A function for tyrosine phosphorylation of type 1 inositol 1,4,5-trisphosphate receptor in lymphocyte activation

Nikhil deSouza,1 Jie Cui,1 Miroslav Dura,1 Thomas V. McDonald,2,3 and Andrew R. Marks1

1Department of Physiology and Cellular Biophysics, Clyde and Helen Wu Center for Molecular Cardiology, Columbia University College of Physicians and Surgeons, New York, NY 10032
2Department of Medicine and 3Department of Molecular Pharmacology, Albert Einstein College of Medicine, New York, NY 10461

Sustained elevation of intracellular calcium by Ca2+ release–activated Ca2+ channels is required for lymphocyte activation. Sustained Ca2+ entry requires endoplasmic reticulum (ER) Ca2+ depletion and prolonged activation of inositol 1,4,5-trisphosphate receptor (IP3R)/Ca2+ release channels. However, a major isoform in lymphocyte ER, IP3R1, is inhibited by elevated levels of cytosolic Ca2+, and the mechanism that enables the prolonged activation of IP3R1 required for lymphocyte activation is unclear. We show that IP3R1 binds to the scaffolding protein linker of activated T cells and colocalizes with the T cell receptor during activation, resulting in persistent phosphorylation of IP3R1 at Tyr353. This phosphorylation increases the sensitivity of the channel to activation by IP3 and renders the channel less sensitive to Ca2+-induced inactivation. Expression of a mutant IP3R1-Y353F channel in lymphocytes causes defective Ca2+ signaling and decreased nuclear factor of activated T cell activation. Thus, tyrosine phosphorylation of IP3R1-Y353 may have an important function in maintaining elevated cytosolic Ca2+ levels during lymphocyte activation.

Introduction

T cell activation is initiated by the engagement of the antigen/major histocompatibility complex with the T cell receptor (TCR), triggering the formation of the immunological synapse (Yokosuka et al., 2005). The immunological synapse is a dynamic, highly ordered structure that includes adaptor proteins and kinases, including the nonreceptor Src tyrosine kinases Lck and Fyn (Monks et al., 1998; Bromley et al., 2001). Once activated, these kinases trigger a phosphorylation cascade that leads to the activation of PLCγ-1, which hydrolyzes phosphotyidylinositol 4,5 bisphosphate into diacylglycerol and inositol 1,4,5-trisphosphate (IP3; Koretzky and Myung, 2001). IP3 triggers Ca2+ release from the ER by activating the IP3 receptor (IP3R; Berridge and Irvine, 1984). ER Ca2+ depletion is sensed by stromal interaction molecule 1 (STIM1), an EF hand containing ER transmembrane protein (Liu et al., 2005; Roos et al., 2005).

ER Ca2+ depletion triggers the redistribution of STIM1 such that STIM1 forms more discrete puncta at functional junctional ER sites near the plasma membrane (Zhang et al., 2005; Luik et al., 2006; Wu et al., 2006). STIM1 communicates the loss of ER Ca2+ to the plasma membrane Ca2+ release–activated Ca2+ (CRAC) channels (Feske et al., 2006; Vig et al., 2006), which colocalize with STIM1 (Luik et al., 2006; Wu et al., 2006). Activation of CRAC channels triggers sustained Ca2+ influx, which is referred to as capacitative Ca2+ entry (Putney et al., 2001). Additionally, plasma membrane–localized IP3Rs potentially contribute to Ca2+ influx upon T lymphocyte activation (Dellis et al., 2006). Sustained elevation of intracellular Ca2+ ([Ca2+]i) causes nuclear factor of activated T cells (NFAT) nuclear translocation, eventually leading to interleukin-2 (IL-2) production (Shibasaki et al., 1996; Lewis, 2001).

During T cell activation, [Ca2+]i elevation persists for hours after the initial activation event (Huppa et al., 2003), and sustained [Ca2+]i elevation requires prolonged IP3R-mediated Ca2+ release to keep the ER Ca2+ depleted, ensuring sustained Ca2+ influx. However, upon lymphocyte activation, global [IP3] is only transiently increased and rapidly decreases within 10 min after stimulation (Guse et al., 1993; Sei et al., 1995). Moreover, IP3R1 channel activity is inhibited by increasing [Ca2+]i (>300 nM Ca2+; Bezprozvanny et al., 1991) and the channel would be

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closed when exposed to the cytosolic [Ca\(^{2+}\)] achieved during lymphocyte activation (Lewis, 2001). Thus, there must be a mechanism that enables IP\(_3\)R channels to remain open when exposed to cellular conditions of globally decreasing [IP\(_3\)] and elevated [Ca\(^{2+}\)].

In neurons, which require elevated [IP\(_3\)] (10–15 \(\mu\)M) to trigger IP\(_3\),R activation and rapid ER Ca\(^{2+}\) release (Khodakhah and Ogdan, 1993; Svoboda and Mainen, 1999), PLC-coupled receptors cocluster with IP\(_3\)Rs, forming “signaling microdomains” to ensure efficient IP\(_3\)R activation by creating locally elevated [IP\(_3\)] (Delmas et al., 2002; Delmas and Brown, 2002). Similarly, in activated T cells, IP\(_3\),R1 cocaps with the TCR at sites of T cell activation (Khan et al., 1992), where the cellular IP\(_3\)-generating machinery, specifically linker of activated T cells (LAT) and PLC\(_{\gamma}\)-1, also accumulate (Douglass and Vale, 2005; Espagnolle et al., 2007).

