N-terminally truncated Vav induces the formation of depolymerization-resistant actin filaments in NIH 3T3 cells

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Abstract The Dbl family proto-oncogene *vav* is a guanine nucleotide exchange factor (GEF) for Rho family GTPases. Deletion of the N-terminus of Vav, harboring the single calponin homology (CH) domain, activates Vav's transforming potential, suggesting an important role of the CH domain in influencing Vav function. Since calponin binds actin, it has been suggested that the CH domain may mediate association with the actin cytoskeleton. In this study we have analyzed the subcellular localization and investigated the putative actin association of the Vav protein using enhanced green fluorescent protein (EGFP) fusion constructs. Our data show that both EGFP-tagged full length Vav and the CH domain-depleted EGFPvav 143–845 construct localize throughout the cytoplasm but fail to colocalize with F-actin. However, the latter construct of Vav was more strongly retained in the Triton-insoluble cytoskeleton fraction than full length Vav. Whereas removal of the CH domain had no apparent influence on the subcellular localization of Vav, deletion of the SH domains caused nuclear localization, indicating that Vav contains a functional nuclear localization signal. Expression of N-terminally truncated Vav constructs caused depolarization of fibroblasts and triggered the bundling of actin stress fibers into parallel arrays in NIH 3T3 cells. Notably, the parallel actin bundles showed prolonged resistance to the actin polymerization antagonists cytochalasin B and latrunculin B. These data point towards a regulatory role for the CH domain in Vav and suggest an actin cross-linking or bundling protein as a downstream effector molecule of *vav*-mediated signalling pathways.

Key words: Vav; Green fluorescent protein; Localization; CH domain; Cytoskeleton; Actin

1. Introduction

Rearrangement of the actin cytoskeleton is largely influenced by the activity of Rho family GTPases, namely Rac1, RhoA and Cdc42, leading to the formation of lamellipodia, stress fibers, and filopodia, respectively [1,2]. Upstream regulators control their nucleotide-bound state by activating the GTPase activity (GAPs) or by facilitating the exchange of GDP for GTP (GEFs). Some of these proteins, like Dbl [3] and Vav [4], exhibit transforming activities, presumably by constitutively activating Rho family GTPases. It is still unclear whether Vav, which is specifically expressed in cells of the hematopoietic system, acts as a specific GEF for Rac1 [5,6], or enhances nucleotide exchange for all three Rho family GTPases [7,8]. Like all known GEFs Vav consists of an arrangement of functional modules [9,10] containing an active core comprising a Dbl homology (DH) domain and an adjacent pleckstrin homology (PH) domain [10–13]. Additional C- and N-terminal domains in the various Dbl family proteins are likely to modulate the specificity of the molecule for certain GTPases by serving as targeting modules or regulators of protein-protein or protein-phospholipid interactions (reviewed in [10,14]).

A module unique to two GTPase regulators, the Cdc42 GAP, IQGAP, and the Rac1 GEF, Vav, is the calponin homology (CH) domain [15]. This 100 residue motif has been proposed to serve as an autonomous actin binding site, directly linking signalling molecules to the actin cytoskeleton. Notably, the first 216 amino acid residues of IQGAP1, embracing the CH domain, have been demonstrated to co-sediment with actin in vitro [16]. However, isolated CH domains from other single CH domain-containing proteins [17] fail to bind to actin, suggesting a subordinate role for this motif in actin binding. Moreover, in the archetypal smooth muscle protein calponin this domain is neither necessary nor sufficient, and indeed dispensable for actin association in vitro and in vivo [18]. In this context, the role of the single CH domain in Vav required reconsideration (see [19] for review).

With the exception of gelsolin [20] and cofilin [21,22] little is known about the ultimate downstream effectors of Rho family GTPase signal cascades which directly interact with the actin cytoskeleton (see [23,24]). It is evident that GTPases themselves cannot modulate the conformation of actin filaments, but rather mobilize actin binding proteins to specialized sites within the cell, which then drive actin polymerization in lamellipodial protrusions, or lead to the stabilization of actin bundles in stress fibers [25–27]. It is thus important to determine the cellular localization of components within the signalling pathway of small GTPases involved in actin cytoskeleton rearrangements and their immediate upstream and downstream effector molecules (see [19]).

Here, using enhanced green fluorescent protein (EGFP)-tagged mutant constructs we show that the CH domain is not a critical determinant for the cellular localization of Vav and that deletion of the CH domain-containing amino-terminus enhances the formation of Rho-dependent stress fibers in NIH 3T3 cells.

