Rho GTPases link cytoskeletal rearrangements and activation processes induced via the tetraspanin CD82 in T lymphocytes

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Summary

Activation of T lymphocytes requires the engagement of the T-cell receptor and costimulation molecules through cell-to-cell contacts. The tetraspanin CD82 has previously been shown to act as a cytoskeleton-dependent costimulation molecule. We show here that CD82 engagement leads to the tyrosine phosphorylation and association of both the Rho GTPases guanosine exchange factor Vav1 and adapter protein SLP76, suggesting that Rho GTPases participate in CD82 signaling. Indeed, broad inactivation of all Rho GTPases, or a specific blockade of RhoA, Rac1 or Cdc42, inhibited the morphological changes linked to CD82 engagement but failed to modulate the inducible association of CD82 with the actin network. Rho GTPase inactivation, as well as actin depolymerization, reduced the ability of CD82 to phosphorylate Vav and SLP76 and to potentiate the phosphorylation of two early TcR signaling intermediates: the tyrosine kinases ZAP70 and membrane adapter LAT. Taken together, this suggests that an amplification loop, via early Vav and SLP76 phosphorylations and Rho-GTPases activation, is initiated by CD82 association with the cytoskeleton, which permits cytoskeletal rearrangements and costimulatory activity. Moreover, the involvement of CD82 in the formation of the immunological synapse is strongly suggested by its accumulation at the site of TcR engagement. This novel link between a tetraspanin and the Rho GTPase cascade could explain why tetraspanins, which are known to form heterocomplexes, are involved in cell activation, adhesion, growth and metastasis.

Key words: Tetraspanin, TM4SF, CD82, Rho GTPases, Cytoskeleton, T lymphocytes, Costimulation

Introduction

During their maturation, lymphocytes migrate through vessel walls, which are home to lymphoid organs, interact with antigen-presenting cells and adhere to target cells. These features suggest that all stages of maturation and activation are dependent upon functional cytoskeleton networks. Indeed, T-cell activation requires contact between the T-cell receptor (TcR) and major histocompatibility complexes (MHC) expressed on antigen-presenting cells (APCs). However, the complete activation that leads to gene transcription (cytokine production and proliferation) also requires a second signal that is mediated by accessory molecules. Recent data indicate that although the T cells and APCs are in contact, the cells reorganize their membrane receptors to form 'supramolecular activation clusters' (SMACs) or 'immunological synapses' that contain the antigen receptor, coreceptors, adhesion and signaling molecules (Monks et al., 1998; Penninger and Crabtree, 1999). In T cells, this spatial reorganization requires an active cytoskeleton network (Wulfing and Davis, 1998) and is involved in the integration of the various signaling pathways that lead to sustained activation and gene transcription.

The cytoskeletal changes are governed by the interplay between various intracellular molecules, such as the Rho family of GTP-binding proteins, which is crucial. In fibroblasts, these proteins regulate various cytoskeletal rearrangements: RhoA controls stress fiber formation and the attachment of bundles of actin and myosin filaments to the cell membrane at points of focal adhesion, where integrin clusters are observed. Rac regulates the formation of membrane ruffles, whereas Cdc42 activation is linked to the extension of filopodia and microspikes. These GTPases are also involved in gene transcription and seem to function sequentially: Cdc42 stimulates Rac activity, which then activates Rho A (Hall, 1998). In T lymphocytes, these proteins are involved in TcR- (van Leeuwen and Samelson, 1999), IL-2- (Arrieumerlou et al., 1998) or CD28-mediated (Kaga et al., 1998) activation. The activity of these GTPases is regulated by the differential binding of guanosine nucleotides. They can be switched to their active state (GTP-linked) by regulatory molecules known as guanosine exchange factors (GEFs). In hematopoietic cells, the main activators of Rho/Rac proteins during signal-transduction processes are thought to be the Vav family of proteins (Bustelo, 1996). Their GEF activity is regulated by tyrosine phosphorylation, which also modulates their association with tyrosine kinases and adapter molecules such as SLP76. SLP76 phosphorylation is also involved in appropriate signal transduction, leading to IL-2 production, and phosphorylated SLP76 interacts with other adapter proteins and could serve

as a scaffold to colocalize the GEF activity of Vav with the Rho GTPases effector proteins.

The tetraspanin family (also known as TM4SF: transmembrane-4 super family) of proteins were first identified in leukocytes, but proteins of this family are now known to be widely expressed in a variety of cells and tissues in many species. These proteins are involved in cell adhesion and motility: the expression of tetraspanins, such as CD9, CD63 and CD82, regulates the metastatic potential of some tumors (Adachi et al., 1998; Hemler et al., 1996; Adachi et al., 1997), and anti-tetraspanin mAbs induce homotypic aggregation and modulate cellular migration (Cao et al., 1997; Domanico et al., 1997; Lagaudriere-Gesbert et al., 1997a; Shaw et al., 1995; Yanez-Mo et al., 1998; Maecker et al., 1997). In addition to their role in adhesion, several studies report the ability of the anti-tetraspanin mAbs to deliver coactivation signals to leukocytes (Tai et al., 1996; Shibagaki et al., 1998; Levy et al., 1998; Worthington et al., 1990; Olweus et al., 1993; Roberts et al., 1995; Lebel-Binay et al., 1995a; Lebel-Binay et al., 1995b). No physiological ligand or counter-receptor has been reported for any of these molecules, and this suggests that tetraspanins may function as coreceptor molecules. Indeed, tetraspanins have been shown to be associated with other tetraspanins in multimeric complexes that also include β1 integrins (Mannion et al., 1996; Radford et al., 1996; Rubinstein et al., 1996; Serru et al., 1999; Tachibana et al., 1997) and MHC molecules (Damjanovich et al., 1998; Lagaudriere-Gesbert et al., 1997b; Szollosi et al., 1996). In T lymphocytes, both CD81 and CD82 are associated with CD4 or CD8 (Imai et al., 1995).

We previously showed that, in T lymphocytes, signaling through the tetraspanin CD82 results in stable adhesion and growth of membrane extensions and acts synergistically with TcR engagement to induce T-cell activation (Lagaudriere-Gesbert et al., 1998). Here, we have used various toxins and dominant-negative mutants to investigate whether these processes depend on the Rho family of GTPases. We found that functional GTPases were required for all the events triggered by CD82 but not for the association of the tetraspanin with the cytoskeletal matrix. However, Toxin B and other cytoskeleton-destabilizing agents diminished the CD82-induced activation of Vav and SLP76 as well as its synergistic activation of early TcR intermediates: the tyrosine kinase ZAP70 and the linker of T-cell activation (LAT). These findings indicate that an amplification loop, involving Rho-GTPases and cytoskeleton mobilization, is initiated by CD82-induced self-association with cytoskeletal components. As we observed that GFP-tagged CD82 accumulated at the site of TcR engagement, we conclude that this cytoskeleton-dependent amplification loop participates in the formation of the immunogical synapse and in the CD82 costimulatory effects.

Materials and Methods

Cell culture, antibodies and reagents

ATCC Jurkat cells and the variant expressing SV40 T antigen, Jurkat TAg, were maintained in culture in RPMI 1640 Glutamax (Life Technologies) supplemented with 7% FCS.

