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GTP-BINDING MEMBRANE PROTEINS IN ACTIVATED AND DIFFERENTIATING T CELLS

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We have earlier reported changes in the GTP binding of several membrane proteins including Gsα and Giα during thymic differentiation of T cells. Using an [α-^{32}P]GTP-photoaffinity labeling technique we have studied the pattern of GTP binding proteins in activated and resting T lymphocytes and in T cells induced to differentiate by TPA. The GTP binding proteins in mitogen-activated T cells resembled those seen in leukemia T cell lines. Treatment of Jurkat, but not of CCRF-CEM, T cells with TPA caused increased GTP-labeling of a 34 kDa protein and Giα. The GTP labeling pattern in TPA-treated Jurkat cells resembled that in resting T lymphocytes. TPA induced *de novo* expression of functional TCR/CD3 on CCRF-CEM and downregulation of TCR/CD3 on Jurkat cells but these changes did not correlate with the altered GTP-labeling patterns.

GTP-binding regulatory (G) proteins comprise a large family of proteins that participate in the transduction of various cellular signals [1]. The best known G proteins include Gs and Gi, involved eg. in hormonal control of adenylate cyclase [2]. The small *ras*- and *rab*-related G proteins are implied in the regulation of cell growth, intracellular vesicle traffic and secretion [3,4]. Many calcium mobilizing receptors apparently activate intracellular phospholipase C via G proteins [2], and this has also been suggested for the T cell antigen receptor/CD3 complex (reviewed in [5]).

G proteins are active when binding GTP and their intrinsic GTPase activity terminates their action towards the target [1,2]. Therefore covalent cross-linking of [α-^{32}P]GTP by uv-irradiation would be expected to primarily visualize active G proteins. We have used this technique to show that during T lymphocyte maturation in the thymus the α subunits of Gs and Gi, together with three unidentified membrane GTP-binding proteins, p26, p30 and p34, increase their affinity for GTP [6]. In parallel, the adenylate cyclase system matures and the cells aquire immunocompetence. Others have also reported differentiation-related changes in the expression or properties of G proteins concomitantly with altered function of the corresponding signal transduction systems [7-10]. Here we study the effect of

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Abbreviations; PKC, protein kinase C; TCR, T cell antigen receptor; TPA, tetradecanoyl phorbol acetate.

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mitogen-induced activation and TPA-induced differentiation on GTP-binding proteins in membranes of T cells.

MATERIALS AND METHODS

Reagents: [α-^{32}P]GTP (400Ci/mmol) was from Amersham International, calf intestinal alkaline phosphatase from Boehringer Mannheim and other reagents from Sigma. Rabbit antisera against Giα-2 (AS/7) and Giα-3 (EC/2) were from New England Nuclear, OKT3 and OKT4 from Ortho Diagnostics.

Cells: All cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum. Human mononuclear cells were isolated from the blood of healthy donors by Ficoll Isopaque gradient centrifugation and T lymphocytes were enriched by passage through nylon wool. T blasts were obtained by stimulating T lymphocytes (1.5×10^6/ml) with 5 μg/ml PHA for 3-4 days and enriched by Percoll gradient centrifugation. Blood granulocytes were isolated from the erythrocyte and granulocyte-containing pellet after the Ficoll Isopaque centrifugation by lysis of the erythrocytes. Blood monocytes were isolated by Percoll gradient centrifugation.

GTP-labeling of membrane proteins: The cells were disrupted by sonication in hypotonic buffer (10 mM Tris, pH 7.6, 0.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF and 10 μg/ml aprotinin, leupeptin and pepstatin) and membranes were isolated as described [6]. The resulting membrane pellet was suspended in the same buffer and stored at -70°C until use.

