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Fyn binds to and phosphorylates T cell Immunoglobulin and mucin domain-1 (Tim-1)

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Abstract

The gene encoding T cell immunoglobulin and mucin domain-1 (TIM-1) is linked to atopy and asthma susceptibility in mice and humans. TIM-1 is a transmembrane protein expressed on activated lymphocytes and appears to have a role as a co-stimulatory receptor in T cells. The protein has not been shown to have enzymatic activity but contains a site within its cytoplasmic tail predicted to be a target for tyrosine kinases. Here, we show that Tim-1 can associate with the kinase Fyn, a member of the Src family of tyrosine kinases. This association does not require Fyn's kinase activity and is independent of the phosphorylation of a conserved tyrosine present within the cytoplasmic tail of Tim-1. Fyn is necessary for phosphorylation of this tyrosine in Tim-1 and the phosphorylation of Tim-1 varies with the levels of Fyn present in cells. These data suggest a role for Fyn in the signaling downstream of Tim-1.

1. Introduction

The ability of cells to respond to external signals requires the presence of a large array of transmembrane proteins that function as receptors. The immune system is reliant on such proteins to help coordinate the cellular responses required for an effective immune response.

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Conflict of Interest

P.D. Rennert is a stockholding employee of Biogen Idec Incorporated. The other authors have no financial or commercial conflicts of interest to disclose.

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One family of such surface receptors, called the TIMs (T cell immunoglobulin and mucin domain), is thought to regulate both physiologic and pathologic immune responses (Curtiss and Colgan, 2007; Freeman et al., 2010; Rodriguez-Manzanet et al., 2009; Su et al., 2008). TIM-1 was initially cloned from African green monkeys (*HAVcr1*) (Kaplan et al., 1996) and humans as a cellular receptor for hepatitis A virus (*HAVCR1*) (Feigelstock et al., 1998). Polymorphism of *TIM-1* have been linked to atopic diseases and asthma in both mice and humans. Using a genetic approach, McIntire et al. demonstrated that a DBA/2-derived genomic segment containing the Tim family of genes (*Tapr* locus) could decrease the susceptibility of BALB/c mice to atopy (McIntire et al., 2001).

The biological function of Tim-1 is unclear. Although originally described as a molecule secreted by kidney proximal tubule epithelial cells upon injury (Ichimura et al., 1998), subsequent studies demonstrated Tim-1 is expressed by several cells of the immune system, including activated CD4⁺ T cells (McIntire et al., 2004; Meyers et al., 2005; Umetsu et al., 2005) and activated B cells (Wong et al., 2010). Tim-1 appears to bind to several ligands, among them LMIR-5/CD300b, phosphatidylserine, and possibly itself and/or Tim-4. Tim-1, Tim-3, and Tim-4 contain IgV domains with a conserved loop in the ligand-binding domain that forms a pocket for phosphatidylserine (DeKruyff et al., 2010; Kobayashi et al., 2007; Miyanishi et al., 2007; Santiago et al., 2007), and overexpression of Tim-1 on a kidney epithelial cell line conferred a phagocytic phenotype, allowing uptake of apoptotic cells (Ichimura et al., 2008). Tim-1 and Tim-4 also bind to LMIR5/CD300b, expressed on granulocytes and other cells (Yamanishi et al., 2010). Other literature suggests Tim-1 can bind Tim-4 or can bind other Tim-1 molecules through homotypic interactions (Santiago et al., 2007; Wilker et al., 2007). Thus, Tim-1 engagement by its various ligands may contribute to both immune and non-immune responses.

Signaling pathways involving Tim-1 have been studied in T cells through overexpression studies in Jurkat cells. A conserved tyrosine residue in the cytoplasmic tail of Tim-1 is situated in a motif that is similar to a consensus sequence recognized by SH2 domain-containing proteins (Songyang and Cantley, 1995). Tim-1 appears to be phosphorylated at a basal level in Jurkat cells, and treatment with pervanadate (de Souza et al., 2005) or TCR activation (Binne et al., 2007) increases the level of Tim-1 phosphorylation. A Jurkat cell line with reduced Lck levels (JCaM1) exhibits reduced Tim-1 phosphorylation in response to pervanadate (de Souza et al., 2008), and overexpression of Lck in this line restored pervanadate-induced Tim-1 phosphorylation. However, the limited tissue distribution of Lck suggests that Tim-1 may be phosphorylated by other Src family kinase members in non-CD4⁺ T cells. For example, B cells upregulate surface Tim-1 in response to BCR stimulation *in vitro* and *in vivo* (Wong et al., 2010) but do not express Lck. We present data that the Src family kinase member Fyn can bind to and phosphorylate Tim-1 in a B cell line, thus implicating Fyn in signaling downstream of Tim-1.