Stimulating mAbs used were CD82 (purified γC11, mouse IgG1 (Lebel-Binay et al., 1994), purified anti-CD3 (OKT3, mouse IgG2a, the kind gift of Orthoclone), anti-CD9 (syb1, mouse IgG1, kind gift of E. Rubinstein), anti-CD81 (Z81, mouse IgG1, kind gift of F. Lanza) and control mouse IgG1 (Sigma). Anti-Cdc42 and anti-Rac mAbs were purchased from Santa Cruz Biotechnology, anti-phosphorylated tyrosine (4G10), anti-LAT (rabbit polyclonal IgG), anti-ZAP-70 (whole rabbit anti-serum), anti-Vav1 (rabbit polyclonal IgG and mouse monoclonal ascite) and anti-SLP76 (sheep polyclonal IgG) were purchased from Upstate Biotechnology and anti-c-myc mAb (9E10, mouse IgG1) from Boehringer Mannheim. Sheep HRP anti-mouse Ig and donkey HRP anti-rabbit Ig were purchased from Amersham, and goat PE anti-mouse IgG, rabbit HRP anti-sheep and goat HRP anti-mouse IgG2b were from Southern Biotechnology. C3 exoenzyme (Popoff et al., 1991) and Toxin B (Hofmann et al., 1997) were prepared as previously described.

The Toxin-B-treated cells were incubated for two hours at 37°C with 0.1μg/ml Toxin B (RPMI + 7% FCS). This produced total inhibition of the PMA-induced homotypic aggregation without affecting cell viability (trypan blue exclusion showed more than 95% cell viability after 10 hours in culture). C3 exoenzyme treatment was performed by electroporation (960 μF, 320 V, in electroporation buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4/KH_2PO_4, 2 mM EGTA, 5 mM $MgCl_2$, 2 mM ATP, 5 mM glutathione, 25 mM HEPES)) supplemented with 1 mg/ml lucifer yellow (Sigma) and 20 μg/ml C3 exoenzyme. After two hours at 37°C, live cells were collected by centrifugation (800 ***g***) for five minutes in 10 ml FCS.

Polystyrene microbeads (Polysciences) were coated by incubating for one hour with anti-CD3 (OKT3, 20 μg/ml) at room temperature and then washed twice in PBS and saturated for 30 minutes at room temperature by incubating with PBS + 2% BSA.

Flow cytometry

For surface labeling, 3.10^5 cells were incubated for 30 minutes in 50 μl of saturating dilution of the different mAbs in PBS + 1% FCS. After washing thoroughly, the cells were incubated for 30 minutes with goat anti-mouse PE mAb (1/250), washed and fixed for 30 minutes in 1% PFA before being transferred to Falcon tubes in 400 μl of PBS. The same protocol was used to produce intracellular labeling of the cells, except that cells were fixed for 30 minutes in 4% PFA, washed and treated for 15 minutes in glycine buffer (Glycine 0.1 M in PBS) before being permeabilized with 0.05% saponin in PBS supplemented with 0.2% BSA and labeling with mAbs. PBS supplemented with saponin and BSA was used for all the labeling and washing steps.

Plasmids and transfections

mycN17Rac1 and pRK5mycN17Cdc42 were kindly donated by D. Cantrell and A. Hall, respectively. pEYFP-C1 and pEGFP were purchased from Clontech. YFP-CD82 was constructed by *Hind*III + *Xba*I digestion. These plasmids were transiently expressed in Jurkat-TAg cells by electroporation. Briefly, Jurkat-TAg cells were adjusted to 10^7 cells/ml in transfection buffer (RPMI + 20% FCS). After 15 minutes at room temperature in the presence of 15 μg of N17-Rho-GTPases or empty vector and 3 μg of GFP-encoding plasmid, 500 μl of cell suspension was subjected to an electric pulse (320V, 960 μFa) in a BioRad electroporator and re-suspended in 5 ml of culture medium. After 24 hours at 37°C, live cells were collected by Ficoll gradient, and protein expression was analyzed by flow cytometry. Maximum expression was reached two days post transfection, and almost all the GFP expressing cells (>95%) also expressed the c-myc epitope. However, N17 mutated proteins were only expressed in two thirds (N17Rac) and one third (Cdc42) of the cells, respectively. Consequently, GFP-expressing cells were sorted (ELITE, Coulter) before most of the experiments. The same protocol was used for YFP-CD82 transfection, except that GFP-encoding plasmid was not added.

YFP-CD82-expressing cells were mixed with antibody-coated beads without previous sorting.

Cell stimulation

The cells were usually stimulated by being cultured on culture plates coated with anti-CD3 (OKT3) and/or anti-CD82 (γC11) mAbs. The coating was performed as followed: mAbs, adjusted to the appropriate concentration in coating buffer (11 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3), were incubated overnight at 4°C on various culture supports. To avoid variations in coating efficiency on the different supports, the doses of mAbs used were well above those required for saturation. After one wash with PBS + 1% FCS, the supports were saturated for 30 minutes at 37°C with PBS + 10% FCS. For tyrosine-phosphorylation and adhesion assays, cells were plated on antibody-coated 96-well microplates in RPMI + 7% FCS at 200×10^3 cells per well. For the tyrosine-phosphorylation analysis of immunoprecipitates, cells were seeded at 20×10^6 cells in 5 ml on 10-cm Petri dishes. For the cytoskeleton rearrangement assays, cells (0.5-2×10^6/ml in 400 µl RPMI 7% FCS) were plated on antibody-coated Thermanox slides (Nunc), which had been pre-cleaned by serial rinses in ethanol, in the bottom of four well Multidishes on sterilized Nunclon plates (Nunc, Inc. Naperville, IL). Stimulation with anti-CD3-coated beads was performed by incubating 2×10^5 beads with 2×10^5 Jurkat cells transfected 48 hours before with YFP-CD82. After 15 minutes of contact at 37°C, the cells were plated on microscope slides, fixed with Fluoromount and analyzed on a Nikon TE300 equipped with Metaview software (Universal Imaging Corporation, Downingtown, PA).

Cytoskeleton rearrangement assays

Cells (0.5-1×10^6/ml in RPMI + 7% FCS) were layered over the coated Thermanox slides and incubated for various times at 37°C. Any non-adherent cells were removed by gentle washing with PBS. The remaining adherent cells were fixed with 4% paraformaldehyde, 2% sucrose in PBS for 30 minutes at room temperature. After permeabilization (one minute at 0°C with 0.1% Triton X-100, 20 mM Hepes, pH 7.4, 300 mM sucrose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 150 mM NaCl), F-actin was labeled by exposure to 2U/ml rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR) for 20 minutes at room temperature. Slides were mounted in Fluoromount (Southern Biotechnology) to prevent photobleaching.

Cells were analyzed by confocal fluorescence microscopy (Bio-Rad MRC1000, Bio-Rad Laboratories, Hercules, CA) equipped with a digital Diaphot 200 system. Digital pictures were analyzed using Comos software and processed using Adobe Photoshop.

Immunoprecipitation and tyrosine-phosphorylation assays

For direct tyrosine-phosphorylation assays, pretreated or untreated cells were washed twice in PBS, resuspended at 5×10^6 cells/ml in RPMI 1640 glutamax supplemented with 0.1% FCS, and 40 µl of this suspension was added to mAb-coated microtiter wells. The cells were incubated at 37°C and lysed at various time intervals by adding 50 µl of a 2× stock of reducing SDS-sample buffer. The samples were boiled for five minutes and the proteins separated by 10% SDS-PAGE. For immunoprecipitations of Vav, SLP76, LAT and ZAP70, proteins from 20×10^6 cells were solubilized in 1 ml of lysis buffer (TRIS pH 7.5, 0.5 mM NP40, 1 mM Na_3VO_4, 1 mM NaF, 1 mM EDTA and protease inhibitors (Bohringer tablets without EDTA) for one hour at 4°C. Protein concentrations were adjusted to 1 mg/ml and 500 µl of each sample were incubated overnight at 4°C with 4 µg of specific mAbs. Immune complexes were collected using protein G Sepharose beads (Fastflow- Sigma, two hours at 4°C) and washed five times with lysis buffer. After adding 60 µl of SDS sample and boiling for 10 minutes, 30 µl of each sample were separated by 7.5% (forVav, SLP76 and

