In vivo dynamics of the F-actin-binding protein neurabin-II

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Neurabin-II (spinophilin) is a ubiquitously expressed F-actin-binding protein containing an N-terminal actin-binding domain, a PDZ (PSD95/discs large/ZO-1) domain and a C-terminal domain predicted to form a coiled-coil structure. We have stably expressed a green fluorescent protein (GFP)-tagged version of neurabin-II in PC12 cells, and characterized the *in vivo* dynamics of this actin-binding protein using confocal fluorescence microscopy. We show that GFP-neurabin-II localizes to actin filaments, especially at cortical sites and areas underlying sites of active membrane remodelling. GFP-neurabin-II labels only a subset of F-actin within these cells, as indicated by rhodamine-phalloidin staining. Both actin filaments and small, highly motile structures within the cell body are seen. Photobleaching experiments show that GFP-neurabin-II also exhibits highly dynamic

behaviour when bound to actin filaments. Latrunculin B treatment results in rapid relocation of GFP-neurabin-II to the cytosol, whereas cytochalasin D treatment causes the collapse of GFP-neurabin-II fluorescence to intensely fluorescent foci of F-actin within the cell body. This collapse is reversed on cytochalasin D removal, recovery from which is greatly accelerated by stimulation of cells with epidermal growth factor (EGF). Furthermore, we show that this EGF-induced relocation of GFP-neurabin-II is dependent on the activity of the small GTPase Rac1 but not the activity of ADP-ribosylation factor 6.

Key words: actins, luminescent proteins, PC12, protein binding, spinophilin.

INTRODUCTION

The actin cytoskeleton is responsible for many of the fundamental processes in cells, from the generation and maintenance of cell morphology to cell movement (for reviews see [1,2]). Actin microfilaments function in separation of daughter cells in mitosis [3], the organization of cell polarity, regulation of cell–cell and cell–substrate contacts [4,5], intracellular traffic [6–8] and localization of intracellular organelles [9,10]. Much of the arrangement of actin filaments within cells, such as nucleation, stability and superorganization, are mediated by a diverse array of actin-binding proteins [2,9,11]. The higher order organization of actin filaments is mediated by actin cross-linking proteins. This family of proteins binds along the side of actin filaments, and, through multivalent actin-binding sites, can bundle actin filaments to supermolecular structures such as stress fibres [12]. These proteins appear to have functions other than actin organization alone. For example, fimbrin is essential for receptor-mediated endocytosis in yeast [13], whereas the 280 kDa actin-binding protein (ABP-280) anchors several different transmembrane proteins, including furin, to the cytoskeleton as well as determining the intracellular localization of endosomes and lysosomes [9].

Neurabin-I and neurabin-II are two recently described actin cross-linking proteins of interesting architecture [14–16]. In addition to an N-terminal actin-binding domain, both proteins contain a PSD95/discs large/ZO-1 (PDZ) protein-binding domain and extensive, C-terminal coiled-coil regions. Neurabin-I is exclusively expressed in the brain, whereas neurabin-II is ubiquitously expressed but is present at the highest levels in brain

tissue. Neurabin-I localizes to synapses of mature neurons and in the lamellipodia of growth cones in developing neurons [15]. Antisense ‘knockdown’ experiments specifically targeting neurabin-I also demonstrated a role for this protein in neurite outgrowth in primary rat hippocampal neurons [15]. Neurabin-II (also called spinophilin) was initially identified as a protein phosphatase I-binding protein [14] that localizes to dendritic spines in neuronal cells. Furthermore, a putative *Drosophila* homologue of neurabin-II, E62, has been identified that is induced following the ecdysone-induced metamorphic pulse at the end of the third larval instar [17]. These data point to a key function for the neurabins, in development and synaptic function.

In recent years, the use of the green fluorescent protein (GFP) from *Aequorea victoria* as a fluorescent protein tag has become a widely used method for real-time, *in vivo* visualization of the dynamic behaviour of proteins in living cells [18]. GFP-tagging has enabled a particular wealth of data to be acquired regarding the function of both the actin and microtubule cytoskeleton in eukaryotic cells [19]. Much work has been focused on the use of GFP-actin as a probe for the dynamics of actin filament organization. Studies using GFP-actin in yeast [20], fibroblasts [21], neurons [22] and migrating melanoma cells [23] have given new insight into the highly dynamic nature of the actin cytoskeleton. Further analysis has been undertaken of filament assembly using GFP-tagged versions of capping protein and actin-related protein 3 (‘Arp 3’) [24]. The actin-binding protein coronin was one of the first to be studied using this approach [25,26] showing accumulation within the endocytic cup and leading edge of *Dictyostelium*. Similarly, the actin-binding domain of ABP-120 [27] has been studied to visualize motility and

Abbreviations used: ABP, actin-binding protein; ARF, ADP-ribosylation factor; ARNO, ARF nucleotide-binding-site opener; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; HA, haemagglutinin; NGF, nerve growth factor; PDZ, PSD95/discs large/ZO-1 (protein-binding domain named for the first three proteins shown to have this domain).

