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The Wiskott-Aldrich syndrome

J. S. Orange[a,*], K. D. Stone[b], S. E. Turvey[c] and K. Krzewski[d]

[a] Division of Immunology, Children's Hospital of Philadelphia, Department of Pediatrics, University of Pennsylvania School of Medicine, 3615 Civic Center Blvd, Philadelphia, Pennsylvania 19104 (USA), Fax: +1 267 426 5727, e-mail: orange@mail.med.upenn.edu
[b] Division of Immunology, Children's Hospital Boston, Department of Pediatrics, Harvard Medical School, 300 Longwood Ave., Boston, Massachusetts 02115 (USA)
[c] Division of Infections and Immunolocal Diseases, Department of Pediatrics, University of British Columbia, 317-2194 Health Sciences Mall, Vacouver, BC VT6123 (Canada)
[d] Department of Molecular and Cell Biology, Harvard University, 7 Divinity Ave., Cambridge, Massachusetts 02138 (USA)

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Abstract. The Wiskott-Aldrich Syndrome (WAS) is an inherited immunodeficiency caused by a variety of mutations in the gene encoding the WAS protein (WASp). WASp is expressed in hematopoetic cells and facilitates the reorganization of the actin cytoskeleton in response to many important cell stimuli. Extensive study of WAS and more recently WASp has given great insight into the relevance of this molecule and related molecules to both basic cell biology and human immune defenses.

Key words. Wiskott-Aldrich syndrome; WASp; cytoskeleton; actin; primary immunodeficiency.

History

The Wiskott-Aldrich Syndrome (WAS) was first recognized in 1937 when Wiskott described the clinical phenotype of three brothers presenting in early life with thrombocytopenia, bloody diarrhea, eczema and recurrent ear infections [1]. Subsequently, Aldrich clearly demonstrated the X-linked mode of inheritance of WAS [2]. The immune deficiency accompanying WAS, however, was not truly appreciated until years later [3, 4] (the history of WAS is thoroughly reviewed in [5]). In the decades since Wiskott's initial observation, advances in technology have afforded clinicians and scientists a remarkable understanding of the molecular mechanisms responsible for this primary immune deficiency.

* Corresponding author.

Clinical and immunologic disease characteristics

Clinical manifestations

The incidence of WAS is estimated at between 1 in 10^5 and 1 in 10^6 live births, although this is likely to be an underestimation, as patients lacking the classic phenotype are often unrecognized [6, 7]. Classic WAS is an X-linked condition characterized by the clinical triad of thrombocytopenia, eczema and recurrent infections (table 1). However, this classic triad is only seen in 1 in 4 patients, and initial clues regarding the diagnosis of WAS are easily overlooked. Consequently, diagnosis is often delayed until around the time of the child's second birthday [8].

Perhaps the most reliable feature of WAS is thrombocytopenia (usually platelet counts of $<70{,}000/mm^3$ in patients who have not undergone splenectomy; normal is ~220,000 to $460{,}000/mm^3$) with low platelet volume (usually <5 fl; normal is ~7–10 fl). As both of these features are present from birth, neonates may present with

Table 1. Summary of the Major Clinical Features of WAS.

Clinical features
Thrombocytopenia with small, defective platelets
Immunodeficiency
Eczema
Autoimmune disease
Malignancy
X-Linked inheritance pattern

bruising, petechiae, rectal bleeding and bleeding following circumcision. The severity of the platelet defect in WAS should be respected, since 30% of patients experience a life-threatening bleeding episode before they are diagnosed with WAS. Mutations in the gene responsible for WAS also cause X-linked thrombocytopenia (XLT) [9, 10], a condition with the same platelet abnormalities seen in WAS, but without significant immune dysfunction.

Recurrent infections, particularly sinopulmonary infections, are a hallmark of WAS. The largest study of the clinical features of patients with WAS is a retrospective multiinstitutional survey of 154 patients published in 1994 [8]. According to this survey, the most common infections that afflict patients with WAS after diagnosis of their condition are otitis media (78%), pneumonia (45%), sinusitis (24%), sepsis (24%) and infectious diarrhea (13%). Viral infections are also problematic, with varicella (16%), herpes simplex I and II (16%), molluscum contagiosum (9%) and warts (7%) being the most common. *Pneumocytis carinii* pneumonia (PCP), often found in other combined immunodeficiencies, only occurs in 9% of WAS patients.

Eczema, one of the classic triad of WAS symptoms, is another early manifestation that develops in more than 80% of affected individuals [8]. Although it has the potential to be debilitating for some patients, there can be marked variability in severity. The relation of WAS to atopy is somewhat more obscure. Greater than 30% of patients have eosinophilia [8], and many have elevated serum immunoglobulin (Ig)E concentrations [11, 12]. Immediate hypersensitivity to food antigens can be found clinically as well as by in vivo assays demonstrating food-specific IgE [13, 14]. The presence of specific IgE, however, does not necessarily correspond to clinical immediate hypersensitivity, which may point to defects in cells that possess IgE receptors (as demonstrated in a murine model of WAS [15]). Despite this, anaphylaxis has been found in a WAS patient in response to double-blind, placebo-controlled challenge with a particular food for which the child had elevated specific IgE [J.S.O., unpublished].

Autoimmune and inflammatory diseases also occur at high frequencies in patients with WAS (reviewed in [16]). Between 40 and 72% of those with WAS are affected by autoimmunity [8, 17], and ~25% of patients have multiple autoimmune phenomena. Autoimmune hemolytic anemia (AIHA) is the most common autoimmune disease observed, affecting as many as 36% of WAS patients and typically developing before the age of 5 years [8, 17]. The development of AIHA is a poor prognostic factor as demonstrated in a multivariate, retrospective analysis of 55 WAS patients [17]. There is also a relationship between AIHA and development of lymphoproliferative disorders in both the general population [18] and WAS patients [8]. High serum IgM before splenectomy is a risk factor for developing AIHA and as such is a marker of poor outcome. Other important autoimmune diseases in this population include neutropenia with anti-neutrophil antibodies (25%), arthritis (10– 29%), vasculitis (13–22%) and inflammatory bowel disease (3–9%). It has been recommended that WAS patients who develop significant autoimmunity, especially AIHA and cerebral vasculitis, should receive definitive treatment with hematopoietic stem cell (HSC) transplantation (HSCT) as early as possible [17].

As seen in other immunodeficiencies, many WAS patients develop malignancies at a young age. Malignancy affects ~13% of boys with WAS, and the average age at diagnosis is 9.5 years [7, 8]. Most malignancies are lymphoreticular in origin [Epstein-Barr virus (EBV)-positive B cell lymphoma is particularly common], but many other types can also occur [8]. Improved diagnosis and patient care technology is resulting in increasing survival of WAS patients, and thus the incidence of malignancy in WAS is also likely to increase.

Platelet abnormalities

Thrombocytopenia with small platelets is a consistent finding in WAS (table 2). Automated blood cell counters rely on preset gates to measure platelet size and volume, and thus the extremely small platelets (often colorfully referred to a 'platelet dust') in WAS patients are usually overlooked. Consequently, non-automated techniques are recommended to count and size platelets in cases

Table 2. Summary of the Major Laboratory Features of WAS.

Laboratory features
Decreased platelet count (< 70,000 /mm^3)
Decreased mean platelet volume (MPV) (3.8–5.0 fl)
Progressive development of lymphopenia after age 6 years
Normal IgG, normal or low IgM, elevated IgA and elevated IgE
Poor antibody response to polysaccharide antigens
Diminished T cell response to a range of stimuli
Decreased NK cell cytotoxicity per NK cell
Decreased expression of CD43
Impaired monocyte chemotaxis and phagocytosis
Non-random maternal X-chromosome inactivation
Mutations in the gene encoding Wiskott-Aldrich syndrome protein (WASp)

of suspected WAS. A platelet count of less than 70,000 platelets/mm^3 is required to meet formal diagnostic criteria [19], although counts may be transiently elevated in the context of infection or inflammation. The mean platelet volume (MPV) is generally half that of healthy children (MPV = 3.8–5.0 fl in patients with WAS vs 7.1–10.5 fl in controls) [20]. Splenectomy improves both platelet number and platelet size [21–24]. The exact mechanism responsible for platelet abnormalities in WAS is still somewhat unclear, but a variety of specific defects are known, including increased Ca++ fluxes, phosphatidylserine expression and microparticle release [25]. Interestingly, the molecular pathways responsible for the platelet defects in WAS may be distinct from those causing defects in other hematopoetic cells [26].

Immunological defects

Most patients with WAS have evidence of both cellular and humoral immune dysfunction (table 2). During infancy lymphocyte number is generally normal, but by the age of 6–8 years lymphopenia is common [8, 20]. Serum immunoglobulin levels are variable, but the general pattern is normal IgG, normal or low IgM, elevated IgA and elevated IgE [8, 17, 19]. More important than the total immunoglobulin level is an individual's ability to produce antibody against relevant antigens. A characteristic feature of WAS is the inability to generate appropriate antibody titers against polysaccharide antigens, as evidenced by low isohemagglutinins and poor response to pneumococcal polysaccharide vaccination [4, 8, 20]. Antibody production to protein antigens also is depressed, and immunization with the neoantigen bacteriophage ϕX174 results in a failure to switch from IgM to IgG [5]. Although administration of live viral vaccines is generally contraindicated in patients with combined immunodeficiencies, the response to live viral vaccines in WAS patients tends to be normal [8].

Lymphocyte function is also generally depressed in WAS patients. Patient T cells respond poorly in vitro to mitogens [4, 8, 27], allogeneic cells [8, 20], immobilized anti-CD3 monoclonal antibody [28] and periodate [27]. T cell function in vivo as assessed by delayed type hypersensitivity skin testing is negative in most patients [8], but results can be difficult to interpret as platelet dysfunction often results in intradermal bleeding at the site of injection [5]. Phenotypically, T cells from WAS patients are often notable for absent or decreased expression of CD43 [29], although this feature is not found in all patients [30]. The function of natural killer (NK) cells has also been evaluated in WAS patients by standard 51Cr release assay and is typically quite variable when assessed as activity per total peripheral blood mononuclear cells [31–34]. The percentage of NK cells in WAS patients, however, is unusually high, and cytolytic activity per NK cell is markedly decreased [35].

Leukocyte chemotaxis in WAS patients is defective [20], and patient monocytes migrate poorly in response to formyl-methionyl-leucyl-phenylalanine or monocyte chemoattractant protein-1 [36, 37]. The chemotactic response of polymorphonuclear leukocytes, however, is more variable and implies that the defect is most pronounced in certain hematopoietic cell lineages. Phagocytic functions can also be defective in cells from WAS patients, and in particular the ingestion of IgG-coated particles is impaired [38]. Finally, based on studies in a murine model of WAS, antigen presentation of particulate, but not soluble antigens appears to be specifically impaired in disease [39]. These features may be of clinical significance and probably contribute to the overall susceptibility to bacteria found in WAS.

