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ADP-Ribosylation of Rho by C3 Ribosyltransferase Inhibits IL-2 Production and Sustained Calcium Influx in Activated T Cells

Vachras Angkachatchai* and Terri H. Finkel**†

Activation of the T lymphocyte induces dramatic cytoskeletal changes, and there is increasing evidence that disruption of the cytoskeleton inhibits early and late events of T cell signal transduction. However, relatively little is known about the signaling molecules involved in activation-induced cytoskeletal rearrangement. The rho family of small GTP-binding proteins, which include rho, rac, and cdc42, regulates the cytoskeleton and coordinates various cellular functions via their many effector targets. In prior studies, the Clostridium botulinum toxin C3 exoenzyme has been used to ADP-ribosylate and inactivate rho. In this study, we demonstrate that treatment of T cells with C3 exoenzyme inhibits IL-2 transcription following ligation of the TCR. Inhibition of IL-2 expression correlated with loss of sustained increase in [Ca^{2+}], and mitogen activated protein kinase (MAPK/Erk) activity, but not with activation of the tyrosine kinase, lck. These findings are the first to show that ADP-ribosylation of rho by C3 ribosyltransferase (exoenzyme) inhibits IL-2 production due, in part, to the requirement for sustained calcium influx and MAPK activation after Ag receptor ligation. The Journal of Immunology, 1999, 163: 3819–3825.

The TCR relies on the signaling capacity of associated CD3 complex (γ, δ, ε) and ζ-chains to convey Ag recognition and activation to the cell interior. Early signaling events that include inductive tyrosine phosphorylation, activation of the mitogen-activated protein kinase (MAPK) cascade, and the influx of calcium are critical for cytokine production and other downstream activation events (1). Another critical component of early T cell activation is the involvement of the T cell cytoskeleton (1, 2). Recently, vav, the rho family guanosine exchange factor (3), has been shown to regulate cytoskeletal reorganization mediated by the TCR via its binding to activated synk family kinases (4, 5). In addition, members of the small GTP-binding rho family, which include rac, cdc42, and rho, have been shown to play a role in regulation of cell growth, differentiation, and signaling (6, 7), as well as in organization of the actin cytoskeleton (8, 9). Cdc42 and rac have been shown to bind to the Wiscott-Aldrich syndrome protein (10) and to stimulate the stress-activated protein kinase/c-jun kinase (SAPK/JNK) (11, 12), and cdc42 has been shown to regulate the polarization of T cells toward their Ag-presenting targets (13).

Previous studies have also shown that several small GTP-binding proteins regulate IL-2 production in T lymphocytes. p21ras activates the MAPK/extracellular signal-regulated kinase (Erk) cascade in response to TCR ligation and synergizes with calcium pathways to regulate IL-2 transcription (14). Rac and cdc42 activate a transcription factor for IL-2, c-jun, by regulating the SAPK/JNK cascade in response to costimulation by CD28 and TCR/CD3 (15). In addition, rac function synergizes with p21ras in the induction of NF-AT; another transcription factor needed for IL-2 production (16).

Though studies on the role of rho in T cells are incomplete, there is evidence that rho activity is necessary for at least some events of T cell signal transduction. The Clostridium botulinum toxin C3 exoenzyme (17) has been used to ADP-ribosylate and inactivate rho in multiple cell types (8, 18–22). In cytotoxic T cells, C3 exoenzyme inhibits exocytosis and release of cytotoxic granules (18). C3 exoenzyme also induces aberrant pseudopodia formation in response to coligation of β1 integrin and CD3 (19) and has been shown to inhibit shape changes and invasive capacity of a T cell line (22). Given these reports, it is likely that rho plays a role in linking the T cell cytoskeleton with T cell activation.

In this study, we used the C. botulinum toxin C3 exoenzyme to inhibit rho activity and investigate its effects on early and late events of TCR-mediated activation. We show that treatment of T cells with C3 exoenzyme leads to inhibition of IL-2 production. Of interest, calcium influx was not sustained in these stimulated cells, and only partial activation of MAPK/Erk was observed. In contrast, inductive tyrosine phosphorylation of major substrates downstream of the TCR, as well as the tyrosine kinase activity of lck, were apparently intact in C3-treated cells. These data suggest that rho is required for a late event of T cell activation, i.e., IL-2 production, via its regulation of calcium influx and MAPK activation.

