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Advances in Brief

Threonine Phosphorylation of the MMAC1/PTEN PDZ Binding Domain Both Inhibits and Stimulates PDZ Binding

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Abstract

Two-hybrid searches with the tumor suppressor MMAC1/PTEN isolated the proteins hDLG and hMAST205. Further two-hybrid analysis and microtiter plate binding assays localized the sites of interaction to PDZ domains from hDLG and hMAST205 and the PDZ binding domain at the COOH terminus of MMAC1/PTEN. A synthetic peptide derived from the MMAC1/PTEN PDZ binding domain (MMAC1/PTEN-PDZBD) was used to coprecipitate proteins from A431 human cell lysate. The recovered proteins were resolved by SDS-PAGE and immobilized on a nitrocellulose membrane. Treatment of this membrane with an anti-hDLG antibody identified a M_r 140,000 band, consistent with the size of hDLG. Treatment of this membrane with the MMAC1/PTEN-PDZBD peptide identified a single prominent band of slightly larger than M_r 200,000 (M_r 200,000 kDa). Threonine phosphorylation of the MMAC1/PTEN-PDZBD peptide inhibited both microtiter plate binding to the hDLG and hMAST205 PDZ domains and coprecipitation of the M_r 140,000 and >200,000 proteins, but promoted coprecipitation of proteins of approximately M_r 90,000 and M_r 120,000 from A431 cell lysate. This result suggests phosphorylation of the MMAC1/PTEN PDZ binding domain can both inhibit and promote PDZ interactions.

Introduction

MMAC1/PTEN/TEP1 is a tumor suppressor frequently disrupted in gliomas and occasionally disrupted in a variety of other tumors. The primary sequence of MMAC1/PTEN contains motifs with significant sequence similarity to protein phosphatases (1–3). Enzymatic studies have confirmed that MMAC1/PTEN is a phosphatase with activity toward both phosphatidylinositol (4) and protein (5) substrates. A number of recent publications have suggested MMAC1/PTEN phosphatidylinositol phosphatase activity may be involved in the regulation of cell survival via the PI3 Kinase/Akt signaling pathway (6–8). Other reports have presented evidence that MMAC1/PTEN may regulate cell migration and focal adhesion formation by dephosphorylation of FAK (9–11).

Our approach to investigate MMAC1/PTEN function was to identify interacting proteins using the yeast two-hybrid system. Two-hybrid screens isolated the PDZ domain containing proteins hDLG (discs-large) and hMAST205 (mircotubule-associated serine-threonine kinase; molecular weight, M_r 205,000). Additional analyses localized the interactions to the PDZ domains of these proteins. DLG, originally identified as a tumor suppressor in *Drosophila*, contains three PDZ domains and a guanylate kinase-like domain (12, 13). MAST205, originally identified in mice, contains a single PDZ domain and shares significant sequence similarity to the A and C families of serine/threonine kinases (14). PDZ domains are ~80 amino acid motifs often found as repeating units in membrane-associated scaffold proteins but are also found in a variety of other proteins (12, 13, 15, 16). PDZ domains interact with PDZBDs[3], which are short sequences found at the COOH terminus of a variety of proteins. Consistent with this, the COOH terminus of MMAC1/PTEN (. . . ITKV-COOH) resembles known PDZBDs. Because many PDZ proteins are believed to organize and regulate signaling complexes (16), the identification of PDZ domain containing proteins that bind to MMAC1/PTEN suggests that PDZ interactions may regulate or localize MMAC1/PTEN activity.

The potential role of the MMAC1/PTEN-PDZBD in tumor suppression has been discussed previously (17, 18). Inactivation of the PDZBD by deletion (17) or fusion with a hemagglutinin tag (19) has no apparent effect on the ability of MMAC1/PTEN to suppress anchorage-dependent growth when transfected into MMAC1/PTEN null cells. Evidence exists that the PDZBD may play a role in anchorage-independent growth (17). However, the ability of the MMAC1/PTEN-PDZBD to bind to PDZ domains has not been addressed. Here, we use biochemical methods to show the MMAC1/PTEN-PDZBD is a bonafide PDZBD and that interactions between this domain and other molecules is both positively and negatively regulated by threonine phosphorylation. Whereas previous work has shown that phosphorylation of PDZBDs can disrupt PDZ interactions (20), we believe this is the first report that phosphorylation of PDZBDs can promote PDZ interactions.