2. Materials and methods

2.1. EGFPvav constructs

Full length human proto-vav (1–845) and vav 143–595 [28,29] in pTag/CMV-neo were a gift of G. Baier (University of Innsbruck). For both constructs the insert (including the N-terminal p18^{HIV} tag encoded by the vector) was cut out with *Bam*HI and *Bcl*I and cloned

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Abbreviations: EGFP, enhanced green fluorescent protein; Dbl, diffuse B-cell lymphoma; SH, src homology; Rho, ras homology; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor

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into the *Bam*HI site of pMW172 [30]. The correct orientation was confirmed by sequencing using a LI-Cor model 4000 automated sequencer (MWG-Biotech, Germany). Both pMW172 constructs were digested with *Pst*I, which cuts once in the vav sequence and once in the ampicillin resistance gene of the vector. To obtain vav 1–595 the fragment of vav 1–845 pMW172 encoding the N-terminal part of vav was ligated to the fragment of vav 143–595 pMW172 encoding the C-terminal part. For vav 143–845 the C-terminal fragment of vav 1–845 pMW172 was ligated to the N-terminal fragment of vav 143–845. Inserts of all four vav constructs in pMW172 were cut out with *Bam*HI and *Eco*RI and cloned in frame into the *Bgl*II and *Eco*RI sites of pEGFP-C1 (Clontech). All constructs were confirmed by sequencing the region of the restriction sites used. EGFPvav 1–143 was generated by the polymerase chain reaction (PCR) using EGFPvav 1–595 as template. A sense primer (GAG AGG ATC CAT GGG ATC CCG TAT CCA G) binding to the N-terminus of the $p18^{HIV}$ tag introducing a *Bam*HI site at its 5′ end and an antisense primer (CTG AAT TCA CTG TAG ATG TCT TCA TC) binding to the region coding for vav 138–143 and introducing an *Eco*RI site at its 5′ end were used. The isolated PCR product was digested with *Bam*HI and *Eco*RI and ligated into the *Bgl*II and *Eco*RI site of pEGFP-C1. The stop codon supplied by the vector was used to terminate translation. The correct sequence was confirmed by sequencing both strands. To express a fusion protein of the CH domain of calponin h1 to EGFP a PCR was performed with calponin h1 1–127 in pCGN [18] as template using primers as follows: sense primer (GAG GAT CCA GCT ACC CTT ATG ACG TG) binding to the N-terminus of the HA tag encoded by pCGN and introducing a *Bam*HI site at its 5′ end; antisense primer (GAG AAT TCT TAC AGA GCC AGG AGA GTG) binding to the region encoding amino acids 122–127 introducing a stop codon and an *Eco*RI site. The PCR product was cloned and sequenced as for EGFPvav 1–143.

2.2. Cell culture, transfection and immunofluorescence microscopy

NIH 3T3 or REF 52 fibroblasts [31] were grown in high glucose (4500 mg/l) DMEM supplemented with 10% FBS (Hyclone, Utah, USA), penicillin/streptomycin (Life Technologies, Austria) at 37°C and 5% CO_2. Cells at 70% confluence were transfected using the Lipofectamine (Life Technologies, Austria) method in the absence (NIH 3T3) or presence (REF 52) of serum, essentially as described [18]. Stable cell lines were passaged after similar transfection times and cells resistant to 1 mg/ml G-418 and expressing the transfected transgene were identified by Western blotting. For transient expression cells were grown and transfected as described [31] and prepared for either immunofluorescence or protein extraction 24 h post transfection. For immunofluorescence microscopy cells were cultured on 15 mm glass coverslips. Cells were washed three times in PBS (138 mM NaCl, 26 mM KCl, 84 mM Na_2HPO_4, 14 mM KH_2PO_4, pH 7.4), fixed in 3.7% PFA (Merck, Germany) in PBS for 60 min and subsequently extracted with 0.3% Triton X-100 in PBS for 1 min. F-actin was visualized using Alexa 568 or Alexa 488 phalloidin (Molecular Probes, The Netherlands). Antibodies to GFP (polyclonal and monoclonal) were from Clontech, polyclonal antibodies to vav from Santa Cruz Biotechnology, and horseradish peroxidase-coupled secondary antibodies from Amersham. Fluorescent images were recorded on a Zeiss Axiophot using a 63× oil immersion lens and Kodak P3200 or P400 Tmax film.

