Leukocyte-specific protein 1 targets the ERK/MAP kinase scaffold protein KSR and MEK1 and ERK2 to the actin cytoskeleton

Rene E. Harrison[1], Barbara A. Sikorski[2] and Jan Jongstra[2,*]

[1]Cell Biology Programme, The Hospital for Sick Children Research Institute, Toronto, Ontario, M5G 1X8, Canada
[2]Toronto Western Research Institute, Cell and Molecular Biology Division, University Health Network, and Department of Immunology, University of Toronto, Toronto, Ontario, M5T 2S8, Canada
Author for correspondence (e-mail: jongstra@uhnres.utoronto.ca)

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Summary

The identification and characterization of scaffold and targeting proteins of the ERK/MAP kinase pathway is important to understand the function and intracellular organization of this pathway. The F-actin binding protein leukocyte-specific protein 1 (LSP1) binds to PKCβI and expression of B-LSP1, an LSP1 truncate containing the PKCβI binding residues, inhibits anti-IgM-induced translocation of PKCβI to the plasma membrane and anti-IgM-induced activation of ERK2. To understand the role of LSP1 in the regulation of PKCβI-dependent ERK2 activation, we investigated whether LSP1 interacts with ERK/MAP kinase pathway components and targets these proteins to the actin cytoskeleton. We show that LSP1 associates with the ERK scaffold protein KSR and with MEK1 and ERK2. LSP1-associated MEK1 is activated by anti-IgM treatment and this activation is inhibited by expression of B-LSP1, suggesting that the activation of LSP1-associated MEK1 is PKCβI dependent. Confocal microscopy showed that LSP1 targets KSR, MEK1 and ERK2 to peripheral actin filaments. Thus our data show that LSP1 is a cytoskeletal targeting protein for the ERK/MAP kinase pathway and support a model in which MEK1 and ERK2 are organized in a cytoskeletal signaling complex together with KSR, PKCβI and LSP1 and are activated by anti-IgM in a PKCβI-dependent manner.

Key words: LSP1, Targeting, Actin cytoskeleton, ERK2, MAP kinase, B-lymphoma

Introduction

The ERK/MAP kinase pathway transmits signals generated at the plasma membrane to a large number of cytoplasmic and nuclear substrates involved in proliferation, differentiation and cell survival. Targeting ERK to appropriate intracellular locations is one way to ensure the correct response of the ERK pathway to different extracellular stimuli. Part of activated ERK translocates to the nucleus to phosphorylate and activate transcription factors (Brunet et al., 1999), while other pools of activated ERK phosphorylate a number of cytoplasmic targets (Pearson et al., 2001). The study of multifunctional enzymes such as PKC and PKA have identified a large number of proteins that target these enzymes to specific cytoplasmic locations (Faux and Scott, 1996; Michel and Scott, 2002; Mochly-Rosen, 1995). However, few targeting proteins for the ERK pathway have been identified. The ERK scaffold protein KSR (kinase suppressor of Ras) binds ERK and its direct activator MEK (Denouel-Galy et al., 1997; Roy et al., 2002; Stewart et al., 1999; Yu et al., 1997). In unstimulated cells KSR is mainly cytoplasmic but translocates to the plasma membrane after growth factor stimulation, thus targeting MEK and ERK to the plasma membrane (Michaud et al., 1997; Muller et al., 2001; Stewart et al., 1999; Xing et al., 1997). A second targeting protein, p14, targets ERK2 to an endosomal location (Teis et al., 2002; Wunderlich et al., 2001) through its interaction with MP-1, an adaptor protein that binds MEK and ERK (Schaeffer et al., 1998). However, it is not clear how ERK is targeted to other cytoplasmic regions, including the cytoskeleton.

The MAP kinases ERK1 and ERK2 are phosphorylated and activated by the MAP kinase kinases MEK1 and MEK2, that are in turn phosphorylated and activated by ERK/MAP kinase kinase kinases such as members of the Raf family (Pearson et al., 2001). The ERK/MAP kinase pathway is activated by RAS-dependent and PKC-dependent mechanisms and these two mechanisms converge at the level of Raf-1. The mechanism of PKC-dependent activation of Raf-1 is not completely understood and is thought to involve PKC-dependent activation of Ras and the formation of Ras/Raf complexes, but evidence for a direct activation of Raf-1 by activated PKC has also been described (Schonwasser et al., 1998; Soh et al., 1999; Marais et al., 1998). Leukocyte-specific protein 1 (LSP1) is an F-actin-binding cytoskeletal protein (Jongstra-Bilen et al., 1992) that is expressed in lymphoid and myeloid cells (Jongstra et al., 1994; Li et al., 1995; Marafioti et al., 2003; Pulford et al., 1999). LSP1 binds directly to PKCβI through LSP1 residues 179-330. Expression of B-LSP1, an LSP1 truncate, consisting of residues 179-330, inhibits anti-IgM-induced translocation of PKCβI to the plasma membrane, which leads to inhibition of anti-IgM-induced activation of ERK2 and to increased anti-IgM-induced apoptosis (Cao et al., 2001). The inhibition of PKCβI-dependent ERK2 activation by