Materials and Methods

Peptides, Antibodies, Cell Lines, and Nucleotide Accession Numbers. The biotinylated peptides MMAC1/PTEN-PDZBD (biotin-ENEPFDEDQHTQITKV), MMAC1/PTEN-pThrPDZBD (biotin-ENEPFDEDQHTQIpTKV where pT is phosphothreonine), and Src-SH3BD (biotin-SGSGILAPPVPPRNTR) were synthesized at Research Genetics Inc. (Huntsville, AL). Anti-DLG monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY). A431 human epidermoid carcinoma cells were purchased from American Type Culture Collection (Manassas, VA) and were grown in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 4 mM glutamine, 0.15% sodium bicarbonate, and 5 mM sodium pyruvate. The following GenBank entries are referred to here: mouse MAST205, U02313; human discs-large, U13897; and human MMAC1, U92436.

Two-Hybrid Screens. DNA sequences encoding either full-length human MMAC1/PTEN (aa 1–403) or the amino acid fragments 1–200, 183–403, 183–277, 277–403, and 277–388, were inserted into the DNA binding domain vector pGBT.C. Each construct was cotransformed into the yeast strain J692 along with activation domain libraries containing either human liver, kidney, or brain cDNA inserts (Clonetech Laboratories, Palo Alto, CA). Transformed yeast were plated on yeast minimal media lacking trytophan, leucine, and histidine and containing 25 mM 3-amino-1,2,4-triazole. After 7–10 days incubation at 30°C, two-hybrid interactions were identified by assaying for

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[3] The abbreviation used is: PDZBD, PDZ binding domain.

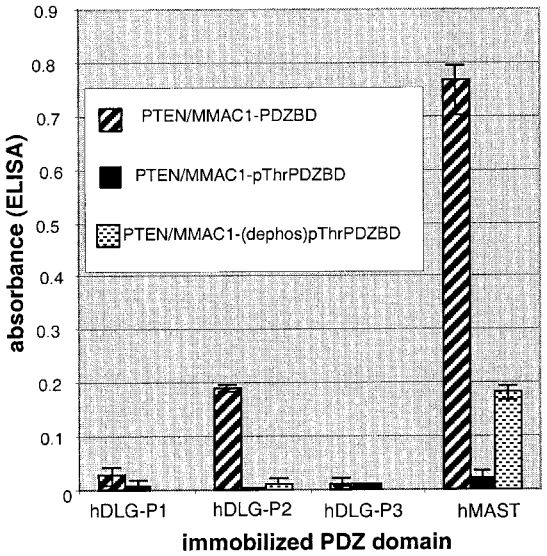

Fig. 1. Microtiter plate peptide binding assay demonstrating binding of MMAC1/PTEN-PDZBD peptides to immobilized PDZ domains. The peptides MMAC1/PTEN-PDZBD, the phosphothreonine version (MMAC1/PTEN-pThrPDZBD), and MMAC1/PTEN-pThrPDZBD treated with PP1 phosphatase (MMAC1/PTEN-(dephos)pThrPDZBD) were prebound to streptavidin peroxidase, then incubated with the indicated GST-PDZ domain fusion proteins preabsorbed to the wells of a 96-well microtiter plate. Following repeated washes, the amount of bound peptide was determined by colorimetetric assay. Shown is the average of three determinations with the extremes indicated by *error bars.*

β-galactosidase expression (21). To confirm the yeast two-hybrid results, positive isolates were assayed for a lack of β-galactosidase expression against a panel of unrelated inserts in pGBT.C. In addition, the cDNA inserts from these isolates were inserted into pGBT.C and screened against pACT2 constructs containing the MMAC1/PTEN residues 277–403 or 277–388, to confirm the interaction.

Microtiter Plate Peptide Binding Assay. The following PDZ domain-expressing fragments were cloned into the GST expression vector pGEX-4T-1 (Promega Corp., Madison, WI): hDLG-P1 (aa 212–318, YVNGT . . . SEKIM), hDLG-P2 (aa: 310–414, RKPVS . . . MNDGY), hDLG-P3 (aa 449–562, PVSKA . . . HDLRE), and hMAST205 (aa 1059–1166, DFLPA . . .IKVGP). These constructs were verified by DNA sequencing, expressed in *Escherichia coli* BL21 cells, and purified with glutathione-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) using the supplied protocols. Fusion protein (1 μg/well) was adsorbed to 96-well microtiter plates (Corning Costar, Acton, MA) in 0.1 M sodium bicarbonate for 1 h at room temperature, then blocked with 1% BSA under the same conditions. The amount of bound fusion protein was verified with an anti-GST antibody (Amersham Pharmacia Biotech). Streptavidin peroxidase (1 μg/well; Sigma Chemical Company, St. Louis, MO) was prebound with 0.2 μg of the appropriate biotinylated peptide in 100 μl wash buffer (1× PBS, 0.1% BSA, and 0.1% Tween 20), the remaining sites blocked with excess free biotin, then incubated in the appropriate wells for 1 h at room temperature. Following repeated washes, the amount of bound peptide was quantitated using ATBS at 405 nM after the supplied protocols (Amersham Pharmacia Biotech). To dephosphorylate the MMAC1/PTEN-pThrPDZBD peptide, 10 μg of peptide were treated with 20 units of PP1 phosphatase (Calbiochem, La Jolla, CA) for 30 min at 30°C in 50 mM Tris (pH 7), 0.1 mM EDTA, 5 mM DTT, 20 μM $MnCl_2$, and 200 μg/ml BSA.

