Differential Localization and Function of ADP-Ribosylation Factor-6 in Anergic Human T Cells: A Potential Marker for Their Identification

Dimitrios Tzachanis, Leonard J. Appleman, Andre A. F. L. van Puijenbroek, Alla Berezovskaya, Lee M. Nadler and Vassiliki A. Boussiotis

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Anergy is a state of immunologic tolerance in which T cells are viable but incapable of responding to antigenic stimulation. Recent data indicate that anergic cells have a distinct gene expression program that determines their unique function. In this study we show that anergic human T cells selectively express the small GTPase ADP-ribosylation factor-6 (ARF6), which is involved in membrane traffic and regulation of the cortical actin cytoskeleton. ARF6 was expressed in the GTP-bound form that localizes at the plasma membrane, resulting in a distinct morphologic appearance of anergic cells. Forced expression of ARF6-GTP in Jurkat T cells prevented TCR-mediated reorganization of cortical actin, extracellular signal-regulated kinase1/2 activation, and IL-2 transcription. Forced expression of ARF6-GTP in primary human T cells inhibited extracellular signal-regulated kinase1/2 activation and proliferative responses. Importantly, T cells with the distribution pattern of ARF6-GTP were detected in peripheral blood, suggesting that anergic T cells may constitutively exist in vivo.


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2 Address correspondence and reprint requests to Dr. Vassiliki A. Boussiotis, Dana-Farber Cancer Institute, Mayer 547, 44 Binney Street, Boston, MA 02115. E-mail address: vassiliki_boussiotis@dfci.harvard.edu

3 Abbreviations used in this paper: SMAC, supramolecular activation cluster; ARF6, ADP-ribosylation factor-6; ERK, extracellular signal-regulated kinase; HA, hemagglutinin; GEF, guanine nucleotide exchange factor; GRF1, general receptor for phophomonoesterase 1.
Materials and Methods

Cells and suppression subtractive hybridization

Allot antigen-specific human T cell clones were rendered anergic by culture with soluble anti-CD3 mAb (100 ng/ml; Research Diagnostics, Flanders, NJ). Control-cultured populations were rescued from anergy induction by culture with the same concentration of soluble anti-CD3 plus anti-CD28 (200 ng/ml; Research Diagnostics) according to described protocol (12). After 6 h of culture, mRNA was isolated from each population, and cDNA was prepared by RT-PCR. cDNA from anergic T cell clones was used as “tester.” cDNA from control cultured cells was used as “driver,” and subtractive suppressive hybridization (18) was done with the use of the PCR-select cDNA subtraction kit (Clontech Laboratories, Palo Alto, CA) as previously described (12). Primary human T cells were isolated from ficoll/paque (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation from leukopacks obtained from the blood banks of the Dana-Farber Cancer Institute and Brigham and Women’s Hospital. T cells were enriched by depletion of plastic-adherent mononuclear cells, positive selection by E rosetting using SRBC (BioWhittaker, Walkersville, MD), and removal of NK and B cells by mAbs and anti-mouse Ig-coated magnetic beads. For experiments in which expression of ARF6 on CD8+ cells was examined, enrichment for CD8+ T cells after E rosetting was achieved by removal of CD4+ cells along with NK and B cells by the use of mAbs and anti-mouse Ig-coated magnetic beads.

Northern blot

For Northern blot analysis, 15 μg of mRNA was analyzed by electrophoresis on 1% agarose gel containing formamide and transferred on Hybond-XL blotting membrane (Amersham Pharmacia Biotech). The ARF6 probe consisted of full-length human ARF6 cDNA. The G3PDH probe consisted of a 1-kb fragment of the cDNA spanning the coding region of human G3PDH. cDNA fragments were labeled by random priming with α-32PdATP using a labeling kit (Amersham Pharmacia Biotech). Blots were hybridized with labeled probes in hybridization buffer (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s protocol. After hybridization, blots were washed four times at room temperature in SSC (2×) and SDS (0.1%), followed by one wash at 50°C. They were then examined by autoradiography.

