A novel 4.1 ezrin radixin moesin (FERM)-containing protein, 'Willin'

A novel 4.1 ezrin radixin moesin (FERM)-containing protein, 'Willin'

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Abstract The 4.1 superfamily of proteins contain a 4.1 ezrin radixin moesin (FERM) domain and are described as linking the cytoskeleton with the plasma membrane. Here, we describe a new FERM domain-containing protein called Willin. Willin has a recognizable FERM domain within its N-terminus and is capable of binding phospholipids. Its intra-cellular distribution can be cytoplasmic or at the plasma membrane where it can co-localize with actin. However, the plasma membrane location of Willin is not influenced by cytochalasin D induced actin disruption but it is induced by the addition of epidermal growth factor.

Keyword: 4.1 ezrin radixin moesin

1. Introduction

Family members of the 4.1 superfamily include: the closely related proteins ezrin, radixin and moesin (ERM), merlin, talin and protein-tyrosine phosphatases. All of these proteins are involved in maintaining the submembrane cytoskeleton. In some cases, they directly link transmembrane proteins to the cytoskeleton or link kinase and/or phosphatase enzymatic activity to the plasma membrane [all reviewed in 1]. Therefore, it has been suggested that these proteins are involved not only in cell–extracellular matrix interactions and cell–cell communication but also in apoptosis [2], carcinogenesis and metastasis. For example, merlin has been shown to function as a tumor suppressor [1], whilst ezrin has a role in sarcoma metastasis [3] and moesin has been implicated in oral squamous cell carcinomas [4].

Despite their physiological significance, the understanding of their molecular mechanisms is still in its infancy. The 4.1 protein family has a conserved region called the FERM (4.1 ERM) domain, which is predominantly situated in the N-terminus [1]. Distal to this FERM domain, the ERM proteins have a 'coiled-coil' α helical central domain, followed by a C-terminal domain. These proteins are capable of intra-molecular head-to-tail interactions between the N-terminal FERM domain and the C-terminal tail. This has the effect of masking the FERM domain, rendering the protein essentially dormant [1]. However, by a poorly understood mechanism involving phospholipids and kinases, the FERM domain can be released, allowing these proteins to bind to other proteins such as other ERMs, various transmembrane receptor proteins, e.g. CD44, ICAM-1/2/3, CD43, membrane-associated proteins, e.g. EBP-50 (ERM-binding phosphoprotein of 50 kDa) and also the cytoskeletal protein, actin [all reviewed in 5].

A recent yeast two-hybrid screen of the transmembrane L1 family member neurofascin, by this laboratory, identified a new FERM-binding motif within neurofascin which bound ezrin [6] and also a novel FERM-containing cDNA. This new protein was given the name 'Willin', and here we describe for the first time some of the features of this novel FERM-containing protein.

2. Materials and methods

2.1. Cloning

Willin was cloned into pEGFP-N3 or pGEX-KG by PCR using Taq polymerase and the full-length cDNA clone Image:3941276 (Accession No.: BC020521). The following primer combinations were used for cloning into pEGFP-N3 to produce GFP tagged full-length Willin (pWillin-GFP): sense 5′-GGAATTCATGAACAAATTGAATTTTC-AT-3′ and anti-sense 5′- CGGGATCCCACAACAAACTCTGGA-AG-3′ containing *Eco*RI and *Bam*HI restriction sites, respectively. The resulting PCR product was digested with *Eco*RI/*Bam*HI and cloned into the same sites of pEGFP-N3. For cloning into pGEX-KG to produce glutathione *S*-transferase (GST) tagged full-length Willin (pGEX-Willin): sense 5′-CGGGATCCATGAACAAATTG-AATTTTCAT-3′ and anti-sense 5′-GGAATTCTTACACAACA-AACTCTGGAAG-3′ were used, containing *Bam*HI and *Eco*RI restriction sites, respectively. The resulting PCR product was digested with *Bam*HI/*Eco*RI and cloned into the same sites of pGEX-KG. For cloning into pCMV-Tag4A to produce FLAG tagged full-length Willin (pFLAG-Willin): sense 5′-CCACCTCGAGCTCTTCAG-3′ and anti-sense 5′-CGGGATCCCACAACAAACTCTGGAAC-3′ containing *Sac*I and *Bam*HI, respectively, were used for PCR using pWillin-GFP as template and Pfu Turbo (Stratagene) polymerase. The resulting PCR product was digested with *Sac*I/*Bam*HI and cloned into the same sites of pCMV-Tag4A (Stratagene). The coding sequence of human moesin was amplified from the full-length cDNA clone Image:4908580 (Accession No. BC017293) by PCR using High Fidelity Taq DNA polymerase (Roche), and the sequence specific primers (sense 5′-CGGAATTCCATGCCCAAAACGATCAGTGTG-3′ and anti-sense 5′-CGCGTCGACTTACATAGACTCAAATTCGTCA-AT-3′) containing *Eco*RI and *Sal*I restriction sites, respectively. The

