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THE LYMPHOCYTE-SPECIFIC PROTEIN LSP1 IS ASSOCIATED WITH THE CYTOSKELETON AND CO-CAPS WITH MEMBRANE IgM[1]

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LSP1 is a lymphocyte-specific intracellular Ca^{2+}-binding protein. We found previously that a fraction of the total cellular pool of LSP1 protein accumulates at or near the cytoplasmic face of the plasma membrane. LSP1 protein was also shown to be present in the cytoplasm. Here we report that approximately 10% of the total intracellular LSP1 protein is associated with the Nonidet P-40 insoluble cytoskeleton of the $mIgM^+$, $mIgD^+$ B lymphoma cell line BAL17. Variation in conditions of extraction did not alter this value. To rule out the possibility that LSP1 associates with the nucleus that is also present in the detergent insoluble pellet, we prepared a separate nuclear fraction essentially free of cytoskeletal material and found only trace amounts of LSP1 protein. After accounting for yield losses during subcellular fractionation by measuring the recovery of ^{125}I-labeled membrane IgM, or of the cytoplasmic marker enzyme lactate dehydrogenase activity, the LSP1 in membrane fractions was calculated to represent approximately 30% of the total cellular LSP1 and cytoplasmic LSP1 accounted for approximately 55% of the total. Approximately 75% of the plasma membrane LSP1 protein was soluble in 1% Nonidet P-40 containing buffer, indicating that the majority of the LSP1 in the plasma membrane fraction was distinct from the cytoskeletal LSP1 protein. The preparation of membrane fractions in the presence of 1 M NaCl, or washing of membranes in 3 M KCl did not diminish the levels of membrane LSP1. These results show the existence of three discrete intracellular LSP1 pools. Double label immunofluorescence studies showed that the peripheral ring-like distribution of LSP1 in BAL17 cells became a distinct cap upon cross-linking the mIgM. These intracellular LSP1 caps were always found to be located directly underneath the mIgM caps.

The LSP1 gene is expressed in a lymphocyte-specific manner (1, 2). Using an LSP1-specific polyclonal rabbit antiserum raised against recombinant mouse LSP1 protein, we showed that normal mouse T and B lymphocytes, as well as transformed B lymphoma lines express an LSP1 protein doublet with an apparent molecular mass of 52 and 50.5 kDa (2). Interestingly, all tested transformed mouse T lymphoma cell lines do not express the LSP1 gene. Closely related cell types such as granulocytes, as well as cells from liver, kidney, and heart tissue also do not express the LSP1 gene (1). Recently we have isolated a cDNA clone from a functional human T cell line that represents the human homologue of the mouse LSP1 gene (3). The human gene appears to have an expression pattern similar to its mouse counterpart. Comparison of the 330 amino acid mouse LSP1 protein with the 339 amino acid human LSP1 protein shows a high degree of identity between the two. The identity is most striking in the C-terminal halves of the LSP1 proteins, which are 85% identical, whereas the N-terminal halves are 53% identical. Both the human and mouse proteins have a high content of basic and acidic amino acid residues that appear clustered in the protein. We have proposed that the LSP1 proteins are divided into two regions, consisting of an N-terminal acidic domain and a C-terminal basic domain (3). The sequence of the mouse LSP1 protein predicts the presence of two EF-hand-like Ca^{2+}-binding sites within the acidic domain (1). Although the sequence for these two sites is not conserved in the human LSP1 protein, a single EF-hand-like Ca^{2+}-binding site might be present overlapping a stretch of amino acids unique to the acidic domain of the human LSP1 protein. This suggests a functional conservation between acidic domains of the mouse and human LSP1 proteins rather than a strict sequence conservation, which in turn suggests that the Ca^{2+}-binding property is important for the biologic function of LSP1 (3). Further studies showed that recombinant mouse LSP1 binds Ca^{2+} when incubated with 1 μM $^{45}CaCl_2$ (2). Inasmuch as the concentration of free intracellular Ca^{2+} varies between approximately 0.1 μM in resting lymphocytes and approximately 1 μM in activated lymphocytes, the binding of Ca^{2+} to rLSP1 suggests that the LSP1 protein in intact lymphocytes may respond to the transient increases in free intracellular Ca^{2+} which occur shortly after activation of B and T lymphocytes through their respective antigen receptors (4–10).

LSP1 is an intracellular protein. Subcellular fractionation and immunofluorescence studies have shown that part of the total cellular LSP1 protein accumulates at or near the inner side of the plasma membrane (2). This peripheral localization of LSP1 can be explained by an association of the protein with the inner side of the plasma membrane, with the underlying cytoskeleton or by a combination of both. In this paper we report that a significant portion of the total cellular LSP1 is associated

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with the detergent insoluble lymphocyte cytoskeleton. In addition, LSP1 is shown to associate with the plasma membrane and to be present in the cytoplasm. We also show that the intracellular LSP1 protein assembles in large caps after capping of the mIgM molecules by anti-μ treatment of B lymphoma cell lines. Furthermore these intracellular LSP1 caps are located directly underneath the extracellular mIgM caps.