UV-affinity labeling of membrane proteins (40 μg) with [α-^{32}P]GTP was performed as described [6], except that the 10 min preincubation was performed at room temperature. To remove free [α-^{32}P]GTP the samples were either passed through 0.5 ml Sephadex G-25 columns [6] or the proteins were precipitated with 20% TCA and the precipitates washed with diethyl ether. The samples were run by SDS-PAGE (12% gels) under reducing conditions. The gels were subjected to autoradiography or alternatively, the proteins were transferred to nitrocellulose filters and the filters exposed to film.

Phosphatase treatment: Membranes were incubated with calf intestinal alkaline phosphatase (10 U/ml) and 100 μM $ZnCl_2$ in sonication buffer at +30°C for 30 min and collected by centrifugation at 100 000 g for 45 min.

Calcium measurements and FACS-analysis: $[Ca^{2+}]_i$ was measured as described [11,12] using the fluorescent calcium indicator Fura-2. Cell phenotype was analyzed by indirect immunofluorescence and flow cytometry using the monoclonal antibodies OKT3, OKT4 and fluorescein isothiocyanate-conjugated goat anti-mouse IgG.

RESULTS

To compare the repertoire of GTP-binding proteins in T lymphocytes to that in other cell types, we separated [α-^{32}P]GTP-photoaffinity labeled membrane proteins by SDS polyacrylamide gel electrophoresis. These experiments revealed marked differences, both in the band patterns and in the intensity of individual bands, between various normal or malignant cell types (Fig. 1). Some bands, such as a 40 kDa protein (identified as Giα in T cells [6]), were seen in all samples, while others displayed certain cell type specificity. GTP-labeled bands similar in size to T lymphocyte p26 and p30 were present in most cell lines, while p34 was seen only in T cells, Raji (lane d) and HL-60 (lane h). The GTP-labeling patterns were reproducible in more than 3 separate experiments (for each cell type).

In comparison with resting blood T lymphocytes the labeling of Giα and a 34-36 kDa (p34) band were significantly lower in mitogen-activated T cells (Fig. 1. lanes b and c). The pattern of GTP-binding proteins seen in T blast membranes resembled that seen in Jurkat and CCRF-CEM T leukemia cell membranes (Fig. 2 c) and in membranes from immature thymocytes [6].

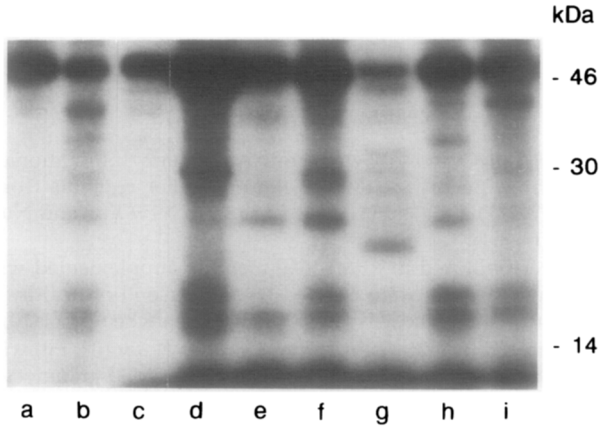

Figure 1. GTP-photoaffinity labeling of membranes from a) Jurkat T cells, b) blood T cells, c) PHA-stimulated T blasts, d) Raji B cells, e) blood monocytes, f) U937 monocytic cells, g) blood granulocytes, h) HL-60 promyeloid cells and i) K562 erythroleukemia cells.

