Tyrosine phosphorylation of WASP promotes calpain-mediated podosome disassembly

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Supplementary Design and Methods

Infections of DCs with lentiviral vectors

Lentiviral vector stocks were produced in 293T cells by cotransfecting the transfer vector pHR'SINcPPT-SFFV-mCherry or SFFVeGFP-WASP, SFFVeGFP-WASPY291E, SFFVeGFP-WASPY291F, SFFV-WIP-mCherry, the envelope plasmid pMD.G, and the packaging plasmid pCMVR8.91. We seeded 3 $\times 10^7$ cells onto 150 cm^2 flasks and transfected them with 10 mg DNA envelope, 30 mg DNA packaging, and 40 mg DNA transfer vector by pre-complexing with 0.125 mMPEI (22 kDa) for 15 min at room temperature in Optimem. After 4 h at 37°C, the medium was replaced with fresh DM¬EM 10% FCS and virus were harvested 48 and 72 h posttransfection. After filtering through a 0.45 mm pore size filter, the virus suspension was concentrated by centrifugation at 50,000 g for 2 h at 4°C. The resulting pellet was resuspended in RPMI (Sigma) and stored at -80°C until use. The desired number of DCs or THP-1 cells were plated in complete culture medium and lentivirus containing supernatant was added to the cells at an appropriate MOI (MOI 100-150 for DCs and MOI 10, 20, 50 for THP-1 cells) and incubated for 24 h. Media were replaced for complete DC culture medium after 24 h, and cells were cultured for another 48 h to allow maximal expression of lentiviral vectors before being used in experiments.

Confocal interference reflection microscopy (IRM) and analysis of adhesion turnover

DCs expressing eGFP-WASP, eGFP-WASPY291E or eGFP-WASPY291F were plated on poly-L-lysine (Sigma, UK) coated glass coverslips in complete culture medium and incubated overnight at 37°C in a 5% CO_2 atmosphere as previously described.[1] Coverslips were mounted onto viewing chambers in culture medium. The podosome turnover in DCs expressing eGFP constructs was performed by simultaneously visualizing the GFP signal and adhesion-substratum interface using a Zeiss LSM Meta confocal scanning head, using the 488 nm line of an Argon laser and a 470-500 nm band-pass filter to detect the eGFP signal, and a 505 nm long-pass filter to detect the interference reflection signal. Interference reflection micrographs were processed using Adobe Photoshop® version 7.0 to threshold the adhesion sites of the cells with the substratum. To analyze the persistence of adhesion sites, 5 IRM images taken 30 s apart were used. Each image was thresholded to produce white adhesions on black background and then inverted as black adhesions on white background. The black value of each image was then divided by 5 to obtain dark gray corresponding to adhesions (i.e. 256/5 on the scale of 1-256). The images were then overlapped using the *difference* function in Adobe Photoshop. We thus obtained a composite image with 5 relevant gray levels. The lightest gray level represented pixels that were present in one of the five images (adhesion points last for 30 s), and the darkest gray level represented pixels that were present in 5 out of 5 images (i.e. adhesion points last for 150 s). Therefore, the areas of lighter gray pixels represent dynamic adhesions whereas areas of dark gray and black pixels represent increasingly stable adhesions during the selected time course of measurement. Using Mathematica™ 5.2 notebooks,[2] we quantified the percentage of pixels corresponding to each gray level per image. This allowed us to calculate a turnover index by dividing the percentage of pixels present in 1 or 2 frames by the percentage of pixels present in 4 or 5 frames.[2] Thus, a ratio of unstable adhesion over stable adhesion in each live cell was obtained. The higher value of the turnover index represents the more dynamic of the cell adhesion. Unpaired Student's t-test was used to assess the significance of experimental results.

Pseudocolor method to analyze distribution dynamics of adhesions

Images of distribution of fluorescent signals in DCs expressing eGFP-WASP variants were obtained using a Zeiss LSM 510 Meta confocal scanning head as previously described[3] using the 488-nm line of an Argon laser and a 470-500 nm band pass filter to detect the eGFP signal. A series of 3 images taken 30 s apart were overlapped using our pseudocolor method for analysis of adhesion turnover. Using Photoshop, the first, second and third images were pasted in the red, green and blue channels, respectively, of a TIF image in RGB mode. Adhesions in red represent adhesions that break down after the first frame of the time course, whereas green or blue adhesions that were not associated with red adhesions would have formed in the second and third frame, respectively. Stable adhesions or where adhesions assemble and break down in the same region without spatial translocation will be colored white or pink.