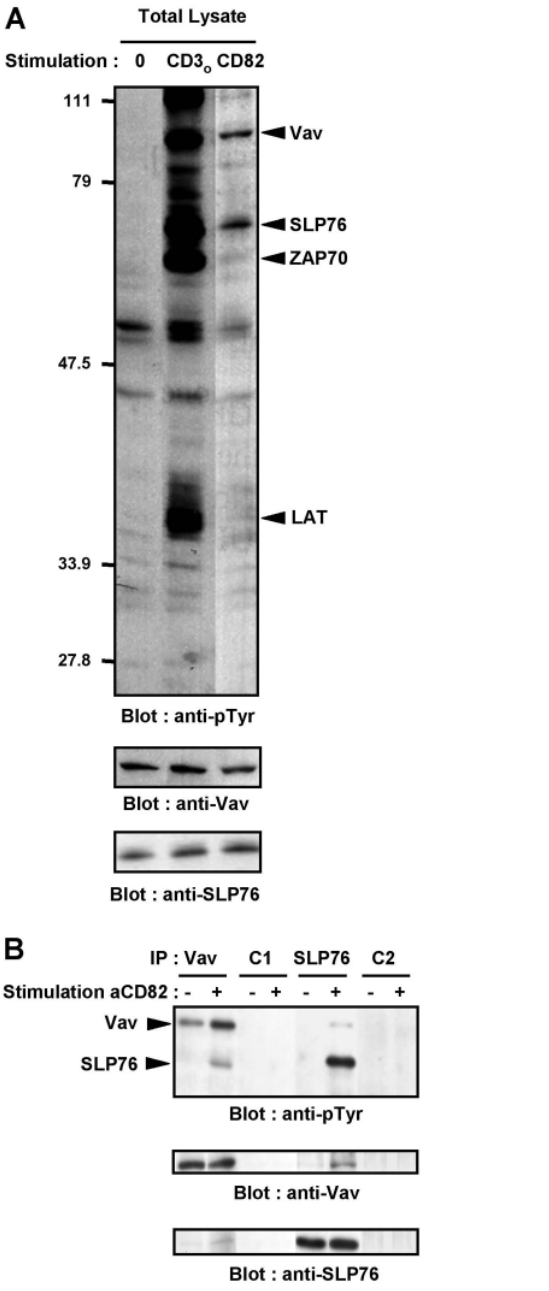

Fig. 1. CD82 engagement triggers tyrosine phosphorylation of Vav and SLP76. Jurkat cells were cultured for 10 minutes at 37°C on uncoated plates or plates coated with anti-CD82 (γC11, 50 µg/ml). (A) upper panel, after lysis (5×10^6 cells) in SDS sample buffer and SDS-PAGE analysis, tyrosine-phosphorylated proteins were analyzed by immunoblotting with 4G10 antibody. Stimulation, lane 1; no mAb, lane 2; anti-$CD3_o$ (optimal OKT3, 20 µg/ml); anti-CD82 (γC11, 50 µg/ml). (A) Middle and lower panels, after dehybridization, the same membrane was re-probed with anti-Vav1 rabbit (middle panel) or anti-SLP76 sheep antisera (lower panel). (B) After solubilization (20×10^6 cells) in lysis buffer, 500 µg of each samples were immunoprecipitated with anti-Vav1 (rabbit polyclonal IgG), anti-SLP76 (sheep polyclonal IgG.) or control antibodies (C1, rabbit IgG; C2, sheep IgG). Immunoprecipitates were resolved by 7.5% SDS-PAGE and blotted with the anti-phosphotyrosine mAb 4G10 (upper panel), monoclonal mouse anti-Vav1 (middle panel) or polyclonal sheep anti-SLP76 (lower panel). The data shown are representative of three independent experiments.

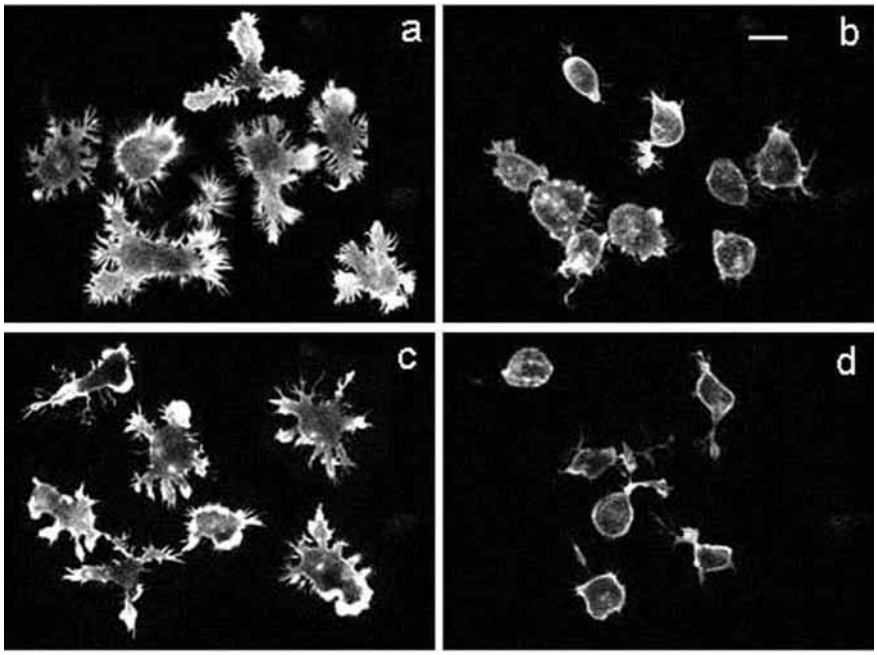

Fig. 2. Rho-GTPase-inactivation inhibits CD82-induced morphological changes. (a,b) Untreated Jurkat cells (a) and Jurkat cells treated (b) for two hours with 0.1 μg/ml of Toxin B from *C. difficile* were cultured for one hour at 37°C, on anti-CD82-coated plates (γC11, 50 μg/ml). (c,d) Jurkat cells were cultured at 37°C on anti-CD82-coated plates (γC11, 50 μg/ml). After one hour, 0.1 μg/ml of Toxin B was added (d), or not (c), to the culture medium and the cells were cultured for another two hours on anti-CD82 coated plates. After removing non-adherent cells, Jurkat cells were fixed, permeabilized and stained with rhodamine-phalloidin. The data shown are representative of five independent experiments. Bar represents 10 μm.

ZAP70) or 12% (for LAT) SDS-PAGE. To detect tyrosine phosphorylation, the proteins were electrophoretically transferred onto a polyvylidene difluoride membrane (Dupont-New England Nuclear). Anti-phosphotyrosine immunoblotting was performed using mAb 4G10 (UBI) followed by horseradish-peroxidase-conjugated goat anti-mouse immunoglobulin G2b (GaM-IgG2b, Southern Biotechnology). Immunoreactive proteins were visualized using the ECL Western Blotting Detection Kit (Amersham).