These data on the intracellular localization and redistribution of the lymphocyte-specific LSP1 protein, together with its potential to respond to transient intracellular Ca^{2+} increases suggest that LSP1 may function as a component of a lymphocyte-specific signal transduction pathway, possibly the mIg signaling pathway.

MATERIALS AND METHODS

Cell cultures. The $mIgM^+$ $mIgD^+$ B cell lymphoma line BAL17 (11) was used for all studies. Cells were grown as previously reported (2).

Antibodies. The mouse LSP1-specific rabbit serum has been described previously (2). RAM μ-H chain-specific antiserum was kindly provided by Dr. C. Paige, Ontario Cancer Institute, Toronto, Ontario, Canada. The FITC-labeled $F(ab')_2$ GAM[3] μ-H chain-specific and the goat anti-rabbit Ig-specific sera were purchased from Cappel. TRITC-labeled GAM μ-specific antiserum was from Kirkegaard and Perry Laboratories Inc. Gaithersburg, MD.

Cell solubilization. Washed cells were resuspended at 2×10^7/ml in buffer 1 (0.02 M Tris-HCl, 0.14 M NaCl, 5 mM $MgCl_2$, pH 7.4) and diluted 1:1 in the same buffer containing the indicated amount of Nonidet P-40 (NP-40). Cells were incubated on ice with frequent resuspension for 10 min, then layered over buffer 1 containing 0.88 M sucrose and centrifuged at either $1300 \times g$ for 8 min at 4°C in an IEC Centra 8R centrifuge (International Equipment Co., Needham Heights, MA) with an IEC 216 rotor (low speed) or at $100,000 \times g$ for 60 min in a Beckman L5-50 ultracentrifuge (Beckman Instruments Inc., Fullerton, CA) with an SW 41 rotor (high speed). The NP-40 soluble supernatant was removed and one-half volume 3 × Laemmli buffer was added (12). The insoluble pellet was resuspended in Laemmli buffer and both samples were immersed in boiling water for 10 min.

Cross-linking of mIgM. Washed cells were resuspended at 5 to 7.5 $\times 10^7$/ml in HBSS minus Ca^{2+} and Mg^{2+}. RAM or $F(ab')_2$ FITC-GAM μ-specific antiserum was added to a final dilution of 1:50 and the cells were incubated at 37°C with frequent mixing for 30 min.

Preparation of nuclear fraction. Cross-linked cells were resuspended at 1×10^7/ml in buffer 1, and disrupted in a Parr cell disruption bomb under a pressure of 400 lb/in^2. The crude cell extract was layered over buffer 1 containing 0.88 M sucrose and centrifuged at $1300 \times g$ for 8 min as outlined above. The resulting pellet was treated with 0.5% NP-40 and centrifuged as described under cell solubilization. The resulting nuclear pellet (designated the N_{cav} fraction) was solubilized in Laemmli buffer and immersed in boiling water for 10 min.

Subcellular fractionation of cells. Cross-linked cells (1×10^7/ml) were disrupted at 500 lb/in^2 in a Parr disruption bomb and intact cells and nuclear material were centrifuged out as above. The resulting low-speed supernatant was centrifuged at $100,000 \times g$ (28,000 rpm in an SW 41 rotor) for 60 min. The high-speed centrifugation supernatant represents the cytoplasmic fraction as shown by the recovery of the cytoplasmic marker enzyme lactate dehydrogenase (2). The plasma membrane containing pellet (2) was solubilized in buffer 1 with 1% NP-40 for 10 min and centrifuged through buffer 1 containing 0.88 M sucrose at $100,000 \times g$ for 60 min.

Surface iodination of BAL17. The 2×10^7 cells (>95% viable, as judged by trypan blue exclusion) were resuspended in 1 ml of ice-cold PBS containing 20 mM glucose. To this was added 500 μCi ^{125}I (Amersham Corp., Arlington Heights, IL), 1.125 U lactoperoxidase, and 0.024 U glucose oxidase (both from Sigma Chemical Co., St. Louis, MO). Cells were gently mixed, then left on ice for 25 min, with resuspension of cells every 5 min. The reaction was stopped by a 15-fold dilution with cold PBS, and centrifuged at $180 \times g$ for 5 min in a Beckman GPR centrifuge with a GH 3.7 rotor. The pelleted cells were then washed three times in PBS to remove any free ^{125}I before use. To prepare iodinated membrane fractions, 1×10^7 iodinated non-cross-linked cells were mixed with 2×10^8 unlabeled mIgM cross-linked BAL17 cells. Cells were then fractionated as outlined above.