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pseudopod extension in these cells. The *Drosophila* homologue of the ABP moesin has been used to generate transgenic flies expressing GFP-moesin and then to visualize changes in cell shape during morphogenesis [28].

In the present study, we have generated a stable PC12 cell line expressing GFP-tagged neurabin-II to investigate the intracellular localization and dynamics of this protein in living cells. We show that GFP-neurabin-II labels a subset of F-actin filaments, and shows highly dynamic behaviour on F-actin fibres as well as on small structures undergoing short-range movement within the soma of differentiated cells. GFP-neurabin-II associates with filamentous actin during cytochalasin-induced depolymerization. It also decorates cortical actin fibres which are rapidly induced upon epidermal growth factor (EGF) treatment following cytochalasin-induced depolymerization. We further demonstrate that such remodelling of neurabin-II-labelled actin fibres is dependent upon the function of the small GTPase Rac1 but not on the function of ADP-ribosylation factor 6 (ARF6). These data suggest that neurabin-II (and by inference neurabin-I) have central roles in actin organization and intracellular transport.

MATERIALS AND METHODS

Reagents and enzymes

All reagents were purchased from Sigma (Poole, U.K.) unless stated otherwise. DNA restriction and modifying enzymes were from Boehringer Mannheim (Lewes, U.K.).

Cell culture and transfection

The *Bgl*II restriction fragment of neurabin-II (amino acids 1–801, lacking the C-terminal 16 amino acid residues) was subcloned into pEGFP-N3 (ClonTech) generating an in-frame fusion with enhanced GFP ('EGFP'), pEGFP-neurabin-II. It was not found possible to amplify the entire coding region of neurabin-II by PCR, presumably due to the unusually high GC content of the coding sequence. Others (Dr. S. L. Milgram, personal communication) have also encountered this problem independently. PC12 cells [29] (generously provided by Dr. Frank Gunn-Moore, University of Bristol, Bristol, U.K.) were cultured on plastic dishes in Dulbecco's modified Eagle's medium (DMEM, Gibco, Paisley, U.K.) containing 10% (v/v) foetal calf serum, 5% (v/v) horse serum, penicillin and streptomycin. Cells were transfected with pEGFP-neurabin-II using Lipofectin (Gibco) according to standard protocols. Stable clones expressing GFP-neurabin-II were selected using 400 $\mu\text{g} \cdot \text{ml}^{-1}$ G418 (Gibco) and maintained in 200 $\mu\text{g} \cdot \text{ml}^{-1}$ G418.

Immunofluorescence

For immunofluorescent staining, cells were grown on collagen-coated glass coverslips (22 mm diameter) and differentiated with 25 ng $\cdot \text{ml}^{-1}$ nerve growth factor (NGF, Santa Cruz Biotechnology) in serum-free DMEM for 2–7 days (as indicated in the Figure legends). Cells were fixed with 4% (w/v) paraformaldehyde in PBS for 20 min. Paraformaldehyde was quenched with 30 mM glycine in PBS, and cells were permeabilized with 0.2% Triton X-100 for 5 min. Coverslips were then blocked for 30 min in PBS containing 3% (w/v) BSA (PBS/BSA), and incubated with 13 nM rhodamine-phalloidin (Molecular Probes, Cambridge, U.K.) diluted in PBS/BSA. Coverslips were then washed three times for 5 min each in PBS/BSA and finally mounted on glass slides with Mowiol solution [10% (w/v) Mowiol 4-88 (Calbiochem, Nottingham, U.K.), 25% (w/v)

glycerol, 100 mM Tris/HCl, pH 8.5, 2.5% (w/v) 1,4-diazabicyclo-(2,2,2)-octane]. Images were acquired using a Leica DM-RBE confocal scanning microscope (Kr/Ar laser, 63 $\times$ magnification, N. A. 1.32 oil immersion lens) controlled by Leica TCS-NT4 software. Images were processed for presentation using Adobe Photoshop 5.0 (Adobe Systems, Uxbridge, U.K.).

