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The B-cell transmembrane protein CD72 binds to and is an *in vivo* substrate of the protein tyrosine phosphatase SHP-1

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Background: Signals from the B-cell antigen receptor (BCR) help to determine B-cell fate, directing either proliferation, differentiation, or growth arrest/apoptosis. The protein tyrosine phosphatase SHP-1 is known to regulate the strength of BCR signaling. Although the B-cell co-receptor CD22 binds SHP-1, B cells in CD22-deficient mice are much less severely affected than those in SHP-1-deficient mice, suggesting that SHP-1 may also regulate B-cell signaling by affecting other signaling molecules. Moreover, direct substrates of SHP-1 have not been identified in any B-cell signaling pathway.

Results: We identified the B-cell transmembrane protein CD72 as a new SHP-1-binding protein and as an *in vivo* substrate of SHP-1 in B cells. We also defined the binding sites for SHP-1 and the adaptor protein Grb2 on CD72. Tyrosine phosphorylation of CD72 correlated strongly with BCR-induced growth arrest/apoptosis in B-cell lines and in primary B cells. Preligation of CD72 attenuated BCR-induced growth arrest/death signals in immature and mature B cells or B-cell lines, whereas preligation of CD22 enhanced BCR-induced growth arrest/apoptosis.

Conclusions: We have identified CD72 as the first clear *in vivo* substrate of SHP-1 in B cells. Our results suggest that tyrosine-phosphorylated CD72 may transmit signals for BCR-induced apoptosis. By dephosphorylating CD72, SHP-1 may have a positive role in B-cell signaling. These results have potentially important implications for the involvement of CD72 and SHP-1 in B-cell development and autoimmunity.

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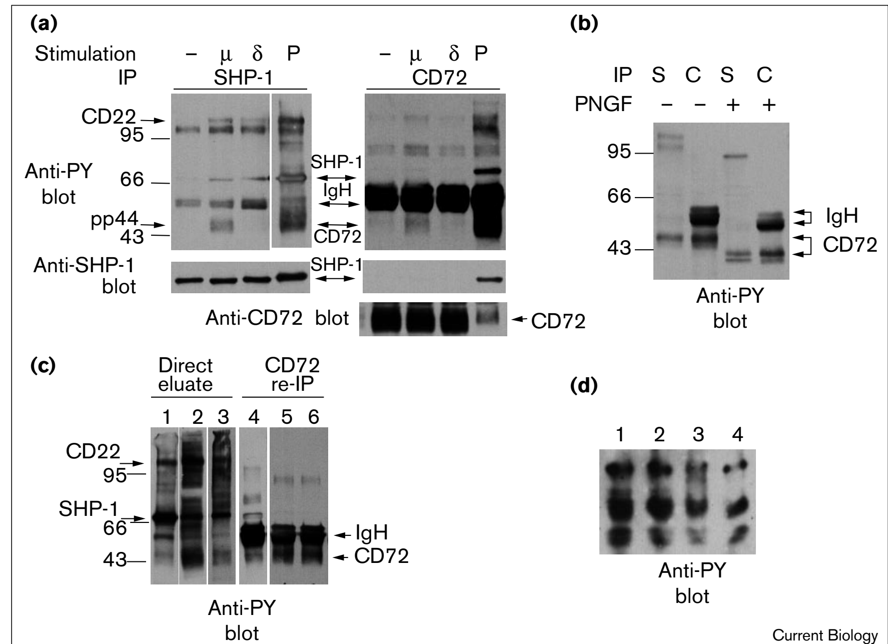
Background

The intensity of signals from the B-cell antigen receptor (BCR) is a key determinant of the outcome of antigenic stimulation. BCR engagement activates several protein tyrosine kinases, leading to the tyrosine phosphorylation of many downstream signaling molecules [1]. Other B-cell surface molecules (for example co-receptors) modulate the BCR signal so that appropriate responses are generated [2,3]. A critical intensity of BCR signaling is required for survival and proliferation (positive selection); more intense signals evoke anergy or deletion (negative selection) [2,3]. As B cells mature, the threshold for deletion is increased, with more intense BCR stimulation required for growth arrest/cell death [4]. The precise mechanism by which early signaling events are integrated and translated into B-lymphocyte responses remains unclear.

The protein tyrosine phosphatase (PTP) SHP-1 is a negative regulator of many hematopoietic signaling pathways, as illustrated by the phenotype of mice lacking SHP-1 expression — *motheaten* (*me*/*me*) — or expressing defective SHP-1 — *motheaten viable* (*me*^{v}/*me*^{v}) [5]. SHP-1 binds to and is activated by members of a family of inhibitory receptors, which deliver SHP-1 to the vicinity of activating receptors and/or their substrates [5,6]. These inhibitory receptors contain at least one copy of the consensus amino-acid sequence I/V/LXpYXXL/V (in single-letter amino-acid code), termed the immunoreceptor tyrosine-based inhibitory motif (ITIM), in their intracellular domains. Upon tyrosine phosphorylation, ITIMs are specifically recognized by the SH2 domains of SHP-1, its relative SHP-2 and/or the inositol monophosphatase SHIP [5–9].