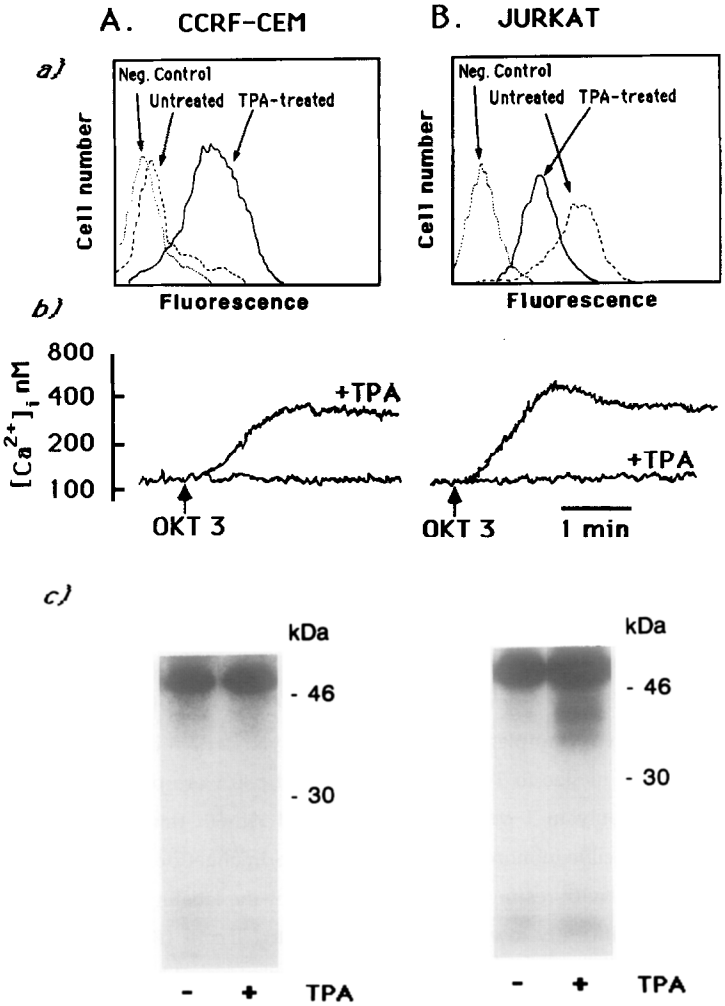

Figure 2. Effects of TPA-treatment (16 nM, 5 d) on the expression of CD3 (a), mobilization of calcium induced by OKT3 (b), GTP-photoaffinity labeling of membranes (c) in CCRF-CEM (A) and Jurkat cells (B).

One of the bands present in all samples was a heavily labeled 49-52 kDa (p49) band (overexposed in the shown autoradiograms). Its intensity correlated with the proliferation rate of the cells. Resting blood T lymphocytes, granulocytes and monocytes had weaker p49 bands than the rapidly growing tumor cell lines (Fig.1). This correlation was also evident in samples of mitogen-activated T lymphocytes, in which this band was increased compared to resting T cells (lanes b and c).

Treatment of CCRF-CEM cells with 16 nM of TPA induced *de novo* expression of TCR/CD3 receptors within 5 days (Fig. 2 a). These receptors were functional as indicated by their ability to induce a rapid rise in the intracellular concentration of free calcium (Fig. 2 b). In Jurkat cells, which constitutively express TCR/CD3, the surface expression of the TCR/CD3 decreased during TPA-treatment (Fig. 2 a). In addition, the reactivity of these cells to TCR/CD3 stimulation was lost rapidly (already within minutes [12]) and irreversibly (Fig. 2 b). The expression of CD4 on both cell lines decreased during TPA-treatment (not shown).

GTP-photoaffinity labeling of the membranes from untreated and TPA-treated CCRF-CEM cells showed no change in their pattern of GTP-binding proteins (Fig. 2c). Notably, no increase in p26, p30 or p34 could be detected in five separate experiments at any time from 1 to 10 days of induced differentiation. In contrast, incubation of Jurkat T cells with 16 nM of TPA induced increased GTP-photoaffinity labeling of p34 and a 40 kDa protein (Fig. 2c). Higher doses of TPA did not increase the labeling further. The 40 kDa band coincided with Giα-2 and Giα-3 detected by immunoblotting. (T cells do not express Giα-1 [13]). The immunoreactivity of these proteins, however, did not increase following treatment with TPA up to 5 days. Inhibition of protein synthesis by addition of 20 μg/ml cycloheximide did not block the TPA-induced increase in GTP-labeling of either protein (data not shown). Antibodies against Goα did not react with Jurkat membranes (data not shown).

