Brief Definitive Report

RELEASE OF THE RAT T CELL ALLOANTIGEN RT-6.2 FROM CELL MEMBRANES BY PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C

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The rat T cell antigen RT-6 (1), formerly designated ART (2), Pta (3), AgF (4), or RTLy-2 (5), exists in at least two allelic forms (RT-6.1 and RT-6.2) and is coded for by genes mapped in linkage group 1 (6). It is a highly immunogenic T cell differentiation antigen but is not expressed on thymic lymphocytes or on any other cell type (our own unpublished data). This antigen is unusual in that it is not glycosylated and is relatively resistant to solubilization by nonionic detergents (7, 8).

Most lymphocyte surface antigens, in common with other integral proteins, are attached to the membrane via hydrophobic peptide domains that penetrate the lipid bilayer (9, 10). However, it has recently been shown (11–18) that there are exceptions to this rule in that certain cell surface proteins seem to be anchored by a covalent linkage with phosphatidylinositol. We report here that a highly purified phospholipase C, specific for phosphatidylinositol, selectively releases the rat T cell alloantigen RT-6.2 from viable lymphocytes and T-T hybridoma cells. This suggests that RT-6.2 is anchored to the cell membrane via phosphatidylinositol or a closely related lipid.

Materials and Methods

Materials. Phosphatidylinositol-specific phospholipase C (PI-PLC) was purified from *Staphylococcus aureus* culture supernatants (19). Suspensions of thymic and lymph node lymphocytes of inbred DA-rats/Han. were prepared as described previously (20). An RT-6.2^+/W3/25^+ rat T cell hybridoma (Ep-D3) was constructed by PEG-mediated fusion of Thy-1.1^+, $HGPRT^-$ rat thymoma C58NT cells (kindly provided by M. Nabholz, Ludwig Institute for Cancer Research, Epalinges, Switzerland) with RT-6.2^+ lymph node cells of an immunostimulated DA-rat (21). The rat RT-6.2 mAb-producing hybridoma Gy1/12 was kindly provided by G. W. Butcher, Institute of Animal Physiology, Medical Research Council, Cambridge, United Kingdom, and propagated in our laboratory. Monoclonal OX7 (anti-Thy-1.1) and W3/25 (anti–gp 48/53 rat helper marker) were gifts from A. F. Williams, Medical Research Council Cellular Immunology Unit, Sir William Dunn School of Pathology, Oxford, United Kingdom. The mouse anti-Thy-1.2–producing hybridoma Tib 107 was from the American Type Culture Collection, Rockville, MD.

Experimental Protocol. Cells (10^6 cells/ml) were incubated for 60 min at 37°C in RPMI

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1640 containing BSA (2 mg/ml), DNase (200 μg/ml), and 2-ME (50 μM) with (*a*) PI-PLC (20 μg/ml Tris-acetate buffer, pH 7.4), (*b*) trypsin (500 μg/ml, PBS, pH 7.4), or (*c*) Tris-acetate buffer or PBS (pH 7.4) only. After washing in PBS (pH 7.4), cells were treated with Gyl/12 (biotinylated), OX7, W3/25, or Tib 107 antibodies, stained with fluorescently labeled second reactants (avidin-FITC for Gyl/12, goat anti-mouse Ig-FITC for OX7 and W3/25, rabbit anti-mouse Ig-FITC for Tib 107), fixed in 1% paraformaldehyde and analyzed in a FACS IV flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

In another series of experiments, surface-radioiodinated cells (7, 22) were exposed to PI-PLC or buffer as described above. Cells were then exposed to 0.5% Triton X-100 (TX-100) for 15 min at 4°C (8). The Triton-resistant material was then resolubilized with 0.5% TX-100 for 15 min at 37°C (8). PI-PLC and TX-100 solubilisates were preabsorbed for 2 h at 4°C with protein A–Sepharose and normal rat or mouse serum, and reacted (2 h at 4°C) with one of the mAbs listed above and protein A–Sepharose (8). Immunoprecipitates were washed, eluted, and analyzed by SDS-PAGE (12% gels) autoradiography as described previously (7).