2.3. Cell extraction and fractionation

Twenty-four hours after transfection cells grown in 60 mm petri dishes were washed twice with ice-cold PBS and extracted with 200 μl extraction buffer (10 mM HEPES pH 7.5, 1.5 mM $MgCl_2$, 1 mM DTE, 0.1 mM PMSF, 0.67 μg/ml pepstatin, 1.67 μg/ml leupeptin, 10 or 100 mM KCl, with or without addition of 0.5% Triton X-100). Cells were scraped off with a cell scraper, transferred to a glass homogenizer and lysed with 40 strokes on ice. The extract was centrifuged at 100 000×*g* for 30 min at 4°C in a Sorvall S100-AT4 rotor. The supernatant was centrifuged again at 100 000×*g* for 20 min. To the supernatant of this latter centrifugation 1/4 volume of 5×SDS sample buffer was added. The pellet of the first centrifugation was washed with 200 μl of extraction buffer and centrifuged again at 100 000×*g* for 20 min at 4°C. The supernatant was removed and the pellet was brought to the original volume and 1/4 volume of 5×SDS sample buffer was added. The distribution of EGFPvav in the pellet and supernatant was estimated by Western blotting using a monoclonal antibody against GFP.

2.4. Electrophoresis and Western blotting

Analytical SDS gel electrophoresis on 8–22% gradient polyacrylamide mini-slab gels and Western blotting onto nitrocellulose (Amersham) was performed as described elsewhere [32]. Transferred proteins were visualized using horseradish peroxidase-coupled secondary antibodies and the ECL chemiluminescence detection system (Amersham).

2.5. Cytoskeleton disruption

Cells grown on 15 mm glass coverslips to 60% confluence, serum-starved for 24 h and then incubated for 30 min with 5 μM cytochalasin B or 200 nM latrunculin B (Santa Cruz Biotechnology, USA) in DMEM. Cells were then processed for fluorescence microscopy as above.

3. Results

For the purpose of this study we constructed Vav mutants (Fig. 1A), fused at their amino-terminal ends to the EGFP lacking either the amino-terminus embracing the CH domain (EGFPvav 143–845), the entire SH2-SH3-SH2 domain region at the carboxy-terminal end (EGFPvav 1–595), or both (EGFPvav 143–595). For comparison of the cellular effects of these mutants we used the full length Vav molecule (EGFPvav 1–845) and the isolated amino-terminus (EGFPvav 1–143). With the exception of the latter construct, all EGFP fusion constructs contained the catalytic core consisting of the DH and PH domains, the cysteine-rich, diacylglycerol-

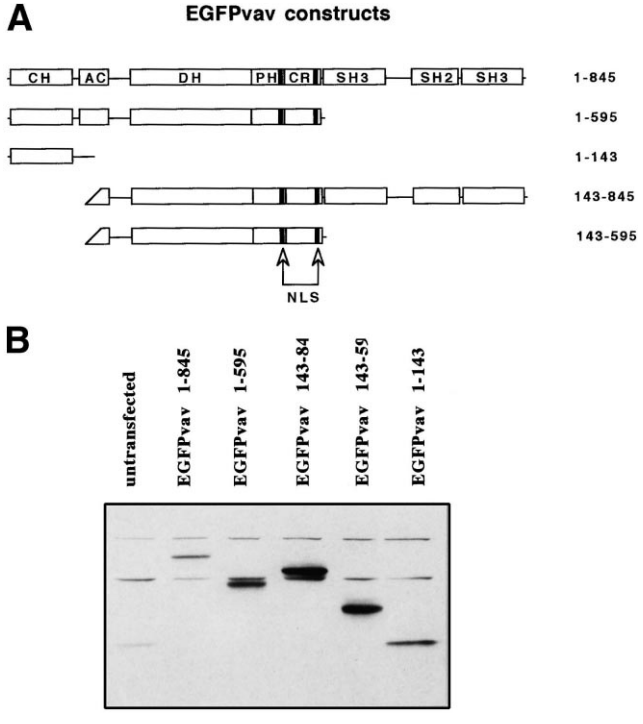

Fig. 1. A: Molecular domain structure of EGFPvav constructs. Abbreviations: CH, calponin homology domain; AC, acidic domain; DH, Dbl homology domain; PH, pleckstrin homology domain; CR, cysteine-rich region, SH2/SH3, src homology domains 2 and 3. The position of the two putative nuclear localization signals is indicated. All EGFP fusions contain the fluorescent protein at their amino-terminus. Numbers indicate amino acid residues according to the sequence of human vav. B: Western blot of whole cell extracts from stable cell lines expressing the indicated EGFPvav constructs, or transiently transfected NIH 3T3 cells (EGFPvav 1–143) probed with a polyclonal anti-GFP antibody.

