Engagement of CD81 induces ezrin tyrosine phosphorylation and its cellular redistribution with filamentous actin

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Abbreviations:
B cell receptor (BCR); ezrin-radixin-moesin (ERM); filamentous actin (F-actin); fluorescein isothiocyanate (FITC); isotype control (IC); liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) monoclonal antibodies (mAbs); peripheral blood mononuclear cells (PBMC); spleen tyrosine kinase (Syk); tetramethyl rhodamine iso-thiocyanate (TRITC).

Summary

CD81 is a tetraspanin family member involved in diverse cellular interactions in the immune and nervous systems and in cell fusion events. However, the mechanism of action of CD81 and of other tetraspanins has not been defined. We reasoned that identifying signaling molecules downstream of CD81 would provide mechanistic clues. We engaged CD81 on the surface of B-lymphocytes and identified the induced tyrosine-phosphorylated proteins by mass spectrometry. This analysis showed that the most prominent tyrosine phosphorylated protein was ezrin, an actin binding protein and a member of the ezrin-radixin-moesin family. We also found that CD81 engagement induces spleen tyrosine kinase (Syk) and that Syk was involved in tyrosine phosphorylation of ezrin. Ezrin colocalized with CD81 and F-actin upon stimulation and this association was disrupted when Syk activation was blocked. Taken together, these studies suggest a model in which CD81 interfaces between the plasma membrane and the cytoskeleton by activating Syk, mobilizing ezrin, and recruiting F-actin to facilitate cytoskeletal reorganization and cell signaling. This may be a mechanism explaining the pleiotropic effects induced in response to stimulating cells by anti-CD81 antibodies or by the hepatitis C virus, which uses this molecule as its key receptor.

Introduction

CD81 is a member of the tetraspanin family of proteins. Tetraspanin family members contain four transmembrane domains flanking two extracellular loops and short cytoplasmic N- and C-termini. One distinctive feature of this evolutionarily conserved family of proteins is their propensity to associate with other proteins within the cell membrane and to form multimolecular complexes, known as tetraspanin enriched microdomains (TEM) (Levy and Shoham, 2005a). Partner proteins include integrins, members of the immunoglobulin superfamily, and lineage specific markers (Hemler, 2005; Levy and Shoham, 2005b). CD81 associates with different partner proteins in different cell types; consequently its engagement activates a wide variety of cell type-specific responses.

Originally, CD81 was identified as a target of an antibody that controlled B cell proliferation (Oren et al., 1990). However, the significance of CD81 to cell physiology and differentiation was subsequently re-discovered in other cell types where anti-CD81 monoclonal antibodies (mAbs) inhibited i) HTLV-induced syncytium formation (Imai and Yoshie, 1993), ii) maturation of mouse T cells (Boismenu et al., 1996) iii) FcεRI-mediated mast cell degranulation (Fleming et al., 1997) and altered the morphology of rat astrocytes (Geisert et al., 1996). In addition, anti-CD81 mAbs inhibited muscle cell fusion (Tachibana and Hemler, 1999), and enhanced HIV-induced syncytium formation (Gordon-Alonso et al., 2006). The mechanisms underlying these diverse effects by the anti-CD81 mAbs are not well understood. Importantly, CD81 was discovered to be a cellular receptor for the hepatitis C virus (HCV) (Pileri et al., 1998).

CD81 engagement activates signal transduction in several cell types. In hepatocytes, the engagement of CD81 by the HCV envelope glycoprotein E2 or by anti-CD81 mAb activated the MAPK/ERK and Rho GTPase pathways (Brazzoli et al., 2008; Carloni et al., 2004; Zhao et al.,

2005). Whereas in T cells the engagement of CD81 by HCV-E2 or by mAb induced the activation of the Src family kinase Lck (Soldaini et al., 2003). Interestingly, the coengagement of CD81 with CD3 in T cells and with CD16 in NK cells resulted in opposing effects; the activation of T cells was enhanced, while that of NK cells was inhibited (Crotta et al., 2006; Wack et al., 2001). However, in both cases, the coengagement led to the reorganization of the actin cytoskeleton (Crotta et al., 2006; Wack et al., 2001). Human B cells are especially susceptible to the engagement of CD81, which induced numerous tyrosine-phosphorylated proteins (Cocquerel et al., 2003; Rosa et al., 2005; Schick et al., 1993). Here we used mass spectrometry to identify the most prominent activated tyrosine phosphorylated protein as ezrin, an actin binding protein and a member of the ezrin-radixin-moesin (ERM) family (Bretscher et al., 2002). CD81-induced ezrin tyrosine phosphorylation required activation of Syk, an important B cell kinase that is also responsible for the initiation of signaling through the B cell receptor (BCR) (Jumaa et al., 2005). Importantly, we report that CD81, ezrin and filamentous actin (F-actin) colocalized following CD81 engagement. The cellular redistribution of ezrin and F-actin may be the underlying mechanism by which CD81 exerts its effect in diverse cell types.

