Liprin β1, a Member of the Family of LAR Transmembrane Tyrosine Phosphatase-interacting Proteins, Is a New Target for the Metastasis-associated Protein S100A4 (Mts1)*

Metastasis-associated protein S100A4 (Mts1) induces invasiveness of primary tumors and promotes metastasis. S100A4 belongs to the family of small calcium-binding S100 proteins that are involved in different cellular processes as transducers of calcium signal. S100A4 modulates properties of tumor cells via interaction with its intracellular targets, heavy chain of non-muscle myosin and p53. Here we report identification of a new molecular target of the S100A4 protein, liprin β1. Liprin β1 belongs to the family of leukocyte common antigen-related (LAR) transmembrane tyrosine phosphatase-interacting proteins that may regulate LAR protein properties via interaction with another member of the family, liprin α1. We showed by the immunoprecipitation analysis that S100A4 interacts specifically with liprin β1 in vivo. Immunofluorescence staining demonstrated the co-localization of S100A4 and liprin β1 in the cytoplasm and particularly at the protrusion sites of the plasma membrane. We mapped the S100A4 binding site at the C terminus of the liprin β1 molecule between amino acid residues 938 and 1005. The S100A4-binding region contains two putative phosphorylation sites by protein kinase C and protein kinase CK2. S100A4-liprin β1 interaction resulted in the inhibition of liprin β1 phosphorylation by both kinases in vitro.

Metastasis-associated protein S100A4 is highly expressed in metastatic tumors and tumor cell lines of different origins, and its expression is tightly associated with poor prognosis in cancer patients (1–6). The direct involvement of S100A4 in the formation of metastases has been shown in transgenic models, where the murine mammary tumor virus-S100A4 transgene provokes malignant progression of mammary tumors in mice (7, 8).

Different attempts have been made to elucidate the mechanisms underlying the role of S100A4 in tumor progression. Modulating the expression level of S100A4 in numerous tumor cell lines revealed its direct role in cell motility, invasiveness, and p53-dependent apoptosis (9–13). In addition, S100A4 is secreted from cells, and its extracellular activity as an angiogenic factor has been described previously (14, 15).

S100A4 belongs to the S100 family of small calcium-binding proteins that possess common EF-hand structures (for review, see Ref. 16). S100 proteins participate in various cellular processes as transducers of calcium signal. S100 proteins may exist in different forms such as monomer, homodimer, or heterodimer, whereas the prevailing form is antiparallelly noncovalently packed homodimer. Recombinant and secreted forms of S100A4 adopt all these conformations including an oligomeric form (15, 17–20). Calcium ions induce conformational changes in the S100 protein structures, allowing interaction with target proteins. Conformational plasticity and structural variations explain the unique ability of members of the S100 family to participate in multiple cellular processes via interaction with different targets (16).

To date, several intracellular target proteins of S100A4 have been identified, such as tropomyosin, actin, heavy chain of non-muscle myosin II A (MHC),1 and p53 (13, 21–23). The binding of S100A4 to its targets was characterized as a calcium-dependent interaction, which was blocked in the presence of EGTA. Recently, using the yeast two-hybrid system, the interaction of S100A4 with another S100A4 family member, S100A1, has been demonstrated (17–18).

Despite the fact that several S100A4 target proteins have been identified, there are still some unknown proteins detected in complexes with S100A4. Using mass spectrometry-based sequencing analysis, we identified liprin β1 as a protein co-immunoprecipitated in a complex with S100A4.

Liprin β1 is a 105-kDa cytosolic protein that belongs to the liprin family of transmembrane tyrosine-phosphatase LAR-interacting proteins (24). Although the involvement of LAR in neuron guidance and mammary gland development has been well documented and its function in tumorigenesis was suggested, the exact biological role of liprin β1 remains unclear (25–27). There are two groups of liprins, α-liprins and β-liprins, that might form homodimers and heterodimers. Liprins have high sequence homology, especially at C termini, where liprin homology (LH) domains are localized. However, only α-liprins interact with LAR and regulate its cellular distribution (28). Liprins form homodimers via the N-terminal coiled-coil region

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1 The abbreviations used are: MHC, myosin heavy chain; LAR, leukocyte common antigen-related; SAM, sterile α motif; LH, liprin homology domain; PKC, protein kinase C; aa, amino acid(s); mAb, monoclonal antibody; DOX, doxycycline.
were transfected with pMV-S100A4 retroviral vector DNA harboring the terminus of liprin. To demonstrate inducible expression of S100A4, we used the GP packaging cell line (34). GP

VMR-Liv-Tet-on-S100A4 cells. Using blot overlay analysis, we mapped the interaction between S100A4 and liprin and heterodimers via three sterile antibodies.