2. Materials and Methods

2.1 Cell lines

293T cells were maintained in DMEM/10% w/v FCS/1000 U/mL penicillin/1000 µg/mL streptomycin/20 mM L-glutamine/10 mM sodium pyruvate and transfected as indicated by the calcium phosphate method (Arimura et al., 2004) or Lipofectamine 2000 (Invitrogen). M12.4.1 (Hamano et al., 1982) and A20.2J (McKean et al., 1981) cells were obtained from Dr. Gail Bishop (University of Iowa) and maintained in RPMI 1640/10% w/v FCS/1000 U/mL penicillin/1000 µg/mL streptomycin/20 mM L-glutamine/10 mM sodium pyruvate/5% w/v non-essential amino acids/50 µM β-mercaptoethanol ("complete medium").

2.2 Antibodies and reagents

Tim-1 specific rat IgG2a monoclonal antibody clones 1H9 and 3A2 have been previously described (Sizing et al., 2007). Abcam anti-Myc rabbit polyclonal antibody, Santa Cruz anti-Myc (clone 9E10) mouse monoclonal antibody, Santa Cruz FYN3 polyclonal antibody, and Biogen anti-Tim1 (clone 1H9) were used for immunoprecipitation. Normal rabbit IgG was purchased from Santa Cruz. Rat IgG2a isotype control was purchased from BD Pharmingen. For M12.4.1 cell immunoprecipitations, rat IgG2a isotype control (clone mAb72) was purified from hybridoma supernatants. Jackson ImmunoResearch Affinipure goat anti-rat IgG (H+L) was used to coat Dynabeads protein G (Invitrogen).

The following antibodies were used for Western blot: Biogen anti-Tim-1 (rat monoclonal, clone 3A2), Abcam anti-Myc (rabbit polyclonal), Millipore anti-Myc (mouse monoclonal, clone 4A6), Invitrogen anti-Xpress (mouse monoclonal), BD Transduction Laboratories anti-Fyn (mouse monoclonal, clone 25), Santa Cruz anti-Fyn (rabbit polyclonal, FYN3), Cell Signaling anti-Lck (rabbit polyclonal), Upstate anti-Src (mouse monoclonal, clone GD11), Upstate anti-Hck (rabbit polyclonal), Roche anti-HA (rat monoclonal, clone 3F10), and Sigma anti-actin (rabbit polyclonal). Millipore anti-phosphotyrosine (4G10) was used to probe immunoprecipitations from 293T cells. Millipore 4G10 Platinum HRP was used to probe immunoprecipitations from M12.4.1 cells.

The following antibodies were used for FACS: PE anti-Tim-1 (clone RMT1-4) and PE anti-rat IgG2b isotype control were purchased from Biolegend. Biotinylated anti-Tim1 (clone RMT1-4), anti-Tim2 (clone RMT2-1), and rat IgG2b isotype control were obtained from eBioscience.

2.3 DNA Constructs

Full-length (isoform a) myc-tagged Tim-1 was constructed by amplification of Tim-1 from BALB/c kidney cDNA with the following primers: forward 5'-CTAGCATGAATCAGATTCAAGTCTTCATTTTCAGGCCTCATACTGCTTCTCCCA GGC-3' and reverse 5'-GGTACCGGTCTATCTTCAACAATGTAGATGTTGTCTTCAGCTCGGG-3'. PCR products were ligated into pCR TOPO BLUNT II (Invitrogen), sequenced and subcloned into pcDNA3.1B (Invitrogen) in frame with the Myc epitope tag using the *KpnI* and *NheI* restriction sites. A Stratagene QuikChange Site-Directed Mutagenesis kit was used according to manufacturer's instructions to generate the Tim1/Myc Y299F construct. pcDNA3.1B Tim1/Myc and Tim1/Myc Y299F were amplified and ligated into pCR TOPO BLUNT II, digested with *EcoRI*, and subcloned into pMIG to generate pMIG Tim1/Myc and pMIG Tim1/Myc Y299F. Myc-tagged Tim-1 isoform b (lacking exon 6 of the mucin domain) was generated by amplification of C57BL/6 kidney cDNA as above, then cloning the PCR product into pcDNA3.1B in frame with the Myc epitope tag.