Immunoprecipitation of iodinated IgM. Aliquots of NP-40 solubilized membrane or total cell samples (1.25×10^7) were diluted to 1 ml with buffer 1 containing 1% NP-40. To each fraction, an excess of RAM anti-μ antiserum (1:40 final dilution) was added, and the tubes rotated for 2 h. A total of 50 μl of a slurry consisting of equal volumes of protein A-Sepharose CL-4B beads (Sigma) and buffer 1 + 1% NP-40 was then added, and the tubes mixed for another 45 min. Beads were harvested by centrifugation in a microfuge for 10 min. Immune precipitates were washed three times by addition of 1 ml RIPA buffer (50 mM Tris-HCl, pH 8.0, with 0.15 M NaCl, 1% NP-40, 0.5% deoxycholate, and 0.1% SDS) followed by centrifugation for 2 min in a microfuge. Beads were then subjected to a final wash in 1 ml Tris-buffered saline (20 mM Tris-HCl pH 8.0, with 0.14 M NaCl). Precipitated protein was recovered by addition of 150 μl of Laemmli sample buffer to the washed pellet, followed by immersion in boiling water for 10 min.

Immunofluorescence of capped and noncapped cells. The 2.5×10^5 BAL17 were incubated for 30 min with TRITC-labeled GAM μ-specific antiserum, either at 37°C (capping), or at 4°C in the presence of 0.1% sodium azide (noncapping). Cytospin preparations and permeabilization of the cells were done as described previously (2). Permeabilized cells were then incubated for 30 min with anti-LSP1 serum diluted in HBSS (1:40), followed by a wash in PBS. FITC-labeled goat anti-rabbit Ig serum was then used at a dilution of 1:40, followed by a final PBS wash.

RESULTS

LSP1 is associated with cytoskeleton. The cytoskeletal framework of a cell consists, in part, of a protein lamina that lies just beneath the plasma membrane (13, 14). A number of membrane proteins such as the FcR or mIg on B lymphocytes and the T lymphocyte TL Ag (CD1) are known to interact with this structure, although the nature of this association is not clear (14). Because of this interaction, the lamina may play a role in transducing or internalizing external signals, through the linkage of membrane receptors such as Ig to the underlying cytoskeleton. A portion of the total cellular pool of LSP1 protein is also localized at the periphery of the cell beneath the plasma membrane. This localization could be due to an association with the plasma membrane or with the cytoskeleton or both. To determine whether LSP1 is associated with the cytoskeleton we analyzed the detergent insoluble fraction of the $mIgM^+$, $mIgD^+$ B lymphoma cell line BAL17 for the presence of LSP1 protein. Figure 1 reveals that NP-40 solubilization of BAL17 cells and low speed centrifugation of the extract resulted in an insoluble pellet that contained a fraction of the total cellular LSP1. The amount of LSP1 in this pellet was quantitated by comparison of the LSP1 signal in increasing increments of the sample to the LSP1 signal in a total cell lysate on Western blots. Approximately 10% of the total cellular LSP1 was present in the NP-40 insoluble fraction (compare Fig. 1*a, lanes 1 to 2* to *lanes 3 to 5*). Analysis of three such experiments found an average of 11% of the total cellular LSP1 present in this fraction, with results ranging from 8 to 13%. Centrifugation of the NP-40 extract at a higher centrifugal force ($100,000 \times g$) gave similar results (not shown).

The detergent insoluble fraction of tissue culture cells contains the cell nuclei as well as the cytoskeleton (15, 16). To exclude the possibility that the LSP1 in the insoluble fraction is associated with the nucleus, we prepared a nuclear fraction essentially free of cytoskeletal material using a nitrogen cavitation protocol (the N_{cav} fraction). As the recovery of intact nuclei obtained with the nitrogen cavitation method may vary from the recovery obtained with the above described NP-40 lysis method, the N_{cav}

[3] Abbreviations used in this paper: mIg, membrane immunoglobulin; GAM, goat anti-mouse; NP-40, Nonidet P-40; RAM, rabbit anti-mouse; TRITC, tetramethylrhodamine isothiocyanate; PI, phosphoinositide.

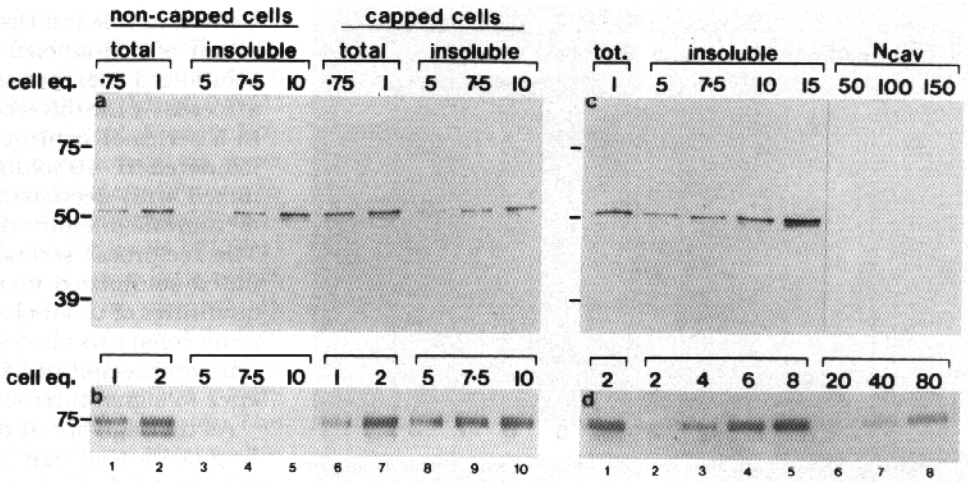

Figure 1. LSP1 is associated with the cytoskeleton. Cross-linked or noncross-linked cells were solubilized in buffer containing 0.5% NP-40. Electrophoresis and Western blotting of BAL17 nuclear and cytoskeletal samples was as described previously (2). Cell equivalence is expressed in units of 10^5 cells. *a* and *c* are Western blots probed with anti-LSP1 serum. *b* and *d* are Western blots probed with anti-mouse μ serum. The samples analyzed are described in the text. Numbers on the left of each panel correspond to the positions of the molecular mass markers in kDa (Bio-Rad low m.w. prestained SDS-PAGE standards).

