Published in final edited form as:

J Proteome Res. 2011 September 2; 10(9): 3983–3992. doi:10.1021/pr200577d.

Proteomics analysis of the ezrin interactome in B cells reveals a novel association with Myo18a α

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Abstract

The molecular regulation of recruitment and assembly of signalosomes near the B cell receptor (BCR) is poorly understood. We have previously demonstrated a role for the ERM family protein ezrin in regulating antigen-dependent lipid raft coalescence in B cells. In this study we addressed the possibility that ezrin may collaborate with other adaptor proteins to regulate signalosome dynamics at the membrane. Using mass spectrometry-based proteomics analysis we identified Myo18a α as a novel binding partner of ezrin. Myo18a α is an attractive candidate as it has several protein-protein interaction domains and an intrinsic motor activity. The expression of Myo18a α varied during B cell development in the bone marrow and in mature B cell subsets suggesting functional differences. Interestingly, BCR stimulation increased the association between ezrin and Myo18a α , and induced co-segregation of Myo18a α with the BCR and phosphotyrosine-containing proteins. Our data raise an intriguing possibility that the Myo18a α /ezrin complex may facilitate BCR-mediated signaling by recruiting signaling proteins that are in close proximity of the antigen receptor. Our study is not only significant with respect to understanding the molecular regulation of BCR signaling but also provides a broader basis for understanding the mechanism of action of ezrin in other cellular systems.

Keywords

B cell receptor; Myo18a α ; ezrin; signal transduction

Introduction

Engagement of the B cell receptor (BCR) on mature B cells by cognate antigen initiates BCR signaling that results in B cell activation and differentiation.^{1, 2} Association of the BCR with specialized membrane microdomains known as lipid rafts is important for the regulation of BCR signaling.³⁻⁵ We have previously reported that antigen-induced lipid raft coalescence is regulated by ezrin, a member of the ezrin-radixin-moesin (ERM) family.⁵ Ezrin mediates the crosslinking of the plasma membrane and actin filaments through its N-terminal FERM and C-terminal actin-binding domains.^{6, 7} Conformational activation of ezrin is regulated through phosphorylation of the conserved threonine residue 567 (T567) in

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Supporting Information Available Supplemental Table 1, Amino acid sequences of peptides identified for murine proteins listed in Table 1.

Supplemental Table 2, Amino acid sequences of peptides identified for murine proteins listed in Table 2.

This material is available free of charge via the Internet at <http://pubs.acs.org>.

the C-terminal actin-binding domain.^{6, 7} In resting mature B cells, ezrin is constitutively phosphorylated on T567 and exists primarily in the active conformation. BCR engagement induces dephosphorylation of T567 and rapidly converts ezrin into the inactive form.⁵ As ezrin provides regulated linkage between the plasma membrane and cortical actin cytoskeleton, it is likely to be involved in the assembly of the BCR signaling machinery.

The importance of ezrin has been highlighted in both non-hematopoietic and hematopoietic cells. Mice deficient in ezrin exhibit neonatal lethality because of defective intestinal villus architecture, indicating that ezrin is required for appropriate localization of apical membrane proteins in the intestinal epithelial cells.⁸ Ezrin is highly expressed in many types of tumors and can promote metastasis.⁹⁻¹³ In breast and prostate cancer cell lines with aggressive metastatic behavior, a complex of podocalyxin and the active form of ezrin was found to be concentrated at cellular boundaries. As podocalyxin acts as an anti-adhesion protein, its association with ezrin may contribute toward increased motility of these tumor cells.¹³ In epithelial cells tyrosine phosphorylated ezrin was shown to play a role in spatial recruitment and activation of the tyrosine kinase Fes, which is essential for transmission of the activation signal.¹⁴ Ezrin recruits the tyrosine kinase Zap70 to the immunological synapse in T cells thereby promoting proximal T cell signaling.¹⁵ Knockdown of ezrin expression in T cells perturbs the organization of microtubule network at the immunological synapse and inhibits the formation of SLP-76 containing microclusters resulting in lower NFAT activation.¹⁶ While T cells from conditional ezrin-deficient mice exhibited lower NFAT activation and reduced IL-2 production, the formation of the immunological synapse and the recruitment of Zap70 were intact.¹⁷ Ezrin creates boundaries by linking the B cell plasma membrane to cortical actin filaments, which limit the steady state diffusion of the BCR. Disruption of the boundaries by treatment of resting B cells with pharmacological agents that alter actin polymerization increased the mobility of the BCR and resulted in antigen-independent activation.¹⁸ Collectively, these studies indicate that ezrin controls cellular functions by regulating the positioning and movement of cell-signaling components. However, as ezrin does not have intrinsic motor activity, how it is directed to appropriate subcellular localizations is unclear.

In this study, we performed proteomics analysis of ezrin in B cells to identify binding partners with a potential to regulate the localization of protein complexes. We identified Myosin 18a alpha (Myo18a α), a member of the unconventional myosin family, which is known to regulate the trafficking and localization of protein complexes.¹⁹⁻²² B cell receptor stimulation increased the interaction of Myo18a α with ezrin and tyrosine phosphorylated proteins and induced its co-segregation with the BCR and phosphotyrosine-containing proteins. Our data raise an intriguing possibility that the ezrin/Myo18a α complex may play an important role in the assembly and localization of BCR signaling complexes.

