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$G\alpha_{16}$ protein expression is up- and down-regulated following T-cell activation: disruption of this regulation impairs activation-induced cell responses

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Abstract The role of heterotrimeric G proteins in T-cell activation is poorly understood. Here we show that in normal, mature human T-cells, expression of $G\alpha_{16}$, the 43 kDa α subunit of G_{16}, varies widely, depending on T-cell activation status. Quiescent blood lymphocytes strongly up-regulate $G\alpha_{16}$ after Leuco A stimulation: protein expression of $G\alpha_{16}$ is maximal at day 4, then decreases. Consistently, in human T-cell clones, expression of $G\alpha_{16}$ is high in the first week following activation and decreases rapidly within the second week. In addition, permanent disruption of regulated $G\alpha_{16}$ expression in Jurkat T-cells by stable overexpression of 43 kDa $G\alpha_{16}$ inhibited Leuco A-induced interleukin-2 production, CD69 up-regulation and cell apoptosis (by 58%, 46% and 74%, respectively), suggesting that coordinate regulation of $G\alpha_{16}$ expression is necessary for optimal activation-induced T-cell responses, and that $G\alpha_{16}$ proteins may be involved in the negative regulation of TCR signalling.

Key words: G protein; $G\alpha_{16}$; TCR activation; Human T-lymphocyte

1. Introduction

Recent studies have suggested that heterotrimeric GTP-binding proteins (G proteins) may play a role in the regulation of T-lymphocyte functions [1]. Abnormal thymocyte migration and maturation was reported in transgenic mice expressing in T-cells the catalytic subunit of pertussis toxin [2,3], a toxin which inhibits the function of G_i proteins; transgenic mice deficient in the α subunit of G_{i2} proteins also have functionally abnormal T-cells [4,5]. The G proteins G_q and G_{11} may also play a role in the regulation of TCR signalling [6,7]. Hence, studies of G protein expression in human T-lymphocytes at different states of activation and differentiation might help understand G protein functions in T-cell regulation. The G protein family includes 20 distinct G proteins, made of α subunits and βγ complexes [8–10] and divided into four major sub-families (G_s, G_i, G_q, G_{12}) based on the degree of amino acid identity of their α subunits. In the G_q family, $G\alpha_{16}$, the α subunit of G_{16}, was originally described as a 43 kDa protein expressed specifically in hemopoietic cells [11]. Because most studies of Gα subunits in blood cells were carried out on mRNAs [11–14], little is known of G *protein* expression in human blood T-lymphocytes. G_s and G_{i2} are ubiquitous; G_q and G_{11} RNAs were found in human thymocytes [14]; in human lymphoid cell lines, G_q proteins, but not G_{11} proteins, were detected [12]. $G\alpha_{16}$ mRNA was found in immature human lymphoid cells of B lineage [13], in two T-cell lines, MOLT and CEM, and in human thymocytes [11,14]; in human T-lymphocytes, protein expression of $G\alpha_{16}$ has been described only in tonsil T-cells [15]. Expression of G_{16} and G_{12} proteins varies according to cell lineage and/or degree of differentiation in myeloid cells [11,16], but nothing is known of G_{12} or G_{16} protein expression during differentiation and activation of normal human T-cells. In this study, we used immunoblotting techniques with polyclonal antibodies specific for Gα subunits to screen human peripheral blood lymphocytes (PBL) and various T-cell clones characterized by their T-cell receptor (TCR) chain rearrangements, CD4/CD8 expression, or Th1 or Th2 cytokine profile [17,18].

2. Materials and methods

2.1. Peripheral blood lymphocyte (PBL) preparation and cultures

PBL were obtained from fresh heparinized blood of healthy donors. After platelet removal, >95% pure lymphocyte preparations (PBL), consisting of at least 70% T-lymphocytes, were obtained by separation of mononuclear cells by Ficoll-Hypaque gradient centrifugation followed by two cycles of adherence to plastic to remove monocytes [16]. PBL were stimulated with 0.5 μg/ml Leuco A (leucoagglutinin A, also called PHA-L, Sigma, St. Louis, MO) and 30 ng/ml of recombinant human interleukin-2 (rh IL-2, Cetus, USA) and grown at a concentration of 5×10^5 cells/ml in RPMI plus 10% fetal calf serum (FCS) for up to 6 days. After two washes with PBS pH 7.5, cell pellets were kept frozen at −80°C until membrane preparation.