BCR responsiveness is markedly enhanced in SHP-1-deficient B cells, indicating that SHP-1 negatively regulates BCR signaling [10,11]. SHP-1 appears to act, at least in part, by binding to the inhibitory co-receptor CD22 [12–14]. SHP-1 binds phosphotyrosine peptides based on

Figure 1



Identification of SHP-1-associated pp44 as CD72. **(a)** SHP-1 or CD72 immunoprecipitates (IPs) from unstimulated (–) Wδ cells or Wδ cells stimulated for 1 min with Bet-2 anti-IgM antibody (μ), anti-Sp6 anti-IgD antibody (δ) or pervanadate (P), as indicated, were analyzed by anti-phosphotyrosine (PY) immunoblotting (upper blots). The positions of known proteins, including immunoglobulin heavy chains (IgH), are indicated between the two blots and numbers on the left are molecular weight markers in kDa. Blots were sequentially reprobed for SHP-1 and CD72 (middle and lower gels). Note that the blotting antibody for CD72 (directed against the CD72 cytoplasmic domain) recognizes tyrosine-phosphorylated CD72 poorly. **(b)** SHP-1 (S) and CD72 (C) IPs from Bet-2-stimulated Wδ cells were treated with or without peptide N-glycanase F (PNGF) before analysis by anti-PY blotting. The positions of IgH and CD72 before and after treatment are indicated. **(c)** Pervanadate-stimulated Wδ lysates were immunoprecipitated with SHP-1 antibody (lane 1) or were allowed to bind to GST–(C/S)SHP-1 (lane 2) or GST–Grb2 SH2 domain (lane 3) fusion proteins. Bound proteins were either eluted directly with SDS–PAGE sample buffer (lanes 1, 2, and 3 respectively), or were eluted with 50 mM free phosphotyrosine, followed by re-immunoprecipitation (re-IP) with anti-CD72 antibodies (lanes 4, 5, and 6 respectively). Direct eluates and CD72 re-IPs were analyzed by anti-PY blotting. **(d)** Pervanadate-stimulated Wδ lysates were immunoprecipitated with SHP-1 (lane 1) or CD72 (lane 2) antibodies, or were allowed to bind to GST–Grb2 SH2 domain (lane 3) or GST–(C/S)SHP-1 (lane 4) fusion proteins. Bound proteins were resolved by SDS–PAGE and the corresponding 44 kDa regions were excised from the gel and then subjected to phosphotyrosine peptide mapping using V8 protease.

the CD22 ITIMs, and preligation of CD22 on B cells (which presumably sequesters CD22 from the BCR complex) markedly lowers the threshold for BCR stimulation [12]. Primary B cells from $CD22^{-/-}$ mice and CD22-deficient B-cell lines exhibit enhanced calcium flux upon BCR stimulation, consistent with a negative regulatory role for the CD22–SHP-1 complex [15–19]. However, the B-cell phenotype in $CD22^{-/-}$ mice and cell lines is much less severe than that produced by SHP-1 deficiency [15–19]. Thus, SHP-1 may also act via other molecules. Recent studies suggest that the transmembrane glycoprotein PIR-B/p91A may be one such molecule [20,21], but additional regulators of SHP-1 function in B cells have not been excluded.

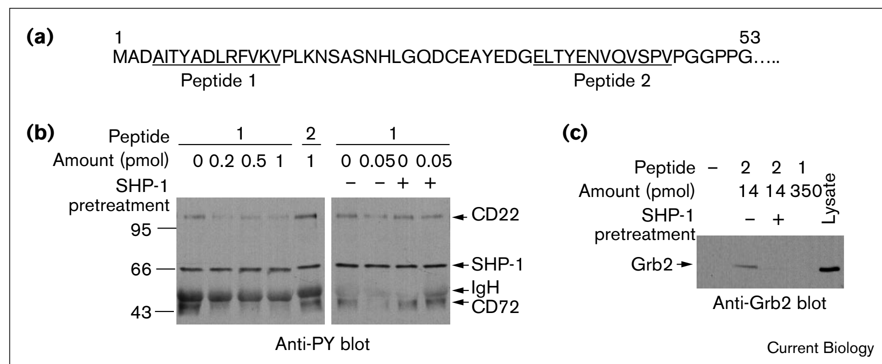
Although several molecules that bind to the SH2 domains of SHP-1 have been defined, no direct targets of SHP-1 in B cells have been identified. The identification of SHP-1 targets is essential for understanding how SHP-1 regulates B-cell activation and how the absence of SHP-1 leads to altered B-cell development and signaling. Here, we have shown that the transmembrane protein CD72 binds SHP-1 and we have identified CD72 as a direct SHP-1 target. CD72 tyrosine phosphorylation strongly correlates with BCR stimuli that lead to B-cell growth arrest/cell death, and antibody ligation experiments suggest that tyrosine-phosphorylated CD72 may transmit signals leading to B-cell death. Moreover, ligation of CD22 and CD72 have opposite effects on the outcome of BCR activation. Our results suggest that the tyrosine phosphorylation status of CD72 may help to determine the outcome of BCR engagement and suggest a new role for SHP-1 in regulating signals from CD72.

Results

Identification of the 44 kDa SHP-1-associated tyrosine phosphoprotein as CD72

A 44 kDa phosphotyrosine protein (pp44) co-immunoprecipitates with SHP-1 only under conditions in which B-cell lines or normal B cells respond to BCR stimulation by undergoing growth arrest/apoptosis [22]. For example, in Wδ, a derivative of the immature B-cell line WEHI-231 which expresses endogenous surface immunoglobulin M (sIgM) and exogenous (transfected) surface IgD (sIgD), crosslinking of sIgM, but not sIgD, leads to growth arrest and apoptosis [23]. Although nearly all early signaling events mediated by sIgM and sIgD are identical in Wδ cells [22–24] (see also below), pp44–SHP-1 association was observed only upon sIgM stimulation ([22]; see also Figure 1a). Notably, the

Figure 2



CD72 ITIMs mediate binding to SHP-1 and Grb2. **(a)** Sequence of the amino-terminal half of the intracellular domain of CD72. Sequences of the two biotinylated tyrosine phosphopeptides (peptides 1 and 2) used in (b) and (c) are underlined. **(b)** SHP-1 IPs prepared from membrane fractions of Wδ cells stimulated with anti-IgM (Bet-2) antibody in the presence of the indicated concentrations of peptide 1 or 2 were analyzed by anti-PY blotting. In the right panel, the indicated amounts of peptide 1 were pretreated with GST (–) or GST–SHP-1 (+) before addition to the IP mixtures. Numbers on the left are molecular weight markers in kDa. **(c)** Unstimulated Wδ lysates were incubated with streptavidin–agarose beads pre-adsorbed with the indicated amounts of biotinylated peptides. Bound proteins and a fraction of the total lysates were analyzed by anti-Grb2 blotting. Where indicated, peptide 2 was pretreated with either GST (–) or GST–SHP-1 (+) as in (b) before adsorption to the beads.