Materials and Methods

Cells, reagents, and mice

The murine B lymphoma cell line CH27 was maintained in DMEM supplemented with 15% heat inactivated fetal bovine serum (Invitrogen) and 50 μ M β -mercaptoethanol at 37° C. Antibodies used for flow cytometry were from BD Biosciences (BD Pharmingen). Purified goat anti-mouse IgM F(ab')₂ antibody was from Jackson ImmunoResearch, and Myo18a antibody (catalog# A301-598A) was from Bethyl Laboratories. The Myo18a antibody recognizes the amino acid sequence encompassing residues 2004-2054 of human myosin 18a (provided on the manufacturer's datasheet). We have compared this sequence (Accession number NP_510880.2 in the NCBI database) to the corresponding region of the mouse protein (Accession number Q9JMH9 in the UniProt database) and found that 47 of 51 amino acids are identical. Biotin- and FITC-conjugated phosphotyrosine (4G10)

antibodies, streptavidin-horseradish peroxidase (HRP), and ezrin antibody (catalog # 07-130) were from Millipore. Ezrin antibody was raised to a KLH-conjugated synthetic peptide corresponding to amino acid residues 479 to 498 of human ezrin. The mouse sequence has 17 identical amino acids out of the 20 residues. When this peptide sequence was subjected to Blast searches using the NCBI and UniProt databases, the “hits” only revealed ezrin out of the three ERM family proteins. PLC- γ 2 antibody was from Cell Signaling Technology. Actin antibody was from Santa Cruz Biotechnologies. Recombinant Protein G-agarose beads and all other chemicals were from Sigma-Aldrich. C57BL/6J mice were maintained in the animal facility of Lerner Research Institute at Cleveland Clinic and used in compliance with the guidelines approved by the Institutional Animal Care and Use Committee.

Stimulation of B cells and immunoprecipitation

B cells were washed and resuspended in DMEM and either left unstimulated or stimulated with 10 μ g/mL of goat anti-mouse IgM F(ab')₂ fragment for the indicated times at 37° C. The cells were centrifuged and incubated in lysis buffer containing 20 mM Tris-HCl, pH 8.3, 30 mM NaCl, 1 mM EDTA, pH 8.0, 0.5% NP-40 and protease and phosphatase inhibitors (0.5 μ M iodoacetamide, 5 mg/ml pepstatin A, 1 mM PMSF, 1 mM sodium metavanadate, and 10 mM sodium fluoride) on ice for 30 minutes, followed by centrifugation for 20 minutes at 22,000 g at 4° C. For immunoprecipitation, cell lysates were pre-cleared using a 20% slurry of Protein G-agarose beads for 1 hour on a rotator at 4° C, followed by overnight incubation with the appropriate antibody. Immunoprecipitates were collected using a 20% slurry of Protein G-agarose beads, the beads washed extensively in lysis buffer, and boiled in appropriate volume of sample buffer.

Western blot analysis

Immunoprecipitates or cell lysates were resolved by SDS-PAGE using either 4-12% or 7% gels, electrophoretically transferred to PVDF membranes (Millipore), and blocked in 5% BSA in TBS-Tween. The membranes were incubated overnight in appropriate primary antibodies at 4 °C with agitation, followed by washing and incubation with HRP-conjugated secondary antibody. The membranes were developed using enhanced chemiluminescence (ECL) reagent (GE HealthCare). For western blotting of sort-purified B cells, protein concentration of each lysate was determined using the Pierce BCA Protein Assay Kit. Densitometric quantification was performed using Image J software.

Sample preparation for mass spectrometry

Ezrin or Myo18a α immunoprecipitates prepared from lysates of unstimulated CH27 cells were resolved by SDS-PAGE using a 4-12% (Fig. 1B) or 7% (Fig. 1D) gel, the gel was washed in Milli Q water and fixed in a solution containing 50% ethanol and 10% glacial acetic acid for 30 minutes at room temperature. After washing in Milli Q water, the gel was incubated overnight in GelCode Blue Staining Reagent (Thermo Scientific), followed by extensive washing in Milli Q water. Prior to digestion, the protein bands were excised as closely as possible, washed/detained in 50% ethanol and 5% acetic acid, followed by dehydration in acetonitrile. The proteins bands were reduced with DTT, alkylated with iodoacetamide, and digested in-gel by adding 5 μ L of 20 ng/ μ L trypsin in 50 mM ammonium bicarbonate buffer overnight at room temperature. The peptides were extracted from the polyacrylamide gel in two aliquots of 10 μ L each using 50% acetonitrile with 5% formic acid. The extracts were combined and evaporated to less than 10 μ L in a Speedvac and resuspended in 1% acetic acid to make up a final volume of approximately 30 μ L for LC-MS analysis.