2.2. T-cell clone cultures

The human T-cell clones used in this study, provided by Drs. Henri Vié and Marc Bonneville (INSERM U463, Nantes, France), have been previously described [17,18]. Clones were grown in RPMI plus 10% FCS and rh IL-2 (30 ng/ml). After co-stimulation with Leuco A (1 μg/ml) and 35 Gy irradiated accessory cells (EBV-transformed B-cells and peripheral blood mononuclear cells) [17,18], clonal T-cells were grown in RPMI plus 10% FCS and rh IL-2 (30 ng/ml), for 7, 14, 21 or 28 days. Cells were then washed twice with PBS pH 7; cell pellets were kept frozen at −80°C until membrane preparation.

2.3. Jurkat cell transfection

Jurkat cells were grown in RPMI plus 10% FCS. Transfection was performed by electroporation at 270 V, 1500 μF, in 800 μl of RPMI in a 4 mm cuvette (Easyject), with pcDNA3 constructs (30 μg of linearized DNA per 5×10^6 cells) containing sense or anti-sense cDNAs encoding human $G\alpha_{16}$ [19]. Cells were allowed to recover in RPMI plus 10% FCS for 24 h, then selected with 750 μg/ml G418-sulfate for 2 weeks. All the studies described below were carried out on pools of transfected, G418-sulfate-resistant cells.

2.4. Protein analysis

After thawing of frozen cell pellets, crude membranes were prepared and protein concentration determined by the BCA method (Pierce), with BSA used as a standard, as previously described [16,20]. Membrane proteins (10 μg or 20 μg) were resolved on 10%

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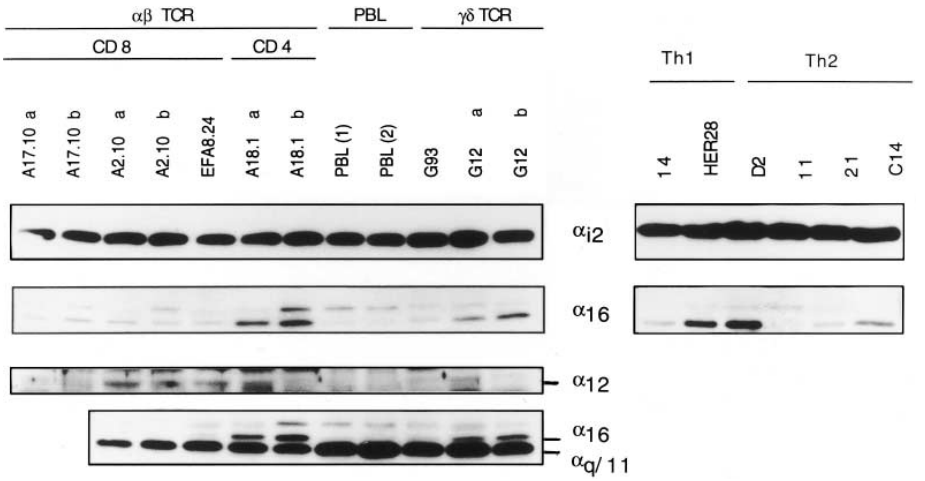

Fig. 1. Expression of Gα subunits in human PBL and T-cell clones. Membrane proteins (10 μg/lane, left panel; 20 μg/lane, right panel) of PBL and T-cell clones characterized by TCR chain, CD4/CD8 expression (left panel) or cytokine profile (right panel) were resolved by SDS/10% PAGE and transferred to PVDF. Duplicate blots were incubated first with antiserum AR ($G\alpha_{16}$), then with AS ($G\alpha_{i2}$) or QL ($G\alpha_{q/11}$), or with QE ($G\alpha_{12}$) first, then AS ($G\alpha_{i2}$) (left panel). Four clones (left panel) were tested on two different cell and membrane preparations (a, b).

SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). For each set of cell and membrane preparations, two identical gels were loaded in order to obtain two identical blots. Immunoblotting was carried out with 0.2 μg/ml of affinity-purified antibodies: AS 7, specific for $G\alpha_{i2}$ [21]; AR, directed against the last 10 C-terminal amino acids (ARYLDEINLL) of the α subunit of human G_{16} [16,21,22]; QL, specific for $G\alpha_{q/11}$ [23]; QE, specific for $G\alpha_{12}$ [16] or RM, specific for $G\alpha_s$ [22]. Antibody-antigen complexes were detected by enhanced chemiluminescence (ECL kit, Boehringer-Mannheim). Blots were exposed to RX Fuji films to visualize immunoreactive bands. For each blot, the complete procedure (incubation with primary and secondary antibodies, ECL revelation) was repeated twice using different affinity-purified antibodies, allowing screening of several Gα proteins on the same membrane preparations.

