Insulin-induced glycosylphosphatidylinositol (GPI) binding to red cell membrane proteins

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In this work GPI binding to membrane proteins from erythrocytes of insulinoma patients for whom prolonged hyperinsulinism and hypoglycemia were characteristic, as well as from normal erythrocytes incubated with supraphysiological concentrations of insulin were analyzed. In the RBCs from insulinoma patients, covalent GPI binding to red cell membrane proteins in the spectrin/ankyrin region, band 4.1 and two proteins of molecular mass of 115 and 110 kD was demonstrated. In erythrocytes incubated with insulin label was associated with band 4.1 and two proteins of molecular mass of 115 and 110 kD. Extraction studies showed that the 100-kD proteins are unrelated to band 3 since they were found in Triton-prepared cytoskeleton. To our knowledge this is the first demonstration of such a modification of red cell skeletal proteins, and the first demonstration of post-translation GPI binding to red cell skeletal proteins in response to insulin. A mechanism proposed for GPI binding to red cell skeletal proteins as well as the relevance of these results for physiological disorders that are characterized by hyperinsulinism are briefly discussed.

Keywords: insulin; GPI; erythrocytes; membranes; hyperinsulinism; hypoglycemia.

INTRODUCTION

Glycosylphosphatidylinositol (GPI) is a complex glycolipid that is covalently attached to surface proteins of many eukaryotic cells; GPI provides an alternative anchoring mechanism to a hydrophobic polypeptide transmembrane domain, enabling stable association of protein with the lipid bilayer.1,2 In addition to GPI-anchors, free GPI units are also expressed in eukaryotic cell membranes.3 The majority of these free GPVs are found at the outer surface in a variety of cell types thus far studied.4 Insulin stimulates hydrolysis of both GPI-anchors5–7 and free GPVs.4 Phosphoinositolglycans and diacylglycerol derived from free GPVs through the action of insulin-activated phospholipase C were proposed as second messengers of insulin.8

Human erythrocytes (RBC) contain highly specific insulin receptors that have structural and functional characteristics similar to those of target tissues for the action of the hor-
mone. Free GPI-lipids from erythrocyte membranes are, as opposed to other cell types, largely cytoplasmatically oriented and insensitive to insulin-induced hydrolysis. We found that exposure of RBC to supraphysiological concentrations of insulin, both in vivo and in vitro, caused covalent glycoinositolphospholipid (GPI) binding to C termini of both hemoglobin (Hb) β-chains which resulted in the formation of a novel, hitherto unrecognized minor Hb fraction (GPI-Hb) (ca. 4% of total Hb).

By applying the same approach and methods our studies were extended in this work to insulin-induced binding of GPI to red cell membrane proteins. Both, red cell membrane proteins from (six) patients with diagnosed insulinoma, for whom chronic hyperinsulinism and hypoglycemia were characteristic, and from normal erythrocytes incubated with supraphysiological concentrations of insulin were analyzed. In the RBCs from insulinoma patients, covalent GPI binding to red cell membrane proteins in the spectrin/ankyrin region, band 4.1 and two proteins of molecular mass of 115 and 110 kD was demonstrated. In erythrocytes incubated with insulin, the label was associated with band 4.1 and two proteins of molecular mass of 115 and 110 kD. To our knowledge this is the first demonstration of such a modification of red cell skeletal proteins, as well as the first demonstration of post-translational GPI binding to red cell skeletal proteins in response to insulin.

RBC membrane proteins were resolved by SDS-PAGE and GPI-modified proteins were identified by overlaying the Coomassie Blue-stained gel on immunoblots obtained by using anti CRD antibodies from polyclonal rabbit antisera raised against soluble form of a variant surface glycoproteins (sVSG). The treatment of GPls with PI-specific phospholipase C (PI-PLC) exposes a carbohydrate epitope known as the cross-reacting determinant (CRD). Anti CRD antibody reacts to a large extent with inositol 1,2-cyclic phosphate epitope (produced by PI-PLC cleavage), and (to a lesser extent) with other GPI epitopes including non-acetylated glucosamine. Thus, detection of the CRD epitope allows the unambiguous assignment of the presence of a GPI anchor on a particular protein. Indeed, immunoblotting of a PI-PLC-treated membrane form of VSG (mVSG) and untreated sVSG gave intensive reaction with anti CRD antibody (results not shown). This is expected for the quantitative binding of anti-CRD antibodies, which requires the presence of all structural elements of the GPI structure. Fig. 1 shows that for GPI-labeled RBC membrane proteins low, but diagnostically significant reaction with the antibody, which did not increase in the PI-PLC treated samples (results not shown) was obtained. This points to the esterification of inositol with palmitic acid, which was shown to make the GPI-anchor resistant towards PI-PLC.

Immunoblots of membrane proteins from control RBC samples show the presence of bands in the 70 kD region, which may be affiliated to the well-known endogenous red cell GPI proteins such as acetylcholinesterase monomer, (Mw 68 kD) and decay accelerating factor (DAF, Mw 70 kD) (Fig. 1. line C). For RBCs from insulinoma patients the of GPI binding to red cell membrane proteins in the spectrin/ankyrin region band 4.1 and two proteins of molecular mass of 115 and 110 kD was characteristic (Fig. 1. line P). GPI binding in the spectrin/ankyrin region is due to spectrin band 2 and spectrin 1 band or ankyrin...
which, in this gel system, migrates with or, sometimes, above the spectrin 1 band. In red cell membrane from erythrocytes of normal healthy volunteers incubated with insulin label binding was associated with band 4.1 and two proteins of molecular mass of 115 and 110 kD (Fig. 1, line 1).

