Introduction

The protrusion of lamellipodia and filopodia is signalled via the Rho family small G-proteins, Rac and Cdc42, respectively (Machesky and Hall, 1997; Nobes and Hall, 1995; Ridley, 2001). Insight into the players downstream of Rac and Cdc42 involved in mediating protrusion is now beginning to emerge from collected information on protein complexes influencing actin polymerisation in vitro (Higgs and Pollard, 2001; Pantaloni et al., 2001) and on the molecular determinants of actin-based pathogen motility (Frischknecht and Way, 2001; Pantaloni et al., 2001), as well as on localisations of proteins in lamellipodia and filopodia in living cells (Small et al., 2002). An understanding of the regulation of actin dynamics during cell motility requires characterisation of the complexes of proteins involved at sites of actin nucleation and turnover. Particularly relevant to the present report is the finding that several candidate proteins implicated in actin polymerisation dynamics in vitro are specifically recruited to the tips of lamellipodia and filopodia, where actin polymerisation for protrusion is initiated and controlled. These proteins include WAVE, a submember of the Wiskott Aldrich Syndrome Protein (WASP) family (Hahne et al., 2001; Nakagawa et al., 2001), VASP/Mena family members (Rottner et al., 1999; Bear et al., 2000) and profilin (Geese et al., 2000).

Summary

The insulin receptor tyrosine kinase substrate p53 (IRSp53) links Rac and WAVE2 and has been implicated in lamellipodia protrusion. Recently, however, IRSp53 has been reported to bind to both Cdc42 and Mena to induce filopodia. To shed independent light on IRSp53 function we determined the localisations and dynamics of IRSp53 and WAVE2 in B16 melanoma cells. In cells spread well on a laminin substrate, IRSp53 was localised by antibody labelling at the tips of both lamellipodia and filopodia. The same localisation was observed in living cells with IRSp53 tagged with enhanced green fluorescence protein (EGFP-IRSp53), but only during protrusion. From the transfection of deletion mutants the N-terminal region of IRSp53, which binds active Rac, was shown to be responsible for its localisation. Although IRSp53 has been reported to regulate filopodia formation with Mena, EGFP-IRSp53 showed the same localisation in MVD* Ena/VASP (vasodilator stimulated phosphoprotein) family deficient cells. WAVE2 tagged with DsRed1 colocalised with EGFP-IRSp53 at the tips of protruding lamellipodia and filopodia and, in double-transfected cells, the IRSp53 signal in filopodia decreased before that of WAVE2 during retraction. These results suggest an alternative modulatory role for IRSp53 in the extension of both filopodia and lamellipodia, through WAVE2.

Movies available online

Key words: IRSp53, WAVE, Mena, Lamellipodia, Filopodia

IRSp53 is colocalised with WAVE2 at the tips of protruding lamellipodia and filopodia independently of Mena

Hiroyuki Nakagawa1,2, Hiroaki Miki3, Motohiro Nozumi2, Tadaomi Takenawa4, Shigeaki Miyamoto2, Jürgen Wehland2 and J. Victor Small1,*

1Department of Cell Biology, Institute of Molecular Biology, Austrian Academy of Sciences, Billrothstraße 11, Salzburg A-5020, Austria
2Department of Biochemical Engineering and Science, Kyushu Institute of Technology, Iizuka, Fukuoka 820-8502, Japan
3Division of Cancer Genomics and 4Department of Biochemistry, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
5Gesellshaft für Biotechnologische Forschung (GBF), Mascheroder Weg 1, 38124 Braunschweig, Germany

*Author for correspondence (e-mail: jvsmall@imolbio.oeaw.ac.at)

