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Biochemical and Biophysical Research Communications 348 (2006) 826–831
BBRC
www.elsevier.com/locate/ybbrc

Magicin associates with the Src-family kinases and is phosphorylated upon CD3 stimulation

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Received 12 July 2006
Available online 31 July 2006

Abstract

We recently identified a novel actin cytoskeleton-associated protein magicin, for merlin and Grb2 interacting cytoskeletal protein. To unravel the cellular functions of magicin, we used a yeast two-hybrid system and identified Fyn tyrosine kinase as a specific binding partner for magicin. Fyn phosphorylates magicin *in vitro*. In addition to Fyn, Src and Lck also interact with magicin. Upon stimulation with anti-CD3 antibody, magicin is phosphorylated in the T lymphocyte leukemia Jurkat cell line. Magicin phosphorylation is not observed in an Lck-deficient line, J.CaM1.6, indicating that Lck is the major Src family kinase for phosphorylating magicin in Jurkat cells. Employing site-directed mutagenesis along with *in vitro* kinase assays, we found that Y64 of magicin is phosphorylated by Lck creating a SH2-Grb2 binding motif. Magicin has also been identified as a Mediator subunit (MED28) in the nucleus involved in transcriptional regulation, therefore we propose that magicin may serve as a multi-faceted adaptor/scaffold to relay cellular signaling to the cytoskeleton and from the cytoskeleton to the nucleus.
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Keywords: NF2; Merlin; Src kinases; EG-1; MED28; Mediator; CD3; T cell receptor; Jurkat cells

We recently identified a novel ~24 kDa cytoskeletal protein, magicin (merlin and Grb2 interacting cytoskeletal protein), which specifically associates with the neurofibromatosis 2 (NF2) tumor suppressor protein merlin, but not with other ezrin–radixin–moesin family members [1]. In addition, magicin also interacts with the SH3 domain of Grb2, an adaptor involved in many receptor tyrosine kinases and other signaling pathways. The association of magicin with merlin and Grb2 suggests the physiological significance of magicin as a scaffold/adaptor protein to convey cellular signals through receptors. Another group also identified magicin as a differentially expressed gene in endothelial cells and named it endothelial-derived gene, *EG-1* [2]. *EG-1* expression is elevated in several malignant cancers of the breast, colon, and prostate [3]. Furthermore, overexpression of *EG-1* increases cellular proliferation [4]. These findings suggest an important function of magicin in cell growth and imply its role in tumorigenesis, which may also be important for merlin function.

Magicin has also been identified independently as MED28, a component of the mammalian multiprotein Mediator complex in the nucleus [5,6]. The Mediator complex, comprising at least 20 protein subunits, was originally identified in yeast. It was subsequently isolated in mammalian cells as a multiprotein coactivator that promotes RNA polymerase II transcriptional activation and is therefore involved in gene regulation [7]. Some of the Mediator subunits directly associate with RNA polymerase II and general transcription initiation factors at the core promoter; others associate with transcriptional activators, which bind to enhancer and upstream promoter sequences [8]. In addition to being a cytoskeletal protein, magicin is also found in the nucleus where it is presumably involved in gene transcription as part of the Mediator coactivator. It is therefore likely that magicin shuttles between the nucleus and the cytoskeleton as part of its physiological role. Thus magicin

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.07.126

could be involved in transcriptional regulation, cell proliferation, and tumorigenesis.

Activation of the T cell receptors (TCR) results in a cascade of signal transduction pathways, including an increase in intracellular calcium, altered patterns of gene expression, cytoskeleton reorganization, and stimulation of cell proliferation [9,10]. The immediate outcome of TCR engagement is the activation of Src tyrosine kinases Fyn and Lck, which phosphorylate the subunits of the CD3 complex as well as other protein tyrosine kinases, most importantly the Syk family tyrosine kinase ZAP70 (ζ chain-associated protein kinase of 70 kDa). These activation events create docking sites for SH2 domain-containing adaptor proteins such as Grb2 to transmit the signals downstream. The present study demonstrates that magicin interacts with Src family members and is phosphorylated upon activation of the T cell receptors in Jurkat cells. Furthermore, phosphorylation of magicin enables binding to the SH2 domain of Grb2. Taken together, magicin could serve as an important adaptor/linker integrating cytoskeletal dynamics and signaling with transcriptional regulation in the nucleus.

Materials and methods

Reagents and antibodies. GST-Src-SH3 domain fusion protein was obtained from Dr. Bruce Mayer (University of Connecticut Health Center, Farmington, CT). Grb2, Lck, and Fyn-GST fusion proteins were purchased from Santa Cruz Biotechnology. Fyn and Lck kinases were purchased from Upstate Biotechnology. The expression and purification of GST and His-tagged fusion proteins of magicin have been described previously [1]. Antibodies used in this study included anti-human CD3-ε (UCHT1) from Ancell Immunology Research Products, Fyn (sc-16), Lck (sc-433 and sc-13) from Santa Cruz Biotechnology, Fyn (610163) from BD Biosciences, 4G10 from Upstate Biotechnology, PY20 from BD Biosciences, anti-His from Qiagen, and anti-GST from Santa Cruz Biotechnology. Horseradish peroxidase (HRP)-conjugated anti-mouse antibodies were obtained from Amersham Biosciences. Western blot analyses were visualized using the ECL system (Amersham Biosciences). The anti-magicin antibodies, Tim3 and 7E1, have been described previously [1].