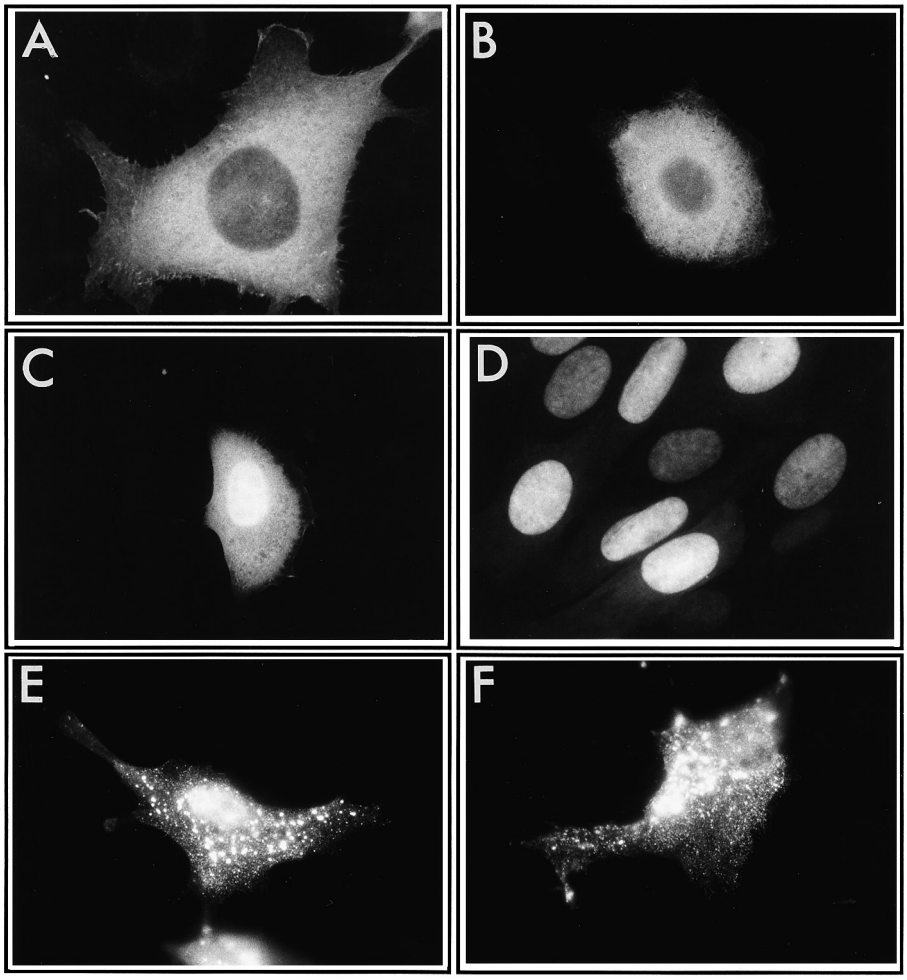

Fig. 2. Subcellular localization of EGFPvav constructs. Full length EGFPvav 1–845 (A) and EGFPvav 143–845 (B) localize throughout the cytoplasm of stable NIH 3T3 cells. Deletion of the C-terminal SH domains causes partial translocation of EGFPvav 1–595 to the nucleus with residual fluorescent signal in the cytoplasm (C). The double truncation EGFPvav 143–595 localizes almost exclusively inside the nucleus (D). Isolated CH domains of Vav (E) and h1 calponin (F) transiently transfected into NIH 3T3 cells are concentrated in spots distributed at the plasma membrane and in the cytoplasm. All direct EGFP fluorescence.

binding region, as well as the two putative nuclear localization signals (NLS).