Results

A unique tyrosine-phosphorylated band is induced specifically in response to engagement of CD81

The engagement of CD81 by antibodies or by the HCV envelope protein E2 induces tyrosine phosphorylation in B cells (Cocquerel et al., 2003; Schick et al., 1993). To delineate these signaling events we stimulated B cells with anti-CD81 mAb and detected the induced tyrosine-phosphorylated proteins by Western blotting (Fig. 1A). One dominant band of approximately 80

kDa was consistently detected after 5 minutes of stimulation (Fig. 1A, arrow, Fig. 2B&E and data not shown). The appearance of this specific phosphorylated band was dependent on the concentration of the stimulating anti-CD81 antibody, down to a limit of 0.2μg/ml (Fig. 1B, lanes 2-5).

Next, we investigated the specificity of the phosphorylation of the ~80 kDa protein. Cells were stimulated with antibodies against CD81, CD19 and the BCR. CD19 is thought to be the signaling partner of CD81 in B cells. This analysis showed that the phosphorylation of the unique ~80 kDa protein was only seen in response to engagement of CD81 (Fig. 1C, arrow) and not via CD19, nor via the BCR. We also stimulated cells via the closely related tetraspanin molecule, CD9, which is expressed in OCI-LY8 cells and was previously shown to associate with CD81 in B cells (Horvath et al., 1998). Engagement of CD9 led to a minor induction of several tyrosine-phosphorylated proteins, but not to a specific effect on the ~80 kDa target (Fig. 1D). Thus, phosphorylation of the ~80 kDa band appeared to be a specific consequence of stimulation through CD81.

Identification of CD81-induced tyrosine-phosphorylated proteins by mass spectrometry

Tyrosine-phosphorylated proteins from cells stimulated with anti-CD81 were immunopurified using an immobilized anti-phosphotyrosine mAb, competitively eluted, and separated by SDS-PAGE. Specific regions of the gel were excised and subjected to in-gel trypsin digestion followed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Within the 80 kDa region, the dominant protein identified was ezrin. Ezrin is a member of the ERM family (ezrin, radixin, moesin) of actin binding proteins (Bretscher et al., 2002; Tsukita and Yonemura, 1997). This finding was of great interest because CD81 has recently been shown to

interact directly with ERM family members (Chang and Finnemann, 2007; Pan et al., 2007; Sala-Valdes et al., 2006).

Ezrin is tyrosine-phosphorylated in response to engagement of CD81

To confirm that ezrin is phosphorylated in CD81 stimulated cells we performed immunoblotting experiments. In anti-phosphotyrosine immunoprecipitates, ezrin was detected in cells stimulated by anti-CD81 but not by an isotype-matched control mAb (Fig. 2A). To further confirm the finding of ezrin phosphorylation, we reversed the steps by first immunoprecipitating ezrin and then blotting for tyrosine phosphorylation. Once again, we found a time-dependent increase in tyrosine-phosphorylated ezrin in CD81 stimulated cells (Fig. 2B). The identity of the immunoprecipitated ezrin in this experiment was re-confirmed by mass spectrometry (data not shown). To establish the generality of this CD81-phosphoezrin pathway we examined other cells. Tyrosine-phosphorylated ezrin was also induced by CD81 in two additional B cell lines, DHL4 and DHL6 (Fig. 2C). We have previously shown that ligation of CD81 by the HCV envelope protein, E2, triggered a number of tyrosine phosphorylated proteins (Cocquerel et al., 2003). To test whether this ligand also induced tyrosine phosphorylation of ezrin, we incubated cells with immobilized HCV-$E2_{661}$ or with control beads, as previously described (Cocquerel et al., 2003). Immunoprecipitation of ezrin and probing with the anti-phosphotyrosine mAb revealed that engagement of CD81 by the viral envelope protein also led to tyrosine phosphorylation of ezrin (Figure 2D).

