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Polyclonal Antibody to Cortactin pTyr466 - Aff - Purified

Catalog No.: BP7033

Quantity: 0.1 ml

Background: Cortactin is an 80/85 kDa cytoskeleton protein that facilitates assembly of cortical actin. Cortactin is widely expressed in most adherent cells and is a prominent substrate of protein-tyrosine kinase Src in vivo and in vitro. The protein sequence of cortactin contains multiple amino-terminal tandem repeats of a unique 37-amino acid sequence, which associates with actin, and a Src homology 3 (SH3) domain at the carboxyl terminus. Between the repeat and the SH3 domain, there is an α helical structure followed by a proline-rich region. Between the proline-rich region and the SH3 domain, there are Src phosphorylation sites at tyrosines 421, 466 and 482 (421, 470 and 486 in the human sequence) that when phosphorylated, attenuate the cross-linking of F-actin into meshworks.

Host: Rabbit

Immunogen: Chemically synthesized phosphopeptide derived from the region of mouse cortactin that contains tyrosine 466 (tyrosine 470 in the human sequence).

Format:
State: Liquid Ig fraction
Purification: Sequential epitope-specific chromatography. The antibody has been negatively preadsorbed using i) a non-phosphopeptide corresponding to the site of phosphorylation to remove antibody that is reactive with non-phosphorylated cortactin protein, and ii) preadsorbed with the phosphopeptides to cortactin tyrosine 421 and 482, to remove cross-reactivity. The final product is generated by affinity chromatography using a cortactin-derived peptide that is phosphorylated at tyrosine 466.
Buffer System: Dulbecco's phosphate buffered saline (without Mg2+ and Ca2+), pH 7.3 (+/- 0.1), with 1.0 mg/mL BSA (IgG, protease free) as a carrier, containing 0.05 % sodium azide.

Applications:
Western blot (0.1 - 1.0 µg/ml).
Positive Control Used: NIH3T3 stably expressing c-Src Y527F mutant protein; NIH3T3 cells treated with 50 ng/mL PDGF for 15 minutes.
Other applications not tested. Optimal dilutions are dependent on conditions and should be determined by the user.

Specificity:
This antibody detects Cortactin.
Species: Mouse.
Other species not tested.

Storage:
Store at -80 şC. Upon initial thawing, aliquot and store at -80 şC. Avoid repeated freezing and thawing.
Centrifuge vial before opening.
Shelf life: one year from despatch.

General Readings:

Head, J.A., et al. (2003) Cortactin tyrosine phosphorylation requires Rac1 activity and association with the cortical actin cytoskeleton. Mol. Biol. Cell. 14(8):3216-3229.
Dudek, S.M., et al. (2002) Novel interactions of cortactin with endothelial cell myosin light chain kinase. Biochem. Biophys. Res. Comm. 29 (4):511-519.
Arderiu, G., et al. (2002) Primary arrest of circulating platelets on collagen involves phosphorylation of Syk, cortactin and focal adhesion kinase: studies under flow conditions. Biochem. J. 364(Pt 1):65-71.
Weed, S.A. and J.T. Parsons (2001) Cortactin: coupling membrane dynamics to cortical actin assembly. Oncogene 20(44):6418-6434.
Weed, S.A., et al. (2000) Cortactin localization to sites of actin assembly in lamellipodia requires interactions with F-actin and the Arp2/3 complex. J. Cell. Biol. 151(1):29-40.
Kapus, A., et al. (2000) Cell volume-dependent phosphorylation of proteins of the cortical cytoskeleton and cell-cell contact sites. J. Biol. Chem. 275(41):32289-32298.
Huang, C., et al. (1998) The role of tyrosine phosphorylation of cortactin in the locomotion of endothelial cells. J. Biol. Chem. 273(40):25770-25776.

Protocols:

