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TCR/CD28-Stimulated Actin Dynamics Are Required for NFAT1-Mediated Transcription of c-rel Leading to CD28 Response Element Activation

Jeffrey C. Nolz,* Martin E. Fernandez-Zapico,† and Daniel D. Billadeau‡∗

TCR/CD28 engagement triggers the initiation of a variety of signal transduction pathways that lead to changes in gene transcription. Although reorganization of the actin cytoskeleton is required for T cell activation, the molecular pathways controlled by the actin cytoskeleton are ill defined. To this end, we analyzed TCR/CD28-stimulated signaling pathways in cytochalasin D-treated T cells to determine the cytoskeletal requirements for T cell activation. Cytochalasin D treatment impaired T cell activation by causing a reduction in TCR/CD28-mediated calcium flux, and blocked activation of two regulatory elements within the IL-2 promoter, NFAT/AP-1 and CD28RE/AP. Treatment had no effect on signaling leading to the activation of either AP-1 or NF-κB. Significantly, we found that NFAT1 is required for optimal c-rel up-regulation in response to TCR/CD28 stimulation. In fact, NFAT1 could be detected bound at the c-rel promoter in response to TCR/CD28 stimulation, and targeting of NFAT1 using RNA interference in human CD4⁺ T cells abrogated c-rel transcription. Overall, these findings establish that disrupting actin cytoskeletal dynamics impairs TCR/CD28-mediated calcium flux required for NFAT1-mediated c-rel transcription and, thus, activation of the CD28RE/AP. The Journal of Immunology, 2007, 179: 1104–1112.

Engagement of the TCR and costimulatory molecule CD28, initiates the activation of multiple signaling pathways leading to changes in gene transcription and, ultimately, T cell activation. After initial TCR engagement by MHC-peptide on an APC, dynamic actin cytoskeletal rearrangement occurs that results in lamellipodial protrusion followed by retraction and formation of a stable immunological synapse. It is believed that the immunological synapse serves as the major signaling component within the T cell–APC conjugate and hours of stable conjugation are required for T cell activation to occur (1). In fact, disruption of immunological synapse formation, using actin-depolymerizing agents such as cytochalasin D, abrogates T cell activation (2–4) and several actin regulatory molecules have been implicated in controlling biochemical signaling cascades leading to transcription of the IL-2 promoter (5).

The promoter of the IL-2 gene has been well characterized, and numerous TCR- and TCR/CD28-stimulated biochemical signaling cascades have been delineated that impact IL-2 promoter activity. The IL-2 promoter contains several transcription factor-binding sites, including NFAT/AP-1, AP-1, and NF-κB, which contribute to the overall enhanced transcription of the gene in response to TCR/CD28 stimulation (6). TCR-stimulated activation of protein kinase C family members as well as Ras exchange factors are involved in the activation of NF-κB family transcription factors (7, 8) and MAPK pathways leading to activation of the AP-1 transcription factor complex (9, 10). TCR-stimulated NFAT-mediated gene transcription is dependent upon increases in intracellular calcium levels leading to activation of calcineurin and subsequent dephosphorylation and nuclear accumulation of NFAT transcription factors (11). In addition to these TCR-stimulated pathways, the costimulatory molecule CD28 has been shown to activate an element within the IL-2 promoter termed the CD28RE/AP (12), which contains both an AP-1 component and is bound primarily by another NF-κB family member, c-Rel (13–16).

TCR-mediated actin reorganization has been intensively studied in recent years and current paradigms suggest that actin cytoskeletal regulators are essential components of T cell activation. In fact, we have shown that the actin regulatory molecule WAVE2 does not affect the activation of MAPK cascades leading to AP-1 activation nor the regulation of NF-κB, but specifically regulates calcium influx following store depletion, and as a result, WAVE2-depleted cells are not able to fully activate NFAT-mediated gene transcription (17). However, it remains unclear whether actin dynamics impact other transcription factor binding sites within the IL-2 promoter. We found that cytochalasin D treatment resulted in defective calcium signaling leading to defects in both NFAT/AP-1 and CD28RE/AP-mediated gene transcription. Importantly, we found that TCR/CD28-stimulated activation of MAPK cascades and AP-1-mediated gene transcription, as well as activation of NF-κB, was unaffected in cells treated with cytochalasin D, but that intracellular calcium mobilization was affected. Interestingly, we identified NFAT1 as an essential regulator of c-rel up-regulation in response to TCR/CD28 stimulation, thereby controlling activation of CD28RE/AP. These findings demonstrate that the integrity of the actin cytoskeleton is required for TCR-mediated calcium flux and, furthermore, identifies c-rel as a direct target of NFAT1-mediated gene transcription.
Materials and Methods