Ezrin, unlike radixin and moesin, contains the known phosphorylation site Y353, allowing direct detection of phosphorylation of this site by a specific antibody. We found that ezrin pY353 was detected as early as 5 minutes after engagement of CD81 (Fig. 2E). Moreover, CD81-induced activation of ezrin was not restricted to B cell lines - the pY353 site was also

induced in peripheral blood mononuclear cells (PBMC) (Fig. 2F), albeit with different kinetics of phosphorylation.

CD81 engagement induces Syk autophosphorylation

Syk, a proximal signal transducing kinase, was also identified by mass spectrometry as a tyrosine-phosphorylated molecule activated by CD81. To confirm the activation of this kinase by CD81, we immunoprecipitated Syk from the stimulated cells and probed with an antibody that recognizes the Syk auto-phosphorylation sites, amino acid positions 525/526 (Fig. 3A). This analysis demonstrated that Syk was activated by CD81. Once again, we tested the generality of this finding by examining other B cell lines. Syk autophosphorylation occurred also in DHL4 and DHL6 B cells in response to engagement of CD81 (Fig. 3B).

CD81-induced ezrin phosphorylation is Syk-dependent

To test if Syk is critical for CD81 signaling, we used three structurally unrelated Syk kinase inhibitors SI 1, SI 2, and R406 (Braselmann et al., 2006). We also included the Src family kinase inhibitor PP2 (Hanke et al., 1996). We analyzed ezrin in cells that were stimulated by anti-CD81 antibody in the presence or absence of these kinases inhibitors. Treatment with the Src kinase inhibitor PP2 resulted in a modest decrease in ezrin tyrosine phosphorylation. However, the Syk inhibitor R406 abolished ezrin phosphorylation, which was also highly reduced by the structurally unrelated Syk inhibitors SI 1 and SI 2 (Fig. 3C). Further analysis confirmed, even more specifically, that R406 inhibited ezrin pY353 (Fig.3D). These data suggest that Syk activity is important for CD81-induced ezrin phosphorylation.

To rule out non-specific effects by the Syk inhibitors we used Syk shRNA to specifically knock down this kinase. This experiment could not be done in B cells because Syk is critical for their survival (Chen et al., 2008; Gururajan et al., 2007). We therefore used the U937-CD81

monocyte cell line to knock down Syk expression by shRNA. Indeed, Syk expression was highly reduced in these knock down cells, as measured by flow cytometry (Fig. 4A, upper panel) and by western blot analysis (Fig. 4B), whereas the levels of ezrin and of CD81 were similar to that of cells that were not transduced by Syk shRNA (Fig. 4A middle and bottom panels, respectively). Syk knockdown almost completely blocked CD81-induced ezrin phosphorylation (Fig. 4C). Thus, both by treatment with the Syk inhibitors and by Syk knockdown we confirmed that tyrosine-phosphorylation of ezrin was Syk-dependent.

Role of the C-terminal tail of CD81

A peptide encoding the N-terminal domain of ezrin was previously shown to interact with a peptide derived from the cytoplasmic C-terminal domain of CD81 (Sala-Valdes et al., 2006). Here we tested whether the C-terminal domain of CD81 is required for the activation of ezrin in living cells. To do so we generated U937 cells in which the C-terminal domain of CD81 was deleted (U937-CD81ΔC). Since these cells express lower levels of CD81, we selected a subclone of U937-CD81 cells expressing comparable CD81 levels for this study (supplemental Fig. 1). We then engaged CD81 on the surface of these two subclones and found that ezrin pY353 was induced in U937-CD81, but not in U937-CD81ΔC cells (Fig. 4D). Thus, the C-terminal domain of CD81 is required for the activation of this site in ezrin.

CD81 engagement induces changes in threonine phosphorylation of ezrin

The phosphorylation of Thr567 in ezrin has been shown to trigger conformational changes enabling binding of its C-terminal domain to F-actin (Bretscher et al., 2002). In resting T and B cells this threonine phosphorylated form of ezrin and moesin has been detected (Gupta et al., 2006; Ilani et al., 2007; Shaffer et al., 2009). In response to engagement of the TCR in T cells or the BCR in B cells, ezrin has been shown to undergo a transient dephosphorylation of Thr567,

followed by increased phosphorylation of this site (Gupta et al., 2006; Ilani et al., 2007; Shaffer et al., 2009). Here we show that engagement of CD81 also induces the dephosphorylation/phosphorylation of this site. As expected, this site was phosphorylated in resting cells, and underwent a transient dephosphorylation upon engagement of CD81 (Fig. 5A). Moreover, three different anti-CD81 mAbs induced dephosphorylation and rephosphorylation of this ezrin threonine residue (Fig. 5A and data not shown).