Tim-2/Myc was generated by amplification of pCMV-SPORT Tim-2 (Open Biosystems) with the following primers: forward 5'-ATAGCTAGCATGAATCAGATTCAAGTCTTC-3', reverse 5'-TTAGGTACCCAGGACTCTTCTTCAGGGTA-3'. Tim-3/Myc was generated by amplification of cDNA from BALB/c CD4⁺ T cells stimulated in Th1-polarizing conditions with the following primers: forward 5'-GCTAGCATGTTTTTCAGGTCTTACCCTC-3', reverse 5'-GGTACCCAGGATGGCTGCTGGCTGTT-3'. PCR products were digested with *NheI* and *KpnI*, then ligated into pcDNA3.1B in frame with the Myc epitope tag.

Src, Fyn-T, and kinase-dead Fyn-B (K296E) expression plasmids were a kind gift from Dr. Gloria Lee (University of Iowa). LYN expression plasmid was obtained from Dr. Dina Alexandropoulos (Columbia University). The LCK expression plasmid was generated by

subcloning an LCK construct (obtained from Dr. Jon Houtman, University of Iowa) into pcDNA3.1B using *NheI* and *XhoI* restriction sites. HCK and YES-1/HA expression plasmids were generated by amplification of HCK and YES-1 cDNAs (both obtained from Open Biosystems) with the following primers: HCK for 5' AAGCTAGCATGGGGGGGCGCTCAAG 3', rev 5' GGTACCTCATGGCTGCTGTTGGTACTGGCTC 3'; YES-1 for 5'-GAGCTAGCATGGGCTGCATTAAG-3', rev 5'-GCGGTACCTAAATTTCTCCTGG-3'. YES-1 (lacking a stop codon) and HCK PCR products were cloned into pCR TOPO Blunt II (Invitrogen), sequenced, and subcloned into pcDNA3.1B (Invitrogen) using the *NheI* and *KpnI* restriction sites. An oligonucleotide encoding the sequence for the HA epitope tag and a stop codon was inserted after the YES-1 coding sequence using the *KpnI* and *XbaI* restriction sites to generate C' HA-tagged Yes-1.

Retroviral constructs expressing mir30-flanked shRNA hairpins targeting murine Fyn were generated following LMP manufacturer instructions using the RNAi Central – RNAi Oligo Retriever website (<http://katahdin.cshl.edu/html/scripts/resources.pl>) to generate oligos (Supplemental Table 1). These oligos were amplified with the following primers: sense 5'-CAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCG-3'; antisense 5'-CTAAAGTAGCCCCCTGAATCCGAGGCAGTAGGCA-3'. The PCR products were digested with *XhoI* and *EcoRI*, then subcloned into the LMP retrovirus (Open Biosystems).

2.4 Retroviral transduction of M12 cells

pMIG, pMIG Tim1/Myc, and pMIG Tim1/Myc Y299F constructs were transiently transfected into 293T cells with the pCL-ECO packing plasmid (Naviaux et al., 1996). Supernatants were harvested at 48 hours post-transfection and M12 cells were resuspended at a concentration of 5×10^5 cells/mL in supernatant with 20 mM HEPES and 8 μ g/mL polybrene, with 1 mL per well of a 24-well plate. Plates were centrifuged at 2000 rpm for 2 hours (30°C). 6 hours after spinfection, 1 mL media was added to each well and cell lines were subsequently maintained at a density of less than 1×10^6 cells/mL. Fyn-deficient M12 cells were generated as above by transduction with LMP retrovirus expressing short RNA hairpins targeting Fyn. This retrovirus contains an IRES and GFP coding sequence downstream of the sequences for RNA hairpins. 24 hours post-transduction, cells were selected and maintained in 4 μ g/mL puromycin. Transduction efficiency was greater than 98%, as assessed by flow cytometry for GFP expression.