Materials and Methods

Cells

Jurkat T cells were obtained from American Type Culture Collection (Manassas, VA) and were grown in RPMI 1640 (Life Technologies, Grand...
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Island, NY), supplemented with 10% FBS (Gemini Bio-Products, Calabasas, CA), 100 U/ml penicillin and streptomycin, and 2 mM l-glutamine (Mediatech, Herndon, VA) in a 37°C, humidified 5% CO2 incubator.

Electroporation of C3 exoenzyme into cells

For the introduction of C3 exoenzyme into cells, C3 exoenzyme (20 μg/ml; Calbiochem, La Jolla, CA) was added to 2 × 10⁷ Jurkat cells/400 μl RPMI in a cooled 0.4-cm gap cuvette. Lower concentrations of C3 exoenzyme produced inconsistent ADP-ribosylation of rho. Thus, 20 μg/ml C3 exoenzyme was used. Cells were subjected to an electric pulse at 960 μl and 260 V, then incubated in 10 ml of complete RPMI for an additional hour. Cells were collected and used for subsequent experiments.

Activation and cell preparation

For assaying early activation events, 10⁷ T cells were incubated with 5 μg anti-CD3 mAb (anti-CD3, OKT3) for 30 min on ice, washed three times, and incubated for another 30 min on ice with 20 μg of the cross-linker, goat anti-mouse Ab (GAM; Jackson ImmunoResearch, West Grove, PA). Cells were washed an additional two times, incubated at 37°C for the indicated times, and then spun down and lysed using 0.5% Nonidet P-40 solution (Calbiochem) containing Tris-buffed saline, protease inhibitors (10 μg/ml PMSF, 10 μg/ml leupeptin, and 10 μg/ml PMSF), and phosphatase inhibitors (10 mM NaF, 10 mM Na3PO4, and 0.5 mM NaVO3) (all from Sigma, St. Louis, MO).

For the production of IL-2, 50 μg/ml OKT3 was coated onto tissue-culture-treated flat-bottom wells at 4°C overnight. Before plating cells, wells were washed twice in complete media, then 2.5 × 10⁶ cells/200 μl were plated/well and incubated for the indicated times. For cross-linking of Ag receptors, 50 μg/ml GAM was coated onto 6-well tissue culture wells overnight at 4°C in PBS and washed three times before use. Pretreated or mock-treated cells were incubated with 20 μg OKT3 for 1 h on ice, then washed three times before plating onto immobilized GAM at 5 × 10⁶ cells/well. Nonactivated control cells were plated onto 50 μg/ml GAM alone and collected in the same manner.

Abs, Western blots, and in vitro kinase assays

For Ick immunoblots, lysates of equal numbers of activated or nonactivated cells were electrophoresed and transferred onto nitrocellulose, then probed with 1 μg/ml anti-Ick mAb (clone LCK 3A5; Zymed, San Francisco, CA) or anti-phosphotyrosine mAb (clone Ab-2; Oncogene Research Products, Cambridge, MA) and detected using GAM/HRP and the chemiluminescent substrate. Similarly, for MAPK immunoblots, equivalent cell lysates were probed using anti-ACTIVE MAPK (Promega, Madison, WI) or anti-phosphotyrosine mAb (clone Ab-2; Oncogene Research Products). For in vitro kinase assays, 1–2 × 10⁵ cells were plated/well and incubated for the indicated times. For cross-linking of Ag receptors, 50 μg/ml GAM was coated onto 6-well tissue culture wells overnight at 4°C in PBS and washed three times before use. Pretreated or mock-treated cells were incubated with 20 μg OKT3 for 1 h on ice, then washed three times before plating onto immobilized GAM at 5 × 10⁶ cells/well. Nonactivated control cells were plated onto 50 μg/ml GAM alone and collected in the same manner.