Conflicting reports by several groups have shown either cytoplasmic [8,33] or nuclear localization for Vav [34]. To determine the subcellular localization of Vav we selected stable cell lines from NIH 3T3 fibroblasts transfected with the EGFP-tagged fusion constructs depicted in Fig. 1A. Expression of the transgene in selected stable cell lines was verified by Western blotting using a polyclonal antibody against GFP or human Vav (Fig. 1B). Phosphorylation of the EGFPvav proteins on tyrosine residues was confirmed by Western blotting employing a specific monoclonal antibody (not shown). The distribution of Vav was analyzed by direct fluorescence microscopy. As shown in Fig. 2, full length Vav (EGFPvav 1–845) was distributed throughout the cytoplasm but excluded from the nucleus. A similar diffuse cytoplasmic localization was observed for the construct EGFPvav 143–845, lacking the CH domain. By contrast, the two C-terminal deletion mutants EGFPvav 1–595 and in particular EGFPvav 143–595, localized to the nucleus. Confocal microscopy confirmed the presence of EGFP fluorescence inside the nucleus (data not shown). Thus, deletion of the carboxy-terminal SH3-SH2-SH3 domain caused the translocation of Vav into the nucleus, while deletion of the CH domain had no detectable effect on the localization of EGFPvav constructs. Identical localization patterns were obtained with untagged versions of either full length or N-terminally truncated Vav transiently transfected into NIH 3T3 cells and visualized by indirect fluorescence microscopy using a polyclonal Vav antibody (not shown). Transient transfection of the construct EGFPvav 1–143 comprising the amino-terminus of Vav including the CH domain into NIH 3T3 fibroblasts revealed a spotty distribution along the plasma membrane and in the cytoplasm (Fig. 2E). A similar pattern was obtained with the isolated CH domain of calponin h1 (Fig. 2F).

Vav is suspected to directly activate RhoA in addition to Rac1 [7]. Therefore, we next compared the effect of EGFPvav overexpression on the organization of the stress fiber actin cytoskeleton in cultured cells. In stable NIH 3T3 cell lines expressing EGFPvav 1–845 the organization of the actin cytoskeleton was unaltered (Fig. 3A,B). CH domain-depleted EGFPvav 143–845, in contrast, induced a characteristic parallel bundling of stress fibers (Fig. 3C,D). In stable cell lines expressing comparable levels of the construct EGFPvav 143–

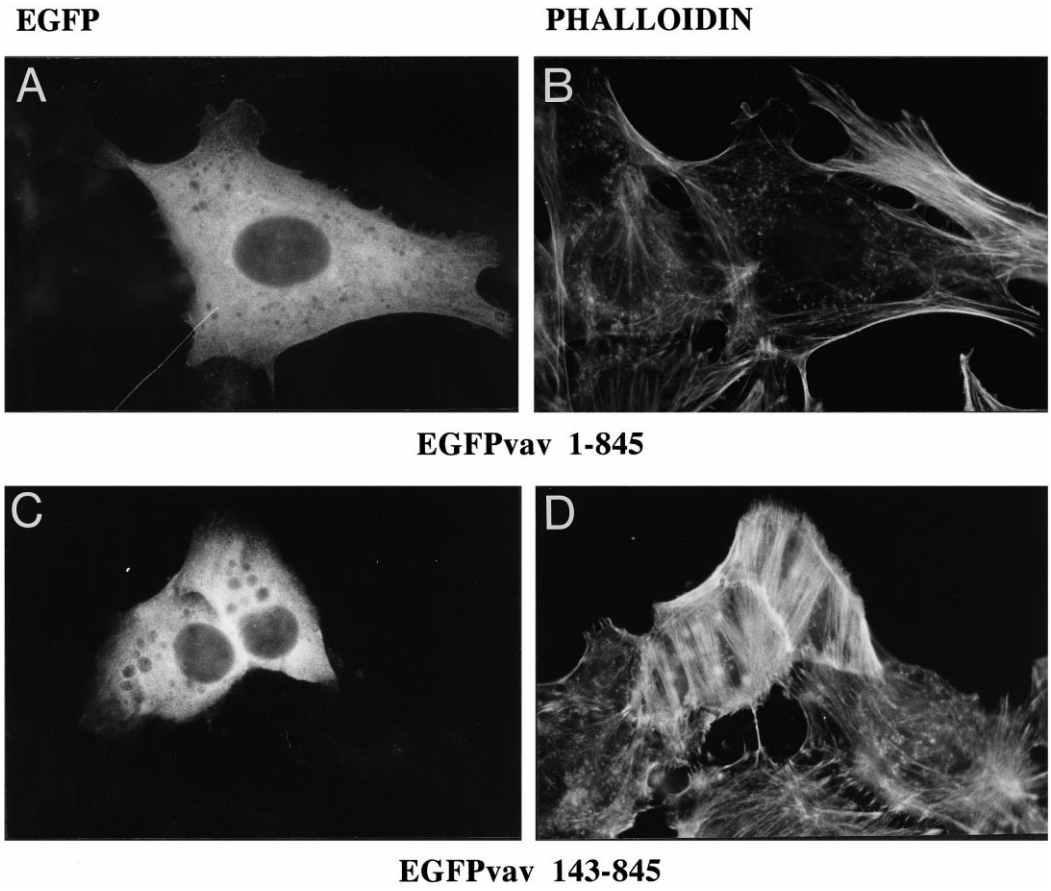

Fig. 3. Bundling of actin stress fibers by EGFPvav 143–845 in stable clonal lines of NIH 3T3 fibroblasts. Cells expressing EGFPvav 143–845 show increased parallel bundling of the stress fibers which traverse the cell.