expression of B-LSP1 or by the PKC inhibitor Gö6976 can be measured 10 and 20 minutes, but not 2 minutes, after anti-IgM stimulation, which shows that the time course of PKCβI-dependent ERK2 activation is slower than the time course of the PKCβI-independent activation of ERK2 (Cao et al., 2001). This suggested to us that the PKCβI-dependent ERK2 may constitute a special pool of ERK2 that is organized in a signaling complex containing PKCβI and other components of the ERK/MAP kinase pathway such as MEK1 and ERK2. Since LSP1 binds to PKCβI, LSP1 could also be part of this signaling complex. Importantly, through its F-actin binding site, LSP1 could target this complex to actin filaments. We identify LSP1 as a targeting protein for the ERK/MAP kinase pathway that targets the ERK scaffold protein KSR and the ERK/MAP kinase pathway components MEK1 and ERK2 to peripheral actin filaments.

Materials and Methods

Cell lines and reagents

The mIgM$^+$ W10 cell line is a single cell subclone derived from the B-lymphoma cell line WEHI-231 (Jongstra-Bilen et al., 1999). The TW10.1 cell line is a stable G418 resistant transfectant derived from W10 cells and expresses the LSP1 truncate B-LSP1 containing LSP1 residues 179-330 (Jongstra-Bilen et al., 1999). The LSP1^{neg} T-lymphoma cell lines BW5147 and T22, an LSP1$^+$ transfectant derived from BW5147 cells have been described previously (Jongstra-Bilen et al., 1992). Cells were grown in RPMI-1640 medium as described previously (Jongstra-Bilen et al., 1992; Jongstra-Bilen et al., 1999). Goat anti-mouse IgM was purchased from Sigma-Aldrich (Oakville, ON, Canada) and used at 10 μg/ml. Goat anti-PKCβI, goat anti-MEK1, goat anti-ERK2, goat anti-KSR and goat anti-caspase 1 were from SantaCruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-LSP1 serum was prepared as described previously (Klein et al., 1989). The MEK1 inhibitor U0126 (Favata et al., 1998) was purchased from Calbiochem (La Jolla, CA), was dissolved in DMSO and added 20 minutes before addition of anti-IgM. GST and GST/LSP1 fusion proteins were expressed using the pGEX-4T2 vector (Amersham BioSciences, Oakville, ON, Canada). The plasmid encoding GST-MEK1 was a gift from Dr K. L. Guan (University of Michigan, Ann Arbor, MI, USA). The GST-ERK2 plasmid was a gift from Dr L. A. Huber (Institute for Molecular Pathology, Vienna, Austria). GST proteins were purified from E. coli BL21 (DE3) pLysS using glutathione-Sepharose beads (Sigma-Aldrich) according to the manufacturer's instructions.

Confocal microscopy

Primary antibodies for ERK2, MEK1 and KSR were from SantaCruz Biotechnology, Inc. Cy3 and Cy5-conjugated donkey anti-goat, anti-rabbit and anti-mouse IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA). Alexa Fluor 488-phalloidin was from Molecular Probes, Inc. (Eugene, OR). Poly-L-lysine was from Sigma-Aldrich. Cells were washed twice in cold medium (DME) and then 5×10^6 cells were spun at 400 ***g*** for 5 minutes onto poly-L-lysine-coated coverslips (100 μg/ml). Cells were fixed with 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, 100 mM glycine in PBS for 20 minutes and blocked for 1 hour in PBS with 5% fetal calf serum (PBS/FCS). The cells were stained with primary antibodies diluted in PBS/FCS directed to either LSP1 (1:150), ERK2 (1:50), MEK1 (1:50) or KSR (1:50). F-actin was visualized using Alexa Fluor 488-phalloidin diluted 1:500 in PBS/FCS. Cells were then washed with PBS and incubated with appropriate fluorescent secondary antibody combinations (1:1000) for 1 hour. Samples were analyzed by confocal microscopy using a Zeiss LSM 510 laser scanning confocal microscope with a 100× oil immersion objective. Images of single mid-cell volume 0.5 μm sections were collected using lasers of 543 nm for Cy3, 488 nm for Alexa 488 phalloidin and 633 nm for Cy5 using the conventional filter sets. Digital images were prepared using Adobe Photoshop® 6.0 (Adobe Systems Inc., San Jose, CA).

Immunoprecipitation and pull down assays

Cells (40×10^6) were washed twice in cold HBSS and then lysed in 500 μl cold CHAPS lysis buffer (50 mM Tris pH 7.5, 5 mM EDTA, 0.5% CHAPS) containing a standard mix of protease and phosphatase inhibitors (Jongstra-Bilen et al., 1992) on ice for 15 minutes. The lysed cells were centrifuged at 13,000 rpm for 15 minutes at 4°C to remove insoluble material. The lysates were transferred to a new microfuge tube and centrifuged again for 15 minutes at 11,500 ***g***. After transfer to new microfuge tubes 10 μl of anti-PKCβI, MEK1, ERK2 or KSR antibody or 10 μl affinity purified anti-LSP1 antibody were added and the soluble lysates were incubated for 1.5 hours at 4°C with complete rotation, then centrifuged twice at 4°C for 15 minutes at 11,500 ***g***. Immune complexes were recovered from the supernatant by incubation with 10 μl of a 1:1 slurry of protein G-agarose beads (Pierce Biotechnology Inc., Rockfort, IL) for 45 minutes at 4°C with complete rotation. Beads were then washed five times in 1 ml of lysis buffer plus inhibitors, and 25 μl 2× Laemmli sample buffer was added to the beads followed by immersion in boiling water for 3 minutes. For pull down experiments 40×10^6 cells were washed and lysed in 0.8 ml of lysis buffer plus inhibitors and cleared from insoluble material as above. After addition of ~2 μg of GST, GST-ERK2 or GST-MEK1 protein the soluble lysates were incubated for 1.5 hours at 4°C with complete rotation followed by centrifugation at 4°C for 15 minutes at 11,500 ***g***. GST and GST fusion proteins were then recovered and washed as above except that 10 μl of a 1:1 slurry of glutathione-Sepharose beads were used.