Reagents and Abs

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich. The antisera against phospholipase C (PLC) γ1 has previously been described (18). Abs against ERK1/2, phospho-ERK1/2 (T202/Y204), phospho-JNK1/2 (T183/Y185), phospho-IκB kinase (IKK)α/β (S180/S181), phospho-IκBα (S32/S36), and phospho-PLCγ1 (Y783) were from Cell Signaling Technology. Abs to JNK (C-17), IκBα, and IκKα/β were obtained from Santa Cruz Biotechnology. The Ab for NFT1 (clone 1) used for immunoblot and chromatin immunoprecipitation (ChIP) analyses was purchased from BD Biosciences. The OKT mAb was obtained from the Mayo Clinic Pharmacy Services (Rochester, MN) and the anti-CD28 mAb was purchased from BD Biosciences.

Cell culture and transfection

Jurkat T cells were grown in complete medium (RPMI 1640 supplemented with 5% FBS, 5% FCS, and 4 mM L-glutamine). Primary human CD4+ T cells were purified from buffy coats from the Mayo Clinic Blood Bank using RosetteSep (StemCell Technologies) according to the manufacturer’s instructions. Cells were then cultured in complete medium in 24-well plates at a concentration of 1 × 10^5 cells/ml each. Reporter plasmids in antibiotic fusion (as previously described (19). Transfected Jurkat T cells were used after 18–24 h. For suppression experiments, 1 × 10^5 cells were transfected with 40 μg of suppression plasmid and 5–10 μg of reporter plasmid. Suppression constructs for WAVE2 have been previously described (17). Cells were used for 48–72 h following transfection. Primary human CD4+ T cells were transfected with 500 pmol of small interfering RNA (siRNA) as previously described and used 48 h following transfection (21). Two siRNA against NFT1 were purchased from Dharmacon to target sequences TCTTAAAGCCGCAACGCCTT and GTGA CCAAAGCGAAGTATT.

Luciferase reporter assays

Jurkat cells (1 × 10^5 cells) were transfected with the indicated reporter plasmids (and suppression constructs, when applicable) and distributed in triplicate into 24-well plates. Thirty minutes before stimulation, cells were pretreated with DMSO, cytochalasin D (10 μM), or cyclosporin A (100 ng/ml). Cells were then stimulated with soluble OKT3 and CD28 Ab at a concentration of 1 μg/ml each as indicated. Reporter plasmids for IL-2 (18), NFAT/AP-1 (19), AP-1 (20), NF-κB (22), and CD28RE/AP (20) have all been previously described. All samples were harvested and prepared for luciferase assays according to the protocol suggested by the manufacturer (Promega), and luciferase activity was measured with a model LB9507 Lumat luminometer (Berthold Systems). All reporter assays were cotransfected with a pRL-TK reporter plasmid to control for intersample variations in transfection efficiency. Firefly and pRL-TK Renilla luciferase activity was measured in each sample with a Dual Luciferase Assay Kit (Promega).

Cell stimulation and immunoblot analysis

For the stimulation time course studies, 2 × 10^5 Jurkat T cells or 10 × 10^6 clonal CD4+ T cells were stained on ice with 5 μg/ml anti-CD3 (OKT3, mAb) and 5 μg/ml anti-CD28 mAb and then cross-linked using goat anti-mouse over the indicated time course at 37°C. After each time point the cells were immediately washed in ice-cold PBS and lysed in Nonidet P-40 lysis buffer (20 mM HEPES (pH 7.9), 100 mM NaCl, 5 mM EDTA, 0.5 mM CaCl2, 1% Nonidet P-40, 1 mM PMSF, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM NaN3) for 10 min on ice. Lysates were clarified by centrifugation at 18,000 × g for 5 min at 4°C. Protein concentrations were quantified and 75 μg of protein was resolved by SDS-PAGE and transferred onto nitrocellulose membranes (Millipore). In most experiments, Jurkat T cells or CD4+ clonal T cells were preincubated for 30 min with either DMSO or cytochalasin D. For immunoblots, mAbs were detected using goat anti-mouse or goat anti-rabbit IgG coupled to HRP (Santa Cruz Biotechnology) and SuperSignal (Pierce).
Cytoskeletal dynamics, actually augment signaling pathways leading to gene transcription, whereas higher doses, which theoretically will have a more profound affect on actin cytoskeletal dynamics, lead to a complete abrogation of T cell activation.

