Regulation of the type 1 inositol 1,4,5-trisphosphate receptor by phosphorylation at tyrosine 353

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Running title: Tyr353 phosphorylation in IP₃R1
Summary

The inositol 1,4,5-trisphosphate receptor (IP₃R) plays an essential role in Ca²⁺ signaling during lymphocyte activation. Engagement of the T cell or B cell receptor by antigen initiates a signal transduction cascade that leads to tyrosine phosphorylation of IP₃R by Src family nonreceptor protein tyrosine kinases, including Fyn. However, the effect of tyrosine phosphorylation on the IP₃R and subsequent Ca²⁺ release is poorly understood. We have identified tyrosine 353 (Y353) in the IP₃ binding domain of type 1 IP₃R (IP₃R1) as a phosphorylation site for Fyn both \textit{in vitro} and \textit{in vivo}. We have developed a phosphoepitope-specific antibody and shown that IP₃R1-Y353 becomes phosphorylated during T cell and B cell activation. Furthermore, tyrosine phosphorylation of IP₃R1 increased IP₃ binding at low IP₃ concentrations (<10 nM). Using wild-type IP₃R1 or an IP₃R1-Y353F mutant that cannot be tyrosine phosphorylated at Y353, expressed in IP3R deficient DT40 B cells, we demonstrate that tyrosine phosphorylation of Y353 permits prolonged intracellular Ca²⁺ release during B cell activation. Taken together, these data suggest that one function of tyrosine phosphorylation of IP₃R1-Y353 is to enhance Ca²⁺ signaling in lymphocytes by increasing the sensitivity of IP₃R1 to activation by low levels of IP₃.
Introduction

The inositol 1,4,5-trisphosphate receptor (IP₃R) is an intracellular calcium (Ca²⁺) release channel located on the endoplasmic reticulum (ER) of mammalian cells. IP₃Rs belong to a family of intracellular Ca²⁺ release channels that include three major isoforms (IP₃R₁, IP₃R₂ and IP₃R₃) as well as the three forms of the related ryanodine receptors (RyR₁, RyR₂, and RyR₃). Hydrolysis of the minor membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP₂) by phospholipase C (PLC) results in the production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃) (1). IP₃ binds to the N-terminal portion of IP₃Rs (2) and causes Ca²⁺ release from the ER via a nonselective cation pore in the C-terminal portion of the channel (3). Earlier work from our laboratory has demonstrated that IP₃R is required for mobilization of Ca²⁺ from ER stores and Ca²⁺-dependent antigen-specific T cell proliferation via interleukin-2 (IL-2) production in the Jurkat T cell lymphoma line (4).

Following engagement of the T cell receptor (TCR) by antigen-presenting complexes, a cascade of tyrosine kinase activation is initiated. The kinases involved include the Src family nonreceptor tyrosine kinases Lck and Fyn, which phosphorylate the TCR (5). Recruitment of other kinases and adaptor proteins by the phosphorylated TCR ζ-chain leads to the phosphorylation and activation of PLC-γ1 to produce IP₃ (6). During T cell
activation Fyn regulates PLC-γ1 activity via these downstream events, and phosphorylates IP3R1 in T lymphocytes (7). Fyn-mediated tyrosine phosphorylation increases IP3R1 open probability by reducing Ca\textsuperscript{2+}-dependent inhibition of the channel (7). Consistent with these findings, T cells from Fyn knockout mice show reduced Ca\textsuperscript{2+} release (8) and reduced IP3R1 tyrosine phosphorylation in response to TCR ligation (7).

The pleiotropic effects of Fyn on the Ca\textsuperscript{2+} signaling cascade are complex and the precise contribution of IP3R1 tyrosine phosphorylation to the Ca\textsuperscript{2+} signaling events that result in T cell activation and proliferation has not been defined.