Live-cell imaging

For live-cell imaging experiments, stably transfected GFP-neurabin-II PC12 cells were grown on 22 mm diameter, collagen-coated glass coverslips. Cells were equilibrated for 30 min in Krebs–Ringer phosphate buffer (136 mM NaCl, 4.7 mM KCl, 1.25 mM MgSO_4 , 1.25 mM CaCl_2 , 5 mM sodium phosphate pH 7.4, 2 mM NaHCO_3 and 25 mM Hepes pH 7.4). Coverslips were placed into a custom-made aluminium holder [30], which was then placed into the centre of a heated jacket attached to a temperature regulator (both from Medical Systems Corp., Greenvale, NY, U.S.A.). During all experiments, cells were kept at 37 °C and imaged in Krebs–Ringer phosphate buffer. Cytochalasin D was used at 2 μM ; latrunculin B (Calbiochem, Nottingham, U.K.) was used at 25 $\mu\text{g} \cdot \text{ml}^{-1}$, and EGF (Santa Cruz Biotechnology) at 100 ng $\cdot \text{ml}^{-1}$. Fluorescence imaging was performed with a Leica DM-IRBE inverted confocal microscope (100 $\times$ magnification, N.A. 1.2 oil immersion lens or 40 $\times$ magnification, N.A. 1.0 oil immersion lens) controlled by Leica TCS-NT4 software. Photobleaching experiments were performed using the 'Photobleach' tool within this software (duration of the photobleach was 5 s). Images were processed for publication using Adobe Photoshop 5.0. Quicktime movies were generated using NIH Image 1.2 (developed at the U.S. National Institutes of Health, Bethesda MD, U.S.A., and available on the internet at <http://rsb.info.nih.gov/nih-image/>) and saved in Quicktime format using Quicktime version 2.1 for Macintosh (Apple). Movies were also saved as animated gifs using GIF Construction Set (Alchemy Mindworks, <http://www.mindworkshop.com/alchemy/alchemy.html>). Specific image details are given in the Figure legends.

ARF6 and Rac1 expression

Expression plasmids encoding haemagglutinin (HA)-tagged wild-type ARF6 and mutant T27N ARF6 [31] were kindly provided by Dr. Julie Donaldson (National Institutes of Health, Bethesda, MD, U.S.A.), and plasmids expressing myc-tagged wild-type and mutant T17N Rac1 [32] were kindly provided by Professor Alan Hall (University of London, London, U.K.) via Dr. Harry Mellor (University of Bristol, Bristol, U.K.). GFP-neurabin-II PC12 cells were transiently transfected with constructs to express either wild-type ARF6, wild-type Rac1, T27N ARF6 or T17N Rac1 using Lipofectin (Gibco). After transfection for 24 h, cells were left untreated, incubated with 2 μM cytochalasin D for 20 min or incubated with 2 μM cytochalasin D for 20 min, briefly washed in serum-free medium, incubated in serum-free DMEM for 10 min and finally stimulated with 100 ng $\cdot \text{ml}^{-1}$ EGF for 5 min. After these incubations, coverslips were fixed, processed for immunofluorescence and subsequently imaged as described above. ARF6 expression was detected with anti-HA (12CA6, kindly provided by Dr. T. Nilsson, EMBL, Heidelberg, Germany) from mouse ascites and diluted 1:1000. Rac1 expression was detected with anti-myc (9E10) used as described [33]. Primary antibodies were detected by incubation with Alexa-594-conjugated anti-mouse secondary antibodies (Molecular Probes) diluted 1:1000 in PBS/BSA.

RESULTS

Steady-state localization of GFP–neurabin-II

We have stably expressed a GFP-tagged version of the F-actin-binding protein, neurabin-II (GFP–neurabin-II), in PC12 cells. Figure 1(A) shows the steady-state localization of GFP–neurabin-II in fixed cells. Figure 1(B) shows the same cell stained with rhodamine–phalloidin to visualize F-actin. It is clear that in these cells GFP–neurabin-II localizes to actin microfilaments, showing the localization expected of an F-actin-binding protein. It is interesting to note that one does not see a complete overlap of GFP–neurabin-II with phalloidin staining, indicating that GFP–neurabin-II labels a subset of F-actin filaments (arrowheads in Figure 1). We see no differences in actin filament organization (as judged by either rhodamine–phalloidin staining or indirect immunofluorescence using an anti-actin antibody) between untransfected PC12 cells and stably transfected PC12 cells expressing GFP–neurabin-II.