MEK1 and ERK2 activity measurements

Both enzyme activities were measured in immune kinase assays exactly as described for the measurement of ERK2 activity (Cao et al., 2001). His-tagged *Xenopus* ERK2 or a peptide containing the MEK phosphorylation sites of human ERK1 (both from SantaCruz Biotechnology Inc.) were used as substrates in the MEK1 activity assays. Myelin basic protein (Sigma-Aldrich) or a peptide derived from the cytoplasmic tail of the EGF receptor (SantaCruz Biotechnology Inc.) were used to measure ERK2 activity. After 15 minutes incubation of the immunoprecipitates at 30°C in the presence of [γ-^{32}P]ATP, peptide substrates were absorbed on Whatman P11 phosphocellulose paper and washed three times in 0.01% phosphoric acid. After drying the filter, incorporation of ^{32}P was analyzed using a personal FX PhosphoImager (Bio-Rad laboratories, Oakville, ON, Canada). Protein substrates were analyzed by SDS-PAGE and the incorporation of ^{32}P was determined by phosphoimaging of the dried gel.

Results

LSP1 targets KSR, MEK1 and ERK2 to actin filaments

To determine whether LSP1 interacts with components of the ERK/MAP kinase pathway, we analyzed anti-MEK1 and anti-ERK2 immunoprecipitates for the presence of LSP1 (Fig. 1). Anti-PKCβI immunoprecipitates were used as positive controls since previous results have shown a direct interaction between LSP1 and PKCβI (Cao et al., 2001). W10 cells were lysed in 0.5% CHAPS and soluble lysates were used to prepare immunoprecipitates. Western blot analyses with anti-LSP1 antiserum show that LSP1 is present in anti-PKCβI, anti-

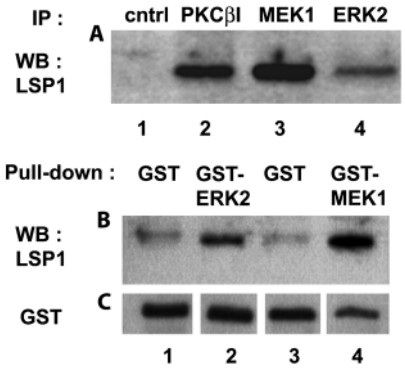

Fig. 1. LSP1 interacts with ERK/MAP kinase pathway components. (A) W10 cells were lysed in 50 mM Tris pH 7.5, 5 mM EDTA, 0.5% CHAPS, and PKCβI, MEK1 and ERK2 were immunoprecipitated and analyzed for the presence of LSP1 by western blotting. No antibodies were added to control assays (cntrl). (B) Pull down experiments were performed using GST, GST-ERK2 or GST-MEK1 recombinant proteins and CHAPS-soluble W10 cell lysates and analyzed for the presence of LSP1. (C) To check recovery of GST and GST fusion proteins the same western blot shown in B was reprobed with an anti-GST antibody. All samples were analyzed on one gel but only the relevant portion of each lane containing the recovered GST or GST fusion protein is shown.

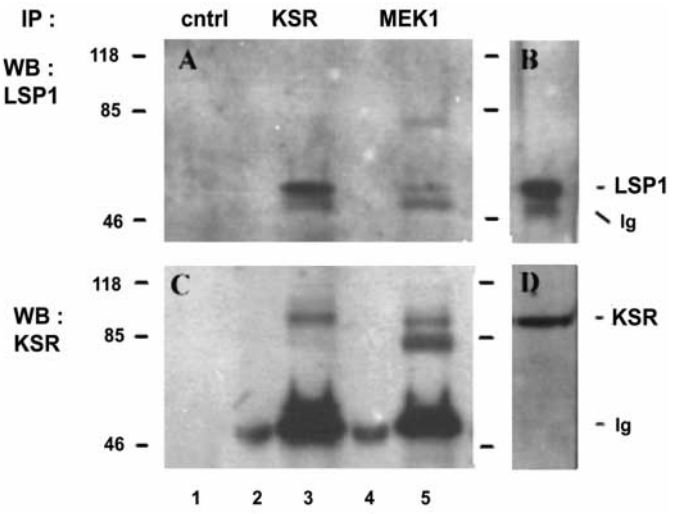

Fig. 2. LSP1 associates with the ERK scaffold KSR. Immunoprecipitates of W10 cell lysates were prepared using anti-KSR (lane 3) or anti-MEK1 antibodies (lane 5). No antibodies were added to the control assay (lane 1). Precipitated proteins were first analyzed by western blotting for the presence of LSP1 (A) and subsequently for the presence of KSR (C). The bands visible in lanes 2 and 4 of A represent spill-over from the anti-KSR immunoprecipitate. The position of LSP1 in the anti-KSR and anti-MEK1 precipitates was identified using an anti-LSP1 immunoprecipitate (B). The position of KSR was identified using a whole cell extract of W10 cells (D). KSR, LSP1 and precipitating IgG are indicated at the right. Molecular mass markers (in kDa) are indicated at the left.