Results

Inactivation of Rho by C3 inhibits IL-2 expression

The C. botulinum toxin C3 exoenzyme has been reported to ADP-ribosylate and inactivate rho by binding to an asparagine (Asn41) in the effector domain of rho (17, 24). C3 exoenzyme has been used previously to inactivate rho in T cells (18–22, 25). Here, C3 exoenzyme was introduced into Jurkat T cells by electroporation, and the cells were allowed to recover for 1 h before assay. To verify that rho was ADP-ribosylated in the intact cell, the mock and C3 pretreated cells were sonicated and subsequently subjected to an in vitro ADP-ribosylation assay using ³²P-NAD and C3 exoenzyme. Samples pretreated with C3 exoenzyme were unable to incorporate ³²P-NAD in vitro, because the majority of the rho proteins had already been ribosylated by C3 exoenzyme expressed in the intact cell. This indicates that rho is ADP-ribo-sylated in T cells electroporated with C3 exoenzyme.

To determine the effects of C3 exoenzyme on TCR/CD3-mediated IL-2 production, C3-treated cells were stimulated with anti-CD3 mAb (OKT3). After 24 h of incubation, the culture supernatant from C3-treated cells was analyzed for IL-2, and compared with supernatant from mock-treated cells. As shown in Fig. 1b,
ADP-ribosylation (data not shown) and is unable to inhibit IL-2 production linked anti-CD3 mAb for 24 h. Total RNA was isolated, quantified, and amplified using specific IL-2 primers and separated in 1.5% Nu-Sieve agarose. Cells treated with 25 ng/ml PMA, and 10 µg/ml ionomycin were used as positive controls for IL-2 expression (lane 4). There is a marked decrease in IL-2 transcript from activated C3-pretreated cells (lane 3) compared with the activated mock-pretreated cells (lane 2). C3 pretreatment does not ablate actin expression (bottom panel, lane 3). These data are representative of four experiments.

C3 exoenzyme partially inhibits MAPK activation, but not inductive tyrosine phosphorylation

Since the T cell-specific tyrosine kinase lck is critical for TCR-mediated signal transduction, we asked whether the activity of this src-family kinase was also inhibited by C3 treatment. Upon T cell stimulation, lck is activated within 1 or 2 min and autophosphorylated (26) as shown by an immunoblot probed first with an anti-phosphotyrosine Ab, then stripped and reprobed with an anti-lck Ab (Fig. 2a). Pretreatment of the cells with C3 exoenzyme did not inhibit lck phosphorylation; this was verified by a more sensitive in vitro kinase assay measuring lck activity (Fig. 2b). Upon CD3 ligation, lck autophosphorylation, as demonstrated by [γ-32P]ATP-labeled lck, was increased in mock- and C3-treated cells. Since basal levels (in nonstimulated cells) of lck activity contribute to overall lck activity, lck activity in stimulated cells was normalized to lck activity in nonstimulated cells from each treatment (data not shown). Although in one representative experiment it appeared that stimulated C3-treated cells contained more activated lck than in stimulated mock-treated cells (see Fig. 2b), based on these normalizations, there was no significant difference in the ability of C3-treated cells to activate lck, in comparison to mock-treated cells (see legend to Fig. 2b). Labeled phosphorylated proteins (at ~56 kDa) were confirmed to be lck by probing a parallel lane with anti-lck mAb (data not shown). These data suggest that the tyrosine phosphorylation of lck lies upstream or parallels that of rho activity and is not compromised by C3 treatment. In addition, there were no apparent differences in the tyrosine phosphorylation profile of proteins following CD3 ligation of mock or C3-treated cells. These data suggest that a very early event of T cell activation, lck tyrosine phosphorylation, is not dependent on intact rho.