We had previously demonstrated that upon T cell activation, IP\(_3\),R1 is phosphorylated by the Src family kinase Fyn. Additionally, in planar lipid bilayer studies, we observed that tyrosine-phosphorylated IP\(_3\),R1 exhibits a higher open probability at \(~\sim 700 \text{nM} [\text{Ca}^{2+}]\) than nonphosphorylated IP\(_3\),R1 (Jayaraman et al., 1996). Thus, IP\(_3\),R1 tyrosine phosphorylation could provide a mechanism that would allow sustained channel activation even as cytosolic [Ca\(^{2+}\)] is in the range of 500–1,000 nM (Lewis, 2001), thereby maintaining a depleted ER Ca\(^{2+}\) store.

We identified IP\(_3\),R1-Y353, located in the IP\(_3\)-binding domain, as a key tyrosine phosphorylation site on IP\(_3\),R1 that is phosphorylated during lymphocyte activation (Cui et al., 2004). We generated IP\(_3\),R-deficient lymphocyte cell lines expressing a recombinant IP\(_3\),R1 mutant (IP\(_3\),R1-Y353F) that cannot be tyrosine phosphorylated at this key regulatory site. This allowed us to assess the effect of IP\(_3\),R1 tyrosine phosphorylation on Ca\(^{2+}\) dynamics upon lymphocyte activation.

We show that in activated Jurkat T cells, Y353-phosphorylated (phosphoY353) IP\(_3\),R1 clusters and colocalizes with the TCR. In cell spreading assays, the clustered Y353-phosphorylated IP\(_3\),R1 forms a distinct ER substructure, facilitating the formation of a TCR–Y353–phosphorylated IP\(_3\),R1 signaling microdomain. Y353-phosphorylated IP\(_3\),R1 staining was clearly detectable for at least 1 h after T cell activation, suggesting that tyrosine phosphorylation of the channel is prolonged. In single channel studies, Y353-phosphorylated IP\(_3\),R1 increased IP\(_3\),R1 channel activity at physiological [IP\(_3\)] and decreased Ca\(^{2+}\)-dependent channel inactivation. IP\(_3\),R-deficient B cells stably expressing IP\(_3\),R1–Y353F exhibited decreased ER Ca\(^{2+}\) release, oscillations, and influx in response to B cell receptor (BCR) activation. Additionally, Jurkat T cells expressing IP\(_3\),R1–Y353F exhibited a blunted NFAT response upon T cell activation, suggesting that phosphorylation of IP\(_3\),R1 at Y353 is important for robust T cell activation.

**Results**

**IP\(_3\),R1 phoshoY353 colocalizes with the TCR upon T cell activation**

Previous studies showed that the TCR cocaps with IP\(_3\),R1 upon T cell activation (Khan et al., 1992). We wanted to examine if phosphorylation at Y353 altered the ability of IP\(_3\),R1 to colocalize with the TCR upon T cell activation. Using an IP\(_3\),R1–Y353 phosphoepitope-specific antibody, we observed uniformly distributed weak basal Y353 phosphorylation in unstimulated cells with the distribution resembling that of IP\(_3\),R1 on the ER. In unstimulated T cells, the TCR/CD3 fluorescence was uniformly distributed on the plasma membrane (Fig. 1 A, top). However, upon T cell activation, both TCR/CD3 and IP\(_3\),R1 phoshoY353 showed a dramatic colocalization to one site in the cell, forming a tight cluster at the site of stimulation (Fig. 1 A). Additionally, the intensity of the immunofluorescence signal for IP\(_3\),R1 phoshoY353 increased significantly upon T cell activation. Preincubation of the phoshoY353 antibody with a blocking peptide decreased the phoshoY353 signal in activated T cells, confirming that the phosphoepitope antibody was specific for IP\(_3\),R1 phoshoY353 (Fig. 1 A, bottom).

Additionally, we wanted to determine if the cocapping event observed upon T cell activation triggered association of IP\(_3\),R1 with upstream regulators of the IP\(_3\)-generating enzyme PLC\(_{\gamma}\)-1. Coimmunoprecipitation assays identified LAT as a protein that associated with IP\(_3\),R1 upon T cell activation (Fig. 1 B). LAT is tyrosine phosphorylated by ZAP-70 in activated T cells and the phosphorylation of LAT is essential for ensuring the robust activation of PLC\(_{\gamma}\)-1 (Sommers et al., 2002). In ZAP-70–deficient T cells, LAT did not coimmunoprecipitate with IP\(_3\),R1 (Fig. 1 B), which suggests that the association of IP\(_3\),R1 with LAT is mediated by phosphorylation of LAT by ZAP-70.

The activation-dependent cocapping of IP\(_3\),R1 phoshoY353 with the TCR and the association of IP\(_3\),R1 with LAT; an essential component of the IP\(_3\)-generating machinery, suggest that a signaling microdomain forms upon T cell activation whereby IP\(_3\),R1 is found in close proximity to the IP\(_3\)-generating machinery.