fraction and the NP-40 insoluble pellet fraction were first analyzed by SDS-12.5% PAGE and the proteins present in these two fractions stained for 30 min with Coomassie brilliant blue. After destaining the gel overnight in a 10% methanol, 10% acetic acid solution, the samples were assessed for nuclear content, as judged by the levels of histones present. The concentration of each major histone species was approximately fourfold higher in the NP-40 insoluble pellet than the N_{cav} fraction, indicating the harsh effect of nitrogen cavitation on the nucleus (not shown). The amounts of LSP1 in each fraction were then quantitated by Western blot. As described above, the detergent-insoluble fraction contained 10% of the total cellular LSP1 (Fig. 1*c*, *lanes 1 to 5*). Less than 1% of the total cellular LSP1 was present in the N_{cav} sample, even after controlling for the diminished nuclear content in these samples (Fig. 1*c*, *lanes 6 to 8*).

A simple but reliable marker for detection of the cytoskeleton is provided by treatment of mIg^+ B cells with anti-Ig serum. This treatment causes an association of mIg with the cytoskeleton and this association remains intact during detergent extraction (16–18). To examine what effect such treatment might have on levels of LSP1 found associated with the cytoskeleton, cells were treated with either an anti-μ serum or an equal volume of HBSS, before NP-40 extraction. Subsequent analysis of the detergent insoluble fractions suggested that cross-linking mIgM had little effect on the amount of LSP1 associated with the cytoskeleton (see Fig. 1*a*, *lanes 3 to 5* vs *lanes 8 to 10*). In contrast, Figure 1*b* shows that approximately 25% of the mIgM from anti-μ-treated cells was associated in the insoluble fraction, as opposed to less than 10% in cells treated with HBSS (Fig. 1*b*, *lanes 3 to 5*, vs *lanes 8 to 10*). Analysis of five experiments found that an average of 10% of the total cellular LSP1 from anti-μ-treated cells was present in the insoluble fraction, with results ranging from 8 to 12%, whereas an average of 30% of the mIgM from these cells was insoluble, with results ranging from 20 to 40%. To better define the percentage of total mIgM present in the insoluble fraction of HBSS-treated cells, Western blots were repeated using a larger cell equivalence of the samples shown in Figure 1*b*, *lanes 3 to 5*. It was estimated that 1% of the total mIgM was present in these fractions (results not shown). Similar analysis of N_{cav} fractions from three experiments using crosslinked BAL17 cells showed the samples to contain from 1 to 4% of the total mIgM, suggesting that the N_{cav} fraction contains little cytoskeletal material (Figure 1*d*, *lanes 6 to 8*).

To determine whether the amount of LSP1 that associates with the detergent insoluble fraction varies with different preparation conditions, we prepared a detergent insoluble fraction using lysis buffer containing 0.5% or 1% NP-40. Both insoluble pellets contained the same amount of LSP1 protein. Similarly, substitution of NP-40 with 1% n-octyl β-D-glucopyranoside, Triton X-100, or digitonin had no effect on levels of LSP1 (results not shown). No effect was seen with minor variation in $MgCl_2$ (from 1.5 to 5.0 mM), pH (7.4 to 8.0), or time of solubilization (5 to 30 min).

These findings show that a well-defined portion (approximately 10%) of the total cellular LSP1 associates with the detergent insoluble fraction of BAL17 cells. The LSP1 protein is present in this fraction due to an association with the cytoskeleton and not due to an association with the cell nuclei that are also present in the detergent insoluble pellet.

Quantitation of membrane and cytoplasmic LSP1 pools. We reported that after subcellular fractionation, a significant portion of the total cellular LSP1 is found in membrane-containing samples. The presence of LSP1 in the cytoskeletal fraction as described above raises the question whether the presence of LSP1 protein in plasma membrane-containing fractions was due an association with the plasma membrane or with the cytoskeleton which is expected to copurify with it (19–21). To address this question, mIgM-cross-linked BAL17 cells were fractionated to obtain cytoplasmic and membrane samples. Quantitation of samples was done by comparing increasing increments to a total cell lysate. Based on an average of seven experiments, approximately 50% of the total cellular LSP1 was recovered in the cytoplasmic fraction. In Figure 2 the LSP1 signal of a total cell lysate prepared from 2×10^5 cells was intermediate to those signals generated by cytoplasmic samples of 2×10^5 and 4×10^5 cell equivalence (compare Fig. 2, *lane 2* with *lanes 3 and 4*), indicating that 50 to 100% of the cellular LSP1 was in this fraction. A similar range was found in one other fractionation. In all other experiments, the cytoplasmic fraction contained 25 to 50% of the total LSP1. As the recovery of cytoplasmic samples during fractionation may not be complete, the activity of the cytoplasmic