In vivo dynamics of GFP–neurabin-II

Figure 2 shows a time-lapse series of images showing the dynamic nature of GFP–neurabin-II in a PC12 cell that has been differentiated with NGF for 2 days prior to imaging. During such a time course (10 min), we see significant changes in the pattern of GFP–neurabin-II fluorescence. Notably, significant changes in the fluorescence pattern occur within the neurites (arrowhead in Figure 2) as well as within the cell body (asterisk in Figure 2). These changes are particularly apparent in the movie of the time-lapse images (<http://www.BiochemJ.org/bj/345/bj3450185add.htm>) associated with Figure 2, which show images acquired every 20 s. We also observe stable structures both within, and at the tips of, neurites. This is particularly evident at points where the neurites alter direction or end. In addition to labelled actin filaments, individual small structures are visible within the soma of this cell. Figure 2(B) shows an enlargement of the area adjacent to the arrow in Figure 2(A) (final three panels) showing the movement of one such structure (the movement of these structures is particularly apparent in the final frames of the associated movie). These structures undergo short range, non-directed movements, implying that they are not being transported along linear actin filaments. They are short lived in this time series, but it is unclear if this is a reflection of their *in vivo* behaviour or if they are merely

Figure 1 Steady-state localization of GFP–neurabin-II in stably transfected cells

Stably transfected PC12 cells were differentiated for 2 days with 20 ng·ml⁻¹ NGF. (A) GFP fluorescence from GFP–neurabin-II. (B) Rhodamine–phalloidin staining of actin filaments. Note that the two patterns of fluorescence overlap but do not completely coincide (see arrowheads). Images shown are single confocal sections. Bar, 5 µm.

passing out of the focal plane of the image (note that the confocal pinhole is fully open for this image series, such that any movement out of the plane of focus during the experiment would further support the highly motile nature of these structures). These structures are only infrequently visible in these cells, further suggesting that they may be highly transient in nature.

Fluorescence recovery after photobleaching (FRAP)

To further visualize the dynamic behaviour of GFP–neurabin-II when bound to F-actin filaments, we performed FRAP analysis (Figure 3 and see also the associated movie). An area within the neurite of the cell (marked with an arrowhead in Figure 3) was bleached for 5 s (frame labelled 0 s, Figure 3) and the fluorescence recovery into this area was monitored by time-lapse confocal microscopy. Images were acquired every 10 s before the bleach and every 5 s afterwards. The highly dynamic nature of GFP–neurabin-II is clearly seen by the rapid refilling of the bleached area of the image, leading to complete refilling within 20 s. This rapid movement of GFP–neurabin-II is particularly apparent in the associated movie. This behaviour was observed for actin structures within the cell body as well as those within neurite outgrowths (results not shown).

Redistribution of GFP–neurabin-II upon actin filament depolymerization

We chose to investigate the fate of GFP–neurabin-II upon actin depolymerization. Cytochalasin D induces depolymerization of actin filaments by binding to the barbed end to prevent addition or removal of G-actin monomers without affecting depolymerization at the pointed end [34,35]. Cytochalasin D (2 µM) caused rapid depolymerization of actin filaments, which was precisely reflected by changes in the pattern of GFP–neurabin-II fluorescence. These results are shown in Figure 4 and the associated movie in which images were acquired every 15 s for 30 min. There are several features of interest to note from this experiment. First, sites of intense GFP–neurabin-II staining at the tips of short processes at the edge of the cell remain stable and unaffected by cytochalasin D treatment. Secondly, extensive actin depolymerization results in the accumulation of focal points of GFP–neurabin-II fluorescence within the cell body. These appear after 2–4 min of treatment and increase in intensity during the remainder of the series (see associated movie). Thirdly, we see retraction of longer cell processes (such as that seen in the top right-hand corner of each frame in Figure 4). This neurite also accumulates small punctate spots of GFP–neurabin-II fluorescence similar to those within the cell body. Quantitatively identical results were obtained using cytochalasin concentrations as low as 10 nM (results not shown). The data presented in Figure 4 are from differentiated cells, and identical results were obtained when undifferentiated PC12 cells were used for these experiments.

