted from IGR 39 cells were incubated with ^{125}I-VIP in the presence of increasing concentrations of VIP, $PACAP_{27}$, helodermin (Hd), hGRF (GRF), PHM, secretin (Sn) or glucagon (Gluc). Results are expressed as the percentage of specifically bound radioactivity. Data are the mean of three independent experiments performed in triplicate. S.E.M. values are $< 10\%$.

3.5. Association of VIPR with lipid vesicles

In order to ensure that VIPR was truly associated with the liposomes we used two different approaches, involving on one hand a discontinuous metrizamide gradient centrifugation, and phospholipase A_2 treatment on the other hand. In a first set of experiments, vesicles containing ^{125}I-VIP-labeled proteins were prepared in the presence of [^{14}C]DOPC as a lipid tracer and subjected to a discontinuous metrizamide gradient (Fig. 7A) as described under the experimental section. Fig. 7B shows the distribution of [^{14}C]DOPC and ^{125}I-VIP radioactivity. About 72% of [^{14}C]DOPC and 78% of ^{125}I-VIP radioactivity floated at the same position between 20% and 40% metrizamide (peak 3). Two small peaks of lower densities were also obtained (peaks 1 and 2); only peak 2 contained ^{125}I-VIP-labeled proteins. Note that the ^{125}I-VIP labeling is dramatically reduced when

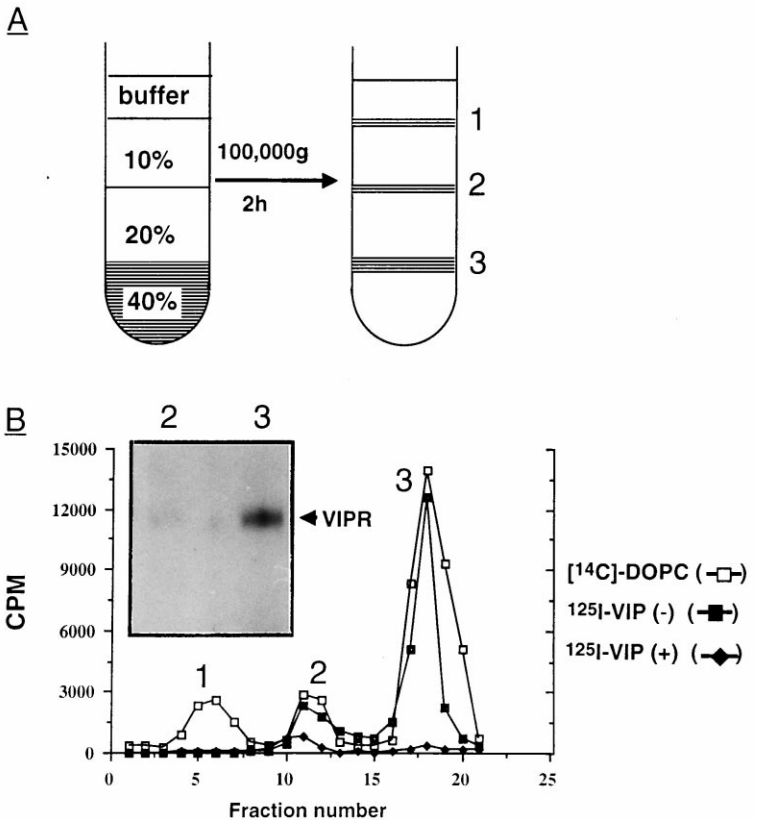

Fig. 7. Separation of vesicles in a metrizamide density gradient. Vesicles prepared with ^{125}I-VIP-labeled proteins and [^{14}C]DOPC were centrifuged in a discontinuous metrizamide gradient. After centrifugation, fractions of 500 μl were collected from the top to the bottom of the tube and radioactivity was determined. (A) Schematic view of the distribution of vesicle populations. (B) Distribution profile for phospholipids ([^{14}C]DOPC) and VIPR (^{125}I-VIP) in the presence (+) or the absence (−) of 0.3 μM unlabeled VIP. Inset: autoradiogram of SDS-PAGE of ^{125}I-VIP affinity-labeled receptor from peak 2 (lane 2) and peak 3 (lane 3). Experimental details are described in Section 2.