To further characterize the GPI-labeled proteins in insulin-treated red cells, the ghosts were extracted with cold Triton X-100. Extraction with Triton X-100 at 4 ºC solubilizes band 3 and the bulk of the glycophorins whereas most skeletal proteins and GPI-anchored proteins, which, similarly to other cell types are clustered in lipid rafts, resist extraction by Triton X-100. Figure 1 (line T) shows that both endogenous GPI-anchored proteins and all insulin-induced GPI-labeled RBC membrane proteins were present in the Triton X-100 pellet. The amount of protein staining in the 100 kD area after Triton X-100 extraction is minimal, which demonstrates that the GPI-labeled 100 kD proteins are clearly unrelated to band 3. Although the 100 kD proteins are minor proteins in terms of amount, they are probably the most highly GPI-labeled species in red cell membranes following their exposure to insulin, as the label incorporated into them was about equivalent to that incorporated into the much more abundant band 4.1 (Fig. 1, lines I, P and T).

The pattern of insulin-induced GPI-labeling seen here, i.e., GPI-labeling of characteristic red cell skeletal proteins, is similar to the pattern of protein kinase C-induced phosphorylation of red cell skeletal proteins. The major substrates for kinase in these studies were...
also two ill-defined skeletal proteins of 100 kD.\textsuperscript{18,21} It would be of considerable interest to identify and determine the function of these two proteins present in the erythrocyte skeleton. It is well documented that addition of GPI anchors to proteins which occurs soon after completion of polypeptide synthesis and translocation involve transamidase-catalyzed reaction mechanism.\textsuperscript{22} GPI-transamidase represents a novel multi-subunit enzyme that removes the carboxy-terminal signal sequence and attaches a GPI molecule to the newly exposed carboxy terminal amino acid.\textsuperscript{23,24} Our recent studies show that formation of GPI-Hb is associated with insulin-induced transpeptidase-like activity in the RBC membrane (unpublished observation). The membrane skeletal proteins, which underlie the lipid bilayer and are associated to it by protein-protein and protein-lipid interactions,\textsuperscript{25,26} come in close contact with free GPI-lipids from the erythrocyte membrane which are largely cytoplasmically oriented.\textsuperscript{4} This permits the ethanolamine from GPI to come in close contact with the C-terminal part of the polypeptide chains of skeletal proteins, which were found to be GPI-modified. Insulin-activated transamidase in RBC membrane then catalyze slowly the transpeptidation, \textit{i.e.}, the replacement of the carboxy-terminal amino acid(s) residues of the polypeptide chains with GPI as an exogenous nucleophile.

The results described in the present work may have relevance to studies of physiological disorders that are characterized by hyperinsulinism.\textsuperscript{27–29} GPI-anchoring of RBC skeletal proteins to the lipid bilayer will cause increased association of the red cell skeletal network with plasma membrane, which is expected to have a profound effect on RBC deformability and stability.\textsuperscript{30} This may, at least partly, explain the decrease of both RBC membrane fluidity and deformability, which were found previously to be characteristic for hyperinsulinism.\textsuperscript{27–29} A GPI adduct of Hb\textsuperscript{31} and GPI-labeled skeletal proteins (data not shown) could be detected even 30 days after pancreatectomy. The detection and monitoring of chronic hyperinsulinism could be improved and/or facilitated by a means of identifying the existence (and consequently levels) of the GPI adducts of red cell proteins.

GPI addition to RBC proteins in response to insulin may be relevant to other cell types as well. In a previous \textit{in vitro} study of Lisanti\textsuperscript{5} it was noted that insulin-induced release of GPI-anchored proteins from cell membrane was followed by their recovery upon prolonged insulin exposure.\textsuperscript{5} The present results may be relevant to both the GPIs turnover in insulin action,\textsuperscript{8} and the role suggested insulin plays in the regulation of GPIs in cell membrane.\textsuperscript{5} Human erythrocytes, which represent an extremely useful and easily accessible cellular model for the study of a variety of proteins, seem to be well suited for studying the insulin-induced GPI-addition to proteins.
ИЗВОД

БЕЗИВАЊЕ ГЛИКОЗИФОСФАТИДИЛИНОЗИТОЛА (GPI-a) ЗА МЕМБРАНСКЕ ПРОТЕИНЕ ЕРИТРОЦИТА ПОДДЕЈСТВОМ ИНСУЛИНА

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У овом раду испитивано је везивање GPI-a за мембранске протеине еритроцита пацијената оболелих од инсулином, за које су карактеристични дуготрајни хиперинсулинзам и хипогликемија, као и у нормалним еритроцитима инкубираним са супрафизиолошким концентрацијама инсулина. Нађено је да у еритроцитима пацијената долази до ковалентног везивања GPI-a за мембранске протеине еритроцита и то у области спектрина и анкирина, за траку 4.1. и два протеина молекулских маса 115 и 110 kD. У еритроцитима инкубираним са инсулином GPI се везује за траку 4.1. и два протеина молекулских маса 115 и 110 kD. Утврђено је да протеин масе 100 kD не потичу од траке 3, јер су детектовани у цитоскелетној фракцији заосталој после екстрахације мембранских протеина раствором Triton-a. У овом раду је први пут детектована модификација цитоскелетних протеина еритроцита везивањем GPI-a, као и пост-трансляционо везивање GPI-a за цитоскелетне протеине еритроцита под утицајем инсулина. Укратко је дискутована механика везивања GPI-a за протеине цитоскелета еритроцита, као и значај добијених резултата за разумевање физиолошких поремећаја у хиперинсулинизму.

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REFERENCES


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