CD81-induced dephosphorylation of Thr567 is Syk-independent and requires the C-terminal domain of the molecule

To test whether Syk activity affects the dephosphorylation of Thr567 we analyzed the effect of Syk inhibition by R406 in OCI-LY8 cells. We found that the dephosphorylation of Thr567 was not affected by the Syk inhibitor, as seen in Fig.5A, right panel. Our data did suggest, however, that R406 may alter the kinetics of rephosphorylation. We also examined whether the C-terminal domain of CD81 was required for the changes in phosphorylation of ezrin Thr567. We found that U937-CD81ΔC engaged by anti-CD81 mAb had no change in pERM whereas in U937-CD81 expressing low surface levels of CD81 (Supplemental Fig.1) pERM was first reduced, and then increased (Fig.5B).

Engagement of CD81 induces its colocalization with ezrin and F-actin

The engagement of CD81 induced the phosphorylation of ezrin, which binds F-actin. To assess the cellular distribution of CD81 relative to both ezrin and F-actin, OCI-LY8 cells were incubated with the anti-CD81 mAb and localization of CD81, ezrin and F-actin was determined by three-color fluorescent microscopy. All three molecules colocalized in a single pole of the stimulated cells (Fig. 6, CD81 stimulation). Tyrosine phosphorylation of ezrin has been associated with its redistribution to the plasma membrane (Jiang et al., 1995; Wu et al., 2000).

To test whether blocking of Syk activity prevents colocalization of CD81 with ezrin and F-actin, we pretreated cells with the most potent Syk inhibitor (R406) for 1 hour, followed by incubation with the anti-CD81 mAb. The co-capping of F-actin with CD81 and ezrin was inhibited by R406 resulting in a dispersed staining of all three molecules (Fig. 6, CD81 stimulation + R406). A similar pattern of CD81 staining can be seen when the anti-CD81 mAb is added to cells at 4ºC (Supplemental Fig. 2).

The engagement of CD81 on lymphoid cells induces cell aggregation (Takahashi et al., 1990). However, inhibition of Syk activity in R406-treated OCI-Ly8 cells did not block CD81-induced cell aggregation. Similarly, anti-CD81 mAbs induced aggregation of U937-CD81-Syk KD cells and of U937-CD81ΔC cells. These cells do not activate ezrin pY353 when Syk is inactive or when the C terminal domain of CD81 cannot interact with ezrin.

Discussion

Here we show that the engagement of CD81 leads to autophosphorylation of Syk (Fig. 3A,B), to tyrosine and threonine phosphorylation of ezrin (Figs 2-5) and to the cellular redistribution of CD81 and ezrin with F-actin (Fig.6). The ability of CD81 to activate ezrin is likely due to their direct interaction. Indeed, CD81 coimmunoprecipitated with ezrin in rat retinal pigment epithelial cells (Chang and Finnemann, 2007; Pan et al., 2007) and a peptide derived from the cytoplasmic C-terminal tail of CD81 bound the N-terminal domain of ezrin (Sala-Valdes et al., 2006). Moreover, ezrin contains an ITAM motif which could enable its direct interaction with Syk (Rozsnyay et al., 1996; Urzainqui et al., 2002). Here we demonstrate that engagement of CD81 specifically facilitates the activation of ezrin by Syk (Figs. 3, 4), and that this activation requires the C-terminal tail of CD81 (Fig. 4D). Importantly, the C-terminal domain of CD81 is

also required for changes in the phosphorylation state of ezrinThr567 in cells responding to CD81 engagement (Fig.5B).

Ezrin and its close homologs, radixin and moesin, are members of the ERM family of proteins that bridge the actin cytoskeleton to membrane proteins (Bretscher et al., 2002). ERM proteins can exist in an inactive form, in which the N-terminal domain (N-ERMAD) interacts with the C-terminal domain (C-ERMAD) of the molecule, masking their respective interactions with membrane proteins and with actin (Gary and Bretscher, 1995). Phosphorylation of threonine at their C terminal end has been shown to trigger conformational changes in ERM enabling the binding of C-ERMAD to F-actin and that of N-ERMAD to the cytoplasmic domain of several membrane proteins [reviewed in (Charrin and Alcover, 2006)]. ERM proteins permit bridging of F-actin and possibly other cytoskeletal proteins to membrane proteins thereby facilitating membrane reorganization. Engagement of the BCR and the TCR on lymphoid cells induce transient changes in dephosphorylation and rephosphorylation of Thr567 (Gupta et al., 2006; Ilani et al., 2007; Shaffer et al., 2009). Here we show that engagement of CD81 i) induces the dephosphorylation and rephosphorylation of Thr567 of ezrin (Fig. 5A); ii) the changes in Thr567 phosphorylation require the C-terminal domain of the molecule (Fig.5B) iii) pThr567 dephosphorylation is not dependent on Syk activity (Fig.5A). Rather, inhibition of Syk results in delay in the rephosphorylation of Thr567 (Fig.5A).