Mass spectrometry analysis

The LC-MS system used was a Finnigan LTQ linear ion trap mass spectrometer system. The HPLC column was a self-packed 9 cm $\times$ 75 μ m Phenomenex Jupiter C₁₈ reversed-phase capillary chromatography column. Ten microliter aliquots of peptide extract were injected and eluted from the column using an acetonitrile/0.1% formic acid gradient at a flow rate of 0.25 μ L/min. The eluted peptides were directly introduced into the ion source of the mass spectrometer, which was operated at 2.5 kV. The digest was analyzed using the data dependent multitask capability of the instrument to acquire full mass spectra to determine molecular weights and product ion spectra to determine amino acid sequence in successive instrument scans. This mode of analysis produces approximately 2500 collision-induced dissociation (CID) spectra of ions ranging in abundance over several orders of magnitude. Peak lists were extracted using Thermo Electron Xcalibur v2.0 subroutine extract.msn.exe and were generated for all peaks with at least 15 product ions in the MS/MS spectra. The peak lists were searched using the program Mascot version 2.0 (Matrix Science) against the mouse Reference Sequence Database (August 25, 2006, total of 44,880 sequences). The search parameters were as follows: species, mouse; enzyme, trypsin; number of missed cleavages allowed, 1; fixed modifications, carbamidomethylated cysteine; variable modifications, oxidized methionine; precursor ion mass tolerance, ± 2.0 Da; fragment ion tolerance, ± 1.5 Da. Subsequent searches to verify the Mascot identifications were performed using the program Sequest that is bundled into Bioworks v3.1. The database used in these searches was manually generated and was composed of all the proteins positively identified in the Mascot searches. The search parameters used in these experiments were the same as those used in the Mascot searches. For MS/MS interpretation, the Mascot search results were filtered using a threshold peptide ion score of 30. All peptides with a score less than 50 were manually validated. For the Sequest searches, the resulting MS/MS identifications were filtered based on the Xcorr value; 1.5 (+1 charge state), 2.0 (+2 charge state), and 2.5 (+3 charge state) were used. Every peptide positively identified in the Sequest searches was manually validated. The false discovery rate (FDR) for these searches was determined by searching the MS/MS data against a decoy database constructed by reversing all of the protein sequences present in the mouse reference sequence database. The same search criteria and filter settings were used in these searches and the resulting FDR for these experiments was determined to be less than 1%.

Purification of B cell subsets

For sorting of different B cell subpopulations, single cell suspensions were prepared from bone marrow and spleens of C57BL/6J mice. Bone marrow cells were incubated with CD16/CD32 (Fc block) antibody for 15 minutes on ice followed by incubation with B220-APC-Cy7, IgM-PE-Cy7, IgD-PE, and CD93-APC antibodies for 30 minutes on ice. The cells were washed extensively, resuspended in PBS containing 0.5% BSA, and sort purified using a FACS Aria cell sorter (BD Biosciences). The different developmental stages of B cells were sorted based on the surface markers as shown in Figures 2A and B. B220⁺ IgM⁻ cells correspond to pro/pre B cells, B220⁺ IgM⁺ CD93⁺ IgD⁻ to immature B cells, and B220⁺ IgM⁺ CD93⁻ IgD⁺ to recirculating mature B cells. Splenic mature B cells were purified by negative selection using CD43 beads and MACS columns from Miltenyi Biotec according to the manufacture's instruction. For sorting of different mature B cell subsets, purified B cells were incubated with CD16/CD32 (Fc block) antibody for 15 minutes on ice, followed by incubation with CD21-FITC, CD23-PE, and CD93-APC antibodies for 30 minutes on ice, washed, and sort purified. CD93⁻ CD21^{lo} CD23⁺ follicular (Fo) B cells, CD93⁻ CD21^{hi} CD23⁻ marginal zone (MZ) B cells, and CD93⁻ CD21⁻ CD23⁻ B1 B cells were sorted using the FACS Aria cell sorter.

Quantitative PCR

Total RNA was isolated from the sort-purified B cells using the Qiagen RNeasy Mini or Micro Kit (Qiagen). cDNAs were generated using qScript™ cDNA SuperMix (Quanta Biosciences) and quantitative PCR was performed using PerfeCta™ SYBR® Green FastMix™ (Quanta Biosciences). The relative expression of Myo18a α was determined based on Pfaffl's model²³ using β -2 microglobulin as the housekeeping gene control. Expression level of Myo18a α in pro/pre cells was used as a calibrator and arbitrarily set to 1. mRNA level of Myo18a α in other B cell subpopulations was calculated relative to that in pro/pre B cells. The following primers were used for amplification, Myo18a α (forward, 5'-AGATGATCCGGCAGTCTGG-3' and reverse, 5'-AGGCCAGATCTTCTAGACGG-3'), and β -2m (forward, 5'-TCAGTCGCGGTTCGCT TC-3' and reverse, 5'-CAAGCACCAGAAAGACTAGGGTC-3'). The Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems) was used to perform the reactions.

Immunofluorescence microscopy

Purified splenic B cells were stimulated in suspension with biotin-conjugated goat anti-mouse IgM F(ab')₂ for 1, 5, and 10 minutes at 37 °C. Unstimulated cells were incubated with the antibody on ice. Subsequently, the cells were fixed in 4% paraformaldehyde, followed by incubation with Alexa Fluor 647-conjugated streptavidin (Molecular Probes) for 30 minutes at 4 °C to detect the surface BCR. The cells were washed, permeabilized, and stained with Myo18a antibody for 1 hour at 4 °C, followed by incubation with Alexa Fluor 568-conjugated anti-rabbit IgG (Molecular Probes) for 30 minutes. After washing, the cells were stained with FITC-conjugated phosphotyrosine antibody for 1 hour at 4 °C, washed, resuspended in PBS, and imaged. All images were acquired in the epifluorescence mode using a Leica-AM TIRF microscope DMI6000 (Leica Microsystems) with an attached Hamamatsu EM-CCD camera, using HCX PL APO 100X oil objective using an additional 1.6X magnification power with numerical aperture of 1.47 and appropriate filter cubes. The images were acquired using the Leica acquisition software LAS AF Version 2.2.0. Metamorph image analysis software was used for data analysis.