Western blot

Equal amounts of protein from each sample were analyzed by SDS-PAGE, transferred on nitrocellulose membranes, and immunoblotted with the indicated mAbs or antiserum. ARF6 mAb was purchased from Alexis Biochemicals (Axova; San Diego, CA). ARF6-specific mAb and actin antiserum were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Immunodetection was done with HRP-conjugated anti-mouse IgG (1:5000) or anti-goat IgG (1:10,000) (Promega, Madison, WI) and developed by chemiluminescence (NEN, Boston, MA). For assessment of ERK1/2 activation, Jurkat cells or primary human T cells resuspended as 107/ml were left untreated or treated with anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) (both from Research Diagnostics) for 30 min on ice followed by crosslinking with rabbit anti-mouse Ig (20 μg/ml; DAKO, Carpinteria, CA) for 2 min. Lysates were analyzed by SDS-PAGE and immunoblot with pERK1/2-specific mAb (Santa Cruz Biotechnology) and subsequently re-blotted with ERK1/2 antisera (Upstate Biotechnology, Lake Placid, NY). Preparation of whole cell lysates, SDS-PAGE, stripping, and reprobing of the immunoblots were done as described (12).

Site-directed mutagenesis

To generate ARF6T27N and ARF6Q67L, specific mutations were introduced in the ARF6 sequence using the QuickChange Multi Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. The following primers were used to generate the mutations:

- primer T27N: GACGCGGCCGGCAAGAACACAATCCTGTACAAG; primer Q67L: TGGGATGTGGGCGGCCTGGACAAGATCCGGCCG.

The fidelity of the mutations was verified by sequencing. Constructs were expressed in pSRαHA vector as fusions to N-terminal hemagglutinin (HA) epitope tag.

Jurkat cell transfection and luciferase assays

For assessment of IL-2 transcription, Jurkat T cells were transiently transfected by electroporation with a constant amount (20 μg) of the reporter constructs of luciferase driven by the 2-kb IL-2 promoter/enhancer and with equal amounts (50 μg) of pcDNA1.1 empty vector or cDNA of the indicated ARF6 plasmids. Forty hours after transfection, 5 × 105 cells were cultured with either medium or with a combination of anti-CD3 plus anti-CD28 mAb for 6 h, and luciferase activity was measured. In all experiments, transfection efficiency was normalized by cotransfection with pEF-lacZ and assay for β-galactosidase.

Transfection or primary human T cells

Primary human T cells were transfected with either empty vector or the indicated ARF6 plasmids by the nucleofection technique and device (19) according to the manufacturer’s instructions (Amxa Biosystems, Cologne, Germany). Twenty-four hours after transfection, T cells were recovered and stimulated as described above for assessment of ERK1/2 activation. At the same time, cells (105 cells per well) were cultured in 96-well plates with either medium or a combination of anti-CD3 (300 ng/ml) and anti-CD28 (500 ng/ml) mAbs, and DNA synthesis was assayed by [3H]thyminidine for the last 16 h of a 72-h culture period. Responses were evaluated by assessment of stimulation index as described before (20).

Confocal microscopy

Cells were isolated from cultures, allowed to attach on poly-l-lysine coated coverslips for 2 min, fixed with 3% paraformaldehyde, and permeabilized with 0.1% Triton X in PBS-1% BSA. Cells were stained with ARF6-specific mAb followed by FITC-conjugated donkey anti-mouse serum and propidium iodide to visualize the nuclei. When detection of double staining for ARF6 and either CD4, CD8, or CD25 was conducted, cells were stained with ARF6 followed by FITC-conjugated donkey anti-mouse serum and were subsequently incubated with the indicated second Ab conjugated with rhodamine. For the bead assay, goat anti-mouse IgG dynabeads M-450 (Dynal Biotech, Great Neck, NY) were coated with anti-CD3 mAb. Coated beads and Jurkat T cells were washed and resuspended in RPMI/HEPES supplemented with 1% FCS. Cells and beads were mixed and incubated for 30 min at 4°C followed by 5 min at 37°C. Cells were allowed to attach on poly-l-lysine coated coverslips for 2 min on ice, fixed, permeabilized, and stained with rhodamine-labeled phalloidin to visualize polymerized actin.

Samples were visualized and photographed using a confocal laser scanner microscope (MRC 1024 Kr/Ar; Bio-Rad, Hercules, CA).