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resulting PCR product was digested with *Eco*RI/*Sal*I and cloned into the same sites of pGEX-4T3 (Amersham Biosciences) vector to express as a GST-fusion protein in bacteria. For red fluorescent protein-ADP-ribosylation factor nucleotide-binding-site opener (RFP-ARNO) production, full-length ARNO was excised from pEGFP-C1 ARNO [7] using the *Eco*RI and *Sal*I restriction sites and ligated into the corresponding sites of pDSRed monomer-C1 (Clontech). The authenticity of all constructs was verified by sequencing prior to use.

2.2. Northern and antibody production

RNA was extracted from the brains and sciatic nerves of 21-day-old mice [8] and polyA mRNA isolated using the polyATtract system (Promega). Four micrograms of brain and sciatic nerve polyA mRNA was denatured and electrophoresed on a 1% agarose formaldehyde gel. After vacuum blotting [9] and baking, the filters were prehybridized at 65 °C then probed for 2 h in QuikHyb (Stratagene) at 65 °C with the 32P-labelled [10] 163SciII clone (Accession No.: AF441249). Filters were washed to a final stringency of 0.2×SSC at 65 °C. Blots were exposed to Agfa RF1 film and developed using Kodak LX24 developer and FX40 fixer.

A rabbit polyclonal antibody was generated against the unique sequence KEASKGIDQFGPPMIIHC of Willin (residues 86–102, Fig. 1A) which was also used to affinity purify the antibody from the resulting serum [11]. Sections of human normal oral mucosa and squamous carcinoma were prepared and immunostained with Willin antibody as previously described [11]. In brief, sections were stained with rabbit polyclonal anti-Willin antibodies (1:200) using an Avidin-Biotin technique. Sections, 4–6 μm thick, were deparaffinized, rehydrated, blocked with 3% hydrogen peroxide, antigen-retrieved with sodium citrate (pH 6.0), blocked with normal goat serum (5%)/bovine serum albumin (2.5%), and then consecutively incubated with the primary antibody (60 min), the biotinylated secondary antibody (30 min), horseradish-peroxide linked avidin-biotin complex (30 min). Finally, diaminobenzidine was added as a substrate (15 min).

2.3. Cell culture

PC12 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, 5% horse serum, 100 μg/ml streptomycin, 100 units of penicillin/ml and 10 mM HEPES, pH 7.4. HEK293 cells were grown in minimum essential medium supplemented with 10 mM non-essential amino acids, 10% foetal calf serum, 2 mM glutamine, 100 μg/ml streptomycin, 100 units of penicillin/ml. All cells were cultured at 37 °C and atmosphere of 5% CO_2.