Statistical analysis

Two-tailed, paired ratio Student *t* tests were performed with Prism 4 (GraphPad Software, Inc.) to determine statistical significance using the α -level of 0.05. For Figure 2C, the values were normalized to pro/pre B cells. For Figure 3, the unstimulated samples were used to normalize the stimulated samples to determine fold induction. These data were then transformed by taking the logarithm of each value. Then, paired Student *t* tests were performed to calculate the *p* values.

Results

Identification and validation of Myo18a α as an interacting partner of ezrin in B cells

To identify potential binding partners of ezrin in B cells, we employed mass spectrometry (MS)-based proteomics analysis of ezrin in the CH27 B cell line (Figure 1A). Ezrin was immunoprecipitated from unstimulated CH27 cell lysates, and the immunoprecipitated material was resolved by SDS-PAGE. The protein bands were detected by GelCode Blue staining and subjected to mass spectrometry-based proteomics analysis. Several proteins of varying sizes were detected in the gel, and eight indicated areas were excised for MS analysis (Figure 1B). The interactome of ezrin contained 37 different proteins that were identified by at least two distinct peptides with Mascot peptide ion scores ranging from 30 to greater than 100. All of the identifications were verified by follow-up Sequest searches. The false discovery rate for these searches was determined to be less than 1%. Two of the

proteins identified were immunoglobulin heavy and light chains corresponding to the ezrin antibody that was used for immunoprecipitation (slice #8), while no proteins were identified from slice #1. The bait protein, ezrin, was identified in slice #7, near the 75 kDa molecular weight marker. Table 1 represents a subset of the identified murine proteins categorized into four groups, including membrane receptors, motor/cytoskeletal/structural proteins, signaling proteins, and proteins involved in trafficking/vesicle formation. All the peptides identified for these proteins, based on the above Mascot peptide ion score criteria, are listed in Supplemental Table 1. The signaling proteins dedicator of cytokinesis 2 (DOCK2) and ras-GTPase-activating protein SH3-domain-binding protein (G3BP1) have been implicated in the regulation of cytoskeletal rearrangement, cell-migration, growth, and survival.²⁴⁻²⁷ Two proteins with intrinsic motor activity were found to be associated with ezrin, Myh9 and Myo18a α (Table 1). The non-muscle myosin Myh9 generates contractile force for cellular movement and morphological changes to regulate cell migration, cell division, and cell adhesion.²⁸ Myosin 18a alpha (Myo18a α), a member of the unconventional myosin family was previously reported to play a critical role in mediating and regulating the localization of protein complexes.^{20, 22, 29} Myo18a α was originally identified in stromal cell lines as a protein that is involved in supporting the growth of hematopoietic stem cells.³⁰ Myo18a has two isoforms, alpha and beta, which differ in the existence of a KE-rich sequence and a PDZ domain at the N-terminus in the alpha isoform.^{30, 31} As Myo18a α can potentially mediate protein-protein interactions through its PDZ and C-terminal globular domains and potentially participate in cell signaling, we focused our study on Myo18a α .

In order to validate the association of ezrin with Myo18a α , we immunoprecipitated ezrin from CH27 cells, followed by western blotting with Myo18a α antibody. Detection of a band of approximately 230 kDa at the expected molecular weight of Myo18a α confirmed the presence of Myo18a α in ezrin immunoprecipitates (Figure 1C). To further confirm the interaction between ezrin and Myo18a α , we performed a reverse immunoprecipitation/MS experiment. Myo18a α was immunoprecipitated from unstimulated CH27 cell lysates and resolved by SDS-PAGE. After staining in GelCode Blue solution, several protein bands that ranged from slightly above 50 kDa to 250 kDa in size were detected (Figure 1D). We excised ten indicated areas of the gel for analysis by MS (Figure 1D). Data analysis revealed 42 different murine proteins that were identified by at least two peptides that have Mascot peptide ion scores ranging from 30 to greater than 100. All of the identifications were verified by follow-up Sequest searches. The false discovery rate was determined to be less than 1%. The murine proteins shown in Table 2 represent those that are involved in maintaining the cytoskeletal network and vesicular trafficking based on the reported functions of myosins. Interestingly, Myh9 and nucleolin were also identified in the Myo18a α immunoprecipitates, as they were in the ezrin interactome (Table 1). The peptides identified in the Myo18a α immunoprecipitates (Table 2) are shown in Supplemental Table 2 based on the above Mascot peptide ion scores. The bait protein Myo18a α was identified in slice #2. Three ERM-derived peptides were identified in slice #7 (Supplemental Table 2). However, as the amino acid sequences of these peptides are completely conserved between ezrin, moesin, and radixin, they could be derived from any of these proteins. Thus, the proteomic analysis of the Myo18a α interactome suggests that it may associate with ezrin, moesin, and/or radixin.

