A neural-specific splicing event generates an active form of the Wiskott–Aldrich syndrome protein

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Actin polymerization is required for cellular events such as podosome, lamellipode or filopode formation in migrating cells, and members of the Wiskott–Aldrich syndrome protein (WASP) family have essential roles in regulating actin dynamics at the cell leading edge. However, WASP proteins need first to be activated in order to be able to target actin polymerization. Here, we show the occurrence of a neural-specific splicing event, which is favoured by the nuclear orphan receptor chicken ovalbumin upstream promoter–transcription factor 1, and generates a truncated WASP protein deleted of exon 2-encoded amino acids. This deletion relocates the protein to the plasma membrane and induces the formation of actin-rich podosome-like structures that also contain paxillin and vinculin. Furthermore, expression of the truncated protein in PC12 cells, as well as in primary neurons, stimulates neuritogenesis. These data underscore the importance of the neural-specific splicing of WASP RNA during development.

Keywords: WASP; podosomes; paxillin; COUP-TFI; WH1

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INTRODUCTION

In the past ten years, proteins of the Wiskott–Aldrich syndrome protein (WASP) family (i.e. WASP, N-WASP and three WASP-family verprolin homologous protein (WAVE)/Scar isoforms) have emerged as key regulators of actin polymerization. These proteins are involved in the formation of cellular structures required for cell locomotion (Linder *et al*, 1999; Takenawa & Miki, 2001), phagocytosis and endocytosis (Lorenzi *et al*, 2000; Kessels & Qualmann, 2002) and vesicle transport (Taunton *et al*, 2000).

WASP proteins are known to interact through distinct conserved domains, with a large variety of cell-signalling molecules implicated in actin filament rearrangement. The amino-terminal part of WASP proteins carries a WASP homology 1 (WH1) domain (aa 39–149), which shows similarity to pleckstrin homology (PH) domains (Miki *et al*, 1996). A central domain has been shown to bind Rho family GTPases and is thus called the

GTPase-binding domain (GBD). The GBD is also able to contact the carboxy-terminal part of WASP, thus generating an auto-inactive form of WASP that is unable to bind the actin nucleation complex Arp2/3 (Kim *et al*, 2000). Activation can, however, occur through binding of Cdc42 and/or PIP₂ (Prehoda *et al*, 2000; Rohatgi *et al*, 2000). WASP proteins share a common C-terminus that consists of one or two verprolin homology regions (V), followed by a cofilin homology and acidic region (CA). V and CA motifs are able to interact with monomeric actin (Miki & Takenawa, 1998) and Arp2/3, respectively (Machesky & Insall, 1998). Thus, the VCA domain is likely to be involved directly in actin nucleation (Yamaguchi *et al*, 2002).

Neuronal actin dynamics involve at least N-WASP and the WAVEs. Indeed, N-WASP is able to enhance neurite outgrowth from differentiating PC12 cells (Banzai *et al*, 2000), whereas WAVE proteins are found in growth cones of NG108 cells (Nozumi *et al*, 2003). As for the WASP gene, it is known to be mainly expressed in the haematopoietic lineage, in which it has an active role, as Wiskott–Aldrich syndrome (WAS) patients show severe phenotypes such as thrombocytopenia and immunodeficiency (Wiskott, 1936; Sullivan *et al*, 1994). In this report, we show that the WASP gene can generate a neural-specific form of WASP that is able to stimulate neuritogenesis.

RESULTS

Skipping of WASP exon 2 is stimulated by COUP-TFI

Previous data have shown that the nuclear orphan receptor chicken ovalbumin upstream promoter–transcription factor 1 (COUP-TFI), when overexpressed during retinoic-acid-induced differentiation of P19 embryonal carcinoma cells, is able to stimulate cell migration as well as neuritogenesis (Adam *et al*, 2000); two phenomena that are linked to actin dynamics and WASP protein activity. We first ran semiquantitative reverse-transcription–polymerase chain reaction (RT–PCR) experiments in control P19 cells, as well as in P19 cells stably expressing COUP-TFI (Adam *et al*, 2000), to examine whether COUP-TFI could influence WASP and/or N-WASP expression. Fig 1A shows that both genes are expressed in P19 cells and that retinoic acid did not modify N-WASP RNA levels. The WASP gene showed a more complex expression pattern because two RNAs were detected, the longer one being mainly expressed in undifferentiated cells, and the shorter one mainly in retinoic acid (RA)-treated cells. In P19 cells stably expressing COUP-TFI, N-WASP RNA levels remained

