Brief Definitive Report

INACTIVATION OF A T CELL RECEPTOR-ASSOCIATED GTP-BINDING PROTEIN BY ANTIBODY-INDUCED MODULATION OF THE T CELL RECEPTOR/CD3 COMPLEX

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Antigen recognition by T lymphocytes initiates a series of events that ultimately lead to expression of functional activities, e.g., lymphokine secretion in helper T cells or delivery of the lethal hit in CTL. The earliest changes detectable after TCR triggering are a rise in the concentration of polyphosphoinositide breakdown products, inositoltrisphosphate (IP3) and diacylglycerol (DAG) (1). IP3 appears to cause a rise in cytosolic Ca^{2+} concentration by release of Ca^{2+} from intracellular stores (2), and DAG is an activator of PKC (3). These consequences of antigen recognition can be artificially induced by mAbs against the TCR or against the TCR-associated CD3 molecules. In addition, mAbs directed to other surface molecules that define "alternative" activation pathways such as CD2 (4) induce the same events (5).

The interaction of mAbs with most of these molecules leads to their transient removal from the cell surface, a process termed modulation. Modulation of CD2 (6) or Tp44 (7, 8) leads to unresponsiveness to the respective mAb but has no influence on other pathways. In contrast, modulation of the TCR/CD3 complex that is also a consequence of antigen-specific triggering results in a transient state of refractoriness to any signal given via a surface molecule (6, 8). The mechanism leading to this general unresponsiveness is not known. Recently it has been shown that in TCR/CD3-modulated Jurkat leukemia cells the early metabolic steps, i.e., formation of IP3 and increase in Ca^{2+} concentration do not take place (9). In this report, we demonstrate that a TCR-associated GTP-binding protein is functionally inactivated by TCR modulation.

Materials and Methods

Cells. Human CD8+ CTL clones or lines were obtained as described (10) and propagated in growth medium consisting of RPMI-1640 with 5% AB serum and 20 U/ml rIL-2.

Reagents. All chemicals were obtained from Sigma Chemical Co., Munich, FRG. Anti-CD3 or anti-CD8 mAbs were purified from the supernatant of OKT3 or OKT8 hybridoma cell lines. The anti-CD8 mAb BMA081 (IgG2a) and the anti-CD3 mAb BMA030 (IgG2a) were a gift from Dr. R. Kurrle, Behring Werke AG, Marburg. Purified goat anti–mouse IgG antibodies were obtained from Tago Inc., Burlingame, CA. α toxin from *Staphylococcus aureus*

was purchased from Calbiochem, Frankfurt, FRG. It was further purified by subsequent precipitations with 65% (vol/vol) and 55% (vol/vol) saturated ammonium sulphate solution. Precipitate was dialyzed against assay buffer. The hemolytic activity was determined with 0.05 ml 2.5% rabbit erythrocytes in PBS as described (11). 1 U of α toxin is defined as the amount of α toxin required to cause halfmaximal hemolysis.

Permeabilization. CTL were permeabilized in a buffered medium (assay medium) consisting of 150 mM potassium glutamate, 5 mM nitriloacetic acid, 10 mM Pipes, and 0.5 mM EGTA, pH 6.9, adjusted with KOH (12). If not specified otherwise, this medium contained 50 μM free Ca^{2+} and 1 mM ATP. The exact free Ca^{2+} concentrations in the medium were calculated by means of a computer program using the stability constants given (13). CTL were permeabilized with α toxin from *S. aureus* by adding 30 U α toxin/10^6 cells directly to the assay.

Measurement of Serine Esterase Release. CD8$^+$ CTL (usually 0.8 × 10^5 cells/well) were incubated in U-shaped 96-well microtiter plates (Nunc, Wiesbaden, FRG) at 37°C. After 45 min, incubation plates were centrifuged at 100 g for 3 min, and 25 μl samples of supernatant were harvested. Activity of serinesterase in the supernatant (14) was measured by a modification of the method of Coleman and Green (15). Briefly, 25-μl samples of supernatant were mixed with 225 μl of substrate solution (0.2 mM BLT and 0.22 mM DTNB in 200 mM Tris-HCl, pH 8.05). The mixture was incubated at 37°C for 20 min. Absorbance at 405 nm was measured in comparison with a blank solution that was treated exactly as experimental. Maximal release was determined by using 0.1% Triton X-100 solubilized cells. Spontaneous release was determined in supernatants of unstimulated nonpermeabilized cells. Data are given as percent esterase release = (experimental release − spontaneous release) : (maximal release − spontaneous release) × 100. Data represent mean of triplicates ± SD.

Modulation. Removal of surface antigens was performed by incubating the cells for 16 h in growth medium containing 4 μg/ml of the respective mAb. The success of modulation was controlled by cell sorter analysis.

