A phosphatidylinositol-linkage-deficient T-cell mutant contains insulin-sensitive glycosyl-phosphatidylinositol

Matías A. AVILA,* Rosa CLEMENTE* and Isabel VARELA-NIETO††
*Instituto de Investigaciones Biomédicas, Consejo Superior de Investigaciones Científicas, and †Departmento de Bioquímica, Universidad Autónoma de Madrid, Arturo Duperier 4, 28029 Madrid, Spain

INTRODUCTION

Glycosyl-phosphatidylinositol molecules, acting as both signal transduction elements and membrane protein anchors, have been proposed to play a role during T-cell activation. The MVB2 cell line is a mutant, derived from the wild-type T-T hybrid YH.16.33, which has a defect in the biosynthesis of PtdIns-protein linkages. As a consequence, MVB2 mutants are defective in activation through the T-cell receptor. Despite the lack of glycosyl-PtdIns anchors in the mutant MVB2 cells, a comparison of the levels and structural features of the insulin-sensitive glycosyl-PtdIns between the MVB2 and YH.16.33 lineages indicates that both cell lines are identical in this respect. The time course for insulin-responsiveness coincides in both cell lines, with maximal hydrolysis 30 s after insulin addition. The ultimate localization of insulin-regulated glycosyl-PtdIn at the outer surface of the cell membrane is also similar. These data indicate that the glycosyl-PtdIns whose hydrolysis is regulated by insulin is not anchoring proteins at the cell surface of T-lymphocytes.

MATERIALS AND METHODS

Radioactive substrates

[6-3H]Glucosamine (32 Ci/mmol), myo-[2-3H]inositol (19 Ci/mmol), [1-3H]ethanol-1-ol-2-amine (19 Ci/mmol), (methyl-3H)-choline chloride (78.2 Ci/mmol) and [3-3H]serine (30 Ci/mmol) were from Amersham. [1-3H(n)]galactose (21.7 Ci/mmol), [9,10-3H]myristic acid (22.4 Ci/mmol), [9,10-3H]palmitic acid (28.5 Ci/mmol) and [5,6,8,9,11,12,14,15-3H]arachidonic acid (238 Ci/mmol) were from New England Nuclear.

Cell culture

The mouse T-T hybridoma YH.16.33 (wild-type hybrid) and the glycosyl-PtdIns-protein-linkage deficient mutant MVB2 cell lines were a gift from Dr. K. L. Rock (Harvard Medical School, Boston, MA, U.S.A.) [6]. Cells were grown in Dulbecco’s modified Eagle's medium containing 10% fetal calf serum, 2 mM L-glutamine, 100 units of penicillin/ml and 100 μg of streptomycin/ml at 37 °C in air/CO2 (19:1). Culture medium and additives were from Flow Laboratories. Cultures were typically seeded at 1 × 105/ml and grown for 48 h to densities of approx. 1 × 107/ml. Before use, lymphocytes were harvested by centrifugation (1000 g for 6 min), washed twice and resuspended in 5 mM-sodium phosphate, pH 7.4, containing 150 mm-NaCl. The integrity of cells was measured by the Trypan Blue exclusion test, and in all experiments was at least 90%.

Cell labelling and glycosyl-PtdIns purification

Cells in exponential growth phase were labelled for 24 h in the

Abbreviations used: PIP, inositol phosphoglycan; PIPs-PLC, PtdIns-specific phospholipase C; TAP, T-cell-activating protein.
†† To whom correspondence should be sent, at: Instituto de Investigaciones Biomédicas CSIC-UAM, c/Arturo Duperier 4, Madrid 28029, Spain.
Fig. 1. Labelling of cells with \([^{3}H]\)glucosamine

YH.16.33 (a, b) and MVB2 (c, d) cells were labelled for 24 h with \([^{3}H]\)glucosamine, and the \([^{3}H]\)glycosyl-PtdIns was purified by three sequential t.l.c. steps as described in the Materials and methods section. (a) and (c), first t.l.c. step, developed in an acidic solvent system; (b) and (d), second t.l.c. step, developed in a basic solvent system. After each chromatography, 1 cm sample fractions were scraped off and the radioactivity associated with each sample was determined by scintillation counting. The positions of marker phospholipid are indicated: Gly-PtdIns, glycospoly-Ins; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; NL, neutral lipids, PtdOH, phosphatidic acid; SF, solvent front.