2.5 Lysis Buffers and Immunoprecipitation

293T cells were harvested 48 hours after transfection and lysed in a buffer containing 0.5% NP-40, 50 mM HEPES, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 4 mM EDTA, 2 mM PMSF, 2 μ g/mL aprotinin, and 10 μ g/mL leupeptin. Lysates were quantified by Bradford assay. 1 mg precleared lysate was immunoprecipitated by 2 μ g of antibody. In some experiments, 293T cells were activated by incubation in 2 mL PBS containing 100 μ M sodium vanadate and 0.068% hydrogen peroxide for 10 minutes at 37°C to serve as a positive control.

Activation and immunoprecipitation of full-length murine Tim-1 from M12.4.1 cells was performed as previously described (Rowland et al., 2007). Dynal protein G beads were conjugated with Jackson anti-rat (10 μ g mAb/10 μ L beads/100 μ L complete medium) for 30 minutes at room temperature. Beads were washed three times in complete medium and incubated with anti-Tim1 clone 1H9 or isotype control antibody (10 μ g/10 μ L beads/100 μ L complete medium) for 30 minutes, washed three times with RPMI complete medium and resuspended in 25 μ L complete medium/10 μ L beads. $3 - 4 \times 10^7$ M12 cells were washed and resuspended at 2×10^8 cells/mL in RPMI complete medium, incubated at 37°C for 30

minutes, and activated by the addition of 25 μ L antibody-coated beads for 5 minutes or with beads and 100 μ M pervanadate for 10 minutes at 37°C. Activation was terminated by addition of cold lysis buffer with a final concentration of 1% Triton X100, 100 mM NaCl, 40 mM Tris pH 7.5, 1.0 mM CaCl₂, 1 mM MgCl₂, 2 mM Na₃VO₄, protease inhibitor (Roche) and 0.05 mg/ml DNase II.

Samples were subjected to SDS-PAGE electrophoresis, transferred to PVDF (Millipore), and probed with the indicated antibodies. To detect phosphorylated Tim-1, membranes were first probed with anti-phosphotyrosine, stripped, and then reprobed for Tim-1. Anti-Myc was used to detect myc-tagged Tim-1, and anti-Tim1 mAb 3A2 (Biogen) was used to detect endogenous Tim-1 from M12.4.1 cells.

2.6 SH2 array and peptide

A tyrosine phosphorylated peptide containing the following Tim-1 cytoplasmic tail sequence was purchased from Quality Control Biochemicals: VVHSRAEDNIYIVEDRP. A Panomics SH2 array was probed with this peptide as per manufacturer's instructions.

3. Results

3.1 Fyn phosphorylates Tim-1

The cytoplasmic tail of Tim-1 contains a conserved tyrosine residue within a motif favorable for recognition by SH2 domains of Src family kinases (de Souza et al., 2008; Songyang and Cantley, 1995). To evaluate the ability of this sequence to interact with SH2 domain-containing proteins, we probed a Panomics SH2 array with a phosphopeptide containing this conserved sequence. We found that this peptide was able to interact with several SH2 domain-containing proteins, among them the Src family kinases Fyn, Yes-1, and Hck (Supplemental Figure 1), as well as GRB2, RAS-GAP1 and the PI3 kinase p85 α and p85 β subunits. These results were consistent with studies performed by others (de Souza et al., 2008) and suggest that Tim-1 may interact with Src family kinases.

To determine which Src family kinase members could interact with and phosphorylate Tim-1 in cells, constructs encoding six of these proteins, (Src, Lck, Fyn-T, Lyn, Yes-1, and Hck) were individually co-transfected into 293T cells with epitope-tagged Tim-1 in 293T cells. Tim-1 was immunoprecipitated and Western blotting was performed on immunoprecipitates and lysates. When we blotted with an anti-phosphotyrosine antibody (Figure 1a) we found that co-expression of Fyn-T with Tim-1 consistently resulted in a high levels Tim-1 tyrosine phosphorylation, suggesting that Fyn-T can phosphorylate Tim-1. In contrast to Fyn-T, the other Src family kinases examined did not induce high levels of Tim-1 tyrosine phosphorylation when expressed in 293T cells. Expression of Src resulted in co-immunoprecipitation of another phosphorylated protein of roughly 100 kDa, which is not consistent with the size of Tim-1. Co-expression of Src, Lck, Lyn, and Fyn-T with Tim-1 resulted in co-immunoprecipitation of phosphorylated proteins of 55–60 kDa, a size consistent with Src family kinases. We confirmed co-immunoprecipitation of Src, Lck, Lyn (data not shown), and Fyn-T (Figure 1a) by stripping and blotting with antibodies to these kinases. Lysates were also blotted for expression of Src, Lck, Yes, Fyn, Lyn, and Hck (data not shown). Although Hck and Yes-1 were able to weakly bind the Tim-1 phosphopeptide by SH2 array, they did not interact with Tim-1 in this overexpression system. Fyn was selected for further study, as it was the only kinase which phosphorylated Tim-1 in 293T co-transfection assays. We also found that immunoprecipitation of Fyn-T resulted in co-immunoprecipitation of Tim-1, indicating that this may be a direct interaction between the proteins (Figure 1b).