We were interested to identify the nature of the cytochalasin D-induced structures within these cells and therefore repeated the cytochalasin treatment, fixed the cells and used rhodamine–phalloidin to identify any remaining F-actin filaments. Figure 5 shows that the large punctate structures that are seen within the cell body persist even after 2 h of cytochalasin treatment of cells. These structures were labelled with rhodamine–phalloidin (Figure 5B), suggesting that they are composed of F-actin. These structures appear identical to those identified in similar experiments using GFP–actin [23], therefore we believe that they are not an artefact of GFP–neurabin-II expression. To confirm this, we chose to depolymerize actin using another reagent, latrunculin B. Latrunculin B depolymerizes actin filaments by

Figure 2 *In vivo* dynamics of GFP-neurabin-II

(**A**) Panels show time-lapse images of a cell expressing GFP-neurabin-II at the time points (in seconds) indicated. Cells were differentiated for 2 days with $20 \text{ ng} \cdot \text{ml}^{-1}$ NGF prior to imaging. Note that there are many stable structures as well as others showing active remodelling (arrowhead and asterisk, see the associated time-lapse movie, <http://www.BiochemJ.org/bj/345/bj3450185add.htm>). We also see infrequent, but clearly defined, structures moving within the cell body (arrow in frames showing 580, 600 and 620 s). (**B**) Enlarged view of the area indicated by an arrow in Figure 2(**A**). The small arrowhead indicates a small structure undergoing defined short range movement. This is more clearly visible in the associated movie. Bar, $10 \mu\text{m}$.

Figure 3 Dynamics of GFP–neurabin-II as revealed by FRAP

Cells were differentiated for 2 days with $20 \text{ ng} \cdot \text{ml}^{-1}$ NGF prior to imaging. Cells were imaged at 10 s intervals prior to a 5 s bleach of the area indicated by the arrowhead. The bleach period ends at 0 s, after which cells were imaged every 5 s. Note the discontinuous time points of the frames shown. The rapid recovery after photobleaching is shown more clearly in the associated movie. Bar, $10 \mu\text{m}$.

Figure 4 Depolymerization of F-actin filaments in differentiated PC12 cells expressing GFP-neurabin-II

Cells expressing GFP-neurabin-II were differentiated for 2 days with $20 \text{ ng} \cdot \text{ml}^{-1}$ NGF, then treated with $2 \mu\text{M}$ cytochalasin D to induce depolymerization of F-actin filaments. Cells were imaged for 30 min after addition of cytochalasin D. Note that frame numbers represent time in min. See also the associated movie. Bar, $10 \mu\text{m}$.

Figure 5 Localization of GFP-neurabin-II and actin after cytochalasin D treatment

Cells expressing GFP-neurabin-II were differentiated for 2 days with $20 \text{ ng} \cdot \text{ml}^{-1}$ NGF. These cells were then treated for 20 min with $2 \mu\text{M}$ cytochalasin D to induce depolymerization of F-actin filaments. Cells were fixed and stained with rhodamine-phalloidin. (A) GFP-neurabin-II fluorescence. (B) Rhodamine-phalloidin fluorescence. Images shown are single confocal sections. Bar, $5 \mu\text{m}$.

sequestration of G-actin monomers [36–38], resulting in an imbalance in the normal dynamic equilibrium of actin polymerization and depolymerization. Latrunculin B-treatment of non-differentiated GFP-neurabin-II-expressing PC12 cells causes complete depolymerization of actin filaments, resulting in a cytosolic localization of GFP-neurabin-II (Figures 6A and 6B). Time-lapse imaging of latrunculin B-treated GFP-neurabin-II PC12 cells shows a rapid redistribution of GFP-neurabin-II to the cytosol (compare cells in Figure 6A after 3 min latrunculin B treatment with untreated cells). This further suggests that the normal dynamic behaviour of actin filaments is unaffected by GFP-neurabin-II expression. In the time-lapse movie associated

with Figure 6(A) (images acquired every 30 s), it is apparent that there is a particularly dramatic redistribution of GFP-neurabin-II to the cytosol between 1 and 2 min of imaging. This is likely to reflect the mode of action of latrunculin B in sequestering G-actin monomers, resulting in a slight time lag before the normal equilibrium of polymerization and depolymerization is affected [36–38].