MEK1 and anti-ERK2 immunoprecipitates (Fig. 1A, lanes 2-4). No LSP1 is present in mock precipitations in which no antibodies were used (lane 1) or in precipitates using an antibody with an irrelevant specificity (anti-caspase-1, not shown). We also performed GST pull down assays in which GST-MEK1 and GST-ERK2 were incubated with CHAPS soluble W10 cell lysates and the GST fusion proteins were recovered on glutathione-Sepharose beads. Western blotting of the recovered proteins showed that LSP1 interacts with GST-ERK2 and GST-MEK1 (Fig. 1B, lanes 2 and 4). No LSP1 was detected when GST protein was used as a negative control (lanes 1 and 3). The protein in lanes 1 and 3 that runs just above the level of LSP1 is a contaminating bacterial protein recognized by anti-LSP1 serum since it is also present when the assay is performed with GST in lysis buffer without addition of cell lysate (not shown). Fig. 1C show the relevant portions of each lane of the filter shown in Fig. 1B after reprobing with an antibody that recognizes GST, to visualize the recovered GST and GST-fusion proteins. These immunoprecipitation and GST-pull down assays show that LSP1 interacts not only with PKCβI, but also with MEK1 and ERK2.

To determine whether LSP1 binds directly to MEK1 and ERK2 we performed in vitro binding assays using GST-tagged LSP1 and HIS-tagged MEK1 or ERK2 recombinant proteins. However, we found no evidence for direct interactions between LSP1 and MEK1 or ERK2 in these experiments (not shown). We then hypothesized that the LSP1 interactions with MEK1 and ERK2 are indirect and involve an intermediary protein, possibly an ERK/MAP kinase pathway scaffold protein. To determine whether LSP1 interacts with the ERK scaffold protein KSR, we analyzed anti-KSR immunoprecipitates from CHAPS-soluble W10 cell lysates. These assays showed that LSP1 is present in anti-KSR precipitates (Fig. 2A, lane 3). Anti-MEK1 immunoprecipitates were used as positive controls (lane 5), and an anti-LSP1 immunoprecipitate (Fig. 2B) was used to identify the position of LSP1 and the precipitating IgG in the anti-KSR immunoprecipitate. Reprobing the filter with anti-KSR antibodies shows the presence of KSR in the anti-KSR and anti-MEK1 immunoprecipitates (Fig. 2C). A total lysate of W10 cells was used to identify the position of KSR in the immunoprecipitates (Fig. 2D). No LSP1 or KSR were detectable in the negative control samples from which the anti-KSR or anti-MEK1 antibodies were omitted (lanes 1 in Fig. 2A,C). The identity of the ~ 80 kDa protein present in the anti-MEK1 immunoprecipitates is unknown. These results show that LSP1 interacts with KSR, and confirms, what has been described in other cell types, namely that in W10 cells KSR interacts with MEK1. A small amount of KSR was also present in anti-ERK2 immunoprecipitates but the observed signal was very weak and variable and hence its significance is unknown.

LSP1 is an F-actin binding protein that accumulates on the peripheral actin filaments underneath the plasma membrane, often referred to as the membrane skeleton or cortical cytoskeleton (Jongstra-Bilen et al., 1992; Klein et al., 1990; Klein et al., 1989). To test the hypothesis that LSP1 targets KSR, MEK1 and ERK2 to the membrane skeleton, we used confocal microscopy to localize KSR, MEK1 and ERK2 in the $LSP1^{neg}$ T-lymphoma cell line BW5147 and an $LSP1^{+}$ transfectant of BW5147, designated T22. The main panels in Fig. 3 show the merged images of cells in which both actin filaments (in green) and either LSP1, KSR, MEK1 or ERK2 (in red) were visualized as indicated at the top of the panels. Co-localization of actin filaments with LSP1, KSR, MEK1 or