**IP\(_3\),R1 phoshoY353 forms a spatially restricted ER substructure colocalized with the TCR**

Because IP\(_3\),R1 is primarily expressed on the ER, we wanted to determine whether IP\(_3\),R1 phoshoY353 clustering represented a general ER reorganization or a specific reorganization of IP\(_3\),R1 phoshoY353. We performed CD3-stimulated cell spreading assays by staining stimulated Jurkat T cells with the luminal ER membrane protein calnexin. Upon T cell activation, calnexin staining remained uniformly distributed throughout the cell, indicating that in Jurkat T cells, the ER does not generally reorganize upon ER Ca\(^{2+}\) release (Fig. 2 A), which is consistent with previously published findings (Luik et al., 2006; Wu et al., 2006). In contrast, the TCR staining showed a strong central focal point with less intense punctate staining extending out to the periphery of the cell (Fig. 2 A). Global phosphoryrosine staining colocalized with the TCR staining is also shown (Fig. 2 A, top). The bottom of Fig. 2 A shows a 3D reconstruction image of the surface immunofluorescence detected in the examined cell slice. Consistent with the 2D images, the calnexin ER staining appears to be uniformly distributed, in contrast to the centrally localized TCR signal, which suggests that no gross changes to the ER occur upon activation. In stimulated cells, IP\(_3\),R1 phoshoY353 colocalized with both the TCR and phosphoryrosine staining (Fig. 2 B).
Figure 1. **Colocalization of IP₃R1 phosphoY353 with TCR upon stimulation.** (A) Jurkat T cells were incubated with mouse mAb against CD3 (OKT3) and a TRITC-conjugated goat anti–mouse antibody. Cells were fixed and stained with either an antibody against IP₃R1 or a phosphoepitope-specific antibody against IP₃R1 phosphoY353. Cells were examined using confocal microscopy and projection images are shown. Unstimulated T cells were examined for IP₃R1 and TCR/CD3 localization. (top) IP₃R1 localization is shown in green and TCR/CD3 localization is shown in red. The far right shows differential interference contrast images. Unstimulated T cells were examined for IP₃R1 phosphoY353 and TCR/CD3 localization. (second from the top) IP₃R1 phosphoY353 localization is shown in green and TCR/CD3 localization is shown in red. (third from the top) The localization of IP₃R1 phosphoY353 and TCR/CD3 in T cells stimulated for 5 min. (bottom) Peptide block–mediated quenching of the IP₃R1 phosphoY353 signal in T cells stimulated for 5 min. Approximately 30% of cells responded by displaying a reorganized, well-formed cap structure. Of that population, a majority of cells showed a robust increase in Y353 phosphorylation signal and colocalization of Y353 with the TCR. Bar, 5 μm. (B) Coimmunoprecipitation of IP₃R1 with LAT in activated Jurkat T cells. Lysate from 2 × 10⁸ cells was incubated with anti-IP₃R1 antibody to immunoprecipitate IP₃R1 and blotted with anti-IP₃R1 (top). [middle] Coimmunoprecipitation of LAT with IP₃R1 with association occurring only in activated Jurkat T cells and not in LAT-deficient cells (LAT⁻/⁻) used as negative control or ZAP70 null cells (ZAP70⁻/⁻), suggesting a phosphorylation-mediated association between LAT and IP₃R1. The arrow designates the IgG signal from the immunoprecipitating antibody. (bottom) The endogenous LAT levels in the various T cell lines used.
3D reconstruction of the surface fluorescence showed that IP$_3$R1 phosphoY353 largely colocalized with the TCR signal, in sharp contrast to the calnexin staining (Fig. 2 B, bottom).

Collectively, these data suggest that upon T cell stimulation, IP$_3$R1 phosphoY353 undergoes a lateral redistribution along the ER membrane, accumulating in spatially restricted domains where it colocalizes with the TCR.

Persistent IP$_3$R1 Y353 phosphorylation is detectable after T cell activation

We had previously determined that Y353 was robustly tyrosine-phosphorylated by 3 min after T cell stimulation (Cui et al., 2004).

To further explore the dynamics of Y353 tyrosine phosphorylation upon T cell activation, we stimulated Jurkat T cells by incubating the cells on CD3-coated coverslips. IP$_3$R1 phosphoY353 was detected as early as 1 min after T cell activation, and the level of phosphoY353 continued to increase up to 30 min after T cell activation (Fig. 3, A and B). In agreement with our previously published data (Cui et al., 2004), Y353 phosphorylation was still detectable at 60 min after the initial T cell activation event (Fig. 3, A and B).

IP$_3$R1-Y353 phosphorylation increases receptor sensitivity to IP$_3$

To determine if the increased affinity of tyrosine-phosphorylated IP$_3$R1 for IP$_3$, (Cui et al., 2004) alters IP$_3$-mediated IP$_3$R1 channel open probability, we conducted single-channel measurements using microsomes generated from human embryonic kidney (HEK) cells transiently cotransfected with either wild-type (WT) IP$_3$R1 and constitutively active Fyn (Fyn Y528F) representing WT-IP$_3$R1 phosphorylated at Y353, WT-IP$_3$R1 and a kinase-dead mutant Fyn (Fyn K296M) representing unphosphorylated WT-IP$_3$R1, or IP$_3$R1-Y353F and Fyn Y528F representing WT-IP$_3$R1, which cannot be phosphorylated at Y353. At [IP$_3$] ranging from 100 nM to 5 μM, tyrosine-phosphorylated IP$_3$R1 exhibited higher open probabilities compared with the mutant IP$_3$R1-Y353F, with an ~4.5-fold decrease in $K_d$ (WT-IP$_3$R1, 0.710 μM ± 0.05, vs. IP$_3$R1-Y353F, 3.264 μM ± 0.05; Fig. 4 A), which is consistent with the increased IP$_3$ sensitivity of Y353-phosphorylated WT-IP$_3$R1. WT-IP$_3$R1 coexpressed with a kinase-dead Fyn mutant exhibited channel activity comparable to IP$_3$R1-Y353F channels (Fig. 4 B). Specifically, the channel activity of phosphorylated WT-IP$_3$R1 was higher than that of IP$_3$R1-Y353F or unphosphorylated IP$_3$R1 at 2 μM IP$_3$ and 173 nM Ca$^{2+}$ (Fig. 4 B). Indeed, phosphorylated WT-IP$_3$R1 exhibited an approximately threefold increase in channel open probability in comparison to both IP$_3$R1-Y353F and unphosphorylated IP$_3$R1 (Fig. 4 B), suggesting that Fyn phosphorylation of IP$_3$R1 at Y353 increases channel activity and that Y353 is a key regulatory Fyn phosphorylation site on IP$_3$R1.
Collectively, these data suggest that IP₃R1-Y353 phosphorylation helps maintain IP₃R1 in the open state, even as [IP₃] levels decline after activation.