2.4. GST-fusion protein expression and purification and lipid-protein overlay assay

The *Escherichia coli* strain BL21 DE3 was transformed with the pGEX (empty), pGEX-Willin or pGEX-Moesin plasmids and grown in LB broth containing 0.1 mg/ml ampicillin to an A_{600} of 0.5–0.6. The cells were harvested and lysed by sonication. GST-tagged protein was then isolated using glutathione Sepharose 4B resin (Amersham)

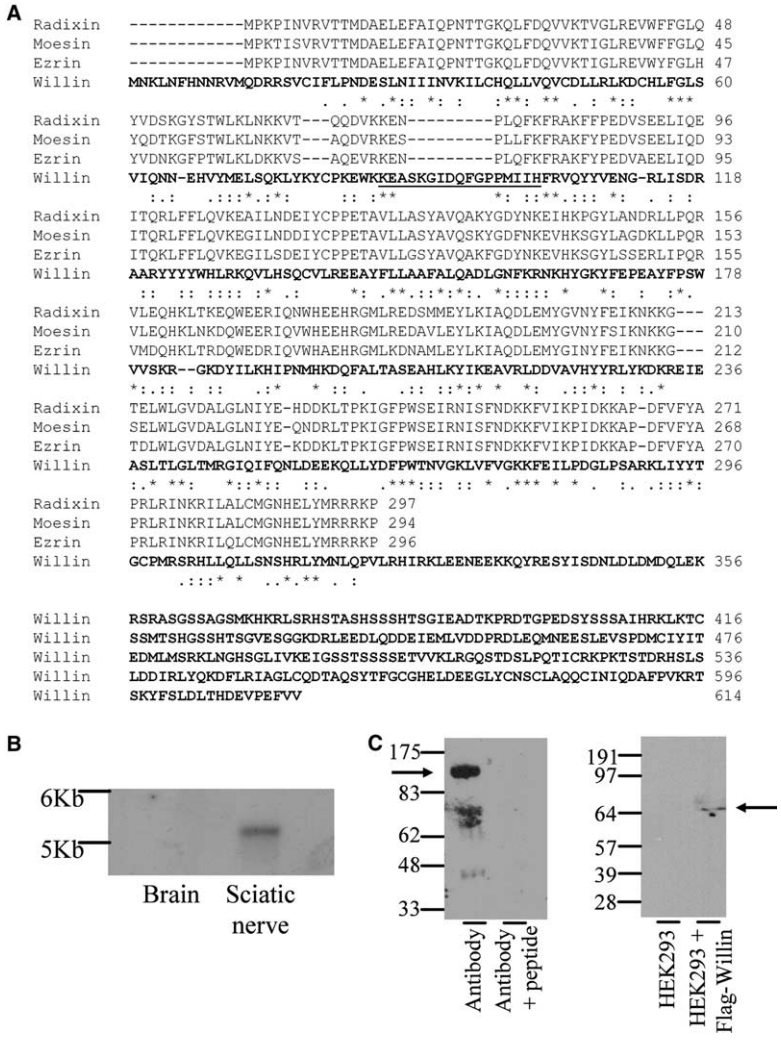

Fig. 1. Willin sequence and size. (A) Sequence of Willin (in bold) and multiple alignment of FERM domains of human Radixin, Moesin and Ezrin. * – identical residue, : – conserved substitution, . – semi-conserved substitution. Underlined region is peptide antibody sequence. (B) Northern blot of Willin in post-natal 12 day rat brain and sciatic nerve. Size markers were 'Millenium markers' (Ambion). (C) Western blots indicating size and expression of both recombinant GST-Willin (left panel) and Flag-Willin in HEK293 (right panel), as indicated by arrows.

and eluted using 0.1 M Tris–HCl, pH 8.0, containing 20 mM reduced glutathione and 0.1% Triton-X-100. Protein concentrations were estimated and purity analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were stored at −20 °C in 50% glycerol until further use [12].