The TPA-induced increase in GTP-labeling of p34 and Giα was detectable within 1h after addition of TPA to Jurkat cells and reached its maximum within 4-6 hours (Fig. 3 A). The increase was 2.5-3-fold as determined by densitometric scanning of the autoradiograms from more than six independent experiments and it remained on this higher level for several days. After removal of TPA the GTP-labeling of p34 and Giα declined slowly and reverted to the basal level within seven days (Fig. 3 B.).

Treatment with other activators of protein kinase C, such as phorbol dibutyrate and mezerein, also caused an increase in GTP-labeling of p34 and Giα (data not shown). The effect of phorbol dibutyrate on GTP-labeling was reversed more rapidly than the effect of TPA (Fig. 3B). To determine whether direct phosphorylation of p34 by protein kinase C might cause its apparent increased affinity for GTP in Jurkat cells, membranes from TPA-induced Jurkat cells were treated with calf intestinal phosphatase prior to GTP-photoaffinity labeling. No significant change in the labeling intensity of p34 was detected. Instead, a striking increase in the GTP-labeling of two smaller bands, 18-19 kDa and 20-21 kDa, occured (Fig 4).

DISCUSSION

A number of reports have suggested that a G protein would serve as a signal coupling agent between the TCR/CD3 complex and phospholipase C (reviewed in [5]). However, this

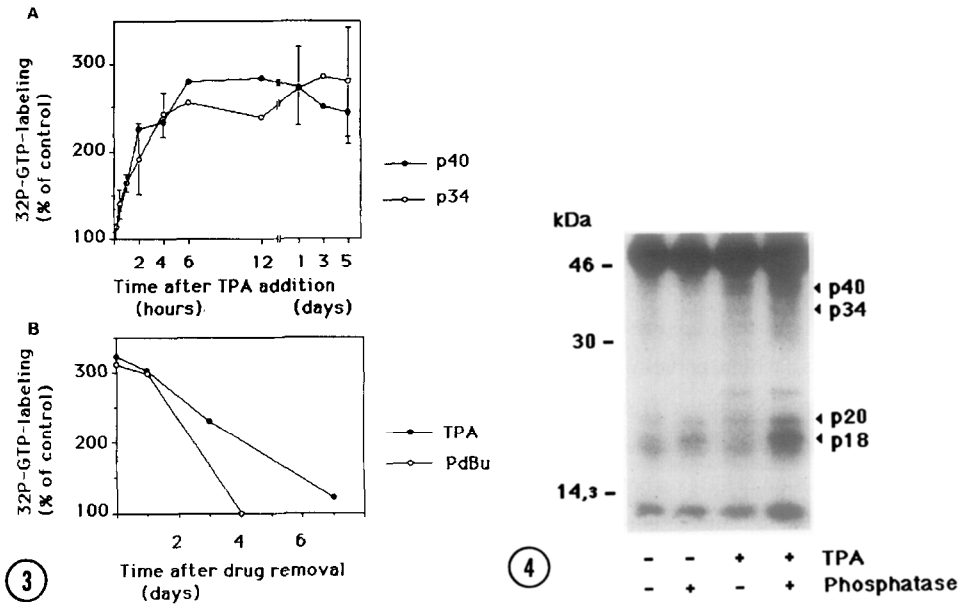

Figure 3.A. Increase in GTP-photoaffinity labeling of p34 and p40 (Giα) in Jurkat cells treated with TPA for various times. The labeling was quantitated by densitometric scanning of autoradiograms and the shown data represent means and SD (where shown) from 3-6 independent experiments. **B.** Reversal of the TPA- or phorbol dibutyrate (PdBu)-induced change in GTP-labeling of both p34 and p40. Cells treated with TPA for 4h, washed and cultured without TPA. The shown data represents densitometric scans from a single experiment.