Immunofluorescence microscopy and quantification of size of podosome cores

Freshly prepared 10 μg/mL poly-L-lysine (PLL, Sigma) solution was incubated over sterile glass coverslips for 1 h at room temperature before plating cells. We then fixed 10^5 DCs plated on substratum-coated coverslips overnight with 4% PFA/3% sucrose for 25 min and permeabilized with 0.05% Triton-X-100/PBS for 10 min. Localization of proteins was achieved by means of an appropriate concentration of antibody diluted in 2.5% BSA/PBS at room temperature for 1 h. After three PBS washes, samples were incubated with appropriate secondary antibodies diluted in 2.5% BSA/PBS. For localization of filamentous actin, cells were incubated with 0.1 μg/mL Alexa 568-phalloidin (Sigma) for 45 min at room temperature. Coverslips were then washed three times with PBS and twice with distilled H_20 before being mounted in Vectashield mounting medium (Vector Laboratories, UK). Confocal images were obtained with a Zeiss LSM 510 Meta confocal laser scanning head attached to a Zeiss META Axioplan 2 microscope. LSM 510 software was used to collect sequential images from optical sections taken 0.2 μm apart along the height of podosomes in the z axis. The same software was used to obtain merged confocal projections along the z axis via maxima fluorescence values. Images were exported from Database Files.mdb to TIFF files by Zeiss LSM Image Browser and processed with Adobe Photoshop 7.0 software. Projected images were also used for quantification of the area of the projection of the podosomal F-actin core using Zeiss LSM Image Browser.

Western vlot

Cell lysates from either untreated or protease inhibitor-treated DCs were obtained by adding Laemmli sample buffer to plated cells. Approximately 20 μg of total cell lysate protein was loaded per lane in a 12% SDS-PAGE gel and subjected to electrophoresis. Proteins were blotted onto nitrocellulose membranes with a Bio-Rad Mini protein II transfer apparatus. Blots were blocked with 5% dried milk/TBS-T for 1 h at room temperature, incubated with indicated antibodies at 4°C overnight. After three washes with TBS-T, immunoprobed proteins were detected by incubation with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. After further washes in TBS-T, immunoprobed proteins were visualized by ECL chemiluminescence kit (Amersham, UK), exposed on Hyperfilm ECL (Amersham, UK), and developed with an Imaging Systems Xograph compact X4 developer. Blots were reprobed after treating with stripping buffer at 50°C for 30 min three times.

Cell transmigration assay

A confluent monolayer of SVEC 4-10 cells was generated by plating 3×10^4 cells on 10 μg/mL fibronectin-coated 13 mm diameter coverslips in 24-well plates overnight. SVEC 4-10 cells were activated to induce maximal expression of cell adhesion molecules by incubation with 100 nM LPS (Sigma) for 6 h. DCs were fluorescently labeled by incubation in CFSE (Molecular Probes, UK) and 25×10^3 cells seeded per well in 0.5 mL RPMI. After 1 h, co-cultures of DCs and SVEC were washed once with PBS at 37°C and fixed for 20 min in 4% w/v paraformaldehyde/3% w/v sucrose in PBS at 37°C. Coverslips were stained with Alexa Fluor 568 phalloidin to detect F-actin, and mounted onto slides. Three sequential confocal optical sections were taken at the top, center and bottom of the SVEC monolayer of randomly chosen fields. We scored the percentage of DCs per coverslip found on either the surface of the monolayer (apical end of the monolayer), spanning the monolayer (transmigrating), or having fully crossed the monolayer (basal end of the monolayer) for 50 DCs chosen at random per coverslip in four coverslips per experiment.

Statistics

Statistical significance was assessed using Sigma Plot software. Student's t-test was applied when all the samples in a given experiment followed a normal distribution. Mann-Whitney-Wilcoxon's test was used for comparison of non-parametric samples.