Results and Discussion

The exocytotic release of the granule enzyme BLT-serine esterase by normal human CD8$^+$ CTL was used as an assay for CTL triggering. Although the role of the various constituents of these granules in CTL killing is unclear, the release of the CTL-specific serine esterase is a useful marker of CTL activation per se, since it is completed within a short time and does not require cellular interactions (16).

To gain access to the interior of the CTL, we permeabilized the plasma membrane with the α toxin of *S. aureus*. This toxin is an ideal permeabilizing agent since upon contact with the target lipid bilayer the α toxin monomers self-associate to form small transmembrane channels. These pores represent a homogeneous population of ring structured hexamers with a defined pore diameter and do not allow passage of molecules with a molecular weight >4,000 daltons (17, 18). Since α toxin monomers cannot pass through these pores, the action of α toxin is strictly confined to the plasma membrane (12, 19). In preliminary experiments, we determined that only low molecular weight components, and not cytosolic enzymes, were released from the permeabilized CTL (not shown).

These pores allowed us to permanently introduce the membrane-impermeable guanosine-nucleotide analogues GTPγS and GDPβS in defined concentrations into the cell. GTPγS is a poorly hydrolysable GTP analogue that is able to irreversibly activate all G proteins, whereas GDPβS inactivates all G proteins (20). As shown in Fig. 1, in the presence of Ca^{2+} and ATP, GTPγS is able to cause release of the serine esterase from permeabilized CTL. That this release is due to stimulation of exocytosis via the action of GTPγS on a G protein and not to nonspecific effects

Additions

permeabilized CTL   non-permeabilized CTL

-

Ca++

Ca++, ATP

Ca++, ATP . GTPγS

- , - . GTPγS

Ca++, ATP . GDP₃S

0 10 20 30 40 50 60 70 80   0 10 20 30 40 50 60 70 80
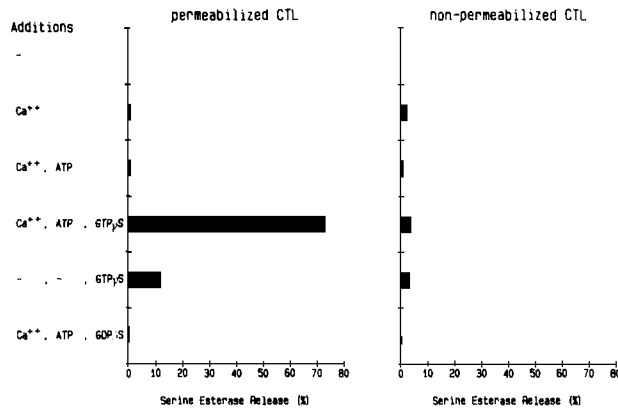Serine Esterase Release (%)   Serine Esterase Release (%)

FIGURE 1.   G protein stimulation leads to exocytosis in permeabilized CTL. Permeabilized (*left*) and non-permeabilized (*right*) CD8+ CTL (0.8 × 10⁵/well) were incubated in assay medium containing 50 μM Ca²⁺, 1 mM ATP, 100 μM GTPγS, or 100 μM GDPβS, as indicated. After a 45-min incubation at 37°C, serine esterase release was determined.

is suggested by the fact that the cytosolic enzyme lactate dehydrogenase is not released (not shown). Under the same conditions, GDPβS has no effect, nor has GTPγS an effect on nonpermeabilized intact cells. Therefore, in CTL, exocytosis can be triggered by activation of a G protein provided Ca²⁺ and ATP are present. The requirement for exogenous Ca²⁺ is probably due to the fact that the assay medium has a high buffering capacity for Ca²⁺ and will buffer all Ca²⁺ released intracellularly by IP3.

We next showed that transmembrane signal transduction via the TCR/CD3 complex was functional also in permeabilized CTL. CTL were permeabilized and incubated with immobilized anti-CD3 mAbs. As shown in Fig. 2, such CTL responded with esterase release in the absence of GTPγS. To show that this triggering via CD3 involves G protein, we used the G protein–inhibiting compound GDPβS. If GDPβS was added to the assay, exocytosis of esterase triggered by immobilized anti-CD3 mAbs was inhibited by 60%. In contrast, the response to the PKC activator 1-oleyl-2-acetyl-rac-glycerol (OAG) that also triggers serine esterase release in the presence of CA²⁺ and ATP was not inhibited. This indicates that GDPβS inhibits a G pro-
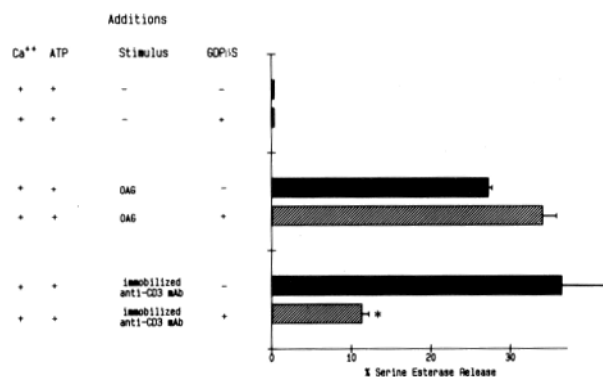
Additions