We have now identified a Fyn-phosphorylated tyrosine residue in IP3R1 (Y353) and shown that phosphorylation of Y353 increases the binding affinity of IP3 to the IP3R at low levels of IP3 (<10 nM), consistent with the previously reported increases in IP3-activated channel activity in response to Fyn phosphorylation (7). DT40 IP3R-deficient B cells stably expressing a Y353-nonphosphorylatable IP3R1 mutant (IP3R1-Y353F) demonstrated altered Ca\textsuperscript{2+} release from intracellular stores in response to B cell receptor activation.
Materials and Methods

Cell culture and transfection. Chicken DT40 B cells were cultured in RPMI 1640 containing 10% FBS, 1% chicken serum, 50 μM β-mercaptoethanol, penicillin and streptomycin at 37°C in a humidified incubator with 5% CO₂. For transfection, 2 x 10⁷ cells in logarithmic phase growth were mixed with 30 μg linearized plasmid cDNA in 400 μL cytomix medium (9). Electroporation was performed in a 4 mm cuvette using Gene Pulser II apparatus (Bio-Rad) at 350 V, 975 μF. For selection of stably transfected clones, 2 mg/mL G418 was added into culture medium 48 h after transfection and cells were incubated for 10-14 days without disturbance. JE6.1 cells were cultured in RPMI 1640 with 10% FBS. HEK293 cells were kept in DMEM supplemented with 10% FBS and transfected with plasmid cDNA as described (10).

DNA constructs. Full-length human T cell type 1 IP₃R cDNA (11) was cloned into the pcDNA3.1 (-) vector using XhoI/KpnI sites. All mutants were generated by PCR using the QuikChange site-directed mutagenesis kit (Stratagene). The human T cell Fyn was cloned by RT-PCR using total RNA from the Jurkat-TAG cell line and the following primers with EcoR1/BamH1 sites: 5’-GGGGAATTCCAGGCTGTGTGCAATGTAAGG-3’ and 5’-CGCGGATCCTTACAGGTTTTCACCAGGTTGG-3’. The cDNA was cloned into p3×Flag-CMV-10 vector (Sigma) to provide an N-terminal Flag epitope. Fusion of EGFP with the N-terminus of IP₃R1 was obtained by introducing the
XhoI/SacII IP3R1 fragment from pcDNA3.1(-) into the pEGFPC3 vector. A series of GST fusion constructs that include different regions of IP3R1 were created by PCR and cloned in-frame into the pGEX-4T1 vector. Purification of the GST fusion proteins was conducted using methods recommended by the manufacturer (Amersham Pharmacia Biotech). All constructs were verified by automated fluorescent DNA sequence analysis.

**Cell stimulation, immunoprecipitation and immunoblotting.** 2 x 10^8 Jurkat cells were stimulated with 3 µg/mL anti-CD3 mAb (OKT-3) on ice for 10 min, followed by 15 µg/ml goat anti-mouse IgG for 3 min at 37°C. DT40 cells were stimulated with 10 µg/mL anti-chicken IgM mAb (M-4) for 2 min at 37°C. Cells used for immunoprecipitation were rinsed once with PBS and lysed in lysis buffer: 150 mM NaCl, 25 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% Nonidet P-40, 0.4% deoxycholic acid, ‘Complete’ EDTA-free protease inhibitor tablets (Roche Molecular Biochemicals), 1 mM Na3VO4, 0.2 mM PMSF, 1 mM NaF. Lysates were incubated with antibodies for 2 h, and the immune complex was isolated by the addition of protein A-Sepharose for 1 h at 4°C. Precipitated proteins were then eluted with Laemmli sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose membranes for immunoblotting with appropriate antibodies.

**[^3H]IP3 binding and in vitro kinase assay.** Binding studies were performed at 4°C in a total volume of 100 µL. Immunoprecipitated IP3R1 bound to protein A-Sepharose beads was washed once with binding buffer containing 50 mM Tris, pH 8.3, 1 mM EDTA and
incubated with 9 nM [3H]IP3 and varying amounts of unlabeled IP3 for 30 min. Reactions were terminated by centrifugation, washed with 2x binding buffer and suspended in scintillation fluid for bound [3H]IP3 determination. The specific binding was defined as total binding minus nonspecific binding that was measured in the presence of 0.01 and 1 µM unlabeled IP3. Equilibrium competition binding curves were fitted assuming a single class of binding site using nonlinear curve fitting equations (Origin Software). For in vitro Fyn phosphorylation, GST fusion proteins bound to glutathione-Sepharose or immunoprecipitates bound to protein A-Sepharose beads were incubated 10 min at room temperature in 20 µL kinase buffer (25 mM HEPES, pH 7.1, 10 mM MgCl2, 5 mM MnCl2, 0.5 mM EGTA, 1 mM Na3VO4, 1 mM DTT, 100 µM Mg.ATP) in the presence of 5 U active Fyn (Upstate Biotechnology), with or without 10% [γ-32P]ATP. Reaction products were analyzed by autoradiography or immunoblotting using antibodies against phosphotyrosine.