Tyrosine phosphorylation of ezrin (Krieg and Hunter, 1992) is associated with its redistribution from the cytosol to the plasma membrane in epithelial cells (Jiang et al., 1995; Wu et al., 2000). We have now extended these observations to hematolymphoid cells; moreover, we show that tyrosine phosphorylation, cellular redistribution, and co-capping of ezrin with F-actin are events induced by the engagement of CD81 (Fig.6). The cellular redistribution of these molecules did

not occur in the presence of the Syk inhibitor R406, which also abolished phosphorylation of ezrin Y353 (Figs 6 and 3, 4, respectively) suggesting that Syk is critical for this process.

Ezrin was previously shown to foster interactions in T cells between membrane and cytoplasmic proteins, facilitating membrane reorganization and immune synapse (IS) formation (Charrin and Alcover, 2006; Ilani et al., 2007; Roumier et al., 2001; Shaffer et al., 2009). Others have demonstrated that CD81 and the tetraspanin molecule CD82 also redistribute to IS of activated B and T lymphocytes (Delaguillaumie et al., 2004; Mittelbrunn et al., 2002). In T cells, co-engagement of CD81 with the T cell receptor induced F-actin capping (Crotta et al., 2006). Based on these observations it is tempting to speculate that TEMs containing CD81 and other tetraspanins play a role in IS formation. The role of CD81 in cytoskeletal reorganization is especially intriguing in the context of HCV infection. CD81 is a key HCV receptor, required for infection by this virus (Cocquerel et al., 2006). Here we show that HCV-E2 induces the activation of ezrin (Fig. 2D). Thus, the engagement of CD81 by the virus might enable the bridging of F-actin to the cell membrane leading to endocytosis (Smythe and Ayscough, 2006). This raises the interesting question of whether the increase in B cell proliferative disorders in HCV-infected patients (Giordano et al., 2007) is linked to a dual engagement of an anti-viral specific BCR and CD81 (Quinn et al., 2001).

Finally, studies in *Cd81*$^{-/-}$ mice have demonstrated the essential role of this molecule in diverse physiological functions, including female infertility due to the inability of eggs to fuse with sperm (Rubinstein et al., 2006), susceptibility to infection by sporozoites of the malarial parasite, *Plasmodium* species (Silvie et al., 2003), and impaired immune and nervous system functions (Levy and Shoham, 2005a). We hypothesize that these diverse functions could be related to the

role of CD81 in facilitating interactions between the cell membrane, intracellular signaling proteins, and the cytoskeleton.

In summary, much evidence is emerging for diverse and important biological roles of the tetraspanin CD81. The underlying molecular mechanisms for its pleiotropic functions are not yet understood. Our observations suggest one model whereby CD81 interfaces between the plasma membrane and the cytoskeleton by activating Syk, mobilizing ERM proteins, and recruiting F-actin to facilitate cytoskeletal reorganization and cell signaling.

Materials and Methods

Cells and reagents

The human B cell lines OCI-LY8 (Oren et al., 1990), DHL4 and DHL6, and the monocyte U937-CD81 (Cocquerel et al., 2003), U937-CD81-Syk KD and U937-CD81ΔC cells were maintained in RPMI, 10% fetal calf serum and 1% penicillin/streptomycin. Human peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Hypaque separation (Ficoll-Paque Plus, GE Healthcare Biosciences AB, Uppsala, Sweden), washed and suspended in RPMI containing 5% fetal calf serum. This study was approved by Stanford University's Administrative Panel on Human Subjects in Medical Research. Purified mAbs used in the study include anti-CD81, 5A6 (Oren et al., 1990) and fluorescein isothiocyanate (FITC)-conjugated 5A6; JS81 (BD Pharmingen, San Diego, CA); 1C1 an anti-TCR Vβ framework, made in our lab, was used as an isotype control; anti-CD9, M-L13; anti-Syk, 4D10 (BD Pharmingen); anti-CD19, 4G7 (Meeker et al., 1984); anti-phosphotyrosine, PY99 (Santa Cruz Biotechnology, Santa Cruz, CA) and 4G10 (Upstate Cell Signaling Solutions, Chicago, IL); anti-actin, C4 (Millipore, Temecula, CA); anti-ezrin, 3C12 (Sigma-Aldrich, St. Louis, MO) and biotin conjugated 3C12