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Recently, the insulin receptor tyrosine kinase substrate IRSp53 (Yeh et al., 1996) was proposed as an adaptor molecule involved in events downstream of Rac and/or Cdc42 (Govind et al., 2001; Krugmann et al., 2001; Miki et al., 2000). IRSp53 was originally identified as the substrate of the insulin receptor tyrosine kinase, although a corresponding function remains outstanding (Yeh et al., 1996). Miki et al. (Miki et al., 2000) found that IRSp53 linked Rac to WAVE2, the ubiquitous member of the WAVE family (Suetsugu et al., 1999). IRSp53 binds to Rac through its N-terminal region named the Rac binding region (RCB) (Miki et al., 2000; Miki and Takenawa, 2002). In a later study, Govind et al. (Govind et al., 2001) reported that IRS-58, a C-terminal alternative splicing variant of IRSp53 (Alvarez et al., 2002), bound to Cdc42 at its centre part. Furthermore, Krugmann et al. (Krugmann et al., 2001) identified IRSp53 as a Rac and Cdc42 binding protein using the yeast two-hybrid method. They defined the Cdc42 binding region as that containing a partial CRIB motif (Cdc42 and Rac binding motif) (reviewed by Hoffman and Cerione, 2000) at the same position as reported by Govind et al. (Govind et al., 2001). IRSp53 binds to the proline-rich regions of both WAVE2 and Mena through its SH3 domain (Krugmann et al., 2001; Miki et al., 2000). From their recent studies, Krugmann et al. (Krugmann et al., 2001) concluded that IRSp53 acts synergistically with Mena in filopodia induction, rather than as a link between Rac and WAVE 2 in signalling lamellipodia formation (Miki et al., 2000). IRSp53 has thus been attributed with apparently alternative roles in the formation of either lamellipodia or filopodia. To gain more insight into IRSp53 function, we investigated the localisation and dynamics of IRSp53 and its target proteins in living cells.

Materials and Methods
Expression contracts and transfection
pEGFP-C1, -N1 and pDsRed1-N1 were purchased from Clontech (Palo Alto, CA). cDNA of full-length IRSp53 was inserted into the BamHI site of pEGFP-C1. The central region of IRSp53 [CNT; 230 to 364 amino acids (aa)] was amplified by PCR and inserted into the BamHI site of pEGFP-N1. A cDNA fragment encoding the Rac binding (RCB; 1 to 229 aa) region was cut out from pEGFP-IRSp53 by SacI and SstI, and inserted between the SacI and Smal sites of pEGFP-C1. WAVE2 cDNA lacking the stop codon was amplified by PCR and inserted between XhoI and AgeI sites of pDsRed1-N1. IRSp53, SH3 and RCB tagged with myc were as reported previously (Miki et al., 2000; Miki and Takenawa, 2002). These constructs were transfected with SuperFect (QIAGEN K.K., Tokyo, Japan) according to the manufacturer’s guide.

Antibodies and immunofluorescence microscopy
Affinity purified polyclonal antibodies against WAVE were characterised as described previously (Miki et al., 1998b; Nakagawa et al., 2001). Rabbit aniserum against green fluorescence protein was purchased from Molecular Probes (Eugene, OR). Monoclonal antibody against myc-tag (clone no. 9E10), FITC-conjugated anti-mouse immunoglobulin G (IgG) goat IgG and rhodamine-phalloidin were obtained from Sigma-Aldrich Japan K.K. (Tokyo, Japan). The immunofluorescence microscopy was performed using a confocal laser scanning microscope (LSM510, Zeiss, Japan) as previously described (Nakagawa et al., 2001). The pinhole of LSM510 was set to adjust the thickness of focal planes at 0.7 μm for immunofluorescence microscopy.

Video microscopy of living cells
Transfected cells cultured on laminin-coated cover glass were mounted in an open heating chamber (Warner Instruments, Hamden, CT) operating at 32°C and 37°C for MVD7 cells and B16 cells, respectively. The treatment with aluminium fluoride was carried out according to the method of Hahne et al. (Hahne et al., 2001). The chamber was mounted on an inverted microscope (Axiovert S100TV, Zeiss, Austria) equipped for epifluorescence and phase contrast microscopy. Data were acquired with a back-illuminated CCD camera (Roper Scientific, Trenton, NJ). The interval time between video frames was set at 7 seconds (Supplemental movie) or 17 seconds. For the double transfections of EGFP-tagged IRSp53 and DsRed1-tagged WAVE2, the time between sequential frames in the two fluorescent channels was 1.5 seconds.