Cytosolic Ca2+ measurement. 2 x 10^6 cells were loaded for 30 min at 37°C with 2 µM Fura-2-acetylmethoxy ester and 0.05% Pluronic F-127 in 400 µL growth medium. Cells were washed twice and resuspended in 2 mL calcium free Hanks’ balanced salt solution (HBSS) containing 1 mM EGTA, 20 mM HEPES and 0.03% BSA. Fluorescence of the stirred cell suspension was measured ratiometrically at 25°C by
emission at 510 nm and excitation at 340 nm and 380 nm, respectively, using a fluorospectrophotometer (PTI). Data were fitted to pulse equations using Origin software from which the first derivative was determined.

**Antibodies.** The following peptides were used to generate specific antibodies against the C-terminus of IP$_3$R1 and phospho-Y353 IP$_3$R1, respectively: (C)-RIGLLGHPPHMNVNPQQPA (12) and (C)-QEKMVpYSLVS. Polyclonal rabbit antibodies were generated at Zymed Laboratories, San Francisco, using the PolyQuik protocol followed by affinity chromatography against epitope peptides. The antibody against the C-terminus of IP$_3$R1 is specific for IP$_3$R1; it does not react with either recombinant IP$_3$R2 or recombinant IP$_3$R3 (data not shown). The following commercial antibodies were also used for immunoblotting and stimulation purposes: mouse monoclonal anti-phosphotyrosine 4G10 (Upstate Biotechnology), anti-GFP (BD Biosciences), mouse anti-chicken IgM M-4 (Southern Biotech), anti-mouse IgG (Sigma) and monoclonal anti-CD3 antibody OKT-3 (Ortho Biotech).
Results and Discussion

**Fyn phosphorylates IP\textsubscript{3}R1 at Y353 in vitro.** We have previously demonstrated that IP\textsubscript{3}R1 is phosphorylated by the tyrosine kinase Fyn during T cell activation (7). We initially studied two predicted phosphorylation sites for Src family tyrosine kinases, based on a consensus motif for these kinases – [R,K]-X(2,3)-[D,E]-X(2,3)-Y (13). These sites were Y482 (in the IP\textsubscript{3}-binding domain) and Y2617 (in the C-terminal cytosolic domain) (11). Mutation of either site or both sites to phenylalanine, however, had no significant effect on the tyrosine phosphorylation pattern in response to Fyn of either full-length recombinant IP\textsubscript{3}R1 containing these mutations or of GST-fusion protein fragments of IP\textsubscript{3}R1 encompassing these residues (data not shown). To find an authentic tyrosine phosphorylation site, fragments of IP\textsubscript{3}R1 (other than those encompassing transmembrane domain regions) were fused to GST and the purified fusion proteins were used as substrates for in vitro Fyn phosphorylation (Fig. 1A). Fragment 305-447 showed the strongest phosphorylation signal. According to the consensus sequence given above, this fragment contains a potential site for Src kinase family phosphorylation, Y353. Mutagenesis of tyrosine to phenylalanine showed that Y353 is the only site in this fragment phosphorylated by Fyn. The Y353F mutant eliminated the phosphorylation signal detected either by autoradiography using radiolabelled [γ\textsuperscript{32}P]ATP, or by the anti-phosphotyrosine antibody 4G10 (Fig. 1B). Y353 is located in the IP\textsubscript{3}-binding
domain and is conserved throughout all three types of IP$_3$R (data not shown). Based on
the 2.2-Å crystal structure of the IP$_3$-binding core of mouse IP$_3$R1 (14), Y353 is located
at the beginning of the β7 β-strand, immediately adjacent to the SI splice variant region
(Fig. 6). The structure indicates that Y353 is highly likely to be present on the exposed
surface of the IP$_3$-binding core, suggesting that Y353 is capable of being phosphorylated
by cytosolic tyrosine kinases.