Cell Lines, Plasmids, and Metabolic Labeling—Two mouse mammary adenocarcinoma cell lines, VMR-Liv (30) and CSML100 (1), and a human osteosarcoma OHS (31) cell line were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in 1 h at room temperature with 30% transfection efficiency. For double staining, the cells were incubated with rabbit anti-S100A4 antibodies, followed by incubation with Alexa Fluor 594-conjugated anti-rabbit IgG from donkey (A-21207, Molecular Probes). Next, chicken anti-N-liprin β1 antibodies were applied, followed by incubation with fluorescein isothiocyanate-conjugated rabbit anti-chicken IgG (F-4137, Sigma). Slides were mounted in Fluormount-G (Southern Biotechnology Assoc.), dried overnight, and analyzed with confocal scanning laser microscope LSM510 (Zeiss).

Immunoprecipitation and Immunoblotting—Cell lysis, immunoprecipitation, and washing steps were carried out in a buffer (0.5% IGE-PAL CA-630, Sigma), 100 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 10 mM β-glycerophosphate, 5 mM NaF, 0.1 mM Na3VO4, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride) at 4 °C. Cell extracts were preclarified on protein G-Sepharose 4 Fast Flow (Amersham Biosciences, Inc.). mAb HM-4 and chicken anti-liprin β1 antibodies were used as a sandwich with rabbit anti-mouse or rabbit anti-chicken antibodies. Immunoprecipitated proteins were collected on protein G-Sepharose, washed, eluted in gel loading buffer, and separated by SDS-PAGE. Gels were dried and exposed, or proteins were transferred to Immobilon-P (Millipore) and analyzed by standard Western blotting procedure with an enhanced chemiluminescence detection system (ECL+ Plus; Amersham Biosciences, Inc.).

Mass Spectrometry Analysis—Bands corresponding to the labeled proteins co-immunoprecipitated with S100A4 were excised from the dried gels, and mass spectrometry analysis was performed as described previously (35).

Synthesis and Expression of the Human Liprin β Fragments—Total RNA from human OHS cells was isolated as described previously (36). Reverse transcription-PCR was conducted using SuperScript reverse transcriptase (Invitrogen). Antisense primer (CTACGCCTAAGAGTTT-GAGTC) corresponding to the liprin β sequence (+3244 to +3264) (24) was used for cDNA synthesis. A full-size and two overlapping liprin β fragments were PCR-amplified using the following sets of primers: (a) liprin β full size (FL-lip), CGCGGATCCATGATGAGTGATGCAAGTGACATG (+241 to +264) and CTACGGTCAAGAATTGAGT (+23244 to +3264), (b) N-terminal fragment (N-lip), CGCGGATCCATGATGCAAGTGACATG (+241 to +264) and AGTGGGCGGAGGGTTGTCGT (+1726 to +1746), and (c) C-terminal fragment (C-lip), CGCGGATCCATGATGCAAGTGACATG (+241 to +264) and TTAGTGCAAGATATCT (+3244 to +3264).