Current clinical management of WAS

Natural history of disease

WAS is fatal without curative bone marrow transplantation, but life expectancy has increased over time. For patients born with WAS before 1935, the median survival was 8 months [7]. Patients born from 1965 to 1978 survived an average of 6.5 years [7], while more recent data demonstrate an average life expectancy of 8 years [8]. Living patients in the more recent survey have an average age of 11 years with a range of 1– 35 years. Several case reports have also described patients with WAS living into their second and third decades [40, 41]. Unfortunately, there is still significant heterogeneity in the clinical management of WAS patients [42], complicating the interpretation of therapeutic interventions. This highlights the desperate need for additional objective clinical data and organized study of this rare, but significantly ill patient population [43].

Platelet dysfunction

With approximately one quarter of WAS patients dying from bleeding complications [8], management of thrombocytopenia is a central issue. Drugs that interfere with platelet function (particularly non-steroidal antiinflammatory medications) must be avoided. Platelet transfusions should be used sparingly for serious bleeding that is not stopped by conservative measures. To prevent transfusion-related graft-vs-host disease (GvHD), all blood products must be irradiated, and the donors should be free of cytomegalovirus. Intravenous immunoglobulin (IVIG) alone rarely increases platelet counts [8, 44], but can be effective if the thrombocytopenia is autoimmune in nature when used in high dose and combined with systemic corticosteroids [5, 8]. The addition of vincristine to IVIG and corticosteroids may also be beneficial in refractory autoimmune thrombocytopenia [45]. Splenectomy, how-

ever, is well established to increase platelet number and size, and reduce bleeding complications [8, 21–23]. Unfortunately, these benefits come at the cost of an increased risk of infection (particularly overwhelming pneumococcal sepsis); accordingly, lifelong prophylaxis is warranted following splenectomy.

Immunodeficiency

Despite the observation that most patients with WAS have relatively normal immunoglobulin levels, prophylactic immunoglobulin replacement therapy is often used in an effort to prevent infections, as antigen-specific antibody production is defective. Prophylactic therapy with oral antibiotics can also serve as an adjunct measure to prevent bacterial infection. A survey of 73 clinical centers caring for 507 WAS patients revealed that the majority of these institutions utilize these therapies [42]. Retrospective review of the utility of immunoglobulin replacement and antibiotic prophylaxis suggests that these measures are effective in maintaining the health of WAS patients [23]. Nevertheless, vigilance must be maintained for the early signs of infections to allow prompt detection and initiation of therapy. Definitive treatment for the immunodeficiency associated with WAS is HSCT and is discussed below.

Eczema

For some boys with WAS, eczema is a major clinical feature. Emollients and topical steroids are effective therapies, and treatment of bacterial superinfection requires systemic antibiotics. Newer topical non-steroidal antiinflammatory medications, such as tacrolimus and pimecrolimus, may be useful; however, if skin damage is severe, there is concern that these medications may be absorbed, resulting in systemic immune suppression. Food allergies may also contribute to the eczema, and clinical benefit resulting from the identification and elimination of the allergenic food protein has been suggested [14]. Interestingly, eczema is worse in WAS patients whose families have an atopic diathesis and thus may be controlled by genes associated with atopy [46].

Hematopoietic stem cell transplantation

HSCT is the only currently available curative option for WAS and has been used effectively for over 25 years [47]. Since patients with WAS have vigorous, although dysregulated, T cell function, pre-transplant conditioning is essential. The outcome of 170 transplantations for WAS, from 1968 to 1996, reported to the International Bone Marrow Transplant Registry and/or National Marrow Donor Program have been subjected to a multivariate analysis of the impact of donor- and treatment-related variables on the outcome [48]. Optimal results are achieved using human leukocyte antigen HLA-matched sibling donors, with 87% survival at 5 years. This confirms the benefits to HLA-matched sibling donors reported in other studies of transplantation for WAS [22, 49, 50]. The 5-year survival for patients receiving cells from HLA-matched unrelated donors is 71%, while the use of other related donors (including haploidentical donors) results in 52% of patients surviving after 5 years. About 75% of patients receiving their cells from HLA-matched sibling donors or HLA-matched unrelated donors are described as ‘cured’ by the transplant, compared to only 57% for haploidentical or other related donor transplants. Some of the discordance between engraftment and clinical effect may be a feature of the mixed chimerism in hematopoetic cell populations that is found post-transplant [51]. Interestingly, there is not a significant difference in the risk of death as a consequence of transplantation in children younger than 5 years who receive HSCs from either an HLA-matched sibling or HLA-matched unrelated donor. Cord blood appears to be a reasonable alternative source of HSCs but is probably best reserved for small, cytomegalovirus-negative recipients [52, 53]. Nevertheless, a direct comparison of the relative efficacy of unrelated cord blood versus unrelated bone marrow transplants has not been reported for this disease.

When compared with contemporary reports of the natural history of WAS, these transplantation outcomes should engender optimism for this potentially curative approach [48]. Despite its shortcomings, the majority of clinical centers caring for WAS patients utilize HSCT if an HLA-matched donor is available [42].

Genetics of WAS

Although Aldrich demonstrated an X-linked pattern of inheritance for WAS [2], 40 years passed before the gene on the X chromosome responsible for disease was identified [54]. WAS was initially linked to Xp11.22-11.23 by several groups [55–57], and the mutant gene, *WASP*, was identified by subsequent positional cloning efforts in this region [54]. The gene contains 12 exons and codes for the WAS protein (WASp), which contains 502 aa [58]. Several hundred mutations associated with disease have been discovered in the *WASP* gene, and a comprehensive database is available at http://homepage.mac.com/kohsukeima/wasp/WASPbse.html. In general, although there are many exceptions, missense mutations affecting the N-terminus result in XLT, whereas C-terminal and more extreme mutations cause WAS (reviewed in [59]). Some *WASP* mutations cause disease outside of typical WAS/XLT. These include very mild missense mutations affecting the C-terminus resulting in an intermittent form of XLT [60] and a gain-of-function missense mutation af-

fecting WASp position 270 that results in X-linked neutropenia without signs of WAS/XLT [61].

In an attempt to quantify the previously discussed clinical characteristics of WAS, a scoring system based upon disease severity has been proposed [62] and is useful in correlating WAS phenotypes to particular genotypes [12, 63]. This system provides a score of 1–5, with 1 being indicative of XLT only and 5 being characterized by the most severe clinical sequelae of WAS, including autoimmunity or malignancy. In general, missense mutations of the first three exons result in detectible WASp and mild disease (score 1–2). In contrast, nonsense mutations, frameshift mutations, splice site mutations, insertions or deletions result in undetectable or unstable WASp and are associated with classic WAS and or severe disease (score 3–5). Although this paradigm is useful, many exceptions exist, including exon 4 missense mutations and certain other N-terminal alterations that result in classic WAS. An additional association between mutation, cellular WASp content and phenotype has also been demonstrated, and more extreme *WASP* mutations result in a lower level of WASp and more severe phenotype [64]. These data have been further substantiated in relation to the presence or absence of WASp by Western blot with respect to the clinical scoring system [12].

Asymptomatic females suspected to be carriers of a mutation responsible for WAS can be identified by examining the pattern of X-chromosome inactivation. Carriers of *WASP* mutations have non-random X-chromosome inactivation with the normal X chromosome being active in all hematopoietic cell lineages [65–69]. Despite this, a number of females with clinical and laboratory findings consistent with WAS have been described. Generally, female carriers of the defective gene have no clinical features of WAS. Their mature blood cells demonstrate a non-random pattern of X chromosome inactivation with the chromosome bearing the normal *WASP* gene active in most hematopoetic cells [65–69]. The non-random pattern of X inactivation in WAS carriers has recently been suggested to result from impaired homing of WASP-defective hematopoietic stem cells to the bone marrow during fetal development [70]. At least two mechanisms appear to be responsible for females developing the clinical phenotype of WAS. First, a impaired in the X-inactivation-specific transcript (*XIST*) gene, which is required to be expressed on the inactive X chromosome, may prevent silencing of the X chromosome carrying the mutated *WASP* gene [71]. Another possible mechanism is that defective selection allows lymphocytes, monocytes and platelets expressing the mutated *WASP* gene to survive and hence contribute to the development of the WAS phenotype [72].

Due to the diversity of *WASP* mutations throughout the length of the gene that causes WAS, sophisticated techniques can be required to identify mutations. Fortunately, a simpler, clinically applicable approach based on directly sequencing amplified exons according to a staggered schedule determined by statistical evaluation of the location of previously identified mutations can be used [73]. In addition, a method for flow cytometric analysis of intracellular WASp expression is useful for diagnosing WAS in patients and carriers, as well as following chimerism longitudinally after bone marrow transplantation [51, 74]. Prenatal genetic screening can be performed on the fetus in a woman known or suspected to carry a *WASP* mutation using chorionic villus samples or amniocytes as the source of DNA [75–77]. Alternatively, fetal platelet analysis is useful, as platelet volume and size in 17- to 21-week fetuses does not differ from those of newborn infants and adults and can be used to identify affected males in utero [75–77].

WASp function

The function of WASp was suggested from observations made in cells from patients with WAS. It was initially noted that lymphocytes from WAS patients have abnormal cell morphologies, decreased number of microvilli and abnormally short microvilli [78, 79]. Since cell shape and microvilli depend upon an intact cytoskeletal structure, these findings strongly suggested that the gene responsible for the WAS would be important in the function of the cytoskeletion. The discovery of WASp subsequently allowed its investigation in cytoskeletal reorganization. Experimental overexpression of WASp results in focal actin polymerization that depends upon the presence of specific regions of WASp that are homologous to actin binding domains found in other proteins [80]. As WASp was ultimately shown to bind actin directly [81], it was clear that the protein serves a critical role in the actin cytoskeleton. The basic biology and biochemistry of the actin cytoskeleton has evolved significantly and has been reviewed extensively [82, 83]. The major utility of WASp in relation to the cytoskeletion is the promotion of actin filament branching. The addition of branches to actin filaments allows them to grow with strength and push overlying membranes. This allows cells to deform, move and reorganize their surfaces [84].

WASp promotes actin branching by approximating an actin monomer and the actin branching complex Arp2/3. Arp2/3 is a group of seven proteins (Arp2, Arp3 and ARPC1–5) that binds to the sides of pre-existing actin filaments in order to initiate an actin branch. Arp2 and Arp3 become the first two subunits of the new actin branch (daughter filament), which is extended at a 70° angle to the existing filament (mother filament). Arp3 is closest to the branch at the 'pointed' end of the new filament, and Arp 2 is at the 'barbed' end to which the actin monomer is added in order to begin filament elongation.

As Arp2 and Arp3 are typically too far apart to initiate this process, the complex needs to undergo a conformational change for a branch to be formed. Using bound WASp and ATP, Arp2 can be approximated to Arp3 to create an actin nucleation site [85, 86]. Importantly, the Arp2/3 complex and the initial actin monomer can specifically localize to sites where actin branching is desired through proteins that bind WASp. These critical functions of WASp necessitate that it be tightly regulated. In this regard, WASp has the characteristic feature of autoinhibition that can be reversed by specific activation signals [87] and is further addressed below.