595 which shows an intense nuclear localization only marginal stress fiber bundling was observed (not shown). Thus, the bundling effects required the cytoplasmic distribution of the expressed transgene. Notably, forced expression of the same construct in transiently transfected NIH 3T3 cells correlated with a significantly increased EGFP fluorescence present in the cytoplasm and lead to the induction of stress fiber bundles, similar to those observed with the EGFPvav 143–845 construct (not shown). No significant colocalization of either EGFPvav construct with these stress fiber arrays, however, was observed.

When the stable NIH 3T3 clonal cell lines were treated with 5 μM cytochalasin B (Fig. 4A–D) or 200 nM latrunculin B (Fig. 4E–H) for 30 min prior to fixation with 4% PFA, the actin stress fiber arrays induced by EGFPvav 143–845 were found resistant to actin depolymerization caused by either of the two toxins (Fig. 4C,D,G,H). By contrast, the actin stress fibers of EGFPvav 1–845 expressing cells were sensitive to both cytochalasin B (Fig. 4A,B) and latrunculin B (Fig. 4E,F). Double immunofluorescence staining further revealed that cells expressing low levels of EGFPvav 143–845 were as sensitive to the action of the toxins as untransfected wild type or EGFP control cells (not shown).

A possible mechanism for the activation of vav's oncogenic potential is the abolition of actin association of the oncoprotein upon disruption of its CH domain [35]. To further test this hypothesis we extracted stably or transiently transfected mouse NIH 3T3 and rat embryo fibroblast (REF 52) cells in the presence or absence of Triton X-100 and varying concentrations of KCl. In the absence of detergent, only a small fraction of the EGFPvav protein was extracted from the cells in the presence of either 10 or 100 mM KCl (Fig. 5, upper panel). Addition of 0.5% Triton X-100 to the extraction buffer led to the quantitative extraction of full length EGFPvav 1–845, but was ineffective in solubilizing the CH domain-depleted EGFPvav 143–845 mutant. Increasing the ionic strength from 10 to 100 mM KCl had only a moderate effect on the extraction of EGFPvav 1–845, but effectively reduced the amount of EGFPvav 143–845 in the pellet by 50%.

4. Discussion

Rho family GTPases are known to modulate the arrangement of F-actin structures [2,36] and their activity is in turn regulated by a variety of GAPs and GEFs, acting as molecular on and off switches [27]. Precise cellular localization is an essential prerequisite for signal transduction molecules to direct their activities to other downstream targets. Thus, the identification of a single CH domain in two GTPase regulators, namely the Cdc42 GAP, IQGAP [37], and the Rac1 GEF, Vav [15], has evoked ideas about a possible interaction of these upstream effectors with actin [16,38,39] potentially providing a direct link for signalling events to the actin cytoskeleton [26]. As a consequence of the above it has been suggested further that the activation of Vav's oncogenic potential, mediated through activation of Rac and Rho is a