**Cytochalasin D treatment impairs activation of NFAT and the CD28RE/AP of the IL-2 promoter**

Within the IL-2 promoter, there are four well-characterized transcription factor binding sites that mediate IL-2 transcription in response to TCR and TCR/CD28 stimulation (Fig. 2A). To test which transcriptional elements were inhibited with cytochalasin D, a series of reporter assays were used that contained only single transcription factor binding sites within the reporter construct. In agreement with early studies (26), TCR/CD28 activation of an NFAT/AP-1 element was impaired with cytochalasin D treatment (Fig. 2B). In contrast, TCR-stimulated activation of both AP-1 (Fig. 2C) and NF-κB (Fig. 2D) reporter constructs was not affected with drug treatment, suggesting that signaling pathways leading to the activation of FOS and JUN (for AP-1) and p65/50 (for NF-κB) are not dependent on actin polymerization downstream of the TCR/CD28 ligation. However, cytochalasin D severely impaired the activation of the CD28RE/AP of the IL-2 promoter (Fig. 2E). These data suggest that inhibition of actin polymerization using cytochalasin D does not affect TCR-mediated signaling pathways globally, rather specifically affects activation of the CD28RE/AP- and NFAT-mediated gene transcription.

**Cytochalasin D blocks conjugate formation required for T cell activation**

TCR/CD28-stimulated actin dynamics drive lamellipodial protrusion and integrin activation leading to immunological synapse formation. In fact, formation of a stable T cell–APC conjugate is required for optimal T cell activation to occur. Because concentrations of cytochalasin D above 2.5 μM were required to inhibit T cell activation in Jurkat, as measured by IL-2 promoter activity, we next determined the concentrations of cytochalasin D required to block TCR-mediated actin dynamics leading to T cell–APC conjugate formation. As seen in Fig. 3A, SEE-pulsed Raji B cells induce a ~3-fold increase in conjugate formation compared with conjugates formed with B cells in the absence of superantigen. Conjugate formation also occurred in cells treated with both 0.5 and 1 μM, whereas concentrations of 5 and 10 μM completely

**FIGURE 1.** The effect of cytochalasin D on IL-2 promoter activity. Jurkat T cells were transfected with an IL-2 promoter luciferase construct and pRL-TK Renilla control. Twenty hours later, cells were pretreated with increasing concentrations of cytochalasin D (0–20 μM) for 30 min. Cells were then left unstimulated or stimulated with anti-CD3/CD28 for 5 h. Luciferase activity was measured and normalized to pRL-TK Renilla readings.

**FIGURE 2.** Cytochalasin D blocks activation of NFAT/AP-1 and the CD28RE/AP of the IL-2 promoter. A, Diagram of the location of the various transcription factor binding sites within the IL-2 promoter along with the transcription factors that drive expression of the gene. Jurkat T cells were transfected with reporter constructs for NFAT/AP-1 (B), AP-1 (C), NF-κB (D), or CD28RE/AP (E) and allowed to recover for 20 h. Cells were then pretreated with 10 μM cytochalasin D for 30 min and then left unstimulated or stimulated with anti-CD3/CD28 for 5 h. Luciferase activity was measured and normalized to pRL-TK Renilla readings.
abolished this actin-dependent process (Fig. 3A). These data demonstrate that actin dynamics are not completely inhibited with drug concentrations below 2.5 μM and that cytochalasin D treatment can completely block TCR-mediated conjugate formation with APCs.

The data in Fig. 2 suggest that not all elements of the IL-2 promoter require TCR/CD28-stimulated actin dynamics for their activation. However, these experiments were performed with Abs against both CD3 and CD28, thereby bypassing the requirement for spatial and temporal signaling at the immunological synapse to be intact. Because T cell–APC conjugate formation is inhibited with cytochalasin D treatment, it seemed reasonable that the activation of all signaling pathways in T cells would be impaired when stimulated with APC. To test this hypothesis, Jurkat T cells were transfected with an AP-1 luciferase reporter construct and stimulated with Raji B cells. Even though signaling events required for AP-1 activation were not affected with cytochalasin D when stimulated with Abs against CD3/CD28, activation of AP-1 was completely inhibited by cytochalasin D when stimulated by APC, probably due to the inability to form a stable conjugate. Overall, these data demonstrate that TCR/CD28-stimulated actin dynamics are required for conjugate formation leading to T cell activation.

