Scar/WAVE is localised at the tips of protruding lamellipodia in living cells

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Received 24 January 2001; revised 9 February 2001; accepted 9 February 2001

First published online 21 February 2001

Edited by Amy M. McGough

Abstract Cell motility entails the extension of cytoplasmic processes, termed lamellipodia and filopodia. Extension is driven by actin polymerisation at the tips of these processes via molecular complexes that remain to be characterised. We show here that a green fluorescent protein (GFP) fusion of the Wiskott–Aldrich syndrome protein family member Scar1/WAVE1 is specifically recruited to the tips of lamellipodia in living B16F1 melanoma cells. Scar1–GFP was recruited only to protruding lamellipodia and was absent from filopodia. The localisation of Scar was facilitated by the finding that the formerly described inhibition of lamellipodia formation by ectopical expression of Scar, could be overcome by the treatment of cells with aluminium fluoride. These findings show that Scar is strategically located at sites of actin polymerisation specifically engaged in the protrusion of lamellipodia.

Key words: Actin; Scar; Wiskott–Aldrich syndrome protein; Cytoskeleton; Motility; Lamellipodium

1. Introduction

Cell motility is initiated via the controlled nucleation and polymerisation of actin filaments to produce sheet- or finger-like projections, termed lamellipodia and filopodia (reviewed in [1]) that are induced, respectively, via activation of the Rho family members, Rac and Cdc42 (reviewed in [2]). The alternative chains of molecular events linking external signals to actin polymerisation have still to be defined, but are thought to terminate with the activation of the Arp2/3 complex, a ubiquitous nucleator of actin filament assembly (reviewed in [3,4]). Current evidence places members of the Wiskott–Aldrich syndrome protein family, WASP, N-WASP and Scar/WAVE (subsequently referred to as Scar) directly upstream of Arp2/3 in the signalling pathway [3]. Thus, the homologous C-terminal regions of these proteins have been shown to activate Arp2/3-induced actin polymerisation in vitro (reviewed in [5]) and studies of living cells have demonstrated the requirement of WASP/N-WASP for filopodia formation [6,7] and Scar for lamellipodia formation [8,9]. A conspicuous difference between WASP/N-WASP and three isoforms of Scar is the presence in the former of a CRIB domain, which mediates the direct binding of Cdc42, and renders WASP/N-WASP competent in the activation of the Arp2/3 complex [10]. Scar does not bind Rac, but recent studies suggest that the insulin receptor substrate IRSp53 acts as the binding intermediate between Scar and Rac [11] in the activation pathway. Other studies have identified the Abl tyrosine kinase and cAMP-dependent protein kinase as WAVE binding partners, thereby implicating WAVE in the recruitment of these kinases to sites of actin cytoskeleton remodelling [12].

The mechanism of protrusion of cytoplasmic processes has been likened to the intracellular propulsion of pathogens that hijack the motile machinery of cells to spread their infection (reviewed in [13,14]). Among these pathogens, both *Shigella* bacteria and *Vaccinia* virus recruit N-WASP to their surface for the purpose of nucleating the formation of the actin comet tail that drives them through cytoplasm. N-WASP is confined to the surface of the bacterium at the head of the tail and, via signaling intermediates that are currently being unveiled [14], recruits and activates the Arp2/3 complex. Subsequent to actin filament nucleation, the Arp2/3 complex is presumed to dissociate from N-WASP, and appears to take up a cross-linking role in the comet tail (reviewed in [13]). To what extent this scenario mimics lamellipodia and filopodia formation has yet to be established. What has been shown is that Arp2/3 localises across the breadth of the lamellipodium [15,16] and that co-partners in actin filament dynamics found at the head of comet tails, including VASP and profilin [17,18] are recruited to lamellipodia and filopodia tips. Significantly, Arp2/3 is apparently absent from filopodia [16].