References

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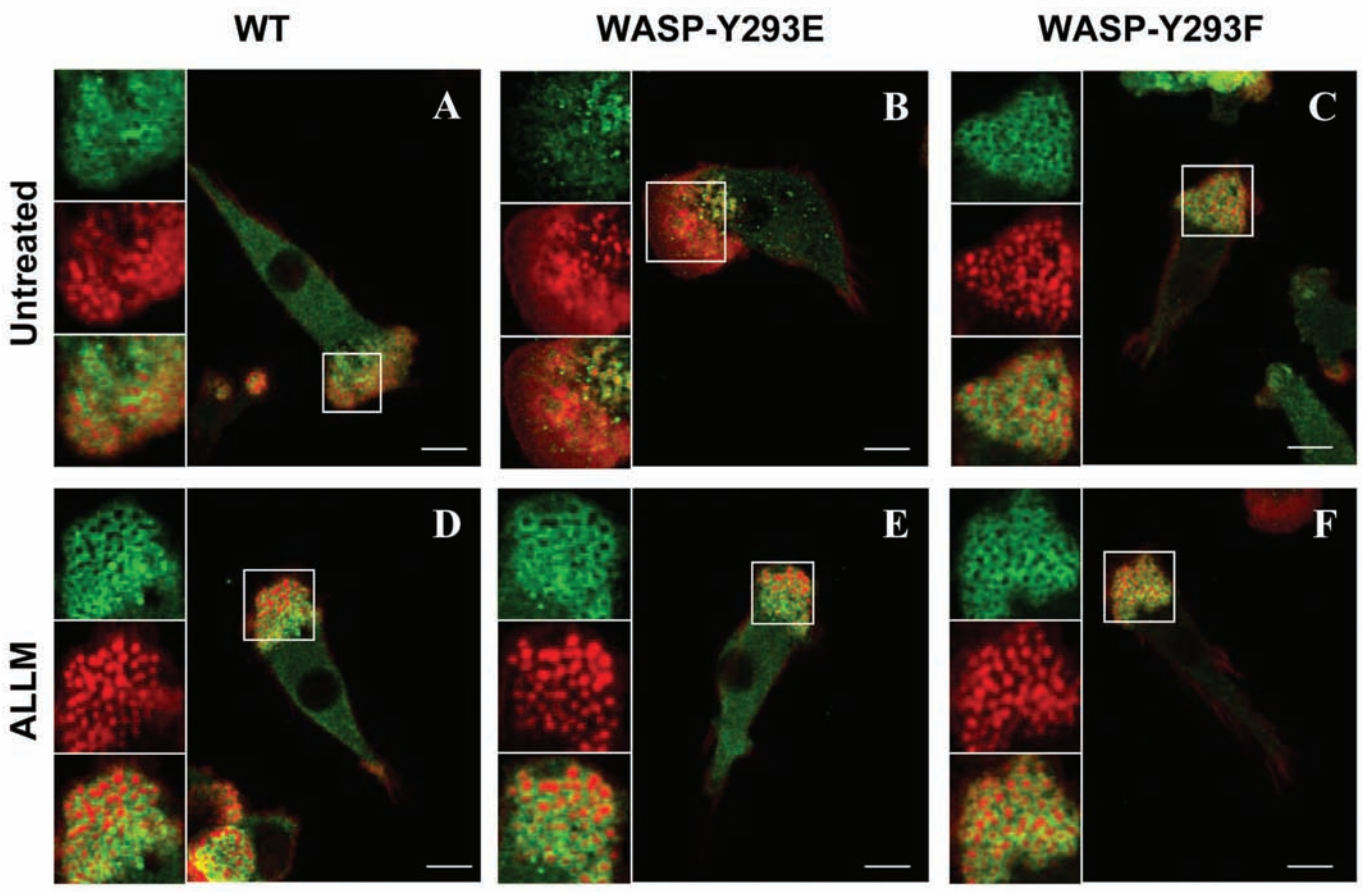

Online Supplementary Figure S1. Phosphorylation of WASP is not required for podosome formation but promotes calpain mediated podosome disassembly. DC were derived from wild-type (WT) mice (A, D). Mice expressing the phosphomimetic version of WASP, WASP-Y293E (B, E) or the phosphodead version of WASP, WASP-Y293F (C, F) and plated overnight on poly-L-lysine coated glass coverslips. DC were left untreated (A-C) or were treated with the calpain inhibitor ALLM at 50 μM for 3 h (C-E) and then fixed with 3% paraformaldehyde, permeabilized with 0.05% Triton X-100 and double-stained to detect vinculin (green) and actin (red). Magnification of the boxed area with vinculin, actin and double staining are shown on the left. Bar 10 μm.

Online Supplementary Video S1. Live confocal time-lapse microscopy imaging of WASP -/- DCs expressing eGFP-WASP seeded on poly-L-lysine-coated coverslips overnight and assembled in viewing chambers. Micrographs were taken 10 s apart and displayed at 10 frames per second. The time lapse video shows that as the margins of the leading edge extend, podosomes assemble at the front of the pre-existing cluster while podosomes at the back of the cluster disassemble. SEE Video 1.gif

Online Supplementary Video S2. Live confocal time-lapse microscopy imaging of WASP -/- DCs expressing eGFP-WASP-Y291E seeded on poly-L-lysine-coated coverslips overnight and assembled in viewing chambers. Micrographs were taken 10 s apart and displayed at 10 frames per second. The time lapse video shows that podosomes in these cells rapidly assemble and disassemble in the same position without coordination with the extending leading edge. SEE Video 2.gif

Online Supplementary Video S3. Live confocal time-lapse microscopy imaging of WASP -/- DCs expressing eGFP-WASP-Y291F seeded on poly-L-lysine-coated coverslips overnight and assembled in viewing chambers. Micrographs were taken 10 s apart and displayed at 10 frames per second. The time lapse video shows that in these cells podosomes form in coordination with the extending leading edge but persist for a longer time compared with eGFP-WASP driven podosomes and are sustained for a longer period of time at the back of the podosome cluster. SEE Video 3.gif