2.5. Cytokine production

Culture supernatants were harvested 18 h after stimulation in triplicate of Jurkat cells with 0.5 μg/ml Leuco A, and kept at −80°C. IL-2 secretion was assessed by ELISA using the IL-2 EASIA kit (Medgenix, France).

2.6. Immunofluorescence studies

Jurkat cells were harvested 18 h after Leuco A stimulation, fixed with 1% paraformaldehyde (PFA) and kept at 4°C until labelling. After two washes with PBS plus 1% BSA, cells were resuspended in 25 μl of the same buffer containing a 1:25 dilution of phycoerythrin-conjugated anti-CD69 antibody (Leu 23, Becton-Dickinson) for 30 min at 4°C. Non-specific fluorescence was determined with isotype-matched irrelevant antiserum. Cells were analyzed on a FACScan cytofluorometer (Becton-Dickinson).

2.7. Detection of Leuco A-induced cell death

Jurkat cells were stimulated in triplicate with Leuco A for 18 h. Apoptotic cells (defined as binding annexin V and excluding propidium iodide) were labelled using an Annexin V-FITC kit (Boehringer-Ingelheim, Germany) and analyzed on a FACScan cytofluorometer.

3. Results and discussion

3.1. Expression of Gα subunits in human T-cells

Expression of Gα subunits in human T-cell clones and in PBL of healthy donors is shown in Fig. 1. Expression of $G\alpha_{i2}$ and $G\alpha_{q/11}$ proteins was high in both PBL and T-cell clones, with only slight variations among clones. $G\alpha_{12}$ proteins were not detected in PBL; in clones, there was some variability in $G\alpha_{12}$ expression, but $G\alpha_{12}$ protein levels were always very low in human T-cells. As in human myeloid cells [16], the anti-$G\alpha_{16}$ AR antiserum recognized two proteins in human T-cells: $G\alpha_{16}$ (43 kDa) and another protein of apparent molecular mass 46 kDa. These two proteins were also revealed by AS 339/3 [16,24], another antibody raised against the C-terminal portion of $G\alpha_{16}$ (data not shown). The levels of expression of both $G\alpha_{16}$ and the 46 kDa protein were variable: PBL and $CD8^+$ clones expressed both proteins at low levels; the $CD4^+$ clone and two γδ TCR clones expressed $G\alpha_{16}$ at high levels, but only the $CD4^+$ clone also expressed the 46 kDa protein at high levels. In Th1 and Th2 clones, expression of 43 kDa $G\alpha_{16}$ proteins varied from very low (clones 14, 11, 21) to very high (clone D2); expression of the 46 kDa protein, also variable, was not detected in clones 14 and C14. Four clones (A17.10, A2.10, A18.1, G12) were tested twice, on different cell preparations (left panel); all expressed $G\alpha_{16}$ and the 46 kDa protein but at different levels on each preparation. In contrast, expression of $G\alpha_{i2}$ and $G\alpha_{q/11}$, tested on the same blots, was identical in both membrane preparations.

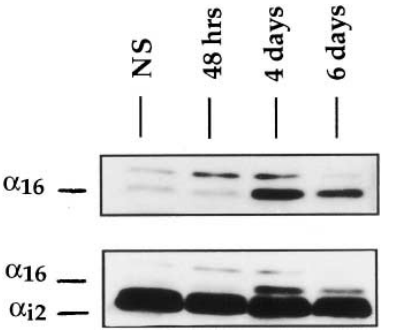

Fig. 2. Expression of $G\alpha_{16}$ proteins in activated PBL. Leuco A/IL-2-stimulated PBL were grown in vitro for the indicated times. Membrane proteins (20 μg/lane) were resolved by SDS/10% PAGE, transferred to PVDF, and blots were incubated first with AR ($G\alpha_{16}$), then AS ($G\alpha_{i2}$) antisera. This experiment was repeated three times, on different PBL and membrane preparations.