(NeoMarkers, Fremont, CA) that was detected using streptavidin tetramethyl rhodamine isothiocyanate (TRITC) (Zymed, Carlsbad, CA). Polyclonal antibodies used include goat F(ab')2 anti-human λ and μ (BioSource, Camarillo**,** CA), Goat anti-rabbit IgG-HRP conjugated; rabbit anti-Syk and anti-Syk pY525/526; anti-ezrin pY353 and anti-pERM (Cell Signaling Technology, Danvers**,** MA); and Goat anti-mouse IgG –HRP conjugated (SouthernBiotech, Birmingham, AL). Immobilized HCV-$E2_{661}$ (HCV-E2) and beads loaded with mock supernatants were prepared as previously detailed (Cocquerel et al., 2003). QuantiBRITE PE (BD Immunocytometry Systems, San Jose, CA) was used to determine the number of antibody molecules bound per cell according to directions by the manufacturer. Phalloidin conjugated to TRITC was obtained from Sigma-Aldrich, and Alexafluor-350 and 488 from Molecular Probes (Carlsbad, CA). Other reagents include paraformaldehyde (Electron Microscopy Sciences, Hatfield**,** PA), cell-Tak (BD Biosciences), and prolong gold anti-fade mounting medium with and without DAPI (Molecular Probes).

Western blot analysis of tyrosine-phosphorylated protein

Detection of phosphotyrosine containing proteins by Western blotting was performed as previously described (Cocquerel et al., 2003). Briefly, 10^6 cells were incubated with antibodies at the concentrations and times specified in each experiment. Whole cell lysates were separated under reducing conditions by 10% SDS-PAGE, transferred to PVDF membrane, incubated with the indicated horseradish peroxidase (HRP)-conjugated antibodies, as specified in each experiment, then detected by ECL reagent (Amersham, Piscataway, NJ). Following probing with antibodies to phosphorylated proteins the membranes were stripped and reprobed with the indicated antibodies.

Immunoprecipitation and kinase specific inhibitors

10^7 cells were suspended in 1 ml RPMI and rested for 1 hour in a 37ºC tissue culture incubator. Cells were then incubated with 1 μg/ml anti-CD81 or isotype control mAb for various times at 37ºC. Where indicated, cells were also incubated with 2.5 μM of the Src family kinase inhibitor PP2, and the Syk inhibitors SI 1, SI 2 (Calbiochem, San Diego**,** CA), and R406 (Braselmann et al., 2006) (Rigel, S. San Francisco**,** CA). Cells were lysed in RIPA buffer followed by immunoprecipitation with anti-ezrin or anti-Syk antibodies. Immunoprecipitated material was washed in RIPA buffer and eluted by boiling for 10 minutes in reducing sample buffer. Eluted material was resolved by 10% SDS-PAGE for Western blot analysis. Ezrin was detected using the anti-ezrin mAb and tyrosine-phosphorylated ezrin was detected using PY99. Syk autophosphorylation was detected by probing with anti-phospho Syk 525/526, then stripping the blot and reprobing for total Syk, following the manufacturers' protocols. The induction of tyrosine phosphorylation was quantified by densitometry using the AlphaImager 2200 (Alpha Innotech Corp., San Leandro, CA).

Generation of U937 cell lines:

i) U937-CD81 stably expressing Syk shRNA (U937-CD81-Syk KD): U937-CD81 were infected as detailed previously (Cocquerel et al., 2003; Schick et al., 1993) with the retroviral plasmid pRSMX_PG (Ngo et al., 2006) expressing both a 21-mer shRNA targeting Syk (starting at nt 243 of the reference sequence NM_003177) and a fusion cDNA for GFP expression and puromycin resistance. GFP positive cells were sorted using FACSCalibur (Becton Dickinson, San Jose, CA), 90% of the sorted cells had reduced Syk expression. The relative expression of Syk, CD81, and ezrin in these cells was determined by FACS using the cyto-perm/cyto-fix kit supplied by BD Biosciences. ii) U937 stably expressing CD81ΔC were generated as detailed

previously for U937-CD81 cells with the exception of the downstream primer -5'-GGTACTCGAGTCTACAGCACCATGCTCAGGATCAT-3' (codon stop is underlined). It is of note that U937-CD81ΔC cells express low levels of CD81. Therefore, in experiments comparing these cells to U937-CD81 cells, we used a subclone of U937-CD81 expressing matching levels of CD81 (Supplemental Fig.1).