In line with the requirement of, respectively, N-WASP and Scar for filopodia and lamellipodia formation, we would expect a differential localisation of these WASP family members in ruffling membranes of motile cells. Specifically, if these proteins are engaged in actin nucleating activity, they would be expected to localise to the very tips of the membrane processes, where actin incorporation occurs [19]. Indeed, Scar has been localised by antibody labelling to ruffling membranes [8] as well as to focal adhesions [12]. However, the precise localisation of Scar in lamellipodia as well as the dynamics of its recruitment were not established. We here report the localisation of Scar1 in living cells and show that it is specifically recruited to the tips of lamellipodia but not of filopodia. The determination of Scar localisation was facilitated by the use of conditions to activate lamellipodia formation that overcome the detrimental effects of the overexpression of Scar noted in former studies [8,9].

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0014-5793/01/$20.00
PII: S0014-5793(01)02239-6

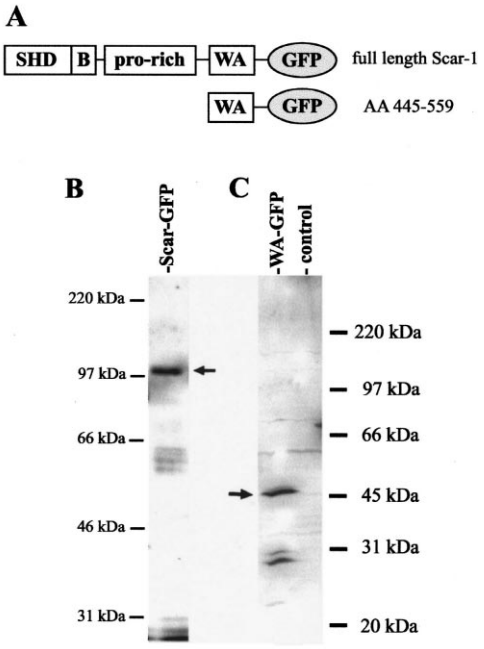

Fig. 1. Constructs of Scar used in this study (A) and their expression in B16F1 melanoma cells as determined by Western blotting with a monoclonal GFP antibody (B,C). The full length Scar–GFP (B) was only weakly expressed, and was therefore enriched by immunoprecipitation from cell extracts with a polyclonal GFP antibody, before immunoblotting. For the WA–GFP construct, total cell extracts were used (C). Abbreviations: SHD; Scar homology domain; B, basic region; pro-rich, proline-rich region; WA, WASP homology and acidic domain [9].

2. Materials and methods

2.1. EGFP–Scar1 constructs

Full length human Scar1 in a pSPORT1 vector was a gift from T. Nagase (Kazusa DNA Research Institute). With this as a template, Scar1–EGFP was generated by polymerase chain reaction (PCR), using a sense primer (GAG AGA ATT CGC CAC CAT GCC GCT AGT GAA AAG) to introduce an *Eco*RI site and an antisense primer (GAG AGA GGA TCC CTC CAA CCA ATC TAC TTC) to introduce a *Bam*HI site, followed by removal of the stop codon. The PCR product was cloned in frame into the *Eco*RI/*Bam*HI sites of an EGFP-N3 vector (Clontech, Germany). Scar–WA was amplified using C ATT CGA GAA TTC TCG CCT GTC ATG GTT ACA GC as a sense primer and GG CCG AAT TCT CTG CAG GAA TCC TAA CTC CAA CC as an antisense primer. The amplified fragments were cloned in frame into the *Eco*RI site of an EGFP-N1 vector and the correct sequences of the constructs confirmed.