Separation of soluble and insoluble fractions

Cells were washed twice in PBS, re-suspended at 10^6 cells/ml in complete medium, and 2 ml of this suspension were added to plastic dishes coated or not with mAbs. The cells were incubated for various times at 37°C cells, solubilized with 0.5% NP-40 in a Tris-buffered saline solution (TBS: 150 mM NaCl, 10 mM Tris (pH 7.3)), containing protease inhibitors (Boehringer cocktail inhibitors tablets) for one hour on ice and centrifuged at 10,000 ***g*** for 15 minutes at 4°C to pellet the insoluble material. Prior to separation on SDS-PAGE, the insoluble pellet was resolubilized by boiling in non-reducing SDS-sample buffer. Proteins were fractionated by 12% SDS-PAGE and transferred onto polyvylidene difluoride membranes (Dupont-New England Nuclear). CD82 was detected using γC11 mAb followed by horseradish-peroxidase-conjugated SaM immunoglobulin (Amersham) and an ECL Western Blotting Detection Kit (Amersham). Kodak films were analyzed and quantified by visible illumination on Bioprofil gel analyzer

Results

CD82 triggering induces Vav1 and SLP76 tyrosine phosphorylations

CD82 had previously been shown to deliver signals to T cells that lead to drastic morphological changes. In an attempt to elucidate the CD82 signaling cascade, we analyzed the primary tyrosine-phosphorylation events. We aimed to establish the pattern of tyrosine-phosphorylated proteins induced by culturing Jurkat cells on anti-CD82 coated plates compared to the pattern induced by saturating doses of immobilized anti-CD3 mAbs. To avoid variations in coating efficiency between the various culture plates used during this study, over-saturating doses of antibody were used. However, coating culture plates with 15-20 μg/ml of γc11 (anti-CD82 mAbs) or 10μg of OKT3 (anti-CD3 mAbs) was generally sufficient to produce the maximum functional effects.

Among the various proteins that were phosphorylated by optimal anti-CD3 stimulation (Fig. 1A, lane 2 aCD3o), two main proteins with bands at 90-100 and 75-80 kDa were also induced by CD82 engagement (Fig. 1A, lane 3, aCD82). These bands comigrated with Vav1 and SLP76 respectively (Fig. 1A, middle and lower panels). To formally identify these two molecules, which are known to be involved in the Rho GTPase activation pathway, immunoprecipitation experiments with

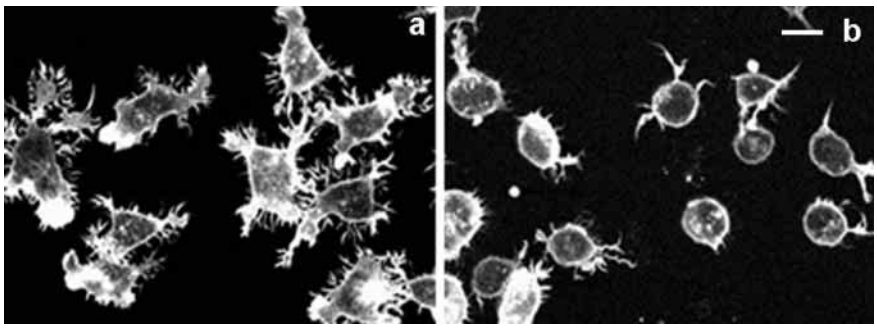

Fig. 3. Inactivation of RhoA inhibits CD82-induced morphological changes. Jurkat cells were electroporated with Lucifer Yellow (1 mg/ml) with or without 20 μg/ml C3 exoenzyme from *C. botulinum.* After two hours in culture, cells were tested for their response to CD82 stimulation, as described in the legend of Figure 1. (a) Jurkat cells electroporated with Lucifer Yellow alone. (b) Cells electroporated with both Lucifer Yellow and C3 exoenzyme. The data shown are representative of three independent experiments. Bar represents 10 μm.

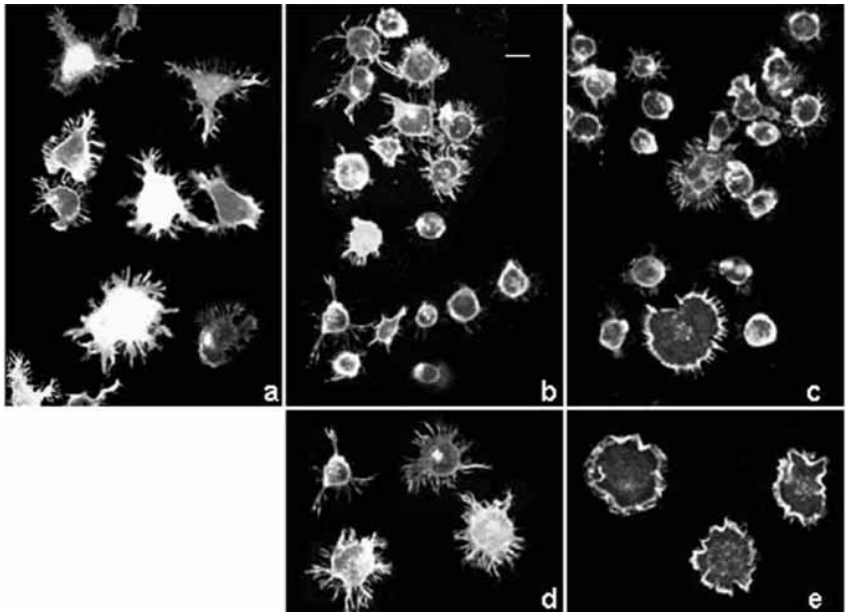

Fig. 4. Expression of N17Rac1 or N17Cdc42 inhibits most of the CD82-induced morphological changes but different cytoskeletal rearrangements still occur. Jurkat cells were transfected with 3 μg GFP and 15 μg of empty vector (a), c-myc-tagged N17Rac1 (b,d) or N17Cdc42 (c,e) as described in Materials and Methods and sorted by flow cytometry by their GFP expression after 48 hours in culture. Cells were stimulated for two hours by culture on anti-CD82-coated plates and assayed for morphological changes as described in the legend of Fig. 1. The data shown are representative of three independent experiments. Bar represents 10 μm.

specific mAbs were performed. As shown in Fig. 1B (upper panel), immunoprecipitation of Jurkat cell lysates with Vav-1-specific antibodies showed that the phosphorylation of Vav-1 was markedly increased by CD82 triggering (Fig. 1B, compare lane 1 and 2, upper band). Interestingly, CD82 triggering led Vav-1 to coprecipitate a 75 kDa tyrosine-phosphorylated protein, which suggests that activated Vav-1 may be associated with SLP76. The CD82-induced association of Vav-1 with SLP76 was confirmed by immunoblotting Vav-1 immunoprecipitates with anti-SLP76-specific antibodies (Fig. 1B, lower panel) and by reciprocal experiments. Two bands, migrating at 95-100 and 75-80 kDa respectively, were precipitated by anti-SLP76 antibodies (Fig. 1B, upper panel, lane 6 compared to lane 5) and were identified by immunoblotting as Vav-1 and SLP76 (Fig. 1B, middle and lower panels).

These experiments demonstrate that CD82 engagement induces activation of the Rho-specific guanosine exchange factor Vav and thr adapter molecule SLP76 and triggers their association.

Morphological changes induced by CD82 are dynamically dependent upon functional Rho GTPases

The observation that CD82 engagement triggers the tyrosine phosphorylation of Vav1, a specific guanine exchange factor of the Rho family GTPases, and its association with the adaptor protein SLP76, led us to investigate whether the CD82-signaling pathway involves the Rho GTPases. We treated Jurkat cells with *C. difficile* Toxin B, an enzyme that uses UDP-glucose as a substrate to inactivate Rho, Rac and Cdc42 (Just et al., 1995). Preliminary experiments revealed that 0.1 μg/ml of Toxin B was sufficient to inhibit the phorbol-ester-induced homotypic aggregation of Jurkat cells without affecting their viability for up to 24 hours. After two hours of pretreatment with, or without, Toxin B, Jurkat cells were cultured on anti-CD82-coated plates for one hour, and cytoskeletal alterations were visualized by actin staining.