We next expressed full length wild-type IP$_3$R1 and IP$_3$R1-Y353F in HEK293 cells, and
immunoprecipitated IP$_3$R1 using a specific C-terminal antibody. The Y353F mutant,
however, was still tyrosine-phosphorylated by Fyn in vitro although the signal was
reduced (Fig. 1C). Thus, in full length IP$_3$R1, other tyrosine residues besides Y353 can
also be phosphorylated by Fyn in vitro. However, Fyn phosphorylation of the GST-
fusion protein fragment of IP$_3$R1 containing Y353 was much stronger than that of any
other fragments, suggesting that Y353 may be preferentially Fyn phosphorylated in vivo
(Fig. 1A).

**Fyn phosphorylates IP$_3$R1 at Y353 in vivo.** To analyze tyrosine phosphorylation of
IP$_3$R1 in vivo, we prepared mutants of T cell Fyn with defective kinase activity (K296M) or
with constitutive kinase activity (Y528F) (15) and co-expressed these active Fyn [Fyn(+)] or inactive Fyn [Fyn(-)] constructs with IP3R1 in HEK293 cells. Fig. 2A shows that when co-expressed with Fyn(+), the N-terminal (1-611) portion of wild-type IP3R1 is tyrosine phosphorylated, but the phosphorylation of the Y353F N-terminal mutant is undetectable. Fig. 2B shows that both wild-type and Y353F full length IP3R1 are phosphorylated by Fyn in vivo, consistent with our in vitro data in Fig. 1. To determine whether Y353 is phosphorylated in full length IP3R1, we generated a phosphoepitope-specific antibody (anti-IP3R1-pY353). Anti-IP3R1-pY353 only recognized wild-type full length IP3R1 when co-expressed with Fyn(+). IP3R1-Y353F co-expressed with Fyn(+), wild-type IP3R1, or IP3R1-Y353F co-expressed with Fyn(-) could not be detected by anti-IP3R1-pY353 antibody (Fig. 2C). These results confirmed that Y353 is an authentic site of phosphorylation by Fyn, but not the only site in full length IP3R1 phosphorylated by Fyn.

The anti-IP3R1-pY353 antibody was next used to determine whether Y353 was phosphorylated in antigen-stimulated lymphocytes. The pY353 level in JE6.1 Jurkat T cells and DT40 B cells were detected by activating cells through ligation of the T cell receptor (TCR) or B cell receptor (BCR), respectively. As shown in Fig. 3, Y353 of
IP₃R1 was phosphorylated in both activated T and B lymphocytes; in contrast, only very weak signals were detected in the control non-stimulated cells. We have observed that IP₃R1 immunoprecipitated from activated and nonactivated lymphocytes and subsequently immunoblotted with general antiphosphotyrosine antibody (4G10) shows no significant increase in total phosphotyrosine content (data not shown). This suggests that other tyrosine residues on IP₃R1 (Fig. 1A) are not being specifically phosphorylated in response to antigen-induced lymphocyte activation. While these studies do not exclude the possibility that other tyrosine residues on IP₃R1 are regulated by signaling mechanisms, our experiments using anti-IP₃R1-pY353 antibody show that Y353 is phosphorylated during activation via TCR or BCR ligation.

**Phosphorylation of Y353 modulates affinity of IP₃ binding to the IP₃R.** Since Y353 is located in the IP₃-binding domain of IP₃R1 we investigated the effects of phosphorylation of Y353 on IP₃ binding. Equal amounts of immunoprecipitated proteins from transfected HEK293 cells (measured by immunoblotting) were used in an equilibrium [³H]-IP₃ competition binding assay (Fig. 4A). Fig. 4B compares the [³H]IP₃ specifically bound to phosphorylated or non-phosphorylated IP₃R1 at an IP₃ concentration of 9.1 nM. Phosphorylation of Y353 on IP₃R1 (via co-transfection of
constitutively active Fyn with IP3R1) significantly increased the affinity of IP3 binding.