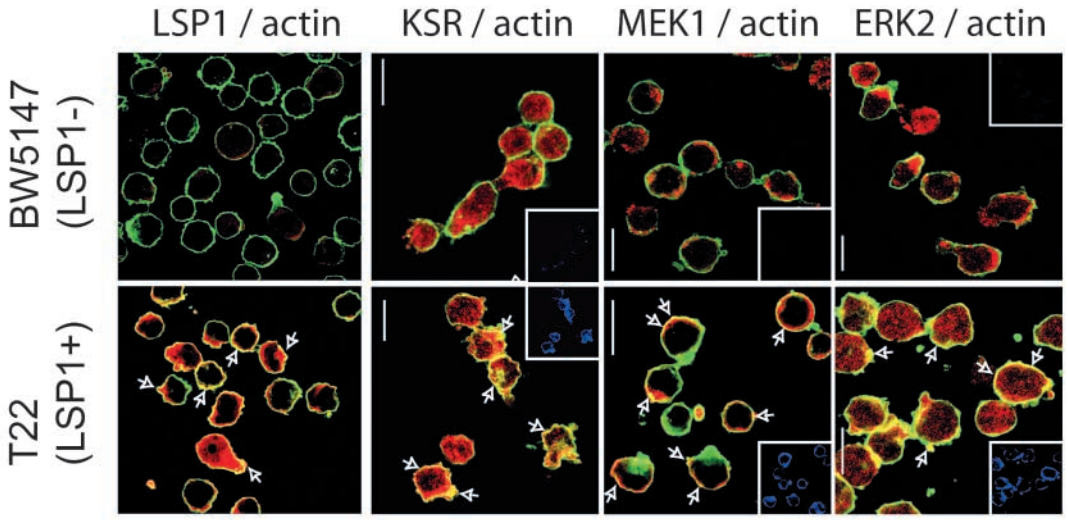

Fig. 3. LSP1 targets KSR, MEK1 and ERK2 to peripheral actin filaments. The LSP1^{neg} BW5147 cells (upper panels) and the LSP1^{+} transfectant T22 (lower panels) were stained for LSP1, KSR, MEK1, ERK2 and F-actin. Merged images show that LSP1, KSR, MEK1 or ERK2 (red) co-localize with F-actin (green) in the presence of LSP1 (lower panels) but not in the absence of LSP1 (upper panels). Some randomly chosen areas of co-localization indicated by a yellow color are identified by arrows. The insets show staining for LSP1 (blue). Scale bar: 10 μm.

ERK2 results in a yellow color. The upper panels show that, as expected, the parental BW5147 cells do not express LSP1 (Jongstra-Bilen et al., 1992). Little or no KSR or MEK1 or ERK2 co-localizes with the peripheral actin filaments in BW5147 cells as shown by a lack of yellow colored areas. In contrast, the bottom panels show that there is easily detectable co-localization of LSP1 or KSR or MEK1 or ERK2 with F-actin in the LSP1^{+} transfectant T22. Several randomly chosen examples of co-localization are indicated with arrows. The insets show staining of BW5147 and T22 cells with LSP1 (in blue) as a control for the appropriate expression of LSP1 in the cells used for each experiment. These data show that the expression of LSP1 leads to co-localization of KSR, MEK1 and ERK2 with peripheral actin filaments and are strong evidence that LSP1 functions as a targeting protein diverting KSR, MEK1 and ERK2 to the membrane skeleton.

LSP1-associated MEK1 is activated by anti-IgM.

The immunoprecipitation, GST pull down and confocal experiments described above show that LSP1 interacts with PKCβI, KSR, MEK1 and ERK2. To determine whether the LSP1-associated MEK1 and ERK2 are activated by anti-IgM treatment we measured MEK1 and ERK2 activity in anti-LSP1 immunoprecipitates. Since LSP1 binds to PKCβI we also determined whether the activation of LSP1-associated MEK1 and ERK2 is dependent on PKCβI. Two predictions derive from a model in which PKCβI is the upstream activator of LSP1-associated MEK1 and ERK2. First, since the anti-IgM-induced, PKCβI-dependent ERK2 activation is slower than the PKCβI-independent activation of ERK2 (Cao et al., 2001), stimulation of W10 cells with anti-IgM should activate LSP1-associated MEK1 and ERK2 with slower kinetics than that of the corresponding enzymes that are not associated with LSP1. Second, the LSP1-associated MEK1 or ERK2 should be activated in anti-IgM-treated W10 cells but not in anti-IgM treated TW10.1 cells, a stable transfectant derived from W10 cells in which activation of PKCβI is inhibited by expression of the B-LSP1 truncate (Cao et al., 2001). To test these predictions we determined the activity of LSP1-associated MEK1 in anti-LSP1 immunoprecipitates and of MEK1 in anti-MEK1 immunoprecipitates (Fig. 4). The latter samples include MEK1 that is not associated with LSP1. To ensure that the enzyme activity detected in anti-LSP1 immunoprecipitates was due to activated MEK1, we used a MEK-specific peptide substrate containing the MEK phosphorylation site of human ERK1 (Fig. 4A,B) or recombinant His-tagged ERK2 (Fig. 4E,F). In addition, we determined whether the observed kinase activity was inhibited by pre-treatment of W10 cells with the MEK-specific inhibitor U0126. MEK1 is activated by anti-IgM treatment of W10 cells and MEK1 activity in anti-MEK1 immunoprecipitates is maximal 5 minutes after addition of anti-IgM and declines thereafter (Fig. 4A). Interestingly, the activation kinetic of LSP1-associated MEK1 is significantly slower, with maximal stimulation 10 minutes after addition of anti-IgM (Fig. 4B). LSP1-associated kinase activity towards the ERK1-derived peptide substrate is inhibited by pre-treatment of the W10 cells with 10 μM U0126 (Fig. 4B), showing that the kinase activity detected in anti-LSP1 immunoprecipitates from anti-IgM-stimulated cells is indeed due to the presence of active MEK1. Furthermore, as predicted, we did not detect any anti-IgM-induced MEK1 activation in anti-LSP1 immunoprecipitates from TW10.1 cells (Fig. 4B). The different kinetics of MEK1 activation in anti-MEK1 or anti-LSP1 immunoprecipitates were confirmed using recombinant His-ERK2 as a substrate (Fig. 4E,F).