**IP₃R1-Y353 phosphorylation reduces Ca²⁺-dependent IP₃R1 channel inactivation**

Having demonstrated that tyrosine phosphorylation of IP₃R1 results in increased channel open probability over a range of [IP₃], we now wanted to determine whether tyrosine phosphorylation modulates the sensitivity of IP₃R1 to Ca²⁺-dependent channel inactivation. IP₃R1 channel activity exhibits a bell-shaped dependence on cytosolic [Ca²⁺], being activated at low [Ca²⁺] (~200–300 nM) and inactivated at higher [Ca²⁺] (Bezprozvanny et al., 1991; Yoneshima et al., 1997; Kaznacheyeva et al., 1998; Picard et al., 1998). In addition to being activated by IP₃, IP₃R1 has to remain open at elevated cytosolic [Ca²⁺] to allow Ca²⁺-dependent lymphocyte activation to proceed.

To determine whether phosphorylation of IP₃R1-Y353 can modulate the Ca²⁺-dependent regulation of the channel, we examined the single channel properties of the channel in response to a physiological range of [Ca²⁺] in the presence of 2 μM IP₃. At [Ca²⁺] that has been shown to inhibit unphosphorylated WT-IP₃R1 (>200–300 nM; Bezprozvanny et al., 1991), tyrosine-phosphorylated WT-IP₃R1 exhibited a significantly higher open...
probability (threefold increase, \(P < 0.05\), \(n > 3\) for each channel type) than IP3R1-Y353F (Fig. 5 A). Mean open times and current amplitudes were similar for both WT-IP3R1 and IP3R1-Y353F (Fig. 5 B). To determine whether the increased open probability of the tyrosine-phosphorylated WT-IP3R1 was influenced by its increased sensitivity to \([\text{IP3}]\) compared with IP3R1-Y353F, we also compared the activity of these channels at 10 and 100 \(\mu\text{M}\) [IP3].

At both 10 and 100 \(\mu\text{M}\) IP3, the difference in channel activity between tyrosine-phosphorylated WT-IP3R1 and IP3R1-Y353F was not significant (unpublished data). Thus, tyrosine phosphorylation of Y353 on IP3R1 increases channel open probability and reduces \(\text{Ca}^{2+}\)-dependent channel inactivation at \([\text{Ca}^{2+}]\) of \(\sim 200-1,000\) nM, which is comparable to the level of sustained \([\text{Ca}^{2+}]\), observed upon lymphocyte activation.

Figure 4. Tyrosine phosphorylation of IP3R1 increases the receptor's IP3 sensitivity. (A) IP3 sensitivity of the channel in the planar lipid bilayer. Channel activity of Fyn-phosphorylated IP3R1 (WT-IP3R1/Fyn Y528F) and phosphorylated IP3R1-Y353F (IP3R1-Y353F/Fyn Y528F) was measured at 173 nM of free cytosolic \(\text{Ca}^{2+}\) in the presence of 2 \(\mu\text{M}\) ruthenium red, 1 mM Na-ATP, and various IP3 concentrations (10 nM to 10 \(\mu\text{M}\)). Open probability values at each IP3 concentration were calculated as a mean from several independent experiments (\(n = 4\) for WT-IP3R1/Fyn Y528F; \(n = 7\) for IP3R1-Y353F/Fyn Y528F; *, \(P < 0.05\) WT vs. Y353F by t test) and fitted by the equation described by Tang et al. (2003). (B) Comparison of open probability for Fyn-phosphorylated WT-IP3R1, Fyn-phosphorylated IP3R1-Y353F, and WT-IP3R1 coexpressed with kinase-dead Fyn (unphosphorylated WT-IP3R1). At 173 nM \(\text{Ca}^{2+}\) and 2 \(\mu\text{M}\) IP3, Fyn-phosphorylated WT-IP3R1 exhibits an approximately threefold increase in channel open probability as compared with both IP3R1-Y353F and unphosphorylated WT-IP3R1 (*, \(P < 0.05\) phosphorylated WT vs. Y353F and phosphorylated vs. unphosphorylated WT by t test). Error bars represent the SEM.

Figure 5. \(\text{Ca}^{2+}\) dependence of WT-IP3R1 and IP3R1-Y353F single-channel activity. (A) A comparison of the open probability of Fyn-phosphorylated WT-IP3R1 and phosphorylated IP3R1-Y353F at varying \(\text{Ca}^{2+}\) concentrations. The activity of the channel was measured in the presence of 2 \(\mu\text{M}\) IP3, 2 \(\mu\text{M}\) ruthenium red, and 1 mM Na-ATP at different \(\text{Ca}^{2+}\) concentrations ranging from 50 nM to 2.5 \(\mu\text{M}\). Each data point represents the open probability calculated as a mean from several independent experiments shown as mean ± SEM. \(\text{Ca}^{2+}\) dependences of tyrosine-phosphorylated WT-IP3R1 (WT-IP3R1/Fyn Y528F, \(n = 3\)) and IP3R1-Y353F (IP3R1-Y353F/Fyn Y528F, \(n = 7\)) were fitted by the bell-shaped equation (*, \(P < 0.05\) WT vs. Y353F by t test). (B) Representative traces of IP3R1 activity at three different \(\text{Ca}^{2+}\) concentrations and the corresponding open probability (\(P_o\)), mean open time (\(T_o\)), and closed time (\(T_c\)) of the channels. Single channel openings are plotted as upward deflections; the open and closed (c) states of the channel are indicated by horizontal bars at the beginning of the traces.
IP₃R₁-Y353 phosphorylation enhances Ca²⁺ entry in B lymphocytes