Fifteen minutes of culture on anti-CD82coated plates was enough to induce the adhesion and spreading of the untreated Jurkat cells and to increase the area in contact with the culture plate. After one hour, the untreated cells developed typical membrane extensions, such as pseudopodia, lamellipodia and filopodia, in which dense actin structures were observed (Fig. 2a,c). Toxin B pretreatment drastically inhibited the CD82-induced cell adhesion. Furthermore, the remaining adherent cells did not spread but retained their spherical shape (Fig. 2b). After culture on control antibodies, anti-CD3 (OKT3) or anti-MHC-I (W6.32), adhesion could be observed albeit weakly and transiently, and the cells never developed the CD82-induced typical membrane extensions (data not shown) (Lagaudriere-Gesbert et al., 1998). In contrast, culturing Jurkat cells on other anti-tetraspanin antibodies led to reduced but typical morphological changes (Lagaudriere-Gesbert et al., 1997a).

We had previously shown that cytochalasin E, an inhibitor of actin polymerization, impairs the CD82-induced development of membrane extensions but does not affect pre-established morphological changes induced by CD82. In the present work, we wondered whether these pre-established morphological changes were sensitive to Rho GTPase inactivation. Jurkat cells, which were cultured for one hour on immobilized anti-CD82 mAbs and which underwent morphological changes, were further incubated with or without Toxin B for two hours. The CD82-induced morphological changes were extremely stable, so that after two hours, untreated control Jurkat cells remained strongly adherent and exhibited a large number of various membrane extensions (Fig. 2c). In contrast, two hours after adding Toxin B, all the membrane extensions had retracted and the Jurkat cells had reverted to their normal shape (Fig. 2d).

These findings indicate that the CD82-induced morphological changes are dynamic processes that require functional Rho GTPases for their initiation and maintenance.

Active Rho A, Cdc42 and Rac are involved in the CD82-induced morphological changes

We next examined the specific role of each of the GTPases in

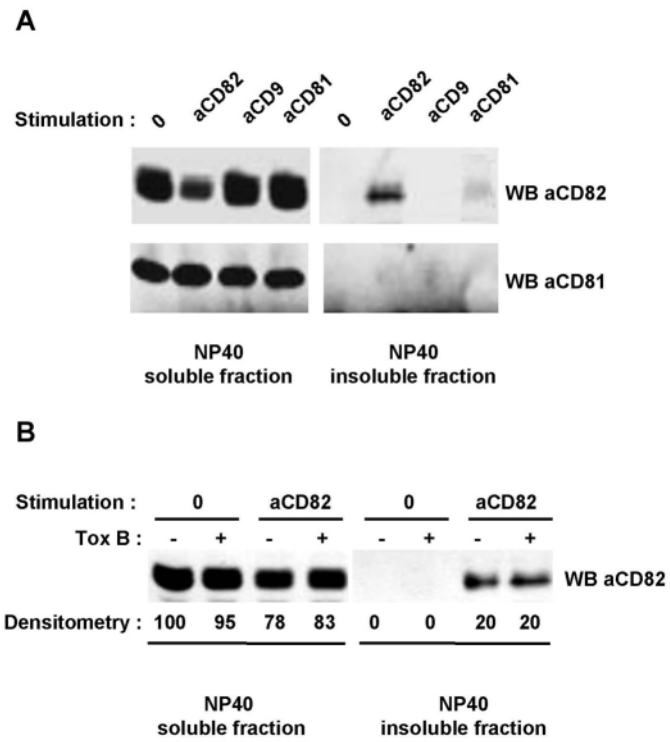

Fig. 5. CD82-specific translocation to the detergent insoluble fraction is independent of Rho GTPases. Jurkat cells, treated with 0.1 μg/ml Toxin B for two hours or untreated, were cultured for 15 minutes at 37°C on antibody-coated plates (anti-CD82: γc11, 50 μg/ml; anti-CD9:Syb1, 50 μg/ml; anti-CD81: Z81, 50 μg/ml). After SDS-PAGE analysis, proteins of the soluble (equivalent to 10^5 cells per lane) and insoluble fractions (equivalent to 2×10^6 cells per lane) were probed by immunoblotting with anti-CD82 or anti-CD81 mAbs. (A) Stimulation: lane 1, 0; lane 2, anti-CD82; lane 3, anti-CD9; lane 4, anti-CD81. The left and right panels represent the soluble and insoluble fractions respectively. The lower and upper panels represent western blots with anti-CD82 and anti-CD81 antibodies, respectively. (B) Stimulation: lanes 1-2, no mAb; lanes 3-4, anti-CD82 mAbs (γC11, 50 μg/ml). Treatment: lanes 1, 3, no addition; lanes 2, 4, 0.1 μg/ml Toxin B. Left panel and right panels represent soluble and insoluble fractions, respectively. The data are representative of three independent experiments.

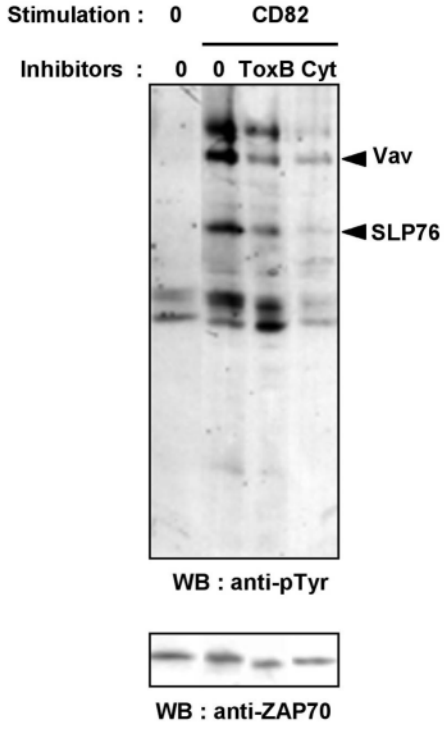

Fig. 6. Rho GTPases and cytoskeleton participate in Vav and SLP76 phosphorylation. Jurkat cells, treated with 0.1 μg/ml of Toxin B for two hours or with cytochalasin for 30 minutes or left untreated were cultured for 15 minutes at 37°C on plates coated with anti-CD82 (γC11, 50 μg/ml) or left uncoated. After lysis (5×10^6 cells) in SDS sample buffer, SDS-PAGE analysis was performed. Upper panel, immunoblot with anti-phosphotyrosine antibodies (4G10). Stimulation: lanes 1, no mAb; lane 2-4, anti-CD82. Cell treatment: lanes 1-2, untreated cells; lanes 3, Toxin B; lane 4, cytochalasin. Lower panel: after dehybridization, the same membrane was re-probed with anti-ZAP70 rabbit antisera.

the morphological changes triggered by CD82 engagement. First we used C3 exoenzyme from *C. botulinum*, which selectively ADP-ribosylates Rho (Aktories, 1994), inhibiting its membrane translocation and effector recognition. In the absence of a functional receptor for the toxin, exoenzyme C3 was electroporated into the cells together with an exclusion dye, Lucifer Yellow, which made it possible to identify the permeabilized cells. Two hours post electroporation, live cells were stimulated by culture on anti-CD82-coated plates before morphological analysis. As shown on Fig. 3a, Lucifer-Yellow-electroporated Jurkat cells spread and developed membrane extensions, whereas exoenzyme-C3-treated cells (Fig. 3b) exhibited a round shape similar to that observed following exposure to Toxin B.

As there is no known toxin that specifically inactivates Rac or Cdc42, these GTPases were inhibited by transient expression of c-myc-tagged dominant inhibitory mutants instead. The N17 mutation induces both Rac1 and Cdc42 to interact with their guanine exchange factor (GEF) and leads to their inactivation. To facilitate the detection of transfected cells, cotransfections of N17-mutated Rac, or Cdc42, and GFP-encoded plasmids were performed. Two days post transfection, cells that expressed the mutated GTPases were enriched by sorting by their expression of the green fluorescent protein. After two hours culture on anti-CD82 coated plates, actin staining was used to visualize any morphological changes.