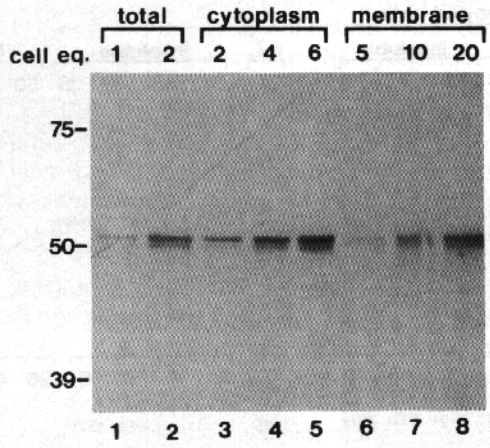

Figure 2. LSP1 associates with the high speed plasma membrane fraction and is present in the cytoplasm. The 1×10^7 iodinated non-cross-linked BAL17 cells were mixed with 2×10^8 unlabeled IgM cross-linked cells and then fractionated as outlined in the text. Electrophoresis and blotting of fractionation samples was as in Figure 1. Cell equivalence is expressed in units of 10^5. Position of the m.w. markers $\times 10^{-3}$ are shown on the *left*.

marker enzyme lactate dehydrogenase was used to estimate the yield of cytoplasmic material (22). Comparison of total lactate dehydrogenase activity in cytoplasmic samples to those in total cell lysates indicated an average recovery of about 90%. Thus after correcting for this recovery, cytoplasmic LSP1 was calculated to represent on average approximately 55% of the total cellular LSP1 pool, with a range of greater than 28% and less than 100%.

Similar quantitation of membrane fractions isolated from mIgM-cross-linked cells found that approximately 10% of the total LSP1 was present in the membrane-containing high speed pellet (Fig. 2, *lanes 6 to 8*). Two other experiments found 10 and 15% of the total LSP1 in this fraction. To calculate the recovery of plasma membrane fragments in the high speed pellet, BAL17 cells were surface labeled with ^{125}I using the lactoperoxidase-glucose oxidase method. A sample of iodinated cells (1×10^7 noncross-linked) was then mixed with 2×10^8 unlabelled cells (crosslinked) and a membrane fraction was prepared exactly as described above. After solubilization of the membrane fraction and of an equivalent number of total cells with NP-40, the ^{125}I-labeled mIgM molecules were immunoprecipitated from both fractions. From three experiments we determined that an average of approximately 40% of the total ^{125}I-labeled mIgM molecules could be recovered from the plasma membrane containing fraction. To ensure that the precipitated IgM was not the intracellular form of the protein (the lower band seen in Fig. 1*b*, *lanes 1* and *2*), we analysed immunoprecipitates resolved on SDS-10% PAGE by both autoradiography and Western blot. After aligning the two, the single band present in autoradiograms was estimated to have the same molecular mass of approximately 75 kDa as the upper band of the Western blot, consistent with the presence of mIgM H chain (results not shown). Thus we estimated that the recovery of plasma membranes in our membrane fraction was approximately 40% (not shown), suggesting that about 30% of the total cellular LSP1 pool is associated with the membrane-containing high speed pellet. To ensure that the determination of plasma membrane yield was valid we had to rule out that the efficiency of immunoprecipitation of ^{125}I-labeled mIgM molecules from the NP-40 solubilized membrane and total cell fractions was not affected by the different composition of the two fractions. In a series of control experiments constant amounts of iodinated NP-40 solubilized total cell lysate (2×10^6) were mixed with decreasing levels of nonlabeled solubilized membrane (membranes of 1×10^8, 1×10^7, or no cells). The reciprocal series, involving constant levels of iodinated solubilized membrane (5×10^6), and decreasing quantities of unlabeled total cell extract (1×10^7, 1×10^6, or no cells) was also done. Autoradiograms of the washed immunoprecipitates from each series revealed no difference in signal intensity.

As the high speed pellet may also contain cytoskeletal fragments that can contribute to the LSP1 present, we solubilized the pellet in NP-40. Such partitioning of LSP1 from the high speed pellet of capped cells into NP-40 soluble and insoluble fractions is seen in Figure 3. Approximately 75% of the LSP1 from this pellet was found to be NP-40 soluble (Fig. 3, *lanes 1* and *2*, vs *lanes 3* and *4*). From five experiments, an average of 75% of the LSP1 was NP-40 soluble, with a range between 50 and 100%. As the membrane-associated LSP1 pool will be present in the NP-40 soluble fraction, then 75% of the LSP1 present in the high speed pellet, or approximately 25% of the total cellular LSP1 is associated with the membrane. Between 12.5 to 25% of the LSP1 associated with the high speed pellet was recovered in the NP-40 insoluble pellet (*lane 1* vs *lanes 5* and *6*). In other experiments this value ranged from 10 to less than 40%, with an average of approximately 17%.