CD22–SHP-1 complex was induced upon engagement of either sIgM or sIgD (Figure 1a). Following treatment with the global PTP inhibitor pervanadate, the pp44–SHP-1 complex was detected in all B cells analyzed (Figure 1a and data not shown), suggesting that tyrosine phosphorylation, not expression, of pp44 is regulated differentially in different B cells.

Biochemical studies indicated that pp44 is a disulfide-linked surface glycoprotein that binds directly to the SH2 domains of SHP-1 and the adaptor protein Grb2 in a phosphotyrosine-dependent manner *in vivo* and *in vitro* (Figure 1c and data not shown). These properties were reminiscent of CD72, a disulfide-linked homodimer of a type II transmembrane glycoprotein which is expressed selectively in B cells and contains two ITIMs [25,26] (Figure 2a).

Multiple lines of evidence indicate that pp44 is CD72. Like pp44, CD72 was tyrosine-phosphorylated in Wδ cells upon anti-IgM crosslinking or pervanadate treatment, but not following anti-IgD stimulation (Figure 1a). CD72 and pp44 co-migrated before and after treatment with N-glycanase F (Figure 1b). SHP-1 was detected in CD72 immunoprecipitates prepared from pervanadate-stimulated cells (Figure 1a). A fusion protein comprising glutathione-S-transferase (GST) fused to catalytically inactive SHP-1 (GST–(C/S)SHP-1) or a fusion protein comprising GST fused to the SH2 domain of Grb2 (Figure 1c, lanes 1–3) bound to pp44. Treatment of these complexes with phosphotyrosine (50 mM) led to elution of pp44, which could be immunoprecipitated with anti-CD72 antibodies (Figure 1c, lanes 4–6). These data and phosphatase protection assays (Figure 3) indicate a direct interaction between the SH2 domain(s) of SHP-1 and one or more CD72 tyrosine phosphorylation sites. Finally, V8 partial peptide maps of pp44 and CD72 were identical (Figure 1d). Together, these results identify CD72 as the SHP-1-associated and Grb2-associated pp44 protein.

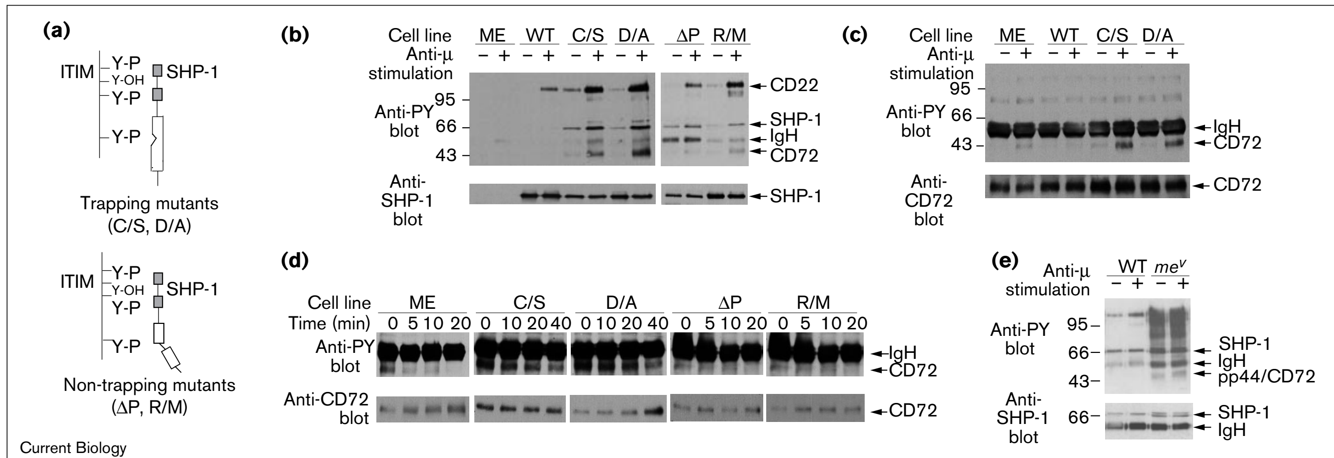
Distinct sequences within CD72 mediate SHP-1 and Grb2 binding

CD72 contains two ITIMs (Figure 2a). The second ITIM is also a potential Grb2-binding sequence (pYXNX) [27]. We identified the binding sites for SHP-1 and Grb2 on CD72 by phosphotyrosine peptide binding and competition studies. A phosphopeptide based on the first ITIM of CD72 (Figure 2a) disrupted the CD72–SHP-1 complex at 50 nM; a phosphopeptide based on the second ITIM (peptide 2, Figure 2a) failed to compete even at 1 μM (Figure 2b). Conversely, when coupled to beads, peptide 2 (14 pmol), but not peptide 1 (at greater than 350 pmol), bound to Grb2 from Wδ lysates (Figure 2c). The respective dephosphopeptides were inactive in these assays (Figure 2b,c). Neither peptide 1 nor peptide 2 alone precipitated SHP-1 from Wδ lysates (data not shown), suggesting that two appropriately oriented ITIMs present in a CD72 dimer may be required for high-avidity binding to the tandem SH2 domains of SHP-1, as reported for SHP-1 binding to killer inhibitory receptors (KIRs) on natural killer (NK) cells [28].