The presence of the various labels at 5 µCi/ml except for \([^{3}H]\)serine (2 µCi/ml), and myo-\([^{3}H]\)inositol (10 µCi/ml). At the end of the incubation period cells were collected, resuspended in 2 ml of phosphate/saline buffer and 2 ml of ice-cold 10%, trichloroacetic acid was added to each sample. After standing for 15 min at 4 °C, cellular lipids were extracted and glycospoly-Ins was purified as indicated in [9]. Briefly, the trichloroacetic acid treatment was followed by a low-speed centrifugation, the pellets were extracted with 1.5 ml of chloroform/methanol (1:2, v/v) containing 0.05 M-HCl. After standing for 15 min at room temperature, the sample was centrifuged (1500 g, 5 min) and the supernatant was transferred to a clean tube. The pellet was re-extracted with 0.75 ml of chloroform/methanol (1:2, v/v)/0.05 M-HCl, centrifuged as above and the supernatants pooled. Chloroform (0.75 ml) plus 0.1 M-KCl (0.75 ml) were added and the organic and aqueous phases were separated by centrifugation (1500 g, 5 min) at 4 °C. The aqueous phases were treated with 0.75 ml of chloroform and again centrifuged; then the organic phases were pooled. Finally, 0.75 ml of 0.1 M-KCl in 50% methanol was added to the organic phases; after standing at -20 °C for 30 min, the organic phases were collected and evaporated to dryness in a Speed-vac concentrator. The dried samples were dissolved into 50 µl of chloroform/methanol (2:1, v/v) and spotted on to a silica Gel G60 TLC (Merck) plate. The plate was developed twice with chloroform/acetone/methanol/glacial acetic acid/water (10:4:2:2:1, by vol.), and the material which remained at the origin of the plate (0.5 cm below to 2 cm above the origin) was scraped off and extracted three times with 1 ml of methanol at 37 °C. The acidic plate extract was evaporated as above and loaded on to a second silica Gel G plate, which was developed once in chloroform/methanol/NH₄OH/water (45:45:4:10, by vol.). Regions of 1 cm were scraped and assayed for radioactivity in a Beckman β-scintillation counter. In some instances, indicated in the text, labelled glycosyl-PtdIns peaks from either the acidic or basic plates were spotted on silica Gel G plates and a two-dimensional chromatography was carried out. Plates were developed first in chloroform/methanol/water (10:10:3, by vol.) and Secondly in chloroform/methanol/NH₄OH/water (90:90:7:20, by vol.). Radioactivity associated with glycosyl-PtdIns was evaluated by fluorography after spraying the plates with En³Hance (New England Nuclear). Verification of the integrity of metabolic labels in sugars was established by acid hydrolysis of lipids in 4 M-HCl at 110 °C for 5 h, followed by t.l.c. analysis of free sugars in pyridine/ethanol/glacial acetic acid/water (5:5:1:3, by vol.).

Glycosyl-PtdIns characterization

To determine the nitrous acid sensitivity of the glycolipids, samples of purified \([^{3}H]\)glycophospholipid obtained from each cell line by metabolic labelling with \([^{3}H]\)glucosamine for 24 h were treated with nitrous acid as described elsewhere [14,15]. Sensitivity to PtdIns-PLC was assessed using a commercial...
Distinct glycosyl-phosphatidylinositol s in T-cells