We previously showed that IP₃R₁-Y353 phosphorylation slows the decay of the Ca²⁺ release transient upon B cell activation (Cui et al., 2004). To further examine both BCR-induced ER Ca²⁺ release and influx, single-cell studies were conducted using IP₃R KO, WT, and IP₃R₁-Y353F–expressing cells. Expression levels of WT/IP₃R₁ and IP₃R₁-Y353F in the stably transfected DT40 cell lines were comparable (unpublished data). IP₃R-KO cells exhibited no BCR-induced ER Ca²⁺ release and the addition of extracellular Ca²⁺ did not trigger Ca²⁺ influx (Fig. 6, A and B). Both WT/IP₃R₁ and IP₃R₁-Y353F cells responded to BCR stimulation manifested as IP₃-induced ER Ca²⁺ release (Fig. 6, A and B). Upon BCR stimulation, the amplitude of the first peak of Ca²⁺ release and the total Ca²⁺ release were increased in DT40 cells expressing WT/IP₃R₁ as compared with IP₃R₁-Y353F–expressing cells (Fig. 6, A and B). Discrete Ca²⁺ oscillations, representing cyclical Ca²⁺ release from and reuptake into ER stores, were observed in ~30% of WT/IP₃R₁–expressing cells, compared with <10% of IP₃R₁-Y353F cells (Fig. 6 B), whereas only a single peak of ER Ca²⁺ release was observed in the remaining cells studied. This suggests that the phosphorylation of IP₃R₁ at Y353 can increase the likelihood that the cell will oscillate upon lymphocyte activation. ER Ca²⁺ loading was similar and the pharmacological emptying of the ER using the SR/ER Ca²⁺ ATPase inhibitor thapsigargin showed that the Ca²⁺ entry machinery was otherwise intact in WT/IP₃R₁–versus IP₃R₁-Y353F–expressing cells (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200708200/DC1). Also, upon addition of extracellular CaCl₂, Ca²⁺ influx was significantly reduced in IP₃R₁-Y353F–expressing cells compared with WT/IP₃R₁–expressing cells in the time measured (Fig. 6 B), suggesting that in cells expressing IP₃R₁-Y353F, the kinetics of Ca²⁺ influx are slower in comparison to WT/IP₃R₁–expressing cells. Collectively, these data suggest that tyrosine phosphorylation of IP₃R₁ at Y353 triggers greater emptying of ER Ca²⁺ stores, thereby causing greater Ca²⁺ influx via capacitative Ca²⁺ entry.

NFAT activity is enhanced by the phosphorylation of IP₃R₁-Y353

Elevation of [Ca²⁺]ᵢ triggers NFAT translocation to the nucleus, eventually leading to IL-2 production (Koretzky and Myung, 2001).
Previous studies revealed that DT40 cells expressing only IP_3R1 exhibited decreased Ca^{2+} oscillations upon IgM stimulation (Miyakawa et al., 1999) and that decreasing the frequency of Ca^{2+} oscillations significantly mutes the NFAT response (Dolmetsch et al., 1998). Also, in lymphocytes, which express all three IP_3R isoforms, there appears to be considerable cooperativity in terms of the Ca^{2+} response upon lymphocyte stimulation (Miyakawa et al., 1999). Thus, to best address whether impaired Ca^{2+} influx observed in IP_3R1-Y353F-expressing cells affected downstream responses, Jurkat cells were transiently cotransfected with an NFAT–luciferase reporter construct and either an empty vector, WT-IP_3R1, or IP_3R1-Y353F. Immunoblotting of transfected cell lysates revealed the levels of WT-IP_3R1 and IP_3R1-Y353F (Fig. 7 A). In stimulated cells expressing WT-IP_3R1, NFAT activity (normalized to the level of IP_3R1 expression as shown in Fig. 7 A) was increased ~1.5-fold compared to vector-transfected cells (Fig. 7 B). This increase was significantly blunted in cells expressing IP_3R1-Y353F (Fig. 7 B). Collectively, these data suggest that phosphorylation of IP_3R1 at Y353 ensures robust NFAT activation.

**Discussion**

In this paper, we demonstrate that IP_3R1-Y353 phosphorylation is important for antigen-induced Ca^{2+} signaling during lymphocyte activation. [Ca^{2+}]_{i} must be elevated for several hours after lymphocyte activation to ensure effective activation of downstream events such as IL-2 production (Huppa et al., 2003). The elevation of [Ca^{2+}]_{i}, is supported by ER Ca^{2+} store depletion, triggering sustained CRAC channel activation. Although recent work has revealed that the ER transmembrane protein STIM1 communicates ER store depletion to CRAC channels (Zhang et al., 2005), thereby triggering Ca^{2+} influx across the plasma membrane, mechanisms used by the cell to maintain depleted ER stores remain unclear. Although IP_3Rs constitute the primary ER Ca^{2+} release channel in lymphocytes, the role of IP_3Rs in mediating sustained ER depletion has been questioned. This is primarily because bulk [IP_3] decreases to basal levels shortly after T cell activation (Guse et al., 1993; Sei et al., 1995) and [Ca^{2+}]_{i} is elevated to levels that inhibit IP_3R1 channel activity in single-channel studies (Bezprozvanny et al., 1991).