As shown in Fig. 4C, Fyn phosphorylated IP3R1 exhibited increased [3H]IP3 binding at low unlabeled IP3 concentrations (less than 10 nM) whereas when the unlabeled IP3 concentration in the assay was above 100 nM, no significant differences were observed between phosphorylated wild-type IP3R1, the IP3R1-Y353F mutant or non-phosphorylated wild-type IP3R1. In the case of phosphorylated wild-type IP3R1, for IP3 binding the EC50 = 8.45 ± 1.36 nM (n=7, p<0.05 compared with non-phosphorylated wild-type IP3R1 or phosphorylated IP3R1-Y353F). The EC50 values of non-phosphorylated wild-type IP3R1 or phosphorylated IP3R1-Y353F were 19.34 ± 1.55 nM (n=7) and 14.44 ± 1.22 nM (n=5), respectively. These results are comparable to published Kd values for IP3 binding for native or recombinant IP3R1 (16-18). These data suggest that the channels are more sensitive to low levels of IP3 when Y353 is phosphorylated. According to the known structure of the IP3 binding domain, Y353 is located in one of the regions required to form the IP3-binding site, although it is not one of the residues that directly interacts with IP3 (2,14,19). Phosphorylation at Y353 could influence the conformation of the IP3-binding region in an allosteric manner. Another possibility is that phosphorylation of Y353 may regulate channel activity by modification...
of the coupling between IP<sub>3</sub>- and Ca<sup>2+</sup>-binding to the channel, as Y353 is also situated in close proximity to two known sites of Ca<sup>2+</sup> binding to the IP<sub>3</sub>R (14). Further experiments are required to test these hypotheses.

Effects of Y353 phosphorylation on BCR-induced calcium release from intracellular calcium stores. To address downstream signaling consequences of Y353 phosphorylation, we examined cytosolic Ca<sup>2+</sup> transients in DT40 cells in response to B cell receptor (BCR) activation. We generated DT40 B cell lines that express only wild-type IP<sub>3</sub>R1 or IP<sub>3</sub>R1-Y353F using the triple IP<sub>3</sub>R-deficient DT40 cell line, which lacks all three types of IP<sub>3</sub>R (20). Expression of recombinant IP<sub>3</sub>R1 at levels comparable to that of IP<sub>3</sub>R1 in wild-type DT40 cells was shown by immunoblotting (Fig. 5A). Fura-2 loaded cells were stimulated with anti-chicken IgM, M-4, and the increase in intracellular free Ca<sup>2+</sup> concentration was measured using an extracellular solution of 1 mM EGTA in HBSS as a nominally Ca<sup>2+</sup>-free buffer. As shown in Fig. 5B, the initial rising phase of the Ca<sup>2+</sup> transient was similar between wild-type and IP<sub>3</sub>R1-Y353F cells. However, the decay of the Ca<sup>2+</sup> transient in wild-type IP<sub>3</sub>R1 cells was significantly slower than that in IP<sub>3</sub>R1-Y353F cells. To compare the Ca<sup>2+</sup> release of wild-type IP<sub>3</sub>R1 cells and IP<sub>3</sub>R1-Y353F
cells, the fluorescent ratio data were fitted to a curve and normalized. The inset of Fig. 5B shows that the increased duration of the Ca^{2+} transient in wild-type IP_{3}R1 cells is caused by a prolonged decay phase. The first derivative of the Ca^{2+} transient is shown in Fig. 5C. In external Ca^{2+} conditions that preclude store-operated Ca^{2+} entry, the first derivative reflects the kinetics of IP_{3}R1 channel activity and ER Ca^{2+} release. The maximum rate of release is determined primarily by the kinetics of channel activation, whereas the maximum rate of decay reflects the kinetics of channel inactivation. The first derivative reveals that the main difference between wild-type and IP_{3}R1-Y353F mutant channels is enhanced inactivation of the channel in the tyrosine phosphorylation deficient mutant (time constant=0.135 ± 0.011 for wild-type IP_{3}R1, 0.266 ± 0.020 for IP_{3}R1-Y353F, $P<0.01$, n=4). This suggests that one role of tyrosine phosphorylation of IP_{3}R1 at Y353 is to decrease inactivation of the channel, particularly as IP_{3} levels are falling following the initial activation of the channel. This finding is consistent with our results showing that tyrosine phosphorylation of IP_{3}R1-Y353 enhances IP_{3} binding when [IP_{3}] < 10 nM. Indeed, the inset of Fig. 5C shows that the maximum rate of decay of IP_{3}R1-Y353F is significantly faster than that of wild-type IP_{3}R1.