PtdIns-PLC purified from *Bacillus cereus* (Boehringer–Mann-hein) or PtdIns-PLC from *Bacillus thuringiensis* (strain 11607). PtdIns-PLC from *B. thuringiensis* was generously provided by donation of Dr. S. Udenfriend (Hoffman–LaRoche, Nutley, NJ, U.S.A.). A sample of purified labelled glycolipid was resuspended in 0.2 ml of 20 mM-sodium borate, pH 7.4, containing 0.16% (w/v) sodium deoxycholate. Glycol-PtdIns was dissolved in detergent-containing buffer by ultrasonication (3 x 1 min) and treated with 1 unit of either PtdIns-PLC for 2 h at 37 °C [9,14,15]. Reactions were terminated by the addition of chloroform/methanol (1:2, v/v)/0.05 M-HCl, and the amount of [3H]glycosyl-PtdIns remaining was determined.

**Glycosyl-PtdIns localization**

MVB2 and YH.16.33 cells were labelled as above with 10 μCi of [3H]galactose/ml and then treated with β-galactosidase from *Escherichia coli* (Sigma, St. Louis, MO, U.S.A.) (25 units/ml) for 90 min at 37 °C, as in [14]. Similar treatment was carried out with 6 units of PtdIns-PLC/ml from *B. thuringiensis* in cells labelled with [3H]glucosamine. One unit of PtdIns-PLC activity is defined as the amount of enzyme that hydrolyses 1 μmol of PtdIns in 1 min at 37 °C. After these treatments, glycosyl-PtdIns was purified as described above.

**Evaluation of insulin binding**

Insulin binding by MVB2 and YH.16.33 cells (1 x 10⁶ cells per assay) was determined at 15 °C for 1 h using [125I]-insulin as described in [11]; the insulin concentration in the assay was 82 μM. Mono-[125I]-insulin with a specific radioactivity of 240–300 μCi/μg was prepared as in [16].

**Biocatalytic labelling of glycosyl-PtdIns-anchored proteins**

Metabolic labelling of T-T hybridomas with [3H]glucosamine or [3H]palmitic acid was performed as previously described [6]. [3H]-labelled proteins were analysed by SDS/PAGE (18 % gels) under reducing conditions. Gels were then fixed, soaked for 30 min in Amplify (Amersham) and dried, and the proteins were visualized by fluorography. All electrophoretic reagents were from Bio-Rad.

**RESULTS AND DISCUSSION**

**Incorporation of [3H]glucosamine into YH.16.33 and MVB2 cell lipids**

Both cell lines were labelled to equilibrium with [3H]-glucosamine for 24 h. Glucosamine has been shown to be incorporated into all glycosyl-PtdIns molecules described so far. After labelling, lipids were extracted and analysed by t.l.c. as already described. Labelling of YH.16.33 and MVB2 cells is shown in Figs. 1(a) and 1(c) respectively. In both cases a major peak, peak I, was detected at the origin of the acid solvent plate which co-migrated with [3H]glycosyl-PtdIns purified from rat liver.

Peak I was eluted from the silica and spotted on to a second t.l.c. plate, which was developed in a basic solvent system as shown in Figs. 1(b) (wild-type) and 1(d) (mutant). Under these conditions peak I was resolved into two distinct peaks, II and III. Peak II co-migrated with the phosphatidylinositol 4-phosphate standard, and its sensitivity to nitrous acid cleavage was low (9.65% and 13.7% cleavage in YH.16.33 and MVB2 cells respectively). The migration of peak III relative to peak II was maintained, and this peak migrated between the phosphatidic acid and phosphatidylcholine standards and its separation from phosphatidylinositol 4,5-biphosphate and phosphatidylinositol 4-phosphate, which are the major known contaminants after chromatography in the first solvent system, was satisfactory. The amount of [3H]-labelled peak III recovered was identical when cells were labelled for 24, 36 or 48 h (results not shown). Peak III co-migrated with authentic insulin-sensitive [3H]glycosyl-PtdIns standard, which was prepared as described in [11] from isolated rat hepatocytes. The chromatographic patterns obtained with the two cell lines were identical. The average amount of radioactivity incorporated into peak II was 546 ± 84 d.p.m./10⁶ cells in MVB2 cells and 611 ± 82 d.p.m./10⁶ cells in YH.16.33 cells.