2.2. Immunoprecipitation and Western blotting

Immunoprecipitation of Scar1–GFP using a polyclonal green fluorescent protein (GFP) antibody was performed as described earlier [20] with minor modifications in the buffer (20 mM imidazole, 120 mM NaCl, 2 mM $MgCl_2$, 5% (v/v) glycerol, 0.5% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaN_3, pH 7.0). Analytical sodium dodecyl sulphate–gel electrophoresis on 8–22% gradient polyacrylamide mini-slab gels and Western blotting onto nitrocellulose (Pall-Gelman, Austria) was performed as described elsewhere [21]. Transferred proteins were visualised using a monoclonal antibody to GFP, horseradish peroxidase-coupled secondary antibodies and the enhanced chemiluminescence detection system (Amersham-Pharmacia Biotech, Austria). Antibodies to GFP (polyclonal and monoclonal) were from Clontech (Germany).

2.3. Cell culture and transfection

Mouse melanoma cells (B16F1) from the American Type Culture Collection were maintained in high glucose (4500 mg/l) Dulbecco's modified Eagle's medium (Sigma-Aldrich, Austria) supplemented with 10% foetal calf serum (FCS; PAA Laboratories, Austria) at 37° in the presence of 5% CO_2. Cells were transfected at about 70% confluence overnight in the presence of serum, using 6 μl Superfect (Qiagen) and 1.5 μg DNA for a 30 mm dish. They were plated onto 15 mm acid-washed glass coverslips coated with 25 μg/ml laminin (Sigma-Aldrich, Austria). For video microscopy, in air, B16F1 cells were transferred to Ham's F12 medium (Sigma-Aldrich, Austria) containing 10% FCS.

2.4. Aluminofluoride treatment, phalloidin staining and fluorescence microscopy

Transfected or untransfected B16F1 cells on laminin were subjected to aluminofluoride treatment by adding 50 μM $AlCl_3$ and 30 mM NaF (final concentration) to full growth medium for 5–40 min. For fixation, cells were washed shortly three times with phosphate-buffered saline (PBS) (138 mM NaCl, 26 mM KCl, 84 mM Na_2HPO_4, 14 mM KH_2PO_4, pH 7.4), extracted with 0.3% Triton X-100 and 4% paraformaldehyde (PFA; Merck, Germany) in PBS for 30 s. After an additional three washing steps they were fixed with 4% PFA for 10 min. F-actin was visualised using Alexa 568 phalloidin according to the manufacturer's protocol (Molecular Probes, The Netherlands). Fluorescent images were recorded on an Axioskop equipped with an Axiocam using a 63× oil immersion lens and Axiovision 2.05 software (all Zeiss, Austria).

2.5. Video microscopy

Cells were mounted in an open heating chamber (Warner Instruments, UK) at 37°C on an inverted microscope (Axiovert S100TV; Zeiss, Austria) equipped for epifluorescence and phase contrast mi-

