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Expression of Lymphocyte ADP-ribosyltransferase in Rat Mammary Adenocarcinoma Cells

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Abstract

The nascent form of glycosylphosphatidylinositol (GPI)-anchored proteins possesses both amino and carboxy terminal hydrophobic signal sequences to direct processing in the endoplasmic reticulum (ER). Following cleavage of the amino-terminal signal peptide, the carboxy-terminal peptide is processed. Previously, mouse lymphocyte NAD : arginine ADP-ribosyltransferase (Yac-1) was cloned and the deduced amino acid sequence of the Yac-1 transferase contained hydrophobic amino and carboxy termini, consistent with known signal sequences of GPI-anchored proteins. This transferase was present on the surface of NMU (rat mammary adenocarcinoma) cells transfected with the wild-type cDNA and was released with phosphatidylinositol-specific phospholipase C. Expression of the mutant protein, lacking the carboxy terminal hydrophobic sequence, resulted in the production of soluble, secreted form of the transferase. This result shows that carboxy terminal sequence is important for GPI-attachment.

Key words : Glycosylphosphatidylinositol-anchored protein, Mouse lymphocyte ADP-ribosyltransferase

Introduction

Mono-ADP-ribosylation of arginine is a reversible modification of proteins, which involves transfer of the ADP-ribose moiety of NAD to protein by specific ADP-ribosyltransferases[1]. The reverse reaction, catalyzed by ADP-ribosylarginine hydrolases, releases the ADP-ribose from proteins, regenerating an arginine. These two enzymatic reactions compose a mono-ADP-ribosylation cycle that may be involved in the regulation of cellular processes. In *Rhodospirillum rubrum*[2,3], this cycle seems to regulate dinitrogenase reductase, a key enzyme in nitrogen fixation.

ADP-ribosyltransferases have been described in viruses, bacteria, and eukaryotic cells[1,3-7]. ADP-ribosylation of cellular proteins by bacterial toxins alters the activity of proteins in critical metabolic or regulatory pathways[5-8]. Diphtheria toxin and *Pseudomonas* exotoxin A ADP-ribosylate a modified histidine residue in elongation factor 2 resulting in inhibition of protein synthesis and cell death[6,9]. Cholera toxin and the related heat-labile enterotoxin of *E. coli* ADP-ribosylate an arginine in $G_{s\alpha}$, the stimulatory guanine nucleotide-binding (G) protein of the adenylnyl cyclase system, resulting in its activation and leading to increased intracellular cAMP[5]. ADP-ribosylation of a cysteine in several members of the family of G proteins (e.g., G_i, G_t, and G_o) by pertussis toxin alters adenylyl cyclase, phospholipid turnover and ion flux, by uncoupling the G protein from its receptor[7].

ADP-ribosyltransferase activity for which arginine is the acceptor amino acid has been detected in numerous animal tissues. The enzymes have been cloned and cha-

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racterized from a few species, including rabbit[10] and human[11] skeletal muscle, chicken heterophils[12] and erythroblasts[13], and mouse lymphocytes[14,15]. The skeletal muscle transferases are glycosylphosphatidylinositol (GPI)-linked exoenzymes[10,11], which, in cultured mouse skeletal muscle (C2C12) cells, modify the adhesion molecule integrin α7[16]. ADP-ribosylation of α7 was proposed to play a role in muscle cell development[17].

The mouse lymphocyte transferase (Yac-1) cDNA contains predominantly hydrophobic amino- and carboxy-terminal amino acid sequences characteristic of GPI-anchored proteins[14]. GPI anchors are found in structurally and functionally diverse proteins, including the hydrolytic enzymes alkaline phosphatase, 5'-nucleotidase, and acetylcholinesterase, the mammalian antigens Thy-1, RT 6, and carcinoembryonic antigen, the neural cell adhesion molecule, and the trypanosomal antigen variant surface glycoprotein[18]. To confirm that mouse lymphocyte ADP-ribosyltransferase is a GPI-anchored protein and to elucidate the importance of carboxy-terminal signal peptide for GPI-anchored proteins, its wild-type and truncated cDNAs were expressed in a rat mammary adenocarcinoma (NMU) cell line.