EGF-induced redistribution of GFP-neurabin-II

The larger F-actin structures induced upon cytochalasin D treatment do not readily reassemble into actin filaments upon washout of the drug (results not shown). However, we were able to fully reverse the effects of cytochalasin D by stimulating cells with EGF, following removal of cytochalasin D. EGF is known to stimulate cortical actin rearrangements [32,39]. Cells were placed on to the microscope stage, treated for 20 min with cytochalasin D, after which, cells were incubated for 10 min in Krebs–Ringer phosphate buffer. Cells were subsequently stimulated by addition of $100 \text{ ng} \cdot \text{ml}^{-1}$ EGF. Figure 7 shows a time-lapse series of images from the point of EGF addition (see associated movie in which images were acquired every 10 s). Minor rearrangements of the actin cytoskeleton are seen within 60 s of this treatment. After 90 s we see areas of cortical F-actin assembly, which become particularly intensely stained for GFP-neurabin-II between 120 and 210 s. The final frame of this series (210 s) represents the maximal reassembly seen, these newly formed filaments do not alter in appearance over a subsequent 5 min of imaging (results not shown). A time-lapse movie of this series shows the dramatic changes in cortical actin assembly very clearly (see movie associated with Figure 7). Repetition of these experiments with subsequent fixing of cells confirms that these newly formed GFP-neurabin-II positive structures also label with rhodamine-phalloidin and therefore do represent

Figure 6 Latrunculin B treatment of undifferentiated PC12 cells expressing GFP–neurabin-II

(A) Images were acquired with a $40\times$ objective and panels represent 1 min intervals during imaging (see the associated time-lapse movie). (B) Still image of undifferentiated PC12 cells treated with latrunculin B for 3 min (acquired with a $100\times$ objective). Bar, $5\ \mu\text{m}$.

Figure 7 EGF induces rapid redistribution of GFP–neurabin-II to cortical actin filaments following cytochalasin D induced depolymerization

Cells were differentiated with $20\ \text{ng}\cdot\text{ml}^{-1}$ NGF for 2 days then treated with $2\ \mu\text{M}$ cytochalasin D for 20 min. Subsequently they were incubated in Krebs–Ringer phosphate buffer for 10 min and stimulated with $100\ \text{ng}\cdot\text{ml}^{-1}$ EGF at 0 s. See the associated time-lapse movie. Bar, $10\ \mu\text{m}$.

F-actin filaments (results not shown). EGF stimulation of cells without prior cytochalasin D treatment resulted in only minor changes in GFP–neurabin-II fluorescence. This is consistent with the absence of GFP–neurabin-II from filopodium-like structures at the extreme edge of the cell (Figure 1), which are most likely to be those undergoing rapid structural changes in response to EGF [32,39]. No gross changes in GFP–neurabin-II localization, other

than those resulting from large changes in cell shape, were observed in EGF-stimulated cells in the absence of prior actin filament depolymerization. This suggests that GFP–neurabin-II underlies, rather than is a component of, those areas of the plasma membrane that undergo membrane ruffling in response to EGF stimulation, analogous to its absence from filopodia in unstimulated cells (Figure 1).

Figure 8 Effect of wild-type and mutant ARF6 or Rac1 on the localization of GFP-neurabin-II following EGF treatment of cells with depolymerized actin

GFP-neurabin-II expressing PC12 cells were differentiated with $20 \text{ ng} \cdot \text{ml}^{-1}$ NGF for 2 days and then transiently transfected with plasmids encoding wild-type ARF6, T27N ARF6, wild-type Rac1 or T17N Rac1. Cells were subsequently treated with cytochalasin D, or cytochalasin D followed by EGF, as indicated in the legends to the previous figures. Epitope tags were used to identify transfected cells. Panels represent single confocal sections. Bars, $5 \mu\text{m}$.

Role of small GTPases in EGF induced redistribution

EGF-induced actin rearrangements are mediated through activation of the small GTPase Rac1 [32,39]. Similarly, the non-Rho-family GTPase ARF6 has been localized to sites of cortical actin rearrangement and plays a role in cell spreading [40]. Consequently, we chose to investigate the molecular requirements for the EGF-induced redistribution of GFP-neurabin-II using 'dominant negative' forms of these small GTP-binding proteins, which have Thr to Asn substitutions in position 27 or 17 of the respective proteins (ARF6 T27N mutant [31], and Rac1 T17N mutant [32]).