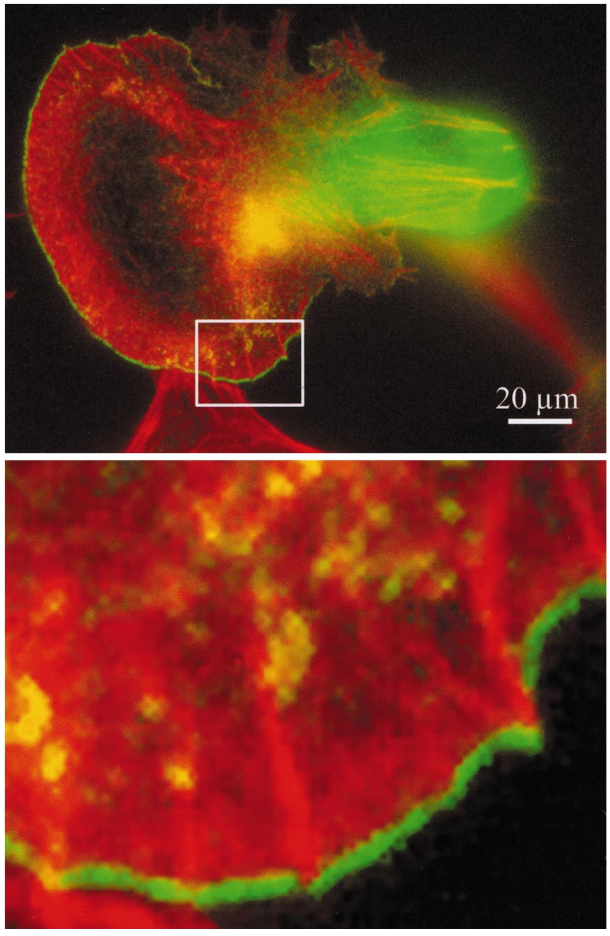

Fig. 2. Fluorescence image of a B16F1 cell expressing Scar–GFP that was fixed and labelled with Alexa 568 phalloidin. (No aluminium fluoride treatment.) Lower panel shows boxed inset in upper panel.

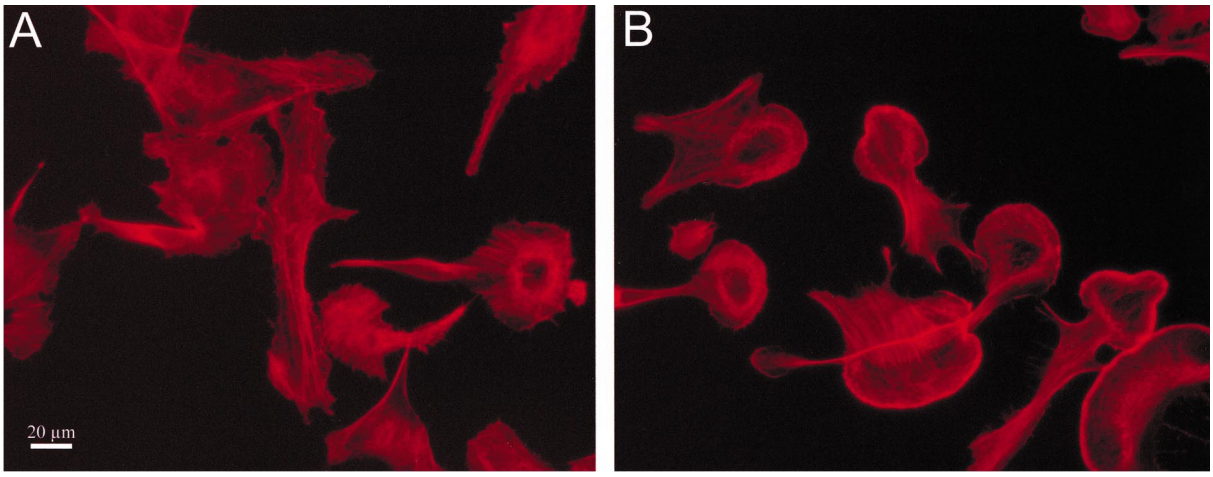

Fig. 3. Activation of protrusion of B16F1 cells by aluminium fluoride. Cells were fixed and then labelled with Alexa 568 phalloidin. A: Control cells. B: Cells treated with aluminium fluoride for 20 min.

croscopy, using a 100×/NA 1.4 plan apochromatic objective. Data were acquired with a back-illuminated, cooled charged-coupled-device (CCD) camera (Princeton Research Instruments, NJ, USA) driven by IPLab software (Scanalytics, MA, USA) and with computer controlled shutters (Optilas, Germany) in the transmitted and epifluorescence light paths to minimise radiation damage to cells.