Further analysis of glucosamine-labelled peak III from either cell line was achieved by its elution from the silica and application to a third thin layer plate for two-dimensional chromatography, followed by fluorographic detection of the labelled glycosyl-PtdIns. As shown in Figs. 2(a) and 2(b), a single spot was apparent, indicating that under these conditions [3H]glucosamine-labelled peak III is homogenous and comparable in YH.16.33 (Fig. 2a) and MVB2 (Fig. 2b) cells.

**Chemical characterization of glycosyl-PtdIns**

Comparison of the structures of glycosyl-PtdIns linkages so
Table 1. Metabolic labelling of peak III glycosyl-PtdIns

<table>
<thead>
<tr>
<th>Labelled precursor</th>
<th>Relative incorporation (%)</th>
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<tbody>
<tr>
<td></td>
<td>YH.16.33</td>
</tr>
<tr>
<td>Myristic acid</td>
<td>100</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>21</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>5</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>100</td>
</tr>
<tr>
<td>Galactose</td>
<td>197</td>
</tr>
<tr>
<td>Mannose</td>
<td>5</td>
</tr>
<tr>
<td>myo-Inositol</td>
<td>7</td>
</tr>
<tr>
<td>Serine</td>
<td>5</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>5</td>
</tr>
<tr>
<td>Choline</td>
<td>5</td>
</tr>
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far elucidated indicates a strict conservation of a phosphatidylinositol-glucosamine-(mannose)3-phosphoethanolamine core among them [17,18]. Rat brain Thy-1 anchor has an extra phosphohexosamine and several additional mannoses residues [19]. Different chemical compositions of insulin-sensitive glycosyl-PtdIns and IPG have been reported [20–22]. In H35 cells and rat liver membranes a compound has been described which contains phospho-chiro-inositol linked glycosidically to one glucosaminy1 and several additional galactosyl residues [21].

Analysis of the chemical composition of glucosamine-containing peak III was performed by metabolic labelling. YH.16.33 and MVB2 cells (1 × 10^6) were labelled by a 24 h incubation with the various precursors, and at the end of this period lipids were extracted and peak III was purified using the acid/base t.l.c. system described in Fig. 1. A comparison between the quantities of [3H]-labelled peak III recovered after labelling of the cells for 24 or 48 h indicated that the labelling with the various precursors was at equilibrium after 24 h. In all experiments, control cells were labelled with [3H]glucosamine and the [3H]glucosamine-labelled peak III was purified in parallel with the samples containing the other labels. As shown in Table 1, selectivity of fatty acid incorporation was observed. The highest incorporation was obtained with myristic acid (C_{14:0}), followed by palmitic acid (C_{16:0}). Little label was recovered when arachidonic acid (C_{20:4}) was used. There were no significant differences in the degree of incorporation between the mutant MVB2 and the wild-type YH.16.33 cells. The heterogeneous fatty acid composition of the glycosyl-PtdIns purified from T-T hybrids agrees with that reported for T-lymphocytes [11]. However, this is in contrast with results from other cell lines and sources [9,20,21].

As also shown in Table 1, YH.16.33 and MVB2 cells were also labelled with myo-inositol, serine, ethanolamine, choline, galactose and mannose. Ethanolamine, inositol and mannose are known to be components of the glycosyl-PtdIns anchor [18,19]. No significant incorporation of any of the above labels was detected in purified peak III, with the exception of galactose. Verification of the integrity of metabolic labels in sugars was achieved by acid hydrolysis of lipids and t.l.c. analysis. In each instance, more than 95% of the label was recovered in the original source. As observed when fatty acid incorporation was studied, no significant differences were obtained between YH.16.33 and MVB2 cells.

Under the same conditions, metabolic labelling of glycosyl-PtdIns protein molecules with [3H]glucosamine or [3H]palmitic acid was carried out. [3H]-labelled glycosyl-PtdIns–proteins were analysed on 18% SDS/PAGE gels and then visualized by fluorography. In agreement with previous data [6], YH.16.33 but not MVB2 cells were able to incorporate both radioactive precursors into proteins with the same molecular masses as TAP and Thy-1 (results not shown).