Cell culture and transfection. CAD cells (mouse neuronal cells) were cultured in D-MEM/F-12 (Gibco-BRL) containing 10% fetal bovine serum (FBS; Hyclone). Human embryonic kidney 293T cells were maintained in DMEM containing 10% FBS. Jurkat wildtype E6-1 and Lck-deficient J.CaM1.6, a derivative mutant line of Jurkat, T cells were obtained from ATCC and cultured in RPMI-1640 medium (Sigma) supplemented with 10% FBS. Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions.

CD3 activation. CD3 stimulation was performed as described [11] with slight modifications. Jurkat cells were cultured in 1% FBS for 24 h prior to stimulation. Cells were then washed three times with serum-free media and incubated with 10 μg/ml anti-CD3 (Ancell) for 15 min on ice. After a wash with RPMI, the pellets were incubated with 10 μg/ml rabbit anti-mouse IgG (Caltag) at 37 °C for various time points, 0 min, 30 s, 1, 2, and 5 min. Stimulation was terminated by the addition of 5 mM EDTA, 10 mM NaF, and 0.4 mM Na_3VO_4. Cells were lysed in 1% NP40 lysis buffer and subjected for immunoprecipitation as described [1]. A separate set of experiments were performed as above, using Jurkat and J.CaM1.6 cells for CD3 stimulation at the 2′ time point.

Yeast two-hybrid system. Full length (aa 1–178), N-terminus (aa 1–100), and C-terminus (aa 79–178) of human magicin were used as baits to screen a human fetal frontal cortex cDNA library as described previously [1]. The control baits used in the two-hybrid screening included bicoid, CDC2 (kindly provided by Dr. Roger Brent, Molecular Sciences Institute, Berkeley, CA), amyloid precursor protein (APP, kindly provided by Dr. Suzanne Guénette, Massachusetts General Hospital, Boston, MA), IQGAP, and neurofibromin (NF1, kindly provided by Dr. André Bernards, Massachusetts General Hospital, Boston, MA).

Site-directed mutagenesis. Mutations of the tyrosine residues of magicin were performed by PCR cloning using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufactuer's instructions. The primers used for the Y to F mutation were Y64F-sense, 5′-GGTGAG TCAGGACTTTGTCAATGGCACCG-3′; Y64F-anti-sense, 5′-CGGTG CCATTGACAAAGTCCTGACTCACC-3′; Y163F-sense, 5′-GCTCCTT GGCCTTCCTGGAGCAGGC-3′; Y163F-anti-sense, 5′-GCCTGCTCC AGGAAGGCCAAGGAGC-3′. Subsequent transformants were sequenced to verify the Y to F mutations.

Blot overlay, pull-down, and immunoprecipitation assays. These procedures were carried out as described previously [1].

In vitro kinase assays. Five micrograms of His-tagged magicin (aa 13–178) was incubated with Fyn kinase (Upstate Biotechnology) and kinase reaction buffer (25 mM Hepes, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 5 mM β-glycerophosphate, 1 mM ATP, and 200 μM Na_3VO_4) for 90 min at 30 °C. The control reactions included omission of Fyn kinase, His-tagged magicin, or ATP, respectively. The reactions were resolved and immunoblotted with anti-phosphotyrosine antibodies. The GST-Grb2(SH2) blot overlay experiment was performed by incubating His-magicin with ATP and kinase buffer, with or without Lck kinase, using conditions described above. After SDS–PAGE, the membrane was overlaid with GST-Grb2(SH2) followed by immunoblotting with an anti-GST antibody.

Results

Identification of Fyn as a specific interactor of magicin

In order to understand the function(s) of magicin, we employed a LexA-based yeast two-hybrid system to identify binding partners of magicin. Three baits, full length (aa 1–178), N-terminus (aa 1–100), or C-terminus (aa 79–178), of magicin were employed to screen a human fetal frontal cortex cDNA library. Full-length Fyn was identified as an interacting partner using the C-terminal bait of magicin, but not with unrelated baits such as APP, Bicoid, CDC2, IQ GAP or neurofibromin (NF1). Subsequently full length and the N-terminus of magicin were compared with the C-terminus for an interaction with Fyn in the yeast two-hybrid system. Full-length magicin revealed the strongest interaction, followed by the C-terminus, and the N-terminus showed a weak interaction (Table 1).

The direct association of magicin with Fyn was further examined by blot overlay assays (Fig. 1). Full length, SH3 domain, or SH2 domain of Fyn, as well as full-length Grb2 or GST controls were overlaid onto membranes containing His-magicin to test the direct interaction. Full-length Grb2 served as a positive control since we had previously shown the magicin interaction with the SH3 domain of Grb2 [1]. The results from blot overlay experiments indicate that magicin can directly bind the SH3 domain of Fyn. GST-Fyn (SH3) revealed a stronger binding with magicin when compared with GST-Fyn (FL) (Fig. 1), suggesting a possible conformational regulation. Since Fyn is highly expressed in the nervous system, we then investigated if magicin associates with Fyn in CAD,

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