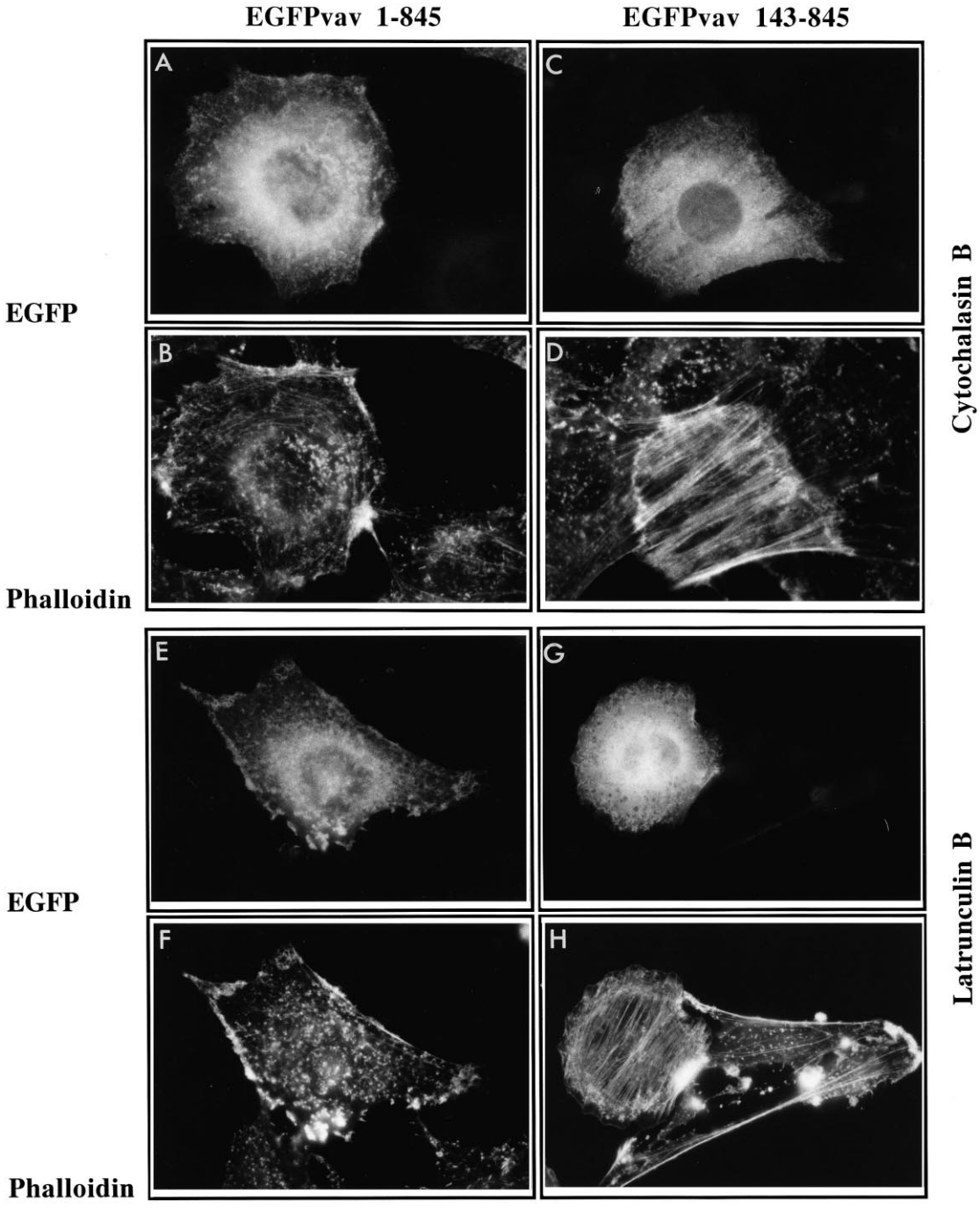

Fig. 4. EGFPvav 143–845-induced actin bundles are resistant to cytochalasin B and latrunculin B. Cells were serum-starved for 24 h and incubated with the indicated toxins for 30 min. Note the presence of actin stress fibers in cells expressing EGFPvav 143–845 (D,H). A,C,E,G: EGFP fluorescence. B,D,F,H: Actin visualized with Alexa 568.

result of inappropriate cellular localization caused by the loss of the actin binding CH domain [14].

Fusion to EGFP did not compromise the localization of the Vav constructs used. The distribution of full length and truncated EGFPvav was similar to that of the untagged Vav counterparts (data not shown) or that reported previously for HA- [40] myc- [8] or T7- [41] tagged Vav. For both EGFPvav 1–845 and EGFPvav 143–845 we observed a diffuse localization in the cytoplasm and along the plasma membrane. We failed, however, to detect any specific enrichment along stress fibers or in lamellipodia. Likewise, a construct comprising the isolated CH domain of Vav transiently transfected into NIH 3T3 cells failed to associate with F-actin containing structures and the resulting, punctate localization pattern was similar to that observed with isolated CH domains of SM22 or calponin [18] indicating that the CH domain alone is ineffective in targeting Vav to the actin cytoskeleton.