Differential expression of Myo18a α in peripheral B cell populations

B cells develop in the bone marrow from hematopoietic stem cells to become immature B cells.³² These immature B cells migrate out of the bone marrow and enter peripheral lymphoid compartments such as the spleen and lymph nodes, where they undergo final maturation. Mature B cells recirculate throughout the body and can re-enter the bone marrow. In the peripheral immune organs, follicular B cells constitute the majority of mature

B cell population and play a critical role in adaptive immunity, whereas B1 and marginal zone B cells comprise a smaller percentage of the peripheral B cells but play a major role in innate-like humoral immunity. To further characterize the regulation of Myo18a α in B cells we examined its expression during B cell development and in peripheral mature B cells.

We sorted different B cell subpopulations from the bone marrow and spleen by flow cytometry (Figures 2A and B). Total RNA was isolated from the purified pro/pre, immature, recirculating mature B cells, follicular, B1, and marginal zone B cells and quantitative RT-PCRs were performed to analyze comparative gene expression of Myo18a α . In the bone marrow, pro/pre cells showed the highest mRNA expression of Myo18a α mRNA and among the splenic B cell populations B1 cells expressed the highest level (Figure 2C). The Myo18a α mRNA was nearly undetectable in immature, recirculating mature B cells, follicular, and marginal zone B cells (Figure 2C). Next, we examined the level of Myo18a α protein expression in the peripheral B cell populations. Cellular lysates of sort-purified B1, follicular, and marginal zone B cells were assayed for the expression of Myo18a α protein by western blotting. Surprisingly, contrary to the high expression level of Myo18a α mRNA in B1 B cells, the level Myo18a α protein in these cells was very low, as shown in two separate experiments (Figure 2D). Follicular and marginal zone B cells expressed 4.8 and 4.4-fold more Myo18a α protein, respectively, as compared to B1 B cells (Figure 2D). Collectively, these data show that Myo18a α gene is expressed at the earliest stages of B cell development. Furthermore, there is an inverse relationship between the expression of Myo18a α mRNA and protein in mature B cell subsets, suggesting additional regulation at post-transcriptional or translational level.

Association of ezrin and Myo18a α increases in response to BCR stimulation

To explore the dynamics of ezrin/Myo18a α interaction in antigen-stimulated B cells, we immunoprecipitated Myo18a α from unstimulated B cells or those stimulated with anti-IgM antibody (surrogate for antigen) for 1, 10, or 30 minutes and examined its association with ezrin. As Myo18a α was previously shown to bind to actin, we also tested its interaction with actin in resting and antigen-stimulated B cells. Myo18a α was found to associate with actin in resting B cells but this interaction did not change upon crosslinking of the BCR (Figure 3A). On the other hand, BCR stimulation induced a significant increase in the association between ezrin and Myo18a α (Figure 3B), which was maximal at 1 and 10 minutes post-stimulation. Constitutive association between ezrin and Myo18a α was also observed in naive splenic B cells purified from mice, which was also significantly increased upon BCR stimulation (Figure 3C). These data demonstrate that ezrin associates with Myo18a α , and that antigen binding to the BCR regulates this interaction.

BCR stimulation induces formation of concentrates containing Myo18a α and tyrosine phosphorylated proteins

Stromal cell lines that express higher amounts of Myo18a α are better capable of supporting the growth of hematopoietic cells.³⁰ As Myo18a α contains protein-protein interaction domains,³⁰ it is likely to be involved in intercellular signal transduction. BCR stimulation induces global tyrosine phosphorylation of many signaling intermediates that participate in B cell activation. To examine whether Myo18a α participates in BCR signaling, we tested whether Myo18a α interacts with tyrosine phosphorylated proteins upon BCR stimulation. Myo18a α was immunoprecipitated from antigen-stimulated purified splenic B cells to examine its co-precipitation with tyrosine-phosphorylated proteins. We observed an activation-induced association of many tyrosine-phosphorylated proteins with Myo18a α that ranged in size from 60-80 kDa and 95-150 kDa (Figure 4A). To examine the proximity of Myo18a α with the BCR and tyrosine-phosphorylated proteins in B cells undergoing activation, we imaged their localization in purified splenic B cells stimulated with anti-IgM.

In resting B cells, the BCR was distributed throughout the plasma membrane and Myo18a α was predominantly located in the submembrane region (Figure 4B). No phosphotyrosine (pY) signal was observed in unstimulated B cells (Figure 4B). At 1 minute of stimulation, phosphotyrosine proteins were detected throughout the plasma membrane and co-segregated with Myo18a α and the BCR, as seen in the overlaid images (Figure 4B). By 5 and 10 minutes of stimulation, both the BCR and phosphotyrosine proteins began to polarize at one end and co-segregated with Myo18a α . These data suggest that Myo18a α may be associating with phosphotyrosine proteins in an activation-dependent fashion and regulating the localization of these proteins.