An important concept intimately associated with the actin cytoskeleton is that of the immunological synapse. The immunological synapse refers to the specific interface created within an immune cell at the point it interacts with another cell or object and has been reviewed in detail [88–90]. The purpose of the immunological synapse is an active area of investigation, but includes the generation, amplification and termination of cell signaling programs as well as directed secretion of cellular contents. A variety of molecules localize to immunological synapses, both on the cell surface and intracellularly in order to enable its function. The actin cytoskeleton is a critical element of cell-activating immunologic synapses and typically contains accumulated actin filaments. As WASp is required for the accumulation of actin clusters induced by the ligation of specific activating receptors [38, 91–93], it comes as no surprise that WASp is also found at the immunological synapse in a variety of hematopoetic cells [35, 94] (fig. 1a). Cell-activating synapse formation requires actin function, and in particular cell types, requires WASp function [95–98] (fig. 1b, c). However, in certain cell systems particular cell-activating surface receptor clustering may be WASp independent [99]. In these cases, there are probably other relevant proteins that compensate to reorganize of the cytoskeleton. Alternatively, some receptors in hematopoetic cells can cluster at the immunologic synapse independent of actin [100]. The biochemistry of WASp (discussed below) demonstrates that it serves both adaptor and effector roles that are fundamental in the organization, rearrangement and function of molecules at and to immunological synapses.

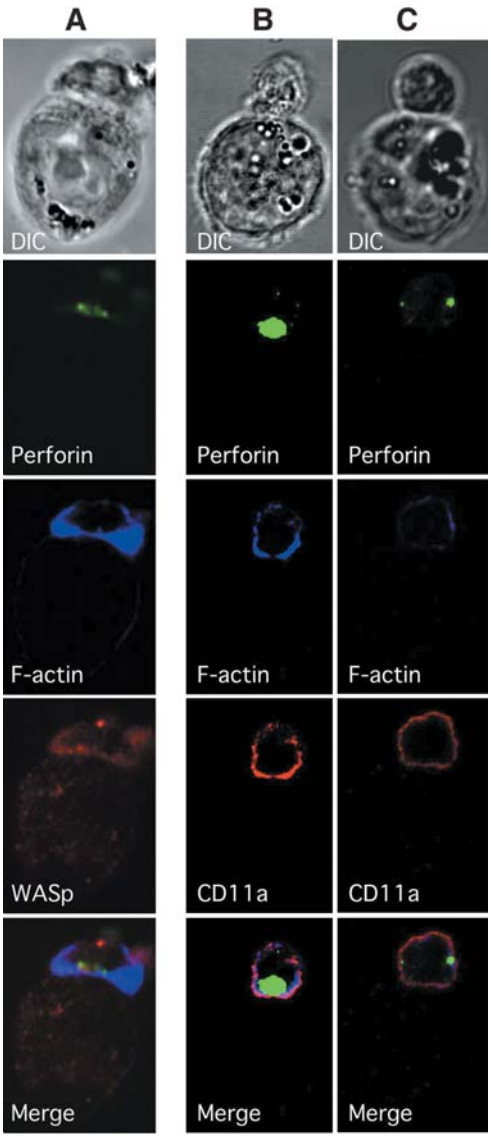

Figure 1. WASp and the immunological synapse. The immunological synapse between ex vivo human NK cells (small cells) and K562 erythroleukemia cells (large cells) was evaluated as described [35], and differential interference contrast images (DIC, top row), as well as fluorescence for the pore-form-molecule perforin (green, second row), filamentous actin (blue, third row) and WASp or the α integrin-CD11a (red, fourth row) are shown. An merged overlay of all fluorescent channels is provided in the fifth row. Evaluation of perforin and filamentous actin relative to WASp (*A*) or perforin and filamentous actin relative to CD11a (*B*) in mature activating immunologic synapses of normal NK cells are shown. The mature activating NK cell immunologic synapse is characterized by the accumulation of CD11a, filamentous actin, WASp and perforin at the interface between the NK cell and target cell. The localization of perforin and filamentous actin relative to CD11a in an NK cell from a patient with a G252A mutation in *WASP* is also shown (*C*). This patient does not have detectable WASp and fails to effectively accumulate perforin, filamentous actin and CD11a at the mature activating NK cell immunologic synapse.

The WASp family of proteins

The WASp family of proteins consists of several members that all possess the ability to interact with the actin cytoskeleton and have three homologous regions: a basic region (BR), a central Pro-rich domain and a C-terminal VCA domain. The BR contains multiple basic residues, the Pro-rich domain contains multiple conserved Pro residues, and the VCA domain is comprised of three regions: a ver-

prolin homology region (V), cofilin homology/central region (C) and acidic region (A). The WASp family can be further divided into two subgroups based upon the N-terminal structure, with the first containing a WASp-homology domain 1 (WH1) and the second a suppressor of cAMP receptor (SCAR)-homology domain (SHD) (fig. 2). The second subgroup is also referred to as the WASp family verprolin-homologous (WAVE) proteins.

Although our focus is upon WASp, SCAR/WAVE proteins also function in actin cytoskeleton rearrangements, but are regulated differently from WASp proteins. First, unlike WASp, SCAR/WAVE proteins are not autoinhibited [101] and obtain negative regulation from other proteins. WAVE1, for example, exists in an inactive form complexed with inhibitory proteins and can be liberated by Rac1 and Nck [102]. Second, SCAR/WAVE proteins participate in Rac signaling and lamellipodia formation, but do not bind Rac directly [103, 104]. In contrast to the interaction between Cdc42 and WASp (discussed in detail below), SCAR requires a specific adaptor protein, the insulin receptor substrate p53 (IRSp53) to bind Rac [104].

A related group of proteins that interact with the actin cytoskeleton and share homology with WASp are the Enabled (Ena) and vasodilator-stimulated phopshoprotein (VASP) family (reviewed in [105]). These proteins serve some similar functions to WASp and contain a WH1 (also known as an enabled VASP homology (EVH1) domain) and Pro-rich domain, but are outside of the WASp family as they do not contain a VCA domain (fig. 2). The WH1 domain of WASp family members has many similarities to the EVH1 domain of Ena/VASP proteins, but binds different motifs [106] as well as substrates in different orientations [107]. Due to the distinctions between the WASp subfamily and the SCAR/WAVE and Ena/VASP proteins, futher discussion will focus on the vast body of work specific to the structure and regulation of WASp subfamily proteins.

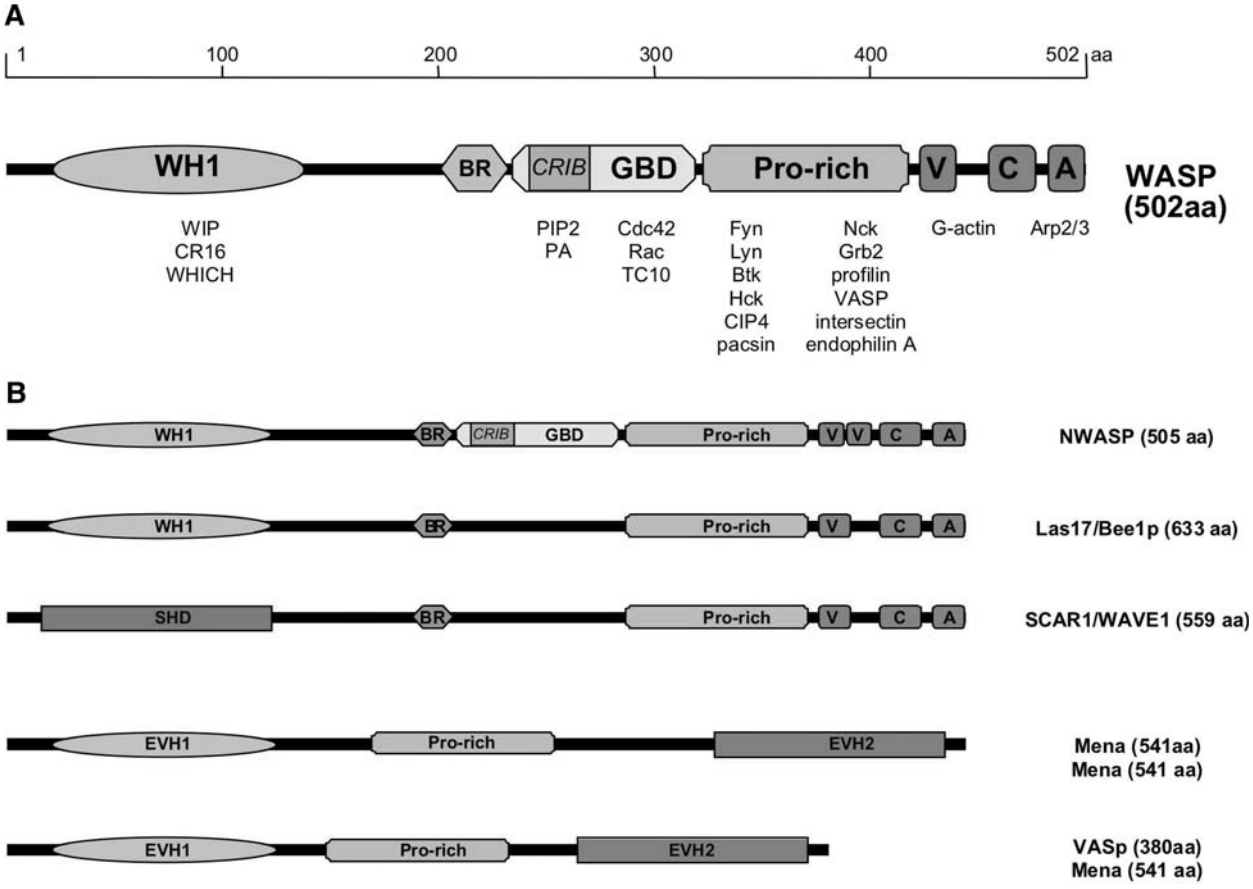

Figure 2. Organization of WASp domains and comparison to selected related proteins. (*A*) WASp is depicted with each major structural domain marked. The scale above the model approximates the length of WASp and its domains as well as their spacing. Molecules known to bind WASp are listed below the domain to which they most likely bind. See text for details and abbreviations. (*B*) The organization of the domains of five WASp-related proteins, labeled on the right, is shown. The length of the proteins is shown in parentheses, and they are not drawn to scale. The distances between domains is not constant, but are aligned for comparison. Only domains relevant for comparison to WASp are pictured.