Results

Anergic cells express high levels of ARF6 mRNA and protein

To identify genes that are selectively expressed in anergic cells, we used suppression subtractive hybridization. Allot antigen-specific human T cell clones were rendered anergic by stimulation via TCR/CD3 alone. Control stimulated cells were rescued from anergy induction by simultaneous costimulation via CD28. Sequence analysis of the confirmed positive clones obtained by subtraction hybridization revealed that ARF6 was selectively up-regulated in anergic cells.

Northern blot analysis showed that ARF6 mRNA was expressed at low levels in unstimulated T cell clones and was up-regulated during culture. Immunogenic culture resulted in transient up-regulation of ARF6 mRNA that peaked at 3 h and gradually declined. In contrast, anergizing culture resulted in sustained and augmented expression of ARF6 mRNA for up to 12 h of culture (Fig. 1A).

Western blot analysis confirmed that higher levels of ARF6 protein were detected in anergic, compared with productively, stimulated cells (Fig. 1B).

ARF6 is expressed in the GTP-bound form in anergic cells

ARF6 is a member of the ARF GTPases and cycles between a GDP-bound and a GTP-bound form (14). The GDP-bound form of ARF6 is present in the cytoplasm and the endosomal membranes. It is converted into the GTP-bound form by the action of guanine nucleotide exchange factors (GEFs), resulting in a conformational change of the protein that allows its intracellular relocation. Subsequently, ARF6-GTP is associated with recycling endosomes on the cell periphery and predominantly with the plasma membrane, resulting in a distinct pattern of intracellular localization between ARF6-GDP and ARF6-GTP (15-17).

One of the GEFs for ARF6 is the general receptor of phosphoinositides 1 (GRP1), a member of the Cytosesins family (21). Notably, GRP1 was shown to be selectively expressed in anergic
mouse T cells. Similar to the expression pattern of ARF6, GRP1 was up-regulated during both anergizing and immunogenic stimulation, but its expression was augmented and sustained only in anergic cells (10). The observations that anergic cells have high and sustained expression of ARF6 and GRP1 suggest that ARF6 may be present in the GTP-bound form. Assessment of GTP load showed that anergic cells preferentially expressed the GTP-bound form of ARF6 (data not shown). Because the GDP and GTP-bound forms of ARF6 are distributed in distinct cellular compartments (14, 16), we hypothesized that a distinct intracellular localization pattern of ARF6 might be detectable in anergic cells. Staining with ARF6-specific mAb revealed that in productively stimulated cells ARF6 was predominantly localized in scattered internal structures (Fig. 1C, top). In contrast, anergic cells had predominantly plasma membrane localization of ARF6, allowing their phenotypic identification (Fig. 1C, bottom). This different localization pattern of ARF6 was detected for up to 4 days of resting culture in medium alone after the induction of productive stimulation or anergy. After this time interval of culture, viability of human T cell clones rapidly declines and does not allow further analysis.

**Forced expression of ARF6-GTP in Jurkat T cells inhibits TCR-mediated actin reorganization and blocks ERK1/2 activation and IL-2 transcription**

ARF6 is involved in regulation of endocytic traffic, but also in remodeling of the cortical actin cytoskeleton (16). The finding that a molecule involved in reorganization of the cytoskeleton is highly expressed in anergic cells suggests that it may play a role in the outcome of TCR ligation by Ag. The link between the actin cytoskeleton and lymphocyte activation has been well documented (13). TCR-mediated stimulation leads to the organization of SMACs at the interfaces of physical contact between T cells and APC. Assembly of the signaling molecules within the SMACs supports signaling synergy, resulting in efficient lymphocyte activation. Formation of SMACs is a dynamic process that depends on movement of actin filaments. Upon binding to an APC, the cytoskeleton of a T cell rapidly polarizes. The accumulation of polymerized F-actin in a tight collar at the T cell-APC interface stabilizes a continuous contact between T cell and APC. T cells also become polarized toward anti-CD3-coated beads and reorganize their actin cytoskeleton as a dense F-actin collar. This approach mimics the actin reorganization taking place during T cell-APC interaction and permits the precise analysis of actin remodeling in T cells (22).