Western Blotting Procedure

1. Lyse approximately 10e7 cells in 0.5 mL of ice cold Cell Lysis Buffer (formulation provided below). This buffer, a modified RIPA buffer, is suitable for recovery of most proteins, including membrane receptors, cytoskeletal-associated proteins, and soluble proteins. Other cell lysis buffer formulations, such as Laemmli sample buffer and Triton-X 100 buffer, are also compatible with this procedure. Additional optimization of the cell stimulation protocol and cell lysis procedure may be required for each specific application.
2. Remove the cellular debris by centrifuging the lysates at 14,000 x g for 10 minutes. Alternatively, lysates may be ultracentrifugedat 100,000 x g for 30 minutes for greater clarification.
3. Carefully decant the clarified cell lysates into clean tubes and determine the protein concentration using a suitable method, such as the Bradford assay. Polypropylene tubes are recommended for storing cell lysates.
4. React an aliquot of the lysate with an equal volume of 2x Laemmli Sample Buffer (125 mM Tris, pH 6.8, 10% glycerol, 10% SDS, 0.006% bromophenol blue, and 130 mM dithiothreitol [DTT]) and boil the mixture for 90 seconds at 100°C.
5. Load 10-30 µg of the cell lysate into the wells of an appropriate single percentage or gradient minigel and resolve the proteins by SDS-PAGE.
6. In preparation for the Western transfer, cut a piece of PVDF membrane slightly larger than the gel. Soak the membrane in methanol for 1 minute, then rinse with ddH2O for 5 minutes. Alternatively, nitrocellulose may be used.
7. Soak the membrane, 2 pieces of Whatman paper, and Western apparatus sponges in transfer buffer (formulation provided below) for 2 minutes.
8. Assemble the gel and membrane into the sandwich apparatus.
9. Transfer the proteins at 140 mA for 60-90 minutes at room temperature.
10. Following the transfer, rinse the membrane with Tris buffered saline for 2 minutes.
11. Block the membrane with blocking buffer (formulation provided below) for one hour at room temperature or overnight at 4°C.
12. Incubate the blocked blot with primary antibody at a concentration of 0.1-1.0 µg/ml in Tris buffered saline supplemented with 3% Ig-free BSA and 0.1% Tween 20 overnight at 4°C or for 2 hours at room temperature.
13. Wash the blot with several changes of Tris buffered saline supplemented with 0.1% Tween 20.
14. Detect the antibody band using an appropriate secondary antibody, such as goat F(ab)2 anti-rabbit IgG alkaline phosphatase conjugate or goat F(ab)2 anti-rabbit IgG horseradish peroxidase conjugate in conjunction with your chemiluminescence reagents and

instrumentation.

Cell Lysis Buffer Formulation:
10 mM Tris, pH 7.4
100 mM NaCl
1 mM EDTA
1 mM EGTA
1 mM NaF
20 mM Na4P2O7
2 mM Na3VO4
0.1% SDS
0.5% sodium deoxycholate
1% Triton-X 100
10% glycerol
1 mM PMSF (made from a 0.3 M stock in DMSO)
or 1 mM AEBSF (water soluble version of PMSF)
60 µg/mL aprotinin
10 µg/mL leupeptin
1 µg/mL pepstatin
(alternatively, protease inhibitor cocktail such as Sigma Cat. # P2714 may be used)

Transfer Buffer Formulation:
2.4 gm Tris base
14.2 gm glycine
200 mL methanol
Q.S. to 1 liter, then add 1 mL 10% SDS.
Cool to 4°C prior to use.

Tris Buffered Saline Formulation:
20 mM Tris-HCl, pH 7.4
0.9% NaCl

Blocking Buffer Formulation:
100 mL Tris buffered saline
5 gm BSA
0.1 mL Tween 20

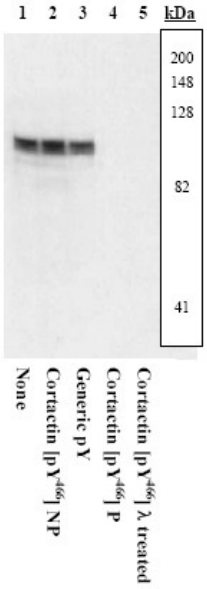
Peptide Competition Experiment
The specificity of a Phosphorylation Site Specific Antibody (PSSA) in each experimental system can be confirmed through peptide competition. In this technique, aliquots of antibody are pre-incubated with peptide containing the sequence of the phosphopeptide immunogen used to raise the PSSA and the corresponding non-phosphopeptide. Following preincubation with the peptide, each antibody preparation is then used as a probe in antibody-based detection methods, such as Western blotting, immunocytochemistry, flow cytometry, or ELISA. With a PSSA specific for the phosphorylated target protein, pre-incubation with an excess of peptide containing the sequence of the phosphopeptide immunogen will block all antigen binding sites, while pre-incubation with the corresponding non-phosphopeptide will not affect the antibody.
In performing the Peptide Competition Experiment, it is important to note that the optimal dilutions of both antibody and peptide should be determined empirically for each specific

application. The optimal dilution of antibody in these procedures is below saturating, as determined by previous experiments in your system.
The optimal dilution of peptide used in these procedures will depend on the overall affinity or avidity of the antibody, as well as the quantity of the target antigen. A 50-150 fold molar excess of peptide to antibody is found to be effective for most peptide competition experiments.
In the example presented below, the PSSA is used as a dilution of 1:1000 and the peptides are used at a concentration of 333 nM. The total volume of the phosphopeptide and nonphosphopeptide pre-incubated antibody preparations is 2 mL, sufficient for probing Western blot strips, as well as for use in other antibody-based detection methods. Under these conditions, the molar excess of peptide to antibody is › / = 50.