This lipid overlay assay was carried out as described previously [13]. Synthetic DiC16 lipids (Cell Signals inc., USA) were dissolved in methanol:chloroform:water (2:1:0.8). Serial dilutions were performed to create lipid concentrations in the range of 1.6–100 pmol/μl. One microlitre of the lipids was spotted onto Hybond-C nitrocellulose filters (Amersham) and left for 1 h at room temperature to dry. The membranes were blocked in Tris-buffered saline (50 mM Tris–HCl, pH 7.5, 150 mM NaCl) containing 0.1% Tween-20 (TBS-T) and 5% skimmed milk powder (BLOTTO) for 1 h at room temperature. The membranes were incubated with 1 μg/ml of GST-fusion protein in BLOTTO, incubated overnight at 4 °C with gentle rocking and washed with TBS-T at room temperature. The protein bound to lipids was detected by an anti-GST antibody raised in goat (Amersham) at 1/2000 dilution in BLOTTO and an HRP conjugated anti-goat IgG antibody (Sigma). The binding was detected using enhanced chemiluminescence reagent (Amersham) and exposing to X-ray film (Amersham) for 1–5 min.

2.5. Transfections, extractions and cell imaging

HEK293 cells were plated onto glass coverslips and transfected with 1 μg of DNA per coverslip using lipofectamine (Invitrogen) according to the manufacturer's instructions. Cells were left for 24 h after which they were washed twice with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde, washed again and then mounted with moviol (Calbiochem). Cells were imaged with a DeltaVision® RT Restoration Imaging System. For Western blot analysis, 1×10^6 HEK293 cells expressing Flag-Willin were suspended in ice-cold PBS with 4% protease inhibitor cocktail (Roche) and boiled in 2× protein sample buffer (125 mM Tris, pH 6.8, 2% (w/v) β-mercaptoethanol, 0.01% (w/v) bromophenol blue, 20% (v/v) glycerol, 4% (w/v) SDS) for 5 min. Whole cell extracts were separated on 4–12% Bis–tris precast gels (Invitrogen) and probed with a monoclonal antibody to the Flag epitope (Sigma).

PC12 cells were plated onto glass coverslips and transfected with 1 μg of DNA per coverslip using Fugene-6 reagent (Roche) according to the manufacturer's instructions. Cells were left to recover for 24 h at 37 °C and 5% CO_2, before serum starvation for 2–3 h prior to imaging. Confocal imaging of Willin-GFP in live cells was performed with a Wallac UltraVIEW confocal microscope (Perkin–Elmer LifeSciences, UK) as described previously [14]. Cells were treated with either 50 ng/ml epidermal growth factor (EGF), 100 ng/ml NGF or 2 μM cytochalasin D for the times indicated in the figure legends.

3. Results

3.1. Willin, a new FERM-containing protein

Recently, by a yeast two-hybrid screening of a rat sciatic nerve library, we discovered that the transmembrane receptor protein neurofascin can bind to the FERM-containing protein ezrin [6]. In this process, another FERM-containing cDNA was identified. This open reading frame was called '163ScII' (Accession No. AF441249) which contained an ATG initiator Met (an in-frame stop codon is present 39 bases upstream) but an unknown 3′ end. This DNA sequence was BLAST-searched against appropriate databases and was found to have 86% identity at the DNA level and 91% identity at the protein level to a full-length cDNA human clone (MGC:17921 image:3941276) which has also been identified as Open Reading Frame 31 Chromosome 14 (Accession No.: BC020521) from the human genome. The cDNA image clone of this protein was obtained from the MRC IMAGE consortium DNA bank and was given the name Willin (after William Dick, founder of the Royal (Dick) School Veterinary College, University of Edinburgh, Scotland, UK). This cDNA clone from a human uterine leiomyosarcoma tissue was fully sequenced and found to contain 614 amino acids predicting a 71-kDa protein. The amino acid sequence of Willin indicated a FERM domain between residues 14 and 322, which showed similarity with the FERM domain proteins: radixin, ezrin and moesin (Fig. 1A). The length and localization of the FERM domain of Willin and the ERM proteins appeared to be almost the same.