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Fig 1 | The WASP gene is subjected to a neural-specific splicing event. RT-PCR analysis of WASP expression in wild-type (wt) P19 cells (A), P19 cells stably expressing wtCOUP-TFI or a mutant COUP-TFI (B) and different tissues (C). Reverse-transcribed RNAs were amplified with primers designed in exons 1 and 3. Note that the 100bp fragments lack exon 2 sequences, as shown by sequencing of the PCR product and by the drawings. PO is a ribosomal phosphoprotein gene used as a control. (D, E) Western blot analysis of P19 cells, P5 cerebellar neurons and GFP-WASP- or GFP-WASPΔEx2-transfected cells, with a WASP antibody. 50 μg of protein were used in each sample except for the spleen extract (10 μg). The arrows indicate the position of molecular weight markers.

unaffected by RA (data not shown), whereas only the short WASP message was detected on addition of RA (Fig 1B). Stable expression of a mutated COUP-TFI unable to bind DNA had no effect (Fig 1B). We next examined WASP expression by RT-PCR on RNAs prepared from different tissues and regions of the

Fig 2 | The nuclear orphan receptor COUP-TFI stimulates exon 2 skipping. A minigene consisting of about 2.5 kb of WASP genomic DNA (A) linked to the SV40 polyadenylation signal was transfected into Cos-7 cells with or without a COUP-TFI expression vector. After reverse transcription, the RNAs were analysed by radioactive PCR (B).

nervous system (Fig 1C). Interestingly, the short WASP RNA was seen only with samples derived from neural tissues. The corresponding complementary DNAs (cDNAs) were cloned and sequenced, revealing that the 250 bp fragment was the expected DNA containing the WASP first three exons, whereas exon 2 was absent from the 100 bp fragment. Thus, exon 2 skipping in WASP RNA is likely to be a hallmark of neural cells. *In silico* translation of WASP RNA deleted from exon 2 revealed that no frameshift or stop codon was introduced by this event, and that the corresponding protein would present a 47 aa deletion (47–93 aa) in the WH1 domain. Western blot analysis revealed the presence of one WASP protein in the spleen extract, migrating at the expected position, whereas P19 cell extracts contained two WASP proteins: the full-length (FL) WASP and a shorter form lacking about 5 kDa when compared with FL (Fig 1D). Cerebellar neurons from a 5-day-old mouse (P5) also expressed both short and FL WASP proteins (Fig 1E). Knowing that the theoretical difference in molecular weight between WASP FL and WASPΔEx2 is 5.3 kDa, the shorter form detected by western blot might result from the exclusion of exon 2.

To determine if exon 2 skipping could be regulated by COUP-TFI, we constructed a WASP minigene containing 2 kb of promoter sequence as well as gene sequence up to intron 4 (Fig 2). When this minigene was transfected alone in Cos-7 cells, the resulting RNA was detected as a single amplicon including exon 2. Notably, co-transfection of the minigene with a COUP-TFI expression vector in Cos-7 cells resulted in a partial exclusion of exon 2 from the transcript, indicating a crucial role for the orphan receptor in regulating splice site selection (Fig 2).

Different subcellular localizations of FL and WASP ΔEx2

Green fluorescent protein (GFP)-fused WASP domains were then expressed in Cos-7 cells to analyse the subcellular distribution of the fusion proteins in comparison with F-actin (Fig 3). Examination of the transfected cells showed that GFP-WASP was mainly found in a large perinuclear domain (first set of GFP-WASP images,

Fig 3 | WASP Δ Ex2 colocalizes with F-actin. Cos-7 cells expressing the various fusion proteins (left column) were analysed by fluorescence microscopy for GFP and F-actin (middle columns). The right column shows the superposition of both labels (scale bars = 20 μ m).