Materials and Methods

Cell culture

NMU (rat mammary adenocarcinoma) cells were obtained from American Type Culture Collection. NMU cells were grown in Eagle's minimum essential media with Earl's balanced salt solution containing L-glutamine (BioWhittaker Inc.). Cells were grown to confluence at 37℃ under 5% CO_2.

Construction of mouse lymphocyte transferase expression vectors

Wild type mouse lymphocyte (Yac-1) and truncated transferase cDNAs were generated using polymerase chain reaction (PCR)-based techniques[19]. Wild-type cDNA was amplified from the purified lambda DNA (100 ng) in a 100 μl reaction containing dNTPs (each 0.2 mM), Taq DNA polymerase (2.5 units), and forward P1 (5'-ACGTACGTACGTGCTAGCATGAAGATT-CCTGCTATGATGTC T-3') and reverse P2 (5'-ACGTA-CGTACGTCTCGAGTCAACATCGGGTAAGTTGCTGGA G-3') primers (100 pmol each). Amplification was performed for 30 cycles of 94℃, 1 min/55℃, 1 min/72℃, 1.5 min, followed by a 7 min extension at 72℃. The truncated pM3'T clone, which lacks the carboxy-terminal 37 amino acids, was generated using forward P1 (5'-ACGTACGTACGTGCTAGCATGAAGATTCCTGC-TATGATGTC T-3') and reverse P3 (5' -ACGTACGTA-CGTCCCGCGGTCAACCCAGCCAGCAGGGCCCAGA-3') primers (100 pmol each). PCR products were purified, digested with Nhe I and Xho I, and subcloned into the pMAMneo vector.

Expression of recombinant proteins in NMU cells

NMU cells were transfected with 15 μg of pMAMneo vector or constructs using the calcium phosphate precipitation method[20] and stable transformants were selected with Geneticin (0.5 mg/ml). Following induction of protein expression with dexamethasone (1 μM) for 24 h, cells were washed with Dulbecco's phosphate-buffered saline (PBS) and incubated for 60 min in PBS (0.7 ml) with or without phosphatidylinositol-specific phospholipase C (PI-PLC, 0.5 unit). After collecting the PBS fraction, the cells were washed with PBS, trypsinized, and lysed in 0.5 ml of lysis buffer (10 mM Tris, pH 8.0/1 mM EDTA) by repeated freeze-thawing. After centrifugation (100,000×g, 1 h) of the lysate, the supernatant (Sup, 0.5 ml) was collected and the membranes (Pellet) were suspended in 0.5 ml of PBS.

Expression of ADP-ribosyltransferase in *E. coli*.

The wild-type transferase was generated as a fusion protein with glutathione S-transferase (GST-Yac-1) by PCR using forward P1 and reverse P2 primers under

PCR conditions described above. Truncated forms of transferase-GST fusion proteins were synthesized in *E coli.* GST-Yac-1-5'3'T, in which the transferase cDNA lacks the N- and C-terminal hydrophobic signal sequences (23 and 37 amino acids from 5' and 3' ends, respectively), was generated by PCR amplification of the Yac-1 transferase cDNA (100 ng) with forward P4 (5'-ACGTACGTACGTCCGCGGAGTTACTCCATCTCACAA-CTA-3') and reverse P3 (5' -ACGTACGTACGTCCGC-GGTCAACCCAGCCAGCAGGGCCCAGA-3') primers (100 pmol each) under PCR conditions described above. GST-Yac-1-5'T, which lacks 23 amino acids from the hydrophobic amino-terminus, was generated using forward P4 and reverse P2 primers. GST-Yac-1-3'T, which lacks 37 amino acids from the hydrophobic carboxy-terminus, was generated using forward P1 and reverse P3 primers. PCR products were gel-purified, subcloned into pGEX-5G/LIC, and expressed in *E. coli* (DH5α). Cells were harvested by centrifugation, suspended in 20 ml of 10 mM Tris, pH 8.0/1 mM EDTA/100 mM NaCl containing protease inhibitors (0.5 mM PMSF, and leupeptin, aprotinin, and pepstatin, each 0.5 μg/ml). After sonification for 1 min, the lysate was centrifuged (5000×g, 30 min). Solubilized GST fusion proteins, purified according to the manufacturer's protocol using glutathione-Sepharose 4B (Pharmacia Biotech Inc.), were assayed for ADP-ribosyltransferase.