Expression of N17Rac or N17Cdc42 did not alter the morphology of unstimulated Jurkat cells (not shown). In contrast with cells transfected with GFP and empty vector, which displayed the usual morphologic changes in response to CD82 engagement (Fig. 4a), many cells expressing N17Rac (Fig. 4b) or N17Cdc42 (Fig. 4c) were not adherent. A large fraction of the remaining adherent cells did not develop any morphological change, but presented phenotypes similar to those observed in response to Toxin B or exoenzyme C3 treatment. However, a small fraction of the adherent cells did develop specific morphological changes. Some N17Rac1-expressing cells developed numerous filopodia (Fig. 4d), but lamellipodia were never observed. Those expressing N17Cdc42 showed the opposite phenotypic outcome, some of the cells were widely spread and displayed very large protrusions such as lamellipodia (Fig. 4c,e) but never developed filopodia.

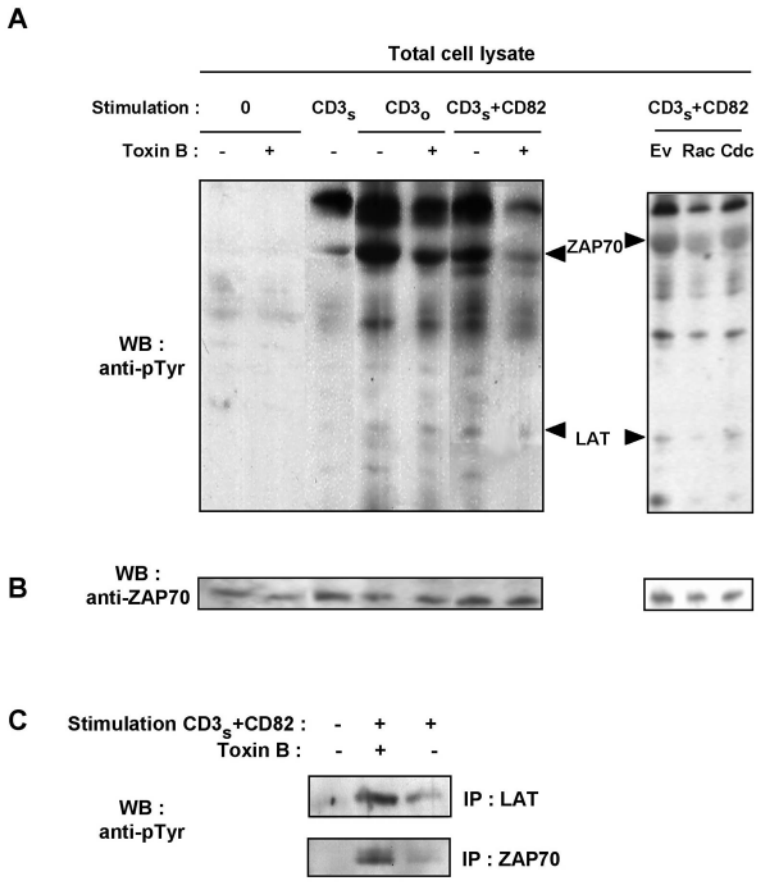

Fig. 7. Inactivation of all the Rho GTPases inhibits CD82 cosignaling activity. (A,B) Jurkat cells (5×10^6 cells) were cultured for 15 minutes at 37°C on antibody-coated or non-coated plates. After lysis in SDS sample buffer, SDS-PAGE analysis was performed. (A) An immunoblot with anti-phosphotyrosine antibodies 4G10. Left panel: cells treated with (lanes 2, 5 and 7) or without (lanes 1, 3, 4 and 6), 0.1 μg/ml Toxin B for two hours. Stimulation: lanes 1-2, no mAb; lane 3, suboptimal doses of anti-CD3 (OKT3: 0.5 μg/ml); lanes 4-5, optimal doses of anti-CD3 (OKT3: 20 μg/ml); lanes 6-7, anti-CD82 and suboptimal doses of anti-CD3 (γC11: 50 μg/ml, OKT3: 0.5 μg/ml). Right panel: before stimulation Jurkat cells were transfected with 3 μg/ml GFP vector and 15 μg/ml of N17Rho GTPases or empty vector and sorted by their levels of GFP expression after 48 hours in culture. (B) After dehybridization, the same membrane was reprobed with anti-ZAP70 rabbit antiserum. (C) Jurkat cells (20×10^6 cells), treated or not with 0.1 μg/ml toxin B for two hours, were cultured for 15 minutes at 37°C on antibody-coated plates. After solubilization in lysis buffer, 500 μg proteins of each sample were immuno-precipitated with anti-ZAP70 or anti-LAT rabbit antisera. Immunoprecipitates were resolved by 7.5% (for ZAP) or 12% (for LAT) SDS-PAGE and blotted with the anti-phosphotyrosine mAb 4G10. The data shown are representative of four independent experiments.

These experiments indicate that Rho, Rac1 and Cdc42 are involved in CD82-induced cytoskeleton mobilization. The specific morphological changes observed in the context of the expression of each dominant-negative GTPase indicate that in T lymphocytes, the expression of one dominant-negative GTPase mimics the effect of the other (Moorman et al., 1999) and confirm that in T cells, as in other cell types, Rac1 governs the development of lamellipodia and membrane ruffling, whereas Cdc42 is mainly involved in filopodium formation. Complete inhibition of morphological change was only observed in the context of Rho inactivation. This specific effect could reflect the ability of exoenzyme C3 to inactivate other upstream GTPases, rather than reflecting true functional discrepancies between Rho A and Cdc42 or Rac1.

Specific and Rho GTPase independent association of CD82 with the cytoskeleton

We had previously observed that a fraction of CD82 is associated with the insoluble cell pellet in response to CD82 engagement (Lagaudriere-Gesbert et al., 1998). Since tetraspanins CD9, CD81 and CD82 were shown to associate with each others and to share biochemical properties as well as signaling functions (Lagaudriere-Gesbert et al., 1998), we compared the abilities of CD9, CD81 and CD82 to induce their own association or that of other tetraspanins with insoluble fractions. Jurkat T cells, cultured for 15 minutes on anti-tetraspanin mAbs, were solubilized for one hour on ice with 0.5% NP-40. After fractionation of the soluble and insoluble material, SDS-PAGE and immunoblotting using anti-tetraspanin mAbs were performed. As shown in Fig. 5A (upper panel) only CD82 could be recovered in the insoluble fractions whatever stimulation was used. A very large amount of CD82 was translocated from the soluble to the insoluble fractions upon CD82 engagement (Fig. 5A, upper panel, soluble and insoluble fractions, lanes 2); however a small amount of CD82 was also recovered from the insoluble pellet upon engagement of CD81 (insoluble fractions, upper panel, lane 4) and sometimes after CD9 triggering. In contrast, whatever the tetraspanin engaged, CD81 (Fig. 5A, lower panel) and CD9 (not shown) were never recovered from the insoluble pellet (middle and lower lanes).

To establish the order of the CD82 signaling cascade, we investigated whether CD82 association with the insoluble fractions depends on Rho GTPase activation using Toxin B pretreatment. As shown in Fig. 5B, Toxin B did not modify the amount of CD82 that translocated to the insoluble fraction at any stimulation time (from five minutes to one hour, not shown).

Since, CD82 translocation with the insoluble fraction was strictly dependent on actin polymerization (Lagaudrière-Gesbert et al., 1998) and independent of cholesterol depletion or solubilization (unpublished results), we concluded that among the tetraspanins known to form large multimeric complexes, CD82 has a specific ability to associate with the cytoskeleton. Furthermore, its independence of the activity of the Rho GTPases, suggests that the CD82-cytoskeleton association is one of the primary events of the CD82 signaling cascade.