To further assess specificity of the interaction between Fyn-T and Tim-1, graded amounts of Fyn-T expression plasmid were co-transfected into 293T cells with a constant amount of Tim-1 expression plasmid. A resultant graded increase in phosphorylation of Tim-1 and in the amount of Fyn-T co-immunoprecipitating with Tim-1 was observed, with a correlation between levels of Fyn co-immunoprecipitation and Tim-1 phosphorylation (Figure 2). These data suggest that Fyn and Tim-1 can interact and that overexpression of Fyn leads to increased tyrosine phosphorylation of Tim-1.

3.2 Fyn binds Tim-1 in the absence of tyrosine 299 in 293T cells

The observed interaction between Fyn and Tim-1 suggests that these proteins may interact to initiate signaling downstream of Tim-1. Thus, we sought to further define the requirements for this interaction. The studies using SH2 arrays suggested that the SH2 domain of Fyn could bind a tyrosine-phosphorylated motif of Tim-1. To assess the requirement for phosphorylation of this tyrosine residue in the interaction of Fyn-T with Tim-1, we used site-directed mutagenesis to introduce a phenylalanine residue in place of the tyrosine at position 299 of Tim-1 (isoform a) within the putative Fyn recognition site. Previous studies indicate that mutation of this tyrosine to an alanine abrogated phosphorylation of human TIM-1 and murine Tim-1 (isoform b) in pervanadate-treated transfected Jurkat cells (Binne et al., 2007; de Souza et al., 2005). Co-expression of the Y299F mutant Tim-1 construct with Fyn in 293T cells abrogated phosphorylation of Tim-1 via Fyn, as well as by pervanadate (Figure 3a). However, the ability of Fyn to co-immunoprecipitate with the Y299F mutant Tim-1 construct was maintained. These data indicate that the interaction between Tim-1 and Fyn may not require phosphorylation of Y299 in the putative SH2 domain binding site.

As a complementary study of the requirement for phosphorylation in Fyn binding to Tim-1, we determined the requirement for Fyn kinase activity in its binding to Tim-1. An expression plasmid for a Fyn mutant lacking enzymatic activity ("kinase-dead") was co-expressed in a graded fashion with a constant amount of Tim-1 in 293T cells. This again resulted in a graded co-immunoprecipitation of the kinase-dead Fyn with Tim-1, although there was no detectable phosphorylation of Tim-1 (Figure 3b). These data suggest that, although there may be enhanced interaction between Tim-1 and Fyn upon tyrosine phosphorylation of Tim-1, the binding of Fyn to the cytoplasmic tail of Tim-1 does not require Fyn kinase activity.

3.3 Tim-1 is phosphorylated in B cells and interacts with Fyn

Recent studies have suggested a role for Tim-1 in lymphoid cells. Therefore, we extended our studies of the interaction between Tim-1 and Fyn to B cells. To identify antibodies that recognize Tim-1 by Western blot and immunoprecipitation, we transfected 293T cells with Myc-tagged Tim-1, Tim-2, and Tim-3. Lysates were subjected to immunoprecipitation with anti-Myc or anti-Tim1 antibodies, and lysates and immunoprecipitates were probed with antibodies to Myc or Tim-1. We identified antibodies that recognize both the full-length isoform a (cloned from BALB/c mice) and the exon 6 deleted isoform b (cloned from C7BL/6 mice) of Tim-1, but not Tim-2 or Tim-3 (Supplemental Figure 2), and used these to probe Tim-1 expression in B cell lines.