Measurement of fluorescence intensity
The fluorescence intensity of the tips of filopodia and lamellipodia were measured using Scion Image 1.62c (Scion Corporation, Frederick, MD). The fluorescence intensity profiles were obtained within a frame of 0.5 μm width along filopodia or of 1 μm width across lamellipodia. The specific fluorescence at the tip was obtained by subtracting the mean background fluorescence on both sides of the tip (see Fig. 7d).

Results
IRSp53 is recruited to the tips of protruding lamellipodia and filopodia through its N-terminal region
To establish IRSp53 localisation, we first transfected B16 melanoma cells with myc-tagged IRSp53 (myc-IRSp53), which preserves the binding activity to Cdc42, Rac, Mena and WAVE2 (Krugmann et al., 2001; Miki et al., 2000) and employed immunofluorescence (Fig. 1a-d) and confocal microscopy. Highly expressing cells formed many branched extensions without clear localisation of myc-IRSp53, as previously reported (Fig. 1a,b, arrowheads) (Govind et al.,

![Fig. 1. Localisation of IRSp53 in B16 melanoma cells.](image)
However, cells expressing moderate levels of myc-IRSp53 extended lamellipodia and filopodia normally. As shown in Fig. 1c,d, IRSp53 was then more concentrated towards the tip of the actin meshwork in lamellipodia and was present at the tips of filopodia.

To analyse how the localisation of IRSp53 was related to cell movement, we observed the dynamics of IRSp53 tagged with EGFP (EGFP-IRSp53) in living B16 cells. In general, transfected cells were reluctant to spread, so cells were cotransfected with dominant active Rac1 (Rac1V12) to promote cell spreading. As shown in Fig. 2a-d, EGFP-IRSp53 was concentrated at the lamellipodium tip similar to myc-IRSp53. In cell edges undergoing retraction, EGFP-IRSp53 was absent but relocated to the tips following extension of lamellipodia (Fig. 2c,d, white lines), as formerly found for other tip proteins (Geese et al., 2000; Hahne et al., 2001; Rottner et al., 1999a; Stradal et al., 2001). The same dynamics of IRSp53 were observed in B16 cells stimulated with aluminium fluoride (Fig. 2e,f). In these cells, EGFP-IRSp53 localised at the protruding tips of lamellipodia and was absent from retracting tips, as well as from retraction fibres (Fig. 2e,f, arrowheads). EGFP-IRSp53 was retained in membrane ruffles at the edge of Rac1V12 cotransfected cells (Fig. 2c,d), as also earlier observed with EGFP-VASP (Rottner et al., 1999a), and was also concentrated at the tips of elongating filopodia (Fig. 6; Supplemental movie). These observations suggest that IRSp53 could function downstream of both Rac and Cdc42.

The localisation of EGFP-IRSp53 in living cells was consistent with the immunofluorescence localisation of myc-IRSp53.

IRSp53 binds to both Rac and Cdc42 through its RCB region and CRIB motif, respectively, (Govind et al., 2001; Krugmann et al., 2001; Miki et al., 2000). The SH3 domain of IRSp53 is reported to interact with WAVE2 and Mena to form lamellipodia and/or filopodia (Krugmann et al., 2001; Miki et al., 2000). To clarify which regions of IRSp53 regulate its localisation, we expressed deletion constructs of IRSp53 in B16 melanoma cells tagged with myc, which does not affect the interaction with Rac (Miki et al., 2000). myc–ΔSH3 and myc-RCB fragments showed the same localisation as the full-length IRSp53, but the SH3 domain...
alone (myc-SH3) failed to localise (Fig. 3d,e). These results indicate that the localisation of IRSp53 could be regulated independently from the interaction with WAVE2 and/or Mena, which are both mediated by the SH3 domain. They also suggest that the N-terminal sequence in the RCB region could tether IRSp53 to the tips of both lamellipodia and filopodia.