3.2. Willin expression

cDNAs corresponding to the sequence of Willin have been found in human uterus (Accession No. BC020521), placenta (Accession No. BX161430) and cervix (Accession No. AL833158). Gene Card analysis also indicates expression in brain, heart, liver, prostate and lung. Northern blot analysis confirmed Willin expression in the rat sciatic nerve as an approximate 5 kb message (Fig. 1B). More directly, an affinity purified peptide antibody to a unique region in the N-terminus of Willin but not in the ERM proteins (Fig. 1A) has confirmed specific expression of Willin antigen in human material from liver, kidney and oral mucosa [11]. However, protein levels of Willin appear to be low, as Western blot analysis failed to detect native expression but it did detect a GST-Willin recombinant protein of ~100 kDa (71 + 27 kDa) protein and also a Flag-tagged Willin construct expressed in HEK293 cells, was shown to be ~70 kDa in size (Fig. 1C), thereby confirming Willin's size.

The overall similarity with the ERM proteins suggested that Willin would bind to phospholipids. Therefore, we tested a GST-Willin chimeric protein for its ability to bind phospholipids immobilized on nitrocellulose. This qualitative technique showed that purified GST-Willin bound to the same profile of phospholipids as purified GST-Moesin (Fig. 2A), namely phosphoinositol-3-phosphate (PI(3)P), phosphoinositol-4-phosphate (PI(4)P) and phosphoinositol-5-phosphate (PI(5)P).

3.3. Intracellular distribution of Willin

Immunohistochemistry studies have indicated that Willin can have different intracellular sites of expression, including the cytoplasm and the plasma membrane [11]. The factors that effect this distribution appear to be complex. For example, Willin localizes predominantly in the cytoplasm of both normal human oral mucosa (Fig. 2B) and squamous cell carcinoma [11]. However, on a rare occasion Willin is found along the plasma membrane in squamous cell carcinoma (Fig. 2C). At present what influences these locations is unknown.

To investigate its intracellular distribution further, we ligated Willin upstream of the green fluorescent protein (GFP), forming the plasmid pWillin-GFP which was then transfected into HEK293 cells. Willin-GFP expression could be observed as punctate staining throughout the cytoplasm but also occasionally at the plasma membrane (Fig. 2D). The plasma membrane location appeared to be enhanced at the mid-point of cleavage furrow of dividing cells (Fig. 2E) where it had a propensity to co-localize with actin (Fig. 2E). PC12 cells expressing Willin-GFP showed a stronger peri-nuclear location (Fig. 3A and C). However, in some cells there was an increase in Willin-GFP expression at the plasma membrane, in particular at places of cell to cell contact, though the level of plasma membrane location varied from not at all, to