Procedure:
1. Prepare three identical test samples, such as identical PVDF or nitrocellulose strips to which the protein of interest has been transferred. The test samples should be blocked using a blocking buffer, such as Tris buffered saline supplemented with 0.1% Tween 20, and either 5% BSA or 5% non-fat dried milk.
2. Prepare 3 mL of a 2x (1 µg/mL) antibody stock solution in a buffer appropriate for the application. Suggested buffer formulations are TBS or PBS supplemented with blocking protein such as BSA or non-fat dried milk.
3. Apportion the unused PSSA into working aliquots and store at -20°C for future use (the stock PSSA contains 50% glycerol and will not freeze at this temperature).
4. Allow the lyophilized control peptides to reach room temperature, ideally under desiccation. 5. Reconstitute each of the control peptides to a concentration of 66.7 µM with nanopure water. (i.e. for a peptide with a molecular mass of 1500, reconstitution with 1 mL water yields a solution with a concentration of 66.7 µM).
6. Apportion the unused reconstituted peptide solutions into working aliquots and store at -20°C for future use.
7. Label 3 test tubes as follows:
- tube 1: water only no peptide control
- tube 2: phosphopeptide
- tube 3: non-phosphopeptide
8. Into each tube, pipette the following components
- tube 1: 2 mL diluted PSSA solution plus 10 µL nanopure water
- tube 2: 2 mL diluted PSSA solution plus 10 µL phosphopeptide
- tube 3: 2 mL diluted PSSA solution plus 10 µL non-phosphopeptide
9. Incubate the three tubes for 30 minutes at room temperature with gentle rocking. During this incubation, the peptides have the chance to bind to the combining site of the antibody.
10. At the end of the incubation step, transfer the contents of each of the three tubes to clean reaction vessels containing one of the three identical test samples.
For Western blotting strips:
Incubate the strips with the pre-incubated antibody preparations for 1 hour at room temperature or overnight at 4°C.
Wash each strip four times, five minutes each, to remove unbound antibody. Transfer each strip to a new solution containing a labeled secondary antibody [e.g., goat F(ab)2 anti-rabbit IgG alkaline phosphatase conjugate or goat F(ab)2 anti-rabbit IgG horseradish peroxidase conjugate.
Remove unbound secondary antibody by thorough washing, and develop the signal using your chemiluminescent reagents and instrumentation.
The signal obtained with antibody incubated with the "Water Only, No Peptide Control" (Tube 1), represents the maximum signal in the assay. This signal should be eliminated by preincubation with the "Phosphopeptide" (Tube 2), while pre-incubation with the "Non-

Phosphopeptide" (Tube 3) should not impact the signal. If the "Phosphopeptide" only partially eliminates the signal, repeat the procedure using twice the volume of water or peptide solutions listed in Step 8. If partial competition is seen following pre-incubation with the "Non-Phosphopeptide", repeat the procedure using half the volumes of water or peptide solutions listed in Step 8.

Pictures:



Antibody-Peptide Competition and Phosphatase Stripping Extracts of NIH3T3 cells stably expressing c-Src Y527F mutant protein were resolved by SDS-PAGE on a 10% Tris-glycine gel and transferred to PVDF. The membrane was left untreated (1-4) or treated with lambda (λ) phosphatase (5), blocked with a 5% BSA-TBST buffer overnight at 4°C, then incubated with the cortactin [pY466] (mouse) antibody for two hours at room temperature in a 3% BSA-TBST buffer, following prior incubation with: no peptide (1, 5), the non-phosphopeptide corresponding to the phosphopeptide immunogen (2), a generic phosphotyrosine-containing peptide (3), or the phosphopeptide immunogen (4). After washing, the membrane was incubated with goat F(ab')2 anti-rabbit IgG alkaline phosphatase and signals were detected using the Tropix WesternStarTM method. The data show that only the phosphopeptide corresponding to cortactin [pY466] (mouse) blocks the antibody signal and that phosphatase treatment removes the signal, demonstrating the specificity of the antibody. While the detected bands appear to run at a somewhat higher molecular weight than expected, identity as cortactin was confirmed by reaction with an antibody to the cortactin protein (data not shown). Cell extracts were kindly provided by Dr. Scott Weed, University of Colorado Health Sciences Center, Denver, CO.