Recent studies have demonstrated Tim-1 surface expression on murine splenic B cells stimulated with anti-IgM or PMA and ionomycin *in vitro*, by germinal center B cells *in vivo*, and on the B cell line A20 (Sizing et al., 2007; Wong et al., 2010; Yamanishi et al., 2010). We confirmed these results and demonstrated that another B cell line, M12.4.1, also expressed Tim-1 on its surface and that this expression was higher than in A20.2J cells (Supplemental Figure 3). Neither cell line expressed detectable surface Tim-2. Both M12.4.1

and A20.2J cells express a protein species of roughly 75 kDa (data not shown), similar to that of Tim-1 when transiently expressed in 293T cells. Furthermore, this species is recognized by antibodies to Tim-1 in immunoprecipitation and Western blot analysis. Tim-1 was expressed at higher levels on M12.4.1 cells, thus experiments were performed in this cell line.

In order to determine if Fyn may play a role in Tim-1 signaling in B cells, we asked if these two proteins could interact in M12.4.1 cells. Cells were incubated with anti-Tim-1 antibodies that were conjugated to beads to mimic ligand:receptor coupling. After 5 minutes, activation was terminated by addition of lysis buffer. We then examined the status of Tim-1 and Fyn in these cells. Immunoprecipitated endogenous Tim-1 did not display basal tyrosine phosphorylation. Treatment with pervanadate or incubation with anti-Tim-1 beads induced Tim-1 tyrosine phosphorylation (Figure 4). Fyn co-immunoprecipitated with endogenous Tim-1 in unstimulated cells, and engagement of Tim-1 by antibody-coated beads or activation with pervanadate resulted in increased co-immunoprecipitation of Fyn with Tim-1 compared to unstimulated cells. These data demonstrate that Fyn and Tim-1 interact in B cells at a baseline state and that this interaction is increased when Tim-1 is activated. Fyn also co-immunoprecipitated with Tim-1, and this increased in response to cross linking with anti-Tim-1 antibodies (data not shown).

Our studies employing 293T cells have defined some of the requirements for interaction between Tim-1 and Fyn. To extend these studies to B cells, we overexpressed epitope-tagged Tim-1 or Tim-1 Y299F in M12 cells to determine the requirement for the conserved tyrosine in Fyn interaction and Tim-1 phosphorylation. When cells overexpressing Tim-1 or Tim-1 Y299F were incubated with anti-Tim-1 conjugated beads, Fyn was co-immunoprecipitated to similar levels. However, we observed tyrosine phosphorylation of both Tim-1 and Tim-1 Y299F (Supplemental Figure 4). Although overexpression of Tim-1 Y299F in 293T cells and the Jurkat T cell line abrogated phosphorylation by pervanadate (de Souza et al., 2008; de Souza et al., 2005), cross linking the overexpressed Y299F mutant receptor in M12 cells still resulted in Tim-1 phosphorylation. Thus, it appears that the membrane-proximal tyrosine residue of Tim-1 may be phosphorylated upon cross linking.

3.4 Fyn is required for phosphorylation of Tim-1 in B cells

The interaction between Fyn and Tim-1 suggests that Fyn may function in the activation of Tim-1. To address whether Fyn is necessary for Tim-1 phosphorylation, we utilized RNAi to knock down Fyn in M12 cells. M12 cells were transduced with retrovirus expressing mir30-flanked shRNA sequences to knock down Fyn (denoted Fyn1, 2, and 3) or a control sequence against luciferase (Luc), and stable cell lines were generated by selection and maintenance in puromycin. Cells transduced with Fyn shRNA 3 expressed approximately 5–10% of the levels found in the Luc shRNA control cells, and cells transduced with Fyn shRNA1 and 2 expressed Fyn at 10–30% of that found in Luc shRNA control cells. (Figure 5a). Activation of Tim-1 with antibody-coated cells resulted in phosphorylation of Tim-1 in cells expressing the control Luc shRNA, and reduced phosphorylation of Tim-1 in cells expressing Fyn shRNA (Figure 5b), with the least phosphorylation in the cells expressing the most potent Fyn knockdown construct (Fyn3). The reduction in phosphorylation appeared to be roughly 10fold in these cells. This data suggests that the presence of Fyn is required for the phosphorylation of Tim-1 in the B cell lines.