The synthesis of the major structural phospholipids was also studied in the two cell lines. A good incorporation of choline into phosphatidylcholine, ethanolamine into phosphatidylethanolamine and serine into phosphatidylserine was observed under the conditions of labelling, indicating that the precursors were entering the cells. The ratio of choline incorporation into PtdCho/glucosamine incorporation into glycosyl-PtdIns was 118:9 in YH.16.33 cells and 117:7 in the mutant MVB2 cell line. Similar ratios between the two cell lines were found when serine or ethanolamine incorporation into their respective phospholipids was compared with glucosamine or palmitic acid incorporation into glycosyl-PtdIns, indicating that MVB2 cells do not show significant alterations in phospholipid metabolism.

To further characterize peak III, its sensitivity to nitric acid deamination and to PtdIns-PLC was tested in cells pre-labelled with [3H]glucosamine for 24 h as described in [8]. Phospholipase treatment was carried out using either B. cereus or B. thuringiensis PtdIns-PLC, for 2 h at 37°C. The amount of [3H]glycosyl-PtdIns recovered over the total period was about 24% and 65% respectively. These data suggest that the glycosyl-PtdIns recovered in peak III might be heterogeneous, despite the fact that it migrates as a single peak in one- and two-dimensional t.l.c. No differences were observed in glycosyl-PtdIns sensitivity to phospholipase between the wild-type and the mutant cells. These results indicate that peak III glycolipid contains PtdIns, despite the failure to find myo-inositol labelling under conditions where other precursors were incorporated. This discrepancy may be explained by the presence of different inositol isomers in peak III, as has been described in H35 hepatoma cells and rat liver membranes [21,22]. Nitrous acid treatment indicated that peak III purified from either cell line was cleaved by about 20%. The glycosyl-PtdIns sensitivity to acid deamination previously reported varied greatly among the sources used [9,11,12].

In conclusion, glycosyl-PtdIns purified from MVB2 cells has all the same characteristics as the lipid purified from YH.16.33 that we have been able to assay for.

Localization of glycosyl-PtdIns at the plasma membrane

TAP and Thy-1 molecules are absent from the cell surface of MVB2 mutants, but there is a normal expression of other T-cell surface proteins [6]. MVB2 cells synthesize low-molecular-mass forms of TAP and Thy-1 proteins which lack the lipid moiety present on the membrane forms of the corresponding wild-type proteins. Insulin-sensitive glycosyl-PtdIns has been localized at the outer surface of human lymphocytes by a combination of chemical labelling and enzymic modifications with β-galactosidase and PtdIns-PLC [14].

A similar approach was used to study whether the final localization of glycosyl-PtdIns was affected in MVB2 mutants. Metabolically labelled MVB2 and YH.16.33 cells (1 × 10^6 cells) were treated with β-galactosidase or PtdIns-PLC. The amounts
of \(^{3}\text{H}\)-labelled glycosyl-PtdIns purified from \(1 \times 10^9\) YH.16.33 and MVB2 cells after incubation with \(\beta\)-galactosidase were 73.1% \((n = 3)\) and 72% \((n = 4)\) respectively of that isolated from control cells. These percentages are lower than those found in rat hepatocytes \([14]\), indicating heterogeneity among different cell types. Also, they suggest heterogeneity within the glycosyl-PtdIns molecules exposed at the cell surface.

In cells treated with PtdIns-PLC from \(B.\) thuringiensis, the amount of \(^{3}\text{H}\)glucosamine-labelled glycosyl-PtdIns was 56% in YH.16.33 cells \((n = 3)\) and 49% in MVB2 cells \((n = 4)\) of that recovered from control cells. Therefore we conclude that both cell lines have analogous distribution of glycosyl-PtdIns molecules at the plasma membrane. Furthermore, the data obtained with PtdIns-PLC treatment indicate that both wild-type and mutant cells are able to efficiently target glycosyl-PtdIns molecules to the cell surface, despite the absence of PtdIns-anchored proteins in MVB2 cells. The different degrees of hydrolysis observed between the two enzymatic treatments further confirm the existence of uncomplexed glycosyl-PtdIns subtypes in T-T hybrids which may have distinct functional roles. Similar results have been obtained in T- and B-lymphocytes \([11,12]\).