CD72 is a substrate for SHP-1

One reason why SHP-1 might be recruited to CD72 would be to catalyze CD72 dephosphorylation. To determine whether CD72 is an SHP-1 target *in vivo*, we used a set of B-cell lines derived from *me/me* mice (ME) and reconstituted with wild-type SHP-1 or various SHP-1 mutants. SHP-1 contains three domains capable of binding phosphotyrosine proteins (its two SH2 domains and its PTP domain), making it difficult to easily distinguish *bona fide* SHP-1 targets from phosphotyrosine proteins that merely bind SHP-1 through its SH2 domains. To resolve this

Figure 3



CD72 is a direct substrate of SHP-1. **(a)** Schematic representation of SHP-1 mutants with different amino-acid substitutions and their predicted effects on a hypothetical ITIM-containing protein. Increased binding/protection is expected with trapping mutants compared with non-trapping mutants. **(b)** SHP-1 IPs from unstimulated or anti-IgM (Bet-2)-stimulated SHP-1$^{-/-}$ B-cell lines reconstituted with expression vector alone (ME) or the various indicated mutant forms of SHP-1 were analyzed by anti-PY blotting and anti-SHP-1 reblotting: WT, wild type. Bands overlapping the IgH chains in the ΔP lanes were not observed reproducibly. **(c)** CD72 IPs from various cell lines were analyzed as in (b). **(d)** *In vitro* PTP protection assays (see Materials and methods and Results) were performed on the indicated Bet-2-stimulated cell lines. CD72 IPs were analyzed by anti-PY blotting and anti-CD72 reblotting. Similar results were obtained when the corresponding SHP-1 IPs were analyzed (data not shown). **(e)** SHP-1 IPs from unstimulated and anti-IgM-stimulated splenic B cells from wild-type or me^v/me^v mice were analyzed by anti-PY blotting and anti-SHP-1 reblotting. Numbers on the left are molecular weight markers in kDa.

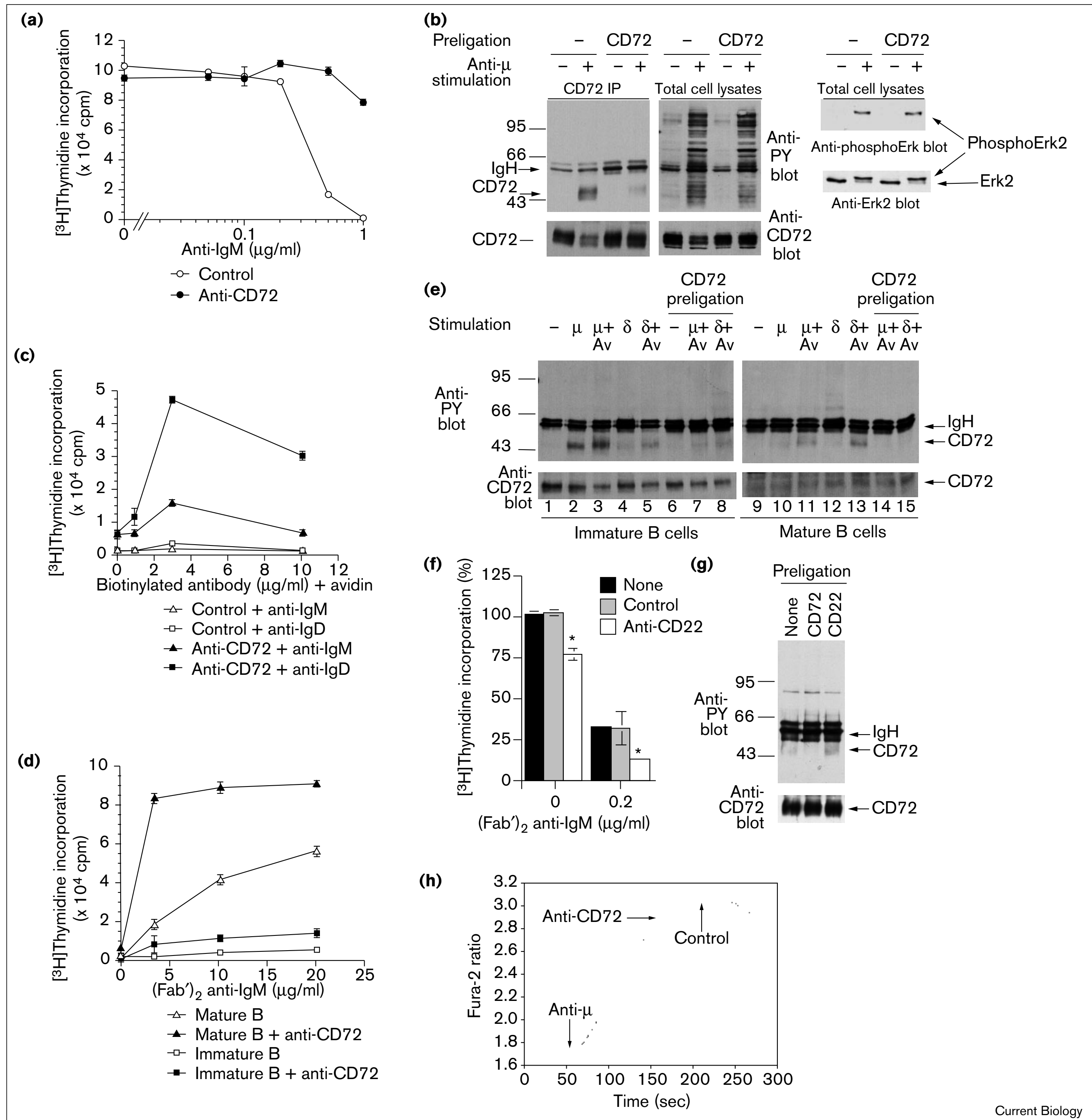
issue, we exploited the distinct properties of different catalytically impaired SHP-1 mutants (Figure 3a). Two of these — C/S (Cys453Ser) and D/A (Asp419Ala) — should retain substrate-binding ability ('trapping' mutants) [29–33]. The other mutants — ΔP, which has an internal PTP domain deletion (residues 451–475), and R/M (Arg459Met), which replaces an arginine residue that is essential for binding the phosphate group of substrates [29–33] — should be unable to bind substrates via their PTP domains (Figure 3a). Phosphotyrosine proteins that bind to the C/S or D/A PTP domains (and therefore represent putative SHP-1 targets) in addition to the SH2 domains present in all of the SHP-1 expression constructs should exhibit higher-avidity binding than those that bind to the SH2 domains alone. Also, tyrosine phosphorylation sites bound by the C/S or D/A PTP domains should be