Ca++ ATP   Stimulus   GDPγS

+   +   -   -

+   +   -   +

+   +   OAG   -

+   +   OAG   +

+   +   immobilized   -
              anti-CD3 mAb

+   +   immobilized   +
              anti-CD3 mAb

0   10   20   30
% Serine Esterase Release

FIGURE 2.   Inhibition of TCR-triggered exocytosis by GDPβS. Permeabilized CD8+ CTL were incubated with medium alone or 60 μM OAG or immobilized OKT3 (anti-CD3) in the presence of 50 μM free Ca²⁺ and 1 mM ATP. To immobilize OKT3, the wells of a 96-well microtiter plate were precoated with goat anti-mouse IgG antibodies. 8 × 10⁴ CTL per well were incubated at 37°C for 45 min. Solid bars represent percent serine esterase release (±SD) in the absence of GDPβS, and hatched bars represent release in the presence of 180 μM GDPβS. Inhibition is significant at *p* <0.005 (*) as determined by Student's *t* test.

TABLE I

Modulation of the CD3 Complex Leads to Inactivation of the G protein

CTL line modulated with*	Percent serine esterase release from cells stimulated with:			
	–	20 μM GTPγS	100 μM GTPγS	60 μM OAG
–	4.2 ± 2.1	15.6 ± 1.2	34.0 ± 0.3	22.2 ± 0.7
Anti-CD3 mAb	1.3 ± 2.3	2.6 ± 1.5[‡]	7.5 ± 2.2[‡]	20.1 ± 2.5
Anti-CD8 mAb	3.3 ± 1.3	16.0 ± 3.6	34.2 ± 1.1	25.9 ± 2.3

Permeabilized CD8^{+} CTL (0.8 × 10^{5} cells/well) were incubated with GTPγS or OAG in the concentrations indicated.

* Cells were incubated for 16 h at 37°C with 4 μg/ml BMA030 (Anti-CD3) or 4 μg/ml BMA081 (anti-CD8), respectively.

[‡] Difference from control was highly significant ($p < 0.001$) based on student's t-test.

tein that acts early in the stimulus secretion coupling, most likely directly after TCR triggering and before the formation of DAG.

To determine the relationship of the G protein to the TCR, we modulated the TCR/CD3 complex from the surface of the CTL. The success of modulation was controlled by cell sorter analysis. As shown in Table I, the response of CD3-modulated permeabilized CTL to GTPγS was reduced by >77%. Modulation of CD8 as a control did not lead to a reduction of GTPγS-stimulated exocytosis. Most importantly, if CD3-modulated permeabilized CTL were stimulated with OAG in the presence of Ca^{2+} and ATP, exocytosis was not inhibited compared with unmodulated permeabilized CTL. This shows that the exocytotic apparatus can still be triggered by second messengers (Ca^{2+}, DAG) that bypass transmembrane signalling.

Taken together, our data suggest that in normal human CTL, a G protein is functionally inactivated after TCR modulation. This G protein is involved in TCR-mediated triggering of exocytosis and can be localized in the sequence of signalling steps after CD3 and before the formation of DAG. It therefore appears to mediate the receptor-induced activation of phospholipase C.

Why does TCR modulation lead to general nonresponsiveness? A possible explanation is that this G protein is also used to transmit signals received by other activating molecules, e.g., CD2 or Tp44. It is likely that the G protein is not only functionally, but also physically, associated with the TCR. Its removal together with the TCR would lead to a general loss of transduction potential.

Summary

TCR modulation induced by anti-TCR or anti-CD3 mAbs leads to a transient state of refractoriness of the T cell to all signals given via cell surface structures. To investigate the underlying mechanisms, we have used human CTL permeabilized with the α toxin of S. aureus. This method of permeabilization allows manipulation of the interior milieu of the cell, but maintains its functional and structural integrity. Introduction of the G protein activator GTPγS into permeabilized CTL leads to triggering of granule exocytosis. The G protein inactivator GDPβS inhibited exocytosis induced by TCR triggering but not that induced by activation of protein kinase C. This indicates that the G protein that triggers exocytosis is localized after CD3 triggering but before formation of the polyphosphoinositol breakdown product

diacylglycerol. In TCR-modulated CTL, GTPγS is no longer able to activate exocytosis. Such CTL, however, still respond to PKC activators. This demonstrates that a TCR-associated G protein has been functionally inactivated by TCR modulation.

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