ADP-ribosyltransferase assay

Reaction was carried out in 0.3 ml of 50 mM potassium phosphate (pH 7.5), with 20 mM agmatine and 0.1 mM [*adenine*-U-^{14}C]NAD (0.05 μCi). After incubation at 30℃, samples (0.1 ml) were applied to columns (0.5×4 cm) of Dowex AG 1-X2. [^{14}C]ADP-ribosylagmatine was eluted with 5 ml of H_2O for liquid scintillation counting.

Results and Discussion

The deduced amino acid sequence of the mouse lymphocyte ADP-ribosyltransferase (Yac-1) had very hydrophobic amino- and carboxy-termini, with a hydrophilic center[14]. This hydrophilicity profile resembled the profiles of glycosylphosphatidylinositol (GPI)-anchored membrane proteins, which are synthesized as nescent polypeptides, containing hydrophobic signal sequences at amino and carboxy termini[18,21]. For GPI-anchored proteins, the amino terminal sequence serves as a signal for translocation into endoplasmic reticulum and the carboxy terminal sequence is a signal for GPI-attachment. Both signal sequences are absent from the mature form of a GPI-anchored protein.

Expression of mouse lymphocyte transferase as GST fusion proteins in *E. coli.*

Based on the assumption that the native mouse lymphocyte transferase has a GPI modification and given the fact that mammalian signal sequences are not recognized in *E. coli,* wild-type and truncated GST-Yac-1 transferase fusion proteins were synthesized in *E. coli.* The full-length (wild-type) or truncated fusion proteins, partially purified by glutathione-Sepharose 4B chromatography, were assayed for transferase activity (Table 1). The enzyme activity was not detected in proteins from vector-transformed *E. coli.* The full-length transferase (GST-Yac-1) was inactive. The GST-Yac-1-5'T construct lacking the hydrophobic amino-terminal signal sequence and the GST-Yac-1-3'T lacking the hydrophobic carboxy-terminal signal sequence had negligible ADP-ribosyltransferase activity. In contrast, the GST-Yac-1-5'3'T construct, which lacks the hydrophobic amino- and carboxy-terminal signal sequences exhibited transferase activity. These results indicate that the amino and carboxy terminal hydrophobic amino acids are not required for enzyme activity.

Table 1. ADP-ribosyltransferase activity in GST-Yac-1 fusion proteins.

Transformant	Specific activity of transferase
	pmol/min/mg
Vector	ND
GST-Yac-1	0.09±0.008
GST-Yac-1-5'T	0.12±0.03
GST-Yac-1-3'T	0.13±0.05
GST-Yac-1-5'3'T	596±12

Data are means±S.E. (n=3). ND, not detectable.

Expression of mouse lymphocyte transferase in NMU cells

More convincing evidence that mouse lymphocyte transferase is a GPI-anchored protein was came from expression experiments in mammalian cells. NMU cells were transformed with the complete coding region of the mouse lymphocyte transferase cDNA using the glucocorticoid-inducible pMAMneo mammalian expression vector. The transformed NMU cells were treated with 1 μM dexamethasone for 24 h. Treatment of the wild-type transfected cells with phosphatidylinositol-specific phospholipase C (PI-PLC) caused almost complete release of the transferase into the PBS fraction (Fig. 1), consistent with extracellular localization of a GPI-anchored transferase. ADP-ribosyltransferase activity was negligible in control NMU cells and in pM (vector) transformants (data not shown).