**Cytochalasin D treatment impairs TCR-mediated calcium flux**

To verify our results in Fig. 2, clonal CD4+ T cells were used for the analysis of signaling pathways leading to T cell activation. PLCγ1 is activated downstream of the TCR and its lipase activity is responsible for the activation of many transcription factors involved in IL-2 transcription through the production of inositol 1,4,5-triphosphate (IP3) and diacylglycerol. In agreement with the reporter assays performed in Jurkat T cells, cytochalasin D treatment had no effect on the activation of PLCγ1 in response to TCR/CD28 stimulation, as detected by immunoblot for phosphorylation of Y783 (Fig. 4A). In addition, MAPK pathways (measured by phosphorylation of ERK and JNK), which are required for AP-1 activity, are unaffected with drug treatment (Fig. 4, B and C).

In resting T cells, NF-κB family members are found primarily in the cytoplasm bound to members of the IκB family, which blocks the nuclear localization signal sequence required for nuclear import and gene transcription. However, the activation of signaling pathways resulting from TCR/CD28 stimulation initiates the phosphorylation and degradation of IκBα, allowing for NF-κB-mediated gene transcription. When compared with control treated cells, cytochalasin D treatment of CD4+ T cells caused slightly augmented activation of the pathway required for NF-κB nuclear accumulation, including prolonged activation of IKKa/IKKβ, as well as increased degradation of IκBα (Fig. 4D). The reason for the elevated activation of this pathway is unknown, but nonetheless, these experiments demonstrate that integrity of the actin cytoskeleton following TCR/CD28 ligation is not required for the activation of signaling pathways leading to IκBα degradation and subsequent transcriptional activity of NF-κB.

Following TCR ligation, a rise in intracellular calcium levels occurs as a result of PLCγ1 activation causing IP3-mediated calcium store release from the endoplasmic reticulum (ER) and subsequent activation of calcium release-activated calcium (CRAC) channels within the cell membrane. This rise in intracellular calcium levels activates the phosphatase calcineurin, which in turn, mediates NFAT dephosphorylation and nuclear translocation leading to gene transcription (11). Because NFAT-mediated gene transcription was impaired with cytochalasin D treatment, we next examined whether calcium signaling dynamics were altered with drug treatment. Consistent with the data shown in Fig. 1, a 1-μM concentration of cytochalasin D had no affect on intracellular calcium levels following TCR/CD28 engagement of CD4+ T cell clones. However, concentrations of both 5 and 10 μM of cytochalasin D significantly impaired calcium flux when compared with the DMSO control (Fig. 4E). Similar results were obtained using Jurkat T cells (J. C. Nolz and D. D. Billadeau, unpublished data). This defect in calcium flux occurred even though activation of PLCγ1 was not affected with cytochalasin D treatment, as evident by tyrosine phosphorylation of PLCγ1 and activation of the ERK and JNK MAPK pathways. In support of the finding that TCR-mediated calcium flux is the only signaling event affected by high concentration cytochalasin D treatment, IL-2 promoter activity is not diminished when Jurkat T cells are stimulated with anti-CD3/CD28 and ionomycin (Fig. 4F).
TCR/CD28-mediated c-rel transcription is impaired with cytochalasin D treatment

Because TCR/CD28-mediated calcium flux was the only signaling pathway identified that was affected by cytochalasin D treatment, it seemed reasonable that the inability to activate the CD28RE/AP of the IL-2 promoter was also the result of this defect. Cyclosporin A inhibits NFAT-mediated gene transcription by preventing activation of calcineurin, which is required for the dephosphorylation of NFAT family members. In agreement with NFAT playing a role in the activation of the CD28RE/AP, treatment of Jurkat T cells with cyclosporin A resulted in an inability to activate the CD28RE/AP with TCR/CD28 stimulation (Fig. 5A). In addition,