Figure 4

CD72 tyrosine phosphorylation correlates with BCR-mediated inhibition of cell growth. **(a)** WEHI-231 cells were pretreated with anti-CD72 ascites or media (control) for 1 h before the addition of varying amounts of anti-IgM Bet-2 antibody, and [^{3}H]thymidine incorporation was determined. In these assays, reduced thymidine uptake correlates with increased apoptosis (Y.W., unpublished observations). Where error bars are not visible, the standard deviation is smaller than the size of the symbol. **(b)** WEHI-231 cells were pretreated with or without anti-CD72 ascites for 1 min before stimulation with Bet-2 for 1 min. CD72 IPs and total cell lysates were analyzed by anti-PY blotting and anti-CD72 reblotting. Total cell lysates were also probed with anti-phosphoErk and anti-Erk2 antibodies, as indicated. **(c)** Mature primary B cells were precultured with or without anti-CD72 ascites as in (a) for 1 h before the addition of varying amounts of biotinylated Bet-2 or Hδ^a/1 antibody and, where indicated, a twofold excess (w/w) of avidin, and [^{3}H]thymidine incorporation was determined. **(d)** Mature and immature Balb/c B cells were pretreated with or without anti-CD72 ascites as in (a) for 1 h before the addition of varying amounts of (Fab′)$_2$ goat anti-mouse IgM antibody. Proliferation was assayed as in (c). **(e)** Immature and mature B cells were subjected to BCR crosslinking by biotinylated Bet-2 (μ) or Hδ^a/1 (δ) antibody alone or hyper-crosslinking with additional avidin (Av) at twofold excess (w/w) for 1 min. Where indicated, cells were pretreated with anti-CD72 ascites as in (b). CD72 IPs were analyzed by anti-PY blotting and anti-CD72 reblotting. **(f)** WEHI-231 cells were pretreated with the indicated reagents (where the control was an isotype-matched non-specific antibody) and monoclonal antibody 2.4G2 to block FcγRIIB binding, before the addition of (Fab′)$_2$ goat anti-mouse IgM antibody. [^{3}H]Thymidine incorporation was determined as in (a). The mean value of the untreated samples was defined as 100%. The asterisk indicates $p < 0.005$ (one-tailed t-test) as compared with the corresponding value for pretreatment with no antibody. Under the same conditions, pretreatment with anti-CD72 antibody antagonized growth inhibition as shown in (a). **(g)** WEHI-231 cells were pretreated with anti-CD72 or anti-CD22 antibody for 1 min before (Fab′)$_2$ goat anti-mouse IgM antibody stimulation for a further minute. CD72 IPs were analyzed by anti-PY blotting and anti-CD72 reblotting. This panel is representative of three independent experiments with similar results. **(h)** CD72 ligation has no effect on BCR-evoked calcium response. Fura-2-loaded WEHI-231 cells were pretreated with anti-CD72 antibodies or with control, isotype-matched non-specific antibodies, followed by BCR stimulation (indicated by anti-μ), and measurement of calcium flux.

protected from dephosphorylation by cellular PTPs. Thus, *in vivo* substrates of SHP-1 should exhibit greater tyrosine phosphorylation in the presence of C/S or D/A mutants than with comparable amounts of R/M or ΔP mutants.

BCR-evoked CD72 tyrosine phosphorylation (Figure 3c,d) and association with SHP-1 (Figure 3b, except in ME cells, which lack SHP-1) were observed in the vector control and all four mutant (but not wild-type) SHP-1-expressing lines. All cell lines except wild-type SHP-1-reconstituted cells exhibited enhanced (Figure 3b) and prolonged CD72 tyrosine phosphorylation ([22] and data not shown). Thus, some of the increased CD72 tyrosine phosphorylation observed in the presence of catalytically impaired mutants of SHP-1 is due to 'protection' by the SHP-1 SH2 domains; this finding provides additional evidence that SHP-1 interacts directly, through its SH2 domains, with tyrosine-phosphorylated CD72. However, CD72 tyrosine phosphorylation was much higher in the cell lines reconstituted with the C/S or D/A mutants of SHP-1 than in those reconstituted with R/M or ΔP mutants, indicating that additional protection was afforded



by ‘trapping’ mutants of SHP-1 (Figure 3b,c). These results suggest that CD72 is an SHP-1 substrate *in vivo*.