Discussion

Although ezrin has been shown to interact with a number of proteins and participate in cell signaling, how it is brought to appropriate subcellular locations is not clear. Here, we sought to identify proteins with motor activity that bind to ezrin, and have the potential to regulate BCR signaling. Using mass spectrometry-based proteomics profiling, we identified Myo18a α , a member of the unconventional myosin family, as a novel binding partner of ezrin. While peptides from ezrin were detected in the Myo18a α immunoprecipitates by mass spectrometry, it was unclear whether they were indeed derived from ezrin as their amino acid sequences are completely conserved with those of moesin and radixin.^{14, 33, 34} As moesin was detected in the ezrin interactome, there exists a possibility that it also interacts with Myo18a α . While radixin is not known to be expressed in B cells, our data do not formally exclude it as a potential interactor of Myo18a α . Detection of ezrin in the immunoprecipitates of Myo18a α by western blotting (Fig. 3B, C) confirmed the association between these two proteins, however, it remains to be determined whether moesin and radixin also interact with Myo18a α . Members of the unconventional myosin family have been implicated in a variety of cellular functions.³⁵ Myo18a α contains a PDZ domain and was reported to be involved in localization of protein complexes. In HeLa cells, Myo18a α was shown to interact with the myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK)/leucine-rich adaptor protein 35a (LRAP35a)/ Myo18a α tripartite complex, which is involved in the Myo2A-dependent assembly of actomyosin network in the lamella.²⁰ Myo18a α was shown to be responsible for bringing the complex to Myo2A. In another study, Myo18a α was reported to complex with and regulate the localization of the p21-activated kinase 2 (PAK2)/beta PAK-interacting exchange factor (β PIX)/G-protein-coupled receptor kinase-interactor 1 (GIT1) complex, although the formation of the PAK2/ β PIX/GIT1 complex did not require Myo18a α .²⁹ Furthermore, Myo18a α connects actin to the complex of Golgi phosphoprotein 3 (GOLPH3) and phosphatidylinositol-4-phosphate (PtdIns(4)P) at the Golgi membrane.²² Disruption of the PtdIns(4)P/GOLPH3/Myo18a α /actin complex by knocking down Myo18a α demonstrated that the complex is essential for maintaining the Golgi complex morphology and vesiculation function. Together, these studies highlight the importance of Myo18a α in mediating localization of these complexes to sustain proper cellular functions. In our study, BCR stimulation led to increased association of Myo18a α with ezrin, and its co-segregation with the BCR and phosphotyrosine-containing proteins, suggesting that Myo18a α may play a role in recruitment and organization of BCR signalosomes. Furthermore, our imaging data showed that Myo18a α localizes primarily at the inner surface of the plasma membrane of B cells. This is in agreement with a previous report in NIH3T3 cells in which EYFP-Myo18a α fusion protein was found to localize to the submembrane region.³⁶ As the earliest events of BCR signaling occur at the plasma membrane where the BCR is located, the subcellular distribution of Myo18a α is ideal for interaction with signaling proteins.

In addition, we also detected proteins such as spectrin, actinin, and tubulin in the immunoprecipitates of ezrin and Myo18a α . As these proteins constitute the cytoskeletal

network, and because both ezrin and Myo18a α are known to interact with proteins of the cytoskeletal network, it is not surprising that these proteins were detected. In erythrocytes, band 4.1 protein which contains FERM domain, as does ezrin, has been shown to interact with spectrins.³⁷ As ezrin is a linker of the plasma membrane and cortical cytoskeleton, and spectrins are found on the cytoplasmic side of the membrane, we believe this association is in agreement with the known interaction profile of ezrin. Similarly the association of Myo18a α with actinin, myosin polypeptide 4, CD2AP, and tubulin was expected as these proteins are known to either constitute or regulate the cytoskeleton.

Identification of clathrin in the ezrin interactome is in agreement with the previous finding that ezrin and clathrin colocalize in human epithelial bronchial cell line in response to human rhinovirus16.³⁸ Interestingly, both clathrin and FcR γ IIb have been reported to localize in lipid rafts in B cells, as does ezrin.⁵ As FcR γ IIb is involved in down regulation of BCR-mediated signal transduction,² it is possible that ezrin sequesters FcR γ IIb to prevent unwanted activation in resting B cells. Mutations in the FcR γ IIb gene lead to its exclusion from the rafts and result in diminished inhibition of B cell signaling components.¹ The coatamer protein complex α and FK506-binding protein 4 are involved in protein transport/trafficking³⁹⁻⁴¹ and may act in concert with Myo18a α .

Myh9 and nucleolin were identified in the interactomes of both ezrin and Myo18a α . Myh9 has also been found in lipid rafts in our previous study.⁵ However, the significance of its presence in lipid rafts is not well understood. Myh9 generates major driving forces for cell motility and perhaps Myo18a α helps to localize Myh9 to the membrane. At this point, however, we do not know whether ezrin, Mo18a α , and Myh9 are all in the same complex. While nucleolin is predominantly found in the nucleus, it has been reported to shuttle between the nucleus and the cytoplasm.⁴² Interestingly, it has been reported to interact with FK506-binding protein,⁴³ which was detected in the Myo18a α immunoprecipitate. Again, it is possible that Myo18a α is involved in the transport of such proteins between the cytoplasm and the nucleus, as it has a KE-rich sequence, which is known to target proteins to the nucleus.³⁰ Collectively, these interactions of Myo18a α are largely novel and unexplored, and the functional consequences of these interactions will require validation and further studies.

Our data showed an increase in the association of ezrin with Myo18a α in response to BCR ligation. We have previously shown that ezrin undergoes dephosphorylation at T567 upon BCR stimulation.⁵ In addition to T567, ezrin contains tyrosine residues that can be phosphorylated upon activation.⁴⁴ The kinetics of the ezrin/Myo18a α interaction coincides with that of ezrin dephosphorylation at T567.⁵ It is attractive to speculate that Myo18a α preferentially associates with the dormant conformational state of ezrin in which it is dephosphorylated at T567. Alternatively, tyrosine-phosphorylated ezrin may bind an SH2 domain-containing signaling protein, which also contains a PDZ domain for interaction with the PDZ domain of Myo18a α .