FIGURE 4. Cytochalasin D impairs TCR-mediated calcium flux, but not activation of ERK, JNK, or NF-κB. A, Clonal CD4+ T cells were preincubated with either DMSO or 10 μM cytochalasin D for 30 min. Cells were then stimulated with anti-CD3/CD28 and goat anti-mouse for the indicated times and immediately lysed. Lysates were clarified and whole cell extracts were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and subsequently blotted for phospho-PLCγ1 (Y783) and total PLCγ1. Whole cell extracts in A were blotted for phospho-ERK1/2 and total ERK1/2 (B), p-JNK1/2 and total JNK1 (C), and phospho-IKKα/β, total IKKα/β, phospho-IκBα, IκBα, and p65 (D). E, Clonal CD4+ T cells were pretreated for 30 min with DMSO or 1–10 μM cytochalasin D. Calcium mobilization in response to anti-CD3/CD28 and goat anti-mouse was measured using Indo-1 staining and flow cytometry in the presence of cytochalasin D or DMSO. F, Jurkat T cells were transfected as in Fig 1 and stimulated for 5 h with either anti-CD3/CD28 or anti-CD3/CD28 and 5 μM ionomycin. Luciferase activity was measured and normalized to pRL-TK Renilla readings.

FIGURE 5. Cyclosporin A treatment and WAVE2 suppression blocks activation of the CD28RE/AP. A, Jurkat T cells were transfected with a CD28RE/AP luciferase construct and pRL-TK Renilla control. Twenty hours later, cells were pretreated for 30 min with DMSO or 100 ng/ml cyclosporin A. Cells were then left unstimulated or stimulated with anti-CD3/CD28 for 5 h. Luciferase activity was measured and normalized to pRL-TK Renilla readings. B, Jurkat T cells were transfected with an CD28RE/AP luciferase construct, pRL-TK Renilla control, and either vector control or suppression vectors against WAVE2 (shWAVE2-A or shWAVE2-B) and allowed to recover for 72 h. Cells were then stimulated and luciferase activity was measured as in A.
we have previously demonstrated that T cells lacking WAVE2 are unable to properly flux calcium in response to TCR stimulation, leading to a defect in NFAT-mediated gene transcription (17). Consistent with our hypothesis, T cells suppressed for WAVE2 are also unable to activate the CD28RE/AP with TCR/CD28 stimulation (Fig. 5B). Overall, these data suggested that NFAT-mediated gene transcription is directly or indirectly linked to the activation of the CD28RE/AP downstream of the TCR and TCR-mediated calcium flux.

Previous studies have demonstrated that c-Rel protein levels increase substantially in response to both CD3 and CD3/CD28 stimulation (28–30). Because many signaling pathways required for activation of the CD28RE/AP were unaffected in cytochalasin D-treated cells, we next analyzed c-rel transcription in cytochalasin D-treated cells. Stimulation of clonal CD4⁺ T cells with anti-CD3/CD28 led to increased c-rel mRNA levels as measured by RT-PCR (Fig. 6A). Levels of c-rel transcript were maximal at ~2 h and declined by 5 h. To determine whether cytochalasin D affected c-rel transcription, CD4⁺ T cells were pretreated with increasing concentrations of cytochalasin D and stimulated with anti-CD3/CD28 for 2 h. Consistent with data in Fig. 1, low concentrations of cytochalasin D (1 μM) led to a slightly enhanced level of c-rel transcripts, whereas higher concentrations completely blocked the increase in c-rel mRNA levels (Fig. 6B).

To more clearly view the kinetics of c-rel gene transcription, clonal CD4⁺ T cells were pretreated with either DMSO or 5 μM cytochalasin D and stimulated over a time course from 0 to 120 min with anti-CD3/CD28. Control T cells up-regulated transcription of c-rel rapidly, and mRNA levels of c-rel continued to climb throughout the time course (Fig. 6C). In contrast, T cells treated with cytochalasin D failed to initiate increased transcription of c-rel in response to TCR/CD28 stimulation. NF-κB family members are known to regulate the IκBα gene (31, 32) and transcription of IκBα occurs in response to TCR-induced NF-κB activation (Fig. 6C). However, kinetics of IκBα transcription differ from that of c-rel because mRNA levels of IκBα increase rapidly and remain relatively constant throughout the time course, whereas c-rel

![FIGURE 6. Cytochalasin D blocks TCR-mediated transcription of c-rel.](image-url)