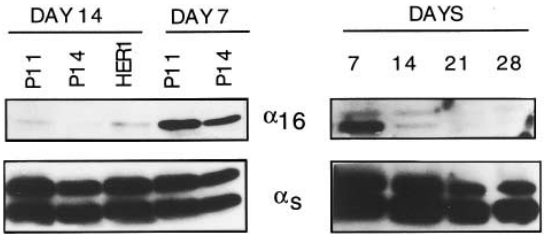

Fig. 3. Expression of $G\alpha_{16}$ proteins in activated human T-cell clones. Two Th2, $CD4^+$ clones (P11, P14), one Th1, $CD4^+$ clone (HER1) and one $CD8^+$ clone (A2.10, right panel) were subjected to co-stimulation with Leuco A, IL-2 and irradiated B-cells (see Section 2) and grown in vitro for 1 month. Membrane proteins (20 μg/lane) were resolved by SDS/10% PAGE, transferred to PVDF and blots were incubated with AR ($G\alpha_{16}$), then RM ($G\alpha_s$) antisera. In clones P11, P14 and HER1, the 46 kDa protein was not detected (left panel).

3.2. *Regulation of $G\alpha_{16}$ protein expression during T-cell activation*

As shown in Fig. 1, protein expression of (43 kDa) $G\alpha_{16}$ was low in PBL, which are presumably quiescent, and in $CD8^+$ clones, which have a low proliferation potential; in contrast, expression of $G\alpha_{16}$ was high in $CD4^+$ and γδ TCR clones, which tend to have a high proliferation potential, suggesting that $G\alpha_{16}$ expression might be associated with T-cell activation or/and proliferation status. We then stimulated freshly isolated PBL with Leuco A and IL-2 and studied $G\alpha_{16}$ expression (Fig. 2). Expression of $G\alpha_{16}$ proteins was strongly increased at day 4 post stimulation, and started to decrease by day 6. Interestingly, expression of the 46 kDa protein also increased in activated PBL, with different kinetics since its expression was already maximal at 48 h, and returned to the pre-stimulation level at day 6. Four T-cell clones were subjected to Leuco A/IL-2 co-stimulation and tested for $G\alpha_{16}$ expression in the following month (Fig. 3). Expression of 43 kDa $G\alpha_{16}$ was high during the first week post stimulation, decreased during the second week and was almost undetectable after 2 weeks. At day 21 and after, expression of the 46 kDa protein was also reduced.

$G\alpha_{i2}$ and $G\alpha_{q/11}$ proteins have been reported to affect T-cell activation or/and differentiation [4–7]. In T-cell clones, expression of neither $G\alpha_{i2}$ and $G\alpha_{q/11}$ (Fig. 1) nor $G\alpha_s$ (Fig. 3) was affected by activation or proliferation status. Similarly, no change in expression of $G\alpha_{i2}$ (Fig. 2) was observed in activated PBL. This, however, does not rule out that changes in the function of G_{i2} or $G_{q/11}$ may occur during T-cell activation. In contrast, expression of $G\alpha_{16}$ was positively correlated with the activated state of human T-cells. Such up-regulation contrasts with the down-regulation of $G\alpha_{16}$ observed during granulocytic, erythrocytic and B-cell differentiation [11,13,16]. Another finding of this study is the existence in T-cells of a 46 kDa protein, which, as in myeloid cells [16], is recognized by antibodies directed against the C-terminal portion of $G\alpha_{16}$. Indeed, in a recent report by Grant et al. [15], anti-$G\alpha_{16}$ antibodies also recognized two proteins in T-cells from human tonsils and in Jurkat cells. Like $G\alpha_{16}$, this 46 kDa protein is up- and down-regulated in activated T-cells. This would be consistent with the existence of a novel Gα subunit closely related to $G\alpha_{16}$, as in the $G\alpha_i$ family. As previous studies of $G\alpha_{16}$ in hemopoietic cells were carried out on mRNAs [11,13], mostly with RT-PCR techniques, a second $G\alpha_{16}$ gene may have been missed.

3.3. *Disruption of the regulation of $G\alpha_{16}$ protein expression with sense or anti-sense $G\alpha_{16}$ constructs: effects on Leuco A-induced activation of Jurkat cells*