cross-linking was carried out in the presence of 0.3 μM unlabeled VIP (Fig. 7B, +), indicating that ^{125}I-VIP-labeled proteins represent VIP receptors. Since ^{125}I-VIP labeling was not displaced in fractions 2 and 3 with the same efficiency, we sought to determine whether VIPR partition between peaks 2 and 3 could be explained by structural heterogeneity of the receptor. To this end, samples from peaks 2 and 3 were analyzed by SDS-PAGE and autoradiography. Fig. 7B (inset) shows that the observed ^{125}I-VIP-labeling pattern of the gradient corresponds to ^{125}I-VIP-labeled VIPR and that no difference could be seen between receptors from bands 2 and 3, with respect to their apparent molecular weight. It seems likely that the WGA/TAC reconstitution technique led to the formation of different size vesicles which may explain the partition of VIPR between a heavy (peak 3) and a light (peak 2) fraction. Further experiments are needed to clarify this point.

Yet, while this result demonstrates the association of VIPR with lipid vesicles, it was of importance to ascertain whether the receptors were merely adsorbed at the surface or had become incorporated as integral proteins. To this end, the TAC fraction was divided in aliquots which were mixed with metrizamide (40% final concentration) at pH 11 or containing 1 M NaCl, conditions designed to remove peripheral (i.e., not integrally inserted) membrane proteins [22]. As a control, an aliquot was mixed with metrizamide containing 1% OG and treated similarly as the other fractions. Distribution of radioactivity was then reexamined by discontinuous metrizamide gradient. As shown in Fig. 8, these conditions have no effect on the profile of the gradient which remained

roughly identical to the profile of untreated TAC fraction (Fig. 8A,B). In contrast, when the vesicles were treated with 1% OG, both [^{14}C]DOPC and ^{125}I-VIP-labeled material was concentrated in the bottom of the tube but a large proportion of radioactivity was randomly distributed throughout the gradient (Fig. 8C). The failure of the lipids to float in the presence of detergent was most likely the result of formation of mixed micelles. Because the association of the ^{125}I-VIP radioactivity with the vesicles was sensitive to detergent and resistant to alkaline or high salt treatments, we conclude that VIP receptors were integrally inserted in the lipid bilayers.

To further ensure that VIPR is present in the TAC pellet as a lipid-associated protein rather than as an insoluble aggregate which retains activity, we studied the effect of phospholipase A_2 on VIP binding. Lipid dependence of VIPR function has been reported earlier by our group [8]. In fact, we found that perturbation of membrane fluidity of HT 29 cells by exogenous short acyl chain phospholipids abolished VIP binding activity [8]. In the present study we demonstrated that ^{125}I-VIP binding to the reconstituted membranes was diminished by 20% after preincubation with 1 IU/ml of phospholipase A_2, and by 40% at an enzyme concentration of 10 IU/ml (Fig. 9). Since phospholipase A_2 has been reported to hydrolyze organized lamellar structures of phospholipids [23], this result strongly suggests that VIPR function is dependent on the organization of surrounding phospholipids. Furthermore, given that VIPR has seven transmembrane (lipophilic) domains, it may cross several times the lipid bilayer of the reconstituted vesicles to be correctly folded and functional.