We repeated the EGF stimulation experiments described above in GFP-neurabin-II PC12 cells transiently transfected with either Rac1 or ARF6 constructs. Transfected cells were identified by virtue of epitope tags incorporated into the ARF6 and Rac1 constructs. Experiments were repeated exactly as described above, followed by processing of cells for immunofluorescence. Cells were stained with anti-myc or anti-HA tag antibodies, as appropriate, and imaged for small GTPase expression and GFP-neurabin-II localization. The results of these experiments are shown in Figure 8; all cells shown in these panels stained positive for either ARF6 or Rac1. Expression of either wild-type or dominant negative ARF6 or wild-type or dominant negative

Rac1 did not result in a significant change in GFP–neurabin-II localization (Figures 8A, 8D, 8G and 8J). Upon cytochalasin D treatment, all cells showed the same collapsed pattern of GFP–neurabin-II as seen in untransfected cells (compare Figures 8B, 8E, 8H and 8K to the later panels of Figure 4). Subsequent EGF-induced redistribution of GFP–neurabin-II fluorescence (as for Figure 7) was observed in all cells expressing wild-type ARF6 (Figure 8C), ARF6 T27N (Figure 8F) or wild-type Rac1 (Figure 8I). No redistribution was observed in cells expressing Rac1 T17N (Figure 8L), implying an inhibition of cortical actin rearrangements in these cells. This block of actin reorganization is seen in all cells expressing T17N Rac1. It is also of note that the EGF-induced redistribution of GFP–neurabin-II to cortical actin filaments is enhanced in cells expressing wild-type Rac1 (Figure 8I), further supporting a direct involvement of this GTPase in this process. This result suggests that formation of cortical actin fibres that bind GFP–neurabin-II is not dependent on ARF6, but is entirely dependent on Rac1 function.

DISCUSSION

The organization of the actin cytoskeleton is crucial to a diverse set of cellular functions from cell division [3], co-ordination of intracellular signalling [41], intracellular organization [9,10] and cell migration [1]. The superorganization of the actin cytoskeleton is dependent upon the function of a large number of ABPs [2,11]. In the present study, we have investigated the *in vivo* dynamics of the F-actin-binding protein neurabin-II. It has previously been shown that neurabin-II is capable of cross-linking actin filaments and localizes to actin filaments [14,16]. Results also exist to suggest a key role for neurabin-II in development; it localizes to dendritic spines in neurons [14] and has been implicated in *Drosophila* development [17]. Neurabin-II belongs to a class of proteins that contain a PDZ domain adjacent to an extensive coiled-coil region [42]. The presence of binding sites for src homology 3 ('SH3') domains, protein phosphatase I and transmembrane proteins such as D2 dopamine receptors [43] and *trans* Golgi network ('TGN')38 [44] suggest that neurabin-II may have a scaffolding role in the cell directly linking membranes to the cytoskeleton and co-ordinating associated signalling events.

We have used GFP-tagging to visualize the *in vivo* dynamics of neurabin-II by confocal fluorescence microscopy. The results presented here show that GFP–neurabin-II undergoes extensive movements within both neurites and the soma of differentiated PC12 cells. We have shown that GFP–neurabin-II localizes to a subset of actin filaments in PC12 cells. This suggests a potential regulation of the actin-binding properties of neurabin-II, which may relate to its function in actin filament cross-linking.

We observed highly dynamic movement of GFP–neurabin-II in transfected PC12 cells, both by direct observation as well as in FRAP experiments. This suggests that GFP–neurabin-II is closely associated with actin filaments undergoing extensive reorganization. In support of this suggestion, we showed that GFP–neurabin-II remains closely associated with actin filaments upon depolymerization induced by cytochalasin D. We observed many small foci of GFP–neurabin-II fluorescence in cytochalasin D-treated cells. It is of note that these structures appeared identical to those observed after cytochalasin B treatment of mouse melanoma cells expressing GFP-actin [23]. Indeed these structures appeared over the same time course in both experiments. These structures persisted even after 2 h of cytochalasin treatment, and labelling with rhodamine–phalloidin confirmed the presence of F-actin. These structures are not an artefact of GFP–neurabin-II expression since we see a complete

relocalization of GFP–neurabin-II to the cytosol on latrunculin B treatment.

The differential effects of these two agents (cytochalasin D and latrunculin B) are likely to result from their modes of action. Cytochalasin D caps the barbed end of filaments preventing monomer addition or loss as well as acting directly to sever filaments [23,34,35]. Latrunculin B, however, acts by sequestering actin monomers thereby preventing further addition and shifting the dynamic equilibrium towards depolymerization [36–38]. Indeed, the lag in redistribution of GFP–neurabin-II to the cytosol (which occurs dramatically between 1 and 2 min after latrunculin B addition) compared to the immediate changes in GFP–neurabin-II distribution on cytochalasin D treatment is likely to be a reflection of the different pharmacological properties of these agents.