In the current study, we have demonstrated that phosphorylation of IP_{3}R1-Y353
increases the affinity of the channel for IP$_3$. The time course of the Ca$^{2+}$ release transient is closely paralleled by the time course of IP$_3$ concentration changes (21). Based on studies from other laboratories, the basal physiological level of IP$_3$ in lymphocytes is about 10 to 60 nM, and reaches 100 to 300 nM during antigen stimulation (21,22). We propose that phosphorylation of IP$_3$R1 at Y353 affects the amount of IP$_3$ bound to the channel only when the IP$_3$ level decreases below 100 nM, a condition that would occur in cells during the same period as the decay phase of the Ca$^{2+}$ transient. During this period, the inactivation time of IP$_3$R1-Y353F was faster than that of wild-type IP$_3$R1. During initiation of Ca$^{2+}$ release, the rapid increase of IP$_3$ via antigen receptor activation of PLC is likely to cause the IP$_3$ concentration to rise above 100 nM. Our data show that IP$_3$ binding to wild-type IP$_3$R1 and IP$_3$R1-Y353F mutant is not significantly different at higher IP$_3$ concentrations in the physiologic range (e.g. 100 nM) (Fig. 4B). Therefore, wild-type IP$_3$R1 and IP$_3$R1-Y353F have similar activation profiles for initial Ca$^{2+}$ release despite their disparate tyrosine phosphorylation states. It has been shown that tyrosine phosphorylation modulates the channel activity of both IP$_3$Rs and RyRs in Jurkat (7,23). However, the precise phosphorylation sites and the importance of specific tyrosine residues have not been previously ascertained. In the
present study, we characterized a tyrosine residue in IP$_3$R1 that is phosphorylated by Fyn. Y353 is located in the IP$_3$-binding domain of IP$_3$R1 and phosphorylation of Y353 modulates the affinity of the channel for IP$_3$. Increased affinity for IP$_3$ results in prolongation of the open state of the channel during the antigen response phase in which the IP$_3$ level decays from its peak toward basal levels. These results support our previous hypothesis (7) that tyrosine phosphorylation of IP$_3$R1 increases channel activity at higher cytosolic [Ca$^{2+}$]$_i$ during antigen receptor activation.

Acknowledgements

This work was supported by grants RO1 AI39794 and RO3 TW00949 (to A.R.M.) from the National Institutes of Health. S.J.M. is supported by an American Heart Association Postdoctoral Fellowship.
References


22. Scharenberg, A. M., El-Hillal, O., Fruman, D. A., Beitz, L. O., Li, Z., Lin, S.,
1961-1972

Chem. 276, 34722-34727
Figure legends

**Fig. 1. In vitro phosphorylation of Y353 of IP$_3$R1 by Fyn.** (A) Tyrosine phosphorylation of GST-fusion protein fragments of IP$_3$R1. Amino acids comprising each fragment are denoted in the figure. Phosphorylation was analyzed by immunoblotting with anti-phosphotyrosine antibody 4G10 (P-Tyr) (upper panel). Fyn is autophosphorylated in this assay, giving rise to the 59 kDa phosphorylated band seen with all treatments. Protein levels were detected by Ponceau S staining (lower panel). (B) Tyrosine phosphorylation of GST-fusion proteins consisting of amino acids 305-449 of IP$_3$R1. Phosphorylation was analyzed by immunoblotting with anti-phosphotyrosine antibody 4G10 (P-Tyr) (upper panel) or autoradiography (lower panel). Protein levels were detected by Ponceau S staining (middle panel). (C) Tyrosine phosphorylation of full length IP$_3$R1 immunoprecipitated from HEK293 cells expressing recombinant IP$_3$R1. Upper panel shows autoradiography of the phosphorylated protein. Lower panel shows the protein level of immunoprecipitated IP$_3$R1. The graph shows the relative phosphorylation level of IP$_3$R1-Y353F (80.0 ± 11.8%, n=4, $P>0.05$) compared to wild-type IP$_3$R1 (100%), which was determined by densitometric quantitation of the autoradiographic signal normalized to the immunoblot signal.
Fig. 2. In vivo phosphorylation of Y353 of IP3R1 by co-expression with constitutively active Fyn [fyn (+)] or inactive Fyn [fyn (-)] in HEK293 cells. Immunoprecipitates were analyzed by immunoblotting with general anti-P-Tyr antibody 4G10 (A and B) or antibody specific for phosphorylated Y353 of IP3R1 (pY353) (C). (A) Tyrosine phosphorylation by fyn(+) of a protein consisting of GFP fused to the N-terminus of an IP3R1 fragment, amino acids 1-611. The band migrating near 50 kDa in all three lanes represents the heavy chain of mouse IgG. (B) Tyrosine phosphorylation by fyn(+) and fyn (-) of full-length IP3R1 (without GFP fusion). (C) Tyrosine phosphorylation at Y353 of full-length IP3R1.