Phase separation in TX-114 was performed according to Bordier (23). In brief, ^{125}I surface-labeled Ep-D3 cells were exposed to PI-PLC as above, or to TX-114. The TX-114-resistant material was reexposed to 0.5% TX-114 at 4°C for another 16 h. The supernatants were cooled to 0°C and mixed with ice-cold solubilization buffer (0.9% TX-114, 1 mM EDTA, 5% NaN_3, 1 mM PMSF, pH 7.4). Aliquots of solubilisates were warmed to 30°C, layered on a 6% (wt/vol) sucrose cushion containing 0.06% TX-114 and centrifuged at the same temperature for 30 min at 300 *g*. Aqueous phases were adjusted to 0.25% TX-114. Detergent phases were mixed (1:100) with Tris-buffered saline (TBS) at 4°C. The single phases were immunoprecipitated with Gyl/12, and precipitates were subjected to SDS-PAGE (12% gel) autoradiography as described.

Results and Discussion

Treatment of DA-rat lymph node cells and the T hybridoma (EPD3) cells with PI-PLC resulted in a distinct decrease of cell surface RT-6.2, as indicated by reduction in the relative fluorescence (Fig. 1). The reduction was substantial in

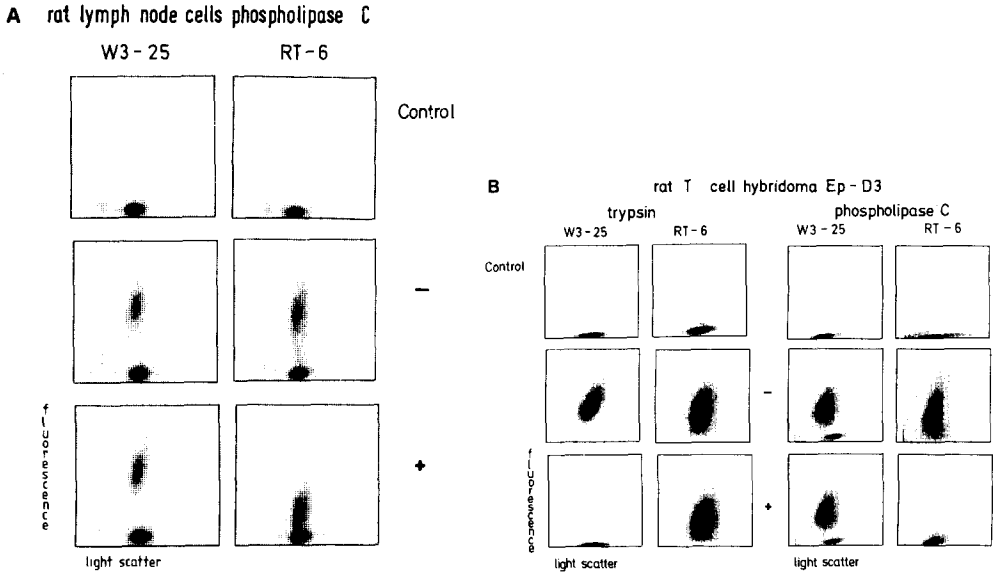

FIGURE 1. FACS analysis of W3/25 and RT-6.2 on viable DA-rat lymph node (*A*) and T hybridoma EP-D3 cells (*B*) before and after treatment with PI-PLC or trypsin.

