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Delivery of an accessory signal for cell activation by exogenous phosphatidylinositol-specific phospholipase C

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Digestion of phosphatidylinositol (PI) or glycosylphosphatidylinositol (GPI) anchors of membrane proteins on the external cell surface with exogenous PI-specific phospholipase C (PIPLC) from *Bacillus thuringiensis* was shown to transmit a signal into the thymocyte to modulate the TCR/CD3 complex-induced signal delivery for cell activation. This was demonstrated for very early protein tyrosine phosphorylation, early c-*fos* transcription and late DNA synthesis. For this effect preincubation of the cells with PIPLC was required, but there was no evidence of involvement of any soluble products released form the cell surface by PIPLC in the signaling, suggesting a crucial role of the membrane-bound counterpart (diacylglycerol or diradylglycerol) of the PI/GPI hydrolysate. A possible role for this accessory signal in the microorganism-linked control of the T cell receptor function is discussed.

Phosphatidylinositol-specific phospholipase C (PIPLC); Glycosylphosphatidylinositol anchor; Signal transduction; Protein tyrosine phosphorylation; c-*fos*

1. INTRODUCTION

An increasing number of proteins are being reported as inserting into the cell membrane with a common glycosylphosphatidylinositol (GPI) anchor [1–3]. They include Thy-1 [4–6], TAP (Ly-6A.2) [7] and Qa-2 [8], and share an activity to transduce a signal for cell activation [5–8] which might be primarily linked to the GPI anchor rather than to protein moieties. Yet the molecular basis of the potentially unique pathway of signal transduction mediated by the GPI anchored proteins or GPI anchors is not known. One hypothetical view is that the extracellular phosphatidylinositol (PI) or GPI anchors are hydrolysed by endogenous PI-specific phospholipase C (PIPLC) [9] or GPI-specific PLC [10] which would generate second messengers [1–3]. The question, however, arises of whether the second message produced at the outer surface of the cell would actually transmit the signal intracellularly. Here we addressed this basic question by use of bacterial PIPLC. The results show that digestion with bacterial PIPLC of PI or GPI anchors on the external surface of the cell does induce the intracellular signal or signals which cooperate with the TCR/CD3-induced signal into murine thymocytes.

Abbreviations: GPI, glycosylphosphatidylinositol; IP, inositol phosphate; PIPLC, phosphatidylinositol-specific phospholipase C; DG, diacyl(diradyl)glycerol.

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2. MATERIALS AND METHODS

2.1. *Cells and reagents*

Single cell suspensions of thymocytes in Eagle's MEM were prepared from C57BL/6 mice as described [11]. MAb specific to CD3 (145-2C11, 1:1,000 of ascites except otherwise noted) and cross-linking anti-Ig (1:50) [6] were used. PIPLC was prepared from the supernatant of a culture of *B. thuringiensis* as described [12]. This highly purified PIPLC gave a single band in SDS-PAGE and was protease-free. For some experiments PIPLC was removed from the PIPLC-containing preparation by passing it through an anti-PIPLC affinity column. The anti-PIPLC column was prepared by use of an Immunoaffinity kit (Bio-Rad Laboratories, Richmond, CA, USA) and PIPLC-specific purified rabbit IgG according to the procedure recommended by the supplier. The column was confirmed to specifically adsorb all the enzyme activity to hydrolyze the GPI anchor.

2.2. *Assay of phosphotyrosine-containing proteins*

Immunoblot assay of phosphotyrosine-containing proteins was carried out as described [6,13] for the lysates of the cells (10^7 cells/200 μl) that had been treated or untreated with PIPLC and/or anti-CD3/anti-Ig. Specificity of the anti-phosphotyrosine antibody used for development of the phosphotyrosine-containing proteins has been extensively studied and reported [13]. Moreover, using phosphotyrosine, phosphoserine and phosphothreonine as inhibitors [6] we confirmed that all the bands we demonstrated were phosphotyrosine-containing proteins.

2.3. *RNA preparation and Northern blot analysis*

Total cellular RNA was prepared by a single-step method and its

Northern blot analysis was carried out as described [14]. The DNA probe used in this study was a 2.4 kb *Bam*HI–*Sal*I fragment of mouse c-*fos* cDNA [15].

2.4. *Assay of DNA synthesis*

Thymocytes (10^6 cells/well) were cultured in 96-well tissue culture plates containing 200 μl/well of RPMI tissues culture medium with 10% FCS [11]. To assess DNA synthesis, 37 KBq/well [^{3}H]thymidine was added during the last 4 h. After harvesting the cells on filter paper the radioactivity (cpm) was determined by liquid scintillation counting.