We have previously observed that anergic T cells have defective ability to reorganize cytoskeletal actin after TCR ligation (23). However, it is currently unclear whether ARF6 might be involved in this event. To address the role of ARF6 in TCR-mediated actin remodeling, we transfected Jurkat T cells with either empty vector, ARF6T27N that remains in the GDP-bound state, or ARF6Q67L that remains in the GTP-bound state (24). Cells were stimulated with anti-CD3-coated beads, and actin remodeling was examined with phalloidin staining. Although ARF6-GDP (ARF6T27N) did not affect actin reorganization and formation of the F-actin collar at the cell-bead contact site, ARF6-GTP (ARF6Q67L) prevented redistribution of F-actin at the contact site (Fig. 2A). Consistent

![FIGURE 1. Differential expression and intracellular localization of ARF6 in anergic and control-activated T cell clones.](image-url)

**FIGURE 1.** Differential expression and intracellular localization of ARF6 in anergic and control-activated T cell clones. A, Northern blot analysis of ARF6. T cell clones were treated for induction of productive immunity, or anergy RNA was extracted at the indicated time intervals, and expression of ARF6 was examined by Northern blot. B, T cell clones were cultured as indicated, lysates were prepared and analyzed by SDS-PAGE and immunoblot with ARF6-specific mAb and actin-specific antisera. C, Productively stimulated (top) and anergic (bottom) T cell clones were fixed, stained with ARF6 mAb and propidium iodide to visualize the nuclei, and subjected to confocal microscopy. This unique expression pattern was detected in >80% of the cells in the productively stimulated and the anergic population (total number of cells counted in multiple fields, n = 50).

![FIGURE 2. ARF6-GTP inhibits TCR-mediated redistribution of F-actin and blocks ERK1/2 activation and IL-2 transcription.](image-url)

**FIGURE 2.** ARF6-GTP inhibits TCR-mediated redistribution of F-actin and blocks ERK1/2 activation and IL-2 transcription. A, Jurkat T cells transfected with the indicated plasmids were stimulated with goat anti-mouse IgG dynabeads M-450 (Dynal Biotech) coated with anti-CD3 mAb. Cells were fixed and stained with rhodamine-labeled phalloidin to visualize polymerized actin. Asterisks indicate beads in contact with cells. Expression of HA-tagged ARF6 was confirmed by staining with anti-HA mAb (data not shown). B, Jurkat T cells, transfected as indicated, were stimulated by CD3 and CD28 cross-linking; and activation of ERK1/2 was examined by immunoblot with an Ab specific for phosphorylated ERK1/2. C, Jurkat cells were transfected with the indicated plasmids along with IL-2-luciferase reporter. Cells were cultured for 6 h as shown in the graph, and IL-2 transcription was examined by luciferase assay.
with the role of actin reorganization in the initiation of signaling cascades that lead to T cell activation, ARF6-GTP prevented TCR/CD3-mediated ERK1/2 activation (Fig. 2B) and inhibited IL-2 transcription (Fig. 2C).

**T cells with membrane localization of ARF6 are detected in peripheral blood of healthy individuals**

The observation that ARF6 intracellular localization provides a detectable morphologic appearance of anergic cells suggests that ARF6 might be a useful marker for the identification of anergic cells in vivo. Enumeration of such cells during treatment for tolerance induction for allogeneic organ or bone marrow transplantation may provide a useful assessment for the efficiency of the treatment approach. It may also provide a means for the evaluation of autoimmune diseases in which tolerance to self-Ags is compromised. For these reasons, we sought to determine whether ARF6 expression was detectable in T cells isolated from peripheral blood of healthy individuals. The majority of peripheral blood T cells had undetectable or very low cytoplasmic expression of ARF6 (Fig. 3). A small number of cells had increased cytoplasmic expression of ARF6 that localized in scattered intracellular structures, similar to that observed in productively stimulated T cell clones. Surprisingly, a number of cells exhibited predominant membrane localization of ARF6, consistent with the expression pattern identified in anergic T cell clones, suggesting that detectable anergic T cells may constitutively exist in vivo. Staining with CD4, CD8, and CD25 mAbs showed that ARF6-positive cells were detected almost exclusively within the CD4+ and CD25+ populations. In contrast, CD8+ T cells had undetectable or very low expression of ARF6 (Fig. 4A).