In various cell types, physiological responses to extracellular agonists elicit sustained IP_3R activation. The formation of signaling complexes bringing IP_3Rs in close apposition to the cellular IP_3-generating machinery is believed to facilitate the activation of the channel even as global [IP_3] decreases. In neurons, coclustering of bradykinin receptors with IP_3Rs creates a locally elevated IP_3 environment upon receptor stimulation (Delmas et al., 2002). Additionally, in kidney cells, IP_3R complexes with Na/K ATPase, Src, and PLC-γ1, creating sites of locally elevated IP_3 production (Yuan et al., 2005). We show that upon T cell activation, clustering of IP_3R1 phosphoY353 with the TCR and association of IP_3R1 with LAT can also create a locally elevated IP_3 environment, ensuring activation of IP_3Rs even as global IP_3 levels are decreasing. Moreover, modeling studies have shown that receptor clustering decreases the activation threshold and increases the response range to ligand (Bray et al., 1998). Collectively, with the phosphorylated receptor’s increased affinity for IP_3 (Cui et al., 2004), the clustering of phosphorylated IP_3R1 upon T cell activation suggests a novel mechanistic solution to the problem of rapidly decreasing [IP_3].

Recently, it has been shown that T cell activation is mediated and sustained by TCR signaling microclusters, which form on the cell surface (Yokosuka et al., 2005). Interestingly the stimulated T cell in Fig. 1 A reveals a colocalization pattern between the TCR and IP_3R1 phosphoY353, which is suggestive of signaling microclusters, specifically in the TCR–IP_3R1 phosphoY353 colocalization observed near the region where the antibody-mediated TCR clustering is the strongest. This is more clearly observed in the cell spreading assay in Fig. 2 B. It appears that both CD3 and IP_3R1 phosphoY353 are coclustered at the center and edges of the stimulated cell. The staining of both
proteins appears more punctuate in the periphery of the cell, with the clusters at the edge of the cell resembling signaling microclusters. The distribution of phosphorylated IP3R1 into microclusters suggests the formation of discrete sites of ER–plasma membrane (PM) junctions, where maximal activation of phosphorylated IP3R1 could occur, which is reminiscent of the ER–PM junctions observed upon thapsagargin-induced ER Ca2+ release in Jurkat T cells (Luik et al., 2006; Wu et al., 2006).

Additionally, because cytosolic Ca2+ levels must remain elevated for 2–10 h after T cell activation (Huppa et al., 2003), persistent phosphorylation of IP3R1-Y353 even 60 min after the initial T cell activation event provides for sustained receptor activation, ensuring both discrete and controlled ER Ca2+ release even as global [IP3] decreases. At the single channel level, phosphorylation of IP3R1 at Y353 increases the channel’s sensitivity to IP3, allowing increased IP3-dependent ER Ca2+ release at [IP3] <10 μM and reduces Ca2+-dependent inactivation of IP3R1 in the physiological range of [Ca2+] that occurs during lymphocyte activation, allowing for more efficient IP3R1 activation at decreasing [IP3]. It should be noted that the difference in Ca2+-dependent activation of tyrosine-phosphorylated channels can be explained in part by the concurrent difference in IP3 sensitivity. We found that at higher [IP3], the difference in Ca2+-dependent activation between phosphorylated and nonphosphorylated channels was reduced. Thus, given the complex relationship between Ca2+- and IP3-dependent effects on IP3R1 channel activity, it is likely that the responses observed in cells represent combined effects. We propose that phosphorylation of IP3R1 at Y353 provides a mechanism for maintaining IP3R1 channel activity in the presence of globally decreasing [IP3] and inhibitory cytosolic [Ca2+]2.

In single cell studies, WT-IP3R1–expressing cells exhibited increased ER Ca2+ release and increased Ca2+ oscillations from the ER upon IgM stimulation in comparison to IP3R1-Y353F–expressing cells. This increased ER Ca2+ release in WT-IP3R1–expressing cells translated to increased Ca2+ influx across the plasma membrane. Current work detailing the communication between depleted ER stores and CRAC channels on the plasma membrane suggests that STIM1 is essential for communicating the level of ER Ca2+ to CRAC channels. Depletion of STIM1 has been shown to trigger decreased store-operated Ca2+ entry and a loss of CRAC channel activity (Liou et al., 2005; Roos et al., 2005). Recent work has shown that upon ER Ca2+ depletion, STIM1 reorganizes into discrete puncta at specific sites on the ER and colocalizes and potentially interacts with CRAC channels on the plasma membrane (Roos et al., 2005; Zhang et al., 2005; Luik et al., 2006; Wu et al., 2006; Yeromin et al., 2006). These closely apposed ER–PM junctions appear to be discrete sites of Ca2+ entry on the plasma membrane surface and imply a locally regulated elementary unit of store-operated Ca2+ entry. These recent findings strengthen the argument for the formation of spatially restricted ER–PM junctions on T cells, where signaling microdomains can occur and local regulation of Ca2+ signaling can be observed. It would be interesting to determine the localization of phosphorylated IP3R1 relative to the STIM1–CRAC channel signaling unit upon T cell activation to determine if the phosphorylated receptor colocalizes with or modulates local Ca2+ signaling dynamics. Also, recent work has suggested that plasma membrane–localized IP3R1s influence Ca2+ influx (Dellis et al., 2006). As such, the contribution of plasma membrane–localized, Y353-phosphorylated WT-IP3R1 to the increased Ca2+ influx observed upon addition of extracellular Ca2+ is a possibility that cannot be excluded.