Analysis of the amino acid sequence of Vav has identified two putative NLS in the C-terminal half of the molecule [35], and Vav is translocated into the nucleus of prolactin-stimu-

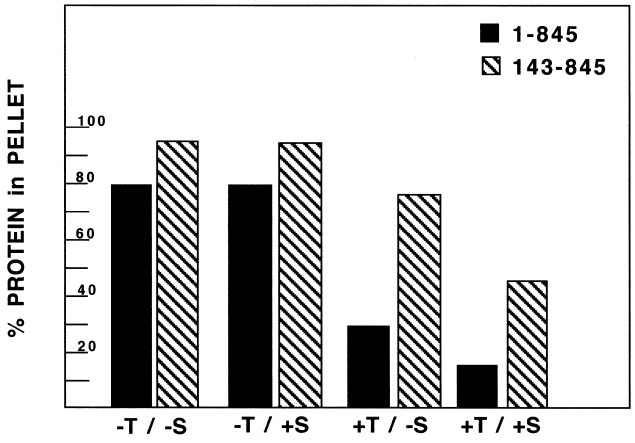

Fig. 5. Retention of EGFPvav 143–845 in the Triton-insoluble cytoskeletal fraction of transiently transfected REF 52 cells. The N-terminally deleted EGFPvav 143–845 construct (hatched bars) shows stronger retention with the Triton-insoluble fraction compared to the full length EGFPvav 1–845 (solid bars). +T, 0.5% Triton X-100; −S, 10 mM KCl; +S, 100 mM KCl.

lated Nb2 cells [42] and UT7 S cells [34]. Moreover, Vav has been shown in vitro to associate with the nuclear protein KU-70, a subunit of the DNA-dependent protein kinase, via its C-terminal SH3 domain [34]. Finally, Bertagnolo and colleagues [43] demonstrated the presence of tyrosine phosphorylated Vav in the nuclear compartment of stimulated HL-60 granulocytes. Here we show that deletion of the C-terminal SH3-SH2-SH3 domains in Vav causes translocation of the corresponding constructs into the nucleus. While EGFPvav 1–595 is partly retained in the cytoplasm, the double truncation EGFPvav 143–595 is almost entirely translocated to the nucleus. This suggests that the C-terminal SH domains can regulate (or mask) the adjacent nuclear localization signals. From our limited deletion studies we are, however, unable to determine if one or both of the two putative NLS are functional in Vav. Site-directed mutagenesis of the NLS sequences should help to delineate the residues involved in nuclear translocation of Vav.

Deletion of the CH domain resulted in an increased resistance to extraction of the EGFPvav 143–845 construct in the presence of Triton X-100 and intermediate ionic strength. Based on the proposed function of the CH domain as an F-actin binding module we expected that deletion of this motif would result in an increased solubility of the corresponding fragment. Surprisingly, however, the opposite was the case. We were also unable to detect co-localization of Vav with actin stress fibers or the EGFPvav 143–845-induced parallel F-actin sheets, arguing against a direct bundling effect of Vav through its localization on actin. The resistance of actin stress fiber arrays induced by EGFPvav 143–845 to actin depolymerization by cytochalasin B and latrunculin B points towards the recruitment of an actin cross linking or bundling protein downstream of the Vav-activated Rac/Rho signalling pathway. These toxins affect actin depolymerization by inhibition of filament growth from the fast growing, barbed ends of the filaments or by reducing the supply of polymerizable monomeric G-actin [44–46]. The resistance of stress fibers to disassembly may thus be due to a markedly reduced rate of actin stress fiber turnover observed in the EGFPvav 143–845 cells. In contrast to our data, Schuebel et al. [47] using a similar approach, observed a disruption rather than an induction of stress fibers in NIH 3T3 cells constitutively expressing a truncated version of Vav (Vav Δ1–67). They, however, reported the formation of abundant stress fibers in clones derived from the same cell line but expressing the Vav-2 oncoprotein. Interestingly, transient transfections resulted in the stimulation of lamellipodial activity at the expense of both filopodial protrusion and stress fiber formation for both Vav variants [47]. In our hands, such strong effects as those leading to a partial disruption of actin stress fibers were observed only in cells with excessive EGFPvav expression. However, such cases were rare and were therefore not considered in this study. Closer control of the expression levels of Rho family GTPases and of the various GAPs and GEFs may thus be required in order to gain reproducible and reliable information about the in vivo effects of these signalling molecules. The EGFP-tagged constructs presented here allow the simultaneous monitoring of expression levels and will serve as valuable tools in future studies using living cells.

In conclusion, we have presented evidence for a possible direct activation of Rho by Vav and that this activity is enhanced by the removal of the CH domain-containing N-terminus, and suggested that translocation of Vav into the nucleus may be regulated by an interaction of the nuclear localization signals with the C-terminal SH domains.

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