The mRNA level of Myo18a α was previously examined in different murine B cell lines that represent pre-B cell and mature B cell stages.³¹ The mature B cell line M12 expressed the Myo18a α mRNA but it was barely detectable in pre-B cell lines 70Z/3 and 18-81. In our study, we detected the Myo18a α mRNA in a mixture of pro/pre B cells isolated from mouse bone marrow. The difference between our data and those reported in the other study could be due to their use of tumor cell lines versus our primary B cell populations. We found a reciprocal expression pattern of mRNA and protein for Myo18a α in mature splenic B cell subsets, wherein the mRNA expression was low but protein expression was high or vice versa. While intriguing, how this relates to BCR signaling and B cell function in different subsets of B cells is currently unclear, and whether Myo18a α plays a distinct role in the

generation or function of different B cell subpopulations will require more detailed examination. Nevertheless, we hypothesize that the differential expression of Myo18a α plays a role in B cell development. Pro/pre B cells, which develop into immature B cells in the bone marrow may need higher Myo18a α expression to receive intercellular signals from bone marrow stromal cells in order to proceed through B cell development. However, once they develop into immature B cells they may not need high Myo18a α expression, as these cells are negatively selected before entering peripheral lymphoid compartments.

A role for ezrin in signal transduction has been noted in many different cellular systems. Ezrin can act as an adaptor molecule to regulate spatial organization of bound complexes to control cellular functions. However, the mechanism of its own localization during BCR signaling was unclear. Our identification of the motor protein Myo18a α as an ezrin-binding protein suggests a possible mechanism of localization of ezrin and BCR signalosomes during B cell activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the Proteomics Core personnel for performing mass spectrometry, and Jennifer Powers for cell sorting. This work was supported by an NIH grant (A1081743) and a Cancer Research Institute Investigation Award to N.G. Author contribution: N.G. conceptualized and designed research, K.M., N.P. and N.B. performed research, N.G., K.M., B.W., and N.P. analyzed data, and N.G. and K.M. prepared the manuscript.

Abbreviations

FITC	fluorescein isothiocyanate
PE	phycoerythrin
APC	allophycocyanin
Cy	cyanine
PMSF	phenylmethanesulfonyl fluoride
PDZ	post synaptic density protein 95, <i>Drosophila</i> disc large tumor suppressor, and zonula occludens-1 protein

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Figure 1.

Identification of Myo18α as a binding partner of ezrin. (A) A schematic representation of sample preparation for mass spectrometry is shown. Cell lysates prepared from unstimulated CH27 cells were subjected to immunoprecipitation using ezrin antibody.

Immunoprecipitates were resolved by SDS-PAGE, and the gel was stained with GelCode Blue. Subsequently, slices of the gel were subjected to in-gel trypsin digestion for liquid chromatography-mass spectrometry (LC-MS) analysis. The data were analyzed by searching mouse Reference Sequence Database using Mascot, and additional searches were performed using Sequest and Blast software. (B) Ezrin immunoprecipitates from unstimulated CH27 cells were resolved by SDS-PAGE using a 4-12% gel. The numbers and boxes indicate the locations of protein bands in the gel that were excised and subjected to MS analysis. (C) Ezrin was immunoprecipitated from lysates of unstimulated CH27 cells, the immunoprecipitate was resolved using a 4-12% gel, and probed with antibodies to ezrin and Myo18α. (D) Myo18α was immunoprecipitated from lysates of unstimulated CH27 cells, resolved by SDS-PAGE using a 7% gel, and the gel was stained with GelCode Blue. The numbers and boxes indicate the locations of protein bands in the gel that were excised and subjected to MS analysis. The data in (B), (C), and (D) are representative of two independent experiments each.

Figure 2.

Differential expression of Myo18α during B cell development and in mature B cell subsets. (A and B) The sorting schemes employed to isolate different B cell subpopulations from the bone marrow and spleen are shown. The forward (FSC) and side scatter (SSC) profiles (left panels) were used to draw gates on live lymphocytes. (A) In bone marrow (BM) cells, the cell surface marker profiles were B220⁺ IgM⁻ for pro/pre B cells (middle panel), B220⁺ IgM⁺ CD93⁺ IgD⁻ for immature B cells and B220⁺ IgM⁺ CD93⁻ IgD⁺ for mature recirculating B cells (right panel). (B) In purified splenic B cells, the cell surface marker profiles (middle and right panels) were CD93⁻ CD21^{lo} CD23⁺ for follicular (Fo) B cells, CD93⁻ CD21⁻ CD23⁻ for B1 B cells, and CD93⁻ CD21^{hi} CD23⁻ for marginal zone (MZ) B cells. (C) Quantitative PCR was performed to determine relative expression of Myo18α mRNA during B cell development and in peripheral B cell subsets. β-2 microglobulin was used as a loading control for the PCR. Expression of Myo18α in different B cell subsets is shown relative to its expression in pro/pre B cells. Mean±SD from three independent experiments is shown. Two-tailed, paired ratio Student *t* tests were performed comparing B1 cells to immature, recirculating mature, follicular (Fo), or marginal zone (MZ) cells. The *p* values are shown. (D) Cell lysates were prepared from sort-purified Fo, B1 and MZ B cells. Equal amount of protein (2–3 μg) from each cell lysate was resolved on a 7% gel for western blotting with antibodies to Myo18α and PLC-γ2. Data from two independent experiments are shown. Densitometry was performed to quantify the expression of Myo18α relative to PLC-γ2.