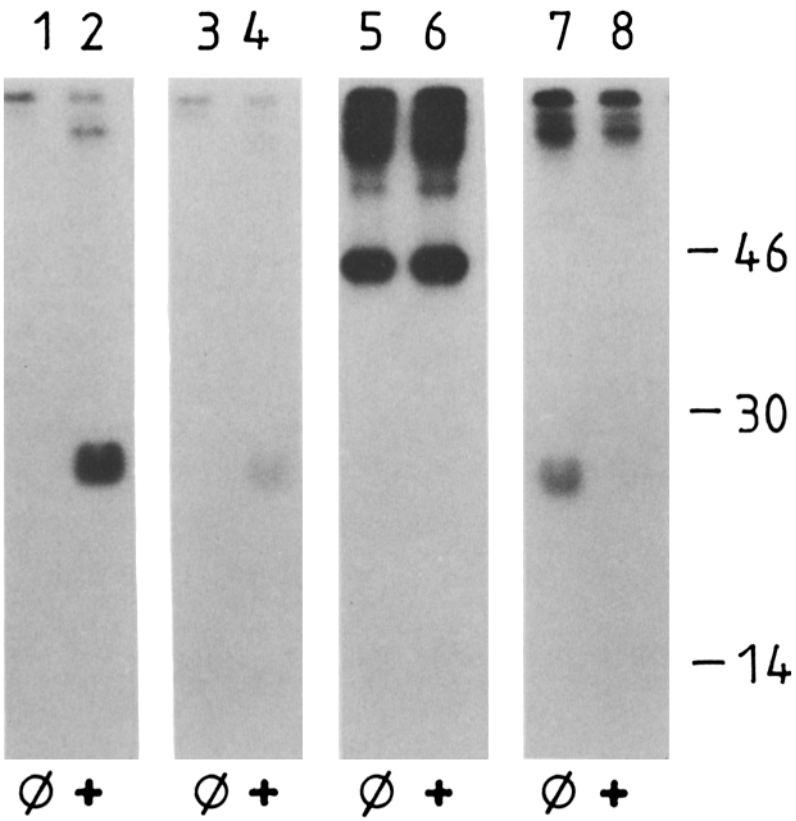

FIGURE 2. Sensitivity of gp W3/25 and RT-6.2 to PI-PLC. T-hybridoma Ep-D3 cells were incubated with (+) or without (Ø) PI-PLC. Supernatants were treated consecutively first with mAb Gyl/12 (lanes *1* and *2*) and then with mAb W3/25 (lanes *3* and *4*). Cells were solubilized by a two-step procedure with 0.5% TX-100 (first 15 min at 4°C, then 15 min at 37°C). First-step solubilisates were reacted with mAb W3/25 (lanes *5* and *6*), second-step solubilisates with mAb Gyl/12 (lanes *7* and *8*). 2.5×10^6 cells; autoradiography by exposing to Kodak X-Omat AR film 20 h at −70°C.

lymph node lymphocytes and almost complete in the T hybridoma cells. The rat T helper marker, as detected by W3/25 mAb (24), was not affected by PI-PLC (Fig. 1). In contrast, treatment of the cells with trypsin at a concentration 25-fold higher than that of PI-PLC caused only a small decrease in the amount of cell-surface RT-6.2, while almost completely removing W3/25-mediated fluorescence (Fig. 1). These findings indicate that RT-6.2 is relatively insensitive to proteolysis (8), and that loss of RT-6.2 from the cell surface as a result of PI-PLC treatment is unlikely to be due to a minor proteolytic contaminant of the highly purified PI-PLC.

Release of RT-6.2 by PI-PLC was also demonstrated by tracing the fate of ^{125}I surface-labeled RT-6.2 (Fig. 2). While RT-6.2 is completely removed from the hybridoma EP-D3 cells by PI-PLC and appears in an antigenically intact form in the supernatant, the W3/25 complementary antigen remains cell membrane associated under these conditions (Fig. 2). The M_r (25–26 kD) of RT-6.2 was not significantly decreased by the PI-PLC treatment (Fig. 2). Previous studies