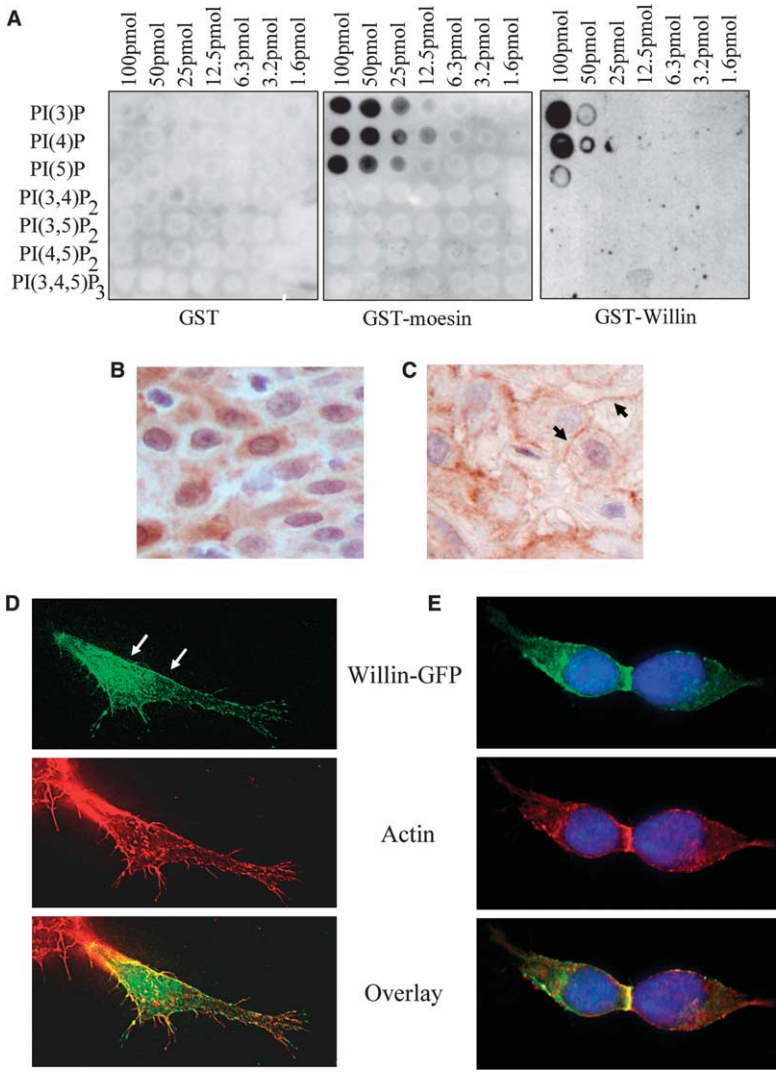

Fig. 2. Phospholipid-binding profile of Willin and its expression in human tissue and HEK293 cells. (A) A lipid blot overlay experiment was performed using purified GST, GST-Moesin and GST-Willin. (B) Immunohistochemistry of Willin in normal human oral mucosa (showing general cytoplasmic staining). (C) A human squamous cell carcinoma (showing membrane localization as indicated by arrows). (D,E) HEK293 cells were transfected with Willin-GFP and then fixed, permeabilized and stained with phalloidin and imaged using fluorescent microscopy (arrows indicate membrane location).

only at points of contact (Fig. 3A), to a more global effect (Fig. 3B).

3.4. Manipulation of the cellular distribution of Willin

The factors controlling this plasma membrane location were unknown. Previous studies from our laboratories have shown that the addition of growth factors to PC12 cells expressing a phospholipid binding pleckstrin homology (PH)-GFP chimeric protein, results in the rapid redistribution of these proteins from the cytoplasm to the plasma membrane [12,15]. Therefore, similar experiments were performed in PC12 cells transfected with Willin-GFP. Following the addition of EGF (Fig. 3C, $t = 0$), the perinuclear pool of Willin-GFP dissipated within 350 s with a resultant increase of fluorescence in the plasma membrane (Fig. 3C) and a subsequent decrease in the cytoplasm as indicated in the change in the ratio of plasma membrane to cytoplasm fluorescence (Fig. 3D). The same phenomenon also occurred after the addition of nerve growth factor (data not shown). However, Willin-GFP redistribution was never seen under these conditions in HEK293 and COS7 cells.

EGF induced redistribution of PH-GFP proteins is inhibited by the addition of wortmannin, implying dependence on PI3 kinase activity [12,15]. Therefore, we performed experiments where 100 nM wortmannin was added either 30 min prior to or after the addition of EGF. Under no conditions was the movement of Willin-GFP to the plasma membrane influenced by the addition of 100 nM wortmannin, though as a control, within the same cells it did block redistribution of the PH domain-containing chimeric protein RFP-ARNO (Fig. 4A). This indicates that PI3 kinase activity is not required for the EGF induced translocation of Willin to the plasma membrane. The presence of a polymerized actin cytoskeleton was also not required for the plasma membrane location of Willin-GFP in PC12 cells, as cytochalasin D induced disruption of the actin cytoskeleton did not prevent Willin-GFP expression at the plasma membrane (Fig. 4B).