Truncated subfragments of the C-terminal fragment or fragments with internal deletions were synthesized by PCR using plasmid C-lip as a template. For subfragments, primers were designed as follows: (a) A-lip, CGG-GGATCCCTGTTTGGGAGCTGCCCCTCCTCC (+1678 to +1695) and ATATTGGAGGGAGGACAT (+2413 to +2430), (b) B-lip, CGCGGATCCACCTGCTAGATGTTG (+2898 to +2940) and CTACGGATCTTTGGTGAT (+23264 to +3264), (c) Ba-lip, CGCGGATCCACCTGCTAGATGTTG (+23264 to +2406) and GGCGGATGTTCAATTCT (+2605 to +2622), (d) Bb-lip, CGCGGATCCCGGAACTGACTTTCG (+2575 to +2592) and CTGGTCCGATATGTTGAT (+23264 to +3264). For internal deletions, primers were designed as follows: (a) Δ1C-lip, AGTCTTATTGGTGGTG (+2788 to +2802) and ATGGAGCTCCG (+2878 to +2889), (b) Δ2C-lip, ATCTGGTCTTTGGTG (2860 to +2874) and GAAATGGTGTCTCAAATG (+2989 to +3006), (c) Δ3C-lip, TTACCACTTCTCTG (+2974 to +2988) and CCTGTTGTGTATG (+3032 to +3066), and (d) Δ4C-lip, ATACCTTCTTCTAGATG (+3031 to +3048) and TCAACGGTCTTCCG (+3247 to +3261).

All synthesized fragments were cloned into pQE30 expression vector (Qiagen) and partially sequenced. Induction and purification of recombinant His-tagged proteins were done according to the recommendations of the manufacturer.

Blot Overlay Analysis—Blot overlay was carried out as described previously (23) in the presence or absence of 1 mM CaCl2 or 1 mM EGTA. PKC and CK2 Phosphorylation Assays—Recombinant PKC and CK2 were expressed in a mixture (25 μl) containing 50 mM Tris-HCl, pH 7.6, 0.1 mM CaCl2, 10 mM MgCl2, 4 mM CaCl2, 2 mM dithiothreitol, 7.5 μM of phosphatidyserine (PKC assay), 50 μM ATP, 2 μCi of [γ-32P] ATP (5,000 Ci/mmol; Amersham Biosciences, Inc.), and 1 μl Bi-anti with or without 5 μl S100A4 for 15 min at 30 °C. PKC was purchased from Roche, and 0.025 μg of PKC was applied per reaction. CK2 was purchased from New England BioSciences, Inc.
BioLabs, and 50 units of CK2 were applied per reaction. Reactions were terminated by the addition of 25 μl of 2× SDS-gel sample buffer, and proteins were separated in 15% SDS-PAGE. Gels were fixed in 5% trichloroacetic acid, dried, and exposed.

RESULTS

Identification of Liprin β1 as a New Target Protein for S100A4—To further investigate the mechanism underlying S100A4 function, we performed a search for proteins interacting with S100A4 in vivo. For this purpose, we generated a S100A4-expressing DOX-inducible clone of the mouse mammary adenocarcinoma cell line VMR-Liv. Cells were metabolically labeled with [³²P]orthophosphate in the absence or presence of DOX (24 or 48 h). Immunoprecipitation was performed with polyclonal antibodies against S100A4 and non-muscle myosin. To control for the specificity of the S100A4-target interactions detected in immunoprecipitation, we used lysates from original VMR-Liv cells. After 24 h of induction, we already observed several phosphoproteins including MHC co-immunoprecipitated with S100A4 (Fig. 1, lanes 8 and 9). These complexes were specific and also detectable in cells maintained in the presence of DOX for 48 h (Fig. 1, lanes 12 and 13). Without DOX stimulation, we observed hardly detectable bands corresponding to the S100A4 co-immunoprecipitated proteins that were absent in lysates from parental VMR-Liv cells (Fig. 1, lanes 1 and 5). That was likely due to the traces of S100A4 present in DOX-untreated VMR-Liv-Tet-on-S100A4 cells. Bands corresponding to the S100A4 co-immunoprecipitated proteins were excised from the gel, and the proteins were studied by mass spectrometry. Protein masses were determined by MALDI mass spectrometry, and final protein identification was achieved by partial sequencing using MS/MS techniques. One of the phosphoproteins with a molecular mass of 113.191 kDa was highly homologous to human liprin β1.