The downstream effect of increased ER Ca2+ release and influx is clearly demonstrated in the increased NFAT activity observed in Jurkat cells expressing WT-IP3R1, a response that is blunted in IP3R1-Y353F–expressing cells. Thus, in a model of sustained receptor activation, as global [IP3] decreases and [Ca2+]2 reaches levels that inhibit nonphosphorylated IP3R, IP3R1 phosphoY353 is clustered on the ER in close proximity to the TCR, creating a spatially restricted signaling microdomain. Additionally, IP3R1 phosphoY353 exhibits increased sensitivity to varying [IP3] and exhibits less channel inhibition at increasing [Ca2+]2, thereby allowing it to remain active at inhibitory [Ca2+]2. This sustained receptor activity contributes to elevating cytosolic Ca2+ levels both by ER Ca2+ release and CRAC channel activation, thereby ensuring robust lymphocyte proliferation (Fig. 8).

Materials and methods
Antibodies
Antibodies that recognize IP3R1 and IP3R1 phosphoY353 have been described previously (Cui et al., 2004). Antiphosphotyrosine 4G10 and mouse anti-LAT (Millipore); mouse anti–chicken IgM M4 (SouthernBiotech); mouse anti–CD3 OKT3 (Ortho Biotech); and anti–mouse IgG (Sigma-Aldrich) were used.

Cell culture and transfection
Jurkat T lymphocytes, ANJ3 LAT–deficient cells (provided by L. Samelson, National Institutes of Health, Bethesda, MD), and ZAP-70 null cells were cultured in RPMI 1640 medium containing 8% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified incubator (Thermo Fisher Scientific) with 5% CO2. Chicken DT40 B cells and stable IP3R1-transfected cell lines were cultured as described previously (Cui et al., 2004). HEK293 cells were maintained in DME supplemented with 10% FBS and transfected with plasmids using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen).

Single channel recording and data acquisition
Microsomes from HEK293 cells expressing WT/IP3R1 cotransfected with Fyn Y528F, WT/IP3R1 cotransfected with Fyn K296M, or IP3R1-Y353F cotransfected with Fyn Y528F were prepared as described previously (Koznacheyeva et al., 1998). In brief, pellets were resuspended in homogenization buffer A (1 mM EDTA, 50 mM Tris-HCl, and 1 mM DTT, pH 8.0) supplemented with protease inhibitors and homogenized with a glass homogenizer (Telfon; DuPont). After a second homogenization step with buffer B (0.5 M sucrose, 1 mM EDTA, 20 mM Hepes, and 1 mM DTT, pH 7.5), the cell lysate was centrifuged at 10,000 g for 10 min. Consecutive supernatants were centrifuged at 10,000 g for 15 min and 100,000 g for 124 min (rotor AH-629; Ultra Pro 80; Sorvall). The pellet from the last centrifugation was resuspended in buffer C (10% sucrose and 10 mM MOPS, pH 7.0). The recombinant IP3R1s were reconstituted by spontaneous fusion of microsomes into the planar lipid bilayer (mixture of phosphatidylcholine and phosphatidylserine in a 3:1 ratio; Avanti Polar Lipids, Inc.). Planar lipid bilayers were formed across a 200-μm aperture in a polysulfonate cup (Warner Instruments), which separated two bathing solutions (1 mM EGTA, 1 mM HEDTA, 250/125 mM Hepes/Tris, 50 mM KCl, and 0.5 mM CaCl2, pH 7.35, as a cis solution; and 53 mM Ba(OH)2, 50 mM KCl, and 250 mM Hepes, pH 7.35, as a trans solution). After incorporation, IP3R1 channels were activated with 2 μM IP3 and the activity was recorded in the presence of 2 μM ruthenium red and 1 mM Na-ATP. Concentration of free Ca2+ in the cis chamber ranged from 80 nM to 2.5 μM by consecutive addition of CoCl2 from 20 mM stock and was calculated with WinMaxC program version 2.50 http://www.stanford.edu/~scapton/maxc.html, Bers et al., 1994).
In IP$_3$ dependence experiments, the activity of the channels was measured in the presence of 173 nM free Ca$^{2+}$, 2 μM ruthenium red, and 1 mM Na-ATP at an IP$_3$ concentration range of 10 nM to 50 μM. Single channel currents were recorded at 0 mV using the Axopatch 200A patch clamp amplifier (MDS Analytical Technologies) in gap-free mode, filtered at 500 Hz, and digitized at 4 kHz. Data acquisition was performed using Digidata 1322A and Axoscope 9 software (both from MDS Analytical Technologies). The recordings were stored on a computer (Pentium) and analyzed using pClamp 6.0.2 (MDS Analytical Technologies) and Origin software (6.0; OriginLab). The data in Ca$^{2+}$- and IP$_3$-dependence experiments was fitted with the curves as described previously (Tang et al., 2003). Only channels that exhibited maximum channel open probability exceeding 2% were included in the data analyses.