A, Clonal CD4⁺ T cells were stimulated with anti-CD3/CD28 and goat anti-mouse for 0, 2, and 5 h and immediately lysed with TRIzol. Levels of c-rel and GAPDH RNA transcripts were then detected by PCR of cDNA generated from RNA isolated from each time point. B, Clonal CD4⁺ T cells were preincubated with DMSO or cytochalasin D (1–20 μM) for 30 min and then stimulated with anti-CD3/CD28 and goat anti-mouse for 2 h. Cells were then immediately lysed with TRIzol, and expression of c-rel and GAPDH was then detected as in A. C, Clonal CD4⁺ T cells were preincubated with DMSO or 5 μM cytochalasin D for 30 min. Cells were then stimulated as in A for 0–120 min and immediately lysed with TRIzol. Expression of c-rel, IκBα, and GAPDH was then immediately detected by RT-PCR.

![FIGURE 7. NFAT1 binding to the c-rel promoter is cytochalasin D sensitive.](image-url)

A, Sequence of the 5' promoter region of the c-rel gene. Transcriptional start site (Txn) and NF-κB (p65/50) binding sites are shown along with a putative NFAT binding site (GGAAA) found within the promoter region. B, Jurkat T cells were pretreated with either DMSO or 10 μM cytochalasin D for 30 min and then left unstimulated or stimulated with anti-CD3/CD28 for 2 h. Binding of NFAT1 to the promoters of either the IL-2 or c-rel gene was then detected by ChIP as described in Materials and Methods. C, Jurkat T cells were transfected with either vector control or WAVE2 suppression vector (shWAVE2), and stimulated with anti-CD3/CD28 for 2 h. ChIP for NFAT1 was performed as in B.
transcription increases with time. In addition, cytochalasin D treatment had no affect on IkBα transcription in response to TCR/CD28 stimulation, confirming our data that NF-κB-mediated gene transcription is not affected by cytochalasin D treatment. Overall, these data suggest that TCR-mediated induction of c-rel is cytochalasin D sensitive and transcriptional events other than those required for IkBα transcription are needed for c-rel up-regulation.

**NFAT1 drives c-rel expression in response to TCR/CD28 stimulation**

The c-rel promoter is unique because it does not contain the standard TATA and CAAT boxes used by many genes to initiate mRNA transcription (33, 34). Rather, immediately upstream of the transcriptional start site lies a ~200-bp GC-rich area, a common feature of many “housekeeping” genes. However, a region between −690 and −190 of the transcriptional start site contains multiple binding sites for NF-κB (33) and also a sequence that fits the consensus sequence for NFAT binding (GGAAA) (Fig. 7A). This putative NFAT binding site is also found within the mouse c-rel promoter (J. C. Nolz and D. D. Billadeau, unpublished observation). Of the different NFAT family members, NFAT1 (NFATp, NFATc2) is the most highly expressed in T cells and accounts for 85–90% of the total NFAT protein in the cells (35). To determine whether NFAT1 bound directly to this region of the promoter, we performed ChIP analysis of the c-rel promoter and the IL-2 promoter. Consistent with our earlier observations, stimulation of Jurkat T cells with anti-CD3/CD28 caused a robust accumulation of NFAT1 at the IL-2 promoter, which was blocked with cytochalasin D treatment (Fig. 7B). In addition, NFAT1 could also be detected bound to the c-rel promoter region in response to TCR/CD28 stimulation and cytochalasin D treatment similarly abolished binding (Fig. 7B). We were unable to detect binding of c-Jun to the c-rel promoter using the same primers used to detect NFAT1 binding, even though we could readily detect c-Jun binding at the IL-2 promoter, suggesting that this NFAT1 site is AP-1-independent (J. C. Nolz, M. E. Fernandez-Zapico, and D. D. Billadeau, unpublished data). Lastly, WAVE2-suppressed Jurkat T cells, which have impaired calcium signaling, are also defective in their ability to accumulate NFAT1 at both the IL-2 and c-rel promoters in response to TCR/CD28 stimulation (Fig. 7C). Taken together, these data confirm that NFAT1 associates with the promoter region of the c-rel gene following TCR/CD28 ligation and treatment of cells with cytochalasin D inhibits NFAT1 accumulation at target genes, including the IL-2 and c-rel promoters.