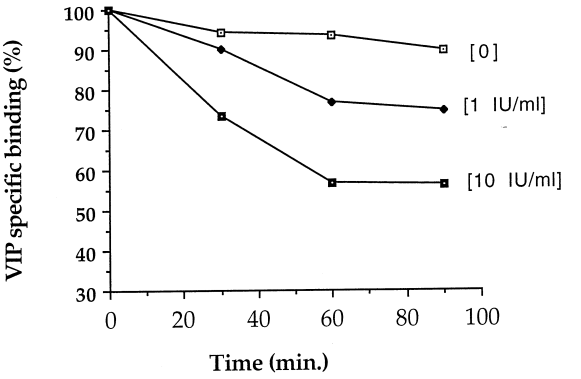

Fig. 9. ^{125}I-VIP binding to phospholipase A_2-treated membranes. Solubilization and WGA/TAC precipitations were performed as described in Section 2. Phospholipase A_2 was added to aliquots of TAC pellet at the indicated final concentrations and samples were incubated for different periods at 25°C. 100 μg/ml aliquots were then assayed for ^{125}I-VIP binding assay as described above. Results are expressed as the percentage of specifically bound radioactivity. Data are the mean of two independent experiments performed in duplicate.

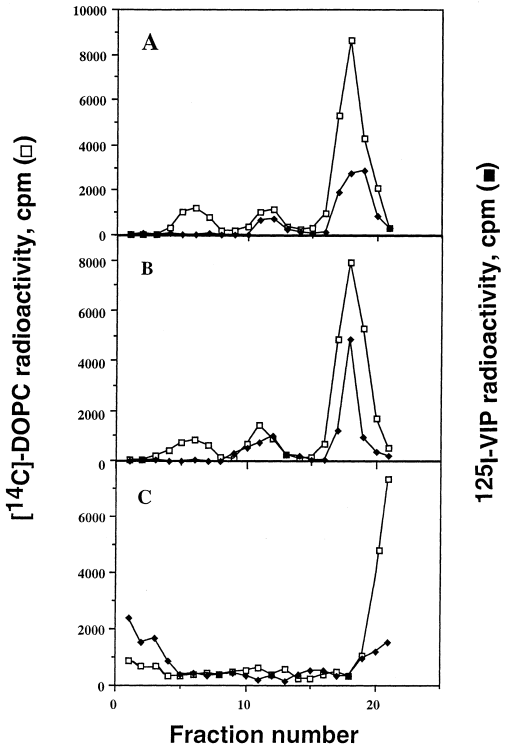

Fig. 8. Insertion of VIPR into liposomal bilayer. Vesicles were prepared with ^{125}I-VIP-labeled proteins and [^{14}C]DOPC, subjected to various dissociation conditions during density gradient centrifugation performed as in Fig. 7. (A) Vesicles treated with 1 M NaCl in 20 mM Hepes/NaOH buffer pH 7.5. (B) Vesicles treated with 20 mM Hepes/NaOH pH 11. (C) Vesicles treated with 1% OG in 20 mM Hepes/NaOH buffer pH 7.5. All buffers contained protease inhibitors (MPI).

3.6. PNGase F-deglycosylation of the reconstituted VIPR

PNGase F is an endoglycosidase cleaving most common mammalian *N*-linked oligosaccharides at the *N*-glycosidic bond [24]. It has been reported that human VIPR carries four potential *N*-glycosylation sites, three of which are located in the N-terminal extracellular domain [4] and we have previously demonstrated that the human VIPR is heavily *N*-glycosylated [5,7,25]. Our previous data have demon-