Structure and functional biochemistry of WASp

WH1 domain

The N-termini of WASp and NWASp form the WH1 domain (WASp aa 35–145), which has a similar tertiary structure to other EVH1 domains [107]. The NWASp WH1 domain (fig. 3A) is composed of six β strands, forming two β sheets, and one α helix. Strands 1, 2, 5 and 6 create the base for a peptide binding groove for the minimal NWASP WH1 domain binding ligand of 25 amino acids (more than twice as long as the typical EVH1 binding peptide). The WH1 domain preferentially binds Pro-

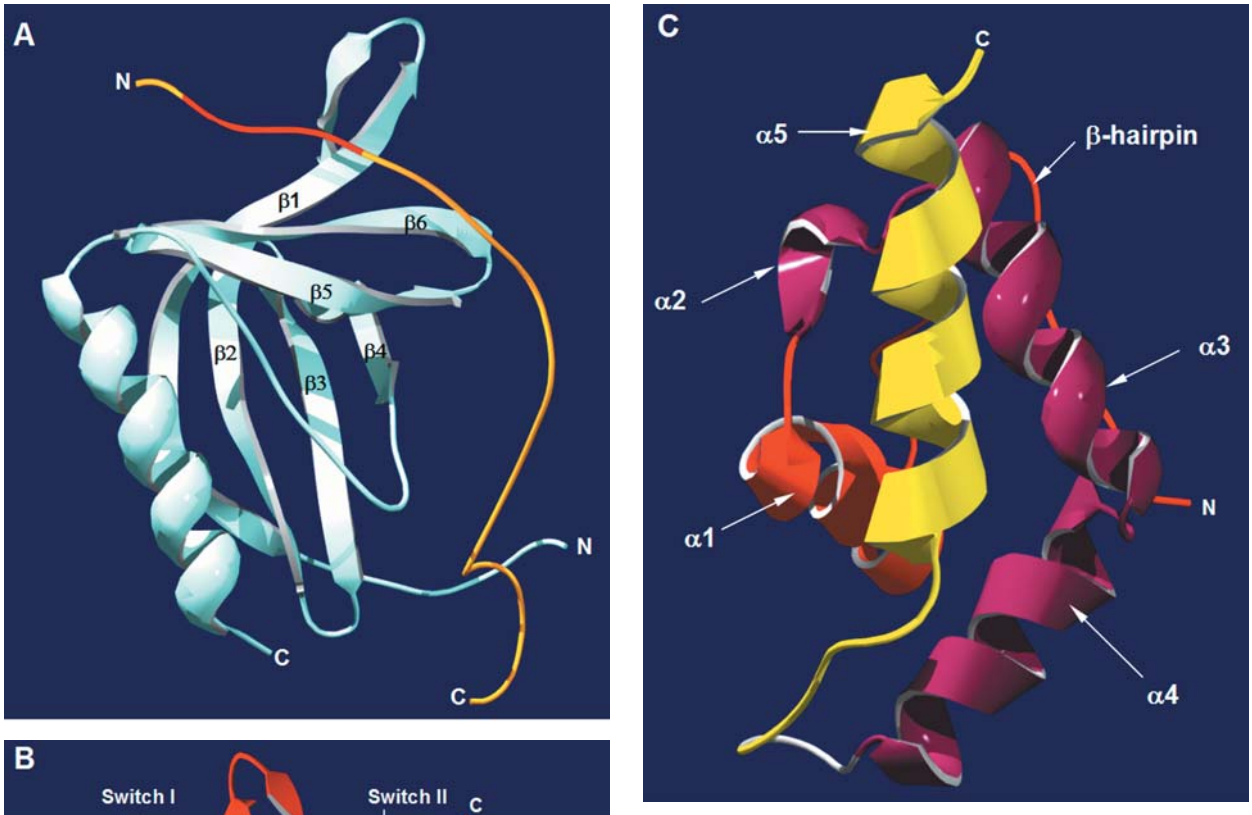

Figure 3. Three-dimensional structures of WASp/NWASp domains. (*A*) Structure of WH1-peptide complex (accession number, 1MKE [107]) is modeled with NWASp WH1 domain in light cyan and the 25-a-long WIP peptide in dark orange. The canonical EVH1 binding sequence (LPPPP) is marked in red. (*B*) Structure of the WASp GBD is shown complexed with Cdc42 (accession number, 1CEE [133]). The Cdc42 molecule is presented in gray with switch I (Sw I) and switch II (Sw II) motifs in green, and the nucleotide is displayed as a stick model. The β2, β3 strands and the α5 helix of Cdc42, and α1 helix of the WASp GBD are marked. Only the N-terminal part of the GBD (CRIB, β-hairpin and α1) is displayed (red), and the CRIB motif is highlighted in yellow. (*C*) Autoinhibited structure of WASp is modeled with a focus upon the C region and GBD interaction (accession number, 1EJ5 [87]). The structural elements creating the autoinhibited fold are color-coded: layer 1, red; layer 2, crimson; layer 3, yellow [87]. For details, see text. The CRIB motif is not shown. All models were created using DeepView/Swiss-PdbViewer v3.7 and POV-Ray v3.5 software and structures from the Protein Data Bank.

rich peptides containing the motif L/FPPPP/E [107, 108]. Proteins with these motifs have the ability to adopt a polyproline II helical conformation and wrap around the WH1 domain. Unlike non-WASP subfamily members, the NWASp WH1 binding peptide is oriented N- to C-terminal [107]. These structural data [107] reveal a novel model of WH1 domain and peptide interaction which provides insight into *WASp* mutations that cause WAS. In this light, several of the WAS-associated N-terminal *WASP* mutations alter residues on the surface of the WH1 domain, opposite the EVH1 binding site, and not in the hydrophobic core or in the Pro-rich peptide-binding groove [109]. As the ligand wraps around the entire WH1 domain, the mutant residues may contact the ligand distal to the canonical binding site, disrupting normal electrostatic forces and destabilizing protein-protein interactions [107]. Thus, the novel wrapping mechanism of peptide recognition, first postulated [108] and later confirmed [107], indicate that mutations of the WH1 surface can disrupt the interaction between NWASp/WASp and its binding partners, possibly affecting the localization and activity of WASp.

At least three proteins directly interact with the WH1 domain. The first is WASp-interacting protein (WIP), a Pro-rich homolog of yeast verprolin involved in regulating actin cytoskeleton rearrangements [106, 108, 110–113]. The second is CR16, a monomeric and filamentous actin-binding protein found in neurons, which is ~25% homologous to WIP [114]. The third is WIP- and CR16-homologous protein (WICH), which participates in actin-microspike formation through cooperation with NWASp [115]. Older reports have also suggested that the WH1 domain binds phosphatidylinositol-4,5-biphosphate (PIP2) [116, 117], but the WH1 domain is not required for PIP2 binding in *Xenopus* egg extracts [118]. Thus, the observed binding of PIP2 to the WH1 domain [116, 117] may be somewhat non-specific. The structure of the EVH1 domain is similar to that of the pleckstrin homology domain involved in PIP2 binding, and the presence of basic residues in the EVH1 domain, therefore, may contribute to binding acidic phospholipids [108]; but the NWASp WH1 domain has been shown to function primarily as a peptide-binding unit [119]. However, as PIP2 activates WASp to induce actin nucleation to a lesser extent in the absence of the WH1 domain, PIP2 binding to the WH1 domain cannot be excluded [120].

Early studies of WH1 domain function demonstrate its involvement in organization of the cytoskeleton, as mutations in N-terminal part of WASp block actin polymerization in COS-7 cells [117]. These findings were furthered by the observation that deletion of NWASp WH1 results in reduced rate of actin polymerization in vitro [121]. A potential mechanism for these activities involves the recruitment of other proteins required for efficient actin polymerization by the WH1 domain [122]. This may be due to the direct binding of proteins to this region or to the influence that proteins bound to this region have on WASp [95, 112]. The WH1 domain may additionally allow for localization of WASp to sites of actin polymerization through protein recruitment [95, 108]. In this regard, VASP and Mena are recruited to the immunologic synapse in Jurkat T cells through the interaction of their EVH1 domain with the SLP-76 associated protein (SLAP) [123]. Although WASp does not bind to SLAP directly, it forms a complex with Nck, SLP76 and SLAP. This complex has been found in non-stimulated cells at low levels, indicating that WASp and proteins bound to the WH1 domain create a possible starting point for cytoskeletal rearrangements [123]. Additional evidence for this model is provided by pathogens that exploit WASp (discussed below), as the WH1 domain is essential for efficient recruitment and function of NWASp induced by vaccina virus, which is independent of Cdc42 [124]. Finally, WASp localization to the immunologic synapse involves WIP through its ability to bind to CrkL, which in turn binds ZAP-70, allowing the recruitment of the WASp complex to the T cell receptor (TCR) signalosome [95]. In this light, WIP-deficient mice have a defect in the ability to polymerize actin filaments, supporting a critical role for WIP in WASp localization and function [125].

The WH1 domain also serves an important regulatory role for WASp function and cell activation through protein binding. WIP, as will be discussed later, can also retard actin polymerization [112] and participates in a complex NWASp regulatory mechanism. The WASp N-terminus can also bind the catalytic domains of Abl, Lck, Fyn, Hck and Src tyrosine kinases and suppress their function in vitro and in vivo [126]. Binding of these kinases to WASp decreased the V_{max} of substrate phosphorylation more than threefold, but not their affinity for peptide substrates or ATP. This may represent a mechanism for control of cellular activation once filamentous networks are established. Thus, the WH1 domain serves to recruit proteins, localize and regulate WASp. The importance of this is highlighted by the observation that >85% of *WASP* missense mutations resulting in WAS affect the WH1 domain of WASp [127]. In addition, many of these disease-causing mutations alter the integrity of the WH1 domain and/or would be predicted to specifically disrupt normal interactions with molecules that bind this region [109].

Basic region and GTPase binding domain

C-terminal from the WH1 domain is a short highly BR containing six lysine residues (fig. 2). Although it was conceived that this region might bind to the acidic region of WASp [128], it was later shown that the BR is not required for WASp binding to the VCA domain in vitro [87]. Furthermore, deletion of the BR fails to reduce the affinity of the N-terminal part of NWASp for the VCA re-

gion [118]. A NWASp BR deletion mutant, however, has a greatly reduced ability to induce actin polymerization in vitro [129], indicating that the BR serves an important role in actin polymerization mediated by NWASp. In this light, the BR participates in Arp2/3 binding, further implicating this region in actin polymerization [119]. The BR also binds PIP2, which is important for WASp/NWASp activation [118–120]. In addition, phosphatidic acid also binds to the BR and enhances NWASp-mediated actin polymerization [130]. Thus, binding of acidic phospholipids (e.g. PIP2, phosphatidic acid) by the BR can contribute to WASp/NWASp activation and function.

The central part of WASp comprises the GTPase binding domain (GBD). This region of 70–80 aa is responsible for binding the Rho family GTPases; Cdc42, which is required for filopodia formation [120, 128, 131–134], Rac, which is essential for lamellipodia development [131]; and Tc10 (NWASp only) [135]. High-affinity binding of Cdc42 depends on it being in the GTP-bound and prenylated form [120, 131]. WASp residues 230–288 maintain high affinity for Cdc42 [133] and contain the highly conserved Cdc42/Rac-interactive binding (CRIB) motif (IGxPxxFxHxxH). This motif is crucial for Cdc42 binding to WASp/NWASp, as its mutation abolishes the interaction of WASP with Cdc42 [128]. The GBD can also interact with the VCA domain to block Arp2/3 complex binding and/or function, thus inhibiting actin nucleation. This 'auto-inhibition' (described in detail below), however, is reversed by Cdc42-GTP and/or PIP2 biding [118, 128] and further indicates that the GBD serves an important role in regulating WASp activity.