Up-regulation of $G\alpha_{16}$ protein expression in activated T-cells could be either a non-essential consequence of T-cell activation or an absolute requirement for efficient T-cell activation. To distinguish between these two possibilities, disruption of the regulation of $G\alpha_{16}$ protein expression in human PBL would be the ideal strategy. However, as PBL cannot readily be transfected, we used Jurkat, a human T-lymphoma cell line commonly used to study TCR-mediated activation. After TCR activation, Jurkat cells, like PBL, produce IL-2 and up-regulate expression of CD69, a cell surface activation marker [25]. Unlike PBL, which proliferate within 5 days of TCR activation, Jurkat cells undergo apoptosis within 24 h [26]. In order to disrupt $G\alpha_{16}$ expression, Jurkat cells were stably transfected with either sense or anti-sense pcDNA3 constructs encoding human $G\alpha_{16}$; the $G\alpha_{16}$ cDNA cloned in the pcDNA3 constructs encodes 43 kDa $G\alpha_{16}$ [11,19]. As shown in Fig. 4, in Jurkat cells as in lymphocytes, the AR antiserum recognizes 43 kDa $G\alpha_{16}$ (weakly expressed in Jurkat cells) and a 46 kDa protein. Expression of 43 kDa $G\alpha_{16}$ is clearly increased in Jurkat cells transfected with sense $G\alpha_{16}$ and is below the detection level in cells transfected with anti-sense $G\alpha_{16}$. Studies of $G\alpha_{16}$ protein expression during activation of Jurkat cells by Leuco A were not possible: indeed, activation-induced apoptosis occurs very rapidly in Jurkat cells, and Western blot studies show evidence of apoptosis-related degradation of membrane proteins as early as 6 h post activation (data not shown).

Responses of transfected Jurkat cells to Leuco A stimulation (IL-2 production, up-regulation of CD69 expression, cell apoptosis) were studied (Fig. 5). In cells transfected with sense $G\alpha_{16}$, responses to Leuco A stimulation were strongly inhibited. In these cells, CD69 expression was inhibited by 40–46%; only $21 \pm 1\%$ of sense $G\alpha_{16}$-transfected cells expressed CD69 (vs. $35 \pm 2\%$ of vector-transfected cells (Fig. 5A), a 40% inhibition) with a mean intensity of fluorescence (MFI) reduced by 46% (Fig. 5B), and IL-2 production was inhibited by 58% (Fig. 5C), compared to vector-transfected cells. Only $6.0 \pm 0.1\%$ of sense $G\alpha_{16}$-transfected cells stimulated for 18 h with 0.5 μg/ml Leuco A underwent apoptosis (vs. $22.7 \pm 1.7\%$ of vector-transfected cells, a 74% inhibition) (Fig. 5D). Transfection with the anti-sense $G\alpha_{16}$ construct had little effect on Jurkat cell responses to Leuco A stimulation. In these cells, CD69 expression was not significantly affected: $31 \pm 1\%$ of anti-sense $G\alpha_{16}$-transfected cells ex-

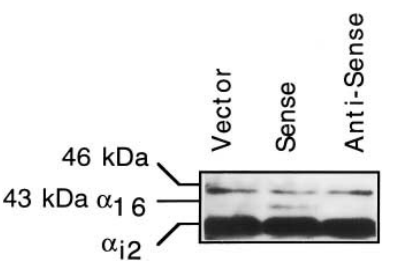

Fig. 4. Expression of $G\alpha_{16}$ in transfected Jurkat cells. Membranes (20 μg/lane) of Jurkat cells transfected with sense or anti-sense $G\alpha_{16}$ constructs were resolved by SDS/10% PAGE, transferred to PVDF and blots were incubated first with AR ($G\alpha_{16}$), then AS ($G\alpha_{i2}$) antisera.