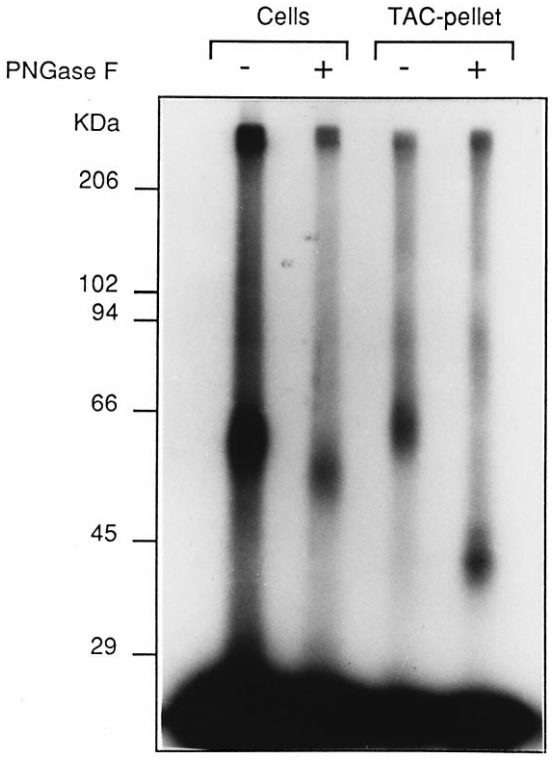

Fig. 10. SDS-PAGE of PNGase F-treated VIPR. After cross-linking with ^{125}I-VIP, IGR 39 cells were either directly treated with 100 U/ml of PNGase F for 3 h at 37°C, or solubilized with OG and reconstituted by the WGA/TAC technique. The reconstituted ^{125}I-VIP-labeled vesicles were then treated with PNGase F under the same conditions. Control samples did not receive the enzyme. Reference protein size markers are indicated on the left of the figure.

strated that under chaotropic conditions, i.e., in the presence of SDS and β-mercaptoethanol, the 63 kDa VIP binding protein could be converted by PNGase F into a 43 kDa species presumed to be the fully deglycosylated receptor. Under native conditions, however, only a partial deglycosylation of VIPR could be observed either with intact cells or membrane preparations, suggesting an inaccessibility of some *N*-glycosidic bonds to the enzyme [7].

In order to get further insight into the molecular characteristics of the reconstituted VIPR, we investigated their sensitivity to PNGase F de-*N*-glycosylation. To this end, ^{125}I-VIP was cross-linked to intact cells and PNGase F-deglycosylation was performed under native conditions either directly on ^{125}I-VIP-labeled cells or on ^{125}I-VIP-labeled lipid vesicles reconstituted from those cells. Fig. 10 shows that the molecular mass of the cellular VIPR was shifted to 53 kDa whereas, under the same conditions, the reconstituted receptor was converted into the fully deglycosylated 43 kDa protein. Interestingly, all the reconstituted VIPR were converted by PNGase F, since no trace of labeled material remained at the position 63 kDa. These data may indicate that native and reconstituted VIPR may be in different lipid environments or differently embedded in the lipid bilayer and hence, differently accessible to the enzyme PNGase F.

4. Discussion

Our previous investigations on the glycosylation of VIP receptor have demonstrated that VIPR bound avidly to the immobilized lectin WGA [5,7]. We are now showing that the OG-solubilized VIP receptor can be precipitated by free WGA and functionally reconstituted by displacing the lectin with the trisaccharide TAC. To our knowledge this is the first time that a lectin precipitation technique is used to reconstitute membrane vesicles. Attempts to reconstitute VIPR using common techniques and different mixtures of exogenous lipids have met with little or no success. The successful reconstitution of active VIP receptors described in this report was probably dependent on the combination of OG as detergent and WGA as a tool to aggregate membrane glycoproteins and their associated lipids. In preliminary experiments the WGA/TAC reconstitution method was tested with other detergents such as Tween 20, Triton X-100 and CHAPS. All these detergents could solubilize active VIPR to a certain extent but OG was, by far, the most effective with respect to solubilization, lectin aggregation, lipid vesicle formation and VIPR functionality, suggesting that the other detergents either caused a conformational change in the VIPR or were more delipidating agents (data not shown). The procedure described herein allowed us to recover more than 96% of VIPR with a slight increase in VIP binding parameters (see K_d values and B_{max}), as well as a typical rank order of potency for VIP-related peptides.

Since VIPR contains seven putative transmembrane domains, one could reasonably assume that receptors become automatically incorporated as integral proteins upon vesicle formation. Nevertheless, it

was of importance to rule out the possibility that VIPR were merely adsorbed at the surface of the liposomes. We demonstrated here that VIPR associated with the vesicles could only be removed by resolubilization in OG and not by high salt or alkaline pH. Furthermore, two lines of evidence argue that the reconstituted VIPR are oriented right side out: (i) VIP binding activity was not increased by OG resolubilization of the TAC pellet and (ii) PNGase-F treatment of vesicles completely de-*N*-glycosylated the receptor.