Figure 3.

BCR stimulation induces an increase in the association of Myo18α with ezrin. CH27 B cells (A and B) or purified splenic B cells (C) were either left unstimulated or stimulated with 10 μg/mL of anti-IgM for 1, 10, or 30 minutes. Myo18α was immunoprecipitated, the immunoprecipitate resolved using a 7% gel, and probed with antibodies to actin, ezrin, and Myo18α. Densitometry was performed to quantify the association between actin and Myo18α or ezrin and Myo18α, and the ratios obtained were used to calculate the fold increase. The fold increase was calculated by dividing the ratios of stimulated samples by the unstimulated samples. Mean±SD of fold increase from four independent experiments is shown. Two-tailed, paired ratio Student *t* tests were performed comparing the unstimulated samples to 1, 10, or 30 minute stimulated samples. *p* values are shown.

Figure 4.

BCR ligation induces the association of Myo18aα with tyrosine phosphorylated proteins. (A) Purified mouse splenic B cells were either left unstimulated or stimulated with 10 μg/mL of anti-IgM for 1, 10 or 30 minutes. Myo18aα was immunoprecipitated, resolved using a 7% gel, and probed with antibodies to Myo18aα or to phosphotyrosine. Arrows indicate major tyrosine phosphorylated proteins associating with Myo18aα. Data are representative of three independent experiments. (B) Purified splenic B cells were either left unstimulated or stimulated for the indicated times as described in Experimental Section. The cells were stained with antibodies to Myo18aα, phosphotyrosine (pY), and BCR and pseudocolored red for Myo18aα, green for pY, and cyan for BCR. Epifluorescence images of the stained cells were acquired using appropriate filter sets and a 100X objective with an additional 1.6X magnification. Scale bar, 5 μM. Overlaid images of Myo18aα and pY, or Myo18aα, BCR, and pY are shown. The data are representative of two independent experiments (n = 15-31 images for each time point).

Table 1

A list of murine proteins identified in immunoprecipitates^a of ezrin by LC-MS.

Functional Category (protein)	NCBI protein accession number ^b	MW (kDa)	Mascot score ^c	Peptide coverage (number of peptides) ^d
Membrane receptors				
FcγRIIb	NP034317	33	147	18% (3)
Motor/cytoskeletal/structural				
Myh9	NP071855	227	4808	45% (76)
Myo18a	NP035716	232	1204	15% (20)
Spectrin α2, isoform 1	NP001070022	279	170	2% (2)
Spectrin β2, isoform 1	NP787030	275	180	2% (2)
Ezrin	NP033536	69	1438	47% (26)
Moesin	NP034963	68	1182	41% (22)
Signaling proteins				
Dedicator of cytokinesis 2	NP203538	213	240	3% (4)
Ras-GTPase activating protein SH3-domain binding protein 1	NP038744	52	145	7% (2)
Trafficking/vesicle formation				
Clathrin heavy polypeptide	NP001003908	194	589	11% (11)
Nucleolin	NP035010	77	955	24% (17)

^a Ezrin immunoprecipitates were prepared from unstimulated CH27 cell lysates.

^b Accession numbers of proteins in NCBI's Reference Sequence database are shown.

^c Mascot version 2.0 (Matrix Science) was used.

^d Sequence coverage was determined by dividing the number of amino acids in a peptide by total number of amino acids, and the number of peptides detected for the identified protein is shown in parentheses.

Table 2

A list of murine proteins identified in immunoprecipitates^a of Myo18aα by LC-MS.

Functional Category (protein)	NCBI accession number ^b	MW (kDa)	Mascot score ^c	Peptide coverage (number of peptides) ^d
Motor/cytoskeletal/structural				
Myh9	NP071855	227	2668	24% (31)
Myosin polypeptide 4	NP034985	223	656	6% (9)
Myo18a	NP035716	232	3721	35% (50)
Tubulin α1c	NP033474	51	199	16% (4)
Actinin α3	NP038484	104	195	13% (5)
Ezrin/Radixin/Moesin ^e	NP033536/CAA43087/AAA39728	69	410	4% (3)
CD2AP	NP033977	71	249	14% (7)
Trafficking/vesicle formation				
Coatamer protein complex subunit α	NP034068	140	669	13% (10)
FK506-binding protein 4	NP034349	52	2064	36% (12)
Nucleolin	NP035010	77	3098	26% (15)

^aMyo18aα immunoprecipitates were prepared from unstimulated CH27 cell lysates.

^bAccession numbers of proteins in the NCBI's Reference Sequence database are shown.

^cMascot version 2.0 (Matrix Science) was used.

^dSequence coverage was determined by dividing the number of amino acids in a peptide by total number of amino acids, and the number of peptides detected for the identified protein is shown in parentheses.

^eThe sequences of the identified peptides (Supplemental Table 2) are completely conserved between ezrin, radixin, and moesin.