Confirming the Interaction between S100A4 and Liprin β1—To clone human liprin β1, we amplified liprin β1 sequences by reverse transcription-PCR using total RNA from human osteosarcoma cells. We cloned a fragment corresponding to the full-length protein (1–1005 aa) as well as two over-
lapping fragments matching the N-terminal coiled-coil region (N-lip, 1–501 aa) and C-terminal region (C-lip, 480–1005 aa) that contains a LH domain (24). Bacterially expressed N-lip was used for the immunization of chickens. Because human and mouse liprin β1 proteins are highly homologous, affinity-purified antibodies against N terminus of human liprin β1 specifically recognized liprin β1 of both mouse and human origin (data not shown).

To verify the interaction between S100A4 and liprin β1 in vivo, we performed immunoprecipitation of S100A4 and liprin β1 from two different cell systems. We chose CSML100 cells, highly metastatic mouse adenocarcinoma cells that expressed a high level of S100A4, and VMR-Liv cells infected with either control retroviral vector or the same vector harboring S100A4. Proteins were immunoprecipitated with antibodies against either S100A4 or liprin β1, resolved in gel, blotted, and analyzed with both antibodies. Anti-S100A4 antibodies immunoprecipitated S100A4- liprin β1 complex from CSML100 and VMR-Liv/S100A4 cells, but not from S100A4-negative cells (Fig. 2, lanes 2 and 4). Antibody against liprin β1 could precipitate the complex less efficiently and only from CSML100 cells, where the level of S100A4 expression is higher than that in VMR-Liv/S100A4 cells (Fig. 2, lane 6). A previously identified S100A4 target, MHC, was co-immunoprecipitated with S100A4 (Fig. 1, lanes 8, 9, 12, and 13). However, we could not detect the presence of the MHC in the liprin β1 precipitation, and, vice versa, liprin β1 was not present in the MHC-containing complexes (data not shown).

To determine whether S100A4 and liprin β1 are co-localized in cells, double immunofluorescence staining of CSML100 cells with affinity-purified polyclonal antibodies was performed. Both proteins appeared to be diffusely distributed in the cytoplasm and displayed enhanced staining in the perinuclear region and at the protrusion sites of the plasma membrane (Fig. 3, A and B). S100A4 and liprin β1 were predominantly co-localized to the lamellipodia-like sites on plasma membrane (Fig. 3C). Incubation with the antigen liprin β1-N completely abolished liprin β1 staining (Fig. 3E), confirming the specificity of the generated anti-liprin β1 antibody. The specificity of anti-S100A4 staining has been confirmed previously (15).

Thus, we considered these data an indication of the direct interaction between S100A4 and liprin β1 in vivo.

To verify this conclusion and to confirm the direct interaction between the two proteins in vitro, we applied blot overlay analysis. Bacterial lysates containing either recombinant full-length liprin β1 or its N- or C-terminal peptides (Fig. 4) were resolved by SDS-PAGE and blotted. Membranes were incubated with S100A4 in the presence of calcium or EGTA and stained with the anti-S100A4 antibodies. Recombinant proteins were synthesized as fused proteins containing His tag at their N termini, and the amount of the liprin β1-derived peptides on the membrane was controlled by staining with the anti-His antibodies (Fig. 5A, lanes 1–3). S100A4 interacted with full-length liprin β1 or its C-terminal fragment in calcium-dependent mode (Fig. 5B, lanes 1–3). Interaction was completely abolished in the presence of EGTA (data not shown).

Mapping of the S100A4 Binding Site on Liprin β1—The non-coiled C-terminal part of liprin β1 contains LH domain between 638 and 867 aa (24) that is composed of three SAM domains located between 638 and 702, 714 and 748, and 798 and 867 aa (Ref. 37 and CD alignment RPS-BLAST) (Fig. 4). To map the S100A4 binding site on liprin β1, we synthesized two overlapping recombinant fragments of the C-terminal region of liprin β1 containing one or two SAM domains, A-lip and B-lip (480–730 and 716–1005 aa) (Fig. 4). Cloning of the recombinant fragments was performed by PCR by using a plasmid containing a C-terminal fragment of liprin β1 as a template (see “Experimental Procedures”). Bacterial lysates expressing A-lip or B-lip were used for blot overlay analysis. Because the all recombinant protein fragments were expressed as 6-His-fused proteins, we could control the amount of loaded peptides in bacterial lysates by Western staining with anti-His antibodies (Fig. 5A). S100A4 interacted with the B-lip peptide containing two SAM domains, but not with A-lip, in a calcium-dependent manner (Fig. 5B, lanes 4 and 5). To further narrow the S100A4-binding region of liprin β1, we generated three new overlapping peptides (Ba-lip, Bb-lip, and Bc-lip) containing sequences corresponding either to the second or third SAM domains or to the C-terminal tip of liprin β1 (716–794, 779–926, and 889–1005 aa, respectively) (Fig. 4). It is clear from Fig. 5B that S100A4 interacted only with the C-terminal tip of liprin β1 (lanes 6–8). We concluded that the S100A4 binding site is localized between 889 and 1005 aa on the liprin β1 molecule.