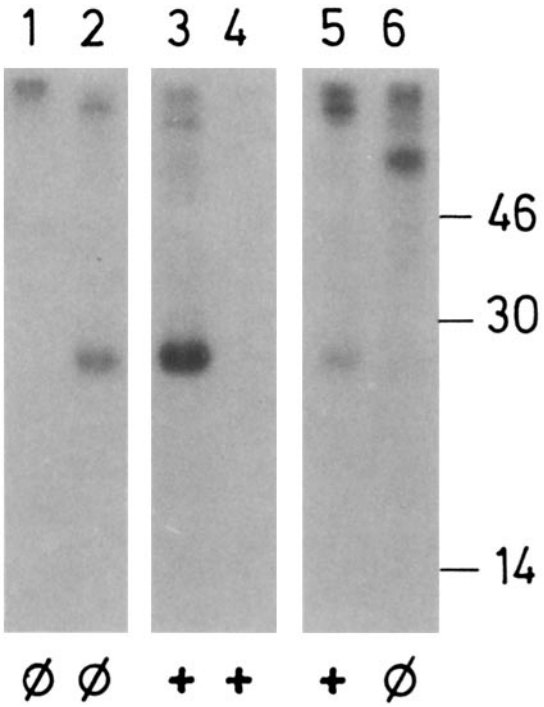

FIGURE 3. Phase separation in TX-114 of RT-6.2 as released from rat T-hybridoma Ep-D3 cells by PI-PLC (+) (60 min, 39°C) or TX-114 (16 h, 4°C). Lanes *1* and *2*, RT-6.2 as solubilized by TX-114 from non–PI-PLC-pretreated cells (lane *1*, aqueous; lane *2*, detergent phase). Lanes *3* and *4*, RT-6.2 as released by PI-PLC (lane *3*, aqueous; lane *4*, detergent phase). Lanes *5* and *6*, controls (lane *5*, TX-114 solubilisate of PI-PLC-pretreated cells; lane *6*, supernatant of non–PI-PLC-pretreated cells). 2.5 × 10^6 cells; autoradiography 72 h at −70°C.

using TX-114 phase separation have suggested that RT-6.2 contains a hydrophobic domain that is responsible for membrane anchoring (8). This technique was therefore used in the present study to determine the effect of PI-PLC treatment on this domain. RT-6.2 released from cells by PI-PLC was found only in the aqueous phase of a TX-114 phase separation, in contrast to detergent-solubilized RT-6.2, which was in the TX-114-enriched phase (Fig. 3). This suggested that the hydrophobic domain in RT-6.2 was completely removed by the PI-PLC treatment, and that this was responsible for its release from the membrane.

The data presented in this report indicate that RT-6.2 is anchored in the membrane by phosphatidylinositol or a closely related lipid. Although the nature of the interaction between the RT-6.2 polypeptide and phosphatidylinositol is unknown, it seems likely that it is covalent, as has been shown for certain other membrane proteins, including Thy-1 (16, 17). The physiological reason for attachment of particular membrane proteins to phosphatidylinositol has not yet been determined (17, 18), but it is of interest that two T cell antigens (i.e., RT-6.2 and Thy-1), which differ quite markedly in their tissue and species distribution, molecular properties, and expression during ontogeny, should share this unusual mechanism of membrane anchoring.

Summary

The mechanism by which the rat T cell alloantigen, RT-6.2, is attached to the membrane was investigated. Treatment of rat lymph node and T-hybridoma cells with phosphatidylinositol-specific phospholipase C (PI-PLC) caused a substantial reduction in the amount of RT-6.2 on the cell surface. No significant release of a rat T helper marker (visualized by the mAb W3/25) was observed in response to PI-PLC treatment. This is in sharp contrast to the effects of trypsin, which removes most of the T helper marker but had little effect on RT-6.2. SDS-PAGE analysis of the RT-6.2 released by PI-PLC indicated that the M_r was not significantly changed by this treatment. Phase separation of the released RT-6.2 in Triton X-114 showed that the PI-PLC had converted it from an amphiphilic membrane form to a water-soluble form, apparently by removing its hydrophobic membrane anchoring domain. These results strongly suggest that RT-6.2, in common with Thy-1 and several other cell surface proteins, is anchored in the membrane by the 1,2-diacylglycerol moiety of a covalently attached phosphatidylinositol molecule.

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