IRSp53 is localised at the tips of lamellipodia and filopodia protruding from Mena/VASP double-knockout cells

Because overexpression of IRSp53 induced filopodial extensions, it was suggested that the complex of IRSp53 and Mena regulates filopodia elongation (Krugmann et al., 2001). This conclusion is inconsistent, however, with the recruitment of both IRSp53 (Figs 1, 2) and Mena (Gerlter et al., 1996; Nakagawa et al., 2001; Rottner et al., 1999a; Rottner et al., 2001) to lamellipodia, as well as filopodia. To provide further insight into the in vivo relationship between IRSp53 and Mena, we analysed the localisation of EGFP-IRSp53 in MVD7, Ena/VASP family deficient cells (Bear et al., 2000). When cotransfected with Rac1V12 and EGFP-IRSp53, MVD7 cells plated on laminin extended lamellipodia and filopodia with EGFP-IRSp53 localised to their tips (Fig. 4). This result showed that the IRSp53:Men complex is not essential for filopodia elongation, and that IRSp53 may interact with WAVE2 rather than with Mena at these tips.

WAVE2, but not WAVE1, localises to filopodia tips with IRSp53

Significantly, IRSp53 binds only WAVE2 of the three WAVE isoforms and specifically enhances WAVE2 activity to induce Arp2/3 complex activation (Miki et al, 2000). These results suggested that WAVE2 might be localised at the tips of both lamellipodia and filopodia, together with IRSp53. To investigate this possibility, we determined the localisation of WAVE2 and compared its dynamics with that of IRSp53 in B16 cells. Before the living cell observation, we localised endogenous WAVE in B16 cell using an anti-WAVE antibody that recognises all three WAVE isoforms. The antibody labelled the tips of both lamellipodia and filopodia (Fig. 5a,b), in contrast to WAVE1, which localises only at the lamellipodium tip (Hahne et al., 2001) when expressed in B16 cells. In the neural growth cone, WAVE2 and 3 localise to both tips of lamellipodia and filopodia but only WAVE1 localises to the lamellipodium tip (Nozumi et al., 2003). Because WAVE1 and 3 are expressed mainly in neural tissues (Suetugu et al., 1999; Benachenhou et al., 2002), the tip label observed by antibody labelling in untransfected B16 cells is most likely to be attributed to the ubiquitously expressing WAVE2 isoform. The localisation of DsRed1-tagged WAVE2 (WAVE2-DsRed) confirmed this possibility (Fig. 5c,d). WAVE2-DsRed-transfected B16 cells were stimulated with aluminium fluoride to enhance lamellipodia formation (Hahne et al., 2001). As shown in Fig. 5c,d, WAVE2-DsRed localised to the edge of extending lamellipodium and the tips of filopodia similar to the immunolabelling with anti-WAVE antibody.

Different dynamics of IRSp53 and WAVE2 at the tip of retracting filopodia

By observing the dynamics of IRSp53 and WAVE2 in the

Fig. 4. Localisation of IRSp53 in MVD7 at the tips of lamellipodia and filopodia of Ena/VASP family deficient cells. The cells were cotransfected with EGFP-IRSp53 and Rac1V12 were cultured on laminin and visualised in fluorescence (a,c) and phase contrast (b,d). Bars, 5 μm.

Fig. 5. Localisation of WAVE2 in B16 cells plated on laminin. (a,b) A cell immunolabelled with an anti-WAVE antibody that recognises all three WAVE isoforms (a) and with rhodamine-phalloidin (b). (c,d) Sequential video frames taken in fluorescence (c) and phase contrast (d) of a cell that was transfected with WAVE2-DsRed. For living cell observation, lamellipodia protrusion was stimulated by treatment with aluminium fluoride 15 minutes before image acquisition. White line indicates region of retraction followed by lamellipodium protrusion; arrows and arrowheads indicate filopodia. Time is in seconds. Bars, 10 μm.
same cell, we could compare the dynamics of these two proteins. B16 cells were cotransfected with EGFP-IRSp53 and WAVE2-DsRed and were microinjected with constitutively active Rac1L61 to promote cell spreading (Fig. 6). Consistent with the single label experiments, EGFP-IRSp53 and WAVE2-DsRed were colocalised at the tips of lamellipodia.