4. Discussion

Tim-1 is a surface receptor expressed by diverse cell types, including kidney proximal tubule epithelial cells, CD4⁺ T cells, and activated B cells. The cytoplasmic tail of Tim-1 contains a conserved tyrosine residue embedded in a motif predicted to be recognized by

SH2 domain-containing proteins. This tyrosine residue was previously reported by be phosphorylated by Lck when Tim-1 was overexpressed in Jurkat cells. Based on our data, we propose that Tim-1 is phosphorylated by Fyn in B cells.

Several lines of evidence indicate that Fyn may bind to Tim-1 in an SH2-dependent manner. The SH2 domain of Fyn was able to bind a phosphopeptide from the tail of Tim-1. Co-expression of Fyn and Tim-1 resulted in phosphorylation of the conserved tyrosine within the tail of Tim-1. However, the ability of Tim-1 and Fyn to interact in phosphotyrosine-independent manner was also detected. Endogenous Tim-1 and Fyn interact in murine B cell lines, and 293T expression studies demonstrate the interaction was maintained even when a “kinase-dead” Fyn was co-transfected with Tim-1 or when the conserved tyrosine 299 of Tim-1 was mutated to a phenylalanine. These results suggest that Fyn recognizes and interacts with the cytoplasmic tail of Tim-1 even in the absence of its phosphorylation. Binding of Fyn-T to the Tim-1 cytoplasmic tail may initially occur at a basal state prior to Tim-1 activation, and upon receptor aggregation Fyn binding may create a docking site to which other signaling molecules may then be recruited, or recruitment of Fyn to Tim-1 may instead be the initial event allowing phosphorylation of tyrosine 299. This suggests a potential sequential model of engagement, whereby low levels of Fyn are associated with Tim-1 in a resting state, and engagement of the receptor results in rapid accumulation of Fyn and phosphorylation of Tim-1.

Our results differ somewhat with experiments performed in Jurkat cell lines. Kane et al. demonstrated reduced phosphorylation of Tim-1 in JCam1 cells, which express reduced levels of Lck. Overexpression of Lck in JCam1 cells restored Tim-1 phosphorylation in response to pervanadate, although a role for Fyn in Tim-1 phosphorylation could not be ruled out (de Souza et al., 2008). Fyn levels in Jurkat cells are roughly 30 fold lower than in peripheral CD4+ T cells, while Lck levels are similar. Moreover, Lck levels are 30 fold higher than Fyn in Jurkat cells (Denny et al., 2000). Fyn and Lck are thought to have partially overlapping roles in TCR signaling in T cells. It is possible that Lck-mediated signaling pathways are regulated differently in JCam1 cells relative to wildtype cells, secondary to the dysregulation of Fyn. TIM-1 has been immunoprecipitated with the CD3 complex from Jurkat cells (Binne et al., 2007), thus it is likely that TIM-1 may be in proximity with both Lck and Fyn during TCR stimulation. It is also possible that in Jurkat cells, Lck phosphorylates Tim-1 as a consequence of its higher expression level relative to Fyn.

The role of Fyn in B cells is not clear. Fyn associates with components of the BCR at baseline and upon receptor engagement (Clark et al., 1992; Clark et al., 1994; Pleiman et al., 1994). However, Fyn deficient mice do not have defects in B cell responses to BCR stimulation by soluble antibodies *in vitro* or in T-dependent antibody production or germinal center formation *in vivo* (Appleby et al., 1995; Cannons et al., 2006; McCausland et al., 2007; Sillman and Monroe, 1994). Fyn deficient B cells exhibit slight hypoproliferation in response to BCR stimulation by T-independent Type II antigens (Horikawa et al., 1999) and in mounting an IgG3 response to T-independent type II antigens (Appleby et al., 1995), possibly due to the multivalent nature of these molecules. Fyn deficient mice on a mixed 129/C57BL/6 background develop spontaneous production of low levels of autoantibodies (Yu et al., 2001), and the locus encoding Fyn is frequently lost in B cell acute lymphoblastic leukemia (Kuiper et al., 2007). This suggests that although Fyn is redundant with other Src family kinases in some settings, in others it may play a unique role in B cell signaling and survival. Tim-1 activation through Fyn may affect B cells in ways that have not been previously characterized.