Although aggregation of hydrophobic glycoproteins with WGA could be easily understood, the process of lipid vesicle formation subsequent to the removal of the lectin is not immediately apparent. One likely explanation is that WGA-glycoprotein-lipid complexes may form a rigid network where lipid-lipid interaction may not be facilitated. Hence, the displacement of the lectin by TAC may 'break' cross-links, leading to free movements of complexes and increasing lipid-lipid interaction and vesicle formation. Furthermore, oligosaccharide moieties of the reconstituted glycoconjugates may contribute to the stability of the lipid vesicles and the maintenance of the three-dimensional structure of membrane proteins. Our studies were performed with a complex mixture of lipids and proteins and the data do not lend themselves to a detailed analysis of how a given glycoprotein, such as VIPR, maintains its function. Nevertheless, Moellerfeld et al. [26] have reported that the stability of lipid membranes could be improved by coating with polysaccharide derivatives bearing hydrophobic anchor groups.

VIPR has been previously purified by Couvineau et al. [27]. However, although the receptor was purified to homogeneity it was unable to bind VIP unless the receptor was re-mixed with the flowthrough fractions of the VIP affinity column, suggesting that without the other membrane constituents the VIPR presumably undergoes a conformational change to a state with an altered affinity for VIP. Besides, it has been shown that the conformation of the ligand VIP itself could be modulated by interaction with membrane phospholipids [28,29]. These data coupled with the results of the present paper suggest that VIP-VIPR interaction in target tissue may depend, in part, on membrane phospholipid characteristics. One could expect a successful functional WGA/TAC reconstitution of a purified VIPR mixed with host cell lipids. In this regard, we have found that the recombinant VIPR from CHO cells behaved as the native VIPR (from IGR 39 cells) with respect to lectin precipitability and reconstitution (ongoing experiments from our group). This first step is encouraging, especially using recombinant VIP receptors which have been engineered (i.e., carrying a polyhistidine tag) to facilitate their purification. Moreover, transfected cells expressing engineered VIPR may provide a feasible system to complete our studies on structure-function relationship of VIPR glycosylation. For instance, addition of particular sugars such as sialic acid or fucose by co-transfection of VIPR cDNA with specific glycosyltransferase cDNAs may provide an interesting system where terminal glycosylation would be controlled to allow precipitation/reconstitution with specific lectins.

Other membrane receptors such as the integrins $\alpha_v\beta_5$ and $\alpha_v\beta_6$, the β-adrenergic and the IGF-1 (insulin-like growth factor-1) receptors have been functionally reconstituted using this procedure (data not shown). The remarkable stability of the WGA/TAC reconstituted receptors (several days at 4°C and up to 24 h at room temperature) is an additional factor which may contribute to lighten experiments in cell adhesion and ligand-receptor assays.

This first successful functional reconstitution of VIP receptors is a step towards its purification and now permits further investigation of receptor structure and function.

Acknowledgements

The authors are much indebted to Mrs. Monique Roccabianca for her expert technical assistance in electronic microscopy and Mrs. Odette Nobili for lipid analyses. This work was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM) grant CRE 92205 and the Association pour la Recherche sur le Cancer (ARC) grant 6122.

References

[1] J.R. Silvius, Annu. Rev. Biophys. Chem. 21 (1992) 323–348.