To address the role of ARF6 on TCR-mediated activation of primary T cells, we transfected freshly isolated peripheral blood human T cells with either empty vector, ARF6T27N that remains in the GDP-bound state, or ARF6Q67L that remains in the GTP-bound state using the nucleofection technique. Cells were rested in medium for 24 h and subsequently stimulated by TCR/CD3 and CD28 cross-linking. Activation of ERK1/2 was determined by immunoblot with phospho-specific Ab. Although forced expression of ARF6-GDP (ARF6T27N) did not affect ERK1/2 activation, forced expression of ARF6-GTP (ARF6Q67L) resulted in defective augmentation of ERK1/2 phosphorylation after TCR/CD3 ligation (Fig. 4B). ARF6-GTP (ARF6Q67L) also prevented optimal proliferative responses of T cells to TCR/CD3-plus-CD28 ligation (Fig. 4C).

**Discussion**

Our present studies identified ARF6 as an unexpected target of T cell anergy. ARF6 is expressed in anergic cells, not only in higher levels compared with productively stimulated cells, but, more importantly, in the GTP-bound form that localizes at the plasma membrane. This distinct localization makes ARF6 an attractive candidate marker for the identification of anergic cells in various clinical conditions in which anergy may have a critical role. Such
conditions include allogeneic bone marrow and organ transplantation, autoimmune diseases, and cancer in which induction of tolerance to tumor Ags may be an early event in the tumor-bearing host (25).

Several studies have provided compelling evidence that central tolerance allows some thymocytes with self-reactivity to slip the deletion process and exit in the periphery. Self-reactive T cells can be isolated from the peripheral blood of healthy individuals, suggesting that they are silenced by different mechanisms (1). Our present data provide evidence that T cells with a phenotypic ARF6 localization of anergic T cell clones can be detected in the blood of healthy individuals, suggesting that active mechanisms for maintaining immune quiescence and peripheral tolerance are constitutively present. The fact that GRP1 GEF (10) and its target, ARF6, are transcriptionally up-regulated in anergic cells confirms once more the active nature of the anergic state.

The mechanism by which ARF6-GTP inhibits redistribution of polymerized actin and blocks TCR-mediated activation is still unclear. Studies of other cell types have shown that ARF6 and Rho GTPases are antagonistic, and ARF6-GTP down-regulates Rho and Rac activation (26, 27). It is possible that in a similar manner in T cells, ARF6-GTP inhibits actin reorganization because it inhibits activation of Rho GTPases. RhoA controls polarization and spread of T cells and potentiates AP-1 transcriptional activity after TCR engagement (28–30). Mouse T cells expressing constitutively active RhoA are hyperresponsive to TCR-induced proliferation (31). Rac1 is activated by TCR, CD28, and CD46-mediated signals (32–34). It is required for activation-dependent actin polymerization (35) and is implicated in the activation of Jun kinase, phosphorylation of c-Jun, and transactivation of AP-1 (32, 36). Constitutively active Rac1 is sufficient to induce T cell development even in the absence of a functional pre-TCR (35). The fact that deletion of Vav, a GEF for Rac1 (37), impairs cytoskeletal actin organization and T cell activation (38, 39) provides evidence for the actin requirement for lymphocyte signal transduction.

In epithelial cells, ARF6-GTP facilitates disassembly of the adherens junctions by decreasing Rac1-GTP levels, but also by promoting clathrin-dependent internalization of E-cadherin to early endosomes (27). In a similar fashion, in T cells ARF6-GTP may mediate internalization of critical transmembrane proteins that participate in the immune response. In this respect, it is noteworthy that HIV-1 Nef down-regulates MHC class I via the ARF6 endocytic pathway, providing a mechanism for HIV-1 immunoevasion (40). Further studies will determine the precise mechanism by which ARF6-GTP inhibits activation of T cells.

Regardless of the mechanism by which it regulates T cell activation, the distinct intracellular localization of ARF6 provides a detectable phenotypic appearance of the anergic cells. Finally, the observation that T cells with the intracellular ARF6 distribution pattern of anergic T cell clones are detected among primary peripheral blood T lymphocytes suggests that anergic T cells may exist in vivo, and their detection may be feasible.

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