The small GTP-binding protein, ras, mediates the synthesis of IL-2, in part, by activating the MAPK/Erk cascade (27). Therefore, we asked whether C3 has an inhibitory effect on Erk activity in CD3-ligated, C3-treated cells. Erk1 (p44) and Erk2 (p42) belong to a class of serine-threonine kinases that are threonine- and tyrosine-rylates (26) as shown by an immunoblot probed first with an anti-phosphotyrosine Ab, then stripped and reprobed with an anti-tyrosine Ab (Fig. 2c). Pretreatment of the cells with C3 exoenzyme did not inhibit Erk activity; this was verified by a more sensitive in vitro kinase assay measuring Erk activity (Fig. 2d). Upon CD3 ligation, Erk1 and Erk2 activation, as demonstrated by [γ-32P]ATP-labeled Erk, was increased in mock- and C3-treated cells. Since basal levels (in nonstimulated cells) of lck activity contribute to overall lck activity, lck activity in stimulated cells was normalized to lck activity in nonstimulated cells from each treatment (data not shown). Although in one representative experiment it appeared that stimulated C3-treated cells contained more activated lck than in stimulated mock-treated cells (see Fig. 2b), based on these normalizations, there was no significant difference in the ability of C3-treated cells to activate lck, in comparison to mock-treated cells (see legend to Fig. 2b). Labeled phosphorylated proteins (at ~56 kDa) were confirmed to be lck by probing a parallel lane with anti-lck mAb (data not shown). These data suggest that the tyrosine phosphorylation of lck lies upstream or parallels that of rho activity and is not compromised by C3 treatment. In addition, there were no apparent differences in the tyrosine phosphorylation profile of proteins following CD3 ligation of mock or C3-treated cells. These data suggest that a very early event of T cell activation, lck tyrosine phosphorylation, is not dependent on intact rho.

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C3 exoenzyme partially inhibits Erk activity, but not inducive tyrosine phosphorylation or lck activity. a, Equivalent numbers (10^6) of mock-treated (lanes 1 and 3) and C3-treated (lanes 2 and 4) cells were activated in the presence (+) or absence (−) of GAM cross-linked anti-CD3 mAb for 2 min at 37°C. Cells were lysed, solubilized in nonreducing Laemmli SDS buffer, separated on 10% SDS-PAGE, and transferred to nitrocellulose. The membrane was first probed with an anti-phosphotyrosine mAb (left), stripped, and reprobed with anti-lck mAb (right). There were no differences in the phosphotyrosine profiles or lck mobilities between C3 and mock treatment. b, Equal numbers of cells per treatment (2 × 10^6 cells) were activated as before in the presence (+) or absence (−) of cross-linked anti-CD3, lysed, and equal amounts of protein were immunoprecipitated using anti-lck mAb. Immunoprecipitates were washed, subjected to [gamma-32P]ATP, electrophoresed in 10% SDS-PAGE, dried, and exposed to film. Densitometric measurements for each treatment were used to evaluate autophosphorylation and activation of lck, calculated as density of phosphorylated lck in stimulated cells divided by density of phosphorylated lck in nonstimulated cells. The mean ratio of pooled experiments indicated that the activation-induced increase of lck activity in C3-treated cells was not significantly different from that of mock-treated cells (2.17 ± 0.86 vs 1.21 ± 0.31, n = 4). c, Cells were treated without (mock) or with C3 exoenzyme, and then incubated in the presence (+) or mock-treated cells was maximal at 5 min, followed by a progressive decrease in activity at 15 and 30 min (Fig. 2c) (28). Although Erk activity in C3-treated cells was also maximal at 5 min, the amount of activity in C3-treated cells was roughly half that of mock-treated cells. This suggests that MAPK/Erk activity is partially inhibited in C3-treated cells.

Inhibition of sustained calcium influx by C3 exoenzyme

Several studies have shown that sustained calcium influx is required for cytokine production (1, 29). We assessed the effect of C3 treatment on calcium influx of T cells following CD3 ligation. Minutes after electroporation, C3-treated cells were loaded with 5 μM of the calcium-binding fluorescent dye, Indo-1 AM, for 30 min, then stimulated with either anti-CD3 (Fig. 3, a and c) or anti-TCR (Fig. 3, b and d) mAb and analyzed flow cytometrically. Although both the mock- and C3-treated cells showed a rapid increase in [Ca^{2+}], following TCR/CD3 ligation, only the mock-treated cells (Fig. 3, a and b) sustained this increase over a period of minutes (Fig. 3, c and d). This was not due to a loss of cell integrity, since addition of ionomycin to C3-treated cells resulted in sustained and maximal calcium flux similar to that of mock-treated cells (data not shown). Furthermore, the decrease in this response was not due to differences in the levels of CD3 or TCR expression between C3 and mock-treated cells (data not shown). Rather, these data suggest that ADP-ribosylation of rho inhibits the early signaling events required for sustained calcium influx.

