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Short communication

The non-redundant role of N-WASP in podosome-mediated matrix degradation in macrophages

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ABSTRACT

Wiskott-Aldrich Syndrome Protein (WASP) is a hematopoietic cell-specific regulator of Arp2/3-dependent actin polymerization. Despite the presence of the highly homologous N-WASP (neural-WASP), macrophages from WAS patients are devoid of podosomes, adhesion structures in cells of the monocytic lineage capable of matrix degradation via matrix metalloproteases (MMPs), suggesting that WASP and N-WASP play unique roles in macrophages. To determine whether N-WASP also plays a unique role in macrophage function, N-WASP expression was reduced using silencing RNA in a sub-line of RAW 264.7 macrophages (RAW/LR5). Similar to reduction in WASP levels, cells with reduced N-WASP levels were rounder and less polarized. Interestingly, podosomes still formed when N-WASP was reduced but they were unable to perform matrix degradation. This defect was rescued by re-expression of N-WASP, but not by over-expression of WASP, indicating that these proteins play distinct roles in podosome function. Additionally, reducing N-WASP levels mistargets the metalloprotease MT1-MMP and it no longer localizes to podosomes. However, N-WASP was only found to co-localize with MT1-MMP positive vesicles at podosomes, suggesting that N-WASP may play a role on the targeting or fusion of MMP-containing vesicles to podosomes in macrophage-like cells.

Introduction

Macrophages play an important role in the immune response through phagocytosis of foreign pathogens and subsequent antigen presentation. However, macrophages may also destroy non-infected cells when they are recruited to sites of inflammation. In tumors, they may speed the progression of metastasis and their recruitment into breast tumors has been correlated with a poor prognosis (reviewed in Pixley and Stanley, 2004).

Wiskott-Aldrich Syndrome and X-linked thrombocytopenia are X-linked diseases caused by mutations in the gene encoding WASP and are characterized by immune system malfunctions including thrombocytopenia, eczema, recurring infections, and autoimmune disorders (reviewed in Thrasher and Burns, 2010). However, while WASP is only found in hematopoietic cells, the ubiquitously expressed WASP homolog N-WASP is also present in these cells, albeit at low levels (Isaac et al., 2010; Snapper et al., 2001; Stamm et al., 2005; Suzuki et al., 2002), suggesting that N-WASP cannot compensate for the reduced WASP protein levels typically seen in WAS patients or in the WASP knockout mouse.

WASP is important in Arp2/3 mediated actin polymerization and, among other things, regulates the formation of podosomes, dynamic adhesion structures that are localized to the ventral surface of cells that mediate matrix degradation by localization of various enzymes including matrix metalloproteases (MMPs) (Linder, 2007; Yamaguchi et al., 2006). It has also been shown that podosomes are absent in macrophages and DCs derived from WAS patients, with disorganized clusters of F-actin lacking the typical vinculin ring (Burns et al., 2001; Linder et al., 1999). Podosome-like structures are also present in non-hematopoietic cells such as endothelial cells, vascular smooth muscle cells, Src-transformed cells and in certain aggressive cancer cells where they are often called invadopodia. These structures have distinct organization and they contain N-WASP in the F-actin core instead of WASP (reviewed in Linder, 2007).

N-WASP has 50% overall sequence homology with WASP, with conserved domain organization, including a WH1 domain that binds WASP interacting protein (WIP), a proline-rich region that binds SH3 proteins, a GBD domain that binds Cdc42-GTP, and a basic sequence that binds PIP_2 (Miki et al., 1996). At the C terminus, both proteins have a VCA domain, which binds actin via the

Abbreviations: WASP, Wiskott-Aldrich Syndrome Protein; N-WASP, neural-WASP; MMP, matrix metalloproteinase; siRNA, small-interfering RNA.

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V domain and the Arp2/3 complex via the CA region but N-WASP has an additional V domain. *In vitro* data using the purified VVCA domain of N-WASP showed that this domain alone was sufficient for actin polymerization and its activity was higher than the isolated VCA domain of WASP (Zalevsky et al., 2001). This suggests that N-WASP is a better inducer of actin polymerization due to its additional V domain. Additionally, WASP and N-WASP are differentially activated by SH3 proteins *in vitro* where Grb2 and Nck1 are more potent activators of N-WASP than WASP (Tomasevic et al., 2007), consistent with potentially different cellular functions. Also, studies using pathogenic microorganisms suggest WASP family proteins may not compensate for each other completely. For example, Shigella outer membrane protein VirG binds to N-WASP and not to WASP and the defect in Shigella motility in the absence of N-WASP cannot be restored by ectopic expression of WASP (Snapper et al., 2001; Suzuki et al., 2002). However, WASP does compensate for N-WASP in cytoplasmic vaccinia virus motility (Snapper et al., 2001) and Mycobacterium marinum can exploit either WASP or N-WASP interchangeably (Stamm et al., 2005). These studies suggest that WASP and N-WASP may serve redundant or non-redundant functions depending on the cellular context.

Due to the differences between WASP and N-WASP in their activation and cellular processes, it is speculated that they may play unique, non-redundant roles. While many studies have focused on WASP, the function of N-WASP in macrophages has not been clearly elucidated and is the subject of this short communication.