Resolution of the GBD structure has helped elucidate the importance of this domain for WASp/NWASp function. Interestingly, the free WASp GBD is largely disordered, but can adopt two different, incompatible conformational states depending on its binding partners (fig. 3B, C) [87, 133]. When bound to the Cdc42-GMPPCP (the non-hydrolysable GTP analogue), GBD adopts a structure characterized by contacts between the N-terminal part of GBD (including the CRIB motif) and the switch I, β2 and α5 regions of Cdc42. The CRIB motif specifically contacts the C-terminal part of switch the I and β2 strand, while GBD residues preceding the CRIB region form a loop that interacts with the β2-β3 turn and α5 helix of Cdc42. The part of the GBD following the CRIB motif folds into an antiparallel β hairpin and α helix that pack against a hydrophobic area of the switch II and β3 strand of Cdc42 (fig. 3B) [133]. Mutations in either the switch I of GTPase [136] or CRIB motif of NWASp [128] decrease the affinity of Cdc42 for WASp/NWASp. These results are elucidated by structural analyses, demonstrating that hydrophobic and polar interactions are responsible for binding between WASp and the nucleotide switches of Cdc42. An illustration of this specificity is derived from the fact that WASp binds strongly to Cdc42 and weakly to Rac, but not to Rho [131]. Rho-family members are almost identical in the fragment of WASp binding site comprising both switches. The main difference is position 38 of switch I, which is Asp in Cdc42 and Rac, but Glu in Rho. Therefore, a conserved difference in the switch I of GTPase may be responsible for specificity to the conserved HxxH motif in the CRIB region [133]. Cdc42 also utilizes hydrophobic interactions to attach its β2-β3 hairpin and α5 helix to the N-terminal part of the WASp GBD. Some of these hydrophobic amino acid residues in the β2-β3 hairpin of Cdc42 correspond to polar residues in Rac and Rho, which may disrupt some contact points between WASp and GTPase, thus imparting specificity to Cdc42 for WASp [133].

The autoinhibitory capability of WASp is a feature of the GBD binding to the VCA domain. The resulting intramolecular interactions between the GBD and VCA domain can prevent the C terminus of WASp from binding the Arp2/3 complex [87]. Upon VCA binding, the GBD, which contains a β hairpin and four α helices, assumes a compacted structure with a hydrophobic core composed of three distinct layers (fig. 3C). The complex of the GBD and VCA domain contains an additional α5 helix formed from the C region of the VCA domain. The short β hairpin and α1 helix form an amphipathic surface that creates the first layer of the hydrophobic core. The second layer is composed of helices α2, α3 and α4, which contact the first layer and are stabilized by hydrogen bonds and hydrophobic interactions. The third layer consists of the α5 helix formed by the C fragment from the VCA domain and is stabilized by electrostatic interactions with the loop between the α2 and α3 helices and by hydrophobic interactions with the α1, α3 and α4 helices (fig. 3C). NWASp most likely shares a similar autoinhibited state with WASp, since the residues at the interface of the three layers are highly conserved [87].

The α1 helix is almost perpendicular to the long axis of the hydrophobic core in the autoinhibited state. However, it becomes parallel upon Cdc42-GTP binding. As a result the surface area buried by interactions between layer 1 and the other two layers is diminished, eliminating many contact points and destabilizing the hydrophobic core. This causes the loss of the VCA domain binding site in the GBD and leads to the release of the C region. This is mimicked to some degree by a naturally occurring human WASp mutant (L270P) that destabilizes the α1 helix, resulting in a loss of autoinhibition and a constituitively active WASp [61]. Formation of the autoinhibited WASp/NWASp structure therefore requires sequences that are C-terminal to the CRIB motif, while binding of Cdc42-GTP and formation of the activated fold requires the N-terminal part of the GBD containing the CRIB motif [87, 133]. Thus, both biochemical and spectroscopic data indicate that the GBD is critical in WASp activation and regulation.

Pro-rich domain

A Pro-rich region of ~100 aa found in all WASp family members (fig. 2) contains several binding sites for SH3 domains (PxxP). Compared with other regions of WASp, the Pro-rich domain binds the largest number of interacting proteins, including several SH3 domain kinases: Fyn [137, 138], Btk [139, 140], Lyn [140, 141] and Hck [142, 143], which can all contribute to regulating WASp activity (discussed below). Other proteins also interact with the Pro-rich domain through their SH3 domains to affect cytoskeletal reorganization and include CIP4, endophillin A, pascin, profilin, intersectin 1/2 and VASP. CIP4 is an adaptor protein linking WASp to microtubules that is required for the formation of actin-rich podosomes [144, 145]. Endophilin A promotes endocytosis and binds dynamin to allow for specific NWASp localization [130]. Pacsin also binds dynamin and recruits other proteins to vesicles for participation in endocytosis [146]. Profilin is a central protein in actin polymerization, as it exchanges ADP bound to actin for ATP [147]. Intersectin-1 is a scaffold protein that participates in formation of clathrin-coated vesicles and also serves as a guanine nucleotide exchange factor for Cdc42 [148]. Intersectin-2 also associates with endocytotic vesicles and can activate Rho GTPases, including Cdc42, to participate in TCR internalization [149]. VASP is a WASp-homologue (discussed above – fig. 2) that can promote actin polymerization, but only binds WASp, as NWASp does not compete with WASp for VASP binding in vitro [150]. The Pro-rich domain also binds other SH3-SH2 adaptor proteins, including Nck [113, 151, 152], Grb2 [113, 116, 153] and PSTPIP [97, 141, 154], which all likely participate in the appropriate localization of WASp.

The Pro-rich domain serves two important functions, the first of which is to affect the WASp activation state and promote interaction with the Arp2/3 complex. The importance of this region is illustrated by WASp mutants lacking the Pro-rich domain, which lose the ability to induce actin polymerization at the plasma membrane in RBL-2H3 cells [150]. Profilin may be a critical link, as it increases the efficiency of Cdc42-induced actin nucleation more than six-fold, probably by increasing local actin concentrations [147]. Alternatively, the Pro-rich region may function in releasing the autoinhibited fold of WASp, as profilin binding to this domain could discourage autoinhibition and favor the activated form of WASp [147]. Grb2 also favors NWASp activation (mediated by Cdc42), as demonstrated by in vitro actin polymerization and *Escherichia coli* motility assays [153]. Nck can activate both NWASp and WASp and activates NWASp in vitro by itself, but requires other proteins to activate WASp in Jurkat T cells [151, 152]. In this light, Nck-induced actin nucleation mediated by NWASp is enhanced by PIP2, but not Cdc42-GTP. Nck is a more potent activator of NWASp than Grb2 or PIP2, but had an additive effect with PIP2 [151]. Additionally, endophilin A also enhances NWASp-mediated actin polymerization and is more potent than the SH3 domain of Grb2 in vitro [130]. Thus, the Pro-rich domain is central to the activation and regulation of WASp/NWASp activity through its multiple binding partners.

The second function of Pro-rich domain is the localization of WASp through interactions with SH3-SH2 adaptor proteins. TCR signaling results in recruitment of both Cdc42 and WASp to the T cell:APC contact site through distinct and independent mechanisms. WASp localization does not depend upon Cdc42, and the WASp GBD, as a WASp mutant lacking the CRIB motif, still polarizes to the contact site [88]. However, the deletion of Pro-rich domain affects WASp recruitment, indicating that interaction of WASp with an SH3-domain protein is responsible for WASp accumulation at the T cell:APC immunologic synapse [88, 94]. One candidate is Nck, which is required for WASp recruitment to the T cell:B cell immunologic synapse by SLP-76 [152]. Another candidate is the proline, serine, threonine-rich, phosphatase-interacting protein (PSTPIP), which links CD2/CD2AP to the WASp Pro-rich domain through its coiled-coil and SH3 domains, respectively, and is required for WASp polarization to the T cell:B cell immunologic synapse after TCR ligation with CD2 costimulation [97]. Thus, distinct costimulatory signals are probably capable of recruiting WASp using different adaptor proteins that bind the Pro-rich domain. This also allows for specific localization of WASp after TCR ligation that is costimulation dependent, as CD28 but not CD2 signaling results in WASp accumulation in lipid rafts [96, 97].

VCA domain

The C-terminal region of all WASp family members consists of a VCA domain of ~80 aa in three segments (fig. 2). The N-terminal part contains one (two in NWASp) 15–20 aa V region, followed by a 20–30 aa C region. The structure of the C region has differences from the corresponding sequence in cofilin [155] and therefore is now referred to as the central or connecting region [122, 155, 156]. The most C-terminal 15–20 residues of the VCA domain contain a highly negatively charged A region (fig. 2). The entire region is also known as the WA domain: 'W' for WASp homology 2 that corresponds to the V region, and 'A' for acidic region. The overall significance of these regions is most importantly highlighted by the severity of clinical disease in boys with WAS due to mutations impairing the C-terminus of WASp [63].

The V region binds monomeric actin [81, 155–158], through its monomeric actin binding motif (QLNK in WASp and QLKK/QLKS in NWASp). Mutations in the V region decrease the affinity of the VCA domain for actin and impair the ability of the mutant WASp to bind actin in vitro [155, 156]. Although the V region is essential for

actin polymerization, it cannot bind filamentous actin nor the Arp2/3 complex, indicating that the monomeric actin binding site is distinguished form the Arp2/3 binding site [155].

The C and A regions contribute to Arp2/3 complex binding and activation [118, 155, 156, 158, 159]. The NWASp VCA domain induces a conformational change in the Arp2/3 complex and makes contact between the ARPC3 and ARPC4 subunits possible [158]. WASp binds to the ARPC3 subunit [159], and NWASp can be crosslinked to Arp2, Arp3 and ARPC1 [160]. The A region of WASp interacts directly with and is required for Arp2/3 complex binding, but does not induce activation [155, 156]. The specificity of the interaction is illustrated by a W500S mutation in the A region that abolishes WASp binding to the Arp2/3 complex [155]. The C region in particular is essential for Arp2/3 activation, as an R477K mutation of WASp impairs the ability of WASp to activate Arp2/3 [155]. The C region may also have the ability to directly bind or increase affinity for actin, as peptides corresponding to the V or CA region of WASp can compete with VCA domain for binding monomeric actin [155].

The C region and more notably the C and A regions together contribute to the interaction with the GBD and WH1 domain [118]. The CA region is minimally required for Arp2/3 binding [118] and has been confirmed by nuclear magnetic resonance (NMR) spectroscopy, demonstrating that the C region and the C-terminal part of the A region bind Arp2/3 [161]. As mentioned above, the α helix formed by the C region closes the hydrophobic core of the autoinhibited domain of WASp and is released from the GBD after Cdc42-GTP binding, enabling the VCA to interact with the Arp2/3 complex [87]. The hydrophobic residues and a conserved Arg477 (required for Arp2/3 activation) are all clustered on one side, and the hydrophobic face of the α5 helix is likely binding the hydrophobic surface of the Arp2/3 complex. Mutations of the hydrophobic residues impair Arp2/3 activation, while mutations on the hydrophilic face of the helix do not alter the activity. Concealing the hydrophobic residues within the autoinhibited structure of WASp/NWASp and their release upon Cdc42 binding to the GBD is central to the regulation of WASp/NWASp activity [161].