Discussion

Several small GTP-binding proteins, including rac, ras, and cdc42, have been shown to be involved in T and B cell activation. In this paper, we used C3 ribosyltransferase (or exoenzyme), which ADP-ribosylates and inactivates rho (18, 19, 25, 30), to investigate the effects of rho inhibition on early and late events of TCR-mediated activation. We demonstrate that C3 treatment inhibits sustained calcium influx and TCR-mediated IL-2 production.

Since early T cell signaling events require the tyrosine kinase lck and the phosphorylation of downstream targets (3, 26, 31, 32), we investigated whether inhibition of rho affected either lck phosphorylation or the tyrosine phosphorylation profile of activated T cell lysates. In our hands, treatment with C3 did not appear to increase or decrease tyrosine phosphorylation, nor did it affect lck activity or the tyrosine phosphorylation profile of activated T cells. These data suggest that rho may function downstream of lck or that lck and rho may be independently regulated. However, recent data regarding lck and its role in activating vav, the guanine nucleotide exchange factor for the rho family, suggest that rho function is dependent on lck activity (3). Data supporting this view derive from studies by Henning and Cantrell (25); expression of constitutively active lck induces increased proliferation of pre-T cells, while, with expression of both activated lck and C3 exoenzyme, the proliferative effects are lost. These data suggest that lck must lie upstream of rho activity, since activated lck was unable to bypass the C3-mediated inhibition of proliferation.

While our results showing a lack of effect of C3 on lck activation were predicted, our results showing inhibition (even partial

absence of (−) of GAM cross-linked anti-CD3 mAb at 37°C for the indicated times. Lysates were prepared, and electrophoresis was performed as above. Duplicate samples were probed with anti-ACTIVE MAPK or anti-Erk1 Ab. The graph shows normalized densitometric measurements (reflecting the ratio of the sum of phosphorylated Erks/the sum of Erk1 and Erk2) of activated Erk in mock (●) and C3 (□) pretreated cells over a 30-min period. These data are representative of four experiments.
inhibition, see Fig. 2B) of MAPK/Erk activation were not. The inhibition of MAPK/Erk activation was surprising since ras, not rho, has been shown to regulate the MAPK/Erk cascade (27, 33). In fact, constitutively active rho, rac, and cdc42 have been shown to synergize with an activated ras effector, raf, to stimulate Erk (5). It is possible that rho inhibition is inactivating a component needed for potentiation of the MAPK/Erk pathway. What effect does this potentiation have on downstream signal transduction? Rho-mediated potentiation of the MAPK/Erk cascade is not responsible for the rho-mediated enhancement of AP-1 activity, since this has been shown to be MAPK/Erk-independent (34). In these studies, expression of a MEK inhibitor had no effect on the augmentation of AP-1 activity mediated by activated rho and PMA (34). These data suggest that the enhancement of MAPK/Erk activity by rho does not lead to enhanced AP-1 activity. Collectively, these data by which inactivation of rho affects IL-2 production.

Our data also show a marked inhibition of sustained calcium influx associated with ADP-ribosylation of rho by C3. Rho has been reported to regulate the synthesis of phosphatidylinositol 4, 5-bisphosphate (PiP2) (35), a lipid that is cleaved by phospholipase C (PLC) into diacylglycerol (DAG), which activates protein kinase C, and inositol 1, 4, 5-triphosphate (IP3), which initiates the elevation of [Ca2+]i. PiP2 is also used as a substrate by PI3 kinase for the production of phosphatidylinositol 3, 4, 5 triphosphate and that, in turn, aids in the breakdown of PiP2 to IP3 and DAG by PLC (36). One direct effect of IP3 production is the transient and substantial rise in [Ca2+]i, via release of IP3 receptor-gated intracellular Ca2+ stores. Sustained increases in [Ca2+]i are due to plasma membrane or store-regulated extracellular Ca2+ entry (37, 38). There are several mechanisms by which extracellular Ca2+ entry may be regulated in T cells, namely via regulation of IP3-regulated intracellular Ca2+ stores and calcium release activated channels (CRACs) (37, 39) and/or involvement of a Na/Ca2+ exchanger (40). The former mechanism requires depletion of IP3-regulated intracellular calcium stores for activation of CRACs (and entry of extracellular Ca2+). Inhibition of calcium influx has been observed when IP3 levels are low, and insufficient IP3 is available within the cell to deplete the intracellular calcium stores (39). In our case, by treating the cells with C3 exoenzyme and inactivating rho, we may have lowered IP3 levels, thereby preventing the depletion of IP3-regulated intracellular calcium stores. Lower levels of IP3 would not affect the initial release of intracellular calcium stores observed after TCR/CD3 ligation, which requires only a low concentration of IP3 (39, 41). Alternatively, it is possible that rho effectors such as p160ROCK are affected and regulate an as-yet-unknown pathway for calcium influx. Indeed, p160ROCK has been shown to activate the Na-H exchanger (42). Inhibition of sustained calcium influx in C3-treated cells is consistent with recent data showing that T cells from vav−/− mice have impaired calcium mobilization in response to stimulation (4). Since vav is a guanosine exchange factor for the rho family, this suggests that rho may mediate vav’s regulation of calcium influx after T cell stimulation.