Mass spectrometry of isolated tyrosine-phosphorylated proteins

10^9 B cells were suspended in 200 ml RPMI with 1 μg/ml of anti-CD81 or an isotype control mAb for 1 hour at 37ºC, and then lysed in 10 ml RIPA buffer for 1 hour on ice. Phosphotyrosine proteins were immunoprecipitated using the 4G10 mAb immobilized onto protein-A agarose (Upstate Cell Signaling Solutions) as recommended by the manufacturer. Immunoprecipitated proteins were eluted with 200 μl of 100 mM sodium phenyl phosphate (Sigma-Aldrich), concentrated by vacuum-spin, separated by 10% SDS-PAGE, and silver stained using the SilverSNAP Stain for Mass Spectrometry kit (Pierce Biotechnology, Rockford**,** IL). Gel pieces containing the stained proteins were subjected to trypsin digestion and identified by LC-MS/MS using the Agilent 1100 LC system and the Agilent XCT plus Ion Trap (Agilent Technologies, Santa Clara, CA) as previously described (Lopez-Avila et al., 2006). The MS/MS spectra were scanned against the SwissProt database using the SpectrumMill software (Agilent), a minimum of 2 peptides was required for protein identification with a $p \leq 0.05$ for each peptide identified.

Fluorescent Microscopy

OCI-LY8 cells were suspended in RPMI and incubated for 10 minutes at the indicated temperature with 1 μg/ml of FITC-conjugated anti-CD81 (5A6) mAb. In some experiments, cells were pre-treated for 1 hour with 2.5 μM R406 to inhibit Syk kinase activity. The stained cells were fixed in 3.7% paraformaldehyde for 10 minutes, transferred to cover slips pretreated

with cell-Tak, permeabilized for 10 minutes with 0.1% Triton-X 100 in PBS, and blocked with 5% goat serum/1% BSA in PBS for 1 hour. To detect ezrin and F-actin, cells were incubated, as indicated, then stained with biotin conjugated anti-ezrin for 1 hour followed by streptavidin-TRITC for 1 hour and with 165 nM Alexafluor-350 conjugated phalloidin for 30 minutes. Cells were mounted in medium with or without DAPI. Images were obtained with a fluorescent microscope (Eclipse E800; Nikon) using a Plan Apochromat 100x objective (numerical aperture = 1.4.) equipped with a CCD camera. Images were acquired with the Spot software, version 4.6 (Diagnostic Instruments Inc., Sterling Heights, MI). U937 cells were also examined by these methods, but they proved unsuitable because F-actin was observed in the absence of any deliberate activation.

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Figure Legends

Fig. 1. A unique tyrosine-phosphorylated protein is induced in response to engagement of CD81. OCI-LY8 cells were incubated with (A) 1 μg/ml of 5A6, an anti-CD81 (αCD81) or isotype control (IC) mAbs for the indicated periods. (B) Cells were incubated with the indicated concentrations of αCD81 or with IC mAb (0) for 30 minutes. (C) Cells were incubated with αCD81 (α81), anti-CD19 (α19), αBCR, or with IC mAb at the indicated concentrations for 30 minutes. (D) Cells were stimulated with 1 μg/ml αCD81, anti-CD9 (αCD9) or IC mAb for the indicated periods. Proteins in cell lysates were separated by SDS-PAGE, Western blotted and probed with an anti-phosphotyrosine mAb. A tyrosine-phosphorylated band of approximately 81 kDa (arrow) is seen in response to treatment with the αCD81 mAb, but not with αBCR, αCD19, or αCD9. Molecular weight markers are depicted on the right.