[2] T. Ishihara, R. Shigemoto, K. Mori, K. Takahashi, S. Nagata, Neuron 8 (1992) 811–819.
[3] S.P. Sreedharan, D.R. Patel, E.J. Goetzl, Biochem. Biophys. Res. Commun. 193 (1993) 546–553.
[4] A. Couvineau, P. Gaudin, J.J. Maoret, C. Rouyer-Fessard, P. Nicole, M. Laburthe, Biochem. Biophys. Res. Commun. 206 (1994) 246–252.
[5] A. El Battari, P. Forget, F. Fouchier, P. Pic, Biochem. J. 278 (1991) 527–533.
[6] A. Couvineau, C. Fabre, P. Gaudin, J.J. Maoret, M. Laburthe, Biochemistry 35 (1996) 1745–1752.
[7] J. Chochola, C. Fabre, C. Bellan, J. Luis, S. Bourgerie, B. Abadie, S. Champion, J. Marvaldi, A. El Battari, J. Biol. Chem. 268 (1993) 2312–2318.
[8] A. El Battari, E. Ah-Kye, J.-M. Muller, H. Sari, J. Marvaldi, Biochimie 67 (1985) 1217–1224.
[9] J.-M. Martin, H. Darbon, J. Luis, A. El Battari, J. Marvaldi, J. Pichon, Biochem. J. 250 (1988) 679–685.
[10] J. Luis, J.-M. Martin, A. El Battari, M. Reynier, J. Marvaldi, J. Pichon, Eur. J. Biochem. 180 (1989) 429–433.
[11] A. El Battari, J.-M. Martin, J. Luis, O. Pouzole, J. Secchi, J. Marvaldi, J. Pichon, J. Biol. Chem. 263 (1988) 17685–17689.
[12] A. Kurrle, P. Rieber, E. Sackmann, Biochemistry 29 (1990) 8274–8282.
[13] U.K. Laemmli, Nature 227 (1970) 680–685.
[14] J. Folch, M. Lees, S.G.H. Stanley, J. Biol Chem. 226 (1957) 497–509.
[15] J. Lindstrom, R. Anholt, B. Einarson, A. Engel, M. Osame, M. Montal, J. Biol. Chem. 255 (1980) 8340–8350.
[16] P.P. Van Veldhoven, G.P. Mannaerts, Anal. Biochem. 161 (1987) 45–48.
[17] P.J. Munson, D. Rodbard, Anal. Biochem. 107 (1980) 220–239.
[18] R. Pabst, T. Nawroth, K. Dose, J. Chromatogr. 285 (1984) 333–338.
[19] K.A. Nalecz, R. Bolli, A. Azzi, in: A. Azzi, L. Masotti, A. Vecli (Eds.), Membrane Proteins: Isolation and Characterization, Springer, New York, 1986, pp. 11–23.
[20] J.B. Fisher, A. Schronbrunn, J. Biol. Chem. 263 (1988) 2808–2816.
[21] E.L.M. Ochoa, A.W. Dalziel, M.G. McNamee, Biochim. Biophys. Acta 727 (1983) 151–162.
[22] G. Fairbanks, T. Steck L, D.F.H. Wallach, Biochemistry 10 (1971) 2606–2617.
[23] E.A. Dennis, Phospholipases Enzymes 16 (1983) 307–353.
[24] A.L. Tarentino, C.M. Gomez, T.H. Plummer Jr., Biochemistry 24 (1985) 4665–4671.
[25] A. El Battari, J. Luis, J.-M. Martin, J. Fantini, J.-M. Muller, J. Marvaldi, J. Pichon, Biochem. J. 242 (1987) 185–191.
[26] J. Moellerfeld, W. Pras, H. Rengsdorf, H. Hamasaki, J. Sunamoto, Biochim. Biophys. Acta 857 (1986) 265–272.
[27] A. Couvineau, T. Voisin, L. Guijarro, M. Laburthe, J. Biol. Chem. 265 (1990) 13386–13390.
[28] R.M. Robinson, R.W. Blakeney Jr., W.L. Mattice, Biopolymers 21 (1982) 1217–1228.
[29] Y. Noda, J. Rodriguez-Sierra, J. Liu, D. Landers, A. Mori, S. Paul, Biochim. Biophys. Acta 1191 (1994) 324–330.