3. RESULTS AND DISCUSSION

Stimulation of murine thymocytes with anti-CD3 mAb or anti-CD3 mAb plus cross-linking anti-Ig induced low levels of increase in tyrosine phosphorylation of 110, 92, 80 and 40 kDa proteins as the very early signal for T cell activation [6,16,17]. The tyrosine phosphorylation of these proteins was definitely promoted by treating the cells previously with PIPLC, and the promotion was most evident for the 40 kDa protein. The intensification by PIPLC was most evident as early as 2 min after anti-CD3 stimulation (Expt. 2 in Fig. 1). Although data are not shown treatment with PIPLC alone without anti-CD3 stimulation was barely active. A concentration of 30 mU/ml was enough either for full promotion of signal transduction or for the maximum cleaving of PIPLC-sensitive Thy-1 from the thymocytes (not shown).

Fig. 2A shows that for full promotion both pre-incubation with PIPLC and its continuous presence during anti-CD3 stimulation were needed (compare lane 4 with lane 3), although pre-incubation alone (lane 4) was partially effective. It could be that IP glycan-linked proteins released during the necessary pre-incubation time acted as the second messenger of the PIPLC activity. The culture supernatant of the PIPLC-treated thymocytes, which contained both the IP glycan-linked proteins and PIPLC, actually promoted the signal delivery (lane 3 in Fig. 2B). However, when the culture supernatant of the PIPLC-treated thymocytes (lane 5) or the original PIPLC solution (lane 4 in Fig. 2B) was passed through an anti-PIPLC antibody affinity column it lost all the activity to promote the protein tyrosine phosphorylation (compare with lane 3). This result argued against the above mentioned view, and further confirmed the close association of the activity with PIPLC.

Fig. 3 demonstrates an extensive acceleration of transcription of c-*fos* as an immediate early gene for cell growth response [18], which occurred following the augmented protein tyrosine phosphorylation in the PIPLC-treated and anti-CD3-stimulated thymocytes. DNA synthesis was actually promoted in these cells at the peak level attained in the culture 2 days after stimulation (Fig. 4). The latter result did not agree with the earlier report by Presky et al. [19], who failed to demonstrate such an effect with PIPLC from *S. aureus*. The apparent discrepancy between their and our data is probably due to the higher accessibility to cell surface