3. Results and discussion

The full length construct of Scar1 showed different levels of expression in B16F1 mouse melanoma cells according to the end to which GFP was tagged, the C-terminal tag giving lower expression. As in former studies [9], we found that overexpression of Scar1 generally compromised cell spreading. We therefore focused on weak expressers carrying the C-terminal GFP tag, that could spread normally. As seen in Fig. 1, Western blotting of immunoprecipitates of extracts from transfected cells showed that the tagged full length construct migrated at the expected molecular weight. When plated on laminin, a small proportion of well spread cells could be found that expressed detectable levels of GFP fluorescence, following fixation. Counterstaining with phalloidin showed that Scar1–GFP was localised to the tips of the actin network of the lamellipodium (Fig. 2). Contrary to observations on other cells [12] and consistent with the findings of Miki et al. [8] we observed no localisation in focal adhesions. Motile B16F1 melanoma cells characteristically show small surface patches in phase contrast that move laterally and which either pinch off from the lamellipodium and move inwards, or form spontaneously on the dorsal surface. These 'rufflets', which were earlier described in 3T3 cells injected with constitutively active Rac [22] also recruit Scar–GFP (see supplementary videos on ftp site (ftp://movies@server4.imolbio.ocean.ac.at, user: movies, password: scar)).

For living B16F1 melanoma cells plated on laminin, the proportion of cells with well developed lamellipodia is highly variable and between 20 and 50%. We found that this proportion could be dramatically and reproducibly increased, by

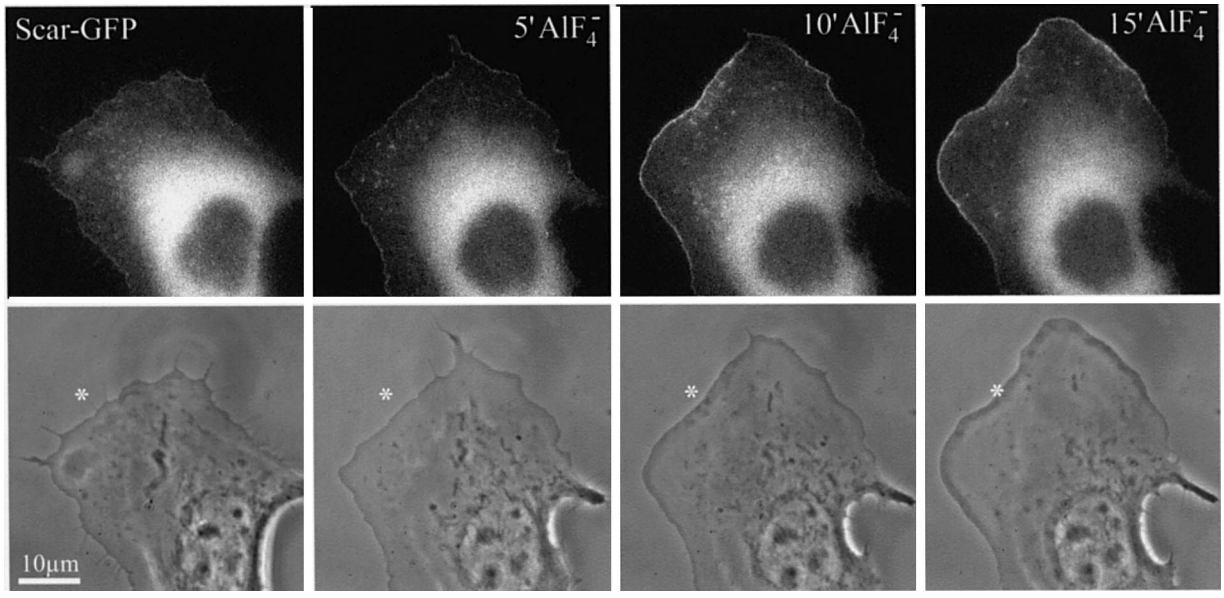

Fig. 4. B16F1 melanoma cell transfected with Scar–GFP, before (far left panel) and after treatment with aluminium fluoride for the times given. Note enhancement of GFP signal at the cell edge (fluorescence image) and the formation of a prominent lamellipodium (denser, peripheral band in phase contrast) in aluminium fluoride.