Because cytochalasin D inhibited TCR-mediated calcium flux required for NFAT-mediated gene transcription, it seemed reasonable that activation of the CD28RE/AP was impaired due to a decrease in the generation of new c-Rel protein in response to TCR/CD28 stimulation. In fact, ectopic expression of c-Rel in Jurkat T cells leads to activation of the CD28RE/AP in unstimulated cells (Fig. 8A), as well as enhances its activation in response to TCR/CD28 stimulation (Fig. 8A). To test whether NFAT1 was required for TCR/CD28-mediated c-rel up-regulation to occur, two siRNA duplexes were created to target endogenous levels of NFAT1 in primary human CD4+ T cells. As shown in Fig. 8B, compared with the control siRNA, transfection of either siRNA against NFAT1 into a polyclonal human CD4+ T cell population efficiently suppressed NFAT1 protein levels 48 h posttransfection. Stimulation of TCR/CD28 in the human polyclonal T cell population yielded similar results as seen with the CD4+ T cell clones, with c-rel mRNA levels substantially increased at the 120-min time point. However, T cells in which NFAT1 had been targeted with siRNA were less efficient in up-regulating c-rel mRNA in response to TCR/CD28 stimulation (Fig. 8C). Overall, these data demonstrate that c-rel is a direct target of NFAT1 and that increased c-rel expression enhances activation of the CD28RE/AP of the IL-2 promoter.

**Discussion**

The importance of the actin cytoskeleton in controlling T cell activation has long been appreciated (5). Although many events

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**FIGURE 8.** NFAT1 is required for TCR-mediated c-rel up-regulation and activation of the CD28RE/AP. A, Jurkat T cells were transfected with a CD28RE/AP reporter construct, pRL-TK Renilla, and increasing amounts of c-rel expression constructs and allowed to recover for 20 h. Cells were then left unstimulated or stimulated with anti-CD3/CD28 for 5 h. Luciferase activity was then measured and normalized to pRL-TK Renilla readings. RNA was isolated from the different reporter construct, pRL-TK Renilla, and increasing amounts of c-rel. (A) Western blot analysis was used to verify that the expected proteins were detected. (B) Primary human CD4+ T cells were transfected with either control siRNA or siRNA against NFAT1 as in C. (C) Primary human CD4+ T cells were transfected with either control siRNA or siRNA against NFAT1 as in B. Cells were then treated with either anti-CD3/CD28 and goat anti-mouse for 120 min and immediately lysed with TRIzol. Expression of c-rel and GAPDH transcripts was then determined using RT-PCR.
preceding the changes in gene transcription that are required for a T cell to become activated, including migration, integrin activation, and conjugate formation, are dependent on the actin cytoskeleton, it has been less clear whether the actin cytoskeleton is required for the maintenance of signaling cascades that are activated downstream of the TCR. The data presented in this study suggest that polymerization of the actin cytoskeleton in response to TCR/CD28 engagement is regulating TCR-mediated calcium flux required for NFAT-mediated gene transcription.

Several actin regulatory proteins have been implicated in controlling T cell activation and gene transcription, including Vav1 (20, 26), Itk (37, 38), WASp (39), and WAVE2 (17, 40). However, it has been difficult to access whether the defects in T cell activation were a result of the decreased de novo actin polymerization, or because other qualities of the protein (such as kinase activity or adaptor function) abrogated the activation of intracellular signaling pathways. For instance, mouse T cells and Jurkat T cells that are deficient for Vav1 are impaired in not only actin cytoskeletal rearrangement, but also the activation of ERK and JNK, as well as TCR-mediated calcium flux (20, 26). However, because the reduced activation of ERK, JNK, and calcium flux in \textit{vav1}−/− T cells is probably at least partially due to inefficient activation of PLCγ1 (41), it makes it difficult to evaluate whether these defects are a cause, effect, or mutually exclusive to the re-diction in cytoskeletal rearrangement.

The exact mechanism by which the actin cytoskeleton regulates TCR-mediated calcium flux remains elusive. We have recently found that WAVE2 is not required for TCR-stimulated MAPK activation, but is required for calcium influx through CRAC channels following IP$_3$-mediated store depletion. Interestingly, the effect of WAVE2 on calcium influx is independent of its VCA domain (J. C. Nolz, and D. D. Billadeau, manuscript submitted), thereby excluding the idea that TCR-stimulated actin-related protein (ARP) 2/3-dependent actin polymerization initiated by WAVE2 controls calcium signaling. Consistent with this notion, suppression of either ARP2 or ARP3 in Jurkat T cells does not affect calcium mobilization following TCR ligation (21). Thus, other proteins involved in the regulation of TCR-stimulated actin dynamics might regulate calcium signaling downstream of the TCR.