FIGURE 3. Sustained [Ca2+]i is inhibited by C3. Mock-treated cells (a and b) and C3-treated cells (c and d) were loaded with Indo-1 AM for 30 min at 37°C, washed, and analyzed for [Ca2+]i. Anti-CD3 (a and c) or anti-TCR (b and d) mAb were added at the indicated times (arrow), and the results are expressed as the ratio of Indo-1 AM violet/blue fluorescence (395 nm/500 nm) plotted against time. With C3 treatment, a substantial percentage of the responding population returned to baseline levels within minutes following Ag receptor ligation. These data are representative of five experiments.
Finally, rho plays a major role in organizing the cytoskeletal framework in many cell types (8, 20, 22). In T cells, an intact cytoskeleton is required for sustained calcium signaling (1). Whether TCR-dependent elevation of [Ca\(^{2+}\)]\(_i\) is due to or causes the reorganization of the T cell cytoskeleton after activation is under investigation. Rac, but not rho, has been shown to accumulate at focal adhesion-like cell contacts in response to CD28 ligation (43). It is possible that rho may be solely involved in TCR/CD3-dependent, and not CD28-dependent, changes in the cytoskeleton. That said, others have shown that JNK, a kinase activated by rac and cdc42, integrates signals mediated by TCR and CD28 (11, 12, 15, 44). Studies are in progress to determine whether inactivation of rho attenuates cytoskeletal polarization or actin polymerization in TCR/CD3-ligated T cells.

Although C3 exoenzyme has been shown to specifically ADP-ribosylate and inactivate rho (8, 18–22), it is possible that other proteins within the cell are targets of C3. Indeed, other rho subfamily members such as rac and cdc42 are subjected to similar ADP-ribosylation by C3 under nonphysiological and denaturing conditions (45). From total Jurkat cell extracts, we observed only one radiolabeled ADP-ribosylated product (see Fig. 1), and this product migrated to the same location as rho (data not shown). While it is possible that the in vitro ADP-ribosylation assay does not detect other lesser C3 targets, our data confirm the work of others suggesting that rho is the primary target of C3.

do to date, little is known regarding a role for rho in T cell signalning. Here, we examined the effects of an inhibitor of rho on early and late events of T cell activation. Our data suggest that rho is required for IL-2 expression, perhaps by regulating sustained calcium influx or by potentiating MAPK/Erk activity. This does not preclude the possibility that other rho-mediated actions may have been compromised by C3 treatment and that these may be responsible for IL-2 production. Others have demonstrated that rho regulates transcriptional activation of c-fos by binding of serum-response element factor to the serum-response element in the c-fos promoter (7). Expression of c-fos is needed for the formation of the AP-1 complex and subsequent binding of the AP-1 complex to AP-1 binding sites present in the IL-2 promoter. Recent reports demonstrate that rho can potentiate AP-1 activity in Jurkat T cells (34). In addition, another small GTP-binding protein, rac, has been shown to potentiate ras-mediated NFAT activity (16), while rho has been shown to potentiate rac-mediated T lymphoma inhibition of a fibroblast monolayer (46). The regulation of IL-2 expression may, therefore, involve a number of pathways that require the activity of several small GTP-binding proteins namely, rho, rac, and ras. Our data suggest that rho, in addition to ras, is a small GTP-binding protein required for optimal activation of T cells.

References