Cytosolic Ca$^{2+}$ measurement

For single-cell calcium imaging, DT40 cells were loaded with 2 μM Fura-2/AM in culture medium at 37°C for 20 min, washed twice with culture medium, and washed once with nominally Ca$^{2+}$-free medium (107 mM NaCl, 2.2 mM KCl, 1.2 mM MgCl$_2$, 10 mM gluoses, and 20 mM Hepes, pH 7.2). Glass coverslips coated with poly-L-lysine were placed in a perfusion chamber (Photon Technology International) mounted on the stage of the microscope. Ca$^{2+}$ images were captured as described previously (Cui et al., 2002). Only WT-IP$_3$R1- and IP$_3$R1-Y353F-expressing lymphocytes that showed Ca$^{2+}$ release upon BCR stimulation were used for the data analysis. For the measurement of the peak of [Ca$^{2+}$], cells that showed only a single peak of Ca$^{2+}$ release and exhibited oscillations were included in the histogram analysis. For oscillating cells, only the first peak of Ca$^{2+}$ release was measured. For total ER Ca$^{2+}$ release measurements, all cells that showed Ca$^{2+}$ release upon IgM addition were measured. The analysis included the multiple Ca$^{2+}$ release events observed in oscillating WT-IP$_3$R1– and IP$_3$R1-Y353F–expressing cells. Additionally, total [Ca$^{2+}$] release upon IgM stimulation was calculated by measuring the area (Fura2 ratio subtracted from baseline and integrated with time) for all cells showing release upon IgM stimulation.

Ca$^{2+}$ entry was calculated by integrating the peak [Ca$^{2+}$], with respect to time 5 min after the introduction of 1.5 mM CaCl$_2$. 1,500 s after addition of 1 mM Ca$^{2+}$, ionomycin and EGTA were added to calculate maximum and minimum Ca$^{2+}$. For cells treated with thapsagargin, no significant difference in Ca$^{2+}$ entry was observed for the WT-IP$_3$R1–expressing cells when compared with IP$_3$R1-Y353F–expressing cells upon addition of extracellular Ca$^{2+}$. All measurements shown are representative of three to five independent experiments.

NFAT luciferase assay

10^6 Jurkat cells were cotransfected with 3 μg WT-IP$_3$R1 or IP$_3$R1-Y353F plasmids with a 2-μg NFAT-luciferase plasmid and a 15-ng pRL-thymidine...
kinase (TK) control plasmid using Lipofectamine 2000 in 24-well plates. 36 h after transfection, cells were split into two groups and were either stimulated using 1 μg/ml OKT3 anti-CD3 antibody or not stimulated as a control for 7 h, respectively. A luciferase assay was performed using a dual-luciferase assay (Promega) according to the manufacturer’s instructions. The NFAT activity was expressed as fold excess over unstimulated cells for all three populations of cells (vector alone, WT-IP3R1-transfected, and Y353F IP3R1) and was normalized for IP3R1 expression. Data are presented as the mean ± SEM of seven independent experiments each performed in triplicate. Statistical significance was determined using a t test.

**Cell stimulation, immunoprecipitation, and immunoblotting**

For the LAT coimmunoprecipitation experiment, 2 × 10⁶ cells of each cell type were stimulated by incubating with 25 μg/ml anti-CD3 mAb (OKT3) at 37°C for 2 min followed by 1.5 μg/ml goat anti-mouse IgG for 5 min at 37°C. 10 μl of ice-cold PBS was added to stop stimulation and cells were harvested and lysed for immunoprecipitation and immunoblotting as described previously (Cui et al., 2004).

**Confocal microscopy**

For capping experiments imaging IP3R1, Jurkat T cells were stimulated with 20 μg/ml OKT3 for 1 h on ice followed by cross-linking with 10 μg/ml of TRITC-coupled goat anti-mouse antibody (Jackson Immunoresearch Labo- ratories) for 1 h on ice. Cells were pipetted onto poly-l-lysine–coated coverslips and incubated at 37°C for 5 min. The cells were then fixed with 3.7% formaldehyde for 10 min at room temperature. Cells were then washed, permeabilized, and stained with an antibody against IP3R1 at 1:250 dilution or IP3R1 phosphoY353 at 1:10,000 dilution followed by a secondary antibody stain of Alexa 488 goat anti–rabbit at 1:300 dilution (Invitrogen). For peptide blocking experiments, the IP3R1 phosphoY353 antibody was preincubated with peptide at 4°C for 1 h before use. Coverslips were mounted on glass slides using Slowfade Gold antifade reagent (Invitrogen). The slides were examined with a laser scanning confocal microscope (LSM 510 META) with a 100× 1.3 Plan-Neofluor objective lens (both from Carl Zeiss, Inc.). All experiments were conducted at room temperature. Alexa 488 and TRITC were excited at 488 and 543 nm, respectively, and emissions were collected at 500–550 and >585 nm, respectively. Software (MetaView; Carl Zeiss, Inc.) was used for both acquisition and image processing. Z stack images were collected at an optical section thickness of 1 μm with maximum intensity projections of these sections computed to yield a projection image using MetaView software.

**Cell spreading assay**

The assay was performed as described previously (Bromley et al., 2003) with minor modifications. Coverslips coated with anti-CD3 stimulatory antibody (clone HIT3a; BD Biosciences) were preincubated at 37°C for 2 min before use. Jurkat T cells were added, the cells were incubated at 37°C for 2 min followed by 15 μl of ice-cold PBS was added to stop stimulation and cells were harvested and lysed for immunoprecipitation and immunoblotting as described previously (Cui et al., 2004).

**Statistical analysis**

Statistical analysis was performed using the t test for paired samples. A computer program (Origin Pharmacology DoseResp; MicroCal) was used for statistical analysis. The EC₅₀ values were calculated using SigmaPlot 8.0 (Systat Software Inc.) from a sigmoidal curve fitting of all the data points.

**Online supplemental material**

Fig. S1 shows that treatment of IP3R-KO, WT-IP3R1, or IP3R1-Y353F DT40 cells with thapsigargin triggers comparable Ca²⁺ mobilization. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200708200/DC1.

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