To confirm these findings, we used a novel *in vitro* PTP protection assay, which compares the ability of SHP-1 mutants to protect CD72 from dephosphorylation by cellular PTPs. ME cells expressing mutant SHP-1 or vector controls were stimulated briefly by BCR crosslinking, then lysed in the absence of PTP inhibitors and incubated for various times (see Materials and methods). The C/S and D/A, but not the ΔP or R/M, mutants of SHP-1 offered significant protection from dephosphorylation by cellular PTPs (Figure 3d), clearly establishing that CD72 is a target of the SHP-1 PTP domain. Consistent with the notion that CD72 is an *in vivo* substrate of SHP-1, tyrosine phosphorylation of CD72 was increased in primary B cells from me^v/me^v mice (Figure 3e). These data also clearly show that the SHP-1–CD72 complex forms in primary B cells as well as in B-cell lines.

Tyrosine phosphorylation of CD72 correlates strongly with BCR-evoked growth arrest/cell death

As nearly all proximal signaling events are comparable following sIgM triggering (leading to tyrosine phosphorylation of CD72) and sIgD triggering (leading to no CD72 tyrosine phosphorylation) in Wδ cells (Figure 1a; [22–24]), the CD72–SHP-1 complex does not appear to act like the CD22–SHP-1, PIR-B–SHP-1 or KIR–SHP-1 complexes [5,6,20,21], which interfere with early signaling events. Instead, the correlation of transient tyrosine phosphorylation of CD72 (and consequent SHP-1 association) with BCR-evoked growth arrest/cell death [22], the enhanced B cell deletion in *me/me* and me^v/me^v mice [11] in which CD72 is hyperphosphorylated (Figure 3e), and our demonstration that CD72 is a direct substrate of SHP-1 (Figure 3), suggested to us that tyrosine-phosphorylated CD72 might transmit growth arrest/cell-death-inducing signal(s) that are antagonized by SHP-1-catalyzed dephosphorylation. If so, specific modulation of CD72 might be expected to alter the outcome of BCR signaling. Indeed, prior ligation of CD72 protected WEHI-231 cells from anti-IgM-induced growth arrest (Figure 4a). Notably, anti-CD72 antibody treatment did not alter BCR-proximal signaling events such as overall tyrosine phosphorylation, mitogen-activated protein (MAP) kinase activation or the Ca^{2+} response (Figure 4b,h). However, under these conditions, tyrosine phosphorylation of CD72 was specifically diminished (Figure 4b), supporting the possibility that tyrosine-phosphorylated CD72 transmits a growth inhibitory signal.

CD72 tyrosine phosphorylation (and SHP-1 association; data not shown) also correlated with BCR-induced growth arrest/cell death in primary B cells. Splenic B cells were isolated from normal mice (mature B cells) and from mice that had been sublethally irradiated and allowed to self-reconstitute (and thus contain predominantly immature B cells) [34,35]. Treatment of mature B cells with unconjugated or biotinylated anti-IgM or anti-IgD antibodies evoked proliferation (Figure 4d and data not shown); under these conditions, CD72 was not tyrosine phosphorylated (Figure 4e, lanes 10 and 12). Delivering a stronger BCR signal to these cells (by crosslinking the biotinylated antibodies with avidin) inhibited proliferation and induced apoptosis [4] (Figure 4c and data not shown). Remarkably, tyrosine phosphorylation of CD72 was observed only under such ‘enhanced’ BCR stimulation conditions (Figure 4e, lanes 11 and 13). In contrast, unconjugated or biotinylated anti-IgM antibodies alone were sufficient to induce apoptosis [35] (Figure 4d and data not shown) and CD72 tyrosine phosphorylation in immature B cells (Figure 4e, lane 2), further strengthening the correlation between CD72 tyrosine phosphorylation and growth arrest/cell death. Preligating CD72 attenuated the inhibitory effect of BCR hyper-crosslinking on mature B-cell growth (Figure 4c), and induced modest proliferation of anti-IgM-treated immature B cells (Figure 4d). In both cases (as in WEHI-231 cells), preligation prevented CD72 tyrosine phosphorylation (Figure 4e, lanes 6–8,14,15). Preligation also enhanced mature B-cell proliferation under normal crosslinking conditions (Figure 4d), suggesting that CD72 may restrain mature B-cell proliferation even when its tyrosine phosphorylation is below the limits of detection.

Opposite functions of CD72 and CD22 in regulating BCR signaling outcome

To test whether CD22 (and by inference, the CD22–SHP-1 complex) and CD72 (and the CD72–SHP-1 complex) have distinct functions in B-cell signaling within the same cellular context, we examined the effect of CD22 ligation on BCR signaling in WEHI-231 cells. Contrary to the effects of CD72 preligation (Figure 4a), preligating CD22 enhanced BCR-induced WEHI-231 cell death (Figure 4f). Remarkably, preligation of CD22 also enhanced the tyrosine phosphorylation of CD72 (Figure 4g). These data are consistent with our proposal that tyrosine-phosphorylated CD72 may mediate growth-inhibitory signals from the BCR, as well as with the previous finding that CD22 ligation enhances BCR signal strength [12]. Furthermore, these results suggest that CD72 may be a target for the CD22–SHP-1 complex.

Discussion

Although several SHP-1-binding proteins have been identified, the physiological targets of SHP-1 in B-cell signaling have remained undefined. Our work clearly identifies CD72 as both an SHP-1 binding protein (Figures 1,2) and a direct substrate for SHP-1 *in vivo* (Figure 3). As tyrosine phosphorylation of CD72 strongly correlates with the ability of the BCR to deliver growth-inhibitory/apoptosis-inducing signals (Figure 4), our results suggest that SHP-1-catalyzed dephosphorylation of CD72 may antagonize these signals. Such a model has potentially important implications for how BCR