To determine if the treatment of BAL17 cells with μ-specific antiserum might alter the level of LSP1 present in membrane or cytoplasmic samples, four additional fractionations were performed using noncross-linked BAL17 cells. No difference in the range of LSP1 present was found for either sample (results not shown).

To determine if levels of LSP1 associated with the cytoplasmic and membrane fractions of the cell might be influenced by the ionic strength of our working buffers,

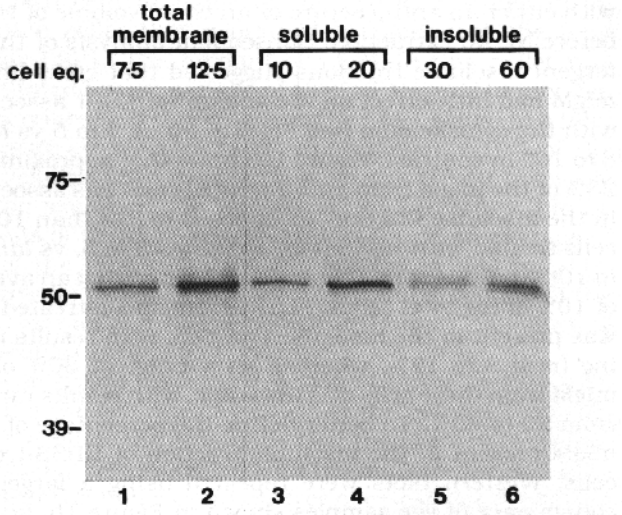

Figure 3. NP-40 solubilization of high speed total membrane pellets. High speed membrane samples prepared from IgM cross-linked BAL17 cells were resuspended and solubilized in buffer containing 1% NP-40. Samples were then recentrifuged at 100,000 × *g* as described in the text, to examine the partitioning of LSP1 between NP-40 soluble and insoluble fractions. Cell equivalence and m.w. markers were as in Figure 1.

we examined the effect of increasing concentrations of NaCl on the amount of membrane-associated LSP1 protein. Membrane fractions from noncross-linked cells were prepared in a buffer containing either 1.0 M, 0.5 M, 0.14 M, or no NaCl, using the protocol described in *Materials and Methods*. Samples from each fraction were first probed with an anti-μ serum by Western blotting to ensure the presence of equal loads of membrane. Standardized samples were then reanalysed for levels of LSP1. Increasing ionic strength was found to have little effect (results not shown). In a similar experiment, membranes were prepared from noncross-linked cells in a buffer devoid of salt (0.02 M Tris-HCl, pH 7.4, with 5 mM $MgCl_2$). Before high-speed centrifugation, the low-speed supernatant was divided into four equal (1×10^8) samples. The resulting membrane-containing pellets were then resuspended and incubated for 20 min in buffer containing either no salt, or 0.14 M, 1.0 M, or 3.0 M KCl. Membranes were then repelleted, and standardized for μ content as before. Figure 4 shows the quantitation of LSP1 in the different samples. LSP1 was found to remain constant over the range of KCl used (*lanes 1 to 4* and *5 to 8*).

The above results suggest that in addition to the cytoskeletal LSP1, which accounts for approximately 10% of the total cellular pool of LSP1, there are two additional intracellular pools of LSP1: a cytoplasmic pool that represents approximately 55% of the total cellular LSP1, and a membrane-associated LSP1 pool that represents approximately 25% of the total cellular LSP1 protein. Cross-linking of membrane IgM does not detectably alter the level of LSP1 in any pool, at least as seen within the time constraints of the fractionation.

LSP1 co-caps with mIgM. Indirect immunofluorescent staining of LSP1 in permeabilized cells results in a peripheral intracellular fluorescence pattern, as well as a diffuse staining throughout the cell. No staining is observed with intact cells (2). To explore the possibility that there might exist an interaction between the mIgM molecules and the intracellular LSP1 protein we determined whether the peripheral LSP1 staining pattern in BAL17 cells changed to a cap-like pattern after capping the mIgM molecules with anti-μ serum. Figure 5*a* shows the staining pattern for mIgM on BAL17 cells incubated under noncapping conditions. The staining is mostly diffuse with a faint peripheral staining. This peripheral staining is much more prominent when FITC-conjugated anti-μ reagents are used (not shown). The peripheral intracellular LSP1 staining is also clearly visible (Fig. 5*b*). Staining BAL17 cells with TRITC-conjugated anti-μ under capping conditions resulted in the formation of large caps on more than 95% of the cells (Fig. 5, *c* and *e*). Approximately 25% of the cells showed the presence of a clearly distinguishable intracellular LSP1 cap (Fig. 5, *d* and *f*). Similar values have been reported for the co-capping of mIg and myosin (14). Changing the anti-μ incubation time to 15 or 60 min did not alter this percentage. In all cases where we have examined the mIgM and the LSP1 cap in the