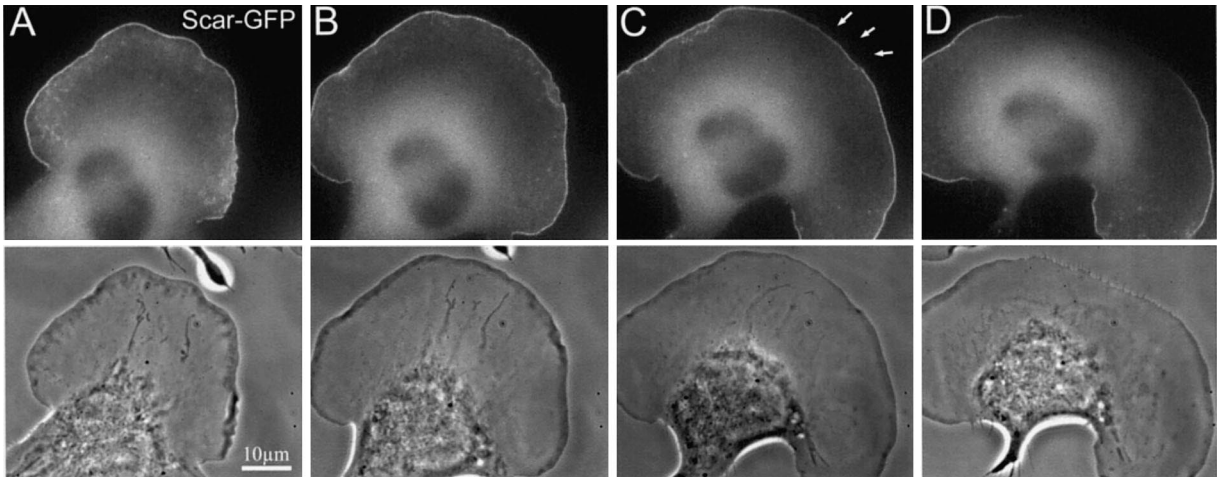

Fig. 5. B16F1 melanoma cell transfected with Scar–GFP and activated with aluminium fluoride 15 min before first frame of sequence (A). Time between frames is 10 min. Upper panels: fluorescence images; lower panels: phase contrast. Note that the retraction of the lamellipodium, initiated in C (arrows) and complete in D (see phase contrast) is associated in D with a delocalisation of Scar from the cell edge.

up to 80%, by the addition of aluminium fluoride to the medium (Fig. 3). Under these conditions, cells developed wide and smooth lamellipodia that were almost devoid of filopodia. As a phosphate analogue, aluminium fluoride binds with high affinity, but reversibly, to sites in proteins occupied by phosphate (reviewed in [23]) and therefore has many putative targets. The enhancement of motility and spreading was, however, Rac1-dependent, as it was markedly reduced in cells expressing dominant-negative N17 Rac (data not shown). We assume that B16F1 cells express Rac at high levels and that Rac becomes activated during aluminium fluoride treatment. However, other targets must also be involved, since the activation of Rac alone, by injection of constitutively active L61 Rac, produces a non-polarised phenotype ([22] and unpublished observations). In particular, aluminium fluoride stabilises actin filaments [24] an effect that could contribute to the potentiation of protrusion.

Fig. 4 shows a cell transfected with Scar–GFP before and after aluminium fluoride was added. The initially fluctuating cell edge was transformed after 15 min treatment into a continuously protruding cell front with a clearly defined lamellipodium in phase contrast. Concomitantly, the Scar–GFP signal at lamellipodium tips increased by up to 30%. This effect was typical for cells exhibiting low levels of GFP fluorescence, whereas high expressors showed almost no response to the compound. A second example, in Fig. 5, shows a cell for which one region of the lamellipodium retracted during the video sequence. In the same manner as observed for VASP–GFP [17] Scar–GFP became delocalised from the cell edge during retraction. Owing to the sensitivity of cells to high levels of Scar expression, it was not readily possible to quantitate the levels of Scar intensity in relation to protrusion rate, as previously achieved with VASP [17]. Thus, for cells that were still competent to protrude lamellipodia, the signal of Scar–GFP at lamellipodia tips was at the limit of the detection level of the rear-illuminated CCD chip, typically 10–20 times lower than that obtained with VASP–GFP. Nevertheless, of the cells recorded (17 in total), all showed the same characteristic localisation, associated with protrusion.