The question addressed here is: do changes of levels or turnover regulation of the glycolipid implicated in insulin function? The data presented above clearly demonstrate that glycosyl-PtdIns levels, their structural features and ultimate localization in MVB2 and YH.16.33 cells are identical. Consequently, we studied whether insulin is able to differentially affect the turnover of peak III glycosyl-PtdIns.

**Insulin-dependent glycosyl-PtdIns hydrolysis in YH.16.33 and MVB2 cells**

Both types of cells bound similar amounts of \(^{125}\text{T}\)-insulin, i.e. 740±91 and 689±102 c.p.m./\(\times 10^6\) cells \((n = 8)\) for the wild-type and mutant respectively. Basal levels of labelled glycosyl-PtdIns were also identical, as shown in Fig. 1. The analogous incorporation of \(^{3}\text{H}\)glucosamine into glycosyl-PtdIns in both cell lines indicates that glycosyl-PtdIns turnover is comparable between the parental line and the anchorage mutant. Previous studies have indicated a correlation between the levels of glycosyl-PtdIns and those of insulin receptors in various cell types \([23,24]\), including T-lymphocytes \([11]\).

To determine the effect of insulin on glycosyl-PtdIns hydrolysis, \(1 \times 10^8\) YH.16.33 and MVB2 cells were labelled with \(^{3}\text{H}\)-glucosamine for 24 h to achieve steady-state labelling of glycosyl-PtdIns. Cells were then washed in insulin-free medium for 90 min, after which reactions were initiated by the addition of 1 nm-insulin. Incubations were carried out for the times indicated in Fig. 3, and glycosyl-PtdIns levels were determined. The addition of insulin to T-T hybrid wild-type cells led to the loss of about 54% of the glycosyl-PtdIns within 30 s. The MVB2 cells responded by hydrolysing 61% of the glycolipid, with the same fast and reversible time course (Fig. 3). Resynthesis of glycosyl-PtdIns began rapidly thereafter, and by 4–5 min levels had reached a new steady-state value. The same results were obtained in cells labelled with either \(^{3}\text{H}\)palmitate or \(^{3}\text{H}\)galactose (results not shown). These data indicate that insulin is a potent regulator of glycosyl-PtdIns turnover in both YH.16.33 and MVB2 cells.

**Biological activity of IPG**

Insulin-dependent hydrolysis of glycosyl-PtdIns by PtdIns-PLC liberates its polar head group, IPG, which displays a variety of insulin-like actions and has been proposed as a mediator of insulin action (for reviews see \([3]\) and \([25]\)). The biological activity of IPG from T-T hybrid cells was assessed in vitro by testing its capacity to inhibit the phosphorylation of histone II A by the cyclic AMP-dependent protein kinase, as described previously \([26]\). IPG was prepared by hydrolysis with bacterial PtdIns-PLC of glycosyl-PtdIns purified from either YH.16.33 or MVB2 cells \((1 \times 10^8\) cells) as in \([15]\). The concentration of IPG was calculated by measuring free amino groups, assuming that each molecule of IPG contains one amino group \([14]\). The addition of IPG \((10 \mu\text{m})\), purified from YH.16.33 or MVB2 cells, to the protein kinase A assay caused respectively 76% and 83%, inhibition of the kinase activity over 10 min. The incorporation of \(^{23}\text{P}\) into washed histone II A in control samples was about 41 000 c.p.m., and the experiment was performed twice in duplicate. These data indicate that both the PtdIns-anchor-deficient mutant and the wild-type T-T hybrid possess a fully active IPG.

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