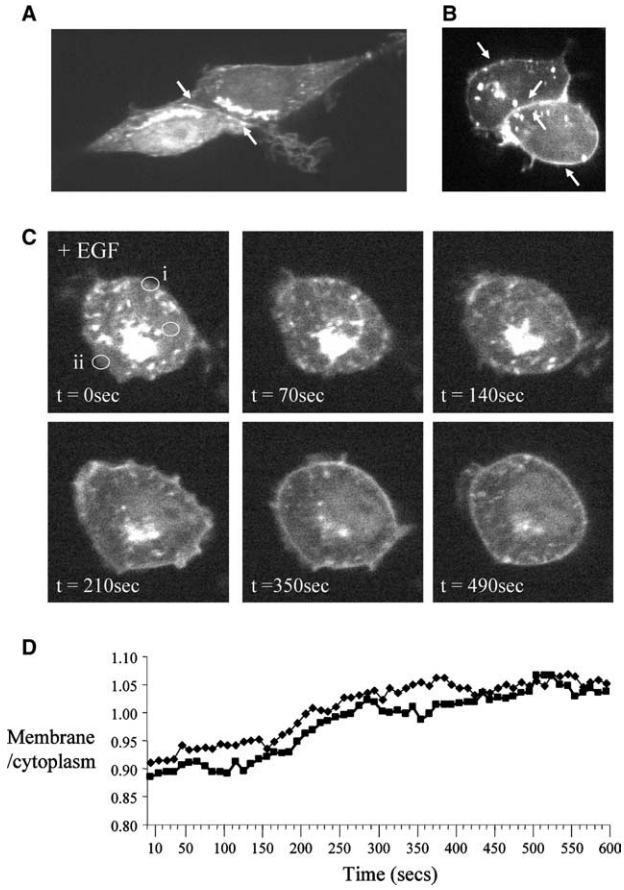

Fig. 3. Cellular localization of Willin in PC12 cells. (A,B) Examples of PC12 cells transfected with Willin-GFP showing varied amounts of membrane location, as indicated by arrows. (C) Time-lapse confocal microscopy of the growth factor-induced translocation of Willin-GFP in PC-12 cells. PC-12 cells were transfected with Willin-GFP. After 24 h, cells were serum-starved and a selected cell was imaged. The number in the panel refers to the time after the addition of 100 ng/ml EGF. (D) Ratio of plasma membrane to cytoplasmic fluorescent intensity of Willin-GFP with time. Each time point is 10 s and the ratio was obtained by comparing fluorescent intensity from two regions of interest in the plasma membrane to a region in the cytoplasm using the UltraView software (Perkin–Elmer LifeSciences, UK). Measured areas are circled and are shown in C and D as **i** is plotted as diamonds; **ii** is plotted as squares, respectively.