Peptide Coprecipitation. A431 cells were grown to 70–80% confluence in 150-mm dishes, washed with cold 1× PBS, and lysed in 1.5 ml of lysis buffer [10 mM Tris-HCl (pH 8.0), 125 mM NaCl, 20% glycerol, 1% Triton X-100, 1 μM leupeptin, 1 μM pepstatin, 0.3 μM aprotinin, 1.0 mM EDTA-Na2, and 100 μg/ml PMSF) on ice for 15 min. The cell lysate was cleared by centrifugation at 15,000 *rpm* for 15 min. For the coprecipitations, 2–4 μg of each peptide was first prebound to 50 μl of streptavidin Dynabeads (Dynal, Oslo, Norway), the remaining sites were blocked with excess free biotin, then incubated with A431 lysate at 4°C for 1 h. The beads were captured with a magnetic support, washed with lysis buffer five times, then boiled in SDS sample buffer for 5 min. The samples were fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, blocked with 5% nonfat dry milk, and probed with either an anti-hDLG antibody or the peroxidase-coupled peptides described above. Following repeated washes, bound antibody was treated with a secondary horseradish peroxidase-linked antimouse IgG antibody. These protein-antibody or protein-peptide complexes were detected using a chemiluminescence reagent kit (Santa Cruz Biotech, Santa Cruz, CA).

Results and Discussion

The yeast two-hybrid system was used to identify proteins that interact with human MMAC1/PTEN. A variety of MMAC1/PTEN fragments were screened against activation domain libraries containing human liver, kidney, or brain cDNA inserts. The COOH-terminal MMAC1/PTEN fragment 277–403 interacted with two PDZ domain-containing proteins: discs-large (hDLG) (12, 13) and hMAST205 (14). Additional two-hybrid studies confirmed that these two-hybrid interactions were due to PDZ binding (see "Materials and Methods").

A microtiter plate binding assay was used to determine whether the MMAC1/PTEN-PDZ interactions identified by two-hybrid analysis could be replicated in a different context. GST-PDZ domain fusions from hDLG and hMAST205 that had been preadsorbed to the wells of a 96-well microtiter plate were probed with a streptavidin-peroxidase-coupled biotinylated synthetic peptide encompassing the COOH-terminal 16 amino acids of MMAC1/PTEN (MMAC1/PTEN-PDZBD). Interactions were observed with the single PDZ domain from hMAST205 and the second PDZ domain but not the first or third PDZ domain from hDLG (Fig. 1). Phosphorylation of PDZBDs *in vivo* has