Figure 4. Effects of phosphatase treatment on the GTP photoaffinity labeling of membrane proteins from Jurkat cells.

putative G protein has remained unidentified and the indications of its existence are rather indirect. Although activation of a G protein by aluminium fluoride or the non-hydrolyzable GTP analogue GTPγS results in inositol phosphate formation in T cells [14-16], this does not prove that this G protein is functionally linked to the TCR/CD3 complex. In fact, when a muscarinic acetylcholine receptor, which is known to use a G protein for signal transduction, was transfected into T cells, it was capable of inducing inositol phospholipid turnover [17]. Inactivation of pertussis toxin-sensitive G proteins, such as Giα, does not impair signalling through the TCR/CD3 [18-20].

We have used a GTP-photoaffinity labeling technique to visualize G proteins and other GTP-binding proteins and study changes in their activity. In membranes from resting human T lymphocytes at least ten different proteins binding GTP with high affinity and specificity can be seen [6]. If any of these is a G protein functionally associated with the TCR/CD3, it should respond to triggering of this receptor by rapidly increasing its affinity for GTP. We have, however, been unable to detect any such changes until 3 days after stimulation. During short time (1-15min) stimulation of T lymphocytes or isolated membranes with mitogenic lectins or anti-CD3 antibodies the GTP-labeling of T cell membrane proteins remained unchanged (data not shown), and there were no changes in membrane GTPase

activity (unpublished observation). Even if all known G proteins have very similar GTP-binding domains we cannot rule out the possibility that some of them escape detection.

To further investigate the possible role of GTP-binding proteins in TCR/CD3 signalling we used the two human T cell leukemia lines CCRF-CEM and Jurkat. The latter expresses high levels of functional TCR/CD3 complexes, while the former normally is TCR/CD3-negative. TPA treatment has opposite effects on the expression and function of TCR/CD3 on these cells. TPA induces expression of functional TCR/CD3 complexes on CCRF-CEM, while it blocks the function of these receptors on Jurkat cells. In neither cell line, however, was there any correlation between functional receptors and GTP-labeling of membrane proteins. Both CCRF-CEM and Jurkat contained very low levels of p26, p30 and p34, and no induction of these proteins occured in CCRF-CEM despite the appearance of functional TCR/CD3 complexes. In Jurkat cells, on the other hand, GTP-binding of p34 (and $G_i\alpha$) was induced following loss of TCR/CD3 function.

The presence of p34 in resting T cells and TPA-induced Jurkat cells correlates with a mature phenotype and growth arrest of these cells. Increased labeling with GTP might reflect functional maturation of a signal transduction system related to mature T cell physiology. The mechanism of induction remains unclear. The effects of TPA, phorbol dibutyrate and mezerein are likely to involve PKC-mediated phosphorylation. We did not, however, find evidence for direct phosphorylation of p34 by *in vitro* phosphatase treatments prior to GTP-labeling. Instead, the labeling of two small GTP-binding proteins increased markedly during phosphatase treatment of membrane samples. Thus, these proteins apparently are phosphorylated and, at least partly, inactivated *in vivo* and removal of the phosphate *in vitro* increases their affinity for GTP severalfold. There are precedents for regulation of G proteins via phosphorylation [21,22]. The *in vivo* phosphorylation of these proteins was not affected by TPA-treatment. The identity of these proteins remains unknown, but they might belong to the the *ras* or *rab* protein families.

Recent findings indicate that an early tyrosine phosphorylation event precedes and is required for activation of phospholipase C in T cells [23, 24]. The TCR/CD3 complex apparently activates a tyrosine kinase, most likely $pp59^{fyn}$ and/or $pp56^{lck}$, which, in turn, might direcly phosphorylate phospholipase C on tyrosine [25] without involvement of a G protein. Although our results do not rule out the possibility that a G protein is involved in TCR/CD3 signalling, they are compatible with such a model.

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