Fig 3, second row) and, in some instances (less than 5% of transfected cells—second set of GFP-WASP images, Fig 3, third row), in perinuclear dots. In any case, FL WASP colocalized with F-actin. GFP-WASP1–254 also accumulated in perinuclear dots, which were devoid of F-actin. GFP-WASP255–521 was mainly distributed around the nucleus, where it colocalized with F-actin. Finally, GFP-WASP Δ Ex2 localized mainly (70% of transfected cells) in F-actin-containing dot-like structures. Furthermore, cells expressing GFP-WASP Δ Ex2 tended to be more spread out on the culture dishes than their neighbours, suggesting a stronger attachment to the surface. These observations led us to postulate that WASP Δ Ex2 could spontaneously relocate to the ventral side of the cell and induce podosome formation. Confocal microscopy experiments allowed us to localize WASP Δ Ex2 in ventral structures approximately 1.5–2.0 μ m high (supplementary Fig S1 online), which is compatible with the size of podosomes observed in other cell types (Evans *et al*, 2003; Moreau *et al*, 2003). Some centrally located structures were higher as they could reach 3.0–3.5 μ m. Fig 4 shows that F-actin, paxillin and vinculin (three known components of podosomes; Linder & Aepfelbacher, 2003) were present in the ventral structures containing WASP Δ Ex2.

Nonetheless, the typical ring-shaped distribution of paxillin and vinculin usually found in podosomes was not observed. Interestingly, WIP, a protein interacting with the WH1 domain of WASP and N-WASP (Ramesh *et al*, 1997; Volkman *et al*, 2002) and which has been shown to localize in podosomes (Moreau *et al*, 2003), was not detected in WASP Δ Ex2-containing structures (Fig 4). These results indicate that WASP Δ Ex2 is able to induce actin polymerization, as well as paxillin and vinculin accumulation, in ventral structures, which may differ from classical podosomes, and that the truncated WASP may not be able to interact with WIP.

WASP Δ Ex2 enhances neuritogenesis

Next, we tested the effects of the different fusion proteins on neurite extension during PC12 cell neuronal differentiation. This cell line produces a homogeneous population of sympathetic neurons when treated with nerve growth factor (NGF), and neurite extension in the differentiating cells is known to involve WASP-like proteins (Banzai *et al*, 2000). This system is thus more suitable for analysing the impact of the various forms of WASP on neurite extension than is the P19 cell system, which produces numerous

Fig 4 | Podosome-like structures are formed in Cos-7 cells on GFP-WASPΔEx2 transfection. Transfected cells were analysed by fluorescence microscopy. F-actin was labelled with rhodamine-phalloidin. Paxillin, vinculin and WASP interacting protein (WIP) were detected by immunocytochemistry. Note that F-actin, paxillin and vinculin colocalized with GFP-WASPΔEx2 (scale bar, 10 μm).

cell types when treated with retinoic acid. Fig 5A, B clearly shows that GFP-WASPΔEx2 was the only fusion protein inducing a higher rate of neurite extension than GFP alone. Similar results were obtained with cerebellar neurons from a 5-day-old mouse. After 3 days *in vitro*, the GFP-WASPΔEx2-transfected neurons showed a higher rate of neurite extension than GFP alone or GFP-WASP-transfected neurons (Fig 5C). Most importantly, 75% of the GFP-WASPΔEx2-transfected neurons that extended neurites showed a punctuated distribution of the fusion protein, whereas only 15% did for GFP-WASP-transfected cells and 3% did for GFP alone (Fig 5C,D). Labelling with rhodamine-phalloidin revealed colocalization of F-actin with GFP-WASPΔEx2 in these dots (Fig 5D). The length of punctuated neurites from GFP-WASPΔEx2-transfected neurons was then compared with the length of neurites from GFP-transfected cells. The results showed a 2.5-fold increase in neurite length when WASPΔEx2 was localized in F-actin-containing dots (Fig 5E). As P5 cerebellar neurons express a short form of WASP (most probably WASPΔEx2; Fig 1E), it would be expected that a large proportion of neurites extending from these neurons would show a punctuated distribution of F-actin. Rhodamine-phalloidin labelling of untransfected cerebellar neurons showed punctuated F-actin in about 50% of neurites (supplementary Fig S2 online). These transfection data provide

evidence that WASP is involved in the control of neuritogenesis and that deletion of the residues encoded by exon 2 has a pronounced effect on the ability of WASP to stimulate neurite outgrowth.