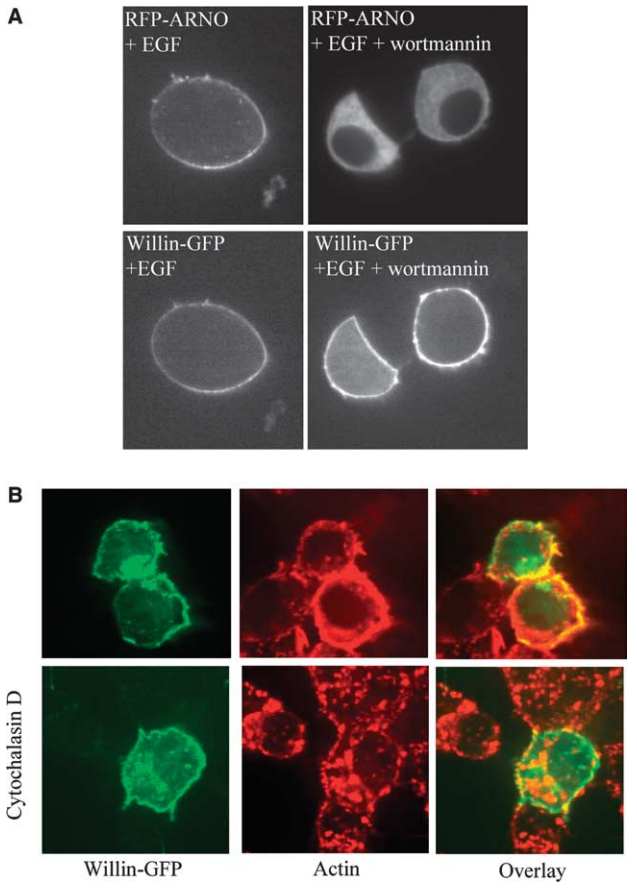

Fig. 4. Plasma membrane location of Willin in PC12 cells is unaffected by addition of wortmannin or cytochalasin D. (A) PC12 cells were co-transfected with RFP-ARNO and Willin-GFP. Cells were treated with or without the addition of 100 nM wortmannin 30 min prior to the addition of 100 ng/ml EGF for 10 min. (B) PC12 cells were transfected with Willin-GFP and stimulated with 100 ng/ml EGF for 10 min, and then treated with cytochalasin D for 10 min resulting in disruption of the actin cytoskeleton but not Willin-GFP distribution.

4. Discussion

Here, we show that a novel FERM-containing protein, Willin, has characteristics associated with the 4.1 super-family of proteins. Firstly, it has a FERM domain situated in its N-terminus. Secondly, a purified recombinant GST-Willin fusion protein can bind to the same phospholipid profile as GST-Moesin. Thirdly, immunohistochemical staining of primary human material and also expression of Willin-GFP in mammalian cell-lines showed that Willin can exist in both the plasma membrane and the cytoplasm. The cytoplasmic expression in HEK293 cells was seen as punctate points throughout these cells, whilst in PC12 cells it appeared more concentrated in a perinuclear location. The amount of Willin in the plasma membrane was variable. Though there was a tendency in both HEK293 and PC12 cells for contacting cells to display Willin expression at points of contact, a very distinct expression was seen in the cleavage furrows of dividing HEK293 cells as was first observed for radixin [16]. Such diverse intracellular distribution has been commonly observed for the 4.1 super-family and is influenced by many differing factors [1]. In particular, for the ERM proteins it has been hypothesized that the cytoplasmic pool is an inactive form caused by a head-to-tail intramolecular binding of the ERM proteins. This inactive form can then be activated by phosphorylation such that it becomes active and can then bind to various transmembrane and membrane-associated proteins, including actin [1,5]. Willin may also exist in such an inactive form within the perinuclear region of PC12 cells. Upon addition of growth factors, Willin may become activated via a tyrosine kinase pathway into an active form that can associate with unknown proteins within the plasma membrane. In the contacting cells, and also possibly in the squamous cell carcinoma, Willin may already be in the active form and hence already is associated with its protein binding partners. The identification of the binding proteins of Willin will help to uncover this mechanism. The ability to stimulate translocation of Willin-GFP by the addition of EGF in PC12 cells but neither HEK293 nor COS7 cells may also reflect differing binding proteins and/or signalling pathways; though we have shown that differences in PI3 kinase activity are not responsible.

Intriguingly, though Willin can colocalize with actin, which is commonly observed for the 4.1 super-family [1], at present the nature of this association is uncertain. Colocalisation of Willin with actin appears to be strongest when Willin is distributed along the plasma membrane. However, cytochalasin D treatment had no effect on the distribution of Willin at this location, implying that the plasma membrane location of Willin is not dependent on the actin cytoskeleton. Future studies will attempt to identify whether Willin does bind actin and also identify its phospholipid binding motifs. However, the determination of these motifs in the 4.1 superfamily is complex as actin binding domains are poorly defined, can exist as multiple sites within a protein, and can be present in either the N- or C-terminus [17]. Phospholipid-binding motifs are equally complex as again more than one site can exist [18,19] which require cooperation between the different sites for binding [19].

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