The fluorescence intensity of EGFP-IRSp53 at the filopodium tip was decreased concomitant with retraction, whereas WAVE2-DsRed was retained at the tip during the early retraction phase (see fluorescence intensity profiles in Fig. 6). As shown in the bottom frame of Fig. 6, when the filopodium was fully retracted, the fluorescence intensity of both EGFP and DsRed at the tip was decreased to the same level. To analyse the difference of EGFP-IRSp53 and WAVE2-DsRed dynamics at the filopodia tips, we measured their fluorescence intensities during retraction. Because the expression time course was different between EGFP and DsRed1 vectors, it was difficult to find cells with moderate fluorescence of both EGFP and DsRed1. Fig. 6 shows an example of three filopodia whose tips could be traced over at least six video frames (35 seconds) sequentially before disappearing in lamellipodia. These filopodia elongated at a rate of 0.19±0.01 μm/min and retracted at a rate of 0.09±0.07 μm/min. The fluorescence intensity of EGFP-IRSp53 decreased to nearly half ($I_R=45.2±11.3$%) within 35 seconds, whereas that of WAVE2-DsRed was preserved ($I_R=88.1±11.2$%) in this period. The fluorescence at lamellipodia edges in the same video frames did not change in the same period; the $I_R$ values of EGFP-IRSp53 and WAVE2-DsRed were $85±16.3$% and $95.6±18.1$%, respectively. To compensate for photobleaching, we estimated the ratios between the $I_R$ of filopodia and lamellipodia. The ratios of EGFP-IRSp53 and WAVE2-DsRed were 53% and 92%, respectively, EGFP-IRSp53 and WAVE2-DsRed showed essentially the same dynamics in other filopodia. These results suggest that EGFP-IRSp53 delocalises from the filopodia tip before WAVE2-DsRed, during retraction.

**Discussion**

Although Krugmann et al. (Krugmann et al., 2001) reported that IRSp53 bound to Rac and WAVE2 in addition to Cdc42 and Mena, they concluded that IRSp53 regulated filopodia formation as its overexpression induced filopodia. Likewise, Govind et al. (Govind et al., 2001) showed that the overexpression of IRS-58, a C-terminal alternative splicing variant of IRSp53 (Alvarez et al., 2002), induced filopodia formation in various cell lines. They observed that endogenous IRS-58 was generally localised in filopodia. We also observed that the overexpression of myc-IRSp53 in B16 cells induced long, multibranched filopodia. Considering that B16 cells express endogenous IRS-58 (H.N. and J.V.S., unpublished), our result suggests that IRSp53 normally localises to the tips of both lamellipodia and filopodia. Because the cotransfection of Rac1V12 recovered filopodium formation of B16 cells on laminin substrata, the predominance of filopodia-like projections in cells overexpressing IRSp53 or the RCB region may be due to the sequestration of Rac by IRSp53 at high expression levels, leading to Rac inhibition.

The observation of Krugmann et al. (Krugmann et al., 2001) that the overexpression of IRSp53 induced the atypical retraction of cell edges, and is dependent on incubation time, is consistent with the onset of deleterious effects at high expression levels. We show here that the N-terminal RCB region of IRSp53 is recruited to the tips of both lamellipodia and filopodia, whereas the central region including the CRIB motif is not responsible for the localisation. Although this N-terminal region has been reported to be essential for Rac binding (Miki et al., 2000; Miki and Takenawa, 2002), it also
recruits IRSp53 to the tips of filopodia, suggesting the involvement of other adaptors determining filopodia localisation. Alternatively, localisation in filopodia could reflect the derivation of filopodia from lamellipodia networks signalled via Rac (see Small et al., 2002). Further exploration of proteins interacting with the IRSp53 N-terminal region should provide information about the additional adaptors or complexes involved.