DISCUSSION

In this study, we show that WASP expression is not only restricted to haematopoietic cells but also significant in neural cells. Indeed, WASP mRNA and proteins are detected in the brain tissue and in differentiating P19 cells. These results are consistent with those of She *et al* (1997), who reported northern blot detection of WASP mRNA in several tissues, notably in the brain. Thus, neuronal cells express several members of the WASP family, namely N-WASP, WASP and WAVEs. *In vitro*, all these proteins act similarly by stimulating actin polymerization. Why do neuronal cells then need so many different WASP proteins if they fulfill similar functions? One explanation would be that each WASP protein exerts specific functions within the cell, either because of a specific subcellular localization or because WASP proteins are specifically activated through individual signalling pathways. Experimental data are in favour of at least the first hypothesis. Indeed, WASP and N-WASP are known to be differentially addressed in Cos-7 cells, whereas WASP is mostly perinuclear (Kato *et al*, 1999) and N-WASP is at the plasma membrane (Miki & Takenawa, 1998; Yamaguchi *et al*, 2000). Furthermore, WAVE1, WAVE2 and WAVE3 show different distributions in the growth cones of NG108 neural cells (Nozumi *et al*, 2003). We now show that exon 2 skipping in the WASP RNA generates a form that has a different subcellular localization. The deletion specifically targets the WH1 domain, a domain that provides important protein-protein and protein-lipid interfaces, including the one allowing WIP recruitment (Prehoda *et al*, 1999; Rohatgi *et al*, 2000; Rong & Vihinen, 2000; Volkman *et al*, 2002). Deleting part of the WH1 domain should thus generate a protein that lacks interaction with WIP. This is substantiated by the fact that WASPΔEx2 is unable to localize WIP in F-actin-containing podosome-like structures in Cos-7 cells. WIP is known to inhibit the N-WASP/Cdc42-activated actin polymerization mediated by Arp2/3 (Martinez-Quiles *et al*, 2001), probably through stabilization of the inactive conformation of WASP. As WIP is expressed in the brain cells (data not shown), one would expect that WASPΔEx2 stimulates neuritogenesis owing to a higher ability to reach the activated state. Our data support such a model and further highlight the complex regulation of actin dynamics in neural cells.

METHODS

Plasmid construction. The mouse WASP cDNA was transferred in-frame from Bluescript to pEGFP-C1 or C2 (Clontech) by *Xho*I digest for GFP-WASP FL, *Hind*III/*Bam*HI double digest for GFP-WASP1–254 and *Bam*HI digest for GFP-WASP255–521. The deletion of exon 2 (47–93 aa) sequence was generated by site-directed mutagenesis and restriction-enzyme-mediated excision of the DNA segment. For this purpose, the QuickChange™ site-directed mutagenesis kit (Stratagene, USA) was used with the following primers: mut1up 5'-GCTTGGCCGAAAGTACTGGA CACTGGCTACC-3'; mut1down 5'-GCCAGTGTCAGTACTTTCC GCCAAGAAGCTCAAAG-3' and mut2up 5'-ATGGCCTACTGTCC GCTACTCTGGGAACAGGAGC-3', and mut2down 5'-CCCAGAG

TAGCCGACAGTACTGTAGGCCATAAAGGCGG-3'. The resulting mutant, which was called WASP Δ Ex2, had a deletion of residues 47–95 as well as two point mutations (C45Y and W46C).

Genomic DNA cloning. The mouse WASP genomic DNA was obtained by screening a mouse genomic library (Mouse 129/SvEvTacFBR Lambda Genomic Library, Stratagene), using the WASP cDNA as a probe. One of the positive clones contained a 3.6 kb DNA fragment with 2.2 kb of proximal promoter and the beginning of the WASP gene (up to intron 4, +1430).

RT-PCR and western blot analysis. A 2 μ g protein of total RNA was used as template for M-MLV reverse transcriptase and Pd(N)6

random hexamer (Amersham Pharmacia). PCR amplification was performed on 2 μ l of the reverse transcription reaction, with 1 μ M of the following primers: mWASP up 5'-CAGAGACTCTTTGAGC-3'; mWASP down 5'-AGTGTGGAAGAACGG-3'; mN-WASP up 5'-AACGAGTCTCTCTTCTCCTCC-3'; mN-WASP down 5'-AGAT GTTGTGACTTGTGAACC-3'; PO up 5'-CAGCTCTGGAGAAAC TGCTG-3'; PO down 5'-GTGTACTCAGTCTCCACAGA-3'. For radioactive PCRs, 2 μ Ci [32 P- α]dCTP was added to the amplification mix. When assaying the activity of the minigene, the constructs were co-transfected in Cos-7 cells together with a β -galactosidase expression vector, and the amount of RNA used for reverse transcription was normalized for β -galactosidase activity. For western blot analysis, whole-cell extracts were run in a 12.5% acrylamide/0.085% bisacrylamide gel. After blotting, the membrane was incubated with a WASP antibody (B-9) from Santa Cruz, Inc.