As indicated, filopodia formation was suppressed by aluminium fluoride treatment. Cells expressing very low levels of Scar–GFP and that spontaneously formed lamellipodia and filopodia were therefore screened to determine whether Scar was also recruited to filopodia. Fig. 6 shows a comparison of lamellipodia and filopodia in B16F1 cells transfected, respectively, with GFP–VASP (Klemens Rottner, unpublished observations) and Scar–GFP. Whereas GFP–VASP was clearly localised to filopodia tips, the filopodia in the Scar-transfected cell, seen in phase contrast, were devoid of GFP label. This result is consistent with the finding of Miki et al. [8] that Scar1 lacking the verprolin homology part of the WA domain blocks lamellipodia but not filopodia protrusion when co-expressed, respectively, with constitutively active Rac or Cdc42.

The C-terminal, WA domain of Scar1 binds both G-actin and the p21-Arc subunit of the Arp2/3 complex [9]. Expression of this domain in fibroblasts and macrophages was found to block lamellipodia formation, and in macrophages also filopodia formation, effects attributed to the disruption of the normal Scar/WASP–Arp2/3 interaction [9]. Using aluminium fluoride, we were able to induce lamellipodia in B16F1 cells expressing WA–GFP (Figs. 1 and 7). That the WA–GFP construct was functionally active was shown in separate experiments in which WA–GFP and the myc-tagged WA construct used by Machesky and Insall [9] were transfected into

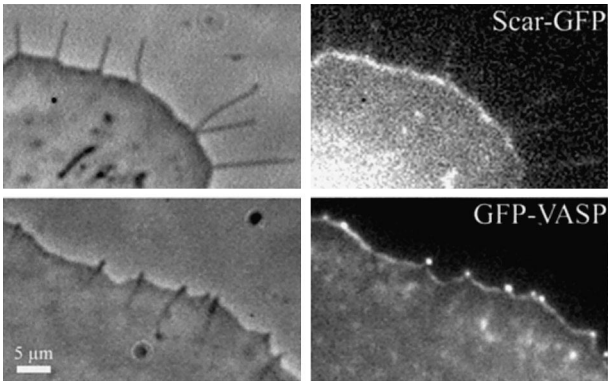

Fig. 6. Correlated phase contrast and fluorescence images of regions of B16 cells that were transfected with either Scar–GFP or VASP–GFP. (No aluminium fluoride treatment.) Filopodia tips recruit VASP, but not Scar.