Cytoskeleton mobilization is part of an amplification loop that enhances CD82-induced Vav and SLP76 phosphorylation

As the CD82 signaling cascade requires an intact cytoskeleton,

we also investigated the functional interplay between CD82 and the cytoskeleton by studying the effects of various cytoskeleton inhibitors on the primary tyrosine-phosphorylation events triggered by CD82. Tyrosine phosphorylations induced by culture on anti-CD82-coated microplates were compared on Toxin B or cytochalasin pretreated Jurkat cells (a strong inhibitor of actin polymerization). As shown in Fig. 6, CD82-induced phosphorylation of both Vav and SLP76 were reduced by pretreatment with cytochalasin or, to a lesser extent, with Toxin B.

These data indicate that CD82 engagement triggers its association with the cytoskeleton and both tyrosine phosphorylation and association of Vav and SLP76. These early events lead to de novo Rho-GTPase-dependent actin polymerization. Such de novo actin polymerization leads not only to morphological rearrangement but also forms part of an amplification loop that contributes to the CD82 signaling cascade by stabilizing and amplifying the CD82-induced Vav and SLP76 phosphorylation.

Rho-GTPase activation is required for CD82 costimulatory activity

We have previously shown that CD82 cooperates with TcR-CD3 complexes to achieve full T-cell activation. To determine whether this interplay also involves Rho GTPases, we used Toxin B pretreatment or expression of dominant-negative N17Cdc42 or N17Rac1 (as described above in) to investigate the impact of Rho GTPase inactivation on primary tyrosine phosphorylations. Control experiments revealed that Toxin B did not modify the IL-2 mRNA induced by optimal stimulation (20 μg of aCD3 + PMA) but totally inhibited the costimulatory activity of CD82 (not shown). This indicated that Toxin B was not toxic at the doses used in these experiments and suggested that cosignaling via CD82 required Rho GTPase activation.

Engagement of CD82 alone induced mainly the tyrosine phosphorylation of two proteins with molecular weights of 95-100 and 75-80 kDa (Vav1 and SLP76, respectively) (Fig. 1A; Fig. 6). In contrast, coengagement of CD82 (Fig. 7A, left panel, CD82+CD3s) combined with a weak TcR signaling (Fig. 7A, left panel, CD3s) to induce strong tyrosine phosphorylation of other proteins, particularly at 70 and 35 kDa, which was not detected with CD82 engagement alone. Similar proteins were tyrosine phosphorylated in response to costimulation (Fig. 7A, left panel, CD82+CD3s) and optimal TcR stimulation (Fig. 7A, left panel, $CD3_o$). The potentiating effect of CD82 on TcR signaling was strongly inhibited when Rho GTPases were inhibited by Toxin B (Fig. 7A, lane 7 compared to lane 6). Using western blotting, two of these proteins could be identified as ZAP70 (70 kDa, Fig. 7B) and LAT (35 kDa) (data not shown). Since these two proteins are essential molecules involved in signaling pathways downstream of the T-cell receptor and are necessary to trigger IL-2 production in T cells (Zhang et al., 1998), immunoprecipitation with specific anti-LAT and anti-ZAP70 antibodies was performed. As expected, a marked reduction in the CD82-induced tyrosine phosphorylation of both ZAP70 and LAT was observed after treatment with Toxin B (Fig. 7C). Inactivation of Rac or Cdc42 also reduced the synergistic effect of CD82 on the tyrosine phosphorylation of various proteins (Fig. 7A, right panel) but did not modify the pattern of tyrosine-phosphorylated proteins in unstimulated cells (not shown). More marked inhibition was observed after the expression of N17Rac1, which may indicate that this GTPase plays a major role in the CD82 costimulatory pathway. However, we cannot rule out the possibility that these effects resulted from lower expression of N17Cdc42 than of N17Rac1. As shown in Fig. 7A (lanes 4-5), Toxin B also partly inhibited the tyrosine phosphorylations induced by optimal CD3/TcR stimulation.

Taken together, these observations indicate that the early tyrosine phosphorylation of the tyrosine kinase ZAP70 and membrane adapter protein LAT, key events in TcR signaling, can be positively modulated by CD82 via mechanisms that involve the cytoskeleton and GTPases of the Rho family.

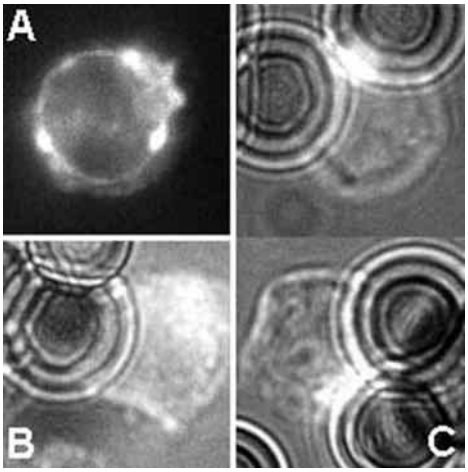

Fig. 8. Surface-expressed YFP-CD82 localized in contact areas with anti-CD3 coated beads. Jurkat cells (2×10^5) were transfected by electroporation with 15 μg of YFP-CD82. After 48 hours in culture, the cells were stimulated, or not (A), by culturing at 37°C with 2×10^5 control IgG2a (B) or anti-CD3-coated (C) beads. After 15 minutes of contact, cells were plated on microscope slides, fixed and analyzed by fluorescent microscopy.

CD82 accumulates at the site of TcR engagement

To address the physiological relevance of the effects induced by aggregation of the molecules using immobilized antibodies more directly, we tried to find out whether engagement of the CD3-TcR complexes modifies the surface distribution of CD82. Anti-CD3 coated microspheres are commonly used as a model for the study of the cellular interactions involved in T-cell activation. Using this system, we analyzed the redistribution of GFP-tagged CD82, overexpressed at the Jurkat cell surface by transient expression. Unstimulated YFP-CD82 expressing cells displayed uniform surface staining (Fig. 8A), whereas YFP-transfected cells exhibited intracellular staining (not shown). As shown in Fig. 8, similar uniform surface staining was observed on the few cells that were in contact with beads coated with an irrelevant antibody (Fig. 8B). In contrast, after 15 minutes of contact with anti-CD3 coated beads, YFP-CD82 molecules were clearly redistributed at contact sites with anti-CD3 coated beads (Fig. 8C).

These findings indicate that, although no ligand has yet been identified for any tetraspanin proteins, the redistribution of the

tetraspanin CD82 toward areas of engaged T-cell receptors strongly supports its functional role in T-cell activation.

Discussion

We have previously shown that CD82 engagement triggers cell adhesion, spreading and morphological changes and contributes to the amplification and stabilization of CD3-TcR signaling on T lymphocytes. In assessing the functional relevance of the effects induced by CD82 aggregation, we showed that CD82 was concentrated in TcR-engaged domains, suggesting that CD82 could be part of the immune synapse that forms rapidly during the cellular interactions involved in T-cell activation. In this study, we analyzed the CD82 signaling pathways in greater detail. We have shown that CD82 triggering induces the tyrosine phosphorylation of the guanosine exchange factor of the Rho GTPases, Vav1, and the cytosol adapter protein, SLP76, as well as the association of these two essential molecules in the Rho GTPase pathway. These findings led us to examine the role of the Rho-GTPases in CD82-mediated events. We were able to show that inhibiting Rho GTPase activity by Toxin B pre-treatment blocks the CD82-induced spreading and development of membrane extensions. Furthermore, in contrast to what is observed in response to treatment with the actin depolymerisating agent cytochalasin, Toxin B can reverse CD82-induced morphological changes. This implies that the morphological changes induced by CD82 are dynamic processes that require functional GTPases for both their initial onset and maintenance. Taken as a whole these findings indicate that Rho family GTPases, through activation by their GEF Vav1, are the main effectors in CD82-induced cytoskeletal rearrangements.