Cell culture and transfections. PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7% horse serum and 3% fetal calf serum (FCS). In all, 2 μ g of each recombinant plasmid was transfected into PC12 cells with FuGENETM 6 transfection reagent (Roche). At 24 h after transfection, differentiation was induced with 50 ng/ml recombinant NGF (ICN) and allowed to proceed for 48 h in DMEM supplemented with 1% horse serum. P19 embryonal carcinoma cells and Cos-7 cells were grown in DMEM supplemented with 10% FCS. Stable P19 cell lines and RA-induced neuronal differentiation were as described (Adam *et al*, 2000). Cos-7 cells were grown in the same conditions as P19 cells. P5 cerebellar neurons were obtained from mice as described (Loeffler *et al*, 1990). Cells were seeded on polylysine-coated coverslips in minimum essential medium (MEM)/10% FCS. At 1 h after plating, cells were transfected with the various vectors and Lipofectamine 2000 (Invitrogen).

F-actin visualization and immunocytochemistry. Cos-7 cells were transfected with the various GFP fusion vectors. After 48 h, cells were fixed with 4% paraformaldehyde and 10% saccharose in phosphate-buffered saline (PBS), and then washed twice with 100 mM glycine in PBS. Labelling of F-actin was performed with 1% Tween 20 and 3 μ M tetramethylrhodamine isothiocyanate (TRITC)-phalloidin (SIGMA) in PBS. For immunocytochemistry, Cos-7 cells were grown on polylysine-coated coverslips and transfected with GFP-WASP expression vectors. Cells were then fixed and incubated with primary antibodies (anti-paxillin from BD Biosciences; anti-vinculin (H-300) and anti-WIP (G-20) from Santa Cruz, Inc.), followed by incubation with rhodamine-

◀ **Fig 5** | Expression of WASP Δ Ex2 results in increased neuritogenesis in PC12 cells and P5 cerebellar neurons. The different GFP fusions were expressed in PC12 cells, which were then induced to differentiate for 48 h by NGF treatment (scale bars, 20 μ m; A). Fluorescent cells were analysed for the presence of neurites longer than the diameter of two cell bodies (B). Statistical differences are shown by asterisks. (C) P5 cerebellar neurons were transfected with the indicated vectors. After 3 days, neurite-bearing neurons were scored as above and the percentage of neurite showing a punctuated GFP fluorescence was calculated. (D) The distribution of F-actin and GFP or GFP-WASP Δ Ex2 was compared in transfected P5 cerebellar neurons. Note that GFP-WASP Δ Ex2 is found in F-actin-containing dots (scale bar, 10 μ m). (E) Neurite lengths were estimated from GFP- and punctuated GFP-WASP Δ Ex2-expressing cerebellar neurons. Statistical difference was analysed by a Student's *t*-test.

coupled secondary antibodies (Jackson Immunoresearch Laboratories, Inc.). Images were acquired on a DMRXA2 microscope (Leica) using a CCD camera (CoolSnap HQ, Roper Scientific) and subsequently processed by the Metamorph software (Universal Imaging). For confocal microscopy, a TCS NT microscope (Leica) was used.

Neurite analysis and statistical methods. PC12 cells were directly observed in culture plates with an epifluorescence microscope coupled to a digital camera. P5 cerebellar neurons were grown on polylysine-coated coverslips and mounted on glass slides before observation. GFP-positive cells with neurites longer than the diameter of two cell bodies were scored as 'neurite bearing'. Neurite length was estimated with the Scion Image software (Scion Corp.). Two separate experiments were carried out, and similar results were recorded. At least 200 cells for each treatment were counted. Statistical analyses were performed using the χ^2 -test, $P < 0.05$ for PC12 cells and the Student's t -test for P5 neurons. **Supplementary information** is available at *EMBO reports* online (<http://www.emboreports.org>).

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