The slow activation of LSP1-associated MEK1 in anti-IgM-induced W10 cells and the absence of activation in TW10.1 cells shown here, suggest that the LSP1-associated MEK1 is involved in the slow PKCβI-dependent anti-IgM-induced activation of ERK2 in W10 cells (Cao et al., 2001). However, we could not demonstrate significant ERK2 activity in LSP1

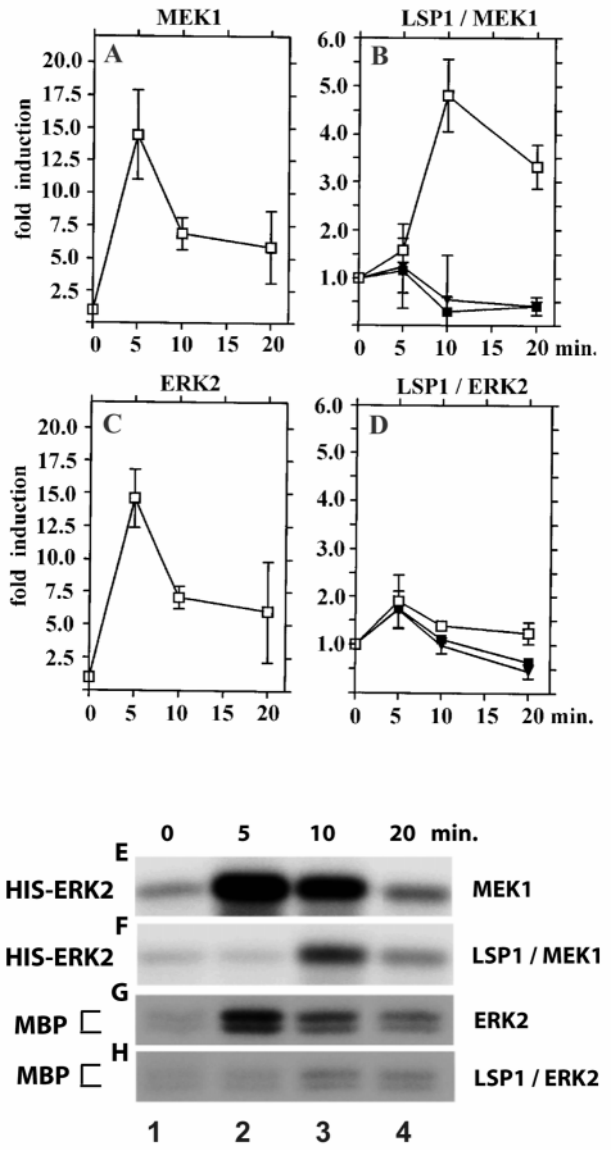

Fig. 4. LSP1-associated MEK1 is activated by anti-IgM. Anti-MEK1 (A) or anti-LSP1 (B) immunoprecipitates were analyzed for kinase activity using a peptide containing the MEK phosphorylation site of human ERK1 as substrate. Similarly, anti-ERK2 (C) or anti-LSP1 (D) immunoprecipitates were analyzed for kinase activity using an EGF-derived peptide. Precipitates were prepared from CHAPS-soluble lysates from unstimulated cells or from cells stimulated for 5, 10 or 20 minutes with 50 μg/ml anti-IgM (□, W10; ■, TW10.1; ▼, W10 cells pretreated for 20 minutes with 10 μM U0126). Results are expressed as the average fold increase over unstimulated cells±s.e.m. of three experiments. Anti-MEK1 (E) and anti-LSP1 (F) immunoprecipitates were assayed for MEK1 activity using recombinant His-ERK2 as substrate. Anti-ERK2 (G) and anti-LSP1 (H) were assayed for ERK2 activity using myelin basic protein (MBP) as a substrate.