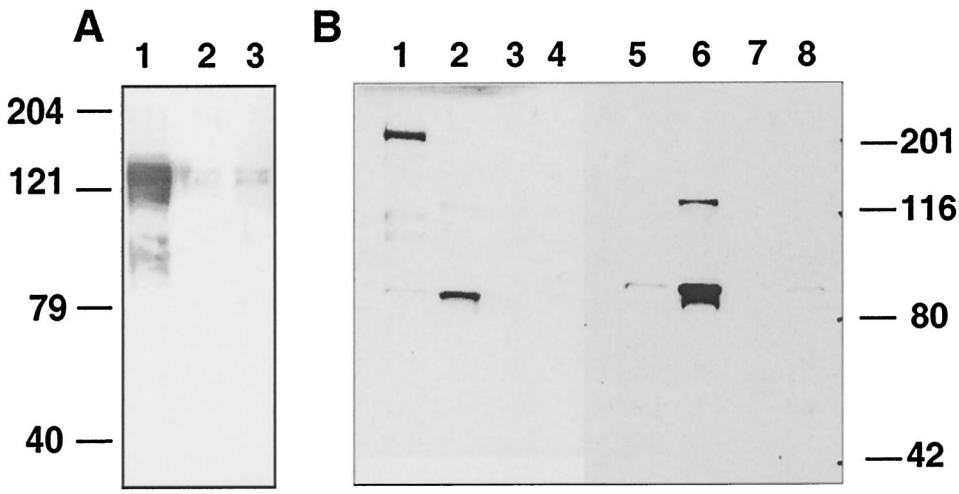

Fig. 2. Coprecipitation experiment using MMAC1/PTEN PDZBD peptides and A431 human cell lyaste. *A,* the biotinylated peptides MMAC1/PTEN-PDZBD *(Lane 1)*, MMAC1/PTEN-pThrPDZBD *(Lane 2)*, and Src-SH3BD *(Lane 3)* were prebound to streptavidin magnetic beads, then incubated with A431 cell lysate. Following repeated washes, the bound molecules were recovered by boiling in SDS sample buffer, resolved by SDS-PAGE, transferred to a nitrocellulose membrane, blocked, then probed with an anti-hDLG antibody. The detected M_r 140,000 band is at the size expected for hDLG. *B,* the peptides MMAC1/PTEN-PDZBD *(Lanes 1* and *5)*, MMAC1/PTEN-pThrPDZBD *(Lanes 2* and *6)*, and Src-SH3BD *(Lanes 3* and *7)* were incubated with A431 cell lysate and resolved by SDS-PAGE along with crude lysate *(Lanes 4* and *8)*, as described above. Unlike *A,* bound proteins were detected with MMAC1/PTEN-PDZBD *(Lanes 1–4)* and MMAC1/PTEN-pThrPDZBD *(Lanes 5–8)* peptides precomplexed to peroxidase.

been shown to negatively regulate PDZ interactions (20). Threonine phosphorylation of the MMAC1/PTEN-PDZBD (. . . IpTKV-COOH) disrupted binding to the hDLG and hMAST205 PDZ domains, which suggests that MMAC1/PTEN may be subject to similar regulation. Treatment of the threonine phosphorylated peptide (MMAC1/PTEN-pThrPDZBD) with PP1 phosphatase partially restored the binding, indicating that the phosphate group is responsible for the lack of binding. Incomplete restoration of activity may be due to low activity of PP1 phosphatase toward the MMAC1/PTEN-pThrPDZBD peptide.

Coprecipitation experiments were used to investigate whether the MMAC1/PTEN-PDZBD peptide can bind hDLG in a human cell lysate. To accomplish this, streptavidin magnetic beads prebound with MMAC1/PTEN-PDZBD, MMAC1/PTEN-pThrPDZBD, or an unrelated peptide were incubated with A431 cell lysate. Following extensive washes, the bound proteins were recovered, resolved by SDS-PAGE, and transferred to a nitrocellulose membrane. Treatment of this membrane with an anti-DLG antibody detected a band of approximately M_r 140,000, the expected size for hDLG, only in the *Lane* derived from the nonphosphorylated MMAC1/PTEN-PDZBD peptide (Fig. 2*A*). The peroxidase-coupled MMAC1/PTEN-PDZBD and MMAC1/PTEN-pThrPDZBD peptides described in the microtiter plate binding assay were used to treat a membrane similar to that described above. Such peptide detection reagents have been shown to be highly specific in similar assays (22, 23). A single prominent band slightly larger than M_r 200,000 (>200 kDa) was detected when the MMAC1/PTEN-PDZBD peptide was used for both the coprecipitation and detection steps (Fig. 2*B*). The size of this protein is consistent with hMAST205, but confirmation of the identity of this protein awaits availability of an appropriate antibody. If the MMAC1/PTEN-pThrPDZBD peptide was used for both coprecipitation and detection steps, the M_r 205,000 band was not observed but two prominent bands of approximately M_r 90,000 and M_r 120,000 are observed. The M_r 90,000 peptide is also detected with the nonphosphorylated peptide. The differential recovery of putative PDZ domain-containing proteins suggests phosphorylation of the MMAC1/PTEN PDZ binding domain may regulate interactions of MMAC1/PTEN with a variety of proteins. However, the phosphorylation status of Thr 401 in the MMAC1/PTEN PDZ binding domain *in vivo* is not known. Whereas previous studies have shown phosphorylation of PDZBDs can disrupt PDZ interactions (20), we believe this is the first report presenting evidence that phosphorylation of PDZBDs promotes binding to PDZ domains.

The lack of a band at the expected size of hDLG was investigated. The four GST-PDZ domain fusion proteins used in the microtiter plate binding assay were subject to SDS-PAGE and immobilization on nitrocellulose. Only GST-hMAST205 was able to bind the MMAC1/PTEN-PDZBD peptide, which suggests hDLG may lose the ability to interact with MMAC1/PTEN following this treatment (data not shown). We also attempted numerous coprecipitation studies to demonstrate a direct interaction of MMAC1/PTEN with hDLG. Whereas the results of these experiments were consistent with this interaction, all MMAC1/PTEN antibodies tested were broadly cross-reactive. Therefore, confirmation of the interaction of MMAC1/PTEN with hDLG or hMAST205 awaits availability of the appropriate antibodies.

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