We selectively inhibited Rho, Rac or Cdc42 in Jurkat cells to study the functional role of each protein separately. Inactivation of Rho A, using exoenzyme C3, Rac1 or Cdc42, by expression of their respective dominant-negative forms, inhibited most of the CD82-induced adhesion and morphological changes. However, when the negative dominant of Rac1 or Cdc42 was expressed, some CD82-stimulated cells exhibited particular phenotypes. Fine extensions similar to filopodia were observed in response to N17Rac, whereas some N17Cdc42-expressing cells developed lamellipodia and membrane ruffling but no filopodia. These findings are in agreement with observations in other cell types: Rac1 activation leads mainly to the development of membrane ruffling, whereas Cdc42 is known to be involved in the extension of microspikes and filopodia. The classical cascade (Cdc42 stimulating Rac that in turn activates RhoA) (Nobes and Hall, 1995) could not account for the different phenotypes observed in our experiments. However, the complete inhibition of the morphological changes that was observed when only RhoA was inactivated may result from dynamic processes (Burridge, 1999). The regulatory activity of RhoA could be important in the contractile events required for maintaining stationary cell spreading, and Rac and Cdc42 could be involved in the regulation of early stages of cell spreading (Sanders et al., 1999). In contrast with the experiments described by Moorman and colleagues (Moorman et al., 1999) in which they demonstrated that viral expression of exoenzyme C3 in the leukemic cell line EL4 led to the appearance of transient membrane ruffles and development of microspikes, our results concerned the long-term induction of adhesion and development of membrane extensions by CD82, where Rho A activity may also be required. Alternatively, it is possible that exoenzyme C3 could modify other Rho GTPases (such as Rnd3 and RhoE), which could be involved upstream in the CD82-signaling pathway.

CD82 has an internalization consensus sequence in its the C-terminus. The presence of CD82 in internalization vesicles (unpublished observations) (Escola et al., 1998; Hammond et al., 1998; Kannan et al., 1995), indicates that this site may be functional. In a previous study, we described that, upon engagement, part of CD82 translocates to the detergent insoluble fraction in an F-actin-dependent manner. In the present study, we demonstrate that CD82 is the only tetraspanin highly expressed on T cells that displays this property independently of Rho-GTPase activation. The association of CD82 with insoluble membrane fractions, which peaks within five minutes of activation, was also observed in response to the engagement of other tetraspanins.

Overall, our results suggest that, upon engagement, CD82 associates with the cytoskeleton, which promotes tyrosine phosphorylation and the association of both the guanine exchange factor Vav and adapter protein SLP76. These early events lead to de novo actin polymerization, which depends on Rho GTPase activation. The observation both that Rho GTPase inactivation and cytoskeletal destabilization decreased Vav and SLP76 tyrosine phosphorylation suggests that actin polymerization facilitates CD82-induced Vav and SLP76-phosphorylation. Association between membrane molecules and the cytoskeleton (Pardi et al., 1992; Rozdzial et al., 1995) have been reported to play a crucial role in cell activation. And the signaling cascade that leads to cytoskeleton mobilization has to be intact for T cell activation (Delon et al., 1998; Lowin-Kropf et al., 1998; Penninger and Crabtree, 1999; Valitutti et al., 1995). Recent data demonstrate that TcR-mediated stimulation leads to a cytoskeleton-dependent reorganization of various membrane proteins, including adhesion molecules, in the contact area of T cells and APC (Monks et al., 1998). On the basis of the reciprocal synergy between TcR and CD82 signaling pathways (Lagaudriere-Gesbert et al., 1998) and the correlation between the intensity of the morphological changes induced by various tetraspanins and their co-stimulating abilities (Lagaudriere-Gesbert et al., 1997a), we have already suggested that cytokeletal mobilization may be the main effector in the tetraspanin-mediated adhesion and activation process. The present data reinforce this hypothesis and suggest that CD82 plays a specific role as a result of its ability to associate with the cytoskeleton. TcR signaling involves src-kinase activation, TcR/CD3ζ chain tyrosine phosphorylation and association with the cytoskeleton as well as the recruitment and phosphorylation of the tyrosine kinase ZAP70. Activation of ZAP70 induces the tyrosine phosphorylation of the integral membrane adapter LAT, and this in turn recruits crucial signaling molecules to the membrane. We demonstrate here that the costimulatory effects induced by CD82 on these early TcR signaling events (tyrosine phosphorylation of the early TcR intermediates ZAP70 and LAT) were strongly reduced by Toxin B and, to a lesser extent, by the expression of the dominant-negative form of Rac or Cdc42. Furthermore, this partial inhibition of early activation stages was correlated with total inhibition of the induction of IL-2 mRNA. We found that

Toxin B (or the expression of N17Rac or N17Cdc42) had marginal effects on the tyrosine phosphorylations induced by the engagement of a large number of TcR-CD3 complexes. Similarly, Toxin-B-treated cells normally produced IL-2 in response to the crosslinking of a large and saturating number of TcR-CD3 complexes in the presence of phorbol esters. These negative results are probably linked to the extreme stimulation conditions used (extensive TcR crosslinking), which avoided the absolute requirement for a functional cytoskeleton.

Various effectors, including receptor tyrosine kinases, Syk/ZAP70 and the Jak family of tyrosine kinases, have been reported to phosphorylate Vav. We failed to detect any significant increase in ZAP70 or LAT tyrosine phosphorylation following CD82 engagement. We are currently investigating whether the tyrosine kinase p56lck, which is constitutively activated in Jurkat cells, is recruited upon CD82 engagement. Indeed, in T lymphocytes, p56lck is known to associate with CD4, which is reported to associate with CD82. However, this association is thought to occur only when CD4 has uncoupled p56lck (Imai et al., 1995). Integrins, which are also reported to associate with tetraspanins, could also link tetraspanin signaling to Vav phosphorylation (Moores et al., 2000; Zheng et al., 1996). Indeed, integrin and tetraspanin functions seem intimately linked. Recently, Yauch and coworkers (Yauch and Hemler, 2000) have shown that some tetraspanins (CD9, CD81, CD151 and A15/TALLA1), but not CD82, associate at the cell membrane with a PtdIns4*P* 5-kinase and are necessary for integrin-mediated lipid-kinase activity. Recent studies have demonstrated that tetraspanins colocalize with and amplify the signaling functions of integrins in various adhesion processes involved in cell development (Berditchevski and Odintsova, 1999; Shaw et al., 1995; Tachibana and Hemler, 1999). However, CD82, which presents one of the weaker associations with integrins among the tetraspanins highly expressed on activated T cells (Serru et al., 1999), was shown to be the more potent at delivering cytoskeletal and coactivation signals to T cells (Lagaudriere-Gesbert et al., 1997a).

To our knowledge, no counter receptor or physiological ligand for tetraspanins has yet been identified. As previously discussed, physiological tetraspanin triggering could occur through their various associated receptors (e.g. integrins, CD4 and MHC). The functional effects observed upon tetraspanin engagement could also reflect their key role in the membrane dynamics as membrane facilitators (Maecker et al., 1997) or membrane organizers (Lagaudriere-Gesbert et al., 1998), as suggested by the rapid redistribution of CD82 observed after engagement of the T-cell receptor. Recently, the tetraspanin-like proteins Rom and RDS expressed in the disk rims of photoreceptor outer segments have been shown to regulate disk morphogenesis (Clarke et al., 2000), and uroplakins have been shown to be directly involved in the quasi-crystalline structure of the bladder membrane (Liang et al., 2001). Through their ability to associate with each other and to link various surface molecules together to form a tetraspanin network (the tetraspanin web), and, in some cases, to bind with and to mobilize the cytoskeleton through Vav and Rho-GTPase activation, these molecules could play an essential role in membrane structure and dynamics. Such properties could explain why these molecules are directly involved in fundamental processes such as cell activation, adhesion, morphogenesis, growth and metastasis.

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