To evaluate the importance of the carboxy terminus in membrane localization, a truncated form of the ADP-ribosyltransferase was made by removing 37 hydrophobic amino acids from the carboxy terminus of the protein. NMU cells transformed with this construct (pM3'T) had no ADP-ribosyltransferase activity in the membrane fraction (Pellet fraction) and little in the cell supernatant, but had 95.6 % (without PI-PLC) or 97.5 % (with PI-PLC) of the activity in the medium (Fig. 2), suggesting that the activity was secreted due to the NH_2-terminal signal sequence, but not retained on the

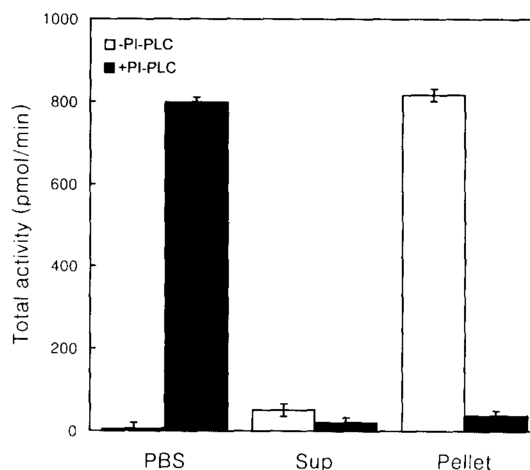

Fig. 1. Effect of PI-PLC on release of ADP-ribosyltransferase activity from NMU cells transformed with the wild-type Yac-1 transferase cDNA. NMU cells were transfected with wild-type (full-length) transferase cDNA, and ADP-ribosyltransferase activity was assayed as described in the Materials and Methods. The ADP-ribosyltransferase activity was released from transformed NMU cells into PBS fraction. Total proteins (mg) were 0.4 (-PI-PLC) and 0.38 (+PI-PLC). (mean±S.E., n=4).

membrane due to the absence of a GPI anchoring sequence. This result is consistent with the view that the C-terminal sequence is required for GPI-attachment and in its absence, the transferase is transported from the endoplasmic reticulum to the plasma membrane, but it is not membrane-anchored.

The hydrophobic aminio- and carboxy-terminal signal peptides of nascent proteins destined for GPI-anchording have rather specific amino acids at their cleavage sites [22]. Based on the similarity between mouse lymphocyte ADP-ribosyltransferase amino and carboxy terminal hydrophobic sequences of other GPI-anchored proteins, as well as amino acid requirements adjacent to the sites of cleavage of signal peptides[22], structures of the nascent and the mature forms of the mouse lymphocyte ADP-ribosyltransferase are proposed (Fig. 3).

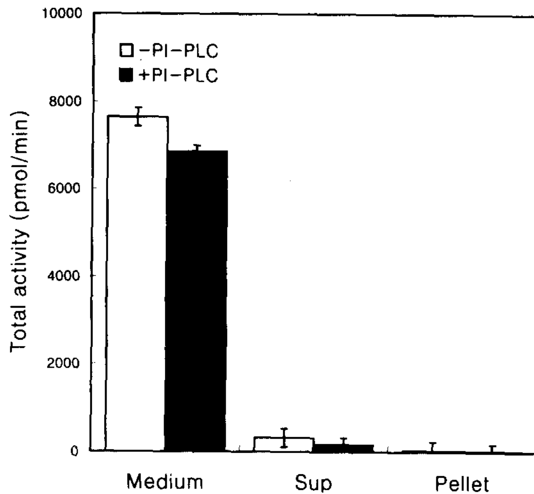

Fig. 2. ADP-ribosyltransferase activity from mutant transferase cDNA transformed NMU cells. Cells were transfected with pM3'T construct lacking C-terminal hydrophobic signal sequence and ADP-ribosyltransferase activity was assayed as described in the Materials and Methods. Total proteins (mg) were 0.45 (-PI-PLC) and 0.4 (+PI-PLC). (mean±S.E., n=3).