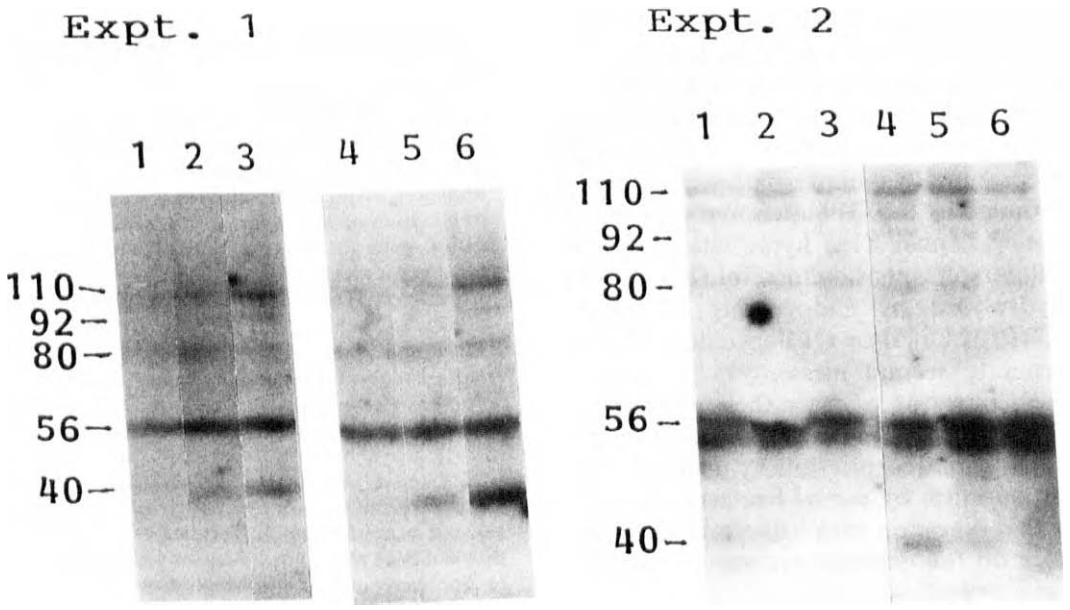

Fig. 1. Promotion by PIPLC of anti-CD3 mAb-induced protein tyrosine phosphorylation. (Experiment 1) Thymocytes were first treated (lanes 3 and 6) or untreated (lanes 2 and 5) with PIPLC (150 mU/ml) for 1 h and were then stimulated with anti-CD3 alone (lanes 2 and 3) or anti-CD3 plus cross-linking anti-Ig at an interval of 1 min (lanes 5 and 6). Cells were incubated at 37°C for 2 min after stimulation. Lanes 1 and 4 show no anti-CD3 control. (Experiment 2) Thymocytes previously treated (lanes 4–6) or untreated (lanes 1–3) with PIPLC (150 mU/ml) for 30 min were stimulated with anti-CD3 plus anti-Ig and incubated for 2 (lanes 1 and 4), 5 (lanes 2 and 5) and 10 (lanes 3 and 6) min. Cells were then lysed for immunoblot assay of phosphotyrosine-containing proteins. Approximate molecular sizes (in kDa) are shown on the left. The heavy band just below the 56 kDa protein corresponds to IgG heavy chain stained with [^{125}I]protein A. Basically the same results were obtained in 5 additional experiments in which thymocytes were stimulated with variable concentrations (1:500–1:5,000) of anti-CD3.

GPI anchors of PIPLC from *B. thuringiensis* than that in *S. aureus* [20].

We conclude from these results that the hydrolysis of PI or GPI anchors of membrane proteins at the external surface of the cell with exogenous PIPLC does deliver an accessory signal into thymocytes, which modulates the TCR/CD3-induced signal for cell activation. Because the bacterial PIPLC hydrolyses both PI and GPI anchors of membrane proteins the mechanism of the PIPLC-mediated signal modification also involves either (or both) of the two pathways through hydrolysis of PI or GPI anchors. Nevertheless, it is possible that the known signaling through cross-linking GPI-anchored proteins [5–8] at least in part involves a similar pathway, possibly activated by endogenous PIPLC [9] or GPI-specific PLC [10]. Whether or not this hypothetical view may be the case our result would suggest a novel host–parasite relationship whereby microorganisms modulate the receptor-mediated intracellular signal delivery of mammalian cells by releasing surface-acting PIPLC, which possibly associates with the pathogenicity of the invasive microorganisms.

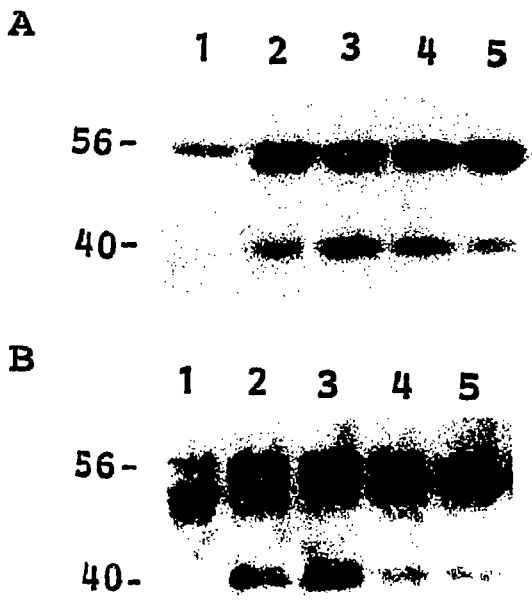

Fig. 2. Requirements for PIPLC-mediated signal promotion. (A) Thymocytes were treated (lanes 3 and 4) or untreated (lanes 2 and 5) with PIPLC (30 mU/ml) for 1 h. They were washed twice with MEM, and then stimulated by anti-CD3 plus anti-Ig in the presence (lanes 3 and 5) or absence (lanes 2 and 4) of freshly added PIPLC (30 mU/ml). Lane 1 shows the no antibody control. (B) Solution of PIPLC (60 mU/ml, and supernatant of culture of the thymocytes that had been incubated with PIPLC (60 mU/ml) at 37°C for 1 h ('culture supernatant') were passed through anti-PIPLC affinity columns and the effluents were adjusted to the volumes of the original preparations. Fresh thymocytes were treated for 30 min with the previously prepared culture supernatant (lane 3), the anti-PIPLC column-passed culture supernatant (lane 5), or the anti-PIPLC column-passed PIPLC solution (lane 4). These cells and untreated control cells (lane 2) were then stimulated with anti-CD3 plus anti-Ig for 2 min, and were lysed for immunoblot assay of phosphotyrosine containing proteins. Only the data around the 40 and 56 kDa proteins are shown.