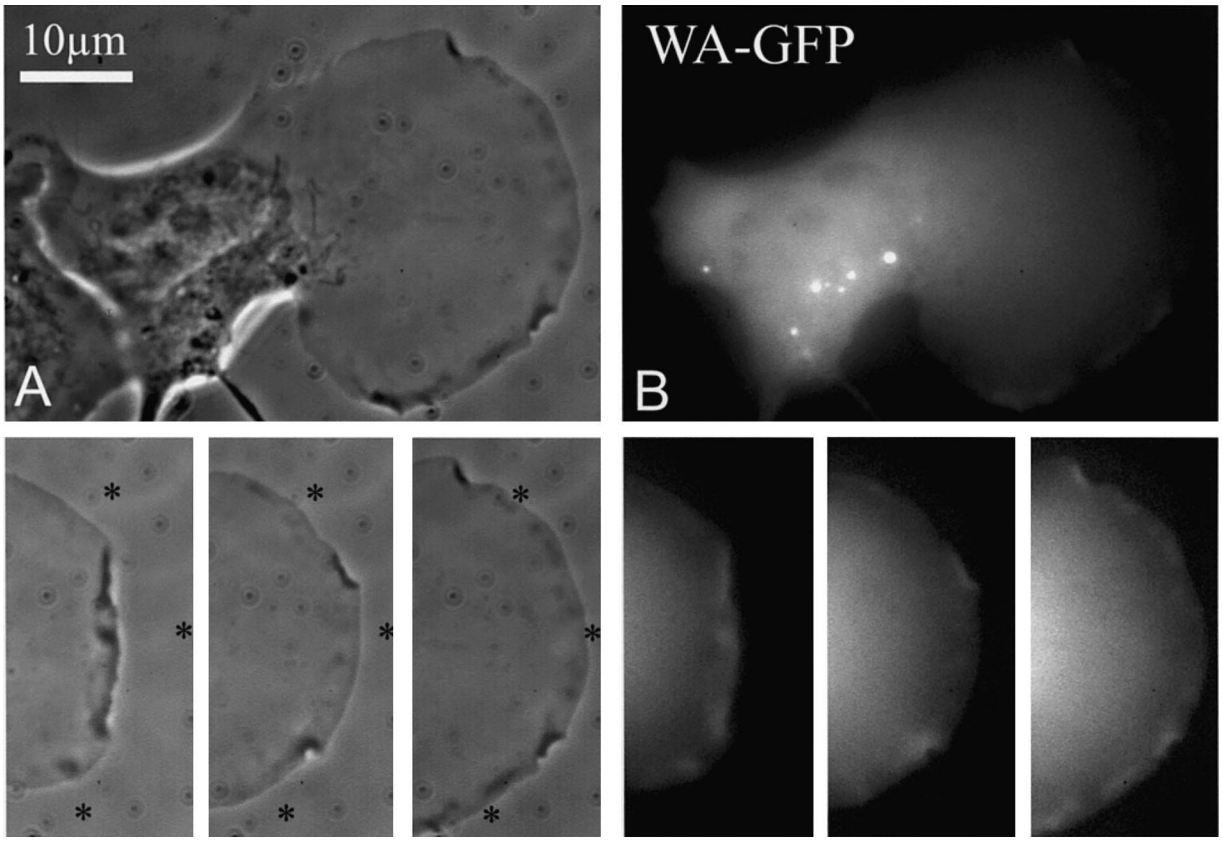

Fig. 7. B16 melanoma cell that was expressing Scar–WA–GFP, after activation of lamellipodia protrusion with aluminium fluoride (15 min in first frame). A: phase contrast, B: fluorescence image. Time between frames is 5 min. Asterisks in lower phase contrast panels mark fixed positions on the substrate. The WA domain does not localise to the lamellipodium edge.

PtK2 cells. After overnight incubation, cells transfected with either construct showed a similar degree of stress fibre disassembly, as compared to untransfected cells (data not shown). As shown in Fig. 7, the WA domain was not recruited to the lamellipodium in B16F1 cells, indicating that the N-terminal region of Scar may be required for targeting it to sites destined to nucleate actin filament growth. Preliminary studies to test this conclusion have been hampered so far, however, by the spontaneous degradation of N-terminal Scar–GFP constructs in B16F1 cells.

Our present findings show that Scar is recruited specifically to sites of assembly of actin filaments for the formation of lamellipodia. VASP and profilin are recruited to the same sites, but in addition to the tips of filopodia, from which Scar is excluded. Pathogens that use actin to move recruit different combinations of molecular intermediates to effect actin polymerisation via the the Arp2/3 complex [14]. By analogy, we suppose that lamellipodia and filopodia are induced and controlled by alternative and more complex assemblies of receptor, adaptor and regulatory molecules, recruited to the membrane–actin interface. A future challenge will be to characterise the molecular composition of these complexes and their mode of control of actin filament assembly. The localisation of molecules in living cells, as here, is going to be one essential requirement to achieve this aim.

Acknowledgements: This work was supported by a grant from the Austrian Research Foundation. We thank Dr. K. Rottner for providing unpublished data on GFP–VASP and Ms. M. Schmittner for excellent technical help.

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