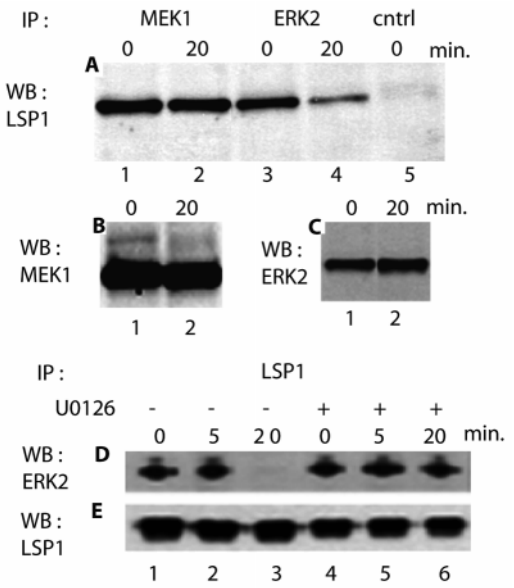

Fig. 5. ERK2 dissociates from the LSP1 complex after anti-IgM stimulation. (A) Anti-MEK1 and anti-ERK2 immunoprecipitates from W10 cells (unstimulated or stimulated for 20 minutes with 50 μg/ml anti-IgM) were analyzed for the presence of LSP1. No antibodies were added to control assays using unstimulated cells (cntrl). Equal recovery of MEK1 or ERK2 in unstimulated and stimulated samples was confirmed by western blot analysis using anti-MEK1 (B) or anti-ERK2 (C) antibodies. (D) Anti-LSP1 immunoprecipitates from unstimulated or anti-IgM-stimulated cells were analyzed for the presence of ERK2 without (lanes1-3) or with pretreatment of the cells with 10 μM U0126 (lanes 4-6). Recovery of LSP1 was determined by western blotting of parallel samples using anti-LSP1 (E).

immunoprecipitates from anti-IgM-stimulated W10 cells using a peptide substrate (Fig. 4D) and only a very low level of ERK2 activity using myelin basic protein as substrate (Fig. 4H), although both assays clearly detected activation of ERK2 in anti-ERK2 immunoprecipitates (Fig. 4C,G). One possible explanation for the absence of active LSP1-associated ERK2 is that activation of ERK2 disrupts its interaction with LSP1. To test this hypothesis we compared LSP1/ERK2 and LSP1/MEK1 interactions in unstimulated and anti-IgM-treated W10 cells. Western blot analyses showed that the amounts of LSP1 or MEK1 present in anti-MEK1 immunoprecipitates do not change after 20 minutes of anti-IgM stimulation (Fig. 5A,B, lanes 1 and 2). In contrast, the amount of LSP1 in anti-ERK2 immunoprecipitates is significantly reduced 20 minutes after addition of anti-IgM (Fig. 5A, lanes 3 and 4). This is not due to differences in the amount of precipitated ERK2 (Fig. 5C). No significant amount of LSP1 was present in the control precipitation without added antibody (Fig. 5A, lane 5). To confirm the effect of anti-IgM stimulation on the LSP1/ERK2 interaction we precipitated LSP1 from W10 cells stimulated with anti-IgM for 0, 5 or 20 minutes. Analyses of these precipitates showed that ERK2 is dissociated from LSP1 after 20 minutes, but not after 5 minutes, of anti-IgM stimulation (Fig. 5D, lanes 1-3). The amount of precipitated LSP1 was not affected by anti-IgM stimulation (Fig. 5E). Thus, after anti-IgM treatment the activation of LSP1-associated MEK1 (Fig. 4B,F) preceeds the dissociation of ERK2 from the LSP1 complex (Fig. 5D). Dissociation of ERK2 from LSP1 is inhibited by treatment of W10 cells with 10 μM U0126 (Fig. 5D, lanes 4-6). Given that the MEK enzymes are the only known direct activators of the ERK enzymes, we conclude that

LSP1-associated ERK2, once activated by LSP1-associated MEK1, no longer interacts with LSP1.

Discussion

The characterization of LSP1 as a cytoskeletal targeting protein for the ERK/MAP kinase pathway significantly increases our understanding of the intracellular organization of the ERK/MAP kinase pathway as it defines a new pool of ERK2 that is characterized by its association with LSP1, a PKCβI-dependent activation and a cytoskeletal location. Co-immunoprecipitation, GST pull-down experiments and confocal microscopy show that LSP1 associates with PKCβI, MEK1, ERK2 and KSR. These experiments do not address the question of whether these five proteins form a single protein complex and it is formally possible that the identified interactions of PKCβI, MEK1, ERK2 and KSR with LSP1 reflect a set of bi- or tri-molecular protein complexes containing different combinations of LSP1 and one or more of its binding partners. However several arguments support the existence of a large complex containing all five proteins. First, studies in several cell types have shown the interactions between KSR and MEK1 and ERK2 (Denouel-Galy et al., 1997; Muller et al., 2001; Nguyen et al., 2002; Roy et al., 2002; Stewart et al., 1999; Yu et al., 1997) and in this paper we have confirmed the interaction of KSR with MEK1 and possibly ERK2 in W10 cells (Fig. 2). Second, although we have not been able to show that the interaction between LSP1 and MEK1 and ERK2 is direct, we have clearly shown the presence of LSP1 in anti-MEK1 and anti-ERK2 immunoprecipitates (Fig. 1) and of MEK1 and ERK in anti-LSP1 immunoprecipitates (Figs 4, 5). Third, we have previously shown direct binding of LSP1 with PKCβI (Cao et al., 2001). Taken together these data suggest strongly the existence of a single protein complex containing LSP1, PKCβI, KSR, MEK1 and ERK2. In this model LSP1 interacts directly with PKCβI, while the interactions of LSP1 with MEK1 and ERK2 are not direct but involve an interaction with the ERK scaffold KSR that binds directly to MEK1 and ERK2. We refer to this protein complex as the LSP1/KSR complex. To estimate the amount of ERK2 present in the LSP1/KSR complex, we compared the results shown in Fig. 5D, with a series of twofold dilutions of a total W10 cell extract. Approximately 2% of the total cellular ERK2 and ~10% of the total cellular LSP1 is present in anti-LSP1 immunoprecipitates (not shown). If all LSP1 is part of the LSP1/KSR complex then ~20% of the total ERK2 would be part of the LSP1/ERK complex. However, in the absence of a reliable estimate of the fraction of LSP1 in the LSP1/KSR complex the fraction of ERK2 in the LSP1/KSR complex may be much smaller than 20%. The role of LSP1 in this LSP1/KSR complex is to provide a binding site for PKCβI and to direct the LSP1/KSR complex to the actin cytoskeleton. KSR associates with ERK/MAP kinase pathway components, several heat shock proteins, the adaptor protein 14-3-3, the kinase C-TAK1 and several as yet unidentified proteins (Muller et al., 2001; Stewart et al., 1999; Xing et al., 1997). Thus LSP1 may interact with MEK1 and ERK2 by directly binding to KSR or to any of the other proteins associated with KSR. KSR interacts with Raf-1, the upstream activator of MEK1/2 (Muller et al., 2001; Roy et al., 2002; Xing et al., 1997), but the interaction is most likely indirect, as KSR and Raf-1 fail to interact in yeast two-hybrid assays (Denouel-Galy et al., 1997; Xing et al., 1997). We have not been able to show the presence of Raf-1 in anti-LSP1 immunoprecipitates, possibly because the interaction of Raf-1 with the LSP1/KSR complex is not stable under the experimental conditions used or is of low stoichiometry. Alternatively, Raf-1-related enzymes such as B-Raf (Barnier et al., 1995; Reuter et al., 1995) may couple activation of PKCβI in the LSP1/KSR complex to the activation of MEK1.