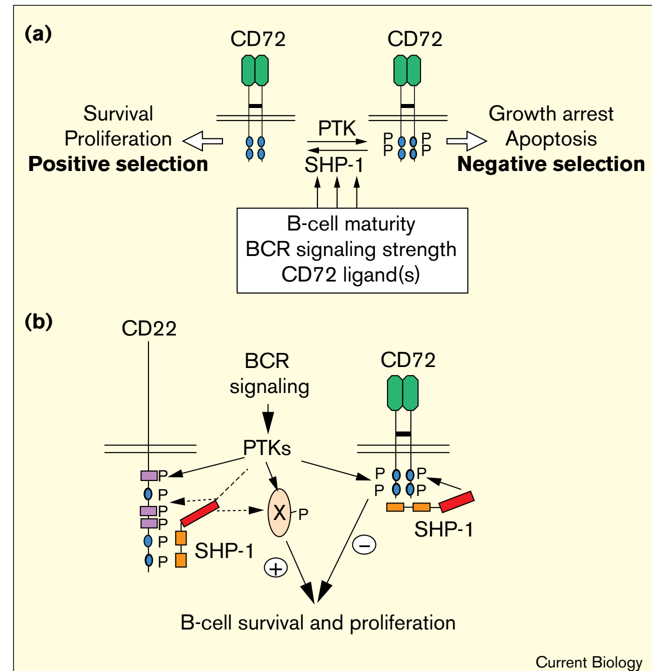
signals lead to distinct outcomes in different cellular contexts, for the role of SHP-1 in this process, and for the severity of the B-cell phenotype in SHP-1-deficient mice.

The use of substrate-trapping mutants to identify SHP-1 targets is complicated by the presence of multiple domains within SHP-1 capable of binding phosphotyrosine-containing sequences. Our results (Figure 3) clearly illustrate the perils of assuming that increased phosphorylation of an SHP-1-binding protein in the presence of a catalytically impaired mutant alone indicates that that protein is a substrate: expression of either non-trapping or trapping mutants of SHP-1 leads to increased CD72 tyrosine phosphorylation, presumably via SH2-domain-mediated protection of binding sites from dephosphorylation by other PTPs. However, trapping mutants cause markedly greater increases in CD72 phosphorylation, because they also bind targets through their (impaired) PTP domains. By comparing the effects of both types of mutant, substrates for SHP-1 can be identified with confidence.

Tyrosine phosphorylation of CD72 strongly correlates with BCR signals leading to growth arrest/cell death in cultured B cell lines (for example WEHI-231) and primary B cells ([22] and Figure 4). More importantly, our antibody ligation studies suggest that CD72 tyrosine phosphorylation is causally important for BCR-induced growth arrest/cell death (Figure 4). Preligating CD72, which results in decreased CD72 tyrosine phosphorylation while having little effect on BCR-induced total tyrosine phosphorylation, MAP kinase activation or calcium response (Figure 4b,h), leads to enhanced survival of WEHI-231 and primary B cells (Figure 4a,c,d). Thus, decreasing the tyrosine phosphorylation of CD72 may contribute to B-cell survival following BCR activation (Figure 5a). Although we favor the notion that CD72 ligation decreases a negative signal that emanates from tyrosine-phosphorylated CD72, we cannot exclude the possibility that CD72 ligation enhances an as yet undefined positive signal generated by unphosphorylated CD72. Likewise, although we observed no differences (except CD72 tyrosine phosphorylation) in early BCR signaling events in CD72-ligated cells, it remains possible that other, unidentified signals (including other, as yet unidentified phosphotyrosine proteins) that antagonize growth arrest and apoptosis are generated. Analysis of CD72$^{-/-}$ cell lines and/or mice is required to resolve this issue.

CD22 ligation has the opposite effect in WEHI-231 cells, leading to enhanced BCR-mediated cell growth arrest/apoptosis. Earlier antibody ligation and genetic studies established that CD22 negatively regulates BCR signal strength most probably through SHP-1. By preventing CD22–SHP-1 complexes from accessing the BCR, CD22 antibody ligation would be expected to increase the effective strength of anti-BCR antibody stimulation, thus

Figure 5



(a) Model depicting the role of CD72 and SHP-1 in B cells. CD72 is shown as a disulfide-linked dimer with a conserved C-type lectin domain and ITIMs (blue). Tyrosine phosphorylation is indicated by P. Parameters that influence the balance between protein tyrosine kinases (PTKs) and SHP-1, which both affect CD72 tyrosine phosphorylation and B-cell fate, are shown in the box. Lack of SHP-1 activity (as in *me/me* or *me^v/me^v* mice) would result in excessive negative selection, whereas defects in protein tyrosine kinase(s) acting on CD72 could lead to altered positive and/or negative selection. **(b)** Distinction between function of SHP-1 bound to tyrosine-phosphorylated CD22 and CD72, respectively. CD22 has three ITIMs and three other tyrosine phosphorylation sites (purple) [12]. SHP-1 is recruited to the ITIMs through its two SH2 domains (orange) before acting on the substrates through its PTP domain (red). Positive signaling molecules targeted by CD22-associated SHP-1 remain to be identified (dashed arrows). Possibilities include BCR-associated protein tyrosine kinases, CD22 itself, and other tyrosine-phosphorylated adaptor or effector molecules (X-P).

leading to enhanced BCR-induced cell death in immature B cells [2]. Remarkably, CD22 ligation also leads to increased CD72 tyrosine phosphorylation (Figure 4g). In addition to further strengthening the correlation between CD72 tyrosine phosphorylation and BCR-induced growth inhibition/cell death, these data suggest that CD22 ligation may exert at least part of its effect by increasing CD72 tyrosine phosphorylation.