Having shown that GFP–neurabin-II follows actin depolymerization in these experiments, we addressed its localization upon actin polymerization and reorganization. We used EGF to induce cortical actin rearrangements [32,39] following cytochalasin D treatment of GFP–neurabin-II expressing cells. We found that removal of cytochalasin D alone was insufficient to result in any major alterations in GFP–neurabin-II distribution during the time course of these experiments. However, EGF caused a rapid and dramatic relocalization of GFP–neurabin-II fluorescence to the cell periphery. This relocalization is likely to be due to activation of small GTP-binding proteins, resulting in cortical actin rearrangements as has been previously described [32,39].

Given the distribution of GFP–neurabin-II to a subset of cortical actin filaments and its absence from peripheral areas of the cell, presumably undergoing rapid remodelling, we decided to address the nature of the small GTP-binding protein responsible for these dramatic changes. It has recently been shown that the guanine nucleotide exchange factor for ARF6, ARF nucleotide-binding-site opener (ARNO), undergoes rapid, EGF-induced translocation to the plasma membrane in PC12 cells [45]. The translocation kinetics in those experiments were identical to the kinetics for the production of phosphatidylinositol-3,4,5-trisphosphate, to which ARNO binds directly [45]. The comparative kinetics in the two sets of experiments (with translocation of ARNO occurring approximately 1 min prior to GFP–neurabin-II redistribution) coupled with the observation that ARF6 itself is involved in a number of diverse actin remodelling processes [40] led us to investigate the role of ARF6 in our system. Similarly, the small GTP-binding protein Rac1 has been shown to mediate a number of EGF-induced changes in actin filament structure [4,32,39] principally membrane ruffling and lamellipodium formation. Rac1 is therefore a likely mediator of GFP–neurabin-II rearrangements seen in our experiments. By using dominant negative mutants of these small GTPases (ARF6 T27N, Rac1 T17N), we were able to show a requirement for Rac1 but not ARF6 in the EGF-induced redistribution of GFP–neurabin-II. We stress that we have no evidence for a direct interaction of Rac1 and neurabin-II and that it is more likely that the GFP–neurabin-II redistribution seen in these experiments is a result of Rac1 mediated actin rearrangements. We were unable to assess the effect of co-expression of the dominant negative forms of Rac1 and ARF6 with GFP–neurabin-II by time-lapse microscopy, since we were unable to identify the cells transiently transfected with these constructs without fixation and subsequent immunostaining. In further support of the relevance of these results, it has been found that Rac1 is important for neurite outgrowth [46,47] and particularly for growth cone function rather than assembly [48]. This is consistent with the probable role of neurabin-II in neuronal

function [14,16]. Furthermore, Rac1 has been shown to be essential for the formation of cadherin-based cell-cell contacts [49,50]. In wild-type, polarized, Madin Darby canine kidney ('MDCK') cells, neurabin-II is localized to adherens junctions; expression of Rac1 T17N results in markedly reduced accumulation of neurabin-II at adherens junctions in these cells [16]. These results are entirely consistent with those presented here.

It has been shown that ARF6 is required for some functions of Rac1 to occur, such as membrane ruffling, but not for other effector functions of Rac1, such as Jun kinase activation or inhibition of transferrin receptor endocytosis [49]. Our results suggest that the EGF-induced redistribution of GFP-neurabin-II is independent of ARF6. This correlates with the observed absence of GFP-neurabin-II from membrane ruffles induced by EGF stimulation of cells. It should be noted that our experiments, unlike those of Radhakrishna et al. [51], were performed following depolymerization of the majority of cellular actin filaments and are a result of growth factor stimulation of cells which directly activates Rac1 [32,39].

It is important to note that in all experiments we have observed exactly the same phenomena with GFP-neurabin-I as with GFP-neurabin-II. Indeed, we have observed no qualitative differences in the behaviour of either of these proteins in any of the experiments described here. Recent results have described Rac1-dependent translocation of another ABP, cortactin, to the cell periphery upon Rac1 activation [52]. It is possible that both cortactin and neurabin-I and -II function in a similar manner, transducing signals, via Rac1, to the actin cytoskeleton.

Many other proteins are able to associate with neurabin-I and -II in addition to actin [14–16,43,44,52]. These include protein phosphatase I [14], the 70 kDa isoform of ribosomal S6 kinase [53] and the integral membrane proteins, *trans* Golgi network protein 38 [44] and the D2 dopamine receptor [43]. This array of interactions suggests that neurabin-I and -II may form the hub of a macromolecular complex co-ordinating and integrating a complex set of intracellular and extracellular signals with the actin cytoskeleton.

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