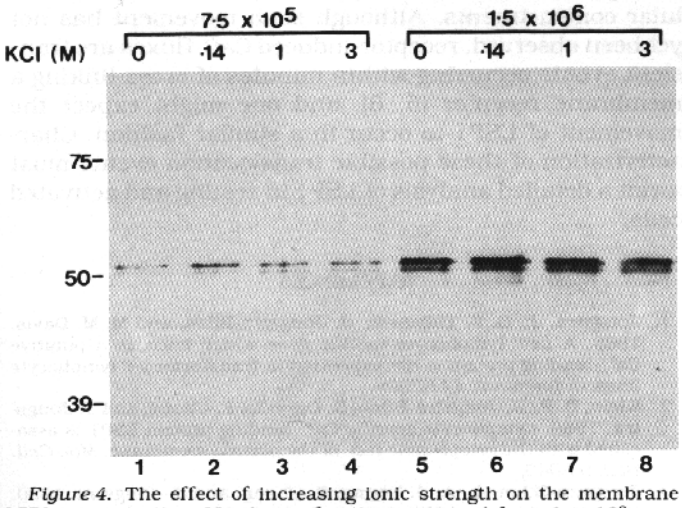

Figure 4. The effect of increasing ionic strength on the membrane LSP1 concentration. Membrane fractions prepared from 1×10^8 noncross-linked BAL17 cells were washed with a buffer containing the indicated concentrations of KCl. Cell equivalence and m.w. markers are as in Figure 1.

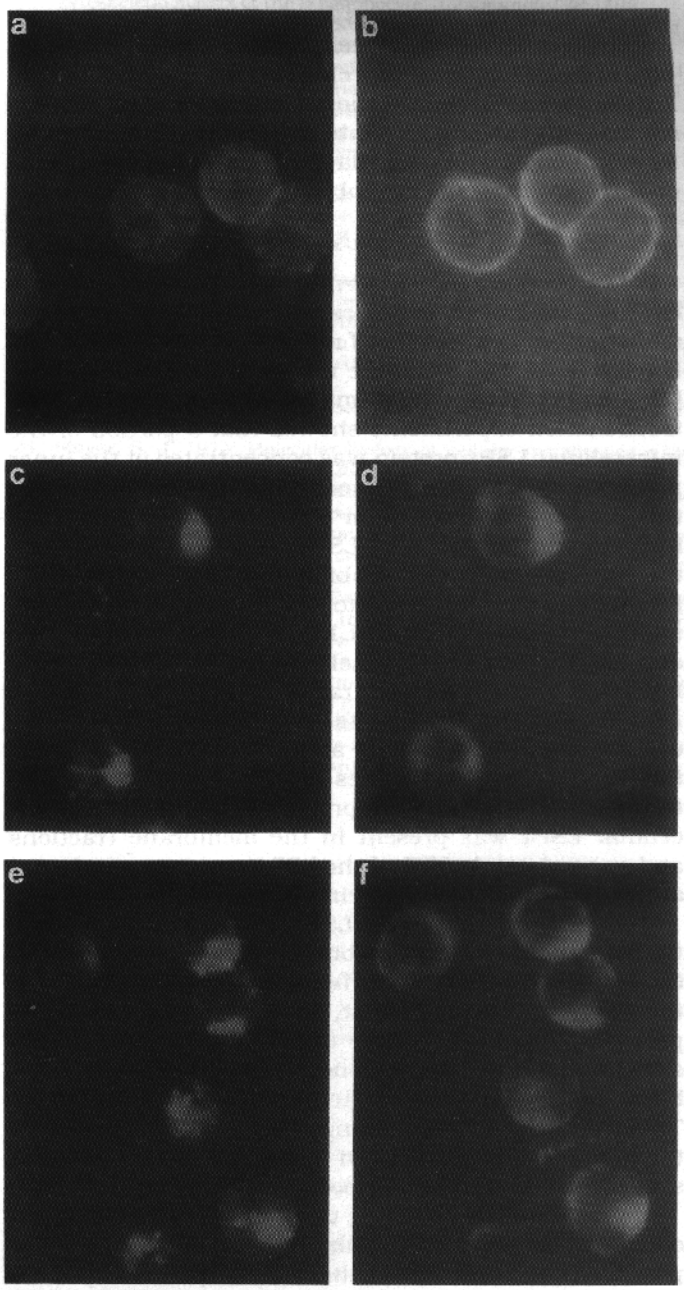

Figure 5. LSP1 co-caps with mIgM. BAL17 cells were stained with TRITC-GAM IgM serum under capping conditions (*c* to *f*) or non-capping conditions (*a* and *b*). Cytospin preparations were then prepared and stained for intracellular LSP1 protein as described in *Materials and Methods*. *a*, *c*, and *e*: mIgM-specific TRITC-fluorescence. *b*, *d*, and *f*: LSP1-specific FITC-fluorescence of the same cells shown in the *panels* directly to the *left*.

same cell the LSP1 cap was located directly underneath the mIgM cap. Few BAL17 cells treated with anti-μ under noncapping conditions had clear LSP1 caps, whereas no IgM caps were present in those cells. These experiments show that there exists an interaction between the transmembrane IgM molecule that serves as the Ag receptor on B cells and the intracellular LSP1 protein.