The role we propose for SHP-1 in regulating the CD72 pathway (Figure 5b) contrasts sharply with how SHP-1 is believed to function upon its association with other ITIM-bearing molecules, such as CD22, PIR-B and KIRs [5,6,20,21]. SHP-1 bound to these molecules antagonizes positive signaling from the BCR (in the case of CD22 and

PIR-B) or activating NK cell receptors (in the case of KIRs) by dephosphorylating one or more as yet unidentified targets. While our manuscript was in preparation, Adachi *et al.* [36] also reported that CD72 binds to SHP-1 and, solely on the basis of this interaction, proposed that CD72 has a negative regulatory role. In contrast, our functional studies (Figure 4) support an alternative model, in which SHP-1 'positively' promotes B-cell survival and expansion by inactivating 'negative' signals from tyrosine-phosphorylated CD72 (Figure 5).

The combined loss of CD72–SHP-1 and CD22–SHP-1 complexes in early B cells may help to explain the marked depletion of B-cell precursors in *me/me* and *me^v/me^v* mice. Loss of CD22 leads to enhanced signaling through the BCR and increased tyrosine phosphorylation of CD72 (Figure 4g). Concomitantly, in the absence of SHP-1, CD72 exhibits enhanced tyrosine phosphorylation, as CD72 is a direct substrate of SHP-1 (Figure 3). This synergistic effect may also help explain why CD22$^{-/-}$ B cells (lacking only the CD22–SHP-1 complex) exhibit a much less severe phenotype than those lacking functional SHP-1 (which lack all functional SHP-1 complexes). Alternatively, as CD22 expression is highest in more mature B cells, the profound depletion of B-cell precursors that occurs in SHP-1-deficient mice may be largely due to loss of the CD72–SHP-1 complex, whereas loss of the CD22–SHP-1 complex may be more important for other aspects of the SHP-1-deficient phenotype, such as the increased activation of more mature B cells. Studies of mice with compound deficiencies in CD22 and CD72 should provide important insights into these issues.

Materials and methods

Cell and cell lines

Balb/c splenic B cells were purified as described [37], except that Ficoll was substituted for Percoll. Immature B cells were obtained from self-reconstituting Balb/c mice as described previously [34,35]. Cell lines were cultured as described [23]. ME cells [38] were transfected with vector alone (ME), wild-type SHP-1 cDNA (wt) or various mutant (Cys453Ser (C/S), Asp419Ala (D/A), residue 415–475-deletion (ΔP) and Arg459Met (R/M)) human SHP-1 cDNAs; these mutants have been described [33]. Selected clones were matched for sIgM expression and comparable levels of SHP-1 protein.

Cell stimulations

Crosslinking of sIgM was carried out using Bet-2 (ATCC) or $(Fab')_2$ goat anti-mouse μ (Jackson Immunoresearch) antibodies. Anti-Sp6 [23] and Hδ^a/1 (provided by F. Finkelman, University of Cincinnati) antibodies were for sIgD activation. BCR hyper-crosslinking was achieved by using biotinylated Bet-2 or Hδ^a/1 antibodies, respectively, followed by addition of a twofold excess (w/w) of avidin. Biotinylated antibodies alone were used for normal crosslinking. Pervanadate was a mixture of 3 mM H_2O_2 and 1 mM sodium orthovanadate. For CD72 preligation, ascites or purified anti-CD72 monoclonal antibody K10.6 [39] was used (CD72.4, Cedarlane Laboratories). Anti-CD22 monoclonal antibody NIM-R6 [15] was used for CD22 ligation.

Immunoprecipitation and immunoblotting

Polyclonal antibodies against SHP-1 were from K. Siminovitch (Samuel Lunenfeld Research Institute, Toronto) or Santa Cruz Biotechnology (Santa Cruz). CD72 immunoprecipitations were performed with K10.6. Antibodies for immunoblotting CD72, Grb2, the kinase Erk2 and phosphoErk were from Santa Cruz Biotechnology. Anti-phosphotyrosine monoclonal antibody (4G10) was from UBI. Peptide N-glycanase F treatment was performed according to the manufacturer (New England Biolabs). Cell lysis, immunoprecipitation and immunoblotting were performed as described previously [22].

Partial proteolytic mapping with V8 protease

Partial proteolytic mapping with V8 protease (Sigma Chemical) was carried out on excised gel pieces containing the 44 kDa region as described [40].

In vitro *binding assays*

GST fusion protein expression constructs were constructed and purified on glutathione–Sepharose (Pharmacia Biotech), as described previously [41,42]. Biotinylated tyrosine-phosphorylated peptides were synthesized as described [43]. Dephosphopeptides were obtained by treating phosphopeptides with GST–SHP-1 bound to glutathione–Sepharose beads in 62.5 mM Tris pH 7.4/6.25 mM EDTA/10 mM DTT for 1.5 h at 37°C. Biotinylated peptides were bound to streptavidin–agarose beads (Life Technologies) and incubated with cell lysates. Bound proteins were eluted in SDS–PAGE sample buffer or, for re-immunoprecipitation experiments, in lysis buffer containing 50 mM phosphotyrosine.

In vitro *PTP protection assay*

Cells were stimulated for 1 min and lysed in NP-40 buffer [22] lacking PTP inhibitors. Lysates were incubated on ice for various times before the addition of 1 mM sodium orthovanadate and processing for immunoprecipitations. For the 0 time point, cells were lysed directly in orthovanadate-containing buffer.

Proliferation and calcium assays

Proliferation was assayed essentially as described [23]. Specifically, cells were cultured in triplicate at 2×10^5/ml for WEHI-231 cells (total of 48 h) and at 7.5×10^5/ml for splenic B cells (total of 42 h) in a total volume of 200 μl. Where indicated, monoclonal antibody 2.4G2 (Pharmingen) was included to block FcγRIIB binding. [^{3}H]Thymidine (NEN), 1 μCi per well, was added to the WEHI cultures for the last 6 h and to the splenic B-cell cultures for the last 18 h. Measurements of BCR-evoked calcium flux in Fura-2-loaded WEHI-231 cells were carried out as described previously [15].

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