Recently, two regulators of the CRAC channel in T cells have been identified. STIM1 was identified as a transmembrane ER protein and regulator of the CRAC channel (42). In unstimulated cells, STIM1 is localized throughout the ER until IP$_3$-mediated calcium release from the ER causes STIM1 to local into puncta within the ER that become closely associated with the plasma membrane (43). It is postulated, that this puncta formation at the periphery permits its association with the CRAC channel, Orai1 (44). However, the mechanism of STIM ER puncta formation and juxtaposition to the plasma membrane remain unresolved, but could involve the actin cytoskeleton. In fact, cytochalasin D treatment has been shown to inhibit store-operated calcium channels in other cell types (45, 46). Clearly, there is a connection between the actin cytoskeleton and TCR-mediated calcium flux, however, it is apparently not an ARP2/3-regulated process (21). Future studies aimed at identifying the actin regulators/nucleators that couple IP$_3$-mediated store release to CRAC channel activation will help in defining the molecular pathways contributing to T cell activation.

Our study identifies a mechanism by which activation of the CD28RE/AP is dependent upon NFAT1-mediated gene transcription. Previous studies have suggested that NFAT may play a role in \textit{c-rel} up-regulation in response to TCR stimulation because it is efficiently blocked with FK506, an immunosuppressant that blocks the activation of calcineurin. However, studies in our lab demonstrate that treatment of both Jurkat as well as primary human T cells with cyclosporin A, which also inhibits activation of calcineurin, blocks not only the activation of NFAT, but also an NF-κB luciferase reporter construct and transcription of IκBα (J. C. Nolz and D. D. Billadeau, unpublished data), indicating that these immunosuppressive drugs may also have off-target effects. In contrast, cytochalasin D inhibited only NFAT and not NF-κB, allowing for specific analysis of the requirement for NFAT in regulating \textit{c-rel} transcription.

Both the human and murine \textit{c-rel} promoters contain multiple consensus sequences for NF-κB binding (33, 34). In addition, the constitutively high \textit{c-rel} expression in B cells is dependent on NF-κB activity (34) and \textit{c-rel} is required for both B and T cell proliferation (47). However, it has been more recently demonstrated that \textit{c-rel} up-regulation downstream of the TCR is p65/50-independent because T cells from p65−/− p50−/− mice are able to up-regulate \textit{c-rel} following TCR/CD28 ligation (48), although the possibility that other NF-κB family members could regulate the gene in a redundant fashion was not determined. The same authors identified an NFAT/AP-1-binding site within the \textit{c-rel} promoter, which was located 2315 bp 5′ of transcriptional start site. However, we have identified an NFAT-binding site much closer to the transcriptional start site (−590) and this binding, as determined by ChIP, was abrogated with cytochalasin D treatment. In addition, we demonstrate that NFAT1, which is the major NFAT family member found in T cells, is required for \textit{c-rel} up-regulation in response to TCR/CD28 stimulation in human T cells. Previously, it was believed that activation of the CD28RE/AP was highly dependent upon CD28 ligation leading to \textit{c-rel} nuclear localization and activity on the IL-2 gene and possibly other promoters including CD40L (49). However, more recently, it was suggested that costimulation through CD28 had a profound impact on NFAT nuclear duration (50), which would agree with our data demonstrating that \textit{c-rel} is a target of NFAT family members. Altogether, these data indicate that TCR/CD28-stimulated reorganization of the actin cytoskeleton is required for optimal binding of NFAT1 to the \textit{c-rel} promoter and suggest that increased nuclear duration of NFAT would lead to a more robust activation of the CD28RE/AP through the direct targeting and transcription of \textit{c-rel} downstream of these receptors.

In summary, the data presented demonstrate that the integrity of the actin cytoskeleton is required for TCR/CD28-mediated calcium flux leading to optimal NFAT1-mediated gene transcription, and \textit{c-rel} up-regulation. Efforts aimed at identifying the TCR/CD28-stimulated actin regulators involved in bridging actin cytoskeletal dynamics with calcium influx will be an important step in understanding the molecular pathways involved in regulating NFAT-mediated gene transcription in T cells.

Disclosures
The authors have no financial conflict of interest.

References
NFAT1 REGULATES TCR-MEDIATED TRANSCRIPTION OF c-rel