Fig. 3. Y353 is phosphorylated in response to TCR or BCR stimulation. JE6.1 cells or DT40 cells were stimulated by 3 µg/mL OKT-3 or 10 µg/mL M-4 for 2-3 min at 37°C, respectively. IP3R1 was immunoprecipitated by antibody against C-terminal of IP3R1 and detected by specific antibody against phospho-Tyr353 of IP3R1 (upper panel). The membrane was stripped and re-probed with anti-IP3R1 antibody (lower panel). The graph shows the relative phosphorylation level at Tyr353 in activated vs nonactivated lymphocytes, which was determined by densitometric quantitation of the
anti-phospho-Tyr353 immunoblot signal normalized to the anti-IP3R1 signal. The IP3R1-Tyr353 phosphorylation level of activated JE6.1 cells was taken as 100%. In comparison, nonactivated JE6.1 cells had 31%, activated DT40 cells had 120% and nonactivated DT40 cells had 14% phosphorylation of IP3R1-Tyr353.

Fig. 4. Phosphorylation at Tyr353 of IP3R1 increases the affinity of IP3 binding. (A) Western blot of the phosphotyrosine level and protein level of IP3R1 used in the binding assay. (B) Specific [3H]IP3 (9.1 nM) binding to immunoprecipitated wild-type IP3R1 or IP3R1-Y353F (* denotes p < 0.05 in comparison with the wild-type coexpressed with Fyn(+)). (C) Competition of [3H]IP3 binding to IP3R1 by varying amounts of unlabeled IP3. Values are normalized to 100% of maximum specific binding. Results are the means ± S.E. from 5-7 independent experiments.

Fig. 5. Effects of the Y353F mutation of IP3R1 on intracellular Ca2+ release by BCR-induced B cell activation. (A) Expression level of IP3R1 in wild-type DT40 cells; triple IP3R-deficient DT40 cells and triple IP3R-deficient DT40 cells stably transfected with wild-type IP3R1 or IP3R1-Y353F. (B) Intracellular Ca2+ response in Fura-2 loaded
DT40 cells in Ca^{2+}-free HBSS monitored by spectrofluorometry after stimulation with M-4 anti-chicken IgM mAb (5 µg/mL). Insert shows the fitting curves for the Ca^{2+} transient. (C) First derivative of Ca^{2+} transient in (A). Inset shows the normalized maximum decay rate. (* Denotes $P < 0.05$ in comparison with the wild-type IP_{3}R1, n=4.)

Fig. 6. Location of Y353 in the IP_{3}-binding core region of IP_{3}R1. The 2.2-Å crystal structure of the IP_{3}-binding core of mouse IP_{3}R1 in complex with IP_{3} is shown as previously published by Bosanac et al. (figure 2A of reference 14). The IP_{3} molecule (shown in purple) is bound at the interface between the α-domain (green) and the β-domain (yellow) and the locations of residue Y353, the SI splice region, the CaI-Ca^{2+} binding region and IP_{3} are marked. The crystal structure is reprinted by permission from Nature 420:696-700, copyright 2002, Macmillan Publishers Ltd (http://www.nature.com/nature).