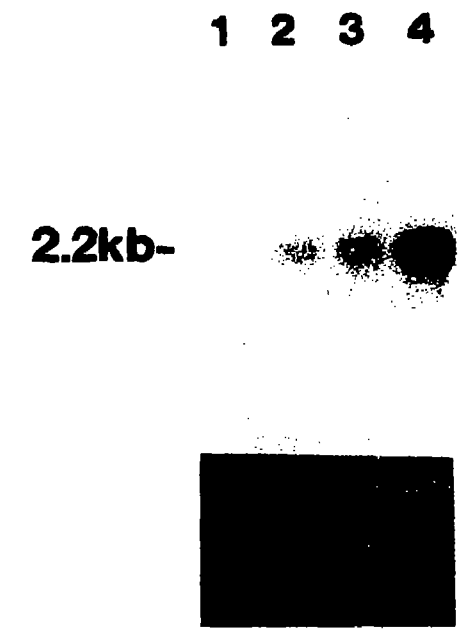

Fig. 3. Acceleration by PIPLC of anti-CD3 mAb-induced c-*fos* transcription. Total cellular RNA was extracted from control thymocytes (lane 1), thymocytes treated with PIPLC (150 mU/ml for 30 min) alone (lane 2), thymocytes stimulated with anti-CD3 alone (lane 3) or thymocytes first treated with PIPLC and then stimulated with anti-CD3 (lane 4). Hybridization was done with a mouse c-*fos*-specific probe. The size of the transcript is shown on the left side. Ethidium bromide staining of the gel containing 28 S and 18 S RNAs is shown below to indicate equal loading.

We failed to demonstrate any second messenger activity in the fraction containing the PIPLC-released soluble factors such as IP glycan-linked proteins (Fig. 2B).

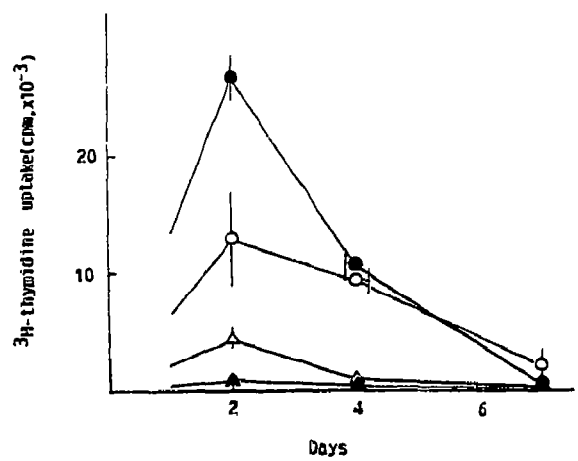

Fig. 4. Augmentation by PIPLC of anti-CD3 mAb-induced DNA synthesis. Thymocytes were first treated (●, ▲) or untreated (○, △) with PIPLC (150 mU/ml) for 30 min and then stimulated (○, ●) or unstimulated (△, ▲) with anti-CD3 (1:5,000) for successive culture. Their proliferation response was tested by [^{3}H]thymidine uptake. Each point shows the mean of triplicate cultures with SD. The difference in the values determined on day 2 (○, ●) was statistically significant ($P < 0.01$ by Student's *t*-test). Basically the same results were obtained in 6 additional similarly designed experiments.

It is, therefore, likely that diacylglycerol or diradylglycerol (DG) with potentially variable fine structures [1–3], which was produced after hydrolysis of PI and/or GPI anchors of membrane proteins with bacterial PIPLC, worked as a second messenger, possibly internalized [9] for activating some isoform of protein kinase C [21,22]. This conclusion might well correspond to the recent observation by Diaz-Laviada et al. [23] that the treatment of Swiss 3T3 fibroblasts with *B. thuringiensis* PIPLC provoked the translocation and activation of protein kinase C through production of DG. However, activation of protein kinase C by DG may not directly explain the pathway for augmented protein tyrosine phosphorylation observed in our experiment. A recent study showed that Thy-1 and other GPI-anchored membrane proteins are associated with a tyrosine kinase $p56^{lck}$ [24]. The signal cascade triggered by PIPLC might therefore involve the functional modification of $p56^{lck}$, possibly through its serine phosphorylation by the DG-activated C-kinase [25], which in turn phosphorylates some distinguished substrates at tyrosine.

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