The VCA domain activates the Arp2/3 complex by inducing a conformational change of Arp2/3 and increasing its affinity for ATP. In this regard, the Arp2/3 complex may exist in three distinct states: (i) inactive; (ii) activated and bound to the WASp VCA domain; (iii) activated and bound to both monomeric actin and the WASp VCA domain (actively branching actin filaments) [162]. The inactive form of Arp2/3 has ATP bound only to Arp3, with the binding site on Arp2 vacant. To branch new actin filaments, Arp2 must first bind ATP. This process is facilitated by binding of the WASp VCA domain to the Arp2/3 complex, which increases the affinity of Arp2 for ATP ~400-fold by closing this second ATP binding domain. Curiously, ATP hydrolysis was not observed in any of the three forms of Arp2/3, but probably is required for actin filament branching [162].

Insight into the function of the VCA domain is derived from the observation that the NWASp VCA domain is more effective than that of other WASp family members. Replacement of the NWASp VCA domain with the corresponding portion of WAVE1 demonstrates that the V region determines potency of Arp2/3-mediated actin polymerization [163]. Furthermore, a WAVE1 VCA mutant, possessing two WAVE1 V motifs, can induce actin polymerization similar to the NWASp VCA [163]. Thus, the two V motifs in NWASp may simultaneously bind two actin molecules, resulting in increased monomeric actin-binding capacity of the VCA domain and more robust Arp2/3 function. Actin nucleation activity of WASp family members, however, also depends in part on the composition of the A region and not on the number of V regions [158]. The fusion of an additional VCA domain to SCAR1 or the removal of one V region from NWASp does not significantly affect the ability to nucleate actin filaments. Conversely, replacement of the SCAR1 A with the A region of NWASp increases the nucleation activity of the chimeric SCAR1 protein. The observed differences are caused by three critical acidic amino acid residues found in NWASp but absent in SCAR1–3 that do not affect Arp2/3 or actin binding affinity [158]. Therefore the mechanism by which the WASp family members maintain their relative potency differs amongst the different proteins.

Arp2/3-mediated actin polymerization is largely a two-step process, with Arp2/3 binding to WASp as a first step and Arp2/3 activation a second [155, 158]. The first step for efficient actin nucleation and polymerization is the recruitment of monomeric actin and WASp to the Arp2/3 complex and the binding of this assembly to the side of an existing filament. The VCA domain probably stabilizes the association of monomeric actin and Arp2/3 and increases the time of interaction to promote actin nucleation [155]. Regardless, Arp2/3 and actin binding is not sufficient for actin polymerization and requires an additional activation step. This is achieved when WASp family proteins induce a conformational change of the Arp2/3 complex and a barbed end of a new branch is created, allowing the daughter branch to elongate [158].

The WASp VCA domain also plays a major role in activating and regulating WASp activity [87, 118–120, 128, 132, 161] and is discussed below.

Regulation of WASp activity

WASp/NWASp autoinhibition

WASp is a likely converging point for a number of signaling pathways, thus necessitating robust control mech-

anisms. WASp/NWASp can adopt the autoinhibitory conformation [87, 119, 134], and data support two existing models for regulation of this structure: the sequestration and direct inhibition models [161] (fig. 4).

In the sequestration model, the GBD hides key portions of the VCA domain required for Arp2/3 complex binding and activation, and thus the VCA domain cannot interact with Arp2/3. This is illustrated by the inhibition of VCA fragment-induced Arp2/3 complex activation by isolated GBD in vitro [118, 120]. The aforementioned structural and biochemical data indicate that the C fragment of the VCA region is sufficient and required for the intramolecular interaction with the GBD and activation of the Arp2/3 complex [87, 161]. The first layer of the autoinhibited intramolecular fold (fig. 3C) becomes incompatible with the rest of the structure after Cdc42-GTP binding, and contacts between the GBD and VCA domains are destabilized allowing full access to Arp2/3 [87, 133, 134]. Cdc42 alone, however, cannot fully destabilize the GBD-C complex, indicating the requirement of additional signals for the full activation of WASp [120, 134]. In this light, PIP2 binding to BR could affect the GBD to destabilize the autoinhibited structure, as evidenced by PIP2 activation of NWASp with Cdc42 in vitro [118]. Both Cdc42 and PIP2 alone are weak activators of WASp/NWASp, but act synergistically for increased potency [118, 120, 122, 132]. Therefore, the sequestration model holds that in the absence of activators (such as Cdc42, PIP2) WASp adopts the autoinhibitory conformation, in which hydrophobic residues of the C region of VCA interact with hydrophobic surface of the GBD and access to Arp2/3 is denied. Destabilization of GBD-VCA contacts by Cdc42 (and PIP2) binding leads to release of the VCA domain and its subsequent binding to the Arp2/3 complex, resulting in Arp2/3 activation and actin nucleation.

In the direct inhibition model WASp/NWASp autoinhibition results from direct interaction of the BR and the

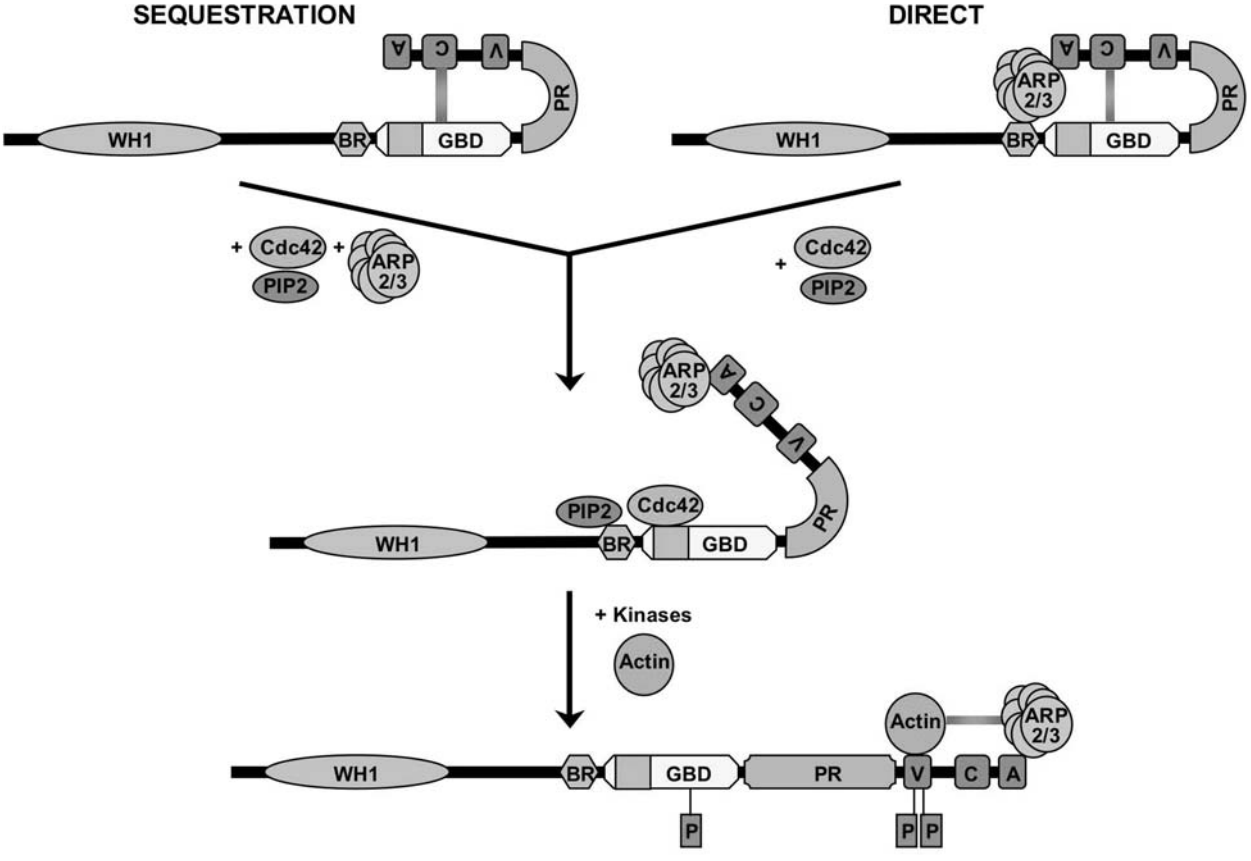

Figure 4. Models for reversal of WASp autoinhibition. In both the sequestration and direct models the C region is bound to the GBD as described in the text and as depicted by a shadowed line, thus stabilizing the folded 'closed' conformation of the protein. In the sequestration model, WASp is not bound to ARP2/3 while it is persisting in the autoinhibited fold, whereas in the direct model Arp2/3 links the A region and BR while the protein is autoinhibited. Upon activation, both models posit that Cdc42 and PIP2 bind WASp, and in the sequestration model this additionally includes Arp2/3. Subsequent phosphorylation of WASp at Y291 by Src- or Tec-family kinases further favors the 'open' activated WASp structure that is capable of linking an actin monomer and Arp2/3. The phosphorlyation of WASp at S483 and S484 is also required for optimal activation and function. Phosphorylation events additionally promote the binding of other proteins that assist in WASp localization and function.

GBD with the Arp2/3 complex [119]. In support of this model the GBD and PIP2-binding motif are necessary for regulation of NWASp. The minimal fragment required for repression of the VCA domain consists of the whole GBD and the BR. Moreover, mutations in the BR eliminate repression, but the GBD alone has no inhibitory effect, although it binds strongly to the VCA domain. In contrast to earlier studies, neither the GBD alone nor the BR and GBD together block the interaction of Arp2/3 and VCA, as Arp2/3 binds two sites in NWASp: the acidic region of the VCA domain and the BR [119]. These results indicate that NWASp can exist in an inhibited state complexed with Arp2/3, ready for instantaneous new actin filament branching upon activation. This model also holds that the GBD and the BR together maintain the VCA-Arp2/3 complex in an inactive state in the absence of stimuli (e.g. Cdc42, PIP2). Binding of either Cdc42 or PIP2 alone to the GBD and BR, respectively, partially destabilizes the repressed NWASp-Arp2/3 complex, favoring binding of the second molecule to induce complete destabilization. Therefore, Cdc42 and PIP2 act cooperatively to disrupt the inhibited conformation of WASp/NWASp, releasing the VCA-Arp2/3 complex to promote actin nucleation and polymerization.

Although existing data support both sequestration and direct models, the former is based mainly upon structural data and the latter upon functional analyses. Furthermore, WASp/NWASp exhibit weak basal activity towards Arp2/3 complex in vitro [118, 120], and the NWASp fragment consisting of the BR, GBD and WH1 domain possess the greatest affinity for the VCA domain and can block the interaction of the VCA domain with the Arp2/3 complex [118]. In this regard, the direct inhibition model better explains the involvement of PIP2 in regulating WASp/NWASp and the observed synergy between PIP2 and Cdc42. Nonetheless, the complexity of both models underscores the intricacy of regulating WASp/NWASp activity, and full activity in vivo probably requires several combined input signals, including lipids, GTPases and adaptors.

WASp/NWASp phosphorylation

WASp phosphorylation occurs in a variety of cell types and plays an important role in WASp function. WASp is phosphorylated by the Src-family kinase Lyn [140] and Tec-family kinase Btk on Tyr291 in NALM6 preB cells [164], in RBL-2H3 mast cells after IgE receptor ligation [140] and in platelets after collagen treatment [165]. In addition, WASp is also phosphorylated on Tyr291 in COS-7 cells by Hck kinase [143]. WASp is dephosphorylated by LAR-PTP phosphatase in vitro [166] and PTP-PEST phosphatase in vivo [141]. PTP-PEST, however, is unable to interact directly with WASp, and the PSTPIP scaffold protein is required to create a link between WASp and PTP-PEST to achieve WASp dephosphorylation [141].