To confirm this conclusion, we prepared four derivatives of the C-terminal fragment of liprin β1 containing internal deletions between 854 and 880, 875 and 917, 916 and 938, and 936 and 1003 aa (see the schema in Fig. 4). Blot overlay analysis with these recombinant proteins confirmed the localization of the S100A4 binding site between 938 and 1005 aa of liprin β1 (Fig. 5B, lanes 9–12). The interaction was calcium-dependent and was not detected in the presence of EGTA (data not shown).

S100A4 Inhibits Phosphorylation of Liprin β1—S100A4 modulates phosphorylation of its target proteins mediated by two protein kinases, PKC and CK2 (13, 35, 36). Liprin β1 is a Ser/Thr-phosphorylated protein in vivo, and, according to the prediction program (Net. Phos 2.0 prediction results), it could be phosphorylated by PKC or CK2. To determine whether S100A4 modulates phosphorylation of the C-terminal fragment of liprin β1 by PKC or CK2 in vitro, a phosphorylation assay with Bc-lip (Fig. 4) in the presence or absence of S100A4 was carried out. Bc-lip contains three putative PKC phosphorylation sites and a single CK2 target site. S100A4 inhibited phosphorylation of Bc-lip by both kinases (Fig. 6A). An excess amount of S100A4 was used in both assays (Fig. 6B). To prove that phosphorylation of S100A4 by PKC
Fig. 6. S100A4 inhibits phosphorylation of Bc-lip by PKC and CK2 in vitro. The Bc fragment of liprin β1 was phosphorylated by PKC (A, lanes 1 and 2) or CK2 (A, lanes 3 and 4) in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of S100A4. Phosphorylation was performed as described under “Experimental Procedures.” Samples were analyzed by 15% SDS-PAGE. A, autoradiography; B, Coomassie Blue staining of the gels.

(Fig. 6, lanes 1 and 2) did not result in the substrate inhibition of the kinase, we also used an excess amount of PKC. The observed level of PKC autophosphorylation was not inhibited by phosphorylation of the added S100A4 protein (data not shown).

DISCUSSION

Metastasis-associated protein S100A4 promotes tumor progression by stimulating tumor cell motility and invasiveness. A recent report (15) describing S100A4 as an angiogenic factor that stimulates motility of endothelial cells indicated another mode of its contribution to tumorigenesis. To elucidate the molecular mechanisms underlying the function of S100A4, we performed a search for proteins interacting with S100A4.

Previously, we have identified in vivo and studied in vitro the interaction of S100A4 with two proteins, MHC and p53 (13, 23, 38, 39). However, analysis of proteins co-immunoprecipitated with S100A4 from cells of different origin clearly indicated the existence of other intracellular targets of S100A4.

In the present study, by using mass spectrometry-based sequencing analysis, we identified liprin β1 as one of the phosphoproteins co-immunoprecipitated with S100A4 from metabolically labeled lysate of mouse adenocarcinoma cells. The interaction was also confirmed by immunofluorescence staining of mouse adenocarcinoma CSML100 cells, which showed co-localization of S100A4 and liprin β1 in the cytoplasm and at the protrusion sites of plasma membrane.