We show here that IRSp53 colocalises with WAVE2 at the tips of both protruding lamellipodia and filopodia, independently of Mena. From these results we propose a schematic model of IRSp53 function (Fig. 7) that could explain the two different models of its role in cell movement (Govind et al., 2001; Krugmann et al., 2001; Miki et al., 2000). Like WAVE1, WAVE2 is recruited to the tips of both lamellipodia and filopodia through its WHD domain (Nakagawa et al., 2001; Nozumi et al., 2003). Mena localisation is regulated synergistically through both the EVH (Ena/VASP homology) 1 and EVH2 domains (Bear et al., 2002). Considering the binding of IRSp53 with both WAVE2 and Mena through its SH3 domain (Fig. 7, solid double-ended arrows) and their colocalisation at the tips of lamellipodia and filopodia, IRSp53 could tie WAVE2 and Mena into a complex. This complex could contribute to the regulation of actin polymerisation at the cell membrane, in an alternative pathway, analogous to the complex of ActA and VASP at the surface of L. monocytogenes (Lanier et al., 1999). As previously reported (Miki et al., 2000), IRSp53 should also enhance WAVE2 activity to promote Arp2/3 complex-induced actin filament polymerisation in lamellipodia (Fig. 7, solid arrow). Because the Arp2/3 complex is absent from filopodia (Svitkina and Borisy, 1999), WAVE2 may regulate actin polymerisation there through the recruitment of actin molecules to filopodia tips by the interaction of its proline-rich region with profilactin (Miki et al., 1998b). IRSp53 localisation is not dependent on WAVE2 or Mena. This is indicated by the observation that IRSp53 disappeared from the filopodia tips before WAVE2 (Fig. 6) and by the peripheral tip localisation of IRSp53 in the absence of Mena. Therefore, IRSp53 may be involved in recruiting WAVE2 and Mena in lamellipodia and filopodia via Rac and Cdc42, respectively.

The localisation of Ena/VASP family proteins to the lamellipodium tip is highly correlated with the protrusion rate (Rottner et al., 1999a), but the roles of these proteins are still controversial (Arguinzonis et al., 2002; Bear et al., 2000; Bear et al., 2002; Nakagawa et al., 2001; Rottner et al., 1999a). It remains to be shown whether the interaction of IRSp53 with Mena influences actin polymerisation dynamics, either in vitro or in vivo. Although N-WASP plays an important role in Cdc42-induced filopodia elongation (Miki et al., 1998; Suetugu et al., 1998), filopodia can also be induced in N-WASP-deficient fibroblastic cells (Lommel et al., 2001; Snapper et al., 2001). These results suggest that, in the process of filopodia formation, N-WASP functions as a modulator of actin cytoskeleton reorganisation, but is not essential. From our present observation, WAVE2 represents an alternative regulator of actin polymerisation in filopodia. Recent studies have shown that WAVE1 exists in an inactive form in a heterotetrameric protein complex, and becomes accessible to Rac activation in the presence of Nck, whereby both Rac and Nck become involved in complex disassembly (Eden et al., 2002; Takenawa and Miki, 2001; Westphal et al., 2002). Because neither Nck nor Rac are present at lamellipodia tips, this activation may precede the engagement of WAVE with IRSp53. Further analysis of the temporal localisation of WAVE-interacting proteins should shed light on the differential regulation of lamellipodia and filopodia protrusion.

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Fig. 7. Interaction between IRSp53, Mena and WAVE2. Mena is recruited to the protruding tips of both lamellipodia and filopodia through the synergetic function of its EVH1 and EVH2 domains. WAVE2 is recruited to the tips through the WHD domain. IRSp53 binding to the proline-rich region of WAVE2 enhances the activity of WAVE2 to stimulate the Arp2/3 complex. Because IRSp53 also binds to the proline-rich region of Mena, IRSp53 may tie WAVE2 and Mena into a complex. The shaded boxes show the proline-rich regions. Double-ended solid arrows between molecules indicate the previously reported interactions. Grey arrows show the tethering activity to the tips of both lamellipodia and filopodia. Arp2/3, Arp2/3 complex; B, basic region; CRIB, Cdc42 and Rac interactive binding motif; EVH, Ena/VASP homology; fil/mns, filopodium/microspike; Iam, lamellipodium; RCB, Rac binding region; SH, Src homology domain; VCA, Verprolin-Cofilin homology and acidic domain; WHD, WAVE/Scar homology domain.

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