Material and methods

Cells, constructs, antibodies and other reagents

All cells were maintained at 37 °C in a 5% CO_2 incubator. Lac Repressor expressing murine monocyte/macrophage RAW 264.7 cell line (RAW/LR5) (Cox et al., 1997) was grown in RPMI medium (Mediatech Inc, Manassas, VA) containing 10% new born calf serum (Cambrex, Walkersville, MD), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, Saint Louis, MO). RAW/LR5 cells were transfected using Fugene HD (Roche, Indianapolis, IN) with bovine Wild-N-WASP and N-WASPΔV in pSRα (Miki et al., 1998), GFP-N-WASP (Yamaguchi et al., 2002), myc-tagged WT human WASP construct in pEFBos. GFP or mCherry tagged MT1-MMP was a generous gift from Jose Bravo-Cordero (Bravo-Cordero et al., 2007). RNA-mediated interference of WASP and N-WASP was performed according to Park and Cox (2009). Chicken anti-N-WASP (Clone AE920) (Isaac et al., 2010) was generated based on a sequence indicated in Miki et al. (1996). The following commercial antibodies were also used: monoclonal mouse anti-β-actin (Clone AC-15) and anti-vinculin (hVin1; Sigma), monoclonal mouse anti-MT1-MMP (Clone Ab-4; Calbiochem), mouse anti-GFP (Roche), rabbit anti-WASP (H250; Santa Cruz). Alexa Fluor 647-, 568-, and 488-conjugated secondary antibodies or phalloidin were from Molecular Probes (Eugene, OR).

Immunofluorescence microscopy

Cells were plated on either 12 mm glass coverslips or 35 mm uncoated glass bottom microwell dishes (MatTek Corporation, Ashland, MA) and were allowed to adhere overnight. Cells were fixed for 7 min in 3.7% formaldehyde in BWD buffer (20 mM HEPES, 125 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM KH_2PO_4, 10 mM $NaHCO_3$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4), permeabilized for 5 min in 0.2% Triton X-100 (in BWD), and blocked in 10% milk in TBS (137 mM NaCl, 24.7 mM Tris, pH 7.4). For MT1-MMP staining, LR5 cells were simultaneously fixed and permeabilized with saponin to preserve membrane integrity, as described (Eddy et al., 2000). Cells were incubated with the primary antibodies of interest in TBS and then with fluorophore-conjugated secondary antibodies. Finally, coverslips were mounted in antifade mounting medium (50% glycerol, 1× PBS, 100 mM n-propyl gallate (Sigma)) for widefield or in PBS for TIRF microscopy. Images were taken by either TIRF or epifluorescence on an Olympus IX71 microscope (Olympus, X150/1.45 NA, oil objective) coupled to a Sensicam cooled CCD camera.

Morphological parameters were analyzed as follows: a cell displaying migratory morphology was defined by an unambiguous leading and trailing edge (Wheeler et al., 2006). The elongation index was determined using ImageJ (http://rsb.info.nih.gov/ij/) by tracing the cells and measuring the ratio of the major axis over the minor axis of the fit ellipse. At least 60 cells per experiment were analyzed for each determination. Podosomes were identified as F-actin punctate structures surrounded by a vinculin ring. F-actin core intensity was obtained by identifying the area inside the vinculin ring from F-actin and vinculin merged image and circling the corresponding area on F-actin alone image for intensity measurement. At least 100 podosomes per cell type were analyzed for each experiment.

Matrix degradation assay

Degradation of fluorescently labeled fibronectin (FN) by RAW/LR5 cells was determined by a protocol detailed in Dovas et al. (2009). Briefly, cells were plated on either Alexa488 or Alexa568 fluorescently labeled fibronectin for 16 h and areas of loss of fluorescence were measured as degradation area per cell. For rescue experiments shWASP or shN-WASP cells, were transfected with the indicated constructs followed by plating on Alex568 labeled fibronectin as described above. Transfected cells were identified by either GFP labeling or staining for Myc. At least 50 cells per experiment were analyzed.

Western blotting

Cells were lysed for 10 min in ice-cold lysis buffer (1% Triton X-100, 25 mM Tris, 137 mM NaCl, 2 mM EDTA, 1 mM sodium orthovanadate, 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, pH 7.4). Clarified whole-cell lysates were mixed with 5× Laemmli buffer and boiled. Samples were run on an 8% SDS-PAGE gel, transferred onto PVDF membranes (Immobilon-P, Millipore), and blotted with appropriate antibodies. Blots were exposed using Kodak IS440 machine and bands were quantified using Kodak ID 3.6 (Kodak Imaging Systems).

Data analysis

Results were considered statistically different when analysis using a Student t-test resulted in differences between two means with a p value of less than 0.05. All graphs indicate mean values and error bars signify standard error of the mean.

Results and discussion

N-WASP is required for macrophage polarization

It has been suggested in the field that WASP and N-WASP have non-redundant functions given the defect associated with the absence of WASP despite the presence of N-WASP (Calle et al., 2006). To determine if N-WASP has a distinct function from WASP, we initially determined N-WASP localization in a macrophage cell line (RAW/LR5) transiently transfected with GFP-N-WASP (Fig. 1). While WASP has been consistently localized to podosomes in human and mouse macrophages, dendritic cells and osteoclasts (Calle et al., 2004a, 2004b; Linder et al., 1999), N-WASP was