We showed previously that expression of the B-LSP1 truncate containing the COOH-terminal residues 179-330 in the $LSP1^+$ B-lymphoma cell line W10 inhibits the anti-IgM-induced translocation of PKCβI, but not of PKCβII or PKCα, which leads to inhibition of ERK2 activation (Cao et al., 2001). ERK2 is stimulated as early as 2 minutes after anti-IgM treatment but the inhibition of anti-IgM-induced translocation of PKCβI by B-LSP1 leads to the inhibition of anti-IgM-induced ERK2 activation that could be measured 20 minutes, but not 2 or 10 minutes, after addition of anti-IgM. This showed that the PKCβI-dependent activation of ERK2 occurs with slower kinetics than the PKCβI-independent activation of ERK2. Two findings documented in this paper strongly suggest that the pool of PKCβI-dependent ERK2 that is inhibited by B-LSP1 expression is associated with the LSP1/KSR complex. First, the LSP1/KSR-associated MEK1 is not activated in TW10.1 cells that express B-LSP1, suggesting that the activation of MEK1 in the LSP1/KSR complex is dependent on activation of PKCβI. Second, in W10 cells, activation of LSP1/KSR-associated MEK1 is detectable after 10 minutes and 20 minutes, but not after 5 minutes, of anti-IgM stimulation, which is similar to the activation of PKCβI-dependent ERK2. These data strongly suggest that the LSP1/KSR associated MEK1 is involved in the slow, PKCβI-dependent activation of ERK2. The fact that ERK2 is present in the LSP1/KSR complex 5 minutes, but not 20 minutes, after anti-IgM stimulation suggests that, once activated by the MEK1 in the LSP1/KSR complex, it dissociates from the complex. These results also show that B-LSP1 functions as a dominant negative construct that interferes with the activation of the LSP1/KSR complex. This dominant negative function of B-LSP1 is most likely due to the inhibition of PKCβI translocation.

The dissociation of activated ERK2 from the LSP1/KSR signaling complex is similar to the release of activated protein kinase A from AKAP79, an anchoring scaffold that binds PKA, PKC and the phosphatase calcineurin. The AKAP79/PKA complex is tethered to the post-synaptic density and it is thought that this promotes the preferential phosphorylation of nearby substrates (Klauck et al., 1996). We speculate that LSP1 has a role similar to AKAP79 and that after anti-IgM stimulation activated ERK2 is released from the LSP1/KSR complex to phosphorylate specific substrates. These substrates may be part of the LSP1/KSR complex or may be located in close proximity to the LSP1/KSR complex at the cytoplasmic side of the plasma membrane or on the cortical actin filaments. We do not yet know which proteins are phosphorylated by ERK2 in the LSP1/KSR complex but we have previously shown that expression of B-LSP1 significantly increases apoptosis of anti-IgM-treated W10 cells. Anti-IgM treatment of W10 cells induces a modest level of apoptotic cell death (~30% apoptotic cells), while anti-IgM treatment of TW10.1

cells that express B-LSP1 induces ~85% apoptotic cell death (Cao et al., 2001; Jongstra-Bilen et al., 1999). Thus failure to activate the ERK/MAP kinase pathway components in the LSP1/KSR complex correlates with increased anti-IgM-induced apoptosis. This leads to the hypotheses that the activation of PKCβI, MEK1 and ERK2 in the LSP1/KSR complex protects W10 cells from anti-IgM-induced apoptosis and that the substrates of the ERK2 in the LSP1/KSR complex mediate the anti-apoptotic role of the LSP1/KSR complex.

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