Fig. 2. Engagement of CD81 induces tyrosine phosphorylation of ezrin. (A) OCI-LY8 cell lysates and immunoprecipitated tyrosine-phosphorylated proteins (αPY IP) following stimulation with αCD81 or IC mAbs were separated by SDS-PAGE and Western blotted with an anti-ezrin mAb. (B) OCI-LY8 cells were incubated for the indicated times with αCD81 or IC mAb. Cell lysates were precipitated by an anti-ezrin mAb and Western blotted with an anti-phosphotyrosine (top panel) and an anti-ezrin mAb (bottom panel). (C) OCI-LY8, DHL4, and DHL6 B cells were incubated with 1 μg/ml αCD81 or IC mAb for 30 minutes. Ezrin was immunoprecipitated and Western blotted with anti-phosphotyrosine (top panel) or anti-ezrin antibodies (bottom panel). (D) OCI-LY8 were incubated for the indicated times with αCD81 or IC mAb, or with immobilized HCV-$E2_{661}$ (HCV-E2) or with beads loaded with mock supernatant (Mock). (E) OCI-LY8 cells and (F) human PBMC were incubated with the anti-CD81 for the indicated times and cell lysates were probed directly with the anti-ezrin pY353 antibody (upper panels) then stripped and reprobed with an anti-ezrin mAb (bottom panels).

Fig. 3. Phosphorylation of ezrin pY353 in B cells is Syk-dependent. (A) OCI-LY8 cells were incubated with 1 μg/ml αCD81 or IC mAb for the indicated times. (B) OCI-LY8, DHL4, and DHL6 B cells were incubated with 1 μg/ml αCD81 or IC mAb for 30 minutes. (A and B) Syk was immunoprecipitated and Western blotted with an antibody specific for the Syk autophosphorylation sites, 525/526 (top panels) and total Syk (bottom panels). (C) OCI-LY8 cells were stimulated with 1 μg/ml αCD81 or IC mAb for 30 minutes in the presence or absence of 2.5 μM of the Src family kinase inhibitor PP2, or the Syk kinase inhibitors, SI 1, SI 2, and R406. Ezrin was immunoprecipitated and probed with an anti-phosphotyrosine (top panel) or an anti-ezrin mAb (bottom panel). (D) Untreated OCI-LY8 cells or cells pre-incubated with R406

for 1 hour were stimulated by αCD81 for the indicated time periods, lysates were probed with anti-ezrin pY353 (upper panels), then stripped and reprobed with anti-ezrin (bottom panels).

Fig. 4. Phosphorylation of ezrin pY353 is Syk-dependent and requires the C-terminal domain of CD81. (A) Flow cytometry analysis of U937-CD81 and U937-CD81-Syk KD. Analysis of Syk (upper panel) and ezrin (middle panel) expression was performed on permeabilized cells, CD81 surface levels (lower panel) are also shown in the two cell lines. (B) Western blot of Syk (left panel) and actin (right panel) in U937-CD81 and U937-CD81-Syk KD cells (lanes 1 and 2, respectively). (C) U937-CD81 and U937-CD81-Syk KD, as indicated, were stimulated with anti-CD81 for the indicated times and cell lysates were probed directly with the anti-ezrin pY353 antibody (upper panel) then stripped and reprobed with an anti-ezrin mAb (bottom panel). (D) U937-CD81 and U937-CD81ΔC cells were stimulated with anti-CD81 mAb for the indicated times. Whole cell lysates were probed with anti-ezrin pY353 (top panels) and anti-ezrin (bottom panels).

Fig. 5. The transient dephosphorylation of ezrin Thr567 requires the C-terminal domain of CD81 and is Syk-independent. (A) Untreated or R406 treated OCI-LY8 cells were stimulated for the indicated times with the indicated anti-CD81 mAbs. (B) U937-CD81 (left panels) and U937-CD81ΔC (right panels) were stimulated with 5A6 for the indicated times. Whole cell lysates were probed with anti-pERM (top panels) and anti-actin (bottom panels).

Fig. 6. CD81 stimulation induces its co-capping with ezrin and F-actin. OCI-LY8 cells were incubated for 10 minutes at 37ºC with a FITC-conjugated αCD81 mAb alone (CD81 stimulation), or in the presence of the Syk inhibitor R406 (CD81 stimulation + R406). Cells were fixed, permeabilized, and stained with biotinylated anti-ezrin (Ezrin), detected by TRITC-conjugated strepavidin, and with phalloidin-Alexafluor 350 (F-Actin). Shown are two

representative images from each treatment condition. Merged images with 10 μm scale bars are shown in the right column.

Supplement Fig. 1. Flow cytometry analysis of CD81 expression in U937-CD81ΔC (blue), a matching U937-CD81 subclone (green) and U937-CD81 expressing higher CD81 levels (red).

Supplement Fig. 2. OCI-LY8 cells were incubated for 10 minutes on ice followed by the addition of FITC-conjugated αCD81 mAb. Cells were processed as detailed in Material and Methods.