Functional studies have demonstrated that mutation of Tyr291 to Glu, which mimics phosphorylated WASp, allows for enhanced actin polymerization in vitro and filopodia formation in vivo [143]. The disruption of the α3 helix and β-hairpin interaction caused by Cdc42 binding has been proposed to increase accessibility of Tyr291 for phosphorylation (fig. 4) [87]. Subsequent phosphorylation of Tyr291 can then destabilize the autoinhibited structure either by introducing a negative charge, which disrupts the contacts between the α3 helix and β-hairpin, or by creating a binding site for SH2 domain-containing proteins. Therefore, GTPase binding acts synergistically with phosphorylation events to enhance WASp activity [87]. This is supported by the observation that efficient phosphorylation of WASp Tyr291 or NWASp Tyr256 in vitro by Lck, Abl or Lyn kinase requires Cdc42-GTP [166]. These tyrosines are buried in the autoinhibited strucuture, but become exposed after binding Cdc42-GTP [87, 166]. Circular dichroism and NMR data suggest that kinase binding and Tyr291 phosphorylation could also destabilize the α5 helix, enabling dissociation of the VCA domain from the hydrophobic core with resulting Arp2/3 complex activation. Thus, Cdc42-GTP binding serves two functions in destabilizing autoinhibited WASp, by first initiating disruption of the autoinhibited fold and second enabling phosphorylation of Tyr291 to further activate WASp.

In contrast to the role of Cdc42-GTP in inducing effective phosphorylation of WASp by Lyn and Btk [143, 166], WASp phosphorylation by Hck is independent of Cdc42 [143]. It is presently unclear as to what substitutes for Cdc42 to allow WASp for phosphorylation. PIP2, however, can enhance NWASp phosphorylation on Tyr256 by Fyn kinase in vitro [167], but a role for PIP2 in Hck-induced WASp phosphorylation has not been determined. Hck, as will be discussed below, also binds WIP [142] and therefore may be introduced to WASp while it is still in its inactive conformation.

WASp/NWASp can be phosphorylated on two serine residues (Ser483 and Ser484 in WASp, Ser484 and Ser485 in NWASp) by a serine/threonine kinase, casein kinase 2 [168]. Endogenous WASp and NWASp are phosphorylated at these residues in a wide variety of cell types [168]. Phosphorylation of these sites is specific, as inhibitors of protein kinase 1, protein kinase A, p38-MAP kinase, MEK or Src kinases do not affect WASp serine phosphorylation in U937 cells. Both serine residues are positioned at the border of the C and A regions of the VCA domain, which is crucial for the formation of the autoinhibited fold [87] and Arp2/3 complex binding and activation [155, 156, 158, 161]. Phosphorylation of S483 and S484 does not affect the interaction between the VCA and GBD domains or the interaction of WASp with

Cdc42, but increases the affinity of the VCA domain for the Arp2/3 complex sevenfold and is required for efficient actin polymerization in vitro and in vivo [168]. Furthermore, the function of these serine residues occurs independent of PIP2 and Cdc42 [168] and introduces novel avenues for regulation of WASp function. Thus, serine phosphorylation of WASp does not disrupt its autoinhibited structure, but is essential for maximal WASp binding of Arp2/3 and optimal WASp function.

Other regulatory mechanisms

WASp activation is also subject to other modes of positive and negative regulation. NWASp can be negatively regulated by WIP, which binds to the WH1 domain and retards Cdc42-induced NWASp-mediated actin polymerization in vitro in a dose-dependent manner [112]. This is not due to actin sequestration by WIP, as WIP does not affect actin polymerization induced by the VCA domain alone. Thus, WIP binding to NWASp likely affects NWASp activation specifically. The inhibitory effects of WIP, however, are negated by PIP2 in vitro [112, 151], illustrating the complex interplay amongst WASp regulatory molecules. One model for the negative regulatory function of WIP involves constitutive binding of WIP to WASp and dissociation of the two molecules after phosphorylation of WIP at Ser488 [95]. In this model WIP, dephosphorylated at Ser488, is bound to WASp to provide negative regulation and is then dissociated from WASp upon Ser488 phosphorylation to alleviate the negative influence. Given the maner in which WIP wraps around WASp (fig. 3 A; [107]), it is feasible that this event may be a necessary prerequisite for the reversal of WASp autoinhibition. The association of WIP and WASp, however, also has the capacity to initiate the process of WASp activation, as WIP recruits, binds and activates Hck kinase [142], which can phosphorylate and activate WASp. WIP also serves the important function of delivering WASp to key sites of actin polymerization at lipid rafts through association with CrkL [95]. Thus, although WIP recruits important proteins to WASp that assist in localizing the complex, it may serve to control WASp function until further activation occurs. WIP, therefore, is a complex WASp regulator that probably serves important roles in inhibiting, activating and localizing WASp/NWASp.

WASp/NWASp activation is also subject to feedback regulation mediated by intersectin-1. Intersectin-1 binds directly to NWASp and Cdc42 via its SH3 domain and Dbl homology domain, respectively, and activates Cdc42 [148]. Binding of Cdc42 by intersectin-1 enhances NWASp-mediated actin polymerization in human neutrophils and induces formation of filopodia in 3T3 fibroblasts. The binding of NWASp by intersectin-1 stabilizes the interaction of intersectin-1 with Cdc42-GDP and allows generation of GTP-bound Cdc42. This creates a model in which binding of NWASp to the SH3 domain of intersectin-1 enhances the ability of intersectin-1 to interact with Cdc42-GDP, favoring the conversion to Cdc42-GTP. Cdc42-GTP can then bind NWASp to stimulate actin polymerization [148]. Thus, binding and upregulation of the guanine nucleotide exchange activity of intersectin-1 by NWASp could create a feedback loop for NWASp/WASp activation by the GTPase.

Exploitation of WASp by pathogens

Microorganisms have developed methods to subvert host cell proteins (including WASp family members) for vital microbial functions. These exploitations have provided significant additional insight into the function and importance of WASp. Intracellular microbes, including *Shigella* spp. and vaccinia virus, have developed methods of linking their motility within host cells and cell-cell spread to utilization of the host NWASp for activation of the Arp2/3 complex for actin-based microbial motility [169, 170]. Protein-protein interactions between microbial proteins, WASp family proteins, and the cytoskeletal machinery are responsible for this linkage. In addition, enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) have developed methods of inducing projections from the host cell surface at the site of bacterial binding, termed pedestals, which are dependent on reorganization of actin at the cytoplasmic surface of the membrane. Pedestal formation is dependent on interactions of EPEC or EHEC derived protein(s) with NWASp and the Arp2/3 complex [171]. Studies elucidating the mechanisms by which microbes recruit WASp family members and use them for activation of actin polymerization have provided important insights into the function of WASp proteins and the pathways for actin polymerization. The common theme with all of these organisms is that they recruit WASp family members, using different adaptor proteins, and activate Arp2/3 independent of Cdc42 (fig. 5).

Vaccinia virus

Vaccinia virus uses actin polymerization for protrusion from the cell surface and cell-cell spread [172]. Following replication of the virus in the perinuclear region of the cytoplasm, the intracellular enveloped viral (IEV) particles are transported to the periphery of the cell on microtubules [173–175]. After the IEV reaches the cell membrane, it fuses to form the cell-associated extracellular enveloped virions (CEV), which remain attached to the membrane at the end of actin-based membrane protrusions [172]. Thus, microtubule-based transport is necessary for transport to the periphery, and actin polymerization is necessary for formation of virally tipped membrane protrusions involved in cell-cell spread [176]. Actin

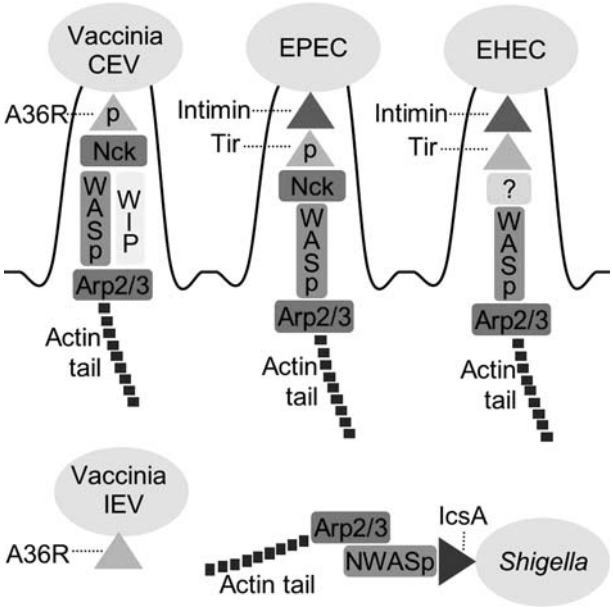

Figure 5. Exploitatation of WASp for microbial-induced actin polymerization. Vaccinia virus, enteropathogenic *E. coli* (EPEC), enterohemorrhagic *Escherichia coli* (EHEC), and *Shigella* spp. have developed methods of recruiting WASp family proteins for activation of the Arp2/3 complex for initiation of actin polymerization. Pathogens are represented by ovals, pathogen-encoded proteins with triangles and host cell proteins with rectangles. The host cell membrane is depicted by the black line. Tyrosine phosphorylation (p) of the vaccinia A36R protein and the EPEC Tir protein is required for recruitment of WASp and the Arp2/3 complex. This schematic presents a model for the mechanisms of recruitment of the WASp family proteins and Arp2/3, as described in the text.

polymerization at the periphery is dependent on the A36R protein of vaccinia. A36R is an integral membrane protein with an ~195 aa residue domain exposed at the cytoplasmic surface at the site of virus binding to the cell surface [177–180]. Tyrosine phosphorylation of the vaccinia A36R protein on residues 112 and 132, mediated by Src-family kinases, is necessary for activation of actin polymerization [181]. Requirement for Src kinases may also explain activation of actin polymerization at the membrane surface. Following tyrosine phosphorylation, Nck, WIP, NWASp, Grb2 and Arp2/3 are recruited to the A36R protein and are required for actin polymerization [124, 181, 182]. Nck is able to bind directly with Tyr112-phosphorylated A36R [181], while Grb2 binds directly with Tyr132-phosphorylated A36R [182]. Grb2 recruitment is disrupted if the polyproline-rich domain of N-WASP is absent, suggesting that efficient recruitment of Grb2 depends on prior recruitment of NWASp by Nck. Thus, Nck and Grb2 may both recruit NWASp to vaccinia particles after tyrosine phosphorylation, but the Nck pathway seems to play a dominant role, with Grb2 primarily increasing the efficiency of recruitment and stability of the complex [182]. The requirement for NWASp for vaccinia-induced actin polymerization is demonstrated by the absence of actin tail formation after infection of NWASp$^{-/-}$ fibroblast-like cells, which can be rescued by ectopic expression of *NWASP* [183]. NWASp also clearly localizes to vaccinia particles at the site of actin tail formation [181]. As demonstrated using dominant negative mutants, WIP is required for actin polymerization and is localized to vaccinia particles at the site of actin tail formation. Furthermore, WIP is recruited to vaccinia in the presence of dominant-negative NWASp mutants, suggesting that WIP binding to Nck is upstream of NWASp recruitment [124]. However, WIP and Nck are not recruited to viral particles in NWASp$^{-/-}$ cells [183]. As previously proposed, WIP and NWASp may actually be recruited as a complex [124]. Actin polymerization in the vaccinia system is not dependent on Cdc42 activity [124]. These results suggest a model in which IEV particles are transported to the cytoplasmic surface of cells where Src kinases tyrosine phosphorylate A36R on residues 112 and 132. The phosphorylated A36R protein then recruits a Nck/WIP/NWASp complex and the Arp2/3 complex, leading to actin tail formation and protrusion of the virus away from the cell.