To study the interaction between S100A4 and liprin β1, we cloned liprin β1 by reverse transcription-PCR and prepared recombinant bacterial fragments of liprin β1 overlapping the whole molecule. We applied blot overlay analysis to study the interaction between recombinant liprin β1 proteins and S100A4 and revealed the calcium-dependent interaction of S100A4 with the C terminus of liprin β1. The S100A4-binding region was mapped between 938 and 1005 aa of liprin β1. The mapping was confirmed by blot overlay analysis of the recombinant liprin β1 fragments containing internal deletions.

Analysis of the S100A4-binding region of liprin β1 using the PSIPred program predicted the presence of a basically charged amphipathic α-helix resembling the S100A4-binding sequences.
revealed in other S100A4-interacting proteins, MHC and p53 (40). This observation is in agreement with the fact that the formation of amphipathic α-helix in target molecules is essential for interaction with other members of the S100 protein family (16).

Members of the S100 protein family were shown to interact with target proteins and inhibit their phosphorylation by PKC, thereby affecting PKC signaling (16). Previously, we have shown that the binding of S100A4 to MHC and p53 inhibits their phosphorylation by PKC at sites located within the sequences directly involved in the interaction with S100A4 (13, 38). In MHC, the residue phosphorylated by another protein kinase, CK2, is located just next to a site directly involved in S100A4 binding. However, the interaction with S100A4 also inhibited CK2-mediated phosphorylation of MHC, either due to conformational changes induced by S100A4 in the substrate molecule or as a result of the binding of S100A4 to the regulatory subunit of the enzyme (13, 38, 39).

Theoretically, Thr-962 located within the S100A4 binding site on the liprin β molecule (938–1005 aa) can be phosphorylated by PKC. The Cb fragment of liprin β (889–1005 aa) used in the PKC phosphorylation assay contains two more putative PKC target residues (Thr-891, Ser-932, and Thr-962). Although we found that the binding of S100A4 induced a significant decrease in the general level of PKC-mediated phosphorylation of the Cb fragment, at present, we do not know which residue’s phosphorylation was most inhibited by S100A4.

Ser-997, a predicted target residue for CK2, is located within the liprin β1 sequence interacting with the S100A4 protein. CK2-mediated phosphorylation of liprin β1 was inhibited by S100A4 either as a result of its direct interaction with a target molecule or via another mechanism involving the modulation of the enzyme activity (39).

Thus, S100A4 interacts with the C terminus of liprin β1 and modulates its intracellular phosphorylation by PKC and CK2. In all three S100A4 targets identified to date, MHC, p53, and liprin β1, there are structural similarities in regions directly interacting with S100A4. Similarly, the interaction with S100A4 results in inhibition of PKC-mediated phosphorylation of each target studied. The phosphorylation by CK2 of two of the three studied S100A4 targets, liprin β1 and MHC, was also inhibited in the presence of S100A4.

Liprin β1 forms heterodimers with another member of the liprin family, α1, via LH domains located at the C termini of the proteins (24). Sequence analysis of the LH domains of liprins revealed the presence of three SAM domains. SAM domains, which represent ~70 amino acid protein modules and are found in various signaling proteins, likely mediate protein homo- and heterodimerization (37). The region of liprin β1 responsible for the heterodimerization (678–1005 aa) overlaps sequence essential for the interaction with S100A4 (938–1005 aa). SAM domains, however, are not involved in the S100A4-liprin β1 interaction.

The LH domain of liprin α1 interacts not only with liprin β1 but also with transmembrane photophosphorylation phosphatase LAR, recruiting the enzyme to focal adhesions (24, 28). Liprin α1 has also been proposed to modulate the substrate specificity of LAR via this interaction (24, 41). LAR is a multifunctional protein that regulates several signal transduction pathways such as insulin or β-catenin signaling and affects cytoskeletal dynamics and cell adhesion (42–47). Our data presented here show S100A4 as a novel component of the LAR-liprin α1-liprin β1 network. We hypothesize that S100A4 modulates liprin α1-liprin β1 complex formation in vivo, thereby affecting LAR function. S100A4 was shown to participate in the regulation of the motility of tumor and endothelial cells. Because LAR-dependent signaling mechanisms are directly involved in the regulation of cell adhesion, we propose that the presently described liprin β1-S100A4 interaction represents a mechanistic explanation of the role that S100A4 plays in tumor progression.

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