In conclusion, we have provided the first methodology to perform biochemical analysis on Tim-1 without the use of epitope tags or overexpression, the first evidence that this methodology can be applied to B cells, and the first evidence that engagement of endogenous Tim-1 by antibody-conjugated beads results in tyrosine phosphorylation of Tim-1 and recruitment of the Src kinase Fyn. These studies may provide an experimental method by which to further study Tim-1 signaling in B cells and other cell types.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Tim-1	T cell and immunoglobulin domain 1 (other names include Timd-1 and Havcr-1)
BCR	B cell receptor
TCR	T cell receptor
SH2	Src homology 2 domain

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Figure 1. Tim-1 interacts with and is phosphorylated by Fyn in 293T cells

A. Myc-tagged Tim-1 expression plasmid was co-transfected into 293T cells with Src family kinase expression plasmids. Tim-1 was immunoprecipitated with anti-Myc and IP samples were immuno-blotted (IB) with antibodies to phosphotyrosine, Myc, and Fyn-T. Positions of Src, Lck, Fyn-T, and Lyn are indicated (*). Representative of two experiments.

B. Myc-tagged Tim-1 expression plasmid was co-transfected into 293T cells with Fyn-T expression plasmid. Fyn-T was immunoprecipitated with anti-Fyn rabbit polyclonal antibodies and IP samples were probed with antibodies to Myc (to detect Tim-1) and Fyn. Representative of two experiments.

Figure 2. Tim-1 phosphorylation correlates with increased expression of Fyn and increased Fyn co-immunoprecipitation

Myc-tagged Tim-1 expression plasmid was co-transfected into 293T cells with increasing amounts of Fyn-T expression plasmid. Pervanadate treatment was used as a positive control for phosphorylation of Tim-1. Tim-1 was immunoprecipitated with anti-Myc and IP samples were probed with antibodies to phosphotyrosine, Myc, and Fyn. Position of Fyn is indicated (*). Representative of three experiments.

Figure 3. Fyn binds Tim-1 in the absence of tyrosine 299
A. Myc-tagged Tim-1 or a Tim-1 Y299F mutant expression plasmid were co-transfected into 293T cells with Fyn-T expression plasmid. Pervanadate treatment was used as a positive control for phosphorylation of Tim-1. Tim-1 was immunoprecipitated with anti-Myc and IP samples were probed with antibodies to phosphotyrosine, Myc, and Fyn. Position of Fyn-T is indicated (*). Representative of three experiments.
B. Myc-tagged Tim-1 was co-transfected into 293T cells with increasing amounts of a “kinase-dead” K296E mutant Fyn (isoform Fyn-b) expression plasmid. Pervanadate treatment or co-transfection with wildtype Fyn expression plasmid was used as a positive control for phosphorylation of Tim-1. Tim-1 was immunoprecipitated with anti-Myc and IP samples were probed with antibodies to phosphotyrosine, Myc, and Fyn. Position of Fyn-b is indicated (*). Representative of three experiments.

Figure 4. Tim-1 is phosphorylated in B cells upon receptor engagement with antibody-coated beads

M12 cells were incubated with beads conjugated to anti-Tim-1 or isotype control antibodies for 5 minutes, then lysed and Tim-1 was immunoprecipitated. M12 cells treated with pervandate and anti-Tim-1 beads for 10 minutes were used as a positive control. IPs were probed with antibodies to phosphotyrosine, Tim-1, and Fyn. Position of Fyn is indicated (*). Representative of four experiments.

Figure 5. Tim-1 phosphorylation is reduced in Fyn-deficient B cell lines

A. M12 cells were transduced with a retrovirus encoding constructs targeting Fyn or a control shRNA to luciferase. Fyn knockdown in stable cell lines was assessed by Western blot using a FujiImager to quantify signal intensity. Mean values of three experiments are shown; error bars represent standard error of mean.

B. M12 cells were incubated with beads conjugated to anti-Tim-1 or isotype control for 5 minutes, then lysed and Tim-1 was immunoprecipitated. IPs were probed with antibodies to phosphotyrosine, Tim-1, and Fyn. Phosphorylated Tim-1 (4G10 relative to anti-Tim1 signal) was assessed by western blot using a FujiImager to quantify signal intensity from immunoprecipitated Tim-1. Representative of two experiments.