DISCUSSION

We have previously shown that the lymphocyte-specific protein LSP1 copurifies with both the membrane and cytoplasmic fractions of normal thymocytes, the pre-B cell line RAW112 and the mIgM$^+$ B cell line WEHI231 (2). Immunofluorescence microscopy and subcellular fractionation experiments showed that a portion of the intracellular LSP1 protein was concentrated at the inner periphery of the plasma membrane. We reasoned that this subcellular localization of the LSP1 protein could indicate an association of LSP1 with the membrane or the cytoskeleton, or a combination of both. To clarify this, we examined cytoskeleton fractions from the mIgM$^+$, mIgD$^+$ B lymphoma line BAL17 for the presence of LSP1 and found that approximately 10% of the total cellular LSP1 was present in this fraction. This percentage did not alter significantly despite variations in solubilization conditions, suggesting that a clearly defined portion of the cellular LSP1 associates with the cell cytoskeletal framework. In addition, approximately 30% of the total cellular LSP1 was present in the membrane fractions and approximately 55% of the LSP1 protein was present as soluble cytoplasmic protein.

We are confident that the LSP1 protein associated with the high-speed plasma membrane-containing sample and the cytoskeleton are two different intracellular pools for the following reasons. First, the membrane-associated pool is approximately three times as large as the cytoskeletal LSP1 pool, and second, the majority of the membrane-associated LSP1 protein was soluble in NP-40. This soluble LSP1, consisting of approximately 25% of the total cellular pool, would therefore represent a cytoskeleton-free membrane-associated pool.

Approximately 55% of the total cellular LSP1 was calculated to be present as soluble cytoplasmic protein. It is possible that a fraction of this LSP1 represents protein removed from other LSP1 pools during cellular fractionation. In particular, the presence of salts in working buffers may dissociate peripheral proteins from the membrane (23, 24). However, isolation of membrane fractions in 1 M NaCl or the incubation of membranes in up to 3 M KCl had little effect on membrane LSP1, indicating that the LSP1 protein is tightly bound to the membrane. Based on the hydrophilic nature of the protein, and the lack of any recognizable trans-membrane regions (1) we do not believe that LSP1 is an integral membrane protein. Thus the tight binding of LSP1 to the membrane may indicate an as yet unknown posttranslational modification of the protein. Although the ineffectiveness of high salt concentrations in dissociating LSP1 from the membrane implies that LSP1 was not removed by physiologic salt concentrations present during sample presentation, it does not preclude dissolution of portions of the membrane by other forces during the nitrogen cavitation rupturing protocol. However, in such an event one would also expect the presence of mIgM in the cytoplasmic samples, which was not the case.

The addition of anti-Ig serum to resting lymphocytes results in the occurrence of a number of biochemical events, including a rapid activation of the PI cycle (8, 25, 26), release of Ca^{2+} from intracellular stores (8, 9), and the translocation and activation of protein kinase C (26, 27). Structural alterations that follow the cross-linking of mIg on B cells include the attachment of a portion of the mIg molecules to the cytoskeleton and a redistribution of mIg to form a distinct cap (14). Several lines of evidence indicate that a cytoskeletal rearrangement concurrent with mIg capping plays a role in mIg signaling. First, the rearrangement of the cytoskeleton and the activation of the PI cycle are dependent on receptor cross-linking, which occurs only in the presence of bivalent anti-Ig reagents (8, 14, 16, 26). Second, using a series of monoclonal anti-IgD antibodies, Goroff et al. (28) showed that only those antibodies that were effective in cross-linking mIgD were mitogenic for normal B cells.

We have postulated previously that the LSP1 protein is part of a lymphocyte-specific signal transduction pathway. This was based on the lymphocyte-specific expression of LSP1, the high degree of conservation between the mouse and human proteins, the subcellular localization of a portion of the total cellular LSP1 along the cytoplasmic side of the plasma membrane and the prediction that the LSP1 protein may respond to the transient increases in intracellular Ca^{2+} which occur in B and T lymphocytes after stimulation of their respective Ag receptors. Our present findings that a defined portion of the intracellular LSP1 protein is associated with the cytoskeleton and co-caps with mIgM molecules after treatment of B cells with anti-μ serum further supports this hypothesis and suggests that LSP1 is part of the mIg signaling pathway in B cells. Whether the LSP1 protein is involved in the biochemical or structural events after anti-μ stimulation is at present unknown. It is possible to imagine that the LSP1 protein plays a part in the regulation of mIgM capping or in cytoskeletal rearrangements. Alternatively the LSP1 protein could serve as an intermediary effector molecule, responding to transient fluxes in intracellular Ca^{2+} brought on by activation of the PI cycle. Such a role for LSP1 suggests a movement or migration of the protein between the various subcellular compartments. Although such movement has not yet been observed, receptor-induced Ca^{2+} fluxes are transient events occurring within minutes of cross-linking a membrane receptor (5, 6), and one might expect the movement of LSP1 to occur in a similar fashion. Characterization of these possible translocation events must await a detailed analysis of LSP1 in resting and activated cells.

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