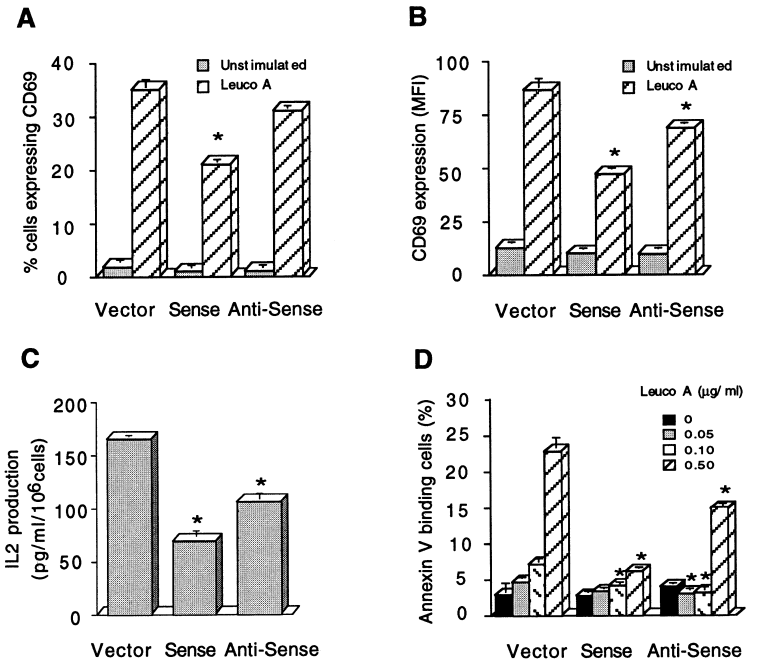

Fig. 5. Response of sense or anti-sense $G\alpha_{16}$-transfected Jurkat cells to Leuco A stimulation. 10^6 Jurkat cells transfected with vector alone, sense or anti-sense $G\alpha_{16}$ were stimulated with 0.5 µg/ml Leuco A in 1 ml wells, in triplicate, for 18 h. Data are presented as means ± S.E.M.; $^*P < 0.05$, Student's *t*-test (compared to vector-transfected cells). All experiments were repeated three times. CD69 expression, prior to and after Leuco A stimulation, and analyzed by FACScan after labelling of PFA-fixed cells with anti-CD69 antibodies, is presented as percentage of cells expressing CD69 (A) and as CD69 MFI of cells expressing CD69 (B); in the absence of stimulation, less than 2% of Jurkat cells (transfected or not) expressed CD69, with a MFI < 12. IL-2 content was measured in culture supernatants by ELISA (C), and cell apoptosis, assessed by propidium iodide exclusion and annexin V binding, is presented as percentage of apoptotic cells after stimulation with 0.05, 0.10 or 0.50 µg/ml Leuco A (D).

pressed CD69 (vs. 35 ± 2% for vector-transfected cells, Fig. 5A) with a MFI reduced by only 21% (Fig. 5B); IL-2 production was inhibited by 35% (Fig. 5C) and 14.9 ± 0.8% of anti-sense $G\alpha_{16}$-transfected cells stimulated with 0.5 µg/ml Leuco A underwent apoptosis (vs. 22.7 ± 1.7% of vector-transfected cells, a 34% inhibition) (Fig. 5C). Hence, inhibition of Jurkat cell responses to Leuco A activation was strong (40–74%) when overexpression of 43 kDa $G\alpha_{16}$ was enforced, and weak (≤ 35% inhibition) when increases in 43 kDa $G\alpha_{16}$ expression were prevented by transfection with the anti-sense $G\alpha_{16}$ construct. These observations suggest that sequential up- and down-regulation of $G\alpha_{16}$ protein expression is necessary for optimal T-cell response to activation. They also suggest that $G\alpha_{16}$ may negatively regulate TCR signalling, since maximal inhibition of Jurkat cell responses to activation was obtained in cells transfected with sense $G\alpha_{16}$. Indeed, survival of activated T-cells requires balancing of cell proliferation and apoptosis and adequate down-regulation of TCR signalling is essential to prevent uncontrolled, excessive apoptosis of activated T-cells. If $G\alpha_{16}$ negatively regulates TCR signalling, $G\alpha_{16}$ expression would logically return to low basal levels once TCR signalling is stopped: this is what was observed in PBL. According to this hypothesis, inhibition of activation-induced apoptosis and IL-2 production was not expected in Jurkat cells transfected with anti-sense $G\alpha_{16}$. However, the inhibition observed was weak (≤ 35%) and might not be significant. One should also take into account that the 46 kDa protein, the most abundant protein recognized by anti-$G\alpha_{16}$ antibodies in Jurkat cells, remained expressed at high levels in anti-sense $G\alpha_{16}$ cells (Fig. 4). Thus, cells may express enough functional $G\alpha_{16}$ proteins to down-regulate activation signals in a manner similar to vector-transfected cells. Alternatively, if TCR signalling cannot be stopped appropriately in cells transfected with anti-sense $G\alpha_{16}$, these cells may be in a (partially) refractory state, and therefore less capable of responding to Leuco A stimulation. These hypotheses are currently being investigated.

In conclusion, $G\alpha_{16}$ proteins can be distinguished from other heterotrimeric G proteins by their specific and tightly regulated expression during activation of human T-cells. Furthermore, this study provides evidence that the coordinated regulation of $G\alpha_{16}$ expression observed in blood T-lymphocytes is required for optimal T-cell activation and suggests a role for $G\alpha_{16}$ proteins in the negative regulation of TCR signalling.

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