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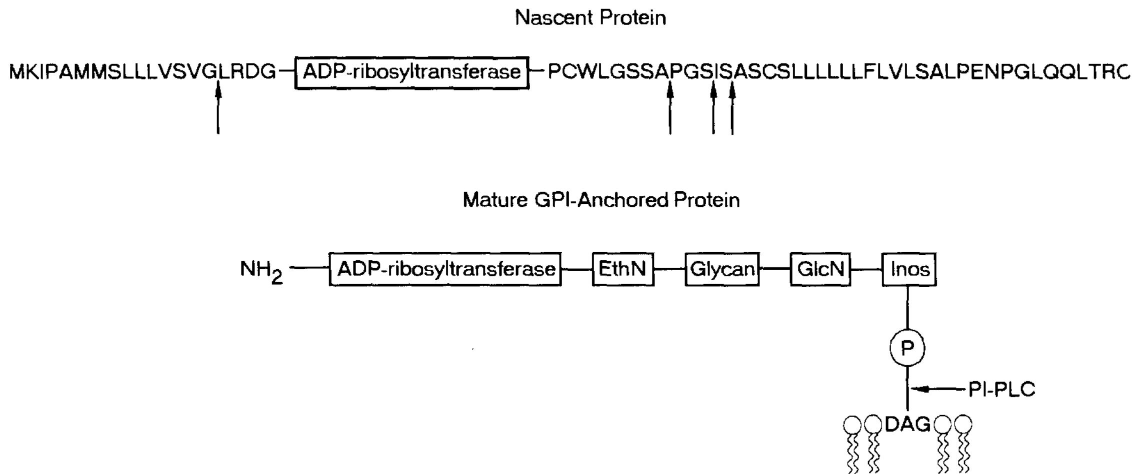

Fig. 3 Proposed structures of nascent and mature forms of GPI-anchored mouse lymphocyte ADP-ribosyltransferase. Top : Possible cleavage sites for amino and carboxy terminal signal peptides in the nascent form of the transferase are indicated with arrows. Bottom : Structure of the mature form of the enzyme is proposed, showing the anchoring into memmbrane via diacylglycerol moiety (EthN : ethanolamine, GlcN : glucosamine, Inos : inositol, DAG : diacylglycerol). The site of the cleavage by phosphatidylinositol-specific phospholipase C(PI-PLC) is indicated.

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초록 : 임파구 ADP-ribosyltransferase의 rat mammary adenocarcinoma cell에서의 발현

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glycosylphosphatidylinositol (GPI)에 의해 고정된 단백질의 초기 형태는 골지체에서의 직접적인 processing을 수행하기 위한 아미노와 카르 복시 말단의 hydrophobic signal sequence를 소유하고 있다. 앞서, mouse 임파구로부터 NAD : arginine ADP-ribosyltransferase (Yac-1)가 클로닝되었으며 Yac-1 transferase의 아미노산 배열을 추정해 본 결과, hydrophobic 아미노와 카르복시 말단을 포함하고 있었으며 이는 GPI-anchored 단백질들의 알려진 signal sequence와 일치하였다. 이 transferase는 야생형의 cDNA로 transfection된 NMU (rat mammary adenocarcinoma) cell의 표면에 존재하였으며 phosphatidylinositol-specific phospholipase C에 의해 방출되어졌다. 카르복시 말단의 hydrophobic sequence가 없는 돌연변이체는 수용성이며 분비성인 transferase를 생산하였다. 이러한 사실은 카르복시 말단의 sequence가 GPI의 부착에 중요함을 나타내준다.