EPEC

EPEC adhere to intestinal epithelial cells in a distinctive pattern, termed attaching and effacing lesions. These result from effacement of microvilli, close adherence of the bacterium to the epithelial cell membrane and formation of filamentous actin structures below the host cell membrane at the site of intimate attachment to form ‘pedestal’ structures [184]. EPEC direct actin reorganization, leading to pedestal formation, from an extracellular position. EPEC insert a receptor protein, Tir, into the plasma membrane of the underlying epithelial cell, where it acts as the receptor for the bacterial outer membrane protein, intimin [185]. Upon insertion into the epithelial membrane and binding to intimin, Tir is phosphorylated on the intracellular C-terminal Tyr474, a requirement for pedestal formation [186]. The minimum sequence required for EPEC Tir tyrosine phosphorylation and signaling has been identified as a 12-aa region, including Tyr 474 [187]. Phosphorylation of Tir on Tyr474 is required for direct interaction with Nck [187, 188], which in turn recruits NWASp and the Arp2/3 complex [188]. Nck$^{-/-}$ cells are resistant to pedestal formation after binding of EPEC and fail to recruit NWASp and Arp2/3 at the site of bacterial adhesion [188]. NWASp is necessary for pedestal formation, as NWASp localizes to the site of pedestal formation [189, 190] and pedestal formation is not supported by *NWASP*$^{-/-}$ embryonic fibroblasts [190]. Reconstitution of *NWASP*$^{-/-}$ fibroblasts with NWASp deletion mutants has identified the N-terminal (residues 1–274) as necessary and sufficient for recruitment to pedestals, while the C-terminal WA domain is necessary for pedestal formation.

Based on the deletion mutants used for reconstitution, the WH1 domain specifically is necessary for recruitment to pedestals [190]. Activation of NWASp by Rho family members is not required for pedestal formation [191, 192]. By analogy to the vaccinia system [124] and with identification of the WH1 domain as being necessary for recruitment to pedestals, it has been proposed that WIP is required to recruit NWASp to Nck at the pedestal tip [171, 193]. A recent study, however, suggests that WIP-WASp interactions are not required for NWASp recruitment to Nck [194]. Thus, the downstream events that lead to NWASp recruitment to Nck remain to be defined.

EHEC

EHEC, like EPEC, induces characteristic attaching and effacing lesions after binding to intestinal epithelial cells. There are clear differences, however, in the mechanism of pedestal formation between EHEC and EPEC. In contrast to EPEC, pedestal formation at the site of EHEC binding is not dependent on Nck, since Nck is not recruited to the sites of pedestal formation [187, 194] and pedestal formation occurs on cells lacking Nck [188]. This is not unexpected since the sequence around the EPEC Tir residue 474 is not conserved in the EHEC Tir protein [186] and EHEC Tir is not tyrosine phosphorylated [195]. While EHEC strains can use either EPEC- or EHEC-derived Tir protein for induction of pedestal formation, EPEC strains do not form pedestals when constructed to express EHEC Tir [187, 196, 197]. Similar to EPEC, however, NWASp is required for pedestal formation by EHEC strains. EHEC does not induce pedestal formation in *NWASP*$^{-/-}$ embryonic fibroblasts, but does in NWASP expressing embryonic fibroblasts. In addition, following bacterial adhesion, NWASp is localized to the tip of the pedestals [194]. Expression of deletion and point mutants of *NWASP* in *NWASP*$^{-/-}$ cells identifies residues 226–274 of NWASp as necessary and sufficient to recruit NWASp to pedestals and identifies the WA domain of NWASp as necessary for actin polymerization [194]. The C-terminal half of the GBD is contained in residues 226–274, but most GTPases require residues 200–219 for binding [119, 133, 198]. Thus, it is unlikely that Rho family GTPases, such as Cdc42, are required for the recruitment. By identifying specific binding partners for residues 226–274 of NWASp, it may be possible to clarify the mechanism of recruitment to the pedestals. While there are suggestions that bacterial factors may be required for recruitment of NWASp to the pedestals formed by EHEC binding [197], the details remain to be elucidated.

Shigella

Shigella is an invasive bacterium that secretes proteins to induce its uptake from the colonic lumen into host cells by actin-dependent phagocytosis. Once the phagosome is formed, it is lysed and the bacterium undergoes actin-based motility, using the host cell actin-polymerization machinery to stimulate actin tail production at the pole of the bacterium. Actin-based motility is required for intracellular and intercellular motility of *Shigella* [199]. The *Shigella* IcsA protein, a 120-kDa outer membrane protein with expression at one pole, is necessary and sufficient for *Shigella* motility [200, 201]. Expression of the IcsA protein in non-motile *E. coli* confers mobility in cytoplasmic extracts [201]. NWASp is required for *Shigella* motility and intercellular spread as demonstrated by in vitro assays and using NWASp dominant negative mutants and NWASp knockouts [183, 190, 202, 203], but is not essential for uptake [183]. IcsA binds directly with NWASp [202–204] and activation of NWASp in this system is also not dependent on Cdc42 [205, 206]. Unlike vaccinia, EPEC and EHEC, which appear to be able to use NWASp or WASp protein for activation of the Arp2/3 complex [181, 183, 189], interaction of the *Shigella* IcsA protein appears to be specific for NWASp [183, 204]. WIP and Nck are recruited to *Shigella*, but do not appear to be necessary for Arp2/3 activation [124, 207].

Prospects for WAS genetic therapy

Although the function and biochemistry of WASp have become increasingly clear, there are still many patients who suffer from WAS. As not all patients have appropriately matched donors for HSCT, gene therapy has been proposed as an alternative for curative treatment. WAS is a candidate disorder for gene therapy based on the severity of its phenotype, inadequate treatment options in some cases, identified single gene defect and tissue-specific expression in cells of hematopoietic origin. In addition, unlike other genes involved in immunoregulation whose expression is tightly regulated during T cell activation, such as *TNFSF5* (CD40 ligand), *WASP* appears to be constitutively expressed.

A continuing limitation of gene therapy, based on current gene delivery vectors, is that transduction efficiencies into HSCs remain low. As a result, successful gene therapy requires that the transduced cells have a selective advantage over untransduced cells. Several findings suggest that transduction of the wild-type *WASP* gene into affected human HSCs would confer selective advantage. First, heterozygous females demonstrate non-random X-chromosome inactivation in hematopoietic cells [65, 67, 69, 208, 209]. Second, spontaneous in vivo reversions of *WASP* mutations have been reported that result in mosaicism of T cells [209–211]. Third, wild-type gene transfer into HSCs from *WASP* knockout mice appears to confer selective survival advantage when transplanted into irradiated mice [212, 213]. Finally, *WASP*-deficient

HSCs from mice are defective in their ability to migrate to hematopoietic tissues, which may provide a further level of selective advantage and may explain the pattern of non-random X-chromosome inactivation in hematopoietic cells [70].

Retroviruses expressing *WASP* have been constructed and used for transduction of the wild-type gene into primary T cells [214] and T cell lines [215] from WAS patients. Transduction of the wild-type gene into T cells results in correction of defects in proliferation, actin polymerization, TCR downregulation and interleukin (IL) 2 secretion in response to TCR stimulation [214, 215]. These studies demonstrate the feasibility of gene therapy approaches for WAS. Infusion of the transduced primary T cells into WAS patients with chronic infections has also been proposed as a potential therapeutic modality [215].

Transduction of the wild-type *WASP* into B cell lines from WAS patients results in correction of defects of actin polymerization [216] and restores normal glycosylation patterns of cell surface proteins [217]. Transduction of dendritic cells from WAS patients with the wild-type *WASP* corrects defective podosome formation [218]. Transduction of the wild-type gene into macrophage from *WASP* knockout mice or macrophage from WAS patients restores phagocytosis, podosome formation and chemotaxis [219, 220]. Thus, defects in several hematopoietic cell lines may be corrected by transduction of the wild-type gene into HSCs from WAS patients.

Proof of principle for gene therapy trials for WAS has been provided using the *WASP* knockout mouse. Following transplantation of *WASP* knockout HSCs that were retrovirally transduced with the wild-type *WASP* gene into irradiated recipient mice, B and T cells develop in normal numbers, and the T cell proliferation defect in response to TCR stimulation is corrected [212, 213]. Correction is not associated with increased expression of activation markers or deleterious effects in the reconstituted mice. In addition, the mice transplanted with HSCs transduced with the wild-type *WASP* gene do not develop colitis, or develop an attenuated form of colitis. This is in contrast to the mice transplanted with HSCs transduced with control vectors who develop colitis and is a further indication of correction of immune dysfunction by gene transfer [212, 214]. Finally, competitive repopulation studies were performed which demonstrated a selective advantage of *WASP*-expressing cells in spleen and lymph node, but not in the bone marrow [212]. The development and function of other hematopoietic cell lineages were not reported from this study. Another study, using a similar approach, demonstrated progressive selective advantage for T cells and B cells, with initial enrichment of neutrophil/myeloid lineage cells by competitive repopulation and retroviral transduction of HSCs. Secondary immune responses to influenza virus were also restored in *WASP*$^{-/-}$ mice by infusion of *WASP*-transduced HSCs [213]. Although these studies provide the necessary encouragement to proceed to human trials, further study of this system is needed to clarify issues pertinent to human gene therapy trials and to develop vectors to optimize control of gene expression. As an example, a recent report describing a revertant mutant demonstrates that WASp expression confers a selective advantage to T cells, but not B cells [221], in contrast to results from murine models.

Based on the initial successes of trials for gene therapy for severe combined immunodeficiency (SCID) caused by mutations in the γ_c chain [222] and adeneosine deaminase deficiency [223], there has been much interest in proceeding with gene therapy trials for other monogenic disorders, including WAS. Unfortunately, with the development of leukemia in two patients in the γ_c chain SCID trial [221, 224], it is clear that there are obstacles to overcome before gene therapy becomes a readily available therapeutic option. While the technical aspects of gene therapy are being refined, continued study of the molecular mechanisms of these disorders is critical, as a thorough understanding is required for the success of the therapy and development of adjunct and/or alternative treatments. In particular, comprehensive knowledge of WASp biology holds the potential for development of novel biochemical and other molecular therapies that could circumvent or compensate for defective WASp function and may provide additional therapeutic advantages.

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